REGULATION OF TUMOUR-ANGIOGENESIS BY PROTEASE INHIBITORS AND RECEPTOR ANTAGONISTS

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DEDICATION

This dissertation is dedicated to my dad, Leslie Naidu, who was recently diagnosed with prostate cancer.

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ABSTRACT

Introduction

Angiogenesis, the growth of new blood vessels from the pre-existing vasculature, is a pre-requisite for tumour growth and metastasis. Tumour-angiogenesis is regulated by various pro- and anti-angiogenic factors released by both endothelial and tumour cells, as well as by the micro-environment. Numerous studies have implicated various systems in the acquisition of the angiogenic phenotype. The present study sought to investigate the role of the kallikrein-kinin system (KKS) in tumour-angiogenesis.

The kallikreins consist of two serine proteases, plasma and tissue kallikrein (TK), involved in the release of kinin peptides by enzymatic cleavage of kininogens. Stimulation of the cognate bradykinin receptors (BKR), B1R and B2R, mediates the mitogenic and vasoactive properties of kinins. In addition, TK activates matrix metallo-proteinases (MMPs) involved in extracellular matrix (ECM) degradation.

The expression profiles of TK and kinins have been found to be dys-regulated in numerous human cancers, and several studies have demonstrated the involvement of the KKS in growth and metastasis of prostate tumours. Further, previous *in vitro* models in our laboratory have established an association between the KKS and prostate tumour-angiogenesis. In those studies it was postulated that the up-regulated TK (produced by endothelial and tumour cells) stimulated endothelial cell proliferation. Thus, the aim of the present study was to define the effects of the KKS and seek a direct correlation with angiogenesis using *in vitro* models with tumour conditioned medium (CM), kinin receptor agonists and antagonists.

Methods

Ethical approval for this project was granted by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (reference number BE152/08). Micro-vascular endothelial cells represent a suitable *in vitro* angiogenic model and dermal micro-vascular endothelial cells (dMVECs) were obtained commercially for this purpose. The tumour model used in this study was an immortalised prostate cancer (DU145) cell line. The CM model involves the treatment of one cell line with the metabolites of another. In the angiogenic model, dMVECs were exposed to increasing concentrations of DU145 CM. Stimulation was further augmented with BKR agonists. Specific BKR antagonists were used to test the specificity of stimulation. In addition, vascular endothelial growth factor (VEGF) was tested as a positive proliferation control. The potential of these agents to induce proliferation and migration was determined using the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and a modified Boyden chamber assay, respectively. Previous studies investigating the pro-angiogenic effects of CM differed, in many respects, in terms of their models and methodologies. In an attempt to fully explore the pro-mitogenic effects of CM on endothelial cells, various modifications, as well as alternate endothelial and tumour cell types, were employed in the present study. The mitogenic and migratory effect of BKR agonists and antagonists on DU145 cells was also assessed. Further, the tumour model was expanded to investigate the autocrine potential of the KKS, by investigating the effect of DU145 CM on DU145 migration.

Results

In the angiogenic model, although the addition of DU145 CM elicited a statistically significant increase in micro-vascular endothelial cell proliferation, this increase was very small (<10%) and not dose-dependent. Pre-incubation of dMVECs with a B1R or B2R antagonist did not influence this small effect of CM on proliferation. In addition, neither

B1R nor B2R agonists, at any concentration, produced any significant proliferative effect on endothelial cells. In contrast to these findings VEGF, a well-known mitogen, was able to stimulate proliferation of dMVECs. Migration assays revealed that DU145 CM failed to stimulate endothelial cell motility. Further, neither BKR agonist displayed any chemo-attractant potential in those assays.

The most important finding was in the tumour model, where stimulation with a B1R agonist significantly enhanced proliferation and especially migration of DU145 cells. In addition, pre-treatment with a B1R antagonist abolished both these effects. B2R agonists could not produce the same positive effect as the B1R agonist on growth and migration of prostate tumour cells. DU145 CM did not prove to be a migratory stimulus for DU145 cells at any concentration.

Discussion

Previous studies in our laboratory have shown prostate-tumour CM to promote proliferation of endothelial cells and have postulated that TK up-regulation may be the reason for this. However, the present study could not reproduce this effect of CM. Further, BKR antagonists had no notable or consistent effect on the minimal promotion of proliferation that had been produced by DU145 CM. In addition, selective BKR agonists failed to induce proliferation or migration of endothelial cells, key events in the angiogenic cascade. Although in contrast to some studies, the present study was unable to implicate the KKS in angiogenesis, tumour neo-vascularisation is a consequence of several angiogenic factors functioning together as opposed to a single, isolated factor. For example, we were able to demonstrate a positive mitogenic effect of VEGF on endothelial cells and it may be this as well as other factors in the CM that are responsible for the small proliferation we observed.

Up-regulation of kallikreins and kinins in tumours may enhance fundamental events in tumourigenesis in an autocrine manner, and bradykinin (BK) has previously been shown to promote tumour growth in mouse models. Our study supported the involvement of the KKS in tumourigenesis. Although CM from DU145 cells did not self-stimulate the migration of these cells, a B1R agonist enhanced both proliferation and migration, an effect that was also abrogated by the relevant antagonist, indicating a role for kinins. In contrast to the findings of another study, stimulation of the B2R failed to significantly promote tumour growth or motility. However, this is not an unexpected finding because it is thought that the ubiquitous B2R mediates physiological effects in the prostate while the inducible B1R plays a role in prostate cancer pathology.

In summary, this study lends support to the ongoing exploration of BKR antagonists as possible candidates in the development of alternate approaches to cancer therapy. This may be particularly beneficial to hormone-independent tumours, such as those of the prostate, for which there exists few effective treatment options.

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ABBREVIATIONS

°C, degrees Celsius

µg, microgram

µl, microlitre

µm, micrometer

µM, micromolar

2-D, two-dimensional

3-D, three-dimensional

ACE, angiotensin I-converting enzyme

AM, acetomethylester

Ang, angiopoietin

ANOVA, analysis of variance

B1R, bradykinin receptor subtype 1

B2R, bradykinin receptor subtype 2

BACE, bovine adrenal cortex endothelial cell

bFGF, basic fibroblast growth factor

BK, bradykinin

BKR, bradykinin receptors

BrdU, bromodeoxyuridine

CAM, chorioallantoic membrane

CDS, Cell-Dissociation Solution

CM, conditioned medium

cm^2, centimetres squared

CO_2, carbon dioxide

COX, cyclooxygenase

CPM, carboxypeptidase M

CPN, carboxypeptidase N

CVEC, coronary venular endothelial cells

DAG, diacylglycerol

ddH_2O, distilled, deionised water

DMEM, Dulbecco's Modified Eagle's Medium

DMSO, dimethylsulfoxide

dMVEC, dermal micro-vascular endothelial cell

DNA, deoxyribonucleic acid

DU145, prostate tumour cell line

EBM, endothelial basal medium

ECM, extracellular matrix

EGF, epidermal growth factor

EGM, endothelial growth medium

ELISA, enzyme-linked immunosorbent assay

ERK, extracellular signal-regulated kinase

FBS, fetal bovine serum

FGF, fibroblast growth factor

g, gram

GA, gentamicin sulphate amphotericin

GPCR, G-protein coupled receptor

HBSS, Hank's Balanced Salt Solution

HBV, hepatitis B virus

HCV, hepatitis C virus

HeLa, Henrietta Lacks (human cervical carcinoma)

HIF, hypoxia-inducible factor

HIV, human immunodeficiency virus

hK, human kallikrein protein

HMWK, high molecular weight kininogen

HRE, hormone response element

HuMMEC, human mammary micro-vascular endothelial cell

HUVEC, human umbilical vein endothelial cell

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

IgG, immunoglobulin G

IL, interleukin

kb, kilobase

kDa, kilodalton

KKS, kallikrein-kinin system

KLK, genes coding for kallikrein protein

L, litre

LMWK, low molecular weight kininogen

lys-BK, lysyl-bradykinin (kallidin)

M, molar

MAPK, mitogen activated protein kinase

$MgCl_2$, magnesium chloride

ml, millilitre

mm, millimetre

mm^3, millimetres cubed

mM, millimolar

MMPs, matrix metallo-proteinases

mRNA, messenger ribonucleic acid

MT-MMP, membrane type matrix metallo-proteinase

MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide

N2α, neuroblastoma cell line

Na_2CO_3, sodium carbonate

NaCl, sodium chloride

$NaHCO_3$, sodium hydrogen carbonate

NEP, neutral endopeptidase

ng, nanogram

nm, nanometre

nM, nanomolar

NO, nitric oxide

PAI, plasminogen activator inhibitor

PAP, prostatic acid phosphatase

PBS, phosphate buffered saline

PC3, prostate tumour cell line

PDGF, platelet-derived growth factor

PIGF, placental growth factor

PK, plasma kallikrein

PKC, protein kinase C

PLC, phospholipase C

pNPP, disodium p-nitrophenyl phosphate

PSA, prostate-specific antigen

PSF, penicillin-streptomycin-fungizone

R3-IGF, insulin-like growth factor

RFU, relative fluorescent unit

rpm, revolutions per minute

RT, room temperature

SCLC, small cell lung carcinoma

SEM, standard error of the mean

serpins, serine protease inhibitors

TGF, transforming growth factor

TIMPs, tissue inhibitors of matrix metallo-proteinases

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

VEGF, vascular endothelial growth factor

VEGFR, vascular endothelial growth factor receptor

VPF, vascular permeability factor

CHAPTER 1

LITERATURE

CHAPTER 1 – LITERATURE

1.1 Cancer

1.1.1 History and definition

The terms "cancer" and "tumour" are of Latin origin (1, 2). It was Hippocrates (460-370 BC) who first associated the radiating veins of a breast tumour with the limbs of a crab, the Greek word for which was *karkinoma* and its subsequent Latin counterpart, *cancer* (2). The word *tumour* initially made reference to "swelling" caused by inflammation (1). In truth, not all swellings are tumours, thus, in the modern sense of the word, the term generally refers to a "new growth" or neoplasm (1, 3).

Cancer encompasses an array of diseases with the capacity to involve virtually any organ system in the extensive animal kingdom (2, 4). Although cancer awareness has progressively increased in the present era, it is by no means a new disease; ancient civilisations made reference to cancer in pictures and writings, while tumours have been detected in dinosaur bones and Egyptian mummies which date to approximately 150 million and 5000 years ago, respectively (2, 5). In approximately 400 BC Hippocrates identified cancer as a disproportion of the black humour (from the spleen) over the three bodily humours - bile, phlegm and blood (6). Although mistaken, his theory was the first to accredit the onset of cancer to natural causes; up until medieval times the belief that cancer was caused by various gods persisted (6). Following the development of the microscope, knowledge of the biological basis of the disease progressed. In the nineteenth century, Rudolph Virchow, a prominent pathologist, proclaimed that "every cell arises from another cell" (2, 5). Although this statement holds true for normal cells in addition to cancer cells, it is only in tumours that cellular growth continues beyond normal development; subsequently, cancer was described

as a “cellular disease, where there was loss of normal control of cell proliferation” (6). Cancer development begins with normal cells undergoing several genetic aberrations, produced by various causative agents that impair their ability to appropriately respond to signals (intracellular and/or extracellular) that are responsible for proliferation, differentiation and irrevocable death (6).

1.1.2 Epidemiology

The worldwide burden of cancer is enormous with consequential human and financial costs (7). In 2008, it was estimated that there were approximately 12.6 million new cancer cases globally (8). After taking into account population size it was determined that cancer risk was twice as high in more developed countries than less developed nations (9).

Prostate cancer is one of the most common malignancies among males in western countries (10, 11). Towards the end of the twentieth century, worldwide prostate cancer incidence rose considerably (12). Essentially, this could be attributed to the increased life expectancy, increased prevalence arising from exposure to carcinogens and/or increased detection through more frequent diagnostic use of prostate-specific antigen (PSA) (10, 12). Asian countries where incidence rates were commonly low have since risen considerably, partly attributable to increasing westernisation of diet and lifestyle (10). Table 1.1 shows the estimated worldwide and South African incidence and mortality rates for cancer.

Table 1.1: Estimated worldwide and SA incidence and mortality for cancer (2008)

Cancer type	Region	Incidence x 1000	Mortality x 1000
All *	**World**	12 663	7565
	SA	75	51
Prostate Cancer	**World**	899	258
	SA	7.6	2.5

(*excluding non-melanoma skin cancer)
Source: Adapted from (8)

1.1.3 Aetiology

1.1.3.1 Genetic, carcinogenic and risk factors

Tumours have the capacity to develop from a single mutated cell (13). The progression from normal cell to cancer cell is a multi-step process that involves both an individual's genetic predisposition to cancer as well as external carcinogenic agents. Carcinogens may be classified as chemical, physical or infectious agents, and can further be classified as genotoxic or non-genotoxic (14-16). Genotoxic carcinogens induce DNA damage while non-genotoxic carcinogens mediate their effects by interference with growth factor expression or signal transduction pathways (17).

Chemical carcinogens are rife and include asbestos, arsenic, constituents of tobacco smoke as well as hormones (13, 17). Physical factors such as ultraviolet (UV) and ionising radiation are well established carcinogenic agents (16). The primary effect of radiation appears to be the introduction of genetic instability, consequently promoting rare mutations and malignant transformation (15). Viruses, eubacteria and helminths are the three types of organisms associated with cancer (13, 14). These infectious agents are the second most prominent cause

of cancer worldwide and are commonly linked to stomach, liver and cervical malignancies (14).

Despite its high incidence and morbidity, prostate cancer aetiology remains to be fully elucidated (10). Its pathogenesis most likely involves interaction of both genetic and environmental factors (18). Established risk factors include familial history, progressing age and ethnicity (12, 18). Genetic factors are thought to contribute about 40% of the risk while progressing age increases the incidence exponentially (10). In addition, factors such as androgens, diet, physical activity, sexual behaviour and obesity have been implicated in the aetiology of prostate cancer; however, their precise roles remain largely unclear (10, 12).

1.1.3.2 Tumour suppressor genes and oncogenes

Transformation of cells to the tumourigenic phenotype is a multi-stage process that involves mutations in two gene classes: oncogenes and tumour suppressor genes (19). These genes are fundamentally involved in the life-cycle of a cell, from cell division to differentiation and ultimately death (20, 21).

A mutation on one allele of a gene transforms the normal 'proto-oncogene' into the activated oncogene (20). Oncogenic dominance may subsequently result in over-production of its growth stimulatory protein or an excessively active form thereof (20, 21). Thus, oncogenes promote malignant transformation by encouraging cellular growth via the activation of growth-enhancing pathways (21, 22). Mutations within oncogenes consequently initiate a gain-of-function (19).

Unlike oncogenes, tumour suppressor genes are recessive (23). They contribute to malignant transformation when both alleles are inactivated by mutations (20, 21, 23). Their resulting loss-of-function means the cell is denied suppressor proteins pertinent to cellular growth (20-22).

Numerous tumour suppressor genes and oncogenes thought to be involved in diverse cancers have been studied. Several have been identified in prostate cancer; however, genes specifically and directly implicated in prostate tumour transformation and growth have yet to be established (24). Tables 1.2 and 1.3 indicate oncogenes and tumour suppressor genes thought to be involved in prostate cancer.

Table 1.2: Oncogenes involved in prostate cancer

Oncogene	Gene Function	Ref
c-myc	regulation of cell proliferation, differentiation and apoptosis	(21, 24, 25)
ERBB2	receptor tyrosine kinases involved in tumour growth	(20, 24)
BCL2	anti-apoptotic protein	(20, 24)
AR	androgen receptor	(24)

Table 1.3: Tumour suppressor genes involved in prostate cancer

Tumour suppressor gene	Gene Function	Ref
TP53	regulation of cell cycle, apoptosis	(26)
PTEN	cell proliferation, apoptosis	(20, 24)
CDKN2A	negative regulator of cell cycle	(24, 27)
CDKN1B	cyclin kinase inhibitor	(24)
GSTP1	prevents DNA impairment due to carcinogenic agents and oxidants	(28)
NKX3.1	homeobox protein	(29)

1.1.4 Tumour development

In normal human adults the balance between cell proliferation and cell death is tightly regulated. Mutations in genes fundamental to cell cycle regulation lead to unrestrained cell proliferation and a disordered cell cycle, the fundamental trademarks of cancer (26). In epithelial cancers tumour progression occurs in stages: (i) begins with a genetically-altered cell, (ii) progresses to hyperplasia, (iii) dysplasia, (iv) *in situ* cancer and (v) ultimately invasive cancer. These stages, as described by Weinberg (1996), are further discussed below (21).

The transformation from a normal to genetically altered cell with an increased capacity to proliferate initiates hyperplasia. At this stage the mass of cells appear healthy but they proliferate excessively. Years later a single cell from the growing mass undergoes an additional mutation that further reduces regulation of cell growth. Hyperplasia subsequently progresses to dysplasia when the cells no longer appear normal. In time, another mutation may further modify cell characteristics. *In situ* cancer refers to a tumour that remains

enclosed. However, with further mutations the growing tumour may acquire characteristics that enable penetration into underlying tissues advancing to invasive cancer. In addition, cells may detach from the original site, enter blood and lymph vessels and metastasise. Secondary tumours may have fatal consequences when the functioning of vital organs is disturbed. Malignant tumours differ from benign tumours in that they possess the capacity to invade adjacent tissue and metastasise. This metastatic ability presents the greatest hindrance to successful cancer treatment (3).

1.1.5 Conventional therapy

Early detection is crucial to successful treatment and possible cure of cancer. The screening and diagnosis of prostate cancer involves rectal examination, biopsy and the measurement of PSA, also known as human kallikrein 3 (hK3) (11, 30). Conventional cancer therapy is dependent on the extent of malignancy and may involve surgery, radiation therapy and/or chemotherapy (3, 31, 32). Since progression of prostate tumours is hormone-dependent, treatment of metastatic disease may also involve androgen-ablative therapy. However, metastatic prostate cancers ultimately transform into hormone-independent forms of the disease (30). In addition, chemotherapy has shown little positive effect in the treatment of prostate cancer (30). Thus, novel, innovative approaches are needed for successful management of progressive prostate tumours.

1.2 Angiogenesis

The term angiogenesis describes the development of new capillary blood vessels from the pre-existing vasculature (33, 34). In a normal adult, the rate of endothelial cell proliferation is lower than that of numerous other cell types in the body (35). Angiogenesis does occur

under normal conditions however physiological angiogenesis is a tightly regulated process essential, and largely confined to embryogenesis, inflammation, wound healing and menstruation (34, 36, 37). Dys-regulated angiogenesis is associated with several pathological conditions such as psoriasis, rheumatoid arthritis, diabetic retinopathy as well as cancer (35, 38, 39).

1.2.1 The angiogenic cascade

Angiogenesis is a continuous process subdivided into several steps: (i) release of angiogenic factors, (ii) basement membrane degradation, (iii) endothelial cell migration, (iv) endothelial cell proliferation and (v) vessel differentiation (36, 39, 40). These sequential events are further discussed below.

(i) Release of angiogenic factors: Angiogenesis begins with the release of pro-angiogenic factors that have the capacity to act directly by binding to endothelial receptors subsequently activating them, or indirectly via stromal cells and macrophages (39-41). In the past two decades, several angiogenic factors have been examined. Their activity is further described in the sections that follow.

(ii) Basement membrane degradation: For endothelial cells to migrate into adjacent tissues the blood vessel basement membrane and surrounding extracellular matrix (ECM) must first be degraded (36, 39). Numerous proteolytic enzymes such as plasminogen activators and matrix metallo-proteinases (MMPs) are involved in this process (36, 39, 41).

(iii) Endothelial cell migration: ECM and basement membrane degradation create a clear course for endothelial cell assembly into a column to form 'sprouts' (37, 39). The migratory process involves chemotactic movement, cytokines [interleukin-6 (IL-6), IL-8 and tumour

necrosis factor-alpha (TNF-α)] and growth factors [vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)] (36, 39).

(iv) Endothelial cell proliferation: Angiogenic factors such as VEGF and basic FGF (bFGF) exhibit mitogenic activity (36). This is essential to angiogenesis as sprouting blood vessels require further endothelial cells to develop (36).

(v) Vessel differentiation: blood vessel differentiation includes the formation of a new basement membrane and the recruitment of fibroblasts, smooth muscle cells and pericytes for vascular stability (34, 39).

1.2.2 Angiogenesis and cancer

While genetic and epigenetic changes are central to malignant transformation, another fundamental hallmark is vital to tumour progression, namely, the onset of angiogenesis which is known as the 'angiogenic switch'(42).

In 1971 Judah Folkman hypothesised that tumour growth was restricted in the absence of angiogenesis and extensive experimental evidence now supports this theory (43, 44). Newly-formed blood vessels are essential to tumour development beyond 1-2 mm^3; they supply the growing mass with nutrients and oxygen while removing metabolic waste products (34, 40). In addition, the blood capillaries present a channel permitting tumour cells to migrate from the primary tumour and form distant metastases (45).

Tumour-angiogenesis differs significantly from physiological angiogenesis (42). Tumour blood vessels lack an organised vascular structure, are characteristically immature and blood flow is irregular (36, 46). They display increased permeability and are consequently termed 'leaky' blood vessels (36). A discontinuous endothelial cell lining, absent or undeveloped

basement membrane and a lack of accessory cells are essentially factors that contribute to hyper-permeability (46). In addition, tumour cells have the capacity to imitate endothelial cells and are often found intermittently in the lining of tumour blood vessels (36).

1.2.3 Regulation of angiogenesis

Tumour-angiogenesis is an elaborate process that requires the involvement of numerous growth factors, cytokines and specific receptors (37). Such factors involved in the regulation of angiogenesis are secreted by both endothelial and tumour cells as well as the surrounding stroma (34, 41). It is this transforming ECM that creates a micro-environment suitable for endothelial proliferation, migration and invasion (30). Pro-angiogenic factors are proteins that stimulate endothelial cell proliferation and motility whilst anti-angiogenic factors, often also secreted by dormant tumours, are inhibitory agents that prevent tumour growth (34). The balance between pro- and anti-angiogenic factors regulates the process of angiogenesis; the angiogenic switch occurs when such equilibrium is disturbed promoting pro-angiogenic expression (42). Factors such as hypoxia, due to a proliferating tissue mass, and tumour suppressor and oncogene mutations tip the scale in favour of angiogenesis (46, 47).

