THE ROLE OF THE KALLIKREIN-KININ SYSTEM IN PROSTATE AND BREAST TUMOURIGENESIS AND TUMOUR-ASSOCIATED ANGIOGENESIS

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DECLARATION

This project represents original work done by the author. It has not been submitted in any other form, part or content to the University of KwaZulu-Natal or to any other university. Where others have made contributions, it has been duly acknowledged in the text.

All of the research described in this dissertation was performed in the Department of Therapeutics and Medicines Management, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Doctor S. Naidoo and co-supervision of Professor J. H. Botha.

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We hereby do certify that the above statements, to the best of our knowledge, are true.

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ABSTRACT

Introduction

The kallikrein-kinin system (KKS) is a protein system in which the serine proteases, tissue kallikrein (TK) and plasma kallikrein (PK), cleave kininogen substrate to release the kinins, kallidin and bradykinin, respectively. Kinins act on bradykinin receptor subtype 1 (B1R) and bradykinin receptor subtype 2 (B2R) to induce vasodilation, increase vascular permeability, promote cell proliferation, regulate cell migration and upregulate proteolytic activity. TK may also cleave and activate matrix metalloproteinases, thereby enhancing proteolytic activity.

The KKS has been shown to play an important role in inflammation and angiogenesis. These processes are vital to the development of cancers, thereby implicating the KKS in cancer pathology. There is evidence that the KKS specifically contributes to the development of prostate and breast cancer. Previous studies have shown involvement of the KKS in the proliferation, migration and invasion of prostate and breast tumour cells, as well in the growth of prostate tumours *in vivo* possibly, in part, via promotion of angiogenesis. In some of these studies, hormone-independent prostate and breast tumour cells were used, thereby implicating the KKS in the progression of such cancers, for which therapy is currently inadequate. Thus, the present study aimed to further elucidate the role of the KKS in prostate and breast tumour progression with particular reference to angiogenesis.

Materials and Methods

Dermal microvascular endothelial cells (dMVECs), DU145 prostate tumour cells and MCF7 breast tumour cells were grown in culture. An *in vitro* challenge model was set up whereby these endothelial and tumour cells were challenged with conditioned medium from tumour and endothelial cells, respectively, at concentrations of 10%, 25% and 50%. The expression of the KKS proteins, TK, B1R and B2R, was then investigated in cells of the challenge

model, while secretion of TK into the surrounding media was also measured. The KKS proteins were localised in unchallenged and challenged cells using the avidin-biotin complex (ABC) immuno-detection system with peroxidase-diaminobenzidine (DAB) as the enzyme-substrate combination. This was followed by quantification of the intensity and extent of KKS staining, using image analysis, to determine the effect of challenge metabolites on intracellular KKS levels. The effect of challenge metabolites on TK secretion was analysed by performing a TK enzyme-linked immunoassay (ELISA) on media collected from unchallenged and challenged cells. Additionally, the effect of challenge metabolites on cell proliferation in culture was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. An endothelial-tumour cell co-culture model was also set up, in which the morphology of cell interactions was observed and in which KKS proteins and cell-specific marker proteins were co-localised using ABC-DAB and alkaline phosphatase-fast red immuno-detection systems.

Results

TK, B1R and B2R were immuno-localised in the cytoplasm, membranes and projections of unchallenged endothelial cells, as well as in the same areas of those endothelial cells challenged by tumour cell metabolites. Such localisation was also evident in small projections of endothelial cells that were involved in initiating endothelial cell-cell contact. There was a significant decrease in intracellular TK in endothelial cells challenged with both types of tumour cell metabolites, and this effect was greater as the challenge concentration increased. Immuno-reactive B1R and B2R levels in endothelial cells were not significantly affected by exposure to tumour metabolites. TK was undetectable in the medium of unchallenged endothelial cells. However, when these cells were challenged with increasing concentrations of tumour metabolites, this protein was detected in increasing amounts. Similarly, endothelial cell proliferation significantly increased with increasing challenge concentrations.

TK, B1R and B2R were immuno-localised in the cytoplasm and projections of prostate and breast tumour cells. While TK, B1R and B2R levels in breast tumour cells were not significantly affected by exposure to endothelial metabolites, in challenged prostate tumour cells there was an overall decrease in intracellular TK, B1R and B2R. Both unchallenged prostate and breast tumour cells secreted TK into their respective media and this activity was not significantly influenced by exposure to endothelial metabolites. Similarly, the proliferation of challenged tumour cells did not differ significantly from that of unchallenged counterparts.

In co-cultures, tumour cells formed footplate-like connections with endothelial cells and occasionally endothelial cell projections formed contact with tumour cells. TK, B1R and B2R were immuno-localised in both endothelial and tumour cells at these points of interaction. In the presence of tumour cells, endothelial cells tended to increasingly connect with one another to form chain-like or circular structures.

Discussion

This study showed that KKS proteins were present in, and TK was secreted by, prostate and breast tumour cells. In addition, the metabolites of both these tumour types increased the secretion of TK from endothelial cells, while there were corresponding decreases in intracellular TK. These findings point to an upregulation of proteolytic activity and kinin generation in prostate and breast tumour environments. Upregulation of these activities may promote tumour-associated angiogenesis by stimulating or facilitating endothelial cell proliferation, migration and invasion of the extracellular matrix (ECM) and capillary tube formation. The role of the KKS in these steps of the angiogenic cascade is additionally supported by the results of the present study. The concomitant increase in TK secretion from, and proliferation of, endothelial cells that were challenged with tumour metabolites, suggests possible involvement of the KKS in endothelial cell proliferation. Further, the presence of the KKS in large projections (i.e. the leading edge of movement) of endothelial

cells may indicate a role for the KKS in endothelial cell migration and invasion of the ECM. The KKS proteins were also localised in small projections of endothelial cells that were involved in initiating endothelial cell-cell contact, which is required for capillary tube formation. Thus, an upregulation of TK in prostate and breast tumour environments may promote prostate and breast tumourigenesis via stimulating angiogenesis in these tumours. Further supporting this hypothesis was the presence of the KKS in tumour-endothelial cell points of contact as well as the fact that, when these two types of cells were cultured together, the endothelial cells formed primitive structures suggestive of angiogenesis.

Upregulation of proteolytic activity and kinin generation in the tumour environment may also enhance tumourigenesis via mechanisms other than the upregulation of angiogenesis. Proteolytic activity and kinins have been shown to promote prostate and breast tumour cell proliferation, migration and invasion. The role of the KKS in migration and invasion of these tumour cells is supported by the localisation of the KKS proteins in tumour cell projections in the present study. Further, since kinin signalling may potentiate that of growth factors important in the development of hormone-independent prostate and breast cancer, increased kinin generation in these tumour environments, as suggested by the present study, supports a role for the KKS in the development of hormone independence. Another consequence of increased kinin generation in prostate and breast tumour environments would be enhancement of inflammation in the stroma of these tumours, which would then additionally contribute to tumour progression.

Conclusion

The study supports an important role for the KKS in prostate and breast tumourigenesis and tumour-associated angiogenesis and there is, as a result, a possibility that KKS antagonists may be useful in the treatment of these cancers, including advanced, hormone-independent forms of these cancers.

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ABBREVIATIONS

°C, degrees Celsius

μg, microgram

μl, microlitre

μm, micrometer

μM, micromolar

2D, 2-dimensional

3D, 3-dimensional

ABC, Avidin-Biotin Complex

aFGF, acidic fibroblast growth factor

AI, androgen independent

ang, angiopoetin

ANOVA, analysis of variance

AR, androgen receptor

AREs, androgen response elements

B1R, bradykinin receptor subtype 1

B2R, bradykinin receptor subtype 2

bFGF, basic fibroblast growth factor

BK, bradykinin

BRC, Biomedical Resource Centre

BSA, bovine serum albumin

CAM, cell adhesion molecule

cAMP, cyclic adenosine monophosphate

cCAM, chick chorioallantoic membrane

CD40L, CD40 ligand

cDNA, complementary deoxyribonucleic acid

CK, cytokeratin

cm^2, centimetres squared

CMI, cell-mediated immunity

CO_2, carbon dioxide

COX, cyclo-oxygenase

DAB, diaminobenzidine

ddH_2O, distilled, deionised water

DMEM, Dulbecco's modification of Eagle's minimum essential medium

DMSO, dimethylsulfoxide

dMVEC, dermal microvascular endothelial cell

DNA, deoxyribonucleic acid

EBM, endothelial basal medium

ECM, extracellular matrix

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

ELISA, enzyme-linked immunoassay

EMEM, Eagle's minimum essential medium

ERK, extracellular signal-regulated kinase

FAK, focal adhesion kinase

FBS, foetal bovine serum

FGFR, fibroblast growth factor receptor

Flk-1, vascular endothelial growth factor receptor 2

Flt-1, vascular endothelial growth factor receptor 1

g, gram

GCSF, granulocyte colony stimulating factor

GMCSF, granulocyte monocyte colony stimulating factor

H&E, haematoxylin and eosin

H_2O_2, hydrogen peroxide

HBSS, Hank's balanced salt solution

HCl, hydrochloric acid

hEGF, human endothelial growth factor

HeLa, human cervical carcinoma

HER2, human epidermal growth factor receptor-2

HIF, hypoxia inducible factor

hK, tissue kallikrein proteins

HMWK, high molecular weight kininogen

HREs, hormone response elements

HUK, human urinary kallikrein

HUVEC, human umbilical endothelial cell

ICAM, intercellular adhesion molecule

Ig, immunoglobulin

IGF, insulin-like growth factor

IGF-R1, insulin-like growth factor receptor type 1

IgG, immunoglobulin G

IL, interleukin

INF-γ, interferon-gamma

KI-CPM, kininase family I carboxypeptidase M

KI-CPN, kininase family I carboxypeptidase N

KII-ACE, kininase family II angiotensin I-converting enzyme

KII-NEP, kininase family II neutral endopeptidase

KKS, kallikrein-kinin system

KLK, genes coding for tissue kallikrein proteins

L, litre

LMWK, low molecular weight kininogen

lys-BK, kallidin

M, molar

MAPK, mitogen activated kinase

$MgCl_2$, magnesium chloride

MIF, macrophage inhibitory factor

ml, millilitre

mM, millimolar

mm^3, millimetres cubed

MMPs, matrix metalloproteinases

mRNA, messenger ribonucleic acid

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MVECs, microvascular endothelial cells

Na_2CO_3, sodium carbonate

NaCl, sodium chloride

$NaHCO_3$, sodium hydrogen carbonate

NF-κβ, nuclear factor of the κ-enhancer in B cells

ng, nanogram

nm, nanometre

NO, nitric oxide

NOS, nitric oxide synthase

$OH^{\cdot}$, hydroxyl radicals

OI, oestrogen independent

$ONOO^{-}$, peroxynitrite

ONOOH, peroxynitrous acid

OR, oestrogen receptor

ORE, oestrogen response element

PAI, plasminogen activator inhibitor

PAP, prostatic acid phosphatase

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PDGF, platelet-derived growth factor

PI3K, phosphoinositide 3-kinase

PlGF, placenta growth factor

PK, plasma kallikrein

PKC, protein kinase C

PLC, phospholipase C

pNPP, disodium p-nitrophenyl phosphate

proTK, human urinary prokallikrein

PSA, prostate specific antigen

PSF, penicillin-streptomycin-fungizone

rhFGF-β, recombinant human fibroblast growth factor beta

RhoGTPases, the Rho family of guanine triphosphatases

RICs, region of interest circles

RNIs, reactive nitrogen intermediates

ROIs, reactive oxygen intermediates

rpm, revolutions per minute

RT-PCR, reverse transcriptase polymerase chain reaction

scuPA, single chain proenzyme of urokinase plasminogen activator

serpins, serine protease inhibitors

SF, scatter factor

TBS, Tris-buffered saline

TGF-α, transforming growth factor alpha

TGF-β, transforming growth factor beta

Th2, T helper 2 cell

TIFF, tagged image format files

TIMPs, tissue inhibitors of matrix metalloproteinases

TK, tissue kallikrein

TNF, tumour necrosis factor

tPA, tissue plasminogen activator

TSP, thrombospondin

uPA, urokinase plasminogen activator

uPAR, urokinase plasminogen activator receptor

UV, ultraviolet

v/v, volume/volume ratio

VCAM, vascular cell adhesion molecule

VEGF, vascular endothelial growth factor

VEGFR, vascular endothelial growth factor receptor

VPF, vascular permeability factor

VWF, von Willebrand's factor

CHAPTER 1

INTRODUCTION

CHAPTER 1 – INTRODUCTION

1.1 Cancer, angiogenesis and the kallikrein-kinin system

1.1.1 Cancer

1.1.1.1 Historical background and definition of cancer

Some of the first references to cancer can be found in Egyptian papyri that were written between 1500-3000 BC (*1, 2*). Since then, ideas of what cancer is and what causes cancer have been changing and developing. The term 'cancer' originated when Hippocrates (460-370 BC) likened the appearance of a breast tumour to that of a crab, the Latin word for which is *cancer* (*3, 4*). Hippocrates thought that cancer was caused by an excess of black bile, which was one of the 4 body humours described at the time – blood, phlegm, yellow bile and black bile (*1*). By the 17th century this theory was no longer accepted and the focus shifted to lymph as the source of cancer (*3, 4*). Following the discovery of cells by Robert Hooke in 1665 and the development of the concept of tissues by Xavier Bichat (1771-1802), it was thought that cancer was derived from cellular tissue (*1, 4*) and this was subsequently established by Johannes Mueller (1801-1858) (*3*). A pupil of Xavier Bichat, René Laennec (1781-1826), first distinguished inflammatory conditions from that of true tumours (*4*). However, the association between inflammation and cancer (first realised by the ancient Greeks) continued to be recognised (*4*) and is still acknowledged today (Section 1.1.1.3). In the nineteenth century, Rudolph Virchow established that each cell arises from the growth and division of other cells (*1, 3*) and he suggested that tumours develop from immature cells in connective tissue (*5*). Remak (1815-1865) extended Virchow's theory by dividing tumours into two classes *viz.* those that arise from epithelial cells (carcinomas; the majority of tumours) and those that arise from mesenchymal/non-epithelial tissue (sarcomas) (*5, 6*). Next, Wilhelm Waldeyer (1836-1921) significantly contributed to our current understanding of cancer by (i) suggesting that tumours arise from cells transformed by external factors, and (ii) establishing that tumour cells metastasise via lymphatic and blood vessels (*4*).

At present, two features define cancer *viz.* (i) unregulated proliferation of cells to form a tumour and (ii) the ability of these cells to invade tissues outside of the original compartment of growth and metastasise to organs distant from the site of origin (*7*). The latter characteristic distinguishes a malignant tumour from a benign tumour (*7*).

1.1.1.2 Tumour initiation, progression and metastasis

The development of a malignant tumour occurs in stages. These stages, using epithelial tissue as an example, are typically: hyperplasia, dysplasia, *in situ* carcinoma and invasive carcinoma. The triggering of this process and the progression from one stage to the next, finally resulting in the malignant phenotype, is the result of genetic mutations in cells. Mutations may be as a result of external factors, such as chemicals, viruses and radiation, or endogenous factors, such as inherited mutations and errors of DNA replication (*7, 8*).

Hyperplasia, an increase in the number of cells, is due to mutations that convert proto-oncogenes to active oncogenes (*8*). Proto-oncogenes usually code for proteins that promote initiation of, and progression through, the cell cycle. These include growth factors or their signalling proteins, transcription factors and cyclins (*7, 9*). Proto-oncogenes become oncogenic when mutations cause their upregulated expression or the expression of an active form of the encoded protein in the absence of its activators. For example, the *erb-b* oncogene encodes a form of epidermal growth factor (EGF) receptor that is activated in the absence of EGF (*9*).

The mutation of proto-oncogenes to oncogenes is not, however, sufficient for tumour progression. This is due to protective cell cycle G_1/S and G_2/M checkpoints that halt progression of the cell cycle from the G_1 to S phase and G_2 to M phase, respectively, when DNA is damaged/mutated. If the damage is too severe to be repaired, apoptosis or

programmed cell death is induced. Mutations in tumour suppressor proteins, that are usually checkpoint proteins, DNA repair proteins or apoptotic regulators, allow for the proliferation and accumulation of cells with oncogenic mutations (*7*). For example, more than 50 % of human cancers have anti-apoptotic mutations in the checkpoint protein p53 (*8*). Accumulation of anti-apoptotic mutations in tumours negatively affects the effectiveness of conventional cancer therapies (*10*).

Increasing dysregulation of cell growth, which results in continued proliferation of cells, prohibits differentiation and results in the buildup of undifferentiated cells (*11*). Mutations may also accumulate in genes that control differentiation, such as those that control cell-cell and cell-extracellular matrix (ECM) interactions (*11*). As these mutations accumulate, the abnormal mass of cells progresses from the hyperplasia to dysplasia stage, in which tumour cells appear different from cells of the surrounding tissue. They are undifferentiated, have a disorganised arrangement and exhibit morphologic changes in their nuclei (*12*). With further mutations, the growth and appearance of tumour cells becomes increasingly abnormal and the tumour acquires invasive capacity. At this stage, if the tumour has not yet grown beyond the borders of its original compartment, it may be classified as an *in situ* carcinoma (*8*).

Several mutations are required before a tumour cell has the capacity to invade tissues outside of the original compartment of growth and metastasise to sites distant from the site of origin. In order to acquire the malignant phenotype, a tumour cell must progressively acquire mutations that sequentially allow it to (i) detach from the solid tumour, (ii) escape anoikis (apoptosis triggered by the loss of cell adhesion to the extracellular matrix [ECM]), (iii) invade and migrate within the ECM, (iv) enter the circulation, (v) escape immune surveillance in the circulation, (vi) adhere to endothelial cells and exit lymph or blood vessels into the tissue of the target organ (*13*). These are discussed in detail below.

(i) Decreased cell-cell adhesion not only results in loss of differentiation but also allows for detachment of cells from the tumour. E-cadherin is the main molecule involved in cell-cell adhesion (*14*) (Section 1.1.2.6.2). Mutations in E-cadherin have been demonstrated in human carcinomas, including breast and prostate cancers, and are associated with invasiveness in these cancers (*15-17*). Disruption of cell-cell contacts may also be achieved by proteolysis of cell adhesion systems by matrix metalloproteinases (MMPs) (*18*) and the urokinase plasminogen activation (uPA) system, which are upregulated in cancer (*19, 20*) (Section 1.1.3.1 and 1.1.3.2).

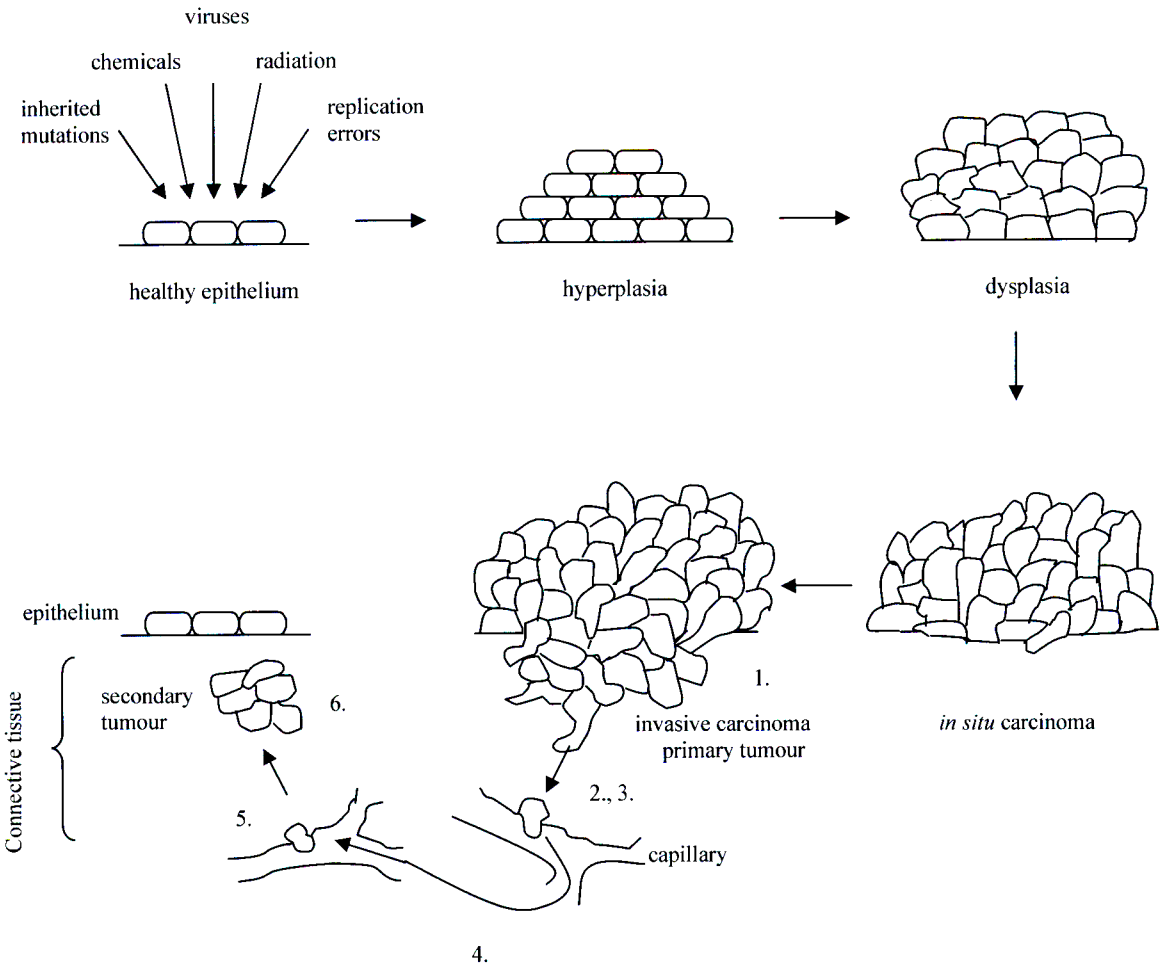
(ii; iii) Invasion of the ECM also requires disruption of cell-ECM adhesions (*21*) (Section 1.1.2.6.1). Integrins are mostly responsible for adhesion of cells to the ECM and disruption of this adhesion usually leads to apoptosis/anoikis (*22*) (Section 1.1.2.6). Cancer cells, including those of breast and prostate, frequently have altered integrin composition and function to allow decreased substrate attachment and survival (*23-25*). Certain integrins, such as $\alpha 6\beta 4$ and $\alpha 5\beta 1$, promote survival of carcinoma cells and may be upregulated by tumour cells (*25-27*). Migration within the ECM also requires removal of ECM barriers to invasion, which is achieved by proteolysis (*28*). Matrix metalloproteinases and the uPA system are important for breast cancer invasiveness (*29*) and MMPs enable invasion of basement membrane by prostate epithelial tumour cells (*30*). Mobility of tumour cells may be promoted by proteases (*31*) (Section 1.1.3.1, 1.1.3.2 and 1.1.3.3.2.2.3) or mutations that upregulate activity of mobility factors. For example, receptors for scatter factor (SF) and autocrine mobility factor are dysregulated in prostate cancer to facilitate mobility of cells (*32, 33*).

(iv) Tumour cells may overexpress vascular endothelial growth factor-C (VEGF-C), which increases the diameter of lymphatic vessels, thereby increasing the surface area for

metastasis (*34*). The structure of tumour vessels that arise from angiogenesis (may be induced by pro-angiogenic mutations) also facilitates extravasation (*35*) (Section 1.1.2.1).

(v) Mutations that increase sialylation of carbohydrates on the tumour cell membrane promote immunologic escape in the circulation (*13*) and increase the binding of circulating tumour cells to endothelial cells (*36*). Downregulated expression of class I major histocompatibility complex, which is involved in antigen presentation, has been identified in prostate cancer (*37*).

(vi) Lastly, tumour cells must further acquire the ability to disrupt endothelial intracellular adhesions in order to exit the circulation into tissues where they may proliferate to form a new tumour. Sandig *et al.* (1997) demonstrated the disappearance of VE-cadherin in endothelial cells in contact with melanoma cells, the intercalation of tumour cells between these endothelial cells and the re-forming of these adhesions once the tumour cells had passed through the endothelium (*38*). Induction of angiogenesis is also required for tumour cells to proliferate in the target tissue (*39*) (Section 1.1.2.1). Once the tumour has invaded the surrounding connective tissue it is classified as an invasive carcinoma (*8*). Refer to Figure 1.1 for a diagrammatic representation of the stages of tumourigenesis.



metastasis

1. invasion of basement membrane and ECM
2. cell detachment from tumour, escape of anoikis
3. intravasation
4. escape of immune surveillance in circulation
5. adhesion to endothelial cells, extravasation
6. establishment of secondary tumour

Factors involved in tumourigenesis

- Growth factors and cell cycle regulators
- Fibroblasts
- Inflammatory cells
- ECM fibres
- Proteases
- Cell adhesions
- Blood vessels

1.1.1.3 The role of the tumour microenvironment and inflammation in tumour progression and metastasis

Solid tumours may be described as having two compartments *viz.* parenchyma (tumour cells) and stroma. Stroma consists of non-cellular ECM components, fibroblasts, inflammatory cells (present as an inflammatory reaction to tumour cells) and blood vessels. Tumour cells induce stroma formation by increasing vascular permeability. This is usually mediated by VEGF, which is secreted by the majority of human tumours. Leakage of plasma results in extravascular clotting and, consequently, the formation of a fibrin network that attracts fibroblasts (a major source of non-cellular ECM) and forms a framework for the development of new blood vessels by angiogenesis (*12*). The crosslinked fibrin formed is chemotactic for inflammatory cells (*31*) and the clotting cascade also results in production of pro-inflammatory products (*40*), resulting in infiltration of inflammatory cells. The stroma plays a vital role in tumour progression (*41-43*). A discussion of the role of these stromal factors, listed in Figure 1.1, in tumourigenesis follows.

Proteases are required for tumour metastasis as described in Section 1.1.1.2. In some cancers, such as breast ductal carcinoma, the source of these proteases is mainly the stromal cells, namely fibroblasts, macrophages and pericytes, rather than the tumour cells (*41*). Experiments performed with transplanted MMP-2 expressing Lewis lung carcinoma cells resulted in reduction in lung metastasis in MMP-2 deficient mice when compared with normal mice, thereby demonstrating the importance of stromally-generated proteases in cancer (*44*).

As discussed in Section 1.1.1.2, the modulation of cell-ECM adhesions occurs with tumour progression and is necessary for metastasis. Modification of the non-cellular ECM and the effect this has on cell behaviour (refer to Section 1.1.2.5) also contributes to

tumourigenesis (*45*). Mutations in genes encoding ECM components are associated with the development of certain types of tumours, such as smooth muscle tumours (*46*). The ECM in conjunction with proteases also regulates growth factor availability (*31, 47, 48*) (Section 1.1.2.5) and growth factors contribute to tumour progression (*49*) (Section 1.1.3.3.2.6.2).

The inflammatory reaction present in the tumour stroma provides a favourable environment for tumour progression (*31, 42, 43*). O'Byrne and Dalgleish (2001) describe the nature of this inflammatory environment as chronic (*43*). If it precedes tumour formation, the risk of random mutations and tumour formation is increased [due to increased reactive oxygen species and a tolerant environment for tumour progression; (*43*)]. Supporting this are the links observed between schistosomiasis and bladder cancer, *Helicobacter pylori* and stomach cancer and fibrosis of the breast and breast cancer (*50-52*). Whether or not a chronic inflammatory type of environment is present prior to tumourigenesis, tumour cells may interact with their environment to induce this favourable state (*42*), which O'Byrne and Dalgleish (2001) argue is necessary for tumour progression (*43*).

An environment of chronic inflammation facilitates tumour progression through decreasing immune surveillance, promoting cell proliferation, decreasing apoptosis and promoting angiogenesis (*43*). In an environment of chronic inflammation, T helper 2 cell (Th2) cytokines predominate over Th1 cytokines (*43*). An initial response is Th1 predominant i.e. T helper 1 cells produce proinflammatory cytokines (*43*). These cytokines upregulate cell mediated immunity (CMI), which is mainly responsible for host attack on tumour cells (*43*). However, Th1 cytokines also induce expression of cyclo-oxygenase-2 (COX-2) by epithelial, mesenchymal and inflammatory cells (*53*). Cyclo-oxygenase-2 results in the upregulation of Th2 cytokines, which then suppress Th1 cytokine production and therefore CMI (*43*). In a Th2-predominant environment, growth factors that promote cell proliferation

are upregulated e.g. insulin-like growth factor 1 (IGF-1) (*43*). Anti-apoptotic factors are also upregulated e.g. macrophage inhibitory factor (MIF) is expressed which represses transcription of pro-apoptotic p53 (*54*). The Th2-predominant environment is also pro-angiogenic as Th2 cytokines stimulate macrophages to release factors that promote vascular endothelial growth (*55*). Tumour cells may induce this environment through constitutive upregulation of COX-2, which has been demonstrated in a wide range of premalignant lesions and tumours (*43*). Supporting the role of chronic inflammation in tumourigenesis are studies that show inhibition of tumour growth by specific COX-2 inhibitors (*53, 56*).

The contribution of cellular and non-cellular ECM components and cell adhesions to blood vessel growth by angiogenesis is described in Sections 1.1.2.4-1.1.2.6, and the role of the blood vessel stromal component in tumourigenesis follows.

1.1.2 Angiogenesis

1.1.2.1 The role of angiogenesis in tumour progression and metastasis

In 1971, Folkman proposed that, in the absence of neovascularisation, primary tumours and metastases cannot expand beyond 1-2 mm^3 (*57*). Since then, considerable evidence has amounted to prove his theory and it has been demonstrated that an increased blood supply, achieved through neovascularisation, is required to provide adequate oxygen and nutrients as well as waste removal in a growing tumour (*39*).

Mechanisms of blood vessel growth include arteriogenesis, vasculogenesis and angiogenesis. Arteriogenesis is a process whereby vessels enlarge and become capable of bypassing occluded vascular segments (*58*). Vasculogenesis involves the differentiation of angioblasts into endothelial cells that then assemble into a vascular network and may occur in both the embryo and the adult (*59*). Angiogenesis may be defined as the sprouting of new capillary

blood vessels from pre-existing postcapillary venules (*39, 58*) and remains the primary mechanism by which new blood vessels arise in the adult (*59*).

In tumours, more than one mechanism of blood vessel growth occurs. While it is not known whether arteriogenesis contributes to the development of a tumour's blood supply (*60*), in some tumours, such as aggressive primary or metastatic cutaneous melanomas, a process similar to vasculogenesis occurs. In these tumours, the tumour cells themselves form a microvascular network. Thus, as in vasculogenesis, blood vessels are formed *de novo* by this mechanism. However, this is not true vasculogenesis, as endothelial cells do not form part of the newly formed blood vessels, hence the term "vasculogenic mimicry" (*61*). Vasculogenic mimicry explains the lack of angiogenesis in melanomas (*59*). Another vasculogenesis-like process is the recruitment of circulating endothelial progenitor cells to tumours with subsequent differentiation in the stroma and incorporation into new blood vessels (*62*). However, this process is angiogenesis-dependent as it relies on angiogenesis-promoting factors to recruit the cells and induce their differentiation (*62*). Angiogenesis is thought to be the main mechanism by which tumours increase their blood supply. It is generally accepted that most tumours are angiogenesis-dependent and are required to make a pro-angiogenic "switch" to progress, as Folkman proposed (*39, 63*).

Angiogenesis not only promotes tumour growth through increasing the blood supply, but also by the production of tumour growth factors by angiogenic endothelial cells. These factors include platelet-derived growth factor (PDGF), IGF-1, SF, granulocyte colony stimulating factor (GCSF) and interleukin 6 (IL-6) (*64*).

Although the lymphatic system is also a route for metastasis (Section 1.1.1.2), blood vessels are the major entry point into the circulation for tumour cells (*39*). Angiogenesis facilitates

the beginning of metastasis by increasing the number of blood vessels, thereby increasing the number of tumour cells that enter the circulation (*39*), a small percentage of which metastasise (*64*). In addition, the entry of tumour cells into the circulation is facilitated by the disrupted basement membranes in tumour vessels that arise by angiogenesis (*39, 65*). Lastly, metastases will remain as micrometastases in a dormant state unless they switch to the pro-angiogenic phenotype and recruit their own blood supply (*66*).

The "angiogenic switch" is of great clinical importance as it is usually metastases (largely angiogenesis-dependent) that cause lethality (*64*). Folkman (2002) suggests that angiogenesis is so central to metastasis that failure of some tumours to make the "angiogenic switch" can probably explain the infrequency of metastasis (*67*).

1.1.2.2 Overview of the angiogenic cascade

The events of the angiogenic cascade, as reviewed by Liekens *et al.* (2001), include i) activation of endothelial cells, ii) release of proteases (MMPs and uPA, Sections 1.1.3.1 and 1.1.3.2) from activated endothelial cells, iii) subsequent degradation of the underlying basement membrane, iv) endothelial cell invasion of, and migration into, the ECM, v) endothelial cell proliferation, and vi) differentiation of endothelial cells into mature blood vessels (*68*). The last step involves the assembly of endothelial cells into cords, the subsequent formation of a lumen and differentiation of endothelial cells into quiescent cells with specialised characteristics. Vascular myogenesis, a process whereby vascular pericytes and smooth muscle cells are recruited, is also part of vessel maturation (*58*).

Angiogenesis occurs during foetal development. In the adult, angiogenesis is induced during exercise-induced muscle growth (*39*), wound healing, the female menstrual cycle, and in a number of pathological processes, such as rheumatoid arthritis, diabetic retinopathy,

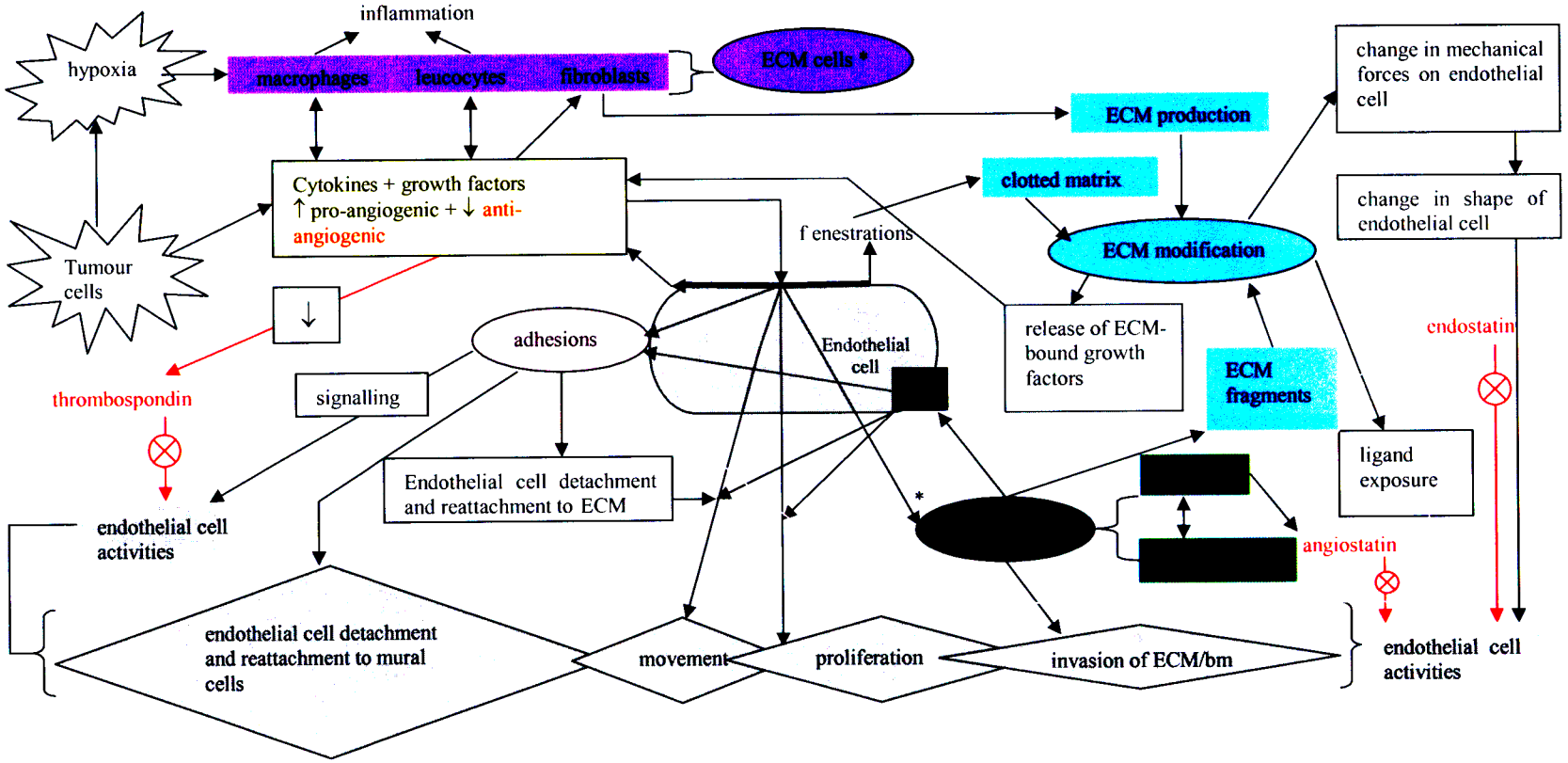
psoriasis, juvenile haemangiomas and tumourigenesis (*68*). Angiogenesis is induced by deficiencies in glucose and oxygen that result from an increased volume of cells (*69*). Maxwell *et al.* (1997) demonstrated that hypoxia is a major trigger of angiogenesis (*70*). These investigators found that induction of angiogenesis by hypoxia is mediated through upregulation of hypoxia inducible factor-1 (HIF-1), which then induces expression of the pro-angiogenic factor, VEGF. Disruption of the balance of angiogenic factors in favour of pro-angiogenic factors (either by an increase of pro-angiogenic factors or a decrease of anti-angiogenic factors) activates endothelial cells and initiates angiogenesis (*63, 66*).

These angiogenic regulatory factors are produced by cells of the ECM and they influence angiogenesis by acting directly on the endothelial cells or through modification of the ECM and cell adhesions. These modifications are mainly mediated by proteases, the production of which (by endothelial cells and cells of the ECM) is under control of angiogenic regulatory factors. This modulation of the endothelial cell environment directs its activities in order to achieve the formation of a new capillary network (*71*). The endothelial cell appears to express all the information necessary to form this network (*71*). A discussion of the regulation of angiogenesis, under the topics of angiogenesis regulators, ECM components, cell adhesions and proteases, follows (Sections 1.1.2.3-1.1.3.2 and 1.1.3.3.2.5) and is summarised in Figure 1.2.

