Proteinases and Extracellular Matrix Degradation in Breast Cancer

by

PHILIP HENDRIK FORTGENS

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from February 1991 to December 1996, under the supervision of Prof. Clive Dennison.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Philip Hendrik Fortgens
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ABSTRACT

A variety of proteases have been shown to promote the progression of cancer by virtue of their ability to degrade extracellular proteinaceous barriers, such as basement membrane and interstitial stroma. At the outset of this study available evidence strongly implicated cathepsin D in breast cancer metastasis. It was envisaged that an antibody inhibitory to the activity of this enzyme might retard invasion, and restrain a tumour from spreading. To this end anti-peptide antibodies were generated against a peptide sequence derived from the substrate capturing "flap" of the enzyme. Inhibition of enzyme activity by these antibodies could not be demonstrated, probably due to the lack of a suitably sensitive enzyme assay. However, the rationale of this study and the expertise gained from it could be applied, in the future, to enzymes that have since been found to be more relevant to tumour invasion.

A feature of many transformed cells is an anomalous lysosomal enzyme trafficking system, and concomitant hyper-secretion of some enzymes. The distribution of low pH compartments and lysosomal enzyme-containing compartments was investigated in human breast epithelial cells, and their c-Ha-*ras*-transformed counterparts. Immunofluorescence and immunoelectron microscopy showed that these compartments have a more peripheral cellular distribution with respect to normal cells, and cathepsins B and D were cell surface-associated.

Studies were undertaken to reveal the extracellular matrix degrading ability of c-Ha-*ras*-transformed cells. Transformed cells exhibited increased degradation of fluorescein-labelled extracellular matrix in serum free medium, and increased

motility, and degradation and disruption of extracellular matrix in serum-containing medium. *In vitro* invasion through artificial basement membrane by transformed cells was investigated using scanning electron microscopy, and was further used to preliminarily identify the proteases involved in invasion by specific inhibition. By this means, greatest inhibition of *in vitro* invasion was obtained using a specific metalloproteinase inhibitor. Overexpression by transformed cells of a metalloproteinase was detected by gelatin zymography. Together these results suggest that the increased invasive capacity of *ras*-transformed breast epithelial cells may be largely due to increased metalloproteinase activity.

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-di-(3-ethyl)-benzthiozoline sulfonic acid
APMA	*p*-aminophenylmercuric acid
AR	analytical reagent
bFGF	basic fibroblast growth factor
BM	basement membrane
BSA	bovine serum albumin
BSA-PBS-E	bovine serum albumin in PBS-E
BSA-PBS-F	bovine serum albumin in PBS-F
C	N, N′-methylenebisacrylamide
C3	third component of complement
CAMOR	coupling agent-modified residues
cDNA	copy deoxyribonucleic acid
dist.H_2O	distilled water
DMEM	Dulbecco's minimal essential medium
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
Fc	crystallisable fragment
FCM	fibroblast-conditioned medium
FITC	fluorescein isothiocyanate
GTP	guanosine triphosphate
Ha-*ras*	Harvey *ras* oncogene
HBSS	Hanks' balanced saline solution
HEPES	N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid
12(S)-HETE	12(*S*)-hydroxyeicosatetranoic acid
HRPO	horseradish peroxidase

IgG	immunoglobulin G
IgY	immunoglobulin Y
k_{cat}	enzyme turnover number
kDa	kilodaltons
LAMP-2	lysosome-associated membrane protein-2
LuECAM	lung endothelial cell adhesion molecule
K_m	Michaelis constant
α_2-M	α_2-macroglobulin
MCF-10A	immortal human diploid mammary epithelial cells (attached)
MCF-10AneoT	MCF-10A cells transformed with c-Ha-*ras*
MCF-10F	immortal human diploid mammary epithelial cells (floating)
MCF-10M	mortal human diploid mammary epithelial cells
MMP	matrix-metalloproteinase
M-6-P	mannose-6-phosphate
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix-metalloproteinase
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PBS-E	PBS for electron microscopy immunolabeling
PBS-F	PBS for immunofluorescence labeling
PEG	polyethylene glycol
pI	isoelectric point
PI 3-kinase	phosphatidylinositol 3-kinase
PLT	progressive lowering of temperature
RER	rough endoplasmic reticulum
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	scanning electron microscopy
serpin	serine proteinase inhibitor
SFM	serum free medium
SV-40	simian virus-40
T	acrylamide
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TIMP	tissue inhibitor of metalloproteinases
tPA	tissue-type plasminogen activator
TPP	three phase partitioning
TRITC	tetramethylrhodamine isothiocyanate
uPA	urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1

CHAPTER 1

INTRODUCTION

1.1 The biology of tumour progression

For many years there has been a tendency to classify cancer as a single disease. It is now clear, however, that cancer is a complex of malignancies many of which appear phenotypically similar, but are characterised by subtle differences in molecular mechanisms. One common denominator to all malignant cancers is metastasis which, despite major advances in surgical techniques and adjuvant therapies, is still the major cause of death in human cancer. This is reflected by the fact that approximately 50% of patients diagnosed with cancer will have distant metastases at the time of diagnosis (Crissman, 1989). Radical therapies directed at the primary tumour will, therefore, not improve survival for this subgroup of patients. Although invasion is an important component of the metastatic process, this process has often already been completed at the time of diagnosis, and is therefore not available for therapeutic intervention. It has been suggested, therefore, that therapeutic developmental efforts be focused on the last steps in metastatic dissemination, i.e., colonisation and angiogenesis (Weinstat-Saslow and Steeg, 1994). However, mapping the proteolytic profile of human cancers may be useful both as a guide to the development of prognostic markers and in the design of anti-proteolytic strategies aimed at controlling the disease. Extracellular proteolysis, for instance, is implicated in growth factor activation and release, which in turn may enhance proliferation of malignant cells or trigger angiogenesis.

Malignancy is a systemic disease in which the tumour cells' interaction with the vasculature forms an integral part of the pathological process. The first step in this process is tumour neoangiogenesis, or the development of new blood vasculature associated with the tumour, accomplished by the secretion of several angiogenic factors by tumour and host cells (Fidler and Ellis, 1994). Failure to recruit and establish new blood vessels to supply essential nutrients and oxygen

for growth, results in small, latent tumours which may remain viable for decades. Metastases are rarely associated with avascular tumours. Upon vascularisation, however, the tumour cells begin to grow in a cylindrical pattern around the new microvessels (Folkman and Shing, 1992), followed by tumour cell detachment from the primary neoplasm. Tumour cells are thought to separate more easily from the solid tumour mass than corresponding normal cells from surrounding tissue (Nicolson, 1988).

For tumour dissemination to occur, the unconstrained malignant cells must gain access to a vasculature, either haematogenous or lymphatic. Tumour interstitium, however, generally lacks lymphatic vasculature and entry of tumour cells into circulation usually takes place via tumour blood vessels. Furthermore, tumour-induced blood vessels are fragile and permeable, thereby increasing the potential for penetration (Blood and Zetter, 1990).

Upon gaining access to the vasculature, cells are subjected to a number of sequential or sometimes concurrent events and processes. These include (Liotta *et al.*, 1986b): transport and survival of tumour cells in the vasculature, interactions of tumour cells with platelets and/or leucocytes, arrest and adhesion to the endothelium of the target organ, tumour cell adhesion to the subendothelial basement membrane (BM), extravasation from the vasculature, secondary tumour growth and angiogenesis. Only a small population of cells from the primary tumour will accomplish all stages of metastasis and form a secondary tumour. A secondary tumour can, in turn, initiate another round of metastasis to give rise to tertiary metastases downstream from the secondary tumour. It is clear that the arrest of the metastatic process at any of its stages will have great therapeutic value in patients with actively metastasising tumours.

The interstitial stroma and BMs of most tissues do not normally contain pre-existing passageways for cells. Only during events such as tissue remodelling, wound healing, inflammation and neoplasia do these matrices become focally permeable to migrating cells. Liotta *et al.* (1986b) have proposed a three-step hypothesis describing the sequence of biochemical events during tumour cell

invasion of the extracellular matrix (ECM). In the first step the tumour cell attaches to components of the matrix such as laminin, fibronectin and vitronectin in BM and fibronectin in stroma, a process mediated by cell adhesion receptors (Zetter, 1993). The anchored cells then secrete hydrolytic enzymes (or induce host cells to secrete enzymes) which locally degrade matrix. The third step is tumour cell locomotion through the region of matrix degraded by enzymatic action.

The vascular endothelium plays dual and opposing roles: it provides a site for receptor-mediated tumour cell adhesion, but also serves as a barrier to extravasation. Kramer and Nicolson (1979) first demonstrated tumour cell adhesion to endothelial cell monolayers, and subsequent endothelial cell retraction (Nicolson, 1982) and tumour cell migration to the exposed underlying subendothelial matrix. Menter *et al.* (1987) demonstrated that tumour cell adhesion to endothelial cells and the subendothelial matrix is enhanced in the presence of platelets. One platelet factor that can transfer from activated platelets to endothelial cells is the lipoxygenase product of arachidonic acid, 12(S)-hydroxyeicosatetraenoic acid, generated during tumour cell-platelet-endothelial cell interactions. This compound has been shown to induce reversible endothelial cell retraction, allowing tumour cells access to the subendothelial matrix (Honn *et al.*, 1989). Directed movement of tumour cells from sites of initial attachment on endothelial cells may be mediated initially by fibronectin associated with the endothelial BM, thereby creating a haptotactic gradient toward the BM (Kramer *et al.*, 1980; McCarthy and Furcht, 1984). Motivation for tumour extravasation through the BM appears to be a function of motility factors or soluble gradients of ECM derived fragments acting as chemotaxins (Hart *et al.*, 1989). Autocrine motility factor and scatter factor have been proposed to a play a major role in the local invasive behaviour of tumour cells and may also facilitate the concerted invasion by groups of tumour cells (Liotta *et al.*, 1986a; Gherardi *et al.*, 1989).

During the development of invasive tumours, tumour cells disobey the social order of organ boundaries and cross into tissues where they do not belong. The

BM acts as an interface between histologically distinct tissues, separating organ cells, epithelia and endothelia from the interstitial stroma. The BM plays a role in cell adhesion, providing a support for growing cells, forming selective permeability barriers for proteins and is important for morphogenesis and mitogenesis. The biochemical components are a BM-specific collagen, type IV collagen, which forms the structural framework; the glycoproteins laminin and fibronectin; a heparin sulfate proteoglycan specific for the BM; entactin and nidogen (Pauli *et al.*, 1983; Liotta *et al.*, 1986b). The interstitial stroma is a complex matrix composed of cells located in a meshwork of collagen fibres (types I and III), glycoproteins and proteoglycans (Tryggvason *et al.*, 1987). This is a widely distributed tissue that has major mechanical and supportive functions and is present in bone, tendons, cartilage, ligaments and as tissue stroma.

It is clear, however, that although mechanisms exist for tumour cell arrest, attachment and migration out of the vasculature, the BM still constitutes a barrier which must be compromised for extravasation to occur. For this reason, degradation of the biochemically complex ECM is a hallmark of the metastatic process. The involvement and relevance of secreted proteolytic enzymes during tumour invasion is now firmly acknowledged. Invasive tumour cells secrete a variety of matrix-degrading proteases, or induce their secretion in host cells (Himelstein *et al.*, 1994), resulting in focal lysis of the BM at regions close to the tumour cell surface (Sloane *et al.*, 1982; Woolley *et al.*, 1984; Danø *et al.*, 1985; Rochefort *et al.*, 1987).

A variety of normal physiological processes also require proteolytic modification of the ECM. Leucocytes extravasate out of the bloodstream in response to stimuli associated with inflammation (Weis, 1989). Resorption of bone by osteoclasts involves the secretion of proteolytic enzymes (Eeckhout, 1990), as does cytotrophoblast invasion of the BM of the uterus during implantation (Librach *et al.*, 1991). Wound healing and tissue remodelling require proteolytic activity directed at ECM (Alexander and Werb, 1989), and angiogenesis by normal endothelial cells shares many properties in common with tumour invasion (Liotta *et al.*, 1991). Rheumatoid arthritis (Weston and Poole, 1973), pulmonary

emphysema (Bieth, 1992) and non-neoplastic invasion by parasites (Doenhoff *et al.*, 1990) are pathologies also characterised by proteolytic degradation of ECM barriers.

A unifying principle of malignant neoplasms appears to be an imbalance of proteolysis which favours invasion. The defect in the tumour cell cannot simply be unbridled production of degradative enzymes, as cell migration requires attachment and detachment of the cell as it moves forward. Lysis of matrix components would remove the substratum necessary for proper cell traction. It is thus probable that the invading tumour cell uses proteolysis in a highly organised manner that is functionally little different from cells that would normally migrate through tissue barriers (Liotta and Stetler-Stevenson, 1991). The difference is that tumour cells achieve invasion by proteolysis and motility at times and places inappropriate for normal cells.

The success of the invasive phenotype, however, is a function of the balance between active proteases and their inhibitors. In an *in vitro* model of tumour cell invasion using amniotic membranes, a bimodal relationship between invasion and plasminogen activator activity was demonstrated. The invasive behaviour of Bowes' melanoma cells, which produce large amounts of tissue-type plasminogen activator (tPA), and HT 1080 fibrosarcoma cells, which produce large amounts of urokinase-type plasminogen activator (uPA), is not blocked but enhanced by the addition of plasmin inhibitors or anti-plasmin antibodies. Conversely, the invasive capacity of cells which produce low levels of these proteases was blocked. Excessive proteolysis in the melanoma and fibrosarcoma cells may cause uncontrolled matrix degradation and the interruption of cell-matrix interactions necessary for invasion. Addition of inhibitors of proteolysis, would reduce degradation to a level compatible with invasion (Tsuboi and Rifkin, 1990).

A large body of evidence shows increased amounts of proteinase activity and secretion in malignant tumour tissue samples and cultured transformed or tumour cells (Liotta *et al.*, 1979; Recklies *et al.*, 1982; Nakajima *et al.*, 1983; Reich

et al., 1988). In some cases a direct correlation between metastatic potential of tumour cells and the production of certain proteinases has been claimed (Liotta *et al.*, 1980; Sloane and Honn, 1984). Proteinases are grouped into four main classes depending on their catalytic site, pH optimum, susceptibility to inhibitors and cation requirements (Table 1). Proteinases that have most frequently been associated with the malignant phenotype are the collagenases, plasminogen activators and cathepsins, but other enzymes such as elastase (Kao and Stern, 1986), chymotrypsin-like proteinases (Kinder *et al.*, 1992) and heparanase (Nakajima *et al.*, 1983) have also been implicated.

A complex set of interactions has been suggested whereby proteases of different classes activate other proteases thus amplifying the degradative process. *In vitro* experiments usually require a high molar excess of the activating protease (Goretzki *et al.*, 1992), but if the proteases were concentrated on the plasma membrane in close proximity to each other, such an excess may not be necessary. In this way the degradation process is localised to sites where the cell surface is in contact with the ECM (Chen *et al.*, 1985). Focal adhesions, responsible for attachment of cells to the ECM, are found at the periphery of the ventral cell surface, whereas invadopodia, cell surface extensions that protrude into the lysed ECM, are found at central sites of the cell, at the leading edge of invasion (Chen and Monsky, 1993). Invadopodia have been found to actively degrade matrix substrates, leading to cell invasion (Chen, 1989), and this interaction may be protected from the effects of protease inhibitors by peripheral focal contacts, thus amplifying the proteolytic cascade (Campbell and Campbell, 1988; Chen and Monsky, 1993). The most closely studied proteases recruited to the cell surface are the plasminogen activators and the matrix metalloproteinases (MMPs).

Table 1. General characteristics of the four classes of proteinases (adapted from Nakajima *et al.*, 1987; Tryggvason *et al.*, 1987).

Class	*Examples	pH range for activity	Inhibitor
Metallo-	*collagenases, gelatinases, stromelysin*	7-9 neutral neutral	metal chelators, TIMP
Serine	trypsin, cathepsin G, chymotrypsin, *plasmin*, thrombin, *chymotrypsin-like proteinases, plasminogen activators, elastase*	7-9	fluoro-phosphates
Cysteine	*cathepsins B, H, L,* S, N, T, O	3-8	iodoacetate, N-ethyl-maleimide, cystatins
Aspartic	pepsin, renin, *cathepsins D*, E	2-7	pepstatin A

*Relationships between tumour invasiveness or metastatic activity and activity of tumour-associated enzymes (in italics) have been reported.

1.2 Matrix-metalloproteinases

The MMPs are a family of zinc metalloenzymes, which have been grouped into three broad categories based on substrate preference: interstitial collagenases, type IV collagenases (gelatinases) and stromelysins (Stetler-Stevenson *et al.*, 1993). Interstitial collagenase and neutrophil collagenase are grouped because of their specificity for type I, II and III collagen degradation. The stromelysins are three related gene products, stromelysin, stromelysin 2, and matrilysin which degrade a variety of matrix components including proteoglycans, non-collagenous glycoproteins such as laminin and fibronectin and the non-collagenous domain of type IV collagen (Liotta and Stetler-Stevenson, 1991; Stetler-Stevenson *et al.*,

1993). The type IV collagenases are so named because of their selective ability to cleave type IV collagen; the 72 kDa (gelatinase B) and 92 kDa (gelatinase A) forms being unique gene products (Liotta and Stetler-Stevenson, 1991).

Gelatinases are secreted as inactive zymogens and enzyme activation is thought to be an important control step in proteolysis. Studies have clearly demonstrated a positive correlation between MMP expression, invasive behaviour and metastatic potential in experimental systems (Liotta *et al.*, 1980; Chen *et al.*, 1991; Pyke *et al.*, 1992; Aimes *et al.*, 1994). These proteases are also an important component of the invasive phenotype in human tumours, including breast, prostate, colon, lung, ovarian and thyroid cancers (Stetler-Stevenson *et al.*, 1993). Furthermore, antibodies (Höyhtyä *et al.*, 1990), and other gelatinase inhibitors (Reich *et al.*, 1988) prevented tumour cell invasion *in vitro*. It is believed, however, that the ratio of active enzyme species to latent proenzyme may provide a better correlation with invasive potential than overall levels of the enzyme.

There is evidence that the latent MMPs bind to the plasma membrane where activation takes place. The human breast cancer cell lines MDA-MB231 and MCF-7 contain a cell surface, high affinity receptor for the 72 kDa MMP (Emonard *et al.*, 1992) and the invadopodia of transformed chicken embryo fibroblasts contain activated 72 kDa MMP (Monsky *et al.*, 1993). Activation of this MMP has recently been elucidated, and is mediated by an integral plasma membrane protein, the first membrane-type MMP (MT-MMP) to be discovered (Sato *et al.*, 1994; Vassalli and Pepper, 1994). Indeed, in human gliomas increased MT-MMP expression correlates with increased expression and activation of the 72 kDa MMP during malignant progression *in vivo* (Yamamoto *et al.*, 1996). In contrast, the 92 kDa MMP is activated by a mechanism which is cell surface associated but independent of the 72 kDa MMP activation mechanism.

Secretion and activation of MMPs is not enough to ensure that they will degrade the target matrix substrate. Tissue inhibitor of metalloproteinases (TIMPs) produced by the tumour or the host can bind to latent and active MMPs (Liotta

and Stetler-Stevenson, 1991). Tight-binding 1:1 complexes form between TIMP-1 and TIMP-2, and 92 kDa MMP and 72 kDa MMP respectively. Exogenously added TIMPs are inhibitors of invasion through reconstituted extracellular matrix (Schultz *et al.*, 1988), and overexpression of TIMP-1 in metastatic melanoma cells suppressed metastasis *in vivo* (Khokha, 1994), indicating that net invasive activity depends upon the balance between the levels of activated enzyme and TIMPs (Kishi *et al.*, 1994). These metalloproteinase inhibitors have also been demonstrated to have an effect on tumour growth. TIMP-1 and TIMP-2 have both been shown, *in vitro*, to have potent growth-promoting activities for a wide range of cells (Hayakawa *et al.*, 1994). In contrast, TIMP-1 overexpressed in B16 mouse melanoma cells resulted in a substantial decline in tumour growth *in vivo* (Khokha *et al.*, 1992) without reducing their ability to extravasate (Koop *et al.*, 1994). Several non-mutually exclusive reasons may exist for the growth inhibitory effect of protease inhibitors. First, many protease inhibitors have been shown to inhibit angiogenesis (Johnson *et al.*, 1994), which is likely to significantly limit growth rate *in vivo*; and secondly, proteinase inhibitors, by exerting control over proteolytic activity, could in turn control the bioavailability of matrix-bound growth factors.

1.3 The urokinase-type plasminogen activator system

The uPA system provides a model for the binding of secreted proteases to the cell surface with subsequent activation. Many cells display specific receptors for uPA and a single class of receptors binds both plasminogen and plasmin. Binding does not involve the active site and receptor bound plasmin and uPA are catalytically active and are not internalised. Co-expression of plasmin/plasminogen and uPA receptors provide tumour cells with a mechanism to generate and localise protease activity to the cell surface (Testa and Quigley, 1990). It has been shown that receptor-bound uPA mediates the activation of plasminogen at the cell surface. The plasmin generated, in turn, activates surface-bound pro-uPA (Stephens *et al.*, 1989). Pro-uPA may also be converted by plasma kallikrein (Schmitt *et al.*, 1992), or the cysteine proteinases, cathepsins B (Kobayashi *et al.*, 1993) and L (Goretzki *et al.*, 1992). Receptor-bound uPA can be inactivated by plasminogen activator inhibitors (PAI), PAI-1 and PAI-

2, but receptor-bound plasmin is inaccessible to serum inhibitors (Stephens *et al.*, 1989) and is active against a variety of ECM molecules including fibrin, fibronectin and laminin (Schmitt *et al.*, 1992). The interaction between receptor-bound uPA and PAI-1 or PAI-2 results in internalisation and subsequent degradation of the enzyme/inhibitor complexes (Testa and Quigley, 1990).

Elevated levels of uPA have been determined in almost all cancer tissues examined, and related to the increased invasive potential of tumour cells (Chucholowski *et al.*, 1991). Tumour cell surface-associated uPA and plasmin mediate the degradation of tumour stroma and basement membrane (Quax *et al.*, 1991; Kobayashi *et al.*, 1992) and the clinical relevance of these findings for breast cancer has been established, indicating uPA to be an independent prognostic factor for early recurrences and shorter overall survival (Chucholowski *et al.*, 1991). Inhibitors of uPA have been shown to inhibit *in vitro* invasion of the ECM by several neoplastic cell lines (Reich *et al.*, 1988; Kobayashi *et al.*, 1995), and anti-catalytic antibodies delayed the onset of pulmonary metastasis by human carcinoma cells in the chicken embryo invasion model (Ossowski and Reich, 1983). In fact, uPA expression alone is sufficient to confer to non-invasive mouse L cells an experimental invasive phenotype (Cajot *et al.*, 1989), while highly invasive cells expressing recombinant PAI-2 were found to have their receptor-bound uPA activity neutralised, resulting in suppression of matrix protein degradation and inhibition of invasion *in vitro* (Laug *et al.*, 1993). In addition, the metastatic process *in vivo* is sensitive to uPA receptor blockade by catalytically inactive uPA mutants (Crowley *et al.*, 1993), as well as antisense inhibition of the receptor in human carcinoma cells (Kook *et al.*, 1994). It is interesting to note that tPA, in contrast to uPA, appears not to play a significant role in tumour invasion, but is mainly involved in intravascular thrombolysis (Schmitt *et al.*, 1992).

A number of studies have shown that both uPA and type IV collagenase are produced by tumour cells as they invade reconstituted basement membrane, and inhibitors of these enzymes block invasion (Mignatti *et al.*, 1986; Reich *et al.*, 1988). There is evidence from these studies that the cleavage of type IV collagen

is the permissive event during *in vitro* invasion because, a) a hydroxamic acid collagenase inhibitor blocks invasion, b) radiolabelled type IV collagen added to reconstituted basement membrane was degraded as cells invaded, c) bacterial collagenase increases invasion of cells, and d) the inhibition of uPA and plasmin could be bypassed using an organomercurial activator of procollagenase. It appears, in this case, that uPA-activated plasmin probably acts as an upstream activator of type IV collagenase, although a contributory effect of plasmin on ECM lysis by virtue of its fibrinolytic and lamininolytic activities, is possible. In contrast, with human melanoma cells that secrete large amounts of uPA and interstitial collagenase, removal of glycoproteins by uPA was found to be a prerequisite and rate-limiting step for the degradation of interstitial collagen (Montgomery *et al.*, 1993). This may reflect the implementation of different strategies for different environments.

An association between levels and activities of the lysosomal cathepsins and invasion has also been observed. A tumour cell has, therefore, at its disposal a whole plethora of hydrolytic enzymes which may act individually, in concert, or as part of a proteolytic cascade (Fig. 1). Based on the heterogenous nature of tissue surfaces and matrices, it is unlikely that any one enzyme is able to remove all ECM barriers. The activity of type IV collagenase would be of major importance for basement membrane disruption, but other enzymes are probably required for further invasion to occur.

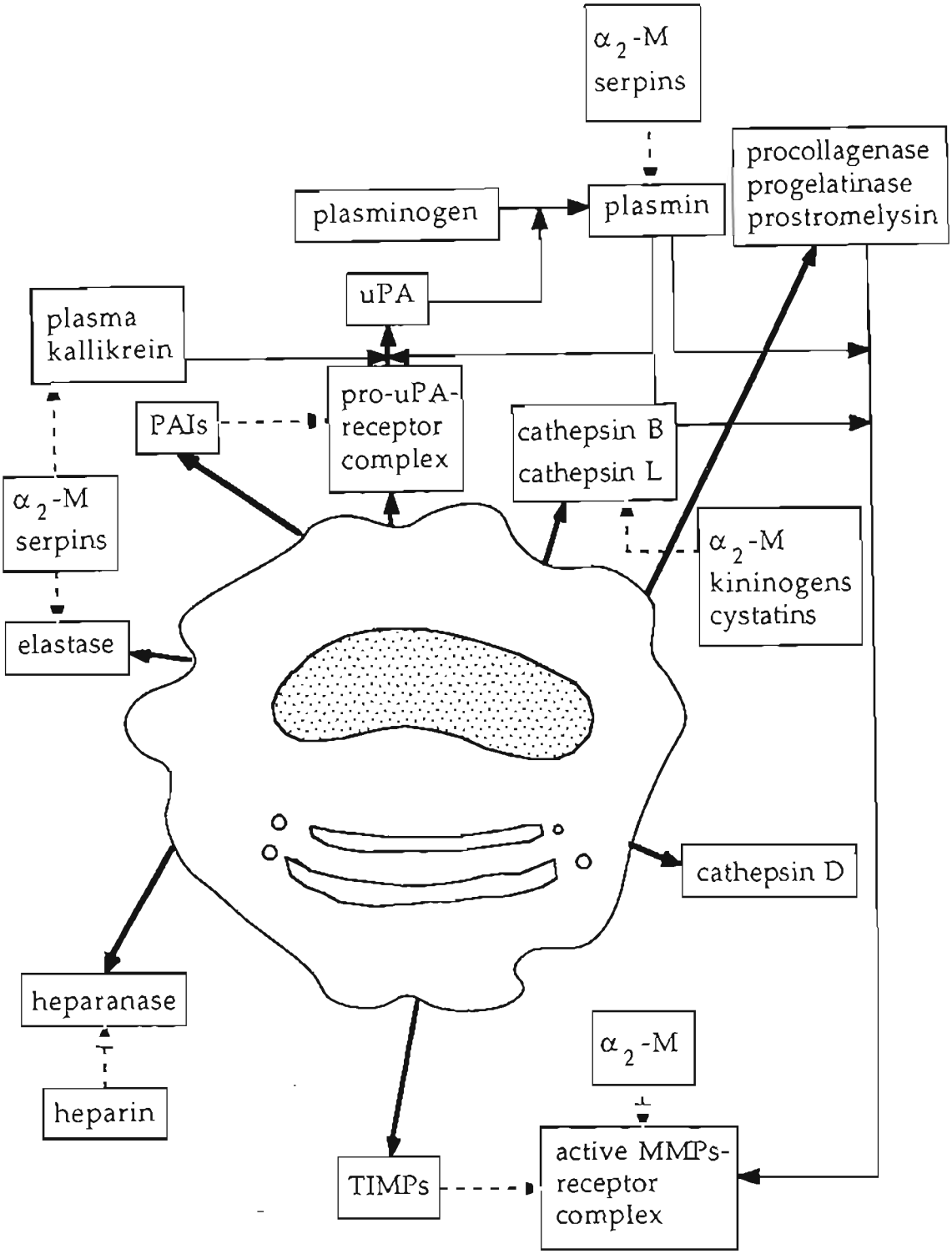

Figure 1 Tumour-associated degradative enzymes and plasma proteinases: their possible roles in metastasis and regulation by tumour or host cell-derived inhibitors.

Bold lines represent the release of enzymes or inhibitors, lines represent activation of proenzymes and broken lines represent possible inhibitory mechanisms (adapted from Nakajima *et al.*, 1987; Alexander and Werb, 1989). α_2M (α_2-macroglobulin); serpin (serine proteinase inhibitor).

1.4 Lysosomal proteinases

A significant body of evidence implicates lysosomal cysteine proteinases in neoplastic progression. In breast carcinoma patients, levels of cathepsins B, L and H are elevated in sera and tissue, when compared with normal sera or tissue (Gabrijelcic *et al.*, 1992). Lah *et al.* (1992a; 1992b) found the activities of cathepsins B and L in breast tumours to be 20- and 50-fold higher, respectively, than in normal matched tissues. Furthermore, cystatins, endogenous protein inhibitors of cysteine proteinases (Barrett *et al.*, 1986), reflected lower activity in two thirds of carcinomas, and higher activity in one third (Lah *et al.*, 1992b). Although cathepsin D levels were also elevated in these studies, no correlation could be found between cathepsin D and cysteine proteinase inhibitor activity, negating the possibility of cathepsin D-mediated inactivation of cystatins (Lenarcic *et al.*, 1988; Lenarcic *et al.*, 1991). In a separate study (Lah *et al.*, 1989), cystatin A from human sarcoma extracts was found to have an impaired ability to inhibit cathepsins B, L and H, thereby providing a mechanism to shift the inhibitor/proteinase balance in favour of proteolysis. In contrast, in human colorectal carcinoma the levels of endogenous cysteine proteinase inhibitors remained constant, while cathepsin B- and L-like activities were elevated (Sheahan *et al.*, 1989).

Of these cysteine proteinases, cathepsin B appears to be most relevant, perhaps due to its ability to digest basement membrane at a pH approaching neutrality (Guinec *et al.*, 1993), as well as its ability to act as an initiator of protease cascades (Fig. 1). Increased cathepsin B activity has been detected in human lung tumours (Krepela *et al.*, 1990) and, significantly, the enzyme was localised to the plasma membrane of a human lung cancer cell line (Erdel *et al.*, 1990). The distribution on the cell surface was not even, but localised to areas of contact with the supporting substrate. This may facilitate directed proteolysis or activation of other plasma membrane-bound proteases such as uPA or type IV collagenase. Surface-bound cathepsin B has also been found in H-*ras*-transformed human breast epithelial cells (Sloane *et al.*, 1994) and murine B16 melanoma variants (Sloane *et al.*, 1986; Rozhin *et al.*, 1987), in the latter characterised by a slightly higher pH optimum and resistance to inactivation by extracellular proteinase

inhibitors. In fact, Sloane *et al.* (1981) have demonstrated a significant correlation with the metastatic potential of *in vivo* grown variants of B16 mouse melanoma and cathepsin B activity.

The possible role of cathepsin B in the degradation of extracellular matrix in tumour invasion is not clear, however. An increased activity in transformed cells may reflect an increase in the general intracellular machinery of the cells, allowing the enzyme to contribute to the process of matrix destruction in lysosomes, after internalisation of fragments generated by hydrolases outside the cell. Furthermore, the bulk of cathepsin B secreted by tumour cells accumulates as latent proenzyme, which would require activation. The only proteinases known to be capable of activating this enzyme, pepsin and cathepsin D, require acidic conditions for activity. However, Keppler *et al.* (1994) have recently described cathepsin B activation by polymorphonuclear leucocyte elastase, and cystatin C (the most potent cathepsin B endogenous inhibitor) inactivation with respect to cathepsin B activity. As tumours are most often infiltrated with polymorphonuclear leucocytes, this would provide a potential source of elastase, allowing activation to occur under physiological conditions. Activated cathepsin B can liberate collagenase, gelatinase and lamininase activities from the central domain of plasma fibronectin (Guinec *et al.*, 1993), thereby initiating another proteolytic cascade.

The lysosomal cysteine proteinase, cathepsin L, has also been ear-marked as a potential candidate for involvement in tumour metastasis. This is mainly due to its superior ability to digest a wide variety of ECM components, including type IV collagen, laminin and fibronectin, when compared to other collagenolytic cysteine cathepsins B, H and S. Furthermore, studies in this laboratory have shown cathepsin L to be active in a medium that mimics extracellular fluid (Dehrmann *et al.*, 1995), although this was only tested against synthetic substrates. However, relative to cathepsin B, less clinical and biochemical data exists demonstrating an involvement of this enzyme in tumour invasion. Nevertheless, cathepsin L has been demonstrated to be cell-surface associated in B16 melanoma cells (Rozhin *et al.*, 1989), and was 7-fold more active in a highly

metastatic compared to a poorly metastatic subpopulation, whereas cysteine proteinase inhibitor activity was 5-fold less. Procathepsin L expression and secretion also correlated with the extent of H-*ras* expression and metastatic potential in H-*ras* transfected mouse NIH 3T3 cells (Denhardt *et al.*, 1987). More direct evidence for a role for cathepsin L in invasion is revealed by the inhibition of invasion of the human amnion by murine mammary and melanoma cells *in vitro* (Yagel *et al.*, 1989a). The cathepsin L-specific peptidyl diazomethyl ketone inhibitor, ZPhePheCHN$_2$ (benzyloxycarbonyl-L-phenylalanyl-L-phenylalanyldiazomethane) was able to inhibit invasion, but not to the extent that inhibitors of MMPs were able, suggesting a role for cathepsin L in MMP activation. Recently, a 41 kDa C3 (third component of complement)-cleaving cysteine proteinase, which is antigenically related to cathepsin L, has been identified as being membrane associated in DM-4 human melanoma cells (Jean *et al.*, 1996). Pretreatment of DM-4 cells with anti-murine procathepsin L antibodies, strongly inhibited their tumourgenicity and significantly decreased their metastatic potential in nude mice. Significantly, these effects are not due to a decrease in the ability of pretreated cells to penetrate the basement membrane, since incubation of DM-4 cells with antibodies did not affect the migration of cells in an *in vitro* invasion assay. It is speculated that inhibition of the cysteine proteinase that cleaves human C3 renders cells more susceptible to complement lysis.

An association between the lysosomal aspartic proteinase, cathepsin D, and cancer has been demonstrated in a number of independent studies. Cathepsin D was found by immunocytochemistry to be widely distributed in a number of carcinomas and lymphomas (Reid *et al.*, 1986), and levels of the enzyme have been found to be elevated in both benign and malignant ductal mastopathies, but are very low in normal or resting mammary glands (Garcia *et al.*, 1986). Furthermore, a number of clinical studies have been undertaken to assess the value of cathepsin D as a prognostic marker in breast cancer (Rochefort, 1990; Roger *et al.*, 1994; Castiglioni *et al.*, 1994) and endometrial adenocarcinoma (Nazeer *et al.*, 1992), but the conclusions remain controversial due to the appearance of various articles offering different opinions. It has been suggested that the establishment of standardised techniques, reagents and diagnostic criteria

would allow for a true assessment of the value of assaying for cathepsin D (Cardiff, 1994).

A variety of reports show the presence in tumours of cathepsin D with unusual biochemical properties. In a comparison between cathepsin D from normal rat liver and that from a transplantable rat sarcoma, it was shown that although the two cathepsins are similar in most respects, their substrate binding sites have different structures (Kazakova *et al.*, 1972). Similarly, cathepsin D isolated from rat Yoshida ascites hepatoma cells showed increased susceptibility to inhibition by pepstatin (a specific fungal inhibitor of aspartic proteases) (Knight and Barrett, 1976), suggesting an alteration in the substrate binding cleft (Bonelli *et al.*, 1988). Anomalous activity was also found in cathepsin D purified from human ascitic fluid. The enzyme showed similar activities against bovine serum albumin (BSA) and haemoglobin (Esumi *et al.*, 1978), whereas normal human liver cathepsin D has an activity against BSA only 5% of that against haemoglobin (Barrett, 1970). Significant elevation of cathepsin D activity against haemoglobin was found in human hepatoma tissues when compared to normal human livers, probably due to increased amounts of enzyme protein, not to the enzyme being more active (Maguchi *et al.*, 1988).

The most compelling evidence favouring the hypothesis that cathepsin D is an important protease facilitating metastasis and invasion, comes from the work of Rochefort and co-workers on human breast cancer. Westley and Rochefort (1980) noted that an oestrogen responsive metastatic human breast cancer cell line (MCF-7), when treated with oestrogen, secreted a 52 kDa glycoprotein into conditioned media. This protein accounts for up to 40% of the secreted proteins of oestrogen-stimulated MCF-7 cells, and was also found to be produced constitutively in large amounts in some hormone-independent breast cancer cell lines, relative to unstimulated oestrogen-responsive cell lines (Garcia *et al.*, 1987). The protein was also found to be inducible in primary cultures derived from metastatic effusions of breast cancer where oestrogen also results in the export of the protein into the medium (Veith *et al.*, 1983). It therefore appears that oestrogen regulation of the 52 kDa protein is not simply an artefact peculiar to

some permanent cell lines, but seems to have some biological relevance to the *in vivo* situation in breast cancer patients.

Monoclonal antibodies against the 52 kDa protein (Garcia *et al.*, 1985) enabled the purification of the protein (Capony *et al.*, 1986), its characterisation and sequencing. A scheme for the glycosylation and post-translational processing was proposed in which a polypeptide of 48 kDa is co-translationally N-glycosylated with two high-mannose oligosaccharide chains to yield a 52 kDa glycoprotein. The protein was found to be phosphorylated mostly on the mannose side chains and mannose-6-phosphate (M-6-P) signals were identified (Capony *et al.*, 1987), suggesting that the protein is normally routed to lysosomes where it exerts its usual metabolic functions (von Figura and Hasilik, 1985). Pulse-chase experiments showed that the 52 kDa protein is a precursor of a lysosomal enzyme where, after oestrogen treatment, about 40% of the cellular pool is secreted and 60% successfully processed into an intermediary 48 kDa and a stable 34 kDa + 14 kDa protein (Morisset *et al.*, 1986a).

Enzymatic activity studies showed that both the purified secreted 52 kDa protein and the corresponding cellular proteins (52 kDa, 48 kDa, 34 kDa + 14 kDa) displayed strong proteolytic activity against haemoglobin at acidic pH, where the 52 kDa proform is autoactivated (Morisset *et al.*, 1986b; Capony *et al.*, 1987). Molecular weights, immunoreactivity, pH and inhibitor sensitivities showed strong homology with cathepsin D.

Cloning and sequencing of cDNA showed complete sequence homology with human kidney procathepsin D (Faust *et al.*, 1985) except for a 5 nucleotide change, involving only one amino acid substitution (A to V) in the profragment (Augereau *et al.*, 1988). The amino acid structure of the 52 kDa cathepsin D of breast cancer thus appears to be almost identical to that of the lysosomal cathepsin D of normal tissues. The structure of the oligosaccharide chains is not yet known, and differences at this level are possible. This is suggested by isoelectric focusing analysis of procathepsin D from normal and mammary cancer cells, the latter containing more acidic forms (Capony *et al.*, 1989).

Major differences in the intracellular processing of the 52 kDa procathepsin D were found in a comparative study between normal human mammary epithelial cells and breast cancer cell lines (Capony *et al.*, 1989). In normal mammary cells, as in human fibroblasts (Hasilik and Neufeld, 1980), negligible amounts of the proform were secreted or accumulated in the cells. The precursor was trafficked to lysosomes and rapidly processed into the mature form (34 kDa+14 kDa) via production of the intermediate form (48 kDa). In several hormone-dependent cell lines (including MCF-7) and hormone-independent cell lines, however, processing was delayed and there was an accumulation of the 52 kDa and 48 kDa forms in cells. Furthermore, secretion of the 52 kDa form was 30 times higher in cancer cells.

Procathepsin D secretion was originally detected due to its oestrogen-specific induction (Morisset *et al.*, 1986a). Oestrogen treatment causes an approximately 10-fold increase in cathepsin D mRNA in MCF-7 cells (Cavaillès *et al.*, 1988). Other mitogens, such as insulin-like growth factor-I, epidermal growth factor (EGF) and insulin are also able to increase cathepsin D mRNA (Cavaillès *et al.*, 1988). Mitogen regulation in breast cancer cells appears to be complex, however, as oestrogen can induce both growth factors and cathepsin D, which in turn can also be induced by these growth factors (Rochefort, 1990).

Evidence, to date, has pointed to an extracellular role for proteolytic enzymes such as uPA and MMPs in invasion. However, this is more debatable for intracellular proteinases, like some cathepsins, which require acidic pH for activation. Briozzo *et al.* (1988) demonstrated pepstatin-sensitive ECM degradation by breast cancer cell-conditioned medium, but only when the medium was acidified to facilitate procathepsin D autoactivation. An extracellular role for cathepsin D was more directly investigated by assessing the inhibition of invasion, by pepstatin, of a series of cathepsin D secreting breast cancer cell lines (Johnson *et al.*, 1993). Neither the addition of pepstatin or chloroquine, which neutralises the pH in acidic compartments of cells, were able to inhibit invasion *in vitro*. Indeed, a novel matrix-degrading,

ethylenediaminetetra-acetic acid (EDTA) and leupeptin inhibitable protease has been identified in the conditioned medium of hormone-dependent breast cancer cell lines (Shi *et al.*, 1993; Dickson *et al.*, 1994), although it remains to be seen to what extent this protease contributes to the metastatic progression of human breast cancer. Extracellular proteolysis by cathepsin D has, in fact, been implicated in proteoglycan metabolism of cartilage matrix (Poole and Mort, 1981), although the nature of this extracellular activity was not resolved.

Matters are far from clear, however, as a direct role in metastasis for cathepsin D has actually been demonstrated. A tumourigenic but low-metastatic transformed rat embryo cell line that does not secrete cathepsin D, was transfected with the enzyme. Overexpression of human cathepsin D in transfected clones increased the malignant phenotype *in vitro*, and the metastatic activity of clones injected into athymic mice was significantly higher than control clones (Garcia *et al.*, 1990). This study was extended to assess the effects on experimental metastasis of retention of the enzyme in the endoplasmic reticulum (Liaudet *et al.*, 1994). The transformed rat cell line was transfected with mutated human procathepsin D that was obtained by an insertion of a KDEL peptide signal responsible for endoplasmic reticulum retention. In contrast to wild-type human procathepsin D transfected clones, procathepsin D-KDEL clones had no ability to metastasise in athymic mice. Furthermore, high producer KDEL clones, where KDEL receptors were saturated, resulting in the secretion of mutated procathepsin D, showed no increase in metastatic activity. This indicates that procathepsin D secretion alone is not sufficient to manifest the invasive phenotype, but maturation of the enzyme into proteolytically active forms is a prerequisite.

Secreted procathepsin D could still, however, play a role in tumour progression by virtue of its proposed mitogenic activity. Vignon *et al.* (1986) first demonstrated autocrinal growth stimulation of MCF-7 breast cancer cells by procathepsin D, and this has been further shown in clones of transformed rat cell lines hyper-expressing procathepsin D (Garcia *et al.*, 1990; Liaudet *et al.*, 1994) and a variety of breast cancer cell lines (Fusek and Vetvicka, 1994). Mitogenic activity cannot be blocked by inhibition of proteolytic activity nor by the inhibition of the

interaction of procathepsin D with M-6-P receptors. The addition of antibodies raised against the propeptide sequence impaired the mitogenic activity of procathepsin D, and a synthetic peptide corresponding to the propeptide of procathepsin D produced a mitogenic effect (Fusek and Vetvicka, 1994). Conflicting results have been reported where no mitogenic effect by procathepsin D was found (Stewart *et al.*, 1994), although no adequate explanation is given for this discrepancy. It remains to be seen if this mitogenic activity has any role *in vivo* in breast cancer, though. Interestingly, a single 20 µg intraperitoneal injection of cathepsin D induced significant stimulation of DNA synthesis and mitosis in the liver but not kidney (Morioka and Terayama, 1984), while the propart peptide was also able to effectively inhibit the enzyme (Fusek *et al.*, 1991; Puizdar and Turk, 1981).

Taken together, the lack of inhibition of invasion of cathepsin D secreting breast cancer cells by pepstatin, a requirement for intracellular maturation of cathepsin D for experimental metastasis to occur, and lack of direct evidence for low pH microenvironments at the cell-substrate interface of breast cancer cells (Montcourrier *et al.*, 1990), mitigates for an intracellular role for the enzyme in tumour progression. This is supported by evidence of large acidic vesicles in breast cancer cells that contain endocytosed extracellular matrix, high cathepsin D concentrations, resemble phagolysosomes and are correlated with an increased ability to invade through reconstituted basement membrane (Montcourrier *et al.*, 1990; Montcourrier *et al.*, 1994). Perturbation of cathepsin D maturation into an active enzyme could, therefore, result in dysfunctional digestive vesicles which become 'constipated' with extracellular matrix, thereby compromising invasion.

Thus it appears that the balance between proteases and their inhibitors, rather than over expression of proteases, is the key determinant in tumour progression. The activity of protease inhibitors is not restricted to inhibition of invasion and metastasis, but preserves the delicate equilibrium between cells and the extracellular matrix, matrix components and matrix-associated growth factors, all of which can have a profound effect on cell behaviour.

In this thesis a study is detailed of some of the proteinases thought to be involved in tumour progression, and some of the characteristics of tumour cells with respect to normal cells. At the outset of this work cathepsin D was viewed as the primary malevolent proteinase in breast cancer metastasis and, as such, attempts were made to develop an anti-peptide antibody to neutralise its activity. Later it became clear that cathepsin D is only part of a complex interaction, and so the study was broadened to encompass other proteinases. This included a study of the distribution of lysosomal proteinases in transformed and normal cells, and initial characterisation of the extracellular degradative processes displayed by these cell lines.

CHAPTER 2

MATERIALS AND METHODS

This chapter will detail methods that are considered in biochemical terms as being fundamental and are used throughout this study. This chapter also contains methods, especially immunological methods, which could be considered relatively specialised but would hinder the intended structure of the relevant chapter. 'Reagents' sections in this chapter only describe those reagents that were prepared before use, reagents that were used as-is are referred to at the relevant point in the 'Procedure' section.

2.1 Materials

The source of all specialised products used in this study is detailed here. Most common chemicals used were from Boehringer Mannheim, BDH or Merck, and were of the highest purity available. Denatured bovine haemoglobin was from Nutritional Biochemicals; Serva Blue G was from Serva; ovalbumin (Grade V), 4-Chloro-1-napthol, pepstatin-diaminohexane-Sepharose, polyacrylhydrazido-agarose, Sephadex G-25 and standard electrophoretic proteins were from Sigma Chemical Co., St. Louis, Mo. Coomassie Brilliant Blue R-250 was from Merck; acrylamide and N,N'-methylenebisacrylamide were from BDH; N,N,N',N'-tetramethyl-ethylenediamine (TEMED) was from Bio-Rad; BSA (Fraction V) and 2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid (ABTS) were from Boehringer Mannheim, SA; Elite fat-free milk powder was from NCD; horseradish peroxidase (HRPO) was from Seravac and enzyme-linked immunosorbent assay (ELISA) plates were Nunc-Immuno Maxisorp F96 plates. Sheep anti-rabbit IgG and rabbit anti-chicken IgY secondary antibodies were prepared by Dr Theresa Coetzer, Department of Biochemistry, University of Natal.

2.2 Protein assays

Protein purification procedures require methods of protein quantification which are rapid, specific and yet sensitive. Spectrophotometry using specific protein

extinction coefficients at 280 nm fulfil these criteria but lack general applicability, whereas assays based on features common to most proteins are often subject to interference, response variation or insensitivity. An assay that to a large extent overcomes such problems, is the Bradford dye-binding assay (Bradford, 1976).

2.2.1 Bradford dye-binding assay

The Bradford assay is based on the observation that Coomassie Brilliant Blue G-250 dye exists in two colour forms, red and blue, the red form predominates at low pH but converts to the blue form upon binding of dye to protein. The high extinction coefficient of the blue protein-dye complex at 595 nm results in great sensitivity in protein measurement. Due to its rapidity, the assay is suitable for processing large sample numbers. Colour development is essentially complete within 2 min of the addition of the dye reagent to the protein sample and remains stable for up to 1 h. The important advantage of the assay is the lack of interference by most chemicals tested. Small effects due to Tris, acetic acid, 2-mercaptoethanol, sucrose, glycerol, EDTA, and trace amounts of Triton X-100 and sodium dodecyl sulfate can be eliminated by using the appropriate buffer blank. Detergents at concentrations of 1% or greater, however, produce abnormalities too great to overcome.

The greatest disadvantage of the dye-binding assay is the wide variation of colour-yield in response to different standard proteins (Read and Northcote, 1981). This would affect the validity of measurements extrapolated from the standard protein. When determining the concentration of purified proteins, this problem is best overcome by using the pure protein as its own standard, although this is seldom feasible due to the wastefulness of such an approach. To reduce response variation, Read and Northcote (1981) modified the method by substituting Coomassie Brilliant Blue G-250 with Serva Blue G, and by increasing the dye concentration or decreasing the phosphoric acid concentration. In this study it was found, however, that modification of dye and phosphoric acid concentrations caused the dye to precipitate. The reagent finally employed in this study was the same as that of Bradford (1976), except that Coomassie Brilliant Blue G-250 was replaced by Serva Blue G. The extent of variation between

ovalbumin, the chosen standard protein, and cathepsin D, a major protein of interest in this study, was tested by comparing the concentration of a suitable sample of purified cathepsin D as derived from the Bradford assay with that derived from its extinction coefficient at 280 nm. The relative concentration difference was found to be 6.6%, which was deemed within acceptable limits.

The only disadvantage of the reagent employed compared to that of Read and Northcote (1981), is evidence of non-linearity at protein levels beyond 25 µg, presumably due to dye depletion. This can easily be overcome by diluting protein samples to within the 5-25 µg working range of the assay. For the determination of lower protein concentrations (1-5 µg), a micro-assay procedure (Read and Northcote, 1981) was used.

2.2.1.1 Reagents

Dye reagent. Serva Blue G (50 mg) was dissolved with stirring in 89% phosphoric acid (50 ml). Absolute ethanol (23.5 ml) was added and stirred for 1 h. The solution was made up to 500 ml with distilled water (dist.H_2O), stirred for a further 30 min and filtered through Whatman No. 1 filter paper. The dye reagent can be stored in a brown bottle at room temperature for several months.

Standard protein solution. Ovalbumin was dissolved at 1 mg/ml in dist.H_2O. This was diluted to 100 µg/ml for the micro-assay.

2.2.1.2 Procedure

Macro-assay. Standard protein solution (0-25 µl), or sample protein, was diluted with dist.H_2O or buffer to a final volume of 100 µl to give the desired concentration levels (0-25 µg). Dye reagent (5 ml) was added to standard solutions, samples and blanks (100 µl), vortexed and allowed to stand for 2 min. The absorbance was read at 595 nm against the buffer blank, for sample, or water for the ovalbumin standard solutions. Assays for a standard curve were carried out in triplicate at five concentrations of ovalbumin. Plastic cuvettes (3 ml) were used as the dye-reagent binds to quartz (Bradford, 1976) although it can be easily removed with dilute sodium hypochlorite.

Micro-assay. Standard protein solution (0-50 µl of the 100 µg/ml solution, i.e. 1-5) or sample was diluted with dist.H_2O or buffer to a final volume of 50 µl. Dye reagent (950 µl) was added to standard solutions, samples and blanks, vortexed and allowed to stand for 2 min. The absorbance was read at 595 nm against the buffer blank, for sample, or water for the ovalbumin standard solutions, in 1 ml plastic micro-cuvettes as above. A standard curve was generated for each batch of reagent made up, and subjected to linear regression analysis from which protein concentrations were calculated.

2.2.2 Spectrophotometry using protein extinction coefficients

Spectrophotometry using extinction coefficients represent a highly specific method of protein concentration determination but is, by definition, inherently impractical for general protein quantification. The extinction coefficient of 1.05 ml/mg/cm at 280 nm for bovine spleen cathepsin D (Lah *et al.*, 1984) was used, where possible, for pure preparations of this enzyme. Determination of the concentrations of pure immunoglobulin G and Y (IgG and IgY) were also facilitated by using extinction coefficients at 280 nm of 1.43 and 1.25 ml/mg/cm respectively (Hudson and Hay, 1980; Coetzer, 1985).

2.2.2.1 Procedure

The absorbance of the relevant sample at 280 nm was read in a quartz cuvette against the appropriate blank and the concentration calculated as follows:

$$A = \varepsilon \times c$$

where A = absorbance at 280 nm
$\varepsilon^{0.1\%}$ = absorbance of a 0.1% solution (i.e. 1 mg/ml) solution in a 1 cm cuvette pathlength
c = protein concentration in mg/ml.

Cathepsin D solutions were generally not diluted prior to reading, while IgG and IgY were diluted 1/40 (Section 2.5).

2.3 Methods of protein concentration

During some procedures it was necessary to concentrate proteins for protein assays, or for further analysis such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.1 Concentrative dialysis

Where concentration of large volumes of dilute protein solutions was required, dialysis against a substance, which has high osmotic pressure when in solution, such as sucrose or polyethylene glycol (PEG) was utilised. Sample concentration is based on the fact that a gradient of water concentration is established between the protein solution in the dialysis membrane and the substance at the exterior surface of the membrane. Water and buffer ions will thus move out of the membrane, slowly saturating the material, while proteins larger than the membrane molecular weight cut-off limit, are retained. The overall volume in which the protein is dissolved is thus reduced, and the process can be halted when the required degree of concentration has been reached.

Sucrose, which is inexpensive, was most often used but suffers from the disadvantage that it is small enough to diffuse into the dialysis tubing. When concentrated sucrose-free protein solutions were required, as is the case of protein assays where a corresponding reagent blank is required, dialysis against PEG (20 kDa) was employed. This substance has a molecular weight greater than the size cut-off of the dialysis membrane and will thus not move into the bag.

2.3.2 Sodium dodecyl sulfate/potassium chloride precipitation

The most often used method of qualitative analysis of proteins was SDS-PAGE and precipitation with sodium dodecyl sulfate (SDS) and KCl provided a quick and simple method to concentrate proteins prior to electrophoretic analysis. The potassium salt of dodecyl sulfate is insoluble, and hence when dodecyl sulfate is bound to a protein molecule, the protein will precipitate with the anion upon addition of KCl.

2.3.2.1 Reagents

5% (m/v) Sodium dodecyl sulfate. Sodium dodecyl sulfate (0.5 g) was dissolved in dist.H_2O by gentle warming, cooled, made up to 10 ml.

3 M Potassium chloride. Potassium chloride (2.24 g) was dissolved in dist.H_2O and made up to 10 ml.

2.3.2.2 Procedure

5% SDS (10 µl) was added to the sample (100 µl) in a polyethylene microfuge tube. The tube was inverted several times, 3 M KCl (10 µl) was added and the tube again inverted. The white SDS-protein precipitate was sedimented by centrifugation (12 000 x g, 2 min, RT), the supernatant fluid discarded and the pellet redissolved in stacking gel buffer (10 µl) and reducing SDS-PAGE treatment buffer (Section 2.4.1). The procedure afforded an approximately 5-fold concentration of protein.

2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Analysis of the composition of protein mixtures is most conveniently performed by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS. This system is designed, in the presence of a disulfide bond reducing agent, to dissociate all proteins into their individual polypeptide subunits. Most proteins interact with and are denatured by SDS in a similar manner, each gram of polypeptide binding a constant 1.4 g of SDS (Reynolds and Tanford, 1970). The SDS-protein complexes assume a rod-shaped random coil configuration and behave as though they have uniform shape. The intrinsic charges of the polypeptide are masked by the negative charge provided by the bound ionic detergent, so that the SDS-polypeptide complexes have essentially identical charge densities and migrate in SDS-PAGE gels, which have molecular sieving properties, according to polypeptide size (Weber and Osborn, 1969). The molecular weight of the sample polypeptides can then be determined by reference to the mobility of polypeptides of known molecular weight separated under the same electrophoretic conditions.

The most commonly-used SDS-PAGE system makes use of the discontinuous PAGE buffer system of Ornstein (1964) and Davis (1964) with the modification of Laemmli (1970) which introduces SDS. Compared to continuous buffer systems, discontinuous systems employ buffer ions of different composition and pH in the gel relative to those in the electrode reservoirs. Furthermore, sample is loaded onto a large pore stacking gel through which it migrates to enter the small pore running gel. The major advantage of such a system over a continuous system, is that relatively large volumes of dilute protein can be applied to the gel without compromising resolution. During migration through the large-pore staking gel, proteins are concentrated into extremely narrow stacks, after which they enter the running gel where band resolution can occur.

2.4.1 Reagents

(A): Monomer solution (30% T; 2.7% C). Acrylamide (58.4 g) and N,N'-methylene-bisacrylamide (1.6 g) were dissolved in dist.H_2O and made up to 200 ml.

(B): 4 x Running gel buffer (1.5 M Tris-HCl, pH 8.8). Tris base (36.3 g) was dissolved in about 150 ml dist.H_2O, adjusted to pH 8.8 with NaOH and made up to 200 ml.

(C): 4 x Stacking gel buffer (500 mM Tris-HCl, pH 6.8). Tris base (3.0 g) was dissolved in about 30 ml dist.H_2O, adjusted to pH 6.8 with NaOH and made up to 50 ml.

(D): 10% (m/v) Sodium dodecyl sulfate. Sodium dodecyl sulfate (50 g) was dissolved in dist.H_2O with gentle warming and made up to 500 ml.

(E): 10% (m/v) Ammonium persulfate. Ammonium persulfate (0.5 g) was dissolved in dist.H_2O and made up to 5 ml. Fresh reagent was made up every two weeks.

(F): Reservoir tank buffer (25 mM Tris base, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3). Tris base (12.0 g), glycine (57.6 g) and 10% SDS (D) (400 ml) were dissolved in dist.H_2O and made up to 4 litres. The pH is automatically 8.3.

Treatment buffer (125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol). Stacking gel buffer (C) (2.5 ml), 10% SDS (D) (4.0 ml), glycerol (2 ml) and 2-mercaptoethanol (1.0 ml) were made up to 10.0 ml with dist.H_2O.

Stain stock (1% (m/v) Coomassie Brilliant Blue R-250). Coomassie Brilliant Blue R-250 (2.0 g) was dissolved in about 150 ml of dist.H_2O with overnight stirring. The solution was made up to 200 ml and filtered through Whatman No. 1 filter paper.

Stain (0.125% (m/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid). Coomassie Brilliant Blue R-250 stain stock (62.5 ml), CP methanol (250 ml) and CP acetic acid (50 ml) were made up to 500 ml with dist.H_2O.

Destaining solution I (50% (v/v) methanol, 10% (v/v) acetic acid). CP Methanol (500 ml) and CP acetic acid (100 ml) were made up to 1 litre with dist.H_2O.

Destaining solution II (5% (v/v) methanol, 7% (v/v) acetic acid). CP Methanol (50 ml) and CP acetic acid (70 ml) were made up to 1 litre with dist.H_2O.

Molecular weight markers (1 mg standard protein/ml). Bovine serum albumin (1 mg), ovalbumin (1 mg), carbonic anhydrase (1 mg) and lysozyme (1 mg) were dissolved in treatment buffer (1.0 ml), placed in a boiling waterbath for 90 s and bromophenol blue tracking dye (0.1% (m/v) in stacking gel buffer C) was added (15 µl).

Table 2. Preparation of running (12.5%) and stacking gels (4.0%) for two 1.5 mm thick polyacrylamide gels.

Reagent	Running gel (ml)	Stacking gel (ml)
A	6.25	0.94
B	3.75	
C		1.75
D	0.15	0.07
E	0.75	0.35
dist.H_2O	3.50	4.20
TEMED	0.075	0.015

2.4.2 Procedure

Protein samples to be electrophoretically analysed were combined 1:1 with treatment buffer, boiled (90 s) and 0.1% (m/v) bromophenol blue tracking dye added to 0.005% (v/v). Typically, pure enzyme preparations were loaded at about 10 µg per well (at least 2 µg of protein per band), crude protein fractions at 50-200 µg per well and molecular weight markers at 4 µl per well.

The running gel pore size and hence the molecular weight resolving range can easily be manipulated by changing the concentration of monomer solution in the gel preparation. For the purposes of this study it was found that a running gel concentration of 12.5% acrylamide was optimal both in terms of molecular weight range (roughly from 12 kDa to 75 kDa) and band resolution.

For all polyacrylamide gel electrophoretic procedures a Hoefer Scientific Instruments SE 250 ('Mighty Small') vertical slab gel unit was employed. The vertical slab gel unit was assembled essentially according to the Hoefer instruction manual. Aluminium plates were separated from glass plates by two vertical 1.5 mm spacers and clamped against both sides of the electrophoresis pod. Two lines of molten agarose (1% (m/v) in dist.H_2O) were poured on a glass base a distance from each other corresponding to the distance between the two gel chambers. The unit was lowered into the agarose, the agarose being drawn up into the gel chamber sandwich to polymerise into a plug gel. The prepared

running gel was gently expelled from a syringe, with a needle attached, into the top of the sandwich to a distance about 3 cm from the top of the glass plates, care being taken not to allow air bubbles to be trapped in the viscous running gel. The running gel was carefully overlayered with dist.H_2O to exclude atmospheric oxygen from the gel as it prevents polymerisation. Completion of running gel polymerisation is indicated by the formation of a visible gel-water interface. The water was poured off and prepared stacking gel was added into the sandwich to the top of the aluminium plates. Combs, either 15 well or 10 well, were inserted forming an oxygen-proof barrier with the atmosphere, allowing the stacking gel to polymerise. Running gel should be allowed to polymerise for a total of at least 2 h, while 30 min is sufficient for the stacking gel.

Upon completion of polymerisation, the combs were carefully removed, the wells rinsed with dist.H_2O and cold reservoir buffer (**F**) poured into the wells and cathodic and anodic compartments. Treated samples were carefully underlayered into wells with a fine-tipped Hamilton syringe and the electrophoresis pod connected to a circulating water-bath set to 10°C. The safety lid was placed on the unit, attached to a power supply and electrophoresis was run at a constant current of 18 mA per gel. When the bromophenol blue tracking dye had reached the bottom of the gel sandwich, the power supply was turned off, the pod disassembled and the gels carefully removed and placed into stain for 4 h. The gels were then placed into destain solution **I** overnight and destain solution **II** thereafter until the background staining had faded to acceptable levels. Gels were photographed and stored in sealed plastic bags at room temperature.

2.5 Fractionation of IgG and IgY

A simple and convenient method of purification of IgG and IgY was found in the protein precipitating properties of PEG, a water-soluble linear polymer. Polson *et al.* (1964) found that relatively low concentrations of high molecular weight polymers were able to precipitate proteins, but high concentrations of low molecular weight species are required to effect the same degree of precipitation. The conclusion drawn was that precipitation by PEG was not due to dehydrating effects of the polymer on the protein molecules as is the case with ammonium

sulfate precipitation. It was apparent, however, that the concentration of the polymer required to precipitate a protein is a function of the net charge on the protein as determined by the pH of the medium in which it is dissolved.

The protocol used to purify IgG from rabbit serum was that of Polson *et al.* (1964) while the method used for IgY purification from chicken egg yolks was as a result of more recent developments in the investigation of antibodies from chicken eggs (Polson *et al.*, 1985; Rowland *et al.*, 1986).

2.5.1 Reagents

10 mM sodium borate buffer, pH 8.6. Boric acid (2.16 g), NaOH (0.2g), 37% (v/v) HCl (0.62 ml) and NaCl (2.19 g) were added to dist.H_2O and made up to 1 litre. The pH should automatically be 8.6.

100 mM phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. NaH_2PO_4 (13.8 g) and NaN_3 (0.2 g) were dissolved in about 800 ml of dist.H_2O, titrated to pH 7.6 with NaOH and made up to 1 litre.