1.2.3.1 Pro-angiogenic factors

VEGF

VEGF, initially known as vascular permeability factor (VPF), is one of the most prominent pro-angiogenic factors central to both physiological and pathological angiogenesis (30, 42). The VEGF family consists of five isoforms: VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E (48). Additional members of the VEGF family include placental growth factor (PlGF-1 and PlGF-2) (36, 48). Produced by diverse cell types, VEGFs are homo-dimeric

glycoproteins that mediate their effects via tyrosine kinase receptors (VEGFR-1, VEGFR-2 and VEGFR-3) found on endothelial cells (36, 37, 49). VEGF-A, commonly referred to simply as VEGF, has been the focus of intense research and is regarded as the most significant isoform in the tumour context (36, 49).

VEGF is a key player in all events of the angiogenic cascade. Important biological functions of VEGF include vascular hyper-permeability and its potent endothelial-cell specific mitogenic capacity (30, 50). In addition, VEGF is involved in elongation, branching and survival of endothelial cells (31). VEGF production is stimulated by cancer cell secretion of hypoxia-inducible factor 1 (HIF-1) which is produced in response to hypoxia (30, 51). Secreted VEGFs stimulate endothelial cells to release various proteases and plasminogen activators consequently resulting in basement membrane degradation (35, 52).

VEGF expression is typically elevated in most human carcinomas and several studies have implicated VEGF in prostate cancer (53-56). Doll *et al.* (2001) found that VEGF is present in low levels if not entirely absent in normal prostate tissues (53). In contrast, raised VEGF concentrations were found in the plasma and urine of patients with prostate cancer (54). In addition, Melnyk *et al.* (1999) found that a VEGF antibody diminished prostate cancer growth and metastasis in mice implanted with a DU145 cell line (57). These findings demonstrate a pathological role for VEGF in the development of prostate cancer.

Other pro-angiogenic factors

Since angiogenesis is a complex process that involves numerous factors and signal transduction pathways, inhibition of one factor may result in angiogenic stimulation via other pro-angiogenic mediators (58). Table 1.4 describes a variety of angiogenic stimulators involved in prostate cancer. Some of these molecules such as those that belong to the VEGF family and the angiopoietins (Ang) are highly specific for endothelial cells. Other angiogenic enzymes, chemokines and bFGF act on a variety of cells types while the angiogenic effect of indirect-acting molecules such as transforming growth factor beta (TGF-β) results from the secretion of other factors by macrophages, endothelial or tumour cells (35).

Table 1.4: Pro-angiogenic factors involved in prostate cancer

Pro-factor	Activity	Ref
VEGF-A	• highly specific endothelial cell mitogen • induces vascular permeability; survival factor for neo-vessels	(37, 59)
VEGF-B	• *in vitro* endothelial cell mitogen	(60)
VEGF-C	• *in vitro* endothelial cell mitogen; induces vascular permeability	(59)
VEGF-D	• *in vitro* endothelial cell mitogen	(61)
VEGF-E (orf virus)	• endothelial cell mitogen; promotes vascular permeability	(59)
PlGF	• weak endothelial cell mitogen • weak inducer of vascular permeability	(59, 60)
bFGF / FGF-2	• potent mitogen for numerous cell types including endothelial cells • induces endothelial cell migration • potentiates VEGF production	(30, 31, 62)
TGF-β	• stimulates angiogenesis *in vivo* *(inhibits endothelial cell proliferation in vitro)* • promotes prostate cancer tumourigenesis by inhibiting host immune function	(30, 35)
Cyclooxygenase-2 (COX-2)	• stimulates angiogenesis by production of prostaglandin E2 which regulates production of VEGF	(31, 63)
PDGF	• induces endothelial cell proliferation & migration	(62)
Ang-1	• growth factor highly specific for endothelial cells • promotes PDGF production by endothelial cells	(64)
Ang-2	• agonist/antagonist activity • combined with VEGF enhances angiogenesis	(64)
Epidermal growth factor (EGF)	• weak endothelial cell mitogen; induces VEGF expression	(62)
Angiogenin	• induces angiogenesis	(31)
IL-8	• endothelial cell mitogen; stimulates endothelial cell migration	(48)

1.2.3.2 Anti-angiogenic factors

Angiogenic inhibitors promote endothelial quiescence in several ways by: (i) inhibiting endothelial cell proliferation and migration, (ii) inhibiting ECM remodelling or (iii) promoting endothelial cell apoptosis (65). Several endogenous angiogenic inhibitors have been discovered. Table 1.5 summarises the anti-angiogenic factors involved in prostate cancer.

Table 1.5: Anti-angiogenic factors associated with prostate cancer

Anti-angiogenic factor	Characteristic / Activity	Ref
Angiostatin	• plasminogen fragment • specific endothelial cell inhibitor • inhibits angiogenesis and metastasis	(31, 34)
Endostatin	• suppresses migration of endothelial cells • inhibits endothelial cell proliferation • induces apoptosis in endothelial cells	(34, 66, 67)
PSA	• converts plasminogen to active angiostatin-like fragment • inhibits proliferation, migration and invasion of endothelial cells	(68, 69)
Thrombospondin-1 (TSP-1)	• ECM protein • inhibits endothelial cell growth	(31, 70)
IL-10	• induces tissue inhibitors of metallo-proteinase (TIMP) expression • inhibits MMP-2 expression • associated with reduced metastatic ability	(71)

1.2.4 Anti-angiogenic therapy

Since angiogenesis is a pre-requisite for tumour growth and metastasis, anti-angiogenic therapy could essentially deprive the tumour of oxygen and growth factors, thereby diminishing its growth and spread (46). Numerous *in vivo* studies using mouse models have demonstrated that tumour growth is diminished with the use of angiogenic inhibitors (72-74). Anti-angiogenic therapies have numerous advantages compared to conventional chemotherapy in that they are unlikely to display the side-effects (hair-loss and gastro-intestinal

complications) and drug-resistance observed with the use of current chemo-therapeutic agents. However, recent studies have demonstrated potential complications with the use of anti-angiogenic agents linked to physiological angiogenesis (75). Currently there are approximately twenty angiogenic inhibitors being investigated in human trials of which three are being tested for their efficacy in prostate cancer (75). These compounds may mediate their effects via different mechanisms which include: (i) inhibiting tumour cell secretion of angiogenic factors, (ii) inhibiting the pro-angiogenic activity of these tumour-secreted compounds, (iii) suppressing the endothelial cell activation in response to pro-angiogenic factors and (iv) stimulation of endogenous anti-angiogenic factors (48).

1.3 Protease systems, cancer and angiogenesis

Angiogenesis, tumourigenesis and metastasis are essentially processes that are initiated by a loss of normal control of cell growth and a shift in the balance of proteolytic and cell motility regulation (76). The release of angiogenic stimuli and proteolytic enzymes that are involved in ECM degradation are crucial to these processes (76, 77). Many of these extracellular proteolytic enzymes belong to either the serine protease family (plasminogen activators and kallikreins) or the MMP family (77). These protease systems are discussed in greater detail in the sections that follow.

1.3.1 The plasminogen-plasmin system

The plasminogen-plasmin system is central to both physiological and pathological processes (78). The system is comprised of plasminogen and two serine proteases, urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), as well as plasminogen activator inhibitors (PAI-1, PAI-2 and 2-antiplasmin) and a urokinase

plasminogen activator receptor (uPAR) (78, 79). Serine protease plasminogen activators convert the inactive pro-enzyme, plasminogen to active plasmin (80). PAI-1, PAI-2 and 2-antiplasmin regulate the expression of plasminogen activators and plasmin, respectively (78). Plasmin activation may initiate numerous pathways involving several activities: (i) it degrades the fibrin and fibronectin components of the ECM, (ii) with or without membrane type MMPs (MT-MMPs), plasmin has the capacity to activate pro-MMPs (collagenases), thereby facilitating the remodelling of a key component of the basement membrane, Type-IV collagen, (iii) it may activate or stimulate release of growth factors such as VEGF, TGF-β and bFGF from the ECM and (iv) it has the capacity to generate kinins from high molecular weight kininogen (HMWK) (78, 81, 82). ECM degradation, growth factor stimulation and bradykinin (BK) generation are key processes in both angiogenesis and tumourigenesis as well as metastasis (81, 83). Several malignancies, including that of the prostate, display uPA and uPAR expression, the levels of which are typically up-regulated in progressive and invasive carcinomas (82).

1.3.2 The matrix metallo-proteinase system

The MMP family comprises more than twenty proteinases with the capacity to degrade elements of the ECM (84). MMPs are released in their inactive form and so must first be cleaved to be activated (85). Their activity can, however, be inhibited by TIMPs (78, 86). Abnormal MMP expression is associated with several pathological conditions including cancer, and several MMPs have been linked to tumour cell invasion and metastasis (84, 87). ECM degradation by MMPs contributes to angiogenesis by facilitating endothelial cell detachment and migration (85). In addition, they release angiogenic stimulatory factors such as VEGF, bFGF and TGF-β (87).

1.3.3 The kallikrein-kinin system

The kallikrein-kinin system (KKS) is an endogenous cascade that is involved in the activation of polypeptides (88). This complex system consists of kallikreins, kininogen, kinins, kininases and kinin receptors. The kallikreins are a family of proteolytic enzymes, some of which have the ability to produce active kinin peptides from precursor kininogen molecules. Kinins mediate their numerous biological effects via their two cognate receptors, bradykinin receptor subtype 1 (B1R) and bradykinin receptor subtype 2 (B2R), and are rapidly metabolised by kininases (89). Each of these components is discussed in greater detail in the following sections, and is further summarised in Figure 1.1.

1.3.3.1 Kallikreins

The kallikreins are a group of serine proteases found in biological fluids, neutrophils and glandular cells (89). This group of enzymes can be divided into two major subsets: plasma kallikrein (PK) and tissue kallikrein (TK) (88, 90). PK and TK differ in many aspects including their function and the type of kinin they generate (91). In their respective roles PK generates BK from HMWK whereas activated TK generates kallidin (Lys-BK) from low molecular weight kininogen (LMWK) (92, 93). However, *in vitro* TK forms kinins from both HMWK and LMWK (89). The plasma KKS is involved in blood clotting, fibrinolysis, vascular tone regulation and inflammation while TK activation occurs during tissue damage, infection, disease and inflammation (89, 94). In the sections that follow hK and KLK refer to the kallikrein protein and its related gene, respectively.

1.3.3.2 Tissue kallikreins

TK (hK1) was discovered in the 1930s as a pancreatic protein found in copious amounts however it was only until 1985 that the gene for this kallikrein (KLK1) was discovered (95).

Also in the 1980s, the human glandular kallikrein gene (KLK2) and PSA gene (KLK3) were identified (95). During the last century a further twelve genes were discovered and designated as part of the kallikrein family (KLK4-KLK15), based on chromosomal location, sequence and structural resemblance to the first three genes (95, 96). The primary focus of the present study, was hK1, and it is hereafter referred to as TK.

Transcriptional regulation of kallikrein gene expression

The kallikrein gene family consists of fifteen homologous kallikrein genes (91, 97). A combination of hormonal and epigenetic factors are thought to be involved in the regulation of kallikrein gene expression (98). KLK1, KLK6, KLK10 genes are up-regulated in response to oestrogens; however, KLK2 and KLK3 are up-regulated by androgens and progestins (99, 100). Steroid hormone receptor complexes control the transcription of genes either directly or indirectly (99). In a direct approach, the steroid hormone receptor complex binds to hormone response elements (HRE) in the promoter/enhancer regions of the gene to assemble co-factors that collaborate with the transcription mechanism and regulate gene expression (99). In contrast, HRE regulates gene expression indirectly by interacting with *trans*-acting transcriptional factors (99). The KLK1 gene promoter bears a suspected oestrogen response element implicated in oestrogenic regulation (99). Dys-regulated transcription of numerous kallikrein genes has been linked to numerous pathologies including cancer (98).

Tissue kallikrein activation

Since proteases catalyse reactions irrevocably, they are synthesised as zymogens and require proteolysis to be transformed into its active state (99). Pro-TK has been reported to be activated by enterokinase, trypsin, trypsin-like kallikreins or by auto-activation *in vitro* (95).

Kallikrein expression and function

KLK1 is expressed in a number of tissues; however, expression is greatest in the pancreas, kidney and salivary glands (81, 91, 101). KLK2 and KLK3 are also expressed in various tissues but at comparatively higher concentrations in the prostate (100, 101). KLK4-15 are not expressed in specific tissue types but many kallikreins have been broadly found to be expressed in endocrine-related organs. Fourteen kallikrein genes are expressed in the breast, eight are expressed in the ovary and ovarian carcinoma cells while most are also expressed in the testis and prostate (96, 100).

Members of the kallikrein family are involved in diverse enzymatic activities; however, only three kallikreins have a specific biological function (89, 96). The presence of TK in diverse sites suggests its function may be different for particular cell types (89, 102). In different cell types, kallikreins may possess one or many functions although the primary function of TK should be considered the release of kinins (89). TK expression in the pancreas, pituitary and other tissues implicates this enzyme in growth factor and peptide hormone processing (99). In addition, TK cleaves several important proteins such as low-density lipoprotein, pro-insulin, atrial natriuretic factor, pro-renin, vasoactive intestinal peptide, angiotensinogen and pro-collagenase (89, 96). Recent studies have implicated newly-discovered kallikreins in numerous physiological processes. KLK4 has been implicated in enamelogenesis, KLK6, 10 and 13 are thought to be involved in hormone processing in the pancreas while KLK6 and 8 may contribute to myelination in the central nervous system (98).

PSA (hK3) and hK2 demonstrate low kininogenase activity compared to TK (99). The co-existence of hK2 and hK3 in tissues is indicative of a functional relationship (96).

Frenette *et al.* (1997) found that hK2 activates uPA *in vitro* (103). Since uPA is associated with metastasis, it is likely that hK2 is also involved in this pathway in prostate cancer (100).

1.3.3.3 Kininogen, kininases and kinins

Kininogen

Kininogens are single-chain glycoproteins synthesized by hepatocytes (89, 102, 104). Thus far, two types of kininogens have been demonstrated in humans: HMWK and LMWK (81). LMWK and HMWK differ in size, structure and cleavage-susceptibility by TK and PK (89). LMWK has a molecular mass of 50-68 kDa (species-dependent) and comprises 409 amino acids while HMWK has a molecular mass of 88 to 120 kDa and comprises 626 amino acids (89).

Kinins (kallidin and bradykinin)

Kinins are active peptides released by enzymatic cleavage of kininogens by kininogenases (89, 105). Cleavage of LMWK by TK releases kallidin while HMWK cleavage by PK produces BK. Aminopeptidases are responsible for the conversion of some kallidin to BK (94, 106). Kinins are multi-functional proteins that exhibit both physiological and pathological roles (107). They are involved in physiological functions such as regulating local blood flow, blood pressure and glucose and electrolyte transport (108). In addition, kinins are regarded as key players in inflammation and produce the fundamental features of inflammation: oedema, vasodilation and pain (81, 89, 108). Further, kinins mediate cellular functions because they activate second generation mediators such as prostaglandins and noradrenaline (89). They display potent mitogenic and chemokinetic potential in numerous

cell types and are thought to stimulate the motility of lymphocytes and tumour cells (11, 109, 110).

Kinin receptors

Kinins mediate their numerous biological functions via their two receptors, B1R and B2R (104). The kinin receptors differ in many respects, for example B2R are found only in some species (111). The B2R is ubiquitous being expressed in respiratory, intestinal, ocular and cardiovascular tissues of mammals. These receptors are thought to mediate most physiological effects of kinins (102). In contrast, the B1R is induced in numerous pathological states including inflammation and cancer (88, 92, 102). B2R have a greater affinity for BK and kallidin while kinin derivatives, desArg^{9}-BK and desArg^{10}-kallidin, preferentially bind to B1R (88, 105).

The genes coding for the two kinin receptors are found in a single locus located on chromosome 14, 12 kb apart and share a 36% homology (104, 112). The B1R and B2R belong to the G-protein coupled receptor (GPCR) family and each consist of a polypeptide chain that traverses the membrane seven times, an extracellular amino terminus, an intracellular carboxy terminus, and three extracellular and intracellular loops (102, 105, 112).

Receptor signalling pathways

Bradykinin receptors (BKR) are involved in mediating most of the biological functions of BK (109). The signalling pathways of B1R and B2R are essentially similar (81). Association of various G-proteins to kinin receptors activates phospholipase C and, therefore, second messengers such as calcium, diacylglycerol (DAG) and inositol triphosphate (105, 113). An

increase in intracellular calcium may stimulate nitric oxide (NO) production, thereby, regulating blood pressure (112). Calcium and/or DAG activates various protein kinase C (PKC) iso-enzymes involved in cell proliferation pathways (105). In addition, activation of mitogen activated protein kinase (MAPK) pathways by GPCRs results in MAPK phosphorylation and subsequent activation of transcription factors involved in mitosis and DNA synthesis (105).

Kininases

Kinins have a short half-life *in vivo* and are quickly destroyed by peptidases soon after their systemic release (89). Kininases are a family of peptidases that deactivate kinins and comprise kininase I [carboxypeptidase N (CPN) and carboxypeptidase M (CPM)] and kininase II [angiotensin I-converting enzyme (ACE) and neutral endopeptidase (NEP)] (81, 89, 108, 114).

1.3.3.4 Inhibitors and antagonists

Tissue kallikrein inhibitors

Serine protease inhibitors (serpins) have a high molecular weight and form specific and covalent bonds with TKs (102, 115). By acting upon their proteases, serpins are involved in diverse physiological processes such as complement activation, coagulation, phagocytosis and fibrinolysis (116). Many serpins exhibit functions separate from proteolytic regulation (116). Kallistatin is a member of the serpin family that binds to TK and is expressed in diverse tissues, cells and fluids where it is involved in the regulation of TK activity (102, 116). It is however also involved in biological activities independent of the KKS (115). No

clinical applications of TK inhibitors have been described in the literature thus far, although TK inhibitors with high specificities are available.

Bradykinin receptor antagonists

In order to determine the activity of cellular molecules specific antagonists are required that show high affinity for their receptors and no inherent activity (89). In 1960, the structure of BK was correctly determined, following which Regoli and colleagues later reported the first B1R antagonist (102). Several antagonists have since been synthesised by creating analogues of the BK molecule (89, 117). Subsequent development of BKR antagonists has produced compounds with greater affinity for their targeted receptors, resistance to enzymic action and increased potency and action (102).

1.3.3.5 KKS in angiogenesis, cancer and prostate cancer angiogenesis

Kallikrein-kinin system in cancer

Research suggests that the kallikrein gene family is dys-regulated in cancer, for example, in ovarian cancer (95, 99). Studies have also reported elevated kallikrein expression at mRNA or protein levels, in tumour cells compared to normal tissues (99, 118, 119). In addition, kallikreins may function synergistically to induce tumour growth; Prezas *et al.* (2006) found that simultaneous expression of kallikreins 4-7 in ovarian tumour cells inoculated into nude mice resulted in a 92% increase in tumour growth compared to the control group which did not express those kallikreins (120). Kallikreins can exhibit both stimulatory and inhibitory effects on cancer cells and their micro-environment and this dual role is likely dependent on the type of tissue and tumour micro-environment (95). Reports suggest that kallikreins may contribute to tumour progression by either direct or indirect stimulation (95). Wolf *et al*.

(2001) demonstrated the involvement of TK in metastasis using *in vitro* Matrigel invasion assays where a TK inhibitor, FE999024, suppressed the invasion of MDA-MB-231 breast cancer cells into Matrigel by 33%. They also found that the TK inhibitor was able to suppress the invasion of breast cancer cells using an *ex-vivo* assay of tumour-cell invasion in explanted rat lungs (121). Previous studies have implicated other kallikreins in hormonal malignancies (96). Steroid hormones have been linked to the aetiology of such malignancies and are also thought to be involved in kallikrein gene expression. Thus, it is likely that kallikreins are linked to the steroid hormone-driven cascade that is triggered during tumour promotion and progression (99). TK is expressed in several tumour cell lines and may be involved in carcinogenesis by enhancing cancer cell proliferation and increasing vascular permeability (99, 119, 122). By releasing active kinin peptides TK stimulates vascular permeability, cell proliferation, metastasis and regulates angiogenesis (123-125). BK demonstrates the potential to stimulate growth diversely; it may directly enhance tumour growth, promote angiogenesis via VEGF and bFGF release, and finally promote tissue permeability to aid tumour invasion by stimulating MMP activity (126). Molina *et al*. (2009) demonstrated that specific stimulation of the B1R induced cell proliferation in breast cancer cells (127). In addition, kallikreins may contribute to cancer independent of kinin production; Hecquet *et al*. (2000) demonstrated that kallikreins directly stimulated B2R (128). BK, prostaglandins and NO are key players in promoting vascular permeability in tumour tissues and nourishing tumour growth (129). Wu *et al*. (2002) found that the B2R is expressed in several human cancers, suggesting a role for BK in the growth and progression of tumours, increasing vascular permeability in the tumour vasculature and the production of NO (130).

Various components of the KKS are also expressed in prostate cancer (101, 119, 124, 131). Using an *in vitro* cell proliferation assay, Barki-Harrington and Daaka (2001) found that BK stimulates the proliferation of prostate cancer, cells thereby, implicating BK in the patho-physiology of prostate cancer (109). Taub *et al*. (2003) demonstrated the expression pattern of kinin receptors in human benign and malignant prostate specimens (11). Their report indicates that the B2R is non-selectively expressed. In contrast, the B1R was only found in prostatic intra-epithelial neoplasia and malignant tissues but not in benign prostate specimens. In addition, Taub and colleagues found that stimulation of the B1R, using a selective agonist, significantly enhanced growth, migration and invasion of prostate cancer PC3 cells. Further, the addition of a B1R antagonist abrogated such effects. These data indicate that the B1R mediates growth, migration and invasion of prostate cancer cells and further implicates the B1R in prostate cancer metastasis. Barki-Harrington *et al.* (2003) suggested the presence of B1R-B2R complexes in prostate cancer cells and found that antagonism of one receptor disturbs the cellular response of the other receptor (132). Thus the interaction between both BKR sub-types may be vital for the proliferation of prostate cancer cells. In addition, BK antagonist peptides have demonstrated anti-cancer activity in athymic nude mice implanted with prostate cancer cells (126).