1.1.2.3 Regulators of angiogenesis

1.1.2.3.1 VEGF

In the 1980s, vascular permeability factor (VPF), a dimeric glycoprotein that increases vascular permeability, was identified in rodent tumour lines and purified from serum-free conditioned medium of a human lymphoma cell line (*72*). Subsequently, Ferrara and Henzel (1989) identified, purified and sequenced a secreted dimeric glycoprotein that potently


inflammation
hypoxia
macrophages
leucocytes
fibroblasts
ECM cells *
ECM production
change in mechanical forces on endothelial cell
change in shape of endothelial cell
Cytokines + growth factors
↑ pro-angiogenic + ↓ anti-angiogenic
clotted matrix
f enestrations
ECM modification
Tumour cells
↓
adhesions
Endothelial cell
release of ECM-bound growth factors
ECM fragments
endostatin
thrombospondin
signalling
ligand exposure
Endothelial cell detachment and reattachment to ECM
*
endothelial cell activities
angiostatin
endothelial cell detachment and reattachment to mural cells
movement
proliferation
invasion of ECM/bm
endothelial cell activities

induced endothelial cell proliferation (*73*). They named it VEGF. The sequence of VEGF was identical to that of VPF i.e. they were found to be the same. Since then five isoforms (*39*) and five homologs of VEGF have been recognised (Table 1.1). Several pro-angiogenic functions of VEGF have been identified. Vascular endothelial growth factor triggers expression of uPA, uPAR (uPA receptor) and PAI (uPA inhibitor), and the MMP, collagenase, in endothelial cells (*74, 75*). (Refer to Sections 1.1.3.1 and 1.1.3.2 for the role of these proteases in angiogenesis.) It also increases vascular permeability by induction of endothelial fenestrations (*76*) and induces vasodilation (*77*). The isoforms of VEGF are also involved in differentially determining the extent of vascularisation and the pattern of connection of new vessels with the existing vasculature (*78*). In addition, VEGF (via activity of different isoforms) is important in the regulation of lumen diameter, survival of endothelial cells and possibly their differentiation into fenestrated endothelium in endocrine glands (*58*). Thus, VEGF acts at all stages of angiogenesis. Vascular endothelial growth factor mediates these angiogenic effects via binding to its receptors, namely Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2) (*68*). Nitric oxide synthase (NOS) activation is mediated by VEGF and is necessary for VEGF-induced angiogenesis (*79*). Several studies using animal models have demonstrated the potent induction of angiogenesis *in vitro* and *in vivo* by VEGF (*80*). Additionally, VEGF inhibitors, including soluble Flt-1 and Flk-1 receptor chimeric proteins, inhibit angiogenesis (*80*).

Evidence suggests that VEGF plays a key role in tumour angiogenesis (*81*). Maxwell *et al.* (1997) demonstrated that HIF-1-mediated induction of VEGF following hypoxia is a major contributor to tumour angiogenesis and therefore tumour growth (*70*). High levels of VEGF expression have been identified in the majority of human tumours (*81*). It is thought that the characteristically leaky tumour vessels with disrupted basement membranes can be attributed to VEGF overexpression in tumours (*65*).

Table 1.1 The role of pro- and anti-angiogenic regulatory factors in angiogenesis

Angiogenic factors	Role in angiogenesis	References
PIGF and VEGF-B	• May form angiogenic heterodimers with VEGF. • Contribute to endothelial mitogenesis.	(*82*) (*83, 84*)
VEGF-C	• Capable of inducing angiogenesis *in vivo.* • Primarily lymphangiogenic.	(*85*) (*86*)
VEGF-D	• May induce angiogenesis *in vivo.*	(*87*)
orf virus VEGF	• Plays a role in dermal angiogenesis that arises when infected with the orf virus.	(*88*)
bFGF and aFGF	• Stimulates endothelial cell proliferation and migration *in vitro.* • bFGF promotes capillary tube formation *in vitro.* • bFGF induces uPA expression by endothelial cells. • bFGF is increased in tumours and may be important for tumour angiogenesis.	(*39*) (*68*) (*89*)
Ang-1	• Promotes maturation of blood vessels • Stimulates endothelial production of PDGF, which mediates recruitment of pericytes and smooth muscle cells	(*39*)
Ang-2	• Destabilises blood vessels by acting as an antagonist of ang-1. • Ang-2 in conjunction with VEGF increases angiogenesis.	(*39*)
Scatter factor	• Promotes endothelial migration, proliferation and capillary tube formation *in vitro.* • Induces angiogenesis *in vivo.*	(*90*)
TGF-β	• Induces angiogenesis *in vivo.*	(*91*)
TNF	• Involved in the activation of endothelial cells *in vitro.* • Necessary for VEGF-induced vascular permeability *in vitro* and *in vivo.* • Pro-angiogenic *in vivo.*	(*92*) (*93*)
IL-6	• Acts synergistically with TNF to stimulate endothelial cell migration.	(*90*)
IL-8	• May contribute to angiogenesis through chemotaxis of inflammatory cells.	(*94*)
oestrogen	• Stimulates angiogenesis *in vitro.* • Upregulates MMPs, VEGF and VEGFR.	(*95*) (*96*)
IGF-1	• Can promote differentiation and mobilisation of endothelial progenitor cells.	(*58*)
TSP	• TSP-1 is considered to be the main physiological angiogenic inhibitor. • Increased TSP-1 production by macrophages inhibits tumour angiogenesis. • TSP-2 potently inhibits tumour angiogenesis and tumour growth.	(*68*) (*97*) (*98, 99*)
Angiostatin	• A plasminogen fragment. • Inactivates ATPases thereby inhibiting endothelial cell migration and proliferation. • Its secretion by primary tumours suppresses growth of micrometastases by inhibiting angiogenesis.	(*100*) (*101*)
Endostatin	• A fragment of collagen. • Inhibits endothelial cell proliferation and migration. • Its secretion by primary tumours suppresses growth of micrometastases by inhibiting angiogenesis.	(*102*) (*103*)

PIGF - placenta growth factor; orf virus/VEGF-(B, C, D) - orf virus/vascular endothelial growth factor-(B, C, D); VEGFR - vascular endothelial growth factor receptor; bFGF - basic fibroblast growth factor; aFGF - acidic fibroblast growth factor; ang-(1, 2) - angiopoetin-(1, 2); PDGF - platelet derived growth factor; TGF-β - transforming growth factor-β; TNF - tumour necrosis factor; IL-(6, 8) - interleukin-(6, 8); IGF-1 - insulin-like growth factor-1; TSP-(1, 2) - thrombospondin- (1, 2); uPA - urokinase plasminogen activator; MMP – matrix metalloproteinase

1.1.2.3.2 COX

Cyclo-oxygenase-1 and COX-2 are enzymes that produce prostaglandins from arachidonic acid. The COX enzymes lie downstream of VEGF and are required for its pro-angiogenic actions (*104, 105*). These enzymes are not only important mediators of angiogenesis, but COX-2 is a key mediator of chronic inflammation and is associated with tumourigenesis (Section 1.1.1.3). Thus, COX-2 links angiogenesis, inflammation and cancer. In Section 1.1.2.4.1, the link between angiogenesis and diseases associated with chronic inflammation [such as cancer (*43*)] is further discussed.

1.1.2.3.3 Other regulators of angiogenesis

The activities of the other pro- and anti-angiogenic factors are summarised in Table 1.1.

1.1.2.4 Cells of the ECM

1.1.2.4.1 Inflammation and angiogenesis

Angiogenesis and inflammation are closely associated (*106, 107*). Not only do blood vessels formed by angiogenesis nurture the inflammatory region but activated angiogenic endothelial cells of these blood vessels produce cytokines, such as IL-8, that promote tissue infiltration by inflammatory cells (*94*) (Table 1.1). Activated endothelial cells also contribute to inflammatory cell recruitment by upregulating expression of leucocyte adhesion molecules (*94*). Inflammatory cells participate in the initiation and maintenance of angiogenesis whether it be in wound healing or chronic inflammatory diseases (*97, 107*). This participation in angiogenesis is described in Section 1.1.2.4.2. The simultaneous existence of pathological angiogenesis and inflammation in several disease states, including psoriasis, rheumatoid arthritis, chronic inflammatory bowel disease and cancer (*97*), and the concomitant attenuation of these processes in arthritis by a single inhibitor, kallistatin, further support the synergistic relationship between the two processes (*108*).

1.1.2.4.2 Inflammatory cells

1.1.2.4.2.1 Macrophages

Macrophages are differentiated monocytes (*109*). They are increased in regions of angiogenesis (*110*) and play a key role in mediating this process (*64*). Hypoxia triggers the expression of angiogenic factors, such as VEGF, in macrophages (*107, 111*). The production of TNF-α and IL-1α by macrophages can stimulate endothelial production of MMPs, contributing to angiogenesis (*112*). Some factors produced by macrophages, namely bFGF, PDGF and TNF-α, may upregulate tumour cell production of VEGF, specifically contributing to tumour angiogenesis (*13*). Macrophages themselves are a source of proteolytic factors, including matrix metalloproteinases and serine proteases such as elastase and uPA, during angiogenesis (*113*). Via proteolytic cleavage of the ECM and also production of ECM components, macrophages can modify the ECM (*113*), thereby influencing angiogenesis (Section 1.1.2.5). Macrophages may also produce angio-inhibitory TSP1 (*97*). Failure of macrophages to switch to an anti-angiogenic phenotype, positively contributes to tumour angiogenesis and vascular proliferation in chronic inflammatory diseases such as psoriasis (*97*). In fact, evidence suggests that macrophages are a large contributory factor to pathological angiogenesis (*97*).

1.1.2.4.2.2 Other leucocytes: T cells and granulocytes

Leucocytes produce several cytokines and *in vitro* studies show that they are capable of inducing vascular proliferation and angiogenesis (*97, 106, 114, 115*). Cytokines produced by leucocytes may activate macrophages, which subsequently promote angiogenesis (*64*). For example, granulocyte monocyte colony stimulating factor (GMCSF; produced by neutrophils) and interferon-γ (INF-γ; produced by T cells) are capable of activating macrophages (*64*). The cytokines, TNF-α, IL-1α and INF-γ (released by leucocytes and

macrophages), upregulate CD40 ligand (CD40L) on vascular endothelium and smooth muscle cells (*116*). Contact between CD40L on activated T cells and CD40L on vascular endothelial cells promotes angiogenesis via upregulated expression of MMPs and also stimulation of capillary tube formation (*117*). Cytokines produced by Th1 cells induce COX-2, which is a key mediator of chronic inflammation and angiogenesis (Sections 1.1.1.3 and 1.1.2.3.2).

Mast cells are connective tissue cells that may be derived from basophils (a type of granulocyte). Mast cells mediate inflammation via the release of vasoactive agents, such as histamine (*109*), thereby contributing to angiogenesis (*107*).

1.1.2.4.3 Fibroblasts

Fibroblasts may participate in inflammation, and thereby promote angiogenesis, via release of cytokines with neutrophil and monocyte chemotactic activity on appropriate stimulation (*118*). Growth factors released during inflammation and angiogenesis, such as TGF-β, stimulate ECM production by fibroblasts (*119*). Fibroblasts are also a source of proteases (MMPs and uPA) (*41*) (Section 1.1.1.3), which are required for angiogenesis (*48*) (Sections 1.1.3.1 and 1.1.3.2). In certain tumours, such as breast carcinoma and colon adenocarcinoma, fibroblasts are a major source of MMPs (*41, 120*).

1.1.2.5 Non-cellular components of the ECM

The non-cellular components of the ECM are ground substance (glycosaminoglycans and extracellular fluid), fibres (collagen and elastin) and glycoproteins (fibronectin, fibrillin, laminin, entastin, tenascin) (*109*). The concentration, structure and arrangement of the various components affects the mechanical forces imposed on cells (by the ECM), cell morphology and consequently cell activities (*45, 121*). Ingber (1990) demonstrated that

increased concentrations of fibronectin exert forces that increase endothelial cell spreading/extension and that this change in morphology leads to increased endothelial cell proliferation (*122*). Similarly, forces imposed on endothelial cells by the fibrin ECM component induce capillary tube formation via influencing cell morphology (*123*). It appears that the ECM influences cell cycle and cell survival-related molecules by spatial organisation of cells within tissues (*45, 124*). Thus, changes in ECM fibre/glycoprotein arrangement could alter cell behaviour. ECM fibres may also contribute to angiogenesis and tumourigenesis through their chemotactic activity. Fibrin is chemotactic for inflammatory cells and also provides a supporting network for the migration of these cells (Section 1.1.1.3), as well as endothelial cells.

Modification of the ECM structure by proteolysis modulates the exposure of different domains that regulate cell behaviour (*121*). For example, proteolysis of collagen I exposes integrin binding sites and thereby regulates cell adhesion and migration (*125*) (Section 1.1.2.6). Fragments of ECM that are produced by proteolytic cleavage can influence angiogenesis and tumourigenesis (*121*). For example, endostatin is an ECM fragment that inhibits angiogenesis (Table 1.1) and laminin-5 fragments (found in tumours) enhance the motility of breast epithelial cells (*126*).

The ECM can also regulate cell function by acting as a ligand for adhesion molecules, such as integrins. Integrin signalling can regulate various aspects of cell behaviour that impact on angiogenesis and tumourigenesis (*25*) (Section 1.1.2.6). The ECM can act as a regulator of angiogenesis and tumourigenesis via modulation of growth factor availability and activity. Several angiogenic growth factors are sequestered in the ECM. For example, bFGF is bound to heparan sulfate (*127*). Other sequestered growth factors include latent TGF-β (*47*) and some isoforms of VEGF (*128*). The factor IGFBP-5 may be ECM-bound, and as bound and

free IGFBP-5 have different affinities for IGF, this allows for regulation of IGF availability (*129*). The interaction of the ECM with adhesion receptors can increase the efficacy of growth factor signalling (*130*) (Section 1.1.2.6).

1.1.2.6 Cell adhesion

1.1.2.6.1 Cell adhesion and motility

Modulation of cell-ECM and cell-cell contacts is essential for the migration and invasion of endothelial cells during angiogenesis (*131*) and invasion of tumour cells (Section 1.1.1.3). It appears that co-coordinated detachment and reattachment at both ends of cells are required for movement (*132*). Reorganisation of the cytoskeleton, which is modulated by adhesion molecules (*25*) (Table 1.2), is necessary for the mobility of cells (*132, 133*). Restricted pericellular proteolysis, in which cell adhesions play a role (Table 1.2), is required to remove the ECM barrier to invasion while still preserving a supportive matrix for migration (*28, 134*). In summary, controlled and balanced cell-ECM adhesion and proteolysis are essential for movement i.e. opposite extremes of these processes inhibit movement (*135*). Cell-cell contacts important for angiogenesis include those between endothelial cells and pericytes. Upon the initiation of angiogenesis these contacts need to be broken (and similarly tumour cells need to dissociate from the tumour for invasion, Section 1.1.1.3) before penetration of the basement membrane (*68*). With capillary tube formation, endothelial-pericyte contacts need to be re-established for normal vascular morphogenesis and to inhibit endothelial cell proliferation (*136*)

1.1.2.6.2 Cell adhesion molecules

The four families of cell adhesion molecules include integrins, cadherins, selectins and immunoglobulin (Ig) superfamily members (*131*). Their function and role in angiogenesis is summarised in Table 1.2.

Table 1.2 The role of CAMs in angiogenesis

CAM	Function and role in angiogenesis	References
Integrins	• Heterodimeric transmembrane proteins with over twenty combinations of fifteen α and eight β subunits. • Present in cellular focal adhesion sites that make contact with the ECM. • Main molecules responsible for ECM-cell adhesion. • Integrin signalling results in FAK phosphorylation which results in recruitment of focal adhesion proteins, formation of focal adhesion complexes and stabilisation of adhesion. • Integrin signalling involves RhoGTPase activation which also promotes adhesion and modulates the cytoskeleton, affecting motility. • Growth factors signal dephosphorylation of FAK, which promotes cell detachment from the ECM. However, growth factors also cause activation of RhoGTPases that activate integrin signalling and promote cell adhesion. • When ligand-bound, increase the effectiveness of growth factor signalling. • Activation of MAPK by integrins may promote growth. • Signal cell survival by upregulation of anti-apoptotic proteins or downregulation of pro-apoptotic pathways. • Integrin $\alpha_v\beta_3$ (which is highly upregulated on activated endothelium and virtually absent from quiescent endothelium) is required for the survival of activated endothelial cells. • Integrin $\alpha_v\beta_3$ binds MMP-2, allowing for restricted pericellular proteolysis during angiogenesis. • Participate in capillary tube formation. • Integrin inhibitors inhibit angiogenesis and tumour growth.	(*22*) (*25*) (*131*) (*137*) (*138*) (*139*) (*140*) (*141*) (*142*) (*143*) (*144*) (*145*) (*146*) (*147*)

Cadherins	• Transmembrane polypeptides that form the cell-cell contacts of adherens junctions.
	• Angiogenic growth factors phosphorylate and thereby loosen VE-cadherin contacts, resulting in :
	  • increased vascular permeability
	  • endothelial cell detachment from neighbouring cells
	  • endothelial cell proliferation
	• Re-formation of VE-cadherin adhesions is necessary for vascular morphogenesis.
	• Important regulators of tumour invasiveness (section 1.1.1.2).
		(*14*)
		(*131*)

Selectins	• Endothelial membrane glycoprotein. • Promotes leucocyte adhesion to endothelium. • Blocking of E-selectin inhibits capillary tube formation *in vitro*. • Blocking of E-selectin inhibits angiogenesis *in vivo*.	(*131*) (*148*) (*149*)
Ig superfamily members	• Mediate heterotypic cell-cell adhesion. • Include the leucocyte adhesion molecules, ICAM-1 and VCAM-1. • ICAM-1 and VCAM-1 are upregulated during angiogenesis. • VCAM-1 can induce angiogenesis *in vivo*.	(*68*) (*149*)

CAM - cell adhesion molecule; ECM - extracellular matrix; FAK - focal adhesion kinase; RhoGTPases - the Rho family of guanine triphosphatases; MAPK - mitogen activated kinase; MMP-2 – matrix metalloproteinase-2; Ig - immunoglobulin; ICAM-1 - intercellular adhesion molecule-1; VCAM-1 - vascular cell adhesion molecule-1

1.1.3 Protease systems, cancer and angiogenesis

1.1.3.1 Plasminogen activation system

The plasminogen activation system includes urokinase/tissue plasminogen activator (uPA/tPA), plasmin, uPA receptor (uPAR), annexin II (tPA receptor) and plasminogen activator inhibitors (PAIs) (*150*). Both uPA and tPA cleave plasminogen to produce plasmin. Tissue PA is primarily involved in intravascular plasmin generation for fibrinolysis of blood clots. This is promoted by the binding of tPA to annexin II. The uPA system functions extravascularly, where it is expressed by cell types such as macrophages and fibroblasts, and is therefore more relevant to tumourigenesis and angiogenesis (*20*).

The factor uPA is a serine protease that is secreted as a single chain proenzyme (scuPA) (*31*). The scuPA binds to uPAR (*151*), where it may be converted to uPA by proteases (*152*). Plasmin cleaves ECM components such as fibrin, fibronectin, laminin and proteoglycans (*153*), proMMPs (including MMP-2 and MMP-9) (*154*), cell adhesion molecules (*20*), and growth factors, for example, cleavage of VEGF to bioactive forms (*155*). Plasmin-mediated proteolysis results in the release of growth factors sequestered in the matrix, including bFGF (*127*). While the uPA system promotes growth factor activity, growth factors upregulate uPA and uPAR (*153, 156*). In addition to plasmin generation, uPA mediates its effects via modulation of uPAR adhesion to vitronectin and also signalling that results from its binding to uPAR (*150, 157*). This signalling affects cell adhesion, motility and growth (*31*). The PAIs control the activity of uPA. Both PAI-1 and PAI-2 bind to uPA, thereby inhibiting its plasminogen cleaving activity (*31, 158*). In addition, PAI-1 displaces uPAR from vitronectin (*150, 159*). The uPA system may promote either fibrinolytic or adhesive activity (in fact, both states may possibly occur at different regions on the same cell) and co-ordinate this to allow the sequential attachment and detachment required for movement (*150, 152*) (Section 1.1.2.6.1). This is achieved by

regulation of uPAR-vitronectin adhesion by uPA, plasmin and PAI-1 and by interactions of uPAR with integrins following uPA binding (*20, 150, 160*).

1.1.3.2 Matrix metalloproteinases (MMPs)

The first MMP, collagenase or MMP-1, was isolated in 1962 by Gross and Lapiere from a tadpole tail and 19 others have since then been identified (*19*). Matrix metalloproteinases are secreted by macrophages, endothelial cells and fibroblasts in a proform that is activated proteolytically by serine proteases, such as plasmin, and MMPs are self-proteolytic (*19, 161*). They are inhibited by tissue inhibitors of MMPs (TIMPs). Matrix metalloproteinase levels are low in normal adult tissues and only become elevated during conditions of tissue remodelling, including wound healing, tumourigenesis and angiogenesis (*19*). However, the induction of arthralgia by MMP inhibitors implies a role for MMPs in normal joint function (*19*). Elevation of MMPs during conditions involving tissue remodelling is as a result MMP expression induced by growth factors that are upregulated in these conditions (*68*), for example VEGF (*162*). The MMPs promote cell migration via ECM proteolysis, e.g. via restricted fibrinolysis (*28*). Collectively, MMPs degrade all ECM components (*163*). Alteration of ECM composition and structure via proteolysis also affects cell migration and other behaviours (adhesion and growth; Section 1.1.2.5). Specifically, MMP-2 cleavage of the laminin-5γ2 chain causes release of a fragment that may bind to malignant breast epithelial cell receptors and induce migration (Section 1.1.2.5), possibly via integrin-mediated signalling that modulates the cytoskeleton (*164*). Furthermore, MMPs cleave cell adhesions mediated by E-cadherin and integrins, thereby affecting cell migration (*18, 165*). The MMPs regulate cell growth by releasing growth factors sequestered in the matrix (*19, 166*), cleaving IGFBPs (*167*), processing growth factors into active forms (*168*) and via cleavage of growth factor receptors, such as

FGFR1 (*169*). Refer to Table 1.3 for the role of MMPs and the uPA system in cancer and angiogenesis.

1.1.3.3 The kallikrein-kinin system (KKS)

The KKS is a protein system that is involved with certain angiogenic and tumourigenic factors that were discussed in Sections 1.1.1 and 1.1.2 and represented in Figures 1.1 and 1.2. This involvement is represented in Table 1.4, which is presented at the end of Section 1.1.3.3, page 45. The KKS consists of kallikreins, kininogen, kinins, kininases and kinin receptors. Kallikreins are a family of serine proteases, some of which have the ability to cleave kininogen to produce kinins, vasoactive peptides. Kinins exert their effects through two G-coupled receptors *viz.* bradykinin receptor subtype 1 (B1R) and bradykinin receptor subtype 2 (B2R), and are metabolised by kininases. A diagrammatic overview of the KKS is presented at the end of Section 1.1.3.3, page 44.

1.1.3.3.1 Overview of the KKS components

1.1.3.3.1.1 Kallikreins

In 1909, Abelous and Bardier discovered the presence of a hypotension-inducing substance, which they named urohypotensine, in human urine (*170*). This substance was subsequently isolated and characterised by Frey and Kraut in 1928 (*170*). Since high levels of this substance were found in the pancreas, Werle and colleagues introduced the term 'kallikrein', derived from the Greek word for pancreas, 'kallikreas' (*170*). Subsequently, a distinction was made between plasma kallikrein (PK) and the tissue kallikrein (TK) multigene family (*171*). The gene for "true" TK or hK1, KLK1, was discovered in 1985, followed closely by the discovery of KLK2 and KLK3, which code for proteins hK2 and hK3, respectively (*172*). During the period 1994-2001, 12 additional KLKs (coding for hK4 to

Table 1.3 The role of the uPA system and MMPs in cancer and angiogenesis.

Protease	Role in cancer and angiogenesis	References
uPA system	• Involved in endothelial cell penetration of vascular basement membrane. • Promotes endothelial cell invasion of the surrounding ECM. • Endothelial migration induced by bFGF and VEGF is dependent on the binding of uPA to uPAR. • Plasmin releases ECM-bound growth factors with angiogenic and tumourigenic activity. • uPAR is involved in mitogenic signalling. • Upregulated in tumours. • Modulation of macrophage migration may contribute to increased macrophages in tumours. • Promotes motility in LnCAP cells (prostate tumour cells), human glioma cells and breast cancer cells. • Promotes metastasis in rat breast tumours, murine B16 melanomas, human melanomas, uveal melanomas and human prostate carcinomas. • PAIs limit excessive proteolysis (excessive proteolysis is detrimental to angiogenesis (section 1.1.2.6.1)). • Generates angiostatin, an anti-angiogenic factor that inhibits the growth of metastases (Table 1.1), from plasminogen.	(*31*) (*68*) (*157*) (*173*) (*174*) (*175*) (*176*) (*177*)
MMPs	• MMP inhibitors inhibit angiogenesis *in vitro* and *in vivo*. • Involved in endothelial cell penetration of vascular basement membrane. • MMP-2 binds to $\alpha_v\beta_3$ on angiogenic endothelial cells and melanoma cells, which allows for localised degradation of the ECM as these cells migrate. • MMPs (expressed by endothelial cells) are important for capillary tube formation. • Upregulate growth factors with pro-angiogenic or tumourigenic activity. • Have been positively correlated with tumour progression and tumour aggressiveness in several tumour types (*68*). • MMP correlation with metastasis is not consistent for lung tumours. • Cleave transglutaminase, an adhesion receptor, at the leading edge of migrating cancer cells, positively contributing to their migration on collagen. • Membrane-type 1 MMP cleaves CD44 (frequently expressed by tumour cells), thereby stimulating tumour motility. • High levels of TIMPs are associated with a poor prognosis, as this may inhibit excessive proteolysis. • Generates angiostatin, an anti-angiogenic factor that inhibits the growth of metastases (Table 1.1), from plasminogen.	(*68*) (*146*) (*178*) (*179*) (*180*) (*181*) (*182*) (*183*) (*184*) (*185*)

uPA system - urokinase plasminogen activation system; ECM - extracellular matrix; bFGF - basic fibroblast growth factor; VEGF - vascular endothelial growth factor; uPA - urokinase plasminogen activator; uPAR - receptor for urokinase plasminogen activator; PAI - inhibitor of plasminogen activator; MMP – matrix metalloproteinase; TIMP - tissue inhibitor of matrix metalloproteinases

hK15) were identified (*172*). The present project focused only on "true" TK. In the literature and in the present project the term TK is used to refer to "true" TK protein. For the purpose of distinguishing TK from other tissue kallikreins, the remaining 14 members of the tissue kallikrein family will be referred to as kallikreins. The location, gene/protein structure and regulation of TK follows.

1.1.3.3.1.1.1 Location of TK

Tissue kallikrein is found in the pancreas, salivary glands, kidneys, intestine, sweat glands, adrenals, spleen, reproductive tissues, plasma (*186*), neutrophils (*187*), macrophages (*188*) and endothelial cells (*189*).

1.1.3.3.1.1.2 Gene and protein structure of TK

The KLK1 gene is clustered together with the other 14 kallikrein genes on chromosome q13.3-13.4. (*170, 190*). All kallikrein genes have 5 coding exons of similar size (*170*). The second, third and fifth coding exons code for histidine, aspartate and serine residues, respectively, that belong to the catalytic triad of all kallikreins (*170, 171, 191*). Kallikreins have a 30-50% amino acid homology with the exception of hK2 and hK3, which have 80% homology (*170*). All proteins are translated as preproenzymes that contain a signal peptide (16-30 amino acids) at the amino terminus (*170, 172, 192*). Cleavage of the signal peptide results in the formation of proenzymes that are subsequently secreted (*170, 172, 192*). Further removal of a propeptide of 4-9 amino acids results in a fully active, mature enzyme, a single chain glycoprotein (*170, 172, 192*).

1.1.3.3.1.1.3 Regulation of TK

1.1.3.3.1.1.3.1 Transcriptional regulation of kallikrein genes: steroid hormones

All kallikrein genes are under direct and indirect transcriptional regulation by steroid hormones. Most are upregulated by androgens, oestrogen and progestin, although to differing extents (*170*). This is the major mechanism of transcriptional control (*172*). Direct hormonal control involves the binding of activated steroid hormone receptors to steroid hormone response elements (REs) in the promoter regions of genes, followed by recruitment of transcriptional machinery (*172*). Functional androgen REs (AREs) have been identified in the promoter/enhancer regions of KLK genes 2 (*193*) and 3 (*194*). AREs have also been identified in KLK4, 14 and 15 (*172*) and an oestrogen response element (ORE) in KLK1 (*195*), although they have not yet been functionally tested (*172*). Steroid hormone receptors may also interact with transcription factors rather than binding to genes directly, thereby indirectly influencing transcription (*172*). Other indirect mechanisms of transcriptional regulation may occur via signalling pathways that crosstalk with steroid hormone response pathways (*196*). For example, growth factor signalling pathways modulate the activity of co-regulators of steroid hormone receptors (*197*) (Section 1.1.3.3.2.6.2).

1.1.3.3.1.1.3.2 Post-transcriptional regulation of TK activity

Pro-kallikreins require proteolysis for activation. Takada *et al.* (1985) demonstrated that human urinary prokallikrein (i.e. proTK) could be activated by serine proteases such as trypsin and PK, and also by the metalloproteinase thermolysin (*198*). Serine proteases, such as plasmin, can activate pro-kallikreins (*199*). Further, kallikreins may have autocatalytic activity or activate one another (*172, 191, 200, 201*). This suggests the possibility that kallikreins become activated in a proteolytic cascade-like manner, as serine proteases of the

clotting pathway (*191*). Supporting this hypothesis is the co-expression of many kallikreins in the same tissue and hormonal regulation of all kallikreins (*191*).

Proteolysis of kallikreins may also be a mechanism of inactivation. Some kallikreins, such as hK6 and 13 have the ability for autoinactivation (*172*). Serine protease inhibitors (serpins) may post-translationally inactivate TK via the formation of a serpin-protease complex. Serpins include kallistatin (first observed to bind to TK; (*202*)), α1-antitrypsin, PAI-1 and antithrombin III, for example (*135*).

1.1.3.3.1.2 Kininogen, kinins and kininases

In the late 1930s, Werle and colleagues found that the hypotensive and gut smooth muscle-contracting effects of kallikrein were not due to kallikrein itself, but rather as a result of a biologically active molecule released by kallikrein-mediated cleavage of its precursor (kininogen) (*171*). This molecule was initially named kallidin by Werle and Berek. Subsequently, Roche e Silva and colleagues identified a substance with activity similar to that of kallidin, which they named bradykinin (BK) (*171*). This name is Greek-derived from its slow ('brady') muscle contracting ('kinin' means movement) action. By the end of the 1960s, the structure of BK and several functionally similar molecules, collectively called kinins, had been solved and their biological effects tested (*203*) (Section 1.1.3.3.2.2). At approximately the same time, kininogens became classified as high and low molecular weight kininogen, HMWK and LMWK, respectively (*171*). Plasma kallikrein and TK cleave HMWK and LMWK differentially to release different kinins. PK cleaves HMWK at lys-arg and arg-ser bonds, releasing the nonapeptide, BK (*171*). Plasma kallikrein may also release BK from LMWK that has been cleaved by neutrophil elastase, although this is a poor substrate for PK (*204*). Tissue kallikrein cleaves the met-lys bond as well as the arg-ser

bond of LMWK to produce kallidin (lys-BK) (*171, 192*). This is the generally accepted kininogen-cleaving activity of TK, but it has also been shown to readily produce kinins (bradykinin and kallidin) from HMWK *in vitro* (*171, 205-207*). Discovery of the kininases, the enzymes that cleave kinins, also began in the 1960s. Kininase family I carboxypeptidase N and M (KI-CPN and KI-CPM) remove the carboxy terminal from kinins, resulting in the release of biologically active des-arg^9-BK and des-arg^9-lys-BK (*171, 208*). Kininase family II angiotensin I-converting enzyme and neutral endopeptidase (KII-ACE and KII-NEP) further degrade these kinin derivatives, inactivating them (*171, 209*). Aminopeptidase is a kininase that can convert kallidin to BK (*192*).

1.1.3.3.1.2.1 Location, structure and regulation

A single human gene, consisting of 11 exons and 27 000 base pairs, encodes kininogen. The HMWK (88-120 kDa) and LMWK (50-68 kDa), single chain glycoproteins, are then produced by alternate splicing (*171, 192*). The primary structures of kininogens, as well as kinins, are represented in Figure 1.3, which is presented at the end of Section 1.1.3.3. Kininogens are synthesised by several cell types *viz.* hepatocytes, platelets, acinar cells of the submandibular gland, human kidney, endothelial cells and neutrophils (*171*). High molecular weight kininogen circulates in the blood stream bound to pre-PK, although this complex is also found bound to the surface of the neutrophil (*171, 210*). LMWK may be found in plasma (*211*), bound to the surface of neutrophils (*210*) or localised in the various tissues mentioned above (*171*).

Generation of kinins occurs when TK and PK become activated and come into contact with kininogens. Plasma kallikrein is activated by blood vessel damage (*212*) and TK is activated by proteases (Section 1.1.3.3.1.1.3.2), which may be released during tissue damage or

inflammation (*213*). Kinins are not exclusively formed by kallikrein action. Snake venom proteases, trypsin and plasmin are also capable of releasing kinins (*171*). Mast cell tryptase and neutrophil elastase, released during inflammation, may act in concert to release BK from kininogen substrate (*207*). This activity may be important in an oxidative environment, such as during inflammation, as oxidation of the methionine residue of kininogen affects kallikrein-mediated kininogen processing. However it does not affect the kininogenase activity of tryptase/elastase. Refer to Section 1.1.3.3.2.2 for biological effects of kinins.

The half-life of kinins is less than 30 seconds due to their rapid degradation by kininases, and this allows for acute regulation of their activity (*214*). Kininase family I carboxypeptidase N is synthesised in the liver and is present in plasma associated with endothelial cells (*171*). Kininase family I carboxypeptidase M is located in plasma membranes in human kidney, lung, fibroblasts, pulmonary artery endothelial cells and placenta (*171*). Kininase family II angiotensin I-converting enzyme resides in the endothelial plasma membranes in the lung from where it is primarily responsible for inactivation of kinins (*214*). Kininase family II neutral endopeptidase is localised on membranes (*209*), or secreted into biological fluids (*171*). An important function of KII-NEP may be to regulate activity of kinins formed on the neutrophil surface as it is found on the surface of neutrophils (*171*). Aminopeptidase P is present in the red blood cells, kidney and lung (*171*).

1.1.3.3.1.3 Kinin receptors

In 1977, Regoli and colleagues presented the first evidence to suggest the existence of two kinin receptor types (*215*). These investigators demonstrated a greater potency of des-arg^9-BK than BK in a rabbit aorta preparation, which contrasted with the typical order of agonist

potency (BK > lys-BK > des-arg^9-BK) in classical preparations (such as rat uterus) (*171, 215, 216*). Further, antagonists based on the structure of des-arg^9-BK were demonstrated to have little effect on classical preparations (*216*). In 1980, Regoli and Barabé proposed that kinin receptors be divided into two types based on the order of agonist potency and the affinity of antagonists (*215*). Those receptors activated most strongly by kinins without the carboxyl arginine, significantly activated by kallidin and responsive to des-arg^9-BK antagonists were named B1R (lys-des-arg^9-BK > lys-BK > des-arg^9-BK > BK), whereas those little affected by these kinin derivatives and antagonists were named B2R (BK > lys-BK > des-arg^9-BK > lys-des-arg^9-BK) (*203, 215*). This classification became firmly established with the development of B2R antagonists based on the structure of BK (*217*). By the late 1980s, several intracellular signalling routes following activation of the kinin receptors had been outlined (*203*). In the late 1990s, advances were made in the identification of the roles of the kinin receptors in disease and non-peptide receptor antagonists, with the potential to treat these diseases, were developed (*203*). More recently, direct activation (independent of kinin generation) of the B2R by TK and other serine proteases was reported (*218*), however a subsequent study failed to support those findings (*219*).

1.1.3.3.1.3.1 Gene and protein structure

The B2R gene is located 5' relative to the B1R, separated by 12 000 base pairs, on chromosome 14q32 (*220*). Both genes have three exons and are 36% homologous (*203*). Both receptors span the membrane seven times, have three extracellular loops with three consensus sites for N-linked glycosylation and three intracellular loops, possess DRY and NPXXY motifs and putative phosphorylation and acylation sites (*172, 221*). The main difference between the two receptors is the C-terminal tail. The B2R has a longer C-terminal

tail than the B1R with clusters of serine and threonine residues, that may be phosphorylated, and cysteine residues, that may be acetylated (*203*).

1.1.3.3.1.3.2 Regulation of receptor expression and activity

Both B1R and B2R may be expressed by vascular cells, nonvascular smooth muscle cells, fibroblasts, epithelial cells and cells of the nervous system. However, the expression of B1R is only induced in pathological conditions, whereas B2R is present physiologically (*203*). The B1R is induced by noxious agents probably as a result of the inflammatory response to these agents (*203*). Inflammatory cytokines, such as interleukin 1 (IL-1), and growth factors, such as EGF, induce B1R expression (*222*). Glucocorticoids have an inhibitory effect on B1R expression (*222*). These effects appear to be linked to the presence of a NF-κβ (nuclear factor of the κ-enhancer in B cells) motif in the promoter region of the B1R gene (*223, 224*). Cytokines and inflammatory mediators may induce B1R expression through activation of the transcription factor NF-κβ (*203*). Glucocorticoids upregulate Iαβα, a factor that inhibits NF-κβ (*203*).

The transcription of B2R is also increased by inflammatory cytokines and growth factors (*225*). Some regulatory elements in the B2R gene promoter include binding sites for IL-6 activator protein, cAMP (cyclic adenosine monophosphate)-response element binding protein, NF-κβ, glucocorticoid, intestinal natriuretic factor and oestrogen (*203*). Therefore, TK as well as B2R may be hormonally regulated. Another mechanism of increased B2R and B1R is IL-1-mediated stabilisation of B2R/B1R mRNA (messenger ribonucleic acid) (*226*). However, while B2R activity is downregulated and desensitised in response to BK exposure (*227, 228*) and may sometimes be downregulated during intense, chronic inflammation (*203*), B1R remains constitutively active (*203, 229*). This may be largely due

to the lack of phosphorylation sites in the B1R C-terminal tail when compared to that of the B2R (*230*) (Section 1.1.3.3.1.3.1). When BK binds to B2R, phosphorylation of threonine and serine residues occurs and this promotes receptor desensitisation (*228*) and internalisation (*231*). Supporting this is the very limited capability of B1R for desensitisation (*229*) and internalisation in response to agonist exposure (*230*). The main difference between B1R and B2R signalling is the significant sustainment of B1R signalling in comparison to B2R signalling (*229*).

There are many other negative feedback pathways induced by BK binding to B2R, including G protein-caveolae associations (*232*) and decreased G protein activation (*203, 233*). Additionally, external factors such as increased proteases in chronic inflammatory conditions may inactivate the B2R by cleavage (*203*).