2.5.2 Isolation of IgG from rabbit serum

Rabbits were bled from the marginal ear vein and the blood allowed to clot overnight at 4°C. Supernatant serum was carefully drawn off the clot, and remaining serum recovered by centrifugation (3 000 x g, 10 min, RT) of the clot. The serum was preserved with NaN_3, added to 0.02% (m/v). Rabbit serum (1 volume) was diluted with borate buffer (2 volumes). Polyethylene glycol (15% (m/v) 6 kDa) was dissolved in the protein solution with stirring and the resulting IgG precipitate sedimented (12 000 x g, 10 min, RT). The pellet was redissolved in phosphate buffer (3 volumes) and the precipitation procedure repeated to remove remaining contaminants. The final pellet was redissolved in half the initial serum volume with phosphate buffer. In determination of IgG concentration, a 1/40 dilution of IgG in phosphate buffer was made and the absorbance read at 280 nm in a quartz cuvette against a buffer blank. To calculate the protein concentration an extinction coefficient of 1.43 ml/mg/cm (Section 2.2.2) was used.

2.5.3 Isolation of IgY from chicken egg yolk

Individual yolks were freed of adhering albumin (egg white) by careful washing in a stream of water. The yolk sac was punctured, the yolk volume measured and phosphate buffer, equivalent to 2 volumes of yolk, was added and thoroughly mixed. Solid PEG (6 kDa) was added to a final concentration of 3.5% (m/v of diluted yolk). The PEG was dissolved with stirring and the mixture was centrifuged (4 420 x g, 30 min, RT) to separate three phases, a casein-like vitellin fraction, then a clear fluid, and a lipid layer on the surface. The supernatant fluid contaminated with some of the lipid layer was filtered through a loose plug of cottonwool in the neck of a funnel. The volume of clear filtrate was measured and the PEG concentration increased to 12% (m/v). The precipitated IgY fraction was centrifuged (12 000 x g, 10 min, RT), the pellet redissolved in phosphate buffer to the volume after filtration and the IgY again precipitated with 12% (m/v) PEG and centrifuged. The final IgY pellet was dissolved overnight in a volume of phosphate buffer equal to one sixth of the original yolk volume. Immunoglobulin Y concentration was determined as in Section 2.5.2 using an extinction coefficient of 1.25 ml/mg/cm (Section 2.2.2).

2.6 Enzyme-linked immunosorbent assay

Immunoassays use the specific interaction of an antibody with antigen to provide information about antibody (or antigen) concentration in unknown samples. In principle, the labelling by chemical conjugation of an enzyme to either antibody or antigen allows the detection of immune complexes formed on a solid phase. This forms the basis of the ELISA, first introduced by Engvall and Perlmann (1971). One of the simplest and most commonly used ELISAs for the detection of antibodies, is a three layer system. Briefly, antigen is coated to the plastic surface of the wells of polystyrene microtitre plates, and the primary antibodies to be quantified allowed to form a complex with the immobilised antigen. After excess antibody has been washed away, the degree or amount of reactivity is quantified with an appropriate detection system. In an ELISA this would take the form of an enzyme conjugated to a secondary antibody which will recognise the primary antibody bound to the immobilised antigen. The enzyme reacts with a substrate

which yields a coloured product which can be measured spectrophotometrically. This quantitative system complements western blotting (Section 2.7), which gives qualitative information about antibody specificity.

During the course of this work, an ELISA was most commonly used as a means to monitor the progress of immunisation of rabbits and chickens, and in enzyme cross-species reactivity studies. It also aided in the determination of suitable antibody dilutions for use in western blot analysis (Section 2.7).

2.6.1 Reagents

Phosphate buffered saline (PBS). NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in about 800 ml of dist.H_2O, adjusted to pH 7.4 with HCl and made up to 1 litre.

0.5% (m/v) Bovine serum albumin in PBS (BSA-PBS). Bovine serum albumin (0.5 g) was dissolved in PBS and made up to 100 ml.

0.1% (v/v) Tween 20 in PBS (PBS-Tween). Tween 20 (1 ml) was diluted to 1 litre in PBS.

Substrate buffer (150 mM citrate-phosphate, pH 5.0). Na_2HPO_4 (2.84 g) and citric acid (2.29 g) were each dissolved in dist.H_2O and made up to 100 ml. The citric acid solution was titrated against the Na_2HPO_4 (50 ml) solution to pH 5.0.

Substrate solution (0.05% (m/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer). ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml), for one ELISA plate.

0.1% (m/v) Sodium azide in 150 mM citrate-phosphate buffer, pH 5.0. For each ELISA plate NaN_3 (15 mg) was dissolved in citrate-phosphate buffer (15 ml).

2.6.2 Procedure

Wells of microtitre plates (Nunc Immunoplate) were coated with antigen (150 µl) at predetermined dilutions (2 µg/ml for cathepsin D and 1.0 µg/ml for peptide (Chapter 3) as determined by a checkerboard ELISA) in PBS overnight at room temperature. Wells were blocked with BSA-PBS (200 µl) for 1 h at 37°C and washed 3 times with PBS-Tween. Dilutions of the primary rabbit antiserum, IgG or IgY solution in BSA-PBS were added (100 µl), incubated for 1 h at 37°C and excess antibody washed out 3 times with PBS-Tween. A suitable dilution of sheep anti-rabbit IgG-HRPO conjugate or rabbit anti-chicken IgY-HRPO conjugate in BSA-PBS, was added (120 µl) and incubated (30 min) at 37°C. The ABTS substrate (150 µl) was added and incubated in the dark for optimal colour development (usually 10-20 min). The enzyme reaction was stopped by the addition of 50 µl of 0.1% (m/v) NaN_3 in citrate-phosphate buffer and absorbances read at 405 nm in a Bio-Tek EL307 ELISA plate reader. Titration curves were constructed from the spectrophotometric values.

2.7 Western blotting

Polyacrylamide gel electrophoresis is a powerful tool for the analysis of complex protein mixtures. The value of this method is, however, restricted in that resolved proteins remain buried within the gel matrix and are not readily available for further investigation. A protein blotting technique devised by Towbin *et al.* (1979) can alleviate this problem.

The technique involves the electrophoretic transfer of proteins from a polyacrylamide gel to an adjacent nitrocellulose filter. This is achieved by placing the polyacrylamide gel-nitrocellulose stack between two electrodes, the gel closest to the cathode and the nitrocellulose closest to the anode. The electrodes and gel-nitrocellulose stack are submersed in a blotting buffer of relatively high pH and also containing SDS, the principles of transfer thus being essentially the same as those in SDS-PAGE. While the presence of SDS in the blotting buffer does give the proteins a high mobility, resulting in very efficient transfer out of the gel, some proteins do not adhere well to nitrocellulose membranes. This is probably due to the detergent action of SDS which disrupts hydrophobic interactions

between protein and nitrocellulose. The absence of SDS, on the other hand, while maximising protein-nitrocellulose binding, compromises transfer. Methanol is often included in the blotting buffer as it improves adsorption of proteins onto nitrocellulose by virtue of its fixing properties.

The accessibility of a resolved protein mixture on the surface of a nitrocellulose membrane allows the use of a variety of detection methods, otherwise limited by the gel matrix structure. In this study it allowed for the targeting of antigens, both pure and in crude fractions, via an antibody specific for the antigen in question. The bound antibody is then recognised by a secondary antibody, conjugated to an enzyme whose coloured reaction product precipitates on the nitrocellulose and can easily be visualised against the white background of the membrane.

Such an immunoblot system is critical for characterising an antibody's specificity for purified antigen when tested alongside a crude preparation, and also for its ability to detect the antigen in the crude sample itself.

2.7.1 Reagents

Blotting buffer. Tris base (27.23 g) and glycine (64.8 g) were dissolved in about 3 litres of dist.H_2O. To this was added 10% (m/v) SDS (4.5 ml) and CP methanol (900 ml) and the volume made up to 4.5 litres with dist.H_2O. The pH is automatically 8.3.

Tris buffered saline (TBS; 20 mM Tris-HCl, 200 mM NaCl, pH 7.4). Tris base (2.42 g) and NaCl (11.68 g) were dissolved in about 900 ml of dist.H_2O, titrated to pH 7.4 with HCl and made up to 1 litre.

Blocking agent (5% (m/v) fat-free milk powder in TBS). Fat-free milk powder (5 g) was dissolved in about 90 ml of TBS and made up to 100 ml.

0.5% (m/v) Bovine serum albumin in TBS (BSA-TBS). Bovine serum albumin (0.4 g) was dissolved in about 50 ml of TBS and made up to 80 ml.

0.1% (v/v) Tween 20 in TBS (TBS-Tween). Tween 20 (0.5 ml) was diluted to 500 ml in TBS.

4-Chloro-1-napthol substrate solution (0.06% (m/v) 4-chloro-1-napthol, 0.0015% (v/v) H_2O_2). 4-chloro-1-napthol (0.03 g) was dissolved in methanol (10 ml). 2 ml of this solution was diluted to 10 ml with TBS, with the addition of 35% peroxide (4 µl).

0.1% (m/v) Sodium azide in TBS. NaN_3 (0.03 g) was dissolved in TBS (30 ml).

2.7.2 Procedure

After the electrophoretic separation of proteins in the presence of SDS (Section 2.4), the polyacrylamide gel was removed, placed on 3 layers of filter paper and the pre-cut nitrocellulose membrane (Schleicher and Schuell, BA 85, 0.45 µm) laid squarely on the gel, care being taken to dislodge all air bubbles. Three further layers of wetted filter paper were placed over the nitrocellulose and placed in the blotting apparatus sandwich. The complete sandwich was transferred to the TE Transphor electroblotting unit (Hoefer Scientific Instruments), placed vertically between two electrodes and submerged in blotting buffer, ensuring that the nitrocellulose is on the anodal side. Electroblotting was accomplished after 1.5 h at 200 mA constant current, the apparatus being cooled to 4°C by a circulating water-bath.

Upon completion of blotting the apparatus was disassembled, the nitrocellulose marked with the positions of relevant wells and hung up to air-dry (1.5-2 h). The membrane was cut with scissors into pieces corresponding to the sample wells, each piece being placed into a separate container and soaked with gentle rocking (1 h) in blocking agent to saturate additional protein binding sites. The membranes were washed with TBS (3 x 5 min) and antibody diluted in BSA-TBS added. Controls consisted of substituting a non-immune preparation for an immune antibody for each different antibody, antibody concentration or blotted protein sample tested. After incubation in the primary antibody (2 h or

overnight), the nitrocellulose was washed with TBS-Tween (3 x 5 min). The presence of detergent decreases non-specific binding of antibody molecules to the nitrocellulose surface by acting as a further blocking agent, or by decreasing non-specific hydrophobic binding of the antibody. The nitrocellulose was then incubated (1 h) in the appropriate secondary antibody-HRPO conjugate (Section 2.6.2) diluted in BSA-TBS, again washed in TBS-Tween (3 x 5 min) and incubated in the dark with freshly prepared substrate solution. Incubation in substrate was allowed to proceed until an optimal colour differential between specifically targeted bands, and non-immune incubations was found. The reaction was stopped by briefly rinsing the membranes in 0.1% (m/v) NaN_3 in TBS and then allowing these to dry on a piece of filter paper. Blots were photographed, and stored in the dark to prevent yellowing.

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF ANTI-PEPTIDE ANTIBODIES AGAINST CATHEPSIN D

3.1 Introduction

Modulation of enzyme activity is normally achieved with enzyme inhibitors, but the binding of antibodies to an enzyme can also influence enzyme activity. The use of specific anti-catalytic antibodies may have important implications in the study of extracellular proteolysis by live cells, as it permits systematic dissection of the reactions utilised by living cells, in particular invading tumour cells, in the degradation of extracellular matrices. Furthermore, a number of novel therapeutic roles can be envisaged for inhibitory molecules. Blockage of proteolytic activity in tumours could stimulate the encapsulation of invasive and unresectable tumours, allowing later resection that would preserve organ structures in the vicinity of the tumour. Inhibitory antibodies could also be tested for their anti-angiogenic activity, and utilised to prevent the growth of micrometastases that require an angiogenic response for macroscopic development.

Although synthetic and naturally occurring inhibitors have been used in such studies, conclusions are not clear. In some studies anti-tumour effects were clearly demonstrated, but in most other studies, limited or no effects were observed. In some instances opposite, or tumour-enhancing, effects of proteinase inhibitors were noted (Nelles and Schnebli, 1982). Furthermore, therapy may require prolonged administration of inhibitors which may lead to unacceptable toxicity (DeClerck and Imren, 1994). Inhibitors to serine proteinases may be problematic if administered systemically, because of their role in blood coagulation. Matrix metalloproteinases are involved in many physiological functions, such as tissue repair and reproduction, and inhibition of these proteinases could have serious consequences. A systemic immune response, however, generating antibodies capable of specific enzyme inhibition, may find application as an adjuvant tumour therapy. Indeed, a number of *in vitro*

experiments have shown significant inhibition of invasion of tumour cells by anti-catalytic antibodies (Ossowski and Reich, 1983; Höyhtyä *et al.*, 1990).

Immunoinhibition of catalysis of a number of enzymes has been studied, including lysozyme (Arnon, 1968), papain (Arnon and Shapira, 1967), a tyrosine-specific protein kinase (Sen *et* al., 1983), type IV collagenase (Höyhtyä *et al.*, 1990), cathepsin L (Coetzer *et al.*, 1992) and cathepsin D (Dingle *et al.*, 1971; Weston and Poole, 1973). Inhibitory antibodies to cathepsin D have been used to elucidate the role of this enzyme in cartilage breakdown (Dingle *et al.*, 1971; Poole *et al.*, 1974) while inhibitory antibodies to uPA and interstitial collagenase indicated a specific role for these enzymes in fibrin and type I collagen breakdown in human HT-1080 cells (Birkedal-Hansen *et al.*, 1989).

A distinction, however, needs to be made between true immunoinhibition and inhibition due to immunoprecipitation. True immunoinhibition occurs either by virtue of a bound antibody interfering directly with the catalytic mechanism of the enzyme, or changing the protein's conformation or flexibility, or by sterically hindering access of a substrate to the active site. An antibody generated to inhibit cathepsin L binds an active-site associated peptide length, including the active-site histidine residue (Coetzer *et al.*, 1992), an interaction which probably disrupts the catalytic machinery of the enzyme, although simple steric hindrance cannot be ruled out. In immunoprecipitation, optimal proportions of antibody and antigen interact to form a cross-linked lattice which may render the enzyme inactive by being locked in an insoluble complex. Precipitation, however, is not always synonymous with inhibition as enzyme in a precipitate may still be active (Branster and Cinader, 1969), at least against soluble substrates. Precipitation requires at least two epitopes and polyclonal antibodies, therefore monoclonal and anti-peptide antibodies will not be precipitating.

A protein's antigenicity refers to its capacity to bind specifically to paratopes (binding sites) of immunoglobulin molecules. The portion of the antigen with which the antibody binds is the epitope or antigenic determinant (Novotny *et al.*, 1983). Not all epitopes are able to elicit an immune response, however, and the

property of an epitope whereby it is able to induce such a response is known as its immunogenicity (Atassi, 1984). Epitopes can be further discriminated on the basis of structural criteria: a stretch of amino acid residues directly linked by peptide bonds constitutes a continuous epitope, while a group of residues brought into apposition by virtue of polypeptide chain folding or interchain interaction is defined as a discontinuous epitope (Atassi and Smith, 1978).

To produce an immune response *in vivo* that generates antibodies inhibitory to selected non-immunogenic proteases, requires a strategy that will break immune self-tolerance. This can be achieved by producing antibodies to a peptide corresponding to an accessible portion of the enzyme, thereby allowing such antibodies to bind to the common, non-immunogenic epitope of the enzyme (Dennison, 1989). This approach also has the advantage of allowing for the site of binding of the antibody to the enzyme to be pre-selected according to criteria maximising the chances of immunoinhibition. Furthermore, prediction algorithms based on empirical observations of the location of continuous epitopes in proteins and structural parameters such as hydrophilicity (Hopp and Woods, 1981; 1983) and segmental mobility (Westhof *et al.*, 1984), can be used to aid peptide selection.

The work described in this chapter was aimed at production of an anti-peptide antibody capable of immunoinhibiting cathepsin D. Cathepsin D, as discussed in the general introduction, has been implicated in breast malignancy. At the outset of this work, an extracellular, proteolytic role for cathepsin D was envisaged, although evidence now seems to indicate that, in the context of tumour progression, the enzyme's proteolytic role may be intracellular (Montcourrier *et al.*, 1994). However, endocytosed immunoinhibitory polyclonal antibodies to cathepsin D have been found to significantly reduce intracellular cathepsin D-dependent proteolysis in lysosomes of macrophages (Dingle *et al.*, 1973). Moreover, ablation by antibodies, of the suggested extracellular mitogenic activity of cathepsin D on breast cancer cells (Fusek and Vetvicka, 1994) is not precluded.

Anti-cathepsin D anti-peptide antibodies were raised in rabbits and chickens, against either free or conjugated peptide immunogens. These antibodies were characterised for binding to the peptide and to cathepsin D purified from various species, as well as for immunoinhibitory activity.

3.2 Purification of cathepsin D

Cathepsin D was purified from human, porcine and bovine spleen for characterisation of the anti-peptide antibodies generated. High levels of the enzyme are present in spleen (Cunningham and Tang, 1976). Its reversible but strong and stable association with pepstatin, a fungally derived aspartic proteinase inhibitor (Aoyagi *et al.*, 1972; Knight and Barrett, 1976), was used to purify the enzyme by affinity chromatography (Kazakova and Orekhovich, 1976; Takahashi and Tang, 1981), a procedure now ubiquitously used. Prior to affinity chromatography, the crude spleen homogenates were subjected to three-phase partitioning (TPP) (Pike and Dennison, 1989a) in *t*-butanol/water/ammonium sulfate (Jacobs *et al.*, 1989), a fractionation technique also successfully used for the purification of cathepsin L (Pike and Dennison, 1989b).

3.2.1 Materials

Spleens. Fresh porcine and bovine spleens were obtained from the Cato Ridge Abattoir. A human spleen was obtained from the University of Natal Medical School, Durban, after a splenectomy due to portal hypertension resulting in congestive splenomegaly. The spleen was human immunodeficiency virus and hepatitis B negative (tested by the Natal Institute of Immunology, Pinetown).

Loading buffer (50 mM sodium acetate, 200 mM NaCl, 0.02% (m/v) NaN_3, pH 3.5). AR acetic acid (2.86 ml), NaCl (11.69 g) and NaN_3 (0.2 g) were dissolved in about 900 ml of dist.H_2O, titrated to pH 3.5 with NaOH, and made up to 1 litre.

Elution buffer (50 mM Tris-HCl, 200 mM NaCl, 0.02% (m/v) NaN_3, pH 8.5). Tris base (6.06 g), NaCl (11.69 g) and NaN_3 (0.2 g) were dissolved in about 900 ml of dist.H_2O, titrated to pH 8.5 with HCl and made up to 1 litre.

3.2.2 Procedures

3.2.2.1 Spleen preparation

Spleens were prepared essentially according to Takahashi and Tang (1981). Porcine and bovine spleens were freed from connective tissue, but the human spleen was found to have almost no connective tissue associated with it. Spleens were diced into 2 x 2 cm cubes and stored frozen (-70°C). Diced spleen was allowed to thaw overnight to 4°C and homogenised in a Waring blendor with cold dist.H_2O. Ratios of 1:1 or 1:2 (mass of spleen to volume of water) were used, and spleens were homogenised for 1 or 2 min. The homogenate was centrifuged (10 000 x g, 30 min, 4°C), the supernatant decanted and adjusted to pH 3.7 with dilute HCl with stirring, and centrifuged as before. The supernatant was poured off and subjected to TPP (Section 3.2.2.2).

3.2.2.2 Three-phase partitioning

All procedures were performed at 4°C. Three-phase partitioning was effected on the pH 3.7 acid supernatant by the addition and mixing in of 2-methylpropan-2-ol (*t*-butanol) to 30% (v/v of final volume).

The volume of *t*-butanol to be added was calculated as follows:

$$\frac{x}{x+y} = 0.3$$

where x = volume of *t*-butanol

y = volume of pH 3.7 supernatant.

t-Butanol was first warmed to 30°C (above its crystallisation temperature of about 25°C). Solid $(NH_4)_2SO_4$ (20% (m/v) based on the volume of pH 3.7 supernatant and *t*-butanol) was added and dissolved with stirring. The mixture was centrifuged (6 000 x g, 10 min, 4°C) in a swing-out rotor and the supernatant *t*-butanol and subnatant aqueous phases decanted, leaving behind the third phase of interfacial protein precipitate, which was discarded. Further $(NH_4)_2SO_4$ was added to the aqueous and solvent phases to bring the concentration to 35% (m/v)

(based on the volume of pH 3.7 supernatant and *t*-butanol) and was dissolved with stirring. The solution was again centrifuged (6 000 x g, 10 min, 4°C) and the interfacial protein layer collected by decantation of the super- and subnatants. The precipitate was redissolved in loading buffer at approximately one-tenth of the volume of the acid supernatant. The solution was centrifuged (25 000 x g, 15 min, 4°C) and filtered through Whatman No. 4 filter paper to remove undissolved protein.

3.2.2.3 Pepstatin affinity chromatography

The sample was loaded onto a column of pepstatin-diaminohexane-Sepharose (1.0 x 3.0 cm = 2.4 ml) that had previously been equilibrated with loading buffer at 10 ml/h. Unbound material was washed off the column with loading buffer and cathepsin D was eluted with elution buffer.

3.3 Assay of cathepsin D

Cathepsin D activity is routinely measured using acid-denatured haemoglobin as a substrate. The distribution of molecular weights of released peptides depends on the nature of the substrate and the specificity of the proteinase, while the extent of proteolysis depends on the amount of enzymatic activity. Activity is measured spectrophotometrically by quantifying the total amount of peptide product separable from the parent substrate protein by acid precipitation. The method of Anson (1938) utilising trichloroacetic acid (TCA), as modified by Takahashi and Tang (1981), is widely used. A linear relationship between change in absorbance and incubation time in the range of 10-120 min has been demonstrated (Barrett, 1970).

3.3.1 Reagents

5% (m/v) Haemoglobin substrate. Bovine haemoglobin powder (0.5 g) was placed on top of dist.H_2O (10 ml) in a small beaker and gently shaken by hand at regular intervals until all protein had dissolved. Excessive stirring entraps air bubbles and slows dissolution of the haemoglobin.

Assay buffer (250 mM sodium citrate buffer, pH 3.2). Citric acid (5.26 g) was dissolved in about 80 ml of dist.H_2O, titrated with NaOH to pH 3.2 and made up to 100 ml.

5% (m/v) Trichloroacetic acid. Trichloroacetic acid (5 g) was dissolved in dist.H_2O and made up to 100 ml.

3.3.2 Procedure

The reaction mixture, consisting of assay buffer (506 µl), substrate (133 µl) and enzyme sample (26 µl), was prepared in a polyethylene microfuge tube. After incubation at 37°C for 30 min, a sample (300 µl) was removed and added to a polyethylene microfuge tube containing TCA (240 µl). For a zero-time control, a sample (300 µl) was removed and mixed with TCA immediately after addition of the enzyme. The precipitated protein, in both reaction mixtures and blanks, was centrifuged to a pellet using a horizontally orientated microfuge tube rotor. The absorbance of the reaction mixture supernatant was read against the blank supernatant in a 1 ml quartz cuvette at 280 nm. Triplicate assays were carried out for both samples and controls. For the purposes of this study, 1 unit of activity is defined as the quantity of enzyme producing an increase of absorbance of 1.0 in excess of the control in 60 min (Barrett, 1970).

3.4 Selection and synthesis of peptide

A comparison of available high resolution crystal structures of aspartic proteinases, indicated a considerable degree of structural similarity (Davies, 1990). This suggested, at the outset of this study, when the tertiary structure of cathepsin D was not yet elucidated, that related aspartic proteinase structures could be used as models of cathepsin D. Penicillopepsin is probably the best characterised aspartic proteinase in terms of catalytic mechanism and structure (Hsu *et al.*, 1977; James *et al.*, 1982), and was thus used for peptide selection.

Aspartic proteinases contain two lobes symmetrical in peptide chain conformation. A large binding cleft (40 Å), which can accommodate about eight substrate amino acid residues, runs across the molecule, separating the two

domains. The active site residues, Asp-215, Asp-32 and Ser-35 (penicillopepsin numbering), are found near the midpoint of the active site cleft (Tang, 1979; Davies, 1990). In the middle of the cleft, the structures of all aspartic proteinases are very similar; superimposition of this core region of human renin and that of three fungal aspartic proteinases shows differences of only 0.45 Å (Sielecki *et al.*, 1989). Unlike some lysosomal cysteine proteinases, the active site lies buried between the two lobes, and would be inaccessible to an antibody. However, another structurally conserved region is the β-hairpin structure known as the "flap", composed of residues 71-83 in penicillopepsin (James *et al.*, 1982; Sielecki *et al.*, 1989; Sielecki *et al.*, 1990). A difference electron density map at 1.8 Å resolution of crystals of a molecular complex between the esterified tripeptide fragment of pepstatin and penicillopepsin, indicated a major conformational change involving the flap as a result of inhibitor binding. There are 82 nonbonded contacts less than 4.0 Å that the atoms of the tripeptide make with 15 residues of the enzyme. Of the 82 contacts, 40 are made by the P_1 statine residue to 10 residues of penicillopepsin, and 3 of these are with the flap, i.e. Tyr-75, Asp-77 and Ser-79. The P_2 amino acid of pepstatin, valine, also makes a major contact with Asp-77.

Mobility of the flap is a prerequisite to accommodate substrates in the binding cleft. A conformational change is indicated by the negative electron density on the difference map between the enzyme and enzyme-inhibitor complex. A hinge-like rotation around an axis connecting the C^{α} atoms of Trp-71 and Gly-83 provides movement to the flap such that the tip of the flap is able to move by about 2.2 Å (James *et al.*, 1982). A recently completed crystallographic study by Baldwin *et al.* (1993) (Fig. 2) and a rule-based model by Scarborough *et al.* (1993), detailing the structure of native and inhibited forms human cathepsin D, confirms the analogous studies done on penicillopepsin.

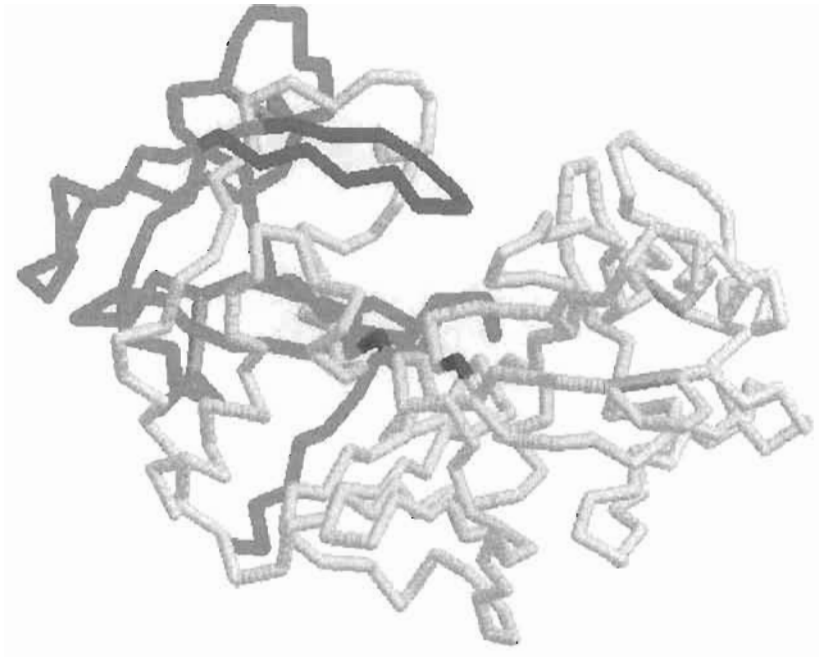

Figure 2 Calpha backbone of two-chained human cathepsin D determined from the crystal structure (Baldwin *et al.*, 1993).
The "flap" (magenta) is situated in the human cathepsin D light chain (cyan). The heavy chain is shown in white, and side chains are shown for the disulfide bonds (yellow) and the two active-site aspartate residues (red). The parent image (reference 1LYB) was retrieved from the Protein Data Bank, Brookhaven National Laboratory, Upton, New York. This image was modified in RasMol version 2.5 for Windows.

Binding of pepstatin to cathepsin D induces residues Gly-79 and Ser-80 at the tip of the flap to move about 1.7 Å toward the inhibitor, accompanied by a decrease of flexibility of the β-bend as result of hydrogen bonding between the backbone of the inhibitor and side-chain atoms of the enzyme (Fig. 3).

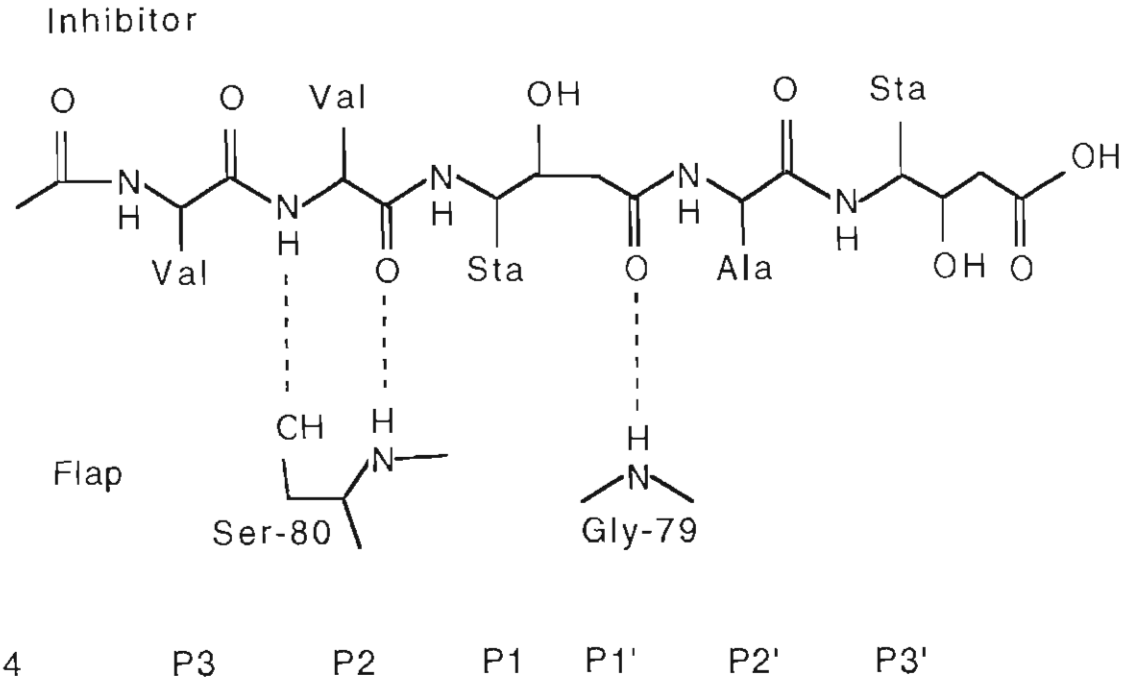

Figure 3 Schematic hydrogen-bonding diagram for pepstatin bound to cathepsin D. Inhibitor side-chain numbering is according to the nomenclature of Schechter and Berger (1967) and flap residues are numbered according to human cathepsin D.

It was envisaged that an antibody binding to the flap would disrupt its flexibility of movement and/or its hydrogen bonding interactions with a good substrate. This approach was independently used with human renin (Bouhnik *et al.*, 1987), where 7 peptide sequences were selected, based on the three-dimensional model of renin. Antibodies against these peptides were tested for immunoinhibitory activity, and the antibody based on the renin flap sequence proved to be the most potent inhibitor.

Based on the considerations outlined above, an amino acid sequence corresponding to residues 73-84 (D73-84) in the human cathepsin D sequence (Faust *et al.*, 1985), was selected (Fig. 4). Despite a high degree of structural conservation, the flap region has a remarkable lack of sequence homology between aspartic proteinases (Fig. 4), and anti-peptide antibodies produced against this sequence would be unlikely to cross-react with other aspartic proteinases. In contrast, the sequence is relatively well conserved across species (Fig. 5), being identical in human, rat and mouse, thereby increasing the versatility of the antibodies produced.

human cathepsin D[1]:	S F D I H Y G S G S L S
human renin[2]:	E L T L R - S T - T V -
human pepsinogen[3]:	T V S - T - - T - - M T
porcine pepsinogen[4]:	E L S - T - - T - - M A
bovine chymosin[5]:	P L S - - - - T - - M Q
penicillopepsin[6]:	T W S - S - - D - - S A

Figure 4. Amino acid sequence homology of the flap region of human cathepsin D with that of other aspartic proteinases.
Dashes in the lower five sequences indicate identity with the human cathepsin D sequence. [1]Faust *et al.*, 1985; [2]Imai *et al.*, 1983; [3]Sogawa *et al.*, 1983; [4]Tang *et al.*, 1973; [5]Harris *et al.*, 1982 and [6]Hsu *et al.*, 1977.

human[1] cathepsin D:	S F D I H Y G S G S L S
porcine[2] cathepsin D:	T - - A - - - - - - - -
chicken[3] cathepsin D:	E - A - - - - - - - - -
rat[4]/mouse[5] cathepsin D:	- - - - - - - - - - - -
mosquito[6] cathepsin D:	A - H - Q - - - - - - -
Schistosoma japonicum[7] aspartic proteinase:	D - S - R - - T - - - -

Figure 5. Cross-species amino acid sequence homology of the flap region of cathepsin D.
Dashes in the lower five sequences indicate identity with the human cathepsin D sequence. [1]Faust *et al.*, 1985; [2]Shewale and Tang, 1984; [3]Retzek *et al.*, 1992; [4]Birch and Loh, 1990; [5]Grusby *et al.*, 1990; [6]Cho and Raikhel, 1992 and [7]Becker *et al.*, 1995.

The flap sequence is more than 10 amino acids long, thereby enhancing immunogenicity (Van Regenmortel, 1988). It is relatively hydrophobic but quite mobile, the region corresponding to the tip of the flap (residues 78-81) being more rigid (Fig. 6). The N-terminus is also relatively constrained, enhancing its suitability for coupling to a carrier molecule prior to inoculation, leaving the more mobile C-terminus free to interact with the immune system. The peptide was, however, also inoculated in the unconjugated, free form.

Peptide D73-84 was custom synthesised by Multiple Peptide Systems, San Diego, Ca. The C-terminal carboxyl residue was amidated to remove the charge associated with the carboxyl group, thereby mimicking more closely the peptide bond in the protein. This modification also ensures a higher yield from peptide synthesis (Multiple Peptide Systems technical bulletin). The N-terminus was left free to allow conjugation to a carrier with glutaraldehyde (Briand *et al.*, 1985).

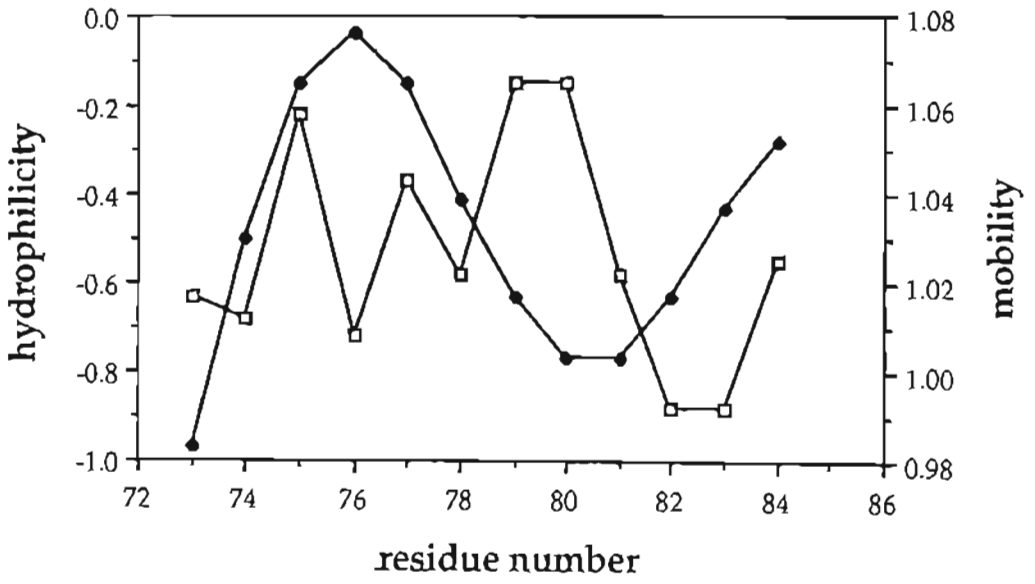

Figure 6. Hydrophilicity and segmental mobility profiles of human D73-84.
Hydrophilicity (□) was calculated according to Hopp and Woods (1981; 1983) and segmental mobility (◆) calculated according to Westhof *et al.* (1984).

3.5 Conjugation of peptide D73-84 to ovalbumin

Conjugation was effected with the homobifunctional reagent, glutaraldehyde, between the N-terminus of D73-84 and the carrier protein, ovalbumin, by the method of Briand (1985). Both tyrosine and histidine, present in D73-84, are capable of secondary reactions with glutaraldehyde (Muller, 1988), while ovalbumin contains 20 ε-NH_2 groups, although not all necessarily available for coupling (Nisbet *et al.*, 1981). A peptide:carrier molar ratio of 1:40 was used and conjugation effected with 1% glutaraldehyde. A 50% coupling efficiency was assumed (Bulinski and Gundersen, 1986).

The peptide was insoluble in buffer at a neutral pH, but NaOH effected solubilisation at *ca.* pH 12. This pH, however, is beyond the optimal range for glutaraldehyde conjugation, so amine-free dimethyl formamide (DMF), which was also able to effect solubilisation, was used as a solvent.

3.5.1 Reagents

Synthetic peptide D73-84. The peptide, SFDIHYGSGSLS, is described in Section 3.4.

Conjugation buffer (100 mM phosphate buffer, 0.02% (m/v) NaN_3, pH 7.0). NaH_2PO_4 (13.8 g) and NaN_3 (0.2 g) were dissolved in about 800 ml of dist.H_2O, titrated to pH 7.0 with NaOH and made up to 1 litre.

Glutaraldehyde (2% (v/v) in conjugation buffer). Glutaraldehyde (80 µl of a 25% solution, Merck, E. M. grade) was made up to 1 ml with conjugation buffer.

3.5.2 Procedure

Peptide (5 mg, 3.9 µmoles, 1270 Da) was dissolved in amine-free DMF (100 µl) and made up to 1 ml with PBS. Ovalbumin (4.6 mg, 0.1 µmoles, 45 kDa) was added to the solution and allowed to dissolve. Glutaraldehyde (1 ml) was added dropwise, with stirring, over a period of 5 min, and allowed to react for a further 2 h at 4°C. The reaction was stopped by the addition of $NaBH_4$ (20 mg) and the mixture incubated for a further 1 h at 4°C. Conjugated peptide was separated from free peptide by dialysis against several changes of conjugation buffer at 4°C.

3.6 Production of anti-D73-84 anti-peptide antibodies in rabbits and chickens

Antibodies to peptide D73-84 were produced in rabbits and chickens. Rabbits were each injected subcutaneously at 4-6 sites on the back with 100 µg conjugated peptide per animal, emulsified (1:1, v/v) in Freund's complete adjuvant (Difco, Detroit, Mi.). Further inoculations were administered in the same manner, in Freund's incomplete adjuvant (Difco) at week 2, followed by monthly boosters. Antibodies to free peptide (200 µg per animal, dissolved in pH 11.25 NaOH) were

similarly raised except that Freund's complete adjuvant was used throughout. Blood was collected from the marginal ear vein of rabbits at 3 and 8 weeks, and by non-lethal cardiac puncture at 12 weeks. Serum was separated from blood clots and IgG isolated (Section 2.5) and stored in glycerol (1:1, v/v) at -20°C.

Chickens were inoculated intramuscularly, at two sites in their large breast muscles, with either conjugate (100 µg conjugated peptide per animal) or free peptide (200 µg free peptide per animal, dissolved in pH 11,25 NaOH). For the conjugate, Freund's complete adjuvant (1:1, v/v) was used at week 0, and Freund's incomplete adjuvant (1:1, v/v) at weeks 2, 4, 6 and for monthly boosters thereafter. A similar schedule was used for free peptide except that complete and incomplete adjuvants were alternated from week 0. Eggs were collected and IgY isolated (Section 2.5) and stored in glycerol (1:1, v/v) at -20°C.

3.7 Enzyme-linked immunosorbent assay with peptide and enzyme

To test anti-peptide antibodies against peptide, an ELISA protocol (Section 2.6) was used, with peptide coated at 1.0 µg/ml. To test the across-species reactivity of anti-peptide antibodies against cathepsin D from human, bovine and porcine spleens, the same protocol was followed using IgY and substituting whole serum in the place of IgG. Cathepsin D, purified from various sources, was coated at 2 µg/ml.

3.8 Competition ELISA for native cathepsin D

The binding affinity of the anti-peptide antibody for native cathepsin D was tested by competition between coated peptide and free enzyme for the anti-peptide antibody. A decrease in the binding of antibody to peptide indicates a positive reaction with the native enzyme. Antibody (2 mg/ml) was pre-incubated (pH 7.2, 30 min, 37°C) with different levels of enzyme (molar ratios of peptide-to-enzyme from 1:32 to 1:0.5) and transferred to peptide coated plates (Section 3.7). The mixture was allowed to incubate for 1 h at 37°C and developed as previously described (Section 2.6).

3.9 Immunoinhibition assays

A major problem associated with cathepsin D is the lack of a sensitive assay. To measure enzyme immunoinhibition by polyclonal anti-peptide antibodies, a large molar excess of antibody over enzyme is required, as only a small proportion of the antibody population will recognise the native enzyme. This necessitates the use of as small an amount of enzyme as possible. Synthetic fluorogenic substrates, which are normally highly sensitive to detection, are not in wide use for cathepsin D, and therefore not well characterised. Use is almost exclusively made of the traditional haemoglobin assay (Anson, 1938), which lacks sensitivity and specificity. In this study, use was made of the haemoglobin assay and the azocasein assay in the presence of urea (Wiederanders *et al.*, 1985).

3.9.1 Modified haemoglobin assay

The routinely used haemoglobin assay was modified to accommodate larger sample volumes, necessitated by the preincubation of enzyme with antibody. Furthermore, it has been observed that maximum pepsin activity was obtained with a substrate concentration of 0.5% haemoglobin, while a concentration of 1.67% elicited 35-40% less activity. A similar inhibition by haemoglobin substrate above a concentration of 1.25% has been reported for cathepsin E (Simonarson *et al.*, 1985). The concentration of haemoglobin was, therefore, maintained at 0.9% (Muto *et al.*, 1988), and not at 1.67% as in the original Anson (1938) assay.

3.9.1.1 Reagents

Assay buffer (250 mM sodium citrate buffer, pH 5.0). Citric acid (5.26 g) was dissolved in about 80 ml of dist.H_2O, titrated with NaOH to pH 5.0 and made up to 100 ml.

1% (m/v) Haemoglobin substrate. Denatured bovine haemoglobin powder (0.1 g) was placed on top of assay buffer (10 ml) in a small beaker and gently shaken by hand at regular intervals until all protein had dissolved.

10% (m/v) Trichloroacetic acid. Trichloroacetic acid (10 g) was dissolved in dist.H_2O and made up to 100 ml.

3.9.1.2 Procedure

Enzyme (400 ng) was preincubated with immune or non-immune antibody (0.1 mg - 9.0 mg) in assay buffer (total volume of 200 µl, 15 min, 37°C). To start the assay, substrate (2000 µl) was added and the mixture incubated in glass test tubes. For a zero-time control, a sample (1100 µl) was removed and mixed with TCA (1000 µl) immediately after addition of the enzyme. After incubation at 37°C (2 h), TCA (1000 µl) was added to the reaction mixtures. The precipitated protein in both reaction mixtures and blanks, was spun to a pellet in a benchtop centrifuge. Supernatants were clarified by filtration through Whatman No. 4 filter paper. The absorbance of the reaction mixture supernatant was read against the blank supernatant in a 3 ml quartz cuvette at 280 nm. Triplicate assays were carried out for both samples and controls. Immunoinhibition can be calculated as the percentage inhibition due to the presence of antibody relative to a parallel non-immune control.

3.9.2 Azocasein assay

Azocasein in the presence of 3 M urea is a more sensitive substrate for cathepsin D than denatured haemoglobin (Wiederanders *et al.*, 1985). Urea has the effect of increasing the susceptibility of azocasein to attack by the enzyme and increases the solubility of hydrolysed products in TCA. Furthermore, the optimum for hydrolysis of azocasein is shifted to pH 5.0 in comparison to that of pH 3.2 in the haemoglobin assay (Anson, 1938). This is advantageous for immunoinhibition assays where pH 5.0 is used to ensure adequate binding of antibody to enzyme. The effect of the presence of urea in the assay system was ascertained by an ELISA (Section 3.9.3). Although there was a reduction in titre, at high antibody levels comparable amounts of antibody bound immobilised enzyme as in urea-free controls.

3.9.2.1 Reagents

Assay buffer (250 mM sodium citrate buffer, pH 5.0). Citric acid (5.26 g) was dissolved in about 80 ml of dist.H_2O, titrated with NaOH to pH 5.0 and made up to 100 ml.

6% (m/v) Azocasein. Azocasein (3 g) was weighed into a glass beaker and dissolved in 50 ml dist.H_2O with gentle magnetic stirring at room temperature for about 1 h.

Azocasein/3 M urea solution. Urea (10.8 g) was dissolved in 6% azocasein solution (10 ml) by stirring on a magnetic stirrer at about 30°C. The volume was made up to 30 ml with assay buffer.

5% (m/v) TCA. Trichloroacetic acid (5 g) was dissolved in dist.H_2O and made up to 100 ml.

3.9.2.2 Procedure

Enzyme (100 ng - 400 ng) was preincubated with non-immune or immune antibody (0.5 mg - 1.0 mg) in assay buffer (total volume of 200 µl, 15 min, 37°C). Sample (200 µl) was mixed with assay buffer (200 µl) and azocasein/urea solution (400 µl), and an aliquot (200 µl) immediately withdrawn and mixed with 5% TCA (1000 µl). This served as a blank for the reaction. After a specified time (30 min-2 h) a further aliquot was withdrawn (200 µl) and mixed with TCA (1000 µl). Samples were centrifuged in a microcentrifuge and the absorbance at 366 nm read in glass micro-cuvettes, with blacked-out sides to prevent transmission of light around the sample. Triplicate assays were carried out for both samples and controls. Immunoinhibition can be calculated as the percentage inhibition due to the presence of antibody relative to a parallel non-immune control.

3.9.3 Enzyme-linked immunosorbent assay in the presence of 3 M urea

The binding of the chicken anti-peptide antibodies in the presence of 3 M urea was established at pH 5.0 and 6.0 using assay buffer (Section 3.9.1) and at pH 7.4 as before (Section 2.6 and 3.7).

3.10 Affinity purification of chicken anti-D73-84 anti-peptide antibodies

Preliminary assays demonstrated a lack of enzyme inhibition by the antibodies tested, which prompted the decision to affinity purify antibodies using bovine cathepsin D immobilised via its carbohydrate groups on polyacrylhydrazido-agarose (Bio-Rad Bulletins 1099, 1298). This ensures, firstly, that the immobilised enzyme is optimally available for antibody binding by virtue of the carbohydrate group acting as a spacer arm and, secondly, that the enzyme remains as native as possible by avoiding immobilisation via protein residues as occurs when cyanogen bromide-activated agarose is used. This should yield an antibody preparation that is essentially monoclonal in its specificity for binding the flap region of the enzyme. Bovine cathepsin D was used due to the similarity of recognition of the bovine and human enzyme in an ELISA, and the availability of suitable source material for its purification.

3.10.1 Reagents

Coupling buffer (100 mM sodium acetate, 150 mM NaCl, pH 5.5). AR acetic acid (5.72 ml) and NaCl (8.76 g) were dissolved in about 900 ml of dist.H_2O, titrated to pH 5.5 with NaOH, and made up to 1 litre.

Bovine cathepsin D. Cathepsin D was purified from bovine spleen as described in Section 3.2.

20.8 mg/ml Sodium periodate. Sodium periodate (20.8 mg) was dissolved in dist.H_2O (1 ml).

Equilibration buffer (20 mM sodium phosphate, 500 mM NaCl, 0.02% (m/v) NaN_3, pH 7.2). NaH_2PO_4 (2.76 g), NaCl (29.23 g) and NaN_3 (0.2 g) were dissolved in about 800 ml of dist.H_2O, titrated to pH 7.2 with NaOH and made up to 1 litre.

Elution buffer (20 mM sodium phosphate, 500 mM NaCl, 3.5 M potassium thiocyanate, pH 7.2). NaH_2PO_4 (0.28 g), NaCl (2.92 g) and potassium thiocyanate

(34.01 g) were dissolved in about 80 ml of dist.H_2O, titrated to pH 7.2 with NaOH and made up to 100 ml.

3.10.2 Procedure

Bovine cathepsin D (10.9 mg) was dialysed into coupling buffer to a volume of 5 ml. Cathepsin D sugar moieties were oxidised with sodium periodate (0.1 volumes) by gentle stirring (1 h, in the dark). To stop the reaction glycerol (8 µl, 20 mM final conc.) was added and mixed in (10 min). Oxidised cathepsin D (5 ml) was applied to a Sephadex G-25 column (2.5 cm x 25 cm = 120 ml), equilibrated in coupling buffer (50 ml/h), to remove excess periodate and glycerol. Eluted cathepsin D (10.2 mg, 14 ml) was coupled in an end-over-end mixer to polyacrylhydrazido-agarose (3 ml, 24 h, RT), previously washed with coupling buffer. The gel was washed alternately with high and low pH buffer (elution and equilibration buffers respectively, Section 3.2.1), coupling buffer and elution buffer and again with equilibration buffer. Determination of the amount of enzyme left uncoupled, gave a coupling efficiency of 95%.

An appropriate antibody preparation (10 mg) was twice passed through the affinity gel (1.0 cm x 3.3 cm = 2.6 ml, 10 ml/h) and unbound material washed off with equilibration buffer. Bound antibody was eluted with elution buffer (5.6 ml), and the eluted fraction desalted into equilibration buffer on a column of Sephadex G-25 as described above.

3.11 Results

3.11.1 Purification of cathepsin D from bovine, porcine and human spleen

Cathepsin D was purified from bovine (200 g), porcine (330 g) and human (250 g) spleens using the procedure described in Section 3.2.2. A comparison of the purification of cathepsin D from the three species is presented in Table 3.

Table 3. The purification of cathepsin D from bovine, porcine and human spleens.

Step	Vol. (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Bovine						
crude supernatant	352	17 632	13 084	0.742	(1)	(100)
acid supernatant	296	5 957	7 556	1.268	1.7	58
TPP	38	92	5 437	59.09	79.6	42
pepstatin-Sepharose	7.7	5.951	4 082	685.94	924.5	31
Porcine						
crude supernatant	540	25 193	15 229.3	0.605	(1)	(100)
acid supernatant	453	9 931	13 798.9	1.389	2.3	90.61
TPP	56	292.8	6 644.32	22.69	37.5	43.63
pepstatin-Sepharose	4.5	4.515	564.02	124.92	206.7	3.7
Human						
acid supernatant	457	3 950	30 384.6	7.69	(1)	(100)
TPP	40	161.6	5 736.6	35.5	4.6	18.8
pepstatin-Sepharose	10.1	4.51	2 518.1	558.3	72.6	8

Freezing of tissue prior to homogenisation is essential to disrupt lysosomes and facilitate the release of lysosomal enzymes. Better recoveries were obtained using twice the volume of dist.H_2O per mass of spleen for homogenisation and twice the homogenisation time recommended by Takahashi and Tang (1981). Different buffers have no effect on cathepsin D extraction (Press *et al.*, 1960).

Cathepsin D eluted from pepstatin-Sepharose in a single peak (Fig. 7). The low pH of the loading buffer allows the enzyme to assume an enzymatically active conformation, and it is thus able to bind to the immobilised inhibitor. By virtue of the reversibility of the enzyme-inhibitor interaction, a change to a higher buffer pH, which induces conformational deactivation, causes the enzyme to

release the inhibitor and it is thus eluted. Enzyme activity in the breakthrough peak was low and probably attributable to non-aspartic protease proteolytic activity against haemoglobin substrate (results not shown). Activity in the eluted peak should be quantitative by virtue of the specificity of the enzyme-inhibitor interaction. Figs 8-10 illustrate the SDS-PAGE analysis of the purification of bovine, porcine and human cathepsin D respectively.

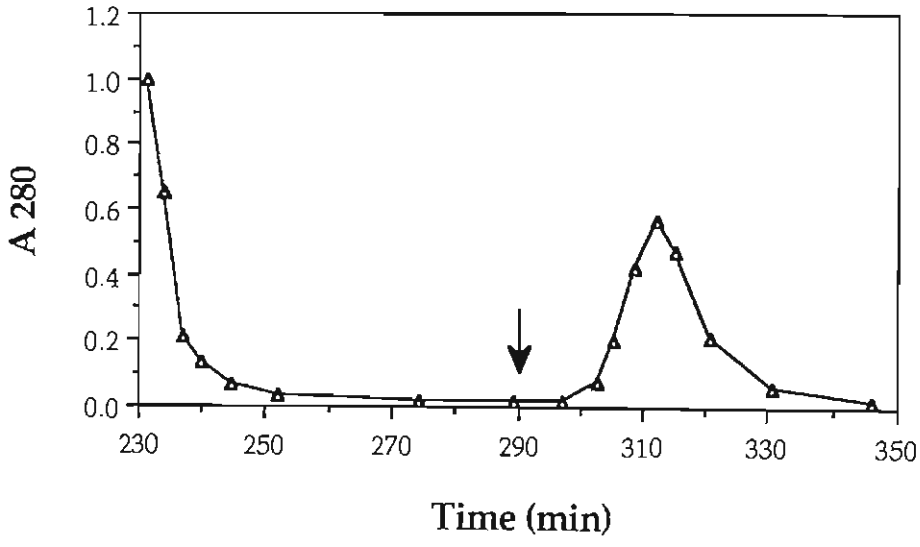

Figure 7. Elution of cathepsin D from pepstatin–Sepharose after application of the TPP fraction.

Pepstatin-Sepharose was equilibrated with loading buffer and bound cathepsin D was eluted with elution buffer, applied at point ↓. The flow rate was 10 ml/h and the eluted peak was collected manually. (Δ) A_{280}.

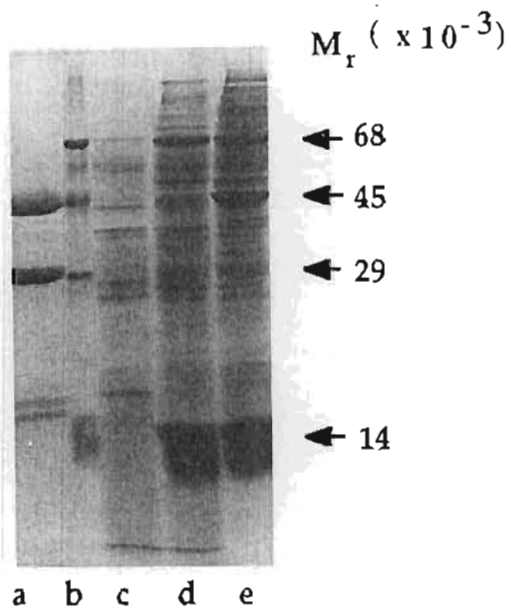

Figure 8 Reducing SDS-PAGE analysis of the purification of bovine spleen cathepsin D. Fractions were reduced and loaded onto a 12.5% gel. (a) cathepsin D; (b) molecular weight markers (BSA, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; lysozyme, 14 kDa); (c) TPP fraction; (d) acid supernatant and (e) crude supernatant.

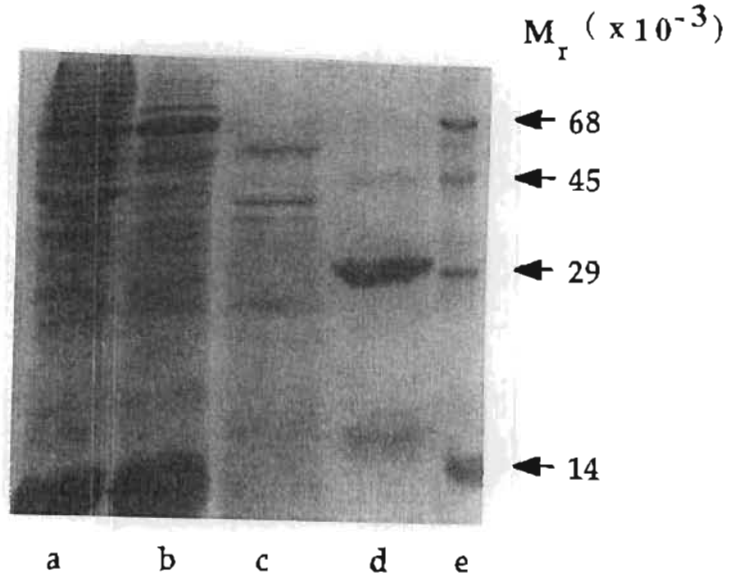

Figure 9. Reducing SDS-PAGE analysis of the purification of porcine spleen cathepsin D. Fractions were reduced and loaded onto a 12.5% gel. (a) crude supernatant; (b) acid supernatant; (c) TPP fraction; (d) cathepsin D and (e) molecular weight markers (as in Fig. 8).

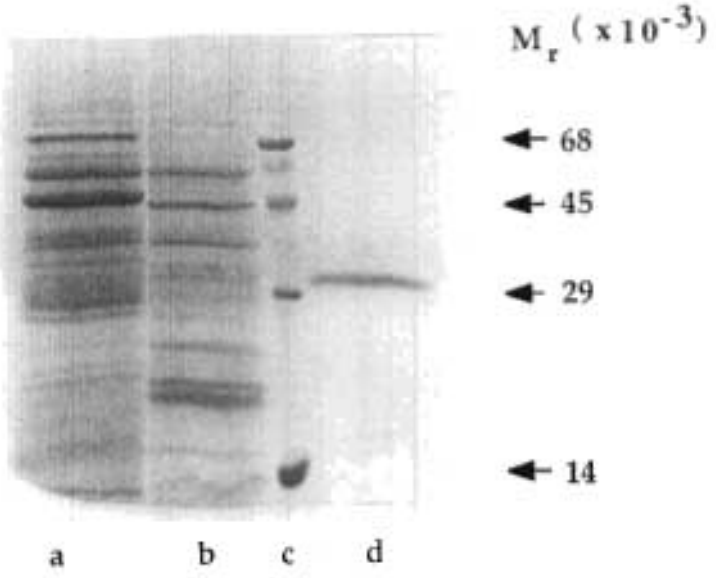

Figure 10. Reducing SDS-PAGE analysis of the purification of human spleen cathepsin D. Fractions were reduced and loaded onto a 12.5% gel. (a) acid supernatant; (b) TPP fraction; (c) molecular weight markers (as in Fig. 8) and (d) cathepsin D.

Lane e of Fig. 8 and lane a of Fig. 9 show the extent of cytosolic proteins in the homogenate supernatant, and lane d and b respectively the corresponding supernatants after the acid precipitation step. Note the degree of purification achieved by the TPP step and the concomitant disappearance of the very prominent band corresponding to haemoglobin.

Bovine cathepsin D was purified as a mixture of single- (45 kDa) and two-chained forms (30 kDa + 15 kDa) (Fig. 8). The light chain component of the two-chain enzyme is present as a doublet, and a band is visible at a molecular weight of about 39 kDa. This band was a feature of all cathepsin D purifications from bovine spleens, and was assumed to be a proteolysis product of the single chain enzyme (Whitaker, 1980).

Cathepsin D from porcine spleen was similarly found to run as a mixture of single- (45 kDa) and two-chain forms on (30 kDa + 15 kDa) on SDS-PAGE (Fig. 9). The proportion of single- to two-chain enzyme appears to be lower relative to bovine cathepsin D. The 15 kDa light chain appeared as a triplet for porcine cathepsin D and as a doublet for human cathepsin D (Fig. 10). The human

enzyme was purified only as a two-chain form with 30 kDa and 15 kDa components.

3.11.2 Production and characterisation of anti-peptide antibodies against the human cathepsin D peptide

Use of glutaraldehyde as a coupling agent can give a coupling yield of 35-50% (Bulinski and Gundersen, 1986). Previous studies in this laboratory have yielded coupling efficiencies of 47.5% (Pike, 1990) and 45% (Coetzer, 1992) for two different peptides coupled by this method. The coupling efficiency for D73-84 was thus assumed to be 50%, and this was used to determine the amount of immunogen required for inoculation.

To monitor the progress of antibody production in host animals, an ELISA was developed in which peptide was coated directly to the wells of the microtitre plate. Many workers prepare conjugate antigens for use in ELISAs, consisting of a carrier and a coupling method different from those used to prepare the immunogen. Most coupling reactions lead to the modification of many residues of the carrier that are not involved in the formation of peptide-carrier bridges. When animals are immunised with peptide conjugates, such coupling agent-modified residues (CAMOR) may elicit the formation of CAMOR antibodies that recognise only the modified carrier molecule, but not the untreated carrier or peptide moiety (Briand *et al.*, 1985). By coating directly with peptide, only the contribution of anti-peptide antibodies is measured and the need to prepare the peptide coupled to a different carrier by means of another coupling agent is obviated.

The chickens responded very similarly during the course of the inoculation programme, and the response of only one chicken is, therefore, shown. Antibody production against conjugated peptide reached a maximum at eight weeks, with the levels at twelve weeks being almost unchanged (Fig. 11A). The antibody did not show appreciable non-specific binding (results not shown) to the unrelated peptide D112-122 from porcine cathepsin D (Coetzer *et al.*, 1991). Free peptide,

however, did not elicit a comparable immune response, although there was some reaction after 12 weeks (Fig. 11B).

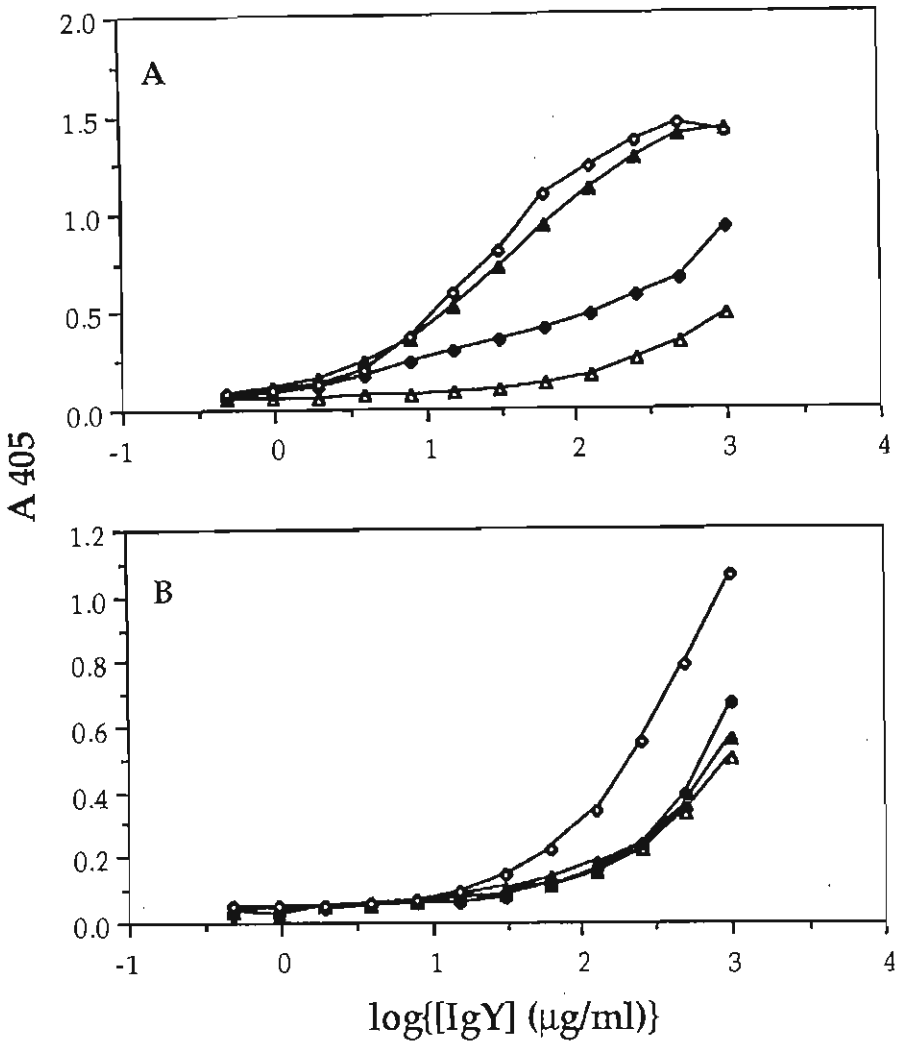

Figure 11. ELISA of the progress of immunisation with conjugated and free peptide D73-84 in chickens.