Kallikreins as cancer biomarkers

Considerably more hK2, hK3 and hK4 are expressed in the prostate compared to other tissues (90). This tissue-specific expression of these kallikreins and their release into biological fluids qualify them as excellent biomarkers for prostatic diseases (90). The most significant and well-established cancer biomarker for the diagnosis and monitoring of prostate tumours is hK3 (PSA). However, PSA is limited in its capacity to differentiate between pre-malignant lesions such as benign prostatic hyperplasia and prostate cancer (97, 133). Recent studies

suggest that other kallikreins may have potential as biomarkers in cancer and other pathologies (97, 98). In addition, KLK5 and KLK11 as well as hK11 show promising potential as prognostic or diagnostic markers in prostate cancer (97, 134).

KKS in angiogenesis

Kallikreins may promote angiogenesis by modulating hallmarks such as the angiogenic switch, endothelial cell proliferation and migration, direct or indirect ECM degradation and blood vessel formation (95). Miao *et al.* (2002) found that kallistatin inhibited VEGF or bFGF-induced endothelial cell proliferation, migration and adhesion (116). In addition, TK indirectly facilitates ECM degradation by activating two Type-IV collagenases (pro-MMP-2 and pro-MMP-9) which are involved in the breakdown of collagen and other elements of the basement membrane (96, 135). TK expressed in angiogenic endothelial cells is involved in the production of kinins which consequently stimulate angiogenesis (125, 136). Emanueli *et al.* (2001) found that upon inducing hind-limb ischemia in mice, capillary density increased and kinin B1R expression was induced (136). That study also demonstrated that human TK gene delivery by injection of adenovirus resulted in a heightened ischemia-induced angiogenic response; and antagonism of the B1R resulted in an attenuated angiogenic response, however, antagonism of the B2R proved fruitless. Recently, Yao *et al.* (2008) demonstrated that TK administration promoted neo-vascularisation in the infarcted myocardium of a rat while Stone *et al*. (2009) demonstrated diminished growth of the vasculature following hind-limb ischemia in TK-knockout mouse studies (137, 138). Growing scientific evidence indicates that BK may be a primary modulator of tumour-angiogenesis and consequential tumour progression; however, the exact mechanism of action remains to be fully elucidated (139). Ishihara *et al.* (2001) demonstrated that BK enhanced tumour-associated angiogenesis and tumour growth in mice implanted with

sarcoma 180 cells (140). That group found that a selective B2R antagonist suppressed tumour-angiogenesis and tumour growth while a B1R antagonist failed to do so. They later demonstrated a role for BK in tumour-angiogenesis in which their results suggest that BK enhanced angiogenesis by promoting tumour vascular permeability (141). Recently, Wright *et al*. (2008) reported the presence of TK, B1R and B2R in the membrane projections of both endothelial and prostate cancer cells *in vitro* and found that the addition of tumour cell metabolites to the endothelial cells stimulated their proliferation (119). That group also demonstrated elevated concentrations of TK released by dermal micro-vascular endothelial cells (dMVECs) when challenged with DU145 conditioned medium (CM). They speculated that the KKS may be central to prostate cancer progression either directly or indirectly by enhancing angiogenesis.

1.4 Angiogenic models

In 1971 Judah Folkman proposed the importance of angiogenesis in tumour growth (142). Almost a decade later, he and a colleague were the first to observe angiogenesis *in vitro* (143). Targeting the micro-vasculature has become the subject of numerous studies in the treatment of cancer. A few frequently-used models are briefly discussed below.

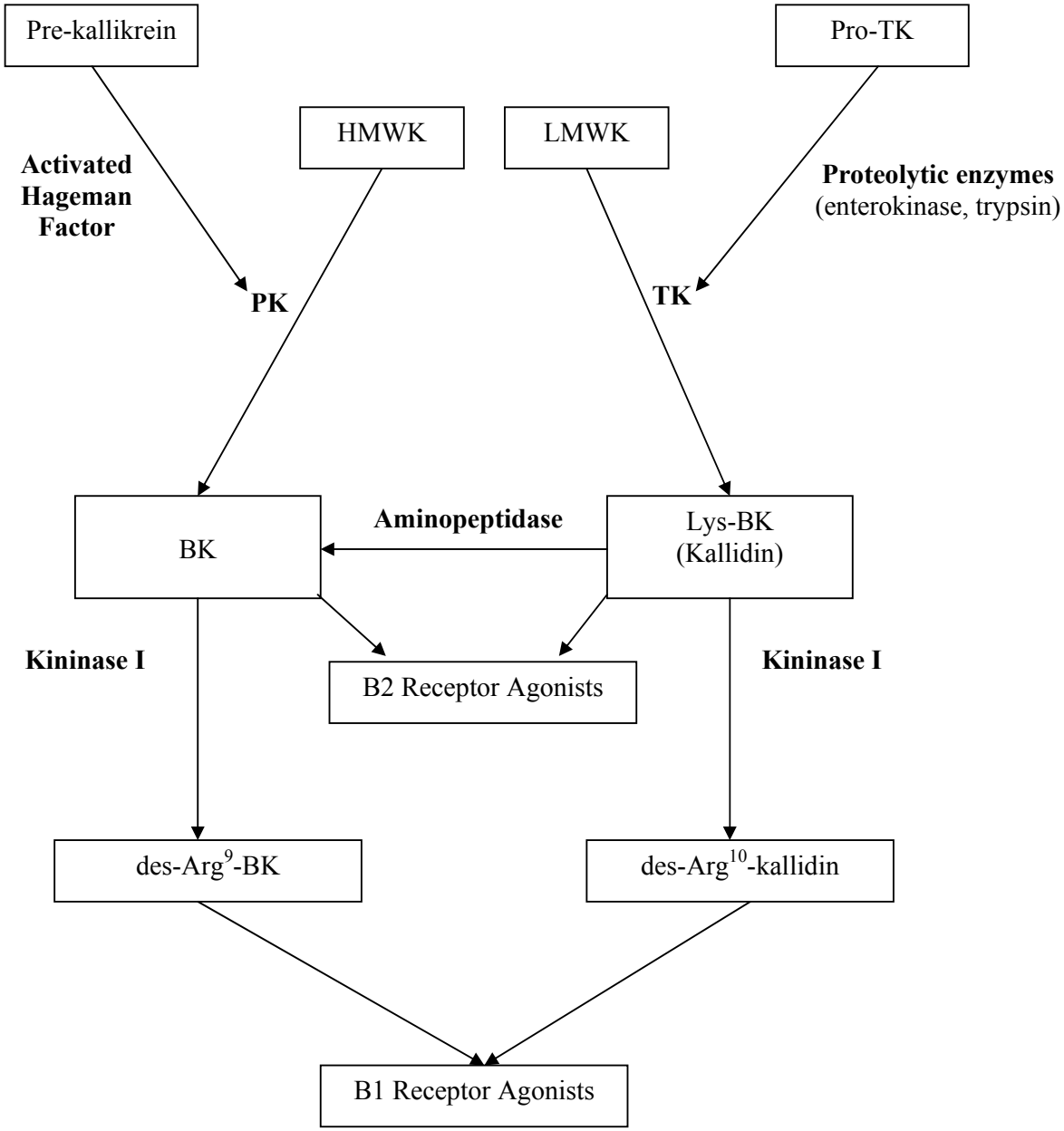


Figure 1.1: Overview of the KKS

Adapted from (92, 94, 102, 104)

1.4.1 *In vivo* models

1.4.1.1 Chorioallantoic membrane assay

The chorioallantoic membrane (CAM) assay is the most commonly used *in vivo* angiogenesis assay to investigate the potential of a test substance to induce the development of blood vessels (144). Since chick embryos that are 7-8 days old lack an established immune system, this model allows for the investigation of tumour-angiogenesis (145). Briefly, it involves an angiogenic or anti-angiogenic test substance within a gelatin or collagen sponge support that is inserted onto the membrane (146). The angiogenic effect of the compound can then be assessed by determining the number of new blood capillaries via visual or computer-assisted methods (146, 147).

1.4.1.2 Corneal assay

The rationale behind the corneal angiogenesis assay is that since the cornea is normally a non-vascular site, any new blood vessels produced would be in response to the experimental substance (148, 149). A 'pocket' is surgically created in the corneal stroma (rabbits, rats or mice models), into which the angiogenic substance is inserted (146, 150). The angiogenic response can then be assessed via visual or computer-assisted methods (146).

1.4.1.3 The dorsal air sac model

The dorsal air sac model is used to assess the angiogenic effect of tumour cells (145, 149). A suspension of tumour cells is inserted into a sub-cutaneous chamber (dorsal air sac) in rats or mice and allowed to form a tumour mass (151). A test substance is usually administered, and the exposed area of the tumour can be assessed in terms of the angiogenic effect by counting the number of new blood capillaries (147).

1.4.2 *In vitro* models

1.4.2.1 Two-dimensional and three-dimensional models

Two-dimensional (2-D) models are those in which endothelial cells grow parallel to the surface of the plastic culture dish on which they are seeded and can be used to evaluate key processes in angiogenesis (152). Proliferation, migration and the formation of cord-like structures by endothelial cells have been the primary focus of *in vitro* angiogenic models (145). Using this model, the effect of stimulatory and inhibitory compounds such as CM, receptor agonists and antagonists can be investigated. .

The proliferative effect of a test substance on endothelial cells can be assessed both directly and indirectly with the use of (i) a haemocytometer, (ii) an electric Coulter counter, (iii) thymidine incorporation or (iv) 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), a yellow tetrazolium salt (145, 153). Proliferation assays, therefore, have the advantage of being simple, reproducible and quantifiable using high-throughput methods (147, 154).

The Boyden chamber assay is the most frequently used model for endothelial cell migration (146, 154). Endothelial cells migrate by chemotaxis in response to angiogenic stimulatory factors (145). Briefly, the upper chamber contains the endothelial cells while the lower chamber contains the chemo-attractant. The upper and lower chambers are separated by a porous polycarbonate filter allowing only the movement of cells (150, 155). Cells that have migrated can further be stained and counted, or high-throughput methods such as fluorescent detection may be employed (146, 155). Alternatively, migration may be evaluated using the "wound healing" assay which involves scraping off the endothelial cell monolayer and subsequent quantification of endothelial cells that have migrated into the disrupted area (147).

The formation of tube-like structures is demonstrative of later events in the angiogenic cascade and can be investigated by seeding endothelial cells onto a matrix such as Matrigel, fibrin or collagen (147, 153). Test substances may be added to the growing vessels to evaluate their angiogenic potential and their effects on tubule length and density can be assessed using image-analysis (146). These differentiation assays can be investigated in both 2-D or three-dimensional (3-D) models depending on the organisation of the tube-like structures within the matrix (146, 152). While 2-D models improve our knowledge of the ECM, 3-D models are a better representation of angiogenesis in the *in vivo* milieu, as endothelial cells have the capacity to proliferate, migrate and differentiate in the matrix of 3-D models (152).

1.4.3 Advantages and limitations of *in vivo* and *in vitro* models

Essentially, angiogenic assays should be simple, inexpensive and reproducible with high-throughput quantitative analysis (146). Although some *in vitro* models meet these requirements, previous studies have demonstrated that a substance regulating events in the angiogenic cascade *in vitro* may not reproduce the same effect *in vivo* (35, 145, 154). The angiogenic effect of a test substance should, therefore, eventually be established with the use of more complex models (146, 148). In addition, while the endothelial cell is the primary element of angiogenesis, several other supporting cells, as well as the blood and ECM contribute to the process (147). Thus, *in vivo* studies investigating angiogenesis are fundamental phases in drug research and development. However, *in vivo* assays have many disadvantages in that they are complicated, often use non-human models, sometimes require surgical skills, are time consuming, expensive and difficult to reproduce (146, 153). Further, tissue injury following implantation of test substances may induce inflammation, thereby altering results (146).

1.4.4 Models used in the present study

Taking into consideration the resources available, *in vitro* models were chosen in the present study because they are simple, relatively inexpensive and reproducible. In addition, *in vitro* models allow for the specific investigation of test substances in an isolated system (154). Micro-vascular endothelial cells were initially selected for use in the angiogenic assays because they are more representative of the *in vivo* environment than endothelial cells of macro-vascular origin; however since dMVECs demonstrated little angiogenic capacity the angiogenic potential of human umbilical vein endothelial cells (HUVECs) was later explored (147). Accordingly, angiogenic 2-D models involving MTT proliferation and Boyden chamber migration assays were selected to investigate the effect of various test substances on the growth and motility of endothelial and tumour cells. A CM approach was used, whereby the medium from one cell type (which contains mediator substances secreted by those cells) is added to other cells (122). Tumour metabolites released by proliferating cancer cells were thereby presented to endothelial cells at increasing concentrations. Using commercial BKR agonists and antagonists, components of the KKS were also tested for their ability to stimulate or inhibit proliferation and migration of endothelial and tumour cells.

1.5 Rationale, aims and objectives

Previous studies using cultivated cells and CM have shown that when endothelial cells are exposed to increasing concentrations of prostate and breast tumour cell metabolites, dMVEC proliferation and TK production was enhanced (156). Thus, those investigators proposed that the KKS may be involved in tumour-angiogenesis. Accordingly, the present study aimed to further investigate the association between the KKS and angiogenesis/tumourigenesis by seeking a direct link that kinins may exert on endothelial and tumour cell proliferation and migration using CM, kinin receptor agonists and antagonists.

The objectives were to establish *in vitro* tumour-angiogenic models and use these to determine the effects of tumour CM and BKR agonists and antagonists on the following angiogenic and tumourigenic processes:

1. endothelial cell proliferation
2. endothelial cell migration
3. tumour cell proliferation
4. tumour cell migration

1.6 Hypothesis

Tumour-induced KKS promotes endothelial and tumour cell growth and motility, fundamental processes in angiogenesis, tumourigenesis and metastasis.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 - MATERIALS AND METHODS

2.1 Ethical approval

Ethical approval for this project was granted by the Biomedical Research Ethics Committee, University of KwaZulu-Natal, reference number BE152/08.

2.2 Cell culture

2.2.1 Cell lines and culture media

2.2.1.1 Endothelial cell lines

The dMVEC line was commercially-obtained from Clonetics (BioWhittaker, Walkersville, USA). The frozen 1 ml aliquot was preserved and transported on dry ice. The primary culture specifications revealed that (i) they were harvested from a 42 year old black female donor in 2007, (ii) the cells were in their third passage with cell viability at 82% and a cell count of 1.2 x 10^6 per ml, and (iii) the human immunodeficiency virus (HIV), hepatitis B virus (HBV) or hepatitis C virus (HCV) was not detected. The cryo-vial was stored at -85^{o}C in an ultra-freezer (Ultralow Freezer, NuAire, USA) for future use. Commercially-obtained HUVECs were kindly donated by the Department of Medical Microbiology, University of Kwa-Zulu Natal.

2.2.1.2 Endothelial cell media

EGM®-2MV Bulletkit (micro-vascular endothelial growth medium-2) cell culture media was purchased from Clonetics. The product consisted of a basal medium (EBM-2) supplemented with fetal bovine serum (FBS, 25 ml), hydrocortisone (0.2 ml), bFGF (2 ml), VEGF (0.5 ml), R3-insulin-like growth factor-1 (R3-IGF-1, 0.5 ml), ascorbic acid (0.5 ml), EGF (0.5 ml) and

gentamicin sulphate amphotericin β (GA-1000, 0.5 ml), under aseptic conditions. Re-constituted medium had a shelf-life of 3 months at 4^{o}C. For clarification, EGM-2 consists of basal medium supplemented with growth factors whereas medium free of growth factors is referred to as EBM-2. EGM-2 and EBM-2 may be serum-free or supplemented with FBS.

2.2.1.3 Tumour cell lines

The human prostate adenocarcinoma (DU145), cervical adenocarcinoma (HeLa) and brain neuroblastoma (N2α) cell lines are immortal cell lines that have been continuously passaged and maintained within the Department of Therapeutics and Medicines Management, University of KwaZulu-Natal, South Africa, for several years. DU145 and HeLa are both aneuploid, epithelial cell lines. DU145, is a metastatic, androgen-independent cell line that was harvested from the brain of a 69 year old Caucasian male while HeLa, was originally isolated from a 31 year old black female (Henrietta Lacks, abbreviated to HeLa) (157, 158). Frozen cryo-vials of tumour cells were stored at -85^{o}C in an ultra-freezer (NuAire, USA) for future use.

2.2.1.4 Tumour cell media

DU145 and HeLa cell culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin-streptomycin-fungizone (PSF, 100 µg/ml) and L-glutamine (2 mM) (BioWhittaker). N2α medium additionally contained sodium pyruvate (2 mM) (BioWhittaker). Tumour media was prepared under aseptic conditions, and thereafter stored at 4^{o}C for a maximum of 2-3 weeks.

2.2.2 Receptor agonists and antagonists in the present study

Selective B1R and B2R agonists were commercially-obtained from Tocris Bioscience, USA. The B1R agonist (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe) has a molecular weight of 1032.21 g/mol while the B2R (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-ψ(CH-NH)-Arg) has a molecular weight of 1046.23 g/mol. The B1R antagonist, des-Arg^9-[Leu^8]-BK acetate salt and B2R antagonist, Hoe 140, were purchased from Sigma-Aldrich, USA. des-Arg^9-[Leu^8]-BK acetate salt and Hoe 140 are selective BKR antagonists that have molecular weights of 870.01 g/mol and 1304.52 g/mol, respectively. des-Arg^9-[Leu^8]-BK is the most extensively used B1R antagonist in experimental studies. Similarly, Hoe 140 has been used in several studies, both *in vivo* and *in vitro*, as a basic prototype of the B2R antagonist (102, 111).

2.2.3 Aseptic technique

Cell culture and media preparation were always performed under sterile conditions in a class II biological safety cabinet (Labotec, Durban, South Africa). Disposable pasteur pipettes (LASEC, Durban, South Africa) and plastic pipette tips (Greiner Bio-One, Austria) were sterilised by autoclaving (Speedy autoclave, Hirayama, Japan) at 121^{o}C for 20 minutes. Pipetting aids consisted of an electronic pipette (Powerpette Plus; Jencons, USA) and mechanical pipettes (Gilson, France). Prior to work commencing the safety cabinet was exposed to UV irradiation for at least half an hour. Although the exposed working surfaces are sterilised by UV irradiation, its effectiveness is, however, limited because it is unable to reach crevices; the sterilising procedure is thus supplemented with the use of alcohol (159). All working surfaces and equipment were swabbed with 1% medical disinfectant solution (Virkon; Antec, South Africa) followed by 70% ethanol solution (Merck, South Africa). Consumables used during cell culture were swabbed with 70% ethanol before being introduced into the safety cabinet.

2.2.4 Thawing, seeding and maintenance of cell cultures

Tissue culture dishes, 60 mm (21 cm^2) or 100 mm (55 cm^2) (Corning-Costar, USA), were pre-primed with appropriate volumes (4 ml and 8 ml, respectively) of cell-specific medium, and incubated for approximately 30 minutes in a humidified CO_2 incubator (Function Line; Heraeus, Germany) calibrated at 37^oC, 5% CO_2. Cryo-frozen cells, stored in 1.8 ml cryo-vials (Corning-Costar, USA), were individually removed from the ultra-freezer and allowed to thaw by regular agitation for a maximum of 3 minutes in a 37^oC water-bath (The Scientific Group). Care was taken not to immerse the cryo-vial below the seal to prevent potential water-borne contamination. The cryo-vial was swabbed, introduced into the sterile cabinet and the contents pipetted into the pre-warmed tissue culture dish. The culture dish was swirled gently to allow even distribution of cells. In order to build cell banks, tumour cells and dMVECs were seeded at a density of 6000 cells/cm^2. Culture dishes were then incubated overnight in a humidified CO_2 incubator (Function Line; Heraeus, Germany) at 37^oC, 5% CO_2 to allow surface attachment. A media change was performed with fresh, pre-warmed medium. The cells were further incubated at 37^oC, 5% CO_2. Nutrients and growth factors were subsequently replenished every alternate day by performing a media change. The following formula was used to calculate the volume of cell-suspension required to determine the appropriate seeding densities:

$$\text{Cell-suspension volume (ml)} = \frac{[\text{seeding density (cells/cm}^2\text{) x surface area of culture dish (cm}^2\text{)}]}{\text{concentration of viable cells (cells/ml)}}$$

Cell cultures were examined using a phase-contrast, bright-field inverted microscope (DMIL; Leica, Germany) and their contaminant-free growth, health and morphology recorded regularly. Upon attaining 70-80% confluency, cells were harvested and subsequently (i) sub-cultured for further cell propagation or (ii) cryo-frozen to build a cell bank.

Figures 2.1 and 2.2 demonstrate confluent dMVECs and DU145 in culture as visualised by phase-contrast microscopy.

2.2.5 Sub-culturing and cryo-storage

Cells were enzymatically disaggregated using trypsin-versene (BioWhittaker, Walkersville, USA). Spent medium was aspirated and discarded. The culture dish was subsequently rinsed and gently swirled for approximately 30 seconds with pre-warmed Hanks Balanced Salt Solution (HBSS; BioWhittaker, Walkersville, USA) to remove cell debris and any remaining serum. The HBSS was aspirated and discarded. Trypsin-versene, (2 ml and 4 ml for a 60 mm and 100 mm culture dish, respectively), pre-warmed to 37^{o}C, was added to the culture dish and incubated at 37^{o}C, 5% CO_2 for approximately 1-3 minutes. When exposed to trypsin-versene cell membranes retract and cells appear round and refractile. At this stage the sides of the culture dish were then gently tapped to dislodge cells. In order to halt the action of trypsin-versene, twice the volume of cell-specific media was added to the culture dish containing cells. Using a Pasteur pipette, the cell-suspension was gently agitated. The cell-suspension was aspirated collected and centrifuged at 100 x g for 5 minutes at room temperature (RT) (Megafuge 1.0 R; Heraeus, Germany). The supernatant was discarded and the pellet re-suspended in 1-2 ml fresh, pre-warmed culture media. The cell-suspension was again gently agitated to disassociate cell clumps. A cell count and cell viability assay, described in section 2.2.5.1, was performed in order to sub-culture and freeze cells.