1.1.3.3.1.3.3 Receptor signalling pathways

The signalling pathways of B1R and B2R, which are initiated by ligand binding, are very similar (*203*). Kinin signalling involves receptor interaction with different types of G proteins (*234*), phosphoinositol hydrolysis by phospholipase C ((*235*) (hydrolysis initiated by BK may also be mediated by kinin-mediated phosphoinositide 3-kinase (PI3K) activation (*236*)), increased intracellular calcium concentration (*235*), nitric oxide synthase (NOS) activation (*237*) (resulting in NO production), eicosanoid production (*238*) and mitogen activated kinase (MAPK) activation (*239*). Activation of MAPK, following B2R stimulation, is protein kinase C (PKC) dependent and involves activation of tyrosine kinases (*240*). Stimulation of B2R also results in activation of RhoGTPases (Rho guanine triphosphatases), focal adhesion kinase (FAK) and several transcription factors (NF-κβ), that induce transcription of cytokines (*203*).

1.1.3.3.2 Physiology and pathophysiology of the KKS

1.1.3.3.2.1 Biological effects of TK

Tissue kallikrein has been implicated in the processing of several growth factors and hormones. Jones *et al.* (1992) reviewed the possible role of TK in the processing of prolactin in the pituitary before its secretion (*186*). Tissue kallikrein is thought to be involved in the turnover of nerve growth factors and hormones that are essential for neurotransmission and normal nerve function (*213*). The proteins processed by TK include pro-insulin, low density lipoprotein, precursor of atrial natriuretic prorenin, vasoactive intestinal peptide and angiotensinogen (*171, 192*). Other kallikreins are implicated in the processing of growth factor and peptide hormones, particularly in the pancreas and nervous system (*172*).

A study by Wolf and colleagues (2001) suggested the involvement of TK in ECM cleavage (*241*). In that study, an inhibitor of TK, FE999024, reduced invasion of matrigel by breast tumour cells and pulmonary extravasation in an *ex vivo* rat model. Whether this was directly mediated by TK is unclear. Other kallikreins, namely hK2, 3, 5, 13, and 14, are capable of directly cleaving the ECM (*172*) and Bõrgono *et al.* (2004) suggest that they may play a role in tissue remodelling comparable to MMPs (*172*). However, only indirect mediation of proteolysis (through upregulation of other proteases) has been clearly demonstrated for TK. Tissue kallikrein has the ability to activate the proteases MMP-2 (*242*) and MMP-9 (*243*). Activation of PAR-1, leading to MMP-12 secretion (*244*), may be another mechanism of TK-mediated increase in MMP activity. Tissue kallikrein has not been demonstrated to activate the plasmin system. However, hk4 has the ability to cleave uPAR, as well directly cleaving and activating uPA (*245*). Proteolysis of the ECM affects cell adhesion and motility (Section 1.1.2.5), thereby implicating an indirect role of

TK in these processes. Despite the many proteolytic-mediated effects of TK, generation of kinins is considered to be its primary function (*170, 172*).

1.1.3.3.2.2 Biological effects of kinins, as mediated by B1R and B2R

1.1.3.3.2.2.1 Vasoactivity

Prostacyclin and NO, which are produced as a result of B1R/B2R signalling (Section 1.1.3.3.1.3.3), act on smooth muscle cells to induce vasodilation (*203*). Rho G protein that is activated by B2R signalling breaks down endothelial intercellular junctions, thereby separating endothelial cells and increasing vascular permeability (*246*) (Section 1.1.3.3.1.3.3). The vasoactivity of kinins has been implicated in the control of renal blood flow and regulation of blood pressure (*171*).

1.1.3.3.2.2.2 Promotion of growth

1.1.3.3.2.2.2.1 NO and tyrosine kinases

Kinin receptor signalling (Section 1.1.3.3.1.3.3) produces NO, which potentiates tyrosine kinase receptor signalling (*247*). Several reactive nitrogen intermediates (RNIs) may be derived from NO, including peroxynitrite ($ONOO^-$) (*247, 248*). Reduction of $ONOO^-$ to form peroxynitrous acid (ONOOH), followed by its decomposition produces hydroxyl radicals (OH^-), which are highly oxidative reactive oxygen intermediates (ROIs). Both RNIs and ROIs modulate signalling pathways (*247*). They can oxidise and thereby inactivate tyrosine phosphatases, indirectly increasing tyrosine phosphorylation. This potentiates the activation of tyrosine kinase receptors, which include the following growth factor receptors: insulin, EGF, IGF, PDGF, VEGF and FGF. Reactive nitrogen intermediates and ROIs may also stimulate tyrosine kinase dependent activation of MAPKs that are usually part of growth

factor signalling pathways (*247, 249*) and also various transcription factors (through oxidation of cysteine residues or iron-sulphur centres) (*247*).

1.1.3.3.2.2.2.2 VEGF

Hayashi *et al.* (2002) have demonstrated an increase in VEGF mRNA in rats following injections of a BK agonist (*250*). Subsequently, BK was found to upregulate VEGF production in stromal fibroblasts in mice and rats with sarcoma 180 and Walker tumours, respectively (*251, 252*). Bradykinin was also shown to upregulate VEGF production in vascular smooth muscle cells (*253*). This occurs via B2R activation followed by PKC activation. Not only do kinins upregulate VEGF production but they also potentiate VEGF signalling. Thuringer *et al.* (2001) demonstrated that activation of B2R by BK could transactivate the VEGFR, Flk-1, in endothelial cells (*249*). Some NOS activation still occurred when Flk-1 was inhibited. Therefore, the NO production due to kinin receptor activation alone would potentiate that produced by Flk-1 activation. Similarly, Miura *et al.* (2003) demonstrated transactivation of Flk-1 by B2R, followed by NOS activation, in coronary endothelial cells (*254*). Other overlaps in the signalling pathways of B1/2R and Flk-1 are PI3K and MAPK activation (*255*) (Section 1.1.3.3.1.3.3).

1.1.3.3.2.2.2.3 bFGF

Bradykinin acts synergistically with bFGF to induce proliferation in coronary postcapillary endothelial cells (*256*). In this instance, activation of the B1R was responsible for the effect of BK. A mechanism by which B1R activation potentiates actions of bFGF was demonstrated by Parenti *et al.* (2001) to be NOS-dependent upregulation of bFGF (*257*). Another possible mechanism is the generation of MAPK by B1R activation as MAPKs are involved in bFGF signalling (*255*).

1.1.3.3.2.2.2.4 EGF

Epidermal growth factor is a mitogen that is found in many tissues (*258*). BK signalling may result in the transactivation of the EGF receptor (EGFR) and in some cases, the actions of BK may be dependent on this transactivation (*259, 260*). Epidermal growth factor signalling involves activation of phospholipase C (*261*) and thus may be potentiated by kinin-mediated phospholipase C activation (Section 1.1.3.3.1.3.3).

1.1.3.3.2.2.2.5 TGF-β

BK may induce TGF-β1 expression via a MAPK-dependent pathway in vascular smooth muscle cells (*262*). Although TGF-β is growth inhibitory in healthy epithelial tissue, it may promote growth in pathological conditions (*263*) (Section 1.1.3.3.2.6.2).

1.1.3.3.2.2.2.6 Cytokine production

Kinin receptor activation results in upregulation of IL-1 production in fibroblasts and IL-8 production in smooth muscle cells (*203*). Kinins may also stimulate the release of IL-1 and TNF by macrophages (*264*).

1.1.3.3.2.2.2.7 Modulation of glucose metabolism

Kinins cause a hypoglycaemic effect (*171*). They improve the transport of glucose to tissues via enhancement of blood flow and they increase tissue uptake of glucose. They may further cause increased glucose oxidation in the presence of insulin, thereby providing energy for growth (*171*).

1.1.3.3.2.2.3 Modulation of cell adhesion and motility

The activity of RhoGTPases and FAK, which affect cell adhesion and motility, may be modulated by kinin receptor activation (Sections 1.1.2.6 and 1.1.3.3.1.3.3). Production of NO (which may occur via kinin receptor activation, Section 1.1.3.3.2.2.2.1) has been demonstrated to promote endothelial cell migration by modulating the phosphorylation status of FAK proteins and the assembly of stress fibres (*133*). Proteolysis of the ECM and cell adhesions, indirectly mediated by kinins, impacts on cell motility (Sections 1.1.2.5 and 1.1.3.3.2.2.4).

1.1.3.3.2.2.4 Indirect proteolytic function

The RNI, $ONOO^-$, derived from kinin receptor signalling, is capable of activating several MMPs (*248*). BK stimulates tissue PA activator release into the circulation (*265*), subsequently resulting in activated plasmin, which may in turn activate MMPs (Section 1.1.3.1). Growth factor signalling, that is enhanced by kinins (Section 1.1.3.3.2.2.2), may upregulate protease systems (Sections 1.1.3.1 and 1.1.3.2).

1.1.3.3.2.3 The role of the KKS in inflammation

The KKS plays a key role in inflammation (*171, 266*): TK upregulates proteolysis (Section 1.1.3.3.2.1) and kinins induce vasodilation, increase vascular permeability, promote cell proliferation and upregulate proteolytic activity (Section 1.1.3.3.2.2). Kinins also induce chemotaxis of neutrophils and monocytes (*118*). This was demonstrated by the release of the chemotactic factors IL-8, GCSF, GMCSF, monocyte chemoattractant protein-1, and TGF-β by lung fibroblasts in response to BK stimulation. Further, kinins are important for neutrophil diapedesis (mediated by vascular permeability) and their subsequent infiltration of tissues (*267*). Kinins also contribute to leucocyte activation, and neutrophils and

macrophages at the site of inflammation display increased sensitivity to kinins, which is due to the upregulation of kinin receptors on these cells (*268*).

The KKS becomes activated as a result of inflammation in response to tissue damage, infection and/or tumourigenic cells (Section 1.1.3.3.1.2.1). Neutrophils appear to be an important source of kinins in inflammation (*266*) since they demonstrate TK (Section 1.1.3.3.1.1.1), HMWK and LMWK (Section 1.1.3.3.1.2.1). Cleavage of the neutrophil kininogen in inflammatory conditions (pneumonia, tuberculous meningitis and rheumatoid arthritis) has been observed (*266*). Other inflammatory cells, namely human macrophages and rat mast cells, possess KKS components, TK and kininogen, respectively (*269*) (Sections 1.1.3.3.1.1.1 and 1.1.3.3.1.2.1). Neutrophil production of cytokines may be largely responsible for the increased kinin receptor expression during inflammation (*203*) (Section 1.1.3.3.1.3.2).

Evidence for the proinflammatory role of the KKS is provided by its involvement in chronic inflammatory disorders, such as arthritis, asthma and inflammatory bowel disease (*171*). There is evidence for increased TK secretion in inflammatory bowel disease, supporting KKS involvement in this pathological state (*188, 270*). Kallistatin (a TK inhibitor) reduced inflammation (and angiogenesis) in a rat arthritis model (*108*). Similarly, administration of HMWK antibodies to Lewis rats with arthritis significantly decreased local and systemic inflammation (*271*). The B1R has been implicated in allergic airway inflammation as suggested by the inhibition of allergen-induced bronchial hypersensitivity in asthmatic rats by B1R antagonists (*203*). A role for B2R in asthma is supported by the improvement of forced expiratory volume achieved by B2R antagonists (*203*). There is also evidence for KKS involvement in cancer (*266*) (Sections 1.1.3.3.2.4 and 1.1.3.3.2.6), another disease in which there is chronic inflammation (Section 1.1.1.3).

1.1.3.3.2.4 The KKS and cancer

Identification of TK and kinin receptors on the leading edge of neuroblastoma cells in an *in vitro* model suggests that KKS-mediated proteolysis contributes to tumour invasion (*272*). The inhibition of matrigel invasion by breast cancer cells due to a TK inhibitor, further supports a role for the KKS in tumour invasiveness (Section 1.1.3.3.2.1). The expression of TK has been demonstrated in human colon epithelial cancer (*273*), human endometrial cancer (*274*), renal carcinoma (*275*) and pancreatic adenocarcinoma (*241*). Upregulation of TK was observed in prolactin-secreting pituitary adenomas and growth-hormone-secreting adenomas (*186*), gastric cancer (*276*) and oesophageal cancer (*277*). The remaining 14 members of the TK family are also implicated in tumourigenesis (*172*). They are either upregulated or downregulated in cancers and may have either protective or tumourigenic functions. They are also an important source of cancer biomarkers, particularly hK3 or prostate specific antigen (PSA) (*172*). This antigen is expressed chiefly in prostatic tissue and is generally elevated in the serum of prostate cancer patients, probably due to the impaired integrity of the prostate gland ducts (*278*). Prostate specific antigen is also expressed significantly in breast tissue and is a marker for breast cancer (*170, 172*). The role of the KKS in prostate and breast cancer is dealt with further in Section 1.1.3.3.2.6.

Kinin generation has been identified in the ascitic fluid of tumours (*279*), with considerable amounts of BK generated in ovarian carcinoma ascites (*280*). Kinins appear to be largely responsible for the increased vascular permeability in ascitic tumours, which supports rapid tumour growth (*248, 279*). Decreased plasma prekallikrein and kininogen levels in patients with various types of cancers suggests that increased kinin generation occurs in cancer (*281*). Further, BK antagonists show potent anticancer activity in lung and prostate cancers *in vitro* and in murine models (refer to Section 1.1.4.2), as well as broad spectrum anticancer activity against colon, cervical, pancreatic and breast cancers *in vitro* (*282*).

The presence of B2R has been identified in human endometrial cancer (*274*), and human adenocarcinoma of the lung and stomach, lymphoma, hepatoma, squamous cell carcinoma of the lung, carcinoid of the duodenum, as well as in several mouse tumours (*283*). The presence of B1R and increased B2R have been observed in cervical cancer and metastases (*284*) as well as in oesophageal cancer (*277*). Although B2R is absent in healthy glial cells, it is expressed by astrocytoma (*285*).

1.1.3.3.2.5 The KKS and angiogenesis

There is abundant evidence that the KKS is involved in the angiogenic cascade (*108, 254, 256, 286-294*). Plendl *et al.* (2000) provided evidence that TK is involved in the angiogenic cascade using an *in vitro* model of angiogenesis (*286*). In that study, increased intensity of immuno-labelling for proTK was observed in microvascular endothelial cells as they proliferated and migrated, eventually differentiating into tube-like structures. Comparatively weak immuno-labelling was observed in non-angiogenic cells. Additionally, intense TK immuno-labelling was observed in endothelial tubular structures and in the end of cellular projections. Subsequently, Emanueli and colleagues (2001) showed that the angiogenic response to ischaemia in a mouse model was enhanced by TK gene delivery (*287*) and that TK gene delivery corrected impaired angiogenesis in spontaneously hypertensive TK deficient rats (*288*). TK gene therapy also promoted angiogenesis in a cerebral ischaemic rat model (*289*) and protected rats from ischaemic injury when administered after the onset of a stroke (*290*). Recently, kallistatin (a TK inhibitor) was shown to inhibit angiogenesis (and inflammation), subsequently leading to clinical improvement in arthritis rat models (*108*). However, it is not clear whether this was due to the inhibition of TK because kallistatin has also been shown to inhibit angiogenic activities induced by VEGF and bFGF (*295*). Bradykinin was reported by Hu and Fan (1993) to potentiate IL-induced angiogenesis *in*

vivo (*291*). In that study, Hu and Fan (1993) also showed that a B1R antagonist abolished this effect. Subsequently, Emanueli and colleagues (2002) showed significant inhibition of vessel growth in ischaemic muscle of a murine model by a B1R antagonist (*292*). These authors suggested that this was due to B1R-mediated endothelial cell proliferation and survival based on these observed effects in an *in vitro* experiment. Further, mitogenic actions of kinins, mediated through the B1R, were shown to contribute to bovine coronary endothelial cell proliferation (*256*). Subsequently, BK, via activation of B2R, was shown to promote human coronary endothelial cell proliferation and tube formation *in vitro* (*254*). Accordingly, HUVEC proliferation and cord formation in *in vitro* assays was inhibited by BK antagonists (*293*). In a recent study, B2R activation promoted endothelial cell proliferation in synovitis (*294*).

Several studies have implicated the KKS in tumour angiogenesis (*250, 251, 272, 296*). Naidoo *et al.* (2004) found that immuno-reactive TK was reduced in HUVECs that were challenged with neuroblastoma metabolites while release of TK into the medium was increased (*296*). This indicated that neuroblastomas increase the secretion of TK by endothelial cells possibly to facilitate tumour angiogenesis. The localisation of TK, B1R and B2R in co-cultures of neuroblastoma cells and HUVECs suggested a synergistic relationship between angiogenic endothelial cells and tumour cells and that B1R and B2R may mediate the invasion of endothelial cells at the leading edge in their movement towards neuroblastoma cells (*272*). A role for BK in tumour angiogenesis was demonstrated by suppression of tumour-associated angiogenesis in mice bearing sarcoma 180 cells by a B2R antagonist (*251*). The suppression of tumour-associated angiogenesis in kininogen-deficient rats and the reversal of this by a FR190997, a mimic of BK, further supports kinin involvement in tumour angiogenesis (*250*). Figure 1.3 and Table 1.4 summarise Section 1.1.3.3.

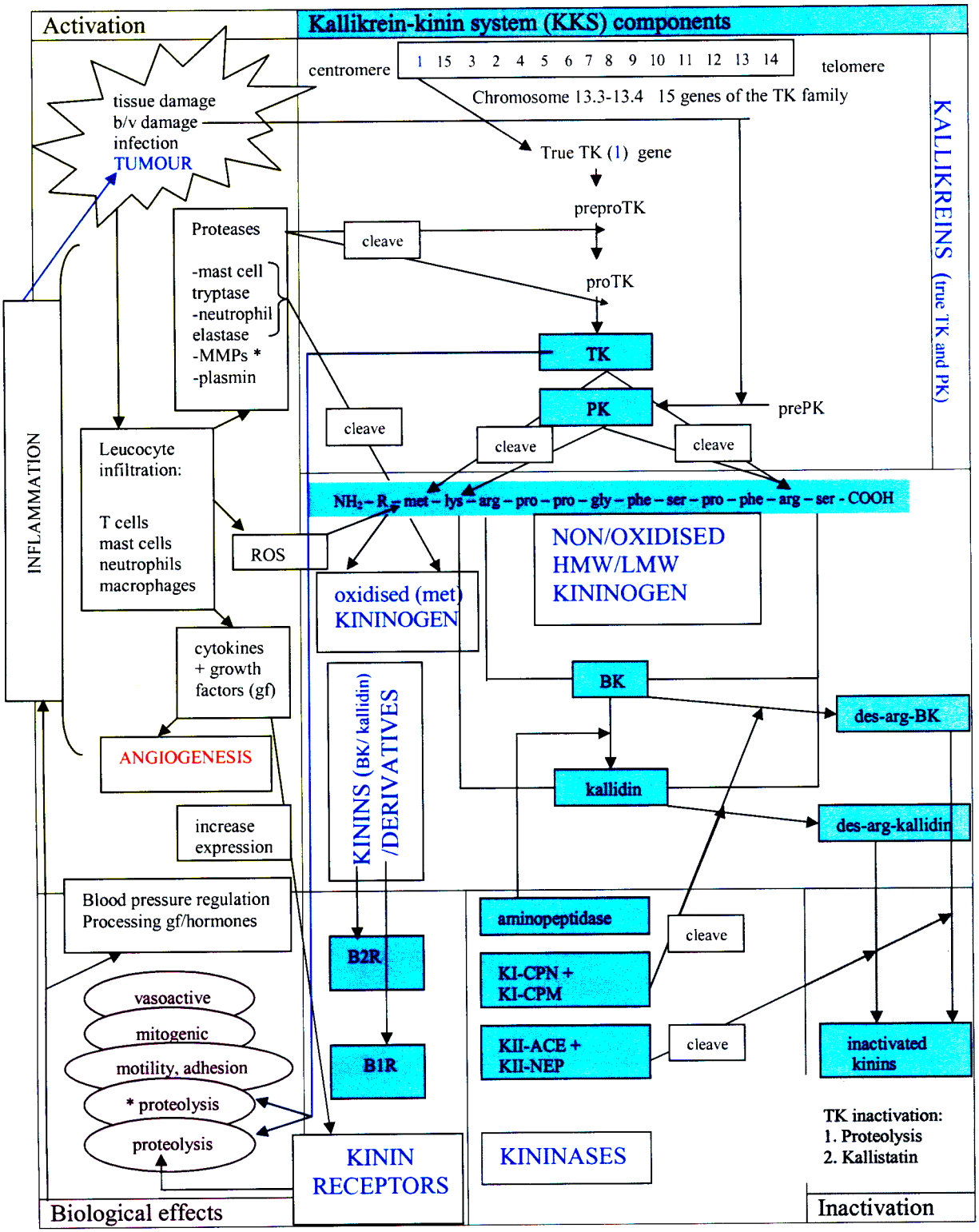

Activation
Kallikrein-kinin system (KKS) components
centromere
1 15 3 2 4 5 6 7 8 9 10 11 12 13 14
telomere
Chromosome 13.3-13.4 15 genes of the TK family
KALLIKREINS (true TK and PK)
tissue damage
b/v damage
infection
TUMOUR
True TK (1) gene
preproTK
proTK
Proteases
-mast cell tryptase
-neutrophil elastase
-MMPs *
-plasmin
cleave
TK
PK
prePK
cleave
cleave
cleave
INFLAMMATION
Leucocyte infiltration:
T cells
mast cells
neutrophils
macrophages
ROS
NH2 – R – met – lys – arg – pro – pro – gly – phe – ser – pro – phe – arg – ser - COOH
NON/OXIDISED HMW/LMW KININOGEN
oxidised (met) KININOGEN
cytokines + growth factors (gf)
BK
des-arg-BK
ANGIOGENESIS
kallidin
des-arg-kallidin
increase expression
KININS (BK/ kallidin) /DERIVATIVES
Blood pressure regulation
Processing gf/hormones
aminopeptidase
cleave
B2R
KI-CPN + KI-CPM
vasoactive
mitogenic
motility, adhesion
* proteolysis
proteolysis
B1R
KII-ACE + KII-NEP
cleave
inactivated kinins
TK inactivation:
1. Proteolysis
2. Kallistatin
KININ RECEPTORS
KININASES
Biological effects
Inactivation

Table 1.4 Association of the activities of the KKS with tumourigenic/angiogenic factors.

Tumourigenic/angiogenic factors		Activities of the KKS
1. GFs/cytokines		• Kinin signalling potentiates that of GFs. Common signalling intermediates: NO, MAPKs, PI3K and PLC (1.1.3.3.2.2.2.1-5). • Kinin signalling upregulates cytokine production (1.1.3.3.2.2.2.6). • TK processes GFs and releases ECM-bound GFs (1.1.3.3.2.1). • Kinins increase glucose metabolism, providing energy for growth (1.1.3.3.2.2.2.7) • Kinins stimulate fibroblasts to release GFs and cytokines (1.1.3.3.2.3).
2. ECM	2.1 fibres (ECM modification)	• Kinins induce vasodilation and increased vascular permeability (1.1.3.3.2.2.1), which leads to extravascular clotting (1.1.1.3). • TK may directly cleave the ECM (1.1.3.3.2.1). • TK may promote the cleavage of ECM by other proteases (1.1.3.3.2.1).
	2.2 inflammatory cells	• Kinins induce vasodilation and increased vascular permeability (1.1.3.3.2.2.1), leading to an extravascular clotted matrix, which is chemotactic for inflammatory cells (1.1.1.3). • Kinins are important for neutrophil diapedesis (1.1.3.3.2.3). • Kinins activate leucocytes (1.1.3.3.2.3). • Kinins stimulate fibroblasts to release factors that are chemotactic for inflammatory cells (1.1.3.3.2.3).
	2.3 fibroblasts	• Kinins stimulate fibroblasts to release GFs and cytokines (that are chemotactic for inflammatory cells) (1.1.3.3.2.3).
	2.4 blood vessels	• The KKS is proinflammatory (1.1.3.3.2.3). Inflammation promotes angiogenesis (1.1.2.4.1). • Kinins and TK may promote angiogenesis by modulating the activity of growth factors/cytokines, ECM components, cell adhesion molecules and proteases – factors which direct angiogenesis (1, 2, 3, 4 of this table, 1.1.2.2, Figure 1.2). Angiogenesis promotes tumourigenesis (1.1.2.1).
3. Cell adhesion molecules		• TK activates MMPs (1.1.3.3.2.1), which have the ability to cleave cell adhesion molecules (1.1.3.2). • Kinins potentiate GF signalling, which may lead to FAK dephosphorylation, resulting in destabilisation of cell adhesion (1 of this table, 1.1.2.6). • Kinins may potentiate integrin signalling, which affects cell adhesion, motility, survival and proliferation. Common signalling pathways: activation of RhoGTPases and phosphorylation of FAK (1.1.2.6, 1.1.3.3.2.2.3).
4. Proteases		• TK cleaves and activates MMP-2 and MMP-9 (1.1.3.3.2.1). • TK may participate in a cascade-like activation of TKs, and some of these TKs have the ability to activate uPA and uPAR (1.1.3.3.2.1).

Numbers in brackets refer to sections of chapter 1.

KKS – kallikrein-kinin system; GF – growth factor; NO – nitric oxide; MAPK – mitogen activated kinase; PI3K – phosphoinositide 3-kinase; PLC – phospholipase C; ECM – extracellular matrix; TK – tissue kallikrein; MMP – matrix metalloproteinase; FAK – focal adhesion kinase; uPA – urokinase plasminogen activator; uPAR – receptor for urokinase plasminogen activator

1.1.3.3.2.6 The KKS in prostate and breast cancer

Prostate and breast tumours are the tumour types investigated in this project. The KKS may be linked to the development and progression of these cancers.

1.1.3.3.2.6.1 Introduction to prostate and breast cancer

Prostate cancer is the most common cancer and second leading cause of death in men of Western society (*49, 297, 298*). Similarly, breast cancer is the most common cancer in women of Western society and accounts for approximately 18% of female deaths in these countries (*298-300*). Androgens and oestrogens drive the growth of prostate and breast cells, respectively (*49, 300, 301*). Thus androgen ablation/anti-oestrogen treatments are the first treatment choices for metastatic prostate/breast cancer, in which other treatment options are ineffective (*49, 299*). However, in both prostate and breast cancer there is an inevitable progression of the disease to an androgen independent (AI) and oestogen independent (OI) state, respectively (*49, 302*). Once prostate/breast cancer has become AI/OI, prognosis is poor and treatment is palliative (*49, 299, 300, 303*). Mechanisms of AI/OI development include enhanced receptor (AR/OR) expression, increased AR/OR sensitivity, AR/OR sensitivity to ligands other than androgen/oestrogen, and ligand independent activation of AR/OR involving co-regulator modulation and upregulation of growth factor pathways (*49, 197, 302, 304*).

1.1.3.3.2.6.2 The role of the KKS in prostate/breast tumourigenesis and AI/OI

Several growth factors, namely EGF, transforming growth factor alpha (TGF-α; a ligand of EGFR), IGF, TGF-β, bFGF and VEGF have been implicated in the development of prostate and breast cancer and their progression to AI and OI, respectively (Table 1.5). The KKS potentiates the signalling of these growth factors (Section 1.1.3.3.2.2.2), thereby suggesting

possible KKS involvement in prostate/breast tumourigenesis and the development of AI/OI. Growth factors increase the activity of the AR (Table 1.5) through the initiation of AR phosphorylation or via phosphorylation of its co-regulators (*197*). Phosphorylation occurs either via protein kinase B or MAPK (*322*). Kinin signal transduction also involves activation of MAPK (Section 1.1.3.3.1.3.3) and therefore may positively influence AR activity by this mechanism. Similarly, growth factors activate the OR independently of oestrogen (Table 1.5) through phosphorylation of the AF-1 subunit of OR by MAPK induction, which may be potentiated by kinin signalling (Section 1.1.3.3.1.3.3). The phosphorylation of AF-1 enhances interaction of the OR with its coactivators, thereby allowing OR activation (*302, 312*).

The KKS can be linked to AI prostate cancer and OI breast cancer by factors other than its interaction with growth factors. (i) Prostatic acid phosphatase (PAP) is a protein tyrosine phosphatase, the downregulation of which is implicated in AI development (*323*). The generation of RNI and ROI, which occurs during kinin signalling (Section 1.1.3.3.2.2.2.1), results in downregulation of tyrosine phosphatases via oxidation (*247*). (ii) Oestrogen signals via myc to promote MCF7 breast tumour cell survival. Phospholipase D and PI3K are upstream of myc and are necessary for this signalling pathway (*301*). Kinins also activate phospholipase D and stimulate phosphoinositol hydrolysis, as does PI3K (Section 1.1.3.3.1.3.3). It is therefore possible that kinins could promote survival in MCF7 cells independently of oestrogen.

Also of interest, is the AI expression of PSA/hK3 in advanced prostate cancer (*196*). It is possible that PSA contributes to prostate tumour progression and AI through cleavage of IGFBP3, which results in increased availability of IGF (*49*).

1.1.3.3.2.6.3 Demonstration of the KKS in prostate and breast cancer

Tissue kallikrein and B2R are both expressed in normal prostatic tissue (*260, 274*). Clements and Muktahr (1997) demonstrated the expression of TK and B2R in prostate cancer, suggesting the presence of a functional KKS in prostate cancer (*274*). Taub *et al.* (2003) showed the expression of B1R in prostate cancer and its absence in normal tissues (*260*). In that study, stimulation of B1R promoted growth, migration and invasion of prostate tumour cells and B1R activation was shown to activate the MAPK, extracellular signal-regulated kinase (ERK). Previously, these investigators demonstrated that kinins transactivate EGFR in prostate cancer cells, resulting in ERK-mediated growth. One mechanism by which B1R could promote tumour cell migration is via activation of FAK and this activation has been demonstrated in prostate cancer cells (*260*). The co-presence of B1R and B2R is necessary for proliferation of prostate cancer cells (*324*). The cell types used in those studies were AI (*260, 324*), supporting a role for the KKS in AI prostate cancer. The presence of TK has been demonstrated both in normal and malignant breast tissue (*325*) and its involvement in the migration and invasion of OI breast cancer cells has also been demonstrated (*241*). B2R is also expressed in normal and malignant breast cells and, when stimulated by BK, promotes cell proliferation (*326-328*). The mitogenic effects of B2R stimulation in normal and malignant breast cells are dependent on ERK1/2, PKC and PLC (*327*).

1.1.4 Angiogenesis and the KKS in cancer therapy

1.1.4.1 Cancer therapy: anti-angiogenesis therapy

Although attention has been given to developing treatments for cancer for centuries, the currently available therapies (surgery, radiation, chemotherapy and hormonal therapy) are sometimes not adequate to save lives (*2*). According to the WHO, of the 15 million it

predicts to be diagnosed with cancer in the year 2020, one third will die (*329*). The major difficulties with available therapies are non-selectivity (resulting in toxicity) and resistance.

Anti-angiogenesis is among the new treatment approaches under development (*330*). These approaches also include photodynamic therapy (*331*), immuno-therapy (*332*) and gene therapy (*333*). These treatments may be used to complement conventional therapies. Targeting of angiogenesis is attractive since most tumours are angiogenesis-dependent (Section 1.1.2.1) and angiogenesis is largely restricted to pathological conditions (Section 1.1.2.2). Several anti-angiogenic agents are currently being tested in clinical trials. These include endogenous inhibitors of angiogenesis, such as angiostatin and endostatin, synthetic inhibitors of endothelial cells, such as TNP-470 and combretastatin, MMP inhibitors and PAIs (*334*). Antibodies against, or synthetic inhibitors of, angiogenic molecules, such as VEGF, VEGFR, Tie-2 and $\alpha_v\beta_3$ are also in trials (*330, 334*). Antibodies against angiogenic molecules that are expressed solely by tumour vessels may be used to deliver immuno-competent cells or toxic agents (*335*). A general lack of toxicity has been observed for anti-angiogenic treatments (*330, 335*). There is also a lack of resistance development for anti-angiogenic therapies that directly target the genetically stable host endothelial cell, rather than tumour cells (*330*).

However, resistance may develop following treatment with indirect anti-angiogenic agents. Indirect anti-angiogenic agents inhibit angiogenesis through either blocking the production of pro-angiogenic agents by tumour cells or blocking the receptors for these on endothelial cells, as opposed to direct anti-angiogenesis inhibitors (*330*). Resistance develops as a result of tumour ability to change the expression of angiogenic molecules. For example, breast tumours become less dependent on VEGF (and unresponsive to VEGF antibodies; (*330*)) and switch to increased FGF expression with progression (Table 1.5). Several targets for

anti-angiogenic treatment need to be investigated in order to provide alternatives to counteract resistance that may develop from indirect anti-angiogenic agents. Furthermore, the molecules involved and their relative contribution to angiogenesis need to be characterised for different tumours at different stages (*330*). This is because the mechanisms of angiogenesis vary depending on the microenvironment of the different organs (*318, 336*). Thus, much work remains to be done before anti-angiogenesis therapies reach the clinic.

1.1.4.2 Cancer therapy and the KKS

The KKS has recently been identified as a potential angiogenic target (Section 1.1.3.3.2.5) that may contribute to the several candidates required for effective therapy (Section 1.1.4.1). Angiogenesis is inhibited by BK antagonists and kallistatin (*108, 293*) (Section 1.1.3.3.2.5). Considering the link between the KKS and OI/AI (Section 1.1.3.3.2.6.2), it is possible that KKS inhibitors may be useful in treating advanced prostate and breast cancers. Evidence suggests that this is the case for both prostate and breast cancer. BK antagonists inhibit advanced AI prostate cancer growth *in vitro* (*293, 337*) and *in vivo* (in nude mice) (*282, 293*), and a TK inhibitor was shown to inhibit invasiveness of OI breast cancer cells in *in vitro* and *ex vivo* models (*241*).

1.2 Models investigating angiogenesis and tumour-associated angiogenesis, and measurement of factors involved

There is a variety of *in vivo* and *in vitro* models available for investigating aspects of angiogenesis or tumour-associated angiogenesis. The various models, and the measurement of angiogenesis and angiogenic factors in these models, are considered below. Advantages and disadvantages of these models for studying angiogenesis are also discussed.

1.2.1 *In vivo* models

1.2.1.1 Chick chorioallantoic membrane (cCAM) assay

The cCAM assay involves implantation of grafts onto the cCAM and monitoring of the subsequent graft vascularisation through a glass window inserted in the shell (*338*). This model may be used to study the effect of substances on angiogenesis by applying coverslips lyophilised with the substances (including tumour-derived substances or tumour cells) to the cCAM (*339*). Angiogenesis (and thereby the effect of factors on angiogenesis) was initially quantified using an observer-dependent, subjective grading system (*57, 340*). However, at present, angiogenesis may be more accurately measured by computer-assisted vessel counting, which may be performed macroscopically or microscopically (on histological sections of the membrane) (*340*).

1.2.1.2 Rabbit corneal neovascularisation model

The corneal neovascularisation model is a similar concept to the cCAM assay. Insertion of substances, tumour cells or tumour extracts into the cornea, allows their angiogenic effects to be determined (*341*) through easily accessible monitoring and measurement (subjective or computer-assisted) of corneal vascularisation using a slit-lamp stereomicroscope (*340*).

1.2.1.3 Pouch/chamber assays

Pouch/chamber assays involve graft implantations or implantation of exogenous materials that are impregnated with angiogenic/test substances into the hamster cheek pouch, anterior chamber of the eye, or subcutaneously created dorsal air sacs (*57, 339*). Exogenous materials include synthetic polymers and natural substances, such as matrigel which is an extraction from the basement membrane matrix of Englebreth-Holm-Swarm tumours (from lathyritic mice) that contains ECM components and growth factors (*340, 342*). Models using transparent chambers allow for subjective/computer-assisted measurement of angiogenesis

over time using transillumination technology, whereas models using exogenous implants allow measurement at one time-point only. Following 1-2 weeks, exogenous implants may be examined for an angiogenic response by measurement of haemoglobin content or by immuno-histochemical analysis using endothelial markers (*340*).

1.2.1.4 Advantages and disadvantages of *in vivo* models

A common disadvantage of *in vivo* models is that tissue injury and subsequent inflammation that occurs in the process of graft/polymer/matrigel implantation may itself induce angiogenesis, interfering with interpretation of results (*340, 343*). The presence of naturally occurring blood vessels in *in vivo* models also obscures results. The corneal model is an exception since the cornea is normally avascular (*338*). *In vivo* models are also generally complicated, expensive and may require specific surgical skills (*340*). Due to the many cell types and factors present, *in vivo* models cannot answer certain questions (*344*). For example, the effect of tumour cell metabolites on endothelial cell activities cannot be determined in an *in vivo* model as metabolites of various cells are present.

1.2.2 *In vitro* models

In vitro models have an advantage over *in vivo* models in that they are generally less expensive, simple, variables are more easily controlled than in *in vivo* models, and they are comparatively easy and quick to execute (*340*). Some *in vitro* models have an added advantage of allowing various aspects of angiogenesis, namely proliferation, migration, invasion, and differentiation, to be studied separately (Sections 1.2.2.1 and 1.2.2.2). *In vitro* models also allow analysis of the effect of a single factor on angiogenic events. In the future, identification of the precise contribution of a specific gene to an angiogenic event will be possible with genetically modified endothelial cells (*345*). However, data derived from *in vitro* models is not as conclusive as that arising from *in vivo* models (*340*).

Therefore, *in vivo* models are required to confirm or test implications of results obtained with *in vitro* models. Specific interactions between particular cell types or factors that cannot be answered by *in vivo* models may be analysed by *in vitro* models that are limited to 1 or 2 cell types. *In vitro* models are either 2D or 3D and involve either the culture of 1 cell type or 2 cell types together (co-culture) or a tissue explant.

1.2.2.1 2D models: endothelial proliferation, migration and differentiation

Cells may be cultured onto a plate and fed with a medium containing nutrients until they form a confluent 2D monolayer. Conditioned medium (i.e. medium that contains cell metabolites derived from a growing mass of cells) may be included in the mixture fed to cells to improve their growth at low cell densities (*346*). A similar concept is the "challenging" of endothelial cells with conditioned medium from a tumour cell culture in order to study the effects of tumour cell metabolites on angiogenesis (*71, 296*).

Proliferation and migration aspects of angiogenesis may be considered separately using 2D models. Various proliferation assays, for example tetrazolium salt assays and assays involving incorporation of thymidine or 5-bromodeoxyuridine into DNA, are available and may be performed on endothelial cells in 2D culture (*340, 347*). Migration may be investigated in isolation with a 2D "wound healing" migration assay (*260*). This involves disruption of a 2D monolayer and counting of cells that subsequently migrated into the "wounded area" after a set period of time. The Boyden chamber assay may also be used to measure endothelial motility and the effect of specific factors on this aspect of angiogenesis. Endothelial cells are plated in upper chambers, which are separated from lower chambers by a filter. The lower chambers contain a test substance and the cells that have migrated through the filter in response to the test substance may be stained and counted (*338, 340*).

