In vivo and *in vitro* analyses of tumour-associated microvascular endothelial cells as a target for the actions of TNF-α

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis. This style is as recommended by the American Association of Cancer Research for journal articles and books.

ACE	angiotensin converting enzyme
Act. D	actinomycin D
aFGF	acidic fibroblast growth factor
APES	3-aminopropyltriethoxysilane
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDEC	brain-derived endothelial cell
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
DA	Dark Agouti
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAMA	Dark Agouti mammary adenocarcinoma
DEPC	diethyl pyrocarbonate
Di-Ac-LDL	1,1'dioctadecyl-3,3,3'3'-tetramethylindo-carbocyanine perchlorate acetylated low density lipoprotein
DIG	digoxigenin
DMEM	Dulbecco's minimal essential medium
DMF	dimethylformamide
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dTT	dideoxythymine triphosphate
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
ECGS	endothelial cell growth supplement
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMAP-II	endothelial monocyte-activating polypeptide II

FADD	Fas-associated death domain protein
FBS	fetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	haematoxylin and eosin
HBBS	Hanks' balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-gamma
Ig	immunoglobulin
Ig-ITIM	immunoglobulin immunoreceptor tyrosine-based inhibitory motif
IL	interleukin
ILP	isolated limb perfusion
IMVS	Institute of Medical and Veterinary Science
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria-Bertani
levamisole	L[-]-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole hydrochloride
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
MQ	Milli-Q (water)(Millipore)
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium salt
NF-κB	nuclear factor kappa B
NSS	normal sheep serum
nt	nucleotide
ON	overnight
OPD	*o*-phenylenediamine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor

PECAM-1	platelet endothelial cell adhesion molecule-1
PPHS	platelet poor human serum
PVDF	polyvinylidene fluoride
RECA-1	rat endothelial cell antigen-1
rATP	riboadenine triphosphate
rCTP	ribocytosine triphosphate
rGTP	riboguanine triphosphate
RIP	receptor-interacting protein
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
rUTP	ribouridine triphosphate
SD	standard deviation
SDS	sodium dodecyl sulphate
SODD	silencer of death domains
SSC	standard saline citrate
sE-selectin	soluble E-selectin
sICAM-1	soluble intercellular adhesion molecule-1
sPECAM-1	soluble platelet endothelial cell adhesion molecule-1
sVCAM-1	soluble vascular cell adhesion molecule-1
sTNF-α	soluble tumour necrosis factor-alpha
sTNF-R55	soluble tumour necrosis factor receptor 55
sTNF-R75	soluble tumour necrosis factor receptor 75
TACE	TNF-α converting enzyme
TAE	Tris-acetate, EDTA
Taq	*Thermus aquaticus*
TAMEC	tumour-associated microvascular endothelial cell
TBS	Tris-buffered saline
TdT	terminal deoxynucleotidyl transferase
TE	Tris-HCl, EDTA
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
TNF-β	tumour necrosis factor-beta/lymphotoxin
tnfr1°	tumour necrosis factor receptor 1 deficient (TNF-R55$^{-/-}$)

tnfr2°	tumour necrosis factor receptor 2 deficient (TNF-R75$^{-/-}$)
TNF-R55	tumour necrosis factor receptor 1/CD120a/p55/TNF-R1/TNF-RI
TNF-R75	tumour necrosis factor receptor 2/CD120b/p75/TNF-R2/TNF-RII
TRADD	tumour necrosis factor receptor 1-associated death domain protein
TRAF	tumour necrosis factor receptor-associated factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
Tween-20	polyoxyethylsorbitan monolaurate
U	units
UEA	*Ulex europaeus* agglutinin-1
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGI	vascular endothelial growth inhibitor
v/v	volume/volume
vWF	von Willebrand factor
WPB	Weibel-Palade body
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

The formation of an effective microvasculature is essential for tumour growth and development. It is this absolute reliance that has lead to the notion of exploiting the vascular component of a tumour as a therapeutic target. Nevertheless the success of this approach depends largely on the identification of phenotypic and/or functional differences between normal and tumour-associated microvascular endothelial cells.

The principal aim of this study has been to test the hypothesis that tumour-associated microvascular endothelial cells are a target for the anti-tumour actions of the cytokine tumour necrosis factor-alpha (TNF-α). This was investigated through the development of a method to isolate and culture microvascular endothelial cells from solid tumour tissues. In parallel, the actions of TNF-α on the tumour-associated microvasculature were examined through the activation of endothelial cell adhesion molecules and TNF receptors *in vitro* and *in vivo*. Analyses were performed at the gene and protein level to assess for any microenvironmental control of the induction and regulation of these molecules. The specific cytotoxic action of TNF-α on tumour-associated microvascular endothelial cells was examined by assays for DNA fragmentation (apoptosis).

Currently much of the data relating to the biology of endothelial cells derives from *in vitro* studies performed on endothelial cells from normal, usually macrovascular, sources. Nevertheless, it is well documented that differences exist between endothelial cells isolated from different vessel types as well as from different tissues. To maintain the phenotype of tumour-associated microvascular endothelial cells *in vitro*, a method to isolate and culture pure populations of endothelial cells from an established rat mammary adenocarcinoma was developed. Potential for drift from the tumour-specific phenotype was avoided by using only primary and passage one cultures. *In vitro* these cells form a confluent, contact-inhibited monolayer of typical cobblestone appearance and are responsive to cytokine stimulation. Cultured cells (referred to as TAMECs) were characterized morphologically (light and electron microscopy), immunohistochemically using a panel of endothelial cell-specific antibodies and functionally by DiI-Ac-LDL incorporation. In these studies, TAMECs were found to retain all of the features exhibited by the corresponding tumour-associated microvascular endothelial cells *in vivo*.

Expression of cell adhesion molecules on endothelial cells is paramount in controlling the migration of leukocytes into tissues. The expression of PECAM-1, E-selectin and ICAM-1 was determined in TAMECs (*in vitro*) and tumour-associated microvascular endothelial cells (*in vivo*). The regulation of adhesion molecules was also investigated by exposing cultures and tumour tissues to TNF-α. PECAM-1 protein was expressed by all tumour-associated microvascular endothelial cells and this expression was not affected by cytokine stimulation. E-selectin mRNA and protein were readily identified in TNF-stimulated TAMECs and tumour tissues by Northern blot and *in situ* hybridization, and immunohistochemistry, respectively. ICAM-1 mRNA was also detected in activated TAMECs and tumour-associated microvessels, however protein expression was not observed immunochemically in TNF-stimulated endothelial cells in culture nor *in vivo*. Strong, specific immunostaining was observed in normal host vessels at the tumour periphery. This suggests that the tumour microenvironment is acting to regulate the post-transcriptional expression of functional ICAM-1 protein. The absence of ICAM-1 has implications in the tumour evading the immune system through reduction in leukocyte emigration.

The actions of TNF-α in inducing cell activation, including adhesion molecule expression, are initiated by binding to its receptors, TNF-R55 (through which signal induction primarily occurs) and TNF-R75. The protein and gene transcript for TNF-R55 were detected in tumour-associated microvascular endothelial cells *in vitro* and *in vivo* at a level which remained unchanged following exposure to TNF-α. Tumour cells, fibroblasts and tumour-associated leukocytes also expressed this receptor. In contrast, nonstimulated endothelial cells (*in vitro* and *in vivo*) consistently failed to exhibit reactivity for TNF-R75. Induction of TNF-R75 only occurred in endothelial cells following treatment with TNF-α. Initial staining for TNF-R75 mRNA and protein was observable after 2 h and reached maximal staining intensity at 16–24 h. These observations support the proposition that TNF-α signalling in tumour-associated microvascular endothelial cells is effected through upregulation of the TNF-R75 receptor. TNF-R75 then transfers TNF-α to TNF-R55, the principal signal-inducing receptor, presumably via a ligand passing mechanism.

The upregulation of TNF-R75 *in vivo* preceded the development of TNF-induced haemorrhagic necrosis within the tumour which was first evident within 4 h of cytokine

treatment and rapidly extended throughout the tumour mass over 24 h. The significant majority of tumour-associated microvessels within the necrotic tissue were congested with erythrocytes but remained intact. In a small proportion of vessels the integrity of the endothelial cell layer was compromised by occasional cells identified by TUNEL assay as undergoing apoptosis. As a functional vasculature is an absolute requirement for tumour growth, the data indicate that exposure to TNF-α is followed by critical vascular disturbance. This may occur by apoptosis, in which loss of only a few endothelial cells occurs, by vascular stasis as a result of erythrocyte congestion and/or thrombosis or by a combination of these processes.

In summary, this study confirms that tumour-associated microvascular endothelial cells represent a unique phenotype. Through the expression of TNF receptors, these cells generate adhesion molecules although microenvironmental factors appear to further influence this process. As a result, the absence of detectable ICAM-1 may promote tumour progression through decreased leukocyte infiltration. The development of haemorrhagic necrosis therefore develops through another mechanism. This appears to intimately linked to the expression of TNF-R75 by tumour-associated microvascular endothelial cells. While the TNF-R55 receptor is constitutively expressed the expression of TNF-R75 requires stimulation by TNF-α. Its induction follows a similar profile to the development of haemorrhagic necrosis within the tumour. The observation that TNF-induced cytotoxicity was restricted to a minority of tumour-associated microvessels suggests that only a few endothelial cells may need to be damaged to induce tumour necrosis. In conclusion, this study demonstrates that the tumour-associated microvasculature represents a target for the anti-tumour actions of TNF-α. Further investigations may identify strageties which may improve its potential as an antitumour therapy.

DECLARATION

I declare this thesis to be a record of original work containing no material that has been accepted for the award of any other degree or diploma.

To the best of my belief and knowledge, no material previously published or written by another person has been included without due reference in the text of the thesis.

I give consent for this thesis to be made available for loan and photocopying.



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PUBLICATIONS IN SUPPORT OF THIS THESIS

Nilsson, T.M., and Woods, A.E. Isolation, culture and characterization of tumour-associated microvascular endothelial cells (in preparation).

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CONFERENCE PROCEEDINGS

Some of the material included in this thesis has been presented at the following scientific meetings

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Alvaro, M.C., **Nilsson, T.M.,** and Woods, A.E. *In situ* hybridization: overcoming the lack of probes. Proceedings of the Australian Institute of Medical Scientists and New Zealand Institute of Medical Laboratory Scientists South Pacific Congress, Christchurch, New Zealand, August 1999, T37.

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CHAPTER 1. INTRODUCTION

1.1 VASCULAR ENDOTHELIUM

For well over a century, endothelial cells were generally thought to be merely an inert barrier lining the inner walls of blood vessels (Kvietys and Granger, 1997). Endothelium is now recognized as a structurally simple but functionally complex tissue (Balconi and Dejana, 1986) comprising a confluent monolayer of thin, flattened cells (Thorin and Shreeve, 1998).

The unique position of the endothelial cells is such that the luminal face is directly exposed to the blood. The abluminal face is attached to the basal lamina which is synthesized and secreted by the endothelial cells and mediates contact with the surrounding tissues (Simionescu and Simionescu, 1984). Pericytes, adjacent to the endothelium, provide the contractile elements of the microvessel wall in conjunction with smooth muscle cells (de Angelis, Moss and Pouton, 1996). Intercellular junctions are formed by a complex network of transmembrane proteins and are described on the basis of morphological and functional characteristics including tight junctions, adherence junctions, gap junctions and syndesmos (Dejana, Corada and Lampugnani, 1995). Junctions play an important role in controlling endothelial permeability, leukocyte migration and maintaining vascular structure.

Several basic functions of endothelial cells are evident in every vessel, including provision of a nonthrombogenic surface, mediation of the passage of nutrients, solutes, and hormones, maintenance of a patent lumen and regulation of vascular tone (Gerritsen, 1987). Microvascular endothelial cells do not merely serve as a passive lining of the vascular system, but are actively involved in a range of vital secretory, synthetic, metabolic and immunologic functions. They detect changes in flow, pressure, inflammatory signals or levels of circulating hormones (Gibbons, 1997). In response, they release or activate substances that regulate four cellular processes: cell growth, programmed cell death (apoptosis), cell migration and modulation of the extracellular matrix (Gibbons and Dzau, 1994).

1.1.1 Endothelial cell heterogeneity

Endothelial cells are as diverse in structural and biochemical characteristics as the organs

and tissues in which they are found (Craig *et al.*, 1998). Heterogeneity in morphology of endothelium derived from various sized vessels and different vascular beds, is a concept that is now widely accepted (Augustin, Kozian, and Johnson, 1994; Craig *et al.*, 1998; Hewett and Murray, 1993; Rajotte *et al.*, 1998; Risau, 1995). The establishment and maintenance of vascular diversity is ultimately controlled at the transcriptional level (Aird *et al.*, 1997).

Heterogeneity is not a result of terminal differentiation, but rather of signals from the environment of the endothelial cell (Aird *et al.*, 1997; Cines *et al.*, 1998; Dellian *et al.*, 1996; Hewett and Murray, 1993). Novel, tissue-specific pathways regulate the function of endothelial cells in response to these signals. The extracellular matrix as well as soluble factors derived from, and in direct contact with, adjacent cells provide these cues. Endothelial cells modify activities in response to alterations in the quantity and/or quality of these signals.

Most of our knowledge about endothelial cell gene expression has been obtained from *in vitro* experiments. However, culturing endothelial cells induces profound changes in gene expression at the transcriptional level (Liaw and Schwartz, 1993; Risau, 1995). A direct comparison of cultured and freshly isolated bovine aortic endothelial cells reveals a dramatic upregulation of basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) B-chain and thrombospondin mRNA *in vitro* while other gene levels are comparable or downregulated (Liaw and Schwartz, 1993). Endothelial cell gene expression is also dependent on the establishment of cell-cell contacts (Garlanda and Dejana, 1997) and the activation state of the cell.

1.1.2 Endothelial cell activation

Endothelial cells represent both a source and a target of cytokines. In general, three separate microvascular responses *in vivo* may be cytokine related: (i) endothelial cells may be injured during immune responses; (ii) endothelial cells migrate, proliferate and form blood vessels; and (iii) endothelial cells may acquire the capacity to perform new functions without evidence of cell injury or division. This process is called activation and is the result of quantitative changes in certain gene products in response to cytokines (Pober and Cotran, 1990). When activated, endothelium is one of the most rapidly proliferating cell

types (Auerbach and Auerbach, 1994).

The five core changes in endothelial cell activation are: (i) loss of vascular integrity; (ii) expression of leukocyte adhesion molecules; (iii) change in phenotype from antithrombotic; (iv) cytokine production; and (v) upregulation of HLA molecules (Hunt and Jurd, 1998). These responses are modified by one of two types of endothelial cell activation. The first, endothelial cell stimulation or activation type I, which does not require *de novo* protein synthesis or gene upregulation and occurs rapidly. This includes retraction of endothelial cells, expression of P-selectin and release of von Willebrand factor (vWF)(Hunt and Jurd, 1998). The second response, endothelial cell activation type II, requires time for the stimulation agent to cause an effect via gene transcription and protein synthesis such as the genes involved in adhesion molecules and cytokines (Hunt and Jurd, 1998).

1.1.3 Angiogenesis

The potential for growth is an inherent property of endothelial cells throughout the vascular system. These cells are not only able to repair pre-existing endothelium but can also give rise to new blood vessels by sprouting from pre-existing endothelium. The term angiogenesis, describing the formation of new vessels, comes from the Greek words, *angeion*, which means *vessels*, and *genesis* which means *birth*.

Angiogenesis is a complex multistep process involving extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation and anastomosis (Blood and Zetter, 1990). Briefly, in response to an angiogenic stimulus, endothelial cells separate from each other and the extracellular matrix is subjected to enzymatic digestion. The endothelial cells migrate into the perivascular space, proliferate and align themselves into patent blood vessels. When sufficient angiogenesis has occurred, the endothelial cells become quiescent and the vessels either remain or regress of no longer needed. The endothelial cells also form branches which then connect to other branches to form capillary loops. The incorporation of pericytes within the basement membrane signals vessel stabilization (Crocker, Murad and Greer, 1970). These adhesion events are likely mediated by endothelial cell adhesion molecules and extracellular matrix molecules that provide instructions to the endothelial cells as they migrate into the perivascular space and

assemble into new vessels with surrouding pericytes. A detailed review of the angiogenic process is available elsewhere (Norrby, 1997).

Endothelial cells possess specific high-affinity cell surface receptors for a number of angiogenic growth factors, in particular bFGF and vascular endothelial growth factor (VEGF). Angiogenic factors act directly by stimulating endothelial cells and/or indirectly via accessory cells including macrophages and/or stromal cells. As well as being produced by various other cell types, endothelial cells secrete growth factors to stimulate their own proliferation (Ko *et al.,* 1999b). These endothelial cells undergo alterations in the expression of cell-cell and cell-matrix adhesive molecules, exhibit reorganisation of the cytoskeletal elements, express cell adhesion molecules and components of the extracellular matrix. The ability of endothelial cells to form capillary-like tubes is regulated not only by specific cytokine/receptor combinations, but also by the extracellular matrix and accessory cells (Cines *et al.,* 1998).

Endothelial cells and pericytes carry the genetic information to form the whole capillary network (Folkman and Shing, 1992; Kräling and Bischoff, 1998). A number of genes are induced during the course of angiogenesis. The transcription factor ETS-1 is activated by growth factors and converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases (MMP)-1, -3, and -9, and integrin β_3 (Oda, Abe and Sato, 1999).

Angiogenesis is intimately associated with a variety of biological processes that include embryonic growth and development, wound repair, increases in body mass, the menstrual cycle and pregnancy. Endothelial involvement is also prominent in various pathological states including atherosclerosis, diabetes, thrombosis, haemorrhagic disorders, inflammation and tumour growth and metastasis.

1.1.3.1 *Tumour angiogenesis*

Tumour growth is angiogenesis dependent (Folkman, 1971; Hanahan and Folkman, 1996; Holash *et al.,* 1999). Many tumours persist *in situ* for months to years in a prevascular stage up to 1–2 mm^3 (Folkman, 1971). The tumour becomes vascularized when a subgroup of cells 'switches' to an angiogenic phenotype, a process which involves an

upregulation of angiogenic factors and a concomitant downregulation of antiangiogenic molecules (Hanahan and Folkman, 1996). Every increase in tumour diameter must be associated with a corresponding increase in new capillaries converging on the tumour (Folkman, 1986). Thus, the growth of a tumour is determined by the degree of tumour cell proliferation which is highest in the immediate vicinity of blood vessels and decreases with increasing distance from the vasculature.

Tumours are vascularized by host-derived vessels expressing host-specific antigenic determinants (Lehr *et al.*, 1997; Lichtenbeld *et al.*, 1998). Tumours acquire their vasculature by infiltration into, and expansion between, a network of newly formed vessels in the surrounding connective tissue (Jain, 1997; Thompson *et al.*, 1987). The tumour microenvironment has a direct influence on vascular morphology and endothelial gene expression (Roberts *et al.*, 1998). While tumours are dependent on a functional vasculature for survival and progression, the relative number of endothelial cells in the tumour-associated microvasculature is lower than with normal tissues (Brenner *et al.*, 1999).

Three stages of tumour growth with distinct microvascular characteristics have been documented following an *in vivo* study of malignant glioma growth (Vajkoczy *et al.*, 1998). These are: (i) the avascular stage (days 0–6), in which there is a lag in glioma growth but initial tumour-induced angiogenesis in peritumoural areas; (ii) early vascular stage (days 6–14), featuring tumour cell proliferation associated with the spatially homogeneous development of a tumour microvasculature; and (iii) late vascular stage (days 14–22), characterized by exponential tumour growth and expansion with high vascular densities in the peritumoural region and reduced vascularization in the tumour centre.

In contrast to established views, Holash *et al.* (1999) have recently reported a new mechanism through which the tumour-associated microvasculature develops. A subset of tumours are initially established by co-opting existing host vessels, rather than inducing host vessels. The co-opted vessels progressively disengage from surrounding support cells and regress, these events precede tumour cell death (Holash *et al.*, 1999). Subsequently, the secondary avascular tumour is rescued through active angiogenesis at the tumour margin, a process mediated by angiopoietins and VEGF.

1.1.4 The tumour-associated microvasculature

Although most solid tumours are highly vascular, these tumour vessels are not identical to normal vessels in mature tissues (Blood and Zetter, 1990). During tumour angiogenesis, primitive vessels appear to lack endothelial cells (Nasu *et al.,* 1999). Once developed, the vessel wall is composed of a layer of endothelial cells and typically lacks smooth muscle cells (Chang *et al.,* 1996), a nerve supply (Mitchell, Schumacher and Kaiserling, 1994) and an organized basal lamina (Lugassy *et al.,* 1997). Aggressive human intraocular (uveal) and metastatic cutaneous melanomas generate vascular channels independent of tumour angiogenesis (Maniotis *et al.,* 1999). These looping, patterned matrix-associated vascular channels are lined by tumour cells which recreate functional, perfusable networks *in vitro* (Maniotis *et al.,* 1999).

The tumour-associated vasculature induced by growth factors is fenestrated and has increased permeability (Roberts and Palade, 1997). High levels of growth factors induce and sustain these vessels in the relative absence of periendothelial cells (Benjamin *et al.,* 1999). The chronic immaturity of tumour-associated vessels led Dvorak to characterize tumours as 'wounds that do not heal' (Dvorak *et al.,* 1986). It may well be that the tumour-associated vasculature is not so much characterized by active angiogenesis but rather by an inability to mature fully (Augustin, 1998).

Tumour-associated vessels form a chaotic disorganized network with thin-walled, dilated, saccular, sinusoidal and tortuous vessels traversing the tumour mass (Denekamp, 1993; Gazit *et al.,* 1997). The vasculature is highly disordered, with numerous vascular shunts, irregular vascular diameters, wide interendothelial junctions, a large number of fenestrae and transendothelial cell channels (Gazit *et al.,* 1997; Jain, 1987). Perfusion rates identify four regions within a tumour; an avascular necrotic region, a seminecrotic region, a stabilized microcirculation region, and an advancing front (Jain, 1997). Like the vasculature, tumour blood flow also displays spatial and temporal heterogeneity. Periods of intermittent flow, reverse flow, or stasis with no consistent direction are commonly observed and occur in different tumour regions at different times (Vaupel, Kallinowski and Okunieff, 1989). Tumour blood flow has a direct influence on tumour angiogenesis (Nasu *et al.,* 1999).

In contrast to normal tissue, many of the endothelial cells that comprise the tumour-associated vasculature are in an immature, cycling and activated state (Jekunen and Kairemo, 1997). In a rapidly growing vascularized tumour, it has been estimated that complete anoxia will be evident at the centre of a tumour cord only 150 μm in radius (Thomlinson and Gray, 1955). Actively dividing endothelial cells are generally found only at the tumour periphery (Denekamp and Hobson, 1982; Fox *et al.,* 1993). In contrast to developmental and physiological angiogenesis, tumour angiogenesis is almost always associated with a prominent inflammatory reaction, which may also contribute to the angiogenic cascade (Augustin, 1998; Polverini and Leibovich, 1984).

1.1.4.1 *Endothelial cell-directed tumour therapy*

The concept of treating solid tumour by inhibiting tumour angiogenesis was first articulated 30 years ago by Folkman (1971). Inhibition of tumour angiogenesis by inflicting severe damage selectively to the tumour-associated vasculature represents a major departure from conventional cancer treatment strategies, which are almost always aimed directly at the tumour cell population itself (Kerbel, 1991). In contrast to tumour cells that are genetically unstable, rapidly mutating and able to generate drug-resistant clones, vascular endothelial cells are genetically stable, homogeneous and have a low mutation rate. Endothelial cells also have an intrinsic amplification mechanism such that elimination of a single endothelial cell can inhibit the growth of up to 100 times more tumour cells (Denekamp, 1993).

Anticancer strategies focussing on the tumour-associated microvasculature fall broadly into two categories: anti-angiogenesis and vascular targeting. Anti-angiogenesis aims to inhibit neovascular formation with the goal of preventing any further growth and metastasis. Vascular targeting is primarily directed towards the established tumour-associated vasculature.

Antiangiogenic agents target at least one of several stages involved in the formation of new vessels, i.e. basement membrane degradation, endothelial cell migration, proliferation and/or tube formation (Chaplin and Dougherty, 1999). These agents can be broadly classified as agents which either (i) block matrix breakdown, (ii) inhibit endothelial cells directly, (iii) block activators of angiogenesis, (iv) inhibit endothelial-cell specific

integrin/survival signalling or (v) have non-specific mechanisms of action. There are numerous antiangiogenic agents currently in clinical trials and these are detailed within Table 1.1 and their mechanisms of action presented in Brower (1999).

There is a growing and structurally diverse family of endogenous protein inhibitors of angiogenesis eg. thrombospondin (Tolsma *et al.,* 1993), interferon α/β (Singh *et al.,* 1995), the 16 kDa fragment of prolactin (Clapp *et al.,* 1993), angiostatin (O'Reilly *et al.,* 1994), endostatin (O'Reilly *et al.,* 1997), vascular endothelial growth inhibitor (VEGI)(Zhai *et al.,* 1999), vasostatin (Pike *et al.,* 1998) and cleavage products of anti-thrombin III (O'Reilly *et al.,* 1999). Some of these are internal fragments of various proteins which normally lack antiangiogenic activity. These precursor proteins are frequently components of the extracellular matrix/basement membrane or members of the clotting/fibrinolytic pathways (O'Reilly *et al.,* 1999).

Angiostatin and endostatin are two antiangiogenic molecules that have shown promise in laboratory animal models and are currently in preparation for Phase I clinical trials. *In vivo,* both agents inhibit angiogenesis and thereby the growth of primary and metastatic tumours (Holmgren *et al.,* 1995; O'Reilly *et al.,* 1994; 1996; 1997). Angiostatic treatments are associated with an increased endothelial apoptotic index consistent with the hypothesis that they act by inhibiting tumour-induced endothelial cell proliferation. Immunity and drug resistance do not occur after prolonged exposure (Boehm *et al.*, 1997).

With the discovery of molecular markers of newly formed blood vessels, therapies have been designed to target characteristics unique to the newly formed capillaries. As a result, these antivascular agents inflict damage to susceptible endothelial cells, resulting in either vessel obstruction or destruction leading to haemorrhagic necrosis. In general, these markers are integrins and cell adhesion molecules and include the significant elevation of extracellular matrix-binding integrin receptors, such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (Brooks *et al.*, 1994). Other 'markers' that are upregulated in activated endothelial cells include E-selectin (Bischoff, 1995), endoglin (Burrows *et al.,* 1995), the ED-B domain of fibronectin (Tarli *et al.*, 1999) and various proteases (Hiraoka *et al.,* 1999). The tumour-associated vasculature is commonly targeted using immunoconjugates (a tumour-associated endothelial cell-specific molecule conjugated to a molecule that can insult the vasculature), gene therapy or

TABLE 1.1

Angiogenesis inhibitors in clinical trials

Data on phases I-III clinical trials of antiangiogenic molecules was collected from the National Cancer Institute Database (http://cancertrials.nci.nih.gov/news/angio/table.html; updated July 2000).

DRUGS THAT BLOCK MATRIX BREAKDOWN

Drug	Sponsor	Trial	Mechanism
Marimastat	British Biotech	Phase I/II glioblastoma	Synthetic inhibitor of MMPs
AG3340	Agouron	Phase III non-small cell lung; Phase III hormone refractory prostate cancer, Phase II glioblastoma multiforme	Synthetic MMP inhibitor
COL-3	Collagenex	Phase I solid tumours, Phase I/II brain	Synthetic MMP inhibitor and Tetracycline® derivative
Bay-12-9566	Bayer	Phase III	Synthetic MMP-2/-9 inhibitor
Neovastat	Aeterna Sainte-Foy	Phase II renal cell cancer, Phase III non-small cell lung cancer	Naturally occurring MMP inhibitor
BMS-275291	Bristol-Myers-Squibb	Phase I	Synthetic MMP inhibitor

DRUGS THAT INHIBIT ENDOTHELIAL CELLS DIRECTLY

Drug	Sponsor	Trial	Mechanism
Squalamine	Magainin Pharmaceuticals	Phase II non-small cellling; Phase II ovarian; Phase I advanced cancers	Extract from dogfish shark liver; inhibits sodium-hydrogen exchanger NHE3
Tropinin	Boston Life Sciences	Phase I	Inhibits endothelial cell growth, may bind to ATP synthase and therefore deplete endothelial cell energy
Combretastatin A-4 prodrug	Bristol-Myers-Squibb/Oxigene	Phase I solid tumours;	From tree bark, binds to tubulin and induces apoptosis in proliferating endothelial cells
Endostatin	EntreMed	Phase I solid tumours	Inhibition of endothelial cells

DRUGS THAT INHIBIT ENDOTHELIAL-SPECIFIC INTEGRIN SIGNALING

Drug	Sponsor	Trial	Mechanism
Vitaxin	IxSys; MedImmune	Phase II	Monoclonal antibody to $\alpha_v\beta_3$ integrin on endothelial cell surface
EMD 121974	Merck kGaA	Phase I advanced or metastatic cancer	Blocks integrin $\alpha_v\beta_5$ on endothelial cell surface

TABLE 1.1 (cont.)

Angiogenesis inhibitors in clinical trials

DRUGS THAT BLOCK ACTIVATORS OF ANGIOGENESIS

Drug	Sponsor	Trial	Mechanism
Angiozyme	Ribozyme	Phase I	Degrades mRNA encoding VEGF receptor
SU-101	Sugen/Pharmacia and Upjohn	Phase III	Blocks PDGF receptor signalling
SU5416	Sugen/Pharmacia and Upjohn	Multiple phase I, II and III trials	Blocks VEGF receptor signalling and MMP formation
SU6668	Sugen/Pharmacia and Upjohn	Phase I advanced tumours	Blocks VEGF, FGF, PDGF receptor signalling
Interferon-α	Commercially available	Phase II/III	Inhibits bFGF and VEGF production
Anti-VEGF antibody	National Cancer Institute; Genentech	Phase I refractory solid tumours, Phase II metastatic renal cancer	Monoclonal antibody to VEGF

DRUGS WITH NON-SPECIFIC MECHANISMS OF ACTION

Drug	Sponsor	Trial	Mechanism
CAI	National Cancer Institute	Phase I solid tumours; Phase II renal cell cancer; Phase III non-small cell lung cancer	Inhibitor of calcium influx
Thalidomide	Celgene	Phase II metastatic prostate, solid tumours, Phase III non-small cell lung cancer	Unknown
Endostatin	EntreMed	Phase I solid tumours	Inhibits endothelial cells
TNP-470	TAP Pharmaceuticals	Phase advanced solid tumours, Phase I solid tumours, lymphomas and acute leukaemia	Synthetic analogue of fumagillin, inhibits endothelial cell proliferation and migration
BeneFin	Lane Labs	Phase II breast, brain, Phase III	Shark cartilage powder
Pentosan polysulphate	Georgetown University	Phase I	Downregulates bFGF production
Interleukin-12	Genetics Institute	Phase I/II Kaposi's sarcoma; Phase I/II IL-12 gene therapy	Upregulation of IFN-γ and IP-10
IM862	Cytran	Phase I ovarian, Phase II colorectal cancer, Phase III Kaposi's sarcoma	Upregulates IL-12, inhibits VEGF and bFGF production

retroviral and adenoviral vectors (Chaplin and Dougherty, 1999). The success of these as therapeutic agents depends upon their specificity for the tumour-associated microvasculature. The most efficacious therapies may be in the synergy of monotherapies directed against both the vascular and tumour compartments.

An 'ideal' antiangiogenic agent is expected to be given on a long term basis to control neoangiogenesis and metastatic spread, without necessarily making the tumour shrink (Harris, 1997). As objective tumour responses are uncommonly seen, assessment of tumour response by classical means (i.e. imaging, clinical examination) which may be inappropriate. One of the major challenges in designing clinical trials for antiangiogenic agents is to define new surrogate endpoints as traditional endpoints used for anticancer agents targeting tumour cells may not be applicable to vascular-mediated therapies. The use of serum of plasma levels of growth factors important in angiogenesis as well as non-invasive imaging (positron emission tomography and magnetic resonance imaging) to directly study tumour blood flow, vascular structure and leaky vessels are now being evaluated. Another concern is that the use of antiangiogenic agents is expected to be chronic and may lead to delayed toxic side effects.

1.2 ENDOTHELIAL CELL CULTURES AS A MODEL OF THE ENDOTHELIUM *IN VIVO*

The endothelial cell plays a major role in general physiology as well as in the development and progression of many diseases. Their involvement has led to increased interest in the vasculature and attempts to further characterize this cell type. While the endothelium is generally inaccessible and difficult to manipulate *in vivo*, the establishment of techniques to isolate and culture endothelial cells *in vitro* has greatly advanced our understanding of this cell type. Their manipulation *in vitro* allows responses to be elucidated in the absence of other cytokines or cell types.

The majority of vascular studies *in vitro* have focused on endothelial cells isolated from macrovascular vessels, particularly human umbilical vein endothelial cells (HUVECs)(Jaffe *et al.*, 1973). However, major functions of endothelium occur at the level of small vessels, where circulating substances and leukocytes have their greatest exposure to endothelial cells (Chen *et al.*, 1995). Pathophysiologic studies should therefore be

performed on microvascular endothelial cells rather than macrovascular endothelial cells and in particular, from the organ of origin. Unfortunately, these specialized endothelial cells are typically difficult to isolate and culture.

1.2.1 Techniques for isolating endothelial cells

Despite recent advances in cell culture techniques, the isolation and culture of microvascular endothelial cells remains technically demanding and time consuming. Low initial cell yields, overgrowth of contaminating cells and long culture periods are the major problems encountered (Carson and Haudenschild, 1986). Selective methods to prevent rapid overgrowth by contaminating cells such as fibroblasts, pericytes and smooth muscle cells are also required (Carson and Haudenschild, 1986; Voyta *et al.*, 1984).

The isolation of organ-derived microvascular endothelial cells can be broken down into two basic steps: (i) dissociation of the blood vessels from the surrounding tissue components and (ii) separation of endothelial cells from non-endothelial cell contaminants. The first requires mechanical and enzymatic dissociation of the tissue into a cell suspension. Endothelial cells are then enriched from the total cell population by exploiting specific endothelial phenotypic and functional characteristics. Endothelial cells are purified by a variety of methods including: (i) differential plating, whereby microvascular endothelial cells attach to the culture dish as capillary fragments and adhere more rapidly than single cells (Chung-Welch *et al.*, 1988; Ohizumi *et al.*, 1998) and other cell types (Diglio *et al.*, 1988; Ohizumi *et al.*, 1998; Utoguchi *et al.*, 1995); (ii) immunomagnetic separation (Jackson *et al.*, 1990), using superparamagnetic Dynabeads® (Dynal) coated with endothelial cell-specific antibodies or lectins — bead-cell complexes are selected for with a magnetic particle concentrator; (iii) panning which purifies endothelial cells based on their binding to culture surfaces coated with endothelial cell-specific antibodies (Gupta *et al.*, 1997); and (iv) density dependent centrifugation which separates cells based on their cell density (Utoguchi *et al.*, 1995).

1.2.2 Characterization of endothelial cells *in vitro*

Once established in culture, the identity of isolates is required to confirm their endothelial origin. This is achieved by using a panel of morphological, immunohistochemical and functional assays. Morphological observations include the development of a contact-

inhibited monolayer of spindle-shaped cells. Ultrastructurally, endothelial cells contain pinocytic vesicles, microfilaments, Weibel Palade bodies (WPB)(in macrovascular endothelial cells) and form tight junctions between adjacent cells. Endothelial cells express a variety of specific markers which can be identified by immunohistochemistry. These include angiotensin converting enzyme, vWF, CD44, endoglin and the endothelial cell adhesion molecules E-selectin, intercellular adhesion molecule (ICAM)-1, platelet endothelial cell adhesion molecule (PECAM)-1 and the integrins. Functionally, endothelial cells form vessels in Matrigel *in vitro* and express scavenger receptors which readily incorporate acetylated low density lipoprotein (LDL) at an accelerated rate compared to other cell types.

1.3 ENDOTHELIAL CELL ADHESION MOLECULES

Endothelial cell adhesion molecules mediate adhesive interactions between cells or cells and the extracellular matrix (Polverini, 1996). Upon stimulation by inflammatory mediators, adhesion molecules are induced or upregulated on endothelial cells and leukocytes. An important role for cell adhesion molecules is in inflammation and their control of leukocyte emigration.

Each step in the migration of leukocytes into tissues is mediated predominantly by a distinct family of cell adhesion molecules. Leukocyte emigration occurs in 4 distinct steps - rolling, activation, tight adhesion and transendothelial migration (Muller, 1995). At sites of inflammation, leukocytes begin to bind weakly and reversibly to the luminal surface of the endothelial cells lining these vessels. As a result, these leukocytes slow down and 'roll' along the vascular wall. Following the low-affinity interactions that concentrate leukocytes at the site of inflammation, the release of cytokines stimulates the expression and activation of cell adhesion molecules. Shortly thereafter, some of these cells adhere tightly to the endothelial surface, cease rolling and spread on the endothelial cell surface. They migrate to a nearby intercellular junction and leave the circulation by squeezing between endothelial cells. Once in the interstitial space, they fulfil their immunological functions. An extensive review of the events in leukocyte emigration is given by Cotran and Mayadas-Norton (1998).

1.3.1 Cell adhesion molecule families

Cell adhesion molecules can be classified into at least four families on the basis of biochemical, structural or functional similarities. These include the selectins, immunoglobulin gene superfamily, integrins and cadherins. Each member is a transmembrane glycoprotein with extracellular binding and cytoplasmic functional domains (Freemont and Hoyland, 1996). To interact with one another or the matrix, two complementary molecules are required, the cell adhesion molecule and its ligand. This occurs through: (i) members of the same group of cell adhesion molecule (homophilic); (ii) members of different groups of cell adhesion molecules (heterophilic); or (iii) a cell adhesion molecule and a molecule that does not belong to any of these groups e.g. integrins binding to matrix molecules (Freemont and Hoyland, 1996).

1.3.1.1 *Selectins*

The selectin family is composed of three members; endothelial (E)-, leukocyte (L)- and platelet (P)-selectin. Selectins mediate adhesive interactions between endothelial cells, leukocytes and platelets, particularly in the early events leading to the initial tethering and then rolling of leukocytes on the endothelium (reviewed in Vestweber and Blanks, 1999).

E-selectin (CD62E; originally called endothelial leukocyte adhesion molecule (ELAM)-1) was first identified as a protein expressed only by activated endothelial cells (Bevilacqua *et al.*, 1987). E-selectin mRNA and protein expression are maximal 4–6 h after stimulus and decline over 12–24 h, even in the continued presence of the inducing agent (Bischoff *et al.*, 1997; Kansas, 1996; Polverini, 1996). Endothelial E-selectin expression is regulated through two distinct pathways. Type I induction is the well-studied cytokine-dependent activation pathway in which cytokines stimulate *de novo* synthesis of mRNA and translation into protein (Bischoff *et al.*, 1997). Type II induction is a noninflammatory mechanism related to the proliferative state of the endothelial cells (Luo, Paranya and Bischoff, 1999). Proliferating endothelial cells *in vivo* and *in vitro* express E-selectin while confluent quiescent endothelial cells do not (Bischoff *et al.*, 1997; Kräling *et al.*, 1996; Luo, Paranya and Bischoff, 1999). This confluence-dependent inhibition is at least partly mediated through PECAM-1 and is related to integrin and cell shape-associated signalling (Litwin *et al.*, 1997).

L-selectin (CD62L) is expressed on essentially all neutrophils and monocytes, the majority of T and B cells and a subset of natural killer cells (Kansas, 1996). P-selectin (CD62P) is stored preformed in the α-granules and WPB of platelets and endothelial cells. It is cytokine inducible and mediates the adhesion between leukocytes and platelets (Freemont and Hoyland, 1996).

1.3.1.2 *Immunoglobulin gene superfamily*

The immunoglobulin gene superfamily is a large and diverse family of molecules containing one or more immunoglobulin (Ig)-like domains. Members include ICAM-1, -2 and -3, PECAM-1 and vascular cell adhesion molecule (VCAM)-1 and each contributes to cell-cell recognition and/or adhesion.

ICAM-1 (CD54) is expressed by several cell types including leukocytes, endothelial cells, epithelium and fibroblasts (van de Stolpe and van der Saag, 1996). Its ligands include the leukocyte β_2 integrin receptors LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18)(Marlin and Springer, 1987). ICAM-1 is constitutively expressed at low levels on endothelial cells and can be induced in a cell-specific manner by cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1 and interferon (IFN)-γ (Faull, 1995; Panés *et al.*, 1995; van de Stolpe and van der Saag, 1996). These stimuli increase ICAM-1 expression primarily through activation of gene transcription. Expression peaks at 18–24 h and remains persistent with ongoing stimulus (Faull, 1995). Cytokines mediate this expression through enhancer elements, among them an inducible nuclear factor (NF)-κB transcription factor, within the ICAM-1 promoter (van de Stolpe and van der Saag, 1996).

VCAM-1 (CD106) is expressed on epithelial cells, follicular dendritic cells, bone marrow stromal cells and some macrophages (Faull, 1995). It supports adhesion through its interaction with $\alpha_4\beta_1$ (VLA-4)(Faull, 1995; Fries *et al.*, 1993). It is not normally expressed on resting endothelial cells and is induced by cytokines, including specifically IL-4 (Faull, 1995). The time course of its induction is similar to that of ICAM-1 and correlates with the influx of lymphocytes and monocytes into inflammatory foci.