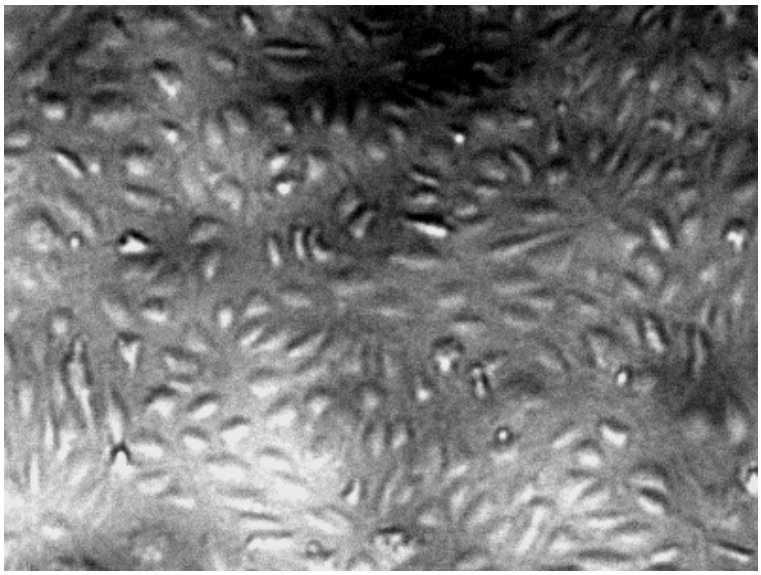


Figure 2.1: Phase-contrast photomicrograph of dMVECs in culture, 100x magnification

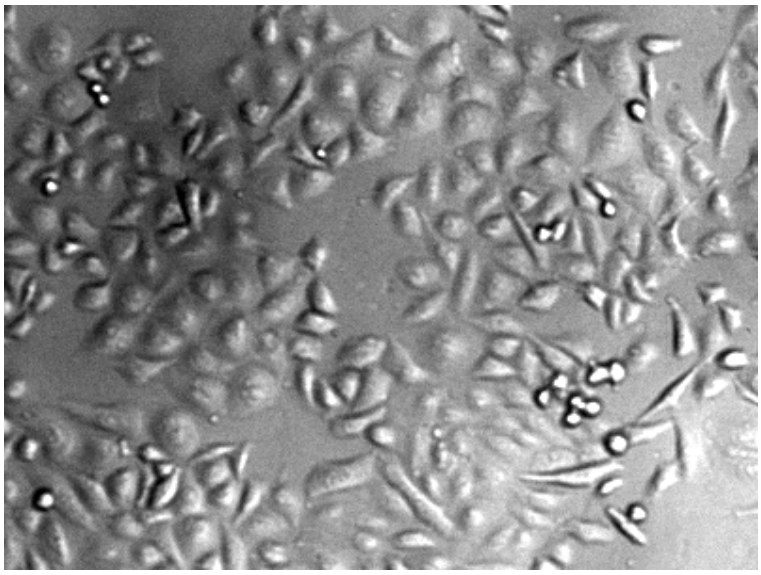


Figure 2.2: Phase-contrast photomicrograph of DU145 cells in culture, 100x magnification

When cells were sub-cultured, the cell-suspension volume necessary to obtain the required seeding density was calculated and tissue culture dishes plated as described in section 2.2.4. For all experimental purposes, endothelial cells were used between passages 4 and 9. If cells were to be frozen, dimethylsulfoxide (DMSO; Sigma), diluted in culture medium, was added to the cell-suspension to yield a final concentration of 10% DMSO (v/v). Freezing densities depended upon cell lines, cell yield and cell numbers required for experimental work but were never less than 200 000 endothelial cells and 300 000 tumour cells. DMSO-cell suspensions were stored in a volume of 1 ml per cryo-vial (Corning-Costar, USA); the cryo-vial was labelled and allowed to settle for a maximum of 30 minutes at RT. They were subsequently transferred and frozen in an ultra-freezer at -85^{o}C. A cell log was maintained to monitor cell banks.

2.2.5.1 Cell count and cell viability

Cell counts and viability assays were performed using a haemocytometer and the trypan blue exclusion test. A 1:1 ratio of cell-suspension and trypan blue was loaded onto the haemocytometer. Cells were counted under a light microscope (DMLB; Leica, Germany). Non-viable cells were distinguished from viable cells as they were blue in colour due to dye uptake. Viable and non-viable cells were counted in a total of eight (1 mm) quadrants to obtain an average score. Due to the toxicity of trypan blue, cell counts were performed between 5 and 10 minutes after the addition of the dye. Cell concentration and viability was determined using the following formulas:

Cell concentration = no. of cells / no. of quadrants x 10^4 x 2

Percentage viability = no. of viable cells / total no. of cells x 100

2.3 Angiogenic and tumourigenic models

2.3.1 Proliferation assays

2.3.1.1 Experiments

The effect of CM from one cell line on the proliferation of another was investigated using the MTT cell proliferation assay. Increasing concentrations of DU145 prostate tumour CM were added to dMVECs and their proliferation determined after 24 hour experimental exposure. BKR antagonists were tested for their ability to inhibit CM-stimulated proliferation, while BKR agonists were investigated for their mitogenic potential on dMVECs. In addition, the effect of VEGF on dMVEC proliferation was examined. As a method control, the effect of serum on dMVEC growth, was also investigated (Appendix B1). CM from other tumour cell lines was also examined for angiogenic potential on dMVECs (Appendix D2) as well as on an alternate endothelial cell line, HUVECS (Appendix E).

In prostate tumour cell proliferation assays, BKR agonists were investigated for their mitogenic potential on DU145 cells. Next, a B1R antagonist was tested for its ability to inhibit agonist-stimulated growth.

2.3.1.2 Background

MTT is a yellow tetrazolium salt that produces water insoluble, blue formazan crystals when cleaved by mitochondria (145, 160). After the addition of a detergent such as DMSO this blue colour can be solubilised and subsequently quantified using a spectrophotometer (161). This method is universally used to evaluate cellular proliferation since metabolically active cells contain mitochondria that enable formazan production (145).

2.3.1.3 Protocol

Endothelial cells (dMVECs or HUVECs) and tumour cells (DU145) were grown to confluency in culture dishes, sub-cultured and re-seeded in 96-well culture plates (Corning) at a density of 4500 cells/cm^2. For the production of CM, tumour cells (DU145, HeLa and N2α) cells were seeded at 4000 cells/cm^2 in P60 culture dishes. CM from tumour cells was generated over a 24 hour period. Upon attaining approximately 60% confluency (typically 2-3 days) in 96-well plates, tumour and endothelial cells were ready for the addition of test substances. The cell-specific growth medium was replaced with CM, BKR agonists, BKR antagonists or VEGF, and incubated at 37^oC, 5% CO_2 for 24 hours following which the MTT assay was performed.

Modification of conditions

Previous studies investigating the mitogenic effects of CM differed in many respects in terms of their CM models. Therefore, the following modifications were employed to the tumour and endothelial cell environment to incorporate the methodologies of previous studies:

1. Tumour cells were grown in their respective cell-specific medium (DMEM) and then gradually weaned onto a common medium (EGM-2), that would facilitate growth of both tumour and endothelial cells.
2. The dMVECs were serum-starved for 24 hours, in 0% FBS/EGM-2, prior to the addition of CM as suggested by a previous report (162). The function of serum-free media appears to be two-fold: 1) to eliminate the effect of FBS - a direct contributor to cell proliferation and 2) to align the cells into the G0 phase of the cell cycle (163).
3. DU145 cells were additionally serum-starved in 0% FBS/EGM-2, as suggested by previous studies (163-165). CM was, therefore, generated in serum-free medium.

4. Low-serum conditions (0.5% FBS/EGM-2) were applied to dMVECs and DU145 cells as suggested by a previous study (151).
5. CM was generated over 24, 48 or 72 hours to explore the possibility that extended time periods of exposure to cultured cells would produce CM containing greater concentrations of angiogenic factors.
6. The concentration range of CM was increased.
7. In addition to the above modifications, the CM model was expanded to include alternate endothelial (HUVEC) and tumour (HeLa and N2α) cell lines to serve as comparative controls.

2.3.1.4 Experimental and control treatments

Endothelial cells

CM of increasing concentrations (0, 10, 25 and 50%) was prepared by filtering the spent media through a 0.22 μm pore syringe filter (LASEC, SA) and, thereafter, diluted accordingly with fresh EGM-2. For each concentration of CM/EGM-2, DMEM/EGM-2 controls were run simultaneously to compensate for the effects of different media on dMVEC growth. Control treatments contained 0, 10, 25 and 50% fresh DMEM, diluted accordingly with EGM-2. Maximum concentrations of 50% CM was tested since it was ascertained in previous work in our laboratories that concentrations greater than 50% proved to be detrimental to cell growth (166).

When the conditions were further modified, 12.5, 25 50, 75 and 100% CM was prepared by diluting accordingly with fresh EGM-2. Fresh EGM-2 added to endothelial cells served as a baseline control.

For agonist experiments, B1R or B2R agonists were added at concentrations of 50, 250, 500 and 1000 nM to EGM-2. EGM-2 served as a relevant baseline control. For antagonist experiments, B1R or B2R antagonists were added at concentrations of 0.5, 1, 2.5, 5 and 10 μM to endothelial cells 30 minutes prior to the addition of 50% CM.

In the method controls, cells were administered serum-free EGM-2 or VEGF at concentrations of 1, 10, 50 and 100 ng/ml. VEGF was added to various basal media:

1. 5% FBS/EGM-2
2. 0% FBS/EGM-2
3. 5% FBS/EBM-2
4. 0% FBS/EBM-2

Tumour cells

B1R or B2R agonists were added at concentrations of 10, 50 or 100 nM to DMEM for DU145 agonist experiments, while for antagonist experiments, 10 μM B1R antagonist was added to DU145 cells 30 minutes prior to the addition of the B1R agonist. VEGF was added at concentrations of 10, 50 and 100 ng/ml. DMEM served as a baseline control.

2.3.1.5 MTT assay

The MTT assay was used to determine dMVEC or DU145 proliferation after exposure to tumour CM, BKR agonists, BKR antagonists or VEGF. MTT salt (Sigma, St. Louis, USA) was dissolved in pre-warmed HBSS at 5 mg/ml by incubation at 37^{o}C for 10 min, and thereafter filtered through a 0.22 μm filter. A volume of 10 μl MTT solution was added to 100 μl of cell-specific medium (EGM-2 or DMEM) for every well to be assayed. The

experimental and control medium in the wells were aspirated and discarded. Next, 110 µl of the MTT-cell-specific medium was added to each well and incubated for 4 hours at 37°C, 5% CO_2. After incubation, the MTT-cell-specific medium was aspirated and discarded and then 100 µl of DMSO was added to each well to dissolve the precipitate. The 96-well plate was further incubated for 1 hour at 37°C, 5% CO_2 following which absorbance was read at a wavelength of 595 nm (reference 655 nm) using a microplate reader (Model 3550; Biorad, UK).

2.3.1.6 Data presentation

For assays in which DMEM CM was diluted with EGM-2, net proliferation was calculated by subtracting the proliferative effect of fresh DMEM/EGM-2 from the proliferative effect of CM. For all other MTT assays, proliferation was expressed in each experimental set as a percentage of the relevant control (expressed as 100%).

2.3.2 Migration assays

2.3.2.1 Experiments

The ability of prostate tumour CM to stimulate another important event in angiogenesis namely endothelial cell migration, was investigated. BKR agonists, in the presence or absence of CM, were also tested for their pro-migratory effect on dMVECs. Additionally, the inhibitory effect of a B1R antagonist was tested by pre-incubation of dMVECs prior to stimulation with basal media, DU145 CM or B1R agonist. VEGF was also tested for its pro-stimulatory effect on dMVEC migration. In method controls the effect of serum and other media types were investigated (Appendix B2).

In DU145 cell migration assays, the ability of DU145 CM to stimulate cell migration in an autocrine manner was tested. BKR agonists were also examined for their pro-migratory potential. Next, a B1R antagonist was investigated for its ability to inhibit baseline, CM or agonist-mediated migration.

2.3.2.2 Background

The modified Boyden chamber assay is frequently used to measure cell motility (146). The chamber consists of an upper (containing cells) and lower compartment (containing test substances) separated by a porous membrane (145). Cells that migrate across the membrane, in response to the test substance, can then be quantified using Calcein-acetomethylester (Calcein-AM). Uptake of Calcein-AM by cells that have migrated causes internal cleavage of the AM component producing free fluorescent calcein (155). The fluorescence can then be quantified using a standard curve and correlated with cell number. For our experiments we used a 96-well HTS Transwell® plate (Corning, NY, USA) containing an 8 μm polyester membrane. Figure 2.3 illustrates the modified Boyden chamber model used in the present study.

2.3.2.3 Protocol

Endothelial or tumour cells were grown to confluence, trypsinised and centrifuged. The supernatant was aspirated and cells were suspended in their respective serum-free, growth factor-free medium. A cell count was performed and the cell density adjusted to 400 000 cells/ml. For dMVEC migration assays test substances (CM, VEGF, BKR agonists and a B1R antagonist) were prepared in 10% FBS/DMEM. For DU145 migration assays, with the exception of CM, test substances (VEGF, BKR agonists and a B1R antagonist) were

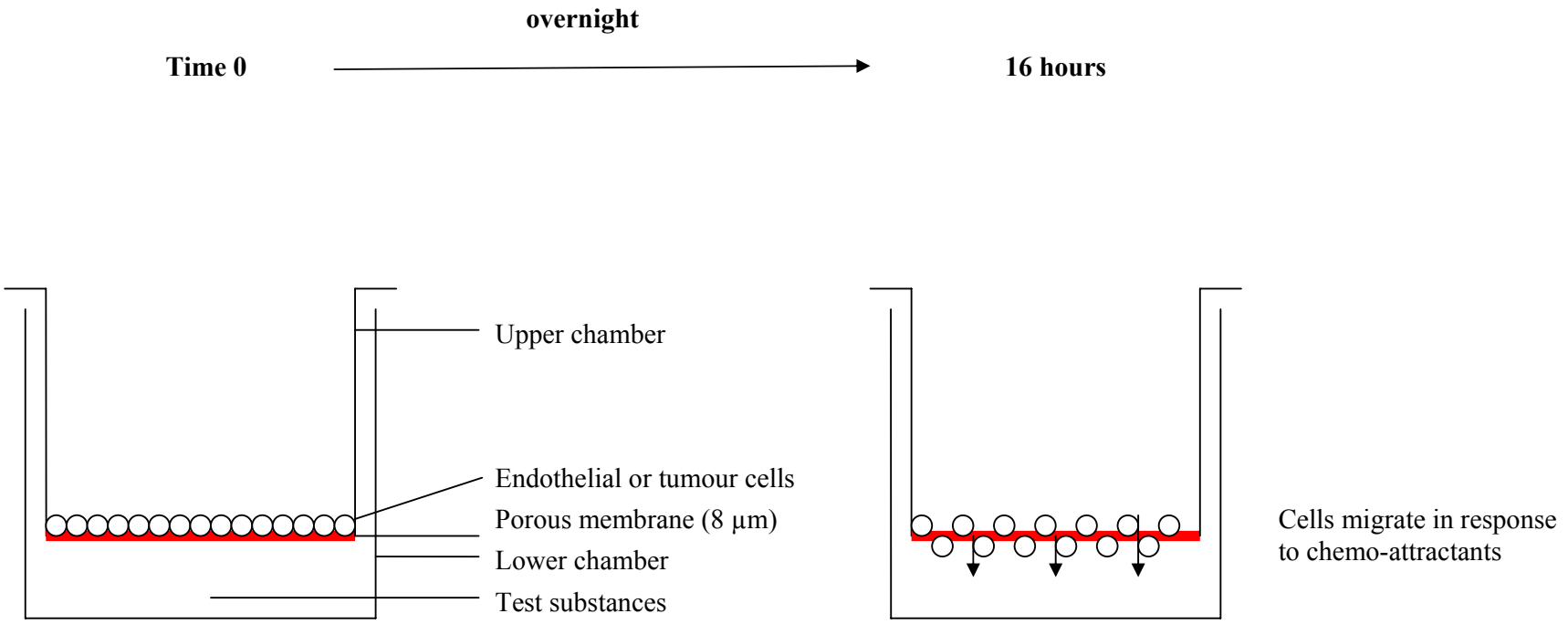


Figure 2.3: Modified Boyden chamber as used in the present study

prepared in 5% FBS/DMEM. These concentrations of FBS were optimised for dMVEC and DU145 cell lines to ensure a baseline migration, over which additional effects of test substances could be measured. Using the access ports, 150 µl of test medium was added to the lower wells of the plate. Thereafter, 20 000 (50 µl) dMVEC or DU145 cells were added to the upper wells of the Boyden chamber. For antagonist experiments, cells were pre-treated with 10 µM BKR antagonist for 30 minutes before addition to the upper chamber. To compensate for background, cells were omitted from at least 3 blank wells. The plate was incubated overnight at 37^{o}C, 5% CO_2.

2.3.2.4 Experimental and control treatments

Endothelial cells

CM was extracted as described for endothelial cell proliferation assays and filtered and diluted to yield test concentrations of 0, 50 and 100%. B1R or B2R agonists were added at a concentration of 100 nM while the B1R antagonist was added at a concentration of 10 µM. In addition, the BKR agonists were added to 100% CM to investigate possible synergistic effects. Serum-free DMEM and 10, 50 and 100 ng/ml VEGF were used as method controls. Due to the large number of endothelial cells required for migration assays, the concentration range of stimulants were limited to those that demonstrated maximal stimulation in these and previous studies. Further, antagonist experiments were performed using only a B1R antagonist due to the lack of effect demonstrated by the B2R in proliferation and migration experiments in the present study.

Tumour cells

CM at concentrations of 0, 10, 50 and 100% was prepared as described for endothelial cells. B1R or B2R agonists were added at concentrations of 10, 50 and 100 nM while the B1R antagonist was added at a concentration of 10 μM. Similarly, serum-free DMEM was used as a method control.

2.3.2.5 Fluorescence detection

Following overnight incubation, cells remaining in the upper chamber were aspirated and each well was washed with 100 μl of HBSS. The medium in the bottom chambers was aspirated and each well washed twice with 200 μl HBSS. DMSO (25 μl) was added to a 50 μg vial of Calcein-AM (BD Biosciences) to make a 2 mM working solution. Calcein-AM (10 μl of 2 mM) was added to 10 ml pre-warmed Cell-Dissociation Solution (CDS; Sigma, St. Louis, USA) and kept in the dark prior to use. Using the access ports, 100 μl of CDS/Calcein-AM was added to each well of the bottom chamber. The device was assembled and incubated for 30 minutes at 37^{o}C, 5% CO_2. It was then gently tapped 10 times on the sides to aid dissociation and incubated for a further 30 minutes. The top chamber was removed, the bottom receiver plate was gently swirled to mix the solution and read using a fluorescent top reader at 485 nm excitation, 520 nm emission (Optima; BMG, Germany).

2.3.2.6 Standard curve

A standard curve was run concurrently with each experiment to extrapolate and quantify the number of cells that had migrated. Cell densities of 20000, 10000, 5000, 2500, 1000 and 500 were prepared by serial dilutions. Cells were seeded in triplicate into the bottom chamber of the 96-well HTS Transwell® plate (Corning, NY, USA) and suspended in 100 μl CDS containing 2 mM Calcein-AM at a ratio of 1 ml CDS: 1 μl Calcein-AM. The plate was

incubated for 1 hour and subsequently read using a fluorescent top reader at 485 nm excitation, 520 nm emission (Optima; BMG, Germany).

2.3.2.7 Data presentation

Values for experimental and standard curve conditions were averaged. The plate background was then subtracted from each value. A standard curve of relative fluorescent units (RFU) versus number of cells was plotted. A trend line was inserted and a straight line equation generated. Using the RFU value for each condition, the number of cells migrated was extrapolated from the graph. This value was expressed as a percentage of the value obtained for the control.

2.4 Conditioned medium analysis

2.4.1 Enzyme-linked immunosorbent assay

2.4.1.1 Background

An indirect sandwich enzyme-linked immunosorbent assay (ELISA) was performed in the present study to determine the concentration of TK in DU145 CM. ELISAs are useful tools for the detection of particular proteins in a sample (167). The underlying principle of an ELISA is the detection of antigen-antibody complexes by enzymatic conversion of a colourless substrate to a measurable coloured product that can then be quantified using a spectrophotometer (168).

2.4.1.2 Samples and controls

Recombinant human kallikrein-1 (TK) was diluted in phosphate buffered saline (PBS) to concentrations of 40, 20, 10, 5, 2.5, 1.25 and 0.625 ng/ml to generate a standard curve

(Figure F1, Appendix F). CM, generated in serum-free EGM-2 or 5% FBS/EGM-2, over 24, 48 and 72 hours comprised the experimental samples while fresh EGM-2 medium (serum-free and 5% FBS) were used as controls. PBS served as a negative control.

2.4.1.3 Protocol

The TK ELISA was performed as described by Naidoo (2005) and Wright *et al*. (2008) with a few modifications (119, 122). Reagents were prepared as described in Appendix A. Goat anti-human TK antibody was loaded into each well of a cold, 96-well polystyrene microtitre ELISA plate (100 µl/well) and incubated at 4^{o}C overnight. Unbound antibody was then removed by washing each well with 250 µl wash buffer, 3 times for 3 minutes each at RT. To prevent non-specific binding, 300 µl milk blocker was added to each well and incubated for 30 minutes at RT. Each well was again washed with wash buffer and incubated with milk blocker as previously. Thereafter, 100 µl of samples, controls and standards were loaded onto the microtitre plate in triplicate. The plate was then sealed with parafilm M (Whatman, UK) and incubated in a shaking water bath (Tecator, UK) bath at 37^{o}C for 1 hour. Wash steps with wash buffer were then repeated to remove unbound antigen. The secondary antibody, rabbit anti-human immunoglobulin G (IgG) TK antibody (100 µl) was added to each well and the plate was then parafilmed and placed in a shaking waterbath at 37^{o}C for 1 hour. Thereafter, the wells were washed with wash buffer, 3 times for 3 minutes each after which anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, St. Louis, USA) was added to all wells. The plate was again incubated in a shaking waterbath at 37^{o}C for 1 hour and the wells subsequently washed with wash buffer, 3 times for 3 minutes. Finally, chromogenic di-sodium paranitrophenyl phosphate substrate (pNPP; Sigma), diluted in substrate buffer was added to all wells. The plate was incubated at RT in the dark until a yellow product developed and the highest absorbance measured ranged between 1 and 1.5 units

(approximately 30-40 minutes). Absorbance was read using a Microplate Reader 3550 (Biorad, UK) at a wavelength of 405 nm.

2.4.1.4 Data presentation

Values for standards, samples, media controls and negative controls were averaged. The mean negative control was then subtracted from each value. A standard curve of absorbance vs concentration of TK was plotted. A trend line generated a straight line equation.