When maintained for long periods, 2D cultures of endothelial cells differentiate to form capillary-like networks (or cord-like structures) representing, and allowing study of, the latter stages of angiogenesis (*71*). This stage of angiogenesis may be analysed by measuring the number and length of tubes, the area covered by the capillary network or the number of branches in the cord-like structures (*340*). The differentiation of endothelial cells into cord-like structures is mostly studied using 3D matrix models (Section 1.2.2.2), which incorporate all aspects of angiogenesis - endothelial proliferation, migration and differentiation, as well as invasion.

1.2.2.2 3D models: endothelial proliferation, migration, invasion and differentiation

Culturing of endothelial cells on collagen, fibronectin or fibrin gels or matrigel results in a 3D angiogenic model as endothelial cells invade the matrix and form networks within these gels (*296, 348, 349*). Collagen gels are useful for identifying factors that induce invasion or cord-like structure formation (*345*). Matrigel represents *in vivo* angiogenesis conditions (in the later stages) more accurately than other 3D gel models and is the most commonly used to investigate endothelial differentiation into cord-like structures (*338, 345*). Matrigel may also be incorporated into the Boyden chamber assay in order to test the effect of factors on migration and invasion aspects of angiogenesis simultaneously (*340, 350*). In addition to staining and counting cells that have migrated and invaded through the gel-coated filter, the option of radiolabelling endothelial cells, followed by scintillation counting, is available to measure migration and invasion (*350*).

A 2D cell culture system has an advantage over a 3D model in that it is much simpler to analyse and visually interpret with microscopy-based methods (*272*), which include immuno-chemistry and polymerase chain reaction (PCR). Immuno-labelling and PCR methods allow detection/quantification of the presence and expression of specific factors in

the angiogenic models, respectively. If thick gels with embedded cells are used in 3D models, they must be fixed, embedded in supporting medium and sectioned prior to immuno-labelling (*351*). Unsectioned, thin gels with cells plated on top do not require this preparation; however, a proportion of cells (that penetrate the gel deeply) are not accessible for labelling or analysis. Another disadvantage of 3D models is that, although they may be analysed by solution PCR or reverse transcriptase (RT-) PCR (*352*), they are not suitable for *in situ* PCR analysis. However, since cellular shape and environment influence gene expression and biological behaviour of cells (*345*) (Section 1.1.2.5), the complexity of 3D models (although disadvantageous in some respects) has the advantage of more closely representing *in vivo* conditions than 2D models (*344*).

1.2.2.3 Co-culture models

Angiogenesis is influenced by many cell types present in the environment of endothelial cells (Section 1.1.2.4). Thus, more accurate representations of *in vivo* conditions are co-culture models and the aortic ring model (*349*). Co-culture models may be used to study the influence of one cell type on another, such as the influence of smooth muscle cells on endothelial cell angiogenic activity (*349*) and the influence of tumour cells on endothelial cells and *vice versa* with respect to expression of KKS components (*272*). The aortic ring model involves culturing of rat or mouse aorta in biological matrices, which results in out-branching of microvessels (*353*). These microvessels may be quantified subjectively or using computer-assisted image analysis. Mural cells and fibroblasts are also present in this model. Thus, the aortic ring model has aspects of both *in vivo* and *in vitro* models and it is also sensitive, reproducible and quantitative. However, large blood vessels used in this type of study are a disadvantage of the aortic ring model as, *in vivo*, new vessels formed by angiogenesis are generated from microvessels (*340*). Additionally, it is not easy to identify the exact role of each cell type in the events leading to capillary tube formation (*345*).

1.2.3 Choice of models for the present study

In the present study, *in vitro* models were chosen in preference to *in vivo* models due to the complicated nature and cost of *in vivo* models, as well as the surgical skill often required in their implementation (Section 1.2.1.4). Further, *in vivo* models are not conducive to the study of specific cell interactions, whereas *in vitro* models are suitable for this purpose.

A 2D "challenge" model (Section 1.2.2.1), involving the challenging of endothelial cells in culture with tumour-conditioned medium and *vice versa*, was chosen to investigate the effect of tumour cell metabolites on endothelial cell expression and secretion of the KKS proteins, as well as their effect on endothelial cell proliferation and *vice versa*. Endothelial and tumour cells were also co-cultured on a 2D surface in order to investigate the role of the KKS in direct endothelial-tumour cell interactions.

1.3 The present study: rationale, aims and hypothesis

Due to the potential tumourigenic and angiogenic role of the KKS (Section 1.1.3.3), and the potential therapeutic benefits of targeting the KKS involvement in tumourigenesis/tumour-associated angiogenesis (Section 1.1.4), the present study aims to further investigate the role of the KKS in tumour angiogenesis. It extends the work of Naidoo (*354*), which focused on neuroblastomas and cervical carcinoma, by using cells of two different tumour types, namely prostate (DU145) and breast (MCF7) cancer. Prostate and breast tumours were chosen as they develop in men and women, respectively, more frequently than any other cancer type, and understanding of their pathogenesis is incomplete (*355*). There is also evidence for the contribution of the KKS to tumour progression and hormone independence in both prostate and breast cancer (Section 1.1.3.3.2.6). Further, the KKS may be a therapeutic target for these cancers, particularly for the AI/OI forms, for which therapy is currently inadequate (Section 1.1.3.3.2.6.1). The present study also extends the work of Naidoo, in which

macrovascular endothelial cells were used, by using microvascular endothelial cells (MVECs). Angiogenesis *in vivo* occurs with microvessels rather than macrovessels (*347*) and therefore the culture of MVECs more closely represents angiogenesis *in vivo*.

Since the activities of the KKS have been linked to the promotion of tumourigenesis and tumour angiogenesis, and since there is some evidence for a tumourigenic role for the KKS in prostate and breast cancer, it is expected that the KKS would promote tumour angiogenesis and therefore tumourigenesis in prostate and breast cancer. To test this hypothesis, the present study aimed to investigate the role of the KKS in tumour angiogenesis, specifically in prostate and breast cancer by:

- determining the effect of tumour cell metabolites on KKS regulation (expression and secretion) in endothelial cells and *vice versa*
- determining the effect of tumour cell metabolites on endothelial cell proliferation and *vice versa*, and relating this to the effect of these metabolites on KKS regulation
- investigating the role of the KKS in direct endothelial cell-tumour cell interactions

To do this, the following objectives were fulfilled:

- optimisation of *in vitro* challenge and co-culture endothelial-tumour cell models in order to investigate MVEC-breast/prostate tumour cell interactions
- investigating the KKS within these models using immuno-cytochemistry
- investigating the KKS within the challenge model using enzyme-linked immunoassay (ELISA)
- measurement of endothelial and tumour cell proliferation within the challenge model using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 - MATERIALS AND METHODS

2.1 Ethics

Ethical approval was obtained from the Biomedical Ethics Committee of the Nelson R Mandela School of Medicine, University of KwaZulu-Natal. Human cell lines were commercially obtained and human salivary gland, kidney and spinal cord control tissues were previously collected from the KwaZulu-Natal Department of Health State Mortuary with the assistance and permission of a forensic pathologist. TK, B1R and B2R antibodies (*354*), used for immuno-chemistry and ELISA, had been previously raised in and collected from animals [by the research unit in the Department of Clinical and Experimental Pharmacology, Faculty of Medicine, former University of Natal, in conjunction with the Biomedical Resource Centre (BRC), former University of Durban-Westville] with the ethical permission of the former University of Durban-Westville Ethics Committee.

2.2 Cell culture

2.2.1 Source of cell lines and constituents of cell culture media

A dermal microvascular endothelial cell (dMVEC) cell line (passage 3) was purchased from Clonetics, BioWhittaker (Walkersville, USA). The frozen 1 ml aliquot was stored in a –85°C ultrafreezer (NuAire, USA) until use. The medium (BioWhittaker) used to culture dMVECs consisted of endothelial basal medium (EBM-2, 500 ml) pre-supplemented with foetal bovine serum (FBS, 25 ml), recombinant human fibroblast growth factor-beta (rhFGF-β, 2 ml), vascular endothelial growth factor (VEGF, 0.5 ml), hydrocortisone (0.2 ml), R^3-insulin-like growth factor (R^3-IGF-1, 0.5 ml), ascorbic acid (0.5 ml), human endothelial growth factor (hEGF, 0.5 ml) and antibacterial agent (GA-1000, 0.5 ml). Medium was re-constituted in a sterile field: a class II microbiological cabinet with vertical laminar airflow and curtain. Once re-constituted, medium was stored at 4°C for not more than 3 months.

Frozen 2 ml aliquots of DU145 prostate tumour and MCF7 breast tumour cell lines were commercially obtained from Highveld Biological, National Repository for Biological Material of the Cancer Association of South Africa (Sandringham, SA). On arrival, they were immediately stored at –85°C. DU145 medium consisted of Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with 10% FBS, penicillin-streptomycin-fungizone (PSF; 200 μg/ml) and L-glutamine (2 mM). Similarly, Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, PSF (100 μg/ml), L-glutamine (4 mM) and sodium pyruvate (1 mM) constituted medium used to culture MCF7 cells. All tumour media constituents were purchased from BioWhittaker. Media were re-constituted in a sterile field, and thereafter stored at 4°C for not more than 2-3 weeks.

2.2.2 Preparation of work area and equipment

Culturing of cells and preparation of media were performed in a sterile biological cabinet. The biological cabinet and all cell culture equipment stored therein (pipettes, pipette tips and culture dishes) were subjected to UV light (100-400 nm) for an hour prior to use. Glass pipette tips (LASEC, SA) and plastic pipette tips (Greiner, Germany) were autoclaved at 121°C for 20 minutes. [The glass pipette tips were used in conjunction with a hand-held electronic pipette (Powerpette Plus, Jencons, USA), whilst plastic pipette tips were used in conjunction with a mechanical pipette (Gilson, France)]. All work surfaces and cell culture equipment were swabbed with 70% ethanol (Merck, SA) and/or antibacterial solution [Virkon (Antec, SA), 0.02 g Virkon in 1ml distilled, deionised water (ddH_2O)] and left to settle for 10 minutes before working. [Water was distilled and deionised by a Milli-U6 water purification system (Millipore, SA).] Items such as media bottles and centrifuge tubes (Greiner) were swabbed before they were introduced into the biological cabinet.

Gloves were sprayed with 70% ethanol immediately prior to opening the incubator, handling the cultures and working in the biological cabinet.

2.2.3 Thawing of cryo-stored cell cultures, plating and maintenance in culture

Cryo-vials (Corning-Costar, USA) were immediately transferred from the –85°C ultrafreezer to a 37°C waterbath and allowed to thaw, with frequent agitation, for 3 minutes (*354, 356*). The contents of the vials were pipetted into either p60 (21 cm^2) or p100 (55 cm^2) culture dishes (Corning-Costar) containing fresh, pre-warmed cell-specific media [warmed for 10-15 minutes in a humidified incubator (Function Line, Heraeus, Germany), at 37°C/5% CO_2]. The final volumes of medium used for a p60 and p100 were 4 ml and 8 ml, respectively. The surface area for plating was determined by the following formula:

Surface area (cm^2) = number of cells per vial / plating density (cells/cm^2)

The plating density of the primary dMVEC cell line and tumour cell cultures was recommended by the respective suppliers. Thereafter, plating densities that yielded approximately 60% confluency after 2 days in culture were used as this was required for challenge experiments (Section 2.2.6.1). These plating densities were 2000 cells/cm^2, 3500 cells/cm^2 and 1500 cells/cm^2 for dMVECs, DU145 cells and MCF7 cells, respectively.

After plating, cells were incubated in the humidified incubator for 24 hours, at which point most cells had attached to the bottom of the culture dish. Following this, the medium from the culture dish was aspirated and cells were supplied with fresh pre-warmed medium. Only one cell line was fed at a time, with a half-an-hour interval in between feeding different cell lines in order to minimise cross-contamination. Subsequently, the medium was changed every 48 hours or when the phenol red in the basal medium turned yellow, indicating a need

to feed the cells fresh medium. The growth of cultures was monitored using a phase contrast, bright field inverted microscope (DMIL, Leica, Germany) that was swabbed with 70% ethanol prior to use.

2.2.4 Passaging

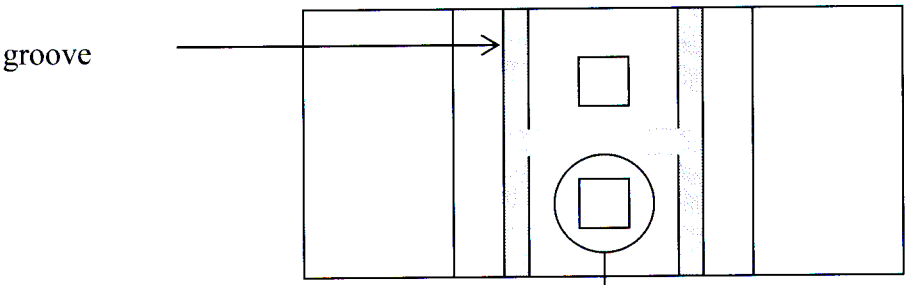
Cells were passaged upon reaching 80-90% cell confluency (*354, 356*). Trypsin-versene (BioWhittaker) was used to enzymatically detach cells from the surface of the culture dish. First, medium was removed from the culture dish and replaced with pre-warmed phosphate buffered saline (PBS, BioWhittaker), swirled gently for 30 seconds and then aspirated and discarded in order to remove serum proteins and debris. The cells were then incubated at 37°C with pre-warmed trypsin. The volume of trypsin used was 2 ml for a p60 and 4 ml for a p100. Upon rounding (i.e. retraction of membrane extensions) of at least 90% of the cells, trypsin was immediately inactivated by adding medium at twice the volume of trypsin used. Cells were not incubated with trypsin for more than 3 minutes (in order to minimise irreversible trypsin-induced cell damage) and were frequently viewed with the inverted microscope during this period in order to determine when to stop the trypsin activity. The trypsinised cells were then centrifuged at 1000 rpm/180 x g for 5 minutes (Megafuge 1.0R, Heraeus, Germany) and resuspended in fresh growth medium. The volume of cell suspension required to plate cells at the density desired (optimised to result in 60% confluency 2 days after plating, Section 2.2.3) was calculated using the following formulas:

number of cells required = plating density (cells/cm^2) x surface area (cm^2)

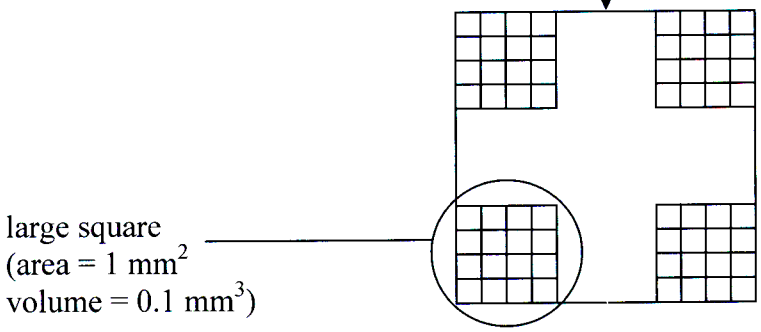
volume of cell suspension (ml) = number of cells required/cell concentration (cells/ml)

The concentration of cells in the cell suspension was determined using a haemocytometer (Neubauer, Assistant, Germany). A haemocytometer is a slide that draws fluid by capillary action over its coverslipped grid when fluid is pipetted into a groove on either side of the grid (Figure 2.1). The grid forms 8 large squares (4 large squares on either side of the slide) each of volume 0.1 mm^3. These are each subdivided into 16 smaller squares. Cells were counted with a phase contrast, bright field microscope (DMLB, Leica, Germany). Cells on the top and left lines of the smaller squares (if lines were adjacent to those of other small squares) were excluded from the count to avoid counting the same cell twice (*356*). If the combined cell number of 4 large squares was less than 100, the 4 squares on the other side of the slide were also counted and an average taken. Since 0.1 mm^3 is equivalent to 10^{-4} ml, the average cell number for the large squares was multiplied by 10^4 to determine the cell concentration of the cell suspension (cells/ml). In order to avoid including non-viable cells in the calculation of cell concentration, a trypan blue viability assay was performed in conjunction with cell counts. This is a rapid, well-established method to test viability, in which trypan blue may be substituted for other dyes (erythrosine and naphthalene black) to which intact cell membranes are also impermeable (*356*). Trypan blue (0.4%, Sigma, St. Louis, USA) was added to cell suspension in an eppendorf tube (Eppendorf, UK) in a 1:1 ratio prior to pipetting 25 µl of the mixture into the groove on either side of the haemocytometer. Cells that had taken up the blue dye (cells with irreversibly compromised membrane integrity i.e. non-viable cells) were excluded from the cell count. Thus, only viable cells that had excluded the dye were counted. Cell counts were performed within 5 minutes since trypan blue is toxic to cells and this results in indiscriminate uptake of the dye after about 10 minutes.

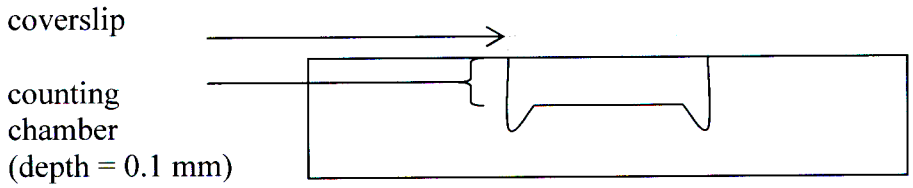
(i)


groove

(ii)


large square
(area = 1 mm2
volume = 0.1 mm3)

(iii)


coverslip
counting
chamber
(depth = 0.1 mm)

2.2.5 Cryo-preservation

A cell bank was built up simultaneously with the passaging of cells to ensure sufficient stocks for performing challenge and co-culture experiments. The proportion of cells stored and the proportion plated depended on the cell yield and the most favourable balance between the quantities of cells required in culture and the level of stock. (A cell log was kept in order to keep track of the amount of stock from each passage.) After performing cell counts and plating, dimethylsulfoxide (DMSO, Sigma) was added to the remainder of the cell suspension such that the final volume comprised 10% DMSO (*356*). The cell count was then repeated for the purpose of recording the cell number per cryo-vial stored. The cryo-vials containing the 10% DMSO-cell suspension mixture were transferred to a polystyrene container in a –85°C ultrafreezer within 20 minutes of adding the DMSO but not before 10 minutes of exposure to DMSO (*354*). This was to ensure enough time for DMSO to penetrate the cells while minimising the toxicity of over-exposure to DMSO. DMSO is a cryo-protectant that penetrates cells and increases the intracellular solute concentration thereby minimising damaging "solution effects" on cooling (*357*). It also either decreases the amount of intracellular ice formed (*357*) or promotes intracellular vitrification (formation of an amorphous protein-sugar-water glass matrix) instead of lethal intracellular ice crystal formation upon cooling (*358*). The cooling rate achieved by insulating cells in a polystyrene container in a –85°C ultrafreezer is approximately 1°C per minute (*356*), a cooling rate found to yield sufficient cell survival for most animal cells (*356, 359*). The day after storing the cells, they were transferred to a cryo-vial freezer box in the –85°C ultrafreezer. When the use of cells in stock was required to perform experiments, thawing of cryo-preserved cells was performed fairly rapidly [in order to minimise damage on thawing, which could result in formation of large lethal intracellular ice crystals if warming is too slow (*358*)] by transferring them to a 37°C waterbath with frequent agitation for no longer than 3 minutes (*356*) (Section 2.2.3). The contents of cryo-vials were pipetted onto a plate of pre-

warmed medium immediately after thawing and medium was changed 24 hours later, thereby minimising cell damage due to DMSO exposure.

2.2.6 Endothelial-tumour models

The endothelial-tumour challenge and co-culture models of Naidoo (2005) were now optimised for dMVECs, DU145 cells and MCF7 cells (*354*). Cells growing in culture were trypsinised and used for challenge/co-culture experiments or cryo-preserved cells were thawed, first plated onto a p60 or p100, grown to confluency, trypsinised and then used for experiments. Only dMVECs of between passages 4 and 12 were used in order to ensure that they retained their characteristics. Since tumour cells are immortal, tumour cells of any passage number were used in experiments.

2.2.6.1 2D Challenge model

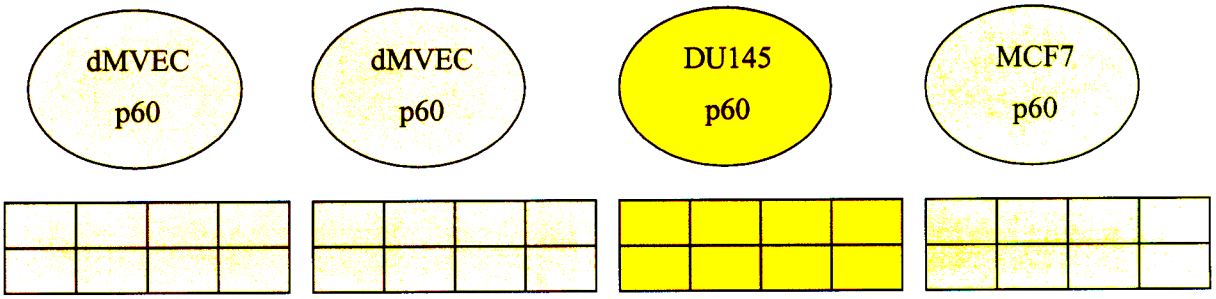
The dMVECs, DU145 cells and MCF7 cells were seeded onto 8-well glass chamber slides (Iwaki, Japan) at 2000 cells/cm^2, 3500 cells/cm^2 and 1500 cells/cm^2, respectively. These optimal densities resulted in cells reaching approximately 60% confluency after 2 days. Cells were simultaneously plated onto a p60 dish for each cell line. Plating and maintenance of cultures is described in Section 2.2.3. After 2 days in culture (at a confluency of approximately 60%), used (conditioned) media were collected from the p60 dishes with dMVEC, MCF7 and DU145 cell lines, respectively. Used media were then filtered through 0.45-μm filter-syringes into 15 ml tubes. Used medium from the dMVEC line was mixed with both fresh DU145 medium and fresh MCF7 medium in ratios of 0%, 10%, 25% and 50% (challenge media). Similarly, used media from the DU145 and MCF7 tumour cell lines were each mixed with fresh dMVEC medium in ratios of 0%, 10%, 25% and 50%. The various challenge media were then warmed in a waterbath at 37°C. Thereafter, the tumour cells and endothelial cells in the chamber slides were challenged with the different ratios of

used/conditioned media (containing metabolites) from endothelial cells and tumour cells, respectively, in duplicate, as depicted in Figure 2.2. After 24 hours, challenge media were aspirated from the chamber slides and saved for the TK ELISA (Section 2.3.3). For each cell line, challenge medium was replaced by serum-free cell-specific medium and cell lines were incubated for a further 24 hours. Thereafter, cells were rinsed in PBS and fixed in acetone (Merck, SA)/methanol (Merck) in a ratio of 1:9 (v/v) for 20 minutes at – 20°C. Thereafter, immuno-cytochemistry was performed on the cells.

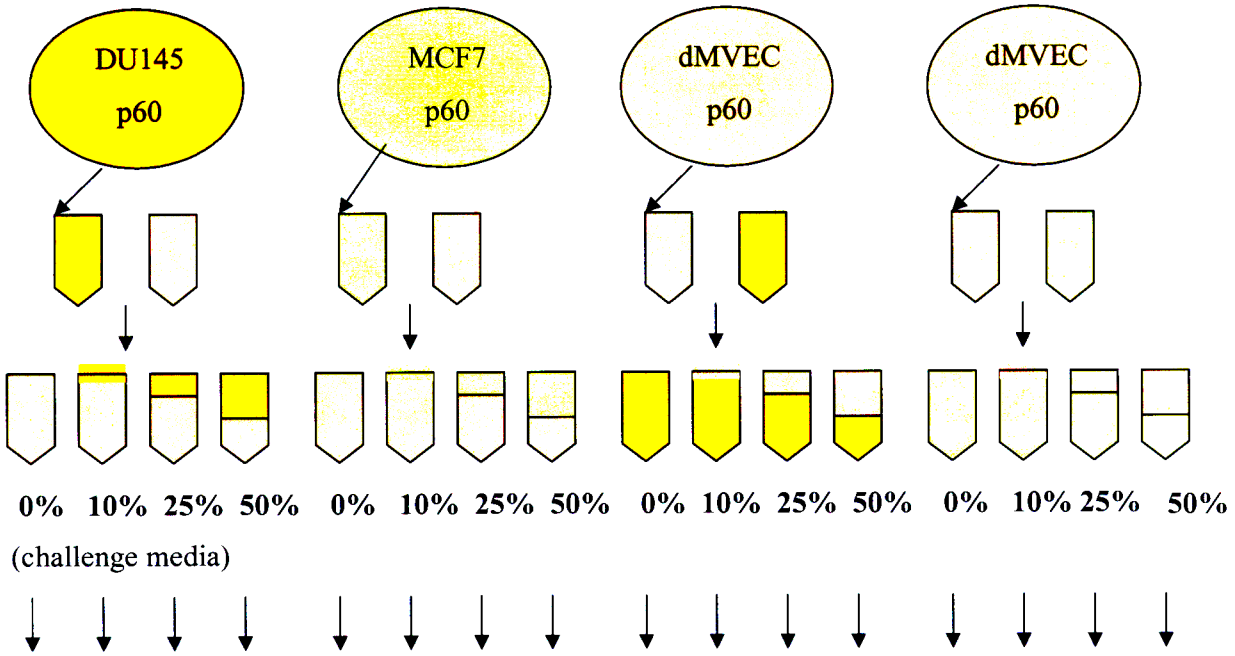
2.2.6.2 2D Co-culture model

Tumour cells were plated onto p60 culture dishes and weaned onto dMVEC medium in order to prepare for co-culture of tumour cells with dMVECs. The dMVECs were seeded onto glass chamber slides at an optimal density of 2000 cells/cm^2 that resulted in their adherence and establishment 24 hours after plating, at which point the tumour cells on the p60 dishes were 80-90% confluent. The tumour cells were then trypsinised (Section 2.2.4) and the DU145 and MCF7 cells added at 2500 cells/cm^2 and 2000 cells/cm^2, respectively, to the glass chamber slides with the dMVECs. Maintenance of these co-cultures is described in Section 2.2.3. Once the tumour cells had begun to proliferate in the co-cultures and heterogeneous cell contact was observed with an inverted microscope (typically within 24 hours), the co-cultures were serum-cleared. After a further 24-hour-incubation, cells were rinsed and fixed as for the challenge model (Section 2.2.6.1), followed by immuno-cytochemistry. Figure 2.3 is a diagrammatic representation of the co-culture model.

1. Plating and maintenance in culture



2. Preparation of different ratios of challenge media



3. Challenging of cells in chamber slides

dMVEC challenged with DU145 medium			
0	10	25	50
0	10	25	50

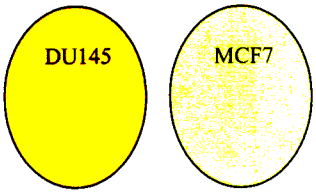
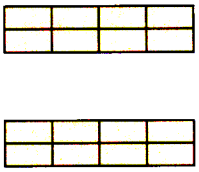
dMVEC challenged with MCF7 medium			
0	10	25	50
0	10	25	50

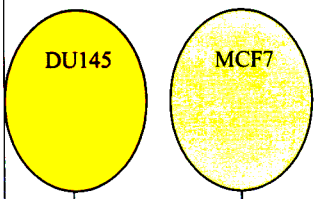
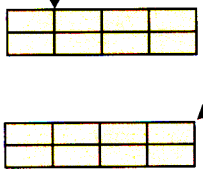
DU145 challenged with dMVEC medium			
0	10	25	50
0	10	25	50

MCF7 challenged with dMVEC medium			
0	10	25	50
0	10	25	50

4. Serum clearing

5. Fixing

Cell line	Day 1	Day2	Day3	Day 4
MCF7 + DU145	Tumour cells plated 	Tumour cells fed 100% cell-specific medium	Tumour cells fed 50% cell-specific medium and 50% dMVEC medium	Tumour cells fed 100% dMVEC medium
dMVEC				dMVECs plated  Chamber slides

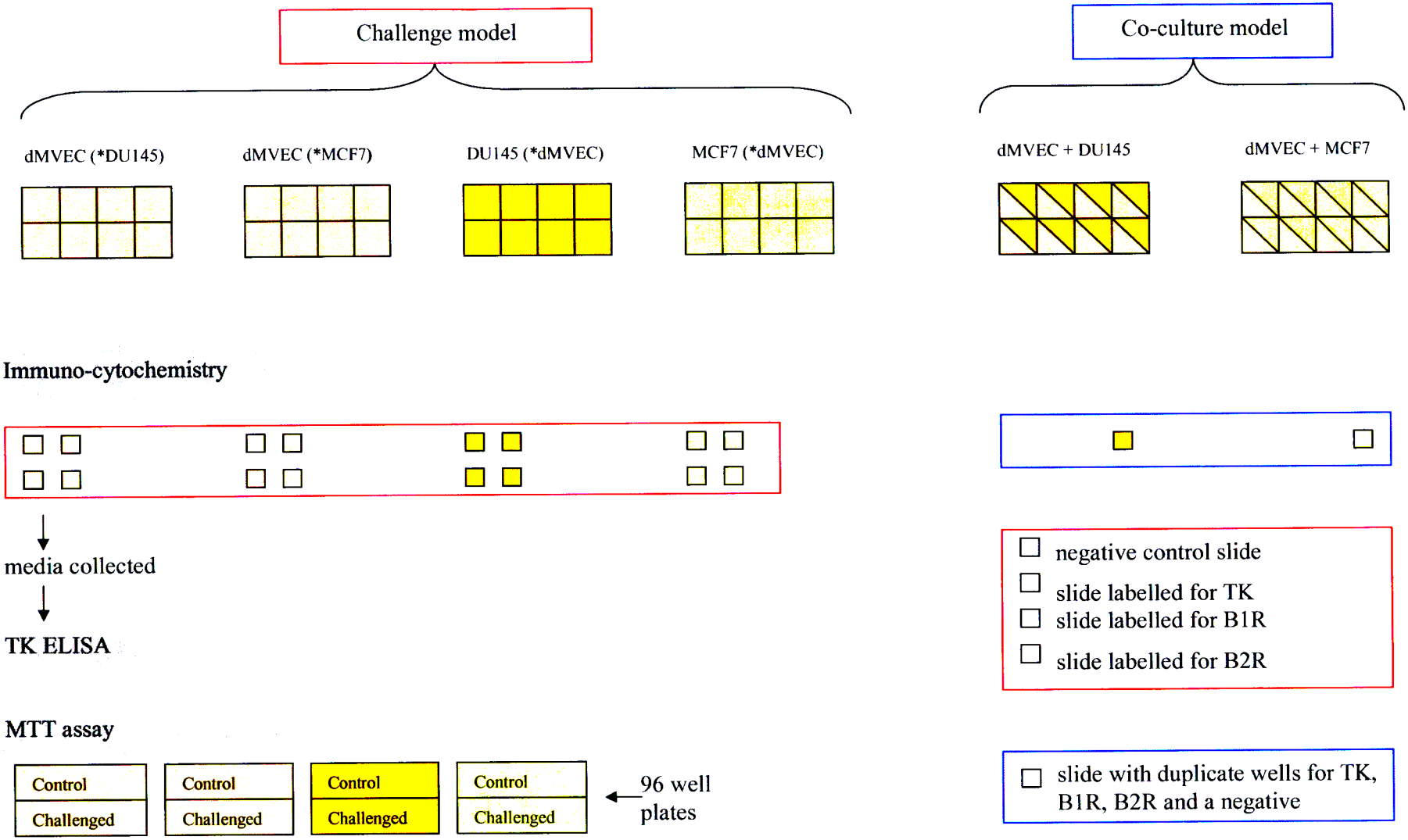
Cell line	Day 5	Day 6	Day 7
MCF7 + DU145	80-90% confluent  Trypsinised and plated	Co-cultures serum-cleared when heterogeneous cell contact observed  dMVEC-DU145 co-culture	Co-cultures fixed
dMVEC	 dMVECs established	 dMVEC-MCF7 co-culture	

2.3 Analysis of challenged cells and co-cultured cells

2.3.1 Overview

Fixed challenged and co-cultured cells were analysed by immuno-cytochemistry (Section 2.3.2.3 and 2.3.2.4) followed by quantification of labelled proteins in challenged cells (Section 2.4). This allowed for both intracellular localisation of TK, B1R and B2R with subsequent relation to their functions, and determination of how the challenge metabolites affected the intracellular levels of these proteins. Additionally, media were collected from slides of the 2D challenge model after challenging and analysed by ELISA for TK protein concentration (Section 2.3.3) in order to relate secretion of TK to intracellular protein levels (measured by immuno-cytochemistry) and the concentration of tumour/endothelial challenge metabolites. The challenge model was also performed in a 96-well plate and these cells were analysed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for cell proliferation (Section 2.3.4) in order to ascertain the effect of challenge metabolites on their proliferation.

A set of 4 slides (including slides labelled for TK, B1R and B2R and a negative control slide) was required for immuno-cytochemistry analysis for each challenge combination. The 4 challenge combinations are depicted in Figure 2.2. For the MTT assay analysis, a 96-well plate for each challenge combination with a respective control (Section 2.3.4.2), performed with 5 replicates for each challenge condition and control, was required. A slide (including duplicate wells for TK, B1R, B2R and a negative) for each co-culture combination (dMVEC-DU145 and dMVEC-MCF7) was required for analysis by immuno-cytochemistry. The overall study design described above is depicted in Figure 2.4. A brief background and detailed description of the methods of analysis used in this project follows.

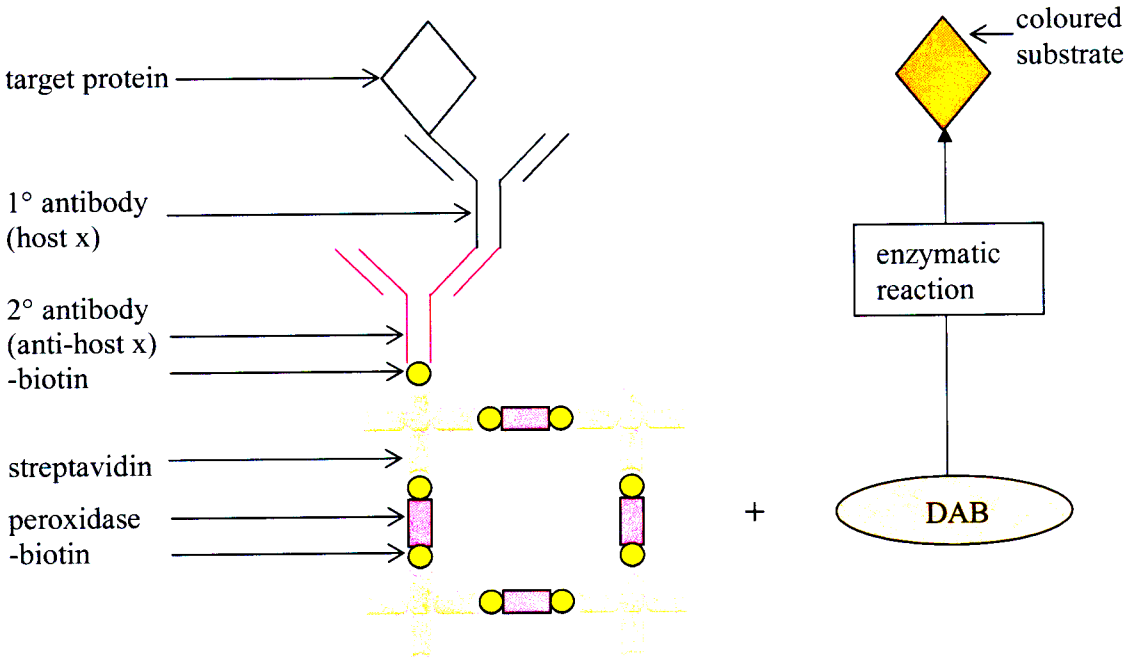

Challenge model
dMVEC (*DU145)
dMVEC (*MCF7)
DU145 (*dMVEC)
MCF7 (*dMVEC)
Co-culture model
dMVEC + DU145
dMVEC + MCF7
Immuno-cytochemistry
media collected
TK ELISA
MTT assay
Control
Challenged
96 well plates
negative control slide
slide labelled for TK
slide labelled for B1R
slide labelled for B2R
slide with duplicate wells for TK, B1R, B2R and a negative

2.3.2 Immuno-chemistry

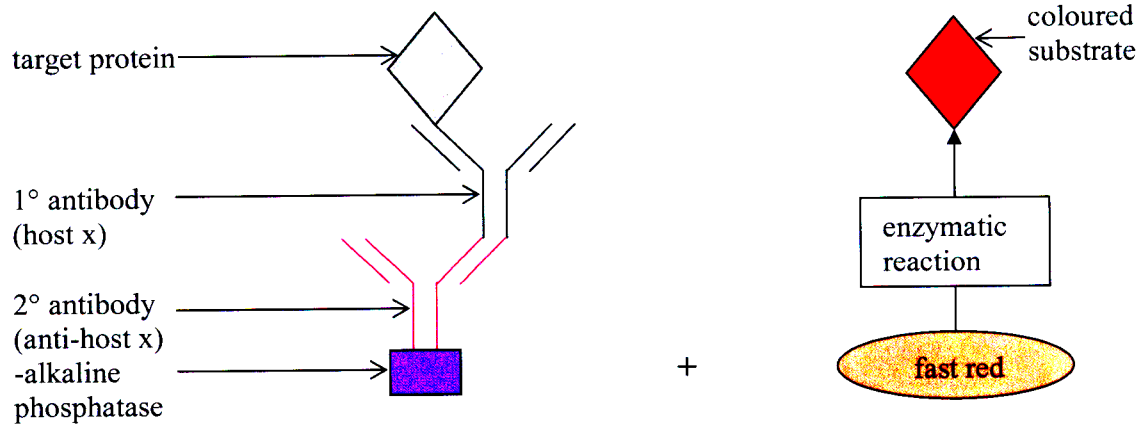
2.3.2.1 Background

Immuno-chemistry allows visualisation of specific cell proteins through the binding of detectable primary antibodies that are specific for these proteins (*360, 361*). These primary antibodies can be detected by the binding of a secondary antibody that is conjugated to a marker. Binding of a secondary antibody to a primary antibody is achieved by the selection of a secondary antibody that will react with antibodies from the host of the primary antibody. For example, if the primary antibody used is rabbit anti-human TK antibody then the secondary antibody used would be an anti-rabbit antibody conjugated to a marker. Markers may be fluorescent dyes (detected by a fluorescent microscope), gold/silver (detected by a light microscope) or enzymes that convert substrate to a coloured product (detected by a light microscope) (*109, 360, 361*). The most common enzyme marker is horseradish peroxidase and the most useful and widely used substrate for peroxidase is diaminobenzidine (DAB) (*361*). This enzymatic reaction is most often employed in the avidin-biotin complex (ABC) method of immuno-chemistry (*360, 361*), as used in the present project [Section 2.3.2.2.4, Figure 2.5(i)]. In this method, secondary antibody is biotinylated and horseradish peroxidase is conjugated to streptavidin, which has 4 binding sites for biotin. Thus, peroxidase becomes localised to the site of the target antigen. Peroxidase then converts DAB to a brown-coloured product. In order to increase the signal, streptavidin molecules are complexed to biotinylated peroxidase, such that there are only biotin-binding sites available on the complex, which bind to the biotinylated secondary antibody, thereby increasing peroxidase number per antigen locus. Another common enzyme marker, which was used in this project, is alkaline phosphatase (*361*). Alkaline phosphatase converts fast red to a bright red-coloured precipitate [Figure 2.5(ii)].