Human cathepsin D peptide D73-84 was coated at 1.0 µg/ml to microtitre plates and incubated with serial two-fold dilutions of chicken anti-human cathepsin D peptide D73-84 IgY collected after 3 (◆), 8 (▲) and 12 weeks (◊) and non-immune IgY (Δ). Immunisation was with (A) ovalbumin-conjugated peptide and (B) free peptide. Binding was visualised by incubation with rabbit anti-chicken IgY-HRPO secondary antibodies as described in Section 2.6.

The reactivity of the IgY anti-peptide antibodies toward whole enzyme was tested in an ELISA where bovine, porcine and human cathepsin D were immobilised (Fig. 12). Using antibodies to the conjugated peptide, the human and bovine enzymes were equally well targeted, while porcine cathepsin D was less well

recognised. A similar trend was found with IgY antibodies against the free peptide except that recognition was relatively weak.

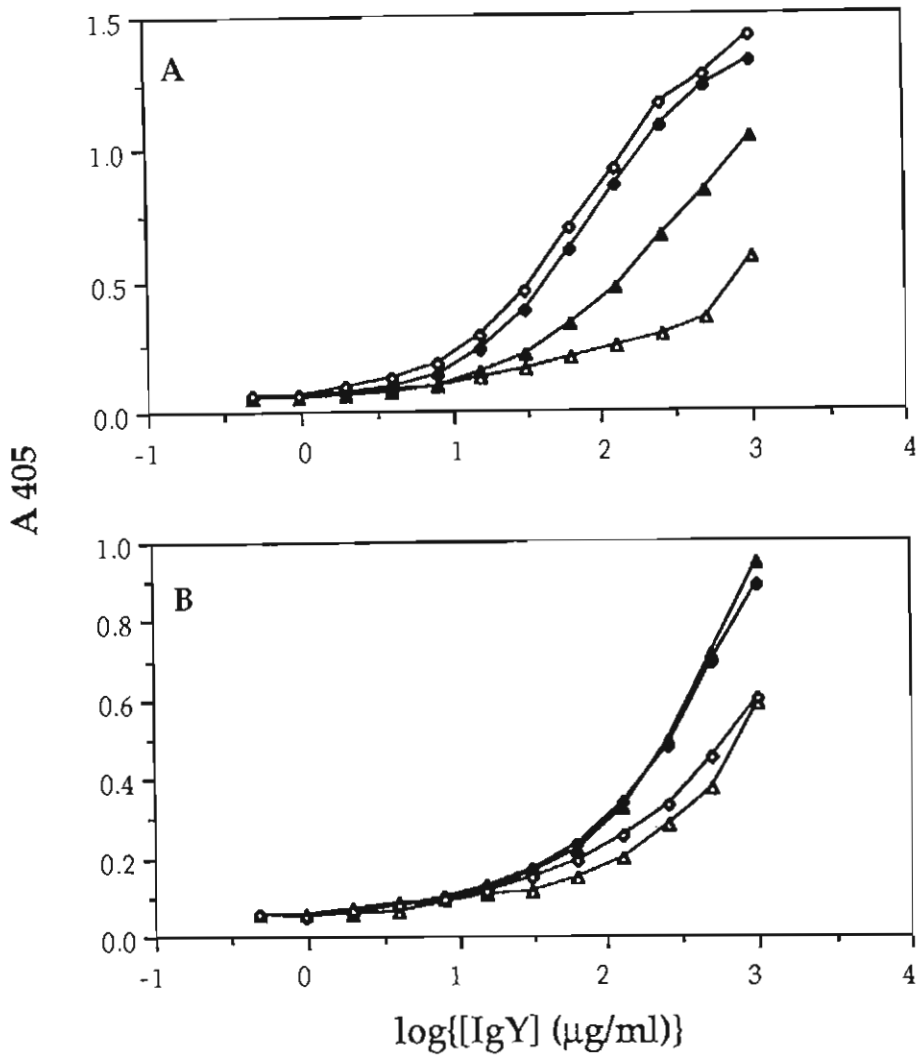

Figure 12. ELISA of the cross-species reactivity of chicken anti-human cathepsin D peptide (conjugated and free) antibodies with bovine, porcine and human cathepsin D.
The enzyme preparations were coated at 2.0 µg/ml to microtitre plates and incubated with serial two-fold dilutions of chicken anti-human cathepsin D peptide D73-84 IgY. Cross-reaction of anti-conjugated D73-84 IgY (A) and anti-free D73-84 IgY (B) with bovine (◆), porcine (▲) and human (◊) cathepsin D. Non-immune IgY (Δ). Binding was visualised by incubation with rabbit anti-chicken IgY-HRPO secondary antibodies as described in Section 2.6.

Immunisation of conjugated peptide into rabbits elicited a similar response that peaked at 8 weeks and remained constant at 12 weeks (Fig. 13A). Free peptide, however, provoked a very rapid response; the titre had reached maximum at

3 weeks, remained unchanged at 12 weeks (Fig. 13B) and was lower than that for the conjugate.

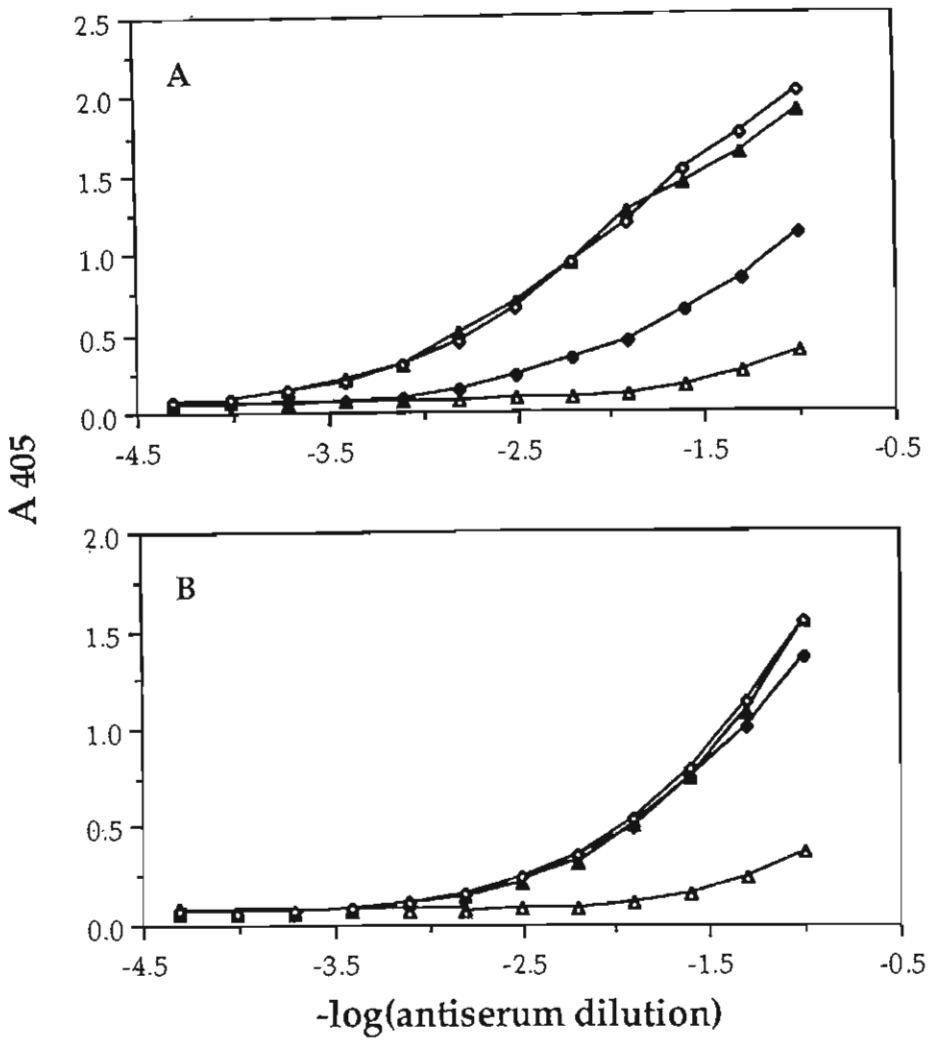

Figure 13. ELISA of the progress of immunisation with conjugated and free peptide D73-84 in rabbits.

Human cathepsin D peptide D73-84 was coated at 1.0 µg/ml to microtitre plates and incubated with serial two-fold dilutions of rabbit anti-human cathepsin D peptide D73-84 serum collected after 3 (◆), 8 (▲) and 12 weeks (◊) and non-immune serum (Δ). Immunisation was with (**A**) ovalbumin-conjugated peptide and (**B**) free peptide. Binding was visualised by incubation with sheep anti-rabbit IgG-HRPO secondary antibodies as described in Section 2.6.

Reactivity of rabbit serum against conjugated or free peptide, in contrast to serum against free peptide incubated with immobilised peptide (Fig. 13B), was similar against bovine, porcine and human cathepsin D (Fig. 14).

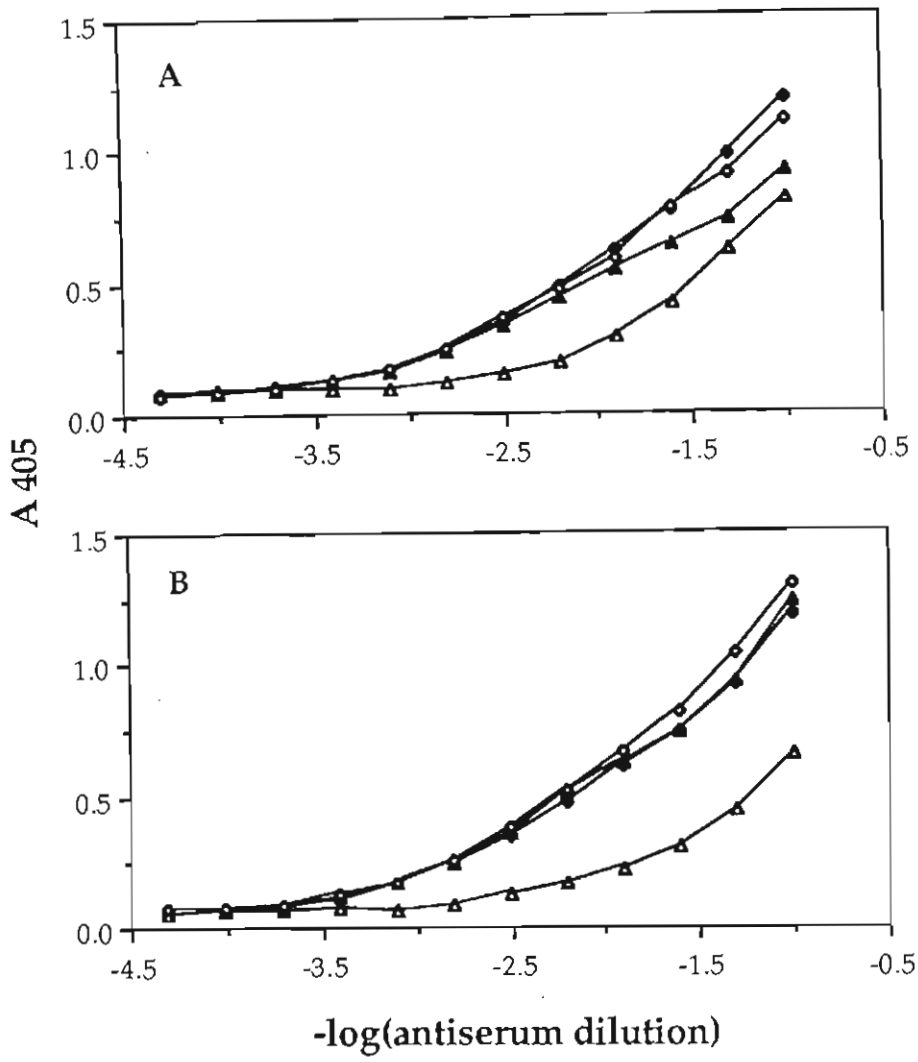

Figure 14. ELISA of the cross-species reactivity of rabbit anti-human cathepsin D peptide (conjugated and free) serum with bovine, porcine and human cathepsin D.

The enzyme preparations were coated at 2.0 µg/ml to microtitre plates and incubated with serial two-fold dilutions of rabbit anti-human cathepsin D peptide D73-84 serum. Cross-reaction of anti-conjugated D73-84 serum (A) and anti-free D73-84 serum (B) with bovine (◆), porcine (▲) and human (◊) cathepsin D. Non-immune serum (Δ). Binding was visualised by incubation with sheep anti-rabbit IgG-HRPO secondary antibodies as described in Section 2.6.

The reactivity of anti-peptide D73-84 antibodies against fully denatured cathepsin D was tested by western blotting (Section 2.7) subsequent to reducing SDS-PAGE, and by a blotting method designed to minimise protein denaturation (Dunn, 1986). To this end non-reducing SDS-PAGE was followed by incubation of the gel in glycerol to facilitate SDS removal and enzyme renaturation. Transfer of the protein to nitrocellulose membrane was effected in the absence of SDS at pH 8.3, and the membrane was not allowed to dry prior to probing. No

reaction was found by either method, suggesting that a conformation-dependent epitope may be recognised on the enzyme.

The recognition of native cathepsin D by anti-peptide D73-84 antibodies at pH 7.2 was evidenced by competitive inhibition by the enzyme of binding of antibody to immobilised peptide (Fig. 15). Maximum competition at 566 µg/ml corresponds to an approximately 32-fold molar excess of enzyme over immobilised peptide. Binding of antibody to native enzyme could not be detected at pH 5.0, the pH used for immunoinhibition studies.

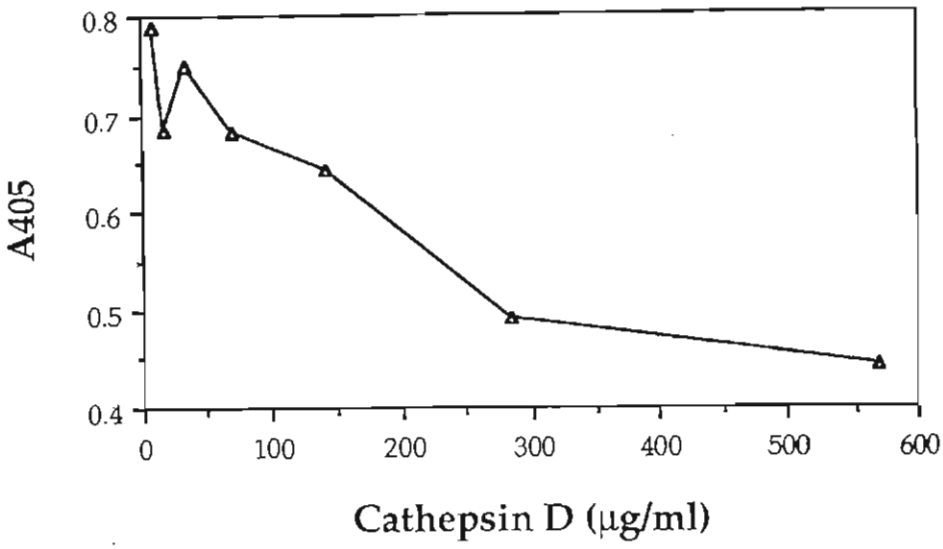

Figure 15. Competition ELISA determination of the ability of anti-peptide D73-84 to bind to native cathepsin D.

Chicken anti-D73-84 was preincubated with different levels of cathepsin D before transfer to peptide-coated microtitre (peptide to enzyme ratio of 1:0.5 to 1: 32) where inhibition of binding of IgY to immobilised peptide was measured at pH 7.0 (Section 3.7). The ELISA was developed as in Section 2.6. Control incubations containing either non-immune IgY or no competing cathepsin D were included.

3.11.3 Cathepsin D immunoinhibition

Immunoinhibition of cathepsin D by anti-peptide antibodies was tested against the protein substrates haemoglobin and azocasein, at pH 5.0. A molar excess of up to 625-fold and 1875-fold of antibody over enzyme was used for the haemoglobin and azocasein assays respectively. No inhibition of enzyme activity was detected in either case. To ascertain whether the presence of 3 M urea in the

assay buffer for azocasein assays affects antibody-antigen interactions, an ELISA was performed at pH 5.0 and pH 7.4 in the presence of 3 M urea (Fig. 16).

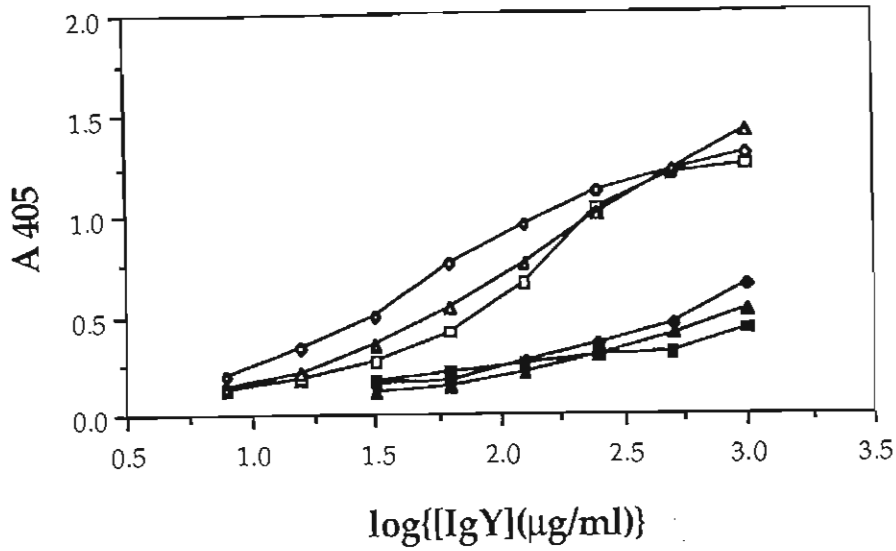

Figure 16. ELISA of the binding of anti-peptide D73-84 to cathepsin D in the presence and absence of 3 M urea.

The enzyme preparations were coated at 2.0 µg/ml to microtitre plates, and incubated with serial two-fold dilutions of chicken anti-human cathepsin D peptide D73-84 IgY in 250 mM citrate buffer, pH 5.0 (Section 3.3) in the presence of 3 M urea, or phosphate buffer, pH 7.4 (Section 2.6). Reaction of cathepsin D with anti-D73-84 at pH 5.0 (Δ), pH 5.0 + 3 M urea (□), pH 7.4 (◊) and non-immune IgY at pH 5.0 (▲), pH 5.0 + 3 M urea (■) and pH 7.4 (◆). Binding was visualised by incubation with rabbit anti-chicken IgY-HRPO secondary antibodies as described in Section 2.6.

Binding of the antibody at pH 5.0 in the presence of 3 M urea was found to be slightly less than at pH 7.4, but still significant. It is unlikely, therefore, that the presence of 3 M urea in the azocasein assay buffer was responsible for perturbing antibody-enzyme interaction, thereby compromising inhibition.

In an attempt to obtain anti-peptide antibodies of a higher potency, antibodies were affinity purified against cathepsin D immobilised by its carbohydrate residues. This procedure yielded approximately 70.0 µg/ml purified antibody per 100 mg IgY preparation applied. The titre of the affinity purified antibody was tested in an ELISA (Fig. 17). Immobilised human cathepsin D was still recognised at four dilutions more than the crude IgY preparation. The affinity purified

antibody, however, still failed to produce enzyme immunoinhibition. Attempts to produce immunoinhibition by using protein substrates with fluorescent leaving groups, such as fluorescein-haemoglobin and fluorescein-casein, were also unsuccessful. These assays did not produce a significantly greater degree of sensitivity than the haemoglobin or azocasein assays.

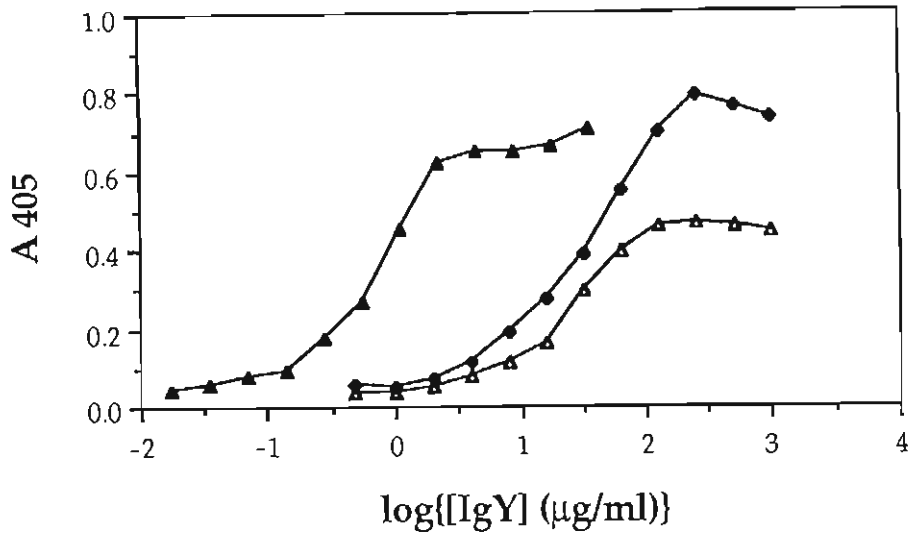

Figure 17. ELISA of the binding of affinity purified anti-peptide D73-84 to human cathepsin D. Human cathepsin D was coated at 2.0 µg/ml to microtitre plates, and incubated with serial two-fold dilutions of chicken anti-cathepsin D peptide D73-84 IgY preparations. Reaction of affinity purified antibody (▲), crude IgY (◆) and non-immune IgY (Δ). Binding was visualised by incubation with rabbit anti-chicken IgY-HRPO secondary antibodies as described in Section 2.6.

3.12 Discussion

This chapter is divided into two complementary parts; first the purification of the aspartic proteinase, cathepsin D, and second, the raising and characterisation of anti-peptide antibodies against a sequence selected from cathepsin D. It was envisaged that anti-peptide antibodies could be generated so as to exhibit inhibition of enzyme activity, as has been demonstrated for other proteinases such as cathepsin L (Coetzer *et al.*, 1991) and renin (Bouhnik *et al.*, 1987). Such antibodies could find applicability in *in vitro* tumour invasion studies and, it was initially envisaged, in tumour immunotherapy.

A major advancement of the cathepsin D purification procedure used, is the use of TPP as the pre-affinity chromatography step. Almost all other procedures encountered appeared to be more costly, and labour and time intensive. The commonly used method of Takahashi and Tang (1981) involves a 40 h dialysis step followed by ion-exchange chromatography and pepstatin-Sepharose affinity chromatography, while the method of Afting and Becker (1981) employs chromatography on concanavalin A-Sepharose and pepstatin-Sepharose affinity chromatography.

Three-phase partitioning was discovered serendipitously during the course of studies on the effects of water-miscible organic solvents on the activity of enzymes (Tan and Lovrien, 1972). It was found that *t*-butanol, normally completely miscible in water, separated from the aqueous phase on the addition of a salt such as ammonium sulfate. Protein in solution was precipitated into a third phase between the *t*-butanol and aqueous phases. A systematic study of TPP (Pike and Dennison, 1989) indicated that proteins precipitate into the third phase most readily at or below their pI, but are most soluble after TPP when carried out above the pI. The amount of salt required to precipitate a protein was found to vary inversely with the protein molecular weight. Three-phase partitioning differs from conventional salting-out with ammonium sulfate in that the protein precipitate is largely dehydrated and has a low salt content. *t*-Butanol, unlike other organic solvents, does not tend to denature proteins, and TPP can therefore be done at room temperature.

Non-covalently bonded proteins such as haemoglobin and myoglobin, are completely denatured by TPP, while single-chain proteins are denatured to a variable extent dependent partly on the pH at which TPP is carried out, relative to the pI of the protein. Three-phase partitioning, therefore, constitutes a method where single chain proteins can be separated from oligomers which, in the case of cathepsin D, results in the removal of haemoglobin, a major contaminant of spleen homogenates, at an early stage of the purification. Indeed, the continuous presence of haemoglobin during the purification is probably the source of a major loss of cathepsin D. Haemoglobin found in tissue homogenates would act as a

substrate for cathepsin D, especially since the purification is performed at a low pH. Procedures where haemoglobin is removed in soluble form probably deplete cathepsin D concomitantly, as is implied by the affinity purification of the enzyme on haemoglobin-Sepharose (Smith and Turk, 1974). The introduction of TPP, which precipitates haemoglobin and, presumably, leaves most cathepsin D in solution, circumvents this problem.

Resolution of the phases requires only low speed centrifugation, resulting in a relatively salt-free fraction, unlike equivalent preparations from ammonium sulfate precipitation. Three-phase partitioning is a cheap and rapid pre-affinity chromatography step that is suited to macro-scale purification of cathepsin D, with competitive enzyme yields. Takahashi and Tang (1981) report the purification of 4.65 mg of cathepsin D/100 g of bovine spleen, while with this procedure up to 5 mg of enzyme/100 g of spleen has been isolated within a far shorter time.

Cathepsin D was purified, using this protocol, from bovine spleens and, for the first time, from porcine and human spleens. Bovine and porcine enzymes were purified as a mixture of single (45 kDa) and two-chained (30 kDa and 15 kDa) forms. This is in agreement with published sizes of bovine cathepsin D (Lah *et al.*, 1984), but differs slightly from the porcine cathepsin D purified by Takahashi and Tang (1981). This group describe the purification of a 35 kDa and 15 kDa two-chain form and a 50 kDa single-chain enzyme. Afting and Becker (1981) have purified from pig myometrium 43 kDa and 30 kDa forms, a result in agreement with that obtained in this study. Human cathepsin D was purified as a two-chained form with 30 kDa and 15 kDa components. These values are the same as those reported by Barrett (1979). The light chain of all species of cathepsin D was consistently detected as a doublet or triplet, a finding also reported by Barrett (1977).

Purified cathepsin D was used either as an immunogen for antibody production (Chapter 4) or, as described in this chapter, for the characterisation of anti-peptide antibodies generated against a peptide sequence derived from cathepsin D.

The use of synthetic peptides as immunogens is an area of immunochemistry characterised by loose terminology and, as a result, is prone to differing interpretation which should be clarified. Some authors, for instance, define the antigenic reactivity of a protein as its capacity to bind specifically to the functional binding sites or paratopes of immunoglobulin molecules (Van Regenmortel, 1988). The epitopal nature of a set of amino acids in a protein can be defined only when an antibody has bound to it. The origin of the antibody used to identify an epitope is, by this definition, irrelevant. Others consider the property of immunogenicity as intrinsic to the concept of epitopes (Atassi, 1984). Immunogenicity refers to the ability of a protein to induce an immune response. An epitope, therefore, refers to a region of a protein that is recognised during the immune response when the whole protein is used as an immunogen. This viewpoint will adhered to in this thesis.

Epitopes have been divided into several conceptual categories that are not easily experimentally shown (Shinnick *et al.*, 1983; Van Regenmortel and Daney de Marcillac, 1988). Sequential epitopes are defined by peptide regions in their random coil form, while conformational epitopes require a specific conformation to be recognised by complementary paratopes. This distinction may be somewhat artificial, however, as it is unlikely that a paratope could recognise a sequence of residues independently of any conformation. A further distinction is between continuous and discontinuous epitopes. Continuous epitopes are defined as a length of contiguous residues in direct peptide linkage while discontinuous epitopes consist of a group of residues that are not contiguous but are brought together by the folding of the polypeptide chain.

In practice, any linear peptide fragment of a protein that is found to react with antibodies raised against the intact protein is termed a continuous epitope. However, this does not imply that the fragment accurately mimics the structure in the protein. The linear peptide may only be part of a larger discontinuous epitope but still reacts with antibodies against the larger discontinuous epitope. Also, not every residue in the continuous epitope will necessarily be able to make

contact with the paratope. The linear epitope is, then, antigenically discontinuous and the division between continuous and discontinuous epitopes is also artificial.

It is generally believed that the majority of epitopes are discontinuous, mainly due to the lack of contiguous stretches in direct peptide linkage at protein surfaces, as ascertained from space-filling models of globular proteins. It is, however, useful to label some antigenic sites as continuous, especially in the context of peptides that cross-react antigenically with a protein.

Most available information on protein immunogenicity is based on the identification of continuous epitopes (Atassi, 1986). This concerns primarily identifying paratope interactions with peptides, which retain only part of their activity after protein fragmentation. This concept has been extended by producing antibodies against synthetic peptides that correspond to linear segments of proteins. These anti-peptide antibodies are often able to cross-react with the complete protein molecule. Furthermore, the immune system can be manipulated to produce antibodies against peptide immunogens that represent 'self' protein antigens, thereby generating an essentially auto-immune response (Dennison, 1989).

Candidates for peptide synthesis may be selected from the protein sequence on the basis of prediction algorithms derived from the observed correlation between the location of continuous antigenic sites and structural parameters. For maximum antibody accessibility, selected sequences should be located on the surface of the protein molecule. Hydrophobic amino acid residues make up the internal part of a protein while hydrophilic side-chains are on the exterior, where they can interact with water. Thus by evaluating relative amino acid hydrophilicities and hydrophobicities in a polypeptide chain, a hydrophilicity profile can be generated. Hopp and Woods (1981, 1983) used such an approach to show that the most hydrophilic portions of a protein tend to correspond with continuous epitopes. Further prognostic information comes from the observed link between peptide chain mobility and antigenicity. Crystallographic

determination of protein structure and nuclear magnetic resonance studies of protein side chains has provided atomic temperature factors or B-values as a measure for mobility. When plotted against residue numbers, B-values provide a profile of high and low mobility, high B-values corresponding to high mobility (Westhof *et al.*, 1984). It appears that the more mobile peptide segments make better candidates for immunisation because recognition of protein epitopes in the form of peptides would occur at such flexible stretches. A variety of other antigenic predictors have been developed, including protein secondary structure, static surface accessibility, sequence variability and chain termination (Van Regenmortel, 1988).

Attention has also been afforded to the role of T cell responses leading to the expression of cytotoxic T lymphocytes, which play an important role in the recovery from viral infection. B and T cells differ in the way they recognise antigens, and the majority of T cells do not recognise the native conformation of protein antigens, but bind to fragments of the antigen (Berzofsky, 1985). Attempts have been made to correlate the structural properties of T cell epitopes with T cell immunogenicity. It appears that T cell epitopes tend to correspond to amphipathic helices and avoid random coiled conformations (DeLisi and Berzofsky, 1985).

Examination of peptide D73-84 indicates generally poor mobility and very poor hydrophilicity, as reflected by its solubility in DMF. According to these predictors peptide D73-84 would be a poor candidate for generating protein-reactive antibodies, yet this was found not to be the case. Indeed, of the 7 different peptide immunogens used in this laboratory, all but one were found to be protein-reactive. In each case, as with peptide D73-84, peptide selection was based on considerations of 3-dimensional structure and function of protein motifs, rather than prediction algorithms. It is now generally believed that a peptide corresponding to virtually any accessible region of a protein can elicit antibodies reactive with that protein (Sutcliffe *et al.*, 1982). An explanation for this is to suggest that in solution a peptide can adopt a variety of interchangeable conformations, some of which mimic those found in the native protein

(Crumpton and Small, 1967). However, since peptides in solution lack any ordered structure (Crumpton and Small, 1967; Atassi and Singhal, 1970), only a small fraction of the fragments can be in the proper conformation. Indeed, the fraction of staphylococcal nuclease fragments in a given conformation at any one time is too small to detect (Sachs *et al.*, 1972). Furthermore, some relatively short-range interactions within a peptide can influence the frequency with which some conformations are adopted. If a peptide can adopt many conformations, what kinds of conformations will elicit antibodies that cross-react with the native protein? A peptide that exactly mimics its native conformation in a protein will bind with high affinity to the immunoglobulin receptor on the appropriate B cell. Conformations resembling the native one may bind with lower affinity, but once in the antibody binding site may be locked into the high affinity conformation. Triggering of an appropriate B cell may thus be accomplished by several of the possible peptide conformations. Also, proteins are not static structures, and individual regions can adopt a limited number of conformations. Therefore, to elicit protein-reactive antibodies, it is probably not necessary to exactly reproduce the native conformation. This may, however, not hold for highly constrained regions in a protein such as a loop, where it may be necessary to mimic conformation more closely.

Peptide D73-84 forms an anti-parallel loop, constituting the active-site associated "flap" of cathepsin D. Despite this obvious conformational restraint, antibodies generated against the peptide were able to recognise the protein coated onto microtitre plates and in free solution, as in the competition ELISA. Lack of targeting of the enzyme on western blots, even under optimally renaturing conditions, may reflect some degree of conformational dependence of antibody binding. Indeed, the better binding found with antibodies generated from conjugated peptide in chickens could, in part, be due to the envisaged higher degree of restraint after conjugation.

Antibodies to peptide D73-84 were also characterised by a lack of detectable inhibition of enzyme activity. This could probably not be attributed to a lack of antibody-antigen binding at the low pH used in the azocasein assay, as shown by

binding under similar conditions in an ELISA. Given the functional importance of the "flap" region in cathepsin D catalysis, and the proven inhibitory activity of antibodies to the "flap" region of renin (Bouhnik *et* al., 1987), lack of detectable inhibition appears to be anomalous. However, when one considers that more than a 450 000-fold molar excess of antibody over renin was required for 40% inhibition, and at best an excess approaching only 2 000-fold was attainable using the azocasein assay, it becomes apparent that enzyme assay sensitivity may be a major limiting factor. This problem may be alleviated by using more potent antibodies. Monoclonal antibodies that putatively recognise the "flap" region of renin (de Gasparo *et al.*, 1987), inhibit the enzyme 100% at an only 8-fold molar excess over enzyme. However, attempts to demonstrate inhibition using high titre affinity purified anti-D73-84 antibodies were unsuccessful.

Highly sensitive lysosomal cysteine proteinase substrates are those based on the amino methyl coumarin leaving group (Barrett and Kirschke, 1981), where a short 2-3 amino acid peptide separates a blocking group from the fluorescent leaving group. These substrates were used to demonstrate 80% inhibition of cathepsin L activity at a 3 235-fold molar excess of antibody over enzyme (Coetzer *et al.*, 1991). Cathepsin D, however, has an extended active site, sufficient to accommodate 7-8 amino acid residues of a peptide substrate (Tang, 1979). Indeed, the rate of peptide hydrolysis shows marked dependence on the amino acid composition and length of the peptide substrate. An induced fit model of catalysis suggests that, within a series of related substrates of varying length, the rate of hydrolysis (k_{cat}) can vary by several orders of magnitude, in contrast with the binding constant (K_m) which is comparatively insensitive (Pearl, 1985). Generally, the rate enhancement for a larger substrate over that observed for a smaller substrate is cumulative.

To accommodate the requirements of the extended substrate binding cleft of cathepsin D, larger peptide substrates, which include the fluorescent methoxy naphthylamine and amino methyl coumarin leaving groups, have been developed. The company holding these substrates, however, no longer exists and no alternative source could be found. Another potential fluorescent substrate is

that utilised for the renin assay. Renin specifically hydrolyses angiotensinogen to form angiotensin I, and a fluorogenic peptide substrate was developed to incorporate the renin cleavage site that occurs in angiotensinogen (Wang *et al.*, 1993). Cathepsin D has been assayed with a radioimmunoassay version of this assay (Takahashi and Tang, 1981), implying that the fluorescent substrate should also be suitable. The cost of this substrate, however, rendered such an approach prohibitive. Proteinaceous substrates chemically modified by the addition of fluorescent groups (haemoglobin-fluorescein isothiocyanate (FITC) and casein-FITC) were also tested for enhanced sensitivity, but were found to be no better than the azocasein in the presence of urea (results not shown). A synthetic chromophoric substrate extensively used for the kinetic characterisation of aspartic proteinases (Dunn *et al.*, 1986) was also found unsuitable due to lack of sensitivity. Radio-labelled protein substrates may have offered a more sensitive alternative, but financial constraints prohibited this avenue being explored.

The rationale used for choosing a peptide which would elicit immunoinhibiting antibodies, was based on sound consideration of the three-dimensional and physico-functional characteristics of cathepsin D. The antibodies generated were ultimately unable to produce demonstrable enzyme inhibition, but this aspect remains inconclusive until a more sensitive assay can be used.

CHAPTER 4

THE DISTRIBUTION OF LYSOSOMAL PROTEINASES IN A HUMAN BREAST EPITHELIAL CELL LINE AND ITS c-Ha-*Ras* TRANSFORMED COUNTERPART

4.1 Introduction

The study of biological processes has been greatly enhanced with the development of *in vitro* cell culture techniques, and indeed the field of cancer biology has profited greatly from such developments. Tumour cell lines can be finely characterised in terms of their genetic make-up, growth characteristics, ultrastructure, metabolism, enzymology and invasiveness. Much of what is known about the significant role played by uncontrolled proteolysis, during tumour metastasis, was elucidated in this way. A singular drawback in breast cancer research has, however, been the lack of a reliable source of normal human epithelial cells to delineate the sequential states of progression to the malignant state, and to act as true control cells under experimental conditions. Until recently the available immortal human (mammary epithelial) cell lines have been altered in some way, e.g. by exposure to benz(*a*)pyrene, by SV-40 (simian virus-40) genetic information, or by isolation of cell lines from patients with tumours (Soule *et al.*, 1990). The establishment of the immortal MCF-10 cell line, that arose spontaneously (Soule *et al.*, 1990) without chemical or viral intervention, has finally provided the ideal "normal" control and a cell line into which various oncogenes may be transfected to allow the progression to the malignant phenotype to be studied.

The work reported in this chapter, as that in Chapter 5, focuses on the use of this cell line, and its oncogenically transformed sister cell line, in studies of the intracellular distribution of proteinases and their role played in invasion. This study was undertaken in collaboration with Dr Bonnie Sloane (Department of Pharmacology, Wayne State University, Detroit, Mi.), who provided the cell lines, and Dr Edith Elliott (Department of Biochemistry, University of Natal, Pietermaritzburg).

Mortal human diploid mammary epithelial cells (MCF-10M) were obtained from a mastectomy performed on a 36 year old parous premenopausal woman with extensive fibrocystic disease, and no evidence of atypia (Soule *et al.*, 1990). Spontaneous immortalisation occurred during growth of these cells in a low calcium medium, giving rise to attached (MCF-10A) and floating (MCF-10F) cell lines, both of which continued to proliferate at normal calcium concentrations. Cytogenetic analysis prior to immortalisation showed normal diploid cells, although later passages showed minimal rearrangement and near-diploidy. The MCF-10A cell line is oestrogen receptor negative, and was characterised as normal breast epithelium by the following characteristics: lack of tumourgenicity in nude mice; growth in culture that is controlled by hormones and growth factors; three-dimensional growth in collagen; lack of anchorage independent growth; and dome formation in confluent cultures (Soule *et al.*, 1990). The MCF-10 cell line is the first spontaneous line of cultured breast epithelium (Tait *et al.*, 1990) for which there is direct evidence that the normal diploid chromosome pattern of the original explanted tissues was retained for over a year of growth in culture.

Transfection of MCF-10A with the activated (mutated) c-Ha-*ras* oncogene resulted in cell transformation (Basolo *et al.*, 1991), the new cell line being designated MCF-10AneoT. A possible link between the expression of the activated *ras* oncogene and the appearance of *in vitro* migratory and invasive capacities was explored. MCF-10AneoT cells exhibited increased transforming growth factor-α expression and secretion (Ciardiello *et al.*, 1990), anchorage-independent growth, hormone and growth factor independence, alterations in the three-dimensional pattern of growth in collagen matrix, increased collagenolytic activity, and tumourgenicity in nude mice (Basolo *et al.*, 1991). Furthermore, another study indicated enhanced chemotactic and chemokinetic activities, an increased ability to invade matrigel barriers, and the ability to form large colonies in matrigel, in contrast to MCF-10A cells transfected with the neomycin resistance gene alone or with the normal *ras* proto-oncogene (Ochieng *et al.*, 1991). The insertion of activated c-Ha-*ras* alone is sufficient to induce these

phenotypes, which is consistent with full malignant transformation. It has been shown that the *ras* gene family induces invasive phenotypes in human breast tissues (Clark *et al.*, 1988) and MCF-7 human breast carcinoma cells transfected with v-Ha-*ras* bypass the oestrogen requirement for tumourgenicity and show higher invasive properties than non-transfected cells (Albini *et al.*, 1986). Moreover, human bronchial epithelial cells transformed with v-Ha-*ras* express more type IV collagenase with a concomitant increase in their invasive properties (Ura *et al.*, 1989). However, transfection of the activated *ras* oncogene does not confer the metastatic phenotype to C127, a non-metastatic mouse carcinoma cell line, indicating the dependence of the developed phenotype and transformation on the recipient cell type (Muschel *et al.*, 1985). The enhancement of the chemotactic potential of *ras*-transformed human breast epithelial cells is probably due to an increase in the signal level of the transducing pathway mediated by the *ras* protein, p21. Chemoattractants bind to receptors on the surface of tumour cells and activate the phosphatidylinositol signal transduction pathway, for which p21 is a likely transducer (Okajima and Ui, 1984).

The transition from normal breast epithelium to fully malignant, invasive tumours occurs via a sequence of stages from benign hyperplasia through atypical hyperplasia to carcinoma *in situ* and eventually to the metastatic cell type, this spectrum of pathological change being termed proliferative breast disease. MCF-10AneoT cells transplanted into nude mice and maintained for more than a year, gave rise to a sequence of histological changes that resemble proliferative breast disease in humans, including the development of carcinomas (Miller *et al.*, 1993). This model for proliferative breast disease has been extended by successive transplantations of cells derived from the xenografts, such that a grading system of proliferative breast lesions could be established, from grade 0 (simple epithelium) to grade 5 (invasive carcinoma) (Dawson *et al.*, 1996). This xenograft model provides a system for the study of the conversion of breast epithelial cells to malignant cells. It must, however, be borne in mind, in the context of the present study also, that MCF-10AneoT cells contain the activated Ha-*ras* oncogene, which is not highly characteristic of female or male breast carcinoma

(Rochlitz *et al.*, 1989), although over-expression of normal *ras* is common in breast cancer (Miller *et al.*, 1993).

Malignant progression, as reflected by the MCF-10AneoT xenograft model, is associated with increases in expression, alterations in subcellular distribution and increases in secretion of lysosomal proteases. The stage at which these alterations occur and whether they are causally linked to progression remains in question. Transfection of murine 3T3 fibroblasts with the *ras* oncogene leads to an increase in the expression of cathepsin L mRNA and protein, and secretion of procathepsin L is observed (Denhardt *et al.*, 1987). In the same cells mRNA transcripts for cathepsin B are increased (Zhang and Schultz *et al.*, 1992), as is the case for murine (Qian *et al.*, 1989) and human tumours (Moin *et al.*, 1992). Similar findings were revealed in an analysis of aspartic and cysteine proteinases in sequentially immortalised and c-Ha-*ras*-transformed rat embryo fibroblasts. The expression of proteinases increased with each degree of transformation, cathepsin D accounting for most of the secreted activity, and cathepsin L and, to a lesser extent, cathepsin B accounting for most of the intracellular activity (Solovyeva *et al.*, 1995). It is notable that the p21 *ras* oncoprotein is a potent inhibitor of cathepsins B and L (Hiwasa *et al.*, 1987), and shows sequence homology with the cysteine proteinase inhibitors, the cystatins, although the significance of this inhibition is unknown.

Changes in the levels of proteases expressed after *ras* transformation is the result of a series of poorly understood events, beginning with the expression of *ras* proteins and their subsequent association with the inner cell membrane, resulting in the induction of *ras*-mediated signal pathways. *Ras*-induced events occur under serum-free conditions (Bar-Sagi and Feramisco, 1986), indicating that ligand-binding is not a prerequisite, a finding substantiated by the growth factor-independence of MCF-10AneoT cells. *Ras* regulates at least two downstream signal transduction pathways. The first induces the constitutive reorganisation of actin (Bar-Sagi and Feramisco, 1986), mediated downstream through members of the *rac* and *rho* family of guanosine triphosphate-binding (GTP) proteins, which are implicated in the control of cell architecture (Symons, 1996). The *rho*

family of proteins increases stress fibre formation (Prendergast and Gibbs, 1993) (stress fibres extend into the cell from cell-surface focal adhesions where integrins make contact with the extracellular matrix), while *rac* induces the appearance of membrane ruffles and fluid-phase pinocytosis (Ridley *et al.*, 1992). Induction of the *rho/rac* pathway by *ras* suggests that actin cytoskeletal rearrangement may play a key role in *ras* transformation. The second, the mitogen-activated protein kinase pathway, regulates a wide variety of cellular processes such as translation, transcription, differentiation and the cell cycle (Prendergast and Gibbs, 1993). Perturbation of any of the events affected by *ras* is consistent with cell transformation.

In this chapter the effects of *ras*-transformation of normal human breast epithelial cells on the distribution of selected lysosomal proteinases and, by association, of lysosomes themselves, will be reported. This, however, should be preceded by an understanding of the trafficking of lysosomal enzymes under 'normal' conditions. Lysosomal enzymes, secretory proteins and plasma membrane proteins, are synthesised by membrane-bound polysomes on the rough endoplasmic reticulum (RER), and are vectorially transported across the RER membrane into the lumen, a process mediated by a signal peptide on the nascent polypeptide (Walter *et al.*, 1984). Lysosomal enzymes, as well as most of the secretory proteins and plasma membrane proteins, undergo cotranslational glycosylation of selected asparagine residues, involving the *en bloc* transfer of a large pre-formed oligosaccharide. In the RER the signal peptide is cleaved and the oligosaccharide is trimmed (Kornfeld and Kornfeld, 1985). The proteins then move, by vesicular transport, to the Golgi stack where they undergo a variety of post-translational modifications and are sorted to their proper destinations. Lysosomal enzymes acquire phosphomannosyl residues (Kornfeld, 1986), the resultant phosphomonoesters serving as a recognition marker that leads to high affinity binding to M-6-P receptors in the Golgi (von Figura and Hasilik, 1986). In this way the lysosomal enzymes are segregated from the proteins destined for secretion. The ligand-receptor complex exits the Golgi via a coated vesicle which fuses with an acidic pre-lysosomal sorting compartment (late endosome) where the low pH facilitates dissociation of enzyme from the receptor (Geuze *et al.*,

1985). The receptor is then free to recycle to the Golgi for further rounds of enzyme transport, while the dissociated enzymes are transferred to the lysosome (Brown *et al.*, 1986). A small proportion of lysosomal enzymes, usually 5-20%, is secreted before delivery to lysosomes. A portion may bind to M-6-P receptors on the cell surface and be internalised and delivered to lysosomes (Valdutiu and Rattazzi, 1979).

In addition to oligosaccharide processing, lysosomal enzymes undergo further proteolytic processing. All lysosomal enzymes studied to date are synthesised as preproenzymes. The pre-piece or signal peptide is cleaved after transport into the RER, while removal of the propiece appears to be initiated in the pre-lysosomal compartments (Gieselmann *et al.*, 1983), a process generally synonymous with enzyme activation.

Studies have shown that transfection of normal breast epithelial cells with the activated *ras* oncogene results in transformed cells capable of growth in nude mice (Miller *et al.*, 1993) and, in some cases progression to invasive carcinoma (Dawson *et al.*, 1996). The intra- and extracellular events leading to full transformation by *ras* are complex and poorly understood. In this chapter an aspect of this event which has been found to be critical to invasion and metastasis, namely abnormal enzyme trafficking that results in inappropriate and uncontrolled proteolysis, is described.

4.2 Cell culture

The MCF-10A and MCF-10AneoT cell lines were cultured exactly as in the Sloane laboratory to ensure a cell phenotype consistent with that laboratory, as this protocol differs slightly from that used by other workers.

4.2.1 Reagents

The MCF-10A and MCF-10AneoT cell lines were donated by Dr Bonnie Sloane (Wayne State University, Detroit, Mi.) as part of a research collaboration. Dulbecco's minimal essential medium (DMEM):Ham's F-12 medium, hydrocortisone, insulin, penicillin G:streptomycin (10 000 units and 10 mg/ml

respectively), trypsin-EDTA solution (X 1) and Hanks' balanced salt solution (HBSS) were from Sigma Chemical Co., St. Louis, Mo.; fungizone (0.25 mg/ml) and equine serum were from Gibco, Paisley, U.K. and EGF was from Biomedical Technologies, Inc., Stoughton, Ma. Where applicable, components were reconstituted and sterilised by filtration through a 0.22 μm filter. Sterile plastic culture-ware was from approved suppliers. Glassware was washed in 7X-PF (ICN Biomedicals Ltd, Irvine, Scotland) detergent solution and copiously rinsed in tap water and double distilled water before autoclaving.

Complete medium (DMEM:Ham's F-12, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), EGF (20 ng/ml), equine serum (5%, v/v), penicillin G:streptomycin (100 units/ml and 100 μg/ml respectively), fungizone (0.25 μg/ml), pH 7.3). Powdered medium and sodium hydrogen carbonate (1.2 g) were consecutively dissolved in about 900 ml of ultra-pure water (Milli-Q Plus, Millipore, Microsep, Durban), adjusted to pH 7.3 with NaOH and made up to 1 litre. Medium was sterile filtered through a 0.22 μm filter and equine serum (52.6 ml), hydrocortisone (2.14 ml), insulin (4.2 ml), EGF (0.43 ml), penicillin G:streptomycin (10.0 ml) and fungizone (1.1 ml) were aseptically added. Complete medium was stored at 4°C.

4.2.2 Procedure

Cells were maintained in 25 cm^2 flasks in complete medium in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were fed every 3-4 days and passaged upon reaching confluence. To this end cells were washed with HBSS (3 X) rinsed in a small amount of trypsin-EDTA, leaving a thin film covering the monolayer, and incubated at 37°C until microscopic inspection showed that cells had detached. Cells were diluted in complete medium and split in a ratio of 1:3.

4.3 Antibodies

Antibodies to cathepsin D were raised in chickens to take advantage of the extremely high antibody yield from eggs and, as found in our laboratory, the relatively higher titre antibody generated by chickens as compared to rabbits.

Two chickens were each immunised with 100 µg of porcine cathepsin D (Section 3.2), 50 µg into each breast muscle. The antigen was triturated in a 1:1 ratio with Freund's complete adjuvant (Difco) at week 0, and in Freund's incomplete adjuvant (Difco) at 1, 2, 4 and 6 weeks and for the monthly boosters, thereafter. Eggs were collected on a daily basis and IgY extracted from the yolks (Section 2.5) at the time points required. Progress of immunisation was monitored by the ELISA (Section 2.6) and specificity ascertained by western blotting (Section 2.7).

Examination of the progress of immunisation curve for a representative chicken indicates a peak of antibody production after 8 weeks (Fig. 18), with a titre of 0.061 µg/ml. Antibody specificity as determined by western blotting indicated specific targeting of single-chain cathepsin D in the TPP fraction, and the 30 kDa heavy-chain component in all fractions (Fig. 19). Non-immune IgY displayed no targeting (not shown).

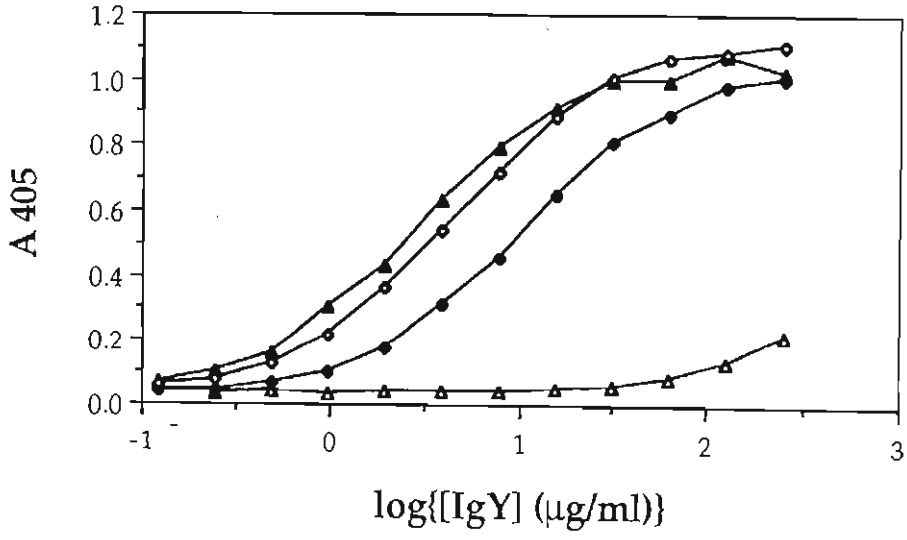

Figure 18. ELISA of the progress of immunisation with porcine cathepsin D in a chicken. Porcine cathepsin D was coated at 2,0 µg/ml to a microtitre plate and incubated with serial two-fold dilutions of chicken anti-porcine cathepsin D IgY collected after 4 (◆), 8 (▲) and 12 weeks (◊) and non-immune IgY (Δ). Binding was visualised by incubation with rabbit anti-chicken IgY-HRPO secondary antibodies as described in Section 2.6.

Figure 19. Western blot of pure and crude fractions of porcine cathepsin D incubated with chicken anti-porcine cathepsin D IgY.
Fractions were electrophoresed on a reducing SDS 12.5% gel (Section 2.4) and electroblotted onto nitrocellulose. The blot was incubated in 20 µg/ml of chicken anti-porcine cathepsin D IgY, and developed with rabbit anti-chicken IgY-HRPO (Section 2.7). Samples were: a) crude supernatant; b) acid supernatant; c) TPP fraction and d) purified porcine cathepsin D (Section 3.2).

For immunofluorescence microscopy (Section 4.5), the following antibodies, which were generous gifts from a number of different researchers, were used: affinity purified rabbit anti-human cathepsin B was from Dr Lukas Mach (Universität für Bodenkultur, Vienna), rabbit anti-peptide antibodies recognising the human cathepsin H active site region, CHKTPDKVNHAV (Coetzer, 1992), was from Dr Theresa Coetzer (Department of Biochemistry, University of Natal), chicken anti-sheep cathepsin L-stefin B complex antibodies were from Frieda Dehrmann (Department of Biochemistry, University of Natal), and rabbit anti-pro cathepsin D was from Dr Bonnie Sloane (Department of Pharmacology, Wayne State University, Detroit, Mi.). Secondary antibodies (FITC-labeled rabbit anti-chicken IgG and tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG) and saponin were from Sigma Chemical Co., St. Louis, Mo.; BSA (Fraction V) was from Boehringer Mannheim and Anti-Fade® immunofluorescence mounting medium was from Molecular Probes Inc., Eugene, Or.

For immunoelectron microscopy (Section 4.9) the following antibodies were used: antibodies to cathepsin D and cathepsin B (as described above); antibodies to LAMP-2 (lysosome-associated membrane protein), a lysosome-specific marker (Chen *et al.*, 1985), were kindly donated by Dr Gareth Griffiths, EMBL, Heidelburg, Germany; and the anti-serum to chicken IgY was prepared by Dr Theresa Coetzer, Department of Biochemistry, University of Natal.

4.4 LysoTracker™ labeling of cells

LysoTracker™ probes are a series of fluorescent acidotropic probes for labeling and tracing acidic organelles in cultured cells. They selectively accumulate in cellular compartments with low internal pH, and exhibit bright fluorescence and good photostability (Bioprobes Bulletin #21, Molecular Probes Inc., Eugene, Or.).

4.4.1 Reagents

Complete medium. Prepared as in Section 4.2.1.

LysoTracker™ in complete medium (1:7157). LysoTracker™ (Red DND-99, Molecular Probes Inc., Eugene, Or.) (2 µl) was added to complete medium (1 ml), and 35 µl of this stock solution added to complete medium (465 µl).

4.4.2 Procedure

Cells were seeded (Section 4.2) at a low density into 12-well Nunc Multiwell plates, each well containing a 15 mm sterile glass coverslip. Before confluence was reached, medium was aspirated from the cells, diluted LysoTracker added to the cells (300 µl) and allowed to incubate (30 min). Incubation medium was aspirated and LysoTracker™ allowed to wash out in complete medium (2 min). Coverslips were inverted onto a small drop of complete medium on a glass slide, sealed with clear nail varnish, and viewed in an Olympus BH2 microscope equipped with a BH2-RFC epifluorescence attachment, fitted with a 460 nm interference filter and a 520 nm barrier filter. Representative cells were photographed with Ilford XP2 400 film (Section 4.11).

4.5 Immunofluorescence microscopy

Immunofluorescence microscopy allows for the assessment of the distribution of an antigen in a tissue or cell, by combining the high selectivity of antigen detection with low magnification light microscopy. This technique was used to determine the relative distributions of proteolytic enzymes in MCF-10A cells. The antibodies used are described in Section 4.3.

4.5.1 Reagents

Phosphate buffered saline (PBS-F) (8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 2.68 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3). Na_2HPO_4 (1.145 g), KH_2PO_4 (0.2 g), NaCl (7.99 g), KCl (0.199 g), $CaCl_2$ (0.147 g) and $MgCl_2$ (0.1015 g) were dissolved in about 800 ml of dist.H_2O, adjusted to pH 7.3 if necessary and made up to 1 litre. The PBS used in immunofluorescence labeling studies differed from that used in electron microscopy immunolabeling, in that calcium and magnesium were included, and it will therefore be referred to as PBS-F.

16% (m/v) Paraformaldehyde stock solution. Paraformaldehyde (BDH, Poole, England) (16 g) was dissolved in dist.H_2O (100 ml), warmed to 60°C and cleared with a minimum amount of 1 M NaOH. The solution was stored at -10°C until required.

3.7% (v/v) Paraformaldehyde in PBS-F. Stock solution (3.47 ml) was added to PBS-F (11.53 ml) and adjusted to pH 7.3 with 1 M HCl.

2% (m/v) Bovine serum albumin in PBS-F (BSA-PBS-F). Bovine serum albumin (0.8 g) was dissolved in PBS-F and made up to 40 ml.

0.1% (m/v) Saponin in PBS-F. Saponin (0.1 g) was dissolved in PBS-F and made up to 100 ml.

5% Foetal calf serum. Foetal calf serum (0.5 ml) was mixed with PBS-F and made up to 10 ml.

Antibodies. Chicken anti-porcine cathepsin D IgY was used at 200 µg/ml, rabbit anti-human cathepsin B IgG at 40 µg/ml, rabbit anti-human cathepsin H peptide at 300 µg/ml, chicken anti-sheep cathepsin L-stefin B complex at 300 µg/ml, rabbit anti-pro cathepsin D at 300 µg/ml, rabbit anti-chicken IgG-FITC at a 1/320 dilution, and goat anti-rabbit IgG-TRITC at a 1/250 dilution.

4.5.2 Procedure

Cells were seeded (Section 4.2) at a low density into duplicate 12-well Nunc Multiwell plates, each well containing a 15 mm sterile glass coverslip. Before confluence was reached, cells were washed three times with warmed (37°C) PBS-F, fixed (10 min., RT) with 3.7% formaldehyde in PBS-F and washed again in PBS-F (3 X). Non-specific binding sites were blocked by incubating cells in BSA-PBS-F (45 min., RT). Coverslips were incubated in the primary antibody (1 h, RT) diluted in saponin in PBS-F. After six rapid washes with saponin in PBS-F, 5% foetal calf serum block solution was applied (30 min., RT). Cells were then incubated in the appropriate secondary antibody (1 h, RT) washed six times in saponin in PBS-F, refixed in 3.7% formaldehyde (10 min., RT), washed three times in PBS-F followed by dist.H_2O and the coverslips allowed to dry. The coverslips were mounted face-down in a minimum amount of Anti-Fade®, sealed with clear nail varnish, and viewed in an Olympus BH2 microscope equipped with a BH2-RFC epifluorescence attachment, fitted with a 460 nm interference filter and a 520 nm barrier filter. Representative cells were photographed with Agfacolour XRG 200 or Ilford XP2 film (Section 4.11). A non-immune antibody preparation was substituted for immune antibody at a concentration equal to the lowest dilution used for the immune antibody. Non-immune incubations were assessed for acceptably low background fluorescence; since this was generally obtained, non-immune controls are not shown in the Results (Section 4.11).

4.6 *In situ* embedding in Lowicryl of cultured cells

The use of immunocytochemical methods has traditionally been the domain of cell biologists, probably due to the apparent complexity and expense of such

methodology. Recent advances in these techniques, and the commercial availability of almost all relevant reagents, has made this technology easily accessible. It remains, however, that many biochemical laboratories are resistant to this fundamental expertise, and for this reason a review of some aspects of immunocytochemistry is offered.

Lowicryl resins are acrylate-methacrylate mixtures that form a vinyl type of carbon backbone during polymerisation (Carlemalm *et al.*, 1982; Carlemalm *et al.*, 1985). The resins were developed to obtain embedding media of low viscosity that can be used at low temperatures to examine the fine structure of minimally fixed tissue by scanning transmission electron microscopy. Subsequently, the Lowicryl resins were found to be eminently suited to colloidal gold methods. Implicit to this approach, is the concept of antigenicity and the belief that maintenance of low temperature during the processing of specimens is a valuable aid to not only the preservation of ultrastructure, but also the retention of soluble components in their *in vivo* positions and conformations.

It is commonly believed that lowering the temperature prevents protein denaturation. In fact, proteins are most stable at a temperature close to that at which they are thermally denatured, and the thermodynamic force which imposes the native conformation upon a protein (hydrophobic "bonding"), is weakened at lower temperatures. This makes proteins more susceptible to the denaturing action of organic solvents (Sjöstrand, 1990). Paradoxically, experiments have revealed that low temperatures do reduce the rearrangement of proteins in non-aqueous liquids (Carlemalm *et al.*, 1982), and have a positive effect on immunolabeling by increasing labeling density and decreasing background staining (Roth *et* al., 1981; Armbruster *et al.*, 1983). This can be explained by taking into account the effects of the increased viscosity of the medium at a lower temperature. Denaturation (unfolding) of a protein is caused by the translational motions of parts of the polypeptide chain, the motion being restricted by the peptide bonds. Denaturation is therefore a diffusion phenomenon, and increasing the viscosity of the denaturing solvent or resin would therefore slow down protein denaturation by slowing down diffusion. It

stands to reason, therefore, that the length of exposure to denaturing media that tissues can tolerate without extensive protein denaturation is limited. The shortest possible dehydration and embedding time should be used to limit diffusion/denaturation. The viscosity of the medium probably delays protein denaturation more than it hinders diffusion of organic solvents and resin molecules, due to the large difference between the mean mass of the mobile parts of the crosslinked polypeptide chain and that of the solvent and resin molecules (Sjöstrand, 1990).

Lowicryl resins have properties that lend themselves well to use at low temperatures: miscibility with different organic solvents, low viscosity, and ability to be polymerised by ultraviolet light. Lowicryl K4M is polar and Lowicryl HM20 is apolar, and these can be used at -35°C and -50°C respectively, while Lowicryl K11M and Lowicryl HM23 are the respectively polar and non-polar ultra-low temperature equivalents (Acetarin *et al.*, 1986). Lowicryl K4M can be kept in a partially hydrated state during dehydration and infiltration, since the resin can be polymerised with up to 5% (m/m) water in the block. Low temperature embedding may be achieved by the progressive lowering of temperature (PLT) technique (Kellenberger, 1985), which involves stepwise reductions in temperature as the concentration of the dehydrating agent is increased. A temperature is selected which is just above the freezing point of the concentration used in the step before, as this is the concentration of the dehydrating agent contained in the specimen when introduced into the next higher concentration of the dehydration series. The PLT method gives good ultrastructural preservation of tissue (Roth *et al.*, 1981); indeed, rat liver embedded in Lowicryl K4M using PLT showed for the first time the presence of an extensive *trans*-Golgi tubular network (Roth *et al.*, 1985). Furthermore, the extraction of proteins and membrane lipids from biological materials embedded at low temperature is reduced (Weibull and Christiansson, 1986), and the Lowicryls can be used with very low glutaraldehyde concentrations for short times (Hobot, 1989).

Although there are good theoretical reasons for the use of low temperature during embedding, when it is desired to preserve the structure and activity of particular proteins in biological specimens, there are also more practical considerations to address. Polymerisation is an exothermic reaction, and a comprehensive study (Ashford *et* al., 1986) showed that there is a significant release of thermal energy during this process. At -38°C there is an 8-12°C rise in temperature in 7.5 ml of polymerising resin. Unexpectedly, however, a further more dramatic temperature rise occurs when the apparently polymerised resin block is taken out of the low temperature polymerisation apparatus. Here the temperature rises rapidly to ambient and overshoots to as much as 37°C. This was unaffected by the length of time that the specimens had been left under ultraviolet light, but was influenced by the temperature at which polymerisation had initially been carried out, being most pronounced after polymerisation at the lowest temperatures. It appears that in Lowicryl K4M one of the polymerisation reactions remains incomplete at low temperatures, and then proceeds rapidly when the temperature rises above a certain level. Such brief but dramatic rises in temperature can be potentially damaging to proteins, but can be avoided by warming specimens up slowly in contact with heat sinks, and is most easily controlled with small volumes of resin (Weibull, 1986). In this study only 3 ml of resin was polymerised in a 35 mm dish, allowing the approximately 0.3 mm deep resin a large surface area in contact with the heat sink.