2.5 Statistical analysis

Proliferation assays were performed in quadruplicate while migration assays were performed in triplicate. The repetitions for each experiment are indicated below each figure. Cell proliferation and migration are expressed as a percentage of the non-stimulated control (regarded as 100%). Results are illustrated in column graphs indicating the mean standard error of the mean (SEM). Error bars are absent where sample sizes were too low. In some experiments inhibition in proliferation or migration was observed after addition of test substances. In order to represent this inhibition, the y-axis of graphs frequently begin with values below 100%. A bio-statistician was consulted regarding the most appropriate data analysis techniques. One-way analysis of variance (ANOVA) was used to test for statistical significance between multiple concentration groups followed by Dunnett's post-hoc testing while Student's t-test was used for comparison between two groups. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using the software package SPSS 18 (IBM, USA).

CHAPTER 3

RESULTS

CHAPTER 3 - RESULTS

3.1 Endothelial cells (dMVECs)

3.1.1 dMVEC proliferation

3.1.1.1 Effect of VEGF

Generally VEGF caused increased proliferation of dMVECs (Figure 3.1.1.1). This effect was greatest in the most rudimentary medium, free of FBS or growth factors (0% FBS/EBM-2), where after a small inhibition at 1 ng/ml, there was a dose-dependent increase with a maximum stimulatory effect of 20.8% at 100 ng/ml (Figure 3.1.1.1D).

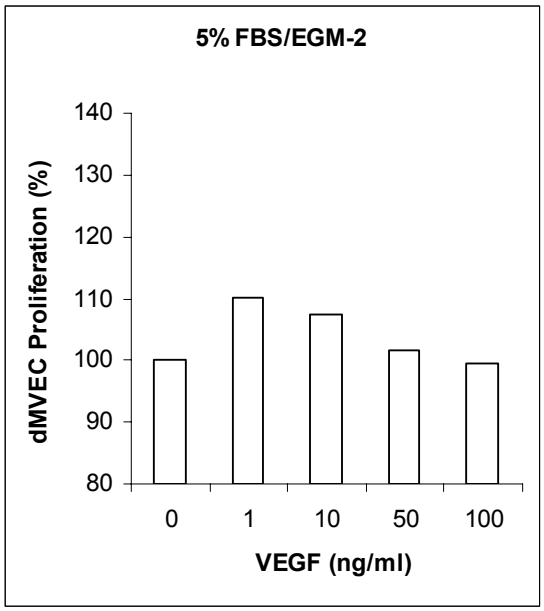
3.1.1.2 Effect of DU145 CM

When dMVECs were challenged with increasing concentrations of DU145 CM, a statistically significant, although small, increase was observed (Figure 3.1.1.2). At a concentration of 50% CM proliferation increased by a mean of only 7.9%. Again this was statistically significant ($p<0.05$); however, the effect was too small to indicate any scientific relevance.

3.1.1.3 Effect of 50% CM on dMVECs pre-incubated with BKR antagonists

Figure 3.1.1.3 shows the effect of increasing concentrations of B1R and B2R antagonists on the proliferation of dMVECs challenged with 50% CM. Only a B2R antagonist at concentrations of 2.5 and 10 μM inhibited any effect of CM, although this was marginal and statistically insignificant. In fact, although to a very small degree, other concentrations marginally increased proliferation.

A



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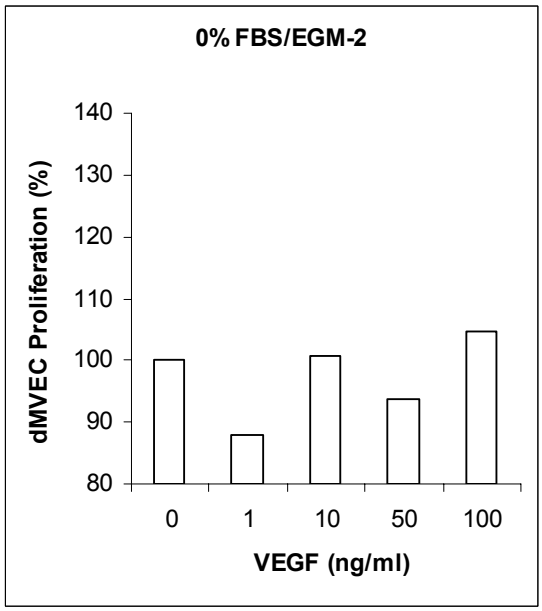

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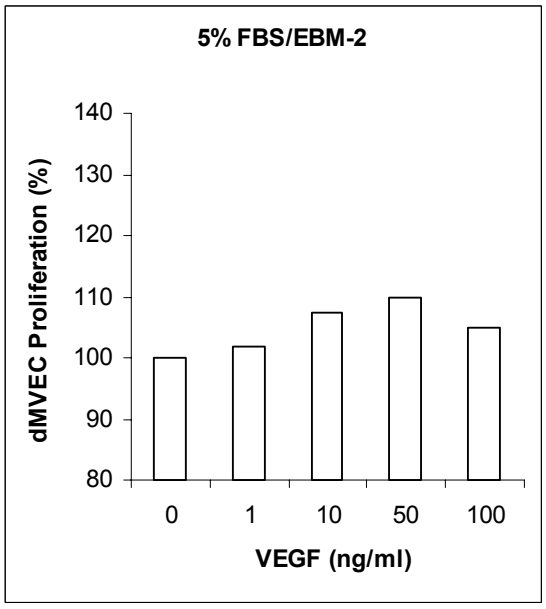

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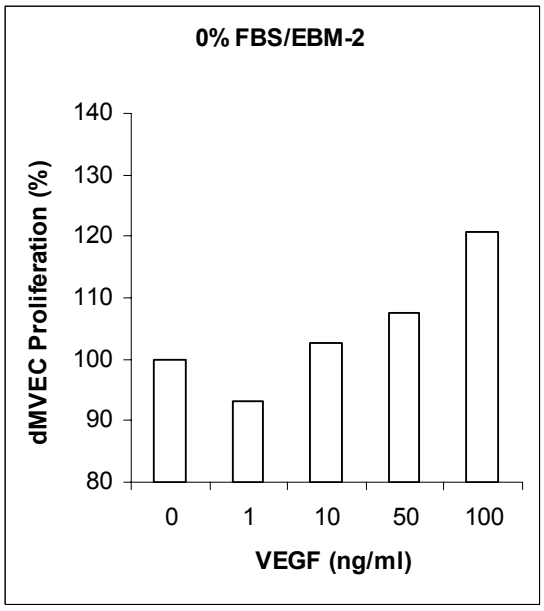


Figure 3.1.1.1: dMVEC proliferation in response to VEGF

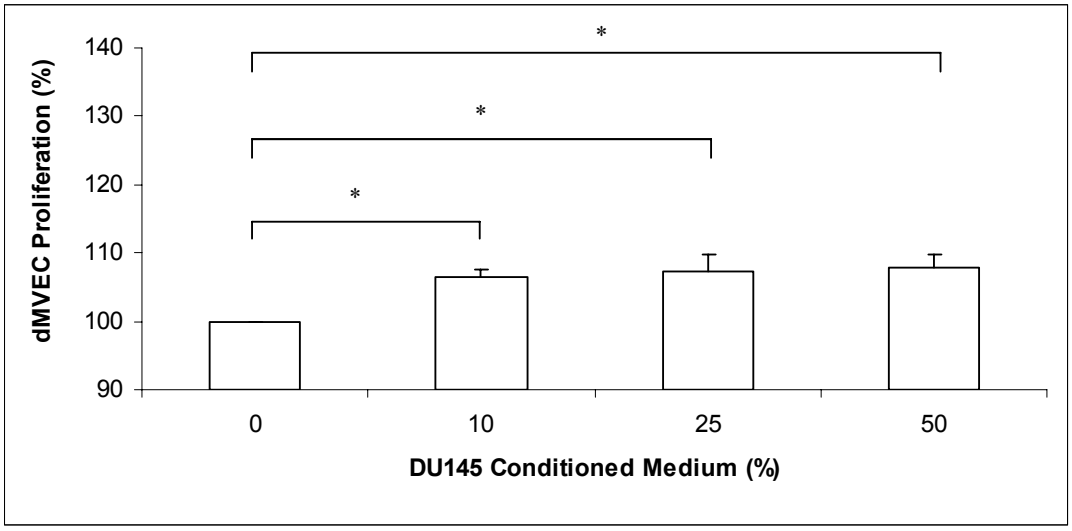


Figure 3.1.1.2: dMVEC proliferation in response to DU145 CM

* $p<0.05$; n=5 (4 replicates each)

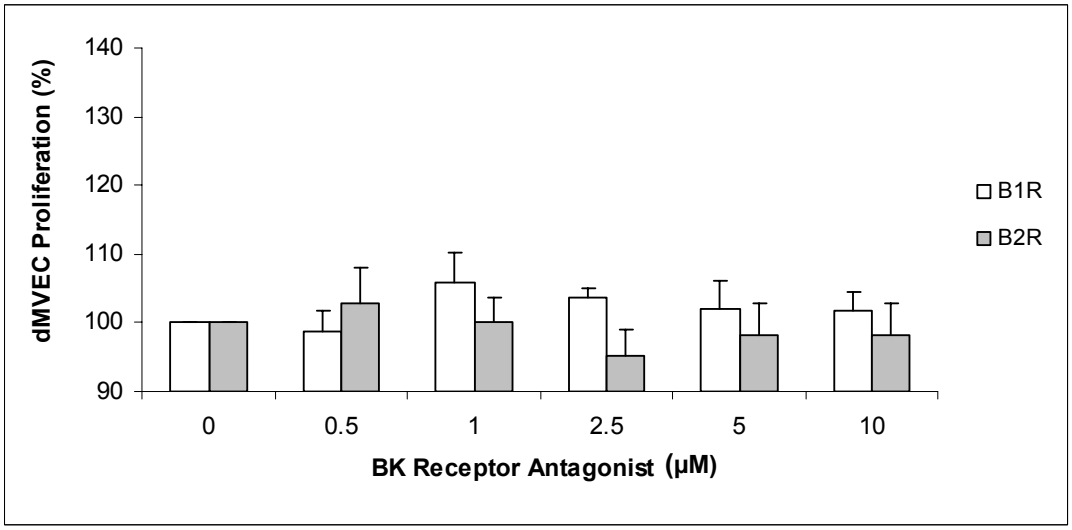


Figure 3.1.1.3: dMVEC proliferation following pre-incubation with BKR antagonists and challenge with 50% CM

Baseline control is dMVEC stimulated by 50% CM (regarded as 100% proliferation).

n=4 (4 replicates each)

3.1.1.4 Effect of BKR agonists

The B1R agonist stimulated endothelial proliferation in the concentration range 250 to 1000 nM but only by 5-6% (Figure 3.1.1.4). The B2R agonist did not stimulate endothelial cell proliferation at any concentration (Figure 3.1.1.4). In fact, neither the B1R nor the B2R agonist had any significant effect on endothelial cell proliferation.

3.1.2 dMVEC migration

3.1.2.1 Effect of DU145 CM with or without an antagonist

Addition of increasing concentrations of DU145 CM to the lower wells of the Boyden chamber did not stimulate dMVEC migration (Figure 3.1.2.1A). In fact, 100% CM significantly inhibited dMVEC migration ($p<0.05$).

Pre-incubation of dMVECs with 10 μM B1R antagonist had little influence on the effect of DU145 CM but it inhibited basal migration (in absence of CM) (Figure 3.1.2.1B).

3.1.2.2 Effect of a B1R agonist with or without an antagonist

Addition of a B1R agonist inhibited migration, while pre-treatment of dMVECs with 10 μM B1R antagonist before the addition of agonist caused further inhibition (Figure 3.1.2.2). When the agonist was added with CM there was statistically significant inhibition which was exaggerated even further by pre-incubation with 10 μM B1R antagonist ($p<0.05$) (Figure 3.1.2.2).

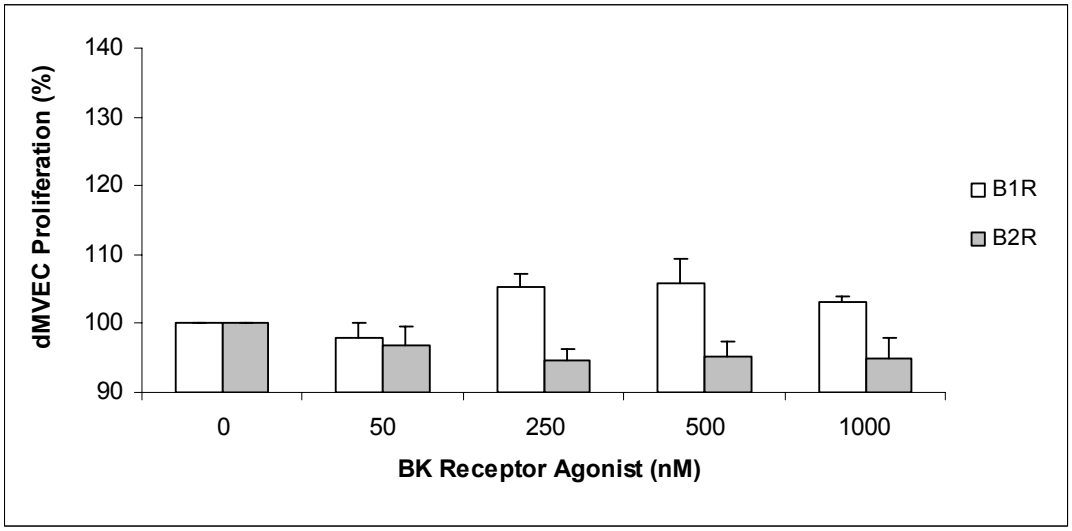


Figure 3.1.1.4: dMVEC proliferation in response to B1R and B2R agonists

n=4 (4 replicates each)

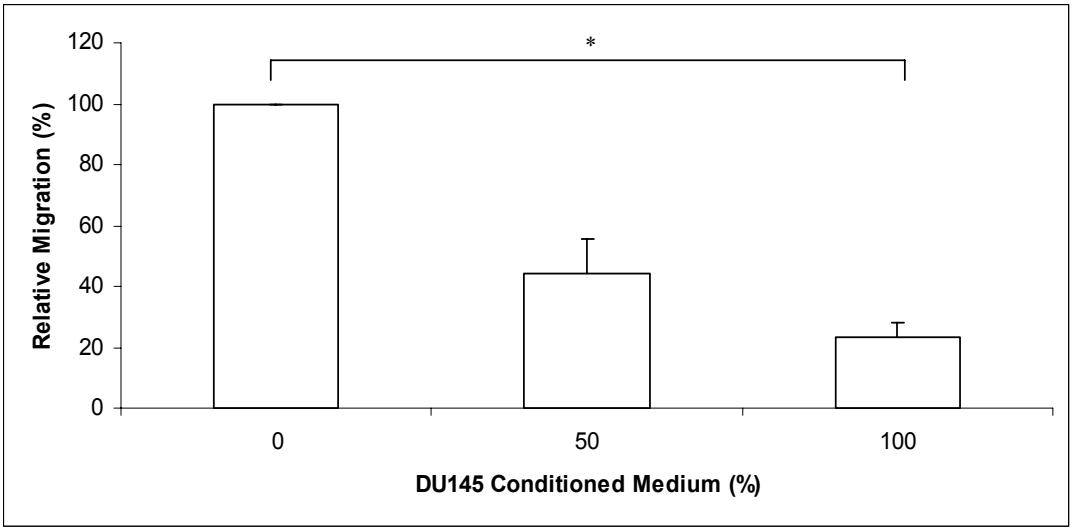


Figure 3.1.2.1A: dMVEC migration in response to DU145 CM

*p<0.05; n=3 (3 replicates each)

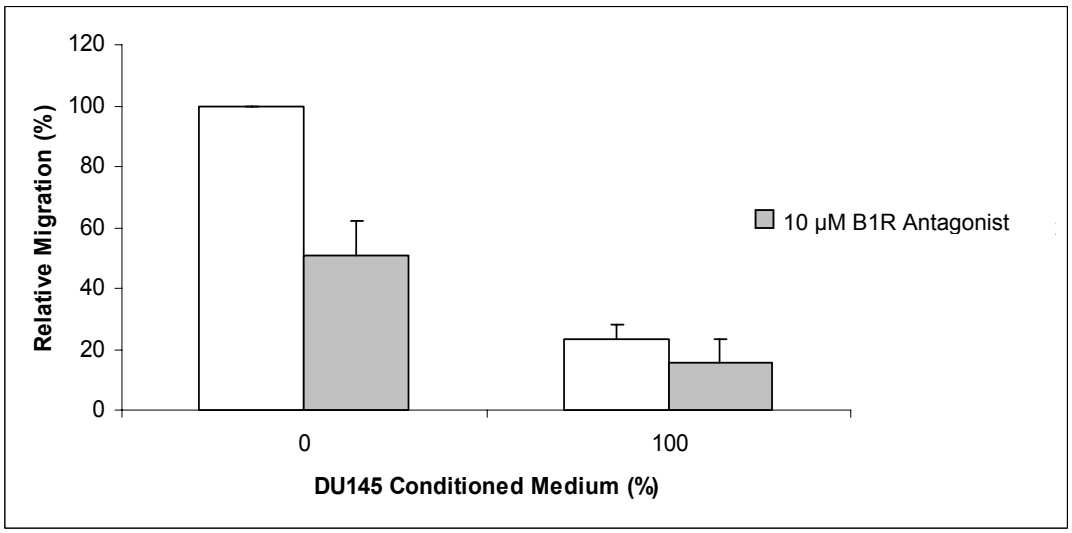


Figure 3.1.2.1B: dMVEC migration in response to DU145 CM and a B1R antagonist

n=3 (3 replicates each)

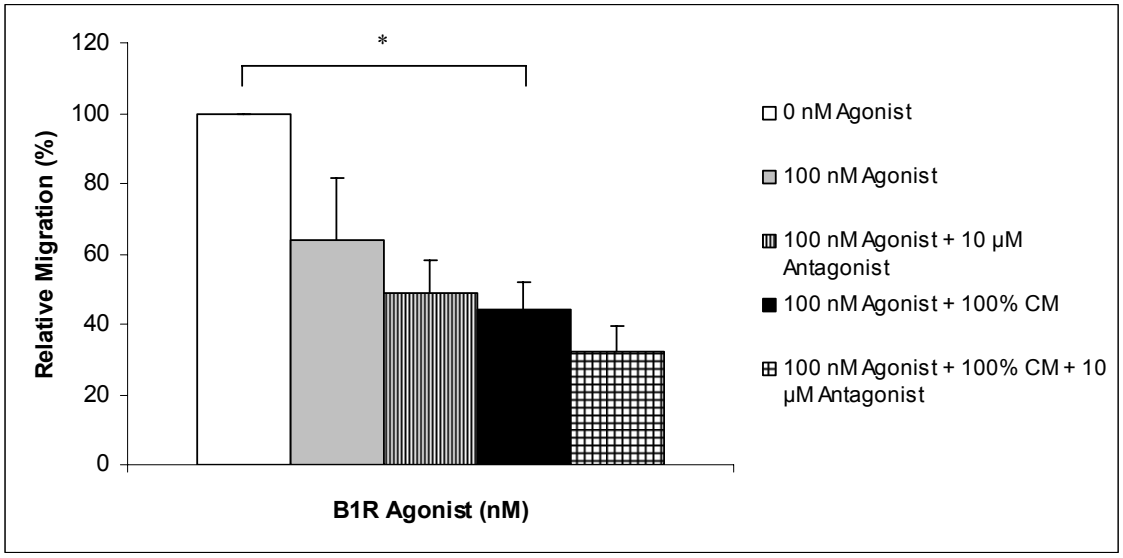


Figure 3.1.2.2: dMVEC migration in response to CM, a B1R agonist and antagonist

*p<0.05; n=3 (3 replicates each)

3.1.2.3 Effect of a B2R agonist

Addition of the B2R agonist to the lower wells of the modified Boyden chamber inhibited dMVEC migration (Figure 3.1.2.3). When 100% CM was also present statistically significant inhibition was observed although this was similar to migration in response to 100% CM ($p<0.05$).

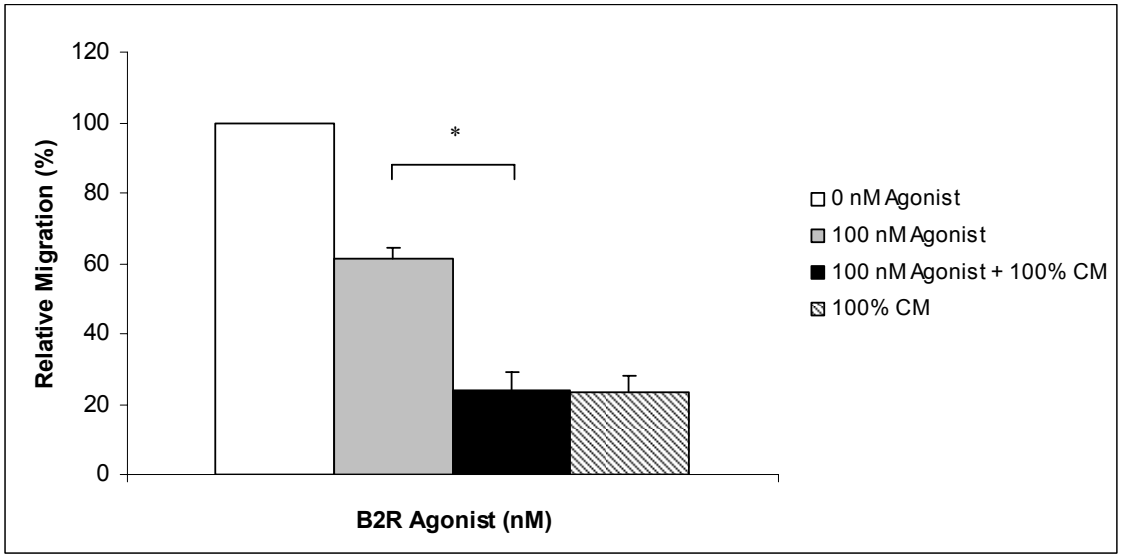


Figure 3.1.2.3: dMVEC migration in response to CM and a B2R agonist

*p<0.05; n=3 (3 replicates each)

3.2 Tumour cells (DU145)

3.2.1 DU145 cell proliferation

3.2.1.1 Effect of BKR agonists with or without an antagonist

All concentrations of the B1R agonist tested stimulated DU145 proliferation. This effect was greatest at a concentration of 10 nM where a statistically significant increase of 11.9% was observed ($p<0.05$) (Figure 3.2.1.1A). Pre-incubation with a specific B1R antagonist significantly inhibited proliferation by 13-18% for the B1R agonist concentration range 10-100 nM ($p<0.05$) (Figure 3.2.1.1A).