(i) **ABC-DAB method**



(ii) **Alkaline phosphatase-fast red method**



2.3.2.2 Immuno-chemistry on tissue controls

Normal human submandibular gland, spinal cord and kidney were used as positive controls for TK, B1R and B2R, respectively. TK is abundant in the submandibular and parotid salivary gland striated and interlobular ducts and is absent in the acini and intercalated ducts of these glands (*362*). B1R has been localised in neurons of the dorsal horn of the spinal cord and was therefore chosen as the control tissue for B1R (*354*). B2R is present in a wide range of tissues; however, kidney was chosen as the control tissue as it is an abundant source of B2R (*363*). In particular, B2R is found in the mesangium of the glomeruli, proximal and distal tubules, loop of Henle, collecting ducts and endothelial cells of blood vessels of the human kidney (*266*). These control tissues were previously collected from the KwaZulu-Natal Department of Health State Mortuary with the assistance and permission of a forensic pathologist (Section 2.1).

2.3.2.2.1 Silanation of slides for immuno-chemistry and haematoxylin and eosin (H&E) staining

Glass slides were silanated in order to enhance the adherence of tissues (*364*). Slides were immersed in a 2% 3-aminopropyltriethoxysilane (Sigma) solution with acetone as the solvent, for 10 minutes. The silane solution was made up fresh and used immediately. Excess silane was washed off in acetone for approximately 30 seconds. Slides were then immersed in ddH_2O for 30 seconds in order to wash off excess acetone. All the above steps were carried out at room temperature. Slides were dried overnight in the oven at 50°C.

2.3.2.2.2 Sectioning of control tissues for immuno-chemistry and H&E staining

Control tissues embedded in wax blocks were cut into sections of 4-5 μm thickness using a rotary microtome (Leica). Sections were then floated in a waterbath at 60°C in order to flatten out creases and were subsequently picked up onto silanated slides (Section 2.3.2.2.1).

Slides were then dried overnight in the oven at 50°C, ensuring proper adherence of sections to slides.

2.3.2.2.3 Selection of non-diseased control tissues – H&E staining

Different salivary gland, spinal cord and kidney blocks were sectioned and picked up onto slides that were previously silanated as described in Sections 2.3.2.2.1 and 2.3.2.2.2. These sections were stained with haematoxylin and eosin using routine methodology (*361*) and viewed with a bright field light microscope in order to ensure the use of normal tissue as controls. Tissues were dewaxed by immersion in 2 changes of xylene (Merck), each for 10 minutes. This was followed by rehydration in a series of increasingly dilute ethanol solutions (100%, 90%, 70% and 50%) for 5 minutes each, followed by 5 minutes in ddH_2O. Tissues were then stained with Mayer's haematoxylin (Sigma) for 5 minutes at room temperature in order to stain nuclei blue. Haematoxylin was washed off in running tap water for 5 minutes. Tissues were then counterstained with eosin (Sigma), which had been diluted to 0.5% with glacial acetic acid (Merck), for 5 minutes at room temperature in order to stain cell membranes and cytoplasmic structures pink. Eosin stain was washed off in running tap water for 5 minutes. Tissues were then dehydrated in a series of increasingly concentrated ethanol solutions (50%, 70%, 90% and 100% ethanol) for 5 minutes each. They were then immersed in xylene for 5 minutes before mounting with glass coverslips (Bectin Dickinson Biosciences) and permanent mountant, DPX (Merck). Only the tissue blocks that were confirmed as normal after analysis of H&E stained sections were sectioned for use as immuno-cytochemistry controls (as described in Section 2.3.2.2.2). The kidney blocks used in the present project were also examined and declared histologically normal by histopathologist Dr. Ashwin Bremdev, Lancet Laboratories, Durban.

2.3.2.2.4 Immuno-chemistry protocol for control tissues

The immuno-chemistry protocol of Naidoo (2005) was followed and is described below (*354*). This protocol was a modification of previous immuno-chemistry protocols (*187, 365, 366*).

Dewaxing and rehydration of tissues- Tissues were dewaxed by immersion in 2 changes of xylene, each for 10 minutes. This was followed by immersion in 100% ethanol twice, each for 5 minutes, in order to clear xylene from the sections. Endogenous peroxidase activity was partially inhibited by immersing tissues in 100% methanol (Merck) (*361, 367*) for 20 minutes. This was done in order to avoid non-specific binding of peroxidase to the streptavidin of horseradish peroxidase-streptavidin conjugate, which would result in non-specific labelling of peroxidase-containing structures such as red blood cells. Tissues were then rehydrated in a series of increasingly dilute ethanol solutions (90%, 70% and 50%) for 4 minutes each, followed by immersion in 100% ddH_2O for 5 minutes. All above steps were carried out at room temperature.

Antigen retrieval- Antigen retrieval refers to the unmasking of antigens that were modified by formalin fixation, in order to improve antigenicity (*368*). This is an important step for satisfactory immuno-staining. Antigen retrieval was performed by heating tissues to approximately 80°C for about 7 minutes in 0.1 M tri-sodium citrate dihydrate (Merck) solution, pH 6. This was achieved by heating a container of the sodium citrate solution with immersed slides on high temperature in a microwave oven (R-4A52; Sharp Corp., Japan) until the solution began to bubble (after approximately 2 minutes). The settings were then immediately switched to low temperature for 5 minutes. The tissues in the boiling sodium citrate solution were allowed to cool to room temperature (for approximately 20 minutes) and were then equilibrated in ddH_2O for 5 minutes.

Quenching of endogenous peroxidase activity and blocking of non-specific sites- Further quenching of endogenous peroxidase activity was carried out in 4 changes of hydrogen peroxide (Merck)-methanol (4 parts 30% H_2O_2 [i.e. 12% H_2O_2] and 6 parts absolute methanol), for 15 minutes each at room temperature. H_2O_2 acts as a substrate for peroxidase and irreversibly inhibits the activity of the enzyme presumably due to structural damage or as a result of the high substrate concentration or accumulation of a toxic product (*360*). Although H_2O_2-methanol inhibits endogenous peroxidase activity, it allows for the activity of the horseradish peroxidase (*369*). The tissues were then washed twice in Tris-buffered saline (TBS)/3% bovine serum albumin (BSA; Boehringer Mannheim, Germany) for 5 minutes each time at room temperature. TBS was prepared as 5% Tris-hydrochloric acid (Tris-HCl; 1 M Trizma Base (Sigma), adjusted to pH 7.5 with 30% HCl [Merck]) and 3% sodium chloride (5M NaCl; Merck). A 3% solution of BSA was then made up using TBS. The function of BSA was to block non-specific antibody/reagent-binding sites. Following the TBS/3%BSA washes, non-specific antibody-binding sites were further blocked for 1 hour at room temperature in 10X concentrated milk blocker (DIG Wash and Block Buffer Set from Roche, Mannheim, Germany) that was diluted to 1X in TBS/3%BSA.

Source of primary antibodies, optimal antibody dilutions and incubation with primary antibodies- Whilst the tissues were incubating with the blocking solution, the primary antibodies were thawed on ice (antibodies were stored frozen at –20°C) and dilutions of these (refer to *Detection of bound primary antibody*), using the 1X milk blocker solution, were made up on ice. Polyclonal anti-human goat TK antibody and polyclonal rabbit B1R and B2R antibodies (refer to Section 2.1) (*272*) were used to label TK, B1R and B2R, respectively. After the tissues had been blocked for 1 hour in milk blocker, they were incubated with the diluted primary antibodies in a humified chamber at 4°C for 18 hours. Negative tissue controls were incubated with 1X milk blocker solution instead of antibody.

Detection of bound primary antibody- Following the 18-hour incubation with antibodies, unbound antibody was washed off in 2 TBS/3%BSA washes, for 2 minutes each. Tissues were then blocked in 1X milk blocker solution twice, each time for 30 minutes at room temperature, in order to block non-specific binding sites for the secondary antibody. Thereafter, kidney tissues were additionally blocked for biotin via the avidin-biotin 'flush' method (*360, 361*) using a biotin blocking kit (Dako, UK) with an avidin solution (incubated for 15 minutes) and biotin solution (incubated for 15 minutes). Biotin blocking was necessary to prevent non-specific binding of streptavidin (horseradish peroxidase-streptavidin conjugate) to biotin, which would result in non-specific labelling of biotin-producing cells of the kidney. Following biotin blocking, all tissues were washed twice for 2 minutes each with TBS/3%BSA. The ABC conjugating kit (LSAB Plus, Dako) was then used on the tissues. This involved incubating the tissues for 20 minutes with a secondary IgG-type multi-species antibody linked to biotin, followed by a 20-minute incubation with streptavidin-peroxidase solution with TBS/3%BSA washes before and after. Tissues were then incubated with DAB in the dark for approximately 5-7 minutes. The tissues were monitored with a bright field light microscope (Leica) and DAB was inactivated by immersing tissues in TBS/3%BSA once adequate specific brown staining of structures was observed. All kits described above were used at room temperature and incubations were in a humidified chamber. Following the incubation with DAB, slides were counterstained with Mayer's haematoxylin for 3-5 min at room temperature to produce a colour contrast between the brown DAB-labelled cellular structures and blue haematoxylin-stained nuclei. The excess haematoxylin was subsequently washed off in running tap water for 5 minutes. Tissues were then dehydrated and mounted as for H&E stained sections as described in Section 2.3.2.2.3. Results were viewed with a bright field light microscope. The optimal dilutions of the TK, B1R and B2R primary antibodies for salivary gland, spinal cord and kidney tissues were determined to be 1:5000, 1:150 and 1:150, respectively.

2.3.2.3 Immuno-cytochemistry on challenged cells and cell culture controls

Firstly, cells were partially permeabilised (in order to allow accessibility of reagents) by treatment with 1% Triton X100 (Merck) for 20 minutes at room temperature. A 2-minute TBS/3%BSA wash was then performed to stop the reaction. Cells were then incubated with 1X milk blocker for 1 hour at room temperature. This was followed by an 18-hour incubation with diluted antibody at 4°C in a humified chamber. The remainder of the immuno-chemistry protocol for cells was the same as that used for tissues following the primary antibody incubation onwards (Section 2.3.2.2.4). Optimal antibody dilutions for cultured cells for TK, B1R and B2R, respectively, were determined to be: (i) 1:50, 1: 100 and 1: 50 for dMVECs, (ii) 1:150, 1:400 and 1:75 for DU145 cells, and (iii) 1:650, 1:750 and 1:200 for MCF7 cells. Negative cell culture controls were incubated with 1X milk blocker solution instead of antibody.

As positive cell culture controls, endothelial cells were labelled for von Willebrand's factor (vWF), a specific marker of endothelial cells (*370, 371*), and DU145 cells and MCF7 cells were labelled for cytokeratin-19 (CK-19), a marker for breast and prostate tumour cells (*372*). The vWF was labelled with rabbit anti-human vWF IgG (Dako) that was raised against vWF isolated from human plasma. CK-19 was labelled with murine anti-human CK-19 IgG (Dako). This antibody was raised against T24 human bladder cancer extract. The antibody dilution used for both vWF and CK-19 was 1:200.

2.3.2.4 Immuno-cytochemistry on co-cultured cells

In order to distinguish between tumour and endothelial cells in the co-culture model endothelial cells were labelled for vWF in addition to KKS proteins. To allow for simultaneous detection of vWF and either TK, B1R or B2R, a double immuno-enzymatic procedure was employed. Mason and Sammons (1978) were the first to perform a

simultaneous double immuno-enzymatic labelling procedure using peroxidase and alkaline phosphatase to label two antigens simultaneously (*373*). For the present project, peroxidase and alkaline phosphatase were used with DAB (Sections 2.3.2.2.4 and 2.3.2.3) and fast red (Sigma) as substrates, respectively, and these chromogen systems were used sequentially, rather than simultaneously, as described below. These chromogen systems could not be used simultaneously for the proteins labelled as the antibodies specific for these proteins shared the same host (a rabbit) with the exception of the TK antibody (derived from a goat). Thus, simultaneous use of the chromogen systems would have resulted in cross-reaction.

Immuno-chemistry on the cells of the co-culture model was performed as described in Sections 2.3.2.2.4 and 2.3.2.3, respectively, up to and including incubation of the cells with DAB chromogen. TK, B1R or B2R were labelled with DAB chromogen using antibody dilutions that were optimal for dMVECs. Following inactivation of DAB in TBS/3%BSA, the cells were blocked for 1 hour with 1X milk blocker. This was followed by an overnight incubation at 4°C with rabbit anti-human vWF IgG (diluted 1:200 with 1X milk blocker). Unbound antibody was washed off in TBS/3%BSA twice for 2 minutes each time. Cells were then incubated with alkaline phosphatase-IgG conjugate (Sigma; diluted 1:50 with 1X milk blocker) overnight at 4°C in a humidified chamber, followed by two 2-minute TBS/3%BSA washes to remove unbound antibody. Next, cells were incubated with Fast Red Solution [Sigma Fast™ Fast Red TR/Napthol AS-MX (containing 1 mg/ml Fast Red, 0.4 mg/ml Napthol AS-MX, 0.15 mg/ml levamisole in 0.1 M Tris, pH 8.2)] in the dark at room temperature for 5-7 minutes. This allowed the development of a red alcohol-soluble precipitate due to the action of alkaline phosphatase on fast red. A 2-minute TBS/3%BSA wash was performed twice to stop the reaction. Next, cells were stained with haematoxylin as described in Section 2.3.2.2.4. The slides were mounted with aqueous jelly (thus sections

were not dehydrated prior to mounting) as the reaction product of alkaline phosphatase and fast red is alcohol-soluble (*361*).

2.3.2.5 Documentation of immuno-chemistry results

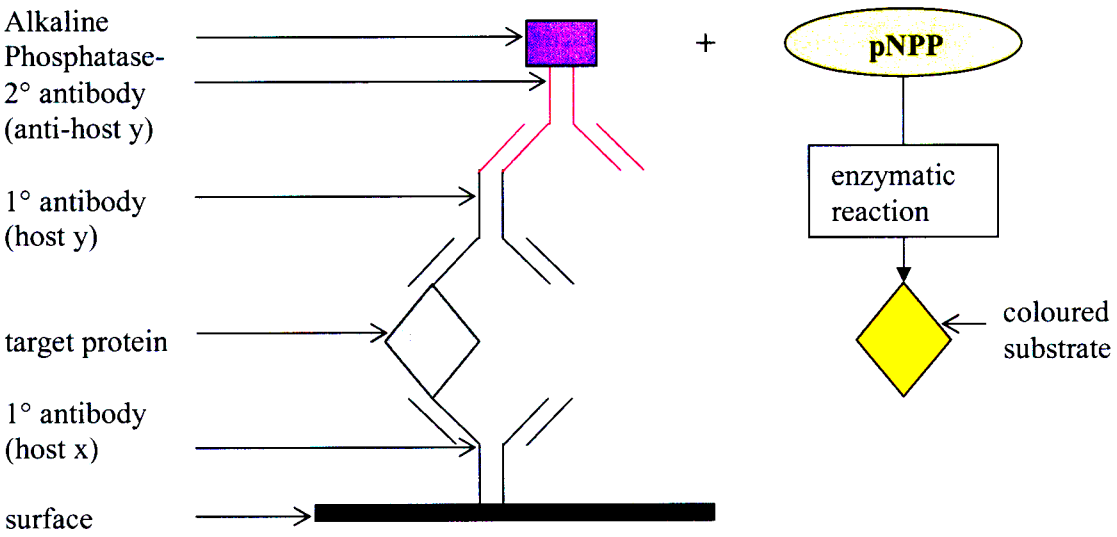
Images of immuno-labelled control tissues and cells were taken with a DC280 digital camera (Leica) that was attached to the phase contrast, bright field microscope. Images were digitised and recorded as 24-bit tagged image format files (TIFF). Magnification of images (taking into account the magnification of the microscope objective and the digital camera) was recorded. The digital camera was interfaced with AnalySIS Pro™ 5 image analysis software (Soft Imaging Systems, Germany), which allowed for analysis of images (Section 2.4).

2.3.3 Enzyme-linked immunoassay (ELISA) for TK

2.3.3.1 Background

The principle of ELISA is similar to that of immuno-chemistry where detectable antibodies bind to target antigens. A brief description of the indirect sandwich ELISA (used for this project) follows (*374*) (Figure 2.6). A primary antibody specific for the target antigen is immobilised on a surface. The primary antibody is then incubated with the source of the target antigen to allow binding. A second primary antibody is then allowed to bind to bound antigen, creating a sandwich of antibody-antigen-antibody. A secondary antibody-marker conjugate that will bind to the second primary antibody (Section 2.3.2.1) is then added to the sandwich. The enzyme markers that may be used for ELISA are the same as those used for immuno-chemistry (Section 2.3.2.1). For this project, alkaline phosphatase conjugated to secondary antibody was used to convert disodium p-nitrophenyl phosphate (pNPP) into a yellow product that could be quantified using a spectrophotometer.

ELISA

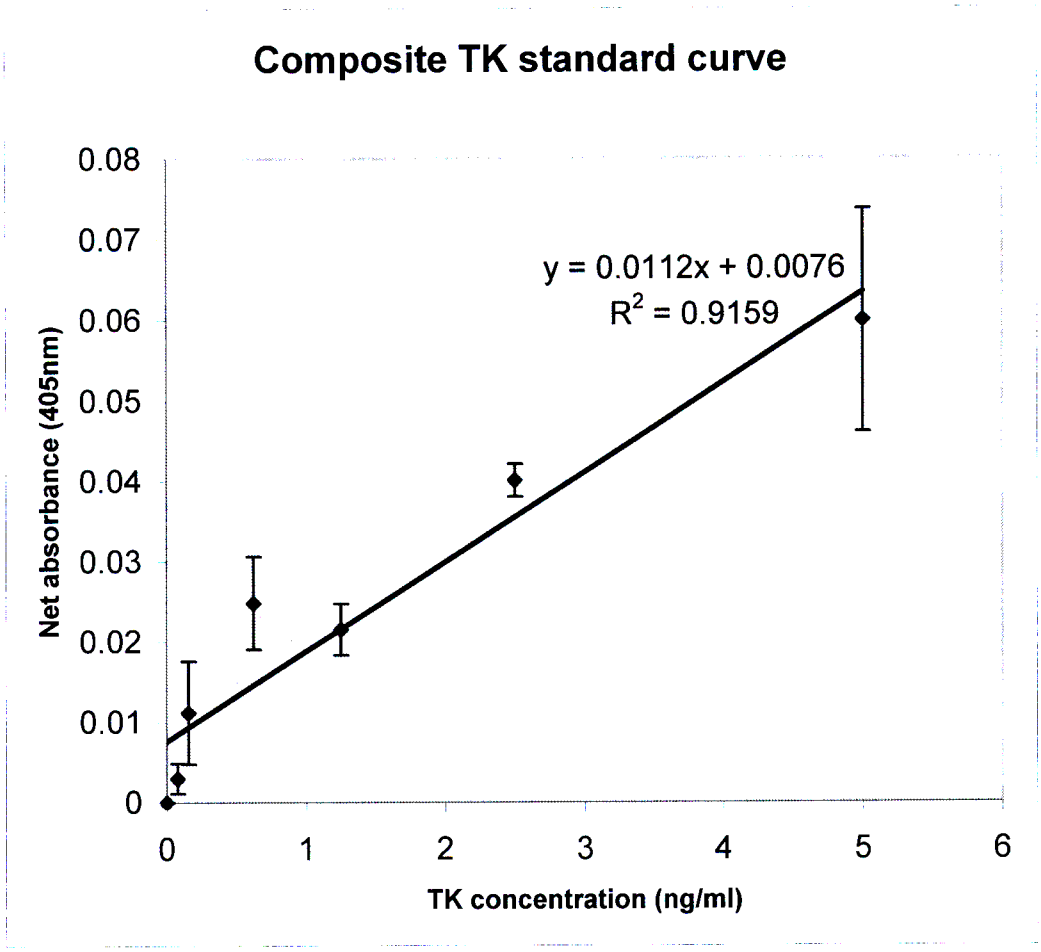

Alkaline
Phosphatase-
2° antibody
(anti-host y)
1° antibody
(host y)
target protein
1° antibody
(host x)
surface
+
pNPP
enzymatic
reaction
coloured
substrate

2.3.3.2 Controls for ELISA

Purified human urinary kallikrein (HUK) was the TK standard used. A composite standard curve was generated from HUK (Calbiochem) standards of 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.3125 ng/ml and 0.156 ng/ml (Figure 2.7). For negative controls, HUK standards and samples were replaced with PBS (Section 2.3.3.3).

2.3.3.3 ELISA protocol

The TK ELISA protocol described by Naidoo (2005) was followed (*354*). Briefly, the goat anti-human TK antibody stock that was used for immuno-chemistry (Section 2.3.2.2.4) was also used as the primary antibody in the TK ELISA. The stock solution of this antibody was 1 mg/ml. TK antibody was diluted to 30 ng/ml with coating buffer. Coating buffer constituted 0.015 M sodium carbonate (Na_2CO_3, Merck) and 0.04 M sodium hydrogen carbonate ($NaHCO_3$, BDH Chemicals, England) in ddH_2O and was adjusted to pH 9.6. Diluted primary TK antibody was loaded into each well (100 µl/well) of a 96-well polystyrene ELISA microtitre plate (Corning Costar), and incubated at 4°C overnight to allow coating of the wells. The wells were then washed with 200 µl 0.01 M PBS/0.05%Tween (Sigma) 3 times for 3 minutes each at room temperature to remove unbound antibody. Wells were subsequently incubated with 200 µl of 0.5X milk blocker for 30 min at room temperature in order to block non-specific binding of antigen to coating buffer. The PBS/Tween wash and 0.5X milk blocker incubation steps were repeated. Next, 100 µl of stored medium from the 2D challenge model (Section 2.2.6.1) was loaded onto the plate. Five replicates were loaded for each sample. As a negative control, 100 µl of PBS were loaded into 3 wells. HUK protein was serially diluted (5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.3125 ng/ml, 0.156 ng/ml) with PBS on ice, and 100 µl of each of these standards was loaded in triplicate onto the plate (Section 2.3.3.2). After loading the samples, controls and standards, the plate was incubated at 37°C for 1 hour in a shaking water bath


Composite TK standard curve
y = 0.0112x + 0.0076
R2 = 0.9159
Net absorbance (405nm)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
1
2
3
4
5
6
TK concentration (ng/ml)

(Tecator, UK). This was followed by 3 PBS/Tween washes of 3 minutes each to remove unbound TK antigen. Next, 100 µl of rabbit anti-human IgG TK antibody (1 mg/ml), diluted to 25 ng/ml in 0.5X milk blocker, was loaded into every well and thereafter the plate was incubated at 37°C for 1 hour in a shaking water bath. This rabbit anti-human IgG TK antibody was also previously raised by the research unit in the Department of Clinical and Experimental Pharmacology (refer to Section 2.1). After washing the wells with PBS/Tween (3 times for 3 minutes each), 100 µl of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1:250 with 0.5X milk blocker was loaded into each well. The plate was sealed with parafilm M (Whatman, UK) incubated at 37°C for 1 hour in a shaking water bath. Following this, wells were washed 3 times with PBS/Tween for 3 minutes each time in order to remove unbound secondary antibody. Next, 100 µl of 1 mg/ml chromogenic substrate, pNPP (Sigma), diluted in substrate buffer, was loaded. Substrate buffer was prepared as 5 mM magnesium chloride ($MgCl_2$,Merck) and 10% diethanolamine (Merck), pH 9.6. The plate was kept in the dark at room temperature until a yellow colour developed and absorbance (405 nm, Biorad (UK) Microplate Reader 3550 using Biorad Microplate Manager 4.0® software) peaked between 1 and 1.5 units. The net absorbance readings for the standards and samples were calculated by subtracting the mean blank (negative control) absorbance from these readings. The TK concentrations of the various samples were calculated from a standard curve of [TK] versus absorbance and expressed in ng/ml (Figure 2.7). Samples with TK concentrations below the range of the standard curve (i.e. with a net absorbance of ≤ 0.0076) were considered to have 0 ng of TK per ml of medium. Graphs of TK concentration (y axis) versus challenge percentage (x axis) were plotted.

2.3.4 MTT cell proliferation assay

2.3.4.1 Background

MTT is reduced in metabolically active cells to form insoluble blue formazan crystals, which may then be solubilised by a detergent (*375*). This blue colour can be quantified spectrophotometrically at 595 nm. Since proliferating cells are metabolically more active than non-proliferating cells, the MTT assay may be used to quantify cell proliferation.

2.3.4.2 Controls in the MTT assay

The MTT assay was performed following the challenging of cells with conditioned medium (as described in Section 2.2.6.1), in order to determine the effect of challenge metabolites (present in conditioned medium) on cell proliferation. In order to control for the effect that cell-specific culture medium for one cell line might have on the proliferation of another cell line, endothelial and tumour cells were also challenged with tumour and endothelial-specific culture medium, respectively, in ratios of 0% (unchallenged), 10%, 25% and 50%. These controls allowed for the net proliferative effect of challenge metabolites to be determined by the subtraction of the proliferative effect of culture medium alone from the proliferative effect of conditioned medium (culture medium + metabolites). Figure 2.8 shows the experiment and control conditions for the MTT assay.

2.3.4.3 MTT assay protocol

Following the challenging of cells, medium was replaced with 110 μl of a mix of MTT solution and cell-specific medium (in a ratio of 1:10), and then incubated at 37°C/5% CO_2 for 4 hours (*354*). MTT solution was prepared as 5 mg MTT (Sigma) per ml of Hank's balanced salt solution (HBSS, BioWhittaker). MTT-medium mix was then replaced by 100 μl DMSO and the plate incubated for 1 hour at 37°C/5% CO_2. The absorbance of cells in each well was read at 595 nm with a reference wavelength of 655 nm at room temperature

Cell line X challenged with conditioned medium from cell line Y	0% conditioned medium (Y) + 100% culture medium for X ○ ○ ○ ○ ○ 10% conditioned medium (Y) + 90% culture medium for X ○ ○ ○ ○ ○ 25% conditioned medium (Y) + 75% culture medium for X ○ ○ ○ ○ ○ 50% conditioned medium (Y) + 50% culture medium for X ○ ○ ○ ○ ○
Cell line X challenged with culture medium for line Y	0% culture medium for Y + 100% culture medium for X ○ ○ ○ ○ ○ 10% culture medium for Y + 90% culture medium for X ○ ○ ○ ○ ○ 25% culture medium for Y + 75% culture medium for X ○ ○ ○ ○ ○ 50% culture medium for Y + 50% culture medium for X ○ ○ ○ ○ ○

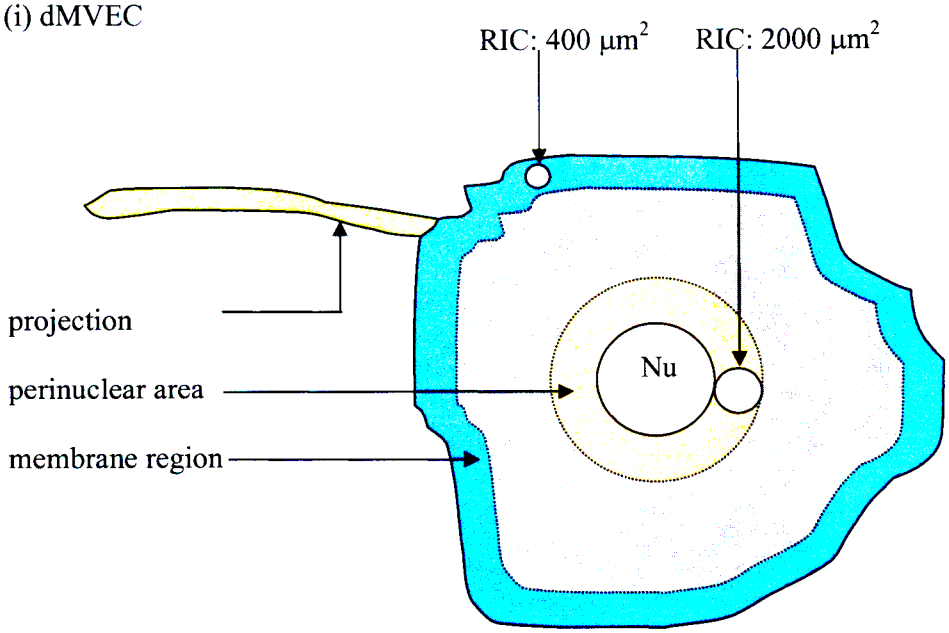
on a Biorad microplate reader using Biorad Microplate Manager 4.0® software. Cell proliferation in the various challenge conditions was expressed as a percentage of the unchallenged control, the mean absorbance of which represented 100% proliferation. The net proliferative effect of challenge metabolites for the various challenge concentrations was then calculated. The mean percentages of increase(+)/decrease(-) in proliferation (relative to the unchallenged control) for the culture medium challenge controls (Section 2.3.4.2) were subtracted from the proliferation percentages for the corresponding conditioned medium challenge experiments. Graphs showing the net proliferative effect of challenge metabolites (y axis) versus challenge percentage (x axis) were plotted.

2.4 Data analysis

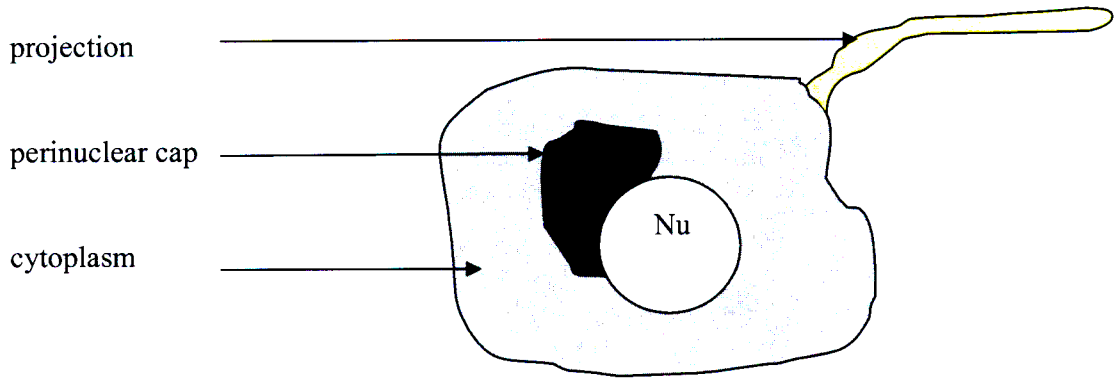
For image analysis of immuno-labelled cells (from the challenge model), fields of view that best represented the *in vivo* setting were chosen. For example, fields within the centre of a large, tightly-packed congregation of dMVECs were not chosen as dMVECs do not form large aggregates *in vivo*. However, images of such fields were not excluded for tumour cells, as tumour cells form aggregates *in vivo*. Fields of view with similar cell shapes were chosen and the 40X objective was used when taking all images in order to minimise the effect of these variables on results. The extreme edges of the well were excluded as stain tended to collect non-specifically in this area. From images taken, 15 cells were analysed for each challenge condition for stain intensity and stain extent in various cell area categories. These categories included (i) perinuclear area, cytoplasm, the membrane region and projections for dMVECs, (ii) perinuclear cap, cytoplasm and projections for DU145 cells, and (iii) cytoplasm and projections for MCF7 cells, as depicted in Figure 2.9.

The stain intensity was quantified using AnalySIS 5 Pro™ image analysis software. Archived 24-bit TIFF images were converted to 8-bit black and white images with a grey

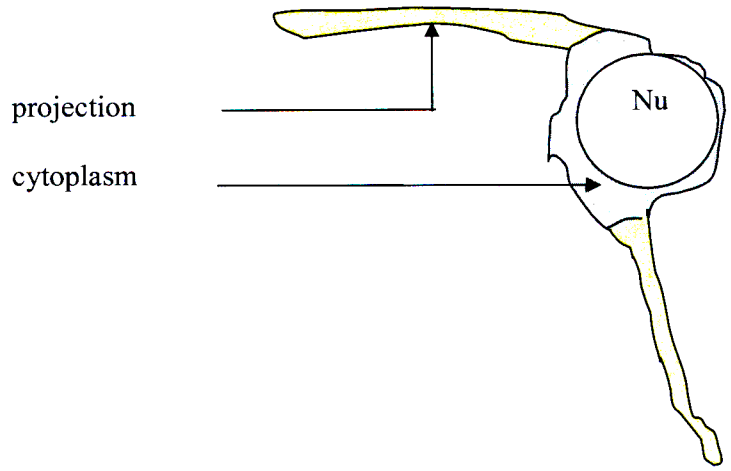
(i) dMVEC



(ii) DU145



(iii) MCF7



scale of 256 phases. The entire stained area for each area category was selected with region of interest circles (RICs). For each RIC, the mean grey scale intensity value and the area of the RIC were calculated. The mean intensity of all the RICs for a particular area category was calculated for each challenge condition. Values below 120 were considered to represent background stain or no stain and were therefore excluded. The total cell area was calculated by selecting the entire cell as a region of interest. Following this, the percentage area of the cell that was stained was calculated as a measure of stain extent. The contribution of staining in each area category to total stain extent was also calculated. Parametric analysis of variance (ANOVA) with Tukey post-hoc testing (*376, 377*) was used to test for significance ($p<0.05$) of difference among the stain intensities and among the stain extents of the different challenge conditions (0%, 10%, 25% and 50 %).

The TK ELISA was performed twice (independently) with 5 replicates for each challenge condition and ANOVA with Tukey post-hoc testing was used to test for significance ($p<0.05$) of difference among secreted TK levels for the various challenge conditions. Similarly, the MTT assay was performed at least twice (independently) with 5 replicates for each challenge condition and ANOVA with Tukey post-hoc testing was used to test for significance ($p<0.05$) of difference among cell proliferation rates for the various challenge conditions.

Parametric ANOVA is based on the assumption that the population to which data belongs is normally distributed, and that the standard deviations of the measurements of each treatment group are similar. Thus, each time ANOVA was performed, the data was subjected to a Kolmogorov-Smirnov normality test and Bartlett's standard deviation test. In cases where the data failed Bartlett's standard deviation test, Kruskal-Wallis (the non-parametric equivalent of ANOVA) with Dunn's multiple comparisons test was used to test for statistical

significance ($p<0.05$). Further, since 5 or more values are required to perform the standard deviation and normality tests, Kruskal-Wallis with Dunn's multiple comparisons test was used to test for significance ($p<0.05$) in cases where there were less than 5 values for the projection area category for one or more of the challenge conditions. [Note: A least 3 or more (3-7 cells) of the 15 cells analysed for each challenge condition for stain intensity and extent had projections, whereas all 15 cells had staining in the perinuclear, cytoplasm and membrane categories.]

CHAPTER 3

RESULTS

CHAPTER 3 - RESULTS

3.1 Immuno-localisation: Controls

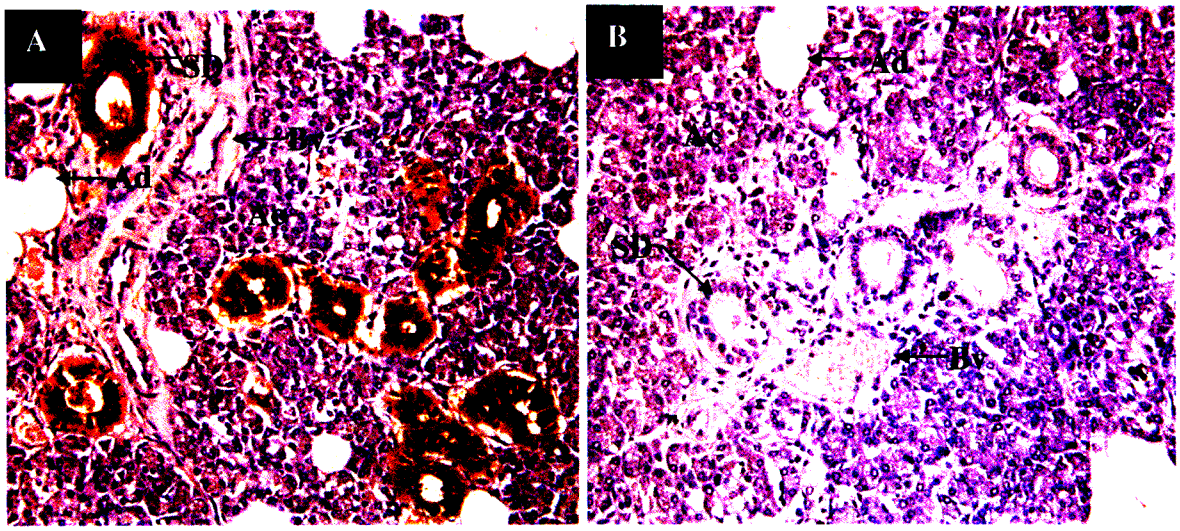
3.1.1 Tissue controls: TK, B1R and B2R

Tissues were immuno-labelled during cell culture immuno-labelling experiments in order to validate the methodology and reagents used (Section 2.3.2.2). In the positive salivary gland tissue control, the striated ducts labelled for TK, while acini, small intercalated ducts and blood vessels with peroxidase-containing red blood cells remained unstained (Figure 3.1(i)A). Neurons of the dorsal horn of the spinal cord were strongly labelled for B1R in the positive spinal cord tissue control (Figure 3.1(ii)A). B2R labelled in the thin limbs, thick ascending limbs and collecting tubules of the kidney medulla in the positive kidney tissue control (Figure 3.1(iii)A). Corresponding negative control tissues were unstained (Figure 3.1(i)B-(iii)B).