An important distinction should be made between Lowicryl K4M (and HM20) and epoxide resins such as Epon, and that is the effect of specimen-related relief in Lowicryl sections (Kellenberger *et al.*, 1986). Thin Lowicryl sections of aldehyde-fixed biological material show a relief estimated to be 2-6 nm, whereas those in Epon are two to three times smoother (Kellenberger *et al.*, 1987). Epoxy resins are able to form covalent bonds with biological material, particularly proteins, and therefore co-polymerisation occurs instead of polymerisation of the resin. Without co-polymerisation, section cutting resembles surface cleavage, which tends to follow the areas of least resistance, i.e, the interfaces between resin and protein, which would expose epitopes. This is probably an important reason for the success of Lowicryl in immunolabeling procedures.

Comparison of labeling after PLT and embedding in Lowicryl K4M with that achieved after optimal embedding in a popular hydrophilic resin, LR White, indicated very little difference in either ultrastructure or gold particle count when high glutaraldehyde (>1%) concentrations were used, even for a short time (15 min). However, this was not true after minimal glutaraldehyde concentrations (<0.1% for 15 min), where PLT and Lowicryl K4M gave superior ultrastructural preservation, and would thus be the choice for labile antigens (Newman and Hobot, 1989). It is interesting to note that theoretical predictions for labeling on melted cryosections, where the biological material is in a fully hydrated and therefore in an antigenically optimal state, are that 10 to 30 times more labeling should be obtained. Yet, for reasons not fully understood, only factors between 0.5 and 2 have thus far been observed (Kellenberger *et al.*, 1987). A newly commercialised hydrophilic, methacrylate resin, Unicryl, has been purported to give superior ultrastructure (Scala *et al.*, 1992), as well as significantly stronger immunolabeling than Lowicryl K4M, perhaps providing a resin alternative to ultracryomicrotomy (Bogers *et al.*, 1996), which still requires a high enough degree of manual skill so as not yet to be considered a routine technique.

During this investigation exclusive use was made of *in situ* flat embedding of cell monolayers in culture dishes. A more accurate picture of protease distribution can thus be obtained, than would be the case if cells were released from the plastic, allowed to round up and processed as a pellet. Flat embedding with Lowicryl, as opposed to embedding in gelatin capsules, however, presents special problems, most notably the inhibition of polymerisation by atmospheric oxygen due to the large resin surface in contact with the air. A variety of methods have been used to overcome this problem, including growing cells on detachable chamber tissue culture slides (Ballou *et al.*, 1985) or in Lab-Tek flaskettes (Bou-Gharios *et al.*, 1986), but the detachment of the polymerised cell layer and resin from the glass is difficult (Carlemalm and Villiger, 1982). For the present investigation, a relatively airtight polymerisation chamber was constructed with N_2 entry and exit ports, so as to create an anaerobic environment within the

chamber itself. It has been suggested (Carlemalm and Villiger, 1982) that routinely used cell culture plasticware is unsuitable for *in situ* embedding, due to the susceptibility of the plastic to corrosion by Lowicryl resin. This problem was not experienced during these investigations, and no corrosion-resistant alternative was used.

In their comprehensive study to find the optimal conditions for specimen processing, embedding and labeling, Robertson *et al.* (1992) addressed the question of fixation, i.e. the concentrations and duration of fixation which will result in the preservation of adequate morphology and antigenicity and hence be suitable for immunocytochemistry. Specimens (including a cell monolayer) fixed for 60 min in a mixture of 2% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde gave uniformly good preservation and labeling characteristics, while specimens fixed for 30 min or 60 min in 4% paraformaldehyde often showed similar but non reproducible results. A mixture of 2% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde for 60 min was therefore chosen for fixation.

Immunolabeling at the electron microscopic level allowed for the precise subcellular localisation of proteinase antigens in MCF-10 cells. This data was used to confirm and expand on that gained from immunofluorescence studies.

4.6.1 Reagents

Phosphate buffered saline (PBS-E, 0.01 mM NaH_2PO_4, 0.15 mM NaCl, 2.5 mM KCl, pH 7.3). NaH_2PO_4 (1.38 g), NaCl (8.76 g) and KCl (0.186 g) were dissolved in about 800 ml of dist.H_2O, adjusted to pH 7.3 if necessary and made up to 1 litre. The PBS used in immunolabeling for electron microscopy is referred to as PBS-E.

400 mM HEPES stock (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid), pH 7.3. HEPES (10.92 g) was dissolved in 90 ml of dist.H_2O, adjusted to pH 7.3 with NaOH and made up to 100 ml. The solution was aliquotted and stored

frozen. Prior to use, the buffer was diluted to 200 mM, and the pH adjusted if necessary.

Fixative (2% (v/v) paraformaldehyde, 0.05% (v/v) glutaraldehyde in 200 mM HEPES, pH 7.3). Paraformaldehyde (1.25 ml of 16% stock, Section 4.5.1) and glutaraldehyde (20 µl of 25% stock, Merck, Darmstadt) were added to about 8 ml of 200 mM HEPES, the pH adjusted to 7.3 if necessary and the solution made up to 10 ml with 200 mM HEPES.

30% (v/v) Ethanol. Ethanol (3 ml, AR) was made up to 10 ml with dist.H_2O.

50% (v/v) Ethanol. Ethanol (5 ml, AR) was made up to 10 ml with dist.H_2O.

75% (v/v) Ethanol. Ethanol (7.5 ml, AR) was made up to 10 ml with dist.H_2O.

95% (v/v) Ethanol. Ethanol (9.5 ml, AR) was made up to 10 ml with dist.H_2O.

Lowicryl K4M resin. Lowicryl K4M resin (Chemische Werke Lowi, Germany) was formulated essentially as described in the manufacturers handbook. Cross-linker A (2.7 g) and Monomer B (17.3 g) were pipetted into a darkened glass container on a balance in a fume-hood. Initiator C (0.1 g of solid) was weighed separately, added to the mixture and dissolved by bubbling N_2 through the resin, which serves also to expel O_2. Care was taken to avoid inhalation or resin fumes and skin contact with the resin as dermatitis is common. It was found that a batch of resin could be stored for at least 3 months in the dark at room temperature.

4.6.2 Procedure

Cells were grown (Section 4.2) in 35 mm petri dishes and upon reaching confluence were washed with PBS-E (3 X), fixed (RT, 60 min) and washed with 200 mM HEPES (3 X). Cells were subjected to progressive lowering of temperature dehydration: 30% ethanol (0°C, ice bath in a cold-room, 10 min, 3 ml), 50% ethanol (-20°C, 3 parts crushed ice + 1 part NaCl placed in a freezer-

room, 10 min, 3 ml), 75% ethanol (-35°C, 1.2 parts ice + 2 parts $CaCl_2.6H_2O$ placed in the pre-cooled polymerisation unit, 10 min, 3 ml), 95% ethanol (-35°C, as before, 10 min, 3 ml), 100% ethanol (-35°C, as before, 10 min, repeated once, 3 ml). Petri dishes were agitated at least twice during each 10 min step. Cells were infiltrated with a 1:1 mixture of resin and 100% alcohol (-35°C, 30 min, 2 ml) followed by a 2:1 mixture of resin and 100% alcohol (-35°C, 1 h, 2 ml), pure resin (-35°C, 1 h, 2 ml) and again pure resin (-35°C, overnight, 2 ml). The following day the petri dishes were placed in heat sinks, resin was replaced (3 ml) and the unit flooded with N_2. Resin was allowed to polymerise in a N_2 atmosphere at -35°C in indirect ultraviolet radiation (360 nm, 24 h) followed by direct ultraviolet radiation (48 h). Petri dishes were placed on a sunny windowsill for at least 3 days until the resin had properly hardened.

Heat sinks consisted of aluminium discs (8 cm ϕ x 2 cm) with a 3 mm deep excavation, slightly larger in diameter than a 35 mm petri dish, turned out of the middle of the disc. Alcohol was used to provide thermal conduction between the dish and heat sink. The polymerisation unit was constructed by the Mechanical Instrument Workshop, University of Natal, Pietermaritzburg. It consists of a small, top-loading chest freezer, fitted with a cooling unit calibrated to -35°C and a slow-speed fan to dissipate heat gradients within the unit. All six inside surfaces of the chest were covered in aluminium foil to provide a reflecting surface for incident ultraviolet radiation and the lid was fitted with two ultraviolet tube lamps (43 cm, Hitachi) lying parallel to each other. The lamps were installed only as they were about to be switched on, as it was found that they would not start if pre-cooled. An N_2 entry port was fitted on the side near the bottom of the chest and an exit port on the opposite side also near the bottom. This port was connected to a vertical hose up to a height corresponding to the top of the interior of the chest. N_2 is heavier than air and will thus fill from the bottom, displacing air out from the top through the tube. A framework with an height-adjustable wire shelf was installed into the unit, and an optimal distance (25 cm) of sample from lamps for resin polymerisation was used. A thin bar was fitted lengthways near the top of the unit to provide a support for an aluminium foil-

covered sheet of metal, bent midway and lengthways to 90°. This acts as a removable shield which provides indirect ultraviolet radiation, when required.

4.7 Sectioning of Lowicryl K4M blocks

Lowicryl K4M is a hydrophilic resin, therefore precautions should be taken to ensure that the block face does not become wet during sectioning. This is accomplished by sectioning with the level of fluid in the knife-trough slightly below normal, while still maintaining a wet knife-edge. Nickel or gold grids are used for labeling, as copper tends to oxidise in the labeling solutions. The stability of sections on the grid during labeling and in the electron microscope can be improved by coating grids with formvar.

4.7.1 Reagents

0.25% (m/v) Formvar in chloroform. Formvar (0.25 g) was dissolved in chloroform (100 ml).

4.7.2 Procedure

A dish (20 cm ϕ x 10 cm) was completely filled with dist.H_2O. A clean glass microscope slide was dipped its length into the formvar solution, the excess allowed to drain and the remaining film dried. The dried film was loosened around the edges of the slide with a razor-blade, and floated onto the surface of the water. Nickel grids (hexagonal, 200 mesh) were placed, shiny side up, on the floating film. Using a rectangular piece of wire mesh as a support, the film was recovered by a scooping motion through the water leaving the film covering the mesh. The grids, adhered to the underside of the film and supported by the mesh were allowed to dry. Individual formvar-coated grids can then be torn away from the film as required.

Small blocks (about 5 mm^3) were cut with a hacksaw from a petri dish containing *in situ* embedded cell monolayers. In most cases the layer of resin did not separate from the dish, and the block was mounted onto a plastic stub with superglue, the layers of resin, cells and dish plastic perpendicular to the stub. The

block was trimmed on a microtome to a pyramidal face and ultrathin sections cut with a diamond knife and collected as soon as possible onto formvar-coated grids. Grids were dried and could be stored indefinitely before immunolabeling.

The level of water in the knife-trough was lower than normal, producing a dark silver reflection along the knife edge. This ensures that the block face does not become wet during sectioning. Sectioning through two plastics of different hardnesses and polarities was not problematic, in fact this double layer helps the rapid location, in the electron microscope, of the resin edge along which the cell monolayer should be found. Furthermore, it protects the cell monolayer in the block from damage, as may be the case if the dish plastic was stripped away from the resin, exposing the cell monolayer at its surface.

4.8 Production of protein A-gold probes by the tannic acid-citrate method

The protein A-gold probes used in this study were either produced in this laboratory or supplied by Dr Jan Slot, University of Utrecht, Utrecht, Netherlands. The production of protein A-gold probes is divided into three stages: formation of the gold colloid, determination of the minimum amount of protein A required to stabilise the colloid and production of the gold probe, and purification and characterisation of the gold probe.

4.8.1 Production of gold colloid

Colloidal gold particles as a marker system have come into widespread use for a number of reasons: (1) high contrast in the electron microscope allows easy detection of small electron dense particles; (2) particles are homogenous in shape and size and this avoids confusion with cell structures; (3) populations of colloidal gold particles of differing size can be produced using relatively simple methods; (4) the non-specific adsorption properties of colloidal gold particles allows for their use as markers for a wide range of molecules differing in physical characteristics (Baudhuin *et al.*, 1989; Leunissen and De Mey, 1989). However, it was the landmark work of Frens (1973) which, because of its simple methodology, allowed the introduction of particle science in cell biology.

Synthesis of colloidal gold is based on the controlled reduction of an aqueous solution of tetrachloroauric acid. The type of reducing agent used and the concentration of the reacting components, largely determine the ratio between nucleation and growth, and thus the final particle size and size distribution. Rapid reduction results in formation of a greater number of nuclei, thereby consuming much of the tetrachloroauric acid and limiting the amount remaining for shell growth (Handley, 1989). Slot and Geuze (1985) used mixtures of a strong reducing agent (tannic acid) and a weaker reducing agent (citrate), to allow the production of particles of different size. For the range 2-10 nm an excess of tannic acid is used, and the reduction is almost exclusively accomplished by tannic acid. In the larger size range (10 nm-20 nm), tannic acid is limiting, so the reduction necessary for particle growth is effected by citrate. Therefore, by varying the amount of tannic acid, there is a corresponding variation in the rate of nucleation and the number of nucleation points. This affects the diameter of the resulting particles, for a constant amount of tetrachloroauric acid. In this way gold particles in the 2 nm-20 nm size range can be produced.

4.8.1.1 Reagents

At all times scrupulously clean, scratch-free glassware was used. All reagents, including water, were of the highest purity available and filtered through 0.22 µm membranes.

1% (m/v) Chloroauric acid. $HAuCl_4$ (Merck, Darmstadt) (0.1 g) was dissolved in dist.H_2O, made up to 10 ml and stored in the dark at 4°C until used.

1% (m/v) Trisodium citrate. Trisodium citrate.$2H_2O$ (1.14 g) was dissolved in dist.H_2O, made up to 100 ml and used freshly prepared.

1% (m/v) Tannic acid. Tannic acid (Mallinckrodt #1764) (0.1 g) was dissolved in dist.H_2O, made up to 10 ml and used freshly prepared.

4.8.1.2 Procedure

To prepare 200 ml of gold sol the following solutions were prepared: A) 1% $HAuCl_4$ (2 ml) was added to dist.H_2O (160 ml), B) 1% trisodium citrate (8 ml) and 1% tannic acid (1 ml for 5 nm probes, 80 µl for 10 nm probes) were added to dist.H_2O (32 ml). Solutions A) and B) were protected from exposure to light, warmed to 60°C and mixed rapidly while stirring. Upon formation of a red colour, the solution was warmed to 90°C and cooled on ice. Larger probes take longer to form and the red colour can take up to 1 h to develop.

4.8.2 Formation of gold probe with a minimum amount of protein A

Colloidal gold particles carry a net negative surface charge; they are composed of an internal core of pure gold surrounded by a surface layer of adsorbed $AuCl_2^-$ ions. It is these ions that confer a negative charge to the colloidal gold and prevent particles aggregating by electrostatic repulsion. The gold colloid is sensitive to electrolytes (chloride > bromide > iodide) that reduce electrostatic repulsion by compressing the ionic layer. This allows the van der Waal's attractive forces between the particles to dominate causing particle aggregation, which is accompanied by a colour change and eventual sedimentation of the gold.

Gold colloids are also lyophobic, the surface of the particles displays not only electrostatic characteristics, but also has hydrophobic properties. The latter may be exploited to bind proteins to the particle surface, and thereby form a specific probe. To achieve this, it is important to know the isoelectric point (pI) of the protein to be coupled. This value, or 0.5 pH units higher, is considered to be the optimum pH for probe preparation. Under these conditions the net protein charge is zero or slightly negative, thus preventing aggregation with gold particles due to electrostatic attraction. To determine whether adsorption has been successful or not, electrolyte is added to a protein/gold colloid mixture. A change in colour (red to blue) upon electrolyte addition indicates incomplete stabilisation. The stabilising amount is the lowest amount of protein added to

the gold colloid that will prevent a colour change upon challenge with an electrolyte.

After the addition of the specific probe protein, bare areas on the surface of particles may be present. Such areas may cause aggregation, and interact with components of biological material in an immunolabeling experiment, giving rise to non-specific background labeling. By adding secondary stabilisers, such as BSA, exposed areas can be masked (Bendayan, 1989; Leunissen and De May, 1989).

The immunolabeling experiments described here make use of the protein A-gold probe. Protein A belongs to a group of proteins, located at the surface of bacteria, that are thought to play a role in pathogenesis and in the host-parasite relationship. Protein A is found in the cell wall of *Staphylococcus aureus*, its most relevant property being its affinity toward IgG through binding to the crystallisable fragment (Fc) (Forsgren and Sjöquist, 1966) on the CH_2 and CH_3 domains (Deisenhofer, 1981). It is, thus, a pseudo-immune reaction that does not interfere with the binding of the immunoglobulin to its antigen. Protein A interacts strongly with most IgG classes of several mammalian species, such as human, rabbit, guinea pig and dog, and less strongly with IgG classes from goat, sheep, rat and chicken (Forsgren and Sjöquist, 1966; Langone, 1982). Where primary antibodies from weaker reacting species are used, a "linker" secondary antibody from a strongly reacting species may be used, followed by the protein A-gold probe. Indeed, immunogold probes, where the secondary antibody is adsorbed onto the gold surface instead of protein A, would also be a viable alternative. This offers the further advantage of signal amplification, because more than one immunogold probe may bind to a single tissue-bound primary antibody (Merighi *et al.*, 1992). Protein A binds immunoglobulin in a one-to-one ratio (Slot *et al.*, 1989), an important factor when quantitative immunocytochemical labeling studies are being undertaken (Griffiths and Hoppeler, 1986).

4.8.2.1 Reagents

1 mg/ml Protein A. Protein A (1 mg, Sigma Chemical Co., St. Louis, Mo.) was dissolved in dist.H_2O (1 ml).

Colloidal gold. Prepared as in Section 4.7.1.

10% (m/v) NaCl. NaCl (1 g) was dissolved in dist.H_2O (10 ml).

0.1 M NaOH. NaOH (0.04 g) was dissolved in dist.H_2O (10 ml).

10% (m/v) BSA in PBS-E. BSA (globulin-free, Sigma Chemical Co., St. Louis, Mo.) (1 g) was dissolved in PBS-E (Section 4.6) (10 ml).

4.8.2.2 Procedure

Protein A was centrifuged at high speed to sediment any precipitate, and diluted to 100 µg/ml with dist.H_2O. The gold colloid was adjusted to pH 6.0 with 0.1 M NaOH using pH paper, as the colloid will block a pH electrode. A dilution series was set up in flat-bottomed microtitre plates (Table 4) and mixed by manual agitation.

Table 4. Dilution series for the determination of the minimum protein A concentration for colloid stabilisation.

protein A (µl)	+	dist.H_2O (µl)	protein A (µl)	+	dist.H_2O (µl)
10		0	5		5
9		1	4		6
8		2	3		7
7		3	2		8
6		4	1		9

Colloidal gold was added to each well and mixed by agitation. Adsorption was allowed to proceed for 2 min and the colloid was then challenged with 10% NaCl

(10 µl). After agitation the plate was allowed to stand (5 min) and the colour change assessed visually. The highest dilution of protein A giving no colour change (pink to blue) represents the end point, and was used to calculate the amount of protein required to stabilise 200 ml of gold colloid. The calculated amount of protein A was added dropwise to the stirred gold colloid suspension. After 5 min, residual protein binding sites were quenched by the addition of 10% BSA in PBS-E, to a final concentration of 0.2% (4 ml added to 200 ml gold colloid).

4.8.3 Purification and characterisation of gold probes

The gold particles are first collected as a loose pellet, resuspended and centrifuged through a continuous glycerol gradient. The colloid fractionates in the gradient on the basis of size, the majority of particles of uniform size forming a band in the gradient and the aggregates passing through. Unbound protein is simultaneously removed.

Once synthesised, colloidal gold preparations need to be evaluated ultrastructurally. This is accomplished by drying a small aliquot of the colloidal gold onto a coated grid, and transmission electron microscopy is used to evaluate the particle size, size distribution and the presence of clusters. As a rule, probes with at least 75% singlets and at the most 5% triplets are considered acceptable (Leunissen and De Mey, 1989).

4.8.3.1 Reagents

0.2% (m/v) BSA in PBS-E (BSA-PBS-E). BSA (globulin-free, Sigma Chemical Co., St. Louis, Mo.) (0.2 g) was dissolved in PBS-E (Section 4.6) (100 ml).

10% (v/v) Glycerol in BSA-PBS-E. Glycerol (10 ml) was diluted in BSA-PBS-E (90 ml).

30% (v/v) Glycerol in BSA-PBS-E. Glycerol (30 ml) was diluted in BSA-PBS-E (70 ml).

0.1% (m/v) Poly-L-lysine. Poly-L-lysine (1 mg) was dissolved in dist.H_2O (10 ml).

4.8.3.2 Procedure

Quenched colloidal gold particles were centrifuged (30 min, 4°C) in a Beckman 70 Ti rotor (5 nm probe at 210 000 x g, 10 nm probe at 75 000 x g). The mobile pellet was resuspended in approximately 1 ml BSA-PBS-E and loaded onto a 15 ml 10%-30% glycerol gradient and centrifuged (45 min, 4°C) in a Beckman SW 40 rotor (5 nm probe at 240 000 x g, 10 nm probe at 65 000 x g). The dark red band in the middle of the gradient was collected and its absorbance (520 nm) adjusted to 2.5-5. Particle size and size distribution was assessed by electron microscopy. A transmission electron microscopy grid was coated with a formvar support membrane (Section 4.7), and further coated with 0.1% poly-L-lysine. A thin layer of gold colloid was allowed to adhere and the photographed particles assessed for size. A size variation of less than 10% was considered acceptable. The binding activity of the probes was evaluated during immunolabeling procedures incorporating the appropriate controls (Section 4.9). Probes were stored stored at 4°C.

4.9 Immunolabeling of Lowicryl-embedded sections

Immunocytochemical labeling of ultrathin sections with protein A-gold, in general follows standard protocols (Merighi, 1992), with the manipulation of variables such as choice of blocking agent, length of incubation times, and length and stringency of washing. The protocol used was slightly modified from that of Roth (1986); foetal calf serum was used as a block and grids were washed in drops rather than by spraying. Double labeling was achieved by using the small probe first, followed by the large. This prevents steric hindrance and accessibility effects if the large probe were used first. Fixation with glutaraldehyde after the first of the two labelings and at the end of the protocol, ensures that the antigen/antibody/protein A-gold complex is securely held together. Furthermore, the first fixation ensures that any antibody not bound to protein A in the protein A-gold complex is inactivated, so that it is not available for binding to the second protein A-gold probe, an event which would cause false positive second labeling. Antibodies used are described in Section 4.3.

4.9.1 Reagents

5% (v/v) foetal calf serum in PBS-E. Foetal calf serum (50 µl) was diluted in PBS-E (Section 4.6) (950 µl).

0.1% (m/v) BSA-PBS-E. BSA (globulin-free, Sigma Chemical Co., St. Louis, Mo., 10 mg) was dissolved in PBS-E (Section 4.6) (10 ml).

1% (v/v) Glutaraldehyde in PBS-E. Glutaraldehyde (40 µl of 25% stock, Merck, Darmstadt) was diluted in PBS-E (Section 4.6) (960 µl).

0.02 M Glycine in PBS-E. Glycine (0.015 g) was dissolved in PBS-E (Section 4.6) (10 ml).

Antibodies. Chicken anti-porcine cathepsin D IgY was used at 250 µg/ml, rabbit anti-human cathepsin B IgG at 20 µg/ml, and rabbit anti-chicken IgY serum at a 1/200 dilution.

4.9.2 Procedure

Immunolabeling was performed by incubation of grids, section-side down, on droplets of reagents on a layer of Parafilm spread onto a bench. Grids were transferred from reagent to reagent with a 1 mm nichrome wire loop on the end of a thin wooden splint. The same loop was used for each system optimised, as the loop carries a small volume of liquid over which introduces a dilution factor.

Single immunogold labeling was performed according to the following regimen:

1) Block: foetal calf serum in PBS-E (20 µl, 15 min),
2) Labeling: 1° antibody diluted in block (15 µl, 2 h),
3) Wash: PBS-E (5 drops, 10 min total),
4) Labeling: 2° antibody (if required) diluted in block (15 µl, 1 h),
5) Wash: PBS-E (5 drops, 10 min total),
6) Labeling: protein A-gold (5 nm or 10 nm, Section 4.8) diluted in BSA-PBS-E (15 µl, 1 h),

7) Wash: PBS-E (5 drops, 10 min total),
8) Fix: glutaraldehyde in PBS (10 µl, 5 min),
9) Wash: 2 drops of PBS-E followed by 5 drops of dist.H_2O,
10) Gently blot grids and allow to dry.

For double labeling, the following regimen was used:

1) Protocol was followed as for single labeling to step 8),
2) Block: glycine in PBS-E (5 drops, 10 min total) followed by BSA-PBS-E (5 drops, 10 min total),
3) Wash: PBS-E (5 drops, 10 min total),
4) Protocol was followed as for single labeling from steps 2)-10).

As a control, all procedures were repeated with immune antibody substituted with a non-immune preparation, at the lowest dilution of immune antibody used. During optimisation of the gold probe level, antibody was additionally substituted with a diluting buffer only control.

Double labeled (5 nm and 10 nm, Section 4.8) grids were incubated first in small probe, then large probe. Controls included single labeling with each probe for each antigen in the absence of primary antibody, single labeling with each probe for each antigen in the presence of primary antibody, and double labeling for both antigens with both sizes of probe.

4.10 Counterstaining of Lowicryl K4M sections

Counterstaining procedures for Lowicryl K4M are similar as those for traditional electron microscope resins. Sections may be stained with either aqueous or alcoholic solutions of uranyl acetate and with lead citrate (Hobot, 1989). The use of Millonig's lead acetate (Millonig, 1961) in combination with uranyl acetate, has been suggested to provide superior quality of contrast and fine granular staining (Roth *et al.*, 1985). Further improvement in the contrast of cellular structures embedded in Lowicryl K4M can be achieved by a contrasting method based on the uranyl acetate/methyl cellulose staining of thawed cryosections (Roth *et al.*, 1990). It was found, however, that for MCF-10 cells staining with uranyl acetate and lead

citrate provided sufficient contrast. Since Lowicryl K4M is hydrophilic, the sections should be incubated on drops of stain for short periods of time. Prolonged staining may cause contamination and distortion of the section.

4.10.1 Reagents

2% (m/v) uranyl acetate. Uranyl acetate (1 g) was dissolved in dist.H_2O (50 ml), the addition of 95% ethanol (1 ml) assists solubility. The uranyl acetate solution was stored in the dark at 4°C.

1 M NaOH. NaOH (0.4 g) was dissolved in dist.H_2O (10 ml).

Lead citrate. Lead nitrate (1.33 g) and trisodium citrate.$2H_2O$ (1.76 g) were added to freshly boiled and cooled dist.H_2O and shaken intermittently for 30 min. NaOH (about 8 ml) was added to clear the milky solution. The solution was adjusted to 50 ml with freshly boiled and cooled dist.H_2O and stored in the dark at 4°C in an air-tight container.

4.10.2 Procedure

Dried grids were inverted onto uranyl acetate (4 min, in the dark), washed with dist.H_2O by gentle pipetting (500 µl) and inverted onto lead citrate (30 s) in a closed dish containing NaOH pellets to remove CO_2. The grids were again washed with gentle pipetting (500 µl), blotted, allowed to air dry and viewed in a Jeol 100CX transmission electron microscope at 80 kV.

4.11 Results

This study was aimed at comparing the relative distributions of lysosomes and lysosomal proteinases in the normal human breast epithelial cell line, MCF-10A, and the *ras*-transformed cell line, MCF-10AneoT.

Examination of the distribution of lysosomes as revealed by LysoTracker™, a fluorescent probe that accumulates in the low pH environment of lysosomes, shows a perinuclear distribution in MCF-10A cells (Fig. 20A), with dark spaces

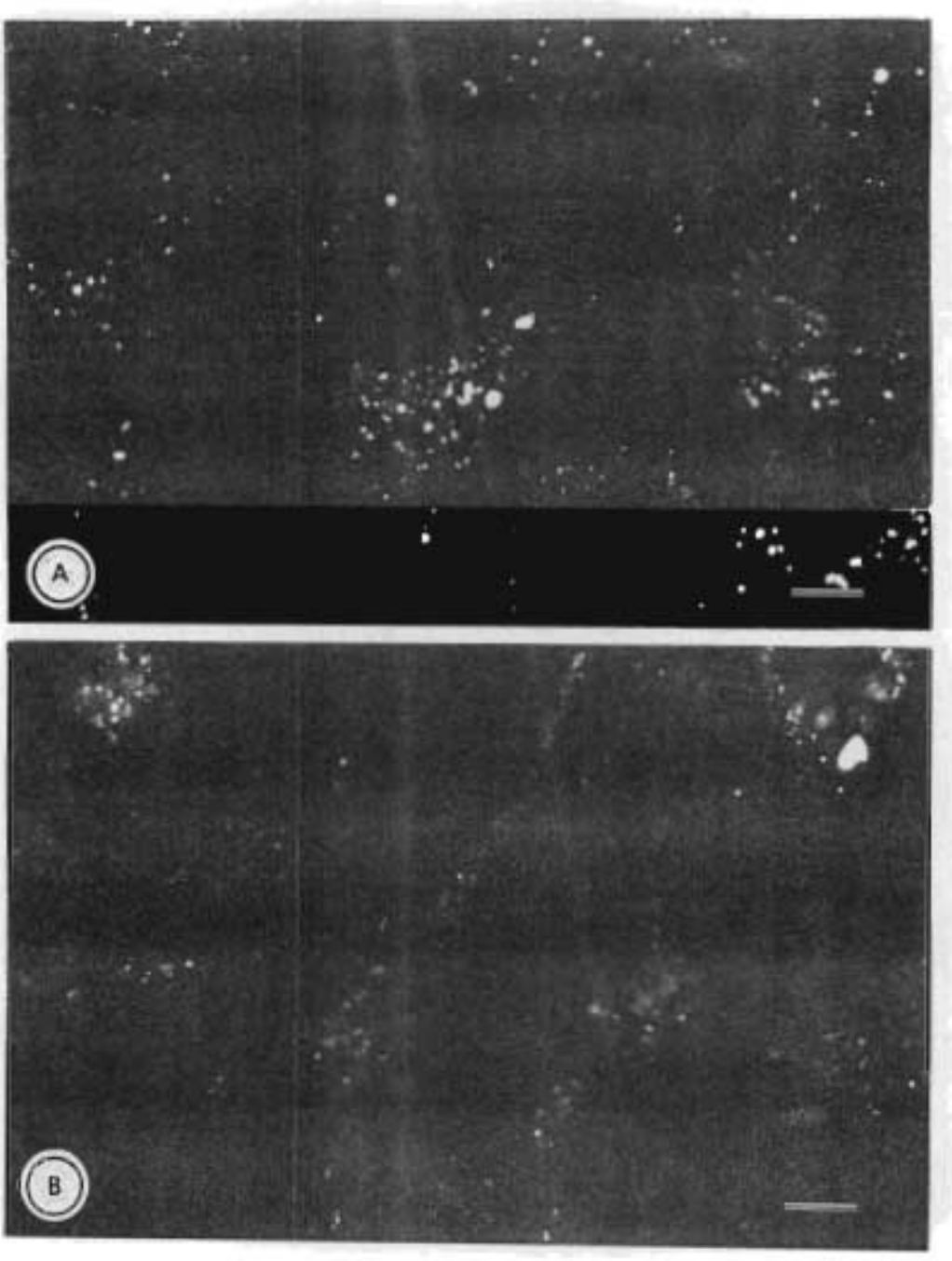
A
B

Figure 20. MCF-10A and MCF-10AneoT cells labeled for low pH compartments. (A) MCF-10A and (B) MCF-10AneoT cells were grown overnight on coverslips, incubated in LysoTracker™ and viewed live by epi-fluorescent microscopy (Section 4.4).

Bar scales: (A and B) 10 μm.

between cells where the cell periphery and borders are located. In MCF-10AneoT cells (Fig. 20B), however, lysosomal labeling can be found in the long, slender cellular processes that extend from the cells and cross each other in the contact uninhibited manner of transformed cells. Labeling for cathepsin D reveals a pattern similar to that of LysoTracker: generally perinuclear in MCF-10A cells (Fig. 21A) and more peripheral in MCF-10AneoT cells (Fig. 21B). Furthermore labeling is profuse and strong, correlating with the abundance of this enzyme in cells and tissues.

Cathepsins B, H and procathepsin D immunofluorescent labeling was detected with a secondary antibody conjugated to TRITC which fluoresces red, a colour to which standard colour film is relatively insensitive. This was overcome by using the more sensitive Ilford XP2 400 film, in which punctate fluorescence is visualised as white dots. Cathepsin B fluorescence was localised around the nucleus on MCF-10A cells (Fig. 22A & B), and more peripherally, to the cell membrane (Fig. 22C) and cellular extensions (Fig. 22D) in MCF-10AneoT cells. There appeared also to be a greater number of fluorescent compartments in the transformed cells (compare Figs 22A & B and Figs 22C & D). Localisation of cathepsin H was characterised by highly defined small or larger compartments with bright fluorescence. In normal cells the distribution was generally perinuclear (Fig. 23B), but examples of a peripheral distribution were found (Fig. 23A). Transformed cells showed a sparse distribution of labeled vesicles which were mostly peripheral (Fig. 23C). In populations of both cell types only the occasional cell showed any labeling at all.

Labeling of cathepsin L was unsuccessful in MCF-10A cells, and very sparce and weak in MCF-10AneoT cells. This is in contrast to similar experiments, using the same antibody, performed on the mouse embryo fibroblast line, NIH 3T3, and the B16-BL6 mouse melanoma cell line, where labeling was abundant (pers. com. with Frieda Dehrmann, Department of Biochemistry, University of Natal). Labeled vesicles appeared primarily perinuclear in the transformed cells (Fig. 24A). Proenzyme labeling was performed to assess any difference in distribution between proenzyme and mature enzyme and between the cell lines.

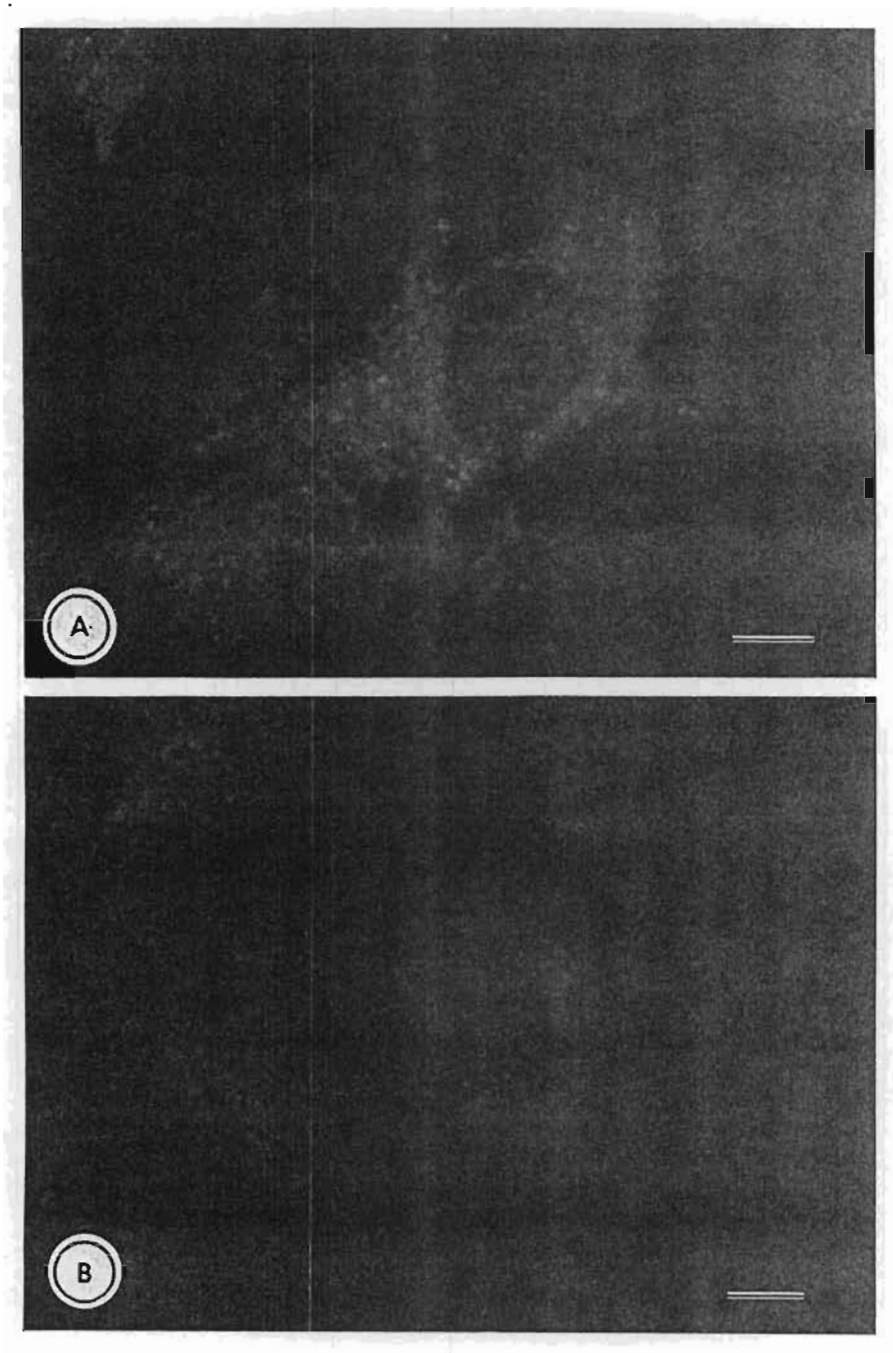
A
B

Figure 21. Immunofluorescent labeling of MCF-10A and MCF-10AneoT cells for cathepsin D.

(A) MCF-10A and (B) MCF-10AneoT cells were grown overnight on coverslips, and labeled (Section 4.5) with chicken anti-porcine cathepsin D (200 µg/ml).

Bar scales: (A and B) 10 µm.

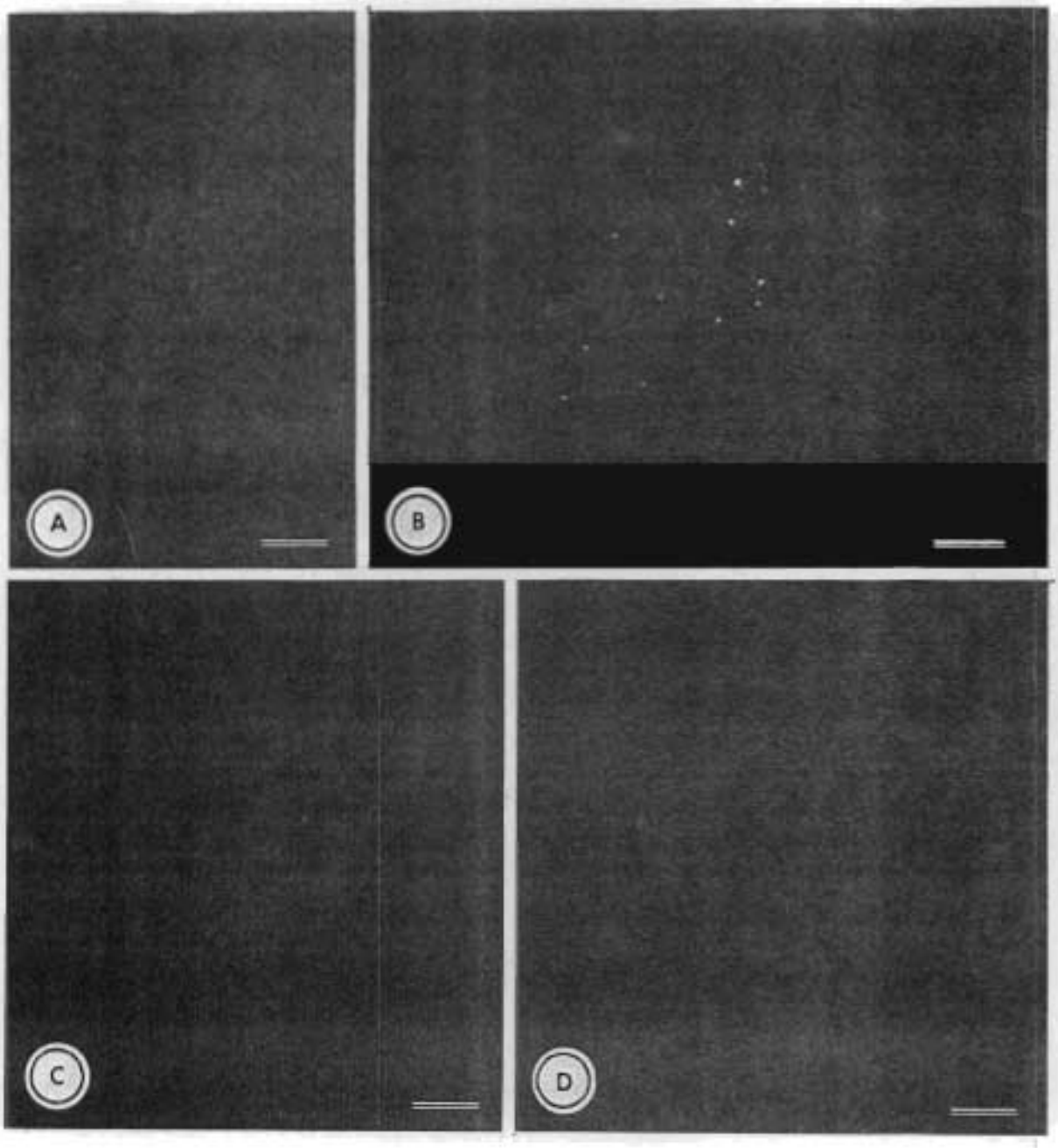
A
B
C
D

Figure 22. Immunofluorescent labeling of MCF-10A and MCF-10AneoT cells for cathepsin B.

(A, B) MCF-10A and (C, D) MCF-10AneoT cells were grown overnight on coverslips, and labeled (Section 4.5) with affinity-purified rabbit anti-human cathepsin B (40 µg/ml).

Bar scales: (A-D) 10 µm.

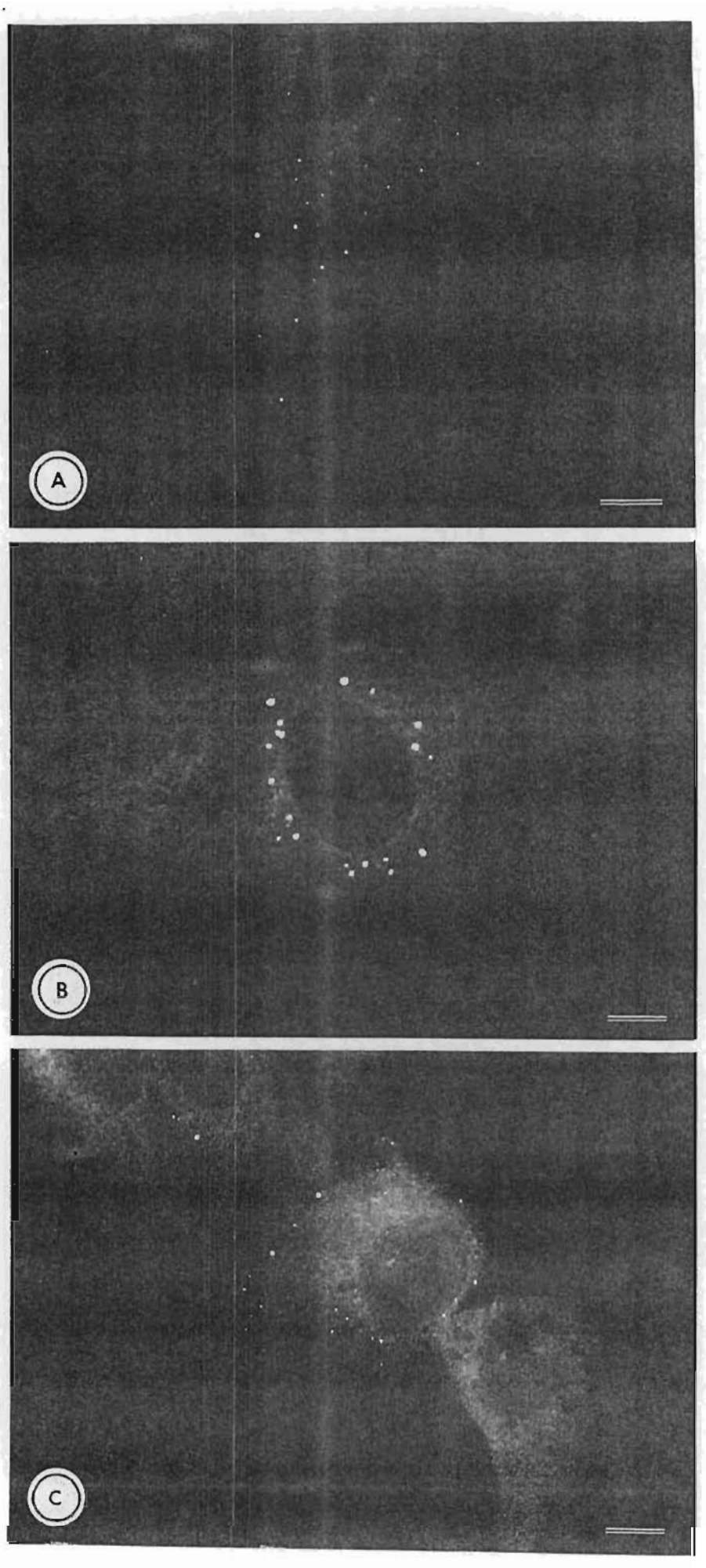
A
B
C

Figure 23. Immunofluorescent labeling of MCF-10A and MCF-10AneoT cells for cathepsin H.

(A, B) MCF-10A and (C) MCF-10AneoT cells were grown overnight on coverslips, and labeled (Section 4.5) with rabbit anti-human cathepsin H anti-peptide antibody (300 µg/ml).

Bar scales: (A-C) 10 µm.

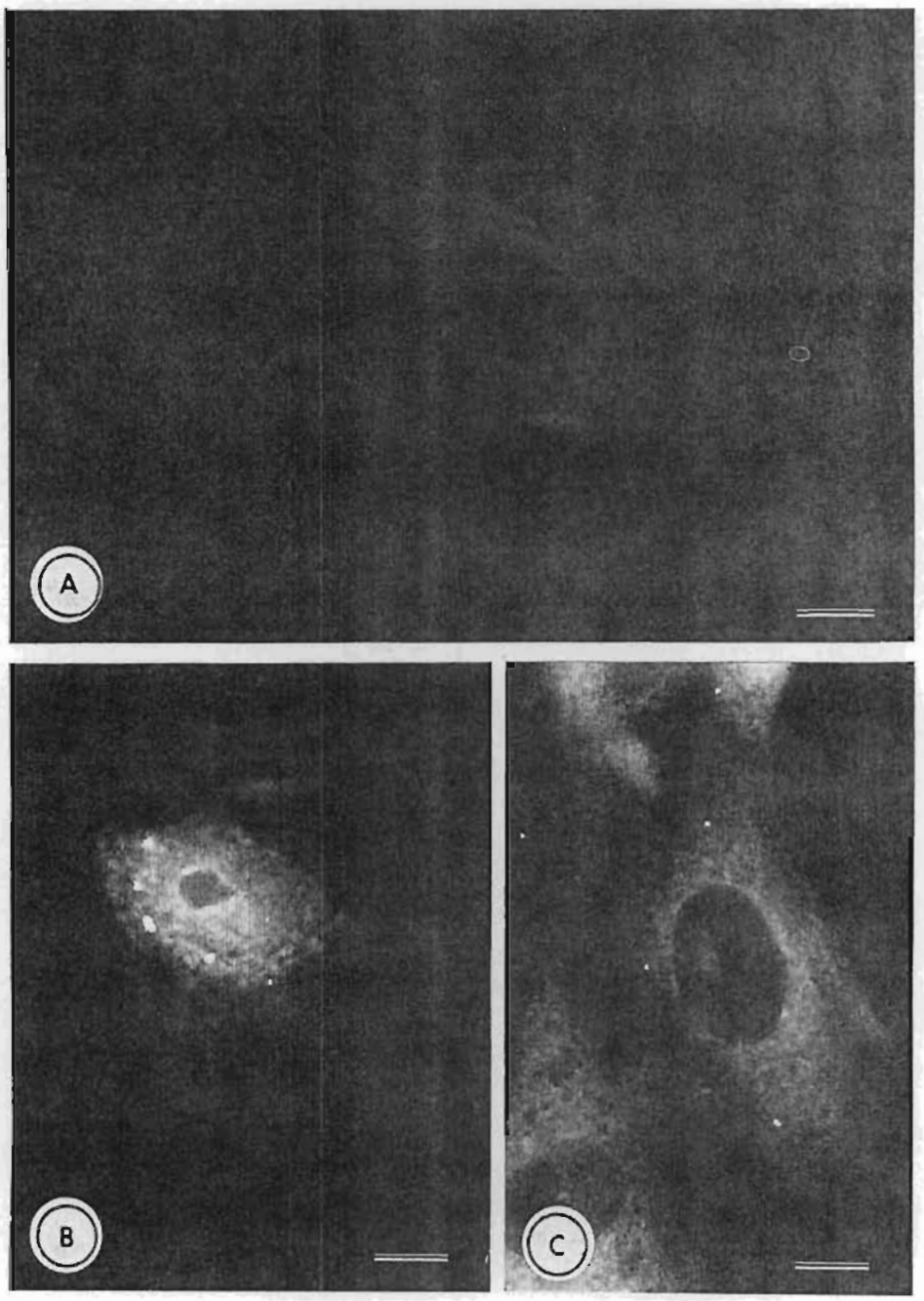
A
B
C

Figure 24. Immunofluorescent labeling of MCF-10AneoT cells for cathepsin L, and MCF-10A and MCF-10AneoT cells for procathepsin D.
MCF-10AneoT cells were grown overnight on coverslips, and labeled (Section 4.5) with (A) chicken anti-sheep cathepsin L (300 μg/ml), and MCF-10A (B) and MCF-10AneoT cells (C) with rabbit anti-human procathepsin D (300 μg/ml).

Bar scales: (A-C) 10 μm.

Labeling for procathepsins B and L was indistinct or absent, and that for procathepsin D was weak and sparse: labeled vesicles were generally near the nucleus or even closely associated with the nuclear membrane, possibly in the RER in normal cells (Fig. 24B), but occasionally also in the cytoplasm. In transformed cells, compartments appeared to be peripheral, near the cell surface (Fig. 24C).

Double-labeling for cathepsins D and B at the electron microscope level revealed two features. First, these enzymes appear to co-localise in the same vesicles more often in MCF-10A than MCF-10AneoT cells and, secondly, both enzymes were found to be cell surface associated in the transformed cells but not in the normal cells. Some of the images presented are of labeled frozen thin sections of cells, work performed by Dr Edith Elliott, Department of Biochemistry, University of Natal.

When MCF-10A cells were labeled for cathepsins D and B, both enzymes were found co-localised in perinuclear vesicles (Fig. 25A, B & C; Fig. 27A). In MCF-10AneoT cells, however, labeling appeared more peripheral and vesicles were generally labeled for either one or the other enzyme, and only occasionally for both (Fig. 26A & B; Fig. 27 B). Labeling for cathepsins D or B in Lowicryl embedded cells always occurred in translucent, asymmetrically rounded organelles, although not all such organelles were necessarily labeled. These vesicles were morphologically identical to vesicles which labeled for LAMP-2 (Fig. 27C), a marker for lysosomes. It is possible, therefore, that most enzyme labeled vesicles are lysosomes, and not endosomes, but this would need to be substantiated with double labeling experiments. Surface, microvillar labeling for cathepsin B was found to occur on the transformed cells (Fig. 28A & B), but this was not widespread. For cathepsin D some labeling was found on the surface of the MCF-10A cells (not shown), but substantially more was observed on the surface of the transformed cells (Fig. 28B). For both enzymes, labeling was localised primarily to the apical surface although, due to the nature of the Lowicryl and ultracryo techniques used, this does not necessarily preclude basolateral labeling.

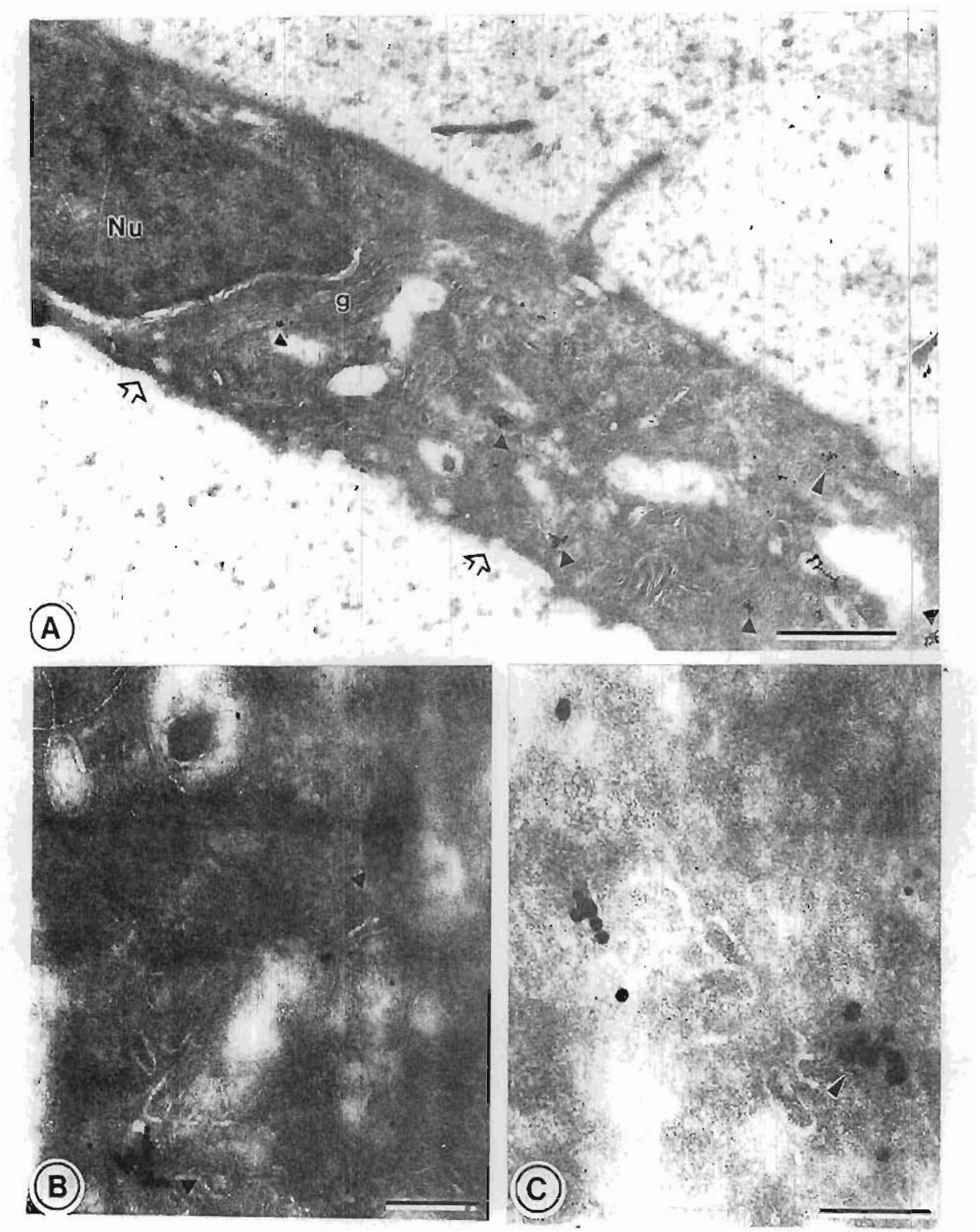
Nu
g
A
B
C

Figure 25. Immunogold double-labeling of cryo-sections for cathepsin B and cathepsin D in MCF-10A cells.

Cathepsin B localisation was performed with a 10 nm gold probe and cathepsin D with a 15 nm probe (Section 4.9). (A) An MCF-10A cell is show in transverse section, the basolateral surface indicated by open arrows. Cathepsins B and D were generally found in a perinuclear location (arrowheads). (B and C) Co-localisation of cathepsins B and D in electron-dense vesicles (arrowheads). Nu = nucleus; g = Golgi apparatus.

Bar scales: (A) 1 μm; (B and C) 0.2 μm.

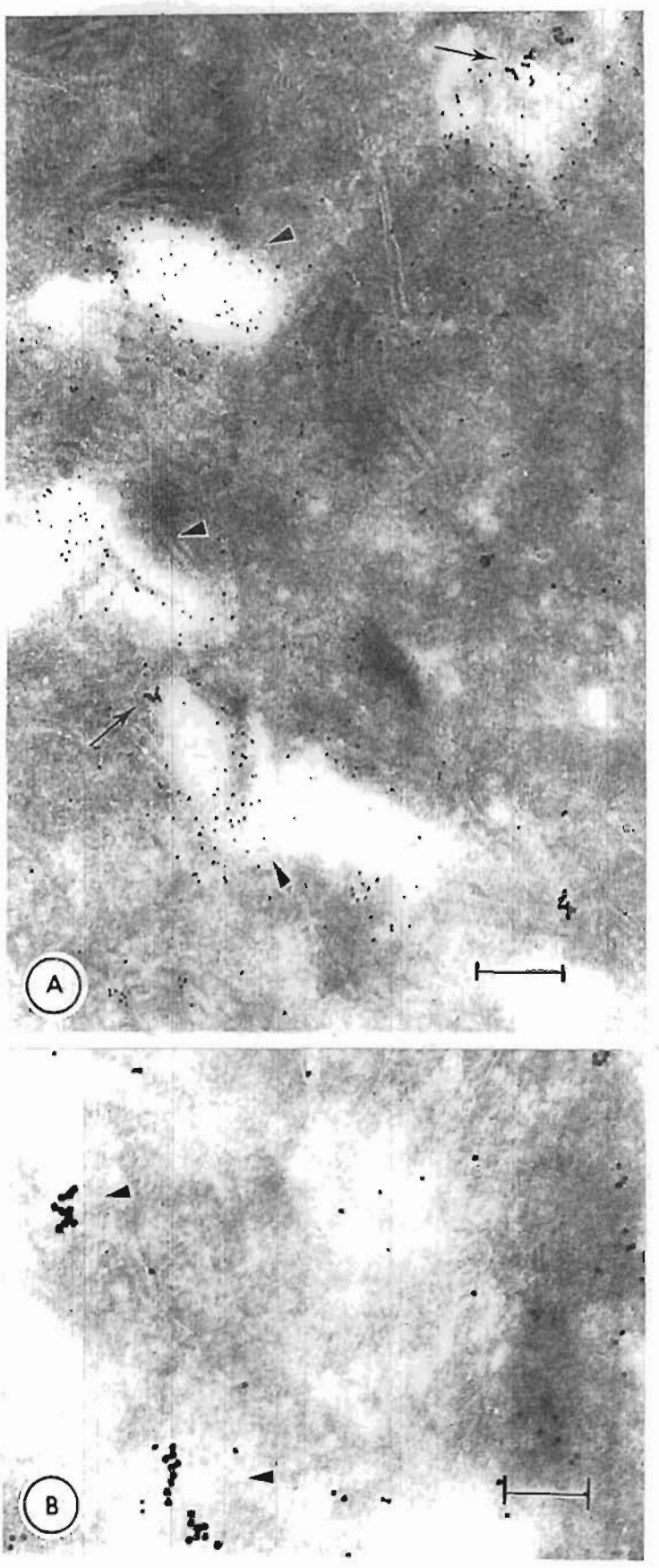
A
B

Figure 26. Immunogold double-labeling of cryo-sections for cathepsin B and cathepsin D in MCF-10AneoT cells.

Cathepsin B localisation was performed with a 10 nm gold probe and cathepsin D with a 15 nm probe (Section 4.9). MCF-10AneoT cells (A and B) indicated less co-localisation of cathepsin B (A, arrowheads) and cathepsin D (A, arrows; B, arrowheads) in vesicles compared to MCF-10A cells (*cf.* Fig. 25).

Bar scales: (A) 0.5 μm; (B) 0.25 μm.

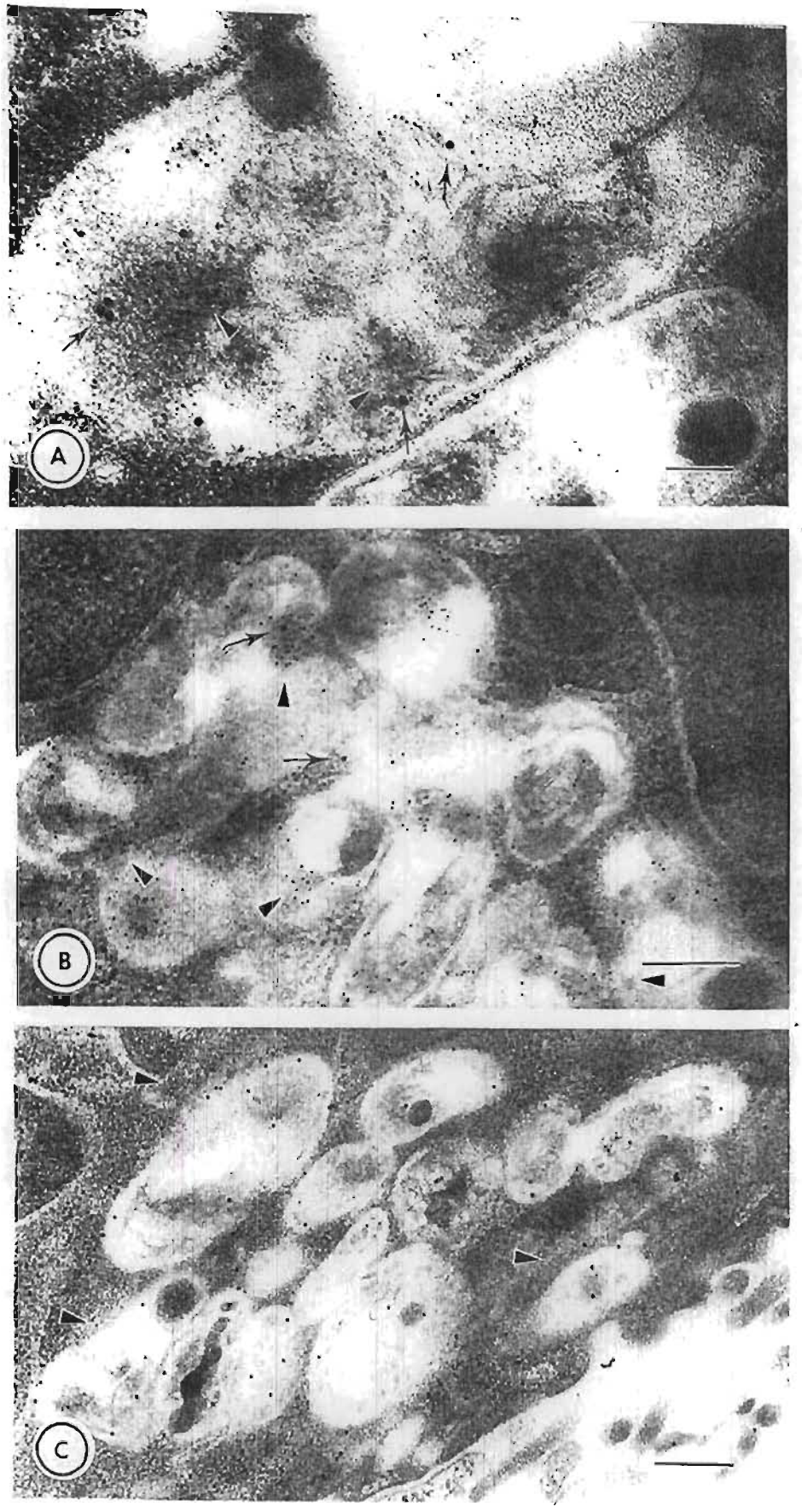
A
B
C

Figure 27. Immunogold double-labeling of lowicryl sections for cathepsin B and cathepsin D in MCF-10A and MCF-10AneoT cells, and labeling for LAMP-2 in MCF-10AneoT cells.