PECAM-1 (CD31) is expressed on circulating platelets, monocytes, neutrophils and selected T cell subsets. Although originally assigned to the immunoglobulin supergene

family, it has recently been reassigned to the immunoglobulin immunoreceptor tyrosine-based inhibitory motif (Ig-ITIM) family (Newman, 1999). Members modulate or attenuate tyrosine kinase-mediated signalling pathways and PECAM's reassignment suggests cell adhesion may not be its primary function. It has recently been proposed that the main role of PECAM-1 is to act as an adhesion-dependent signalling receptor (Bird *et al.,* 1999).

In vivo, PECAM-1 is uniformly expressed on the plasma membrane of all vascular endothelial cells (DeLisser, Newman and Albelda, 1994; Romer *et al.,* 1995; Scholz and Schaper, 1997) and correlates with endothelial surface area (Eppihimer *et al.,* 1998). While it was previously accepted that PECAM-1 was constitutively expressed and non-inducible, cytokines produce dose-dependent changes in the surface distribution in HUVECs (Ferrero *et al.,* 1996; Romer *et al.,* 1995). Upon cytokine exposure, PECAM-1 protein is redistributed away from intercellular junctions (Ferrero *et al.,* 1996; Romer *et al.,* 1995), cytoskeletal connections are disrupted, phosphorylation and permeability increase (Ferrero *et al.,* 1996) and the half-life of PECAM-1 mRNA decreases (Stewart, Kashour and Marsden, 1996). Protein redistribution appears to regulate leukocyte migration across the vascular endothelium (Romer *et al.,* 1995; Stewart, Kashour and Marsden, 1996). Homophilic interactions between leukocyte PECAM-1 and endothelial PECAM-1 are involved in the migration of leukocytes between adjacent endothelial cells (Muller and Randolph, 1999).

1.3.1.3 *Integrins*

Integrins are membrane-spanning heterodimers consisting of non-covalently associated α and β chains. They are grouped according to 8 β chains associated with a variable number of α chains (Springer, 1990). They primarily mediate divalent cation-dependent cell-cell and cell-substratum adhesion, and function as molecular bridges between the intercellular cytoskeleton and adjacent extracellular structures. Integrins exist in (at least) two different functional states, a non ligand-binding 'inactive' and a ligand-binding 'active' state (Schwartz, Schaller and Ginsberg, 1995). Interactions between integrins and their ligands are regulated at several different levels including changes in expression, function and integrin activation which are controlled by intracellular events (inside-out signalling)(Schwartz, Schaller and Ginsberg, 1995).

1.3.1.4 *Cadherins*

Cadherins are integral transmembrane glycoproteins which mediate Ca^{2+}-dependent cell-cell adhesion among most tissues (Suzuki, 1996). They typically consist of five tandem repeated extracellular domains, a single membrane-spanning segment and a cytoplasmic region. Cadherins depend on calcium for their function: removal of calcium abolishes adhesive activity and renders cadherins vulnerable to proteases. There are 4 subfamilies of cadherins, the epithelial (E)-, neural (N)-, vascular endothelial (VE)- and desmosomal cadherins (Suzuki, 1996). Monolayer permeability and neutrophil transendothelial migration are modulated by VE-cadherin-mediated cell-cell adhesion, which in turn is controlled by the dynamics of the actin cytoskeleton (Hordijk *et al.,* 1999). E-cadherin is gaining a lot of interest in tumour biology however, studies into endothelial cell expressed VE-cadherin are limited.

1.3.2 Cell adhesion molecules in angiogenesis

As well as mediating leukocyte migration, cell adhesion molecules appear to play a role in angiogenesis (Jain *et al.,* 1996). Soluble E-selectin (sE-selectin) and soluble VCAM-1 (sVCAM-1) induce endothelial cell chemotaxis *in vitro* and are angiogenic in the rat cornea (Koch *et al.,* 1995). After leukocyte binding, membrane-bound E-selectin and VCAM-1 are shed and free to activate and recruit adjacent endothelial cells. Soluble ICAM-1 (sICAM-1) stimulates endothelial cell migration, tube formation and sprouting *in vitro* and angiogenesis *in vivo* (Gho, Kleinman and Sosne, 1999). Soluble PECAM-1 (sPECAM-1) also appears to be involved in angiogenesis through its localization in angiogenic blood vessels (DeLisser *et al.,* 1997). The involvement of soluble cell adhesion molecules in angiogenesis suggests a possible link between inflammation and neovascularization (Gho, Kleinman and Sosne, 1999).

1.3.3 The expression and regulation of endothelial cell adhesion molecules

The regulation of cell adhesion molecules, whether it be stimulatory or inhibitory, may be directed at the molecule itself or at its functional pathways. At least three mechanisms modulate the expression and function of adhesion molecules on the surface of endothelium and leukocytes (Cotran and Mayadas-Norton, 1998, Freemont and Hoyland, 1996). The first involves the redistribution of cell adhesion molecules to the cell surface, such as P-selectin which is stored in WPB of endothelial cells and quickly redistributed to the

plasma membrane. The second is cell adhesion molecule induction, a process which requires new protein synthesis, most frequently by NF-κB and transcriptional activation. The third mechanism is increasing binding avidity of the cell adhesion molecules, such as in the integrins which undergo a conformational change when activated and therefore increases the affinity for their ligand.

Each adhesion molecule shows a specific time-dependent course of appearance and disappearance in accordance with their physiological role *in vivo* (Scholz *et al.*, 1996). Expression is both related to the dose of stimulant and the time elapsed between introduction and observation. Adhesion molecule expression is generally induced or upregulated by inflammatory cytokines including TNF-α (see Section 1.4.7), IL-1 and IFN-γ (Gerritsen *et al.*, 1993; Haraldsen *et al.*, 1996; Karmann *et al.*, 1995). Endothelial cell adhesion molecule upregulation is a characteristic feature of inflammatory status (Springer, 1990). Exposure to cytokines demonstrates a dose- and time-dependent activation of NF-κB, followed by the upregulation of cell adhesion molecules (Wilson *et al.*, 1999). Upon stimulation, cell adhesion molecules are redistributed to cell junctions, contributing to the recruitment of leukocytes to sites of inflammation (Bradley and Pober, 1996).

1.3.4 Cell adhesion molecule expression on the tumour-associated microvasculature

The tumour-associated microvasculature demonstrates a varied cell adhesion molecule phenotype (Tables 1.2 and 1.3). Large variations in adhesion molecule expression occur between tumour types as well as between individual cases of the same tumour type. Considerable heterogeneity exists within a tumour, within a single vessel and also between primary and metastatic sites (Jain *et al.*, 1996; Kuzu *et al.*, 1993; Suzuki *et al.*, 1995; Ye *et al.*, 1995). Generally, this expression is not restricted to a particular vessel type. Fox *et al.* (1995a) have detected adhesion molecule immunolabelling in tumour-associated arterioles, capillaries and venules in breast carcinoma specimens.

Compared to normal tissue-matched vessels, adhesion molecule expression is reportedly higher in tumour vessels (Tables 1.2 and 1.3). These observations have typically been confined to immunohistochemical and intravital observations. Recently, a dual-radiolabelled monoclonal antibody technique has been performed to assess endothelial cell

TABLE 1.2

E-selectin expression by tumour-associated microvascular endothelial cells

Tumour type	Expression[a]	Tumour vessels[b]	Regulation[c]	Reference
Squamous cell carcinoma	+	↑	ND	Groves *et al.*, 1993
Metastatic melanoma	–	ND	↔[1]	Berger *et al.*, 1993
Melanoma-in-transit metastases	+	↑	↑[2]	Nooijen *et al.*, 1996
Melanoma-in-transit metastases	+	↑	↑[2]	Renard *et al.*, 1994
Basal cell carcinoma	–	↔	ND	Verhaegh *et al.*, 1998
Basal cell carcinoma	+	↑	ND	Groves *et al.*, 1993
Non-small cell lung carcinoma	+++, especially at borders	ND	ND	Staal-van den Brekel *et al.*, 1997
Lung carcinoma	+ at tumour periphery	ND	ND	Zocchi and Poggi, 1993
Lung carcinoma	–	ND	ND	Molema *et al.*, 1997
Colon carcinoma	+ at invasive margin, – within tumour	↑	ND	Suzuki *et al.*, 1995
Colorectal cancer	++	↑	ND	Maurer *et al.*, 1998
Colorectal cancer	++	↑	ND	Nelson *et al.*, 1994
Colon carcinoma	+ (primary), +++ (hepatic metastases)	↑	ND	Ye *et al.*, 1995
Gastric cancer	–	↑	ND	Mayer *et al.*, 1998
RM-1 murine prostate carcinoma	+	↑	↔[3]	Langley *et al.*, 1999
Breast carcinoma	++, especially at tumour periphery	↑	ND	Fox *et al.*, 1995a; 1995b
Renal cell carcinoma	+ at tumour periphery	ND	ND	Zocchi and Poggi, 1993
Renal cell carcinoma	++	↑	ND	Brenner *et al.*, 1999
Renal cell carcinoma	–	↓	ND	Griffioen, 1997
Haemangioma (proliferative)	+	↑	ND	Kräling *et al.*, 1996
Sarcoma	+++	↑	↑[2]	Renard *et al.*, 1994
Sarcoma	+	↑	↑[2]	Nooijen *et al.*, 1996

[a]Expression on tumour-associated microvascular endothelial cells, – = absent; +/– = infrequent; + = expressed at low levels; ++ = moderate expression; +++ = high expression.
[b]Compared to normal tissue-matched vessels, ND = not determined; ↔ = unchanged; ↓ = decreased; ↑ = upregulated expression.
[c]Regulation by leukocytes or cytokines, [1]after dinitrofluorobenzene; [2]after ILP with TNF-α, IFN-γ, melphalan and hyperthermia; [3]after TNF-α stimulation.

TABLE 1.3

ICAM-1 expression by tumour-associated microvascular endothelial cells

Tumour type	Expression[a]	Tumour vessels[b]	Regulation[c]	Reference
Breast carcinoma	+++	↑	ND	Fox *et al.*, 1995a; 1995b
Basal cell carcinoma	–	↑	ND	Verhaegh *et al.*, 1998
Metastatic melanoma	ND	ND	↔[1]	Berger *et al.*, 1993
Melanoma metastases	+++	↑	↑[2]	Renard *et al.*, 1994
Hepatocellular carcinoma	+++	ND	ND	Yoong *et al.*, 1998
Colorectal hepatic metastases	+/–	ND	ND	Yoong *et al.*, 1998
Colon carcinoma	+ invasive margin, – within tumour	ND	ND	Suzuki *et al.*, 1995
Colorectal cancer	++	↑	ND	Maurer *et al.*, 1998
Colorectal cancer	+	↑	ND	Nelson *et al.*, 1994
Non-small cell lung carcinoma	+++	↑	ND	Staal-van den Brekel *et al.*, 1997
Lung carcinoma	+	ND	ND	Zocchi and Poggi, 1993
Lung carcinoma	+/–	ND	ND	Molema *et al.*, 1997
Lung adenocarcinoma	+	↔	ND	Roussel *et al.*, 1994
Renal cell carcinoma	+	ND	ND	Zocchi and Poggi, 1993
Renal cell carcinoma	+	↓	ND	Griffioen *et al.*, 1996a; 1996b
Renal cell carcinoma	++	ND	ND	Anastassiou *et al.*, 1996
RM-1 murine prostate carcinoma	+	↓	↑[3]	Langley *et al.*, 1999
Rodent mammary adenocarcinoma	–	↔	↔[4]	Nilsson *et al.*, 1996
Glioblastoma multiforme	+	↑	ND	Lossinsky *et al.*, 1995
Angioma	+	↑	ND	Lossinsky *et al.*, 1995
Sarcoma	+++	↑	↑[2]	Renard *et al.*, 1994

[a]Expression on tumour-associated microvascular endothelial cells, – = absent; +/– = infrequent; + - expressed at low levels; ++ = moderate expression; +++ = high expression.

[b]Compared to normal tissue-matched vessels, ND = not determined; ↔ = unchanged; ↓ = downregulated; ↑ = upregulated expression.

[c]Regulation by leukocytes or cytokines, [1]after dinitrofluorobenzene; [2]after ILP with TNF-α, IFN-γ, melphalan and hyperthermia; [3]after TNF-α stimulation, [4]after LPS stimulation.

adhesion molecule expression in normal and tumour-associated microvessels (Langley *et al.*, 1999). When corrected for endothelial surface area (PECAM-1 labelling), the vessels in a RM-1 murine prostate carcinoma model express higher constitutive levels of selectins and ICAM-1 and VCAM-1 levels comparable with other vascular beds (Langley *et al.*, 1999).

After cytokine stimulation, tumour-associated endothelial cells are activated and upregulate adhesion molecule expression (Tables 1.2 and 1.3). Generally, this upregulation is to the same levels as endothelial cells in other tissues (Langley *et al.*, 1999). Inflammatory mediators increase leukocyte adhesion in tumour vessels, although at a lower magnitude than normal vessels (Borgström *et al.*, 1997; Fukumura *et al.*, 1995; Wu *et al.*, 1992). Metastases typically demonstrate reduced endothelial cell adhesion molecule expression compared to primary tumours (Ye *et al.*, 1995; Yoong *et al.*, 1998).

1.3.5 Suppression of adhesion molecules on the tumour-associated microvasculature

Elevated cell adhesion molecule expression by tumour-associated microvasculature is in contrast to the decreased leukocyte-endothelial cell interactions observed *in vivo*. While some tumours express elevated adhesion molecule levels, in a variety of tumours expression is actively suppressed. As a functional vasculature is an absolute requirement for tumour growth, evidence suggests that mechanisms have evolved in tumours to protect against increased exposure to leukocytes.

The tumour microenvironment can actively suppress the expression of adhesion molecules due to the presence of growth factors, hypoxic conditions and active angiogenesis. In a subset of tumours including colon adenocarcinoma, melanoma, renal cell carcinoma and small cell lung carcinoma, the expression of cell adhesion molecules by the tumour-associated microvasculature is actively suppressed. As a result, adhesion of leukocytes to microvascular endothelial cells is decreased (Griffioen *et al.*, 1996a; 1996b; Hellwig *et al.*, 1997; Melder *et al.*, 1996; Pilai *et al.*, 1995). In addition, the tumour-associated microvasculature has a decreased sensitivity to activation with TNF-α, a response termed endothelial anergy (Griffioen *et al.*, 1996a). As a consequence of these alterations, the tumour mass avoids the immune response.

Decreased endothelial cell activation appears to be mediated by angiogenic growth factors. For instance bFGF is known to suppress endothelial E-selectin, ICAM-1 and VCAM-1 expression (Griffioen *et al.*, 1996a; 1996b; Kitiyama *et al.*, 1994; Melder *et al.*, 1996). It modulates this through decreasing cell adhesion molecule gene expression and as a consequence, protein expression and ultimately leukocyte adherence are decreased (Griffioen *et al.*, 1996a; 1996b; Kitiyama *et al.*, 1994; Melder *et al.*, 1996). The sensitivity of endothelial cells to cytokine stimulation, and subsequent adhesion molecule induction, is also influenced by angiogenic factors. Pretreatment of HUVECs with bFGF prior to cytokine stimulation results in E-selectin, ICAM-1 and VCAM-1 levels 30–60% less when compared with cytokine stimulation only (Griffioen *et al.*, 1996a; 1996b; Kitiyama *et al.*, 1994). VEGF has similar but less prominent effects (Griffioen *et al.*, 1996a). Within tumours, this suggests that growth factors support tumour growth by inducing vascular expansion as well as decreasing the infiltration of leukocytes. Angiogenesis inhibitors prevent growth factor-induced endothelial cell anergy by restoring endothelial cell adhesion molecule expression responsiveness to inflammatory cytokines (Griffioen *et al.*, 1999). Antiangiogenic therapy in conjunction with immunotherapy may provide a combination that leads to enhanced anti-tumour responses.

Tumour cells themselves are also able to influence adhesion molecule expression. Melanoma and small cell lung carcinoma metastases suppress endothelial VCAM-1 expression via gene transcription (Piali *et al.*, 1995). Tumour cells also synthesize and secrete proteolytic enzymes (Duffy, 1992) which cleave adhesion molecules from the endothelial cell surface (Piali *et al.*, 1995).

Soluble adhesion molecules are generally elevated in cancer patients (Banks *et al.*, 1993; Liu *et al.*, 1999; Ye *et al.*, 1995; Zhang and Adachi, 1999). In some tumour types, serum and/or urine concentrations significantly relate to the number of metastatic sites, response to therapy and overall survival (Ye *et al.*, 1995; Zhang and Adachi, 1999) while in other types and studies no correlation is observed (Liu *et al.*, 1999). The presence of soluble adhesion molecules may enhance tumour growth through the promotion of tumour angiogenesis and escape from immunodetection (Gho, Kleinman, and Sosne, 1999).

1.4 TUMOUR NECROSIS FACTOR

1.4.1 Cloning, structure and release of TNF-α

TNF-α was first cloned and sequenced by Pennica *et al.* in 1984. It is mainly produced by monocytes and macrophages (Bebo and Linthicum, 1995), however, other sources include lymphocytes, endothelial cells, mast cells, neutrophils, keratinocytes, astrocytes, smooth muscle cells and tumour cells (Sarraf, 1994). Human TNF-α has 30% amino acid homology to lymphotoxin (Gray *et al.*, 1984), also termed TNF-β, a cytokine produced by lymphocytes with similar biological properties.

In its native form TNF-α is a homotrimer (Smith and Baglioni, 1987; Wingfield, Pain and Craig, 1987) with a molecular mass of 52 kDa. The shape of the trimer has the appearance of a triangular cone in which each of the three subunits has a typical jelly roll-β structure and are arranged edge to face (Figure 1.1)(Eck and Sprang, 1989). Each subunit of the trimer has a receptor-binding site located in the lower half of the triangular pyramid in the groove between two subunits (Eck and Sprang, 1989).

The production of TNF-α is under complex transcriptional, translational and post-translational control (Aggarwal and Natarajan, 1996; Balkwill, 1992). TNF-α exists in a membrane-bound (precursor, type II transmembrane) form of molecular mass 26 kDa. There is evidence to suggest that this molecule is assembled intracellularly as a homotrimer before translocation into the cell membrane (Tang, Hung and Klostergaard, 1996). Transmembrane TNF-α is proteolytically cleaved on the cell surface by the metalloproteinase TNF-α-converting enzyme (TACE)(Black *et al.*, 1997) to produce the soluble (mature) 17 kDa form. TNF-α mRNA is highly unstable and the protein inactivated and rapidly cleared from the circulation (Beutler, Milsark and Cerami, 1985).

1.4.2 TNF receptors

For TNF-α to transmit a signal and hence exert an effect, it must initially bind to specific surface receptors on the target cell. Two structurally related, but functionally distinct, membrane receptor proteins have been identified and cloned: TNF-R55 (also referred to as TNF-R1, TNF-RI, p55, CD120a) and TNF-R75 (TNF-R2, TNF-RII, p75, CD120b), with molecular masses of 55 and 75 kDa, respectively (Brockhaus *et al.*, 1990; Engelmann, Novick and Wallach, 1990; Himmler *et al.*, 1990; Hohmann *et al.*, 1989; Lewis *et al.*,

FIGURE 1.1

TNF-α engaged with TNF-R55

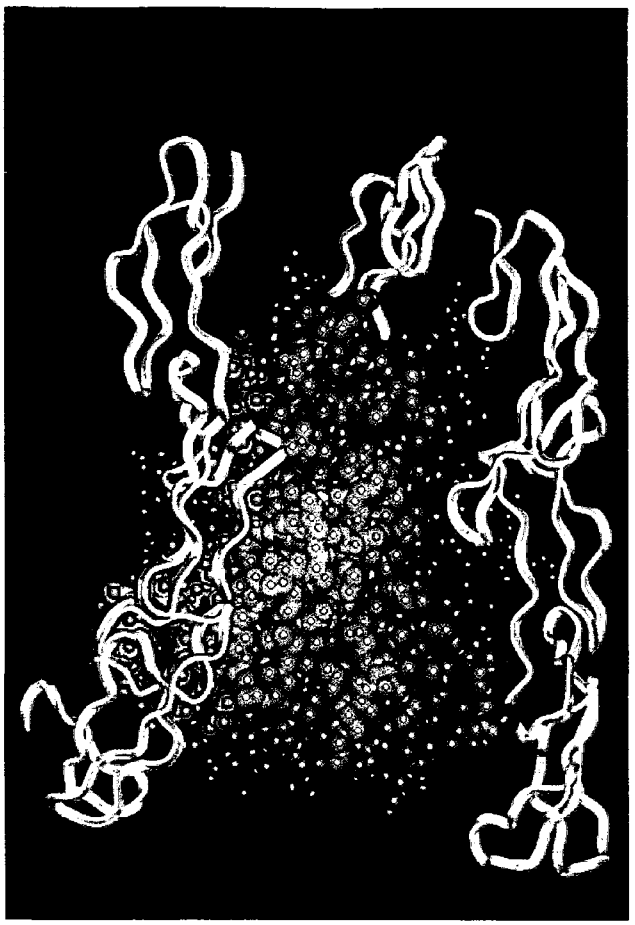
The TNF-α homotrimer is shown binding to three TNF-R55 molecules. The individual TNF-α monomers are coloured in red, blue and brown, and each TNF-R55 in yellow. The balls represent all atoms other than hydrogen.

A. Side view of TNF-α with the cell surface out of the picture at the top of the page.

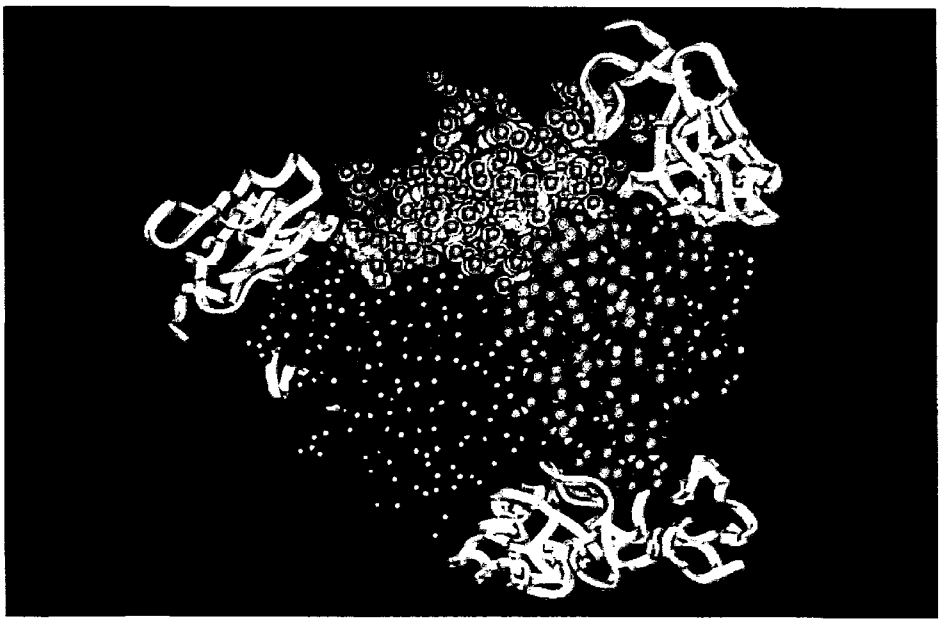
B. Basal view of TNF-α (view from beneath its pyramidal base) showing TNF-R55 in the binding pockets.

Reproduced from Barbara J.A.J, van Ostade, X., and Lopez, A.F. Tumour necrosis factor-alpha (TNF-a): The good, bad and potentially very effective. *Immunol. Cell Biol.*, **74:** 434-443, 1996, with the permission of Dr J.A.J. Barbara and the publisher Blackwell Science Asia.

A



B



1991; Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990). These two receptors are members of the TNF/nerve growth factor receptor superfamily, which also includes lymphotoxin β receptor, Fas, WSL-1, DR4, CD27, CD30, CD40, 4-1BB, OX40 and p75 nerve growth factor receptor (Smith *et al.*, 1990). The two TNF receptors share 28% homology in their extracellular domains (Tartaglia and Goeddel, 1992). In contrast, their intracellular domain sequences are entirely unrelated, suggesting distinct signal transduction pathways (Fiers, 1991). TNF-α and TNF-β interact with the same receptors (Vandenabeele *et al.*, 1995a).

TNF receptors are constitutively shed *in vivo* and *in vitro*. Soluble TNF receptors (sTNF receptors - also referred to as TNF-binding proteins) are generated by proteolytic cleavage of the extracellular portion of the membrane-bound receptor. TACE has recently been identified as the enzyme responsible for sTNF-R75 processing (Solomon *et al.*, 1999). While the soluble forms of the receptors lack intracellular domains, they bind specifically to TNF-α and can compete with cell-associated receptors for the ligand (Aderka *et al.*, 1992; van Zee *et al.*, 1992). Soluble TNF receptors can bind to transmembrane-expressed TNF-α however this interaction does not prevent processing and release of soluble TNF-α (Watts *et al.*, 1999a). Functionally, soluble receptors act as: (i) TNF-α antagonists, capable of neutralizing TNF-α bioactivity at high concentration; (ii) TNF-α agonists, acting as a slow release of biologically active TNF-α when present at low levels; (iii) TNF-α carrier proteins (between body compartments); (iv) stabilizers of TNF-α bioactivity or (v) TNF-α 'buffers', inhibiting the effects of high TNF-α concentrations and presenting it to the cells at low, and well-controlled levels (Aderka, Engelmann and Wallach, 1993). Soluble TNF receptors also interact with membrane-bound TNF-α and can induce 'reverse signalling', whereby the transmembrane ligand acts as a receptor (Watts *et al.*, 1999b). Circulating receptors clear TNF-α from the circulation, principally to the kidney (Beutler, Milsark and Cerami, 1985).

To further elucidate the role of TNF receptors in TNF-α biology, TNF receptor-deficient mice have been generated (Erickson *et al.*, 1994; Peschon *et al.*, 1998; Rothe *et al.*, 1993). The observation that TNF-R55 deficient mice (*tnfr1*$^{-/-}$) and TNF-R75 deficient mice (*tnfr2*$^{-/-}$) are overtly normal indicates that neither TNF-R55 nor TNF-R75, and hence TNF-α, are essential for normal (mouse) development and homeostasis under basal conditions

(Peschon *et al.*, 1998). In addition, TNF-α mutants with selective receptor binding characteristics have provided considerable information on the contribution of each receptor to TNF-α signalling (Barbara *et al.*, 1994; van Ostade *et al.*, 1993).

1.4.3 Expression and regulation of TNF receptors

The majority of cell types and tissues express one or both TNF receptors (Bigda *et al.*, 1994; Fiers, 1991; Leeuwenberg *et al.*, 1995; Rink and Kirchner, 1996), notable exceptions being erythrocytes and unstimulated T-lymphocytes (Aggarwal and Natarajan, 1996). In contrast with TNF-R55 which is expressed on almost every cell type, TNF-R75 expression is restricted to endothelial cells and cells of haematopoietic origin (Slowik *et al.*, 1993). While the presence of TNF receptors is a prerequisite for a biological effect, there is no correlation between receptor number and the magnitude and/or type of response (Aggarwal and Natarajan, 1996; Balkwill, 1992; Fiers, 1991; Grell *et al.*, 1993). Ligand binding and internalization have been shown to occur in both TNF-sensitive and TNF-resistant cells (Sarraf, 1994).

TNF-α can, directly and indirectly, modulate its own receptors through transcriptional control (Aggarwal and Natarajan, 1996; Kalthoff *et al.*, 1993; Winzen *et al.*, 1996) and receptor internalization/shedding (Higuchi and Aggarwaal, 1994b; Kalthoff *et al.*, 1993; Olsson *et al.*, 1993). Regarding the latter, ligand-induced down-modulation of TNF receptors occurs through internalization of TNF-R55 and shedding of TNF-R75 (Higuchi and Aggarwaal, 1994b). The signal for both of these actions is mediated through TNF-R55 (Higuchi and Aggarwaal, 1994b).

Strict regulation of TNF receptor gene expression by inflammatory cytokines represents one level of controlling TNF-α responsiveness. The TNF receptor encoding genes are differentially regulated for each receptor (de Kossodo, Critico and Grau, 1994; Hohmann *et al* 1990; Vandenabeele *et al.*, 1995a). This differential regulation accounts for the variability observed in the numbers of TNF-R55 and TNF-R75 expressed on a cell and between cell types, ranging from 200 to more than 10000 receptors/cell (Fiers, 1991). Low, constitutive TNF-R55 and inducible TNF-R75 expression lead to different physiological responses (Vandenabeele *et al.*, 1995a). TNF-R55 levels are generally maintained at a low level due to a tendency to self-associate spontaneously and signal

independently of ligand interaction (Boldin *et al.*, 1995; Grell *et al.*, 1993; Vandenabeele *et al.*, 1995a). This is controlled through low levels of TNF-R55 encoding genes and the 'silencer of death domains' (SODD) protein (Jiang *et al.*, 1999).

1.4.4 Ligand/receptor interactions

In vivo, TNF-α is able to bind to both receptors, but the subsequent effects are modulated, if not determined, by the receptor to which TNF-α binds (Sarraf, 1994). The stability of TNF-TNF receptor complexes appears to influence directly their differential signalling capability (Grell *et al.*, 1998). TNF-R75 has a higher affinity (K_d 100 pM) and faster dissociation rate ($t_{1/2}$ 10 min) than TNF-R55 (K_d 500 pM; $t_{1/2}$ 3 h), preferentially binding TNF-α at low concentrations (Tartaglia, Pennica and Goeddel, 1993). Cells that produce TNF-α are principally able to autostimulate via a transmembrane-bound TNF-mediated pathway (Haas *et al.*, 1999).

The rapid kinetics of TNF-TNF-R75 association and dissociation have been taken as a basis to postulate a mechanism of ligand passing (Tartaglia and Goeddel, 1992). When TNF-α has access to both receptors on the same cell, the presence of TNF-R75 greatly enhances the rate of association of TNF-α with TNF-R55 (Tartaglia and Goeddel, 1992). TNF-R75 participates in regulating TNF-TNF-R55 interactions possibly by increasing the effective concentration of TNF-α at the cell surface through rapid ligand association and dissociation (Slowik *et al.*, 1993; Tartaglia and Goeddel, 1992; Tartaglia, Pennica and Goeddel, 1993). This association allows TNF-α to exert its effects at a much lower concentration and without generation of an intracellular signal by TNF-R75 (Tartaglia, Pennica and Goeddel, 1993). Additionally, ligand passing, through TNF-R75, appears to be the most effective at triggering the signalling cascade (Grell *et al.*, 1995).

1.4.5 TNF receptor signalling

The TNF-α ligand-receptor system is unique in that one ligand is able to interact with two receptors containing very different intracellular domains (Vandenabeele *et al.*, 1995a). While both receptors are active in signal transduction, their signalling is largely nonredundant (Haridas *et al.*, 1998; Tartaglia and Goeddel, 1992). TNF-α activates signalling systems at multiple subcellular compartments, including the plasma membrane, mitochondria, endosomes, cytosol and nucleus (Ledgerwood, Pober and Johnson, 1999).

The trimeric structure of TNF-α with its three receptor-binding pockets indicates that receptor clustering occurs as part of receptor activation and signal transduction. Activation occurs through ligand-mediated clustering of monomeric TNF receptors into a dimer or trimer (Banner *et al.,* 1993; Fiers, 1991; Pennica *et al.,* 1992). It has been suggested that all receptors are present on the cell membrane as preformed dimers, a dimer allosterically modified by engagement of the ligand constituting the minimal unit of receptor activation (Bazzoni and Beutler, 1995). Aggregation of the intracellular domains subsequently induces a conformational change in the extracellular domains of the receptors resulting in receptor-receptor interactions (Song, Dunbar and Donner, 1994). In most cells, the ligand-receptor complex is rapidly internalized by clathrin-coated pits and degraded in lysosomes (Mosselmans *et al.,* 1988). After receptor triggering, no additional activities of TNF-α are required (Bigda *et al.,* 1994); such that the only function of TNF-α may be to cross-link the receptors on the cell membrane (Natoli *et al.,* 1998; Vandenabeele *et al.,* 1995a).

As TNF-R55 lacks any inherent kinase activity (Vandenabeele *et al.,* 1995a), accessory protein molecules are recruited upon receptor aggregation and couple the receptor to signalling pathways to form a multimolecular signal transducing complex (Hsu *et al.,* 1996; Natoli *et al.,* 1998; Rothe *et al.,* 1994). The intracellular portion of TNF-R55 contains a 'death domain' of about 80 amino acids that is required for the signalling of apoptosis and NF-κB activation (Tartaglia *et al.,* 1993). Following ligand binding, TNF receptor 1-associated death domain protein (TRADD) associates with the death domain of TNF-R55 and subsequently binds and recruits other signalling molecules. The N-terminus of TRADD recruits TNF receptor-associated factor (TRAF)-2 and the death domain recruits receptor-interacting protein (RIP) and Fas-associated death domain protein (FADD)(Hsu *et al.,* 1996; Rothe *et al.,* 1994) to the TNF-R55 complex. TRADD initiates distinct signalling cascades for NF-κB activation through TRAF-2 and programmed cell death through FADD (Chainy *et al.,* 1996; Hsu *et al.,* 1996; Hsu, Xiong and Goeddel, 1995). TRAF-2, in contrast, induces NF-κB activation and increased transcription of genes containing NF-κB consensus sequences. In TNF-R75-associated signalling, ligand binding induces TRAF-1 and TRAF-2 association with the TNF-R75 cytoplasmic tails, followed by NF-κB activation (Rothe *et al.,* 1994). In general, TNF-R75 is involved in antiapoptotic effects of TNF-α, whereas TNF-R55 is involved in both apoptotic and antiapoptotic

signalling (Aggarwal, Gollapudi and Gupta, 1999). For a complete, current review of TNF-α signalling, the reader is referred to Goeddel (1999), Ledgerwood, Pober and Bradley (1999) and Wallach *et al.* (1999). A schematic representation of the subcellular distributions of TNF-α, the TNF receptors, and associated signalling molecules, as presented in Sections 1.4.2–1.4.5, is reproduced from Ledgerwood, Pober and Bradley (1999) in Figure 1.2.

1.4.6 Cellular actions of TNF receptors

Both TNF receptors are capable of mediating, either in cooperation or independently, a wide spectrum of cellular responses ranging from cellular proliferation and differentiation to direct cytotoxicity (Aggarwal and Natarajan, 1996; Vandenabeele *et al.*, 1995a). Which of these cellular responses prevails upon exposure to TNF-α depends on a number of factors including cell type, functional state, TNF-α concentration, presence of other cytokines, protein synthesis inhibitors, drugs and the presence of TNF receptors.

TNF-R55 appears to bind TNF-α and monopolize TNF-mediated signalling whenever it is expressed sufficiently. Most TNF-mediated biological responses such as gene induction, antiviral activity, inflammation, tumour necrosis, cell proliferation, differentiation, apoptosis and cytokine production have been attributed to TNF-R55 (Brockhaus *et al.*, 1990; Fiers, 1991; Lewis *et al.*, 1991; Smith *et al.*, 1990).

In a limited number of cell types, TNF-R75 induces cellular responses including production of granulocyte-macrophage colony-stimulating factor (GM-CSF), growth inhibition of early haematopoietic stem cells, induction of cytokine secretion in T cells and thymocyte proliferation — responses independent of TNF-R55 (Grell *et al.*, 1995; 1998; Jacobsen *et al.*, 1995; Tartaglia *et al.*, 1991; Vandenabeele *et al.*, 1992). In contrast to TNF-R55, there is an essential requirement for TNF-R75 to reach an induced density state in order to transmit an independent biological signal (Haridas *et al.*, 1998; Vandenabeele *et al.*, 1995a). In association with its inducibility, it has been proposed that the major function of TNF-R75 upregulation may be to constitute a rapidly mobilizable pool of sTNF-R75 which neutralizes excessive TNF-α (Alsalameh *et al.*, 1999a).

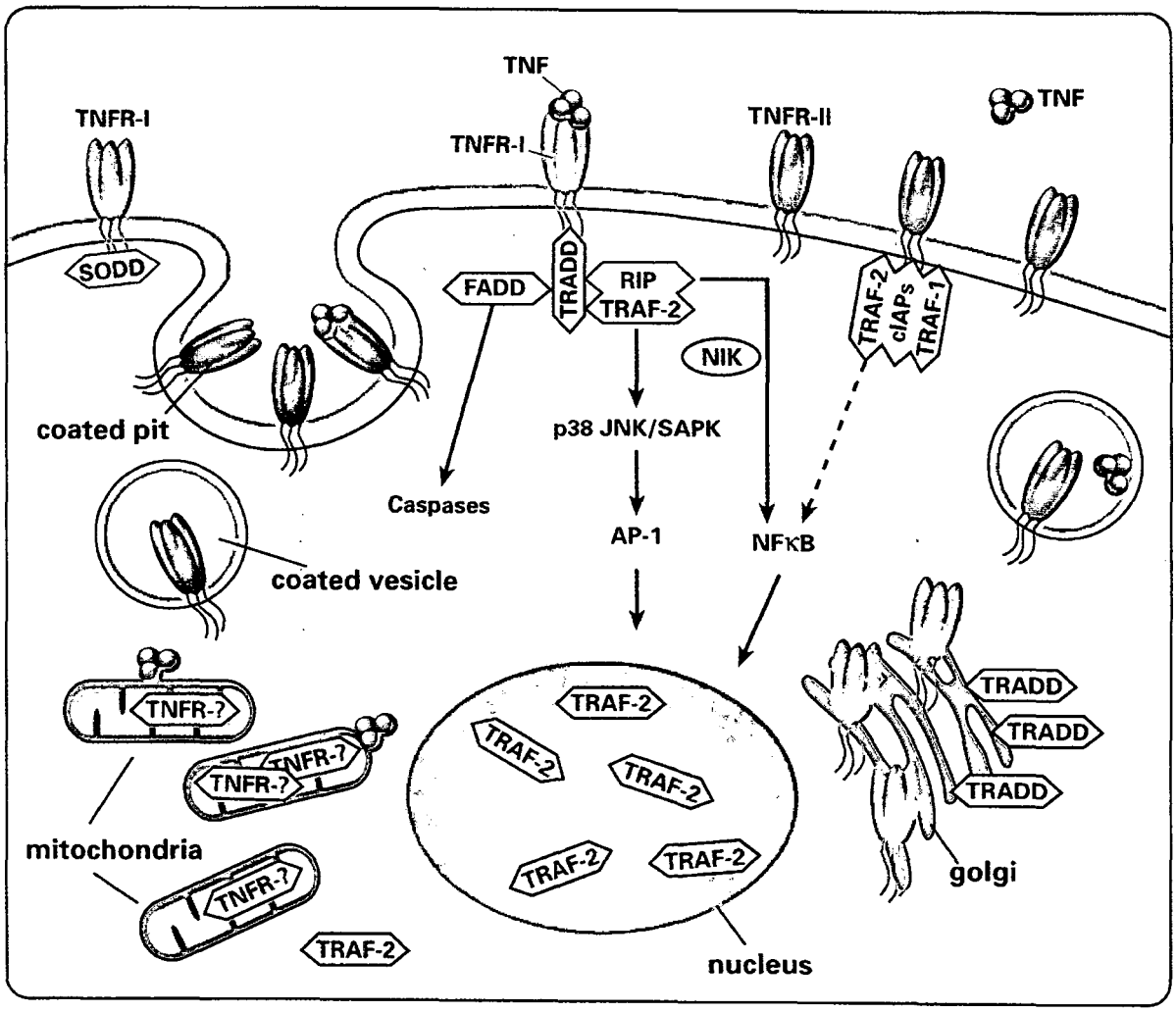
TNF-R75 has an important physiological role when membrane-bound TNF-α, which does

FIGURE 1.2

Schematic representation of the subcellular distributions of TNF-α, the TNF receptors, and associated signalling molecules

The reader is referred to the text within Sections 1.4.2–1.4.5 for discussion of the details contained within the figure. In this figure, TNF-α is represented as TNF, TNF-R55 as TNFR-I and TNF-R75 as TNFR-II.

Reproduced from Ledgerwood, E.C., Pober, J.S., and Bradley, J.R. Recent advances in the molecular basis of TNF signal transduction. *Lab. Invest.*, **79**(9): 1041-1050, 1999, with the permission of the publisher Lippincott, Williams & Wilkins.


TNF
TNFR-I
TNFR-I
TNFR-II
TNF
SODD
FADD
TRADD
RIP
TRAF-2
TRAF-2
cIAPs
TRAF-1
NIK
coated pit
p38 JNK/SAPK
Caspases
AP-1
NFκB
coated vesicle
TNFR-?
TNFR-?
TNFR-?
TRAF-2
TRAF-2
TRAF-2
TRAF-2
TRAF-2
TRADD
TRADD
TRADD
mitochondria
TNFR-?
golgi
TRAF-2
nucleus

not undergo ligand passing, is present (Lucas *et al.*, 1997b). Receptor activation by membrane-bound TNF-α is a juxtacrine process restricted locally by cell-to-cell contact (Grell *et al.*, 1995; Perez *et al.*, 1990). Membrane-expressed TNF-α is an early and very effective stimulus that leads to stronger (and qualitatively different) cellular responses (Alsalameh *et al.*, 1999b). Very small amounts of membrane-bound TNF-α are sufficient to activate TNF receptors, especially in the simultaneous presence of sTNF-α. This allows for simultaneous and efficient activation of both types of receptors on the same cell (Grell *et al.*, 1995). Transmembrane TNF-α may also make sensitive cell types resistant to the necrotic effects of sTNF-α due to the differential signalling pathways operating through each of the two transmembrane TNF-α receptors (Watts *et al.*, 1999a) and the different signals transduced through TNF-R75 by transmembrane TNF-α compared to sTNF-α (Grell *et al.*, 1995). The functional significance of TNF-R75 therefore appears to be in its inducibility, in terms of: (i) its own signalling; (ii) synergism with TNF-R55 dependent signal transduction; (iii) TNF-R75:TNF-R55 ratio and hence ligand-passing function and (iv) the level of soluble receptors (Vandenabeele *et al.*, 1995a).