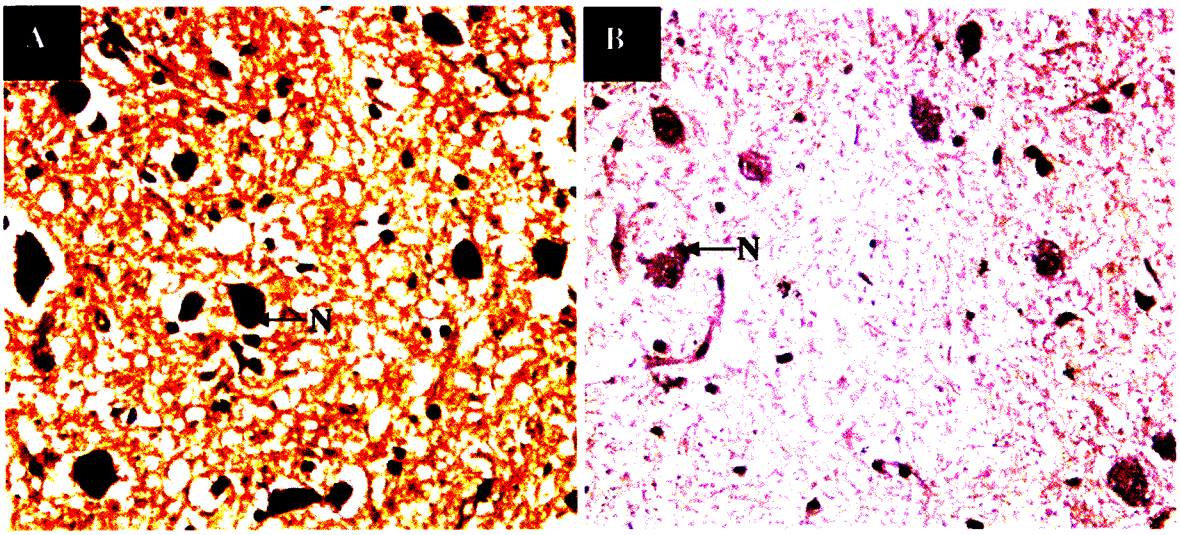
3.1.2 Cell culture controls: von Willebrand's factor (vWF), cytokeratin-19 (CK-19)

For positive cell culture controls, the different cell lines were each labelled for proteins typically expressed by their respective cell types (Section 2.3.2.3). The dMVECs labelled for vWF in their cytoplasm, membranes and small projections (Figure 3.2(i)A). The presence of vWF in dMVECs indicated retention of function in these cells. Both DU145 and MCF7 cells labelled for CK-19 in their cytoplasm and projections (Figure 3.2(ii)A-(iii)A). Negative cell culture controls showed no staining (Figure 3.2(i)B-(iii)B).

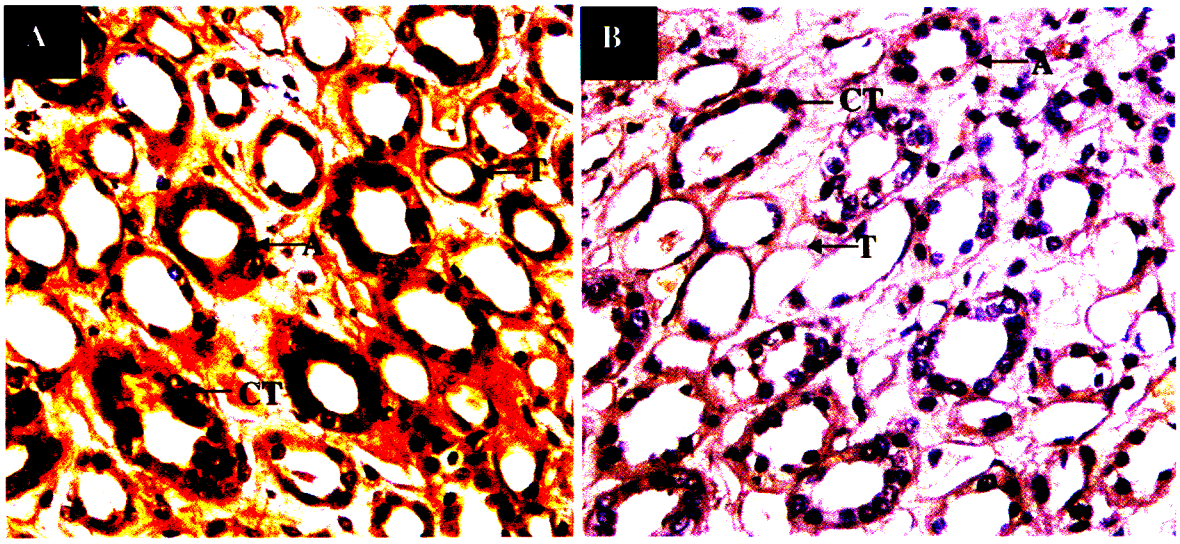
(i) TK – salivary gland



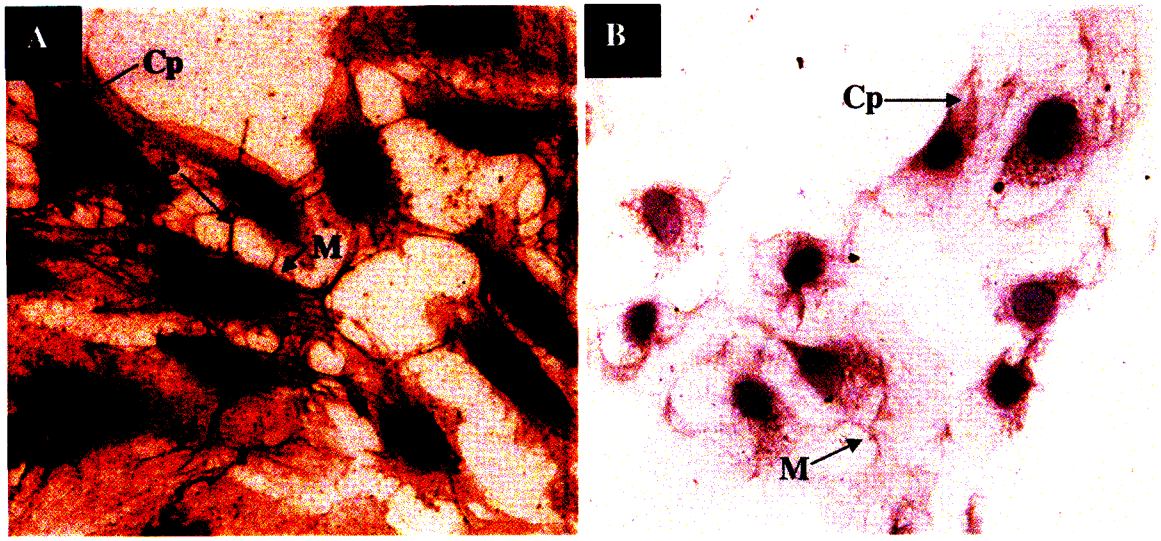
(ii) B1R – spinal cord



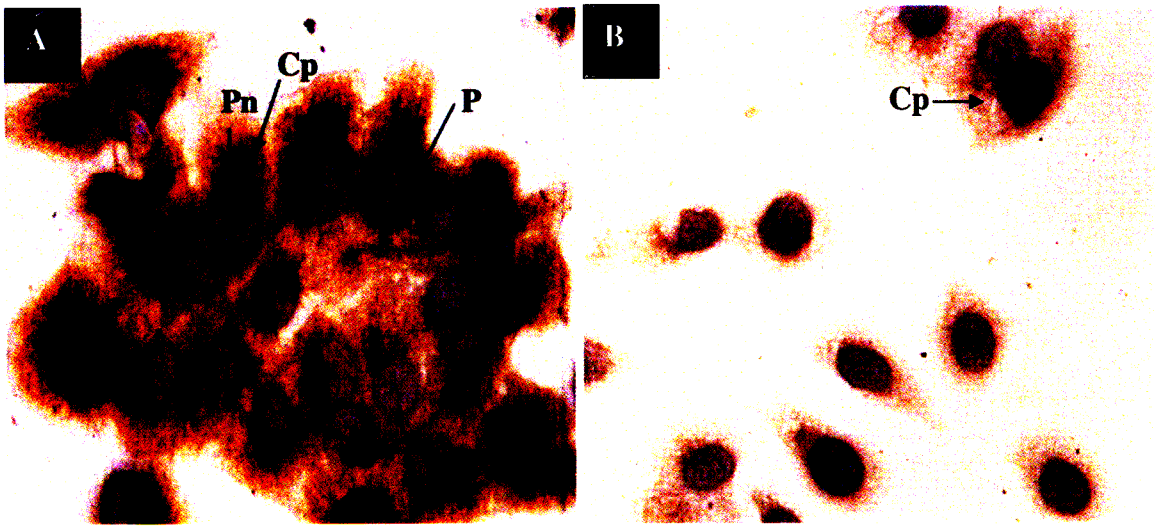
(iii) B2R - kidney



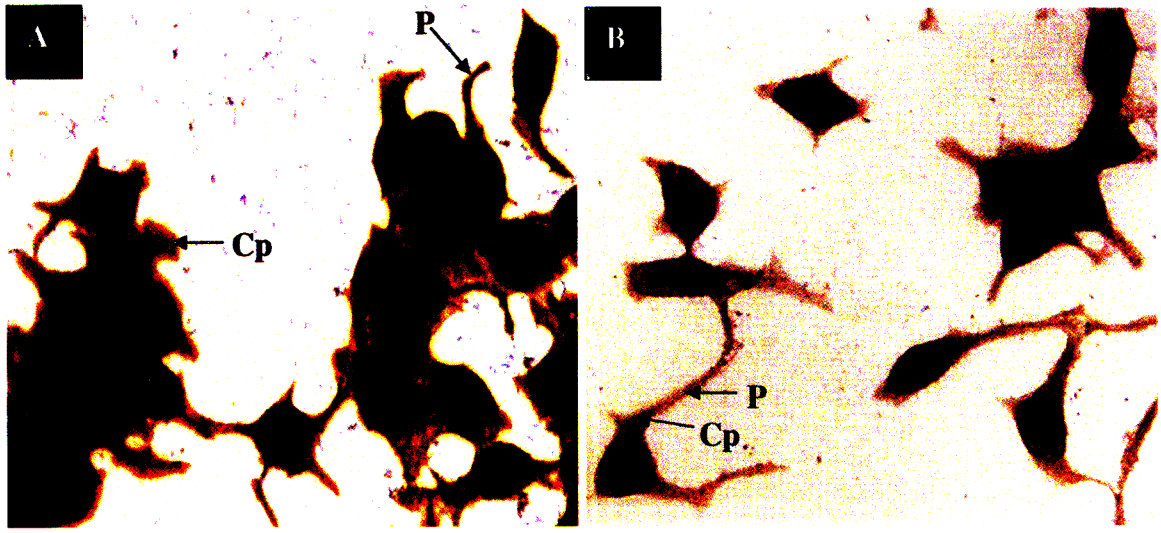
(i) dMVECs - vWF



(ii) DU145 cells – CK-19



(iii) MCF7 cells – CK-19



3.2 Immuno-localisation: TK, B1R and B2R and cell morphology in unchallenged and challenged cells

3.2.1 Dermal microvascular endothelial cells (dMVECs)

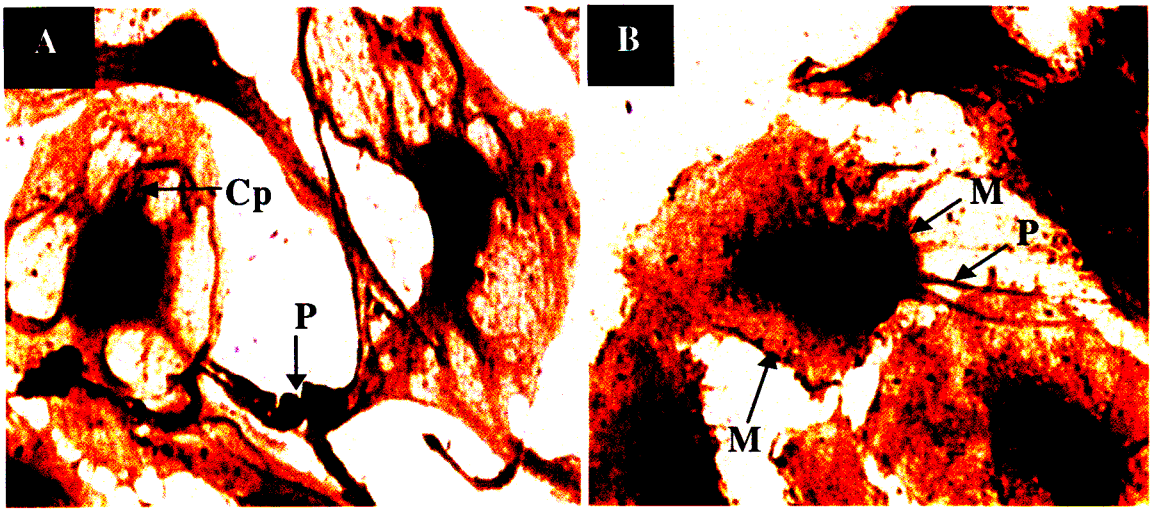
Localisation of TK, B1R and B2R in both unchallenged and challenged dMVECs was similar. Visually, these KKS proteins were labelled strongly in the perinuclear region and large projections, less intensely in the surrounding cytoplasm and to varying degrees of intensity in different regions of the membrane (Figure 3.3(i)-(iii)). There was a tendency to stain in those areas of the membrane that were adjacent to, but not yet in contact with, another cell (Figure 3.3(i)B; (ii)A; (iii)B). KKS-labelled, small projections were frequently present, reaching from these stained membrane regions towards another cell. Stain was generally less or absent where the membranes of cells had already made contact.

3.2.2 DU145 prostate tumour cells

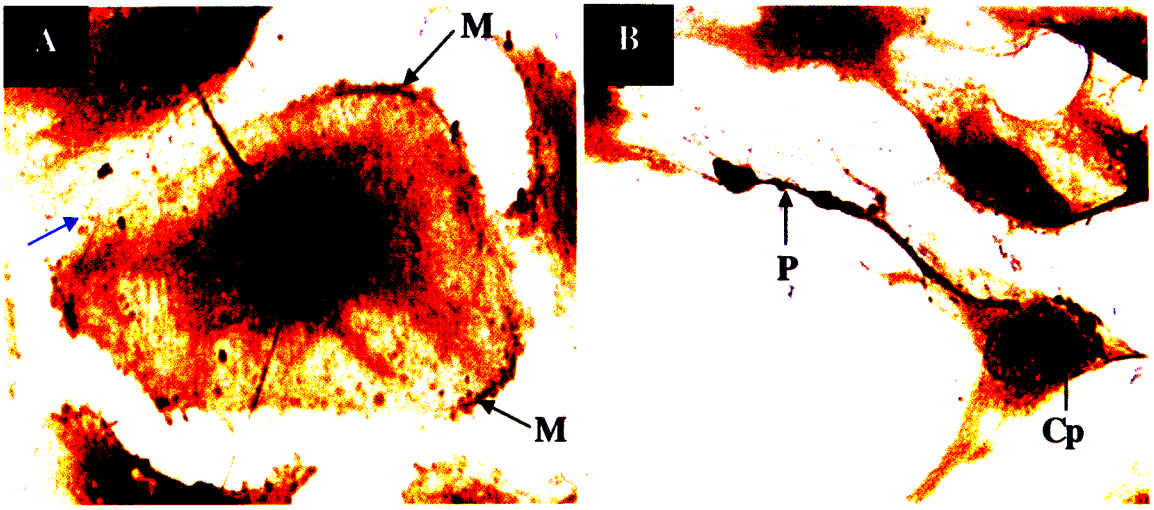
The staining pattern for TK, B1R and B2R was similar in DU145 cells whether unchallenged or challenged with conditioned medium from dMVECs *viz.* a perinuclear cap of visually intense stain and less intense stain in the surrounding cytoplasm and projections (Figure 3.4(i)A-C). However, in some cells with long thin projections, there was visually intense staining throughout the cell (Figure 3.4(i)C).

In addition to observed staining patterns, some observations of DU145 growth patterns were made. The DU145 cells tended to form small clumps in which cell nuclei were positioned centrally and the cytoplasm of cells was directed outwards (Figure 3.4(i)B). Multinucleate cells (Figure 3.4(i)D) and extremely large cells (Figure 3.4(i)E) occurred in the control condition and all challenge conditions but more frequently in the 25% challenge condition.

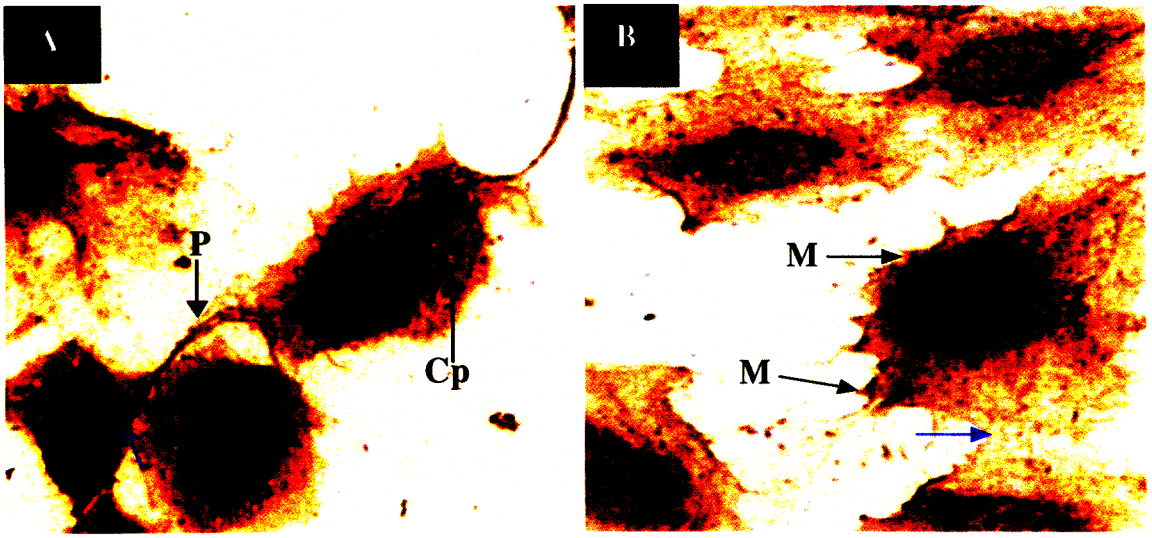
(i) TK



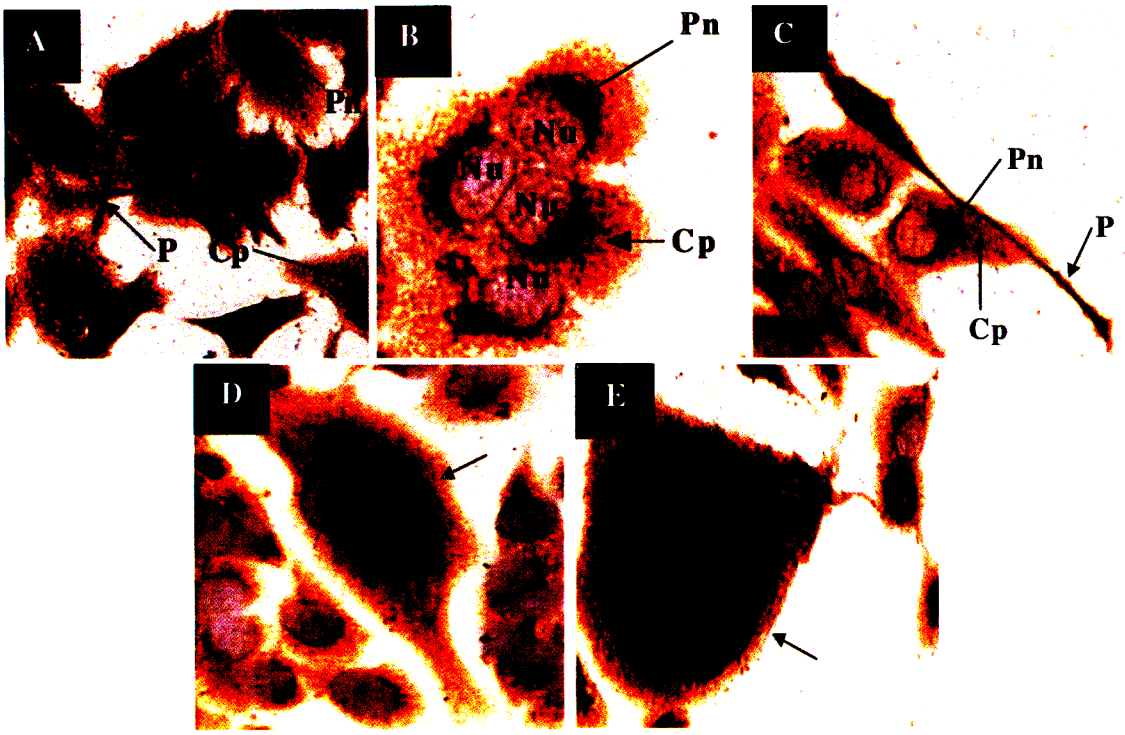
(ii) B1R



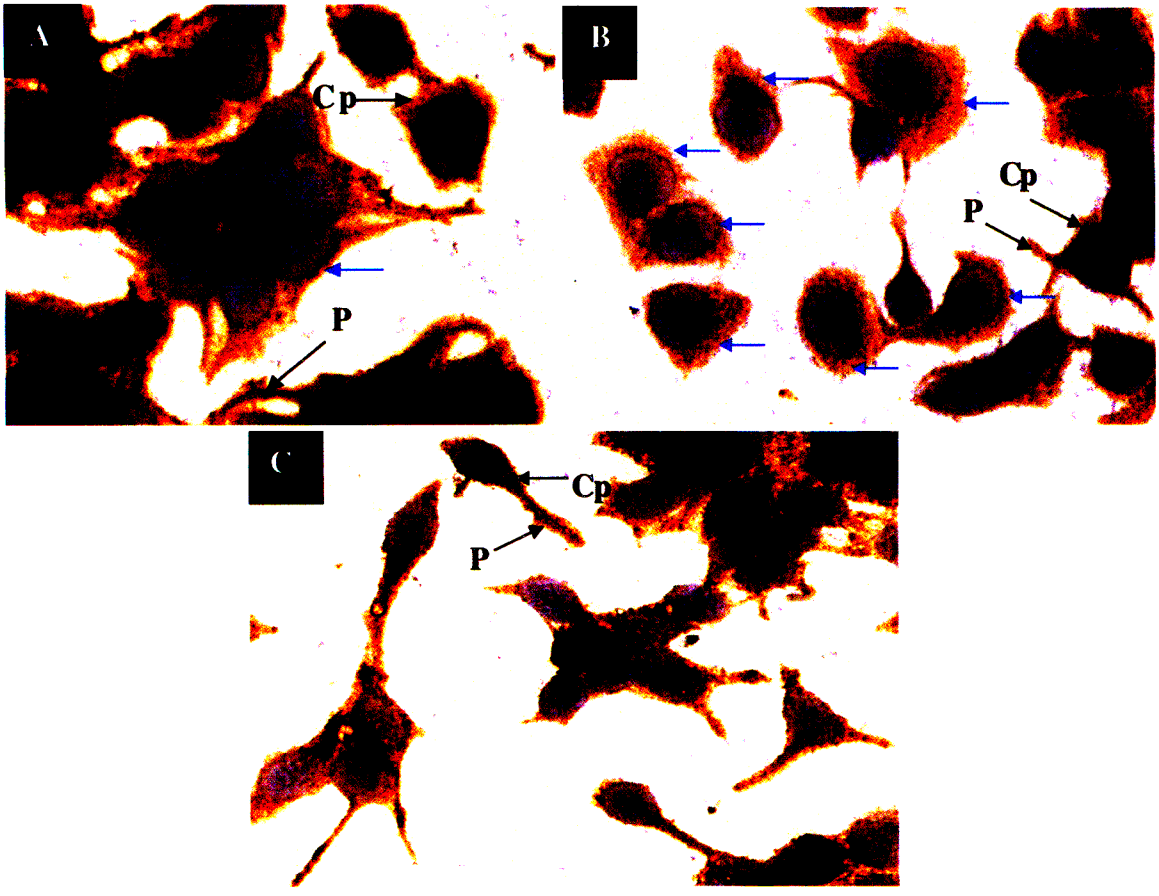
(iii) B2R



(i) DU145 cells



(ii) MCF7 cells



3.2.3 MCF7 breast tumour cells

MCF7 cells characteristically had proportionately large nuclei and the little cytoplasm that surrounded the nucleus formed long thin projections, which typically stained across the entire length for TK, B1R and B2R (Figure 3.4(ii)A-C). The cytoplasm of MCF7 cells stained entirely for the KKS proteins regardless of challenge condition.

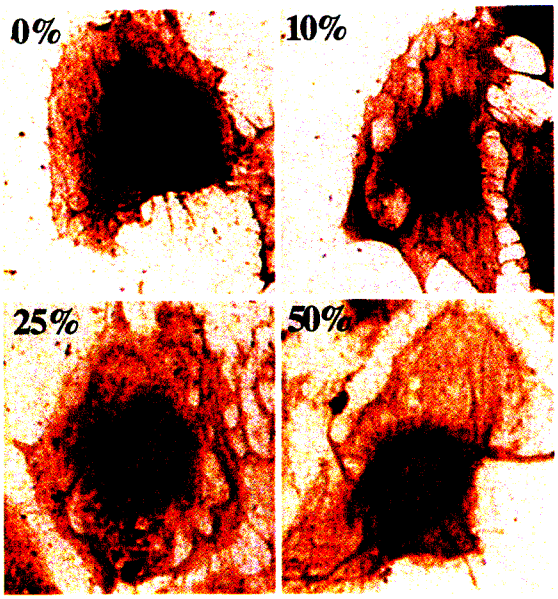
Similar to DU145 cells, MCF7 cells tended to form clumps. In some of these clumps, cell nuclei formed rings (Figure 3.4(ii)A). In contrast with the typical phenotypic characteristics of MCF7 cells described above, a few cells had an extended area of cytoplasm (Figure 3.4(ii)B). These cells were more frequent in the challenge conditions when compared with 0% control condition. Multinucleate cells occurred occasionally, but the frequency of these cells did not appear to be linked to challenge conditions.

3.3 Immuno-localisation: Image analysis in unchallenged and challenged cells

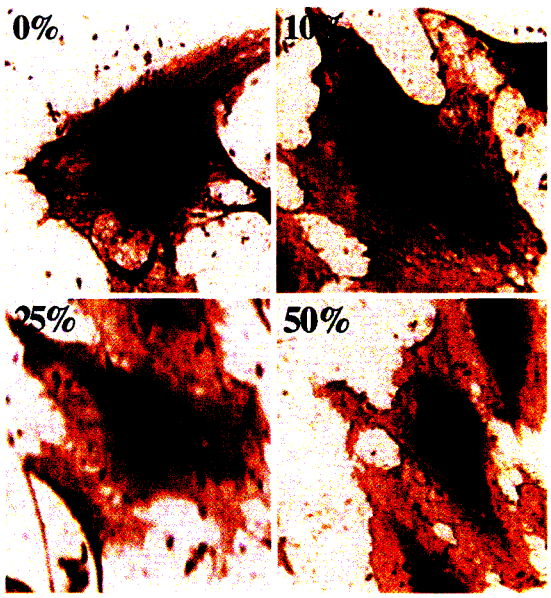
3.3.1 TK in dMVECs

Challenging dMVECs with tumour-conditioned medium (containing tumour cell metabolites) resulted in a decrease in the intensity of stain for TK with increasing challenge percentage, with the exception of the staining in dMVEC projections (Figure 3.5). There was a highly significant decrease in stain intensity in the perinuclear and cytoplasm areas of dMVECs challenged with DU145 metabolites (Figure 3.5(i)A-(ii)A, ANOVA p=0.0003 and p=0.0007, respectively). Similarly, there was a highly significant decrease in stain intensity in the perinuclear and cytoplasm areas of dMVECs challenged with MCF7 metabolites (Figure 3.5(i)B-(ii)B, ANOVA p<0.0001 for both regions). Additionally, there was a significant decrease in dMVEC membrane stain intensity when dMVECs were challenged with MCF7 metabolites (ANOVA p=0.0057). In contrast, there was a tendency for increased stain intensity for TK in the projections of dMVECs with increasing challenge

(i)A. dMVEC–DU145, sample images



B. dMVEC–MCF7, sample images



(ii)A. dMVEC–DU145, stain intensity

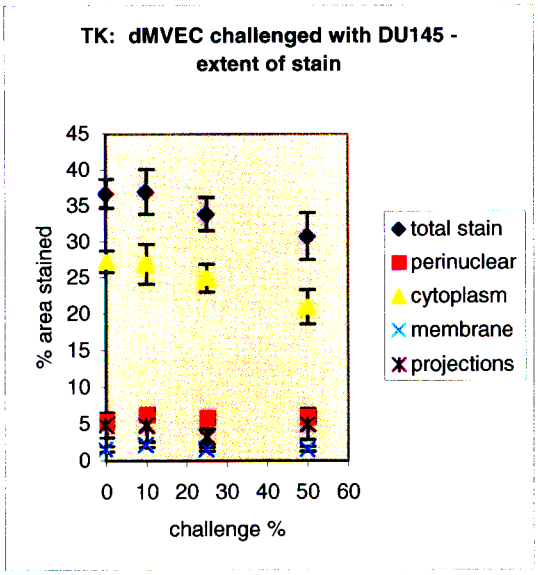
dMVEC-DU145	0%	10%	25%	50%
perinuclear *	100 (2.98)	89.17 (4.07)	81.08 (4.33)	72.44 (5.60)
cytoplasm *	100 (3.69)	94.90 (4.61)	78.27 (5.63)	79.03 (2.86)
membrane	100 (11.76)	80.28 (8.30)	80.48 (9.56)	93.28 (8.12)
projections	100 (14.34)	113.63 (18.31)	114.21 (5.11)	123.21 (11.08)

B. dMVEC–MCF7, stain intensity

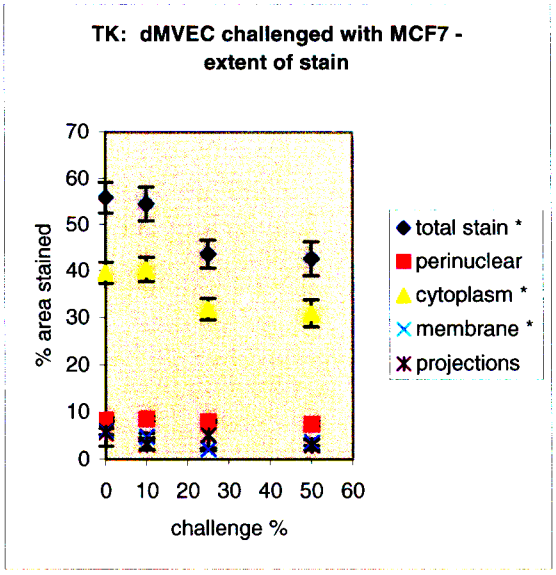
dMVEC-MCF7	0%	10%	25%	50%
perinuclear *	100 (2.31)	100.99 (2.28)	82.33 (4.12)	76.66 (4.88)
cytoplasm *	100 (4.04)	96.60 (4.93)	74.03 (3.17)	75.76 (4.43)
membrane *	100 (6.55)	101.76 (8.28)	84.79 (7.29)	68.97 (6.15)
projections	100 (10.89)	124.95 (7.59)	133.85 (12.39)	121.34 (12.27)

* ANOVA, $p \leq 0.0057$

(iii)A. dMVEC–DU145, stain extent



B. dMVEC–MCF7, stain extent



* ANOVA, $p \leq 0.013$

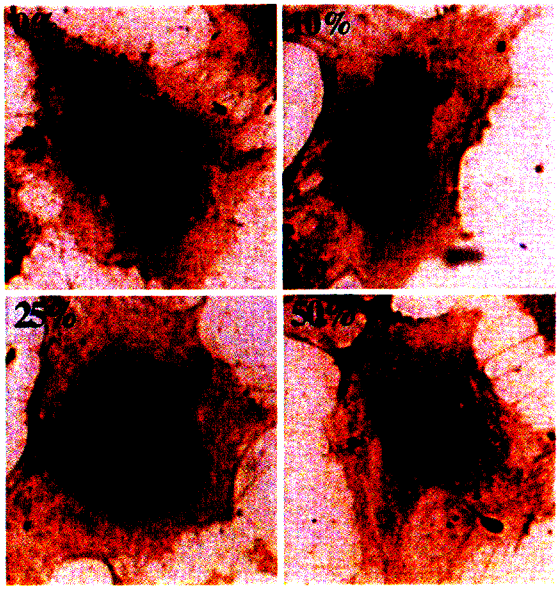
ratios for both DU145 and MCF7-challenged dMVECs (with the exception of the 50% MCF7-challenged dMVEC cells, which had a greater projection stain intensity than cells of the 0% condition but less than that of the 25% challenge condition), however this trend was statistically insignificant (Figure 3.5(ii)).

The extent of stain for TK tended to decrease in dMVECs that were exposed to tumour metabolites (Figure 3.5). The extent of cytoplasmic stain (and therefore total stain extent) diminished slightly with increasing exposure to DU145 metabolites, although this was not statistically significant (Figure 3.5(iii)A). The extent of cytoplasmic and membrane stain significantly decreased in MCF7-challenged dMVECs (ANOVA p=0.013 and p=0.0008, respectively), thereby resulting in a significant decrease in total stain extent (Figure 3.5(iii)B, ANOVA p=0.0104).

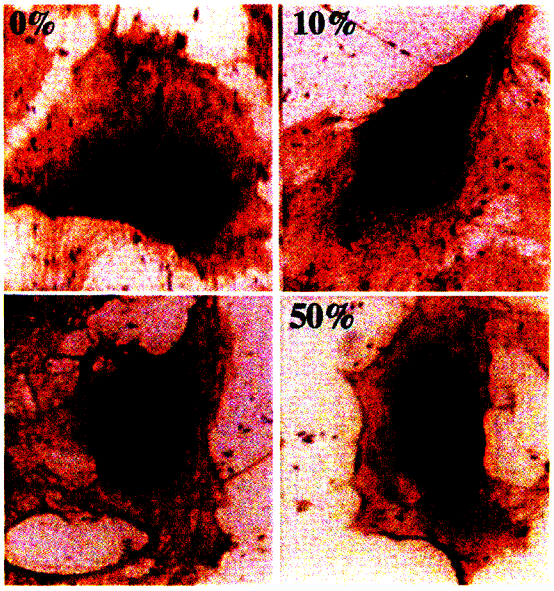
3.3.2 B1R in dMVECs

There were no significant changes in the intensity of stain for B1R in dMVECs that were challenged with tumour cell metabolites when compared with their unchallenged counterparts (Figure 3.6(i)-(ii)). The extent of stain for B1R in DU145-challenged dMVECs decreased slightly with increasing challenge percentage, although this was not statistically significant (Figure 3.6(iii)A). The differences in the extent of B1R in the membrane category of DU145-challenged dMVECs for the various challenge conditions was overall borderline statistically significant (Kruskal-Wallis p=0.045), however there was no significant differences found between individually compared groups (i.e. with the Dunn's multiple comparisons post-hoc test). There were no significant changes in the extent of stain for B1R when dMVECs were challenged with MCF7 metabolites (Figure 3.6(iii)B).

(i)A. dMVEC–DU145, sample images



B. dMVEC–MCF7, sample images



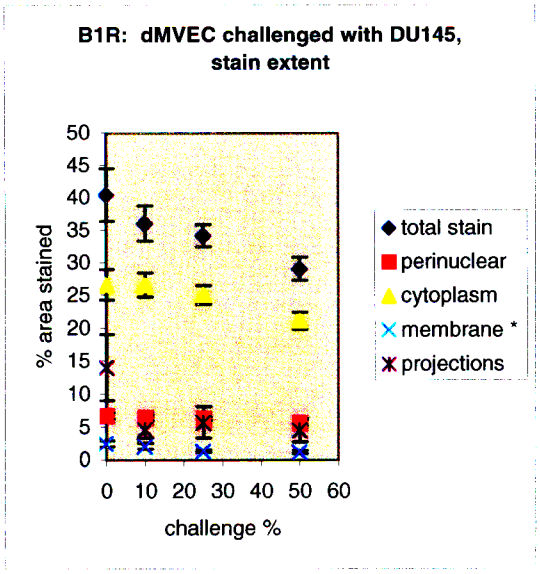
(ii)A. dMVEC–DU145, stain intensity

dMVEC-DU145	0%	10%	25%	50%
perinuclear	100 (2.06)	98.85 (3.35)	98.46 (3.19)	105.48 (2.34)
cytoplasm	100 (5.62)	94.14 (4.42)	81.00 (4.23)	101.59 (6.01)
membrane	100 (12.29)	80.42 (9.66)	64.22 (8.33)	80.79 (12.98)
projections	100 (6.19)	92.13 (11.95)	98.90 (3.02)	98.68 (3.22)

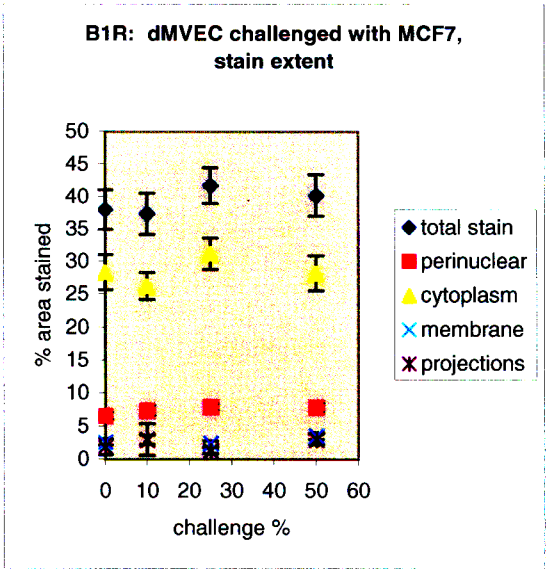
B. dMVEC–MCF7, stain intensity

dMVEC-MCF7	0%	10%	25%	50%
perinuclear	100 (2.63)	94.92 (2.84)	101.62 (1.82)	94.11 (3.17)
cytoplasm	100 (5.49)	97.04 (5.45)	105.29 (4.84)	100.24 (5.05)
membrane	100 (9.79)	136.71 (15.21)	100.03 (9.62)	124.06 (10.42)
projections	100 (7.69)	109.57 (6.25)	105.23 (24.08)	99.29 (10.28)

(iii)A. dMVEC-DU145, stain extent



B. dMVEC-MCF7, stain extent



* Kruskal-Wallis, $p = 0.045$

3.3.3 B2R in dMVECs

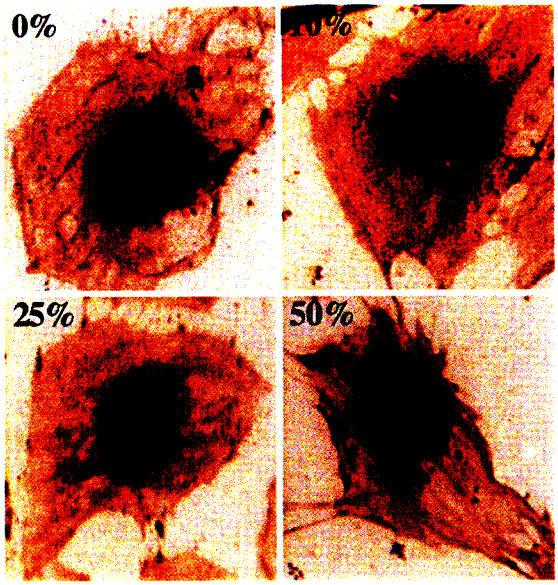
There were no statistically significant changes in the intensity or extent of stain for B2R in dMVECs that were challenged with tumour cell metabolites when compared with their unchallenged counterparts (Figure 3.7). The mean intensities of staining in the projections of MCF7-challenged dMVECs (10%, 25% and 50%) were greater than that of unchallenged dMVECs; however, large variations in projection stain intensity occurred for all conditions and this result was not statistically significant (Figure 3.7 (ii)B).