Cathepsin D was detected with a 5 nm gold probe, cathepsin B with a 10 nm probe (A and B) and LAMP-2 with a 5 nm probe (C) (Section 4.9). Cathepsins B (arrows) and D (arrowheads) were found to typically co-localise in vesicles of MCF-10A cells (A), while in the *ras*-transformed cells (B) little co-localisation was evident. Translucent vesicles, similar to those which typically label for cathepsins B and D (*cf.* B), were labelled for the lysosomal marker, Lamp-2 (C, arrowheads).

Bar scales: (A) 0.1 μm; (B) 0.2 μm; (C) 0.25 μm.

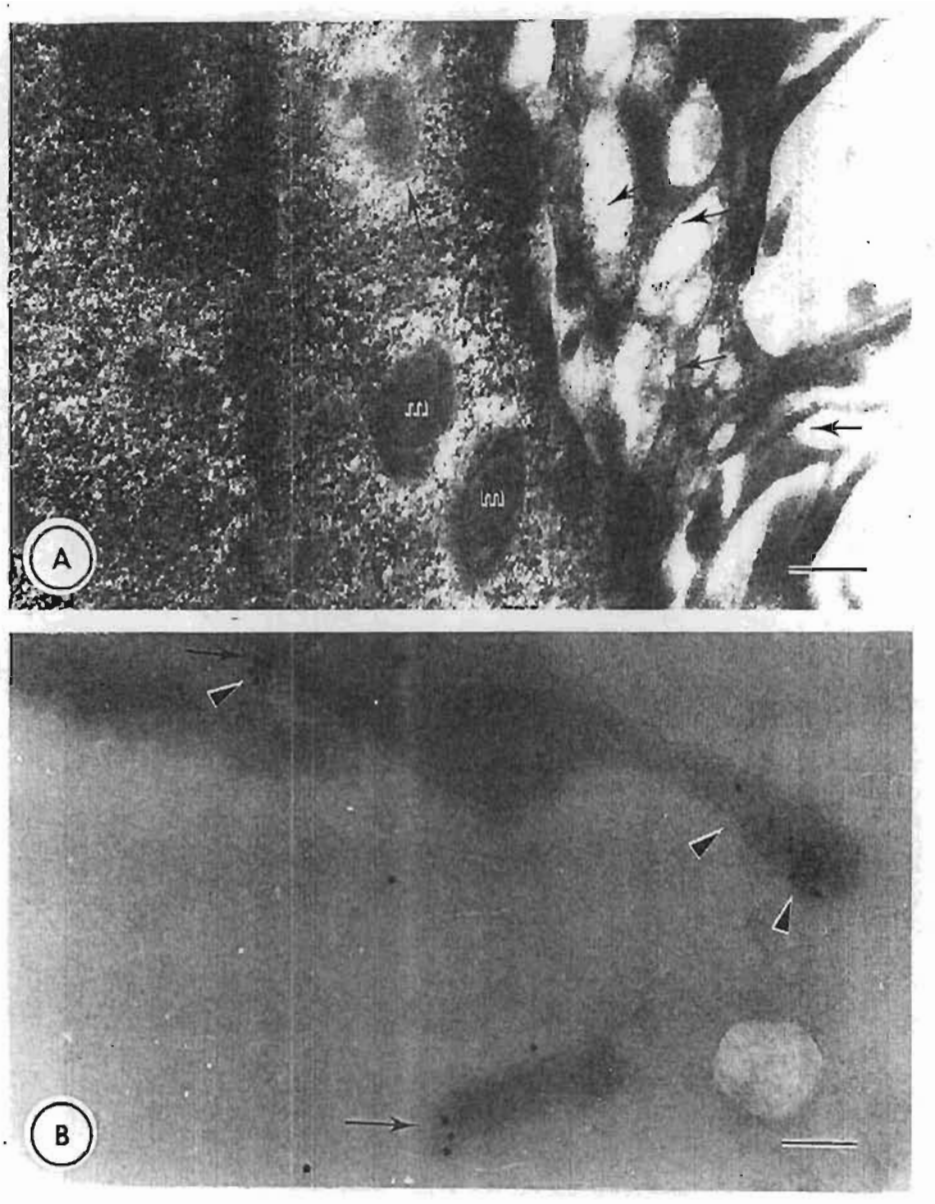
A
B

Figure 28. Plasma membrane labelling for cathepsin B (lowicryl) and cathepsins D and B (cryo-section) in MCF-10AneoT cells.

Single labelling for cathepsin B was achieved with a 5 nm probe, and double labelling for cathepsin D with a 10 nm probe and cathepsin B with a 15 nm probe (Section 4.9). Association of (A) cathepsin B (arrows) and (B) cathepsins B (arrows) and D (arrowheads) with the microvillar projections of the plasma membrane of *ras*-transformed cells is evident. m = mitochondrion.

Bar scale: (A and B) 0.25 μm.

4.12 Discussion

Transfection of MCF-10A breast epithelial cells with oncogenic *ras*, produces altered trafficking of at least some lysosomal enzymes. Three important anomalous phenomena were found: 1) in *ras*-transformed cells lysosomal enzyme-containing vesicles were more peripherally distributed in the cytoplasm than in normal cells, where vesicles were predominantly perinuclear; 2) in transformed cells, labeled enzymes did not always co-localise in vesicles as would be expected of lysosomal enzymes; and 3) both cathepsins B and D were found to be membrane-bound in transformed cells. Indeed, membrane-associated cathepsin B is the mature double-chain form, and therefore functionally active (Sloane *et* al., 1994). Further studies have shown that the constitutive cathepsin B secretion is ~2.5-fold greater in the transfected cells (Rozhin *et al.*, 1994) and cathepsin L is also found more peripherally (Moin *et al.*, 1994). These events are consistent with a metastatic phenotype. Peripherally situated lysosomes (labeling for LAMP-2 of morphologically identical vesicles as those labeled for enzymes, seems indicative of lysosomes, although other compartments cannot be ruled out) may be poised to secrete enzymes or deliver enzymes to the cell surface. Such altered trafficking is typical of metastatic tumour cells, and this may further be reflected by alterations in the enzyme complement of different lysosomes, leading to lysosomal sub-populations. Furthermore, membrane-bound cathepsin B is well situated for focal digestion of extracellular matrix or the activation of other proteases. Cathepsin D, on the other hand, may exert a mitogenic effect on the cell (Fusek and Vetvicka, 1994).

These studies confirm those of Sloane *et al.* (1994) where, in MCF-10AneoT cells, a more peripheral distribution of immunofluorescent staining for cathepsin B was demonstrated, and by immunogold labeling cathepsin B was localised to the cell membrane. Similar observations have been made in other models of malignant progression (Rempel *et al.*, 1994; Rozhin *et al.*, 1994): in the murine B16 melanoma lines that differ in their lung colonisation potential, and human KM12 colorectal carcinoma cell lines that differ in their metastatic capabilities, a more peripheral distribution of cathepsin B was found to be associated with the

more invasive phenotype. Cell surface labeling for cathepsin B has been observed in human lung carcinoma cells (Erdel *et al.*, 1990), and in murine B16a cells (Honn *et al.*, 1994). The association of cathepsin L with the surface of human colorectal cells has also been confirmed (Maciewicz *et al.*, 1989), although in this study cathepsin L labeling proved too weak to confirm this for *ras*-transformed human breast epithelial cells. The localisation of cathepsin D to the plasma membrane may, however, be a phenomenon unique to these cells as no similar observations have been made in other cells lines. Alterations in trafficking that lead to secretion of other lysosomal cathepsins have been widely reported and may be a feature of malignant transformation. Transfection of murine 3T3 fibroblasts with the *ras* oncogene elevates secretion of procathepsin L (Denhardt *et al.*, 1987), transformation of murine BALB/3T3 fibroblasts by Moloney murine sarcoma virus elevates secretion of both procathepsin L and procathepsin B (Achkar *et al.*, 1990), and in MCF-7 human breast cancer cells procathepsin D constitutes the major excreted proteinase (Rochefort, 1990).

Indeed, the results presented in this study show transformed human breast epithelial cells elaborating a perinuclear and also a significantly more peripheral distribution of compartments labeling not only for specific proteinases, but for low pH compartments in general This, and the lack of *rab*11 labeling of peripheral cathepsins B and D labeled vesicles (a marker for the constitutive and regulated pathways) suggests that they are not part of the secretory pathway (Elliott *et al.*, 1995). In contrast, in MCF-7 human breast carcinoma cells peripheral procathepsin B-containing vesicles are of a secretory nature (Elliott *et al.*, 1995). This is consistent with the findings of Rochefort (1990) that MCF-7 cells secrete a high proportion of cathepsin D in the precursor form. This indicates that cathepsins B and D may be trafficked by different pathways, dependent upon cell line or tissue.

Lysosomal enzyme trafficking can occur in a M-6-P-independent manner as has been demonstrated in macrophages (Diment *et al.*, 1988), HepG2 cells (Rijnbout *et al.*, 1991), I-cell disease (Glickman and Kornfeld, 1993) and U937 monocytes (Braulke *et al.*, 1987), and may explain the differential trafficking of cathepsins D

and B in MCF-10 cells. This hypothesis is reinforced by the recent findings of Capony *et al.* (1994), who showed that cathepsin D secretion in some metastatic human breast cancer cell lines, contrary to normal mammary cells including MCF-10, is not increased by ammonium chloride treatment which totally abolishes the M-6-P-dependent pathway by preventing acidic pH dissociation of M-6-P receptor/lysosomal enzyme complexes. Procathepsin D, but neither cathepsin B nor β-hexosaminidase, accumulated in vesicles postulated to be phagosomes or large acid vesicles (Montcourrier *et al.*, 1990). The routing of these enzymes was shown to involve interaction with membranes, but in the present study both cathepsins D and B were found membrane-bound. The presence of cellular lysosomal proenzyme receptors for cathepsins D and L has been proposed (McIntyre and Erickson, 1991) and active membrane-associated cathepsins D and B have been found in rat liver endosomes (Authier *et al.*, 1995). It remains to be seen by which mechanism enzyme/membrane association occurs in *ras*-transformed MCF-10 cells, which may also have altered pH regulation (Sloane *et al.*, 1994) and perhaps also modified procathepsin/membrane interactions.

Evidence seems to suggest that the redistribution of lysosomes and the altered localisation of enzymes in tumour cells parallels malignant progression. Lysosomes are normally localised in the perinuclear region of cells. However, a redistribution of lysosomes to the cell periphery often occurs in cells that participate in invasive or degradative processes. Activated macrophages secrete lysosomal enzymes (Etherington *et al.*, 1981) including cathepsin L (Reilly *et al.*, 1989); activated osteoclasts transport lysosomes toward the apical surface, where enzymes, particularly cathepsins B and L, are secreted into extracellular resorption lacunae formed between an osteoclast and bone (Vaes *et al.*, 1992). The universality of this phenomenon is reflected in the observation that recruitment of lysosomes and fusion with the plasma membrane is required for the invasion of epithelial cells by trypanosomes (Tardieux *et al.*, 1992). Movement of lysosomes away from the cell centre can be experimentally induced by protocols that produce significant cytoplasmic acidification, to the range of ~pH 6.8 (Heuser, 1989). In these studies macrophages and fibroblasts were used to demonstrate pH-

dependent perturbation of microtubule-based motors, this effect being reversed by alkalisation. Similar results have been obtained with polarised MDCK cells (Parton *et al.*, 1991), where lowering the cytosolic pH causes the movement of late endosomes to the base of the cell. It is well known that many tumours have a more acidic interstitial pH than normal tissues (Gerweck and Seetharaman, 1996), primarily due to the poorly organised vasculature of tumours, which gives rise to poor tissue oxygenation. This necessitates reliance on glycolysis, producing lactic acid which is inefficiently removed (Wike-Hooley *et al.*, 1984). It would be interesting to speculate that such a low pH environment would be able to potentially induce a redistribution of lysosomes to the tumour cell periphery. Indeed, an acidic pericellular pH induced a redistribution of cathepsin B positive vesicles toward the cell periphery, and enhanced secretion of active cathepsin B (Rozhin *et al.*, 1994). The pH values in human tumours are on average approximately 0.4 units lower than those observed in normal tissues, a value which appears to be low enough for redistribution to occur.

Such a phenomenon, however, would only be relevant in the environment of the tumour mass, and not in the case of cells in culture or extra- or intravasation of single tumour cells. As has been demonstrated, however, transformation with the *ras* oncogene causes a more peripheral distribution of lysosomes. In the light of the findings of Heuser (1989), it may have been expected that if transformation resulted in intracellular pH perturbation, it would tend towards acidification. However, the opposite is seen: microinjection of oncogenic p21 into fibroblasts induces a rapid rise in intracellular pH (Hagag *et al.*, 1987), and transformation of fibroblasts with *ras* results in significant alkalisation of lysosomes (Jiang *et al.*, 1990). A similar increase was found in the *ras*-transformed epithelial cells (Sloane *et al.*, 1994) and, in NIH 3T3 fibroblasts, is a result of *ras*-dependent activation of the Na^+/H^+ antiporter (Maly *et al.*, 1989). These effects suggest that the events demonstrated by Heuser (1989) are either as a result of a short-circuit of normal cellular controls, or the *ras*-induced effects demonstrated operate by a stronger and independent mechanism. Such a mechanism may be the downstream regulation by *ras* of the *rac* and *rho* pathways, which are known to affect the actin cytoskeleton. Indeed, in HEp-2 cells the movement and

localisation of endocytic compartments depends on microtubules and actin microfilaments (van Deurs *et al.*, 1995), suggesting that oncogenic *ras* may perturb trafficking by affecting the cytoskeleton.

The lysosomal alkalisation (pH 5.0 to 6.1) detected in Kirsten sarcoma virus (*ras*) transformed fibroblasts is comparable to that induced by chloroquine (pH 6.3) (Jiang *et al.*, 1990). Treatment of cells with alkalisation agents such as chloroquine (or ammonium chloride), causes default misrouting of newly synthesised lysosomal enzymes to the cell exterior via the constitutive secretory pathway (Braulke *et al.*, 1987). Yet, in a separate study, similarly transformed fibroblasts were reported to retain almost all lysosomal enzymes except procathepsin L, whose secretion is enhanced due to an intrinsic low affinity for the M-6-P receptor (Dong *et al.*, 1989). This apparent contradiction again serves to underscore the variable trafficking options available to cells, and the further complexity introduced by transformation.

Perhaps the strongest candidate for mediating altered trafficking due to *ras*-transfection, is phosphatidylinositol-3-kinase (PI 3-kinase). *Rho*, a downstream target of *ras*, appears to mediate the activation of PI 3-kinase (Kumagai *et al.*, 1993), which in turn has been implicated in trafficking of M-6-P receptor-ligand complexes from the *trans*-Golgi network towards lysosomes (Davidson, 1995). Wortmannin, a PI 3-kinase inhibitor, causes almost complete extracellular mistargeting of procathepsin D which, in the cell line studied, is normally trafficked to lysosomes in a M-6-P receptor-dependent manner. The most probable explanation for this is that wortmannin directly interferes with M-6-P receptor trafficking by either the prevention of its recruitment into clathrin-coated buds on the *trans*-Golgi network, inhibition of budding or fusion events, or the prevention of receptor recycling (Davidson, 1995). Presumably wortmannin has corresponding effects on similarly sorted lysosomal enzymes, implying a more general role for *ras* in lysosomal enzyme mistargeting.

The work reported in this chapter has provided evidence for the association of some lysosomal proteinases with the plasma membrane of *ras*-transformed

human breast epithelial cells, and an abnormal distribution of compartments containing lysosomal proteinases in these cells. This, and other work already addressed, provides circumstantial evidence for a role of these enzymes in invasion and metastasis. The following chapter addresses the issue of the degradative events effected by the normal and transformed cell lines, as well as the proteinases implicated.

CHAPTER 5

DEGRADATION AND INVASION OF EXTRACELLULAR MATRIX BY c-Ha-*Ras* TRANSFORMED BREAST EPITHELIAL CELLS

5.1 Introduction

The interaction of tumour cells with the microvasculature constitutes a rate regulator for haematogenous metastasis (Weiss *et al.*, 1989). Chapter 4 was concerned with changes brought on by *ras*-transformation which, at this point, are only circumstantially related to invasive ability. In this chapter studies are described that investigate more directly one aspect of this interaction, namely the proteolytic destruction of ECM proteins by the MCF-10 cell lines, a prerequisite for breaching the BM.

Tumour cell adhesion to the endothelial cells of the target organ is the first important event in tumour cell-vessel wall interactions, and plays a decisive role in determining the organ preference of metastasis. The concerted action of a large array of cell surface adhesion molecules mediates the adhesive interactions of blood-borne tumour cells and endothelial cells (Honn and Tang, 1992). An orderly and reproducible sequence of events has been defined using the experimental metastasis of three murine tumour cell lines (Crissman *et al.*, 1988). First, initial tumour cell arrest with intimate contact between tumour cell and endothelial cell plasma membranes during the first 8 h; second, rapid platelet thrombus formation and association with arrested tumour cells (from 2 min. to 1-4 h); third, endothelial cell retraction and extension of the tumour cell to the sub-endothelial matrix commencing at 4 h; fourth, dissolution of the tumour-associated thrombus starting at 8 h and completed by 24 h to re-establish circulation; fifth, intravascular proliferation after 24 h, as reflected by the presence of mitotic figures, and the formation of intravascular tumour nodules; and sixth, dissolution of the BM by the attached tumour cells after 24-72 h. The final outcome of this interaction is the liberation (extravasation) of tumour cells from circulation into the target organ stroma.

The function of platelets and tumour cell-associated thrombosis in the extravasation steps remains unclear, although platelet involvement was indicated by experiments where induced thrombocytopenia resulted in decreased numbers of lung colonies after tail veil injection of tumour cells (Gasic *et al.*, 1973). Platelets are able to form adhesive bridges between tumour cells and the subendothelial matrix (Menter *et al.*, 1987). It has been proposed that the platelet thrombus may be protective against mechanical forces which would tend to dislodge the apparently weakly attached tumour cell from the endothelial monolayer (Weiss *et al.*, 1985). This is supported by the observation that the platelet thrombus remains until the tumour cell achieves contact with the subendothelial matrix (Crissman *et al.*, 1988), presumably to secure a more firm attachment (Kramer *et al.*, 1980). Another possibility is the facilitation of endothelial cell retraction and exposure of the subendothelial matrix (see below). *In vitro* studies have shown, however, that endothelial cell retraction in the presence of tumour cells can occur in the absence of platelets (Kramer and Nicolson, 1979).

The physical and functional integrity of endothelial cell monolayers is critical to retarding the dissemination of metastasising tumour cells. The integrity of the endothelial layer can be perturbed as a result of destructive cell damage in response to a number of stimuli. These include vasoactive substances (e.g., bradykinin, histamine, serotonin), lipid mediators (e.g., platelet-activating factor, leucotriene C_4), thrombin, tumour necrosis factor, serine proteases, γ-radiation and endotoxins (Lafrenie *et al.*, 1992). Most of these stimulants are implicated in increasing vascular permeability and facilitating leucocyte transmission through endothelial monolayers during inflammation. Some types of tumour cells may extravasate by intravascular proliferation resulting in rupture of the vessel wall. However, the majority of studies both *in vitro* and *in vivo* have shown that endothelial cells retract prior to extravasation. Using an *in vitro* system, Kramer and Nicolson (1979) showed that tumour cells were able to attach to endothelial cell monolayers and cause morphological changes, such as a rupture of endothelial-endothelial cell interactions, leading to endothelial

cell retraction and exposure of ECM, and subsequent invasion and underlapping of retracted endothelial cells. Similar observations were made after the experimental metastasis of three tumour cell lines (Crissman *et al.*, 1988). In contrast, entry into circulation of malignant cells from two metastasis forming tumour cell lines, occurred by penetrating the intact endothelial cell body (De Bruyn and Cho, 1982). The migration pore formed by the migrating tumour cell closed after the cell had reached the intravascular space.

The factors responsible for endothelial cell retraction are not clearly defined, although a strong candidate is a lipoxygenase metabolite of arachidonic acid, 12(*S*)-hydroxyeicosatetranoic acid (12(S)-HETE) (Honn *et al.*, 1994a), which induces reversible, non-destructive and non-denuding endothelial cell retraction. Endothelial cell monolayers retract in response to the adhesion of tumour cells, but not in response to normal fibroblasts, suggesting a specific interaction between tumour cells and endothelial cells. Tumour cells synthesise 12(S)-HETE in response to adhesion to endothelial cells, but a lack of detectable metabolite in the culture medium suggests metabolic transfer of 12(S)-HETE to endothelial cells. Indeed, this hypothesis is supported by the observation that tumour cells can establish gap junctional channels between their cytoplasms and those of endothelial cells, and easily transfer small molecules from the tumour cells to endothelial cells (El-Sabban and Pauli, 1991). These authors speculate that the transfer of mediators, such as 12(S)-HETE, may play a critical role in tumour extravasation. *In vivo*, host platelets are an abundant source of 12(S)-HETE, which may be locally released. Platelet activation and aggregation to tumour cells occurs within minutes of tumour cell arrest (Crissman *et al.*, 1988), and platelet-derived 12(S)-HETE may potentiate tumour cell-induced endothelial cell retraction. It is interesting to note that feeding a high fat diet, rich in linoleic acid, enhanced metastasis of a mouse mammary tumour cell line, compared with a diet containing the same quantity of fat, but of low linoleic acid content. Linoleic acid is metabolised to arachidonic acid, which is available for conversion to eicosanoids (Rose *et al.*, 1994). It is interesting to speculate that a mechanism involving enhanced production of 12(S)-HETE may be at work.

The finding that 12(S)-HETE is an activator of protein kinase C in tumour cells (Liu *et al.*, 1991), and that endothelial cell retraction induced by 12(S)-HETE is also protein kinase C-dependent (Tang *et al.*, 1993), has lead to the formulation by Honn *et al.* 1994a of a hypothetical mechanism. Tumour cell adhesion to endothelial cells activates 12-lipoxygenase in tumour cells, leading to arachidonic acid metabolism to 12(S)-HETE which activates protein kinase C and translocates it to the plasma membrane (Liu *et al.*, 1991). Protein kinase C phosphorylates and reorganises cytoskeletal elements which mobilise $\alpha_{IIb}\beta_3$ integrin-containing vesicles to the cell surface, where the integrin is required for tumour cell matrix adhesion (Honn *et al.*, 1992). 12(S)-HETE also modulates tumour cell spreading on matrix and tumour cell motility (Timar *et al.*, 1993). By its action on endothelial cells, 12(S)-HETE enhances $\alpha_v\beta_3$ integrin surface expression and tumour cell adhesion (Honn *et al.*, 1989), and protein kinase C mediated endothelial cell retraction (Honn *et al.*, 1994a). Also, 12(S)-HETE can promote the release of cathepsin B in cancer cell lines, including the *ras*-transformed MCF-10A line (Honn *et al.*, 1994b). Thus, 12(S)-HETE is able to mediate each of the three steps of invasion: adhesion, degradation and migration.

Successful intravasation or extravasation requires directed migration of tumour cells towards or from a blood vessel, while simultaneously overcoming endothelial cell and BM barriers. During extravasation, this process is probably initiated by the preferential adherence of tumour cells to the ECM underlying the vascular endothelial cells. Fibronectin, associated with the endothelial BM may, in part, be responsible for establishing an adhesive gradient that could be important for tumour cell extravasation (Kramer *et al.*, 1980). Similar haptotactic activity has been demonstrated for laminin, a glycoprotein also found in BM (McCarthy *et al.*, 1983). Interestingly, both type I and IV collagenases have been found to manifest significant chemokinetic (random motility) and chemoattractive (directed motility) activity respectively (Terranova *et al.*, 1989). Although an *in vivo* significance for this finding has not been established, even a small amount of collagenase in an extracellular compartment at the leading edge of a tumour cell can imply a high specific activity. Type I and IV collagens and their breakdown products are also chemoattractive (Terranova *et al.*, 1989),

therefore the collagenolytic activity may provide an additional mechanism for *in situ* enhancement of tumour cell motility in a collagen-rich matrix. The migration of tumour cells can also be enhanced by the presence of uPA and plasminogen (Terranova *et al.*, 1989). Immunohistochemical findings have shown that plasminogen is present throughout the thickness of capillary walls in tumour areas and in tumour stroma (Burtin *et al.*, 1985). With this localisation plasminogen could guide tumour cells toward and into capillaries, and promote the spread of cancer. Changes in enzyme and matrix components can thus offer a fine control mechanism which elicits specific responses in tumour cells which can potentiate invasive and metastatic behaviour.

It is clear that adhesive interactions play a critical role in the process of metastatic tumour dissemination. This topic is beyond the scope of this work, but it should be borne in mind when considering metastasis. Adhesion molecules can act as both positive and negative modulators of the metastatic process. Intercellular contacts that confine cells to the primary tumour site are mediated by homotypic adhesion molecules, such as E-cadherin, which are negatively correlated with metastatic potential (Takeichi, 1991). Tumour cells already in circulation may specifically adhere to endothelial cells in selected organs, a process mediated by molecules such as the VLA-4 ($\alpha_4\beta_1$) integrin or molecules containing the Sialyl Lewis X carbohydrate determinant (Zetter, 1993), which bind to endothelial adhesion molecules such as E-selectin (Bevilacqua *et al.*, 1989) or the vascular cell adhesion molecule, VCAM-1 (Osborn *et al.*, 1989). Preferential adhesion and subsequent site-specific metastasis of particular tumour types, may be specified by endothelial determinants such as lung endothelial cell adhesion molecule, LuECAM. After adherence to endothelial cells and endothelial cell retraction, metastatic cells must adhere to elements of the subendothelial BM such as laminin and type IV collagen (Terranova *et al.*, 1982), interactions frequently mediated by a laminin receptor (Castronovo *et al.*, 1991) and members of the β_1 and β_4 integrin families (Zetter, 1993). Finally, for movement into the subendothelial stroma, tumour cell adhesion to connective tissue elements such as fibronectin, type I collagen and hyaluronan is mediated by molecules such as

the β_1 integrins (Zetter, 1993) and the CD44 cell surface adhesion molecule (Gunthert *et al.*, 1991).

5.2 Cell culture

The MCF-10A and MCF-10AneoT cell lines were maintained as described in Section 4.2. Serum free medium (SFM) was prepared as before (Section 4.2) except that equine serum was replaced with sterile 0.1% (m/v) BSA and medium with human serum was prepared as before except that equine serum was replaced with 5% (v/v) complement-inactivated (56°C, 30 min) human serum (volunteer donor). Mouse embryo fibroblasts (NIH 3T3) were cultured as previously described (Section 4.2), except that RPMI-1640 medium (Sigma Chemical Co., St. Louis, Mo.) and 10% (v/v) foetal calf serum (Gibco, Paisley) were used as the culture medium. Fibroblast-conditioned medium (FCM) was prepared by switching the NIH 3T3 cells to MCF-10 cell culture medium (Section 4.2) for 3 passages, and then to conditioning medium (50 µg/ml ascorbic acid in SFM) for 48 h. The conditioned medium was adjusted to pH 7.4 with sterile 0.1 M NaOH, cell debris removed by centrifugation, and stored at -70°C. Where required, cells were assessed for viability and counted by trypan dye exclusion using an Improved Neubauer haemocytometer.

5.3 *In vitro* matrix degradation

A number of *in vitro* assays for tumour cell-dependent proteolytic activity rely on the degradation of insoluble ECM proteins. These are usually quantitative, as in the measurement of released radiolabel from metabolically labeled subendothelial matrix, produced by endothelial cell monolayers (Kramer *et al.*, 1982). A less frequently used approach is fluorescent microscopy of tumour cell-mediated matrix proteolysis. Fibronectin, immobilised as a uniform layer on an appropriate substratum, has been used as a substrate for tumour cell adhesion and proteolysis, degradation being detected by immunofluorescent microscopy (McCarthy *et al.*, 1985; Chen and Chen, 1987; Campbell and Campbell, 1988; Wakabayashi and Kawaguchi, 1992). Chen *et al.* (1994) have also similarly used rhodamine-labeled ECM produced by chicken embryonic fibroblasts, or fluorescein conjugates of type I and IV collagen, laminin (McCarthy *et al.*, 1985;

Kelly *et al.*, 1994) and matrigel (Monsky *et al.*, 1994) co-coupled to the surface of a cross-linked gelatin film, while Montcourrier *et al.* (1990) used fluorescein-labeled ECM produced by endothelial cells. In the present study direct fluorescent microscopy of FITC-labeled ECM was used to characterise the degradative characteristics of MCF-10A cell lines. Biomatrix I, a proprietary name and equivalent to the reconstituted BM, matrigel, was used as ECM.

5.3.1 Preparation of FITC-matrix

Biomatrix I labeled with FITC was produced by the method of Homer and Beighton (1990), where the method was used to generate protein substrates with fluorescent leaving groups.

5.3.1.1 Reagents

75 mM Sodium carbonate, pH 9.5. $NaHCO_3$ (0.315 g) and Na_2CO_3 (0.387 g) were each dissolved in dist.H_2O (50 ml). The Na_2CO_3 solution was titrated with $NaHCO_3$ to pH 9.5.

5.3.1.2 Procedure

Biomatrix I (5 mg in 511.2 µl, Biomedical Technologies, Inc., Stoughton, Ma.) was diluted with sodium carbonate buffer (1 ml), and FITC-celite (1.7 mg, Sigma Chemical Co., St. Louis, Mo.) added and stirred (2 h, 4°C). Celite was removed by centrifugation and the supernatant applied to a column of Sephadex G-25 (1,0 x 19 cm = 15 ml) equilibrated (10 ml/h) with ultrapure water. The V_0 fraction was collected as FITC-matrix, which was aliquoted and stored at -70°C.

5.3.2 FITC-matrix degradation assay

5.3.2.1 Materials

Serum free medium. Prepared as in Section 5.2.

Complete medium with equine serum. Prepared as in Section 5.2.

Complete medium with human serum. Prepared as in Section 5.2.

5.3.2.2 Procedure

Round coverslips (22 mm) were rinsed in ethanol, dist.H_2O and ultrapure water, and allowed to dry under ultraviolet light in a laminar flow hood. FITC-matrix was diluted to 30 µg/50 µl with ultrapure water, and deposited as a drop (50 µl, *ca.* 13 mm in diameter) on the coverslips and allowed to dry under ultraviolet light in a laminar flow hood. Coverslips were placed in 35 mm cell culture petri dishes, and the FITC-matrix allowed to rehydrate in the appropriate medium (5 min; SFM, complete medium with equine serum or complete medium with human serum). Cells (MCF-10A and MCF-10AneoT) were trypsinised (Section 4.2), suspended in complete medium, washed three times in HBSS, and resuspended in the appropriate medium. Protease inhibitors used are indicated in the results (Section 5.6.1), and were made up as in Section 5.4.2. Cells were seeded into petri dishes at low density (5 x 10^3) and incubated (16 h) as before (Section 4.2). A coverslip was placed cell-side down into a custom-designed holder (Mechanical Instrument Workshop, University of Natal, Pietermaritzburg) which is able to accommodate pre-warmed medium. This allows cells to be viewed live under the epifluorescent microscope (Olympus BH-2 equipped with an Olympus BH-2 RFC epifluorescence attachment). Representative cells were photographed under phase-contrast and epifluorescence using an Olympus PM-20 exposure control unit, with Ilford XP-2 film.

5.4 *In vitro* tumour invasion models

Evidence for the involvement of proteinases in tumour cell invasion, is derived from various experimental approaches quantifying the degradation (using metabolically radiolabeled ECM formed by cultured cells) and/or invasion of BM and interstitial stroma by tumour cells. The effect of specific inhibitors or antibodies to selected proteinases is measured in these assays. The modified Boyden chamber invasion system used in some of the SEM studies (Section 5.4.1) and used for quantifying the effects of various proteinase inhibitors, is discussed in Section 5.4.2.

5.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) has been used recently to visualise the penetration of invasive cells through proteinaceous barriers. Human amniotic membrane was used to demonstrate the invasion of B16-BL6 mouse melanoma cells (Mignatti *et al.*, 1986) and polymorphonuclear leucocytes (Bakowski and Tschesche, 1992), and matrigel-coated filters were used to investigate invasion of human cytotrophoblasts (Librach *et al.*, 1991) and hamster melanoma cells (Thomas *et al.*, 1993). In the present study the methods of Neumüller (1990) and Arro *et al.* (1981) were used. These are designed for cultured cells and to minimise artefacts due to imperfect fixation, shrinkage during dehydration and drying, or uneven metal coating.

5.4.1.1 Reagents

Complete medium with equine serum. Prepared as in Section 5.2.

Serum free medium. Prepared as in Section 5.2.

Fibroblast conditioned medium. Prepared as in Section 5.2.

Wash buffer (0.1 M sodium cacodylate, pH 7.2). Sodium cacodylate (5.35 g) was dissolved in about 200 ml of dist.H_2O, titrated to pH 7.2 with HCl and made up to 250 ml.

Fixative (0.1 M sucrose, 2% (v/v) glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2). Sodium cacodylate (1.07 g) and sucrose (1.71 g) were dissolved in about 40 ml of dist.H_2O, glutaraldehyde (4 ml of 25% stock, Merck, Darmstadt) was added, the solution titrated to pH 7.2 with HCl and made up to 50 ml.

0.2 M Sodium cacodylate, pH 7.2. Sodium cacodylate (2.14 g) was dissolved in about 40 ml of dist.H_2O, titrated to pH 7.2 with HCl, and made up to 50 ml.

Post-fixative (1% (v/v) osmium tetroxide in 0.2 M sodium cacodylate, pH 7.2). Osmium tetroxide (1.875 ml of 4% stock) was added to 0.2 M sodium cacodylate (3.75 ml) and made up to 6 ml with dist.H_2O.

30% (v/v) Ethanol. Ethanol (6 ml, AR) was made up to 20 ml with dist.H_2O.

50% (v/v) Ethanol. Ethanol (10 ml, AR) was made up to 20 ml with dist.H_2O.

70% (v/v) Ethanol. Ethanol (14 ml, AR) was made up to 20 ml with dist.H_2O.

80% (v/v) Ethanol. Ethanol (16 ml, AR) was made up to 20 ml with dist.H_2O.

90% (v/v) Ethanol. Ethanol (18 ml, AR) was made up to 20 ml with dist.H_2O.

95% (v/v) Ethanol. Ethanol (19 ml, AR) was made up to 20 ml with dist.H_2O.

5.4.1.2 Procedure

Biomatrix I (Biomedical Technologies, Inc., Stoughton, Ma.) was thawed on ice and diluted with cold ultrapure water (20 µg in 50 µl). Polycarbonate filters (Nuclepore, Costar, Cambridge, Ma., 13 mm, 8 µm pores) were placed on a plastic petri-dish, coated (dull side up) with diluted Biomatrix I (50 µl), allowed to dry in a laminar-flow bench with the ultraviolet light on, and the coating procedure repeated. For chemotaxis control experiments, filters were coated (dull side up) with type VI collagen (10 µg in 10 µl, prepared in this laboratory by Dr Theresa Coetzer). Coated filters were covered with a large drop of SFM (5 min) to reconstitute the ECM proteins. The bottom-half of the Boyden chambers was filled with FCM (200 µl, chemotaxis control or invasion) or complete medium (morphological characterisation), and the filters carefully placed over the medium, taking care not to trap air-bubbles. The upper chamber was screwed on, and cells (MCF-10A and MCF-10AneoT) were trypsinised (Section 4.2), suspended in complete medium, washed three times in HBSS, resuspended in SFM or complete medium, assessed for >98% viability, counted and added (5×10^4 in

800 µl SFM or complete medium) to the upper chamber. Boyden chambers were placed in the cell culture incubator (16 h), and at timed intervals (40 min, 2 h, 16 h) a chamber was removed, disassembled and processed for SEM. Control chambers (16 h incubation) were assessed for chemotaxis and invasion (Section 5.4.2).

For SEM processing, removed filters were gently placed in an equal volume mixture of SFM and fixative and allowed to stand undisturbed (5 min, 37°C). The mixture was replaced with fixative (1 h, 37°C) which was periodically agitated and allowed to cool to RT. Fixative was replaced and cells fixed overnight (4°C). Filters were rinsed with wash buffer (3 x 2 min.), post-fixed (1 h, RT), rinsed again (3 x 2 min.) and dehydrated in an increasing ethanol series. Cells were critical point dried (Hitachi HCP-2) from liquid CO_2, filters cut into ~3 mm^3 squares and mounted, upper surface exposed, onto copper stubs. Some of the filter squares derived from invasion experiment were mounted bottom side up, and others were stripped of cells by gently laying a filter onto the adhesive on a stub and pulling away. This leaves cells, ventral-side up, on the stub. Filters were sputter-coated (Polarson SEM coating unit) with gold/palladium (80/20). Cells were viewed and representative photographs taken in an Hitachi S-570 scanning electron microscope at 8 kV.

5.4.2 Modified Boyden chamber invasion assays

In vitro invasion studies utilise either natural BM, or reconstituted BM. Three different types of natural BM have been used: the human amniotic membrane (Thorgeirsson *et al.*, 1982), the bovine lens capsule (Starkey *et al.*, 1984) and the chorioallantoic membrane of the chicken embryo (Ossowski and Reich, 1983). The amniotic membrane, obtained from human term placentas, is the most commonly used of these natural substrates for cell invasion studies. Soluble ECM from the Englebreth-Holm-Swarm murine sarcoma cell line (matrigel or Biomatrix I), containing laminin, type IV collagen, fibronectin, elastin, entactin and nidogen (Orkin *et al.*, 1977; Kleinman *et al.*, 1986), is the most widely used substrate for invasion studies, as it is considered to be biochemically almost identical to BM.

The assay is conceptually, but not practically, simple. In general, polycarbonate filters with 5-8 μm diameter pores are coated with the matrix, to form an artificial BM. The filters are inserted into chemotaxis chambers consisting of a top and bottom compartment separated by the matrix-coated filter. Culture medium is added to both compartments, and the tumour cells seeded onto the upper, matrix coated, surface of the filter. Protease inhibitors may be included in the media to investigate the contribution of various proteases to invasion. During incubation (6-24 h) the cells infiltrate the matrix coating, migrate and adhere to the bottom surface of the filter. At the end of the incubation the material on the top surface is removed, the filter stained and the number of cells on the bottom surface counted with a microscope (Albini *et al.*, 1987). The blind well Boyden chambers used in this study were manufactured by the Mechanical Instrument Workshop, University of Natal, Pietermaritzburg. The protease inhibitor concentrations used in this study were based on the work of Mignatti *et al.* (1986) and Yagel *et al.* (1989b).

5.4.2.1 Reagents

HBSS. Prepared as in Section 4.2.

Complete medium with equine serum. Prepared as in Section 5.2.

Serum free medium. Prepared as in Section 5.2.

Fibroblast conditioned medium. Prepared as in Section 5.2.

1, 10-Phenanthroline (10 mg/ml) in dimethyl sulfoxide (DMSO).

1, 10-Phenanthroline A (Sigma Chemical Co., St. Louis, Mo.) (5 mg) was dissolved in DMSO (0.5 ml) and diluted to a final concentration of 10 μg/ml in SFM and FCM (Section 5.4.2.2).

Pepstatin A (7.0 mg/ml) in DMSO. Pepstatin A (Sigma Chemical Co., St. Louis, Mo.) (1.05 mg) was dissolved in DMSO (150 µl) and diluted to a final concentration of 10 µg/ml in SFM and FCM (Section 5.4.2.2).

L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) (7.5 mg/ml) in DMSO. E-64 (Sigma Chemical Co., St. Louis, Mo.) (0.75 mg) was dissolved in DMSO (100 µl) and diluted to a final concentration of 36 µg/ml in SFM and FCM (Section 5.4.2.2).

Fixative (2 mg/ml malachite green in methanol). Malachite green (2 mg) was dissolved in methanol (1 ml).

Stain 1 (0.1% (v/v) eosin Y, 0.1% (v/v) formaldehyde, 0.4% (m/v) Na_2HPO_4, 0.5% (m/v) KH_2PO_4). Na_2HPO_4 (0.2 g) and KH_2PO_4 (0.25 g) were dissolved in dist.H_2O, eosin Y (50 µl) and formaldehyde (50 µl) were added and the solution made up to 50 ml.

Stain 2 (0.047% (m/v) methylene blue, 0.4% (m/v) Na_2HPO_4, 0.5% (m/v) KH_2PO_4). Na_2HPO_4 (0.2 g) and KH_2PO_4 (0.25 g) were dissolved in dist.H_2O, methylene blue (23.5 mg) was added and the solution made up to 50 ml.

5.4.2.2 Procedure

For invasion and control random migration and chemotaxis experiments, polycarbonate filters were coated as described in Section 5.4.1. Coated filters were covered with a large drop of SFM with protease inhibitor, DMSO or HBSS solvent when appropriate, and placed in a cell culture incubator (60 min). Cells (MCF-10A and MCF-10AneoT) were trypsinised (Section 4.2), suspended in complete medium, washed three times in HBSS, resuspended in SFM, assessed for >98% viability and counted. The bottom-half of the Boyden chamber was filled with SFM (200 µl, random migration control) or FCM (200 µl, chemotaxis control or invasion), and the filters carefully placed over the medium, taking care not to trap air-bubbles. The upper chamber was screwed on, and cells (3×10^5 in

800 µl SFM, with inhibitor, DMSO or HBSS when appropriate) added to the upper chamber. Boyden chambers were placed in the cell culture incubator (16 h) for migration and invasion to occur. Filters were removed, the upper surface allowed to adsorb onto paper towelling, and the filter gently laterally pulled away to remove adherent cells. Cells on the filter underside were fixed (5 x 1 s), drained, and stained (5 x 1 s) in each of Stain 1 and Stain 2, rinsed in dist.H_2O and allowed to dry. An image analysis system (Kontron Vidas, Kontron Elektronik, Germany), in tandem with a light microscope, was used to determine cell surface area, which was used as a measure of the number of invaded cells The use of cell surface area as a representation of cell number, was validated by comparison with scores obtained by manual cell counting. A total of 10 fields of vision per filter, 4 bisecting the invasion area and 3 on either side, were used to determine cell surface area. Image analysis was not able to discriminate between cells and pores in the filter, and so the program was modified to eliminate any surface area smaller than 200 pixels, which was found to be larger than a pore but smaller than a cell. Invasion was determined by calculating cell surface area of a treated experiment as a percentage of an untreated positive control, which was taken as 100% invasion. Each assay was performed in duplicate, repeated either two or three times.

5.5 Gelatin-substrate SDS-PAGE

Substrate zymograms combine the molecular weight-dependent resolving power of SDS-PAGE, with detection based on the activity of the enzyme of interest. Incorporation of gelatin (denatured type I collagen) into gels allows for the detection of a variety of proteinases (Heussen and Dowdle, 1980). Following SDS-PAGE, proteinases are renatured by substitution of SDS in the gel by Triton X-100. Gels are then incubated in assay buffer containing the relevant activators and inhibitors, to ensure specific enzyme activity. Proteinase activity is visualised, after staining of gels, by clear bands of digested gelatin. The assay buffer of Brooks *et al.* (1996) was used in this study.

5.5.1 Reagents

1% (m/v) Gelatin in running gel buffer. Gelatin (0.1 g) was dissolved in running gel buffer (solution B, Section 2.3.2) (10 ml) with gentle heating.

2.5% (v/v) Triton X-100. Triton X-100 (5 ml) was diluted to 200 ml with dist.H_2O.

Assay buffer (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5. Tris (3.03 g), NaCl (5.84 g) and $CaCl_2$ (0.74 g) were dissolved in 450 ml of dist.H_2O, titrated to pH 7.5 with HCl and made up to 500 ml. When required the metalloproteinase inhibitor, 1, 10-phenanthroline, was added to the assay buffer to a concentration of 10 µg/ml from a 100 mg/ml stock solution (Section 5.4.2).

0.01 M *p*-aminophenylmercuric acid (APMA) in 0.05 M NaOH. *p*-aminophenylmercuric acid (35 mg) was dissolved in 10 ml of 0.05 M NaOH (0.02 g dissolved in 10 ml of dist.H_2O) just before use.

0.1% (m/v) Amido black. Amido black (0.1 g) was dissolved in methanol:acetic acid:dist.H_2O (30:10:60) (100 ml), and filtered through Whatman No. 1 filter paper before use.

5.5.2 Procedure

Serum free medium (8 ml) was conditioned (72 h) by confluent MCF-10A and MCF-10AneoT cells grown in 75 cm^2 flasks, and concentrated to 1 ml. The total number of cells in each flask was counted (Section 5.2), and the relative volumes of conditioned medium added to treatment buffer adjusted to account for differences in cell number. This allows direct quantitative comparison of clearing by enzyme activity from the two cell lines. The procedure for preparing SDS-PAGE gels (Section 2.3.2) was modified by adding 1% (m/v) gelatin (1.5 ml) to the running gel buffer (2.25 ml), and reducing and increasing the respective volumes of acrylamide monomer (solution A) and dist.H_2O, to produce a 7.5% gel. When necessary, latent collagenases were activated with APMA (0.01 M stock diluted to 0.5 mM in conditioned medium, 37°C, 30 min). Samples were

prepared in treatment buffer (Section 2.3.2) without β-mercaptoethanol and without boiling, and run as normal.

After electrophoresis, the running gel was soaked in two changes of 2.5% (v/v) Triton X-100 (50 ml) over 1 h at RT. The gel was incubated in assay buffer (16 h) at 37°C, stained in 0.1% (m/v) amido black (1 h) and destained in several changes of methanol:acetic acid:dist.H_2O (30:10:60).

5.6 Results

It is established that MCF-10AneoT, in contrast to MCF-10A cells, are able to invade artificial basement membrane *in vitro* (Ochieng *et al.*, 1991), and are able to form sustainable xenografts in mice (Miller *et al.*, 1993). No attempt, however, has been made to characterise the proteolytic episodes, elaborated by *ras*-transformed breast epithelial cells, which occur during these events, and the present study begins to address this question.

5.6.1 Extracellular matrix degradation

There are a few reports that growth of transformed cells on a substratum of fluorescently-labeled extracellular matrix results in discrete spots lacking fluorescence beneath the cells (McCarthy *et al.*, 1985; Chen and Chen, 1987; Campbell and Campbell, 1988). To determine whether *ras*-transformation of human breast epithelial cells increases the expression of proteolytic enzymes at the cell surface or in the extracellular milieu capable of degrading artificial BM, the effect of cells grown on FITC-matrix was investigated by visualising degradation under the ventral surface of cells. After 16 h in SFM, MCF-10A cells had effected degradation mostly corresponding to the cell circumference and pseudopodial projections (Fig. 29A & B). Transformed cells, in contrast, were capable of extensive matrix degradation (Fig. 29C & D and Fig. 30A & B), which appeared to originate at the cell periphery, proceeding inwards (results not shown) (initial degradation was almost always observed peripheral to a cell). Degradation associated with cellular motility was observed (Fig. 29D), and matrix removal was also visible under phase-contrast optics (Fig. 29C). It was common to observe phagocytosed matrix in the form of bright fluorescent spots, where

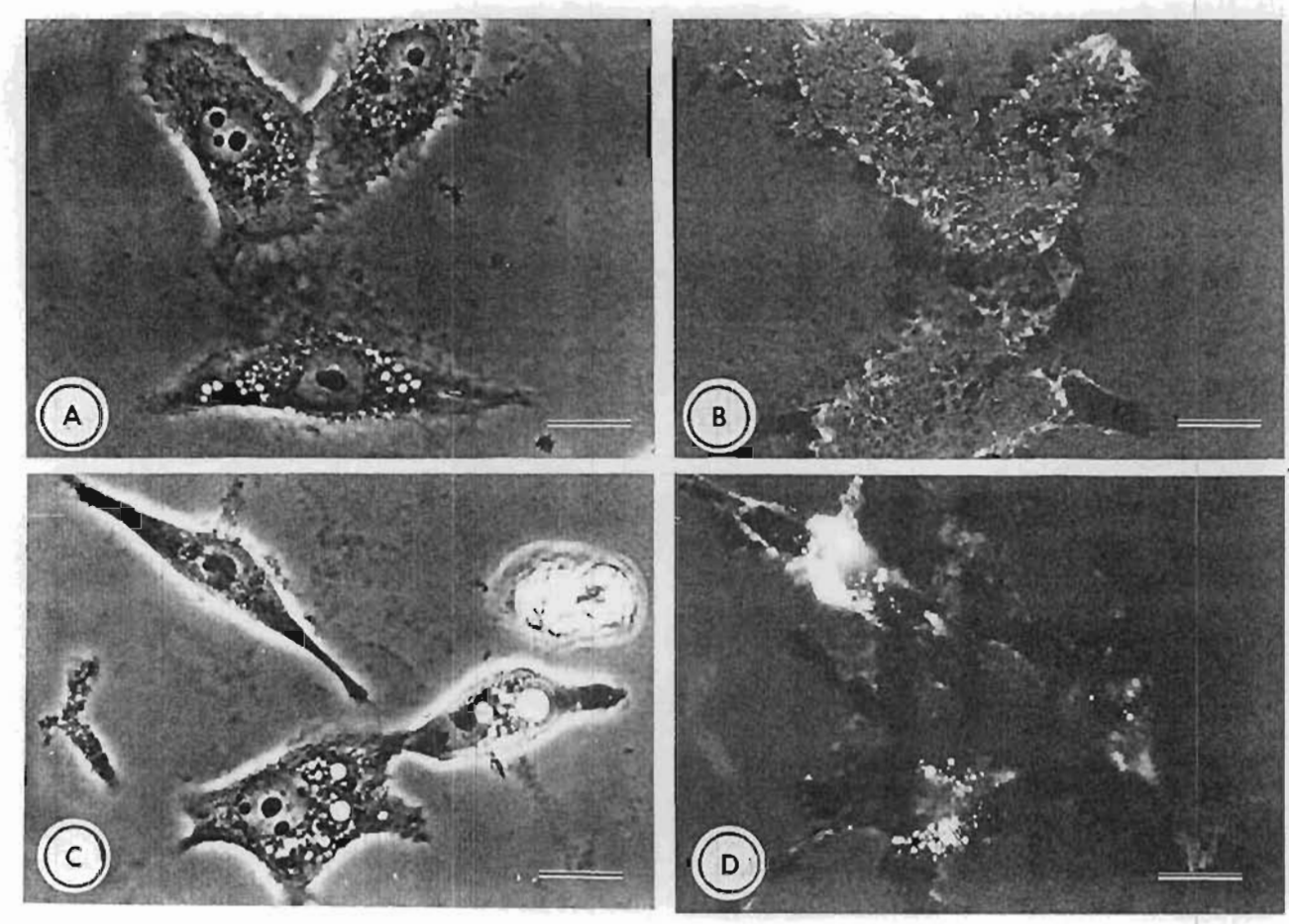
A
B
C
D

Figure 29. Behaviour of MCF-10A and MCF-10AneoT cells, in SFM, on fluorescently labeled Biomatrix I coated substrata.
(A, B) MCF-10A and (C, D) MCF-10AneoT cells were seeded onto FITC-matrix, incubated (16 h) and observed live by (A, C) phase contrast and (B, D) epifluorescent microscopy (Section 5.3.2).

Bar scales: (A- D) 40 μm.

A
B

Figure 30. Behaviour of MCF-10AneoT cells, in SFM, on fluorescently labeled Biomatrix I coated substrata.

MCF-10AneoT cells were seeded onto FITC-matrix, incubated (16 h) and observed live by (A) phase contrast and (B) epifluorescent microscopy (Section 5.3.2).

Bar scales: (A and B) 10 µm.

matrix has been concentrated in intracellular vesicles (Fig. 29C & D and Fig. 30A & B).

Tumour cells, during *in vivo* metastasis, are in contact with interstitial tissue fluids or plasma which are rich sources of proteinase inhibitors, and it was therefore of interest to ascertain the behaviour of the transformed and normal breast epithelial cells in the presence of serum. The most notable effect is the increase in cellular motility and the concomitant destructive effects on the matrix. This effect, in equine serum, is manifest even by the MCF-10A cells (Fig. 31A & B), but is more pronounced with the transformed cells (Fig. 31C & D). Human serum accentuates the effect in the transformed cells (Fig. 31E & F), and cells appear very rounded, probably due to their increased motility. A dominant feature is the accumulation of matrix around motile cells (Fig. 31A & B and Fig. 31E & F), especially at the leading edge of the cell, and the presence of strands of matrix emanating from cells, suggesting that mechanical events may also play a role in matrix disruption in this assay system. Cells may be invading under the matrix layer and by moving along the surface of the cover-slip may disrupt and become entangled in matrix. In the presence of human serum, MCF-10A cells were observed to contain phagocytosed matrix, in the form of many small dim perinuclear vesicles, while transformed cells demonstrated fewer, larger and brighter vesicles (see Fig. 32C & D). Both cell lines were able to 'track' over the substratum leaving a path denuded of matrix, but MCF-10A cells were able to accomplish this only when the matrix was thin and patchy (results not shown). Transformed cells, in contrast, effectively cleared matrix in their path (Fig. 31G & H).

The effect of various protease inhibitors on degradation was tested. All inhibitors used (pepstatin A, 1, 10-phenanthroline, E-64, leupeptin, antipain, ε-amino caproic acid, aprotinin, soybean trypsin inhibitor) were unable to extinguish degradation to any noticeable degree. In the presence of the metalloproteinase inhibitor, 1,10-phenanthroline, transformed cells were as able to disrupt the matrix layer (Fig. 32A & B), and in the presence of leupeptin many instances of matrix phagocytosis were seen (Fig. 32C & D), typical, in this case, of the extensive

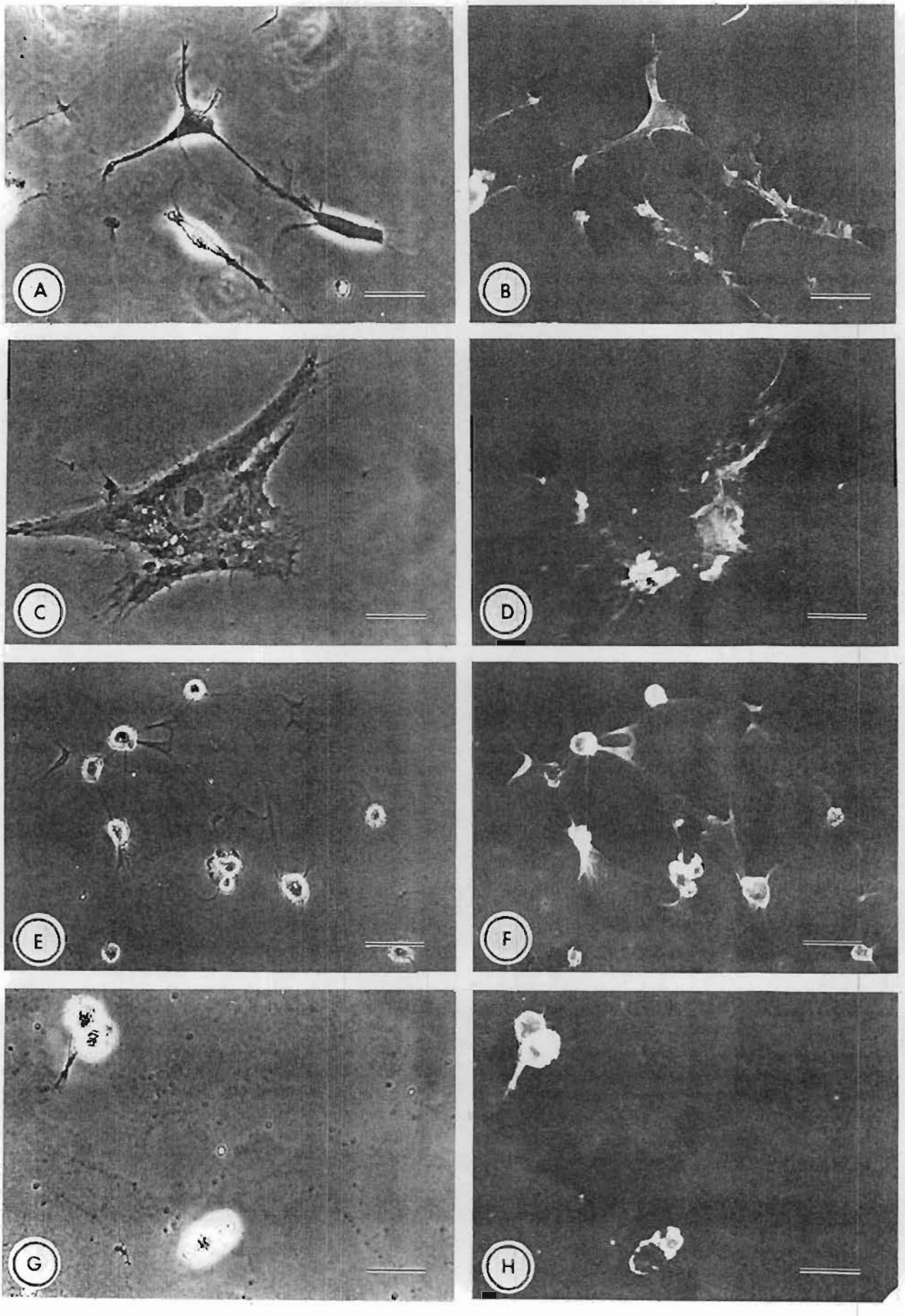
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Figure 31. Behaviour of MCF-10A and MCF-10AneoT cells, in medium supplemented with horse or human serum, on fluorescently labeled Biomatrix I coated substrata.

(A, B) MCF-10A and (C-H) MCF-10AneoT cells were seeded onto FITC-matrix, incubated (16 h) and observed live by (A, C, E, G) phase contrast and (B, D, F, H) epifluorescent microscopy (Section 5.3.2). Cells were grown in the presence of (A-D) equine serum and (E-H) human serum.

Bar scales: (A-H) 40 μm.

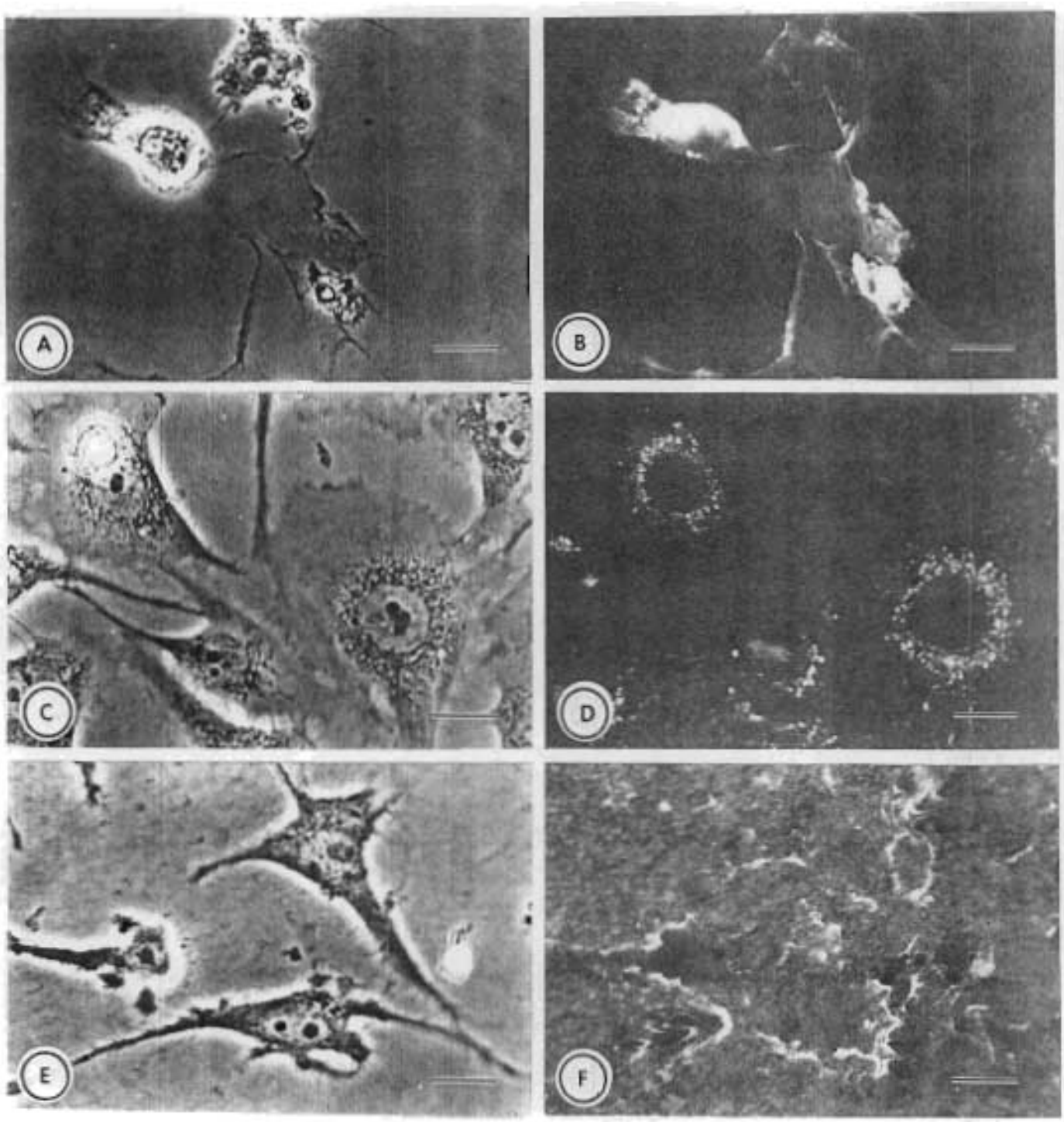
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Figure 32. Behaviour of MCF-10AneoT cells, in the presence of protease inhibitors, on fluorescently labeled Biomatrix I coated substrata. MCF-10AneoT cells were seeded onto FITC-matrix, incubated (16 h) and observed live by (A, C, E) phase contrast and epifluorescent (B, D, F) microscopy (Section 5.3.2). Cells were grown in the presence of (A, B) 1, 10-phenanthroline, (C, D) leupeptin and (E, F) aprotinin.

Bar scales: (A-F) 40 μm.

phagocytosis that appears to occur in areas where matrix is deposited sparsely. In the presence of the serine proteinase inhibitors aprotinin and ε-amino caproic acid, cells were occasionally observed beneath the layer of matrix, as reflected by the blurred regions corresponding to the position of cells when focus is in the plane of the deposited matrix layer, in some cases without disrupting it (Fig. 32E & F). A similar lack of proteolytic inhibition was observed with normal breast epithelial cells in the presence of inhibitors (results not shown).

To ascertain whether any matrix-degrading activity was released into the culture medium, conditioned medium (48 h) from normal and transformed cells, adjusted to pH 5 or 7.2, was incubated (72 h) with FITC-matrix coated cover-slips, and the supernatant tested for released matrix by fluorimetry. No significant increase in fluorescence over control medium was detected for either cell line (results not shown).

5.6.2 SEM characterisation of cell morphology and invasion

A SEM morphological characterisation of the MCF-10A cell line has been reported (Tait *et al.*, 1990), but no such study has been performed on the *ras*-transformed counterpart, MCF-10AneoT. MCF-10A cells grown, in the presence of serum, on Biomatrix I do not differ appreciably from those grown on glass (Tait *et al.*, 1990). The cells form flat monolayers, are closely apposed and roughly polyhedral in shape, and the surface is covered by sparse short microvilli (Fig. 33A). The transformed cells are irregularly shaped, ranging from dome to spindle, to stellar shaped. They are thicker in the centre, have well defined cell borders and at high density overlap one another and thereby do not form a continuous monolayer (Fig. 34B). The surface is covered with abundant long, slender microvilli or filopodia and occasional cells have membrane blebs (Fig. 33B & C). Long, thin horizontal extensions, up to one cell diameter long, project from the cells (Fig. 33B and C). In SFM, MCF-10AneoT cells lack long extensions, but still possess numerous microvilli and blebs (Fig. 33D).

Little is known about the morphological changes undergone by metastasising tumour cells during extravasation. Although this question cannot be adequately

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Figure 33. Morphology of MCF-10A and MCF-10AneoT cells, grown on Biomatrix I, determined by SEM (Section 5.4.1).
(A) MCF-10A cells, grown in the presence of equine serum, form a flat monolayer of which the surface is covered with short microvilli. (B) MCF-10AneoT cells, also in the presence of equine serum, elaborate long cellular extensions and microvilli, while some cells were seen to feature large blebs (upper cell). (C) Enlargement of the lower cell in (B). When the cell density is high, the transformed cells are able to overlap one another (see Fig. 34B). (D) An MCF-10AneoT cell grown in SFM, showing numerous microvilli and blebs.