1.4.7 TNF-α and endothelial cells

Normal vascular endothelium is an important target for the actions of TNF-α. In addition, endothelial cells produce and secrete TNF-α. Production is tightly regulated (in HUVECs) as it is only activated in the presence of multiple inflammatory signals (IFN-γ, IL-1β and lipopolysaccharide (LPS))(Ranta *et al.*, 1999). This suggests that *in vivo*, several signalling pathways need to be activated before molecules as potent and potentially harmful as TNF-α are released (Ranta *et al.*, 1999). The release of TNF-α is also influenced by sTNF receptors, such that sTNF-R55 inhibits its production by activated endothelial cells (Björnberg and Lantz, 1998).

Most endothelial cells express one or both types of the TNF receptors (Bradley, Thiru and Pober, 1995; Leeuwenberg *et al.*, 1995; Loetscher *et al.*, 1993; Lucas *et al.*, 1998; Mackay *et al.*, 1993; Slowik *et al.*, 1993; Weiss *et al.*, 1997). TNF-R55 is expressed at low levels on the endothelial membrane but is more abundant overall, within the Golgi apparatus, cytoplasmic vacuoles (Bradley, Thiru and Pober, 1995; Jones *et al.*, 1999) and caveolae-like domains (Ko *et al.*, 1999a). TNF-R75 is abundant on the endothelial membrane (Bradley, Thiru and Pober, 1995) and has recently been localized within mitochondria

(Ledgerwood *et al.*, 1998). TNF-R75 undergoes endocytosis and transport to lysosomes through coated pits and vesicles (Bradley, Thiru and Pober, 1995). The distribution of each receptor is not altered by TNF-α (Bradley, Thiru and Pober, 1995). TNF-α accelerates receptor clustering and internalization of TNF-R75 whereas the cellular distribution of TNF-R55 is largely unaffected (Bradley, Thiru and Pober, 1995).

The interaction of TNF-α with its cell surface receptor and receptor-mediated endocytosis leads to either new gene expression (endothelial cell activation) or apoptosis (endothelial cell injury)(Lin *et al.*, 1998). The endothelium must adapt its phenotype in response to signals released by other cells. TNF-induced endothelial cell activation results in increased permeability (Stolpen *et al.*, 1986), cell adhesion molecule expression (Pober and Cotran, 1990), leukocyte adhesion (Wahl, Feldman, and McCarthy, 1996), and the release of TNF-α and other cytokines (Bebo and Linthicum, 1995; Paleolog *et al.*, 1994; Pober, 1988). TNF-α may also be injurious through the initiation of endothelial cell apoptosis (Karsan, 1998; Polunovsky *et al.*, 1994; Spyridopoulos *et al.*, 1998). This action is selective as it has been demonstrated to destroy diseased cells, while hardly affecting the viability of normal endothelial cells (Wallach, 1997).

TNF-α possesses bimodal, dose-dependent effects on angiogenesis *in vivo* and endothelial cell proliferation *in vitro*. This may be due to differences in the microenvironment as well as the proliferative state of the endothelium. The local concentration of TNF-α may also be critical in this respect. Low TNF-α doses (0.01–0.1 ng) induce, whereas high doses (1 and 5 μg) inhibit angiogenesis *in vivo* (Fajardo *et al.*, 1992; Norrby, 1996). As TNF-α is unlikely to reach the local tissue concentration in the microgram quantities necessary for antiangiogenesis, it is most likely that TNF-α stimulates tumour angiogenesis (Lewis *et al.*, 1995; Sato *et al.*, 1986). These responses are reflected *in vitro* with TNF-α inhibiting endothelial cell proliferation at high concentrations (Fráter-Schröder *et al.*, 1987; Sato *et al.*, 1986; Schweigerer, Malerstain and Gospodarowicz, 1987) while it appears to stimulate angiogenesis at low concentrations (0.05–0.5 U/ml)(Schweigerer, Malerstain and Gospodarowicz, 1987).

Proliferating endothelial cells express high affinity TNF receptors. In the growing and/or motile state, endothelial cells have an enhanced responsiveness to and increased affinity for

TNF-α (Gerlach *et al.,* 1989; Lucas *et al.,* 1998). This has been associated with a confluence-dependent decrease in receptor numbers as cells establish contacts (Gerlach *et al.,* 1989; Pocsik *et al.,* 1994). The correlation between receptor number and the magnitude and/or type of response appears conflicting. Pocsik *et al.* (1994) have reported that decreased receptors levels (associated with increasing cell density) correlate with a decreased biological response. In contrast, a correlation between receptor number and the magnitude and/or type of response has not been established in other investigations (Aggarwal and Natarjan, 1996; Balkwill, 1992; Fiers, 1991; Grell *et al.,* 1993).

Associated with the effects of TNF-α on proliferation and migration, TNF-α induces changes in endothelial cell morphology. Cultures incubated with TNF-α generally assume morphology consistent with growth arrest, while essentially no cell death is observed (Sato *et al.,* 1986; Schweigerer, Malerstain and Gospodarowicz, 1987). These cells become elongated, overlap, rearrange their actin filaments, form intracellular gaps and lose their fibronectin matrix (Sato *et al.,* 1986; Stolpen *et al.,* 1986; Wójciak-Stothard *et al.,* 1998). These changes are reversible upon removal of TNF-α.

1.4.8 TNF-α and neoplasia

TNF-α is capable of killing certain neoplastic cells *in vitro* as well as causing haemorrhagic necrosis of established tumours *in vivo* (de Kossodo *et al.,* 1995; Pagliacci *et al.,* 1993). The mechanism(s) by which TNF-α mediates this effect is not completely understood. It was previously assumed that the action of TNF-α *in vivo* was based on its ability to kill tumour cells directly however, it may also be due to the ability of TNF-α to destroy the tumour-associated microvasculature. The effects of TNF-α are related to tumour size with small tumours less susceptible to TNF-α than larger tumours (Mulé *et al.,* 1988). This, in turn, relates to the requirement of an established vasculature.

1.4.9 TNF-α as an antineoplastic agent

Although the historical background and preclinical data of TNF-α suggest otherwise, systemic therapy with TNF-α in human neoplasia has proved disappointing (Mueller *et al.,* 1998). Phase I clinical trials of TNF-α in advanced disease began in 1985 shortly after the gene was first cloned with TNF-α being administered in many different dosing regimens and administration routes. Severe systemic side effects were observed before therapeutic

doses could be reached.

Until recently, it was generally accepted that TNF-mediated toxicity in human clinical trials was induced by TNF-R75 mediating the proinflammatory effects of TNF-α (Barbara, van Ostade and Lopez, 1996). It now appears that TNF-R55 is primarily responsible for the induction of systemic toxicity (Erickson *et al.*, 1994; Marr *et al.*, 1998). A TNF-R75-specific mutant form of murine TNF-α (expressed in an adenovirus vector) induces an antitumour response with reduced toxicity (Marr *et al.*, 1998).

It is only when administered locally that TNF-α has shown any of the promise of preclinical studies. Isolated limb perfusion (ILP) with TNF-α, IFN-γ, melphalan (an alkylating chemotherapeutic agent) and mild hyperthermia (40°C) for locally advanced soft tissue sarcoma and *in transit* melanoma metastases produce complete remissions in 50–90% of cases (Nooijen *et al.*, 1998; Renard *et al.*, 1994). ILP has also been successfully adapted to organs, including isolated single-lung (Pass *et al.*, 1996) and hepatic (Alexander *et al.*, 1998) perfusion. During dual targeting ILP, tumour-associated microvascular endothelial cells are activated by the cytokines and induce ICAM-1, E-selectin and VCAM-1 expression (Nooijen *et al.*, 1996; Renard *et al.*, 1994). These activated endothelial cells swell and detach from the subendothelial matrix through the deactivation of $\alpha_v\beta_3$ (Renard *et al.*, 1994; Rüegg *et al.*, 1998). Rapid and specific necrosis occurs in the tumour while normal adjacent tissues remain unaffected (Renard *et al.*, 1994). Hyperthermia, in leukaemia cells, induces TNF-α expression and secretion and is associated with TNF-R55-mediated apoptosis (Katschinski *et al.*, 1999).

Despite encouraging results for ILP, very little is known regarding the mechanism by which TNF-α exerts its effects on tumours. High TNF-α doses improve drug accumulation 3–6-fold by increasing the permeability of the tumour-associated vasculature (ten Hagen *et al.*, 1999). In locally advanced soft tissue sarcomas, a good clinical response after ILP is associated with a decrease in the number of mitotic figures and the tumour proliferation rate and increased apoptosis (Plaat *et al.*, 1999).

Differential sensitivity towards TNF-α between normal and tumour-associated microvascular endothelial cells is a major factor in the promise of TNF-α as a tumour

therapeutic agent. It has been proposed that the release of a novel tumour-derived antiangiogenic cytokine, endothelial monocyte-activating polypeptide II (EMAP-II)(Kao *et al.*, 1992; Schwarz *et al.*, 1995), renders tumour-associated microvascular endothelial cells sensitive to TNF-α (Kao *et al.*, 1992; Wu *et al.*, 1999). EMAP-II has the unique ability to induce tissue factor production by tumour-associated microvascular endothelial cells (Wu *et al.*, 1999). These cells are also activated by EMAP-II and express TNF-R55 (TNF-R75 was not investigated)(Libutti *et al.*, 1997; Wu *et al.*, 1998), E-selectin and P-selectin (Gnant *et al.*, 1999). *In vitro*, EMAP-II induces apoptosis in proliferating microvascular endothelial cells, an effect enhanced by oxygen deprivation (Schwarz *et al.*, 1995). Recently, *in vivo* transfer of the gene encoding EMAP-II using recombinant vaccinia virus sensitizes TNF-α resistant melanomas with subsequent tumour regression and growth inhibition (Gnant *et al.*, 1999). *In vitro* studies suggest that high doses of TNF-α and IFN-γ are not directly cytotoxic to the tumour-associated microvasculature (Yilmaz *et al.*, 1998). While these cytokines affect endothelial cell proliferation and morphology, the cells do not undergo apoptosis (Yilmaz *et al.*, 1998).

1.4.10 TNF receptors in disease

Endothelial mRNA and protein levels for each TNF receptor are significantly upregulated in various disease states. These include cerebral ischaemia (Botchkina *et al.*, 1997), cerebral malaria (Lucas *et al.*, 1997a; 1997b), acute respiratory distress syndrome (Lucas *et al.*, 1997a; 1997b), rheumatoid arthritis (Alsalameh *et al.*, 1999b), experimental autoimmune encephalomyelitis (Bebo and Linthicum, 1995), inclusion body myositis, polymyositis and dermatomyositis (De Bleecker *et al.*, 1999) and experimental allergic encephalomyelitis (Kahn *et al.*, 1999). In these conditions, one or both of the receptors are upregulated. Areas of intense inflammation are associated with strong TNF-R55 expression and increased TNF-R75 expression on the luminal surface of endothelial cells (De Bleecker *et al.*, 1999). In association with membrane-expressed TNF receptors, sTNF receptors are commonly elevated during processes such as systemic therapy with recombinant human TNF-α (Boehme *et al.*, 1996), human experimental endotoxaemia, congestive heart failure (Nozaki *et al.*, 1998) and in critically ill patients (van Zee *et al.*, 1992).

1.4.11 TNF receptors in neoplasia

A wide variety of cell types within the tumour population express TNF receptors, including tumour cells, fibroblasts, infiltrating leukocytes and endothelial cells. TNF-R55 is generally expressed to varying degrees by neoplastic cells (Chen *et al.*, 1997a; Maruno *et al.*, 1996; Naylor *et al.*, 1993; Pusztai *et al.*, 1994) and to a less extent on infiltrating leukocytes particularly T lymphocytes and tumour-associated macrophages (Miles *et al.*, 1994; Naylor *et al.*, 1993). TNF-R75 is predominantly confined to infiltrating leukocytes and stromal cells (fibroblasts and endothelial cells)(Miles *et al.*, 1994; Naylor *et al.*, 1993; Pusztai *et al.*, 1994).

Tumour cells have a greater tendency than nonmalignant cells to shed sTNF receptors from the cell surface (Aderka *et al.*, 1991). Loss of TNF receptor expression has been demonstrated in advanced non-small cell lung carcinoma, suggesting a downregulation and/or shedding of TNF receptors during tumour progression (Tran *et al.*, 1998). Elevated sTNF receptor concentrations are also detected in the serum and urine of cancer patients (Aderka *et al.*, 1991; Engelmann, Novick and Wallach, 1990; Rink and Kirchner, 1996; Schall *et al.*, 1990) and correlate with the staging of disease (Aderka *et al.*, 1991; Rink and Kirchner, 1996).

1.4.12 TNF receptors on the tumour-associated microvasculature

Both TNF receptors have been localized to the endothelium of malignant tissues (Table 1.4). In general, most tumours express both TNF receptors however, the percentage of TNF receptor-expressing vessels varies within each tumour and also between tumour types. For instance, receptor-expressing vessels range from 7.7–74.4% (based on TNF-α binding sites) on glioma-associated microvascular endothelial cells (Maruno *et al.*, 1996). TNF-α binding sites are observed within the tumour-associated microvasculature and vessels adjacent to the tumour but not in remote areas with normal histology (Maruno *et al.*, 1996). Stimulation with TNF-α upregulates endothelial TNF receptor expression (Maruno *et al.*, 1996; Pusztai *et al.*, 1994).

1.5 MECHANISMS OF TNF-MEDIATED CYTOTOXICITY

1.5.1 TNF receptors and TNF-mediated cytotoxicity

TNF-mediated cytotoxicity is dependent on the form of both the TNF receptors, TNF-R55

TABLE 1.4

TNF receptor expression by tumour-associated microvascular endothelial cells

Tumour type	TNF-R55		TNF-R75		Reference
	Expression[a]	Level of expression[b]	Expression	Level of expression	
Glioma	+[c]	↑	+[c]	↑	Maruno *et al.*, 1996
Ovarian cancer	–	ND	–	ND	Naylor *et al.*, 1993
Breast carcinoma	+	↑	+	↑	Pusztai *et al.*, 1994
Invasive breast carcinoma	+[c]	ND	+[c]	ND	Leek *et al.*, 1998
Invasive breast carcinoma	+	ND	+	ND	Miles *et al.*, 1994

[a]Expression on tumour-associated microvascular endothelial cells, – = absent; + = present.
[b]In relation to normal tissue-matched vessels, ND = not determined; ↑ = upregulated.
[c]TNF-α binding sites detected on the endothelium, does not discriminate between TNF-R55 and TNF-R75.

and TNF-R75, as well as their ligand, TNF-α. Both the soluble and membrane-associated forms of TNF-α are capable of inducing cytotoxicity in susceptible cells (Monastra *et al.*, 1996). The expression of mTNF-α is rapid and parallels the acquisition of cytotoxic activity (Monastra *et al.*, 1996).

While the cytotoxic actions of TNF-α appear to primarily mediated by TNF-R55, in certain conditions, TNF-R75 also contributes in signalling TNF-mediated cytotoxicity. This can either occur independently or through cooperation between both TNF receptors. While TNF-R75 lacks the death domain, it can mediate TNF-dependent apoptosis, NF-κB activation and c-Jun activation (Haridas *et al.*, 1998). This occurs when the receptor is overexpressed and still requires activation by the ligand to signal.

1.5.2 TNF-mediated cytotoxicity in endothelial cells

The induction of endothelial cell apoptosis by TNF-α was first described by Robaye *et al.* (1991) and by Polunovsky *et al.* (1994). Exposure of endothelial cells to TNF-α results in typical features of apoptosis, inlcuidng ultrastructural changes, DNA strand breaks, positive TUNEL labelling and the induction of caspases (Spyridopoulos *et al.*, 1997). While the specific mechanism of induction of apoptosis by TNF-α in endothelial cells is not known, previous studies have demonstrated inhibition of $\alpha_v\beta_3$ integrin expression by HUVECs exposed to TNF-α (Defilipi *et al.*, 1993). Thus, prevention of adhesion and spreading of endothelial cells may play a role in the advent of apoptosis in these cells *in vitro*, similar to the effect seen *in vivo* (Brooks *et al.*, 1994).

TNF-α is also capable of inducing the expression of genes which inhibit the advent of endothelial cell apoptosis (Opipari *et al.*, 1992), and of promoting angiogenesis (Friendlander *et al.*, 1995). It is possile, therefore, that at high levels, TNF-α is capable of inhibiting angiogenesis (Fajardo *et al.*, 1992) by its effects on the expression of other molecules, adversely effecting the expression of matrix proteins required for endothelial cell survival, or inhibiting the expression of growth factors or their receptors which would normally promote angiogenesis (Spyridopoulos *et al.*, 1997). There is also a close mechanistic link between TNF-mediated prpgrammed cell death and cell-cycle regulation (Spyridopoulos *et al.*, 1998). TNF-α induces G_1 cell-cycle arrest in endothelial cells, a phase in which they are uniquely susceptible to TNF-induced apoptosis. The capability of

the same stimulus (TNF-α) to result in both cell death and survival signals, illustrates the complex regulatory programs which govern endothelial biology, and which remain to be clarified.

1.5.3. TNF-mediated cytotoxicity within tumours

Disruption of the tumour vascular supply represents a major contributing pathway for tumour necrosis. While the specific mechanism by which TNF-α inhibits tumour growth has not been clarified, inhibition of angiogenesis and tumour growth by a direct action on endothelial cells represents one potential explanation of this effect. For example, TNF-α and IFN-γ (in combination with melphalan and hyperthermia) in ILP insult the tumour-associated microvasculature by endothelial cell activation and subsequent apoptosis through detachment from the extracellular matrix (Nooijen *et al.,* 1996; Renard *et al.,* 1994; Rüegg *et al.,* 1998).

The tumour microenvironment differs markedly from that of surrounding tissues primarily due to insufficient vascular supply and decreased blood flow within the central regions of the tumour (Vaupel, Kallinowski and Okunieff, 1989). Within the tumour tissue, oxygen levels can be as low as 2% (hypoxic) compared to other tissues which are oxygenated at 21% (Sampson and Chaplin, 1994). Within tumours, TNF-mediated haemorrhagic necrosis begins from the central region and expands, leaving only a thin rim of viable tumour cells at the periphery (Nilsson, Woods and Rofe, 1996). Therefore, tumour-associated microvascular endothelial cells in the hypoxic centre of the tumour appear to be resistant to hypoxia-mediated apoptosis yet have an increased sensitivity towards TNF-mediated cytotoxicity. This suggests that other factors within the tumour microenvironment contribute towards sensitizing the endothelial cells to TNF-induced apoptosis under hypoxic conditions. Indeed, growth factor-stimulated microvascular endothelial cells are selectively sensitive to TNF-α (Schweigerer, Malerstain and Gospodarowicz, 1987). This may be a possible mechanism through which hypoxic tumour-associated microvascular endothelial cells (which essentially are growth factor-stimulated and rapidly proliferating endothelial cells) are more sensitive to TNF-α than normal microascular endothelial cells. In some situations, bFGF and VEGF also protect endothelial cells from TNF-induced apoptosis by inducing the anti-apoptotic protein Bcl-2 (Gerber, Dixit and Ferrara, 1998; Nör *et al.,* 1999) and enhancing endothelial cell adhesion

to matrix proteins through fibronectin and β_3 integrin expression (Spyridopoulos *et al.*, 1997).

1.6 SCOPE OF THIS STUDY

As detailed in this chapter, microvascular endothelial cells are an active participant in the immune reaction. In response to TNF-α these cells are activated and undergo a number of structural, functional and phenotypic changes. Within tumours, tumour-associated microvascular endothelial cells are also targets for the actions of TNF-α. Evidence suggests that microenvironmental factors within the tumour contribute to responses unique to the tumour-associated microvasculature following exposure to TNF-α. This includes the suppression of endothelial cell adhesion molecule expression as well as the proposal that these cells have an increased sensitivity to TNF-mediated cytotoxicity.

The biology of tumour-associated microvascular endothelial cells, particularly in relation to TNF-induced activation, may play a key role in mediating the actions of TNF-α. As microvascular endothelial cells demonstrate considerable heterogeneity it may well be that the vasculature induced during neoplastic growth also represents a unique endothelial subtype and thus responds differently to TNF-α. Differences in the structure, phenotype and function of tumour-associated microvascular endothelial cells may be one mechanism through which TNF-α mediates haemorrhagic necrosis in malignant tumours while other tissues are not affected.

The principal aim of this study is to test the hypothesis that the anti-tumour actions of TNF-α are mediated through tumour-associated microvascular endothelial cells. More specifically, the following questions will be addressed:

a) Is it possible to isolate and culture microvascular endothelial cells from solid tumour tissue and are these isolates a representative *in vitro* model of the tumour-associated microvasculature?
b) Are the endothelial cell adhesion molecules E-selectin, ICAM-1 and PECAM-1 upregulated by TNF-α?
c) Are one or both of the TNF receptors expressed by tumour-associated microvascular endothelial cells and does their expression and/or regulation mediate the anti-tumour action of TNF-α? And is the expression of one or both TNF receptors linked to the

expression of adhesion molecules?

d) Is TNF-α directly cytotoxic to tumour-associated microvascular endothelial cells *in vivo* and *in vitro*?

It is known that microvascular endothelial cells isolated from different tissues exhibit differing characteristics. To investigate the phenotypic profile of tumour-associated microvascular endothelial cells *in vitro*, a method to isolate and culture pure populations of microvascular endothelial cells from an established rat mammary adenocarcinoma was developed. These endothelial cells were then used as an *in vitro* model in which the specific effects of TNF-α on tumour-associated microvascular endothelial cells were studied. The establishment of these cultures and their characterization is documented within Chapter 3.

One of the major responses of endothelial cells to TNF-α stimulation is the induction or upregulation of endothelial cell adhesion molecules. This in turn, mediates the infiltration of leukocytes into tissues which stimulate an inflammatory response. The expression of the cell adhesion molecules E-selectin, ICAM-1 and PECAM-1 in tumour-associated microvascular endothelial cells was assessed *in vitro* and *in vivo* in both non-activated and TNF-α exposed samples. To examine mRNA expression by Northern blot analysis and *in situ* hybridization, specific cRNA probes were designed and produced for E-selectin and ICAM-1. Protein expression and regulation by TNF-α were analyzed by enzyme immunoassay and immunohistochemistry for E-selectin, ICAM-1 as well as PECAM-1. As there are numerous endothelial cell adhesion molecules which may be studied, this thesis focussed on three of the four adhesion molecules which play a key role in the migration of cytotoxic leukocytes. VCAM-1 expression and regulation studies were unable to be performed as an anti-rat VCAM-1 antibody was not available during the experimental period of this study. The analysis of the above mentioned endothelial cell adhesion molecules forms the basis of the material presented in Chapter 4.

The actions of TNF-α are ultimately controlled by signal transduction mediated through the TNF receptors, TNF-R55 and TNF-R75. Their expression and regulation by TNF-α within tumour-associated microvascular endothelial cells was examined *in vivo* and *in vitro* using Northern blot hybridization, *in situ* hybridization, enzyme immunoassay and

immunohistochemistry. Riboprobes specific for each TNF receptor mRNA species were constructed and the findings of this study are documented in Chapter 5.

As well as activation, interactions between TNF-α and one or both of the TNF receptors can also trigger cytotoxic processes. It is this selective action which produces the haemorrhagic necrosis observed after TNF-α administration in tumours and is the basis for the proposition that tumour-associated microvascular endothelial cells represent a unique target for TNF-α. While it is generally thought that destruction of the tumour-associated microvascular endothelial cells initiates the necrosis, the particular role of TNF-induced cytotoxicity within the tumour-associated microvasculature remains unclear. To investigate the contribution of the vascular component of the tumour to the mediation of haemorrhagic necrosis, cultures of tumour-associated microvascular endothelial cells and tumours were exposed to TNF-α. Vascular changes and DNA fragmentation were examined morphologically and are documented within Chapter 6.

The major observations and conclusions of the separate chapters are brought together in the final chapter. This includes a discussion of the absence of ICAM-1 protein expression on tumour-associated microvascular endothelial cells compared to normal microvascular endothelial cells. The association between the induction of TNF-R75 on tumour-associated microvascular endothelial cells and the development of TNF-induced haemorrhagic necrosis is also discussed as well as its significance to TNF-mediated tumour therapies.

CHAPTER 2. GENERAL METHODS

This chapter details general methods that are applicable to most experimental chapters. Included is a description of the tumour model, specimen processing, immunohistochemical staining and electron microscopy.

2.1 REAGENTS

All chemicals were AnalaR grade or of the highest grade available and purchased from established suppliers. The addresses of suppliers of reagents and equipment are documented within Appendix A.

2.2 ANIMAL STUDIES

2.2.1 Animals

Inbred female Dark Agouti (DA) rats obtained from the South Australian Government Department of Agriculture were used in all experiments. The genetic profile of the strain is checked yearly by the supplier. Animals weighed between 150–200 g and were housed on sawdust in perspex cages in a holding facility maintained at 22±1°C with a 14 h light/10 h dark cycle. The rats were caged in groups of up to five with food (M&V mouse cubes, Milling Industries) and water available *ad libitum*. Approval for the research protocol and animal experimentation was given by the Institute of Medical and Veterinary Science (IMVS) Animal Ethics Committee (Approval numbers 38/96 and 47/98).

2.2.2 Source and type of tumour

The tumour implanted was a Dark Agouti mammary adenocarcinoma (DAMA) which originated from the Department of Pathology and Immunology, Monash University Medical School. The DAMA was passaged in syngeneic rats by injecting a sterile suspension of tumour cells as previously described (Nilsson, Woods and Rofe, 1996). Briefly, subcutaneous tumours of approximately 5 g were excised and dissected free of necrotic and connective tissue. The viable tumour tissue was diced, washed in sterile phosphate-buffered saline (PBS; 10 mmol/l sodium phosphate buffer, pH 7.0, 150 mmol/l NaCl), homogenized in a motor-driven Potter Elvehjen homogenizer (radial clearance 0.5 mm; Wheaton Scientific) and crude debris removed by filtration through sterile gauze. The suspension was washed in 10 volumes of PBS (400 × g/3×1 min) and the cell concentration and viability determined by trypan blue exclusion (trypan blue in 0.5% (w/v) NaCl; Trace

Biosciences) in an Improved Neubauer counting chamber (Weber; depth 0.1 mm, 1/400m^2). The final concentration was adjusted to 4×10^6 viable cells in 150 µl of sterile PBS. Tumour cell suspensions (150 µl) were injected into each hind flank of recipient rats under light fluothane (Zeneca Ltd.) anaesthesia.

2.2.3 *In vivo* investigations with TNF-α

Rats bearing 8 day old tumour implants were injected with a single intratumoural injection (150 µl) of recombinant rat TNF-α (1 µg/ml in PBS, 5×10^4 U/ml, sp. act. 5×10^6 U/mg; endotoxin <0.1 ng/µg, Batch nos.: 141098, 150798 and 181199; Serotec). TNF-α studies included the following time points: 30 min, 1, 2, 4, 8, 12, 16 and 24 h post-injection. Four rats were studied at each time point.

2.2.4 Development, observations and TNF-α treatment of the DAMA

Analysis of DAMA growth following implantation, the development of the tumour-associated microvasculature and macroscopic and microscopic observations of tumour tissues following the administration of TNF-α or LPS have been previously documented (Hopkins, 1996; Nilsson, 1995; Nilsson, Woods and Rofe, 1996). Briefly, this model has several characteristics in common with the human counterpart. In particular, it displays histological features typical of an adenocarcinoma, being characterized by a lack of glandular differentiation, a high mitotic index and tetraploidy (Coyle *et al.*, 1990). A lack of metastases in the host also ensures that single lesion studies and animal responses are not compounded by secondary tumour deposits. Thus, DAMA is an appropriate model for studying histological responses in malignant tissues, including those associated with the effects of cytokines in tumour-bearing hosts. It should be noted however, that the findings from analysis of this tumour model may not be applicable to all tumours.

Subcutaneous injection of the tumour cell suspension produces a neoplasm which becomes palpable 4–6 days after transplantation. The tumour grows subcutaneosly as a solid tissue enclosed within a connective tissue capsule. At day 8 post-implantation, the tumour tissue is pallid and viable and at day 12, the tumour represents 5% of bodyweight. At this time, the centre of the tumour is beginning to undergo necrosis.

The tumour is composed of nests of large, polymorphic cells surrounded by a vascular stroma. Neoplastic cells contain abundant, eosinophilic cytoplasm and exhibit lack of glandular differentiation. Nuclei are large, vesicular and contain prominent nucleoli. Mitotic figures are common.

The tumour-associated microvasculature of the DAMA consists of irregular blood vessels forming extensive networks. The vessels are distributed irregularly throughout the tumour mass and composed of a thin layer of endothelial cells. The tumour-associated microvascular endothelial cells are rarely surrounded by a thin, disrupted smooth muscle cell layer (Hopkins, 1996). Formation of the vasculature is first observed microscopically within 3–4 days of implantation (Nilsson, 1995). The relative vascular volume increases exponentially to reach a plateau of approximately 6% 12 days post-implantation (Nilsson, 1995). As the size of the tumour increases, the diameter of some blood vessels also increases. In particular, the vessels at the centre enlarge.

Intratumoural injections of TNF-α in day 8 old implants were performed by the author as well as by Hopkins (1996). Histological changes include vascular congestion and pockets of necrotic tissue which become increasingly evident in 8–12 day old tumours 24 h after TNF-α injection. Necrosis is detected as early as 4 h after TNF-α exposure in 8 day old lesions. In most cases, necrosis is marked and extends throughout the tumour mass with viable cells being confined to the outer 1–2 mm of tissue within 16–24 h post-TNF-α administration. All other non-treated control tumours remain pallid. Normal vessels in the host tissue surrounding the tumour mass are not affected by the TNF-α treatment as were organs distant from the tumour.

2.2.5 Specimen collection

At the completion of the animal studies, tumour-bearing rats were killed by cervical dislocation. Viable tumour tissues were dissected free from connective and necrotic tissue and processed according to experimental requirements. For the isolation of TAMECs, tumour tissues were collected and placed in cold transport solution (Chapter 3, Section 3.2.1). Histological studies were performed on tumour tissues treated with TNF-α *in vivo* and fixed in 10% (v/v) buffered formol saline for immunohistochemistry (Section 2.3) and *in situ* hybridization (Chapter 4, Section 4.2.11). Brain, liver and lung tissues were also

collected from rats and fixed in 10% (v/v) formol saline. These served as experimental controls for immunohistochemical studies.

2.3 SPECIMEN PROCESSING

After fixation (6–24 h) in 10% (w/v) buffered formol saline, tumour tissues were placed in a Histokinette Tissue-Tek II processor (Sakura Finetechnical Co. Ltd.), processed through graded alcohols, cleared in chloroform and embedded in paraffin wax. Sections were cut at 4 μm using a rotary microtome (Microm Rotary Microtome HM330, Microm Laborgeräte GmbH). Depending on immunostaining requirements, two to three serial sections were mounted on glass microscope slides coated with 3-aminopropyltriethoxysilane (APES; Section 2.3.1). This permitted control sections to be run on the same slide and under the same conditions as the test section. Mounted sections were allowed to adhere to the slides for 60 min at 60°C.

2.3.1 *Coating of glass slides*

Glass microscope slides were coated with APES (Sigma Chemical Co.) to promote the adherence of tissue sections. Clean slides were coated with APES (1:50 in acetone) for 1 min, rinsed in distilled water (3×1 min), dried ON and stored in a dry, dust-free environment.

2.4 HISTOLOGICAL STAINING PROTOCOLS

2.4.1 Haematoxylin and eosin

Haematoxylin and eosin (H&E) staining was performed on all formalin-fixed, paraffin-embedded tumour sections to observe tissue morphology. Briefly, rehydrated specimens were stained in Ehrlich's haematoxylin (5–10 min), differentiated in 1% (v/v) acid alcohol and 'blued' in ammonia water (0.04% aqueous ammonia; 1 min) prior to counterstaining in eosin (2–5 min). Dehydrated sections were mounted in PIX medium.

2.4.2 Immunoperoxidase staining

The primary antisera used in this study are described in each respective chapter and fully documented in Appendix B. The secondary antisera applied were a biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories), biotinylated horse anti-mouse IgG (H+L) (Vector),

biotinylated rabbit anti-goat IgG (H+L)(Vector). Primary and secondary antisera were diluted in TBS with 2% (v/v) normal goat (DAKO Corporation) or rabbit (Vector) serum.

In preparation for staining, slide-mounted sections were dewaxed in Histoclear and taken through graded alcohols to water followed by Tris-buffered saline (TBS; 50 mmol/l Tris, pH 7.6, 150 mmol/l NaCl). Primary antisera were diluted in TBS with 2% normal serum and added to slide-mounted sections encircled with a pap pen (Zymed Laboratories, Inc.) to prevent the mixing of test and control reagents. Slides were incubated in a humid chamber for either 60 min at 37°C, followed by 60 min at RT or where indicated in the text at 4°C ON. Sections were washed in TBS (3×5 min) following each incubation. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide in methanol prior to incubation with the appropriate secondary antisera (1:300 in TBS with 2% normal serum) followed by horseradish peroxidase-conjugated streptavidin (DAKO; 1:500 in TBS), each for 30 min. Bound antibodies were visualized with freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution (Zymed): the reaction was terminated by washing with distilled water. Finally, sections were stained with Ehrlich's haematoxylin, blued, dehydrated, cleared and mounted in PIX medium. Sections were viewed with an Olympus BH-2 microscope (Olympus Optical Co., Ltd.) and photomicrographs taken with a camera attached to an exposure control unit (Model PM-CBAD; Olympus).

2.4.2.1 *Antigen retrieval*

Antigen retrieval (microwave irradiation or boric acid treatment) was performed to expose antigenic sites of proteins that may have been masked during formalin fixation. Boric acid treatment was performed in sections where non-specific background immunostaining was present (Peston and Shousa, 1998). The treatments were performed, where indicated in the text, after bringing the sections to water and prior to applying the primary antibody. At the completion of each retrieval the slides were rinsed in running water followed by TBS.

2.4.2.1.1 *Microwave irradiation*

Dewaxed slide-mounted sections were transferred to a solution of 10 mmol/l tri-sodium citrate (Ajax Chemicals), pH 6.0, in distilled water and placed in a microwave oven (Panasonic, model NN-S546, power 930W). Slides were heated on HIGH until the solution began to boil (2–3 min) after which the setting was changed to LOW for 10 min.

Rapid cooling of the sections was performed as described by Negoescu *et al.* (1996) as it was found to increase the intensity and specificity of immunostaining. Immediately after irradiation, the pot was filled with distilled water to cool the retrieval solution, then the slides were immersed in TBS.

2.4.2.1.2 *Boric acid treatment*

Dewaxed slide-mounted sections were treated in a solution of 200 mmol/l boric acid, pH 7.0, in distilled water overnight at 60°C (Peston and Shousa, 1998).

2.4.2.2 *Immunoperoxidase controls*

To assess non-specific binding, primary antisera were substituted with TBS, normal serum or an irrelevant antibody. In every other respect, controls and test sections were treated identically.

2.5 ELECTRON MICROSCOPY

All solutions for electron microscopy processing were prepared using Milli-Q (MQ) water from a MilliQ Plus system (Millipore Pty. Ltd) with an electrical resistance greater than 18MΩ/cm.

Cell cultures were fixed in 2.5% (w/v) glutaraldehyde (TAAB Laboratories Equipment Limited) in 100 mmol/l sodium cacodylate buffer, pH 7.4. After fixing for 15 min, specimens were washed in 100 mmol/l cacodylate buffer (2×10 min), and postfixed in 1% (w/v) osmium tetroxide (Johnson Murray Chemicals Ltd) in 50 mmol/l cacodylate buffer for 30 min with constant mixing. Sections were stained in 2% (w/v) aqueous uranyl acetate (TAAB; 15 min), dehydrated in a graded ethanol series (70%, 2×10 min; 90%, 2×10 min; absolute, 2×10 min) and cleared in 1,2-epoxypropane (propylene oxide; BDH; 2×5 min). Resin monomer (Durcupan ACM, Fluka Chemika-BioChemika) was gradually introduced into samples (50% (v/v) monomer in propylene oxide, 60 min; 75% (v/v) monomer in propylene oxide, 60 min; 100% monomer, ON; 100% monomer, 60 min, all at 50°C), prior to embedding with fresh activated monomer in BEEM capsules. Samples were allowed to polymerize for 24–48 h at 50°C under vacuum.

After polymerization, resin-embedded specimens were trimmed and 1 μm survey sections cut on an OmU2 microtome (Reichert) using glass knives (LKB 7800B knifemaker, LKB Produkter AB). Sections were mounted on glass microscope slides and stained with 1% (w/v) toluidine blue in 5% (w/v) borax. The resin block was further trimmed and ultrathin sections showing pale gold interference colour (80–100 nm) were cut. Sections were mounted on plastic (2% (v/v) collodion in amyl acetate, Polaron M152-1)- and carbon-coated copper grids (200 mesh, Graticules Ltd.) and stained with 2% (w/v) aqueous uranyl acetate (5 min) and Reynold's lead citrate (2×3 min). Ultrastructural observations were made using a Philips EM 400 transmission electron microscope (Philips Electron Optics) operated at an accelerating voltage of 100 kV. Electron micrographs were recorded on Kodak ESTAR Thick Base electron microscope film 4489 (Eastman Kodak Company). Negatives were printed on Ilford MG-Glossy III photographic paper using a Variscop enlarger (Agfa).

CHAPTER 3. ISOLATION, CULTURE AND CHARACTERIZATION OF TUMOUR-ASSOCIATED MICROVASCULAR ENDOTHELIAL CELLS

3.1 INTRODUCTION

As an integral part of the tumour microenvironment, the vasculature is critical for neoplastic growth. Although originating from host vessels (Lehr *et al.*, 1997), tumour-associated vasculature shows important differences, in particular, lacking the features which may be necessary for controlling or modulating immune, inflammatory and coagulation responses (Fajardo, 1989). For example, as a consequence of abnormal morphogenesis and the influence of tumour-derived growth factors, the vasculature in solid tumours consists of structurally abnormal channels lined by rapidly proliferating, immature endothelial cells in the relative absence of periendothelial cells (Benjamin *et al.*, 1999; Blood and Zetter, 1990). These endothelial cells form vessels which are thin-walled, chaotically arranged, lack innervation and are more permeable than their normal counterparts (Denekamp, 1993).

As detailed in Chapter 1, Section 1.1.4.1, the tumour-associated microvasculature has recently gained interest as a potential site for anti-tumour therapies. To target this tissue effectively, unique structural, phenotypic and/or functional characteristics need to be identified between normal and tumour-associated microvascular endothelial cells. While information can be obtained *in vivo*, variability in the tissue microenvironment often gives rise to conflicting observations. Culture of endothelial cells *in vitro* thus represents a valuable tool for studying the endothelium in isolation and also permits the effects of specific agents such as cytokines to be examined.

The majority of studies into the morphology and function of vascular endothelial cells have focused on cells isolated from large vessels, particularly HUVECs. However, macrovascular endothelial cells are not an ideal model for studying physiological or pathological conditions which occur at the microvascular level. It has been established that great heterogeneity exists not only between endothelial cells from vessels of differing size but also that the phenotype, structure and function of microvessel endothelia from different tissues are quite diverse (Craig *et al.*, 1998; Hewett and Murray, 1993) — it may well be

therefore that the vasculature induced during neoplastic growth also represents a novel endothelial subtype.

A major obstacle in developing a representative *in vitro* model of the tumour-associated microvasculature has been that it is difficult to isolate and establish in culture (Alessandri *et al.,* 1998a; 1998b; Evans *et al.,* 1994; Modzelewski *et al.,* 1994). The few laboratories that have reported on the successful culture of tumour-associated microvascular endothelial cells (TAMECs[a]; Table 3.1) have each relied on a different endothelial cell specific feature and protocol to isolate these cells. Once established in culture, isolates displayed varying morphology and expressed a range of endothelial cell markers.

Many factors contribute to the low success rate for isolating and culturing tumour-associated microvascular endothelial cells. Firstly, the number of microvascular endothelial cells recovered from tumour tissues appears to be relatively low and dependent on the tissue and tumour type. Reports range from 0.02% in astrocytoma (Alessandri *et al.,* 1998a; 1998b), to 2.3% in meningioma (Alessandri *et al.,* 1998a; 1998b) to 4–7% in the rat RIF-1 fibrosarcoma (Modzelewski *et al.,* 1994). Another obstacle is that not all preparations successfully produce primary cultures (Alessandri *et al.,* 1998a). Cells which have been established *in vitro* exhibit a much lower rate of proliferation and age more rapidly than normal tissue-derived endothelial cells (Alessandri *et al.,* 1998b; Chirivi *et al.,* 1999). While these cells retain many characteristics typical of endothelial cells, other features are modified by the tumour microenvironment and it is these changes that may provide a possible target for therapeutic exploitation.