The B2R agonist did not have any marked effect on DU145 proliferation at any concentration (Figure 3.2.1.1B).

3.2.2 DU145 cell migration

3.2.2.1 Effect of DU145 CM with or without an antagonist

DU145 CM in the lower wells of a modified Boyden chamber did not prove to be a migratory stimulus for the DU145 cells (Figure 3.2.2.1). In fact, DU145 CM significantly inhibited migration at 50% CM ($p<0.05$). Pre-treatment of the cells with a B1R antagonist significantly inhibited cell migration at 0% CM but had no inhibitory effect when the concentration of CM was increased; however, at 100% CM the B1R antagonist stimulated migration ($p<0.05$) (Figure 3.2.2.1).

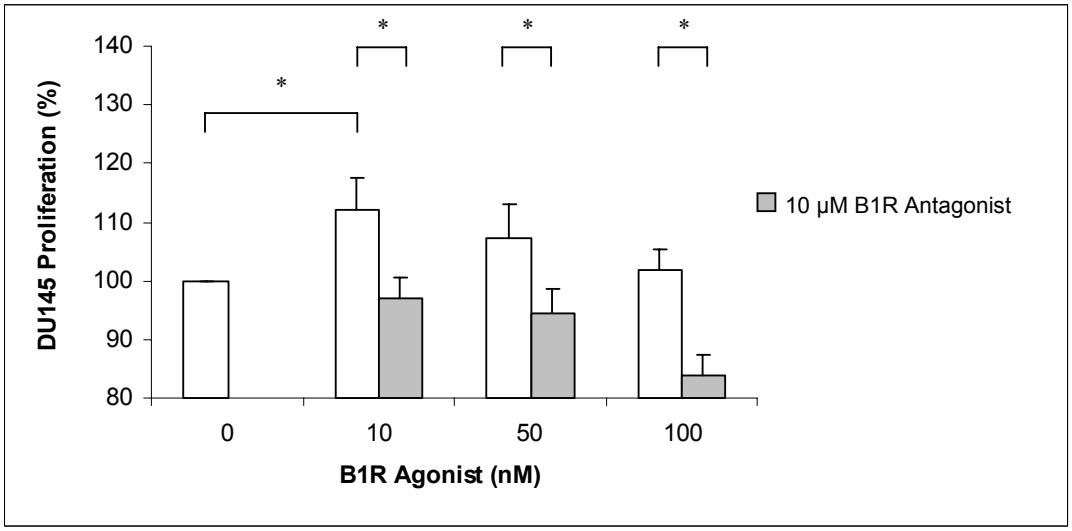


Figure 3.2.1.1A: DU145 proliferation in response to a B1R agonist and antagonist

* $p<0.05$; n=6 (4 replicates each)

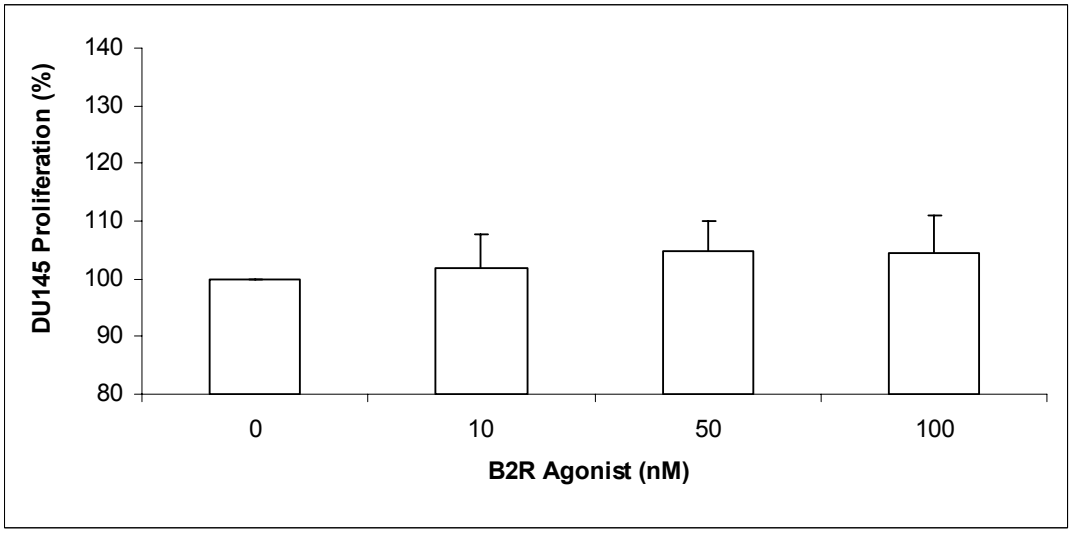


Figure 3.2.1.1B: DU145 proliferation in response to a B2R agonist

n=6 (4 replicates each)

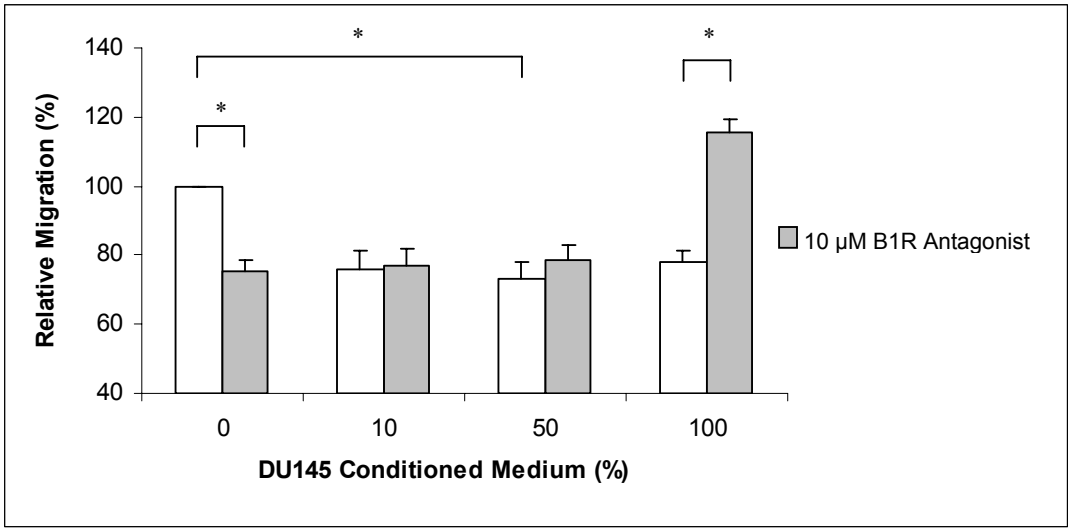


Figure 3.2.2.1: DU145 migration in response to DU145 CM and a B1R antagonist

* $p<0.05$; n=6 (3 replicates each)

3.2.2.2 Effect of BKR agonists with or without an antagonist

The B1R agonist stimulated cell migration in the concentration range 10-50 nM (Figure 3.2.2.2A). A statistically significant, maximum increase of 23.9% was observed at 50 nM ($p<0.05$). Pre-incubation of cells with a B1R antagonist (10 μM) abolished the stimulatory effect of the B1R agonist at concentrations of 10 and 50 nM. This inhibitory effect was found to be statistically significant ($p<0.05$) (Figure 3.2.2.2A).

All concentrations of B2R agonist stimulated cell migration although not significantly. A maximum increase of 16.6% was observed at a concentration of 100 nM (Figure 3.2.2.2B).

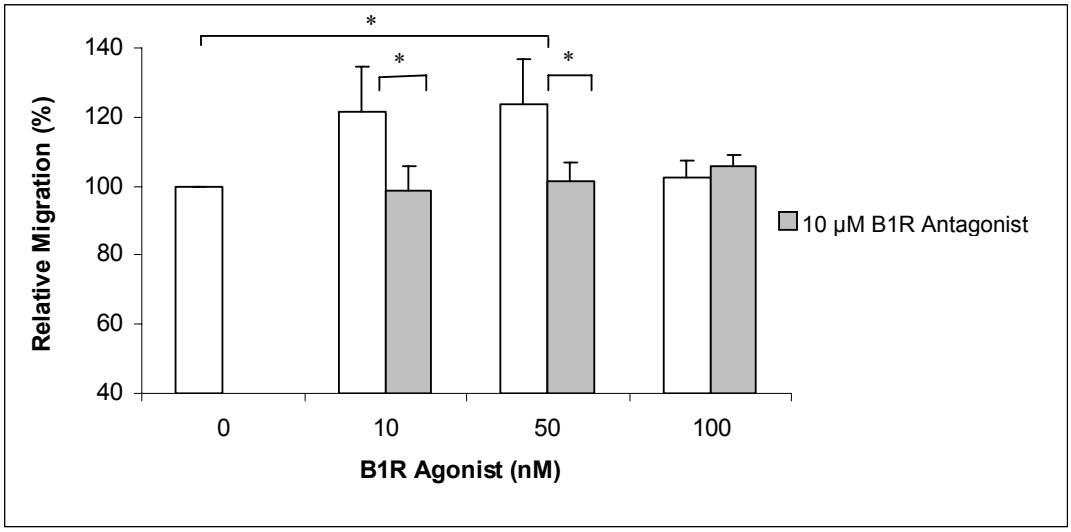


Figure 3.2.2.2A: DU145 migration in response to a B1R agonist and antagonist

* $p<0.05$; n=8 (3 replicates each)

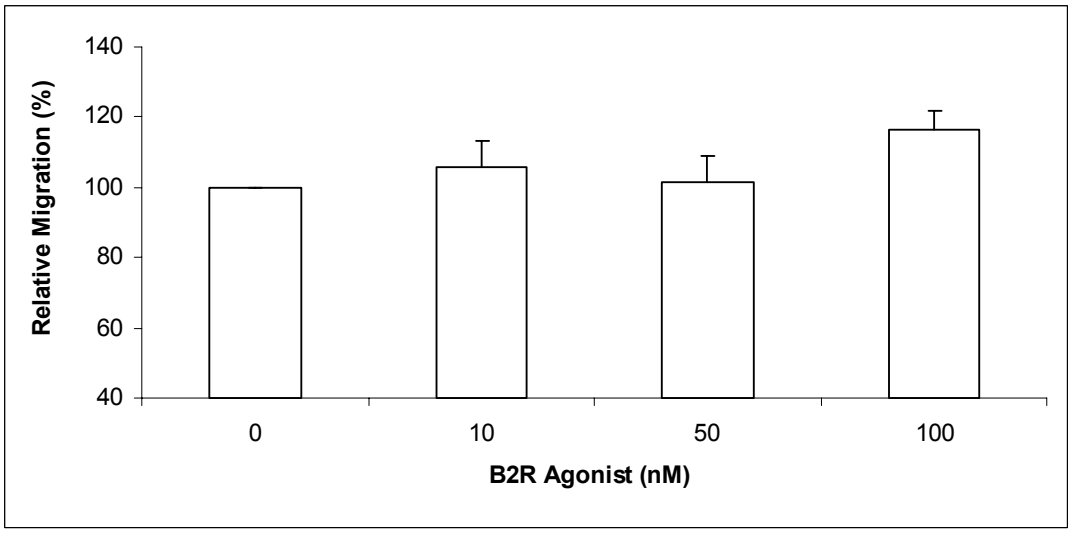


Figure 3.2.2.2B: DU145 migration in response to a B2R agonist

n=8 (3 replicates each)

CHAPTER 4

DISCUSSION

CHAPTER 4 - DISCUSSION

The KKS in endothelial cells

The principal aim of this study was to investigate the kinin system within a controlled angiogenic environment. Previous work on CM models in our laboratory found that increasing the concentrations of prostate tumour CM up to 50% significantly stimulated micro-vascular endothelial proliferation by as much as 40% (119). In addition, they showed increased TK production by dMVECs challenged with CM and speculated that the KKS may be involved in up-regulated endothelial cell activity in angiogenesis. That postulate provided the impetus for the present study.

In the present study, although the addition of DU145 CM elicited a statistically significant increase in dMVEC proliferation, it was very small (<10%). This result was not consistent with the findings of Wright *et al*. (2008). In addition, modification of the endothelial and tumour growth environment with the use of low- and serum-free medium (as suggested by previous studies) did not result in a more significant positive effect on dMVEC proliferation (see Appendix D1). This inability to reproduce the findings of Wright *et al*. (2008), despite experiments being performed under the same laboratory conditions as well as consequential attempts to modify experimental conditions, was surprising. The reason for this lack of reproducibility remains unclear; however, no other previous studies appear to confirm the work of Wright *et al*. (2008), and it would have been advantageous had the data set of that project been larger.

On careful examination of the literature, similar studies using the CM model to investigate the effects of soluble substances secreted by tumours have also produced conflicting data (119, 122, 151, 163, 164, 169, 170). For example, Hewett (2001) tested the CM from various

carcinomas on human mammary micro-vascular endothelial cells (HuMMEC), reporting that just one CM (breast carcinoma MDA-MB-231) from eight different tumour types caused significant endothelial proliferative activity (169). Hepburn *et al*. (1997) found that DU145 CM had no proliferative effect on the micro-vascular cell line, bovine adrenal cortex endothelial cells (BACE) (164). That group further tested five other prostatic tumour cell lines on BACE and found that only one (Ten 12) stimulated proliferation. The fact that those investigators have shown that the CM of only one prostatic cell line stimulated micro-vascular endothelial cell growth whilst CM from numerous other prostatic cell lines did not, is supportive of our findings.

While we initially selected micro-vascular cells (dMVECs) for use in proliferation assays, as they provide a more suitable *in vitro* model to mimic angiogenesis *in vivo*, they showed little angiogenic activity in the present study. Therefore, in an attempt to fully explore the pro-angiogenic effects of tumour CM, other endothelial and tumour cell lines were also investigated. In these experiments we used an alternate endothelial cell line (HUVEC) and found that (i) cervical carcinoma (HeLa) CM showed no positive proliferative effect, (ii) DU145 CM demonstrated marginal increases, and (iii) brain neuroblastoma (N2α) showed the most promising mitogenic profile. Similarly, other studies have found that neuroblastoma CM resulted in marked HUVEC proliferation compared to other tumour cell lines tested (122, 163). Several other studies have also examined the proliferative effect of tumour CM on HUVECs (118, 122, 151, 164, 169). It seems overall that micro-vessels are less responsive to tumour CM than large vessel endothelia (164, 169). Naidoo (2005) reported that CM from HeLa cells increased proliferation by 93%, Hepburn *et al*. (1997) demonstrated that of six prostatic cell lines tested, two showed no endothelial proliferative activity, while Hewett (2001) found that of the tumour CM extracted from four small cell

lung carcinomas (SCLC) and four breast adenocarcinomas, three SCLC and two breast adenocarcinoma cell lines did not significantly enhance endothelial proliferation (122, 164, 169).

Numerous *in vitro* studies have implicated the KKS in angiogenesis, Wright *et al*. (2008) and Naidoo (2005) postulated that TK secreted by both endothelial and tumour cells, cleaved kininogens to form active kinin peptides, subsequently mediating their pro-mitogenic effects via their cognate receptors (119, 122). The possible role of the KKS in angiogenesis is further substantiated by several *in vivo* reports involving mouse models (136, 139-141). One particular study demonstrated that TK gene delivery resulted in a heightened ischemia-induced angiogenic response (136). Further, angiogenic suppression was reported in that and other mouse models with the use of selective BKR antagonists (136, 140).

In the present study, the small proliferative effect of DU145 CM on dMVECs is not likely to be due to the KKS. Specific blocking of both the membrane-bound B1R and B2R with antagonists failed to alter the effects of the CM, suggesting this effect was not directly kinin-mediated. Our investigations also revealed that stimulation of either the B1R or B2R with agonists did not significantly induce endothelial cell proliferation. Further, selective BKR agonists as well as DU145 CM did not demonstrate any influence in endothelial cell migration, another important event in the angiogenic cascade. In fact, BKR agonists inhibited migration. This inhibitory effect was further exaggerated when combined with CM and/or antagonists suggesting possible interference of compounds with basal migration. Interestingly, although a B1R agonist failed to stimulate endothelial cell migration, a B1R antagonist was found to inhibit basal migration. It may be possible that a B1R antagonist inhibits FBS induced basal migration by some mechanism yet to be elucidated. DU145 CM was also found to inhibit endothelial cell migration. Since FBS directly stimulates migration,

this inhibitory effect is most likely due to the depletion of FBS in CM due to uptake by proliferating DU145 cells in culture. In support of this hypothesis, it was found that when CM was generated in serum-free medium and subsequently supplemented with FBS the inhibitory effect was not as marked (See Appendix B2).

Other studies have demonstrated different endothelial cell proliferation results in response to BKR agonists when compared to the present study result. Emanueli *et al.* (2002) and Morbidelli *et al*. (1998) reported increases in endothelial cell proliferation with the addition of a B1R agonist (110, 171). However, consistent with our findings, Morbidelli *et al*. (1998) also demonstrated that a B2R agonist did not cause any significant increase in proliferative activity (110). Similarly, that group showed that endothelial cell migration was not stimulated by selective BKR agonists or BK, a finding consistent with ours. Interestingly, those studies that have demonstrated increased proliferative activity with the use of B1R agonists, were based on the larger blood vessels HUVECs and coronary venular endothelial cells (CVEC), with increased exposure periods of 48 and 72 hours compared to the 24 hour period in the present study.

Kinin receptor mediation in angiogenesis has been proposed in numerous previous studies (110, 171, 172). It is known that the B2R is ubiquitously expressed on endothelial cells, while the B1R is pathologically induced (110, 173). Parenti *et al*. (2001) demonstrated that B1R activation is involved in endothelial cell proliferation, but B2R activation indirectly contributes to angiogenesis via mediation of inflammatory processes (172). Further, the commercially-obtained dMVECs used in the present study were isolated from a 42 year old female, and, since adult endothelium is maintained in a quiescent state, there is little or no angiogenesis in normal tissues (174). Interestingly, Naidoo (2005) was able to demonstrate

TK secretion from HUVECs although Wright *et al*. (2008) was unable to do so in a similar study involving dMVECs (119, 122). The Wright group postulated that while endothelial cells of micro-vascular origin are involved in angiogenesis *in vivo*, they secrete little TK physiologically. These various limitations and unclear implications may provide a plausible explanation for the lack of pro-angiogenic effects demonstrated by kinins in our model.

Tumour CM consists of a myriad of pro- and anti-angiogenic factors that remains to be fully elucidated (164). As a result, tumour neo-vascularisation may be a consequence of several angiogenic factors functioning synergistically as opposed to a single, isolated factor (175). In the present study we were able to demonstrate the proliferative potential of VEGF, a universally accepted mitogen, on dMVECs thereby verifying their physiological integrity. Studies have shown that tumour cell cultures comprise elevated concentrations of VEGF, IL-8 and bFGF, pro-angiogenic factors with potent mitogenic potential (48, 55, 163, 164, 175). Hepburn *et al*. (1997) reported the expression of bFGF and VEGF in the CM of numerous prostatic cell lines including DU145, while Ferrer *et al*. (1998) found significant levels of VEGF and IL-8 immuno-staining in prostate cancer tissue compared to normal prostate tissues (164, 175). Further, Hepburn (1997) found that the level of bFGF in the CM of the only tumour cell line (Ten 12) that stimulated micro-vascular growth was at least 3-fold greater when compared with other prostatic cell lines tested and postulated that the up-regulation of this growth factor was responsible for the resultant endothelial stimulation (164). In addition, other angiogenic factors such as TGF-α and TGF-β are produced by prostate cell lines (48, 164). mRNA expression of TGF-α and TGF-β has been reported in several prostatic cell lines (176, 177). Previous studies have reported stimulatory effects on endothelial cells with TGF-α while TGF-β inhibited endothelial cell proliferation *in vitro* (164, 178). Interestingly, bFGF can alter the inhibitory effect of TGF-β on endothelial cells,

functioning synergistically to stimulate invasion (179). Therefore, it appears that our results and other studies concur with the generally-accepted principal that initiation of angiogenic events requires regulation of various systems that work in concert to induce endothelial growth.

In contrast to some previous studies, the present study was unable to implicate the KKS in angiogenesis, and we speculate that the ideal pathological *in vivo* conditions may not have been adequately reconstructed in the *in vitro* tumour CM model and, therefore, the participation of the KKS in angiogenesis could not be entirely defined. Thus, it appears that the marginal mitogenic potential of DU145 CM demonstrated in the present study probably relates to the balance of pro- and anti-angiogenic factors in DU145 CM as well as to endogenous production by endothelial cells and to the synergism of such factors.

KKS in tumour cells

Up-regulated expression of kallikreins and kinins in various cancers, including prostate cancer, have implicated the KKS in tumourigenesis (109, 127). We used DU145 cells as a model of hormone-insensitive prostate cancer in the present study, and postulated that BK may be involved in tumour cell growth and migration. This project is the first to show, in DU145 cells, that a specific B1R agonist could significantly increase proliferation. In order to prove the effect to be B1R-regulated we used a B1R antagonist which effectively abbrogated this pro-angiogenic effect. This result is consistent with other prostate cancer reports (11, 109, 132). Although expression of BKR has been demonstrated in both normal and malignant tissues, numerous studies have shown that kinin expression is specifically up-regulated in pathological states (11, 101, 124, 180). Further, Taub *et al*. (2003) found that pre-malignant and malignant prostate tissues exhibit an altered kinin expression profile

compared to their normal counterparts (11). Various researchers have shown that the addition of BK and specific BKR agonists enhances growth of PC3 prostate cancer cells. This effect could then be abrogated following pre-incubation of cells with a specific B1R or B2R antagonist (11, 109, 132). Based on similar results, some researchers have suggested that specific inhibition of the B1R may be of significant value to advanced prostate disease (11, 109).

In contrast to the effect of B1R agonism, we found that the addition of a specific B2R agonist, at any concentration, resulted in no significant stimulation of DU145 cells. This finding is inconsistent with another study where it was found that specific stimulation of the B2R enhanced the growth of prostate cancer PC3 cells (132). However, our finding was not an unexpected outcome; indeed, Taub *et al*. (2003) also reported that while the B2R is ubiquitously expressed in both human benign and malignant prostatic tissue, the B1R is found only in pre-malignant and malignant prostate specimens (11).