Bar scales: (A) 21 μm; (B) 10 μm; (C) 4.1 μm; (D) 2.1 μm.

addressed *in vitro*, it is important to characterise the events occurring during modified Boyden chamber invasion assays, as the quantitative results from these assays are used to help define proteolytic mechanisms which may be relevant *in vivo*. To this end the invasive MCF-10AneoT cell line was allowed to invade *in vitro* and then subjected to SEM characterisation. Cells were firmly attached to the Biomatrix I 40 min after seeding and had begun to spread (Fig. 34A). 2 h after seeding, cells were fully spread (Fig. 34B) but there were no signs of invasion, while at 4 h invasion had begun (Fig. 34C). In response to the chemoattractant, cells were able to deform their leading edge into a narrow extension that projected into a pore in the filter (Fig. 34C). On emerging on the filter underside the extension immediately spread over the surface into a thin sheath already elaborating some microvilli (Fig. 34D). After 16 h many cells had emerged underneath the filter, and all appeared firmly attached and spread (Fig. 34E). In an attempt to visualise the pseudopodial extensions which are, presumably, responsible for breaching the Biomatrix I barrier, cells were stripped from their filter after 4 h and viewed upside down. At the point of adherence to the stub, cells appear to be damaged, but the putative pseudopodia appear intact, and display microvilli and membrane folds (Fig. 34F).

5.6.3 *In vitro* invasion assays

The *in vitro* invasion assay was used to study the role of proteinases in *ras*-transformed cell invasion by attempting to inhibit specific proteases. As the proteases mediating cell invasion are probably localised in regions of close contact between the cells and matrix substratum, inhibitors were pre-adsorbed onto the filter-borne matrix, to ensure the presence of inhibitors in regions of cell substratum adhesion. Furthermore, unlike the case with the FITC-matrix assays, inhibitors have access from the lower half of the Boyden chamber to the underside of the matrix, *via* the pores in the filter. This assumes that the inhibitors are able to penetrate through matrix in and coating the pores. The validity of invasion experiments is dependent upon pores of the filter being evenly and completely coated with reconstituted BM matrix. It has been reported that complete coating is attainable down to 25 µg/13 mm filter (Mackinnon *et al.*, 1992), thus proper coating was assumed at the 40 µg/13 mm filter used in this

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Figure 34. Attachment of MCF-10AneoT cells to Biomatrix I and subsequent invasion as demonstrated by SEM (Section 5.4.1).
(A) 40 min after seeding cells are firmly attached and beginning to spread. (B) After 2 h cells are spread and (C) invasion begins. (D) After 6 h a cell emerges through a pore in the filter and attaches to the underside. (E) After 16 h many cell have invaded and are adherent to the filter under-surface. (G) Cells stripped from a filter in the process of invasion (4 h), showing the dorsal aspect revealing invadopodia (F) which were, presumably, extracted from a pore in the filter.

Bar scales: (A) 6.6 μm; (B) 10.1 μm; (C) 7.5 μm; (D) 5.5 μm; (E) 40 μm; (F) 5.5 μm.

study. Coating heterogeneity appears to be the most significant problem, and manifests itself as a heterogenous population of stained cells across the underside of a filter. This makes cell quantification difficult, and sometimes impossible, stressing the need for quantification in many fields of vision across a filter surface.

As shown in this study, both cathepsins B and D are present on the surface of MCF-10AneoT cells. To ascertain if their presence assisted *in vitro* invasion, inhibitors to aspartic and cysteine proteinases, i.e. pepstatin A and E-64, were used in Boyden chamber assays. Neither inhibitor effected significant inhibition of invasion relative to control invasion in the absence of an inhibitor (100% invasion), indeed inhibition of cathepsin D potentiated invasion (Fig. 35).

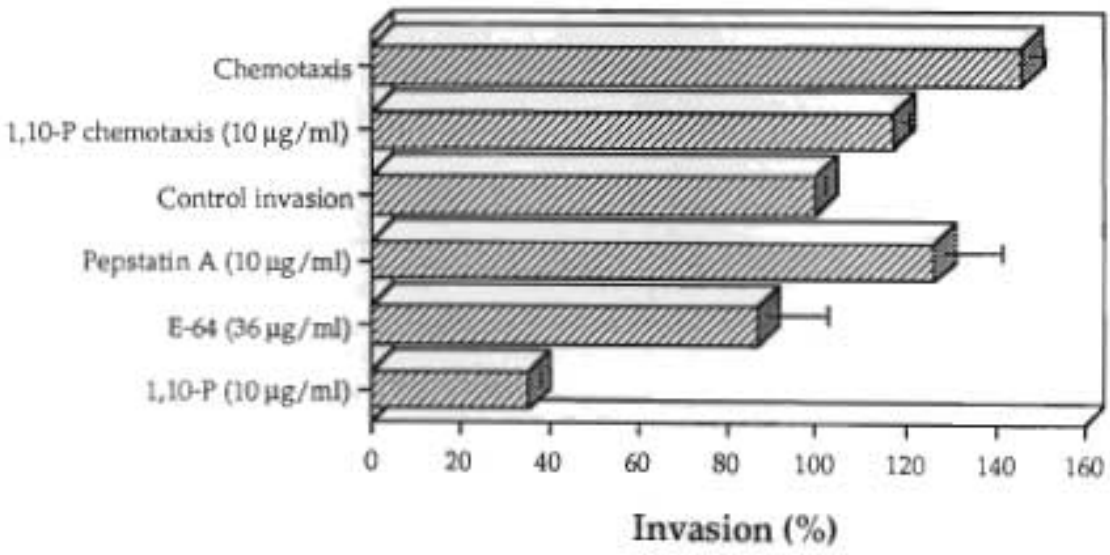

Figure 35 Effect of aspartic, cysteine and metalloproteinase inhibitors on *in vitro* basement membrane invasion by MCF-10AneoT cells.
Inhibitors (at the concentration indicated in the figure) were incubated with Biomatrix I coated filters 1 h prior to cells being added to the chambers, as described in Section 5.4.2. Invasion in the absence of inhibitor was normalised to 100%. Error bars represent the standard deviation of the invasion mean of the assays performed. Each assay was performed in duplicate and each experiment repeated either two or three times.

Significant inhibition of invasion of *ras*-transformed cells was achieved with the MMP inhibitor, 1, 10-phenanthroline, implicating this class of enzymes as the

rate-limiting proteolytic facilitators of *in vitro* invasion (Fig. 35). To ensure that 1, 10-phenanthroline does not perturb cell viability and motility at the concentration used, cell chemotaxis was measured in the presence and absence of inhibitor, and each assay normalised to control (100%) invasion. The presence of the inhibitor only slightly decreases the rate of chemotaxis (Fig. 35), indicating that the inhibitory effect it has in the invasion assay is specific to proteolytically mediated invasion.

5.6.4 Metalloproteinase gelatin-substrate gel electrophoresis

Gelatin-substrate gel electrophoresis was used to ascertain the extent to which normal and *ras*-transformed breast epithelial cell lines express MMPs into the culture supernatant and, in the light of inhibition of *in vitro* invasion by the MMP inhibitor, 1, 10-phenanthroline, the degree to which this expression differs. Both cell lines, in the absence of the metalloproteinase activator APMA, express a minor band of activity at 120-150 kDa, and a major band at 100-110 kDa (Fig. 36A). A major band at 80-85 kDa is, however, significantly more active in the supernatant of the transformed cells. A further minor band of activity is present at 55-60 kDa in the MCF-10A cell line supernatant.

In the presence of APMA (Fig. 36B), the 100-110 kDa band migrated as a doublet, consistent with activation of type IV collagenases (Marbaix *et al.*, 1996). The upper band represents the latent proform and the lower band the activated enzyme. The latent proform is rendered active under the conditions of this assay, probably as a result of the activating power of SDS, which presumably induces a conformational change in type IV collagenases, removing the inhibiting propiece from the active site (Birkedal-Hansen and Taylor, 1982). A faint band of activated 80-85 kDa protease is present in the MCF-10AneoT lane, probably due to incomplete activation under the assay conditions used. Incubation of gels in assay buffer in the presence of 1, 10-phenanthroline completely abolished all clearing, indicating that all activity is due to MMPs (Fig. 36C).

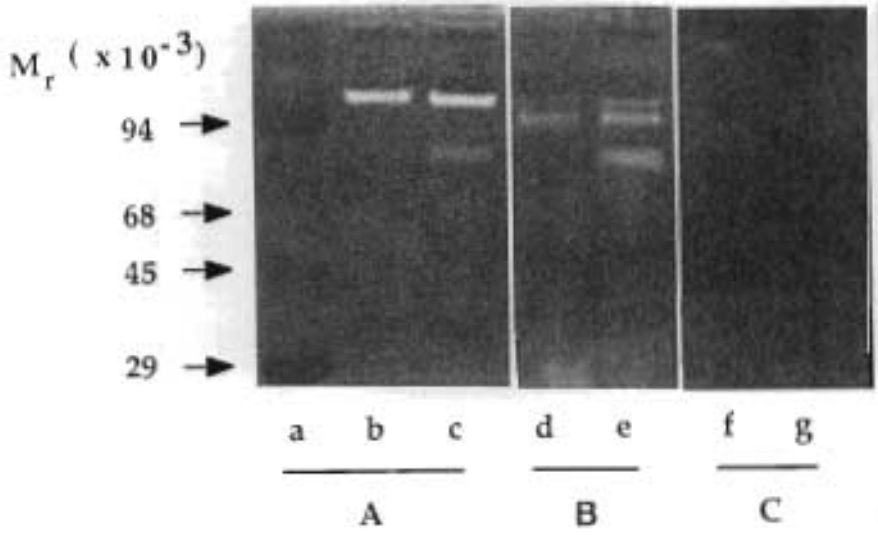

Figure 36 Gelatin-substrate SDS-PAGE of SFM conditioned by MCF-10A and MCF-10AneoT cells.

Non-reduced samples (normalised by cell number) were loaded onto a 7.5% gelatin-containing gel, and following electrophoresis proteases were renatured, the gels incubated in assay buffer, stained and destained as in Section 5.5. **A)** Samples prepared in the absence of APMA and the gel incubated with assay buffer (a) molecular weight markers (phosphorylase b, 93 kDa; BSA, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa). (b) MCF-10A conditioned medium; (c) MCF-10AneoT conditioned medium. **B)** Samples prepared in the presence of APMA and the gel incubated with assay buffer, (d) MCF-10A conditioned medium; (e) MCF-10AneoT conditioned medium. C) Samples prepared in the absence of APMA and the gel incubated with assay buffer in the presence of 1, 10-phenanthroline, (f) MCF-10A conditioned medium; (g) MCF-10AneoT conditioned medium.

5.7 Discussion

Tumour cell metastasis is dependent upon breaching the BM, which implies ECM degradation, a process facilitated by localised proteolysis. Two morphological approaches have been used to access cell invasion in the present study. 1) By using a substratum of immobilised ECM coupled to a fluorescent marker, it has been shown that *ras*-transformed human breast epithelial cells produce proteases that are capable of degrading ECM. 2) An SEM investigation has shown that these cells are capable of ECM invasion by elaborating a pseudopodial structure by virtue of which, presumably, invasion may occur. Furthermore, it has been demonstrated that a metalloproteinase is largely responsible for this invasive process.

Up-regulation of proteolytic capacity due to *ras*-transformation is clearly evident from the more profound clearing of immobilised matrix, and the higher invasive ability of these cells. Normal breast epithelial cells do, however, exhibit some degradative and invasive ability, but this is probably a reflection of a requirement for mammary gland involution (Larsson *et al.*, 1984) and tissue remodelling, which is essential for morphogenesis during development and wound healing.

In the presence of SFM, zones of proteolysis appear as sharply demarcated areas directly subjacent to the cells. Larger zones represent the confluence of a number of smaller zones of proteolysis. The mechanism giving rise to this pattern may: 1) be as a result of the release of soluble proteases from vesicles, and/or 2) occur at sites of close contact between the cell and the matrix substratum. Evidence for an intimate association between the cell and the matrix comes from the fact that inhibitors of all classes of proteases were unable to inhibit degradation of coated ECM.

Similar phenomena have been observed with other invasive cells. Chen and Chen (1987), using Rous sarcoma virus-transformed chicken embryo fibroblasts, showed that a 6 h preincubation with the metalloproteinase inhibitor 1, 10-phenanthroline was required to inhibit clearing. Polymorphonuclear neutrophils were shown to exclude α-1-proteinase inhibitor from areas subjacent to the cells (Campbell and Campbell, 1988). Unlike in this case, the inhibitors used by Chen and Chen (1987) and in the present study were almost all of a very low molecular weight, which emphasises the closeness of the contact between cell and substratum.

The nature of such close contacts is not clear. Cells could form, at their periphery, a band of close contact to delineate an area under the cell which could serve as an "extracellular lysosome", as has been suggested for macrophages (Reilly *et al.*, 1989) and osteoclasts (Vaes *et al.*, 1992). Alternatively, cancer cells, at their basolateral surface, may elaborate invasive pseudopodia or "invadopodia" (Kelly

et al., 1994) which are formed at contact sites (Chen *et al.*, 1994). Focal contacts, in fibroblasts, have a 10-15 nm separation between cell membrane and substrate (Izzard and Lochner, 1980). Given that cathepsin D, a relatively small molecule at 45 kDa, has a length of about 7 nm along its longest axis (Cantor *et al.*, 1992), it would be reasonable to assume that cell membrane- or receptor-associated proteases would be intimately accommodated in such a gap but, due to steric hindrance, small inhibitors would have difficult access. Indeed, uPA has been localised to discrete focal adhesion contact sites in the human fibrosarcoma cell line, HT-1080 (Pöllänen *et al.*, 1987), while the 72 kDa type IV collagenase (Monsky *et al.*, 1993) and seprase, a neutral gelatinase (Monsky *et al.*, 1994), were localised on invadopodia.

The present studies have shown that medium conditioned by MCF-10AneoT cells and adjusted to pH 5 or 7.2, was unable to release more ECM than a control. This indicates that either insufficient or no low or neutral pH proteolytic activity capable of ECM degradation, under the assay conditions used, is released. It would appear, then, that the proteolytic activity is either released as a latent proform, or is cell-associated and not released as soluble enzyme.

The involvement of pseudopodial extensions, which may, in the context of the leading edge of an invasive cell, form specialised invadopodia, is apparent from SEM micrographs of invading cells. It is difficult to discern, under the experimental conditions used, to what degree the chemoattractive forces are responsible for these structures. A cell would not be able to pass through the narrow pores of the filter without constricting at that point, but it is likely that proteases required for degradation of ECM prior to entering the pores are elaborated on the invadopodia. Proteases expressed on invadopodia, are found in close contact with planar ECM substratum. The mechanism for this preferential targeting is, at present, unknown, but may be by virtue of higher concentrations of MMP receptors at the ventral surface of cells. Indeed, integrins have been found to localise to the invadopodia of Rous sarcoma virus-transformed chicken embryo fibroblasts (Mueller and Chen, 1991), and the 72 kDa type IV collagenase can be localised in a proteolytically active form on the surface

of invasive cells, based on its ability to directly bind integrin $\alpha_v\beta_3$ (Brooks *et al.*, 1996). Furthermore, a novel family of membrane proteins has been identified containing both integrin binding sequences and a metalloproteinase catalytic domain (Wolfsberg *et al.*, 1995).

It was shown that *ras*-transformed cells (Fig. 29), as well as normal cells but to a lesser degree, often display vesicles which contain phagocytosed ECM. This is unusual in that this activity is thought to occur mostly in specialised phagocytic cells, such as polymorphonuclear neutrophils and macrophages. However, other instances of this activity are found: the oestrogen-dependent breast cancer cell line MCF-7 (Montcourrier *et al.*, 1990), and murine melanoma and fibrosarcoma cell lines (McCarthy *et al.*, 1985) were found to phagocytose ECM, while the hormone-independent breast cancer cell line, MDA-MB 231, phagocytosed ECM and latex beads during *in vitro* invasion (Montcourrier *et al.*, 1994). Some breast cancer cell lines (Montcourrier *et al.*, 1994), as well as paraffin sections of invasive ductal breast carcinoma (Roger *et al.*, 1994), have shown the presence of large acidic vesicles in which ECM can be digested. It has been suggested that these compartments may be important for the complete digestion of phagocytosed material which, in turn, may be of critical importance for successful invasion (Montcourrier *et al.*, 1994). The ECM-containing compartments in the transformed cells in this study, were generally larger than those of the normal cells.

In addition to proteolysis, it is well known that cell motility is closely related to the pathogenesis of cancer. It is of interest, therefore, that the addition of equine serum and, even more successfully, homotypic human serum greatly enhanced cellular motility on ECM. This was associated with increased destruction of ECM, apparently by both proteolysis and mechanical action. Although the increase in motility was most likely due to an increase in random migration, it is interesting to note that a preliminary experiment showed that serum-containing medium was very chemoattractive in a Boyden chamber assay (results not shown). It may be speculated that blood plasma in capillaries could have similar activity, and could contribute to tumour extravasation, assuming the active molecule is

present in lower amounts in interstitial tissue fluid. Furthermore, breaching the basement membrane may, mechanistically, be as a result of a combination of proteolysis and mechanical disruption, although the nature of the interaction *in vitro*, between the cell surface and the matrix, which causes this disruption is unknown, and nor is it known whether it has any physiological equivalent.

Although localised degradation assays are able to provide insight into cell-matrix interactions, such as the proteolytic capacity of cells, it must be remembered that this is a purely experimental system, perhaps with little direct physiological relevance. The addition of protease inhibitors, for example, had little effect on ECM proteolysis, probably because of a lack of access from underneath the matrix layer. *In vivo*, BM is bathed in extracellular fluid from both sides. This is also a feature of the *in vitro* Boyden chamber system, hence its success as a method for helping determine the proteases involved in invasion, with the use of protease inhibitors.

This approach was used to begin to determine the proteinase requirement for *in vitro* invasion through reconstituted BM by *ras*-transformed breast epithelial cells. Type IV collagen appears to be one of the major barriers to invasion of the BM, since the extent of invasion often correlates well with the extent of collagen type IV degradation (Liotta *et al.*, 1980). This implies the involvement of type IV collagen-degrading proteinases, such as type IV collagenases and cathepsins L and B (Maciewicz *et al.*, 1987). It is not surprising, therefore, that the highest inhibitory activity was displayed by an inhibitor of MMPs, which is consistent with the findings of other workers using a variety of cell lines (Mignatti *et al.*, 1986; Reich *et al.*, 1988; Yagel *et al.*, 1989b).

However, due to the complexity of proteolytic events, it is unlikely that only a single extracellular proteinase can be responsible for invasion. It appears that, in many cases, the uPA system is linked to type IV collagenase activation, as inhibitory antibodies to this enzyme produce a partial inhibition of invasion which can be circumvented by the addition of organomercurial activators of type IV collagenases (Reich *et al.*, 1988). Furthermore, α_2-antiplasmin, a specific

inhibitor of plasmin, was also found to significantly inhibit invasion (Mignatti *et al.*, 1986). This has led to the proposal of a proteolytic cascade: cell-associated uPA activates plasminogen (found in abundance in plasma), and the resulting plasmin activates type IV procollagenase. In the present study, preliminary experiments using serine proteinase inhibitors, indicated no apparent inhibition of invasion, although this remains to be confirmed. Also, the addition of plasminogen did not potentiate invasion (results not shown). Were this the case, another mechanism of type IV collagenase activation would be required. Cathepsin B, a cysteine proteinase, is able to activate latent collagenase (Sloane and Honn, 1984), and Yagel *et al.* (1989a) have shown that inhibitors of cathepsin L, also a cysteine proteinase, retard *in vitro* invasion in a murine melanoma and mammary carcinoma cell line, but to a lesser extent than metalloproteinase inhibitors. They suggest that this may reflect a role for metalloproteinase activation. In the present study however, E-64, a class inhibitor of cysteine proteinases, afforded no significant inhibition of invasion. Similar results have been demonstrated with mouse melanoma B16-F10 cells, where the serine/cysteine proteinase inhibitor, leupeptin, afforded no significant decrease in invasion (Persky *et al.*, 1986). This is surprising as secretion of both uPA (Wang *et al.*, 1980) and cathepsin B (Sloane and Honn, 1984) has been correlated with metastatic potential in this cell line.

Similarly, pepstatin A, a class inhibitor of aspartic proteinases, caused no inhibition, but rather a small increase in invasion (Fig. 35), a result similarly found by Johnson *et al.* (1993) with the human breast carcinoma cell line, MCF-7. This may be due to the inhibition of cathepsin D-dependent inactivation of other proteinases that are important in invasion. This data appears to support evidence that cathepsin D does not have an important extracellular role in the invasive phenotype of invasive cells, at least not in Boyden chamber assays. Such a conclusion should, however, be treated with circumspection as a role for the enzyme in metastasis has been shown (Garcia *et al.*, 1990), although it is unknown what its function is.

The demonstration of the involvement of an MMP in *in vitro* invasion prompted the question whether such activity could be detected in cell culture supernatants. Zymographic analysis indicated three MMP activities, two of which were significant at a M_r of 100-110 kDa and 80-85 kDa and a third minor band at 120-150 kDa. A high molecular weight MMP has been identified in extracts of a human melanoma cell line, which runs as a doublet at 170 and 150 kDa on gelatin substrate gels (Monsky *et al.*, 1994). Significantly, the 80-85 kDa band appears more active in the supernatant medium of the transformed cells than in that of the normal cells, which could be a reflection of the higher invasive activity of the transformed cells. It is unknown at present whether this increased activity is due to induction of the enzyme, or other events such as reduction in secreted endogenous inhibitor levels. At present, the identity of these MMPs is unknown. H-*ras*-transformed human bronchial epithelial cells have been shown to secrete the 72 kDa type IV collagenase (Collier *et al.*, 1988), and the 92 kDa type IV collagenase can be induced in a squamous cell carcinoma cell line by fibroblasts (Lengyel *et al.*, 1995). The molecular weights of the identified bands do not correspond to these enzymes, but it is possible that these enzymes form complexes with other molecules.

The metastatic potential of neoplastic cells is most commonly assessed by injection into a host animal, with subsequent monitoring of tumour growth and metastasis. Invasion is pathologically considered the hallmark of a malignant tumour, hence the development of *in vitro* assays which specifically test this process. However, while this assay is appealing because it is rapid and quantitative, it should not be used as the sole criterion for predicting *in vivo* invasion, as cells derived from normal tissues are able to penetrate the matrix, and some cells with *in vivo* invasive capacity cannot do so *in vitro* (Mackinnon *et al.*, 1992). Furthermore, tPA (McGuire and Seeds, 1989) and the 72 and 92 kDa type IV collagenases (Mackay *et al.*, 1993) have both been found to be associated with laminin in the basement membrane-like matrix from the Englebreth-Holm-Swarm tumour. Although the effect of the presence of these enzymes on invasion is unknown, these findings should be borne in mind in the interpretation of invasion results.

The present study has clearly demonstrated that *ras*-transformation of breast epithelial cells confers a phenotype characterised by increased extracellular proteolytic activity, which is reflected in ECM degradation and *in vitro* invasion. A preliminary study has shown that MMPs are probably the rate-limiting proteases in this invasion assay system, which is corroborated by the increased activity of an MMP in the culture supernatant of transformed cells. The precise role of other proteases, modes of activation of proteases and the contribution of cell derived inhibitors, such as TIMPs, has yet to be determined.

CHAPTER 6

GENERAL DISCUSSION

Proteases and cancer

The aggressiveness of a tumour is primarily dependent on its ability to invade adjacent tissue and then metastasise to distant sites. During these processes natural barriers such as interstitial connective tissue and BM must be degraded. It is now widely believed that enzymes released from the primary tumour are an absolute requirement for the successful development of most cases of metastatic disease. There is substantial evidence implicating proteases in invasion and metastasis, including the following:

- proteases are involved in normal tissue destructive events,
- levels of specific proteases correlate with metastatic potential in model systems,
- inhibitors and antibodies to various proteases inhibit invasion in model systems,
- transfection of cells with cDNA encoding specific proteases increases the metastatic activity of these cells and,
- transfection of cells with cDNA encoding specific protease inhibitors decreases the metastatic activity of these cells.

These enzymes, by virtue of the nature of the required degradative activity, must be active extracellularly, in close proximity to the malevolent cells. Indeed, there are now tangible clues as to the identity of some of these proteases, although it would be misrepresentative not to include the potential role of endogenous protease inhibitors. A protease/protease inhibitor imbalance could just as easily be as a result of suppression of inhibitor activity as of the induction of protease activity. Nevertheless, knowledge of the identity of rate-limiting proteases provides a potential avenue for the development of a treatment strategy aimed at preventing the life-threatening metastatic process.

Invasive cells probably have many ways of degrading ECM. They are able to increase proteolytic activity without necessarily increasing their own production and secretion of proteases, probably by cytokine-mediated recruitment of enzymes from adjacent stromal cells (Danø *et al.*, 1996). Optimal matrix degradation is also achieved by producing a variety of proteases, which can be concentrated and activated in the pericellular space. Thus, tumour cell proteolysis could probably only be controlled by targeting more than one family of proteases.

Protease inhibitors

For many years, prevention of cancer cell dissemination has been the primary potential target for the therapeutic use of protease inhibitors in cancer. Inhibitors of proteases are either physiological inhibitors naturally present in tissues, or non-physiological inhibitors produced by micro-organisms or chemically synthesised. Endogenous inhibitors appear always to be proteins, and thus have the advantage that they can be produced in recombinant form, their activity modified by site-directed mutagenesis, and their expression in cells altered by genetic manipulation. Moreover, as the three dimensional structure and active-site configuration of many proteases is unveiled by X-ray crystallographic studies, synthetic inhibitors can be engineered to optimise potency and bioavailability.

Unfortunately, the application of protease inhibitors to prevent cancer spread is limited, as most patients, at the time of cancer diagnosis, already have detectable or microscopic metastases. Possibly inhibitors could be used prophylactically in high risk groups, such as with patients after resection of tumours. However, as understanding of the biological activity of proteases and their inhibitors has increased, new therapeutic roles have been suggested. The mitogenic action of proteinases is well known, and includes trypsin which cleaves a domain of the thrombin receptor resulting in G-protein-dependent cellular signalling (Bratt and Scott, 1995). A similar explanation has been offered for the mitogenic action of uPA and tPA. The lysosomal proteinases, cathepsins L and D, have also been associated with growth promotion (Berquin and Sloane, 1994). This may occur by the release of growth factors from extracellular matrices (Whitelock *et al.*, 1996), or by a direct mitogenic effect as has been proposed for cathepsin D (Fusek and

Vetvicka, 1994). In favour of a role of cysteine proteinases in growth promotion, E-64-d, a membrane permeable derivative of E-64, causes arrest of epidermoid cancer cells in the metaphase (Shoji-Kasai *et al.*, 1988). It is clear that protease inhibitors could have a significant cytostatic activity on a primary tumour and established metastatic lesions. Furthermore, the ability to block extracellular proteolytic events in tumours could stimulate encapsulation of invasive tumours, which would allow later resection. Tumour angiogenesis could also be retarded by inhibitors, as proteases are well known to play a role in this process.

A further area of importance of protease inhibitors in cancer, is their ability to prevent carcinogenesis *in vivo* and *in vitro* (Kennedy, 1994). The Bowman-Birk soybean proteinase inhibitor, a serine proteinase inhibitor, has been shown to suppress carcinogenesis induced by several different carcinogens in different species, tissues and involving different types of tumours. The mechanism of action is unknown, but is thought to stop the initiating event in carcinogenesis. This is probably related to the ability of this inhibitor to inhibit the expression of the c-*fos* and c-*myc* proto-oncogenes (Kennedy, 1994). Indeed, the Bowman-Birk soybean proteinase inhibitor (Bratt and Scott, 1995), α_1-antitrypsin, hirudin, a thrombin inhibitor (DeClerck and Imren, 1994) and batimastat (Brown, 1994), a metalloproteinase inhibitor, have undergone limited clinical trials as anti-tumour drugs.

However, it is likely that these approaches would require prolonged administration of inhibitors which may lead to toxicity. For example, serine protease inhibitors, due to their involvement in blood coagulation, would be difficult to administer systemically. Matrix metalloproteinases are involved with tissue remodelling and repair, reproduction and menstruation (Marbaix *et al.*, 1996), and would be susceptible to similar problems. Therefore, targeting inhibitors either specifically to tumours or to specific proteinases may be important. This approach is most readily realised by directing antibodies to predominantly tumour antigens, and loading such antibodies with a cytotoxic compound such as a radioisotope (Larson, 1990). A novel approach utilises prodrugs designed for activation by proteases (Panchal *et al.*, 1996). This approach

exploits one of the phenotypes of malignant cancer cells, the presence of cell surface proteases, in this case cathepsin B, to trigger activation of a pore-forming toxin, a mutation of α-haemolysin. α-Haemolysin causes tumour cell permeability leading to cell death. Another interesting approach is the use of drugs that prevent protease secretion instead of inhibiting protease activity. One such drug, estramustine (Wang and Stearns, 1988), prevents invasion thorough ECM barriers of a human prostatic carcinoma and mouse melanoma cell line, by preventing secretion of type IV collagenase due the effects of the drug on the microtubular system which is involved with vesicular trafficking. Other anti-proliferative drugs and alkylating agents (5-fluoracil, cisplatin and L-phenylalanine mustard) were ineffective blockers of invasion.

Inhibitory antibodies targeting cathepsin D and other proteases

In the present study a different approach to protease inhibition has been pursued. By recruiting the immune system to produce antibodies against "self" antigens, such as proteases thought to be relevant to malignancy, these proteases should be neutralised with concomitant benefit (Dennison, 1989). This is done by challenging the immune system with a peptide derived from a sequence of a relevant protease. This effectively "tricks" the immune system into producing antibodies which should cross-react with the protease. The peptide chosen is usually part of a sequence important to the activity of the enzyme, thereby optimising the chances of inhibition of activity. Of course, although preferred, inhibition, is not absolutely necessary for the success of this approach. Opsonised protease molecules, although possibly still active, will be removed by phagocytic cells of the immune system.

Cathepsin D was used, in this study, as the protease of choice as a target in breast cancer therapy. Much circumstantial evidence had indicated, at the outset of the present study, that this enzyme was involved in breast cancer dissemination (Rochefort, 1990; Rochefort, 1992). Currently, the contribution that cathepsin D makes to this process is uncertain. Overexpression of human cathepsin D in rat embryo cells appeared to correlate with an increased propensity to form liver metastases when injected into athymic mice (Garcia *et al.*, 1990). In subsequent

experiments cathepsin D was modified in an attempt to change the cellular compartment in which it is expressed (Liaudet *et al.*, 1994). Cathepsin D containing the endoplasmic reticulum retention signal peptide, KDEL, at the C terminus was expressed in 3Y1-Ad12 cells. Cells expressing cathepsin D with the control KDAS peptide were highly metastatic, while the metastatic potential of cells expressing the KDEL cathepsin D construct was significantly reduced concomitant with a dramatic reduction of the processing of procathepsin D into mature enzyme. The present study suggests that the involvement of cathepsin D in metastasis requires mature (proteolytically active?) enzyme. In contrast, Johnson *et al.* (1993) used *in vitro* invasion experiments, in the presence of the aspartic protease inhibitor pepstatin, to show that secretion of procathepsin D by MCF-7 cells is not correlated with invasion. Rather than indicating that cathepsin D has no role in invasion, these results may emphasise the importance of the processing and maturation of cathepsin D. In fact, the observation that secreted procathepsin D levels are not correlated with invasion is in agreement with the data of Liaudet *et al.* (1994), which shows the ineffectiveness of intracellular unprocessed procathepsin D in promoting metastasis. It is evident that cathepsin D does have a major influence on metastasis in some cell lines. What remains in question, however, is how, and is the effect intracellular, extracellular or both, bearing in mind the acidic pH optimum for the enzyme? Until such time as these questions are addressed, it is difficult to know whether cathepsin D would make a good target for enzyme immunoinhibition.

Cathepsin D may, however, possess mitogenic activity extracellularly (Fusek and Vetvicka, 1994), although the overall importance of this in relation to growth factors remains in question. The anti-peptide antibodies raised during this study could not be shown to inhibit the enzyme, probably as a result of the insensitivity of available enzyme assays for cathepsin D. Inhibition of mitogenic activity does not necessarily require abolishment of protease activity, but rather the perturbation of binding of the enzyme to a putative receptor. It would be of interest to ascertain whether the antipeptide antibodies against cathepsin D, which successfully bind cathepsin D, would have an effect on mitogenic activity.

Despite the lack of immediate success of the immunoinhibition approach with cathepsin D as a tool for cancer therapy, it is proposed that this approach could, nevertheless, be used to target enzymes that have been shown to be involved in the promotion of invasion and metastasis, such as the MMPs and uPA. Judiciously chosen peptides could target anti-peptide antibodies to determinants on the enzyme critical for activity, while simultaneously ensuring a high degree, if not complete, specificity for the chosen enzyme. This would alleviate, to a large degree, cytotoxicity and other metabolic problems that would probably be associated with the prolonged administration of broad spectrum inhibitors, as has been previously discussed. Furthermore, the action of the immune system, being systemic and continued, is probably more powerful than using exogenous drugs. Indeed, peptides or a cocktail of peptides specific to different proteases or even more than one region on the same protease, could be used as an anti-cancer vaccine.

To make such an approach feasible, an intimate knowledge of trafficking and behaviour of proteases in transformed cells, and the inter-relationship between proteases and transformation is required. The remainder of this discussion will address aspects of these questions in the context of the normal and *ras*-transformed human breast epithelial cells used in this study.

Ras-transformation

It is clear, from the present studies and others, that *ras*-transformation of MCF-10A cells induces a number of changes in the proteolytic capacity of these cells, which is, in part, reflected in the induction of the invasive phenotype. Furthermore, not only is an altered *status quo* of proteolysis required, but an overall change in the disposition of the transformed cell. Transformation with the *ras* oncogene causes alterations in disparate cellular systems, including cell architecture (Symons, 1996), translation, transcription, the cell cycle and differentiation (Prendergast and Gibbs, 1993). Of particular interest in the context of invasion and metastasis, are the effects of *ras* on the cytoskeleton, as the cytoskeleton is integral to processes such as cell motility, the formation of specialised structures at the invasive front of invasive cancer cells, and the

expression and transport of proteolytic enzymes at times and to places which are inappropriate.

Normal epithelia, as has been shown by SEM in this study, are organised into closely associated, largely immobile cells. During normal development, transitions from epithelia to fibroblastic cells or mesenchyme can occur, and mesenchyme can differentiate into new epithelia (Birchmeier and Birchmeier, 1993). Such transitions are not confined to development. Malignant carcinomas lose their epithelial character, which results in the appearance of invasive, motile cells, resembling the fibroblastic phenotype and morphology, as was evident in *ras*-transformed cells in this study. It is believed that soluble mesenchymally derived factors lead to the dissociation and scattering of epithelial colonies. The factor, identified as hepatocyte growth factor or scatter factor, in addition to EGF, induces these effects by elevating protein tyrosine phosphorylation, by virtue of their tyrosine kinase receptors (Weidner *et al.*, 1991).

The intercellular junctions of epithelia are composed of a complex of cell surface and cytoskeletal elements which stabilise cell-cell adhesion. These junctions, termed adherens, are specialised regions of the epithelial plasma membrane where transmembrane cadherin molecules located on opposing cells contact each other (Takeichi, 1990). The cytoplasmic portion of E-cadherin is associated with a group of proteins, catenins, that interact with components of the actin cytoskeleton (Birchmeier and Birchmeier, 1993). Together, these proteins form the adherens-type junctions, which encircle the apical perimeter of epithelial cells thereby stabilising them.

Focal adhesions are sites of cell adhesion to ECM, and these increase in number with a concomitant decrease in adherens junctions during the change from epithelial to fibroblastic morphology (Burridge and Connell, 1983). Where in adherens junctions the primary cell adhesion molecule is cadherin, in focal adhesions it is generally an integrin.

Ras-transformed MCF-10AneoT cells reveal increased numbers of focal adhesions and altered cell-cell adhesions, which is accompanied by elevated levels of protein phosphotyrosine (Kinch *et al.*, 1995). The modification of the adherens-type junctions is, in most part, due to the decreased interaction between E-cadherin, β-catenin and the actin cytoskeleton. Furthermore, elevated levels of tyrosine-phosphorylated β-catenin and p120 Cas, sequentially similar to β-catenin, are found. Instead of tyrosine-phosphorylated β-catenin being detected in E-cadherin complexes, tyrosine-phosphorylated p120 Cas is found. It is suggested that elevated tyrosine phosphorylation of proteins such as β-catenin and p120 Cas contribute to the altered junctions of the *ras*-transformed epithelial cells, as both isoforms show decreased association with the actin cytoskeleton (Kinch *et al.*, 1995). Indeed, herbimycin A, an inhibitor of protein tyrosine kinase activity, abolishes tyrosine phosphorylation of β-catenin and restores binding to E-cadherin. This, in turn, restores adherens junctions and reverts the morphology from fibroblastic to epithelial (Kinch *et al.*, 1995).

In question, however, is how *ras*-transformation is able to mediate these events, considering that *ras* is thought to act downstream of tyrosine kinases, such as receptors for growth factors. It has recently been found that MCF-10AneoT cells display increased levels of amphiregulin, a member of the EGF family (Normanno *et al.*, 1994). Amphiregulin is able to bind to and activate the EGF receptor, which exhibits tyrosine kinase activity. It is possible, therefore, that amphiregulin may be acting through an autocrine mechanism to stimulate tyrosine phosphorylation.

Another intriguing relationship is that between focal adhesions and, proteases and phosphorylated proteins localised in these structures in some transformed cell types. Chen (1989) has proposed a scheme to explain the alterations found to occur at fibroblast-ECM contact sites upon Rous sarcoma virus-induced transformation. In normal cells, the formation of the closest contacts with ECM, focal adhesions, involves end-on and lateral attachments of both microfilaments and ECM fibres to membrane associated proteins. Transformation induces cell surface changes, such as the reorganisation of microfilaments and adhesion

plaques, as well as the formation of invadopodia. Cells may then recruit membrane-bound proteases to invadopodia for local ECM degradation. This would allow protrusive activities for directed migration and invasion of cell into the surrounding ECM. The recruitment of these membrane-bound proteases may be regulated by tyrosine kinase activity, as a Rous sarcoma virus *src* gene product, the protein tyrosine kinase $pp60^{v\text{-}src}$, is localised to invadopodia. Indeed, both the degradative and motile activities of the invadopodia were inhibited by genistein, an inhibitor of tyrosine-specific kinases (Mueller *et al.*, 1992).

Given that *ras* transformed breast epithelial cells elaborate elevated phosphotyrosine in many proteins, it would be interesting to speculate that a similar mechanism may be applicable. Although proteases have not yet been localised to invadopodia in MCF-10AneoT cells, circumstantial evidence, collected in this study, seems to suggest that these structures are important in ECM degradation by MCF-10AneoT cells. First, in non-transformed cells matrix degradation was localised in discrete patches predominantly at the cell periphery, where focal adhesions are found (Monsky and Chen, 1993). In the transformed cells, degradation was evident beneath the whole lower surface of the cell, which is consistent with the localisation of invadopodia (Monsky *et al.*, 1993). Secondly, the lack of demonstrable inhibition of coated ECM degradation by protease inhibitors suggests a close association between the cell membrane and the ECM, also consistent with invadopodia-ECM interaction (Chen *et al.*, 1994). Thirdly, invadopodia-like structures were found beneath MCF-10AneoT cells invading through ECM-coated filters. Interestingly, the focal adhesion kinase is tyrosine-specific and has, as the name suggests, been identified in focal adhesions (Hanks *et al.*, 1992), structures which are more numerous in MCF-10AneoT cells (Kinch *et al.*, 1995).

Rho, one of the *ras*-related GTPases essential for *ras*-transformation, appears to be involved in the control of focal adhesion kinase (Symons, 1995), thereby providing a link between *ras*-transformation and tyrosine kinase activity in focal adhesions. An alternative link is that $pp60^{v\text{-}src}$, a tyrosine kinase associated with focal adhesions in normal cells and, as the virally-derived counterpart, with

invadopodia of transformed cells (Mueller *et al.*, 1992), has been shown to suppress N-cadherin-mediated cell-cell adhesion in virally transformed cells (Hamaguchi *et al.*, 1993). At present, however, the c-*src* family of kinases appears to act by a pathway different from that of *ras* (Prendergast and Gibbs, 1993).

Focal adhesions provide not only the starting point for invadopodium formation, but are areas where integrins connect to the ECM and provide the traction that is required for migration. *Ras*-transformation probably affects cell motility through *rho*, which initiates the formation of filopodia, lamellipodia and focal adhesions at the leading edge of the cell and is implicated in adhesive release at the rear of the cell (Huttenlocher *et al.*, 1995).

Degradation and motility are both essential to invasion. Integrins participate in the adhesion of invadopodia during cellular invasion, and it is likely that integrins and membrane-associated proteases co-localise in invadopodial membranes (Mueller and Chen, 1991). The 72 kDa type IV collagenase interacts, at the surface of invasive cells, with an integrin (Brooks *et al.*, 1996), and integrin-ECM binding and integrin aggregation at the cell surface stimulates production of MMPs (Werb *et al.*, 1989). Furthermore, membrane protein trafficking through the cell such as endocytosis (Glenney *et al.*, 1991), has been associated with tyrosine phosphorylation.

Ras-and its related GTPases could also be implicated in other aspects of the malignant phenotype. *Rac* has been shown to be necessary for the activation of arachidonic acid metabolism by EGF and insulin (Symons, 1995). Release of arachidonic acid and subsequent production of leucotrienes leads to *rho*-dependent stress-fibre formation. It is further possible that the induction of leucotrienes may result in the production of 12(S)-HETE, a lipoxygenase metabolite of arachidonic acid, which has been proposed to modulate various events during tumour cell extravasation from blood circulation (Honn and Tang, 1992). Transformation with oncogenic *ras* could also provide potential tumour angiogenesis inducers. Vascular endothelial growth factor expression is enhanced in v-Ha-*ras* transformed NIH 3T3 fibroblasts (Grugel *et al.*, 1995), and

has been localised to the plasma membrane of tumour-associated microvascular endothelial cells (Hong *et al.*, 1995). Vascular endothelial growth factor is one of the most potent inducers of angiogenesis known, and probably by acts as an endothelial cell mitogen. It also enhances microvascular hyperpermeability. Angiogenesis is vital for tumour growth and dissemination, while vascular hyperpermeability, characteristic of the microvasculature of tumours, may aid the escape of tumour cells from the mass into circulation.

It is clear that transformation of cells with the *ras* oncogene has ramifications in many aspects of cellular control, which together gives rise to an invasive phenotype. Activated forms of the p21 *ras* gene product have been found in 20% of all types of human cancer, and in 90% of pancreatic and >50% of colon carcinomas (Gibbs, 1991). This suggests that manipulation of the *ras* signal transduction pathway may provide a useful approach to control cancer.

The *ras* gene product, p21 *ras*, is cell membrane associated, a localisation required for efficient transforming activity. Preceding membrane association, p21 *ras* is post-translationally modified. The C-terminal sequence CAAX (where C is cysteine, A is any aliphatic amino acid, and X is any amino acid) determines which lipid moiety is to be added to the protein. Farnesyltransferase mediates farnesylation of the cysteine residue if X is methionine or serine, while if X is leucine or isoleucine, a geranylgeranyl moiety is added. Normal *ras* proteins are farnesylated, and this has led to the development of farnesyltransferase inhibitors (Gibbs, 1991).

Farnesyltransferase inhibitors can be divided into two classes, based on the two substrates of the reaction. For example, L-739, 749 is a competitive inhibitor of the farnesyltransferase substrate, farnesyl diphosphate, and suppresses the growth of tumours arising from *ras*-transformed Rat1 cells in nude mice by 66% (Kohl *et al.*, 1994). The second substrate is the CAAX tetrapeptide, the portion of the *ras* protein that is sufficient for interaction with the enzyme. Analogs of CAAX have been synthesised that are potent inhibitors of farnesyltransferase, because they are able to block *ras* farnesylation. They have been shown to modulate critical

aspects of *ras*-transformation, including tumour cell growth (Kohl *et al.*, 1994; Garcia *et al.*, 1993). A question arises as to the biological specificity of these compounds, as other proteins such as retinal transducin and nuclear lamin are also farnesylated (Gibbs, 1991).

Achievements of this study

Embarking on new areas of investigation often requires the development of new methodologies, a process which can be hampered by technical difficulties. The present study has made a number of methodological contributions, among which are PLT embedding in Lowicryl resin, the use of fluorescently-labeled ECM for cell-mediated degradation studies, *in vitro* cell invasion assays and scanning electron microscopy of invading cells.

To assess the role of proteolytic enzymes in invasive cells, an immunocytochemical approach was decided upon. Dr Edith Elliott (of this laboratory) was central to establishing the hydrated ultracryo-section technique, and the refinement of labeling methodologies. However, this technique although sensitive and rapid, suffers from several drawbacks. It requires highly skilled manipulations, requires sample blocks to be permanently stored in liquid N_2, and limits the degree to which a sample can be orientated prior to sectioning, when special requirements are needed. *In situ* sample embedding in Lowicryl resin overcomes these problems, simplifying cell orientation when transverse sections are required for protease distribution studies. To this end a PLT embedding system was devised, which required the design of a cabinet which could maintain a temperature of -35°C under a N_2 environment in the presence of ultraviolet radiation. Optimal resin polymerisation conditions required that the resin was not too rubbery, due to slow polymerisation, but without bubbles and distortions due to too rapid polymerisation. This could be attained by adjusting the distance between the sample and the ultraviolet radiation lamps. Labeling protocols also had to be adjusted from cryo-section labeling protocols, especially as Lowicryl K4M sections are susceptible to high background.

The use of *in vitro* invasion assays became central to the present study to morphologically characterise invasion by *ras*-transformed cells, and to investigate the proteases involved in this process. This technique appears simple, but due to the large number of variables involved, is temperamental. Invasion within an experiment can vary a great amount, probably due to factors such as stage of the cell cycle at which cells are used, homogeneity of the coated ECM barrier which appears to be dependent upon the consistency of the coating technique and batch-batch variation of ECM. Indeed, the method has associated with it many small "tricks", some which were learned first hand in the laboratory of Dr Josef Glössl (Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna), but remains somewhat tedious and intensive. However, with perseverance and some help this powerful assay has been developed in this laboratory to the stage where it can now be used routinely to address questions relating to the invasive phenotype.

A number of important elements emerged from the present study, in terms of scientific impact. The first is the formulation of a strategy for an immuno-therapeutic approach to combating cancer. This involves synthesising and raising antibodies to peptides chosen from proteases thought to be involved in tumour progression. Although not necessarily a prerequisite, peptides generating antibodies inhibitory to protease activity, could be tested in *in vivo* tumour metastasis models for inhibition of metastasis. Since most of the methodological and conceptual pioneering work for this strategy has already been completed, this avenue can now be rapidly pursued.

The second is the demonstration, by immunofluorescence and immunoelectron microscopy, of a more peripheral cellular distribution of low pH and protease-containing compartments in *ras*-transformed breast epithelial cells compared to normal cells. Furthermore, cathepsins B and D were found to be cell surface associated. These results give rise to the question of the involvement of lysosomal proteinases, especially cathepsins B and D, in the metastatic phenotype of *ras*-transformed cells.

Thirdly, studies were undertaken to assess the matrix degrading and invasive capabilities of the *ras*-transformed cells. These cells exhibited increased degradation of ECM in SFM, and increased motility and disruption of ECM in serum-containing medium. Protease inhibitors were used to preliminarily identify the proteases involved in *in vitro* invasion by transformed cells. An inhibitor of MMPs was found to give the most prominent inhibition, and overexpression of an MMP in the *ras*-transformed cells was demonstrated by zymography. These results demonstrate that transformation of human breast epithelial cells with the *ras* oncogene confers characteristics of the metastatic phenotype. Cells are morphologically fibroblastic, appear to have lost contact inhibited growth, and appear to demonstrate increased motility in the presence of serum. They have an enhanced disposition for ECM proteolysis which, in part, translates into an accentuated invasive capacity.

Remaining questions

As is often the case when one sets out to address specific issues, the result is that more questions than answers are generated. These may include:

- what is the role of cathepsin B in tumour invasion, and is the redistribution of this enzyme merely an epiphenomenon of *ras*-transformation? This question probably cannot be simply answered by *in vitro* invasion studies, but may ultimately require a more elaborate approach such as gene knockout or gene antisense technology.
- similarly for cathepsin D, does the enzyme have an extracellular function in cancer, or does it function intracellularly by digesting phagocytosed ECM?
- do *ras*-transformed breast epithelial cells elaborate invadopodia, and are proteolytic enzymes concentrated on the surface of these structures? This may be best addressed by confocal immunofluorescent microscopy of cells invading ECM *in vitro*. In confocal microscopy cells can be viewed in the z axis. This may help to identify proteases found at the invasive edge of invading cells.
- what role do protein tyrosine kinases play in the invasive and proteolytic capacities of these cells? Tyrosine kinase-specific inhibitors could be used in conjunction with *in vitro* invasion assays and ECM degradation assays to address this question.

- what is the identity and cellular location of the MMP shown, in this study, to be overexpressed in the transformed cells, and what is the nature of this overexpression, i.e is it at the transcriptional or translational level or due to a reduction in endogenous inhibitor levels? This could be pursued by western and northern blotting and immunofluorescent microscopy with specific antibodies against the most likely candidates, namely the 72 kDa and 92 kDa type IV collagenases.
- what is the status of endogenous MMP inhibitors, namely the TIMPs? This could be ascertained by reverse zymography.
- what role do serine proteinases play in *in vitro* invasion? This question could be addressed with serine proteinase inhibitors and immunoinhibitory antibodies against uPA.
- based on the proteinase profile of invading cells, these enzymes could, first *in vitro* and then *in vivo,* be targeted with anti-peptide antibodies designed to inhibit activity to assess the feasibility of an immunotherapeutic approach to cancer intervention.

Perhaps, from a more philosophical perspective, the most significant outcome of work in this field over the last decade is a collective paradigm shift giving rise to a new model for cancer therapy. Scientists now speak not of necessarily killing cancer cells, but of reining them in, perhaps rehabilitating them and tricking them into dying naturally. After all, no cure exists for diseases such as hypertension and diabetes, yet these are eminently controllable - why not cancer?

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PUBLICATIONS

POSSIBLE SECONDARY FUNCTION OF SPLENIC ENDOTHELIAL STAVE CELLS REVEALED BY IMMUNOLABELLING STUDIES

E. Elliott, P.H. Fortgens and C. Dennison

Department of Biochemistry, University of Natal, Pietermaritzburg

The reticular meshwork of the cord cells and the phagocytic macrophages in the sinuses of the red pulp of the spleen (fig. 1), are thought to be responsible for the removal of aged erythrocytes from the blood. Phagocytosis, and hence removal of ageing, more rigid, erythrocytes may be favoured by prolonged exposure to the high concentrations of hydrolytic enzymes in extracellular fluids in the sinuses[1]. Such exposure may lead to the observed loss of sialic acid residues and the exposure of galactose sugars on outer membranes of ageing cells, possibly leading to phagocytosis by phagocytic cells[1]. In the present study, immunolabelling of human splenic tissue for elastase and cathepsin D has revealed that regulation of the levels of extracellular hydrolytic enzymes may be achieved by pinocytosis by splenic endothelial stave cells.

Human spleen (2 x 2 mm) was fixed in 8% paraformaldehyde in 0,1 M sodium cacodylate (pH 8,0, 20 min), cyroprotected by infusion with 2,1 M sucrose (30 min), mounted on a copper stub and frozen in liquid nitrogen[2]. Sections were double immunolabelled for elastase and cathepsin D using 3 nm and 10 nm protein A gold probes. Control labellings consisted of omitting the primary antibody, substituting pre-immune or pre-adsorbed serum or IgG at the same concentration as the test, and performing labellings for one antigen at a time (single labelling). After labelling, sections were contrasted by a "positive-negative" contrast procedure, using uranyl acetate and methyl cellulose[3].

Cathepsin D has not previously been localised in neutrophils by immunocytochemistry. The present labelling indicates that cathepsin D is present at a concentration approximately ten fold lower than that of the enzyme elastase, and is co-localised with elastase in the azurophil granules of neutrophils (figs 2 and 3). This concurs with biochemical findings. The presence of elastase and cathepsin D, in some pinocytotic vesicles of endothelial stave cells, adjacent to stimulated neutrophils (fig. 3), suggests the co-secretion of these enzymes by neutrophils and simultaneous uptake by endothelial stave cells. The smaller stave cell vesicles, are probably pinocytotic vesicles (figs 2 and 3), due to their size, relatively low cathepsin D content (non-lysosomal nature), and, in the absence of neutrophils, their usually low content of the enzyme elastase. The relatively high content of cathepsin D (usually a lysosomal marker enzyme) in larger stave cell vesicles, present adjacent to the pinocytotic vesicles, indicate that the larger organelles are possibly lysosomes. The uptake of enzymes and hence regulation of the level of enzyme concentration in extracellular fluids in the splenic sinuses, by pinocytosis, may be a mechanism by which endothelial cells may perform a secondary function, the control of levels of extracellular enzymes.

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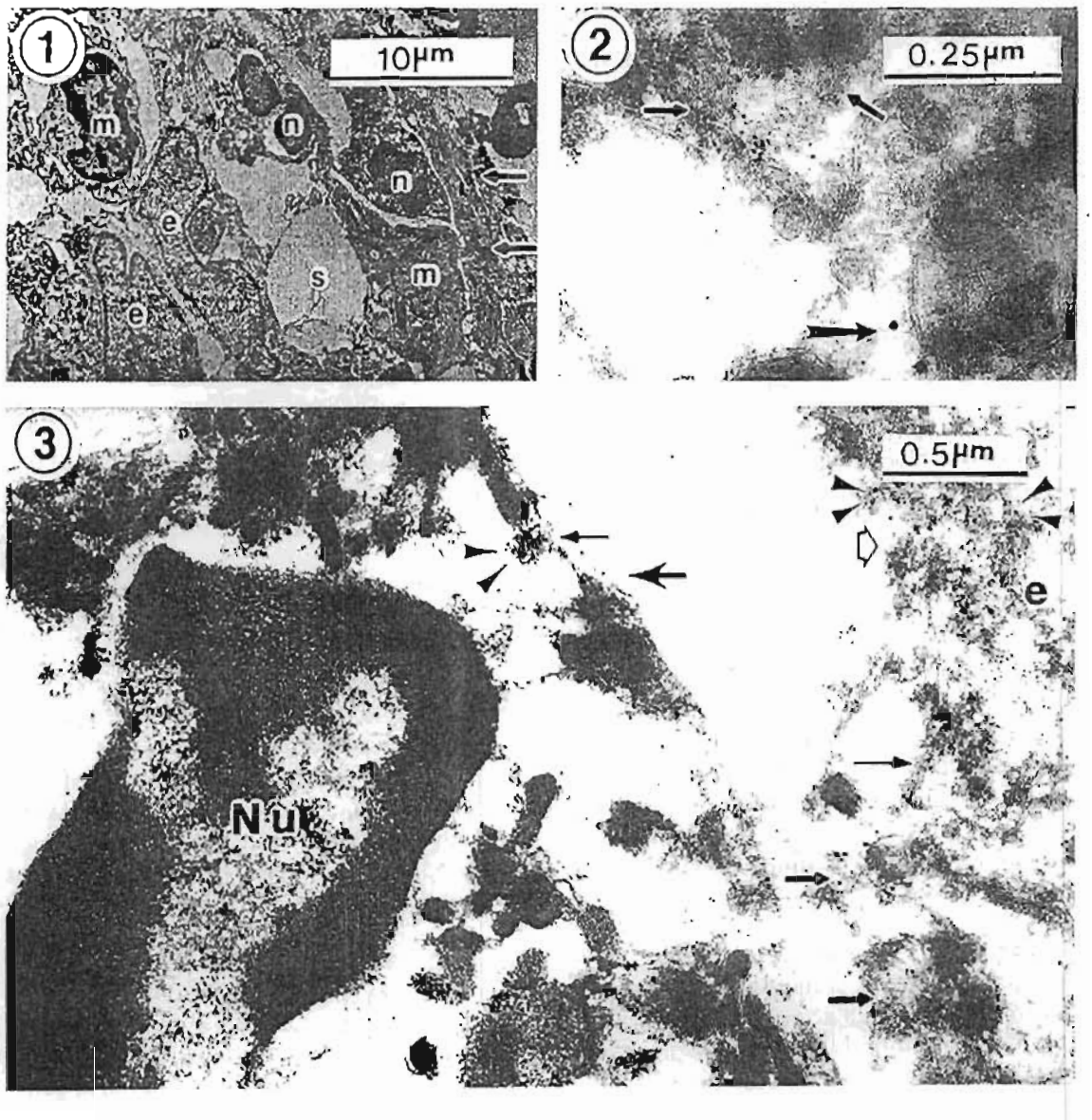

Fig 1. An area of red pulp from a human spleen showing endothelial stave cells (e), macrophages (m), basement membrane (arrows), sinuses (s) and neutrophils (n).

Fig 2. Enlargement of vesicle populations seen in the section of the endothelial stave cell (lower right, fig. 3 below). The smaller, pinocytotic vesicles contain mainly elastase (3 nm gold label, small arrow), while larger vesicles (lysosomes) contain only cathepsin D (10 nm gold label, large arrow).

Fig 3. Neutrophil (Nu) and adjacent endothelial stave cell (e) labelled for cathepsin D (10 nm gold label, large arrow) and elastase (3 nm gold probe, small arrow). Note pinocytotic vesicles (open arrow head) and lysosomal compartments (medium arrow) and co-localisation of elastase and cathepsin D in azurophil granules of Nu and some pinocytotic vesicles (double arrow head).

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Anti-peptide antibodies to cathepsins B, L and D and type IV collagenase

Specific recognition and inhibition of the enzymes

Theresa H.T. Coetzer, Edith Elliott, Philip H. Fortgens, Robert N. Pike and Clive Dennison

Department of Biochemistry, University of Natal, P.O.Box 375, Pietermaritzburg 3200, Republic of South Africa

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Anti-peptide antibodies were raised against synthetic peptides selected from the sequences of human cathepsins B and L, porcine cathepsin D and human type IV collagenase. Sequences were selected from the active site clefts of the cathepsins in the expectation that these would elicit immunoinhibitory antibodies. In the case of type IV collagenase a sequence unique to this metalloproteinase subclass and suitable for immunoaffinity purification, was chosen. Antibodies against the chosen cathepsin B sequence were able to recognize the peptide but were apparently unable to recognise the whole enzyme. Antibodies against the chosen cathepsin L sequence were found to recognise and inhibit the native enzyme and were also able to discriminate between denatured cathepsins L and B on Western blots. Antibodies against the chosen cathepsin D sequence recognised native cathepsin D in a competition ELISA, but did not inhibit the enzyme. Native type IV collagenase was purified from human leukocytes by immuno-affinity purification with the corresponding anti-peptide antibodies.

Key words: Anti-peptide antibody; Cathepsins B, L, D; Type IV collagenase; Immunoinhibition

Introduction

Cathepsins B, L and D and type IV collagenase have been implicated in tumour invasion and metastasis (Liotta et al., 1980; Sloane and Honn, 1984; Denhardt et al., 1987; Spyratos et al., 1989). The role of these enzymes in tumour invasion may be explored using specific antibodies and in this context anti-peptide antibodies (Briand et al., 1985) have many advantages. A sequence of ten or more amino acids has a very high probability of being unique to a particular protein and the corresponding anti-peptide antibody is, therefore, also likely to allow highly specific detection of the protein. Moreover, for immunocytochemistry, for example, with polyclonal anti-peptide antibodies against a linear peptide sequence, there is an intrinsically lower probability of the epitope(s) being destroyed during tissue processing, than in the case of a monoclonal antibody which may be targeted at a single, labile, discontinuous epitope.

Correspondence to: C. Dennison, Department of Biochemistry, University of Natal, P.O. Box 375, Pietermaritzburg 3200, Republic of South Africa.

Abbreviations: ABTS, 2,2′-azino-di(3-ethyl)-benzthiozoline sulphonic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbens assay; HRPO, horseradish peroxidase; KLH, keyhole limpet haemocyanin; MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; M_r, relative molecular weight; NHMec, 7-(4-methyl)coumarylamide; PBS, phosphate-buffered saline; SBTI, soybean trypsin inhibitor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Z-, benzyloxycarbonyl.

The utility of anti-peptide antibodies may be increased if these are additionally able to inhibit enzymic activity. In the case of the cysteine cathepsins, B, H and L, for example, inhibiting anti-peptide antibodies might constitute tools with a unique ability to discriminate between these enzymes, and might thus aid in their identification. It has also been suggested (Dennison, 1989) that inhibiting anti-proteinase anti-peptide antibodies might be therapeutically useful.

To raise anti-peptide antibodies against the cathepsins, peptide sequences were selected from their primary sequences, mainly by consideration of their 3-dimensional structure, but also with reference to the mobility and hydrophilicity of the chosen peptide sequence. The cathepsins are involved in antigen processing (Takahashi et al., 1989; Van Noort and Van der Drift, 1989) and consequently may be regarded as integral parts of the immune system. The question thus arises as to whether there is any prejudice against production of anti-peptide antibodies to these proteinases, especially against their conserved sequences. As a basis for comparison, therefore, anti-peptide antibodies were also raised against a sequence in a non-lysosomal proteinase, type IV collagenase, similar to that previously shown to successfully elicit anti-peptide antibodies (Höyhtyä et al., 1988). We report here our observations on raising antibodies to the selected peptides and on the effectiveness of the resulting antibodies in binding to, and inhibiting, the target enzymes.

Materials and methods

Reagents

KLH and MBS were obtained from Sigma. Glutaraldehyde (E.M. grade) and cyanogen bromide were from Merck and ABTS was from Boehringer Mannheim. Human liver cathepsin B was a gift from Dr. D. Buttle, Strangeways Laboratory, Cambridge, U.K. Sheeps' liver cathepsin L was isolated by a modification of the method of Pike and Dennison (1989); chromatography on S-Sepharose, at pH 4.5, being substituted by chromatography on Sephadex G-75. Human spleen cathepsin L was similarly isolated, though in the form of a complex with cystatin, in a study to be reported elsewhere. Human kidney cathepsin L was purchased from Novabiochem, U.K. Cathepsin D was isolated from human, porcine and bovine spleens by the method of Jacobs et al. (1989). Type IV collagenase was purified from human leukocytes by immunoaffinity chromatography with the anti-peptide antibody immobilised on CNBr-activated Sepharose 4B. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were obtained from Cambridge Research Biochemicals.

Selection of peptides

The peptide sequences from cathepsins B and L (Table I) were selected by considerations of 3-dimensional structure, based on a published structure of the analogous enzyme, papain (Wolthers et al., 1970). The 3-dimensional structures of cathepsins B, H and L have been deduced, from amino acid sequence information, to be comparable to that of papain (Kamphuis et al., 1985; Dufour, 1988).

The sequence selected for cathepsin B, corresponds to residues 13–22 in the structure of human liver cathepsin B (Turk et al., 1986). This sequence is in an accessible position, at one end of the substrate-binding cleft of the enzyme (Wolthers

TABLE I

THE PEPTIDE SEQUENCES SELECTED FOR THE GENERATION OF ANTI-PEPTIDE ANTIBODIES, FROM THE AMINO ACID SEQUENCES OF THE PROTEINASES INDICATED

Peptide	Sequence	Corresponding proteinase
B13–22	Q-C-P-T-I-K-E-I-R-D (+C) [a]	Human cathepsin B
L153–165	E-P-D-C-S-S-E-D-M-D-H-G-V	Human cathepsin L
D112–122	T-K-Q-P-G-L-T-F-I-A-A (+C)	Porcine cathepsin D
COL476–490	M-G-P-L-L-V-A-T-F-W-P-E-L-P-E	Human collagenase IV

[a] The selected peptides were modified for synthesis by the substitution of the cysteine residues in peptides B13–22 and L153–165 with α-amino butyric acid and by the addition of an extra cysteine residue to the C termini of B13–22 and D112–122 respectively, in addition to the acetylation of the N terminus of B13–22 and amidation of the C terminus of L153–165.