The establishment of TAMECs as an *in vitro* model of tumour-associated microvascular endothelial cells will allow large numbers of cells to be studied under different conditions as well as single agent studies to be performed. It also enables potential endothelial cell-directed therapies to be screened for their effects on endothelial cell proliferation, migration and viability.

[a]TAMECs will be used to describe tumour-associated microvascular endothelial cells which have been isolated and analyzed *in vitro*. When referring to the endothelial cells *in vivo*, the complete description will be used.

TABLE 3.1

Isolation, culture and characterization of TAMECs

Tumour tissue	Purification method	Morphology	Immunohistochemistry[a]			DiI-Ac-LDL[b] incorporation	Reference
			ACE[b]	PECAM-1	vWF		
Rat KMT-17 fibrosarcoma	Percoll gradient centrifugation, ACE activity and differential plating	Homogeneous monolayer	+	ND	+	ND	Utoguchi *et al.*, 1995
Murine RIF-1 fibrosarcoma	Fluorescence-activated cell sorting based on anti-ACE binding	Did not exhibit a cobblestone appearance, WPB present	+	ND	+	+	Modzelewski *et al.*, 1994
Human renal cell carcinoma	Flow cytometry with anti-EN4 or DiI-Ac-LDL incorporation	Not cultured	ND	+	ND	+	Griffioen *et al.*, 1996b
Various human neoplasms[c]	Differential plating followed by UEA-1[b] or PECAM-1-conjugated Dynabeads®	Heterogeneous	ND	+	+	ND	Alessandri *et al.*, 1998b
Solid human tumours	Immunomagentic separation	ND	ND	ND	ND	ND	Lehr *et al.*, 1998

[a]ND = not determined; + = expressed by endothelial cells.

[b]Abbreviations: ACE - angiotensin converting enzyme; DiI-Ac-LDL - 1,1'dioctadecyl-3,3,3'3'-tetramethylindo-carbocyanine perchlorate acetylated low density lipoprotein; UEA-1 - *Ulex europaeus* agglutinin-1.

[c]Tumours = astrocytoma, fibrosarcoma, glioblastoma, lung carcinoma, mammary carcinoma, meningioma, neuroblastoma, ovarian carcinoma and angioma.

This chapter aims to develop an *in vitro* model with which to study whether the anti-tumour actions of TNF-α are mediated through tumour-associated microvascular endothelial cells. Specifically, whether it is possible to isolate and culture microvascular endothelial cells from solid tumour tissue and are these isolates a representative *in vitro* model of the tumour-associated microvasculature?

Described herein is a method developed to isolate and culture pure populations of TAMECs which were subsequently characterized using morphological, immunohistochemical and functional observations. These cells will be used as an *in vitro* model for studies into the expression and regulation of endothelial cell adhesion molecules (see Chapter 4) and TNF receptors (see Chapter 5) within the tumour-associated microvasculature and to assess the contribution of tumour-associated microvascular endothelial cells in TNF-mediated haemorrhagic necrosis in tumours (see Chapter 6).

3.2 MATERIALS AND METHODS

The following method used to isolate and culture TAMECs has been developed from techniques described for the isolation and culture of microvascular endothelial cells from different organs, in particular, Chung-Welch *et al.*, (1988), Modzelewski *et al.* (1994) and Utoguchi *et al.* (1995). All tissue manipulations were performed using aseptic technique in a biological class II laminar flow cabinet (Email Westinghouse Pty. Ltd.). Dissection instruments were sterilized and allowed to stand in a beaker of 70% ethanol to maintain sterility. Unless stated, cell culture media and reagents were purchased from Sigma Chemical Co.

3.2.1 Isolation of TAMECs

At the time of sacrifice, tumours were aseptically removed and dissected free of peripheral and necrotic tissues. The remaining viable tumour was minced into 1–2 mm pieces and placed in cold transport solution (calcium/magnesium-free Hank's Balanced Salt Solution (Ca/Mg-free HBSS), pH 7.4, containing 100 U/ml penicillin G, 100 μg/ml streptomycin, and 50 IU/ml heparin (Trace)). The tissue was dissociated in 1 mg/ml collagenase/dispase (from *Vibrio Alginolyticus/Bacillus polymyxa*; Roche Molecular Biochemicals) in Ca/Mg-free HBSS with 25 mmol/l N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 100 U/ml penicillin G, 100 μg/ml streptomycin and 50 IU/ml heparin, for 60

min at 37°C, with gentle agitation. The suspension was centrifuged (200 × g/5 min) and further digested with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) for 15 min at 37°C. Large fragments were removed by filtration through 100 μm nylon mesh (Falcon; Becton Dickinson Labware) and isolates plated out for culture.

3.2.2 Culture of TAMECs

Cells were plated into 25cm^2 tissue culture flasks (Falcon). Complete culture medium consisted of Dulbecco's Modified Eagles' Medium (DMEM) with 4500 mg/l glucose, 110 mg/ml sodium pyruvate, pyroxidone-HCl and sodium bicarbonate, 25 mmol/l HEPES, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 5% (v/v) heat-inactivated platelet poor human serum (PPHS), 5 IU/ml heparin, 2 mmol/l l-glutamine, 30 μg/ml endothelial cell growth supplement (ECGS)(from bovine pituitary glands), 100 U/ml penicillin G, 100 μg/ml streptomycin and enriched with 20% (v/v) tumour-conditioned medium (see Section 3.2.2.1). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Fresh growth medium was added every 2–4 days.

3.2.2.1 *Preparation of tumour-conditioned medium*

Tumour-conditioned medium (Folkman, Haudenschild and Zetter, 1979) was prepared from DAMA cells isolated in the same manner as TAMECs prior to purification. The cells were cultured in 1% (v/v) gelatin-coated (see below) 25cm^2 flasks in complete culture medium. Every second day, the medium was decanted, centrifuged (200 × g/5 min), the supernatant passed through a 0.22 μm filter (Sartorius AG.) and added to the TAMEC cultures at 20% (v/v) with freshly prepared complete culture medium.

3.2.2.2 *Gelatin coating of culture surfaces*

To aid the attachment and proliferation of endothelial cells, culture surfaces were coated with 1% gelatin solution (Type B: from bovine skin). Culture dishes were flooded with gelatin, left for 60 min at RT, excess solution aspirated and left to dry for at least 2 h before cells or medium were added.

3.2.3 Enrichment for TAMECs

TAMECs were enriched from the total tumour cell population 4–6 days after isolation. Non-adherent cells were decanted and the adherent population washed with Ca/Mg-free

HBSS. After detaching with trypsin/EDTA, the adherent cells were washed and enriched based on their preferential adhesion to plastic (Chung-Welch *et al.,* 1988; Diglio *et al.,* 1988). Adherent cells were plated into (uncoated) tissue culture dishes and supplemented with complete culture medium. Thirty minutes after seeding, non-adherent cells were decanted, the adherent population washed and complete culture medium added. Twenty four hours after selection, enriched cells (designated primary culture) were plated in gelatin-coated 16-well chamber slides (Lab-Tek, Nalge Nunc International), 24-well plates or 25 cm^2 flasks depending on experimental requirements. Cultures were incubated with Ca/Mg-free HBSS at 37°C for 20 min 2–3 days after plating to improve culture purity (Abbott *et al.,* 1992).

3.2.4 Isolation and culture of brain-derived microvascular endothelial cells

Rat brain-derived microvascular endothelial cells (BDECs) were cultured as a 'normal' microvascular endothelial cell population. BDECs were isolated using a modification of the method of Abbott *et al.* (1992). Each preparation involved the brain from one tumour-bearing DA rat. After removal, the brain was cut in half longitudinally and the mid and hind brain and white matter were removed. The remaining grey matter was chopped into 1-2 mm pieces and dissociated in 1 mg/ml collagenase/dispase for 60 min at 37°C. After enzymatic dissociation, the suspension was centrifuged (200 × g/5 min), resuspended in 15 ml 25% (w/v) bovine serum albumin (BSA)(in Ca/Mg-free PBS) and centrifuged (1200 × g/15 min) to separate the capillary fragments (heavy) from myelin, neurons, astrocytes and other single cell contaminants (lighter) (Abbott *et al.,* 1992). Capillary fragments were washed in Ca/Mg-free PBS (200 × g/5 min) and resuspended in a second collagenase/dispase solution for a further 2 h at 37°C. The cells were filtered through 100 μm nylon mesh, centrifuged and resuspended in complete culture medium as described for TAMECs (Section 3.2.2). The fragments were plated into 4 wells of a gelatin-coated 24-well plate (Falcon) and incubated in a humidified CO_2 incubator at 37°C. Two hours after plating, non-adherent cells were decanted, the adherent population washed with Ca/Mg-free HBSS and supplemented with fresh complete culture medium.

3.2.5 Subculture of cells

Endothelial cells were subcultured 24 h after enrichment (for TAMECs) and on reaching confluence (after approximately 10–16 days in culture). For subculture, the cells were

washed with Ca/Mg-free HBSS and detached with trypsin/EDTA. Washed cells were plated into freshly gelatin-coated 16-well chamber glass slides or 24-well plates (passage one) at a dilution of 1:2 or 1:4 depending on experimental requirements.

3.2.6 Characterization of endothelial cell isolates

The endothelial origin and purity of cultures was confirmed using a comprehensive range of morphological, ultrastructural, immunological and metabolic criteria. All characterization studies were performed on primary or passage one cultures to minimize drift from the tumour-specific phenotype.

3.2.6.1 *Morphology*

TAMECs and BDECs were routinely studied by phase contrast microscopy using a CK-2 inverted microscope (Olympus).

3.2.6.2 *Electron microscopy*

Endothelial cells grown in 16-well gelatin-coated glass chamber slides were processed for electron microscopy as described in Chapter 2, Section 2.5, with the following modifications. Monolayers were washed in 100 mmol/l sodium cacodylate buffer, fixed in 2.5% (w/v) glutaraldehyde (15 min), washed and postfixed in 1% (w/v) osmium tetroxide (30 min). Specimens were stained, dehydrated and resin monomer gradually introduced into samples as previously described. However, clearing in propylene oxide was omitted: monomer was gradually introduced into the chambers with absolute ethanol. TAMEC monolayers were embedded in fresh activated monomer and allowed to polymerize for 24–48 h (50°C, under vacuum). After polymerization, resin-embedded monolayers were recovered from the glass by immersion in liquid N_2. Resin blocks were trimmed, sectioned and stained as described in Chapter 2, Section 2.5.

3.2.6.3 *Immunohistochemical staining*

TAMECs, in 16-well glass chamber slides, were characterized with the following primary antibodies (Appendix B): mouse anti-rat ACE (dilution 1:200; a gift from Dr R Auerbach, University of Wisconsin); mouse anti-goat E-selectin (1:100; Santa Cruz Biotechnology, Inc.); mouse anti-rat ICAM-1 (1:100; Serotec), mouse anti-rat endothelial cell antigen (RECA)-1 (1:150; Serotec) and rabbit anti-vWF (1:100; DAKO). Immunostaining with

mouse anti-human α-smooth muscle actin (1:500; DAKO) was also performed to assess contamination of cultures by smooth muscle cells. In addition to TAMECs, BDECs and formalin-fixed, paraffin-embedded sections of the DAMA, liver, lung and brain were also assessed for immunoreactivity with the panel of antibodies.

In preparation for staining, monolayers were washed twice with Ca/Mg-free PBS, fixed with ice-cold methanol-acetone (3:1) for 5 min (-20°C) and air-dried. Permeabilized cells (0.1% (v/v) Triton X-100 (Sigma) in TBS, 5 min) were washed and placed in TBS. Tissue sections were rehydrated and placed in TBS. Boric acid or microwave irradiation-mediated antigen retrieval (Chapter 2, Sections 2.4.2.1.1 and 2.4.2.1.2) was performed on paraffin-embedded sections for E-selectin and ICAM-1, respectively. Immunohistochemistry was performed as described in Chapter 2, Section 2.4.2 with the application of the primary antisera. At the completion of the procedure, the chamber wells were removed from the slide and the slide mounted with glass coverslips.

3.2.6.3.1 *Cytokine stimulation*

Twenty-four hours prior to cytokine stimulation, culture medium was removed and the cells washed twice with Ca/Mg-free HBSS. Medium lacking ECGS, heparin and tumour-conditioned medium was then added back to the cells. The following day, monolayers were stimulated with 300 U/ml TNF-α (4 h for E-selectin; 24 h for ICAM-1), fixed and processed for immunohistochemistry as described above.

E-selectin staining was performed on tumour specimens treated with TNF-α (5×10^4 U/ml) for 4 h *in vivo* and ICAM-1 on specimens exposed to TNF-α (5×10^4 U/ml) for 24 h *in vivo*. This analysis would provide evidence of tumour- and/or culture-induced alterations to the endothelial cell phenotype.

3.2.6.4 *In vitro uptake of DiI-Ac-LDL*

The presence of scavenger receptors for acetylated low-density lipoprotein on TAMECs was assessed using DiI-Ac-LDL. Cells in 16-well glass chamber slides were incubated with 10 μg/ml DiI-Ac-LDL (Biomedical Technologies) in growth medium for 4 hours at 37°C as previously described (Voyta *et al.*, 1984). Cells were washed, fixed in 3% (v/v) formaldehyde in PBS for 20 min, mounted in 90% (v/v) glycerol/PBS and sealed.

DiI-Ac-LDL incorporation was examined by confocal fluorescence microscopy with the assistance of Dr Peter Kolesik, Department of Horticulture, Viticulture and Oenology, University of Adelaide. Fluorescence was observed using a Bio Rad MRC 1000 confocal microscope coupled with a Nikon inverted Diaphot 300 microscope fitted with a krypton/argon laser tube with rhodamine excitation; 568 nm excitation light, 605 nm emission filter. Resident peritoneal macrophages, isolated from DA rats, and human embryonic lung fibroblasts were used as positive and negative controls, respectively.

3.2.6.4.1 *Macrophage isolation*

Macrophages are known to possess scavenger receptors and, therefore, were used as a positive control for DiI-Ac-LDL incorporation (Voyta *et al.*, 1984). To isolate resident peritoneal macrophages, the peritoneal cavity of a DA rat was washed with PBS and peritoneal fluid aspirated. The contents were centrifuged (200 × g/5 min), resuspended in culture medium and seeded into a chamber slide. One hour after plating, the medium was decanted and the adherent cells washed and supplemented with fresh culture medium. Resident peritoneal-harvested macrophages were studied for DiI-Ac-LDL incorporation 1–2 days after harvest.

3.2.6.4.2 *Human embryonic lung cell culture*

Human embryonic lung fibroblasts (Commonwealth Serum Laboratories) do not possess the scavenger receptors which are required for DiI-Ac-LDL incorporation. They were used as a negative control and were kindly provided by Dr Susan Semple (University of South Australia). Embryonic lung fibroblasts were cultured in DMEM with 4.5 g/l glucose, 3.7 g/l sodium bicarbonate and 15 mmol/l HEPES, 2 mmol/l l-glutamine, 10% (v/v) heat-inactivated fetal calf serum, 16 μg/ml gentamicin and 12 μg/ml penicillin G (all from Commonwealth Serum Laboratories). Cells were studied for DiI-Ac-LDL incorporation at passage five.

3.3 RESULTS

3.3.1 Isolation and culture of TAMECs

TAMECs were isolated from DAMA tissue by enzymatic and mechanical digestion. This treatment produced small capillary fragments which establish *in vitro* more favourably than single cells. After 4–6 six days in culture, TAMECs were purified from the total tumour

cell population based on their preferential adhesion to plastic (Chung-Welch *et al.*, 1988; Diglio *et al.*, 1988) — these cells represented primary TAMEC cultures.

The composition of the culture medium was important for cell growth. Cells were cultured with DMEM in the presence of FBS, PPHS, ECGS, tumour-conditioned medium and heparin. These supplements provided the most suitable conditions for endothelial cell proliferation and viability in culture while minimizing the proliferation of non-endothelial contaminants.

3.3.2 Morphology of TAMECs and BDECs

The morphology of isolates was routinely observed by phase contrast microscopy. When first plated out, the endothelial cells appeared in small clusters which after 3–5 days began to grow and divide as small colonies of spindle shaped cells. The cells remained in contact with the original colony and divided from the perimeter, expanding radially to form typical swirling patterns. In association with endothelial cells, tumour cells were also present in the cultures initiated from DAMA tissue. These were identified as large, round cells (25–55 μm) which remained in suspension. At this stage of culture, these cells were retained to provide a source of tumour-conditioned factors which enhance endothelial cell proliferation. As the tumour cells were non-adherent, they were progressively removed during each replacement of culture medium.

Ten to fourteen days post-purification, the cells formed endothelial colonies as they attached to the substrate. TAMECs *in vitro* formed confluent contact-inhibited monolayers composed of elongated, closely associated cells (Figure 3.1A). These cells grew out as a population of adherent cells containing oval nuclei and prominent nucleoli. BDECs displayed a similar morphology (Figure 3.1B).

3.3.3 Subculture of endothelial cells

When the cells reached confluence, they were either utilized for experimental purposes or subcultured. Growth of subcultures mimicked that seen in primary cultures. The time taken to reach confluence was dependent on seeding densities. Passaged cells had a similar morphology to that of the primary cultures.

3.3.4 Characterization of isolates

3.3.4.1 *Ultrastructural features of TAMECs*

Ultrastructurally, TAMECs were observed as elongated cells with overlapping processes. Endothelial cells grew in close contact with each other and microfilaments were concentrated at areas of contact with the culture surface (Figure 3.2A). The cytoplasm was dense and contained prominent rough endoplasmic reticulum, Golgi apparatus, frequent mitochondria and scattered intermediate filaments (Figure 3.2B). Pinocytic vesicles were common. Typical WPB were not observed in any of the specimens examined.

3.3.4.2 *Immunohistochemical analysis of endothelial isolates*

Positive immunolabelling was detected for a range of the endothelial cell antibodies investigated. Staining for ACE (Figure 3.3A) and vWF (Figure 3.3B) was distributed diffusely throughout the cytoplasm and along the cell surface. PECAM-1 was immunolocalized diffusely along the plasma membrane (Figure 3.3C). E-selectin was not expressed constitutively by TAMECs, however, it was induced by TNF-α (300 U/ml) after 4 h (Figure 3.3D). ICAM-1 protein expression was not detected in unstimulated or TNF-α (300 U/ml, 24 h)-treated TAMECs (Figure 3.3E). Strong immunolabelling for ICAM-1 was observed in TNF-treated BDECs (Figure 3.3F). TAMECs did not stain with antibodies against RECA-1 or *Bandeiraea simplicifolia* BS-1 isolectin B_4, however cultured brain microvascular endothelial cells and vessels within formalin-fixed paraffin-embedded normal tissues (lung, liver and brain; not shown) did react with all immunolabels (Table 3.2). Cultures did not express α-smooth muscle actin (not shown) and all negative controls (biotinylated horse anti-mouse (Figure 3.3G) and goat anti-rabbit (Figure 3.3H) secondary antibodies) demonstrated no immunoreactivity.

3.3.4.3 *Functional analysis of endothelial isolates*

Intense granular fluorescence was observed within the cytoplasm of TAMECs after incubation with DiI-Ac-LDL-supplemented media (Figure 3.4A). Peritoneal-isolated macrophages rapidly accumulated the fluorescent probe while human embryonic lung fibroblasts did not incorporate DiI-Ac-LDL.

TABLE 3.2

Immunohistochemical reactivity by endothelial cells *in vivo* and *in vitro*

The expression of various endothelial cell antigens was assessed in TAMEC and BDEC cultures by immunohistochemistry. In parallel, formalin-fixed, paraffin-embedded sections of tumour, brain, liver and lung were analyzed to determine whether any antigens were lost through tumour and/or culture-induced alterations.

Antibody	TAMECs	DAMA[a]	BDECs	Brain[a]	Liver[a]	Lung[a]
α-smooth muscle actin	–[b]	–	–	+	+	+
ACE	+	+	+	+	+	+
Bandeiraea simplicifolia BS-1 isolectin B_4	–	–	+	+	+	+
E-selectin[c]	+	+	ND	ND	ND	ND
ICAM-1[d]	–	–	+	ND	ND	ND
PECAM-1	+	+	+	+	+	+
RECA-1	–	–	+	+	+	+
vWF	+	+	+	+	+	+

[a]Formalin-fixed, paraffin-embedded tissues.

[b]ND = not determined, – = not detected; + = immunohistochemically detected on endothelial cells.

[c]Protein was induced *in vitro* or *in vivo* by exposure to TNF-α for 4 h

[d]Protein was induced *in vitro or in vivo* by exposure to TNF-α for 24 h.

3.4 DISCUSSION

Tumour vasculature displays structural and functional differences from those in normal tissues, however, the significance of these changes is not completely clear nor is the mechanism whereby these changes are induced by the tumour microenvironment. For some time it has been hypothesized that the tumour vasculature would make an ideal target for therapeutic intervention either through inhibition of tumour angiogenesis or selective destruction of tumour-associated microvascular endothelial cells *in situ* (Boehm *et al.*, 1997; Kerbel, 1991). Elucidation of the unique features of the tumour-associated microvascular endothelial cells will greatly enhance this approach.

Two significant problems encountered in endothelial cell isolation from most tissues are the low proportion of microvascular endothelial cells and the diversity of cell populations. The vascular content of tumours has been reported to range from 0.02–7% (Alessandri *et al.*, 1998a, 1998b; Modzelewski *et al.*, 1994). In an attempt to maximize yield as well as obtain relatively pure cultures, cell suspensions were purified through their preferential adhesion to plastic (Chung-Welch *et al.*, 1988; Diglio *et al.*, 1988). This relatively straightforward tactic allows for a specific selection of microvascular endothelial cells over cell types such as fibroblasts, smooth muscle cells and tumour cells that commonly contaminate TAMEC preparations.

It is widely accepted that the constitutive endothelial cell phenotype is unstable and lost once endothelial cells are taken out of their environment (Augustin, Kozian and Johnson, 1994). Knowledge of culture-induced changes in phenotype and function is thus essential for the interpretation of data obtained from *in vitro* models of endothelial cell physiology and pathology. Utoguchi *et al.* (1995) have observed that TAMECs (isolated from a rat KMT-17 fibrosarcoma) at passage four no longer express their tumour-endothelial phenotype and appear to revert to a phenotype typical of normal tissue endothelium. This was demonstrated by decreased permeability and increased leukocyte adhesion (comparable with rat epididymal fat pad-derived capillary endothelial cells) in contrast to primary and passage one tumour-derived endothelial cell cultures. It was suggested that this may be due to the absence of interactions between the tumour and endothelial cells or omission of essential growth factors within the culture medium. Notably, tumour-conditioned medium was not used (Utoguchi *et al.*, 1995).

Inherent in the approach of obtaining pure TAMEC cultures was the maintenance of characteristics that may be specific to the tumour microenvironment. To minimize potential phenotypic drift *in vitro*, as well as including tumour-conditioned medium, only primary and passage one TAMEC cultures were used. While this requires the frequent supply of fresh tumour samples, the maintenance of the tumour-specific phenotype, essential for experimental procedures studying the unique characteristics of tumour-associated vasculature, is assured. In addition, large numbers of endothelial cells can be generated and tested in a wide range of experimental systems. When using a laboratory animal tumour model, the only animals used are those maintaining the tumour *in vivo* as a source of tissue.

TAMECs have been shown to proliferate poorly and age rapidly in standard culture media (Alessandri *et al.,* 1998a). In the present study, TAMECs were cultured with DMEM in the presence of FBS, PPHS, ECGS, tumour-conditioned medium and heparin. The growth of endothelial cells is, in part, dependent upon the ability of these cells to use serum components to produce conditioning factors for their own growth (Gajdusek and Schwartz, 1982). ECGS and tumour-conditioned medium selectively stimulate endothelial cell proliferation and viability and provide growth factors present within the tumour microenvironment. PPHS selectively supports the growth of endothelial cells in preference to non-endothelial cells as it does not contain the platelet products which stimulate fibroblast, pericyte and smooth muscle cell proliferation (Gitlin and D'Amore, 1983). Heparin inhibits exponentially growing and growth-arrested smooth muscle cells *in vivo* and *in vitro* (Clowes and Karnovsky, 1977). In combination with heparin and ECGS, PPHS improves initial attachment and spreading (Carson and Haudenschild, 1986; Thornton, Mueller and Levine, 1983) and helps to achieve confluency faster (Bowman *et al.,* 1990; Gitlin and D'Amore, 1983; Thornton, Mueller and Levine, 1983). When used in combination, this selective growth medium provided the most suitable conditions for TAMEC proliferation and viability *in vitro*.

Steps taken to improve the purity of the culture both at the time of the isolation and culture were reasonably effective. Cultures that started with a high percentage of endothelial cells tended to grow slowly initially, presumably because of the absence of endothelial growth

factors from contaminating cell types, in particular tumour cells. Paradoxically, these cultures sometimes showed greater contamination by non-endothelial cells at later times, apparently because the small percentage of contaminants proliferated faster than the endothelial cells. The best compromise was a high density of endothelial cells, with growth stimulated by a smaller population of non-endothelial cells, yet with the endothelium able to proliferate fast enough to confluence to exclude contaminants. This was best achieved by the presence of tumour cells prior to purification. Selection of endothelial cells through their preferential adhesion to plastic was also successful in eliminating the major contaminants and producing cultures of pure TAMECs.

Once established in culture, it is essential that the origin of isolates be confirmed. The ability to identify and distinguish between microvascular endothelial cells and non-endothelial cell contaminants is based on the structural and functional properties of endothelial cells *in vitro*. There are many well-characterized morphological, ultrastructural, biochemical, immunological and functional criteria which can be routinely used. It is also advisable to demonstrate the absence of markers for common contaminants such as pericytes and smooth muscle cells. Of, perhaps, greater importance is to determine whether the isolated cells retain their tissue-derived properties in culture and therefore accurately reflect the *in vivo* environment being reproduced.

The various characterization procedures undertaken confirm that the cultured cells are endothelial. Morphologically, the growth pattern of microvascular endothelial cells is unique and identified by colonies of cobblestone appearance comprising squamous epithelial cells that always express strict contact inhibition. This growth parameter distinguishes endothelial cells from smooth muscle cells, which tend to produce typical 'hills and valleys' as a consequence of piling up, and from fibroblasts that also tend to multilayer when they reach confluence (Jaffe, 1984). A distinguishing ultrastructural feature of the endothelial cell cytoplasm is the relative richness of vesicular and tubular membranous structures and membrane-bound organelles (Hormia and Virtanen, 1986). WPB were not detected ultrastructurally. While they are frequently observed in large vessel endothelium, WPB are reported to be scarce in microvascular endothelial cells *in vitro* (Dorovini-Zis and Huynh, 1992). Additionally, vWF antigen has been localized in cultured human brain endothelial cells (although WPB were not observed) to cytoplasmic

vesicles in close association with the rough endoplasmic reticulum and Golgi apparatus (Dorovini-Zis and Huynh, 1992).

Immunolabelling for ACE and vWF demonstrated typical endothelial cell staining patterns. Both of these proteins are synthesized and secreted by endothelial cells and play a role in haemostasis (Auerbach *et al.*, 1982; Dorovin-Zis and Huynh, 1992). While the expression of ACE or vWF does not confirm endothelial cell identity exclusively (as they are expressed by other cell types), as a part of a panel of markers examining the endothelial cell phenotype they further strengthen the analysis. There are numerous other endothelial cell markers which may have been included within the analysis to identify the cell isolates. These include the expression of CD35, CD44, endoglin and EMAP-II as well as morphological features including the presence of a basal laminar (Augustin. Kozian and Johnson, 1994; Thorin and Shreeve, 1998). The combination of morphological observations (light and ultrastructural) with ACE, vWF, PECAM-1 and TNF-induced E-selectin immunolabelling are routinely used as the parameters examined to identity endothelial cells *in vitro* (Alessandri *et al.*, 1998a; 1998b; Griffioen *et al.*, 1996; 1996b; Hewett and Murray, 1993).

The endothelial cell markers PECAM-1 and TNF-stimulated E-selectin expression are also routinely investigated as indicators of endothelial origin. While PECAM-1 was not immunolocalized to cell junctions, the diffuse labelling over the plasma membrane is supported by Scholz and Schaper (1997). The selective and specific E-selectin expression on stimulated TAMECs is the strongest evidence for the endothelial cell origin of isolates as it is exclusively restricted to activated endothelial cells (Bevilacqua *et al.*, 1987). As Chapter 4 specifically examines endothelial cell adhesion molecule expression within the tumour-associated microvasculature, a detailed discussion of these adhesion molecules and their relative role within the tumour-associated microvasculature is contained within Chapter 4, Section 4.4.

The absence of ICAM-1 protein expression by TNF-stimulated TAMECs is supported by the lack of immunodetectable protein in tumour-associated microvessels *in vivo* (see Chapter 4, Section 4.3.4; Nilsson, Woods and Rofe, 1996). These findings and possible explanations for the absence of detectable ICAM-1 protein in tumour-associated

microvascular endothelial cells *in vivo* and *in vitro* is discussed in detail within the discussion of Chapter 4 (Section 4.4). In addition, the profile of ICAM-1 expression within the tumour-associated microvasculature includes examination of ICAM-1 gene and protein in TAMECs as well as *in vivo*. Briefly, the lack of ICAM-1 protein on TAMECs may be due to a downregulation of the translation of ICAM-1 mRNA into protein, growth factors within the tumour microenvironment suppress ICAM-1 protein expression and/or ICAM-1 is being cleaved from the endothelial cell membrane and released into the circulation.

The absence of staining for *Bandeiraea simplicifolia* BS-1 isolectin B_4, RECA-1 (Duijvestijn *et al.*, 1992) and TNF-induced ICAM-1 by TAMECs, while detected on cultured BDECs and microvessels in formalin-fixed, paraffin-embedded normal rat tissues, highlights the antigenic heterogeneity that exists between microvascular endothelial cells. The loss of these particular antigens on the tumour-associated vasculature may be due to the influence the tumour microenvironment has on their structure, function and/or phenotype.

3.5 SUMMARY

A method for isolating and culturing a purified preparation of tumour-associated microvascular endothelial cells has been developed. Microvascular endothelial cells were purified by their preferential adhesion to plastic and cultured in media supplemented with factors to enhance endothelial cell proliferation and minimize the growth of contaminating cell types. Morphological and immunohistochemical characterization of isolates confirms that the cells isolated and cultured are endothelial in origin. TAMECs grew as a homogenous monolayer that exhibited contact inhibition. Ultrastructurally the endothelial cells contained many organelles indicating a proliferative state as well as pinocytic vesicles and microfilaments. Using a panel of endothelial cell-specific antibodies, positive immunolabelling was observed for ACE, vWF, PECAM-1 and E-selectin (after TNF-α stimulation). ICAM-1 protein was not expressed by TAMECs constitutively or after TNF-α exposure and appears to be regulated by the tumour microenvironment. The absence of Bandeiraea simplicifolia BS-1 isolectin B_4 and RECA-1 from the tumour-associated microvasculature and their expression in normal microvascular endothelial cells demonstrates that particular antigens may be suppressed within tumour tissues. These

analyses demonstrated that TAMECs retain some of the phenotype and functions of endothelial cells in vivo, and in particular, of the tumour-associated microvasculature. TAMEC cultures will provide a readily available in vitro model to study the biology of tumour-associated microvascular endothelial cells as well as to evaluate potential anti-vascular therapies.

FIGURE 3.1

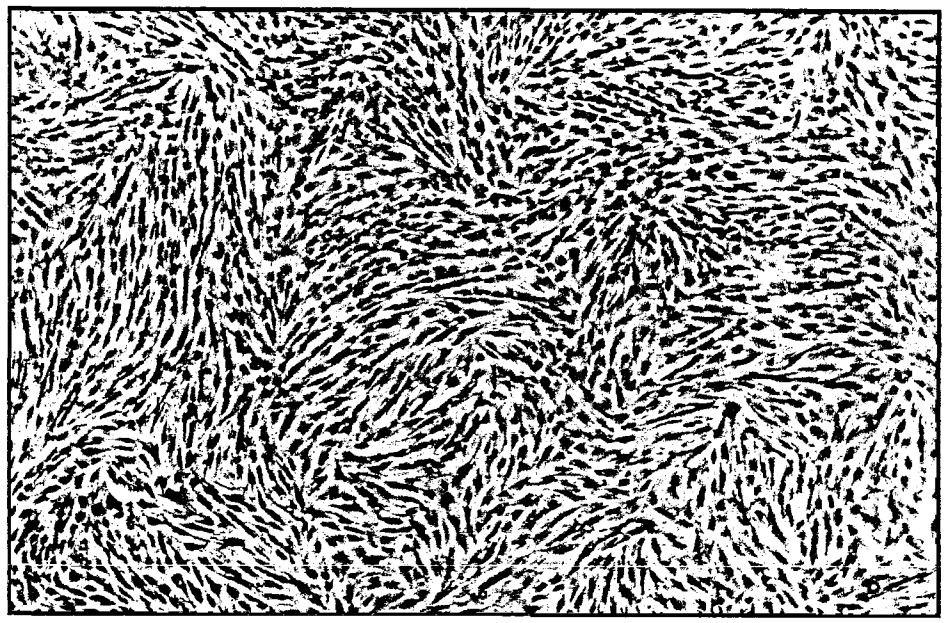
Morphology of TAMECs and BDECs

Morphological characterization of TAMECs and BDECs *in vitro*. Endothelial cells were cultured to confluence and assessed for growth patterns and purity by phase contrast microscopy.

A. Phase contrast micrograph of TAMECs displaying characteristic cobblestone morphology and radial growth patterns, ×120.

B. Phase contrast micrograph of BDECs displaying characteristic cobblestone morphology, ×120.

A



B



FIGURE 3.2

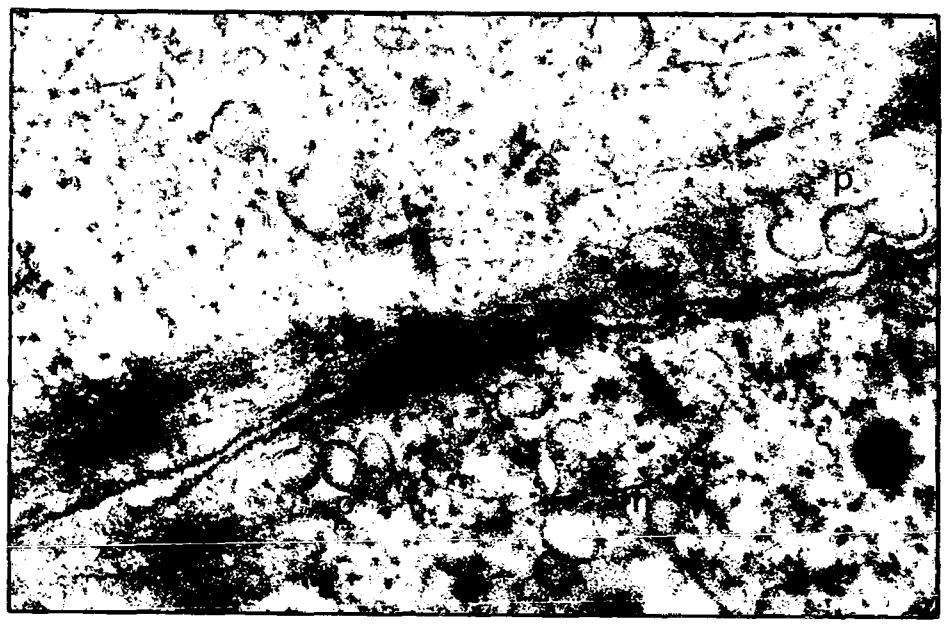
Ultrastructural features of TAMECs

Morphological characterization of TAMECs *in vitro*. Monolayers were processed and embedded in resin for ultrastructural analysis by electron microscopy as described in Section 3.2.6.2.

A. Electron micrograph of two apposed TAMECs containing pinocytic vesicles (p) and microfilaments (m), ×76000.

B. Electron micrograph of a TAMEC in which the nucleus (N), mitochondria (M), free ribosomes and microfilaments (m) are seen, ×20000.

A



B



FIGURE 3.3

Immunohistochemical and functional analysis of TAMECs

TAMECs were assessed for endothelial antigen expression by immunohistochemistry as described in Section 3.2.6.3. E-selectin and ICAM-1 expression were assessed on cultures stimulated with TNF-α for 4 and 24 h, respectively, as described in Section 3.2.6.3.1. In addition, ICAM-1 expression was examined in TNF-treated BDECs.

A. Anti-ACE antibody: label is distributed throughout the cytoplasm and along the surface of the endothelial cells, ×300.

B. vWF immunoreactivity is observed throughout the cytoplasm of TAMECs, ×450.

C. PECAM-1 immunostaining is localized throughout the cell cytoplasm, ×450.

D. Strong, vascular E-selectin immunostaining following stimulation of TAMECs with TNF-α (300 U/ml) for 4 h, ×300.

E. ICAM-1 staining is not immunodetectable on TAMECs stimulated with TNF-α (300 U/ml) for 24 h, ×300.

F. Strong immunoreactivity for ICAM-1 in BDECs stimulated with TNF-α (300 U/ml) for 24 h, ×300.

G. Negative control – mouse secondary antibody. A representative micrograph of a negative control in which the primary antibody was omitted and the immunohistochemical reaction performed with a biotinylated horse anti-mouse secondary antibody, ×300.

H. Negative control – rabbit secondary antibody. A representative micrograph of a negative control in which the primary antibody was omitted and the immunohistochemical reaction performed with a biotinylated goat anti-rabbit secondary antibody, ×300.

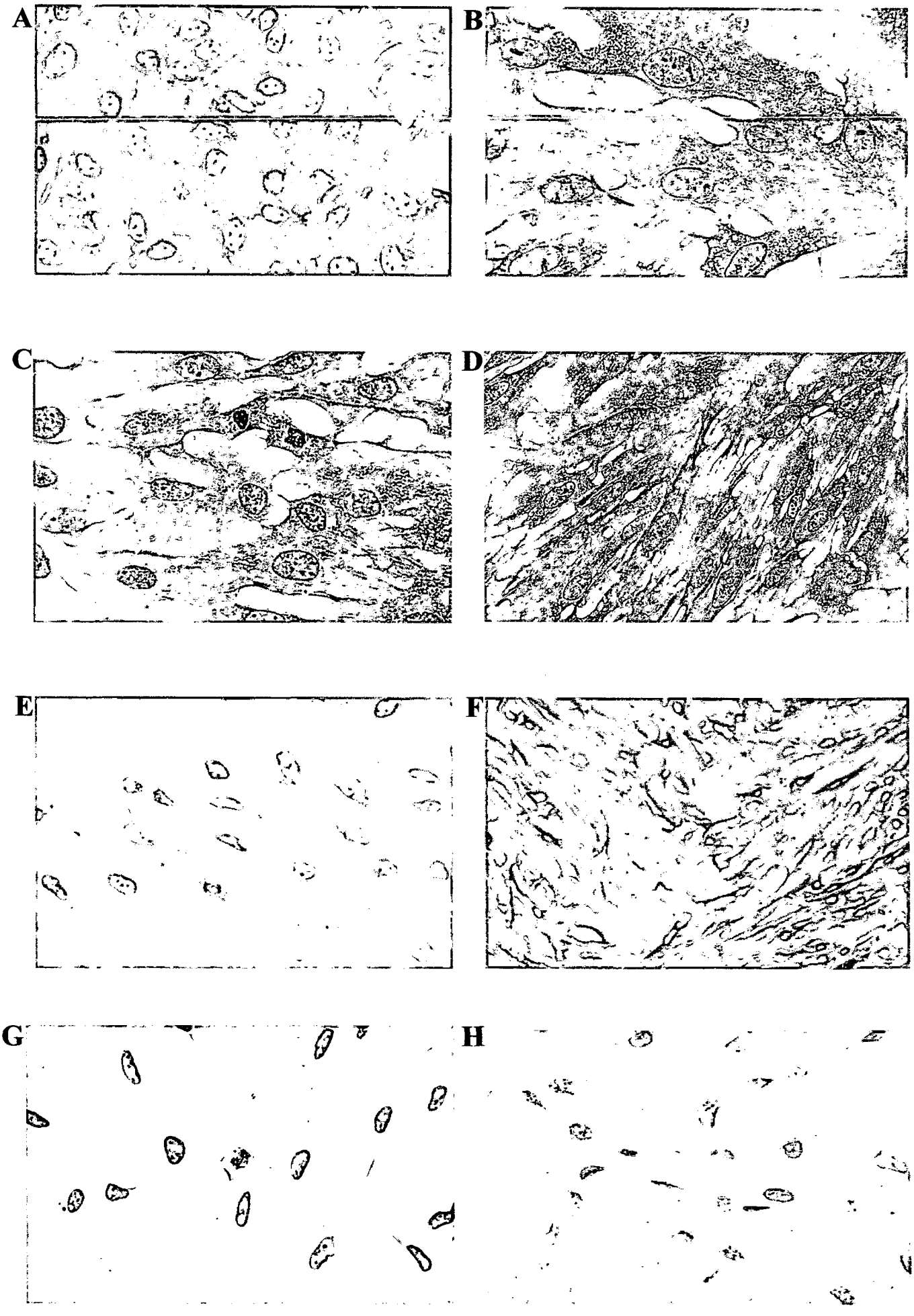
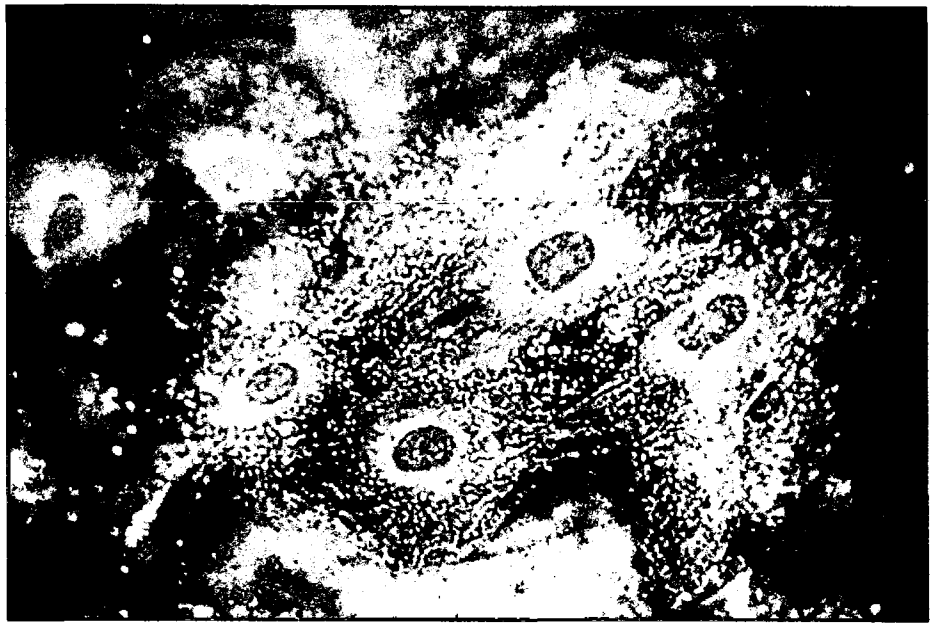

A
B
C
D
E
F
G
H

FIGURE 3.4

In vitro accumulation of DiI-Ac-LDL

TAMECs were assessed for the incorporation of DiI-Ac-LDL as described in Section 3.2.6.4.