In the present study, we did note some variation in the extent to which a B1R agonist stimulated tumour cells when compared to previous work (11, 132). The effect of BKR agonists on tumour cells *in vitro* are likely to be dependent on a number of factors; for example, the tumour type and conditions of experimental models. It is possible that such variation could also be attributed to the different prostatic cell lines. DU145 and PC3 cell lines are both classical epithelial cell lines of prostate cancer; however, they differ in their DNA profiles and cytogenetic analysis (158). In addition, the exposure period in other experiments were notably longer (48 hours compared to 24 hours in the present study). In a similar way, Barki-Harrington and Daaka (2001), Barki-Harrington *et al*. (2003) and Taub *et al*. (2003) demonstrated that BK or BKR agonists were able to stimulate proliferation of PC3

cells; however, this could not be reproduced by Srinivasan *et al*. (2004) where neither PC3 cells nor LNCaP cells (2 metastatic prostatic carcinomas) could be stimulated by the addition of BK (11, 109, 132, 181). Srinivasan and colleagues attributed these discrepancies to differences in passage numbers of cells between the studies, reporting that BKR expression may be dependent on passage number and culture conditions. Interestingly, Molina *et al*. (2009) found that proliferation measured by bromodeoxyuridine (BrdU) incorporation, increased 2-fold when MCF-7 breast cancer cells were stimulated with BK, while the addition of a B1R agonist induced a proliferative response by almost 4-fold compared to the untreated control (127). This leads one to speculate that kinins may be involved in both androgen-independent (DU145) and oestrogen-sensitive (MCF-7) carcinomas. Because kinins may potentiate the effects of other growth factors (bFGF and EGF) implicated in the development of hormone-independence, and up-regulated TK and kinins have been shown in several human cancers, the KKS is likely to play a role in the transformation of hormone-dependent to hormone-independent tumour subtypes (110, 119, 182). Wright *et al*. (2008) postulated that increased TK in the tumour micro-environment would support the mutation into a hormone-independent form (119).

Tumour cell migration is fundamental to advanced cancer progression and metastasis (11). In addition to expressing receptors for BK, tumour cells also promote the production of these receptor ligands, and BK has been identified as an autocrine growth factor in tumours (126). In the present study, DU145 CM did not stimulate DU145 migration. In fact, it inhibited DU145 migration, an effect that is once again likely due to FBS depletion in the CM. Interestingly, the B1R antagonist inhibited basal DU145 migration but stimulated migration when pre-incubated with cells exposed to 100% CM. It may be that the B1R antagonist inhibits FBS induced migration, whilst when FBS concentration is at its lowest (100% CM)

the antagonist stimulates migration by binding to some other pre-migratory receptor. Alternatively, perhaps the antagonist has the capacity to stimulate mitogenic pathways in a similar manner compared to receptor agonists. Interestingly, a study performed by Drube *et al*. (2000) found that in some lung, colon and breast tumour cell lines a B2R antagonist acts as an agonist stimulating DNA synthesis (183). These researchers suggested that the B2R antagonist may trigger the same signalling pathways as BK.

Previous studies, including some in our laboratory, have shown increased expression of TK in prostate cancer as well as the presence of kinin peptides in ascitic tumour fluid (119, 124). Since DU145 CM did not stimulate DU145 migration and the level of TK was found to be undetectable in the CM of DU145 cells by ELISA (see Appendix F), the present study was unable to positively link CM, the KKS and migration. However, we were able to demonstrate that the addition of a B1R agonist induced a positive chemotactic response, significantly stimulating DU145 cell migration. Subsequent use of a B1R antagonist inhibited migration, further illustrating kinin receptor mediation. These findings are consistent with previous work performed by Taub *et al*. (2003) where stimulation of the B1R was found to promote PC3 migration in the wound healing assay (11). Pre-treatment with an antagonist abrogated that effect. That study observed a 6-fold increase in tumour cell migration when stimulated with a B1R agonist whereas the present study noted an approximate increase of just 20%. This variation may be largely attributed to differences in assays used to investigate migration as well as the cell lines that were tested. The PC3 cell line is a highly metastatic prostatic carcinoma compared with DU145 which exhibits moderate metastatic potential (158). Taub *et al*. (2003) also demonstrated the ability of kinins to induce prostate cancer PC3 invasion, with the use of a modified Boyden chamber, and showed specific stimulation of the B1R enhanced invasion by 3-4-fold (11). In addition,

other components of the KKS have been shown to be involved in metastasis; Wolf *et al*. (2001) implicated TK in the metastasis of a breast carcinoma and found that this process could be significantly attenuated with the use of a TK inhibitor (121). Naidoo (2005) also postulated that TK, released by a tumour mass, contributes to ECM remodelling thereby allowing expansion of the tumour. Thus, the KKS may contribute to tumour invasion via TK activated MMPs as well as kinin receptor stimulation.

Summary and conclusions

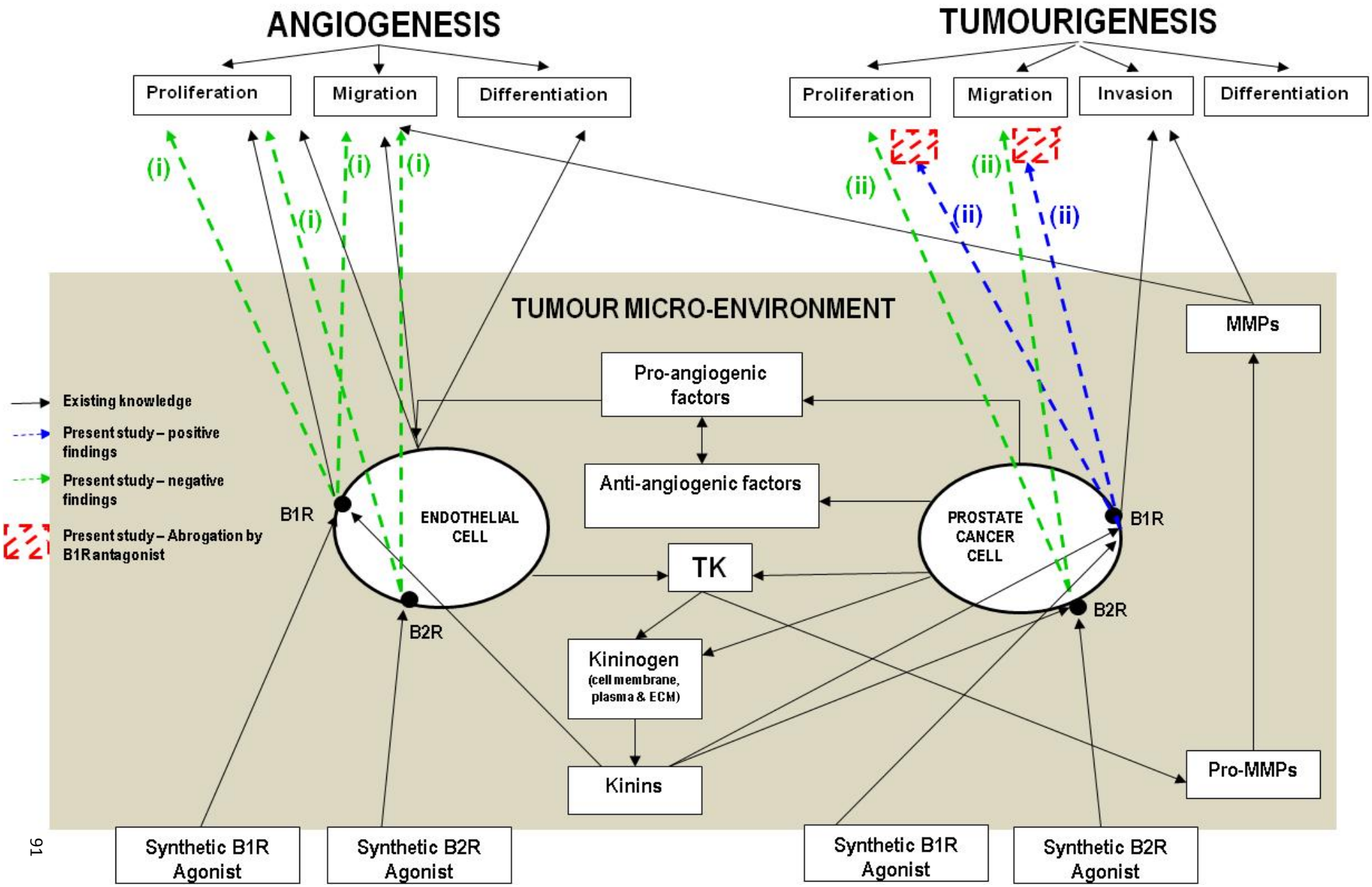
In summary, it appears that CM from some prostate cancer cell lines stimulates micro-vascular endothelial cell proliferation whilst that from other prostatic cell lines does not. In the present study the extent to which DU145 CM stimulated dMVEC proliferation was small. Although our results did not support a role for the KKS in dMVEC proliferation and migration and hence angiogenesis, previous studies suggested that the KKS has a multifunctional role in angiogenesis and tumourigenesis.

The present study was the first to show the involvement of the KKS in DU145 tumourigenesis; we demonstrated that specific stimulation of the B1R increased tumour cell proliferation and migration, while pre-incubation with the relevant BKR antagonist abrogated this effect (Figure 4.1). Therefore our results do support previous studies and suggest a possible role of BK in prostate pathology. There is, therefore, compelling evidence for the involvement of the KKS in cancer and prostate cancer. This may be more so in PC3 than DU145 tumours. PC3 tumours are more aggressive and metastatic and it appears, therefore, that the effect of BK on growth and migration may be even greater. However, even in hormone-independent cancers such as DU145, because few effective treatments are available, BKR antagonists should be considered plausible candidates in the development of alternate approaches to cancer therapy.

Figure 4.1: Proposed role of the KKS in prostate angiogenesis and tumourigenesis

According to the prevailing concepts of angiogenesis and tumourigenesis (solid black lines) prostate tumour cells secrete both pro-and anti-angiogenic factors required for angiogenic stimulation. In addition, TK, secreted by both endothelial and tumour cells, contributes to the activation of MMPs involved in ECM degradation, and cleavage of kininogens (LMWK and HMWK) to generate active kinin peptides. Kinins mediate their mitogenic and invasive properties via their cognate receptors present on endothelial and tumour cells. Tumour cells may also facilitate their own growth via endogenous kinin production.

Results (broken blue, green and red lines) demonstrate that (i) BKR agonists did not appear to induce proliferation or migration in endothelial cells and (ii) specific stimulation of the of the B1R, but not the B2R, increased tumour cell proliferation and migration, while antagonism of the relevant BKR abrogated this effect.


ANGIOGENESIS
Proliferation
Migration
Differentiation
TUMOURIGENESIS
Proliferation
Migration
Invasion
Differentiation
(i)
(ii)
TUMOUR MICRO-ENVIRONMENT
MMPs
Pro-angiogenic factors
Anti-angiogenic factors
Existing knowledge
Present study – positive findings
Present study – negative findings
Present study – Abrogation by B1R antagonist
B1R
ENDOTHELIAL CELL
PROSTATE CANCER CELL
B1R
TK
B2R
B2R
Kininogen
(cell membrane, plasma & ECM)
Kinins
Pro-MMPs
Synthetic B1R Agonist
Synthetic B2R Agonist
Synthetic B1R Agonist
Synthetic B2R Agonist

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APPENDIX

APPENDIX A

A1. TK ELISA reagents

1. Coating Buffer - 0.015 M Na_2CO_3 (AR, Merck) and 0.04 M $NaHCO_3$ (BDH Chemicals, England) dissolved in distilled, deionised water (ddH_2O) and adjusted to pH 9.6
2. PBS - PBS tablets (Sigma) dissolved in ddH_2O to 0.01 M
3. Wash Buffer - Tween 20 (Sigma) dissolved in 0.01 M PBS to PBS/0.05% Tween 20
4. Milk Blocker - 5% fat free milk powder (Elite, SA) dissolved in 0.01 M PBS
5. Substrate Buffer - 5 mM magnesium chloride ($MgCl_2$,AR, Merck) and 10% di-ethanolamine (Sigma) dissolved in ddH_2O and adjusted to pH 9.6
6. pNPP - pNPP tablets (Sigma) dissolved in substrate buffer to 1 mg/ml
7. Goat anti-human TK IgG - diluted to 30 ng/ml in coating buffer
8. Rabbit anti-human TK IgG - diluted to 25 ng/ml in milk blocker
9. Anti-rabbit IgG alkaline phosphatase (Sigma): diluted 1:5000 in milk blocker

Goat anti-human TK IgG and Rabbit anti-human TK IgG antibodies were previously raised by researchers in the Department of Therapeutics and Medicines Management, UKZN (122).

APPENDIX B

B1. dMVEC proliferation assay control

Effect of serum

When the effect of cell dependency on serum as a control was tested, it was shown that when serum is removed from the growth medium, there was a significant reduction in proliferation by 42.2% ($p<0.05$) (Figure B1).

B2. dMVEC migration assay controls

Effect of serum and other media

The effect of cell dependency on serum as a control was tested and it was demonstrated that when serum is completely removed from the stimulatory medium, migration was significantly inhibited by 82.1% ($p<0.05$) (Figure B2a). Complete EGM-2, that which contains 5% FBS supplemented with growth factors, proved to be the optimal medium for migration, resulting in a 2.5-fold increase compared to 10% FBS/DMEM.

Effect of VEGF

All concentrations of VEGF tested stimulated migration of dMVECs. This effect was greatest (58%) at 10 ng/ml (Figure B2b).

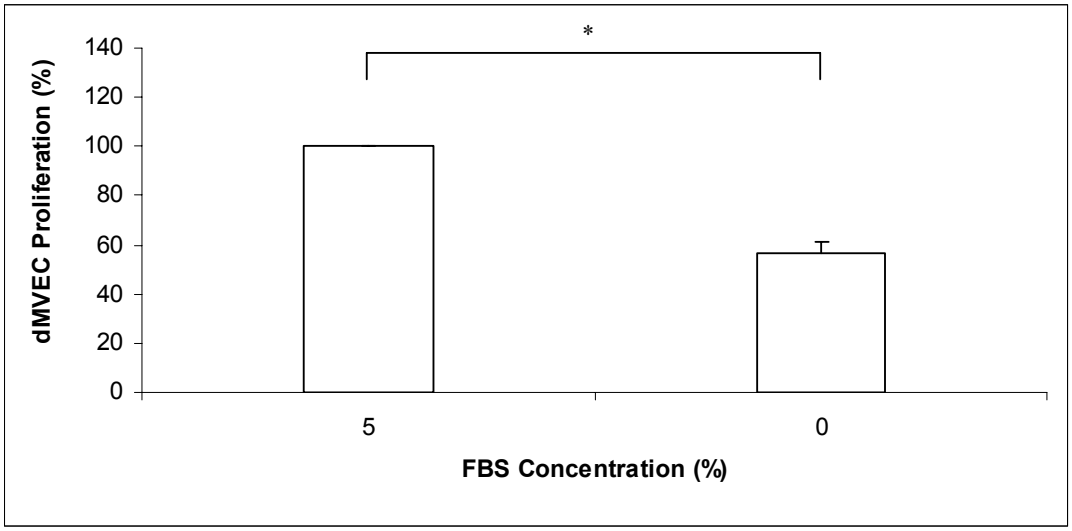

Figure B1: dMVEC proliferation in response to serum

*$p<0.05$; n=4 (4 replicates each)

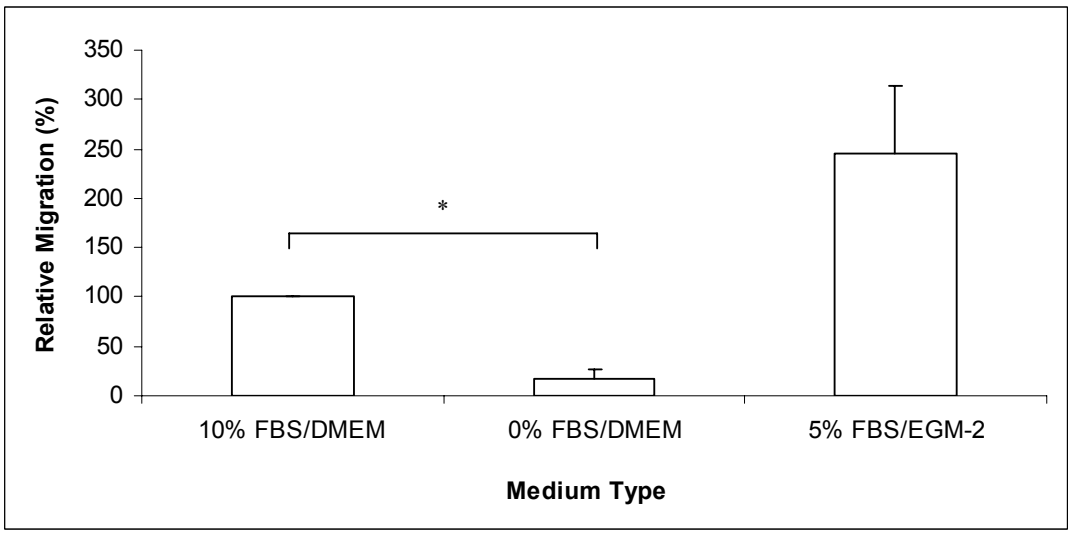

Figure B2a: dMVEC migration in response to serum and other media

*$p<0.05$; n=3 (3 replicates each)

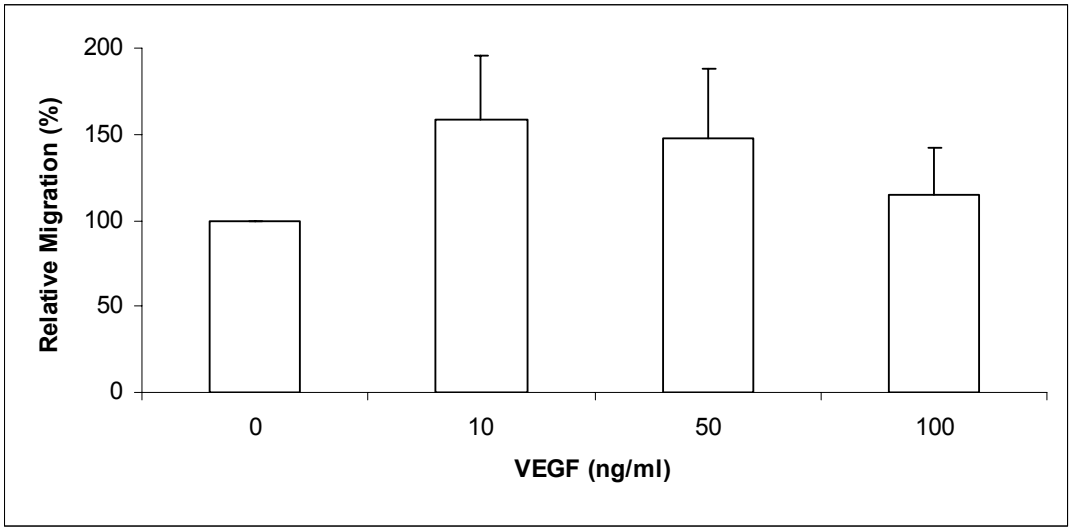

Figure B2b: dMVEC migration in response to VEGF

n=3 (3 replicates each)

Effect of serum-free CM post-supplemented with FBS

DU145 CM was generated in serum-free DMEM over 24 hours to prevent FBS being depleted by the proliferating tumour cells. Prior to addition to the bottom wells of the modified Boyden chamber, CM was extracted and supplemented with 10% FBS. DU145 CM did not stimulate endothelial cell migration (Figure B2c), however the inhibitory effect was not as marked compared to when CM was generated in 10% FBS/DMEM (Figure 3.1.2.2A).

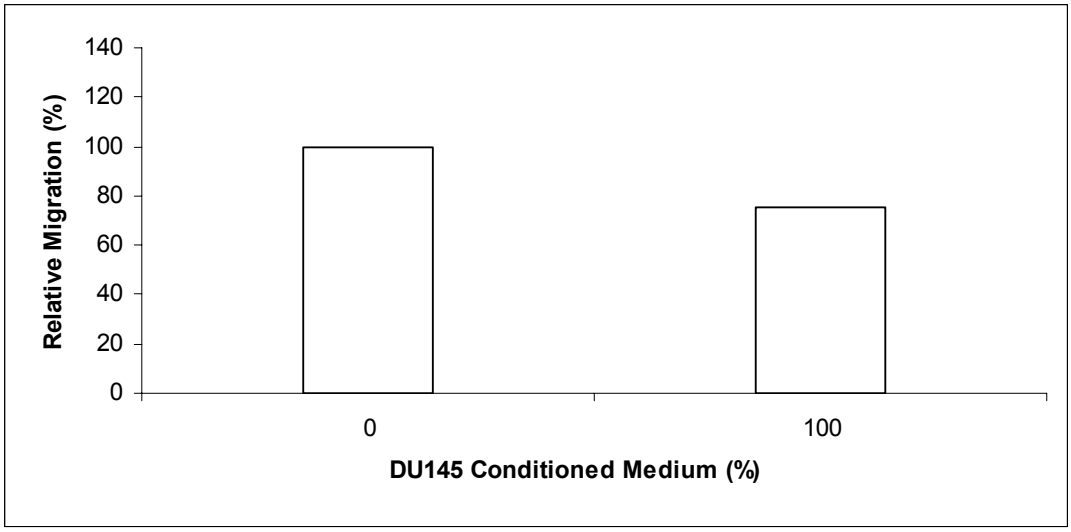

Figure B2c: dMVEC migration in response to serum-free CM post-supplemented with FBS

n=1 (3 replicates each)

APPENDIX C

C1. DU145 cell proliferation assay control

Effect of VEGF

The mitogenic effect of VEGF on DU145 was tested at 10, 50 and100 ng/ml (Figure C1). At concentrations of 10 and 100 ng/ml no stimulation was observed. At 50 ng/ml an increase of 1.2% was noted.

C2. DU145 cell migration assay control

Effect of serum and VEGF

In order to control for serum as an endogenous stimulator, the effect of cell dependency on serum was tested (Figure C2). It was shown that when serum is removed from the stimulatory medium, there was a significant reduction in relative migration by up to 47.5%. Spiking the system with 100 ng VEGF enhanced the positive migratory effect of FBS by 17.9%.