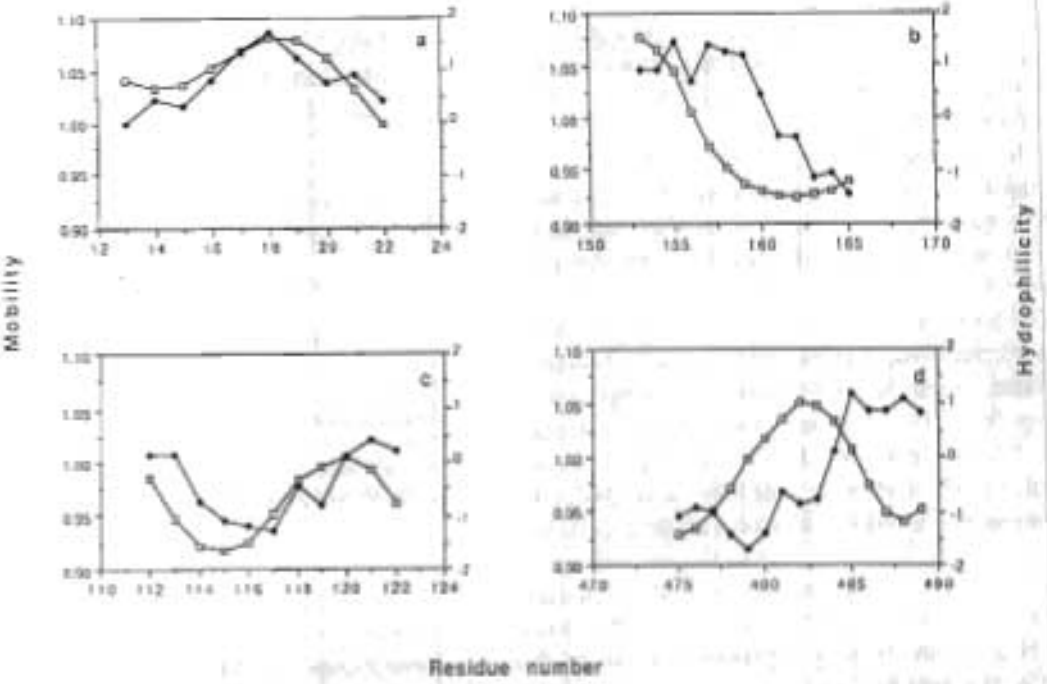

Fig. 1. Hydrophilicity and segmental mobility profiles of the selected peptides. ◆, hydrophilicity, calculated according to Hopp and Woods (1981, 1983); □, segmental mobility, calculated according to Westhof et al., (1986). Profiles indicated are for the peptides: (*a*) B13–22; (*b*) L153–165; (*c*) D112–122; and (*d*) COL476–490.

et al., 1970). It also corresponds to a peak of both hydrophilicity (Hopp and Woods, 1981, 1983) and segmental mobility (Westhof et al., 1984) (Fig. 1*a*).

A sequence different from that for cathepsin B was chosen for cathepsin L, to potentially maximise the information gained from the experiments. Also, the region chosen for cathepsin B is not a suitable choice for human cathepsin L since the human cathepsins L and H have analogous sequences in this region, with seven out of the 11 amino acids being similar or identical (Ritonja et al., 1988). There is thus an increased probability that an anti-peptide antibody to the sequence in cathepsin L may cross-react with cathepsin H.

By contrast, the loop of amino acids containing the active site histidine is also accessible (Wolthers et al., 1970), and there are marked differences in the sequences in this region between the different cysteine cathepsins. The presence of the active-site histidine in this sequence was also thought to increase the probability that antibodies targeting this region might be inhibitory. The chosen sequence corresponds to residues 153–165 in the amino acid sequence of human cathepsin L (Ritonja et al., 1988); in papain the comparable residues are 150–161. The sequence is largely hydrophilic but has a cluster of hydrophobic residues towards its C terminus (Fig. 1*b*). The L153–165 sequence is also relatively conserved between species and may be expressed as Glu-Pro-Asx-Cys-Ser-Ser-A-Asx-B-Asp-His-Gly-Val, where Asx is either Asp or Asn, A is Glu or Lys and B is Met or Leu (Dufour et al., 1987; Ishidoh et al.,1987; Ritonja et al., 1988). An additional criterion in its selection, therefore, was its potential to target cathepsin L across species.

The sequence chosen for cathepsin D (Table I) was based on the 3-dimensional structure of a related aspartic proteinase, penicillinopepsin (Hsu et al., 1977), since no 3-dimensional structure of cathepsin D has been published. The sequence corresponds to residues 112–122 in porcine cathepsin D (Faust et al., 1985), and corresponds to a loop on the rim of the substrate-binding groove of penicillinopepsin. It has low hydro-

philicity and mobility (Fig. 1c) and differs from human cathepsin D in a single, conservative, substitution of leucine for isoleucine at position 117 (Faust et al., 1985).

The sequence chosen for human type IV collagenase (Table I) is based on the sequence of a CNBr-generated fragment of this enzyme from human melanoma A2058 cells (CB4 peptide), reported by Höyhtyä et al. (1988) to elicit antibodies which bind only to type IV collagenase and not to related, secreted, extracellular matrix metalloproteinases, such as interstitial collagenase and stromelysin. The sequence corresponds to residues 476–490 in human type IV procollagenase (Collier et al., 1988) and is hydrophilic towards its C terminus and mobile in its centre (Fig. 1*d*). In the present study the C terminal Lys was omitted from the CB4 peptide to ensure that glutaraldehyde conjugation was effected exclusively through the N terminus, thereby exposing the hydrophilic part of the peptide.

Synthesis of peptides

The selected peptides were modified, before synthesis, by the substitution of the cysteine residues in peptides B13–22 and L153–165 with α-amino butyric acid and by the addition of an extra cysteine residue to the C-termini of B13–22 and D112–122 respectively. The resulting peptides were custom synthesised by Multiple Peptide Systems, San Diego, CA.

Conjugation

All four peptides were conjugated to KLH, using two different conjugation methods. Peptides B13–22 and D112–122 were conjugated, through their C termini to KLH, using MBS (Robertson and Liu, 1988). The maleimide content of KLH-MBS was determined by the addition of mercaptoethanol and subsequent assay for reduced thiol content (Kitagawa and Aikawa, 1976). Due to their solubility differences, it was necessary to treat B13–22 and D112–122 differently. B13–22 was dissolved in 200 mM sodium phosphate buffer, pH 8.0, and D112–122 was dissolved in the same buffer, but containing 8 M urea, before reduction and conjugation. The method of Sedlak and Lindsay (1968) was used to determine the peptide reduction. Peptides L153–165 and COL476–490 were conjugated to KLH, through their N termini, using 1% (v/v) glutaraldehyde, according to Briand et al. (1985). A carrier protein-to-peptide ratio of 1 : 40 was used.

TABLE II

INOCULATION PROTOCOL

Week	Freund's adjuvant	Site	Dose
0	Complete	s.c. [a]	200 μg conjugated peptide
2	Incomplete	s.c.	200 μg conjugated peptide
3			Bleed
6	Incomplete	s.c.	200 μg conjugated peptide
8			Bleed
10	Incomplete	s.c.	200 μg conjugated peptide or
		i.v. [b]	1 mg free peptide
12			Bleed
Monthly boosters as indicated for 10 weeks			

[a] s.c. = subcutaneous injection on the back at each of five sites.
[b] i.v. = intravenous in marginal ear vein.

Inoculation protocol

For each peptide two rabbits were inoculated with peptide conjugate according to the protocols summarized in Table II. For comparison the protocol of Richardson et al. (1985) was followed, in which conjugate was replaced by free peptide from week 10. B13–22 was only subjected to the latter protocol.

ELISA for anti-peptide antibodies

Wells of microtitre plates (Nunc Immunoplate) were coated overnight at room temperature with peptide solution in PBS, pH 7.2, at 5 μg/ml (B13–22 and L153–165), 0.5 μg/ml (D112–122) and 1 μg/ml (COL476–490). Wells were blocked with 0.5% BSA in PBS for 1 h at 37°C and washed 3 × with 0.1% Tween 20 in PBS (PBS-Tween). Dilutions of the primary antiserum in 0.5% BSA-PBS were then added, incubated at 37°C for 2 h, and excess antiserum was again washed out 3 × with PBS-Tween. A 1/200 dilution of sheep anti-rabbit IgG-horseradish peroxidase conjugate, in 0.5% BSA-PBS, was added and incubated for 30 min at 37°C. The ABTS substrate (0.05% in 150 mM citrate-phosphate buffer, pH 5.0, containing 0.0015% H_2O_2) was added and incubated for 15 min. The enzyme reaction was stopped by the addition of 0.1% NaN_3 in citrate-phosphate buffer

and the absorbance was read at 405 nm in a Bio-Tek EL307 ELISA plate reader.

ELISA for immobilized enzyme

The ability of anti-peptide antibodies to cross-react with the respective whole enzymes (not necessarily in their native form) was measured by coating the wells of microtitre plates with either cathepsin B or L (5 μg/ml and 1 μg/ml, respectively, in 50 mM carbonate buffer, pH 6.0, for 3 h at 37°C, followed by overnight at 4°C) or cathepsin D (2 μg/ml in PBS, pH 7.2, overnight at room temperature). The remainder of the procedure was as outlined above except that IgG was purified from serum, by the method of Polson et al. (1964), to remove serum inhibitors of the enzymes (e.g. cystatin). Species cross-reactivity of anti-peptide antibodies was measured using the same ELISA by coating with cathepsins purified from various sources.

Competition ELISA for native enzyme

The binding of the anti-peptide antibodies to the native cathepsins was tested in an ELISA in which free enzyme was permitted to compete with immobilized peptide for binding to the antibody and thus prevent a fraction of the antibody from being immobilized. Microtitre plates were coated with peptide as described above. Various amounts of antibody (between 10 and 450 μg/ml IgG) were pre-incubated at 37°C for 30 min with different levels of enzyme (molar ratios of peptide-to-enzyme from 1 : 24 to 1 : 0.5), before the incubation mixture was transferred to the peptide coated wells. After a further 1 h incubation at 37°C, the ELISA was developed as described above.

Removal of anti-KLH antibodies

KLH was coupled to cyanogen bromide activated Sepharose-4B according to Kohn and Wilchek (1982). Anti-KLH antibodies were removed from immunoglobulin fractions, purified from serum according to Polson et al. (1964), by passage through KLH-Sepharose.

Immunoblotting

The different enzymes were subjected to reducing SDS-PAGE (Laemmli, 1970), before transfer to nitrocellulose membranes (Schleicher and Schull, BA 85,0.45 μm) essentially as described by Towbin et al. (1979). Following electro-blotting for 16 h, the nitrocellulose membrane was air dried for 1.5 h and non-specific binding sites were blocked with low-fat dried milk powder (5% in TBS) for 1 h. After this, and at all subsequent steps, the membrane was washed (3 × 5 min) with TBS. Anti-peptide antibodies, from which anti-KLH antibodies had been removed, were diluted in 0.5% BSA-TBS and incubated with the membrane (2 h), followed by sheep anti-rabbit IgG-HRPO conjugate (1 h). All incubation steps were carried out at room temperature. The HRPO reaction was detected with 0.06% 4-chloro-1-naphthol in TBS, containing 0.0015% H_2O_2. The reaction was stopped by rinsing in TBS containing 0.1% NaN_3. Targeting of sheep and human cathepsin L by anti-L153–165 antibodies was also visualised by protein A-gold labelling with silver amplification (Moeremans et al., 1984).

Immunoinhibition assays

Assays for the immunoinhibition of cathepsins B and L were carried out using the substrates Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, respectively, as described by Barrett and Kirschke (1981). Cathepsin B (250 ng) or cathepsin L (25 ng) were incubated at 30°C for 15 min with anti-peptide IgG, or normal rabbit IgG, at the appropriate concentration in 400 mM Na-phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.1% Tween 20. Assays against the Z-Phe-Arg-NHMec substrate revealed that the IgG fractions had intrinsic activity against this substrate, which is probably attributable to contaminating plasma kallikrein which cleaves this substrate (Barrett and Kirschke, 1981). This activity was controlled by the addition of 40 μg/ml of SBTI, and by subtracting the residual activity in the antibody fractions from the measured cathepsin L activity. SBTI inhibits kallikrein but not cathepsin L. Stopped time assays were carried out over the range of IgG concentrations, and the inhibition by anti-peptide antibodies was calculated in comparison to normal rabbit IgG. Immunoinhibition of cathepsin D was carried out using acid denatured hemoglobin as substrate, essentially as described by Dingle et al. (1971).

Results

Anti-peptide antibody production

All four peptide conjugates elicited antibodies, which reacted with the corresponding immobilized peptides in an ELISA (Fig. 2). In each case, it appears that the antibody titer peaked at about 8–12 weeks. No significant difference could be observed in titer obtained with the two inoculation protocols (using conjugate throughout or changing to free peptide after 10 weeks) when tested against immobilized peptide. Anti-B13–22 antibodies showed a decline after 12 weeks, but this could not be attributed to changing to inoculation with free peptide since anti-D112–122 antibodies, for instance, showed a similar decline in titer after 8 weeks with both inoculation protocols.

Recognition of enzymes coated to ELISA plates

The anti-B13–22 antibodies, although able to recognize the peptide B13–22, were unable to recognize the whole enzyme, coated to a multititer plate at pH 6.0, 7.2 or pH 9.6 (results not shown). By contrast, anti-L153–165 antibodies were able to recognize both human and sheep cathepsin L, immobilised on ELISA plates (Fig. 3*a*). They apparently reacted more strongly with the sheep than the human enzyme, from which the peptide sequence was selected, but this may merely be a concentration phenomenon. Human spleen cathepsin L, used in this test, was complexed to cystatin and the measured protein concentration was therefore not a true reflection of the amount of cathepsin L present per se. Anti-porcine cathepsin D, was able to recognize whole human, porcine and bovine cathepsin D enzymes, immobilised on an ELISA plate (Fig. 4). The peptide antibodies, raised against D112–122 (a sequence from porcine cathepsin D), apparently reacted better with human than with porcine or bovine cathepsins D. In the region corresponding to the chosen peptide, the human cathepsin D sequence shows a single substitution of leucine for isoleucine, at position 117, compared to the porcine

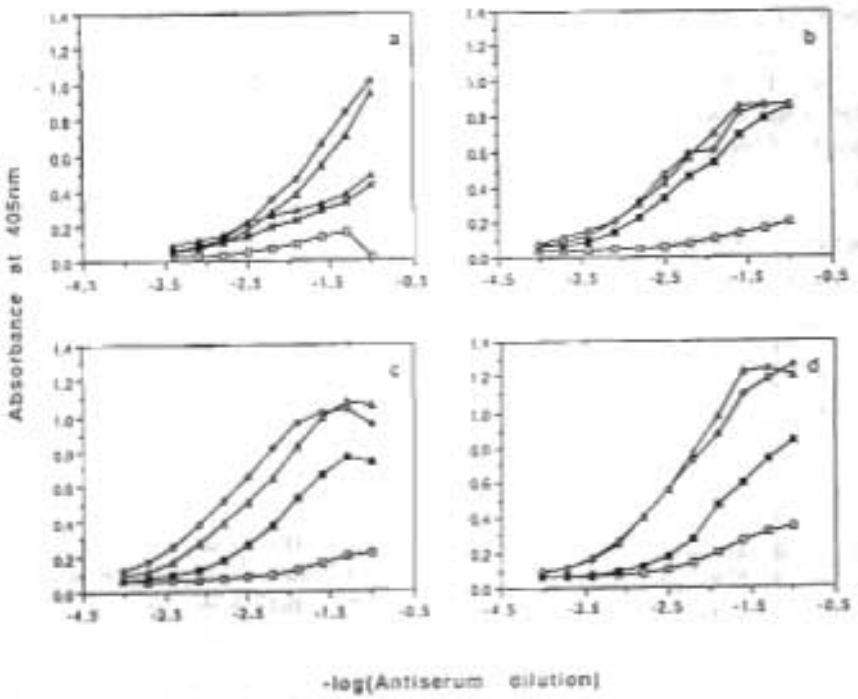

Fig. 2. Progress of immunisation with peptide conjugates as determined by ELISA. Peptides were coated to microtitre plates, (*a*) B13–22; (*b*) L153–165; (*c*) D112–122; and (*d*) COL467–490 and incubated with serial two-fold dilutions of antisera collected after 3 (■), 8 (○), 12 (△), 30 (▲) and 32 weeks (×). Normal rabbit serum control (□). This was followed by incubation with HRPO- linked secondary antibody and ABTS as a chromogenic substrate, as described under materials and methods section. Each point is the mean absorbance at 405 nm of duplicate samples.

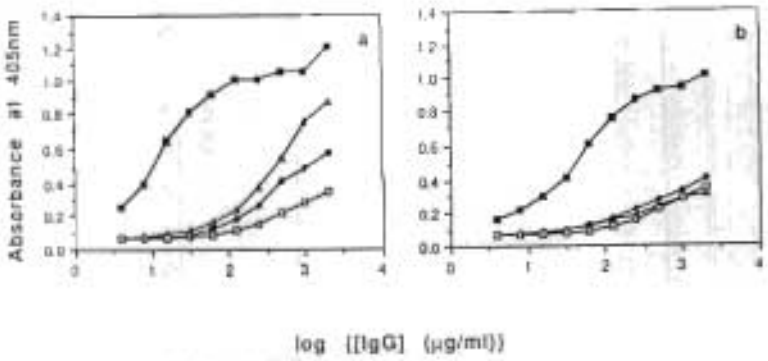

Fig. 3. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin L. Cross-reaction of anti-L153–165 antibodies with human (◆) and sheep (▵) cathepsin L, and peptide L153–165 (■). Normal rabbit IgG (□). Experimental procedure as in Fig. 2 and in the materials and methods section. (*a*) anti-L153–165 antibodies elicited by use of conjugated peptide throughout. (*b*) anti-L153–165 antibodies elicited by use of conjugated peptide followed by free peptide in the inoculation procedure.

enzyme, and it may be inferred from the results that the bovine enzyme must also be very similar in this region. Due to the lack of sufficient enzyme, the anti-COL476–490 antibodies could not be tested against the collagenase IV enzyme, in an ELISA.

Although antibodies raised using the two different immunisation protocols apparently had the same titer against immobilised peptide, a clear difference was sometimes seen in their ability to target the immobilised whole enzyme. In the case of anti-L153–165 peptide antibodies, for example, where conjugate was used throughout, the resulting antibodies cross-reacted with the whole protein to a much higher degree (Fig. 3). This phenomenon was less marked in the case of cathepsin

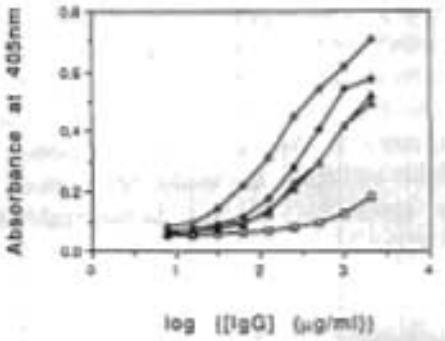

Fig. 4. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin D. Cross-reaction of anti-D112–122 antibodies with human (◆), porcine (▵) and bovine (▲) cathepsin D, and peptide D112–122 (○). Normal rabbit IgG (□). Experimental procedure as in Fig. 2 and in the materials and methods section.

D (result not shown). Anti B13–22 antibodies, tested against whole cathepsin B, did not give a positive reaction at any stage, including at 8 weeks, before the switch to free peptide.

Specificity of anti-peptide antibodies: Western blot analyses

In Western blot analyses it was found that a more specific reaction was obtained if anti-KLH antibodies were removed by passage through a column containing immobilised KLH. Anti-L153–165 antibodies targeted human cathepsin L to a much higher degree than the sheep enzyme and protein A-gold labelling with silver amplification was required to show the targeting of sheep cathepsin L (Fig 5*A*). The specificity of this targeting was evidenced by the fact that there was no cross-reactivity with human cathepsin B. Anti-B13–22 and D112–122 antibodies did not show any reaction with the corresponding enzymes on a Western blot (result not shown). The anti-COL476–490 antibodies detected a M_r 66,000 band of type IV collagenase purified from human leukocytes (Fig. 5*B*).

Recognition of native enzymes

Anti-B13–22 antibodies did not interact with the native form of cathepsin B when tested in a competition ELISA and immunoinhibition assays, all at pH 6.0 (results not shown). Cathepsin D inhibited the binding of anti-D112–122 antibodies (250 μg/ml) to the peptide coated to multititer plates, in a dose-dependent manner, up to 60% at

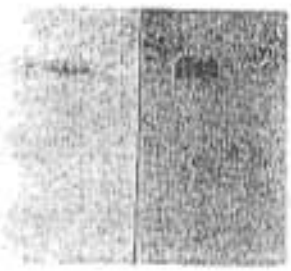

Fig. 5. Targeting of cathepsin L and type IV collagenase by anti-peptide antibodies on Western blots. *A*: samples ((1) sheep cathepsin L; (2) human cathepsin L; (3) human cathepsin B) were subjected to 12.5% reducing SDS-PAGE, electroblotted onto nitrocellulose and then incubated with anti-KLH-purified anti-L153–165 IgG, before developing with (a) protein A-gold with silver amplification or, (b) sheep anti-rabbit-HRPO conjugate as described in the materials and methods section. *B*: human type IV collagenase was electrophoresed on a 7.5% SDS-polyacrylamide gel with reduction, transferred to nitrocellulose and immunologically stained with anti-KLH-purified anti-COL476–490 IgG as described in the materials and methods section.

446 μg/ml (Fig. 6), suggesting that the antibody recognizes the native enzyme. Because of the relatively high concentrations of enzyme required for

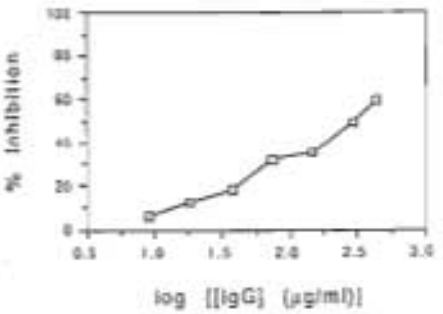

Fig. 6. Competition ELISA for native cathepsin D. The ability of cathepsin D to inhibit the binding of anti-D112–122 antibodies to immobilised D112–122 was measured by pre-incubating various amounts of IgG with different levels of enzyme before transfer of the incubation mixture to peptide coated plates. The ELISA was developed as in the materials and methods section. The percentage inhibition was calculated from control incubations containing either normal rabbit IgG or no competing cathepsin D.

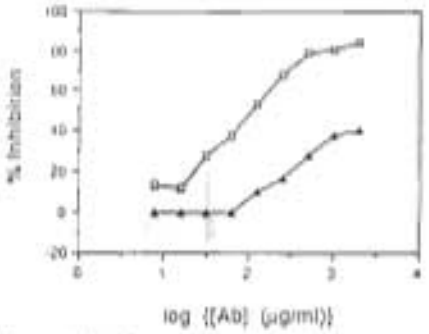

Fig. 7. Immunoinhibition of human and sheep cathepsin L by anti-L153–165 antibodies. Stopped time assays were carried out using human (□) and sheep (▵) cathepsin L as described in the materials and methods section and the percentage inhibition calculated relative to control assays with normal rabbit IgG.

this assay, cathepsin L and type IV collagenase were not included in these tests. Cathepsin D activity was, however, not inhibited by anti-D112–122 antibodies in the enzyme immunoinhibition test.

An indication that anti-COL476–490 antibodies bind to native type IV collagenase is given by their effectiveness in immunoaffinity purification of the enzyme. Type IV collagenase thus purified from human leukocytes showed gelatinolytic activity on a gelatin zymogram (result not shown).

The L153–165 antibodies almost completely inhibited human cathepsin L at high antibody concentrations and inhibition decreased with decreasing antibody concentration until a plateau was reached at low antibody concentration (Fig. 7). Sheep liver cathepsin L was also inhibited, but to a lesser extent than the human enzyme. These results therefore show that the antibody was able to bind to and inhibit native human and sheep cathepsin L. Anti-L153–165 antibodies did not inhibit cathepsin B (results not shown), showing the specificity of this immunoinhibition for cathepsin L.

Discussion

The failure of the anti-B13–22 peptide antibodies to recognize whole human cathepsin B was not expected since the peptide corresponds to peaks of both hydrophilicity and mobility in the sequence

of cathepsin B (Fig. 1*a*) and, from a consideration of the 3-dimensional structure of papain, it would also appear to be on the surface of the molecule. It has been reported that, in general, segmental mobility is an important criterion for the recognition of the native protein by anti-peptide antibodies (Van Regenmortel, 1988a). This does not appear to hold for the peptide B13–22, and it may be speculated that the presence of a disulfide bridge might, perhaps, constrain the peptide in a particular way in the native protein. There is thus an apparent conflict between the high mobility value assigned to Cys-14, by Westhof et al. (1984) antigenicity prediction profile, and its participation in a constrained disulfide bridge. Comparison with the results obtained for the cathepsin L peptide, L153–165, may be instructive. The peptide L153–165 was conjugated through its N terminus, which is close to the Cys residue involved in a disulfide bridge, and in this case antibodies to the peptide were able to recognize the native protein. In both cases, the Cys residue was substituted by an α-amino butyric residue, but since the peptide L153–165 elicited competent antibodies, this substitution per se is probably not the reason why the peptide B13–22 failed to raise antibodies able to recognize the native enzyme. It may be interesting to examine the possible recognition of the native protein by antibodies raised against B13–22, but conjugated through its Cys residue, or its N terminus.

It must be noted that cathepsin B is generally a refractory enzyme with regard to antibody production and normal polyclonal antibodies, raised against whole cathepsin B, are only able to recognize denatured forms of the enzyme (Barrett, 1973). Monoclonal antibodies against native cathepsin B have been reported (Wardale et al., 1986). Monoclonal antibodies are produced in vitro, however, and it may be speculated that there is a prejudice against production of anti-cathepsin B antibodies in vivo, due to its involvement in antigen processing. By contrast, antibodies are easily raised against native cathepsin L, and it is interesting to note, in this regard, that Takahashi et al. (1989) have concluded that cathepsin B, and not cathepsin L, might be the major enzyme involved in antigen processing. Therefore, if human B13–22 shares sequence homology with its rabbit counterpart, any rabbit B cell clones producing anti-B13–22 antibodies capable of recognising native cathepsin B may be suppressed.

The cathepsin L peptide, L153–165, is much less hydrophilic and mobile at its exposed C terminus, than at its N terminus (Fig. 1*b*), but it was decided to conjugate it through its N terminus so as to expose the active site histidine. This stratagem appears to have been successful in eliciting anti-peptide antibodies able to inhibit native human and sheep cathepsin L (Fig. 7). Due to the specificity of this inhibition, anti-L153–165 peptide antibodies may be useful research tools, since the inhibitors currently in use are unable to discriminate qualitatively between cathepsins B and L (Kirschke et al., 1988). Anti-L153–165 antibodies also discriminate very specifically between cathepsins B and L on Western blots (Fig. 5*A*), which suggests that they may also be useful in immunocytochemistry. They may also be useful as therapeutic agents in pathologies arising from excessive cathepsin L activity.

A criterion in the selection of the peptide L153–165 was the potential of antibodies to this peptide to target cathepsin L across species. The cross-reactivity between anti-L153–165 antibodies and sheep cathepsin L, immobilised in ELISAs and on Western blots, and in the enzyme immunoinhibition assays (Figs. 3, 5*A* and 7), confirms this expectation.

Anti-D112–122 antibodies recognized the peptide as well as whole human, porcine and bovine cathepsin D enzymes, immobilised on multititer wells (Fig. 4). Nevertheless, the colour took a relatively long time (about 1 h) to develop in the ELISA assay against immobilised whole enzymes. There is evidence (Van Regenmortel, 1988b) that proteins become partially denatured or undergo conformational changes when adsorbed to solid phases, so the slow colour development may indicate that anti-D112–122 antibodies recognize the partially denatured enzymes only weakly or that only a small percentage of the enzyme adopts a conformation suitable for antibody binding. The antibody clearly recognizes the native form of human cathepsin D, as evidenced from the competition ELISA results (Fig. 6), but does not target the fully denatured enzyme on a Western blot. It may be inferred, from these results, that the epi-

tope in the native enzyme, recognized by anti-D112–122 antibodies, may be a continuous but conformationally specific epitope which is destroyed by reducing SDS-PAGE. Consideration of the 3-D structure of penicillinopepsin reveals a prominent spiral turn in the region corresponding to the D112–122 sequence and suggests that this may constitute such a conformational epitope.

Conjugation of the peptide D112–122, to KLH, was effected through its C terminus, since these residues appear to be less accessible in the native protein; a situation which may therefore be mimicked in the conjugate by the presence of the carrier protein. The more exposed N terminal residues proved to be antigenic and the resulting anti-peptide antibodies were able to bind to the native protein, but were not able to inhibit the enzyme. The paratope-epitope interaction is possibly too distant to occlude the substrate binding cleft.

From a methodological point of view it is of interest that although peptide D112–122 is largely hydrophobic, and was initially insoluble in all solvents tested except 8 M urea, it remained in solution after subsequent removal of the urea and could successfully elicit antibodies able to recognize the native enzyme. The significance of this is that many enzymes apparently have hydrophobic binding sites. From the results obtained using peptide D112–122, it would appear that generation of peptide antibodies against such hydrophobic sites is not impossible.

Omission of the CB4 C terminal Lys-residue (Höyhtyä et al., 1988), in COL476–490, used in the present study, ensured glutaraldehyde conjugation exclusively via the N terminus. This presentation, exposing the more hydrophilic C terminus, proved to be sufficiently immunogenic to elicit anti-peptide antibodies which are able to interact with native type IV collagenase from human leukocytes in immunoaffinity purification. This result confirms the finding of Höyhtyä et al. (1988) that the anti-CB4 antibody specifically immunoprecipitated native type IV collagenase from a mixture of metalloproteinases secreted by human melanoma cells and also recognized the denatured proteinase (M_r 68,000) on Western blots following SDS-PAGE. Targeting of a 67,000 band on a Western blot by anti-CB4 antibodies was also used by Spinucci et al. (1988) to positively identify the proteinase purified from c-Ha-*ras* oncogene transformed mouse NIH 3T3 fibroblasts. In the present study anti COL476–490 antibodies similarly recognized the denatured M_r 66,000 proteinase from human leukocytes (Fig. 5*B*). From these results it may be inferred that these anti-peptide antibodies recognize native and denatured type IV collagenase from both normal (leukocyte) and malignant (melanoma) human sources as well as oncogene transformed mouse NIH 3T3 fibroblasts.

From this study it is clear that there is as yet no reliable basis on which to predict which peptides will successfully elicit antibodies capable of recognising the native target protein. Consideration of the 3-D structure, when this is available, appears to be the most promising approach and was successful with cathepsin L, though not with cathepsin B. It will be interesting, in future, to further explore the structures of cathepsins B and D with a view to finding inhibitory peptide antibodies to these proteinases.

Acknowledgements

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Original Article

Paraformaldehyde Fixation of Neutrophils for Immunolabeling of Granule Antigens in Cryoultrasections[1]

EDITH ELLIOTT,[2] CLIVE DENNISON, PHILIP H. FORTGENS, and JAMES TRAVIS

Department of Biochemistry, University of Natal, Scottsville, Pietermaritzburg, South Africa (EE,CD,PHF), and Department of Biochemistry, University of Georgia, Athens, Georgia (JT).

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Paraformaldehyde (PFA) fixation was optimized to facilitate the immobilization and labeling of multiple granule antigens, using short fixation regimens and cryoultramicrotomy of unembedded neutrophils (PMNs). In the optimal protocol, extraction of azurophil granule antigens (especially of the abundant elastase) was obviated by manipulating the polymeric state of PFA, and hence its rate of cross-linking, by altering its concentration and pH in a multistep process. Primary fixation conditions used (4% PFA, pH 8.0, 5 min) favor fixative penetration and rapid cross-linking. Stable cross-linking of the antigen was achieved in a secondary fixation step using conditions that favor larger, more cross-linking polymeric forms of PFA (8% PFA, pH 7.2, 15 min). Immobilization of granule antigens was enhanced by flotation of cut sections on fixative (8% PFA, pH 8.0) before labeling and by using post-labeling fixation with 1% glutaraldehyde. The optimized protocol facilitated immobilization and immunolabeling of elastase, myeloperoxidase, lactoferrin, and cathepsin D in highly hydrated, unembedded PMNs. (*J Histochem Cytochem 43:1019–1025, 1995*)

KEY WORDS: Paraformaldehyde fixation; Cryoultramicrotomy; Elastase; Cathepsin D; Myeloperoxidase; Lactoferrin; Neutrophils.

Introduction

The invasive movement of activated polymorphonuclear leukocytes (PMNs) and monocyte/macrophages through barrier membranes is believed to be facilitated by hydrolytic enzymes, including proteases (Faurie et al., 1987; Johnson and Varani, 1981). Initially our investigations, aimed at identifying and defining the origin of such proteases, were frustrated by extraction and translocation of azurophil granules and their antigens, reportedly a common problem in immunolabeling studies of PMNs (Hibbs and Bainton, 1989; Damiano et al., 1986; Cramer et al., 1985; Ganz et al., 1985). This problem prompted the present investigation of paraformaldehyde (PFA) fixation regimens.

In this study, brief fixation with PFA, one of the least cross-linking (Tokuyasu, 1986) and denaturing fixatives (Larsson, 1988; Tokuyasu, 1986), was used to preserve maximal antigenicity and allow the detection of low levels of antigens in some granule populations. Complete immobilization of all antigens and the preservation of the integrity of all granule types is also required, however. To achieve this, the concentration (Griffiths, 1993; Walker, 1964) and pH of the fixative (Griffiths, 1993; Bourgnon and Chardon, 1987; Eldred et al., 1983; Berod et al., 1981) were varied to alter the degree of penetration and cross-linking, and hence optimal conditions were found.

Infiltration of the fixative through the newly cut side of the section, by flotation of the section on fixative before labeling, was also found to improve the immobilization of granule antigens and preservation of granule morphology.

Materials and Methods

Different preparations of stock PFA were used in repeat experiments, and cell isolation, processing, and immunolabeling procedures were performed at least five times for each protocol described.

Separation of PMNs. Blood drawn from healthy volunteers was collected into lithium–heparin tubes and the PMN fraction was separated within 1 hr by the Percoll (Pharmacia; Uppsala, Sweden) gradient method of Haslett et al. (1985). Cells were washed (400 × g, 10 min, 20°C) with 2% bovine serum albumin (BSA, fraction V; Boehringer, Mannheim, Germany) in PBS, pH 7.2 (Slot and Geuze, 1985), and prepared for specificity testing of antisera by Western blotting, or were fixed for immunolabeling.

Preparation of Paraformaldehyde Fixative. Paraformaldehyde powder (BDH; Poole, UK) (16 g) was added to distilled water (100 ml), heated to 60°C, and cleared by the dropwise addition of 1 M NaOH (Hayat, 1981). The stock solution was diluted with concentrated buffer solution, titrated to the required pH, diluted to yield the required strength of both buffer (0.2 M HEPES) and fixative, and stored frozen until required.

[1] Supported by grants from the University of Natal Research Fund and the Foundation for Research Development.

[2] Correspondence to: Dr. E. Elliott, Dept. of Biochemistry, University of Natal, Pvt Bag X01, Scottsville, Pietermaritzburg 3209 South Africa.

Table 1. *Protocols tested for fixation of neutrophils*[a]

Protocol no.[b]	Primary fixation conditions			Secondary fixation conditions		
	pH	%PFA	Min	pH	%PFA	Min
1	7.2	4	30	–	–	–
2	8.0	4	30	–	–	–
3	7.2	8	30	–	–	–
4	8.0	8	30	–	–	–
5	7.2	4	5	7.2	8	15
6	8.0	4	5	7.2	8	15
7	7.2	4	5	8.0	8	15
8	8.0	4	5	8.0	8	15

[a] In every case, post-labeling fixation with 1% glutaraldehyde was additionally used to stabilize antigen-antibody-label complexes.

[b] If flotation fixation was additionally used, this is indicated in the text by a "+" after the protocol number and means flotation on the highest concentration of PFA used, at the pH at which the primary fixation was carried out.

Fixation and Processing for Electron Microscopy. PMN pellets were fixed at room temperature in either 4% or 8% PFA (30 min) or in 4% PFA (5 min) followed by 8% PFA (15 min), at pH 7.2 or pH 8.0. This gave eight combinations, denoted protocols 1 to 8 (Table 1). When reference to concentration and pH is made in the text, the above values will be described as either "high" or "low" concentration or pH.

After fixation the pellet was infiltrated with 2.1 M sucrose in PBS, frozen in liquid nitrogen, and sectioned with a tungsten-coated (Roberts, 1971) glass knife (Morewood et al., 1992) in an RMC-MT-6000XL ultramicrotome fitted with a CR-2000 cryoattachment. Sections (100–150 nm thick) were retrieved on 2.3 M sucrose (Tokuyasu, 1986; Griffiths et al., 1983), placed on a 100-mesh hexagonal, formvar- and carbon-coated grid, and floated out onto chilled PBS or fixative for 30 min. This procedure of extending fixation by flotation of the section onto fixative will be termed "flotation fixation" (see Table 1, Footnote b).

Antibodies. The specificity of all antibodies was assessed by Western blotting of crude white-cell homogenates and pure antigen. These were probed with immune and pre-immune sera/antibodies at the same level as used in the electron microscopy immunolabeling procedure.

The rabbit anti-human cathepsin D antibody, raised against human spleen cathepsin D purified by the method of Jacobs et al. (1989), was tested against homogenates of human PMNs (~80 μg) and purified spleen fractions (3 μg), separated by SDS-PAGE on 12.5% gels, and electroblotted as described previously (Coetzer et al., 1991). The blot was probed with the anti-cathepsin D antiserum [diluted 1:40 in 5% fetal calf serum (Gibco; Paisley, UK) in PBS], and labeling located with a sheep anti-rabbit peroxidase label and an H_2O_2/4-chloro-1-naphthol detection system (Hudson and Hay, 1980).

The rabbit anti-human leukocyte elastase (HLE) antibody, obtained from Athens Research and Technology (Athens, GA), was similarly tested with Western blotting of HLE (3 μg), isolated as described by Baugh and Travis (1976), and a human PMN homogenate (~80 μg). Binding of the anti-HLE antibody (88 μg/ml) was detected with a biotinylated goat anti-rabbit and streptavidin–biotin–peroxidase system (Amersham; Poole, UK).

The anti-human cathepsin D antiserum located the 30 KD heavy chain of the two-chain form of human cathepsin D (Figure 1A, a) and the anti-HLE antibody located the characteristic closely associated subunits of elastase (M_r 27,000 and 29,000) in the purified fractions (Figure 1B, a). Neither antibody located equivalent bands in the crude PMN homogenate, even when crude extracts were overloaded (~80 μg of total protein) and over-developed to reveal any reactivity against proteins present at very low concentrations. Control blots probed with pre-immune rabbit serum or IgG showed no targeting of the purified antigen or the crude extract.

The anti-cathepsin D antiserum, pre-adsorbed with 1 nmol/ml of human cathepsin D, showed almost total extinction of labeling (Figure 1A, b). Because of limits on the amounts of HLE antigen available, adsorption controls were performed only in the immunolabeling procedures. All control labelings confirmed the specificity of the antibody and labeling systems used and indicated that the Western blotting systems used were too insensitive to detect the target antigens in crude PMN fractions.

Antibodies used to confirm labeling results included a chicken anti-porcine cathepsin D antibody raised and characterized as described by Sameni et al. (in press) and a sheep anti-HLE antibody from Serotec (Oxford, UK). Other antibodies used included a rabbit anti-human lactoferrin IgG from Sigma Chemical (St Louis, MO) and a rabbit anti-human myeloperoxidase IgG from Dakopatts (Glostrup, Denmark).

Immunogold Labeling for Electron Microscopy. Gold labeling was performed with 5-nm protein A-gold labels purchased from Janssen Pharmaceutica (Beerse, Belgium) or produced according to the method of Slot and Geuze (1985). Antibodies and gold labels were diluted in globulin-free bovine serum albumin (Sigma) or 1% fetal calf serum (Gibco) in PBS.

Sections were blocked by incubation in 0.02 M glycine (10 min) and by 2% BSA or 1% fetal calf serum in PBS (15–30 min). Incubation (1 hr) in either rabbit anti-HLE (0.88 μg in 10 μl), rabbit anti-human lactoferrin

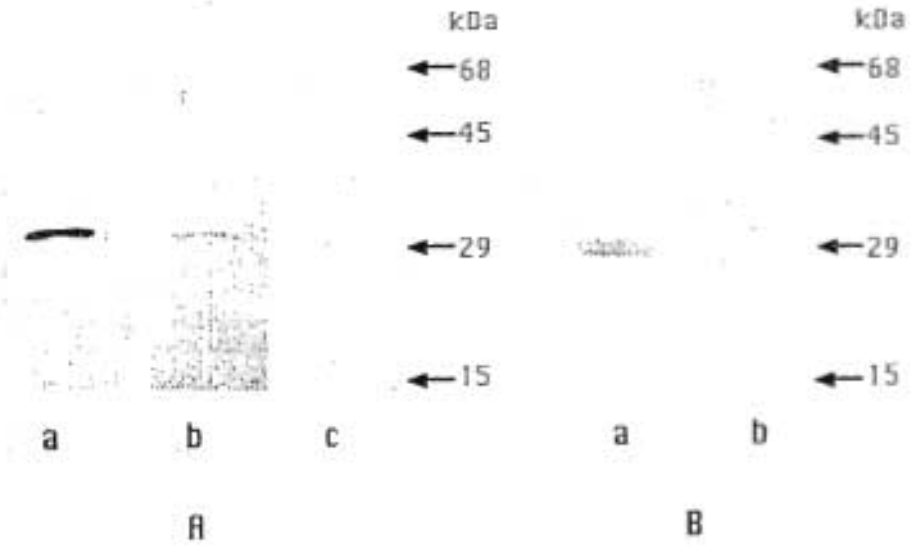

Figure 1. Western blot for (A) the anti-human cathepsin D antibody and (B) the anti-human leucocyte elastase (anti-HLE) antibody. (A) Purified human spleen cathepsin D probed with the anti-cathepsin D antibody (a), pre-adsorbed anti-cathepsin D (b), and pre-immune serum (c). (B) Purified HLE probed with the anti-HLE antibody (a) and pre-immune serum (b).

(0.36 μg in 10 μl), rabbit anti-human myeloperoxidase (0.5 μg in 10 μl), or rabbit anti-cathepsin D antiserum (1:40) was followed by washing in PBS (20 min total), and incubation with a 5-nm protein A–gold probe (10 μl, A_{520} 0.1–0.5). Post-labeling fixation to stabilize the binding of the final antigen–antibody–gold label complexes was effected with 1% glutaraldehyde in PBS (5 min) (Tokuyasu, 1986).

Double labeling was performed as described by Slot et al. (1991). Sections were blocked, incubated in primary antibody and 5-nm protein A–gold probe as described above, and treated with 1% glutaraldehyde in PBS (5 min) to eliminate reactivity between the primary antibody and the secondary (10-nm) protein A–gold probe. Residual reactive fixative groups were quenched by blocking as before and sections were incubated in the secondary antibody and gold label (10 nm) as described above. Sections were re-fixed with 1% glutaraldehyde, washed, and contrasted in acidic uranyl acetate/methyl cellulose (Tokuyasu, 1986), and viewed in a Jeol 100CX transmission electron microscope at 80 kV.

Controls included the substitution of pre-immune IgG or serum for antibodies used in the labeling scheme, omitting the primary antibody and, in the case of the anti-HLE antibody, labeling was also performed with pre-adsorbed antibody (88 μg/ml antibody adsorbed with 1–5 nmoles of HLE).

In double-labeling procedures, the adequacy of quenching of reactivity between the primary antibody and the secondary gold probe was established by performing the labeling procedure but omitting the secondary antibody. Labeling was also validated by varying the order in which antigens were labeled.

Cathepsin D and elastase labeling results were confirmed using a chicken anti-porcine cathepsin D and a sheep anti-HLE antibody, respectively.

Results

In assessing different fixation regimens, their success in meeting the following criteria were considered: (a) preservation of the antigenicity of multiple granule antigens; (b) preservation of granule ultrastructure (especially of elastase-containing azurophil granules); and (c) immobilization of target antigens, especially HLE. All protocols mentioned refer to those outlined in Table 1.

Representative micrographs for presentation were selected from at least 10 micrographs of random sections of PMNs, each fixed with one of the different fixation regimes.

Preservation of Antigenicity and Verification of Labeling Specificity

In all PFA fixation protocols, high-density immunolabeling was observed for all antigens tested except where extensive extraction of antigen occurred (e.g., with protocol 1; Figure 2a) or where antigen was over fixed (e.g., using Protocol 8; results not shown).

The labeling system used was judged to be free of nonspecific interactions, as substitution of pre-immune IgG at the same level of antibody used or omission of the primary antibody gave low background counts (6–10 gold probes/cell section, assessed on a minimum of 10 cells for each fixation protocol). In double-labeling regimens, only five large (10 nm) gold labels were seen to be nonspecifically bound per cell (averaged over 10 cells), indicating the significance of the double labeling shown in Figure 3b.

The labeling specificity of the anti-HLE antibody used was confirmed by adsorption of the antibody with 1 nmole or 5 nmoles of HLE, which resulted in a reduction in immunolabeling for elastase (by 75% or 99%, respectively), and by similar granule labeling patterns seen using an unadsorbed antibody raised in sheep (results not shown). The anti-human cathepsin D antibody labeling specificity was confirmed using a chicken anti-porcine cathepsin D antibody that crossreacts with the human antigen (Sameni et al., in press) (results not shown) and by adsorption controls on Western blots. The specificity of the antibodies and labeling systems used in double and single labelings was thus confirmed by all controls.

Effect of PFA Concentration and pH on Ultrastructure and Antigen Immobilization

The apparent shape of granules may be influenced by the plane of sectioning but, on the basis of morphology and elastase content, three types of azurophil granules were distinguishable: approximately spherical, electron-dense, elastase-containing granules of ~350 nm diameter; elongated, dumbbell-shaped granules of approximately 200 × 600 nm; and smaller, less electron-dense spherical granules 100–200 nm in diameter. These morphologically distinct "azurophil" granule types resemble those described by Pryzwansky and Breton-Gorius (1985). All three granule types were best preserved and the elastase antigen best immobilized by Protocol 6+, using a combination of rapid fixation with a low concentration of PFA at a high pH, followed by longer fixation at high concentration and at a low pH, and flotation fixation (Figure 3c).

All protocols using fixation only at a low pH gave poor immobilization of the elastase antigen (Protocols 1, 3, and 5; Figures 2a, 2c, and 2e, respectively). High pH fixation for more than 5 min (Protocol 2 or 4, results not shown; Figure 2d, arrowheads) generally gave poor preservation of small and dumbbell-shaped granules (and hence poor antigen immobilization) but better granule preservation (and hence superior antigen immobilization) in larger azurophil granules than equivalent low pH fixation protocols. In the high pH double-fixation Protocol 8, however, all granules were well preserved but labeling density was extremely low, possibly due to overfixation of membranes resulting in limited antibody penetration (results not shown).

Lactoferrin-containing specific granules were adequately preserved and labeled in all fixation regimens used in this study. Single labeling for myeloperoxidase- (Figure 3a, inset) and cathepsin D-containing granules (Figure 3b) was similar to that observed for elastase. Cathepsin D-labeled granules appeared to decrease in number from ~8 granules/section in less mature PMNs (assessed on sections of 10 cells showing two nuclear lobes or less), to 2–4 granules/section in more mature cells (with two or more nuclear lobes). These results, however, confirm the observations of Ishikawa and Cimasoni (1977), Barabasi and Nitsberger (1994), Levy et al. (1989), and Reid et al. (1986) that cathepsin D occurs in PMNs.

Flotation Fixation and Post-immunolabeling Fixation

Where the initial fixation was relatively weak, e.g., Protocols 1, 5, and 6, immobilization of the elastase antigen was improved by pre-labeling flotation fixation (compare Figures 2a and 2b, Figures 2e and 2f, and Figures 3a and 3c, respectively). The preservation of dumbbell-shaped granules and antigen immobilization in Protocols

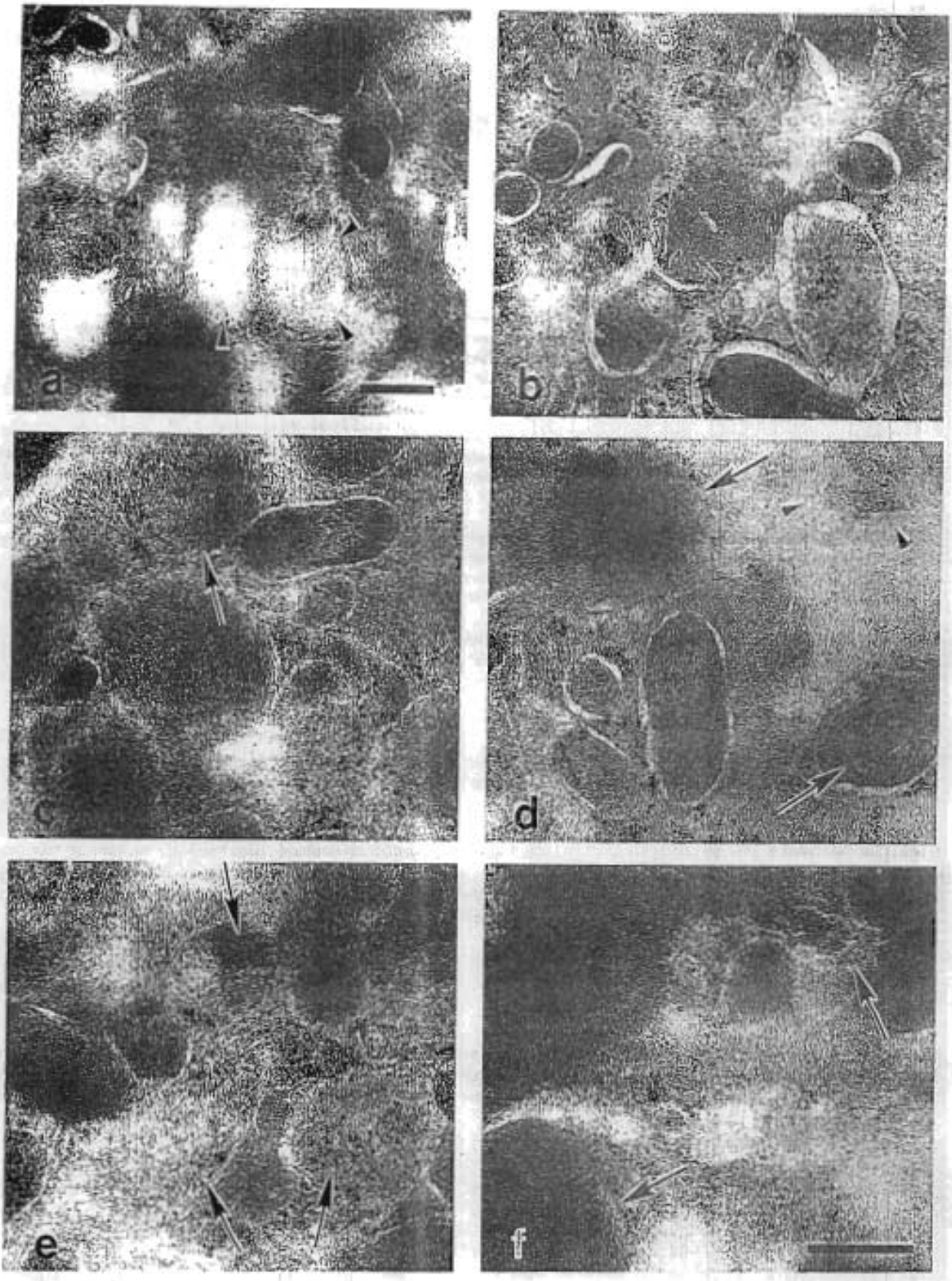

Figure 2. Cryoultramicrotomy sections of unembedded PMNs labeled for HLE after fixation (for 30 min unless otherwise indicated) with (a) 4% PFA, pH 7.2, (b) 4% PFA, pH 7.2, followed by pre-labeling lateral fixation, (c) 8% PFA, pH 7.2, (d) 8% PFA pH 6.0, (e) 4% followed by 8% PFA, pH 7.2 (for 5 and 15 min, respectively), and (f) 4% followed by 8% PFA pH 7.2 (for 5 and 15 min, respectively) and lateral fixation of the section. Small arrowheads indicate extracted granules (a) or translocation of elastase due to inadequate membrane fixation (d). Large arrows indicate reasonably well-preserved dumbbell-shaped or large elastase-containing granules (c–f). Original magnifications: a × 35,000; b–f × 50,000. Bars = 0.2 μm.

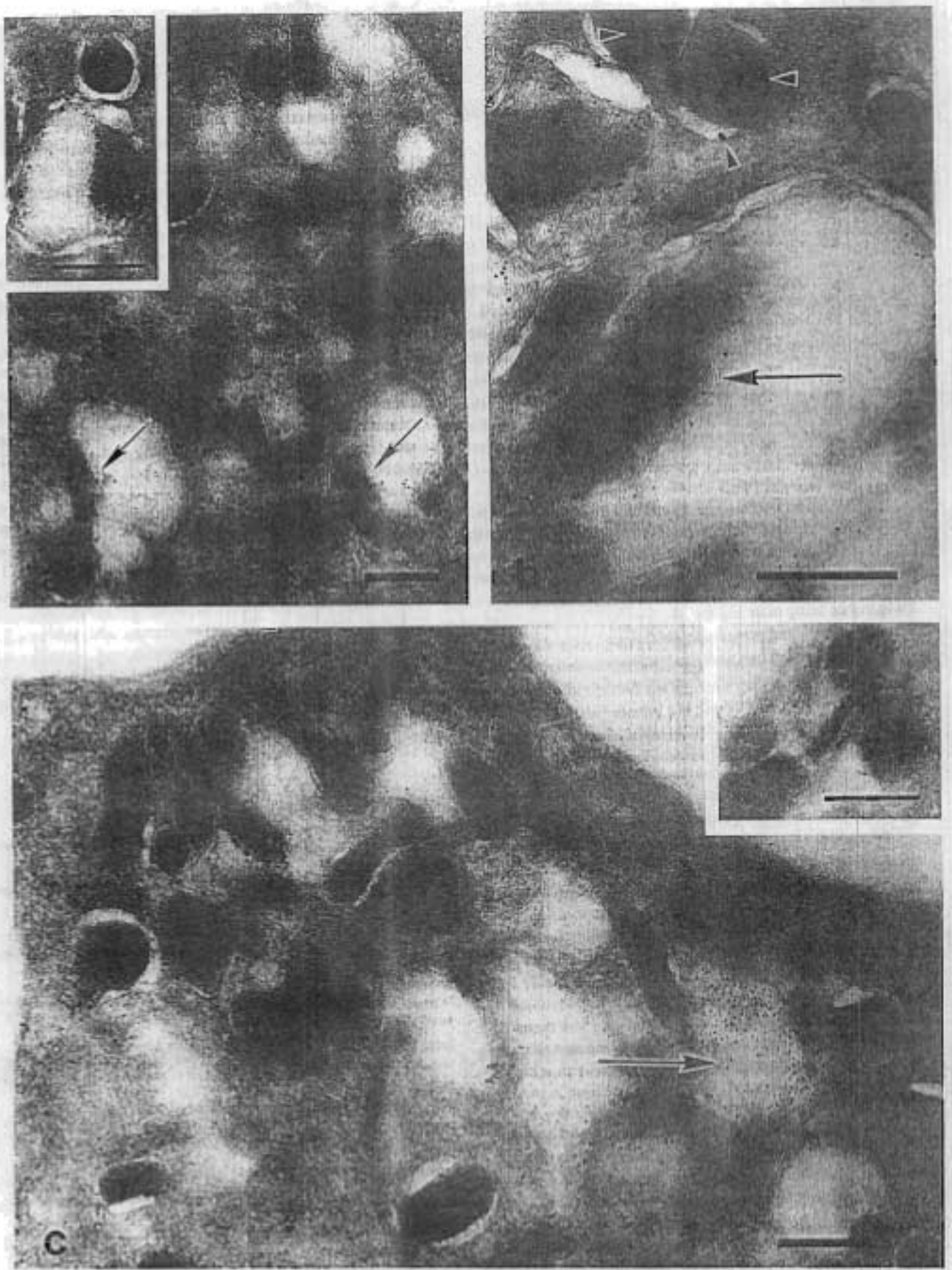

Figure 3. (a–c) Cryoultramicrotomy sections of unembedded PMNs fixed with 4% PFA, pH 8.0 (5 min) followed by 8% PFA, pH 7.2 (15 min), (c) with and (a,b) without flotation fixation prior to labeling. (a) Large azurophil granules labeled for elastase (5-nm gold probe) appear shrunken, partially extracted (arrows), and show antigen translocation. Inset shows similar extraction of myeloperoxidase. (b) Large (possibly azurophil) granules labeled for cathepsin D (5-nm gold probe) (large arrow) show partial extraction, whereas smaller cathepsin D-labeled granules and specific granules labeled for lactoferrin (10-nm gold probe) (arrowheads) remain unextracted. (c) Flotation fixation allows most precise immobilization of elastase, even in swollen, yet unextracted granules (large arrow). Inset shows labeling of specific granules for lactoferrin. Original magnifications: a,c × 26,000; b × 50,000; insets × 33,000. Bars = 0.25 μm.

6+ and 5+, however, was enhanced by flotation fixation (compare Figures 3a and 3c and Figures 2e and 2f, respectively).

Discussion

In this study, conditions required for optimal fixation of HLE-containing granules appeared to vary among the three types of such granules. This may reflect differences in membrane composition and hence in reactivity with the PFA polymers, which vary in length and reactivity at the different concentrations and pHs used.

Solid PFA consists largely of polymeric species that dissolve in alkaline solutions by slowly depolymerizing to smaller, more penetrating polymers but which are less able to cross-link residues some distance apart (Larsson, 1988; Walker, 1964). Increasing dilution has a similar effect on polymer size. Above a 2% (w/v) concentration the degree of polymerization of PFA increases progressively and the fixative molecules become less penetrating, more able to cross-link distant residues (Walker, 1964).

At low concentrations, PFA reacts mainly and most rapidly with deprotonated primary or secondary amines to form hydroxymethylene bridges (Kallen and Jencks, 1966), and hence should react best at higher pHs. At higher concentrations, however, PFA reacts with both protonated and unprotonated amines (Kallen and Jencks, 1966), hence being more reactive over a wider pH range, including physiological pH, and presumably produces a more extensive cross-linking of reactive residues owing to the increased polymer lengths.

In this study, as predicted, fixation was facilitated by the increased length of PFA polymers and the greater reactivity of PFA at high concentrations. High concentrations of PFA alone, at physiological pH, however, appear not to be adequate to prevent the translocation of azurophil antigens, possibly indicating that fixation is too slow and reversible at this pH, or that polymers are too large to penetrate tissues quickly to effect fixation.

Fixation with a low concentration of PFA, to ensure rapid penetration of fixative, followed by a high concentration of PFA, to ensure adequate tissue cross-linkage (a 5 + 15-min fixation procedure), was found to give better containment of elastase and slightly better ultrastructural preservation of granules than any single concentration of fixative (applied for 30 min). PFA's affinity for protonated amines is, however, two to three orders of magnitude less than for the non-protonated amine (Kallen and Jencks, 1966). Fixation with a low concentration of PFA at high pH was therefore also attempted. Under these conditions the fixative was more penetrating but fixation was more rapid and effective than at higher concentrations and lower pH. This gave the best antigen immobilization in large but not in small azurophil granules.

To prevent premature fixation of the outer membranes (e.g., of the smaller granules) impeding fixative penetration, the pH shift method of Berod et al. (1981) and Eldred et al. (1983) has been proposed. This uses fixation with low PFA at a low pH to allow rapid penetration, followed by fixation with the same low concentration of PFA but at a high pH to facilitate rapid cross-linkage and immobilization of the antigen. In the present study, however, a reverse pH shift strategy, a high to low pH shift, combined with an increase in PFA concentration, was most successful in immobilizing azurophil antigens in all granule types, provided the high pH fixation period was kept brief. In this case, the low penetration caused by increased efficiency of fixation of the azurophil granule membranes at the higher pH was possibly partially offset by the short period of fixation and the low concentration of PFA and the decreased size of the polymers present at the higher pH used for initial fixation.

Lastly, the results of flotation of the newly cut section onto fixative suggest that this is an effective way of additionally fixing PMN granule contents and preserving the ultrastructure of certain granules and stabilizing cytoskeletal elements responsible for the maintaining of granule shape.

Initially, PMN granules were classified into two groups, larger "primary" or "azurophil," peroxidase-positive, hydrolytic enzyme-containing granules, and smaller "secondary" (specific) lactoferrin-containing granules (Bretz and Baggiolini, 1974). Subpopulations of azurophil granules, differing in levels of myeloperoxidase and elastase (Damiano et al., 1988; Przywansky and Breton-Gorius, 1985), and specific granules, varying in their gelatinase content (Hibbs and Bainton, 1989), have since been discovered. Immunocytochemical classification of granules is difficult, however, if granule antigens are extracted. The optimal fixation regimen described here (4% PFA at pH 8.0, 5 min, followed by 8% PFA, pH 7.2, 15 min, followed by flotation fixation) preserves maximal antigenicity of a wide range of PMN antigens, facilitating the simultaneous labeling of myeloperoxidase, lactoferrin, elastase, and cathepsin D. Antigens are immobilized without compromising antibody penetration and the structure of three azurophil granules is preserved, hence opening the way for multiple simultaneous labeling studies on PMNs and accurate classification of granule subtypes.

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ARTICLE

Cathepsin B and D are Localized at the Surface of Human Breast Cancer Cells*

Mansoureh SAMENI,[1] Edith ELLIOTT,[2] Grace ZIEGLER,[1] Philip H. FORTGENS,[2] Clive DENNISON[2] and Bonnie F. SLOANE[1]

[1]Department of Pharmacology, Wayne State University, Detroit, USA
[2]Department of Biochemistry, University of Natal, Pietermaritzburg, South Africa

Alterations in trafficking of cathepsins B and D have been reported in human and animal tumors. In MCF-10 human breast epithelial cells, altered trafficking of cathepsin B occurs during their progression from a preneoplastic to neoplastic state. We now show that this is also the case for altered trafficking of cathepsin D. Nevertheless, the two cathepsins are not necessarily trafficked to the same vesicles. Perinuclear vesicles of immortal MCF-10A cells label for both cathepsins B and D, yet the peripheral vesicles found in *ras*-transfected MCF-10AneoT cells label for cathepsin B, cathepsin D or both enzymes. Studies at the electron microscopic level confirm these findings and show in addition surface labeling for both enzymes in the transfected cells. By immunofluorescence staining, cathepsin B can be localized on the outer surface of the cells. Similar patterns of peripheral intracellular and surface staining for cathepsin B are seen in the human breast carcinoma lines MCF-7 and BT20. We suggest that the altered trafficking of cathepsins B and D may be of functional significance in malignant progression of human breast epithelial cells. Translocation of vesicles containing cathepsins B and D toward the cell periphery occurs in human breast epithelial cells that are at the point of transition between the pre-neoplastic and neoplastic state and remains part of the malignant phenotype of breast carcinoma cells. (Pathology Oncology Research Vol 1, No1, 43–53, 1995)

Key Words: aspartic proteases, breast cancer, cathepsins, cysteine proteases, oncogenic *ras*

Introduction

Expression, redistribution and/or secretion of the lysosomal proteases cathepsins B, D and L have been reported to parallel malignant progression.[1,2] Redistribution of cathepsin B has been observed in human colon carcinomas,[3] prostate carcinomas[31] and gliomas,[27] this redistribution parallels increased malignancy and/or decreased patient survival. Recently, the distribution of cathepsin D in phagolysosomes has been suggested to be a prognostic indicator for human breast carcinoma.[4,30] In macrophages and osteoclasts, i.e., cells that like tumor cells participate in degradative or invasive processes, lysosomes undergo translocation from the perinuclear region to the cell periphery. This redistribution is induced by cytoskeletal alterations associated with membrane ruffling. Lysosomes redistribute toward the ruffling membrane of activated osteoclasts and lysosomal enzymes are secreted.[5]

The study of breast cancer progression has been facilitated by the development of the diploid MCF-10 human breast epithelial cell lines. These cells were obtained during reduction mammoplasty from a patient with fibrocystic breast disease and underwent spontaneous immortalization in culture.[35] Transfection of immortal MCF-10A cells with mutated *ras*[6] results in cells (neoT) that have some of the characteristics of atypical breast epithelial stem cells. *In vitro* they are capable of indefinite proliferation and invade

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Correspondence: Bonnie F SLOANE; Department of Pharmacology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, Michigan 48201 USA. Fax: (313) 577-6739. E-mail: bsloane@med.wayne.edu

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The Confocal Imaging Core was supported by the Center for Molecular and Cellular Toxicology with Human Application and the Karmanos Cancer Institute.