A. Strong, granular cytoplasmic accumulation of DiI-Ac-LDL by endothelial cells, ×.



CHAPTER 4. EXPRESSION AND REGULATION OF THE CELL ADHESION MOLECULES, E-SELECTIN AND ICAM-1, ON THE TUMOUR-ASSOCIATED MICROVASCULATURE *IN VIVO* AND *IN VITRO*

4.1 INTRODUCTION

Interactions between leukocytes and endothelial cells are generally reduced in tumours compared to normal microvessels (Borgström *et al.*, 1997; Fukumura *et al.*, 1995; Wu *et al.*, 1992). It is widely acknowledged that tumour-associated microvascular endothelial cells are able to express cell adhesion molecules, however, in a number of tumours endothelial cell adhesion molecule expression appears to be suppressed possibly as a means to escape immune detection. Many factors within the tumour microenvironment are known to influence expression of cell adhesion molecules including cytokines, angiogenic growth factors and hypoxia.

Relatively little is known about the regulation and kinetics of endothelial cell adhesion molecule expression within the tumour-associated microvasculature *in vitro* or *in vivo*. In general, *in vitro* studies have utilized HUVECs (Griffioen *et al.*, 1996a; 1996b) or microvascular endothelial/endothelioma cell lines (Piali *et al.*, 1995; Stoelcker *et al.*, 1996) with the results of these studies being directly extrapolated to tumour-associated microvessels. Currently, only two groups have studied the expression of adhesion molecules by endothelial cells isolated from various neoplastic specimens (Table 4.1)(Alessandri *et al.*, 1998a; Chirivi *et al.*, 1999; Griffioen *et al.*, 1996a; 1996b). These groups report differing results in endothelial cell adhesion molecule expression compared to normal microvessels and also in their response to TNF-α stimulation. While ICAM-1 suppression has been demonstrated by Griffioen *et al.* (1996a; 1996b) in renal tumour-derived microvascular endothelial cells, studies to investigate the regulatory mechanisms influencing these differences were performed on HUVECs and dermis-derived endothelial cells. While the source of tissue for isolations may be a limiting factor, the validity of these endothelial cell populations as a model of the tumour-associated microvasculature should first be determined for each particular condition. Only once this has been confirmed are these cells able to be used as a routine model *in vitro* and their results discussed in terms of conditions and responses within the tumour microenvironment.

TABLE 4.1

E-selectin and ICAM-1 expression and regulation by TAMECs

Source of TAMECs	Adhesion molecule	Expression[a]	Tumour vessels[b]	Regulation[c]	Reference
Various human tumours	E-selectin	+	↔	↑[3]	Alessandri *et al.*, 1998a
Various human tumours	E-selectin	+	ND	ND	Chirivi *et al.*, 1999
Various human tumours	ICAM-1	+	↔	↔[3]	Alessandri *et al.*, 1998a
Various human tumours	ICAM-1	+	↓	ND	Chirivi *et al.*, 1999
Ovarian carcinoma	ICAM-1	+	↓	ND	Griffioen *et al.*, 1996b
Renal cell carcinoma	ICAM-1	+	↓	↓[3]	Griffioen *et al.*, 1996a

[a]Expression on tumour-associated microvascular endothelial cells, + = positive immunostaining detected.
[b]Compared to normal tissue-matched vessels, ND = not determined; ↔ = unchanged; ↓ = decreased; ↑ = upregulated expression.
[c]Regulation after exposure to TNF-α.

Diminished leukocyte adhesion in primary and passage one cultures of TAMECs isolated from a rat KMT-17 fibrosarcoma has been reported by Utoguchi *et al.* (1995). This resembles the effects observed *in vivo*, however, by passage four, these tumour-specific adhesion features were lost and the cells had reverted to a 'normal' endothelial adhesion profile (Utoguchi *et al.*, 1995). Notably, tumour-conditioned medium was not supplemented into these cultures and thus factors normally present in the tumour microenvironment which influenced the suppression of adhesion molecules may not have been available. The specific adhesion molecule(s) affected *in vitro* were not identified.

Few *in vivo* studies assessing the regulation of adhesion molecules within the tumour-associated microvasculature have been reported. Overall, constitutive and induced adhesion molecule expression appear to be quite variable and related to the tumour type. In the RM-1 murine prostate carcinoma model, both constitutive and TNF-induced cell adhesion molecule expression do not differ between normal and tumour-associated microvascular endothelial cells (Langley *et al.*, 1999). Following ILP with TNF-α, IFN-γ, melphalan and hyperthermia, endothelial E-selectin, ICAM-1 and VCAM-1 expression are induced/upregulated within the vasculature of sarcoma and melanoma specimens (Lejeune, Rüegg and Liénard, 1998; Renard *et al.*, 1994). In addition, morphological changes including endothelial cell swelling are observed as is neutrophil recruitment (Renard *et al.*, 1994).

Based on observations of the distribution of adhesion molecules in tumours, Suzuki *et al.* (1995) have classified tumour vessels into two types; those for inflammatory cell infiltration and those for nutrient supply. Immunologically inactive vessels comprise the majority of blood vessels within a tumour mass. Immunoreactive vessels, which express cell adhesion molecules, are generally found at the invasive margin. These vessels are characterized by endothelial cell swelling and narrowing of the lumen, features of immaturity (Suzuki *et al.*, 1995).

This chapter aims to examine whether the antitumour action of TNF-α are mediated through its regulation of endothelial cell adhesion molecules on tumour-associated microvascular endothelial cells. Specifically, are the endothelial cell adhesion molecules

E-selectin, ICAM-1 and PECAM-1 induced and/or upregulated by TNF-α? Using TAMEC cultures (documented in Chapter 3), the expression of E-selectin and ICAM-1 mRNA will be assessed by Northern blot analysis. The expression and TNF-stimulated regulation of the translated proteins will be analyzed in TAMECs by cell-based enzyme immunoassay. These *in vitro* observations will be extended to *in vivo* studies investigating the cell types expressing E-selectin and ICAM-1 in TNF-treated tumour tissues. mRNA and protein expression and regulation will be assessed by *in situ* hybridization and immunohistochemistry, respectively. In addition, the expression and inducibility of PECAM-1 protein will be examined *in vitro* and *in vivo*.

As there are numerous adhesion molecules expressed by endothelial cells, this study has been limited to those which play a key role in recruiting cytotoxic leukocytes to the tumour tissue. In addition, it is focussed ons those which are regulated by TNF-α. VCAM-1 was unable to be included due to the absence of an available antibody during the experimental period of this thesis. PECAM-1 was included within some of the studies as a marker of endothelial cells as it is constitutively expressed.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of RNA probes

RNA probes were designed in order to assess the expression of adhesion molecule mRNA by tumour-associated microvascular endothelial cells *in vitro* and *in vivo* by Northern blot analysis and *in situ* hybridization, respectively. Each molecular technique was performed as described in Sambrook, Fritsch and Maniatis (1989) or by the manufacturer of commercial kits. All molecular biology procedures used sterile MQ water. For RNA manipulations, MQ water was treated with diethyl pyrocarbonate (DEPC; Sigma) to prevent ribonuclease (RNase) degradation of RNA samples.

4.2.2 mRNA preparation

As a template for cDNA synthesis, mRNA was extracted from cultures of BDECs. mRNA for E-selectin amplification was isolated from BDECs stimulated with TNF-α (300 U/ml) for 3 h and for ICAM-1 from BDECs stimulated with TNF-α (300 U/ml) for 12 h. All cells for mRNA extraction were harvested (with trypsin/EDTA for adherent BDECs), washed in Ca/Mg-free HBSS, adjusted to 8×10^6 cells and pelleted (12000 × g/2 min).

4.2.2.1 *mRNA extraction*

mRNA was extracted using the QuickPrep™ *Micro* mRNA purification kit (Amersham Pharmacia Biotech Pty. Ltd.) according to manufacturer's instructions. Briefly, the cell pellet was homogenized in extraction buffer, elution buffer and centrifuged (12000 × g/1 min). The supernatant was transferred to a prespun oligo(dT)-cellulose tube, mixed gently by inversion for 3 min and centrifuged (12000 × g/10 s). The resin was washed five times in high salt buffer and then twice in low salt buffer. The slurry was transferred to a microspin column, washed in low salt buffer (3×) and mRNA eluted in prewarmed elution buffer by centrifugation (12000 × g/10 s).

4.2.3 Reverse-transcription polymerase chain reaction

cDNA was transcribed from each source of mRNA as directed in the First Strand cDNA synthesis kit (Amersham Pharmacia Biotech). mRNA (200 ng) was heated at 65°C for 10 min, chilled on ice and first strand buffer, 400 ng $pd(N)_6$ primer, 200 mmol/l dithiothreitol (DTT) and DEPC-treated water added and incubated for 2 h at 37°C. Reverse transcriptase was inactivated at 65°C (15 min).

cDNA was amplified by PCR using specific primers. Each PCR reaction contained template cDNA (200 ng), 200 ng of each primer (Table 4.2), 2.5 mmol/l each of dATP, dCTP, dGTP, dTTP (Promega), 2 (E-selectin) or 3 (ICAM-1) mmol/l $MgCl_2$ (Life Technologies Inc.), 2.5 U *Thermus aquaticus* (*Taq*) polymerase (Life Technologies), 10× *Taq* reaction buffer (Life Technologies) and sterile MQ water, to a final volume of 50 µl. The reaction mixture was overlaid with sterile mineral oil (Sigma) to ensure minimal evaporation during amplification of the template. cDNA was amplified in a Perkin Elmer Cetus TC1 thermal cycler (Perkin Elmer) using 35 cycles of 94°C for 1 min (denaturation), 58°C for 1 min (primer annealing) and 72°C for 1 min (primer extension). Positive (β-actin) and negative control (water) reactions were included in all amplifications.

4.2.3.1 *Design of synthetic oligonucleotides*

Synthetic oligonucleotides (Table 4.2) were produced using published primer sequences or designed to amplify regions containing the highest degree of conservation based on nucleotide homologies between the human, mouse and rat sequences. β-actin primers were

TABLE 4.2

Cell adhesion molecule PCR primer pair sequences

	Primer sequence	Size	Reference(s)
β-actin	$^{5'}$TAA AGA CCT CTA TGC CAA CAC AGT$^{3'}$	240 bp	Gaudette *et al.*, 1991
	$^{5'}$CAC GAT GGA GGG GCC GGA CTC ATC$^{3'}$		
E-selectin	$^{5'}$CAG TGT TCA AGC CTT GAC CT$^{3'}$	320 bp	Wang *et al.*, 1995
	$^{5'}$AAG ACT TTG GCA GCT ACT AGC$^{3'}$		Billups *et al.*, 1995
ICAM-1	$^{5'}$GCT GCC TAT CGG GAT GGT GAA GTC$^{3'}$	264 bp	
	$^{5'}$AGT TTT AGG GCC TCC TCC TGA GCC$^{3'}$		

kindly supplied by Ms Heather Rickard (University of South Australia) and were included in all PCR amplifications as a positive control. They were designed to amplify a 240 bp fragment of β-actin (GenBank accession number J00691) representing bases 2750–2990 of the published sequence as reported by Gaudette and Crain (1991). A 320 bp fragment of E-selectin (L25527) representing bases 1288–1608 of the published sequence, was amplified using primers based on the primers used by Billups *et al.* (1995) and Wang *et al.* (1995). A 264 bp fragment of ICAM-1 (D00913) representing bases 1379–1643 of the published sequence was amplified using primers designed to cross a transmembrane domain. Primer pairs were synthesized by the Molecular Pathology Unit, IMVS, using an Applied Biosystems 381A DNA synthesizer. Primers were extracted once with butanol to remove residual salts, dried and reconstituted in MQ water.

4.2.3.2 *Agarose gel electrophoresis*

To detect the presence of target DNA, a 1% (w/v) horizontal gel was prepared containing ultra pure DNA grade agarose (Promega) in 40 mmol/l Tris-acetate, 1 mmol/l EDTA, pH 8.0 (1× TAE). Gels were stained internally by addition of ethidium bromide (0.5 μg/ml) to the agarose prior to casting. Before loading, samples and appropriate DNA size markers were mixed with 0.1 volumes of DNA loading buffer (0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol FF, 0.4% (v/v) orange G, 10% (v/v) Ficoll® 400, 10 mmol/l Tris-HCl and 500 mmol/l EDTA; Promega) and electrophoresed in 1× TAE buffer. DNA fragments were visualized under longwave ultraviolet light using a transilluminator (UltraLum Inc.) with a CCD video camera module (Sony). Gel images were analyzed using the iPhotoPLUS imaging system, version 1.1 software (Ulead Systems Inc.), operating on an IBM compatible computer.

4.2.3.3 *Estimation of sizes of DNA*

The size of PCR products was estimated by comparing relative mobility to molecular weight markers. The 100 bp DNA ladder (Life Technologies) consisting of 15 fragments ranging in length from 100 to 1500 bp, at 100 bp increments, and an additional fragment at 2072 bp was used.

4.2.3.4 *Spectrophotometric determination of DNA concentrations*

DNA concentrations were determined using the GeneQuant II RNA/DNA Calculator

(Amersham Pharmacia Biotech). At 260 nm an absorbance of 1 corresponds to 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA and ~20 μg/ml for single-stranded oligonucleotides (Sambrook, Fritsch and Maniatis, 1989).

4.2.4 Ligation of PCR products into cloning vectors

PCR products were cloned into pGEM®-T (ICAM-1) or pGEM®-T Easy (E-selectin) vector systems (Promega) which incorporate both T7 and SP6 RNA polymerase sites. The PCR products were ligated using 50 ng vector, 7 μl PCR product, 3 Weiss U T4 DNA ligase, T4 DNA ligase 10× buffer and water, to a final volume of 10 μl. The reaction mixture was combined and incubated at 4°C ON. In parallel, positive (SPP-1 bacteriophage DNA restricted with *Eco*R I) and negative (water) control reactions were performed for each ligation.

4.2.5 Transformation of plasmid DNA into competent bacteria

4.2.5.1 *Cultivation of bacteria*

Solid and liquid Luria-Bertani (LB) media were used for the growth of bacterial cultures. All media were sterilized by autoclaving for 15 min at 121°C.

Broth

LB medium consisted of 1% (w/v) Bacto-tryptone (DIFCO Laboratories), 1.5% (w/v) Bacto-yeast extract (DIFCO) and 1% (w/v) NaCl, pH 7.0. Where necessary, 125 μg/ml ampicillin (Sigma) was added immediately before use.

Solid medium

The solid medium used for plating of cells was LB agar (LB containing 1.5% (w/v) Bacto-agar (DIFCO)). Ampicillin (100 μg/ml) was added to cooled molten media before pouring plates to culture bacteria containing recombinant plasmids.

4.2.5.2 *Transformation with competent E. coli*

Cells were transformed with plasmid DNA using either calcium- or electro-competent bacteria.

Electrocompetent DH5α cells were prepared for transformation with E-selectin plasmid

DNA according to the electroporator manufacturer's instructions (EC Apparatus Corp.). An ON shaken culture in SOB (2% (w/v) Bactotryptone, 0.5% (w/v) Bacto-yeast extract, 10 mmol/l NaCl, 2.5 mmol/l KCl, 10 mmol/l $MgCl_2$ and 10 mmol/l $MgSO_4$, pH 7.0.) was diluted 1:100 in prewarmed SOB and grown, with vigorous agitation, to mid log phase (OD_{550} 0.8). Cells were chilled on ice for 15 min and harvested by centrifugation (2500 × g/15 min/4°C). The pellet was washed twice in ice-cold sterile MQ water, recovered by centrifugation (2500 × g/15 min/4°C) and washed twice in ice-cold sterile 10% (v/v) glycerol (2500 × g/15 min/4°C). Electrocompetent cells were resuspended in sterile 10% glycerol to an optical density of 100-200_{550} U.

Prior to transformation, the E-selectin ligation mix was desalted in 2.5 volumes absolute ethanol, precipitated at -20°C, pelleted (12000 × g/5 min/4°C), washed (70% ethanol) and resuspended to the original volume with water. Desalted ligation mixture (2.6 µl) was added to electrocompetent cells (40 µl), gently mixed and the cell-DNA mixture added to a pre-chilled electroporation cuvette. The cuvette was placed in an EC100 electroporator (EC Apparatus) and an electric current (2800 V) pulsed through the cells. The cells were suspended in LB broth and allowed to recover, with shaking (225 rpm), at 37°C for 60 min. For colour selection of recombinant plasmids, 50 µmol/l isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, in water) and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Promega; in N,N'-dimethylformamide (DMF)) was added to the cells which were plated on LB/ampicillin plates and incubated at 37°C ON. Bacteria carrying recombinant plasmids were identified as white colonies. The typical transformation frequency obtained with electroporation was 1.2×10^5 colonies/µg DNA. Positive (pGEM) and negative (water) transformation controls were run in parallel to each reaction.

DH5α *Escherichia coli* (*E. coli*) cells were made calcium-competent for transformation with ICAM-1 plasmid DNA as follows (Brown *et al.*, 1979). An overnight shaken culture (in LB) was diluted 1:20 in prewarmed LB and grown to mid log phase (OD_{600} 0.6). Cells were chilled on ice for 15 min and harvested by centrifugation (6000 × g/15 min/4°C). The pellet was resuspended in 10 ml ice-cold 100 mmol/l $MgCl_2$, recentrifuged and resuspended in 2 ml ice-cold 100 mmol/l $CaCl_2$ and left on ice for 60 min before transformation.

Ligated ICAM-1 PCR product (2 µl) was added to calcium competent DH5α cells (100 µl), gently mixed and placed on ice for 30 min. The cell-DNA mixture was heated at 42°C for 2 min and placed on ice for a further 2 min. The cells were suspended in LB broth and allowed to recover at 37°C for 60 min, pelleted and resuspended in 100 µl LB and plated out on LB/Amp plates for colour selection. The typical transformation frequency obtained was 1×10^5 colonies/µg DNA.

4.2.6 Small scale plasmid purification

Small scale plasmid purification was employed for the isolation and purification of plasmid DNA from transformed bacteria. Single, white ampicillin-resistant bacterial colonies were inoculated in LB broth (5 ml), with ampicillin, in a rotary incubator/shaker (37°C/500 rpm/ON). Plasmid DNA was purified using reagents from the QIAGEN plasmid purification kit (QIAGEN Pty. Ltd.). Bacterial cells were centrifuged (3500 × g/10 min) and the pellet resuspended in resuspension buffer using a vortex mixer. The cells were lysed in lysis buffer, the tube inverted and incubated at room temperature for no longer than 5 min. The tube was placed on ice and 3 mol/l potassium acetate, pH 5.5, added to precipitate chromosomal DNA, RNA and protein. After centrifugation (12000 × g/5 min), the supernatant was extracted in an equal volume of phenol:chloroform (1:1), equilibrated in 10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0 (TE). After centrifugation (12000 × g/5 min), the upper aqueous layer was collected, extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged (12000 × g/5 min). Plasmid DNA was precipitated with 2.5 volumes chilled absolute ethanol (12000 × g/5 min/4°C), washed (70% ethanol), air dried and resuspended in sterile MQ water (50 µl).

4.2.6.1 *Restriction endonuclease digestion of plasmid DNA*

Cloned PCR fragments were restricted from pGEM®-T vector plasmid DNA using *Apa* I (Life Technologies) and *Pst* I (Life Technologies) restriction endonucleases and from pGEM®-T Easy vector plasmid DNA using *Eco*R I (Promega). DNA (1 µg) was added to a tube along with restriction endonuclease(s) (3 U), the appropriate restriction buffer (10× REACT 4 or 10× buffer H, respectively) and sterile MQ water, to a final volume of 20 µl. The samples were incubated for 4–16 hours at 37°C and the restriction digest product visualized on a 1% (w/v) agarose gel. One kb DNA ladder (Life Technologies) molecular

TABLE 4.3

Sequencing PCR primer sequences

M13 Universal sequencing primers

	Primer sequence	Source
Forward	$^{5'}$GTAAAACGACGGCCAGT$^{3'}$	Geneworks Pty. Ltd.
Reverse	$^{5'}$CACACAGGAAACAGCTATGACCATC$^{3'}$	Geneworks Pty. Ltd.

weight markers, which produce fragments of 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134, and 75 bp, were used to estimate the size of restricted DNA inserts.

4.2.7 Sequencing

Plasmid DNA was sequenced, in both the forward and reverse directions, by the Molecular Pathology Unit, IMVS, using an Applied Biosystems Model 370/373A DNA Cycle Sequencing System, GeneScan®. DNA (500 ng) was sequenced with 8 µl ABI PRISM™ Dye Terminator cycle sequencing ready reaction mix (Perkin Elmer Cetus), 3.2 pmol/l specific sequencing primers (Table 4.3) and sterile water, to a final volume of 20 µl. Target DNA was amplified in a thermal cycler using 30 cycles of 96°C for 30 s (denaturation), 50°C for 15 s (primer annealing) and 60°C for 4 min (primer extension).

To precipitate the sequencing product, absolute ethanol and 3 mol/l sodium acetate, pH 4.6, were added to 20 µl sequencing reaction. Sequencing products were precipitated on ice for 15 min, pelleted (12000 × g/15 min/4°C), washed and the DNA dried at 65°C for 10 min. Sequences were analyzed using the MacVector™ (Version 6.0) computer programme (Oxford Molecular Limited) to identify the origin of cloned DNA inserts.

4.2.8 mRNA detection by Northern blot and *in situ* hybridization

All steps up to posthybridization were performed in solutions treated with DEPC. Separate glassware was used for pre- and post-hybridization steps. Gloves were worn and changed frequently throughout the procedure. Glassware was RNase-treated by heating at 180°C overnight.

4.2.9 Probe labelling by *in vitro* transcription

RNA probes were synthesized with either ^{32}P-UTP or digoxigenin (DIG)-UTP for Northern blot and *in situ* hybridization, respectively.

4.2.9.1 *^{32}P-RNA probe generation by in vitro transcription*

The Riboprobe® *in vitro* transcripton system (Promega) was used to incorporate [α-^{32}P]dUTP (Geneworks) and was performed as detailed in the associated Promega technical manual. Antisense probes were generated from *Spe* I-linearized E-selectin and

ICAM-1 DNA with T7 RNA polymerase. Negative controls were sense probes generated from *Nco* I-linearized E-selectin and ICAM-1 DNA transcribed with SP6 RNA polymerase. Linearized template DNA (1 μg) was purified by phenol-chloroform extraction, ethanol precipitated and resuspended in DEPC-treated water. For transcription, the following components were added in the following order at RT: 5× transcription optimized buffer, 100 mmol/l DTT, 20 U RNasin® ribonuclease inhibitor, 2.5 mmol/l rATP, rCTP and rGTP, 100 μmol/l rUTP, 1 μg linearized DNA template DNA, 6 μmol/l [α-^{32}P]UTP (10 μCi/μl), and 20 U SP6 or T7 RNA polymerase. The transcription reaction was allowed to proceed for 60 min at 37°C. Template DNA was digested with 1 U RQ1 RNase-free DNase for 15 min at 37°C. Unincorporated nucleotides were removed using the QIAquick nucleotide removal kit (QIAGEN). Briefly, 10 volumes of buffer PN was added to the reaction buffer, applied to a QIAquick column, centrifuged (6000 × g/1 min) and washed with buffer PE (6000 × g/2 × 1 min). The column was centrifuged (12000 × g/1 min) and RNA eluted with 50 μl buffer EB (12000 × g/1 min). The incorporation of ^{32}P was assessed by adding 1 μl of labelled probe to 2 ml aqueous counting scintillant and counting in a 2200CA Tri-Carb® liquid scintillation analyzer (Packard). Probes giving a specific radioactivity of greater than 1×10^5 cpm from this dilution were used.

4.2.9.2 *DIG-RNA probe generation by in vitro transcription*

To incorporate DIG-UTP for *in situ* hybridization, the DIG RNA labelling kit (Roche Molecular Biochemicals) was used according to manufacturer's instructions. Antisense probes were transcribed from *Spe* I-cleaved E-selectin and ICAM-1 using T7 RNA polymerase. Sense probes were generated from *Nco* I-cleaved E-selectin and ICAM-1 using SP6 RNA polymerase. One μg of purified, linearized template DNA was added to 10× concentrated DIG RNA NTP labelling mix, 10× concentrated transcription buffer, 20 U RNase inhibitor, 40 U SP6 or T7 RNA polymerase and MQ water, to a final volume of 20 μl. The contents were mixed gently and incubated for 2 h at 37°C and 200 mmol/l EDTA, pH 8.0, added to inactivate the RNA polymerases. DIG-labelled transcripts were precipitated with 4 mol/l LiCl and prechilled absolute ethanol, mixed well and left for at least 60 min at -70°C. RNA was pelleted (13000 × g/15 min/4°C), washed (70% ethanol) and resuspended in DEPC-treated water for 30 min at 37°C. DIG-labelled RNA probes were divided into 15 ng aliquots in DEPC-treated water and stored at -70°C.

The incorporation of DIG-labelled nucleotides was assessed by dot blot as described in the Boehringer Mannheim (Roche Molecular Biochemicals) DIG user's filter hybridization technical manual (Grünewald-Janho *et al.*, 1996). Briefly, ten-fold dilutions from a 15 ng tube of labelled probe were prepared and spotted onto a polyvinylidene fluoride (PVDF) nylon membrane (Micron Separations Inc.) and UV cross-linked to the membrane in a Hoefer UVC 500 UV crosslinker (Amersham Pharmacia Biotech) at 1.2×10^6 μJ/cm^2. DIG-labelled riboprobes were detected by immunohistochemical and colourimetric development essentially as described for *in situ* hybridization (Section 4.2.13.3). DIG-labelled riboprobes producing colourimetric detection at less than or equal to 10 pg/μl were used for *in situ* hybridization studies.

4.2.10 RNA preparation and Northern blot analysis

Total and messenger RNA were extracted from TAMECs stimulated with TNF-α (500 U/ml) for 2 (E-selectin) or 4 (ICAM-1) h. mRNA was harvested using the QuickPrep™ *Micro* mRNA purification kit as previously described (Section 4.2.3.1). Total RNA was extracted from TAMECs using the QuickPrep™ Total RNA extraction kit (Amersham Pharmacia Biotech) according to manufacturer's instructions. Briefly, cells were detached with trypsin/EDTA, washed with PBS and resuspended in LiCl solution by vortexing. β-mercaptoethanol and extraction buffer were added, the sample homogenized to disrupt the cells, followed by cesium trifluoroacetate. After chilling, RNA was collected by centrifugation (12000 × g/ 15 min) and the protein coat and DNA in solution removed by vacuum aspiration. The RNA was washed in extraction buffer, LiCl solution and cesium trifluoroacetate, (12000 × g/10 min/4°C) followed by 70% ethanol. The pellet was air-dried, resuspended in DEPC-treated water and heated at 65°C for 10 min. RNA samples were concentrated with glycogen solution, 2.5 mol/l potassium acetate and absolute alcohol overnight at -20°C.

For electrophoresis, 5 μg mRNA or 20 μg total RNA was added to 10× MOPS (200 mmol/l 3-(N-morpholino)propanesulfonic acid, 50 mmol/l sodium acetate, 10 mmol/l EDTA, pH 7.0), 2.2 mol/l formaldehyde (BDH), 50% (v/v) formamide, deionized (Sigma), 1 mg/ml ethidium bromide and water, to a final volume of 10 μl. Samples were heated at 65°C for 10 min and snap cooled on ice for another 10 min prior to adding RNA loading dye (50% (w/v) glycerol, 1 mol/l EDTA, 0.25% (v/v) bromophenol blue and 0.25% (v/v) xylene

cyanol). Samples were separated by electrophoresing horizontally in a 1% (w/v) agarose/2.2 mol/l formaldehyde gel in 1× MOPS for 4 h at 40 V. The mRNA was transferred by capillary action onto a PVDF membrane with 10× SSC; the blot dried and UV cross-linked.

The Northern blot hybridization procedure was performed essentially as described in the Riboprobe® *in vitro* transcription kit (Promega) and Sambrook, Fritsch and Maniatis (1989). The membrane was prehybridized in 50% formamide, deionized, 6× SSC, 5× Denhardt's solution (Sigma), 0.5% (w/v) sodium dodecyl sulphate (SDS) and 100 mg/ml denatured (95°C, 10 min) and sheared herring sperm DNA (Sigma) for 15 min at 60°C min. Subsequently, prehybridization solution containing each ^{32}P-labelled RNA probe (denatured, 95°C, 5 min) was added and the membranes hybridized at 60°C ON. Blots were washed (2× SSC, 0.5% (w/v) SDS, RT, 5 min; 2× SSC, 0.1% SDS, 2×30 min, RT), covered with plastic wrap and exposed to X-ray film (Curix Blue HC-S Plus, Agfa-Gevaert N.V.) in a Hypercassette™ (Amersham) at -70°C for 1–10 days. Membranes were developed using an automated Agfa CP1000 X-ray developer (Agfa-Gevaert). If non-specific hybrids were detected, membranes were washed with increasing stringency (0.1× SSC, 0.5% SDS, 30 min, 37°C; 0.1× SSC, 0.5% SDS, 20 min, 65°C; 0.1× SSC, 20 min, 22°C). The size of the detected mRNA species in TAMECs was determined based on the relative migration of the 18S and 28S ribosomal RNA within the total RNA sample.

4.2.11 *In situ* hybridization

The *in situ* hybridization protocol is essentially as described by Komminoth (1996) with modifications described in the text.

4.2.11.1 *Preparation of slides*

Clean glass microscope slides were acid washed in Glassware cleaning solution (Ajax) for 10 min, rinsed in distilled water and coated with APES as described withiin Chapter 2, Section 2.3.1.

4.2.11.2 *Tissue preparation*

Formalin-fixed, paraffin-embedded sections were cut at 4 μm and 2 serial sections mounted side by side on acid-washed and APES-coated glass microscope slides. Paraffin

sections were dewaxed and rehydrated through graded alcohols to DEPC-treated MQ water.

4.2.11.3 *Hybridization*

Rehydrated sections were washed with DEPC-treated PBS (2×5 min), treated with DEPC-treated PBS containing 100 mmol/l glycine (BDH; 2×5 min) followed by DEPC-treated PBS containing 0.3% (v/v) Triton® X-100 (15 min). After washing in DEPC-treated PBS, tissues were transferred to a solution of DEPC-treated 10 mmol/l tri-sodium citrate, pH 6.0, and placed in a microwave oven and heat treated (Chapter 2, Section 2.4.2.1.1). At the completion of irradiation, the solution was rapidly cooled with DEPC-treated water, the slides washed in DEPC-treated PBS (2×5 min) and acetylated with 100 mmol/l triethanolamine (BDH) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (BDH; 2×5 min). Prehybridization buffer (4× SSC, 50% (v/v) formamide, deionized) was applied to sections for at least 10 min at 37°C.

Hybridization buffer (40% formamide, deionized, 10% (w/v) dextran sulfate (Sigma), 1× Denhardt's solution, 4× SSC, 10 mmol/l DTT (Progen Industries Ltd.), 1 mg/ml yeast t-RNA (Life Technologies) and 1 mg/ml denatured (95°C, 10 min) and sheared herring sperm DNA was prepared prior to draining prehybridization buffer from slides. Sections were incubated with 30 µl of hybridization buffer containing 5–10 ng DIG-labelled RNA probe, covered with RNase-free plastic coverslips and incubated at 52°C ON in a humid chamber.

Coverslips were removed by immersing slides for 5–10 min in 2× SSC. For post-hybridization washes (2× SSC, 2×15 min, 37°C; 1× SSC, 2×15 min, 37°C; 0.1× SSC, 2×30 min, 55°C), sections were agitated in a shaking water bath. For immunological detection, sections were washed in buffer 1 (100 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl) then blocked for 30 min with buffer 1 containing 0.1% (v/v) Triton® X-100 and 2% (v/v) normal sheep serum (NSS; Sigma). The blocking solution was decanted and the sections incubated with buffer 1 containing 0.1% (v/v) Triton® X-100, 1% (v/v) NSS and alkaline phosphatase conjugated sheep anti-DIG antibody (Fab fragments, 1:500; Roche Molecular Biochemicals) for 2 h. The slides were washed in buffer 1 (2×10 min), incubated with buffer 2 (100 mmol/l Tris-HCl, pH 9.5, 100 mmol/l NaCl, 50 mmol/l $MgCl_2$) and colour

solution (75 mg/ml nitroblue tetrazolium (NBT; Sigma) in 70% (v/v) DMF (Sigma)/50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) in 100% DMF), containing 1 mmol/l levamisole (L[-]-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole hydrochloride), added to the slides. Colour development was allowed to proceed in a humid chamber in the dark for 16–48 h: the reaction was stopped with buffer 3 (TE). The slides were rinsed in distilled water, lightly counterstained in 0.1% (w/v) nuclear fast red, rinsed and mounted in 90% (v/v) glycerol/TBS. Negative controls included the DIG-labelled sense riboprobe, omission of probe, omission of anti-DIG antibody and tissues known not to express the mRNA being probed for.

4.2.12 Enzyme immunoassay

The expression and regulation of the cell adhesion molecules E-selectin and ICAM-1 by TAMECs was quantitated by enzyme immunoassay as described in Klein *et al.* (1995). TAMECs were seeded into a 96-well plate (Sarstedt) and cultured as previously described. After TNF-α stimulation (50–500 U/ml, 0–48 h), monolayers were fixed with ethanol/methanol for 45 min, washed with PBS and stored at 4°C until analysis the next day. After washing, the cells were blocked with 1% (w/v) blocking buffer (Roche Molecular Biochemicals) in PBS containing 1% hydrogen peroxide for 60 min at 37°C. Adhesion molecule expression was assessed used the following antibodies (Appendix B): mouse anti-rat E-selectin (dilution 1:100) and mouse anti-rat ICAM-1 (1:100). Each primary antibody was applied, followed by the appropriate biotinylated linking antibody (1:300) and then horseradish peroxidase-conjugated streptavidin (1:500), each for 30 min. Monolayers were washed with PBS (3×5 min) following each incubation. Substrate development was performed with 2 mg/ml *o*-phenylenediamine (OPD; Sigma) with 0.015% (v/v) hydrogen peroxide in PBS for 45 min — to stop the reaction 3 mol/l sulphuric acid was added. Photometric evaluation was performed using a Titertek Mulitskan® MCC enzyme-linked immunosorbent assay (ELISA) plate reader (Flow Laboratories) at 492 nm.

Controls included cultures incubated in the absence of TNF-α and omission of the primary antibody. The constitutive expression of ICAM-1 on BDECs was used as an internal standard as detailed in Klein *et al.* (1995). This amount is defined as the 100% standard value and data obtained for each cytokine treatment and for each adhesion molecule was

related to this constitutive expression. Each dose response and time course experiment was performed three times (n=3) from TAMECs from three independent isolations.

4.2.13 Immunohistochemistry

The immunoperoxidase technique (Chapter 2, Section 2.4.2) was used to localize cell adhesion molecules *in vitro* and *in vivo*. Anti-E-selectin (1:200), anti-ICAM-1 (1:100) and goat anti-mouse PECAM-1 (1:200, Santa Cruz)(Appendix B) were applied to the tissue sections or TAMECs. *In vitro* studies were performed on TAMECs stimulated with TNF-α (300 U/ml) for 0–24 h. *In vivo* analyses were on formalin-fixed, paraffin-embedded tissues processed from rats injected intratumourally with TNF-α (5×10^4 U/ml, 0–24 h). Microwave-induced (Chapter 2, Section 2.4.2.1.1) antigen retrieval was used to expose ICAM-1 antigens and boric acid-mediated antigen retrieval (Chapter 2, Section 2.4.2.1.2) for E-selectin and PECAM-1.

4.3 RESULTS

4.3.1 Adhesion molecule mRNA expression and regulation in TAMECs *in vitro* – Northern blots

The mRNA expression of E-selectin and ICAM-1 by TAMECs was assessed by Northern blot hybridization. Specific ^{32}P-labelled RNA probes were used to detect cell adhesion molecule mRNA.

The E-selectin gene migrated at a size of approximately 3.9 kb and was detected in TAMECs treated with TNF-α (500 U/ml) for 2 h (Figure 4.1A). ICAM-1 gene expression was detected in TAMECs after exposure to TNF-α (500 U/ml) for 4 h. The gene transcript was detected at approximately 3 kb, with minor transcripts also at 1.9 and 2.4 kb (Figure 4.1B). Hybridization using the sense probe did not detect any E-selectin or ICAM-1 mRNA species — non-specific hybrids were removed with stringent washing.

4.3.2 Adhesion molecule mRNA expression and regulation *in vivo* – *in situ* hybridization

The spatial and temporal expression and regulation of the genes encoding E-selectin and ICAM-1 was assessed within tumour tissues by *in situ* hybridization. Gene expression was visualized as purple/black cytoplasmic staining in positive cells.

The *in situ* hybridization protocol was performed essentially as described by Komminoth (1996) with some variations to increase probe penetration and the detection of specific hybrids. Microwaving sections in tri-sodium citrate buffer (Chapter 2, Section 2.4.2.1.1) was more effective in facilitating probe penetration than permeabilizing tissues with proteinase K (20 μg/ml, 30 min, 37°C). In addition, increasing the hybridization temperature from 42°C to 52°C enhanced specific hybridization of probes with target mRNA. During post-hybridization washes, the RNase step (20 μg/ml RNase A in 500 mmol/l NaCl, 10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0), 30 min, 37°C, between stringent washes with 1× SSC and 0.1× SSC) was omitted as it was found that it decreased specific hybridization signals and it was not necessary to digest any single-stranded (unbound) RNA probe. The hybridization conditions described above were found to be appropriate for all of the DIG-labelled RNA probes applied for *in situ* endothelial cell adhesion molecule studies.

E-selectin mRNA was not detected in tumour-associated microvascular endothelial cells within unstimulated tumours (Figure 4.2A). Following intratumoural injection of 5×10^4 U/ml TNF-α, reactivity for E-selectin mRNA was detected within 1-2 hours. Expression was maximal at 4h as strong, linear staining of the tumour-associated microvasculture. Positive labelling was not evident in every endothelial cell within a given vessel or in every vessel of the tumour. Expression was mainly localized to microvessels within the outer portion of tumour tissue and close to the tumour/host interface (Figure 4.2B). Hybrids were not detected in the vessels within the central regions of the tumour. Expression was no longer observed in the sections exposed to TNF-α for greater than 4 h. The antisense probe did not hybridize with any cell types other than tumour-associated microvascular endothelial cells. This localization signifies the specificity of the riboprobe since E-selectin is exclusively expressed by (activated) endothelial cells (Bevilacqua *et al.*, 1987). Sense E-selectin probes did not hybridize to the tumour-associated microvascular endothelial cells (Figure 4.2C).

Constitutive ICAM-1 mRNA was not detected in untreated tumour-associated microvascular endothelial cells. Intratumoural administration of TNF-α (5×10^4 U/ml) for 24 h, induced strong, uniform staining for ICAM-1 mRNA within tumour-associated

microvascular endothelial cells (Figure 4.3A). Staining was first observed 8 h post-TNF-α administration and increased reaching maximal intensity at 16–24 h. ICAM-1 mRNA vascular reactivity was observed in all tumour-associated microvascular endothelial cells of every vessel, irrespective of location within the tumour. In addition to vascular staining, ICAM-1 mRNA was also induced in fibroblasts and tumour-infiltrating leukocytes (Figure 4.3C). Sense ICAM-1 probes did not strongly hybridize to any cell type within the tumour population (Figures 4.3B, 4.3D).