3.3.4 TK in tumour cells

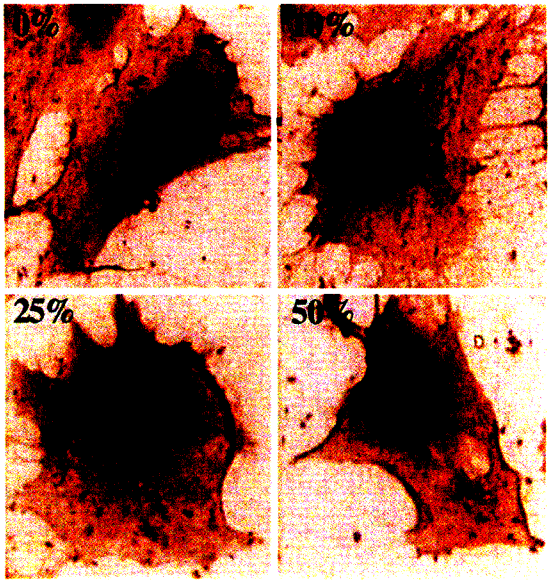
The stain intensity for TK in the perinuclear caps and cytoplasm of DU145 cells was similar for the 10%, 25% and 50% challenge ratios and significantly less than the stain intensity for the 0% condition (Figure 3.8(i)A-(ii)A, ANOVA p=0.0093 and p=0.0039, respectively). The intensity of stain in DU145 projections followed the same trend although it was not statistically significant. The area of DU145 cytoplasmic stain for TK significantly decreased with increasing challenge percentage (ANOVA p=0.012) and this largely contributed to a significant decrease in total stain extent (Figure 3.8(iii)A, ANOVA p=0.0053). There were also slight, statistically insignificant decreases in the extents of perinuclear cap and projection stain. Overall, there was a tendency for decreased staining for TK in challenged DU145 cells.

Although the mean intensities of stain for TK were greater in challenged MCF7 cells when compared with unchallenged counterparts, no statistically significant changes in stain intensity occurred (Figure 3.8(i)B-(ii)B). Similarly, challenging MCF7 cells with dMVEC metabolites did not significantly affect the extent of TK labelling in these cells (Figure 3.8(iii)B).

(i)A. dMVEC–DU145, sample images



B. dMVEC–MCF7, sample images



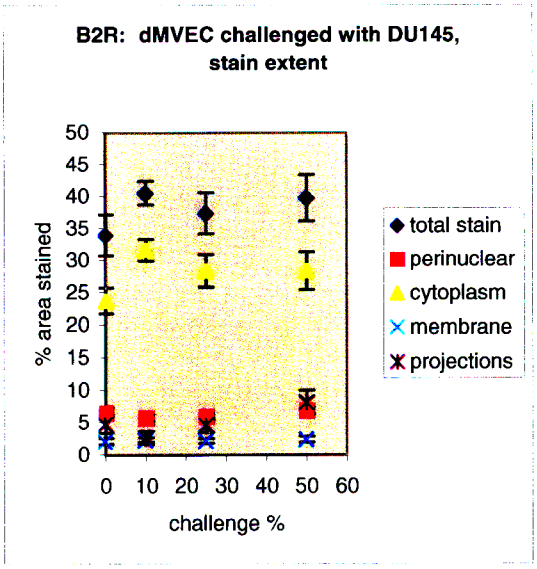
(ii)A. dMVEC–DU145, stain intensity

dMVEC-DU145	0%	10%	25%	50%
perinuclear	100 (2.74)	101.85 (3.72)	96.86 (3.76)	96.03 (2.6)
cytoplasm	100 (4.17)	96.64 (4.01)	90.04 (5.55)	97.94 (7.12)
membrane	100 (9.92)	92.61 (9.52)	81.59 (9.42)	111.73 (13.90)
projections	100 (2.37)	81.42 (8.46)	97.25 (3.25)	93.79 (2.51)

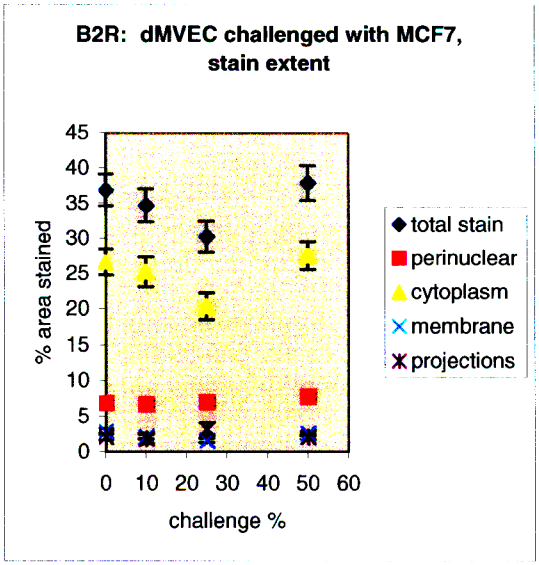
B. dMVEC–MCF7, stain intensity

dMVEC-MCF7	0%	10%	25%	50%
perinuclear	100 (3.24)	99.51 (3.04)	97.14 (4.68)	95.85 (3.38)
cytoplasm	100 (6.29)	93.38 (4.24)	103.86 (8.36)	96.48 (3.74)
membrane	100 (8.79)	105.46 (7.37)	84.54 (8.85)	95.48 (8.83)
projections	100 (15.93)	116.50 (7.91)	120.77 (17.32)	110.47 (8.31)

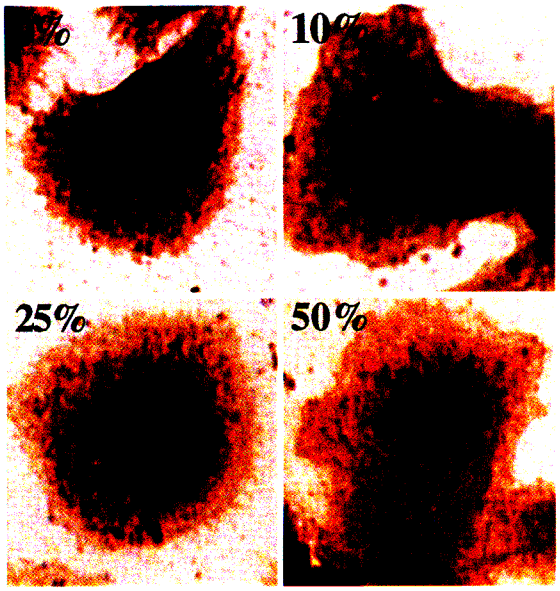
(iii)A. dMVEC–DU145, stain extent



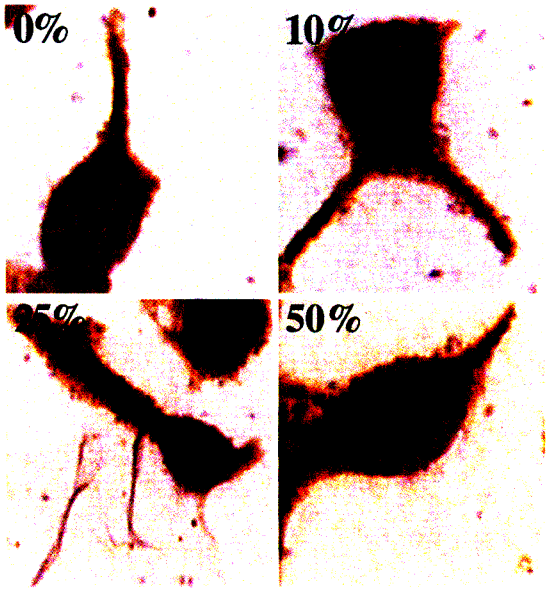
B. dMVEC–MCF7, stain extent



(i)A. DU145–dMVEC, sample images



B. MCF7-dMVEC, sample images



(ii)A. DU145–dMVEC, stain intensity

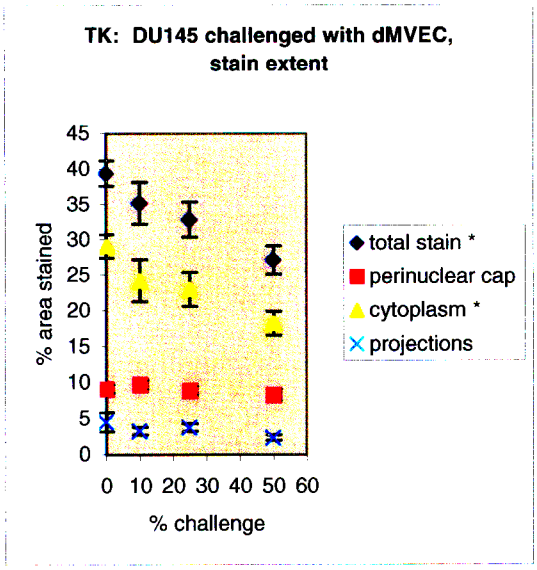
DU145 – dMVEC	0%	10%	25%	50%
perinuclear cap *	100 (1.52)	84.91 (4.01)	86.99 (3.56)	89.51 (3.40)
cytoplasm *	100 (5.19)	68.58 (7.53)	75.13 (7.24)	70.53 (6.33)
projections	100 (16.37)	59.78 (11.60)	65.93 (4.71)	66.10 (20.35)

B. MCF7-dMVEC, stain intensity

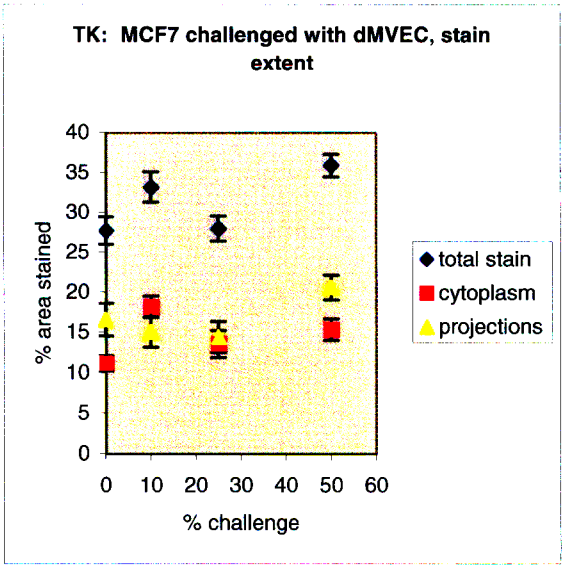
MCF7 - dMVEC	0%	10%	25%	50%
cytoplasm	100 (8.47)	123.43 (4.87)	118.99 (6.55)	117.14 (6.76)
projections	100 (6.18)	119.69 (6.87)	100.87 (5.70)	129.43 (5.09)

* ANOVA, $p \leq 0.0093$

(iii)A. DU145–dMVEC, stain extent



B. MCF7–dMVEC, stain extent



* ANOVA, $p \leq 0.012$

3.3.5 B1R in tumour cells

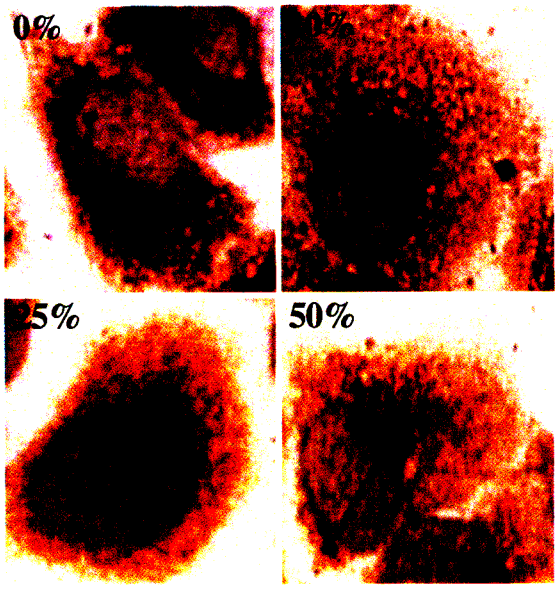
Decreases in the stain intensity for B1R occurred in dMVEC-challenged DU145 cells (Figure 3.9(i)A-(ii)A). There was a statistically significant decrease in the perinuclear cap stain intensity in cells of the 10% challenge condition when compared with that of unchallenged cells (ANOVA p=0.0025). Challenged DU145 cells (10%, 25% and 50% challenge conditions) had decreased cytoplasmic stain intensity (when compared with unchallenged cells) that was most marked in the 10% challenge condition (ANOVA p=0.014). The extent of stain for B1R was diminished in the cytoplasm of challenged cells (ANOVA p<0.0001), resulting in a significant decrease in total stain that was most diminished for the 50% challenge condition (Figure 3.9(iii)A, ANOVA p<0.0001). Overall, there is evidently a trend for decreased stain intensity and extent for B1R in DU145 cells that were exposed to dMVEC metabolites.

Challenging MCF7s with dMVEC metabolites did not result in significant changes in stain intensity or extent of stain for B1R (Figure 3.9(i)B-(iii)B).

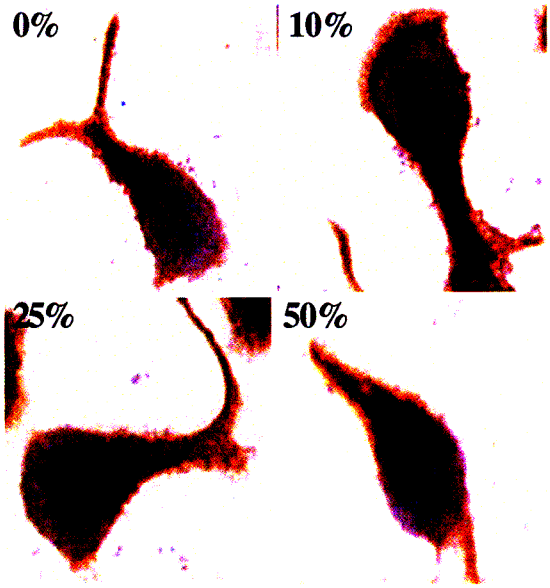
3.3.6 B2R in tumour cells

Staining in the cytoplasm of DU145 cells that were challenged with dMVEC metabolites diminished with increasing challenge ratio, however this was not statistically significant (Figure 3.10(i)A-(ii)A). Similarly, the stain intensity in the projections of DU145 cells of the 25% and 50% challenge conditions was decreased, although this was statistically insignificant, when compared with unchallenged cells. There was a stepwise statistically significant decrease in the extent of cytoplasmic stain in challenged DU145 cells (ANOVA p=0.0004) that resulted in an overall significant decrease in total stain extent (Figure 3.10(iii)A, ANOVA p=0.0003).

(i)A. DU145–dMVEC, sample images



B. MCF7-dMVEC, sample images



(ii)A. DU145–dMVEC, stain intensity

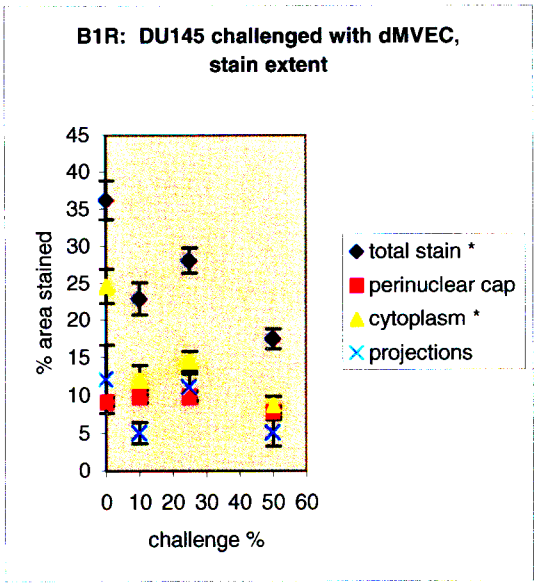
DU145 – dMVEC	0%	10%	25%	50%
perinuclear cap *	100 (3.14)	82.20 (4.46)	94.66 (3.66)	98.31 (2.4)
cytoplasm *	100 (8.90)	62.43 (12.62)	93.55 (10.36)	75.15 (6.79)
projections	100 (18.56)	84.45 (13.99)	66.65 (13.52)	104.97 (44.53)

B. MCF7-dMVEC, stain intensity

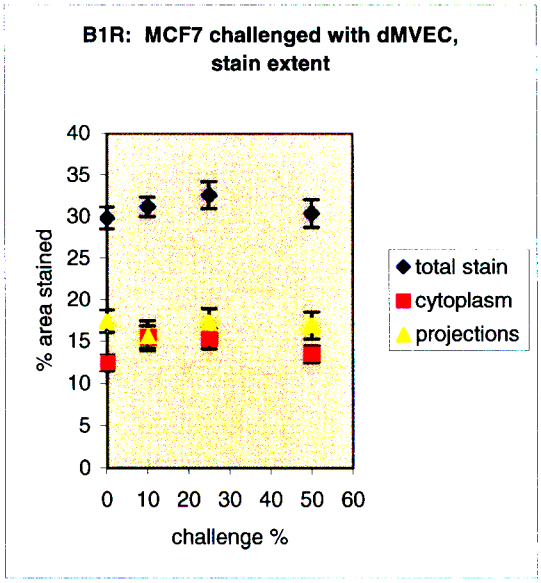
MCF7 - dMVEC	0%	10%	25%	50%
cytoplasm	100 (4.80)	99.42 (4.90)	102.54 (4.83)	105.72 (3.68)
projections	100 (9.58)	100.73 (11.26)	92.73 (8.77)	109.13 (6.89)

* ANOVA, $p \leq 0.014$

(iii)A. DU145–dMVEC, stain extent

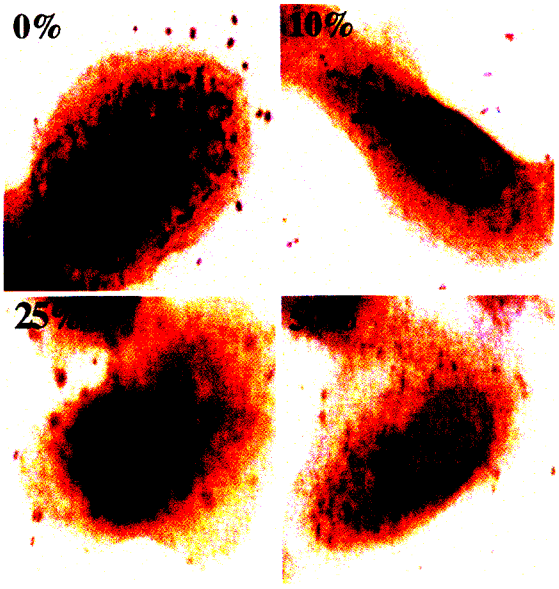


B. MCF7–dMVEC, stain extent

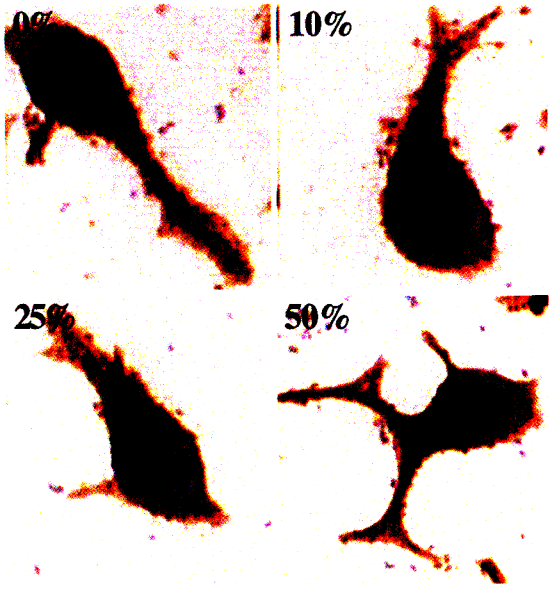


* ANOVA, $p < 0.0001$

(i)A. DU145–dMVEC, sample images



B. MCF7-dMVEC, sample images



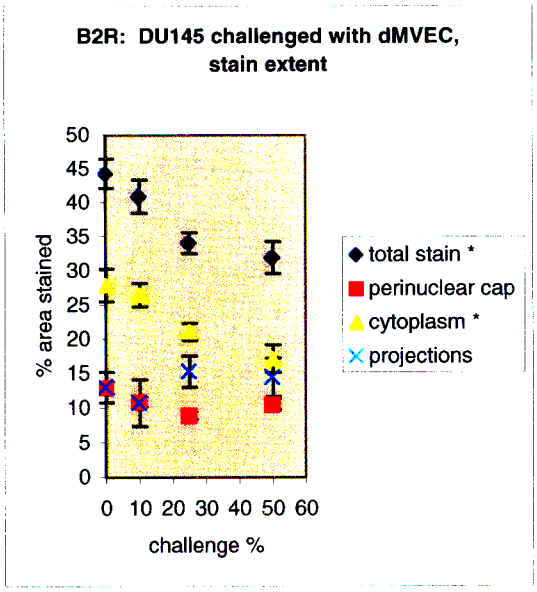
(ii)A. DU145–dMVEC, stain intensity

DU145 – dMVEC	0%	10%	25%	50%
perinuclear cap	100 (2.10)	100.31 (1.64)	97.56 (1.97)	97.61 (2.09)
cytoplasm	100 (11.88)	87.45 (8.58)	79.5 (7.24)	71.09 (7.31)
projections	100 (13.59)	117.35 (16.22)	73.96 (9.28)	80.91 (13.72)

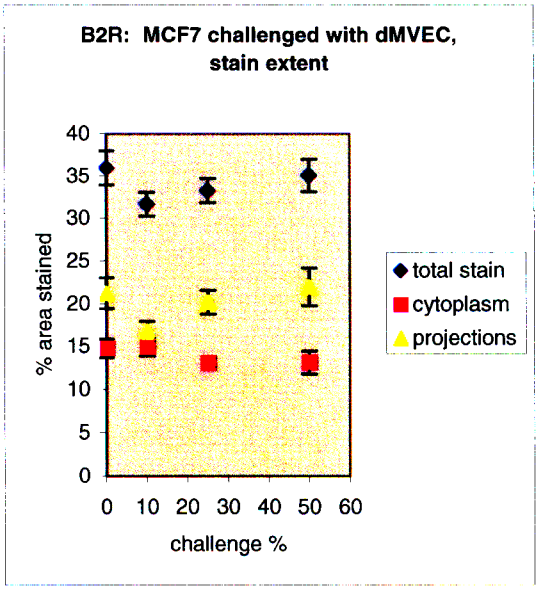
B. MCF7-dMVEC, stain intensity

MCF7 - dMVEC	0%	10%	25%	50%
cytoplasm	100 (6.58)	98.45 (6.16)	98.26 (5.15)	107.46 (6.56)
projections	100 (8.24)	112.31 (11.42)	100.98 (9.77)	126.77 (7.98)

(iii)A. DU145–dMVEC, stain extent



B. MCF7–dMVEC, stain extent



* ANOVA, $p \leq 0.0004$

Challenging MCF7s with dMVEC metabolites did not result in any statistically significant changes in the stain intensity or extent of stain for B2R (Figure 3.10(i)B-(iii)B). Although the mean intensity of stain in the MCF7 projections of the 50% challenge condition was considerably greater than that of unchallenged cells, no overall trend was observed for projection staining intensity and no statistically significant differences were found between the various challenge conditions (Figure 3.10(ii)B).

3.4 Immuno-localisation: TK, B1R and B2R in endothelial-tumour co-cultures

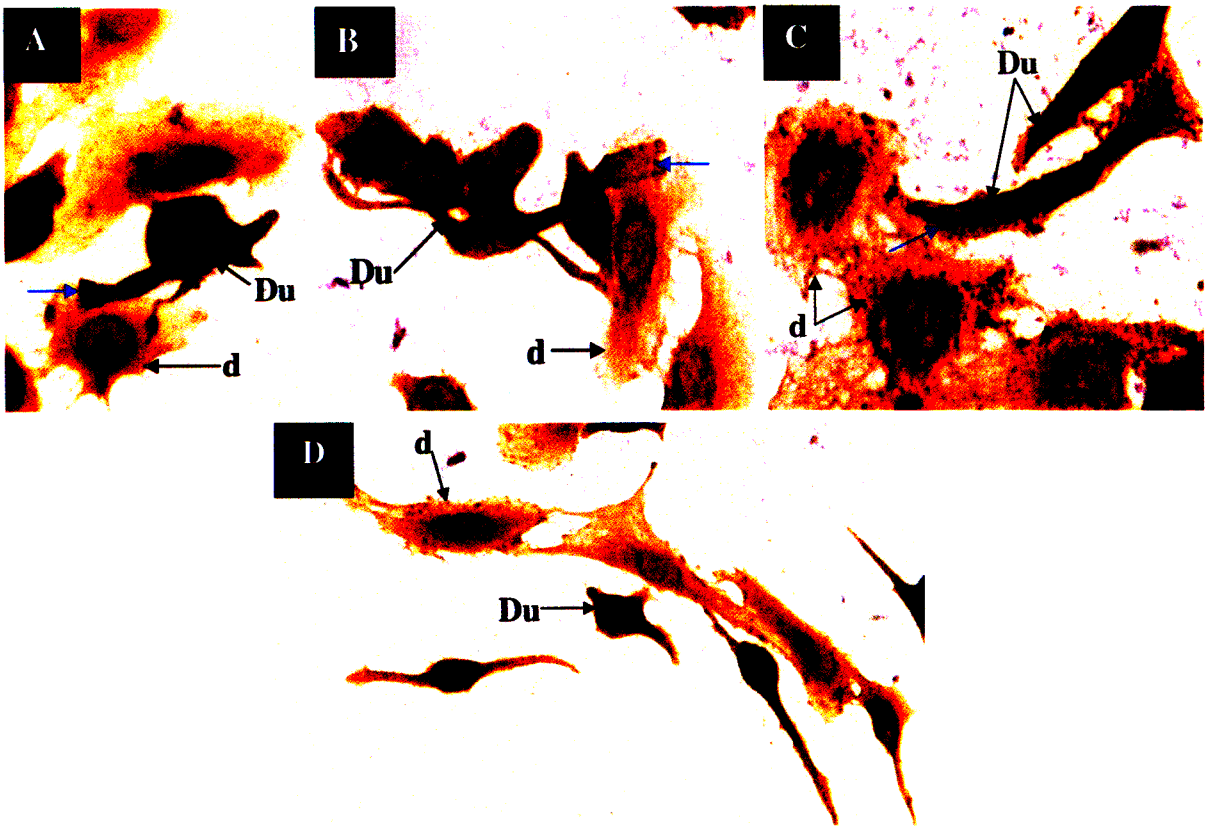
3.4.1 dMVEC-DU145 co-culture

DU145 cells stained intensely over the entire cell for TK, B1R and B2R when co-cultured with dMVECs while the staining pattern for these proteins in dMVECs co-cultured with DU145 cells was similar to that in mono-cultured dMVECs (Figures 3.3 and 3.11(i)A-C). TK, B1R and B2R were present in DU145 projections that formed footplate-like connections with dMVECs (Figure 3.11(i)A-C). Staining for these KKS proteins was also present in the few dMVEC projections that connected with DU145 cells. There was a tendency for dMVECs to increasingly form connections with one another, resulting in long chain-like structures, when co-cultured with DU145 cells (Figure 3.11(i)D).

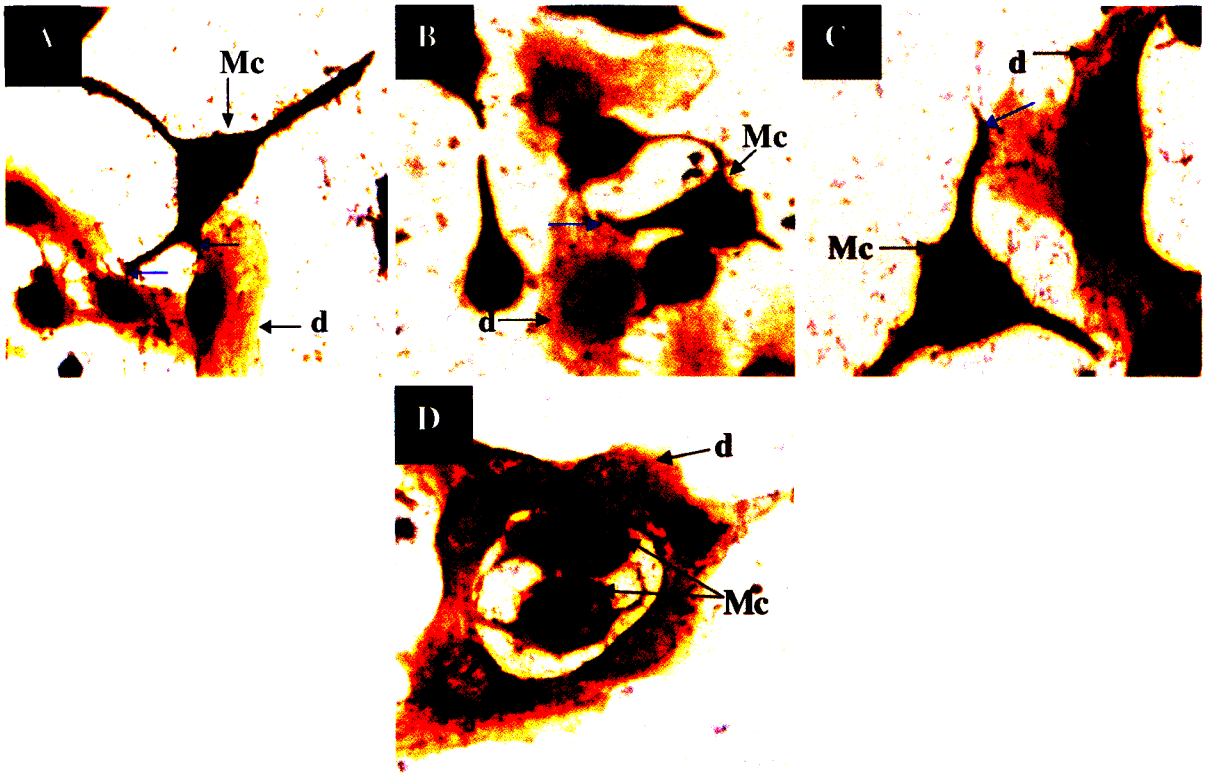
3.4.2 dMVEC-MCF7 co-culture

Similar to the dMVEC-DU145 co-cultures (Section 3.4.1), the MCF7 tumour cells labelled intensely over the entire cell for TK, B1R and B2R while the staining pattern for these proteins in dMVECs remained similar to that of dMVECs in mono-culture (Figures 3.3 and 3.11(ii)A-C). Staining for the KKS proteins was present in MCF7 projections that made contact with dMVECs in the form of footplate-like ends (Figure 3.11(ii)A-C). Occasionally dMVECs formed connections (that stained for TK, B1R and B2R) with MCF7 cells. As in

(i) dMVEC-DU145 co-culture



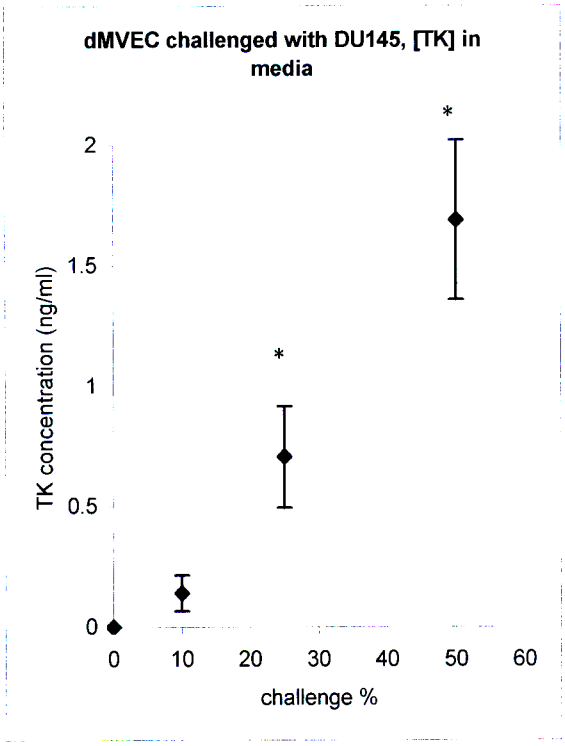
(ii) dMVEC-MCF7 co-culture



The TK concentrations in the conditioned media of unchallenged DU145 cells and cells of the 25% and 50% challenge conditions were similar (Figure 3.12C). Although, the TK concentration for the 10% challenge condition was increased when compared with that for unchallenged cells, this was not statistically significant. Similarly, the media of 10% challenged MCF7 cells had an increase in TK concentration when compared with that in the media of unchallenged MCF7 cells and cells of the other challenge conditions, however this was not statistically significant (Figure 3.12D).

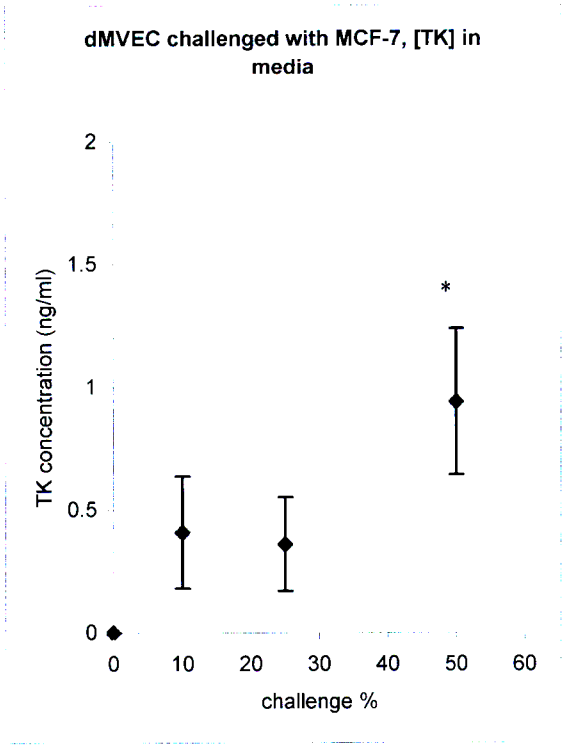
TK concentration in the conditioned media of unchallenged tumour cells was considerably greater than that in the media of unchallenged dMVECs (Figure 3.12). However, 50% DU145 and MCF7-challenged dMVECs secreted more TK than tumour cells and similar amounts of TK when compared with tumour cells, respectively.