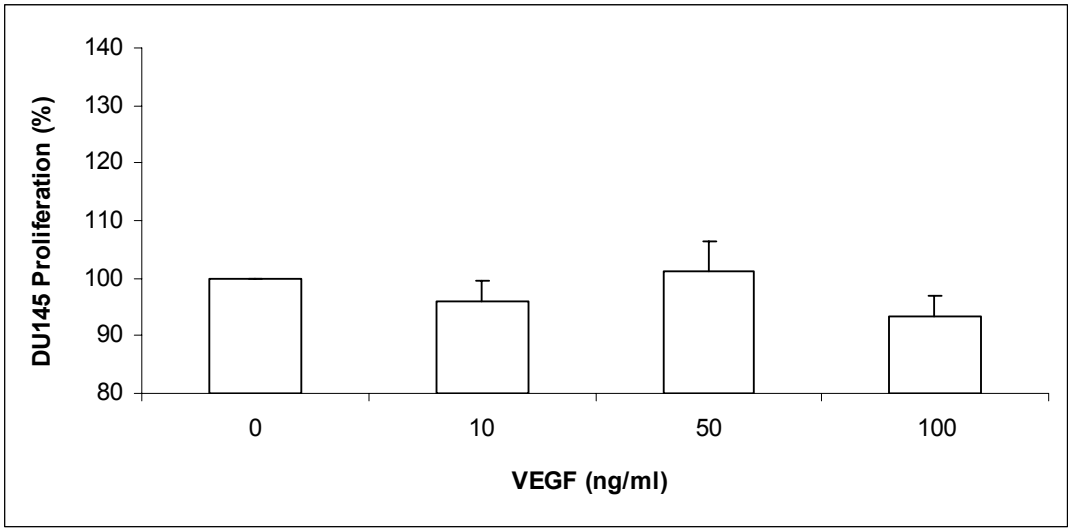

Figure C1: DU145 proliferation in response to VEGF

n=6 (4 replicates each)

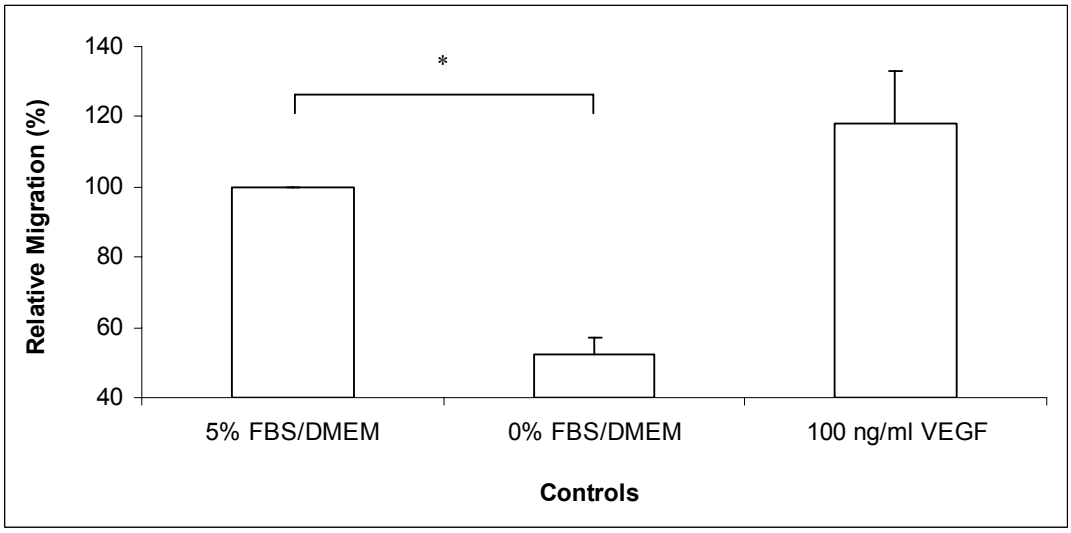

Figure C2: DU145 migration in response to serum and VEGF

*p<0.05; n=8 (3 replicates each)

APPENDIX D

D1. Modification of conditions – Effect of DU145 CM on dMVEC proliferation

Effect of EGM-2

Under normal conditions, CM was generated in tumour cell-specific medium (DMEM) over a defined period of time (24 hours). DMEM was replaced with a common medium EGM-2 that was sustainable for both the metabolite-inducing cell cultures and the fastidious endothelial cells. We applied the rationale that an increased generation time of CM (24 hours compared to 48 hours) would yield a more concentrated, pro-angiogenic blend. Modifying these conditions did not, however, result in a positive trend in dMVEC proliferation (Figure D1a).

Effect of dMVEC serum-starvation

Since no effect on dMVEC proliferation was observed with EGM-2, the protocol was amended such that the dMVECS were serum-starved in 0% FBS/EGM-2 as suggested by a previous study (162). There was, however, still no significant effect of DU145 CM (generated over 24, 48 or 72 hours) on dMVEC proliferation (Figure D1b).

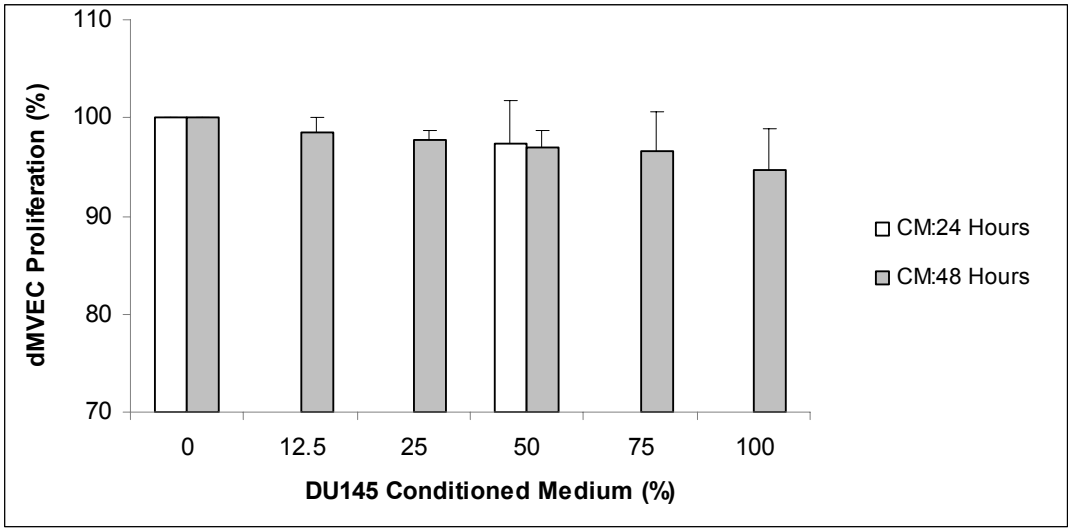

Figure D1a: dMVEC proliferation in response to DU145 CM generated in EGM-2

n=3 (4 replicates each) except for CM generated over 48 hours where n=2 (4 replicates each) at concentrations of 12.5% and 75%.

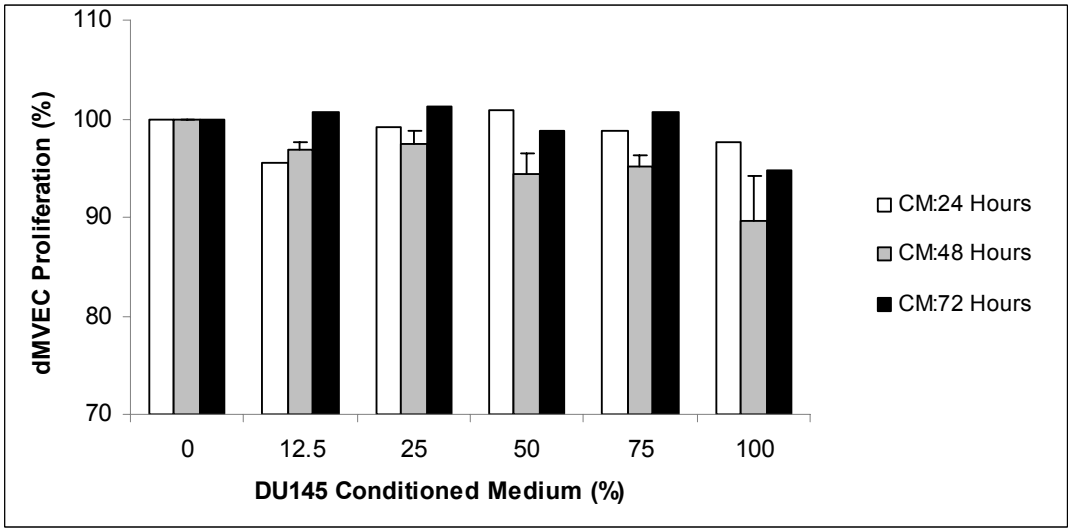

Figure D1b: dMVEC proliferation following serum-starvation of endothelial cells

n=1 (4 replicates each) except for CM generated over 48 hours where n=2 (4 replicates each).

Effect of dMVEC and DU145 serum-starvation

As there was still no significant effect on dMVEC proliferation when the dMVECs were serum-starved, we then serum-starved DU145 cells as suggested by previous studies (163, 164, 169). CM was, therefore, generated in serum-free medium over increasing time periods. No significant effect of DU145 CM (generated over 24, 48 or 72 hours) on dMVEC proliferation was observed (Figure D1c).

Effect of low-serum

Since serum-starvation had no effect on dMVEC proliferation, low-serum conditions (0.5% FBS/EGM-2) were applied to endothelial and tumour cells, as suggested by a previous study (151). No proliferative effect of CM was demonstrated. Cellular inhibition was, however, observed between 75% and 100% CM (Figure D1d).

D2. Use of alternate tumour cell lines as controls

Effect of HeLa CM

Other tumour CM were tested for their pro-angiogenic properties on micro-vascular endothelial cells. The addition of increasing concentrations of Hela CM, generated in EGM-2, had no effect on dMVEC proliferation (Figure D2a).

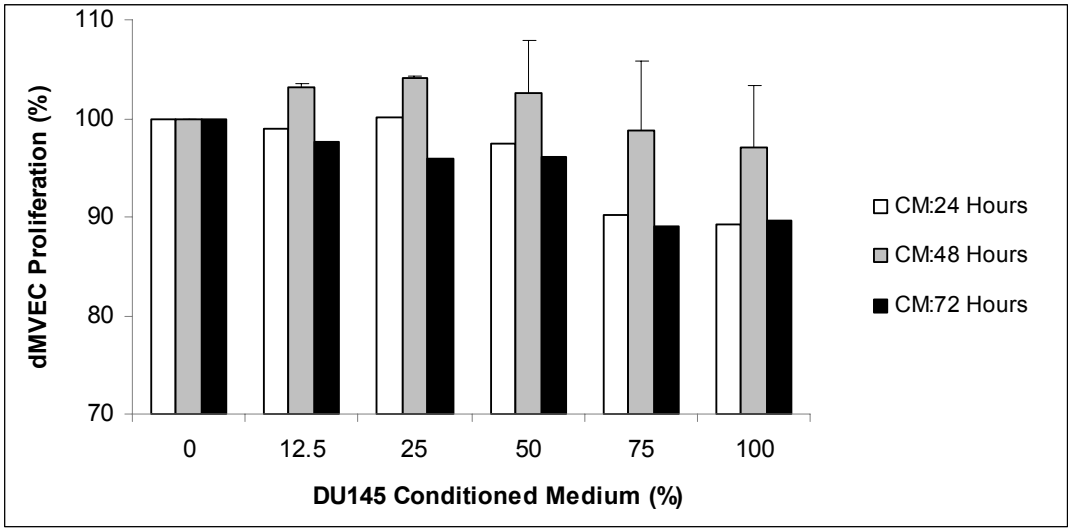

Figure D1c: dMVEC proliferation following serum-starvation of endothelial and tumour cells

n=1 (4 replicates each) except for CM generated over 48 hours where n=2 (4 replicates each).

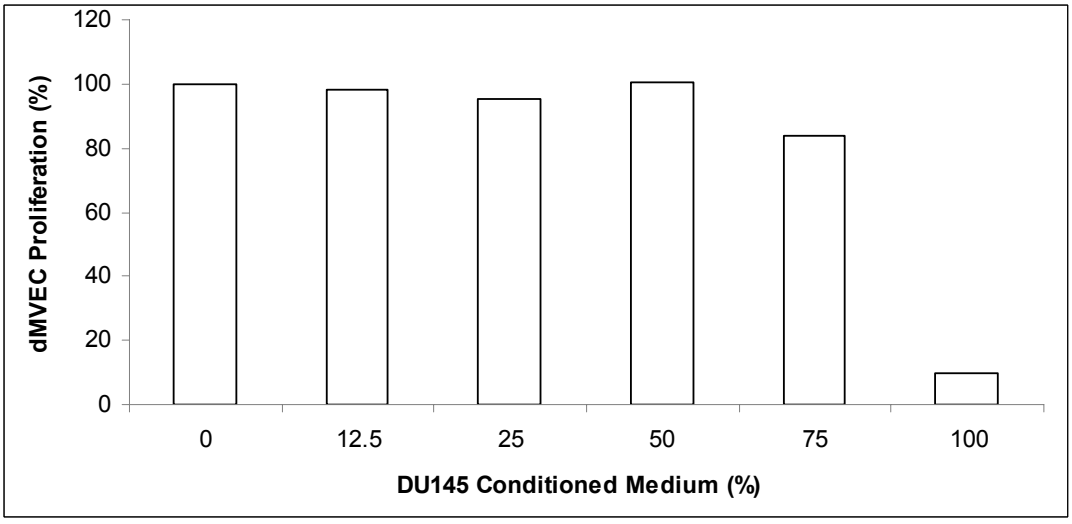

Figure D1d: dMVEC proliferation following growth of endothelial and tumour cells in 0.5% FBS/EGM-2

n=1 (4 replicates each)

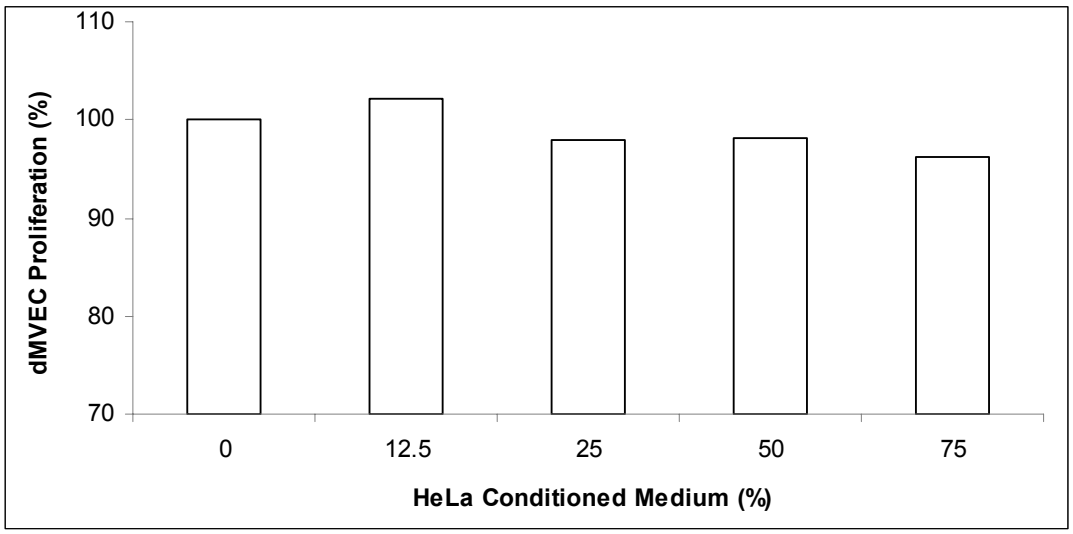

Figure D2a: dMVEC proliferation in response to HeLa CM

n=1 (4 replicates each).

Effect of N2α CM

An additional control, a neuroblastoma cell line (N2α) was tested for its effect on dMVEC proliferation. Increasing concentrations of N2α CM had no effect on dMVEC proliferation (Figure D2b).

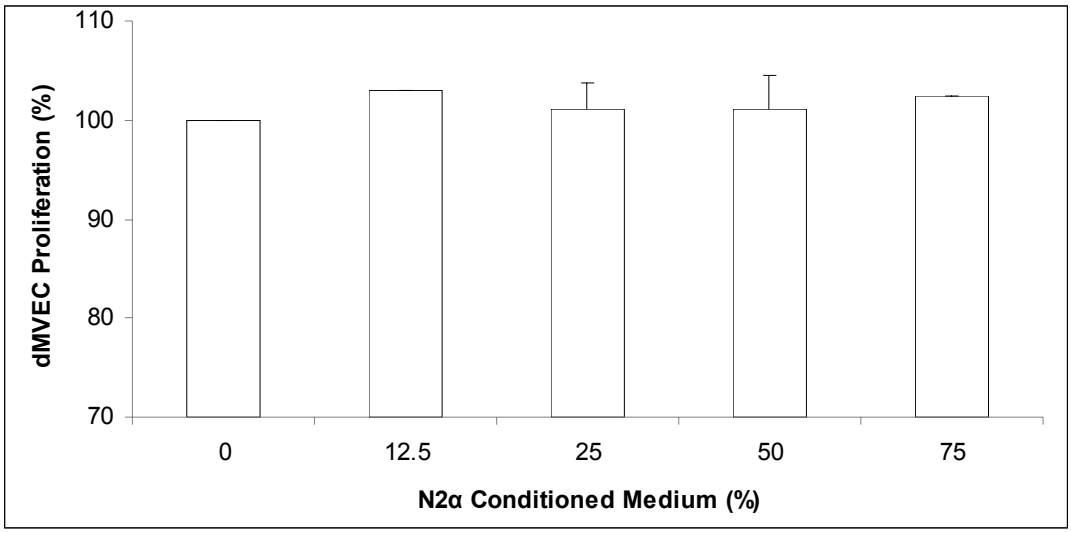

Figure D2b: dMVEC proliferation in response to N2α CM

n=2 (4 replicates each) except at concentrations of 12.5 and 75% where n=1 (4 replicates each)

APPENDIX E

E1. HUVEC proliferation results

Effect of DU145, HeLa and N2α CM

When an alternate endothelial cell line (HUVEC) was tested with DU145, HeLa and N2α CM, no positive proliferative effect was observed with HeLa CM. DU145 CM showed a marginal increase of 4.9% at 25% CM. CM from N2α cells showed the most promising proliferative profile producing a dose-dependent curve, demonstrating a maximum increase of 17.6% at 50% CM (Figure E1).

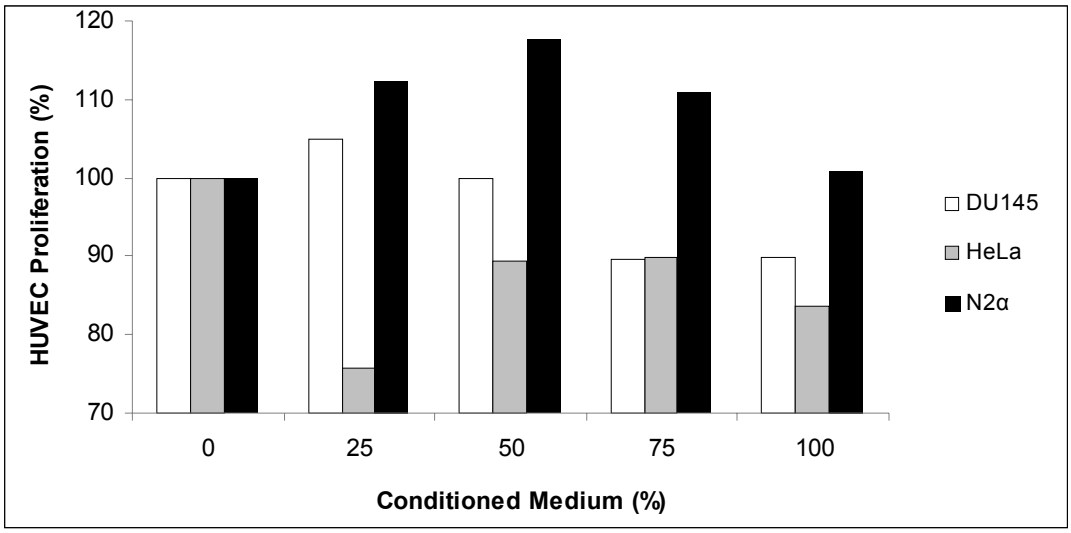

Figure E1: HUVEC proliferation in response to DU145, HeLa and N2α CM

n=1 (4 replicates each)

APPENDIX F

F1. TK measurement (ELISA)

The amount of immune-reactive TK was measured in the CM of DU145 cells. It was observed that when data were extrapolated using the TK ELISA standard curve, TK concentrations were below zero, leading us to conclude that the level of TK released by DU145 cells was below the lower limit of sensitivity of the assay and could, therefore, not be quantified in the present system (Figure F1).

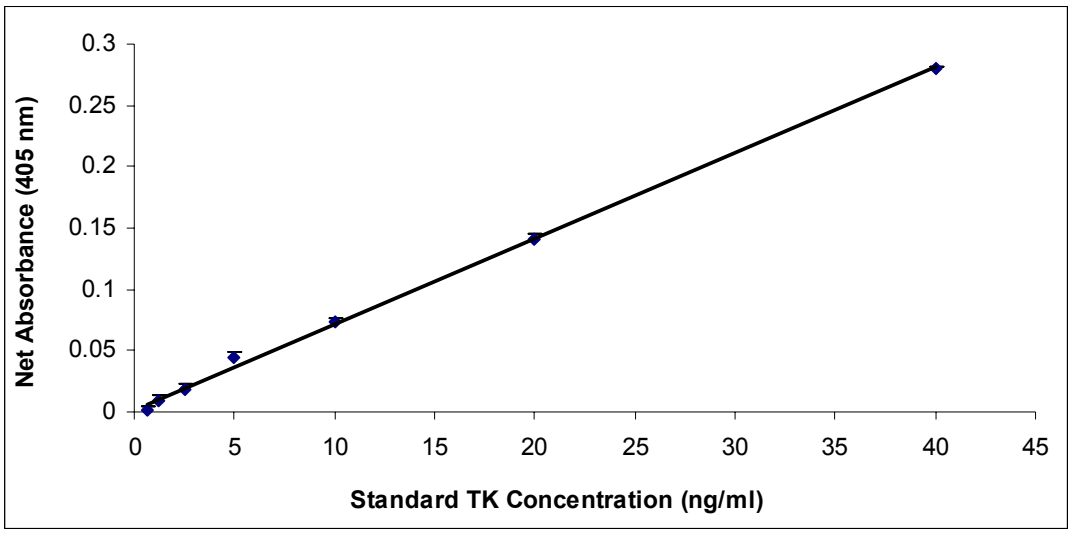

Figure F1: ELISA standard curve for TK

Absorbance = 0.007 (concentration) + 0.0018

Correlation co-efficient = 0.9984