4.3.3 *In vitro* expression and regulation of cell adhesion molecules in TAMECs following TNF-α administration

The *in vitro* regulation of E-selectin and ICAM-1 protein expression by TNF-stimulated TAMECs was assessed by immunohistochemistry and cell-based enzyme immunoassay. The expression of PECAM-1 by TNF-stimulated TAMECs was included in the immunohistochemical investigations. These results have been previously presented and discussed in Chapter 3, Section 3.3.4.2. In the latter, bound antibodies were developed with OPD and expression analyzed on an ELISA plate reader.

Constitutive E-selectin protein was not detected in TAMECs by immunohistochemistry or enzyme immunoassay. Following stimulation with TNF-α (300 U/ml) for 4 h, E-selectin was identified immunohistochemically in TAMECs (discussed in Chapter 3, Section 3.3.4.2, see Figure 3.3D). By cell-based enzyme immunoassay, E-selectin was detected within 2 h of exposure to TNF-α (300 U/ml) with maximal expression occuring at 4 h post-TNF-α administration. Protein levels remained elevated up to 8 h and then declined over the 24 h experimental period (Figure 4.4A) – following a similar profile of expression as observed *in vivo*. After exposure to TNF-α for 4 h, detectable levels of E-selectin were consistent across a dose range of 100–500 U/ml (Figure 4.4B).

ICAM-1 expression was not detected in untreated TAMEC cultures by immunohistochemistry or enzyme immunoassay. Stimulation with TNF-α (50–500 U/ml) for 0–48 h *in vitro* was ineffective at inducing ICAM-1 expression as detected by immunohistochemistry (discussed in Chapter 3, Section 3.3.4.2, see Figure 3.3E). Minimal expression was detected on TNF-treated TAMECs by cell-based enzyme immunoassay (Figures 4.5A, 4.5B). ICAM-1 expression was readily detected on BDECs constitutively

and also after exposure to TNF-α for 24 h.

4.3.4 *In vivo* expression and regulation of cell adhesion molecules on the tumour-associated microvasculature following TNF-α administration

Adhesion molecule protein expression was localized *in vivo* by immunohistochemistry. Boric acid (E-selectin and PECAM-1) or microwave-citrate (ICAM-1)-mediated antigen retrieval prior to primary antibody application was performed to expose antigenic sites. Bound antibodies were visualized with DAB and highlighted as a brown deposit.

Constitutive E-selectin protein expression was not detected in any vessels of the untreated tumour tissues examined (Figure 4.6A). After stimulation with TNF-α (5×10^4 U/ml), expression was first evident within 2 hours and was stained intensely after 4 h (Figure 4.6B). Immunostaining was present in every endothelial cell of each stained vessel, irrespective of size or vessel type. There was no variation in staining within areas of the tumour, including the tumour centre and tumour periphery nor in the host tissue surrounding the tumour. Endothelial cells in necrotic areas reacted strongly even though few viable tumour cells surrounded the vessels (Figure 4.6C). By 16 h after treatment with TNF-α, E-selectin was no longer detectable. Negative controls on a serial section on the same slide demonstrated no positive immunoreactivity (representative section in Figure 4.8B).

Constitutive ICAM-1 expression was not detected within the endothelial cells of untreated tumour tissues. After TNF-α stimulation (5×10^4 U/ml), strong, specific ICAM-1 staining was detected within host microvessels in the connective tissue surrounding the tumour (Figures 4.7A, 4.7B). In contrast, staining for ICAM-1 in tumour-associated microvascular endothelial cells was not detected at any of the time points studied (30 min–24h)(Figures 4.7A, 4.7C). Also, in tumours undergoing TNF-induced haemorrhagic necrosis, strong, granular ICAM-1 staining was observed in tumour-infiltrating cells which displayed large nuclei containing prominent nucleoli. These cells were located within the necrotic tissue and especially at the margin between the viable and necrotic tumour cells (Figure 4.7D). Although not specifically identified, these cells are likely to be activated tumour-associated macrophages, which are known to express ICAM-1 protein (van de Stolpe and van der Saag, 1996). Negative control sections demonstrated no immunoreactivity (representative

section in Figure 4.8C).

PECAM-1 immunolabelling was present in all tumour-associated microvascular endothelial cells. Expression was strong in every endothelial cell of every vessel, irrespective of location within the tumour tissue (Figure 4.8A). The expression and distribution of PECAM-1 was not influenced by TNF-α stimulation *in vivo* (5×10^4 U/ml, 30 min–24 h). Negative control sections demonstrated no immunoreactivity with the detection reagents (Figure 4.8B).

4.4 DISCUSSION

Endothelial cells generally exist in the vasculature as a quiescent population with a very low turnover rate. In response to an inflammatory stimulus, various genes are induced or upregulated by endothelial cells. One of the major gene groups involved in this process is that which regulates expression of the cell adhesion molecules, which themselves play a key role in the infiltration of leukocytes into tissues. Within tumours, enhanced surface expression of adhesion molecules potentially provides a means for mounting an immune response against the tumour.

E-selectin mRNA was readily detected by Northern blot analysis in TNF-stimulated TAMECs and was restricted to tumour-associated microvascular endothelial cells at the tumour periphery by *in situ* hybridization. The size of the transcript is consistent with previous reports in microvascular endothelial cells (Bevilacqua *et al.*, 1989). The induction and disappearance of cell surface E-selectin is primarily regulated by the initiation and termination of E-selectin gene transcription (Whelan *et al.*, 1991). *In vitro*, E-selectin gene transcription and mRNA accumulation closely parallel the expression of protein on the endothelial cell surface (Boyle *et al.*, 1999). Immunohistochemical staining of tissue sections for E-selectin revealed rapid and specific protein induction within 4 h of TNF-α administration both *in vivo* and *in vitro*. Immunostaining in the absence of mRNA signals, as observed in the central tumour-associated microvessels in the present study suggests a difference in the respective half-lives of the protein and mRNA species — low mRNA levels are not detectable by *in situ* hybridization, and high protein levels are readily detected by positive immunostaining. To enhance E-selectin gene detection within the tissue sections it may be necessary to amplify the mRNA contained within the tumour-

associated microvascular endothelial cells by *in situ* RT-PCR (Nuovo, 1995).

The accumulation of ICAM-1 on the cell surface is a delayed event requiring new RNA and protein synthesis (Dustin *et al.*, 1986; Springer, 1990; Wertheimer *et al.*, 1992). The rate of endothelial ICAM-1 transcription and translation are similar *in vitro* and *in vivo* as well as in cells isolated from different tissues (Fries *et al.*, 1993; Gerritsen *et al.*, 1993; Panés *et al.*, 1995; Wertheimer *et al.*, 1992). In the current study, ICAM-1 mRNA was detected in TNF-treated specimens *in vitro* by Northern blot analysis as three transcripts. Their sizes are consistent with the published size of the ICAM-1 gene (Wertheimer *et al.*, 1992). The significance of the individual ICAM-1 transcripts is unknown as they do not give rise to different proteins (van de Stolpe and van der Saag, 1996). *In vivo*, transcripts were first detected 4 h post-TNF-α administration and at 24 h levels still remained high. Nevertheless ICAM-1 protein could not be demonstrated in TAMECs or in tumour-associated microvascular endothelial cells constitutively or after exposure to TNF-α.

The transcription of both E-selectin and ICAM-1 genes is mediated by NF-κB (Wilson *et al.*, 1999). Since in the present model TNF-α induces E-selectin, it appears that the control of ICAM-1 expression is occurring posttranscriptionally. This is in contrast to the majority of mechanisms regulating vascular ICAM-1 expression which predominantly control production at the transcriptional level (Renkonen *et al.*, 1992; van de Stolpe *et al.*, 1994). Growth factor-induced ICAM-1 suppression in TAMECs occurs through downregulation of the steady-state of mRNA level of the gene (Griffioen *et al.*, 1996b).

There are several potential mechanisms that may prevent the efficient translation of TNF-induced ICAM-1 transcripts. The 3'-untranslated region (UTR) of ICAM-1 mRNA participates negatively in protein expression (Shida *et al.*, 1999). This region contains multiple destabilizing AU-rich elements (AU-R, typically AUUUA repeats) which affect mRNA steady-state level in two ways; by post-transcriptional regulation (most likely mRNA instability) and transcriptional regulation (Shida *et al.*, 1999). However, the precise relationship between inhibition of ICAM-1 translation but not its transcription within the tumour remains unclear.

Novel tissue-specific pathways regulate the function of endothelial cells in response to

signals derived from their local microenvironment (Aird *et al.*, 1997). Within the tumour these may be controlled through tumour-host interactions, cell-cell contacts, the release of tumour-derived factors and conditions within the tumour microenvironment. Within the tumour-associated microvasculature of hepatic (Liliensiek *et al.*, 1998) and lung (Piali *et al.*, 1995) cancers, gene expression is under the control of tumour cells and the tumour-mediated immune response, in particular T cells (Liliensiek *et al.*, 1998). Dedifferentiation and metastasis also contribute to the phenotype of tumour and tumour-associated microvascular endothelial cells (Chott *et al.*, 1999; Dorudi *et al.*, 1993). Hypoxia reduces adhesion molecule expression and facilitates tumour cell detachment (Hasan *et al.*, 1998). As a consequence, the induction or suppression of genes and/or protein species occurs within tumour tissues.

Environmental control of transcription and translation occurs at multiple levels and modulates the expression of mRNA and protein species together or independently. Often alterations to protein expression occur in response to alterations to gene transcription (Fenhalls *et al.*, 1999). In other circumstances, substantial amounts of mRNA are transcribed yet there is a failure to synthesize and/or express functional protein (Luss *et al.*, 1997). Within tumours, post-transcriptional regulation suppresses the production and/or expression of the γ2 subchain of laminin 5 during the progression from premalignant prostate tissue to invasive carcinoma (Hao *et al.*, 1996) and IL-2 expression in tumour-infiltrating lymphocytes by human breast cancer cells (Lopez *et al.*, 1998).

Neoplasms produce many factors that support progression by stimulating angiogenesis as well as protecting the tumour from exposure to the immune system. For instance, along with angiogenic growth factors bFGF and VEGF, tumours are known to produce transforming growth factor (TGF)-β which facilitates tumour progression through angiogenesis, immunosuppression and changes in the extracellular matrix (de Visser and Kast, 1999). Reduction in endothelial cell adhesiveness is a critical component of the immunosuppressive action of TGF-β (Fabry *et al.*, 1995). Cytokine-induced endothelial cell activation and lymphocyte migration are inhibited by TGF-β_1 and TGF-β_2 (Chen *et al.*, 1997a; 1997b; Doreduffy *et al.*, 1994; Fabry *et al.*, 1995). Additionally, TGF-β downmodulates adhesion molecule expression by decreasing endothelial TNF receptors (Weiss, Cuff and Berman, 1999). Previous reports demonstrate that TGF-β does not

influence endothelial ICAM-1 expression (Chen *et al.*, 1997b; Gamble, Khew-Goodall and Vadas, 1993).

It is also conceivable that ICAM-1 may be translated but is subject to modification or rapid degradation. Reduced translation and/or degradation of the newly translated protein result in a rapid disappearance of newly synthesized receptor protein (Seth *et al.*, 1999). ICAM-1 protein expressed at the endothelial cell surface can also be rapidly cleaved, resulting in the release of sICAM-1. Basal cell carcinoma cells shed ICAM-1 receptors that are never detectable on the cell surface (Kooy *et al.*, 1998). Therefore, ICAM-1 may be produced and rapidly cleaved from the endothelial cell surface to fulfil a role in tumour angiogenesis (Gho, Kleinman and Sosne, 1999) and not for mediating the infiltration of leukocytes. Once the vasculature has been established/expanded, endothelial cell adhesion molecule expression may be downregulated as angiogenesis is no longer occurring to the same extent. This reduction would also prevent the infiltration of leukocytes and promote the expansion of the tumour. The involvement of this mechanism could be determined by measuring sICAM-1 levels *in vivo* and *in vitro*.

The absence of ICAM-1 protein expression by tumour-associated microvascular endothelial cells represents a possible means to decrease the infiltration of cytotoxic leukocytes into the tumour tissue. This study represents the first to directly examine ICAM-1 gene and protein expression within the tumour-associated microvasculature by *in situ* hybridization and immunohistochemistry, respectively. Previously, analyses have been limited to immunohistochemistry and when mRNA was examined it was through RT-PCR and/or Northern blot hybridization. Direct examination of gene and protein expression in serial sections has revealed that post-translational mechanisms within the tumour appear to be regulating the absence of functional ICAM-1. In renal cell carcinoma-derived microvascular endothelial cells, growth factors suppress the steady state levels of ICAM-1 mRNA and results in decreased ICAM-1 protein expression (Griffioen *et al.*, 1996b). In this tumour model, ICAM-1 mRNA is responsive to TNF-α stimulation It is only when the mRNA is translated into functional protein is its expression within the tumour-associated microvasculature disturbed.

As these observations were made *in vitro* as well as *in vivo* further strengthen these

observations regarding ICAM-1 gene and protein expression and regulation. Previously the majority of cell adhesion molecule studies of the tumour-associated microvasculature were performed on normal microvascular endothelial cells with attempts to induce the tumour microenvironment through the addition of angiogenic growth factors and/or tumour-conditioned medium (Griffioen *et al.,* 1996a; 1996b). In the limited adhesion molecule studies to be performed on cultures of tumour-associated microvascular endothelial cells, ICAM-1 protein was expressed but generally at a lower level compared to normal microvascular endothelial cells (Alessandri *et al.,* 1998a; Chirivi *et al.,* 1999; Griffioen *et al.,* 1996a; 1996b). Further examination into the mechanisms regulating this suppression were performed on normal microvascular endothelial cells (Griffioen *et al.,* 1996a; 1996b). This study represents the first report on the possible mechanisms regulating the lack of detectable ICAM-1 within the tumour-associated microvasculature.

From the absence of ICAM-1 expression, it may be expected that the infiltration of leukocytes into this tumour model may be decreased. In contrast, previous studies using this tumour model have demonstrated significant numbers of tumour-associated macrophages (Nilsson, 1995; Nilsson, Woods and Rofe, 1996). Following LPS administration, the number of tumour-associated macrophages (detected with an anti-macrophage antibody) significantly increased over 24 h. This observation was associated with a similar absence of ICAM-1 expression within the tumour-associated microvasculature. This suggests that these cells gain entry into the tumour cells via TNF-damaged endothelial cells or by interaction with the normal host vessels incorporated in or peripheral to the tumour mass. TNF-induced disruption of the tumour-associated microvasculature has been demonstrated in this current study and these findings are presented in Chapter 6. Briefly, tumour-associated microvascular endothelial cells are susceptible to TNF-induced apoptosis and this is associated with the observation of erythrocytes and leukocytes moving into the tumour tissue.

4.5 SUMMARY

Analysis of the cell adhesion molecules E-selectin, ICAM-1 and PECAM-1 reveals differential expression for each molecule by tumour-associated microvascular endothelial cells. E-selectin mRNA and protein are detected within tumour-associated microvessels in culture and in situ. Expression was induced by TNF-α and was maximal 4 h after cytokine

treatment. While E-selectin mRNA expression was restricted to vessels close to the tumour periphery, protein was immunodetected in all endothelial cells. This observation may reflect differences in the half-life of each respective species. Post-transcriptional regulation appears to influence ICAM-1 protein expression within the tumour-associated microvessels. While ICAM-1 mRNA was readily detected in vivo and in vitro, after TNF-α stimulation, protein expression was not demonstrated within tumour-associated microvascular endothelial cells and distribution was restricted to normal host vessels surrounding the tumour mass. The posttranscriptional control of ICAM-1 protein has not been previously demonstrated, in normal or tumour-associated microvascular endothelial cells.

FIGURE 4.1

E-selectin and ICAM-1 mRNA expression by TAMECs *in vitro*

TAMECs were stimulated *in vitro* with TNF-α (500 U/ml) for 2 (E-selectin) or 4 (ICAM-1) h. Total and messenger RNA were extracted as described in Section 4.2.10. RNA samples were separated on a 1% agarose/2.2 mol/l formaldehyde gel and transferred to PVDF membrane. The nylon was probed with specific ^{32}P-labelled RNA probes for E-selectin and ICAM-1 and hybrids detected by exposure to X-ray film.

A. Expression of E-selectin mRNA by TAMECs after exposure to TNF-α for 2 h. A single gene transcript is observed at approximately 3.9 kb.

B. Expression of ICAM-1 mRNA by TAMECs after exposure to TNF-α for 4 h. A major gene transcript is detected at approximately 3 kb, with minor transcripts also at 1.9 and 2.4 kb.

A

Total mRNA



B

Total mRNA



FIGURE 4.2

E-selectin mRNA expression by tumour-associated microvessels *in vivo*

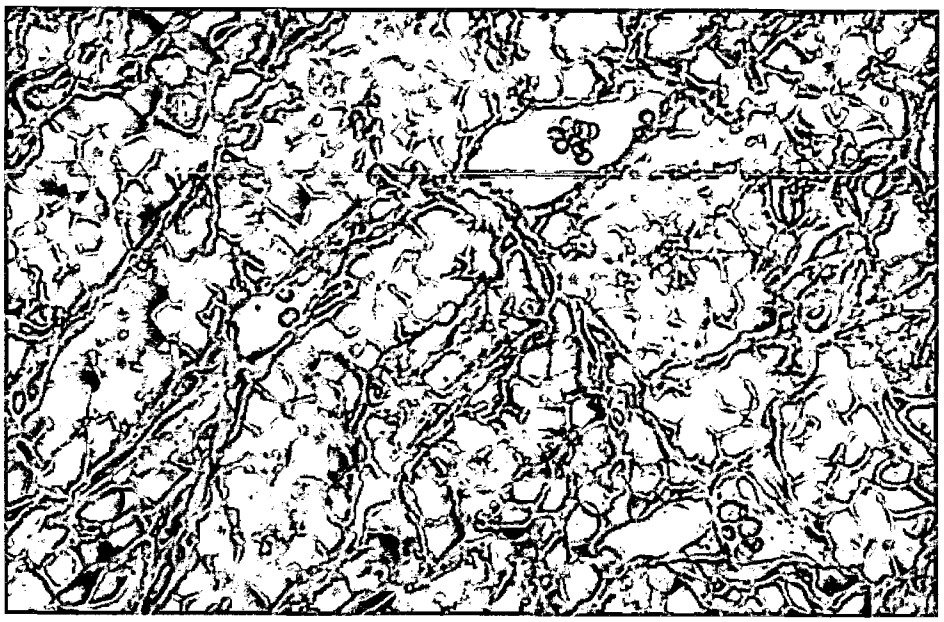
E-selectin mRNA was immunolocalized within formalin-fixed, paraffin-embedded tumour tissues by *in situ* hybridization. Tumour sections were treated with an anti-sense DIG-labelled probe generated against E-selectin. A control serial section on which the sense probe was applied was run in parallel on each slide — this serves as the negative control. Probe-mRNA hybrids were revealed with an anti-DIG antibody and visualized with NBT/BCIP.

A. Untreated tumour treated with the anti-sense E-selectin probe. Constitutive E-selectin mRNA expression is not detected within tumour-associated microvascular endothelial cells, ×460.

B. Tumour tissue stimulated with TNF-α for 4 h *in vivo* treated with the anti-sense E-selectin probe. Specific mRNA expression is detected within tumour-associated microvascular endothelial cells, ×460.

C. Serial section of the tissue in Figure B treated with the sense E-selectin probe. No non-specific hybrids are detected, ×460.

A



B



C



FIGURE 4.3

ICAM-1 mRNA expression by tumour-associated microvessels *in vivo*

ICAM-1 mRNA was immunolocalized within formalin-fixed, paraffin-embedded tumour tissues by *in situ* hybridization. Tumour sections were treated with an anti-sense DIG-labelled probe generated against ICAM-1. A control serial section on which the sense probe was applied was run in parallel on each slide — this serves as the negative control. Probe-mRNA hybrids were revealed with an anti-DIG antibody and visualized with NBT/BCIP.

A. Tumour tissue stimulated with TNF-α for 24 h *in vivo* hybridized with the anti-sense ICAM-1 probe. Strong, linear labelling of tumour-associated microvascular endothelial cells is detected, ×120.

B. Serial section of the tissue in Figure A hybridized with the sense ICAM-1 probe. No non-specific hybrids are detected, ×120.

C. Tumour tissue stimulated with TNF-α for 24 h *in vivo* hybridized with the anti-sense ICAM-1 probe. Strong linear labelling is seen in the tumour-associated microvascular endothelial cells as well as in fibroblasts and tumour-infiltrating leukocytes, ×650.

D. Serial section of the tissue in Figure C hybridized with the sense ICAM-1 probe. No non-specific hybrids are observed, ×650.

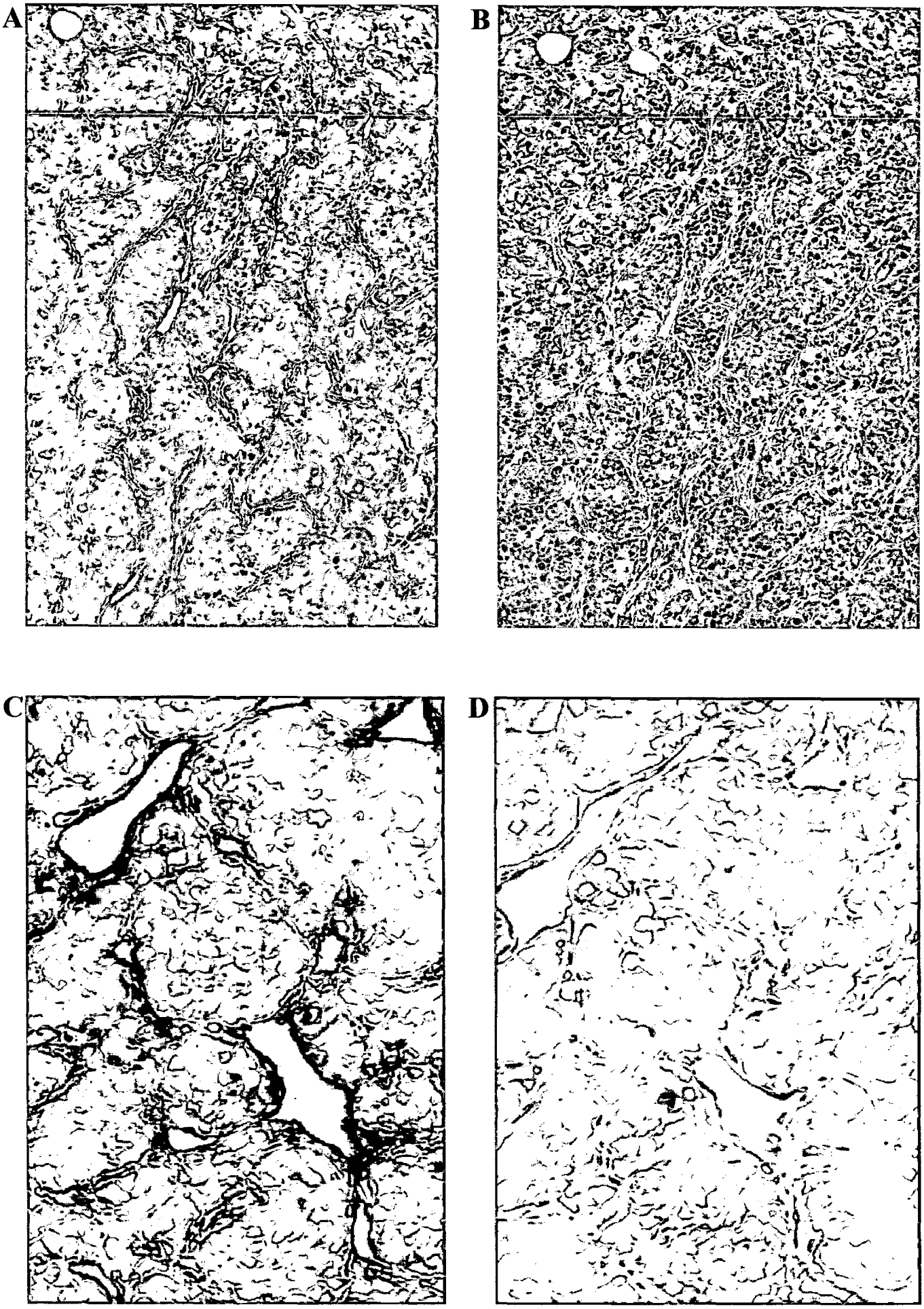

A
B
C
D

FIGURE 4.4

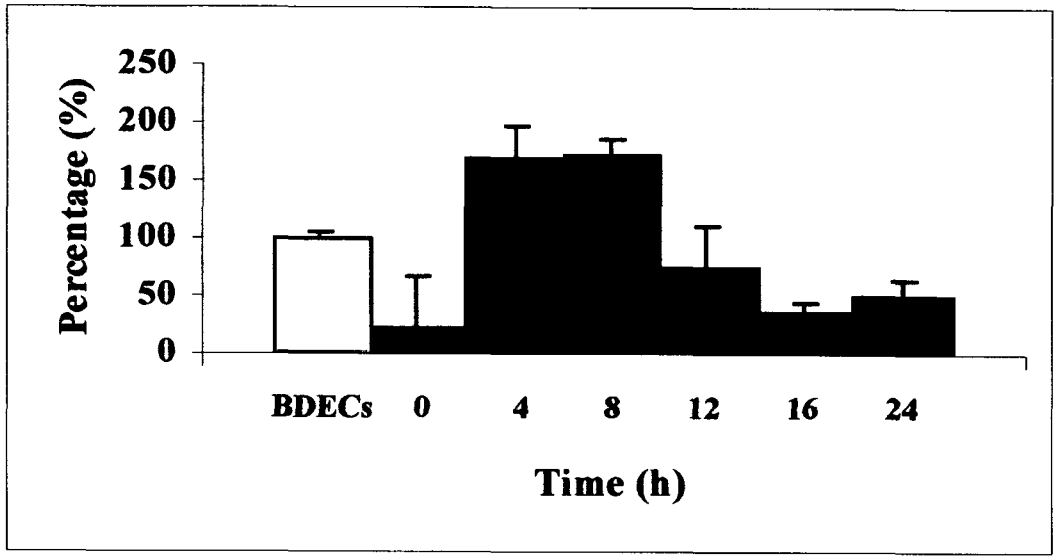
Expression and regulation of E-selectin by TAMECs *in vitro*

E-selectin protein expression and regulation was assessed by cell-based enzyme immunoassay on TAMECs stimulated with TNF-α (50–500 U/ml) for 0-24 h. Each treatment was performed in triplicate and protein levels quantitated in an ELISA plate reader at 492 nm. The results of all experiments are expressed as a percentage of the absorbance reading of the internal standard (constitutive expression of ICAM-1 on BDECs, represents 100%), and are presented as the mean + 1 SD (n=3).

A. Time course of E-selectin protein regulation in response to TNF-α (300 U/ml)

B. Dose response of E-selectin protein induction in TAMECs to varying concentrations of TNF-α for 4 h.

A


250
200
150
100
50
0
Percentage (%)
BDECs
0
4
8
12
16
24
Time (h)

B

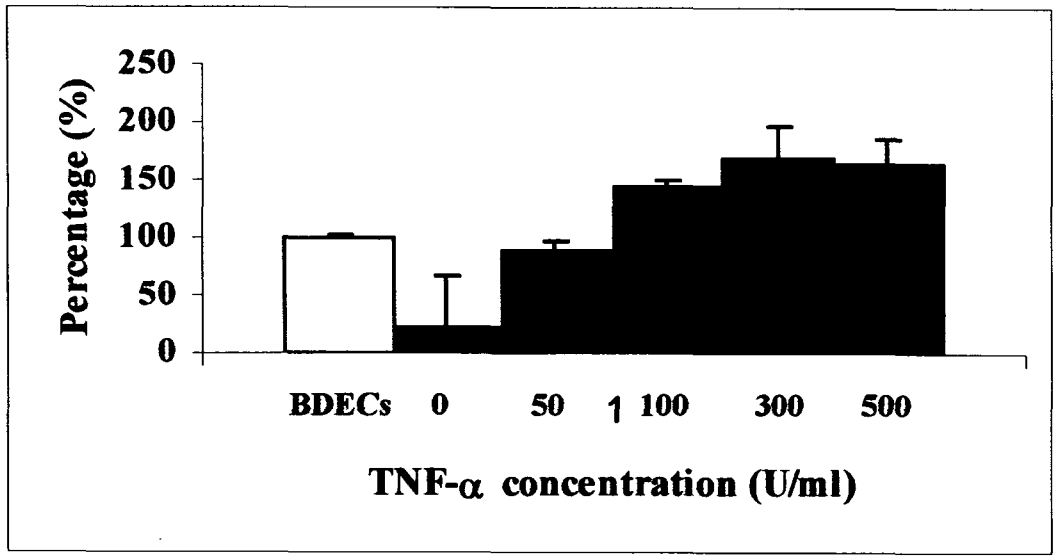

250
200
150
100
50
0
Percentage (%)
BDECs
0
50
100
300
500
TNF-α concentration (U/ml)

FIGURE 4.5

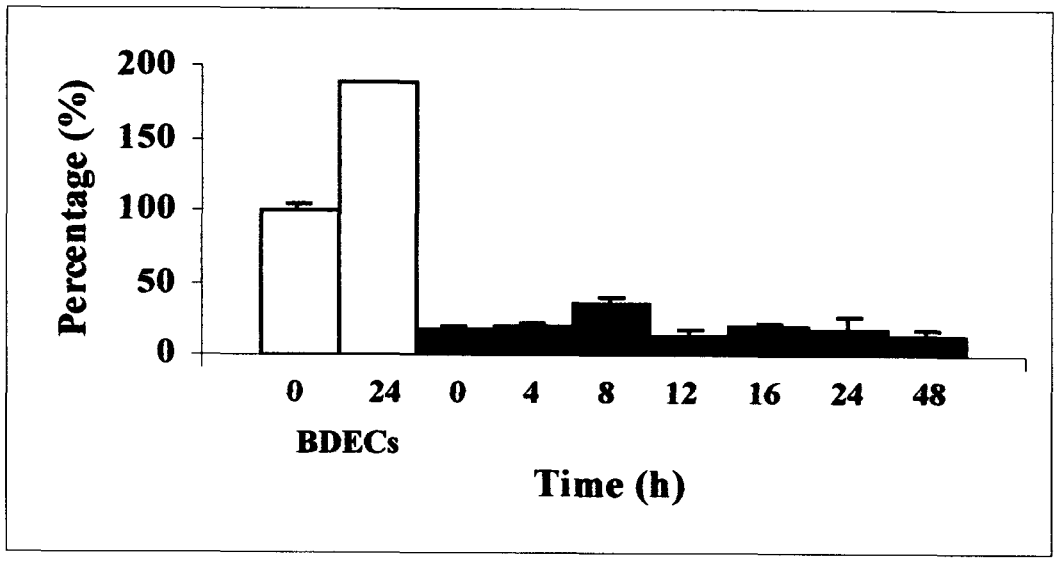
Expression and regulation of ICAM-1 by TAMECs *in vitro*

ICAM-1 protein expression and regulation was assessed by cell-based enzyme immunoassay on TAMECs stimulated with TNF-α (50–500 U/ml) for 0-48 h. Each treatment was performed in triplicate and protein levels quantitated in an ELISA plate reader at 492 nm. The results of all experiments are expressed as a percentage of the absorbance reading of the internal standard (constitutive expression of ICAM-1 on BDECs, represents 100%), and are presented as the mean + 1 SD (n=3)..ICAM-1 expression in TNF-stimulated BDECs (24 h, 300 U/ml) was included in the assay to assess the validity of low ICAM-1 regulation in TNF-stimulated TAMECs.

A. Time course of ICAM-1 protein regulation in response to TNF-α (300 U/ml).

B. Dose response of ICAM-1 expression in TAMECs to varying concentrations of TNF-α for 24 h.

A


200
150
100
50
0
Percentage (%)
0
24
0
4
8
12
16
24
48
BDECs
Time (h)

B

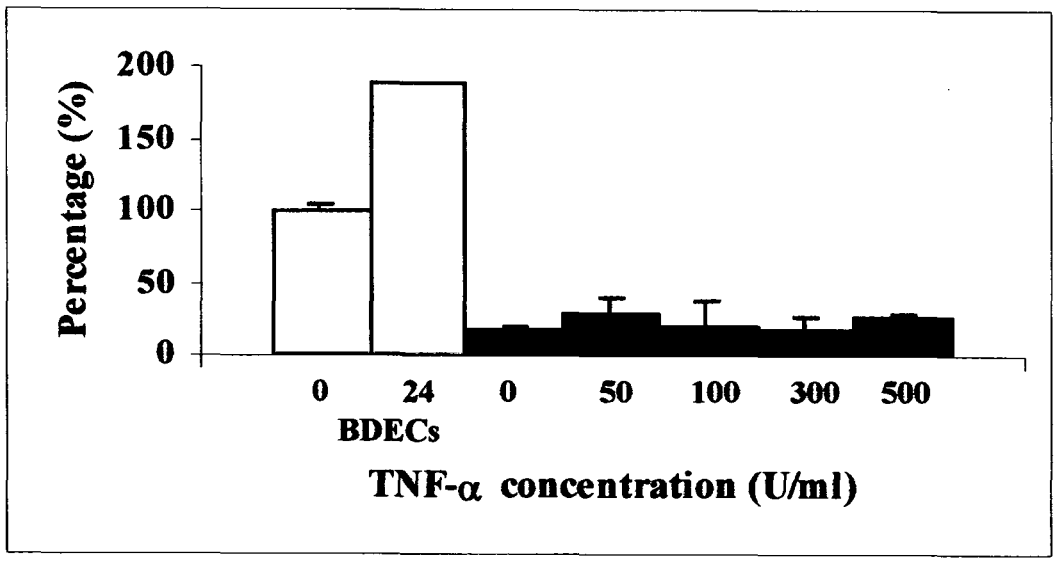

200
150
100
50
0
Percentage (%)
0
24
0
50
100
300
500
BDECs
TNF-α concentration (U/ml)

FIGURE 4.6

E-selectin protein expression by tumour-associated microvessels *in vivo*

E-selectin protein expression was evaluated on formalin-fixed, paraffin embedded tumour tissues by immunohistochemistry. The expression of E-selectin was also assessed after a single, intratumoural injection of TNF-α (5×10^4 U/ml) for 30 min-24 h. Antigen retrieval was performed on all sections with boric acid (200 mmol/l, 60°C, ON) prior to applying the primary antibody. Sites of E-selectin expression were developed with DAB and observed as a brown deposit.

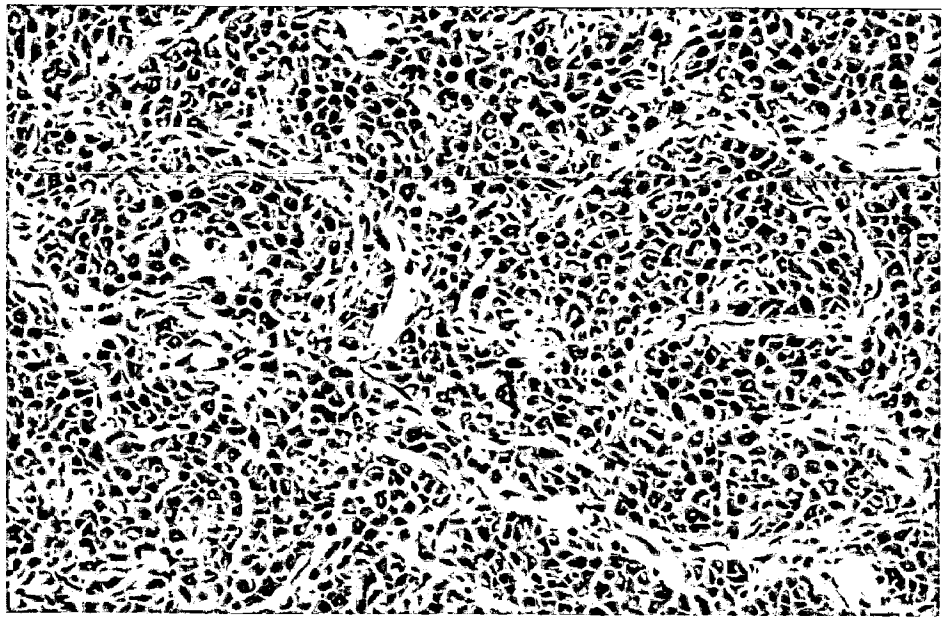
A. Untreated tumour. Constitutive E-selectin protein expression is not detected in tumour-associated microvascular endothelial cells, ×220.

B. Tumour tissue stimulated with TNF-α for 4 h *in vivo*. Strong, linear positive immunoreactivity for E-selectin is observed within tumour-associated microvascular endothelial cells, ×460.

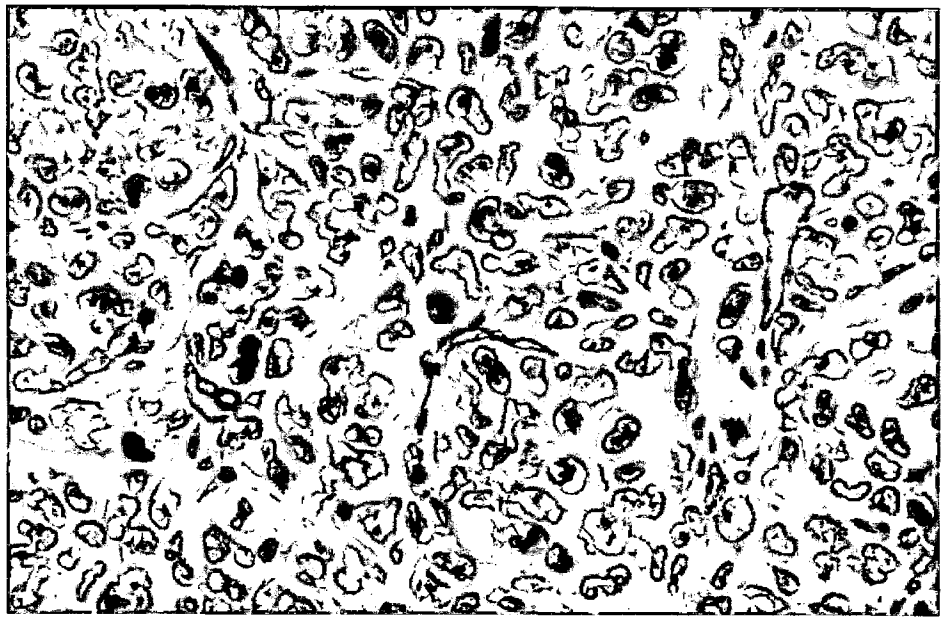
C. Tumour tissue stimulated with TNF-α for 4 h *in vivo*. Tumour-associated microvessels within the viable and necrotic/apoptotic areas of the tumour are still intact and express E-selectin protein, ×220.

Note. A representative negative control section for E-selectin immunostaining (section exposed to boric acid-mediated antigen retrieval and a biotinylated rabbit anti-goat secondary antibody) is presented in Figure 4.8B.

A



B



C

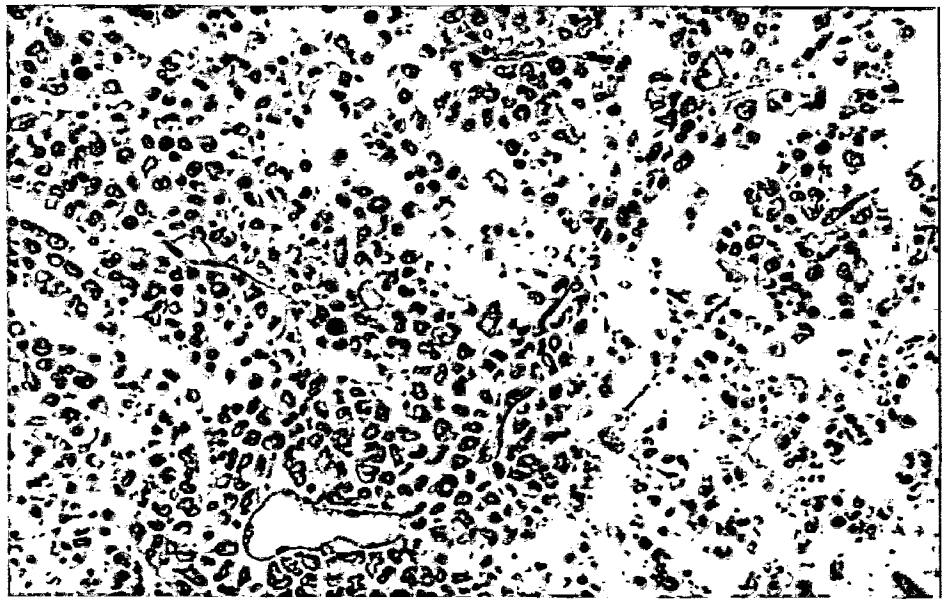


FIGURE 4.7

ICAM-1 protein expression by tumour-associated microvessels *in vivo*

ICAM-1 protein expression was evaluated on formalin-fixed, paraffin embedded tumour tissues by immunohistochemistry. The expression of ICAM-1 was also assessed after a single, intratumoural injection of TNF-α (5×10^4 U/ml) for 30 min–24 h. Antigen retrieval was performed on all sections with microwave heat treatment prior to applying the primary antibody. Sites of ICAM-1 expression were developed with DAB and observed as a brown deposit.