Abbreviations: BSA: bovine serum albumin; HEPES: N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid; IgG: immunoglobulin G; IgY: immunoglobulin Y; MPR: mannose 6-phosphate receptor.

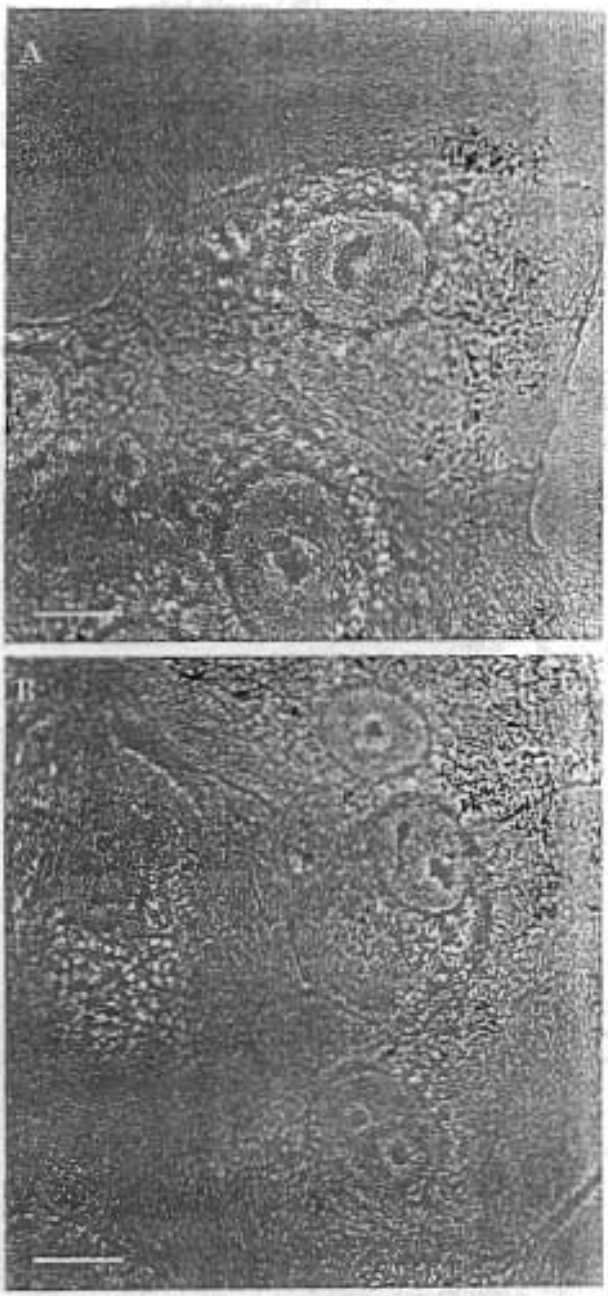


Figure 1. Immunocytochemical localization of intracellular cathepsin D in parental 10A cells (A) and their neoT counterpart transfected with mutated ras (B). The primary antibody was mouse anti-human cathepsin D IgG1. Cathepsin D staining in the 10A cells (A) was concentrated in the perinuclear region, whereas in the ras-transfected cells (B) the distribution of cathepsin D staining was more peripheral. The secondary antibody was Texas red-conjugated donkey anti-mouse IgG. The staining for cathepsin D has been repeated ten times to date with comparable results. Only a weak background fluorescence was observed in controls in which mouse or rabbit pre-immune IgG replaced the primary antibodies (not illustrated). Bars, 10 μm.

through Matrigel[29] and *in vivo* they form persistent palpable nodules that exhibit three pathologic entities: 1) benign ductal aggregates, sometimes with mild hyperplastic changes; 2) atypical hyperplastic lesions; and 3) carcinoma *in situ* and invasive carcinomas.[30] *ras*-transfection of the MCF-10 lines results in altered trafficking of cathepsin B,[19] such that this enzyme is localized in the cell periphery and on the cell surface. As *ras*-transfection of breast cancer cells increases their invasiveness[1] and metastatic ability,[13] the observations on altered trafficking of cathepsin B in *ras*-transfected MCF-10 cells may be of functional significance in the early progression of breast cancer. In the present study, we determined whether transfection of MCF-10 human breast epithelial cells with the c-Ha-*ras* oncogene affects the trafficking of cathepsin D as well as that of cathepsin B, whether cathepsins B and D are trafficked to the same vesicles and whether altered trafficking of these enzymes also is characteristic of fully malignant human breast carcinoma cell lines.

Materials and Methods

Materials

Saponin, Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, minimal essential medium, HEPES, BSA, insulin, hydrocortisone, antibiotics, fish skin gelatin and methylcellulose were from Sigma (St Louis, MO); equine serum and fetal bovine serum from GIBCO (Grand Island, NY); epidermal growth factor from UBI (Lake Placid, NY); and 4,6-diamidin-2-phenylindol-dihydrochloride from Boehringer-Mannheim (Indianapolis, IN). A monoclonal antibody to human breast cancer cathepsin D was purchased from BioSys (Compiegne, France). Fluorescein-conjugated affinity-purified donkey anti-rabbit IgG, Texas red-conjugated affinity-purified donkey anti-mouse IgG and normal donkey serum were obtained from Jackson ImmunoResearch (West Grove, PA); formaldehyde from Polysciences (Warrington, PA); and SlowFade anti-fade reagent from Molecular Probes (Eugene, OR). The microbiological grade gelatin used for embedding of tissues and glutaraldehyde were purchased from Merck (Darmstadt, Germany); paraformaldehyde from BDH (Poole, United Kingdom); fraction V BSA from Boehringer-Mannheim (Mannheim, Germany); and 10 and 15 nm protein A-gold probes from Drs. Slot and Posthuma, Department of Cell Biology, University of Utrecht, The Netherlands. The rabbit anti-chicken IgY used in immunogold labeling was raised against IgY isolated from eggs of non-immunized chickens using polyethylene glycol precipitation.[20] Rabbit anti-chicken IgY-horseradish peroxidase was prepared as previously described.[8] All other chemicals were of reagent grade and were obtained from commercial sources.

Cell lines and culture

MCF-10 is a diploid human breast epithelial cell line derived from a patient with fibrocystic breast disease. This line underwent spontaneous immortalization in culture and grows attached in the presence of calcium or floating in the absence of calcium.[31] Transfection and cotransfections

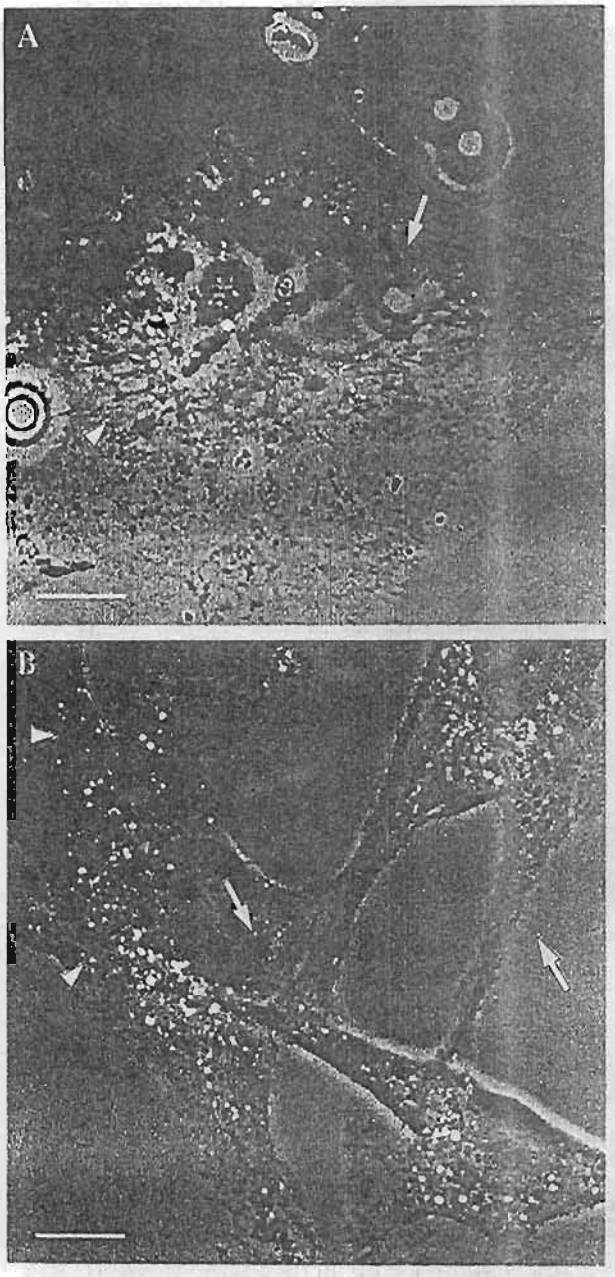


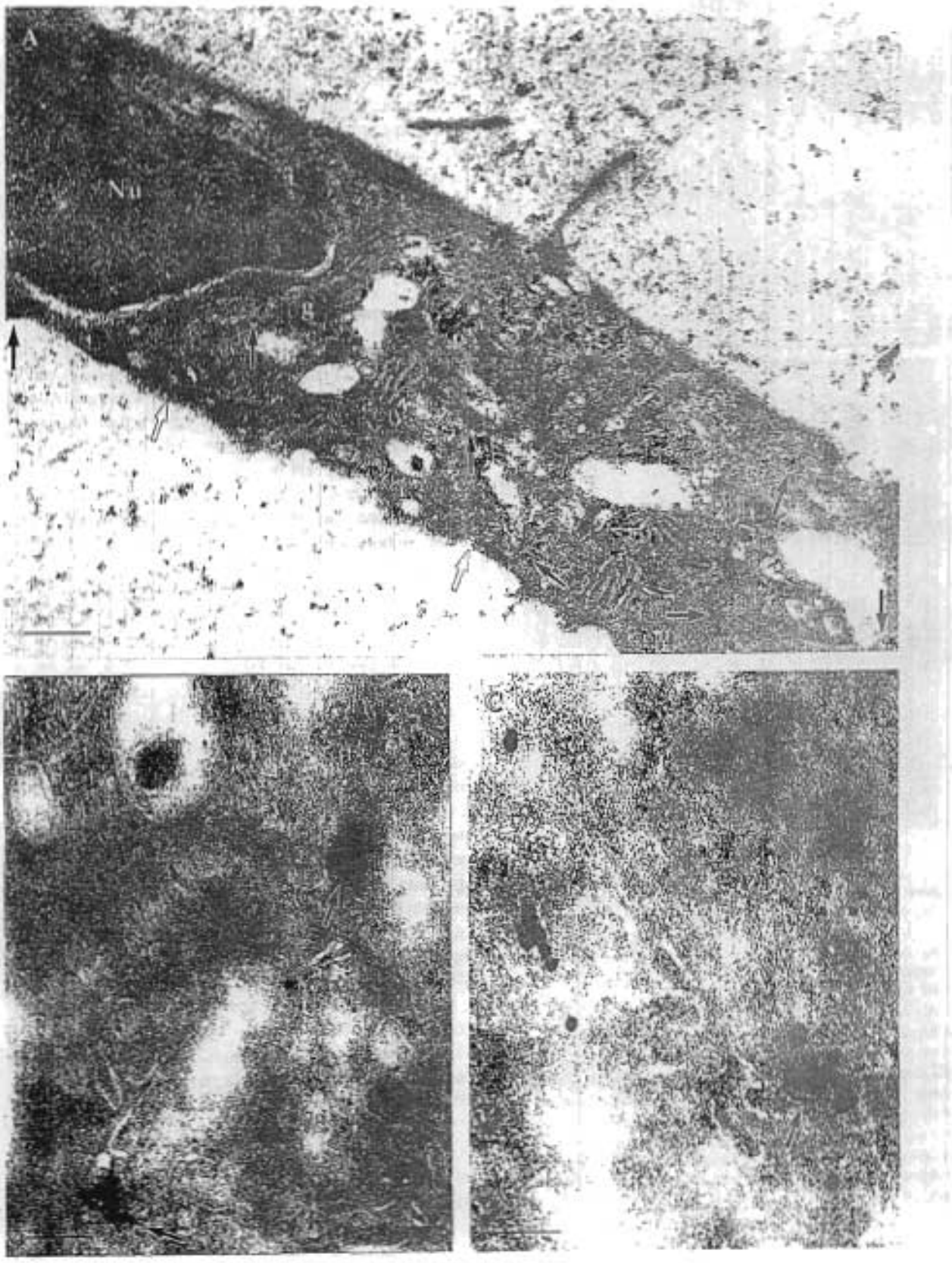
Figure 2. Immunocytochemical colocalization of intracellular cathepsin B and cathepsin D in immortal MCF-10A cells (A) and their counterpart transfected with oncogenic ras (B). Vesicles staining for cathepsin B alone are indicated with arrowheads and those staining for cathepsin D alone are indicated with arrows. Vesicles staining yellow indicate possible colocalization. Primary antibodies were rabbit anti-human cathepsin B IgG and mouse anti-human cathepsin D IgG1. Fluorescein-conjugated affinity-purified donkey anti-rabbit IgG and Texas red-conjugated affinity-purified donkey anti-mouse IgG were used as secondary antibodies. The double labeling for cathepsins B and D has been repeated six times to date with comparable results. Only a weak background fluorescence was observed in controls in which rabbit or mouse pre-immune IgG replaced the primary antibodies (not illustrated). Bars, 10 μm.

were performed using the calcium phosphate method with a plasmid containing the neomycin resistance gene as a transfection vector either alone (MCF-10Aneo) or with constructs containing wild-type (MCF-10AneoN) or mutated (MCF-10AneoT) c-Ha-*ras*.[4] The MCF-10 lines were grown in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, containing 5% equine serum, supplemented with insulin, hydrocortisone, epidermal growth factor, antibiotics and fungizone,[4,25,35] but without amphotericin and cholera toxin.[35] The MCF-7 and BT20 human breast carcinoma lines were grown in minimal essential medium containing 10% fetal bovine serum as recommended by the ATCC (Rockville, MD). All cell lines were screened on a routine basis with 4',6-diamidin-2-phenylindol-dihydrochloride and shown to be free of *Mycoplasma*.

Immunochemical studies

Preparation of monospecific anti-cathepsin B IgGs and anti-cathepsin D IgY: Cathepsin B antisera were raised in rabbits as described.[21] An IgG fraction was purified and stored at -20°C. The specificity of the IgG used for immunofluorescence labeling of cathepsin B has been confirmed by slotblotting and immunoblotting against crude and purified cathepsin B fractions from human liver and sarcoma,[21] acetone fractions of human colonic mucosa and colon tumors[5] and cell homogenates of human breast epithelial cells.[33] Immunogold labeling for cathepsin B was performed using an affinity purified anti-human liver cathepsin B antibody, kindly supplied by Drs. Lukas Mach and Josef Glössl, Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna, Austria. The production and specificity of this antibody was reported previously.[17] Cathepsin D was purified from porcine spleen according to the method of Jacobs et al.[14] Laying hens were immunized with 100 μg of porcine cathepsin D (50 μg into each breast muscle). The antigen was triturated in a 1:1 ratio with Freund's complete adjuvant at 0 wk and in Freund's incomplete adjuvant at 1, 2, 4 and 6 wk and for monthly boosters thereafter. Eggs were collected on a daily basis. IgY was isolated from the yolks by precipitation with polyethylene glycol as described.[26] The specificity of the IgY for cathepsin D was confirmed by immunoblotting against crude and purified cathepsin D fractions (data not shown).

Immunofluorescent staining: Intracellular cathepsins B and D and surface cathepsin B were localized using a modification[33] of the general immunocytochemical methodologies described by Willingham.[36] Cells grown to 60–80% confluence on glass coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline, pH 7.4. Fixation and subsequent steps were performed at 25 °C for intracellular labeling and at 4 °C for surface labeling. After washing with phosphate-buffered saline, cells were


A
Nu
B
C

blocked with phosphate-buffered saline-2 mg/ml BSA. For intracellular labeling, all subsequent antibody and wash solutions contained 0.1% saponin; saponin was not used in the surface labeling studies. Cells were incubated with primary antibody (rabbit anti-human liver cathepsin B, mouse anti-human breast cancer cathepsin D IgG1) for 2h and washed. Surface labeling was performed on cells incubated with primary and secondary antibodies at 4°C prior to fixation for the breast epithelial cells. For the breast carcinoma cells, surface labeling was performed subsequent to fixation at 4°C as these cells detached from the substratum at 40°C. In controls, preimmune serum (rabbit or mouse) was substituted for the primary antibody. After blocking with normal donkey serum (5% in phosphate-buffered saline-0.1% saponin for intracellular staining and without saponin for surface staining), cells were incubated for 60 min with fluorescein-conjugated affinity-purified donkey anti-rabbit IgG or Texas red-conjugated affinity-purified donkey anti-mouse IgG at 20 µg/ml. After washing, the coverslips were mounted upside-down on slides with SlowFade anti-fade reagent and observed with a Zeiss LSM 310 confocal microscope.

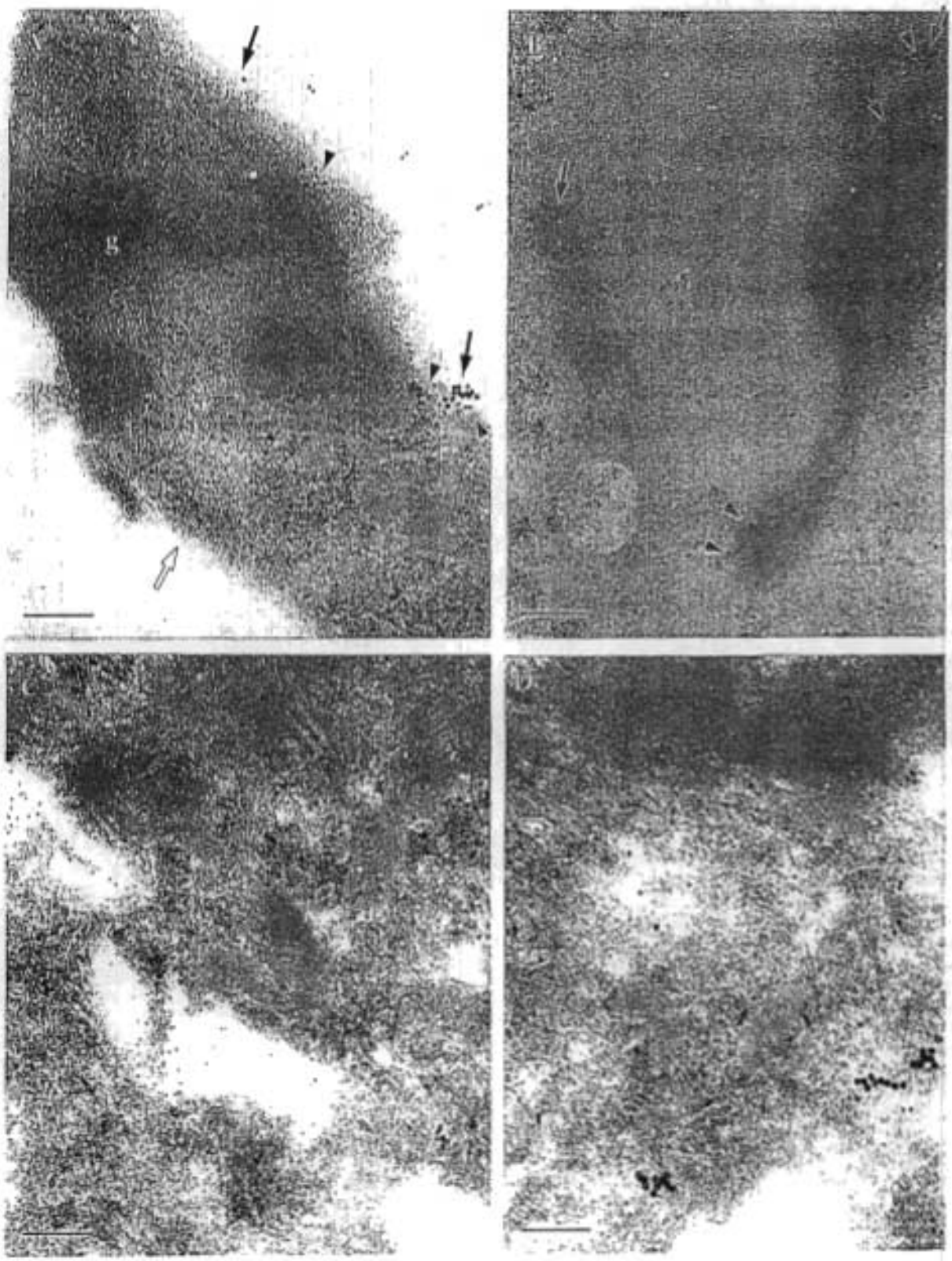
Immunogold staining: Cells grown to 60-80% confluence in T-25 flasks were fixed and processed by a modification of the method of Griffiths et al.[35] Cells were fixed in 2% paraformaldehyde containing 0.02% glutaraldehyde in 200 mM HEPES buffer, pH 7.3, at 4°C for 1h. After washing in phosphate buffer containing 20 mM glycine, the fixed monolayers were infiltrated with gelatin [10% (mass/vol) bacteriological gelatin phosphate buffer, 1h at 37°C] and crosslinked with the primary fixative (10 min at room temperature). The crosslinked, gelatin-infiltrated cell layers were stripped off the plastic and cryoprotected by infiltration with 2.1 M sucrose. Blocks were cut, oriented for vertical sectioning of cells, frozen in liquid nitrogen and ultra-thin frozen sections were cut using an RMC MT6000XL ultramicrotome fitted with a CR2000 cryo-attachment. Sections were collected on 2.3 M sucrose, thawed and mounted on 100 mesh hexagonal copper grids, previously formvar- and carbon-coated and glow-discharged. Thawed, grid-mounted sections were collected on 0.1% fraction V BSA in phosphate buffer prior to labeling. The grids were labeled as described by Slot et al.[34] Non-specific binding sites on the sections were blocked by incubation in 2% fish skin gelatin and 20 mM glycine in phosphate buffer. Incubation on primary antibody (chicken anti-porcine spleen cathepsin D IgY or rabbit anti-human liver cathepsin B IgG, 10 µg/ml) was for 1h at 25°C. Incubation in the anti-porcine spleen cathepsin D IgY required an additional incubation step with a rabbit anti-chicken linker antibody (1:100 dilution for 1h at 25°C). For single labeling, the grids were then incubated for 30 min at 25°C with a 1:40 dilution of protein A-gold probe (mean particle size of 10 nm) before being washed, fixed with 2% glutaraldehyde, counterstained and sealed in a uranyl acetate/methyl cellulose mixture as described by Slot et al.[34] Double labeling was performed by repetition of the blocking and labeling regime described, the detection of antibody-binding to the second antigen being detected using a 1:55 dilution of a second protein A-gold probe (mean particle size of 15 nm). Labeling specificity was verified by the omission of primary and secondary antibodies in various labeling schemes, and the performance of labeling for the two different antigens in different orders, using detection with first the small and then the large gold labels, according to Slot et al.[34] Grids were viewed and photographed in a Jeol 100 CX transmission electron microscope, at 100 kV.

Results

We have previously established that the lysosomal cysteine protease cathepsin B is distributed more peripherally in MCF-10A human breast epithelial cells transfected with oncogenic *ras*.[33] Rochefort and colleagues have shown an association between the presence of cathepsin D in phagolysosomes near the cell surface of human breast carcinomas and prognosis.[30] Therefore, we determined whether the subcellular localization of cathepsin D also was altered. In the parental 10A cells, the staining for cathepsin D was localized primarily to the perinuclear region *(Fig.1 A)*; a localization consistent with cathepsin D being distributed in

Figure 3. Immunogold labeling for cathepsin B and cathepsin D in immortal MCF-10A cells. Cell monolayers were fixed with 2% paraformaldehyde containing 0.02% glutaraldehyde, embedded with gelatin, refixed, and the gelatin-infiltrated monolayer, cryoprotected with 2.1 M sucrose, was stripped off the plastic, cut into blocks, mounted for vertical sectioning of cells and frozen for cryoultramicrotomy. Immunolabeling for cathepsin B and D on the sections was performed using an affinity purified rabbit anti-human liver cathepsin B antibody and a chicken anti-porcine cathepsin D antibody. For protein A gold labeling using the chicken anti-porcine D antibody, a linker (rabbit anti-chicken antibody) was used. Labeling was performed for cathepsin B and then cathepsin D, and vice versa, labeling being detected using a small (10 nm) followed by a larger (15 nm) protein A gold probe, in each case. Similar colocalization results were observed in either case. Controls for double labeling indicated adequate blocking of sections between double labeling steps. In the micrographs illustrated, localization of cathepsin B was performed first and detected with the 10 nm protein A gold probe and labeling for cathepsin D was performed subsequently and detected with a 15 nm gold probe. A transverse section of an MCF-10A cell is shown (A; basolateral surface indicated with open arrows). Generally, cathepsins B and D were found to colocalize in the more electron-dense, larger vesicles (presumably late endosomal or lysosomal compartments) situated in a perinuclear location (arrowheads). Vesicles selected for enlargement (B and C) indicate colocalization more clearly. Nu = nucleus, g = Golgi apparatus. Bars, 1 µm (A) and 0.2 µm (B and C).

lysosomes. In the *ras*-transfected neoT cells (*Fig. 1 B*), both perinuclear and peripheral staining for cathepsin D was observed. Thus, cathepsin D exhibited a more peripheral subcellular distribution in the neoT cells, a pattern similar to that observed previously for cathepsin B.[23] In order to assess whether the two enzymes were distributed in the same vesicles, we performed double labeling studies. In the immortal 10A cells, cathepsins B and D were found to be primarily



colocalized in perinuclear vesicles *(Fig.2 A)*. A different pattern was observed in the neoT cells transfected with mutated *ras (Fig.2 B)*. The distribution of both enzymes was more peripheral and three patterns of vesicular staining were observed: 1) vesicles staining for both enzymes, 2) vesicles staining for only cathepsin B, and 3) vesicles staining for only cathepsin D. Vesicles staining for cathepsins B and D, cathepsin B or cathepsin D appeared to be of similar sizes.

The peripheral vesicles staining for cathepsin D may be endosomes as endosomes containing cathepsin D have been observed in macrophages[29] and hepatocytes[7] or may be the phagolysosomes described by Rochefort and colleagues.[33,30] In order to determine the localization of cathepsins B and D at the ultrastructural level, we employed immunogold double-labeling. In the immortal 10A cells, cathepsins B and D were largely colocalized in perinuclear vesicles *(Figs.3 A, B, and C, arrowheads)*. In contrast, in the neoT cells transfected with mutated *ras*, a more peripheral distribution of the gold labeling for both enzymes was observed, including increased labeling on the cell surface *(Figs.4 A and B)*. The majority of peripheral vesicles exhibited label for only one of the two cathepsins *(Fig.4 A, C and D)*. Gold particles representing cathepsin D protein could be observed apparently in the process of being secreted from surface protrusions of the neoT cells *(Fig.4 B)*. The most numerous cathepsin D- and B-labeled organelles in the immortal 10A cells *(Fig.3)* were of the order of 0.14-0.19 μm in diameter, whereas in the *ras*-transfected neoT cells these vesicles were 0.1-0.13 μm in diameter *(Fig.4)*. In the 10A cells, occasional vesicles (0.5-0.54 μm in diameter) were observed that resembled phagolysosomes and labeled heavily for cathepsin D. Larger phagolysosomes (0.5-1 μm in diameter) were observed in the neoT cells where they labeled more heavily for cathepsin B than for cathepsin D *(Fig.4 C)*.

Cell surface labeling for cathepsin B has been observed by immunofluorescence techniques in human lung carcinoma cells[3] and murine B16 amelanotic melanoma cells.[12] In order to evaluate whether the immunogold labeling for cathepsin B observed in *ras* transfected neoT cells[33] *(Fig.4)* was on the external surface of the cells, we performed immunofluorescence staining in non-permeabilized cells. Staining for cathepsin B was not observed on the surface of the immortal 10A cells *(Fig.5 A)*, but was present on the surface of the *ras*-transfected neoT cells *(Fig.5 B)*. In these latter cells, the staining for cathepsin B was localized to discrete regions on the basal surface. For cathepsin D, some cell surface labeling was observed on immortal 10A cells, yet substantially more cell surface labeling on the *ras*-transfected neoT cells (data not shown). As indicated above, in these confocal studies, the cell surface labeling was localized primarily to the basal surface of the cells with apical labeling only in a few cells. By the immunogold method, apical labeling was observed rather than basal *(Figs.3 and 4)*. However, this latter technique may not be optimal for examining basal membrane expression of cathepsins as the surface-bound cathepsins may be lost when the cells were stripped off the plates (see Materials and Methods). By contrast, in the immunofluorescence method, the cells were examined without removal from the coverslips.

Studies in human colon carcinomas,[5] prostate carcinomas[31] and gliomas[27] suggest that altered trafficking of cathepsin B is part of the malignant phenotype. Rochefort and colleagues.[30] have shown that altered trafficking of cathepsin D may be of prognostic significance in human breast carcinomas. As similar studies have not been performed for cathepsin B, we analyzed the intracellular and surface distribution of cathepsin B in two human breast carcinoma lines, MCF-7 and BT20. In both lines, cathepsin B was found to be distributed throughout the cytoplasm rather than being restricted to the perinuclear region *(Fig.6 A and C)*. The sizes of the vesicles labeling for cathepsin B could not be accurately determined in these immunofluorescent images. In both MCF-7 and BT20 cells, surface labeling for cathepsin B was observed *(Fig.6 B and D)*. As in the *ras*-transfected MCF-10AneoT cells *(Fig.5 B)*, surface staining for cathepsin B was found at discrete regions on the basal surface of the cells.

Discussion

Trafficking by two distinct pathways might be responsible for cathepsins B and D being localized in separate peripheral vesicles in the *ras*-transfected neoT cells *(Figs.2 and 4)*. Studies to date suggest that cathepsin B is trafficked to the lysosomes via a MPR-dependent pathway.[11,17] On the other hand, cathepsin D has been shown to be trafficked by both MPR-dependent[2,14,19] and MPR-independent

Figure 4. Immunogold labeling for cathepsin B and cathepsin D in MCF-10AneoT cells transfected with oncogenic ras. Cell monolayers were fixed, embedded, and processed for vertical cryoultramicrotomy sectioning and immunolabeling for cathepsins B and D was performed, in both orders, as described in the legend to Fig. 3. In the micrographs illustrated, localization of cathepsin D was performed first and detected with a 10 nm protein A gold probe (A and B) and labeling for cathepsin B was performed subsequently and detected with a 15 nm gold probe. Immunolabeling was also performed in the reverse sequence (C and D), cathepsin B being detected with the small gold probe and cathepsin D with the larger probe. A transverse section of a cell, shows less colocalization of cathepsins B and D in peripheral regions (A) [basolateral surface indicated with an open arrow (A)] and in subperinuclear regions of the cell (C and D), than in the parental MCF-10A cells (cf. Fig. 3). Some peripheral vesicles appear caught in the process of secretion (A). Association of cathepsin B (arrows) and cathepsin D (arrowheads) with the surface of cells is also evident in the ras-transfected neoT cells (A and B). g = Golgi apparatus. Bars, 0.25 μm (A, B and D) and 0.5 μm (C).

pathways.[28,37] Although the peripheral vesicles labeling for either cathepsin B or cathepsin D might represent two different vesicular compartments, this would appear to be unlikely as the sizes of the vesicles are similar. Another possibility is that one compartment might contain only pro forms of the two cathepsins and the other mature forms. However, as the antibodies used in the present study recognize both pro and mature forms of cathepsins B and D, both compartments should stain for the two enzymes. Thus, at present the identity of the peripheral vesicles staining for only cathepsin B or for only cathepsin D is unknown. Studies to establish the molecular forms of cathepsins B and D associated with the cell surface and these peripheral vesicles and the identity of these peripheral vesicles are in progress.

Rochefort and colleagues have proposed that intracellular cathepsin D plays a functional role in breast carcinoma, specifically in the degradation of extracellular matrix proteins in a peripheral compartment of phagolysosomes.[23] In the present study by immunogold labeling, we localized

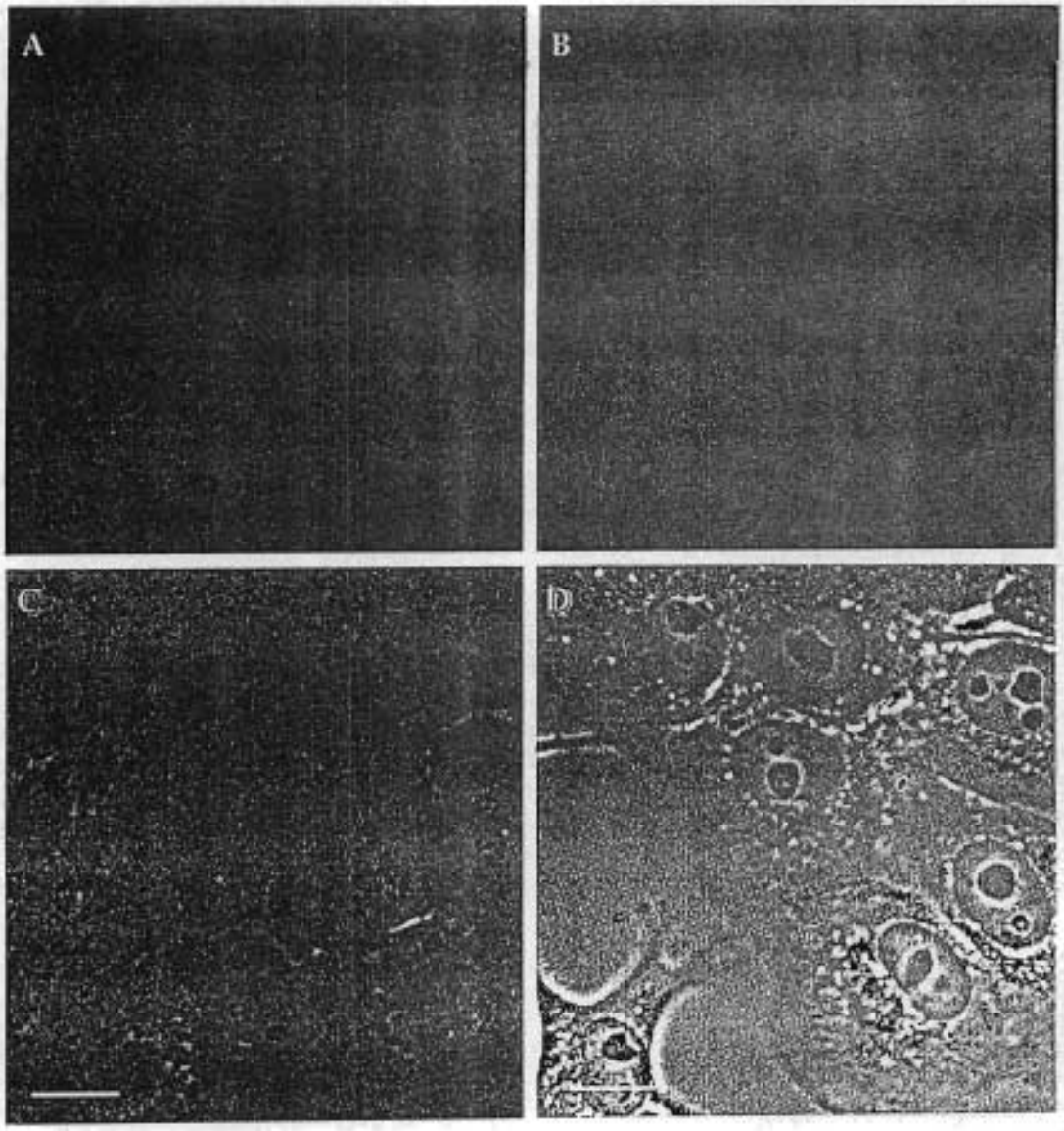


Figure 5. Immunocytochemical localization of cathepsin B on the surface of non-permeabilized MCF-10 human breast epithelial cells. Surface staining for cathepsin B was present in discrete regions on the basal surface of the cells transfected with mutated ras (B). Surface staining could not be visualized on the immortal 10A cells (A). The coverslips were mounted upside-down on slides. Thus, the labeling observed in panel B is underneath the cells. The primary antibody was rabbit anti-human cathepsin B IgG and the secondary antibody Texas red-conjugated donkey anti-rabbit IgG. The staining for cathepsin B has been repeated three times to date with comparable results. Only a weak background fluorescence was observed in controls in which rabbit pre-immune IgG replaced the primary antibody (not illustrated). Panels C and D are the phase contrast images corresponding to the fluorescence images of panels A and B, respectively. Bars, 10 μm.

cathepsin D to phagolysosomes primarily in immortal 10A cells and cathepsin B to phagolysosomes primarily in *ras*-transfected neoT cells. We also localized both cathepsins B and D to smaller peripheral vesicles and to surface membranes of the *ras*-transfected neoT cells. To our knowledge, the present study is the first to localize cathepsin D to the cell surface by immunogold techniques. Three lysosomal proteases have now been localized to the surface of malignant cells: cathepsin D to the surface of *ras*-transfected neoT cells by immunogold microscopy (present study); cathepsin B to the surface of 1) human lung adenocarcinoma cells by immunofluorescence microscopy,[9] 2) murine B16 amelanotic melanoma cells and *ras*-transfected neoT cells by immunofluoresence microscopy and flow cytometric analysis,[12] 3) *ras*-transfected neoT cells by immunogold microscopy[33] (present study), and 4) *ras*-transfected neoT cells and MCF-7 and BT20 human breast carcinoma cells by immunofluorescence microscopy (present study); and cathepsin L to the surface of human colon adenocarcinoma cells by immunofluores-

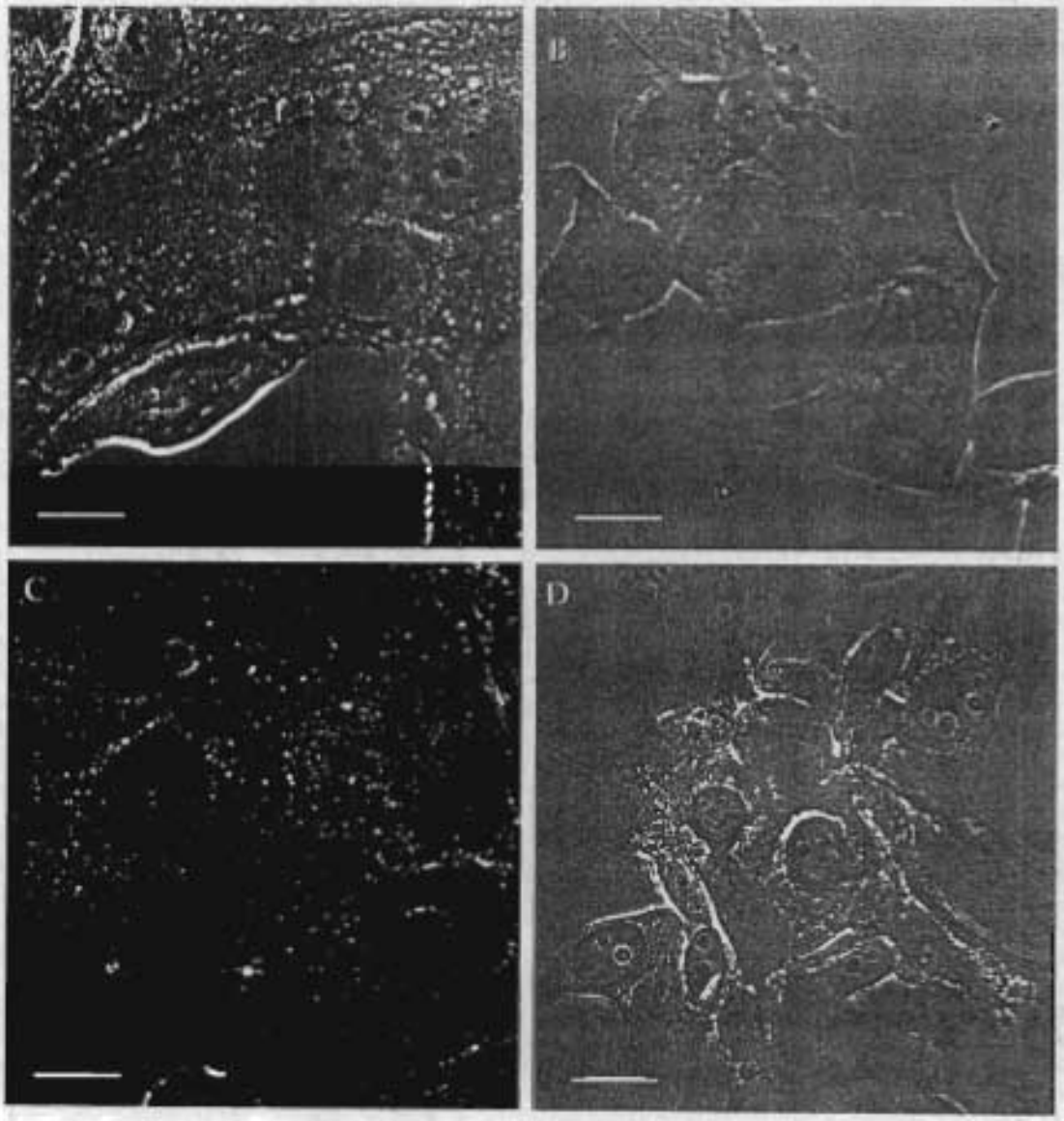


Figure 6. Immunocytochemical localization in MCF-7 (A, B) and BT20 (C, D) human breast carcinoma cells of intracellular cathepsin B (A and C) and cell surface cathepsin B (B and D). Intracellular cathepsin B staining in the breast carcinoma lines was present throughout the cytoplasm and at the cell periphery. Surface staining for cathepsin B was present on the basal surface of both cell lines (see legend to Fig. 5). The primary antibody was rabbit anti-human cathepsin B IgG and the secondary antibody Texas red-conjugated donkey anti-rabbit IgG. The staining for cathepsin B has been repeated three times to date with comparable results. Only a weak background fluorescence was observed in controls in which rabbit preimmune IgG replaced the primary antibody (not illustrated). Bars, 10 μm.

cence microscopy.[18] We do not yet know whether surface associated lysosomal proteases play a functional role in tumor progression. This possibility is suggested by the ability to induce concomitantly in malignant cells the surface expression of cathepsin B, the integrin $\alpha_{IIb}\beta_3$, and the autocrine motility factor receptor. These three proteins could mediate the three putative steps in tumor invasion: adhesion, local degradation and migration.[13] Furthermore, the localization of cathepsin B to discrete regions on the basal surface of *ras*-transfected human breast epithelial cells and breast carcinoma cells resembles the localization of proteases to the invadopodia described by Chen and colleagues,[22] a structure shown to be involved in cell adhesion, focal degradation and invasion.[24]

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Anti-cathepsin D chicken IgY antibodies: Characterisation, cross-species reactivity and application in immunogold labelling of human splenic neutrophils and fibroblasts

Philip H. Fortgens, Clive Dennison, Edith Elliott *

Department of Biochemistry, University of Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Hyperexpression, alteration of trafficking and secretion of cathepsin D has been linked with tumour invasion and inflammation. To study these phenomena in a variety of cells large quantities of anti-cathepsin D antibodies and the appropriate immunogen are required. As the human immunogen for studies on human tissue is less easily accessed, antibodies to both human and porcine cathepsin D were raised in chickens, as high levels of antibody may be recovered from egg yolks, and the potential cross-reactivity of the anti-porcine cathepsin D IgY antibody was assessed. This preparation cross-reacted strongly with human cathepsin D, comparing favourably with the reactivity of the chicken antibody to the human immunogen. The necessity for isolating human immunogen can thus be circumvented. The cross-species-reacting chicken IgY was successfully used to localise cathepsin D in immunogold labelling of human tissues. To our knowledge, IgY antibodies have not previously been used by other researchers for this purpose. Application of the cross-reacting antibody to human splenic neutrophils (PMNs) has confirmed the presence of cathepsin D in some granules. Double labelling has shown these to be novel subpopulations of azurophil granules. Cathepsin D may, therefore, be relevant in the invasive and inflammatory activities of PMNs and a target for therapeutic strategies.

Keywords: Chicken anti-cathepsin D IgY; Cross-species reactivity; Immunogold labelling; Neutrophils; Fibroblasts

1. Introduction

Our interest in cathepsin D, a lysosomal aspartic proteinase, centres on the possible common role that this enzyme may play in the invasion process of metastatic tumours (Liaudet et al., 1994; Rochefort, 1992) and in inflammatory leucocytes such as macrophages (Bever et al., 1989) and polymorphonuclear neutrophils (PMNs) (Ishikawa and Cimasoni, 1977; Barabasi and Nässberger, 1994; Elliott et al., 1995). We have elected to study the relevance of cathepsin D in this phenomenon, by screening a large variety of tumour and inflammatory tissues, using immunocytochemical techniques. For such a screening programme a primary requirement is a large quantity of anti-cathepsin D antibody, capable of strong, specific recognition of the enzyme in human tissue.

Human tissue, as a source material for immuno-

Abbreviations: ABTS, 2,2 azino-di-(3-ethyl)-benzthiazoline sulphonic acid; BSA-PBS, bovine serum albumin in PBS; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; PBS, phosphate buffered saline; PBS-Tween, Tween 20 in PBS; PEG, polyethylene glycol; PMN, polymorphonuclear neutrophil.

* Corresponding author. Tel.: +27-331-2605455; fax: +27-331-2605462; e-mail: elliott@sun1.cc.unp.ac.za.

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gen isolation, is problematic with respect to accessibility and disease risk. This provided the motivation for the present investigation of the cross-reactivity of anti-cathepsin D antibodies, raised against the enzyme isolated from animal tissues, with the human enzyme.

In this study antibodies against human and porcine cathepsins D raised in chickens (IgY) were compared with respect to their ability to react with human cathepsin D. The rationale of the latter combination was that the source (pig) and target (human) species are relatively close, in phylogenetic terms, which should favour antibody cross-reactivity, but are distant from the species (chicken) in which the antibodies were raised, which should favour immunogenicity. Chicken anti-porcine IgY was subsequently used to probe for cathepsin D in human spleen tissue, using immunogold. To our knowledge, IgY has not previously been used for immunogold labelling studies. An advantage of IgY antibodies is that milligram amounts of IgY can be isolated from each egg.

Cathepsin D has previously been demonstrated to occur in PMNs (Ishikawa and Cimasoni, 1977; Barabasi and Nässberger, 1994; Elliott et al., 1995) and fibroblasts (Mort et al., 1981). This study confirms the presence of cathepsin D in fibroblasts. It also describes double labelling studies of PMNs, using immunogold probes of different size, the anti-porcine cathepsin D IgY described in this paper, and a previously characterised anti-elastase antibody (Elliott et al., 1995). Labelling results suggest that cathepsin D occurs in subpopulations of azurophil granules. How such subpopulations may arise and the possible relevance of cathepsin D in the invasive activities of inflammatory PMNs is discussed.

2. Materials and methods

2.1. Cathepsin D purification

Cathepsin D was purified from human, porcine and bovine spleens as previously described (Jacobs et al., 1989).

2.2. Inoculation protocol

Purified human and porcine cathepsins D (100 μg) were each triturated with adjuvant and injected into the breast muscle of two laying hens (Polson et al., 1980). Freund's complete adjuvant (Difco) was used for the first immunisation and Freund's incomplete adjuvant (Difco) at 1, 2, 4, 6 and 10 weeks and for monthly boosters thereafter. Eggs were collected on a daily basis.

2.3. Isolation of IgY

Egg yolks were freed of adhering albumin, the yolk sac was punctured and its contents diluted in 2 volumes of 100 mM Na-phosphate buffer (pH 7.6) and precipitated with PEG-6000 (polyethylene glycol-6000), added to 3.5% (w/v). After centrifugation (4420 × g, 30 min, room temperature) the supernatant was filtered through a loose plug of cotton-wool and the concentration of PEG-6000 in the clear filtrate was increased to 12% (w/v). The precipitate was harvested by centrifugation (12000 × g, 10 min, room temperature) and redissolved in phosphate buffer (Polson et al., 1985; Rowland et al., 1986). An extinction coefficient of 1.25 ml/mg/cm at 280 nm was used to determine IgY concentration. Chicken antibodies were diluted 1:1 with glycerol and stored at −20°C. Exposure to low temperature often resulted in the formation of a lipid precipitate which could be filtered off, with no loss of antibody yield or titre.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The progress of inoculation or the ability of antibodies to cross-react with cathepsin D from different species was measured by ELISAs. Microtitre plate wells (Nunc Immunoplate) were coated overnight at room temperature with enzyme (2 μg/ml) in phosphate-buffered saline (PBS), pH 7.2. Wells were blocked with 0.5% (w/v) bovine serum albumin-PBS (BSA-PBS) (200 μl) for 1 h at 37°C and washed 3× with 0.1% (v/v) Tween 20 in PBS (PBS-Tween). Dilutions of the primary antibody in BSA-PBS (100 μl) were added, incubated at 37°C for 2 h and excess antibody washed out with PBS–Tween. A 1/750 dilution of rabbit anti-chicken IgY-horseradish peroxidase (120 μl) was added and incu-

bated at 37°C for 30 min. The ABTS substrate (0.05% (w/v) in 150 mM citrate-phosphate buffer, pH 5.0, containing 0.0015% (v/v) H_2O_2) (150 μl) was added and incubated for 15 min. The enzyme reaction was stopped by the addition of 0.1% (w/v) NaN_3 in citrate-phosphate buffer (50 μl) and the absorbance read at 405 nm in an ELISA plate reader.

2.5. Immunoblotting

Antibody specificity was ascertained by immunoblotting, essentially as previously described (Coetzer et al., 1991).

2.6. Immunogold labelling of human splenic tissues

Gold probes were produced by the method of Slot and Geuze (1985) and before use were diluted to an absorbance of between 0.05 and 0.1 at 520 nm. Samples of red pulp from a spleen provided by the Department of Surgery, University of Natal, were fixed (20 min) in a mixture of 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in 100 mM sodium cacodylate, pH 7.1, or in 8% (w/v) paraformaldehyde in 10 mM Na-phosphate, 150 mM NaCl, 2.5 mM KCl, pH 9.0. Tissue blocks (ca. 1 mm cubes) were infiltrated with 2.3 M sucrose and rapidly frozen in liquid nitrogen for cryoultrasectioning (Griffiths et al., 1983). Sections (approximately 100 nm thick) were cut using an RMC MT6000XL ultramicrotome fitted with a CR2000 cryoattachment. Sections were collected on 2.3 M sucrose, thawed and mounted on 100 mesh hexagonal copper grids, previously formvar- and carbon-coated and glow-discharged. Thawed, grid-mounted sections were collected on 20 mM glycine in PBS and labelled as described by Slot et al. (1991). Non-specific binding sites on the sections were blocked by incubation in 10% (v/v) foetal calf serum (FCS) and 20 mM glycine in PBS. For single labelling, incubation on primary antibody (226 μg/ml chicken anti-porcine cathepsin D IgY diluted in 5% (v/v) FCS in PBS, 1 h) was followed by incubation on a rabbit anti-chicken IgY linker antiserum (1/200 dilution in 5% FCS in PBS, 1 h). After washing, grids were incubated (30 min) on a goat anti-rabbit immunogold probe.

Double labelling was performed using the method of Slot et al. (1991). The first and linker antibodies (chicken anti-porcine cathepsin D IgY and rabbit anti-IgY antiserum, diluted as before) were detected using a 3 nm protein A-gold probe. A fixation step (1% glutaraldehyde in PBS, 5 min) was included before the blocking and labelling regime were repeated, this time with rabbit anti-human elastase (Athens Research and Technology, Athens, GA) diluted to 88 μg/ml and detected using a 10 nm protein A-gold probe. Grids were washed, counterstained and sealed in a uranyl acetate/methyl cellulose mixture (Griffiths et al., 1983). Sections were viewed and photographed in a Jeol 100CX transmission electron microscope, at 100 kV.

For controls for single labellings, pre-immune IgY antibodies were substituted into the labelling regime, at the same level of IgY as in the test. For double labelling, specificity was verified by the omission of primary and secondary antibodies and by labelling for the two different antigens in different orders, using detection with first the small and then the large gold labels (Slot et al., 1991).

3. Results

3.1. Chicken anti-human and anti-porcine cathepsin D IgY production

Immunisation of hens with human cathepsin D resulted in a peak of antibody production at 8 weeks and a plateau until 12 weeks (Fig. 1). Porcine cathepsin D elicited a rapid IgY response, with a

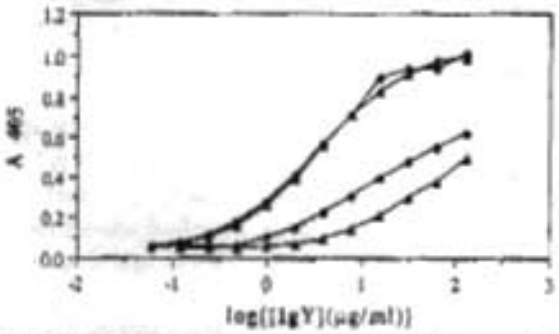

Fig. 1. ELISA of the progress of immunisation of a representative chicken inoculated with human cathepsin D. Human cathepsin D was coated at 2 μg/ml, incubated with serial two-fold dilutions of chicken anti-human cathepsin D and the microtitre plate developed as described in Section 2. Chicken IgY from week 4 (□), 8 (▲), 12 (×) and pre-immune IgY (○).

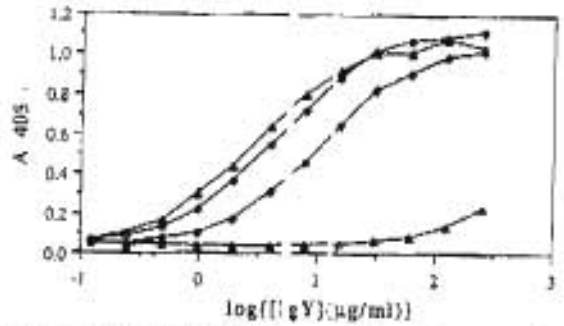

Fig. 2. ELISA of the progress of immunisation of a representative chicken inoculated with porcine cathepsin D. Porcine cathepsin D was coated at 2 µg/ml, incubated with serial two-fold dilutions of chicken anti-porcine cathepsin D and the plate developed as described in Section 2. Chicken IgY from week 4 (u), 8 (s), 12 (×) and pre-immune IgY (D).

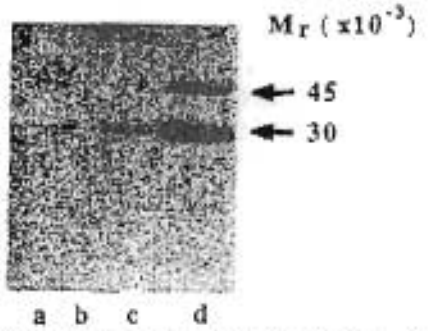

Fig. 3. Western blot of pure and crude fractions of porcine cathepsin D incubated with chicken anti-porcine cathepsin D. The blot was incubated in 20 µg/ml of immune IgY. Samples were: (a) crude supernatant; (b) acid supernatant; (c) TPP fraction and (d) purified porcine cathepsin D (Jacobs et al., 1989). The pre-immune control showed no targeting.

significant titre apparent after 4 weeks (Fig. 2). The titre peaked at 8 weeks, with a slight reduction at 12 weeks. Between 50 and 150 mg of IgY could be purified per yolk, depending on the yolk size and differences between individual chickens.

3.2. Specificity of chicken anti-porcine cathepsin D IgY

Western blot analyses of the antibody revealed high potency and specificity: the 45 kDa single-chain cathepsin D was targeted in the TPP fraction and the 30 kDa heavy chain of two-chain cathepsin D was recognised even in the most crude fraction (Fig. 3).

3.3. Homologous chicken anti-human cathepsin D reactivity compared to the cross-species reactivity of chicken anti-porcine cathepsin D

Human and bovine cathepsin D were strongly targeted by chicken anti-porcine cathepsin D IgY in an ELISA and only slightly more weakly than the porcine antigen, or the human antigen when probed with chicken anti-human cathepsin D IgY (Fig. 4).

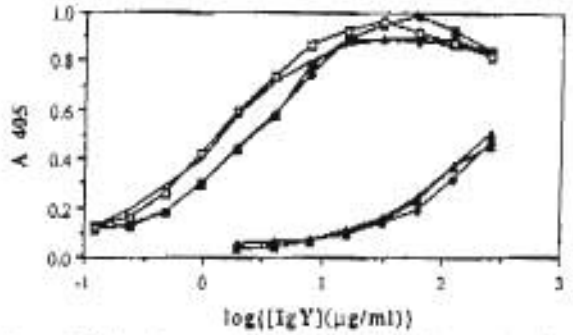

Fig. 4. ELISA of cross-species reactivity of chicken anti-porcine cathepsin D with bovine, porcine and human cathepsin D and a comparative reaction of chicken anti-human cathepsin D with human cathepsin D. The enzyme preparations were coated at 2 µg/ml, incubated with serial two-fold dilutions of antibody and the microtiter plate developed as described in Section 2. Cross-reaction of chicken anti-porcine cathepsin D IgY with bovine (n), porcine (z) and human cathepsin D (×); chicken anti-human cathepsin D with human cathepsin D (–) and pre-immune IgY with bovine (u), porcine (s) and human cathepsin D (D).

3.4. Immunogold labelling of human splenic tissues

Lysosome-like vesicles in many cells of the red pulp tissue labelled for cathepsin D, using the chicken anti-porcine cathepsin D IgY preparation. Some sub-populations of neutrophil granules (Fig. 5a) show

double labelling for cathepsin D and elastase, while fibroblasts (Fig. 5b) labelled for cathepsin D, show the highest labelling density. Labelling density is apparently not affected by the fixation regime used (compare Figs. 5 and 5(b)).

4. Discussion

Cathepsin D immunolocalisation studies based on the cross-species reactivity of antibodies, have been previously reported (Dean et al., 1986; Andujar et al., 1989; Saku et al., 1990) and the phylogenetic distance between mammals and chickens has also previously been exploited to generate high titre chicken antibodies (Vieira et al., 1984; Song et al., 1985; Stuart et al., 1988).

A previous extensive cross-species study of anti-cathepsin D antibodies (Weston and Poole, 1973) revealed no cross-reaction where the immunogen and antigen were from distantly related species, but complete identity among different bird species when the immunogen was chicken cathepsin D. These studies employed immunodiffusion techniques, which have now been superceded by the more sensitive ELISA and western blotting technologies used here. Nevertheless, the general conclusions are supported by the results of the present study.

In the present study, a rabbit anti-bovine cathepsin D antiserum targeted the human enzyme more weakly than the porcine enzyme, presumably because of the greater evolutionary distance of bovines from humans than porcines (result not shown). However, when the immunogen and antigen are from more closely related species, and phylogenetically distant from the inoculated species, there seems to be significant recognition of enzymes across species. Thus, chicken anti-porcine cathepsin D recognised both the bovine and human enzymes essentially to the same degree and similarly to the homologous reaction between chicken anti-human cathepsin D and human cathepsin D. Furthermore, the chicken anti-porcine cathepsin D IgY cross-reacts with mouse cathepsin D on western blots, and with rat cathepsin D in immunofluorescent labelling (unpublished results). Comparison of the amino acid sequence of the

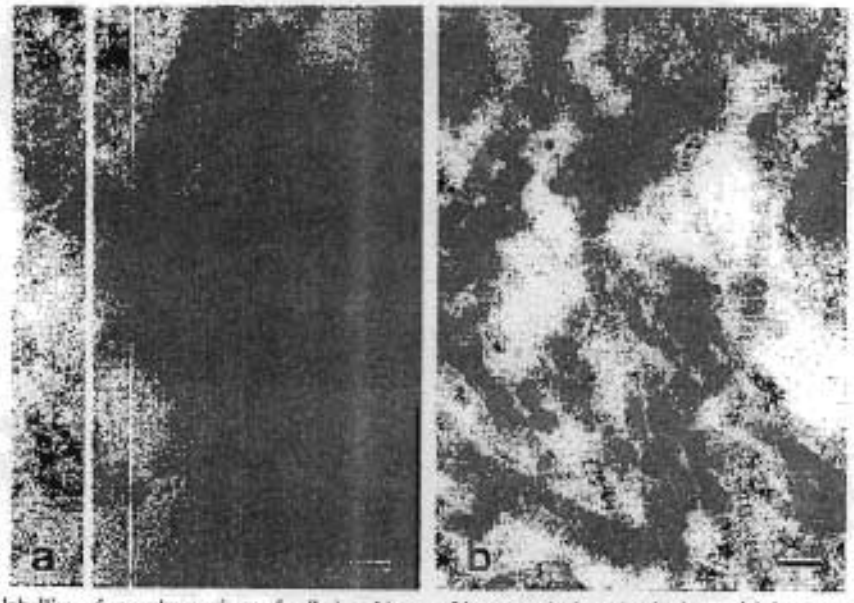

Fig. 5. Immunogold labelling of cryoultrasections of cells in a biopsy of human splenic red pulp tissue. (a) Section of a human neutrophil showing double labelling of an azurophil granule for cathepsin D (3 nm particle) and elastase (10 nm particle). Tissue fixed using 8% paraformaldehyde at pH 9.0, 20 min. Original magnification 50,000×. (b) High magnification micrograph of collagen fibres and a lysosome-like vesicle in a splenic fibroblast labelled for cathepsin D (10 nm particle). Tissue was fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde at pH 7.0, 20 min. Original magnification 66,000×. All controls (not shown) were satisfactory, indicating labelling specificity. Bar scale: 0.1 μm.

human enzyme with those of other species, are consistent with these results, with 87% sequence homology obtaining between the human and porcine cathepsins D (Faust et al. 1985) 81% homology with mouse cathepsin D (Gresby et al., 1990) and 83% with rat cathepsin D (Faarch and Lok, 1990). The sequence of bovine cathepsin D has not been published.

Cathepsin D has previously been reported in fibroblasts (Mort et al., 1981) and PMNs (Ishikawa and Cimasoni, 1977; Barrabasi and Nässberger, 1994; Elliott et al., 1995) and is often used as a marker enzyme for the lysosomes of the cell (Araki et al., 1995). The organelles immunolabelled in the collagen-secreting fibroblast are, by this definition, lysosomal compartments. In the neutrophil, however, the double labelling, using the anti-porcine cathepsin D IgY and anti-elastase IgG, reported here, has provided the first indication of the identity of the cathepsin D-containing granule.

Initially, PMN granules were classified into two groups, the larger 'primary' or 'azurophil', myeloperoxidase-containing granules and the smaller 'secondary' (specific) lactoferrin-containing granules (Bretz and Baggiolini, 1974). Sub-populations of azurophil granules, differing in levels of myeloperoxidase and elastase (Damiano et al., 1988; Pryzwansky and Breton-Gorius, 1985) and specific granules, varying in their gelatinase content (Hibbs and Bainton, 1989), have since been discovered, however. We have used elastase as a marker enzyme for a subpopulation of the azurophil granule.

We have previously shown that cathepsin D is present in early myeloid cells (unpublished data) and using an anti-human cathepsin D antibody, we have also reported that cathepsin D-labelled granules appear to decrease, relative to the total number of other granules, with maturation of PMNs (Elliott et al., 1995). In this study, we show that these cathepsin D-labelled granules are subpopulations of the elastase-labelled subpopulation of the azurophil granules. As only some populations of elastase-labelled granules also labelled for cathepsin D and cathepsin D labelled granules seem to decrease with cell maturation, we also suggest that cathepsin D expression may slowly decrease as myeloid differentiation progresses and elastase expression increases. The subpopulation of cathepsin D-containing azurophil granules may, therefore, arise at some early period when the first population of azurophil granules is synthesised during the early promyelocytic-PMN differentiation phase. At such a time, cathepsin D expression may be diminishing as elastase expression is increasing. The overlapping synthesis of these marker enzymes for these different states of differentiation may, therefore, co-exist in these early granules.

The existence of other subpopulations of the different granule types would suggest that this may be a phenomenon common to all granule types. Our preliminary data supports such a hypothesis, but further quantitative data and cell maturation studies are required to substantiate these results. It is important to identify the different enzymes present in various granule populations and hence classify the populations and to study the order of their release in response to various stimuli in order to devise therapeutic strategies for the control of PMN infiltration and hence some inflammatory conditions. As neutrophils and cancer cells may invade via a common mechanism, involving proteolysis, the enzyme involved may be a commonly expressed enzyme such as cathepsin D and this enzyme may, therefore, constitute a target for therapeutic intervention. This manuscript reports the first of the multiple labelling experiments which will be utilised to classify the various subpopulations of PMN granules and an additional cross-species-reacting antibody that will be used in further studies.

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