A



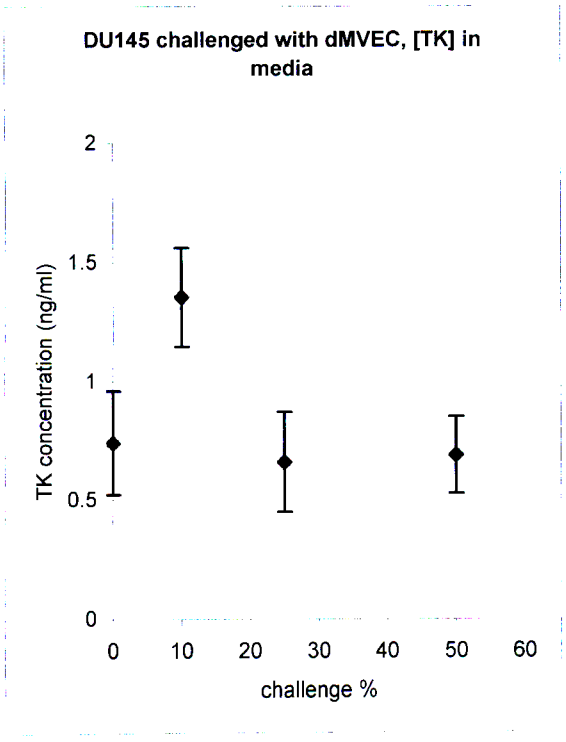
Kruskal-Wallis, p=0.0003

B

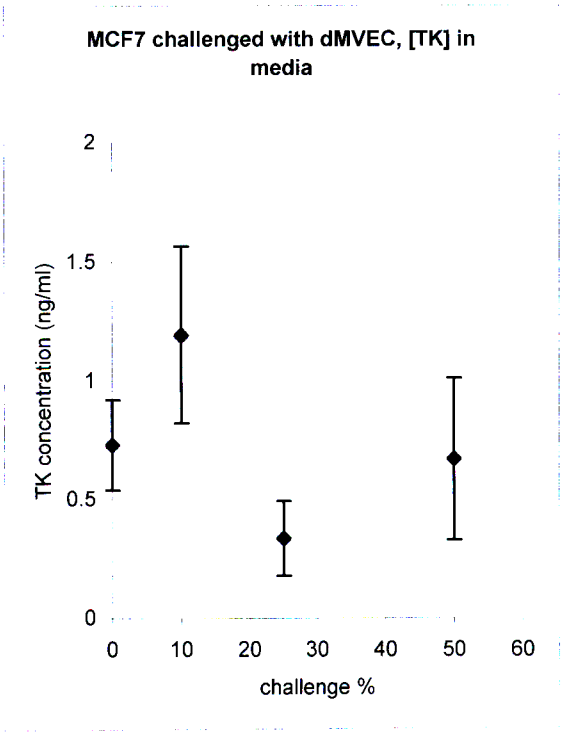


Kruskal-Wallis, p=0.0017

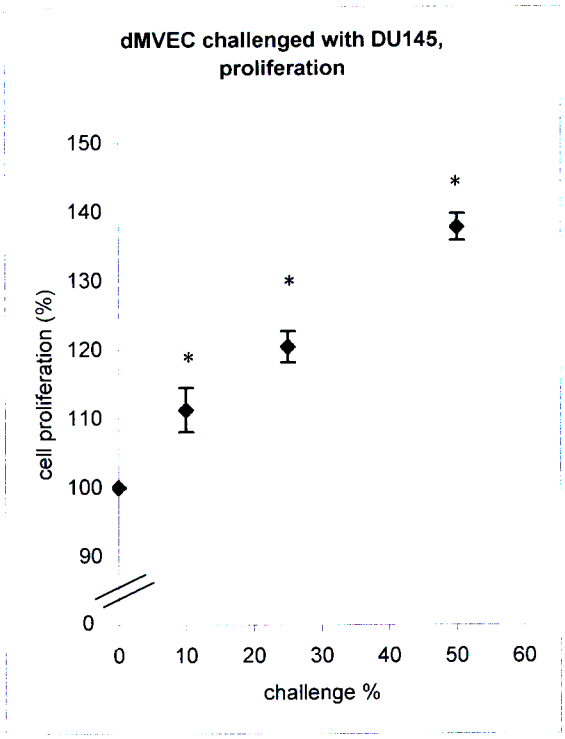
C



D

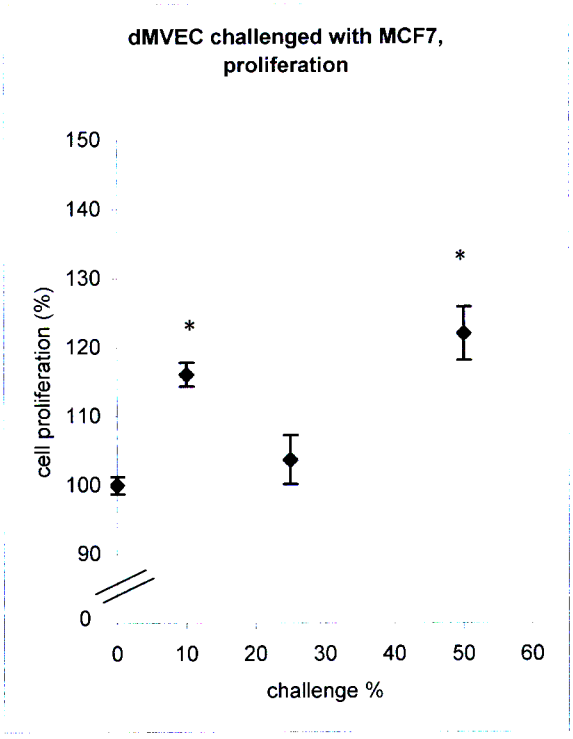


A



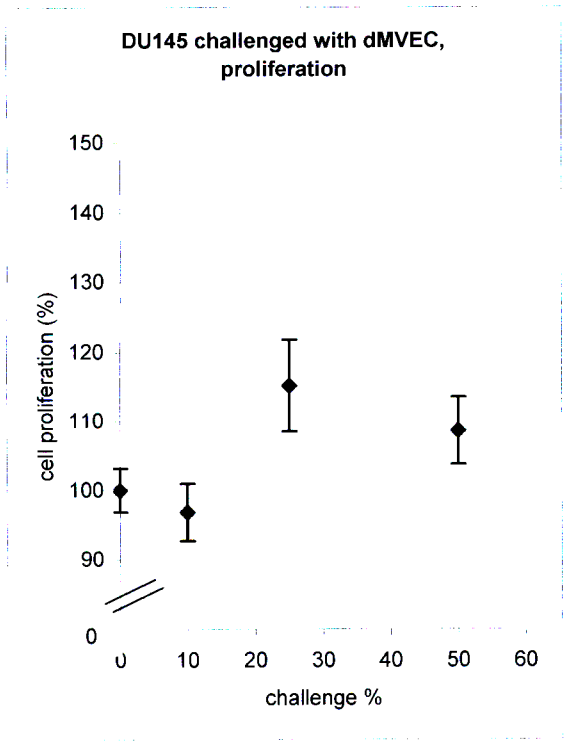
ANOVA, $p< 0.0001$

B

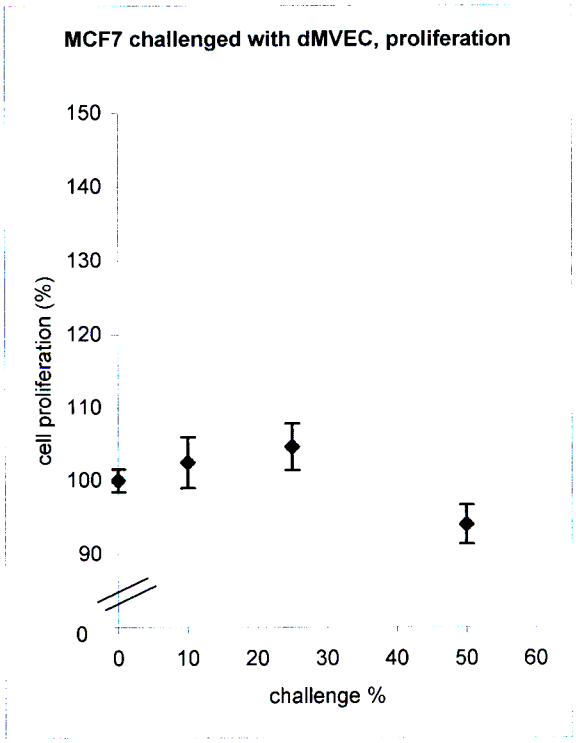


ANOVA, $p< 0.0001$

C



D



CHAPTER 4

DISCUSSION

CHAPTER 4 - DISCUSSION

4.1 The KKS in endothelial cells

4.1.1 Unchallenged endothelial cells

The present study found that TK, B1R and B2R were expressed in cultured human endothelial cells. While previous studies have shown TK and B2R to be consistently expressed in cultured endothelial cells (*286, 354, 378*) and endothelial cells *in vivo* (*189, 203*); the expression of B1R in cultured endothelial cells has not been consistently demonstrated (*286, 378*) and *in vivo* expression of B1R only occurs to a significant extent in pathological conditions (*203*). Expression of B1R in cultured endothelial cells in the present study, and in some endothelial types in previous studies (*256, 292, 354, 378*), could possibly be explained by the phenomenon of post-isolation induction. B1R induction following isolation of tissues from the body is a well-known event that was first shown to occur in rabbit aortic tissue (*203*). Therefore, it appears that, while TK and B2R are expressed in endothelial cells physiologically, B1R is not significantly expressed in these cells although it may be expressed in endothelial cells in a pathological environment, such as that of a tumour.

In the present study, unchallenged endothelial cells (dMVECs) did not appear to secrete TK, as measured by ELISA. However, this may be due to sensitivity limitations of the assay rather than an absence of TK secretion from endothelial cells. Yayama *et al.* (2003) demonstrated secretion of TK from HUVECs by western blotting of HUVEC-conditioned medium using anti-human TK antibodies (*379*). Similarly, Naidoo (2005) demonstrated TK secretion from HUVECs using the same methodology as that used in the present study (*354*). Therefore, it seems likely that the unchallenged microvascular cells (dMVECs) used in the present study secreted TK, although to a much lesser extent than HUVECs.

Yayama *et al.* (2003) showed that the TK secreted by endothelial cells was active. In that study, TK activity in the conditioned medium of HUVECs was demonstrated by cleavage of the synthetic substrate Pho-Phe-Arg-4-methyl-coumaryl-7-amide and generation of kinin-like contractile activity on rat uterus when incubated with kininogen and HUVEC-conditioned medium, followed by inhibition of these effects by a specific TK inhibitor, apronitin. This strongly suggests that TK secreted by endothelial cells is in an active form and has kinin-generating ability. This is consistent with reports of protease conversion from the inactive to active form occurring intracellularly (within the secretory pathway) as well as extracellularly (*203*). It is possible that active TK secreted from endothelial cells may act on localised kininogen, which is found bound to the surface of endothelial cells (HMWK) and in plasma surrounding endothelial cells (LMWK) (*171, 211*), to produce kinins that then act on endothelial cell B2R/B1R. However, it should be noted that although TK has been shown to readily produce kinins from HMWK *in vitro* (*171, 205-207*), the well-accepted route of its action is the generation of kallidin from LMWK (*171*). While both kallidin and bradykinin may activate B2R, kallidin may also significantly activate B1R (*203*). In addition to kinin generation followed by B1R/B2R signalling, active TK has been reported to directly activate B2R, possibly via proteolytic cleavage (*218*), although this was not supported by a later study (*219*). Endothelial cell B1R and B2R signalling following the secretion of active TK may enhance angiogenic activities (*354*), as is discussed in detail later in Section 4.1.2. Aside from kinin generation, TK may also cleave and activate other proteases involved in angiogenesis (*48, 242, 243*) (Section 4.1.2). Since, in adults, angiogenesis is mainly a pathological process that is controlled by a balance of pro and anti-angiogenic factors (*39*), it appears reasonable that microvascular endothelial cells, which participate in angiogenesis *in vivo* (*347*), secrete very little TK (a pro-angiogenic factor) under normal physiological conditions.

While TK, B1R and B2R immuno-labelled in the cytoplasm region of endothelial cells in the present study [and in previous studies (*286, 354*)], it was of particular interest that these proteins were also localised in (i) large projections of endothelial cells and (ii) in specific endothelial cell membrane regions in relation to surrounding cells and in small projections arising from these membrane regions, as discussed below. It should be noted that while labelling for B1R and B2R was observed in various regions of the cell, these receptors are actually present in the plasma membrane (i.e. on the cell surface) (*260*).

(i) The intense labelling of TK, B1R and B2R in the large projections of endothelial cells was consistent with the finding of Plendl *et al.* (2000) who demonstrated intense TK labelling in projections of bovine aortic endothelial cells (*286*), and Naidoo (2005) who demonstrated intense labelling for TK, B1R and B2R in HUVEC projections (*354*). Since invasive cell migration is initiated by the growth of long cellular extensions/projections (*380*), the localisation of the KKS proteins in large projections of endothelial cells suggests a role for the KKS in the migration and invasion of these cells, which occurs during angiogenesis (*39*). Although specific involvement of the KKS in endothelial cell migration has not been demonstrated thus far, there are many possible mechanisms by which the KKS may promote migration and invasion of endothelial cells during angiogenesis. For example, TK secreted from endothelial cell projections may cleave and activate MMP-2 (*242*) and MMP-9 (*243*). These MMPs may then cleave ECM components thereby facilitating cellular invasion of the ECM (*19*). In particular, MMP-2, which is bound to $\alpha_v\beta_3$ on the surface of angiogenic endothelial cells allows for localised degradation of the ECM as these cells migrate (*68*). Further, secretion of TK from the endothelial cell projections, followed by activation of MMPs would localise ECM-degrading activity to the leading edge of movement (i.e. the projection). It is also possible that TK secreted from endothelial cell projections may directly cleave the ECM at the leading edge

of movement, thereby facilitating endothelial cell invasion of the ECM. Direct cleavage of the ECM by TK has not been demonstrated thus far, however, a previous study has suggested TK involvement in ECM cleavage (*241*) and several kallikreins have been shown to directly cleave the ECM (*172*). TK secreted from endothelial cell projections may also generate kinins from localised kininogen bound to the surface of endothelial cells or in plasma surrounding endothelial cells resulting in B1R/B2R signalling in endothelial cell projections (as discussed earlier). B1R/B2R signalling results in the production of NO (*203*) which, via modulation of FAK phosphorylation, is important in the regulation of endothelial movement (*133*). FAK phosphorylation [which may be mediated by B1R (*260*)] results in recruitment of focal adhesion proteins, formation of focal adhesion complexes and stabilisation of adhesion (*25*). Localisation of this activity to the endothelial cell projection would result in attachment of the projection to the ECM. Attachment to the ECM is required at the leading edge of movement (the projection) in order to provide traction to pull the cell forward while detachment of adhesions at the trailing edge allows forward motion (*20, 380*). Thus, the localisation of KKS proteins in large projections of endothelial cells supports the role of the KKS in angiogenesis.

(ii) In the present study, KKS labelling was also observed in endothelial cell membrane regions that were adjacent to, but not yet in contact with, other endothelial cells as well as in small projections that extended from these labelled areas towards other endothelial cells. This suggests that the KKS is involved in the initiation of cell-cell contact in endothelial cells. Accordingly, KKS proteins were generally absent or less in regions of the cell periphery that already had well-established contact with another cell. Folkman and Haudenschild (1980) demonstrated that the alignment and connection of endothelial cells with one another, such that their autolytic vacuoles (present in the cytoplasm) form a continuous tube, is necessary for capillary tube formation (*71*). Additionally, the formation

of endothelial cell contact with pericytes is necessary for vessel formation (*136*). Thus, the involvement of the KKS in initiating endothelial cell contact with other endothelial cells, and possibly with other cell types, suggests that the KKS could potentially play a role in establishing capillary tube formation during the latter stages of angiogenesis. In support of this, a previous study has shown inhibition of HUVEC cord formation on matrigel by BK antagonists (*293*).

4.1.2 Challenged endothelial cells

A major finding in the present project was that when endothelial cells were challenged with prostate and breast tumour metabolites, intracellular TK significantly decreased, as indicated by the diminished intensity and extent of stain for TK, and this was accompanied by a significant increase in TK in the conditioned media. This indicates that the decrease in intracellular TK in challenged endothelial cells was most likely due to tumour-induced secretion of TK by endothelial cells into the medium.

Previous studies support tumour-induction of TK secretion by endothelial cells. HUVECs that were challenged with neuroblastoma and cervical carcinoma metabolites had decreased intracellular TK and increased extracellular TK (secreted into the medium) when compared with unchallenged HUVECs (*354*). Stadnicki *et al.* (1998, 2003) have shown, although not conclusively, increased TK secretion by intestinal goblet cells and macrophages with a concomitant decrease in TK in intestinal tissue in both rat enterocolitis and human inflammatory bowel disease (specifically Crohn's disease) (*188, 270*). Those studies provide evidence that TK secretion increases in chronic inflammatory conditions, which include cancer (*43*). Contrary to these findings, Plendl *et al.* (2000) found increased proTK in bovine angiogenic endothelial cells of the mature corpus luteum in comparison to non-angiogenic cells and no significant difference in intracellular TK levels between them (*286*).

However, TK secretion from these cells was not quantified. It is possible that increased proTK in angiogenic cells indicated increased expression of the TK gene; however, no corresponding increase in intracellular TK could suggest increased secretion of TK. Other studies have shown that tumour cells may induce the release of angiogenic factors other than TK from various cell types, including endothelial cells. Examples of this are the increased VEGF levels which occurred in the culture medium concomitant with decreased VEGF levels in hepatocytes when these cells were co-cultured with neuroblastoma cells (*381*), and the finding that microvascular endothelial cells secreted significantly increased amounts of MMP-2 when co-cultured with prostate tumour cells (*382*).

Since TK may be secreted from endothelial cells in an active form (*379*), the upregulation of TK secretion from endothelial cells exposed to prostate and breast tumour metabolites implies increased extracellular TK activity in these tumour environments. As discussed earlier (Section 4.1.1), active TK may generate kinins from local kininogen resulting in the activation of endothelial cell kinin receptors, and TK may also directly activate endothelial cell B2R. Previous *in vitro* studies have shown that the activated endothelial cell kinin receptors are involved in signalling angiogenic activities such as endothelial cell proliferation (*256, 292, 293*) and cord formation (*254, 293*). Kinin receptor signalling has also been shown to potentiate that of VEGF receptors and bFGF receptors in endothelial cells (*249, 254, 256*). Since VEGF is involved in mediating all steps of the angiogenic cascade (*39, 58, 78, 81*) and since bFGF has been shown to stimulate endothelial proliferation, migration and capillary tube formation *in vitro* (*39, 68*), involvement of the kinin signalling in all stages of angiogenesis is likely. The role of kinin receptor signalling in angiogenesis is further supported by *in vivo* studies using mouse models that have shown significant suppression of angiogenesis by both a B1R antagonist (*292*) and a B2R antagonist (*383*). Endothelial cell kinin receptor signalling also results in increased capillary

permeability (*203, 246*), which is important for the infiltration of inflammatory cells and therefore initiation of inflammation (*266, 267*). Kinins produced by active TK in the tumour environment may also act on stromal cells to enhance chemotaxis and activation of inflammatory cells (*118, 268*). Since inflammation is a promoter of angiogenesis and these two processes often occur concomitantly (*97, 106, 107*), it is likely that the pro-inflammatory effects induced by kinins in the tumour environment would potentiate angiogenesis. Aside from the generation of kinins, active TK secreted by endothelial cells may upregulate proteolytic activity in the tumour environment either directly or via the activation of MMPs (*242, 243*). Proteolytic activity not only facilitates the migration and invasion of endothelial cells via enhancing ECM cleavage, but also plays a role in other aspects of the angiogenic cascade. For example, MMPs play an important role in capillary tube formation (*180*) and they may cleave and activate growth factors and growth factor receptors that are involved in endothelial cell proliferation and capillary tube formation (*48, 68*). Further supporting the role of TK as a promoter of angiogenesis, TK gene therapy promoted angiogenesis in ischaemic mouse (*287*) and rat models (*289*), corrected impaired angiogenesis in spontaneously hypertensive rats (*288*) and protected rats from ischaemic injury when administered after the onset of a stroke (*290*). Therefore, it is postulated that a tumour-mediated increase in TK secretion by endothelial cells would upregulate angiogenesis, thereby promoting prostate and breast tumour progression. The TK secreted by endothelial cells exposed to tumour metabolites may also promote tumour progression by directly influencing tumour cell activities and this is discussed in detail in the following section on tumour cells (Section 4.2.1).

Although there was a significant overall decrease in intracellular TK in challenged endothelial cells and TK decreased in the cytoplasm, perinuclear and membrane regions of these challenged cells, this did not apply to TK staining in the projections of challenged

endothelial cells. It was interesting that TK tended to increase in endothelial cell projections with increasing exposure to prostate and breast cancer cell metabolites, although this was not a statistically significant trend. This finding suggests that tumour metabolites may recruit TK to the endothelial cell projections. Considering the presence of all the KKS components in the region of the endothelial cell projections and the implication of KKS activity in cell migration, it appears that tumour metabolites could promote endothelial cell migration via this mechanism. This would further contribute to tumour angiogenesis and therefore tumour progression.

Although intracellular TK decreased significantly in challenged endothelial cells, intracellular B1R and B2R levels in endothelial cells were not significantly affected by exposure to prostate and breast tumour cell metabolites. These findings are consistent with the unchanging intracellular B1R and B2R levels in HUVECs challenged with neuroblastoma metabolites in a previous study (*354*). However, since inflammatory cytokines and growth factors upregulate the expression of B1R and B2R (*203*), it is likely that the expression of B1R and B2R would be increased in endothelial cells exposed to the inflammatory environment present in the stroma of prostate and breast tumours (*12, 43*).

Exposure of endothelial cells to prostate and breast tumour cell metabolites significantly increased their proliferation (with the exception of the 25% MCF7-challenged dMVECs). These findings are consistent with those of a previous study in which HUVEC proliferation was greatly and significantly increased by neuroblastoma and cervical carcinoma cell metabolites (*354*). Also in support of increased endothelial cell proliferation induced by tumour cells, are the results of a previous study showing increased proliferation of endothelial cells following exposure to conditioned medium of progestin-treated breast cancer cells (*384*).

TK secreted from challenged endothelial cells [as well as TK secreted by prostate and breast tumour cells (Section 4.2.1)] may have contributed to the observed increase in proliferation of challenged endothelial cells. As discussed earlier, tumour-induced TK secreted from endothelial cells may activate endothelial cell kinin receptor signalling. The contribution of increased extracellular TK to upregulation of endothelial cell proliferation is supported by *in vitro* studies that demonstrate a role for kinin receptors in endothelial cell proliferation (*256, 292*). Further, it has been demonstrated in an *in vivo* study that B2R contributes to endothelial cell proliferation (*294*). Since endothelial cell proliferation is a necessary step of angiogenesis, it appears that tumour-induced endothelial cell proliferation (possibly involving TK) is a mechanism of promoting angiogenesis in prostate and breast tumours. In the present study, endothelial cell proliferation may also have been stimulated by VEGF, an important endothelial cell growth factor and initiator of angiogenesis (*39, 81*), which may be released by both DU145 and MCF7 cells (*385, 386*).

4.2 The KKS in tumour cells

4.2.1 Unchallenged tumour cells

The present study found that TK, B1R and B2R were expressed in cultured DU145 prostate tumour cells. This is consistent with previous reports of TK and B2R in cultured prostate cancer cells (*274*) and the expression of B1R in malignant prostate tissues and cultured prostate cancer cells (*260*). While TK and B2R are expressed in both the normal prostate and in prostate cancer (*260, 274*), B1R is only expressed in prostate cancer (*260*), suggesting a role for B1R in prostate tumourigenesis. It has been suggested that B1R may be used as a marker for pathological growth of the prostate in addition to other kallikrein markers already in use (*260*), the most important clinical marker being PSA/kallikrein 3 (*172*).

TK, B1R and B2R were also expressed in MCF7 breast tumour cells in the present study. The presence of TK in breast cancer tissue has been demonstrated previously (*325, 387*), and B1R and B2R have been detected in cultured breast tumour cells (*327, 388*). TK and B2R are also expressed in normal breast tissue (*325, 326*), although B1R is not. Specific expression of B1R in breast cancer cells indicates that B1R is involved in breast tumourigenesis.

In contrast to unchallenged endothelial cells, both prostate and breast tumour cells secreted amounts of TK that were detectable by ELISA in the present study. The secretion of TK by these tumour cells in conjunction with tumour-induced TK secretion from endothelial cells (Section 4.1.2) and B1R and B2R expression in tumour cells, suggests the presence of an active KKS in these tumour types. Although the kininogen component of the KKS was not demonstrated in the present study, it is expected that kininogen is abundant in the tumour environment. LMWK and HMWK in tumours could be provided by extravascular fluid (*171, 211*), which is abundant in tumours due to the high permeability of tumour blood vessels (*12*). Neutrophils present in the inflammatory reaction in the tumour stroma (*266*), are a source of both HMWK and LMWK (*210*). Additionally, endothelial cells in the tumour environment could act as a source of HMWK (*171*). Therefore, tumour-derived TK, or TK derived from endothelial cells exposed to tumour metabolites (in the tumour environment), may generate kinins from kininogen present in the tumour environment and thereby activate tumour cell kinin receptors. This, in addition to TK-mediated upregulation of proteolytic activity, may have pro-tumourigenic effects, a discussion of which follows.

TK-mediated kinin generation from kininogen present in the tumour environment may signal the proliferation of prostate and breast tumour cells (*260, 327*), and the migration and invasion of prostate tumour cells (*260*), which are necessary steps in the process of tumour

progression (*13*). KKS involvement in prostate tumour cell migration and invasion was suggested by B1R-mediated migration of these cells on a 2D surface and invasion of fibronectin by these cells in a previous study (*260*). This is additionally supported by immuno-labelling for TK, B1R and B2R in DU145 prostate tumour cell projections in the present study. Likewise, the presence of TK, B1R and B2R in the projections of MCF7 breast tumour cells in the present study indicates probable KKS involvement in breast cancer cell invasion and migration. TK participation in breast cancer invasion and migration was demonstrated in a previous study, in which an inhibitor of TK, FE999024 reduced invasion of matrigel by breast tumour cells and pulmonary extravasation in an *ex vivo* rat model (*241*). However it is not known whether TK acted directly in this instance, through MMP activation, through kinin generation followed by kinin receptor activation, or via more than one mechanism. Due to the presence of B1R and B2R in the MCF7 projections in the present study, it would appear that kinin receptor activation could be a mechanism by which TK promotes breast cancer cell migration and invasion. TK present in the tumour environment may also activate MMPs (*242, 243*), which may promote the growth, migration and invasion of tumour cells (*19*). Involvement of MMP-2 specifically in breast cancer epithelial cell migration has been suggested by a previous study (*164*). Further, the enhancement of inflammation in the tumour environment resulting from upregulated extracellular TK activity (Section 4.1.2), facilitates tumour progression via promoting cell proliferation amongst several other mechanisms (*43*) (discussed in detail in Section 1.1.1.3).

It is also postulated that the upregulation of TK in prostate and breast tumour environments (via tumour-induced endothelial cell TK secretion in addition to TK secreted by tumour cells) may be a mechanism for progression of these tumours to AI and OI forms, respectively. Kinin receptor signalling as a result of TK-mediated kinin generation, may potentiate the signalling of bFGF receptors (*256, 257*) and EGF receptors (*259, 260*). FGF

and EGF, are important in the development of prostate and breast cancer, respectively (*308, 311, 389*), and have also been implicated in the development of AI and OI forms of these cancers, respectively (*49, 197, 302, 311*). There are also other examples of KKS potentiation of growth factors that are involved in the development of AI and OI cancers and this was reviewed extensively in Section 1.1.3.3.2.6.2. Therefore, it is plausible that increased TK in the environment of prostate and breast tumours would contribute to the development of hormone-independence. Although the MCF7 breast cancer cells used in the present study were oestrogen-dependent (*390*), the DU145 prostate cancer cells used were AI (*260, 391*). In addition, (i) demonstration of kinin-mediated (via the B1R) growth, migration and invasion of AI prostate cancer cells (*260*), (ii) B2R involvement in AI prostate tumour cell proliferation (*324*), (iii) the suppression of AI prostate tumour growth by BK antagonists in a nude mice model (*293*), and (iv) TK-mediated invasion of OI breast cancer cells (*241*), support this hypothesis.

The contribution of increased TK activity in prostate and breast tumour environments, as indicated by the present study, to prostate and breast tumour-associated angiogenesis (Section 4.1.2), tumourigenesis and possibly progression to hormone-independent cancers, has therapeutic implications. KKS antagonists may be useful in treating prostate and breast cancer, particularly hormone-independent forms of these cancers for which there is currently no satisfactory therapy (*49, 299, 300, 303*). A BK antagonist, B9870, is already in pre-clinical development for treatment of prostate cancer at the US National Cancer Institute in their RAID program (*392*). Considering the upregulation of endothelial cell TK secretion by breast cancer cell metabolites, BK antagonists might also be effective in inhibiting breast tumour growth. Further, considering (i) the success of kallistatin (a TK inhibitor) in suppressing angiogenesis and inflammation [both of which are necessary for tumour progression, (*39, 43*)] *in vivo*, (ii) the effectiveness of TK in inducing

angiogenesis *in vivo* (*287-290*), and (iii) the upregulation of extracellular TK by breast and prostate cancer cells in the present study, specific TK antagonists may be useful in treating these cancers.

4.2.2 Challenged tumour cells

TK, B1R and B2R levels in breast tumour cells, as well as TK secretion from and proliferation of these cells, were not significantly affected by exposure to endothelial cell metabolites. However, in challenged prostate cancer cells there was an overall decrease in intracellular TK, B1R and B2R. The reason for this is not clear. However, it would appear that this result is of limited significance since (i) the output of secreted TK from prostate tumour cells was not significantly diminished by endothelial cell metabolites in the present study [supporting this, HUVEC metabolites had no significant effect on TK secretion by neuroblastoma and cervical carcinoma cells (*354*)], (ii) other factors in the *in vivo* environment of the tumour, such as inflammatory growth factors and cytokines [these may increase B1R and B2R expression (*203*)], could counteract the endothelial-mediated decrease in the prostate tumour cell kinin receptors, rendering it insignificant *in vivo*, and (iii) several previous studies (*in vitro* and *in vivo*) have shown that kinin receptors play an important role in the growth of prostate tumours (*259, 293, 324*) and in the present study prostate tumour cell proliferation was not significantly affected by exposure to endothelial cell metabolites.

4.3 The KKS in endothelial-tumour co-cultures

Although KKS proteins were localised in the same regions of endothelial and tumour cells in co-culture as in these cells in mono-culture, the presence of KKS proteins in the endothelial cell projections that were in contact with tumour cells and in the projections of tumour cells that connected with endothelial cells was a noteworthy finding of the present study. Naidoo

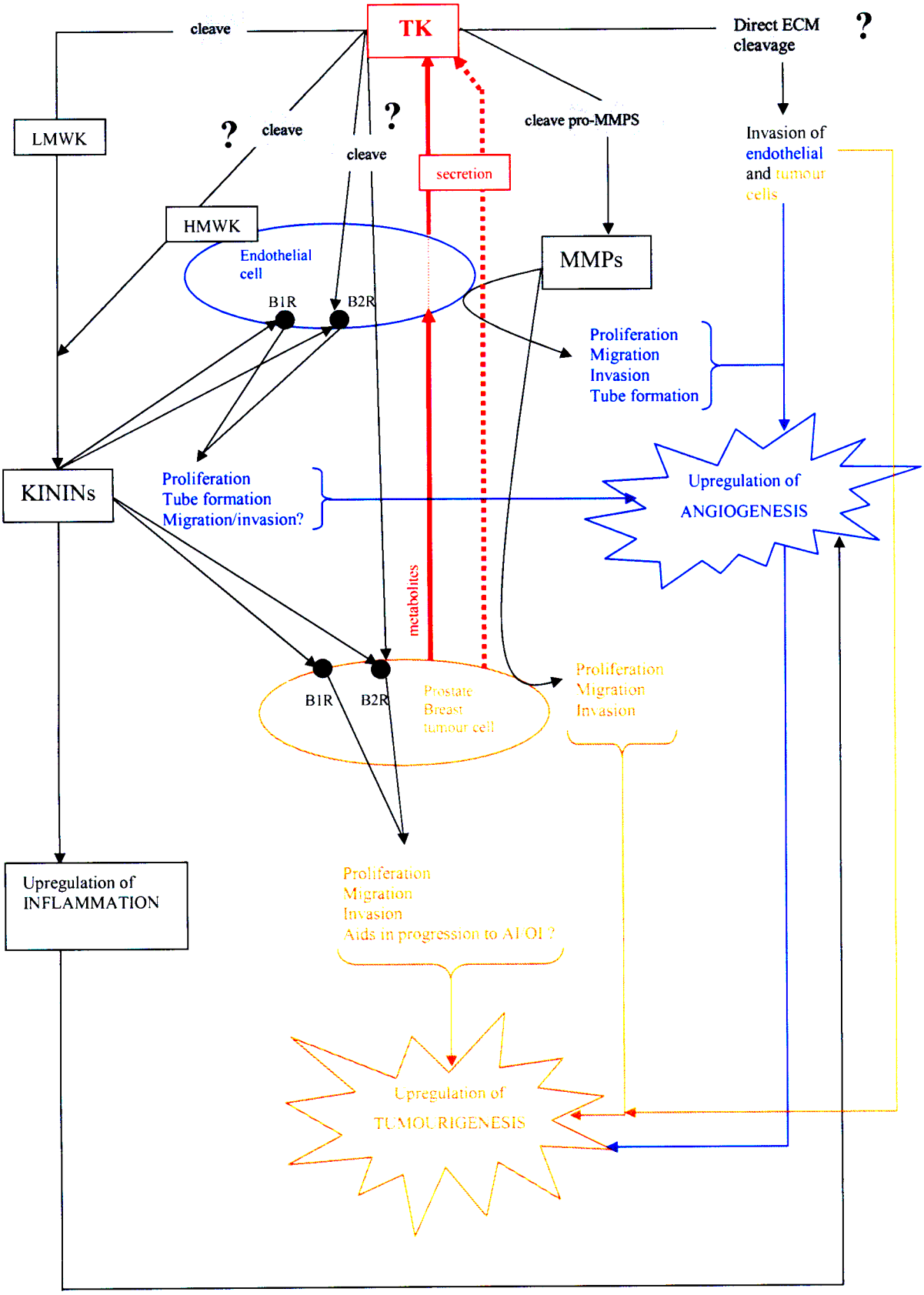
(2005) also demonstrated KKS proteins in the contact points between endothelial cells (HUVECs) and tumour cells (neuroblastoma cells) (*272, 354*). The presence of the KKS in contact points between tumour and endothelial cells indicates that the TK secreted by tumour cells and endothelial cells (stimulated by exposure to tumour cell metabolites) results in localised activation of the B1R and B2R on both cell types. As discussed earlier, it is plausible that kininogen is present in the vicinity of the tumour-endothelial contact point, allowing for localised kinin generation, and TK may also directly activate the B2R. The signalling from endothelial and tumour cell kinin receptors (B1R/B2R) that follows may result in several pro-angiogenic and pro-tumourigenic activities, as was discussed in detail in Sections 4.1.2 and 4.2.1, respectively.

The increased connection of endothelial cells with one another to form chain-like structures in endothelial-tumour co-cultures, as well as circular structures in dMVEC-MCF7 co-cultures, was an interesting observation in the present study. Although this phenotypical arrangement of endothelial cells was not observed when endothelial cells were challenged with tumour cell metabolites, in the co-cultures this arrangement was observed when tumour cells were not necessarily in direct contact with endothelial cells. Thus, this finding in the co-cultures may be due to endothelial exposure to increased tumour cell metabolites secreted at close range, and is suggestive of the ability of tumour metabolites to stimulate angiogenesis. The ability of tumour cell metabolites to induce angiogenesis was shown by the study of Folkman and Haudenschild (1980), in which endothelial cells, cultured in tumour-conditioned medium, arranged themselves over a period of weeks into capillary-like structures (*71*). An additional observation made in the present study, was that occasionally the circular endothelial structures in dMVEC-MCF7 co-cultures enclosed breast tumour cells. This hints at the ability of breast tumour cells to attract differentiating endothelial cells, which may then form blood vessels and provide a route for metastasis (*39*).

4.4 Summary and conclusions

In summary, the present study shows that prostate and breast tumour cell metabolites increase the secretion of TK from endothelial cells, and that these tumour cells also secrete TK. This indicates upregulation of TK extracellular activity in prostate and breast tumour environments. The activities of TK including (i) proteolytic activation of MMPs, (ii) possible direct cleavage of ECM components, and (iii) direct activation of kinin receptors or indirect activation of these receptors via kinin generation, may result in endothelial cell proliferation, migration, invasion and capillary tube formation. The involvement of the KKS in these angiogenic activities is supported by the following findings of the present study: (i) the localisation of TK, B1R and B2R in large projections of endothelial cells and (ii) in small projections that establish endothelial cell-cell contact, and (iii) the increase in endothelial cell proliferation in conjunction with increased endothelial cell TK secretion (in response to tumour metabolites). The activities of TK may also promote tumourigenesis by stimulating the proliferation, migration and invasion of tumour cells, as is supported by localisation of KKS proteins in the projections of prostate and breast tumour cells in the present study. The localisation of KKS proteins in heterogeneous tumour-endothelial cell contacts in the present study also suggests a role for these proteins in tumour-endothelial cell interactions, which may be involved in promoting angiogenesis and tumourigenesis. Another consequence of increased extracellular TK activity would be enhancement of inflammation in these tumours, thereby further stimulating angiogenesis and tumourigenesis. It is also possible that upregulated TK activity in these tumour environments may assist in the development of AI (as supported by the AI characteristic of DU145 prostate tumour cells used in the present study) and OI forms of prostate and breast cancer, respectively, for which therapy is currently inadequate. Thus, KKS antagonists, some of which are already in pre-clinical development for the treatment of prostate cancer, have potential for use in the treatment of prostate and breast cancer, including hormone-independent forms of these

cancers. The main findings and implications of the present study, in relation to previous studies, are summarised in Figure 4.1. In conclusion, the findings of the present study support the hypothesis that the KKS promotes tumour-associated angiogenesis and tumourigenesis in prostate and breast cancers.


TK
cleave
Direct ECM cleavage
?
LMWK
?
cleave
?
cleave
cleave pro-MMPS
Invasion of endothelial and tumour cells
secretion
HMWK
Endothelial cell
MMPs
B1R
B2R
Proliferation
Migration
Invasion
Tube formation
KININs
Proliferation
Tube formation
Migration/invasion?
Upregulation of
ANGIOGENESIS
metabolites
B1R
B2R
Prostate
Breast
tumour cell
Proliferation
Migration
Invasion
Upregulation of
INFLAMMATION
Proliferation
Migration
Invasion
Aids in progression to AT/OI ?
Upregulation of
TUMOURIGENESIS

ANNEXURE: FUTURE STUDIES

The results of the present study, in conjunction with previous studies, provide evidence for upregulated KKS activity with pro-angiogenic and pro-tumourigenic effects in prostate and breast tumours; however, this could be more definitively established by studies using KKS antagonists.

Firstly, an increase in endothelial cell kinin receptor signalling due to prostate and breast tumour metabolites could be confirmed by measurement of ERK phosphorylation and inositol phosphate production in: (i) unchallenged endothelial cells with no exposure to antagonists, and (ii) endothelial cells challenged with tumour metabolites and not exposed to antagonists or exposed toTK/B1R/B2R-specific antagonists or exposed to all 3 KKS antagonists. Specific TK, B1R and B2R antagonists are available (*203, 241*). ERK phosphorylation and inositol production may be used as indicators of kinin receptor signalling (*324*). These events are also part of other signalling pathways; however, ERK phosphorylation and inositol production due to other signalling pathways would be a background constant and comparison with controls not exposed to antagonists would account for this. This experimental design should allow assessment of the effect of tumour metabolites on the activity of each component of the KKS separately and as a whole. However, the activity of TK apart from its indirect or direct activation of kinin receptors cannot be assessed by this proposed study.

After establishing/confirming the effect of prostate and breast tumour metabolites on endothelial cell KKS activity, the role of tumour-induced upregulated KKS activity in the angiogenic events of endothelial cell proliferation, migration and invasion and cord/tube formation could be further confirmed using microvascular endothelial cells. This could be achieved by performing a proliferation assay (such as the one used in the present study), as

well as a migration assay [e.g. the wound healing assay, (*260*)], invasion assay [e.g. Boyden chamber assay, (*338*)] and cord formation assay [using matrigel, (*338, 345*)] on microvascular endothelial cells with and without exposure to TK, B1R and B2R antagonists. Some *in vitro* studies have already shown involvement of the some of the KKS components in these endothelial cell activities, although not necessarily using microvessel cells (*254, 256, 292, 293*). The contribution of TK to specific steps of the angiogenic cascade, and the specific involvement of B1R and B2R in endothelial cell migration and invasion have yet to be established.

Similarly, to confirm a role for the KKS in the proliferation, migration and invasion of prostate and breast tumour cells, these assays could be performed on tumour cells with and without KKS antagonist exposure. Such a study has been conducted with a B1R antagonist and AI prostate cancer cells using proliferation, migration and invasion assays, confirming the role of B1R in prostate cancer and possibly the development of AI cancer (*260*). If the proposed study were to be conducted with OI breast cancer cells it could provide evidence for a role of the KKS in the development of OI cancer. Evidence has already been provided for TK involvement in the invasion of OI breast cancer cells (*241*). The involvement of B2R in breast cancer proliferation (*326*) and the proliferation of AI prostate cancer cells (*324*) has also been demonstrated. However, the roles of the B1R in breast tumourigenic activities, and B2R and TK in other prostate/breast tumourigenic activities have yet to be confirmed. The proposed studies discussed above could also be performed for other cancer types in which KKS expression has been demonstrated (*273-277, 279, 280, 283, 284*).

Evidence provided by the present study and previous studies for the involvement of the KKS in prostate and breast tumour-associated angiogenesis and tumourigenesis, particularly in AI and OI forms of these cancers, has therapeutic implications (Section 4.2.1). *In vivo* studies

that test the effectiveness of KKS inhibitors in inhibiting development of breast and prostate cancer are required. Promising *in vivo* studies that show potent inhibition of prostate cancer by BK antagonists, have recently led to pre-clinical development of one of these antagonists (Section 4.2.1). Considering the upregulation of endothelial TK secretion by breast cancer cells, *in vivo* studies using these BK antagonists in an attempt to inhibit breast cancer development should be conducted. Additionally, the results of the present study, as well as the previous demonstration of the effectiveness of kallistatin (a TK inhibitor) in inhibiting angiogenesis and inflammation *in vivo* (Section 4.2.1), suggest that the effectiveness of TK inhibitors in inhibiting growth of breast and prostate tumours should be tested in an *in vivo* model.

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