A. Tumour tissue stimulated with TNF-α for 24 h *in vivo*. ICAM-1 immunolabelling of endothelial cells is present in the host microvessels (left half) but not in tumour-associated microvessels (right half), ×120.

B. Strong labelling for ICAM-1 protein by microvascular endothelial cells within the normal, host tissue surrounding the tumour tissue (from the same tissue section as Figure A), ×360.

C. Positive immunoreactivity for ICAM-1 is not detected within tumour-associated microvascular endothelial cells (from the same tissue section as Figure A), ×360.

D. Strong, granular labelling for ICAM-1 in tumour-infiltrating leukocytes within areas of the tumour undergoing TNF-induced necrosis. Tumour-associated microvascular endothelial cells in this section do not express ICAM-1 protein, ×460.

Note. A representative negative control section for ICAM-1 immunostaining (section exposed to microwave-mediated antigen retrieval and a biotinylated horse anti-mouse secondary antibody) is presented in Figure 4.8C.

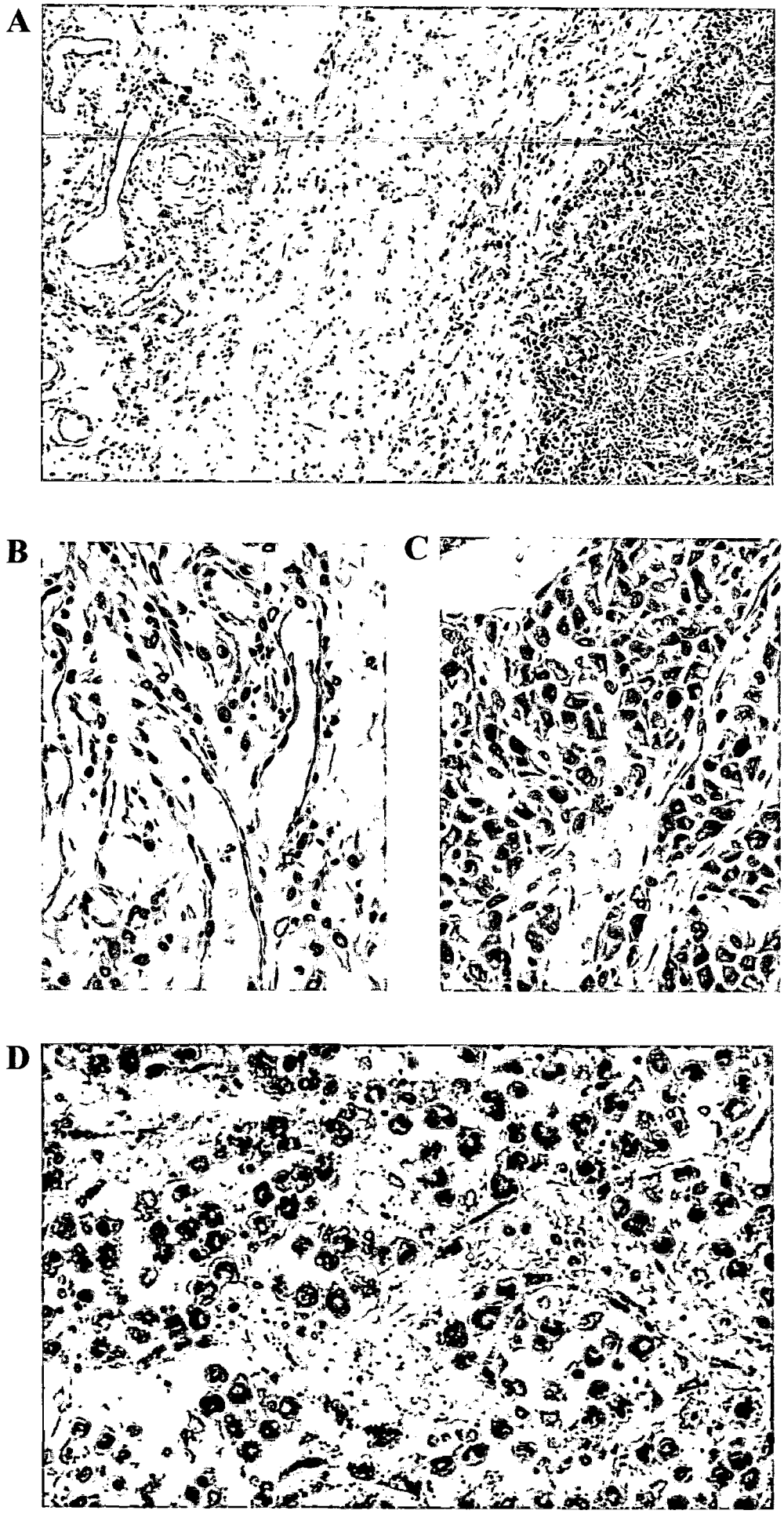

A
B
C
D

FIGURE 4.8

PECAM-1 protein expression by tumour-associated microvessels *in vivo*

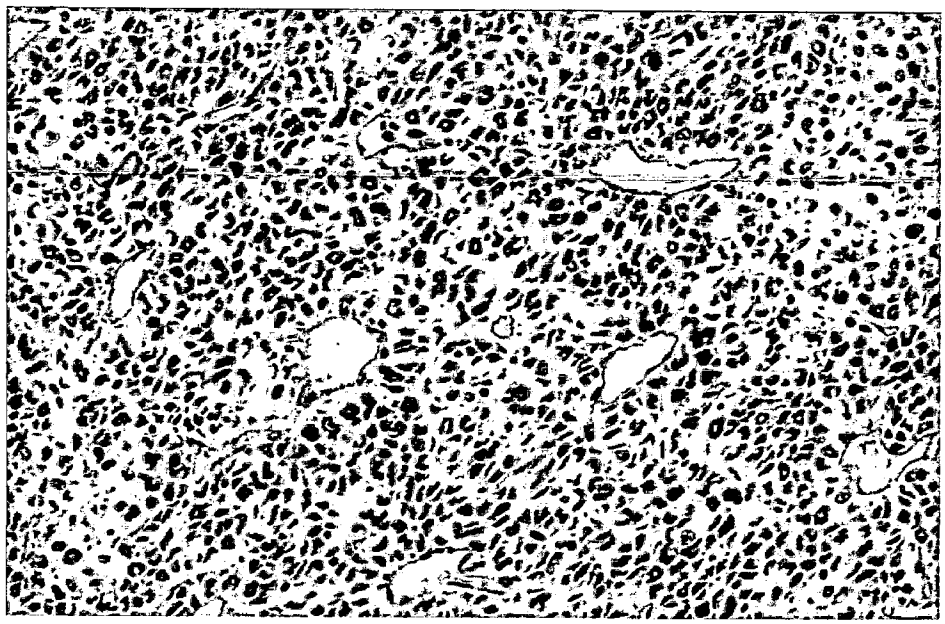
PECAM-1 protein expression was evaluated on formalin-fixed, paraffin embedded tumour tissues by immunohistochemistry. The expression of PECAM-1 was also assessed after a single, intratumoural injection of TNF-α (5×10^4 U/ml) for 30 min–24 h. Antigen retrieval was performed on all sections with boric acid (200 mmol/l, 60°C, ON) prior to applying the primary antibody. Sites of PECAM-1 expression were developed with DAB and observed as a brown deposit.

A. Untreated tumour. Constitutive PECAM-1 expression is observed as strong, linear immunolabelling of tumour-associated microvascular endothelial cells, ×220.

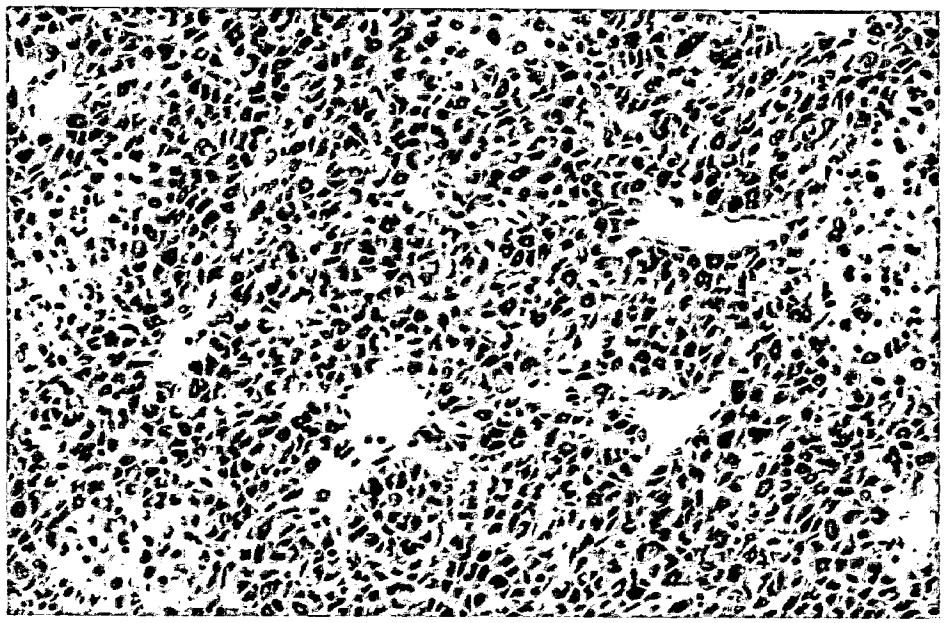
B. Negative control – goat secondary antibody. Negative immunocontrols were performed in each staining run and consisted of the primary antibody being replaced with non-immune serum. This is representative of all immunohistochemistry negative controls for paraffin-embedded specimens exposed to boric acid-mediated antigen retrieval and incubated with a biotinylated rabbit anti-goat secondary antibody. Immunostaining for both E-selectin and PECAM-1 were performed under these conditions, ×220.

C. Negative control – mouse secondary antibody. Negative immunocontrols were performed in each staining run and consisted of the primary antibody being replaced with non-immune serum. This is representative of all immunohistochemistry negative controls for paraffin-embedded specimens exposed to microwave-mediated antigen retrieval and incubated with a biotinylated horse anti-mouse secondary antibody. Immunostaining for ICAM-1 was performed under these conditions, ×220.

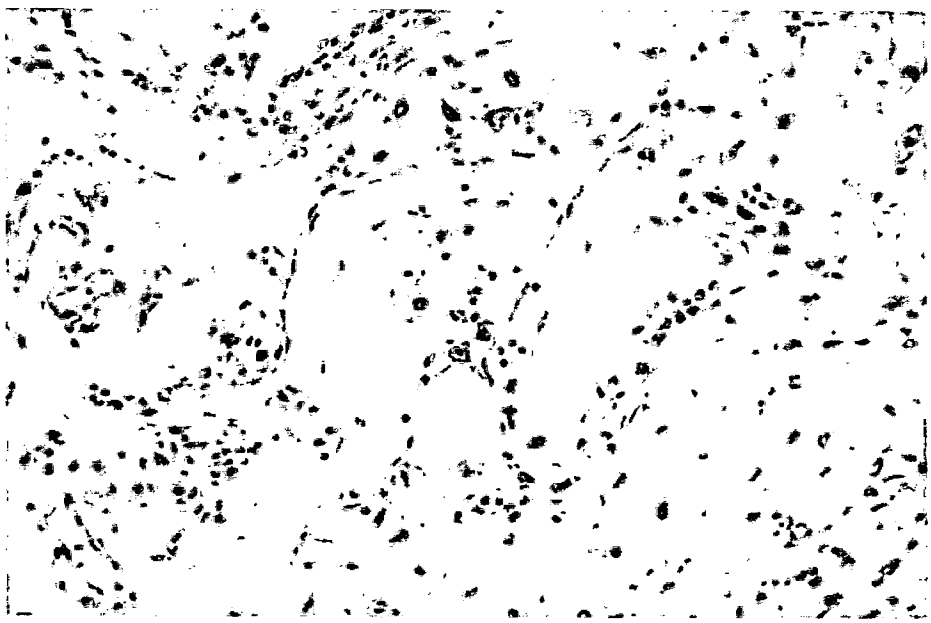
A



B



C



CHAPTER 5. THE EXPRESSION AND REGULATION OF THE TNF RECEPTORS, TNF-R55 AND TNF-R75, ON THE TUMOUR-ASSOCIATED MICROVASCULATURE *IN VIVO* AND *IN VITRO*

5.1 INTRODUCTION

The contribution of each TNF receptor in controlling the expression of adhesion molecules on endothelial cells is controversial. Many groups report that adhesion molecule induction is controlled exclusively by TNF-R55 whereas others acknowledge that TNF-R75 does participate, if only through ligand passing.

TNF-dependent regulation of ICAM-1, E-selectin and VCAM-1 on endothelial cells is predominantly regulated by TNF-R55 (Barbara *et al.*, 1994; Loetscher *et al.*, 1993; Mackay *et al.*, 1993; Neumann *et al.*, 1996; Paleolog *et al.*, 1994; Slowik *et al.*, 1993). This relationship is further strengthened by the presence of κB elements within the ICAM-1, E-selectin and VCAM-1 gene regulatory sequences (Mackay *et al.*, 1993). Activation of TNF-R55 (by TNF-α) leads to increased NF-κB expression (Chainy *et al.*, 1996; Mackay *et al.*, 1993). It has been suggested that the only role TNF-R75 plays in adhesion molecule expression is as an enhancer of TNF-R55-mediated activation (Barbara *et al.*, 1994; Leeuwenberg *et al.*, 1995).

Further evidence for the association between endothelial cell adhesion molecules and TNF receptors is provided by *tnfr2*° mice in which mice are generated deficient in TNF-R75 by gene targeting. These mice are protected against cerebral malaria-associated brain damage through an absence of ICAM-1 upregulation (Lucas *et al.*, 1997b). Since TNF-R55 is still functional, this illustrates that, in this model, TNF-R75 is crucial for TNF-mediated ICAM-1 upregulation, presumably mediated through the transmembrane form of TNF-α (Lucas *et al.*, 1997b).

Along with receptor expression, responsiveness to TNF-induced regulation in TNF receptor positive cells is another means of controlling endothelial cell function. Brain microvascular endothelial cells cultured from CBA/J and BALB/c mice exhibit differential sensitivity to TNF-α and susceptibility to cerebral malaria (Lou *et al.*, 1998). While the constitutive expression of both TNF receptors is comparable between strains, an

upregulation of both TNF-R55 and TNF-R75 mRNA and protein on the microvascular endothelial cells of CBA/J, but not BALB/c mice, mediates their susceptibility to malaria through upregulation of ICAM-1 and VCAM-1 expression (Lou *et al.*, 1998).

In addition, the form of TNF-α and each receptor (i.e. soluble or membrane-bound) has a significant influence on adhesion molecule modulation. When both TNF receptors are expressed at comparable levels, TNF-R55 mainly mediates ICAM-1 upregulation (Lucas *et al.*, 1997a). In situations where only TNF-R75 is present, membrane-bound TNF-α can upregulate ICAM-1 on microvascular endothelial cells, while soluble TNF-α cannot (Lucas *et al.*, 1997a). TNF-induced upregulation of ICAM-1 and VCAM-1 is reduced by sTNF-R55, which can bind to and inactivate TNF-α (Chen *et al.*, 1997a; 1997b).

This chapter aims to examine the contribution of TNF receptors expressed by tumour-associated microvascular endothelial cells in mediating the antitumour actions of TNF-α. Specifically, are one or both of the TNF receptors expressed by tumour-associated microvascular endothelial cells and does their expression and/or regulation (by TNF-α) mediate the anti-tumour action of TNF-α? Using the TAMEC cultures described in Chapter 3, mRNA expression for TNF-R55 and TNF-R75 are analyzed by Northern blot analysis. The translation of these genes into expressed protein is assessed by cell-based enzyme immunoassay. These *in vitro* observations are then compared and validated in tumour sections by *in situ* hybridization and immunohistochemistry. This also extends the investigation by identifying other cell types within the tumour which are potential targets for the actions of TNF-α. In addition to analyzing expression, the regulation of the TNF receptors by their ligand, TNF-α, is examined.

5.2 MATERIALS AND METHODS

5.2.1 Probe preparation

The methods for RNA probe synthesis were documented in Chapter 4, Section 4.2. Alterations to conditions and procedures for the generation and application of TNF receptor riboprobes are detailed below.

Template mRNA was extracted from cultures of DAMA cells for TNF-R55 (Chapter 3, Section 3.2.1) and resident peritoneal-isolated macrophages for TNF-R75 (Chapter 3,

Section 3.2.6.4.1). cDNA was subsequently transcribed and amplified by RT-PCR using the primers detailed in Table 5.1. TNF-R55 (GenBank accession number M75862) primers were designed to cross a transmembrane domain to amplify a 294 bp fragment (601–894 nt). TNF-R75 (U55849) primers were synthesized using the published primer sequence of Bader and Nettesheim (1996) to amplify a 259 bp fragment corresponding to the cytoplasmic part of the protein from nucleotides 1–259. $MgCl_2$ was included in the PCR reaction at a final concentration of 2 mmol/l for TNF-R55 and 3 mmol/l for TNF-R75.

5.2.1.1 *Cloning of PCR products*

PCR products were cloned into pGEM®-T (TNF-R55) or pGEM®-T Easy (TNF-R75) vector systems and ligated as previously described (Chapter 4, Section 4.2.5). TNF-R55 was transformed into DH5α *E. coli* using calcium competent cells and TNF-R75 with electrocompetent cells. Cloned DNA was purified using small scale plasmid preparation. Sequence analysis confirmed the identity of cloned DNA inserts.

5.2.2 Reporter molecule labelling

Cloned DNA probes were labelled with either ^{32}P-UTP or DIG-UTP as described in Chapter 4, Sections 4.2.9.1 and 4.2.9.2, respectively. *Nco* I-linearized TNF-R55 and *Apa* I-linearized TNF-R75 templates were transcribed with SP6 RNA polymerase to generate anti-sense probes. *Spe* I was used to linearize TNF-R55 and TNF-R75 templates for the transcription of sense riboprobes with T7 RNA polymerase. Probe labelling efficiency with ^{32}P and DIG was assessed by scintillation counting and dot blot, respectively.

5.2.3 Northern blot hybridization

Total and messenger RNA were extracted from TAMECs stimulated with TNF-α (500 U/ml) for 2 (TNF-R55) or 4 (TNF-R75) h. RNA was resolved in a denaturing gel, transferred to a PVDF membrane and UV cross-linked. Membranes were probed with anti-sense and sense ^{32}P-labelled RNA probes to detect TNF-R55 and TNF-R75 mRNA expression in TAMECs and exposed to X-ray film.

5.2.4 *In situ* hybridization

Detection of TNF receptor mRNA transcripts by *in situ* hybridization in tumour tissues was performed as described in Chapter, 4, Section 4.2.11. Modifications made to the protocol

TABLE 5.1

TNF receptor PCR primer sequences

	Primer sequence	Size	Reference
TNF-R5	$^{5'}$GAC ACC GTG TGT GGC TGC AAG AAG$^{3'}$	294 bp	
	$^{5'}$GAA GAT AAC CAG AGG CAA CAG CAC$^{3'}$		
TNF-R7	$^{5'}$GAT GAG AAA TCC CAG GAT GCA GTA GG	259 bp	Bader *et al.*, 1996
	$^{5'}$TGC TAC AGA CGT TCA CGA TGC AGG$^{3'}$		

of Komminoth (1996), as discussed in Chapter 4, Section 4.3.2, were also applied for the detection of TNF receptor mRNA transcripts *in situ*.

5.2.5 Enzyme immunoassay

TNF-R55 and TNF-R75 protein expression and regulation were quantitated *in vitro* in TAMECs stimulated with TNF-α (0–500 U/ml) for 0–48 h. The polyclonal antibodies (Appendix B) used were a goat anti-mouse and rat TNF-R55 (1:100; Santa Cruz) and goat anti-mouse and rat TNF-R75 (1:100; Santa Cruz) and the assay performed as described in Chapter 4, Section 4.2.12. The constitutive expression of TNF-R55 on BDECs was used as an internal standard for both TNF-R55 and TNF-R75 studies. This amount was defined as the 100% standard value and data obtained for each cytokine treatment and for each TNF receptor was related to this level of expression.

5.2.6 Immunohistochemistry

The standard immunoperoxidase technique (Chapter 2, Section 2.4.2) was used to localize each TNF receptor within tumour tissues *in vivo*. Analyses were performed on paraffin-embedded tissues from tumour-bearing rats injected intratumourally with TNF-α (5×10^4 U/ml) for 30 min–24 h *in vivo*. Receptor proteins were immunolocalized with anti-TNF-R55 (1:250) and anti-TNF-R75 (1:250) antibodies (Appendix B) and colour development mediated by DAB. Boric acid-mediated antigen retrieval (Chapter 2, Section 2.4.2.1.2) was performed for each receptor. Primary antibodies were applied to the sections at 4°C ON in a humid chamber.

5.3 RESULTS

5.3.1 TNF receptor mRNA expression and regulation in TAMECs *in vitro* – Northern blots

TNF-R55 mRNA was detected as two gene transcripts at approximately 2.5 and 1.6 kb in TAMECs stimulated with TNF-α (500 U/ml) for 2 h (Figure 5.1A). TNF-R75 was detected in TAMECs treated with TNF-α (500 U/ml) for 4 h. Three gene transcripts specific for TNF-R75 were detected (Figure 5.1B). The first was a major transcript at 5.7 kb and two minor bands at 3.5- and 4.8 kb. Hybridization with the sense probes did not detect specific TNF-R55 or TNF-R75 mRNA species — any non-specific hybrids were removed with stringent washing.

5.3.2 TNF receptor mRNA expression and regulation *in vivo – in situ* hybridization

The spatial and temporal expression of each TNF receptor gene within tumour tissue was detected by *in situ* hybridization as purple/black cytoplasmic staining. DIG-labelled RNA probes specific for the sense strand of each gene (negative control) were applied to serial sections on the same microscope slide as the test (anti-sense) probe.

Constitutive TNF-R55 gene staining was localized within tumour cells, fibroblasts, tumour-infiltrating leukocytes and tumour-associated microvascular endothelial cells (Figure 5.2A). Upon stimulation with TNF-α (5×10^4 U/ml) for 30 min–24 h, TNF-R55 mRNA staining was observed within the same cell types, with no significant TNF-R55 upregulation above constitutive levels (Figure 5.2C). Sense TNF-R55 probes did not hybridize to any cell type within the tumour population (Figures 5.2B, 5.2D).

Staining for TNF-R75 mRNA in untreated tumours was observed within tumour-infiltrating leukocytes and fibroblasts. After TNF-α administration, staining for TNF-R75 was also immunolocalized within tumour-associated microvascular endothelial cells (Figure 5.3A). This was first evident within 2 hours of exposure to TNF-α and increased in intensity and distribution over 24 h after TNF-α administration. Staining was uniform and observed within every endothelial cell of each tumour-associated microvessel. In the central areas of tumour undergoing TNF-induced haemorrhagic necrosis, strong labelling for TNF-R75 occurred in the necrotic tumour cells as well as in tumour-associated microvascular endothelial cells (Figure 5.3C). In some areas, a breakdown in the composition of the endothelial cell layer was also observed in tumour-associated microvessels in necrotic areas. The application of sense TNF-R75 probes did not result in staining in any of the cell types within the tumour population (Figures 5.3B, 5.3D).

5.3.3 *In vitro* expression and regulation of TNF receptors in TAMECs following TNF-α administration

The expression and regulation of TNF receptor protein on TNF-stimulated TAMECs was assessed using a cell-based enzyme immunoassay. TNF-R55 protein was expressed by unstimulated TAMECs. Values changed little after stimulation of TAMECs with TNF-α (300 U/ml) over the 48 h experimental period (Figure 5.4A). Constitutive TNF-R55

expression in TAMECs was at a similar level to constitutive levels in BDECs. After 4 h, TNF-R55 levels were not altered by increasing doses of TNF-α (50–500 U/ml)(Figure 5.4B).

TNF-R75 protein expression was barely detected on unstimulated TAMECs. After stimulation with TNF-α (300 U/ml), TNF-R75 levels in TAMECs gradually increased to reach maximal expression at 24 h, still remaining elevated (although at a lower level) 48 h post TNF-α administration (Figure 5.5A). After exposure to TNF-α for 24 h, detectable levels of TNF-R75 were consistently raised across a dose range of 50–500 U/ml (Figure 5.5B) with maximum expression occurring in response to a dose of 300 U/ml.

5.3.4 *In vivo* expression and regulation of TNF receptors on the tumour-associated microvasculature following TNF-α administration

TNF receptor protein expression was immunolocalized within TNF-treated tumour sections by immunohistochemistry. Bound antibodies were visualized by DAB and antigenic sites detected as a brown deposit.

TNF-R55 protein was immunolocalized within tumour cells, tumour-infiltrating leukocytes, fibroblasts and tumour-associated microvascular endothelial cells in untreated tumour tissues (Figure 5.6A). Upon stimulation with TNF-α *in vivo* (5×10^4 U/ml), immunostaining also localized within each cell type at each time point studied (Figure 5.6B). Vascular staining was strong, uniform and confined to tumour-associated microvascular endothelial cells (Figure 5.6C). Immunoreactivity was detected within every vessel irrespective of vessel origin, size or location within the tumour mass. Negative controls demonstrated no non-specific binding (representative section in Figure 5.7C).

TNF-R75 protein expression was detected within the tumour-infiltrating leukocytes of untreated tumour tissues. After stimulation with TNF-α (5×10^4 U/ml), positive vascular immunoreactivity was first observed within 4 hours as faint labelling of tumour-associated microvascular endothelial cells. Staining intensity and also the number of positive tumour-associated microvessels increased from 12 to 16 h post-TNF-α administration. At 24 h, strong, specific immunolabelling of the tumour-associated microvascular endothelial cells was observed (Figure 5.7A). Isolated tumour cells expressed TNF-R75, but the greater

majority were negative, even in TNF-stimulated specimens. The incidence of haemorrhagic necrosis in the tumours correlated positively with the induction of TNF-R75 expression (Figure 5.7B). Negative control sections failed to stain (Figure 5.7C).

5.4 DISCUSSION

Endothelial cells are a primary target for the actions of TNF-α. In response to activation, these cells normally would release cytokines, express endothelial cell adhesion molecules and proliferate but TNF-α appears to exert some different effects in normal compared with tumour-associated microvascular endothelial cells. It has been proposed that the anti-tumour action of TNF-α is mediated through a direct cytotoxic action on tumour-associated microvascular endothelial cells. Subsequently, tumour cells undergo apoptosis and necrosis as a result of losing their vascular supply. For TNF-α to initiate these anti-tumour effects, tumour-associated microvascular endothelial cells must express one or both of the TNF receptors.

Despite the interest in TNF-α as an anticancer agent, the expression and contribution of each TNF receptor in mediating the anti-tumour actions of TNF-α has not been fully determined. Limited reports have localized TNF-R55 and TNF-R75 to various cell populations within tumours by *in situ* hybridization, immunohistochemistry and ligand binding studies. For instance, both receptors have been detected within the microvasculature of glioma (Maruno *et al.,* 1996) and breast cancer (Leek *et al.,* 1998; Miles *et al.,* 1994; Pusztai *et al.,* 1994) tissues (reviewed in Chapter 1, Table 1.4). Not all tumour-associated microvessels express TNF receptors as demonstrated in human glioma sections in which receptor positive microvessels ranged from 7.7–74.4% (Maruno *et al.,* 1996). Differences in receptor expression between central and peripheral vessels was not reported.

While limited studies have reported on the expression of TNF receptors within tumour tissues (Chapter 1, Table 1.4), de Kossodo *et al.* (1995) is the only study to report on the regulation of TNF receptors by cytokines. TNF-α and IFN-γ were injected into mice bearing a breast cancer xenograft and receptor expression analyzed from tumour digests by RT-PCR. Maximal mRNA expression for TNF-R55 and TNF-R75 was observed 3 h after cytokine exposure and receptor levels had declined to constitutive expression within 24 h.

The contribution of microvascular endothelial cells within the tumour to the receptor levels could not be determined due to whole tumour digests being used as the source of mRNA. In association with receptor upregulation, selective destruction of the tumour-associated microvasculature and death of tumour cells was observed histologically. These findings support the proposal that TNF-α (in association with IFN-γ) mediates, at least in part, tumour necrosis through a cytotoxic action on the tumour-associated microvasculature. To support and extend these observations, the regulation of TNF receptors by TNF-α (and other cytokines) within tumour tissues needs to be assessed histologically (by immunohistochemistry and/or *in situ* hybridization). This will determine the responsive cell types within the tumour and therefore identify the cell population(s) which may be contributing to the antitumour actions of TNF-α.

In general, *in vitro* observations regarding TNF receptor expression within tumour-associated microvasculature have been extrapolated from HUVECs or microvascular endothelial cells (Wu *et al.*, 1998). While a number of studies have demonstrated TNF receptor expression in cultures of normal endothelial cells (Bradley, Thiru and Pober, 1995; Lucas *et al.*, 1998; Mackay *et al.*, 1993), investigation of receptor regulation by cytokines is limited to a study by Bebo and Linthicum (1995). Murine cerebrovascular microendothelial cells were stimulated with IL-1β, IFN-γ and/or TNF-α to induce TNF-R55 and TNF-R75 mRNA. While IL-1β and IFN-γ upregulated receptor expression, TNF-α had no effect on either receptor. Notably, Bebo and Linthicum (1995) also demonstrated that cytokine regulation of TNF receptor mRNA in a transformed endothelial cell line (EOMA) differs from non-transformed cerebrovascular microvascular endothelial cells. To date, *in vitro* studies of TNF receptor expression in tumour-associated microvascular endothelial cells have not been performed and, as discussed in Chapter 1, the heterogeneous nature of endothelial cells indicates that studies on non-tumour-derived microvascular endothelial cells may not reflect the nature of the tumour-associated microvasculature.

This study has provided for the first time an analysis of TNF receptor gene and protein expression within tumour-associated microvascular endothelial cells *in vitro*. TNF-R55 expression was observed in TNF-stimulated TAMECs by Northern blot analysis and cell-based enzyme immunoassay. Gene transcripts were observed at 2.5- and 1.6 kb. The 2.5

kb transcript is in agreement with published values (Lewis *et al.,* 1991), however the presence of another transcript at 1.6 kb has not been previously reported in endothelial cells. The significance of this mRNA species is unknown, as is its cellular distribution. By enzyme immunoassay, TNF-R55 protein was constitutively expressed by TAMECs and levels remained unchanged after exposure to TNF-α.

In vitro, the predominant TNF-R75 gene transcript in TAMECs was the 5.7 kb species, with minor bands at 3.5- and 4.8 kb. The predominant TNF-R75 gene transcript varies between cells and the activation state of the cells. In microglia it is the 3.5 kb species, whereas in RAW 264.7 cells (a murine peritoneal macrophage cell line) it is the 4.8 kb transcript (Dopp *et al.,* 1997). The 3.5 kb transcript is also constitutively expressed in murine spleen cells while the 4.8- and 5.7-kb transcripts are induced during clinical experimental allergic encephalomyelitis (Kahn *et al.*, 1999). The significance of the three transcripts and the strong expression of the 5.7 kb species in TNF-stimulated TAMECs remain unclear. The *in vitro* induction of TNF-R75 protein expression by TNF-stimulated TAMECs (assessed by ELISA) demonstrated similar kinetics to that observed in the tumour-associated microvasculature *in vivo*.

This study has attempted to define further the relevant target cells for the anti-tumour activity of TNF-α *in vivo*. The expression and distribution of TNF-R55 and TNF-R75 mRNA an d protein were localized to individual cell types within the tumour population and receptor regulation was assessed *in vivo* following induction by TNF-α. TNF-R55 mRNA and protein were detected in tumour cells, fibroblasts, tumour-infiltrating leukocytes, and tumour-associated microvascular endothelial cells. This is consistent with previous observations based on immunodetection of TNF-R55 in breast carcinoma tissues (Miles *et al.,* 1994; Pusztai *et al.,* 1994). A positive association between expression of TNF-R55 by breast carcinoma-associated vessels and the number of leukocytes has been reported (Pusztai *et al.,* 1994). In the present investigation, strong constitutive TNF-R55 expression was observed however, *de novo* synthesis and the amount of immunoreactive receptor protein was not upregulated by TNF-α. mRNA expression parallelled the distribution of protein product *in vivo*.

Constitutive TNF-R75 mRNA expression within the tumour was limited to tumour-infiltrating leukocytes. Upon stimulation with TNF-α, receptor mRNA expression was detected within tumour-associated microvascular endothelial cells as well as in tumour cells. The distribution of TNF-R75 mRNA was more widespread than for the translated protein detected by immunohistochemistry. Immunoreactive TNF-R75 was restricted to tumour-associated microvascular endothelial cells and tumour-infiltrating leukocytes after stimulation with TNF-α. Previous studies have immunolocalized TNF-R75 reactivity in breast cancer and ovarian carcinoma specimens to fibroblasts, infiltrating lymphoid and mononuclear cells while tumour cell expression has not been observed (Miles *et al.,* 1994; Naylor *et al.,* 1993; Pusztai *et al.,* 1994). These findings have been confirmed in this study.

Most cells coexpress both TNF receptors however, they may differ in the level of expression or degree of inducibility. The influence of TNF-α on the induction/regulation of each receptor observed in this study is consistent with previous observations. Alsalameh *et al.* (1999a) and Kalthoff *et al.* (1993) have also noted that TNF-R55 is not responsive to cytokine stimulation while TNF-R75 is transcriptionally upregulated by exposure to TNF-α. There is also a clear correlation between TNF-R75 mRNA upregulation and an increase in the corresponding protein level.

The limited expression of TNF-R55 and absence of TNF-R75 protein expression on tumour cells suggests that TNF-α might not be responsible for direct cytolysis of tumour cells in the present model. Strong vascular expression and regulation supports the proposal that the tumour-associated vascular endothelium may mediate the anti-tumour action of TNF-α. Indeed, the induction of vascular TNF-R75 protein expression correlates with the appearance of haemorrhagic necrosis within TNF-treated tumour tissues. Areas of tumour cell necrosis were first observed within 4 h after the administration of TNF-α. While TNF-R75 was not constitutively expressed by tumour-associated microvascular endothelial cells, strong vascular immunostaining was first observed within 4 h post-TNF-α. This suggests that the expression of TNF-R75 by microvascular endothelial cells may be required for the induction of TNF-mediated cytotoxicity. The significance of this observation is discussed in detail within Chapter 6.

Although TNF-R55 protein expression was more widespread than TNF-R75, this may have no impact on the actions and signalling of TNF-α. Within the tumour, strong induction of TNF-R75, in addition to constant TNF-R55 levels, appears to be important in mediating the anti-tumour action of TNF-α. As noted in Chapter 1, TNF-R75 can significantly reduce the concentration of TNF-α required for cell killing through the mechanism of ligand passing (Tartaglia and Goeddel, 1992). When TNF-α levels are low, upregulated TNF-R75 may capture TNF-α and pass it over to TNF-R55 to induce signalling and cellular responses. The lack of TNF-R55 upregulation on tumour-associated microvascular endothelial cells may also be a mechanism for the cell to escape its destruction by avoiding highest stimulation. Alternatively, TNF-induced upregulation of TNF-R75 may increase the release of sTNF-R75. Soluble TNF receptors reduce sensitivity by neutralizing TNF-α as well as impairing ligand presentation to TNF-R55 (Aderka *et al.*, 1998).

5.5 SUMMARY

TNF receptors mediate the effects of TNF-α, however, the contribution of the TNF receptors (TNF-R55 and TNF-R75) in mediating TNF-induced tumour necrosis is not completely understood. TAMECs express both receptor species as assessed by Northern blot analysis and enzyme immunoassay. Tumour cells, tumour-associated macrophages and tumour-associated microvascular endothelial cells constitutively express TNF-R55. mRNA levels parallelled TNF-R55 protein expression in the same cell types and were not upregulated by TNF-α. Constitutive TNF-R75 expression was restricted to tumour-infiltrating leukocytes while TNF-α rapidly induced expression within tumour-associated microvascular endothelial cells. The appearance of haemorrhagic necrosis correlated with the induction of TNF-R75 expression on the tumour-associated microvasculature. TNF-R55 was constitutively expressed and not regulated by exposure to TNF-α, whereas TNF-R75 was only expressed by tumour-associated microvascular endothelial cells after induction by TNF-α. Overall, the expression of TNF-R55 was much more widespread within the tumour than TNF-R75. These observations support the proposition that TNF-α signalling in tumour-associated microvascular endothelial cells is effected through upregulation of the TNF-R75 receptor. TNF-R75 then transfers TNF-α to TNF-R55, the principal signal-inducing receptor, presumably via a ligand passing mechanism. The data suggest that the expression of TNF-R75 is intimately linked to the anti-tumour action of TNF-α.

FIGURE 5.1

TNF-R55 and TNF-R75 mRNA expression by TAMECs *in vitro*

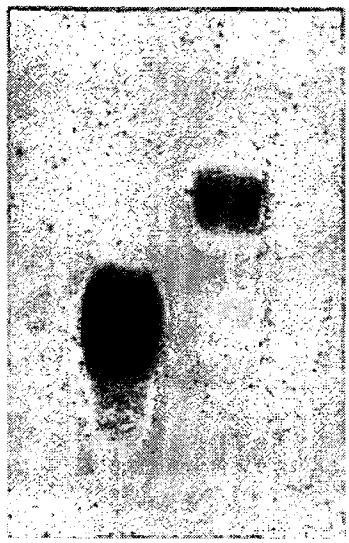
TAMECs were stimulated *in vitro* with TNF-α (500 U/ml) for 2 (TNF-R55) or 4 (TNF-R75) h. Total and messenger RNA were extracted as described in Section 4.2.10. RNA samples were separated on a 1% agarose/2.2 mol/l formaldehyde gel and transferred to PVDF membrane. The nylon was probed with specific ^{32}P-labelled RNA probes for TNF-R55 and TNF-R75 and hybrids detected by exposure to X-ray film.

A. Expression of TNF-R55 mRNA by TAMECs in response to TNF-α for 2 h. Two gene transcripts at approximately 2.5- and 1.6 kb are detected.

B. Expression of TNF-R75 mRNA by TAMECs in response to TNF-α for 4 h. Three gene transcripts (approximately 3.5-, 4.8-, and 5.7 kb) specific for TNF-R75 are detected.

A

Total mRNA



B

Total mRNA

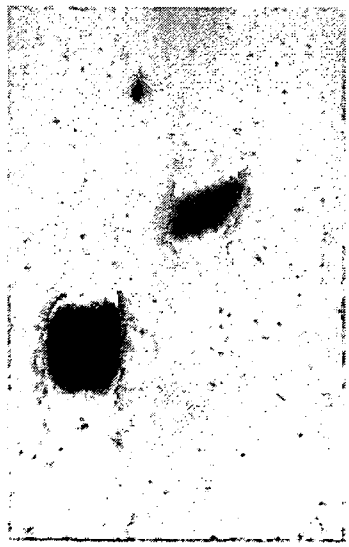


FIGURE 5.2

TNF-R55 mRNA expression by tumour-associated vessels *in vivo*

TNF-R55 mRNA was immunolocalized within formalin-fixed, paraffin-embedded tumour tissues by *in situ* hybridization. Tumour sections were treated with an anti-sense DIG-labelled probe generated against TNF-R55. A control serial section on which the sense probe was applied was run in parallel on each slide. Probe-mRNA hybrids were revealed with an anti-DIG antibody and visualized with NBT/BCIP.

A. Untreated tumour hybridized with the anti-sense TNF-R55 probe. Constitutive TNF-R55 mRNA expression is observed within tumour-associated microvascular endothelial cells, tumour cells and tumour-infiltrating leukocytes, ×240.

B. Serial section of the tissue in Figure A hybridized with the sense TNF-R55 probe. No non-specific hybrids are detected, ×240.

C. Tumour tissue injected intratumourally with TNF-α for 4 h hybridized with the anti-sense TNF-R55 probe. Intense staining for TNF-R55 mRNA is detected within tumour-associated microvascular endothelial cells, tumour cells and tumour-infiltrating leukocytes, ×460.

D. Serial section of the tissue in Figure C hybridized with the sense TNF-R55 probe. No non-specific hybrids are detected, ×460.

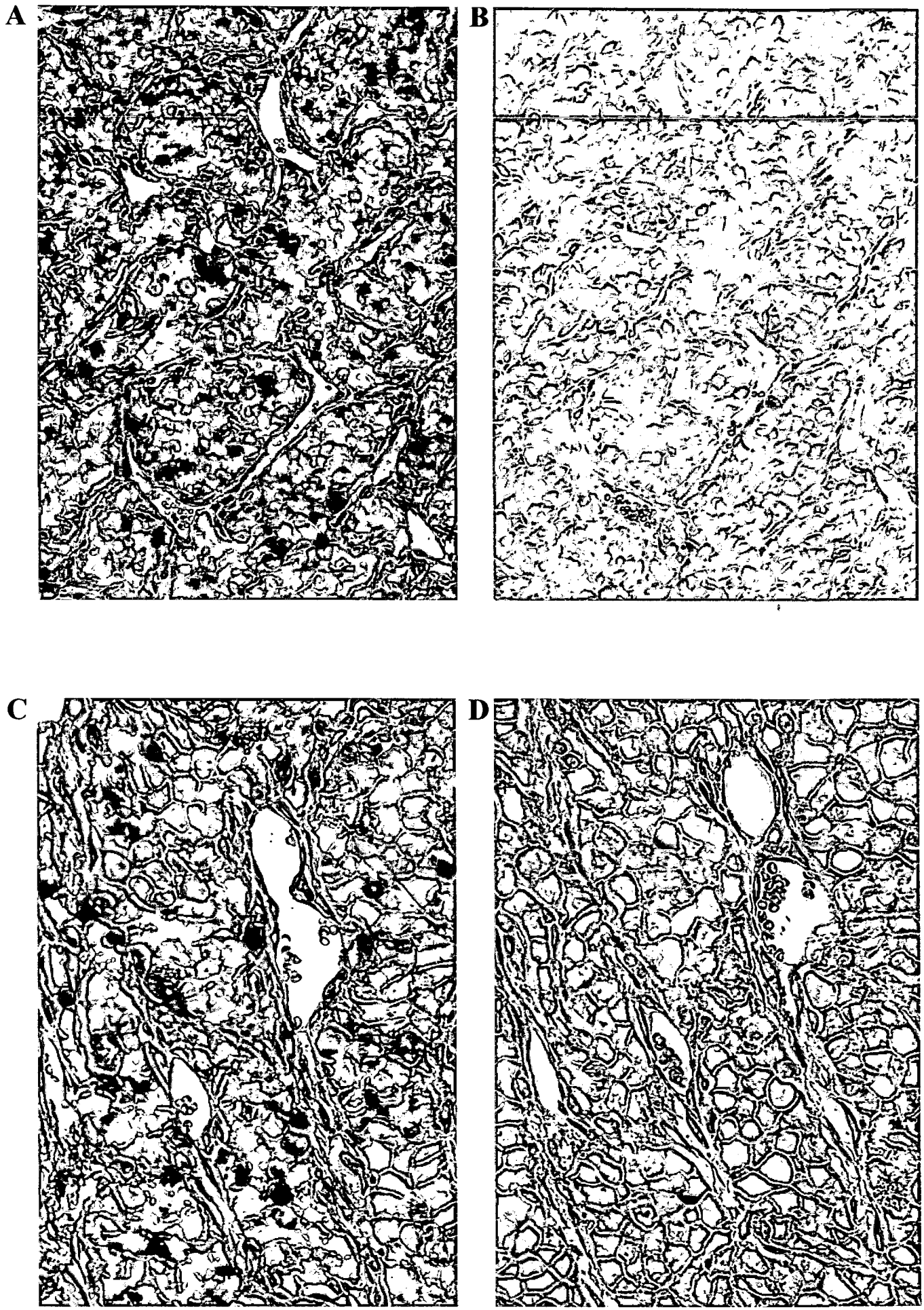

A
B
C
D

FIGURE 5.3

TNF-R75 mRNA expression by tumour-associated vessels *in vivo*

TNF-R75 mRNA was immunolocalized within formalin-fixed, paraffin-embedded tumour tissues by *in situ* hybridization. Tumour sections were treated with an anti-sense DIG-labelled probe generated against TNF-R75. A control serial section on which the sense probe was applied was run in parallel on each slide. Probe-mRNA hybrids were revealed with an anti-DIG antibody and visualized with NBT/BCIP.

A. Tumour tissue stimulated with TNF-α for 24 h *in vivo* hybridized with the anti-sense TNF-R75 probe. Strong staining is observed within tumour-associated microvascular endothelial cells, tumour cells and tumour-infiltrating leukocytes, ×460.

B. Serial section of the tissue in Figure A hybridized with the sense TNF-R75 probe. No non-specific hybrids are detected, ×460.

C. Tumour tissue stimulated with TNF-α for 24 h *in vivo* hybridized with the anti-sense TNF-R75 probe. In areas of TNF-induced haemorrhagic necrosis, strong staining of necrotic tumour cells is observed as well as vascular staining in tumour-associated microvascular endothelial cells. Note that there appears to be a loss of preservation of the vasculature in some areas (arrow), ×720.

D. Serial section of the tissue in Figure C hybridized with the sense TNF-R75 probe. No non-specific hybrids are detected. Note that there appears to be a loss of preservation of the vasculature in some areas (arrow), ×720.

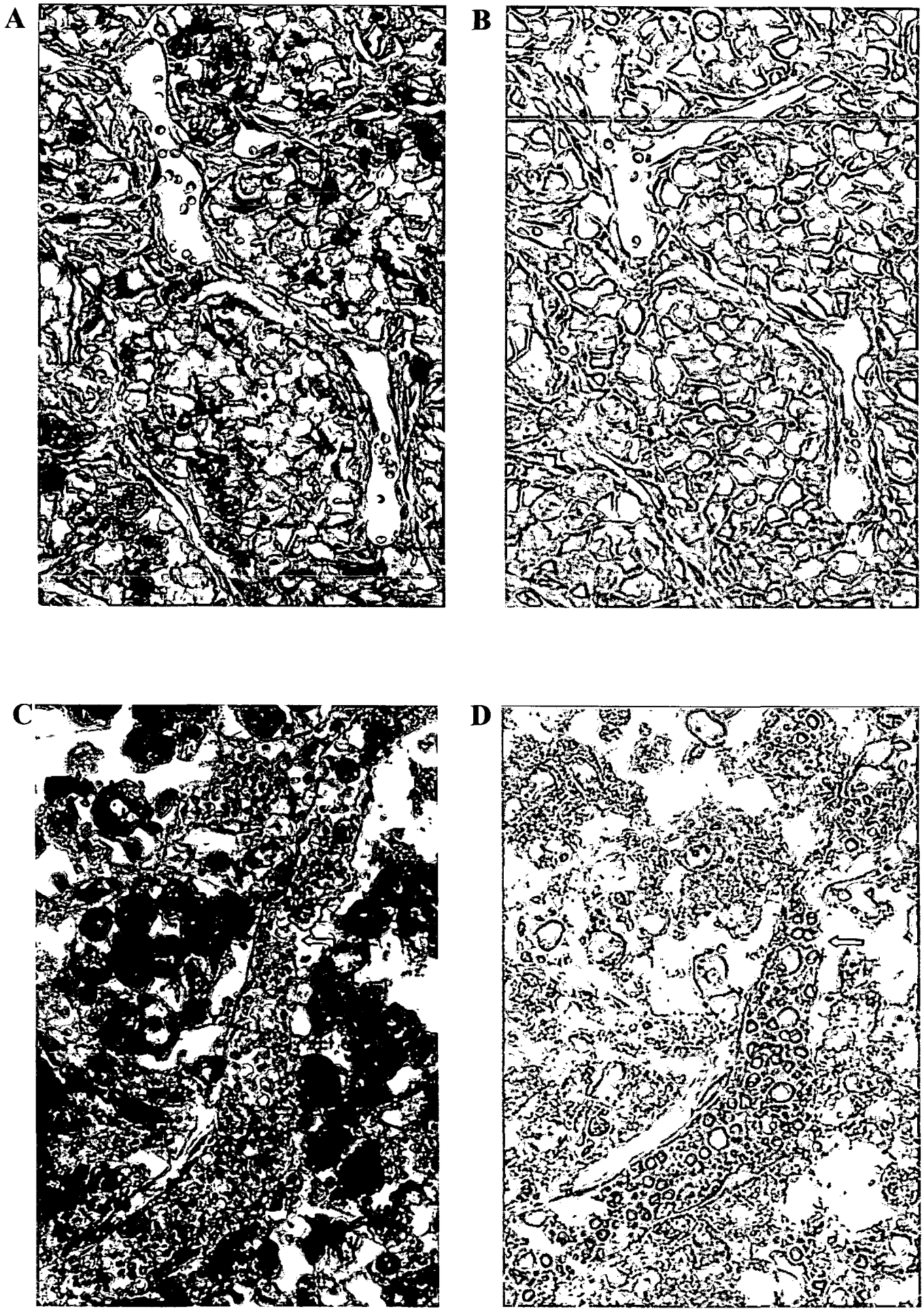

A
B
C
D

FIGURE 5.4

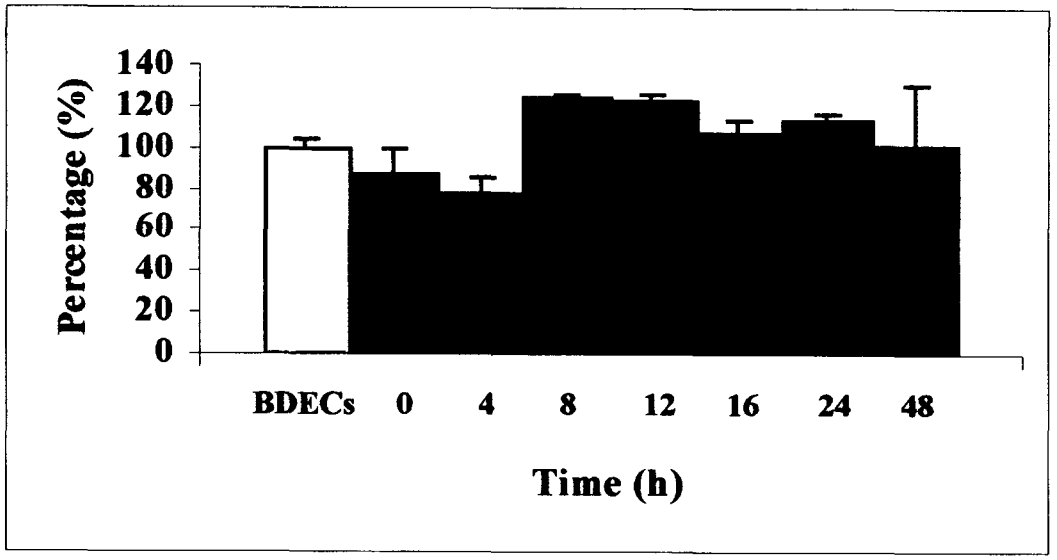
Expression and regulation of TNF-R55 by TAMECs *in vitro*

TNF-R55 protein expression and regulation was assessed by cell-based enzyme immunoassay on TAMECs stimulated with TNF-α (50–500 U/ml) for 0–24 h. Each treatment was performed in triplicate and protein levels evaluated by enzyme immunoassay as described in Chapter 4, Section 4.2.2. TNF-R55 levels were quantitated in an ELISA plate reader at 492 nm. The results of all experiments are expressed as a percentage of the absorbance reading of the internal standard (constitutive expression of TNF-R55 on BDECs, represents 100%), and are presented as the mean + 1 SD (n=3).

A. Time course of TNF-R55 protein regulation in response to TNF-α (300 U/ml).

B. Dose response of TAMECs to varying concentrations of TNF-α for 4 h.

A


Percentage (%)
140
120
100
80
60
40
20
0
BDECs
0
4
8
12
16
24
48
Time (h)

B

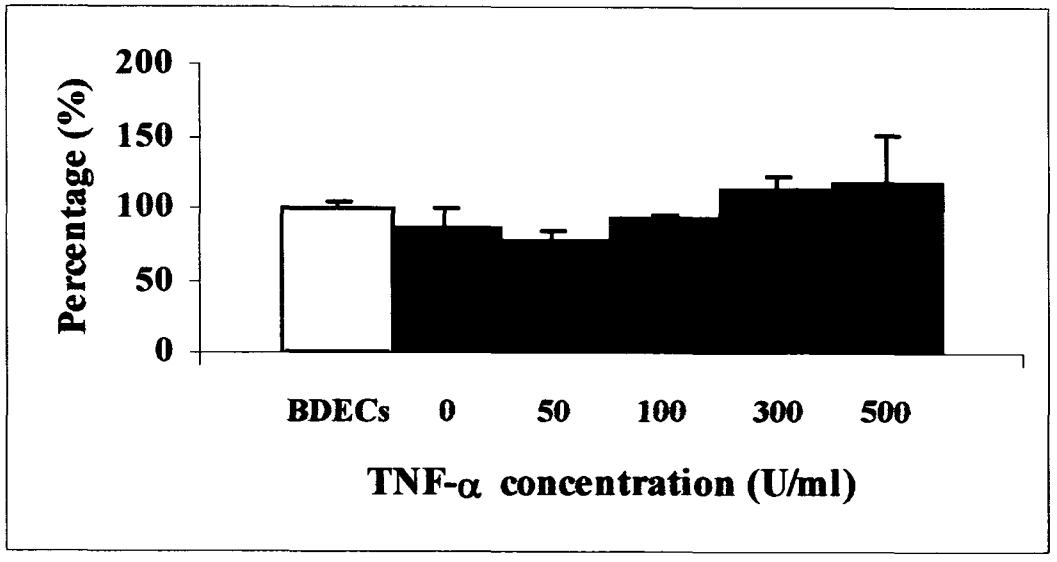

Percentage (%)
200
150
100
50
0
BDECs
0
50
100
300
500
TNF-α concentration (U/ml)

FIGURE 5.5

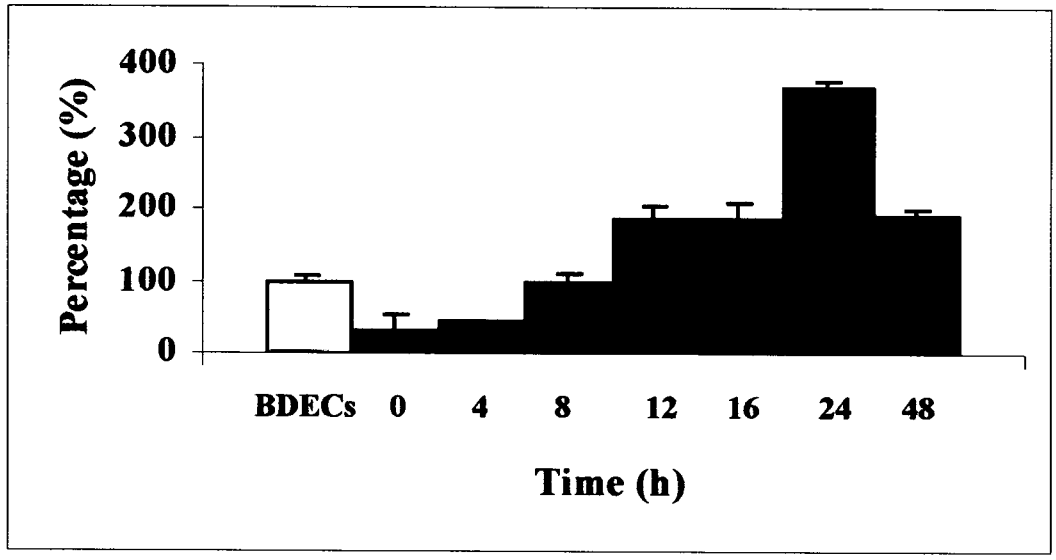
Expression and regulation of TNF-R75 by TAMECs *in vitro*

TNF-R75 protein expression and regulation was assessed by cell-based enzyme immunoassay on TAMECs stimulated with TNF-α (50–500 U/ml) for 0–24 h. Each treatment was performed in triplicate and protein levels evaluated by enzyme immunoassay as described in Chapter 4, Section 4.2.2. TNF-R75 levels were quantitated in an ELISA plate reader at 492 nm. The results of all experiments are expressed as a percentage of the absorbance reading of the internal standard (constitutive expression of TNF-R55 on BDECs, represents 100%), and are presented as the mean + 1 SD (n=3).

A. Time course of TNF-R75 protein regulation in response to TNF-α (300 U/ml).

B. Dose response of TAMECs to varying concentrations of TNF-α for 24 h.

A


400
300
200
100
0
Percentage (%)
BDECs 0 4 8 12 16 24 48
Time (h)

B

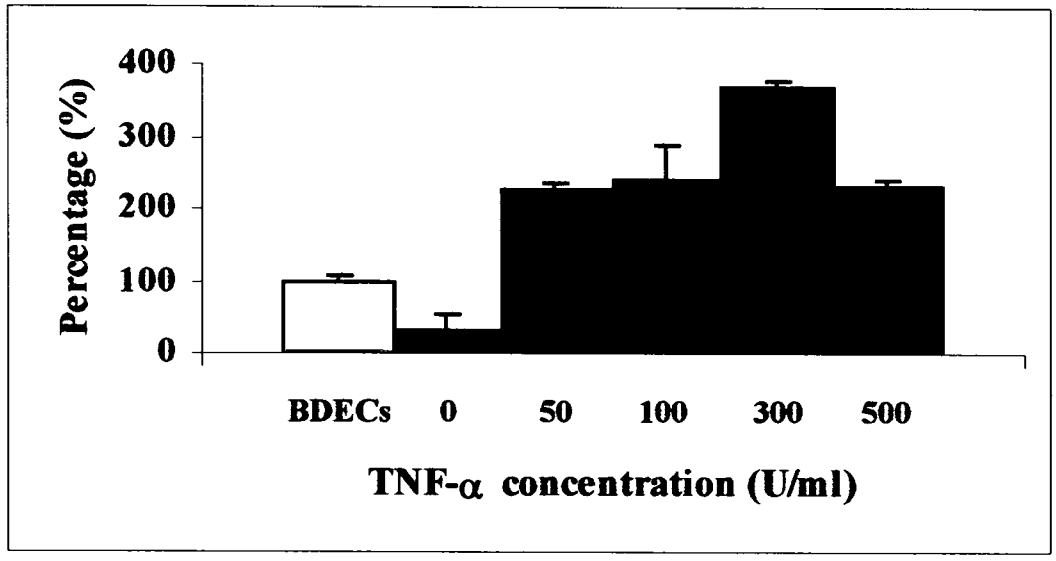

400
300
200
100
0
Percentage (%)
BDECs 0 50 100 300 500
TNF-α concentration (U/ml)

FIGURE 5.6

TNF-R55 protein expression by tumour-associated microvessels *in vivo*

TNF-R55 protein expression was evaluated on formalin-fixed, paraffin embedded tumour tissues by immunohistochemistry. The expression of TNF-R55 was also assessed after a single, intratumoural injection of TNF-α (5×10^4 U/ml) for 30 min–24 h. Antigen retrieval was performed on all sections with boric acid (200 mmol/l, 60°C, ON) prior to applying the primary antibody ON at 4°C. Sites of TNF-R55 expression were developed with DAB and observed as a brown deposit.

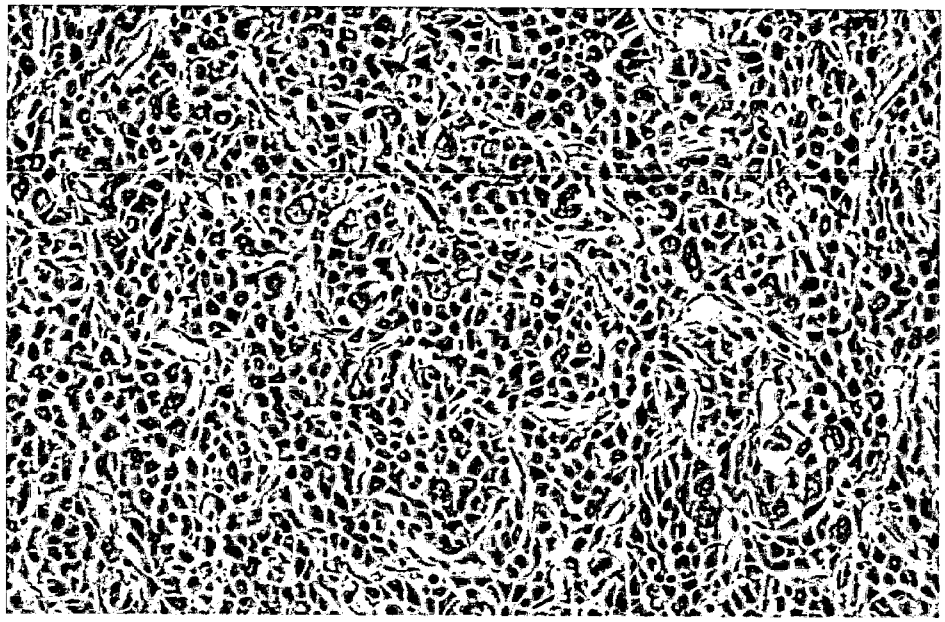
A. Untreated tumour. TNF-R55 is constitutively expressed by tumour cells, tumour-infiltrating leukocytes and tumour-associated microvascular endothelial cells, ×220.

B. Tumour tissue stimulated with TNF-α for 4 h *in vivo*. Immunostaining for TNF-R55 protein is intense in tumour cells, tumour-infiltrating leukocytes and tumour-associated microvascular endothelial cells, ×220.

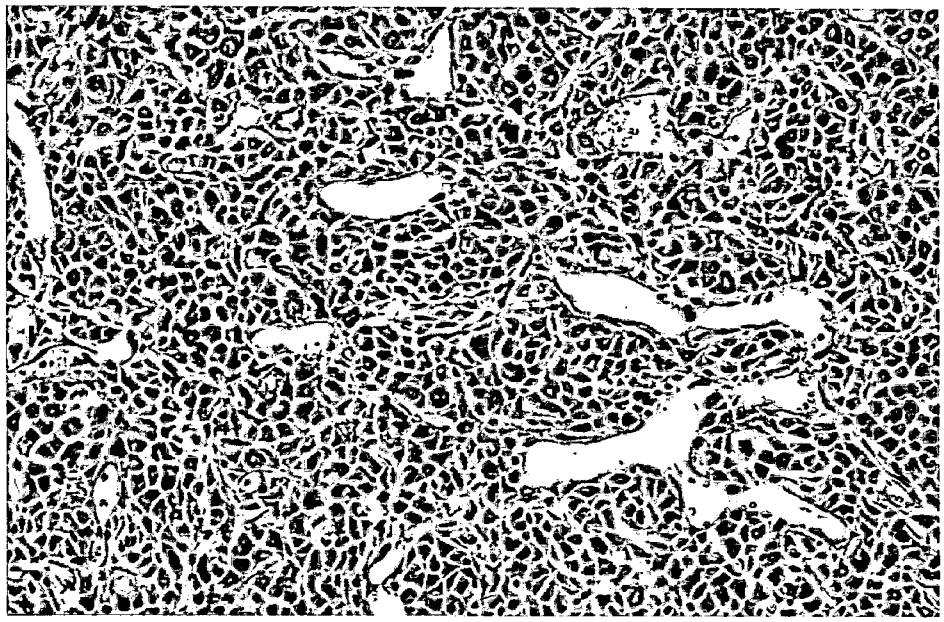
C. Tumour tissue stimulated with TNF-α for 24 h *in vivo*. Strong, uniform labelling for TNF-R55 protein is evident within tumour-associated microvascular endothelial cells as well as tumour cells, ×460.

Note. A representative negative control section for TNF-R55 immunostaining (section exposed to boric acid-mediated antigen retrieval and a biotinylated rabbit anti-goat secondary antibody) is presented in Figure 5.7C.

A



B



C

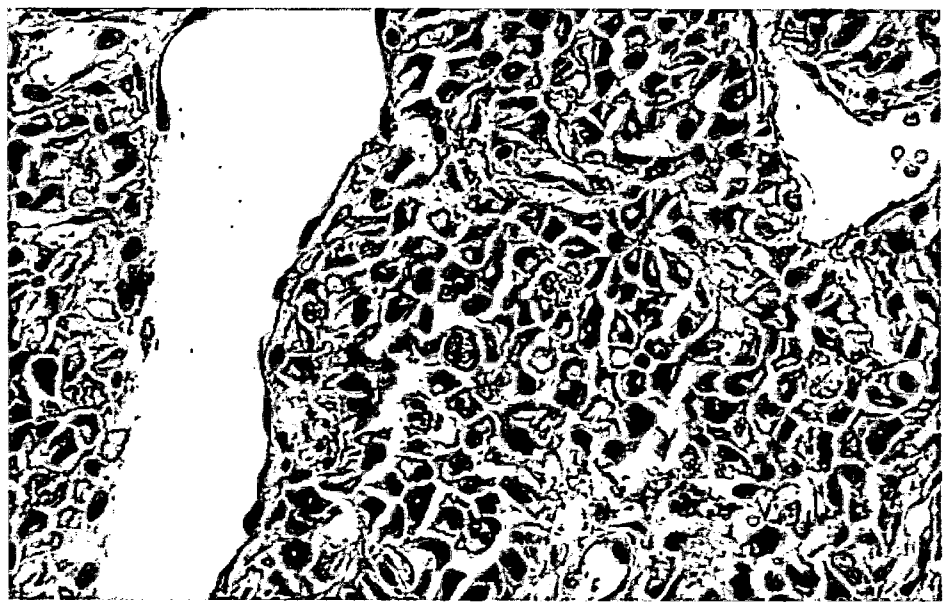


FIGURE 5.7

TNF-R75 protein expression by tumour-associated microvessels *in vivo*

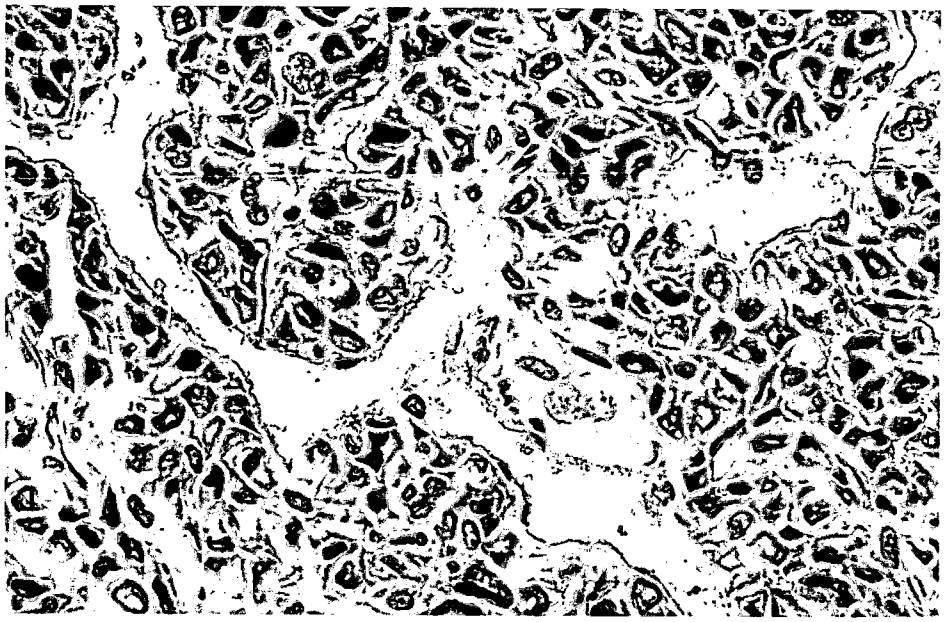
TNF-R75 protein expression was evaluated on formalin-fixed, paraffin embedded tumour tissues by immunohistochemistry. The expression of TNF-R75 was also assessed after a single, intratumoural injection of TNF-α (5×10^4 U/ml) for 30 min–24 h. Antigen retrieval was performed on all sections with boric acid (200 mmol/l, 60°C, ON) prior to applying the primary antibody ON at 4°C. Sites of TNF-R75 expression were developed with DAB and observed as a brown deposit.

A. Tumour tissue stimulated with TNF-α for 24 h *in vivo*. Immunodetectable TNF-R75 protein is localized to tumour-associated microvascular endothelial cells, ×460.

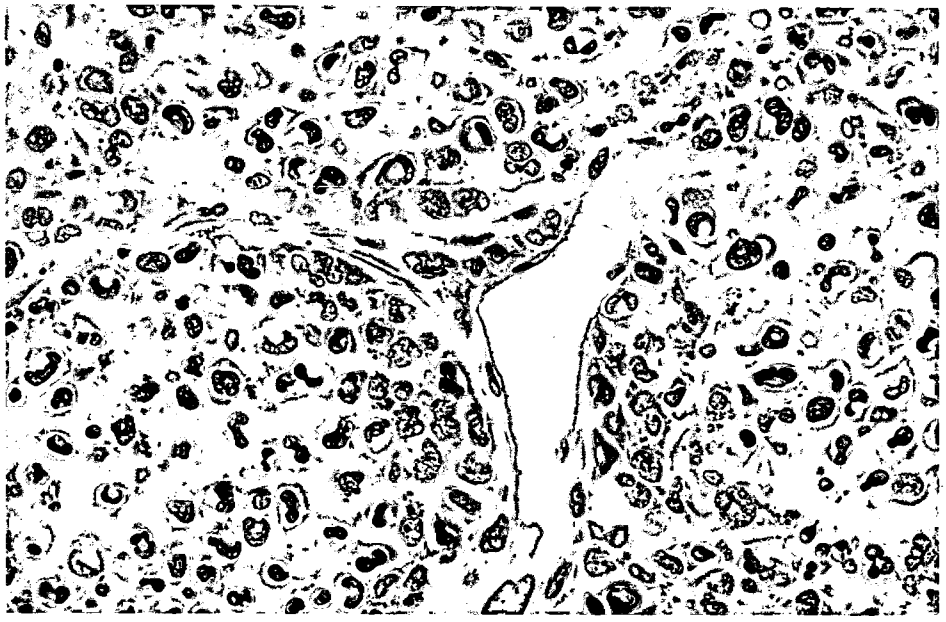
B. Tumour tissue stimulated with TNF-α for 24 h *in vivo*. The expression of TNF-R75 protein by tumour-associated microvascular endothelial cells is associated with the development of TNF-induced tumour cell necrosis, ×460.

C. Negative control – goat secondary antibody. Negative immunocontrols were performed in each staining run and consisted of the primary antibody being replaced with non-immune serum. This is representative of all immunohistochemistry negative controls for paraffin-embedded specimens exposed to boric acid-mediated antigen retrieval and incubated with a biotinylated rabbit anti-goat secondary antibody. Immunostaining for both TNF-R55 and TNF-R75 were performed under these conditions, ×220.

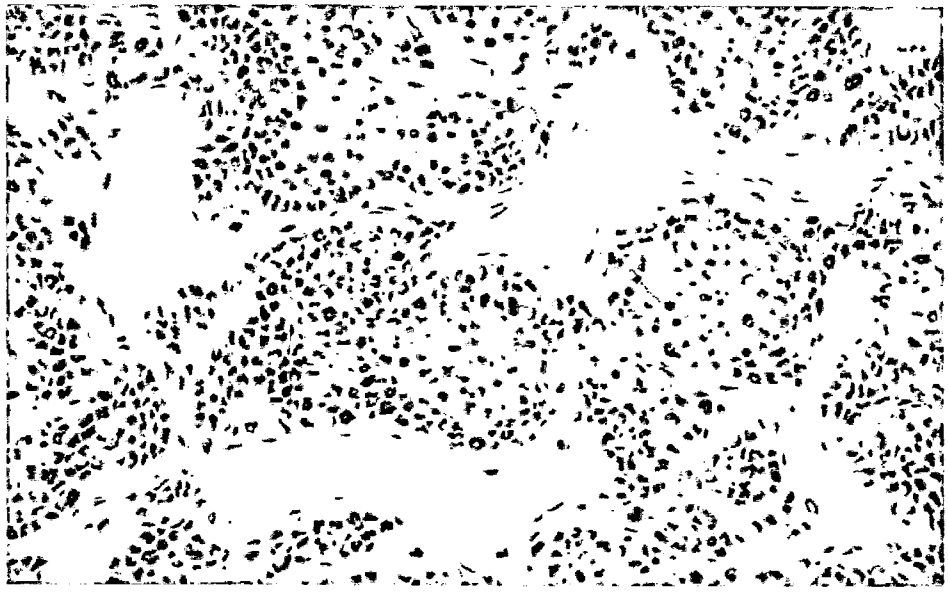
A



B



C



CHAPTER 6. TUMOUR-ASSOCIATED MICROVASCULAR ENDOTHELIAL CELLS AS A TARGET FOR TNF-MEDIATED CYTOTOXICITY

6.1 INTRODUCTION

TNF-α was first identified as a molecule responsible for inducing haemorrhagic necrosis in susceptible tumours (Carswell *et al.*, 1975). As first discussed in Chapter 1, it is well established that TNF-α is capable of killing certain neoplastic cells *in vitro* as well as causing haemorrhagic necrosis of established tumours *in vivo* (de Kossodo *et al.*, 1995; Pagliacci *et al.*, 1993). In tumours it has been suggested that TNF-mediated cytotoxicity occurs through an increased sensitivity of tumour-associated microvascular endothelial cells to TNF-α. One potential mechanism is through the release of the cytokine EMAP-II within tumour which acts on microvascular endothelial cells (Kao *et al.*, 1992; Wu *et al.*, 1999).

While TNF-α can be cytotoxic to tumour cells as well as tumour-associated microvascular endothelial cells, it has been proposed that TNF-mediated tumour necrosis and regression may occur in response to disruption of the tumour-associated microvasculature following endothelial cell apoptosis. In response, tumour cells undergo necrosis and apoptosis as a result of losing their vascular supply. This response would be intimately linked to the expression of one or both TNF receptors, TNF-R55 and TNF-R75, on tumour-associated microvascular endothelial cells. Tumour cells may therefore undergo apoptosis either through direct TNF-mediated cytotoxicity (through TNF receptor expression) or secondary as a result of disruption of the tumour-associated microvasculature.

From the observations made in Chapter 5, the tumour-associated microvasculature appears to be a target for TNF-α. TNF-R55 is constitutively expressed by tumour-associated microvascular endothelial cells and TNF-R75 is induced in these vessels after exposure to TNF-α. Low levels of TNF-R55 and absence of TNF-R75 protein on tumour cells further support the notion that tumour-associated microvascular endothelial cells may be a primary target for the anti-tumour actions of TNF-α.

The ability of TNF-α to induce cytotoxicity is ultimately controlled by the presence of one or both TNF receptors. As noted in Chapter 5, the induction of TNF-R75 on the tumour-associated microvasculature was followed by the appearance of necrosis in TNF-treated tumours. While many cell types within tumour tissues express one and/or both TNF receptors, the particular cells responsible for mediating the anti-tumour action of TNF-α remains unclear. Tumour-associated microvascular endothelial cells have been shown to express both receptor species (Chapter 5) and identifying their contribution in TNF-mediated apoptosis may lead to the development and/or refinement of strategies to deliver TNF-α as a means of tumour therapy.

This chapter aims to determine the role tumour-associated microvascular endothelial cells play in mediating the anti-tumour actions of TNF-α. Specifically, is TNF-α directly cytotoxic to tumour-associated microvascular endothelial cells *in vivo* and *in vitro* and mediates tumour apoptosis due to disruption of the tumour vascular supply? To investigate the mechanism(s) through which TNF-α induces haemorrhagic necrosis in this tumour model, TNF-induced DNA fragmentation will be assessed *in vitro* and *in vivo*. In particular, the involvement of tumour-associated microvascular endothelial cells in TNF-induced apoptosis willl be examined in detail. Using TAMEC cultures (see Chapter 3), TNF-induced apoptosis will be analyzed morphologically by electron and fluorescence microscopy and TUNEL staining. *In vivo*, the development of haemorrhagic necrosis will be observed by light microscopy (H&E staining) as well as TUNEL staining to identify apoptotic cells within the tumour.

6.2 MATERIALS AND METHODS

6.2.1 TNF-induction of apoptosis in TAMECs and tumour tissues

To induce apoptosis *in vitro*, TAMECs were exposed to TNF-α (0–5000 U/ml) for 1, 2, 3 or 4 days. For incubations greater than 2 days, fresh growth medium with freshly prepared TNF-α was added every second day. *In vitro* studies were performed on cultures of subconfluent and confluent TAMECs to assess differences in sensitivity between proliferating and quiescent endothelial cells. TAMECs induced to undergo apoptosis through serum deprivation for 24 h (Gerber, Dixit and Ferrara, 1998) served as a positive control for each *in vitro* investigation.

TNF-induced apoptosis *in vivo* was studied in formalin-fixed, paraffin-embedded TNF-treated tumour sections (Chapter 2, Section 2.2.3). Tumours were exposed to TNF-α (5×10^4 U/ml) for 30 min–24 h.

6.2.2 Morphology of TNF-induced apoptosis

Morphological features of TNF-induced apoptosis were studied in TAMECs by fluorescence microscopy and in paraffin-embedded tumour tissues by light microscopy.

6.2.2.1 *Fluorescence microscopy*

For observations of apoptotic cell death, the fluorescence staining method of Ellerby *et al.* (1999) was performed. TAMECs were treated with TNF-α (0–5000 U/ml) for 1, 2, 3 or 4 days, washed with PBS and a 20-fold dilution of the dye mixture (100 μg/ml acridine orange (Sigma) and 100 μg/ml ethidium bromide) in PBS added. Cultures were examined with an Olympus BX60 fluorescence microscope. Cells with nuclear morphology including chromatin condensation and/or nuclear fragmentation which had incorporated acridine orange were categorized as early/mid apoptotic cells and those that had compromised membranes and were ethidium bromide-positive were considered to be in the late stages of apoptosis (Ellerby *et al.*, 1999).

6.2.2.2 *Light microscopy*

Formalin-fixed, paraffin-embedded sections of TNF-treated tumours (5×10^4 U/ml, 30 min–24 h) were stained with H&E (Chapter 2, Section 2.4.1) to observe the development and progression of TNF-induced necrosis.

6.2.3 Detection of apoptosis by TUNEL staining

DNA fragmentation was visualized by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) as described in the ApopTag® Plus peroxidase *in situ* apoptosis detection kit (Intergen Company).

6.2.3.1 *Staining protocol for TAMECs*

For *in vitro* analysis of TNF-induced apoptosis (5000 U/ml; 0–4 days) in TAMECs, monolayers in 16-well chamber slides, were collected from suspension (following TNF-induced separation from substrate), fixed in 1% (w/v) paraformaldehyde in PBS for 10

min, washed and postfixed in ethanol:acetic acid (2:1) for 5 min at -20°C. TAMECs were washed in PBS and microwave heat-treated in citrate buffer (Chapter 2, Section 2.4.2.1.1). After washing in PBS (2×5 min), equilibration buffer was applied to the cells prior to the addition of TdT enzyme for 60 min at 37°C. Excess enzyme was removed (stop/wash buffer/10 min; PBS, 3×1 min) followed by the anti-DIG-peroxidase conjugate for 30 min. Colour development with DAB was monitored microscopically. TAMECs were counterstained with 0.5% (w/v) methyl green for 10 min, dehydrated and mounted. TUNEL-positive cells exhibited brown cytoplasmic deposits. Controls were run in each experimental batch and included a positive control slide (normal female rodent mammary gland, 3–5 days after weaning) included within the kit. Negative controls included omitting the TdT enzyme or anti-DIG antibody.

6.2.3.2 *Staining protocol for tissue sections*

TUNEL staining was performed on formalin-fixed, paraffin-embedded sections of tumours exposed to TNF-α (5×10^4 U/ml) for 30 min–24 h as described for TAMECs (Section 6.2.3.1) with the following modifications: after rehydration tissues were digested with proteinase K (20 μg/ml; Sigma) for 15 min, washed in PBS and endogenous peroxidase activity quenched with 3% (v/v) hydrogen peroxide in methanol for 5 min. Microwave heat-treatment was not performed on tumour sections. TUNEL staining was then performed as described in Section 6.2.3.1 with the application of equilibration buffer.

6.3 RESULTS

6.3.1 Observations of TNF-induced apoptosis in TAMECs by fluorescence microscopy

TNF-treated TAMECs were stained with acridine orange and ethidium bromide to observe the morphological aspects of apoptosis by fluorescence microscopy. Control (untreated) TAMECs did not demonstrate any morphological features of apoptosis. TAMECs induced to undergo apoptosis through serum deprivation demonstrated nuclear changes consistent with apoptosis including cytoplasmic condensation, formation of surface protuberances, chromatin condensation and fragmentation and in, the later stages, a compromised plasma membrane (Figures 6.1A). Apoptotic nuclear changes were associated with detachment of TAMECs from the culture surface.

6.3.2 TUNEL analysis of TNF-induced apoptosis in TAMECs

TNF-induced apoptosis was analyzed in TAMECs by TUNEL staining. TNF-α doses of up to 5000 U/ml for 1, 2, 3 or 4 days were unable to initiate apoptosis in cultures (Figure 6.1B). TAMECs induced to undergo apoptosis through serum deprivation were consistently positive for DNA fragmentation as observed by DNA fragmentation and surface blebbing (Figures 6.1C, 6.1D).

6.3.3 Morphological aspects of TNF-mediated tumour necrosis

The DAMA grew as nests of tumour cells interspersed with vascular channels (Figure 6.2A). TNF-induced necrosis was first evident within 4 h post-TNF-α administration. Necrosis was initiated in the tumour cells at the centre of a nest and furthest from blood vessels. Often, only a thin layer of 4-6 tumour cells surrounded these vessels (Figure 6.2B) which progressively disappeared as the treatment continued. Tumour cell necrosis and associated haemorrhage became increasingly widespread over 24 h. At this time, viable tumour cells were confined to the peripheral rim of the tumour. The central area contained necrotic tumour cells and tumour-associated microvessels filled with erythrocytes. The architecture of these tumour-associated microvessels varied from those that were intact to those that appeared to be disrupted with loss of erythrocytes (Figure 6.2C). In some tumour-associated microvessels, extensive degeneration and loss of the endothelial cells lining the vessel was seen (Figure 6.2D).

6.3.4 *In situ* detection of apoptosis in TNF-treated endothelial cells

The TUNEL technique was used to detect apoptosis within tumour-associated microvascular endothelial cells *in vivo*. Apoptosis positive tumour and endothelial cells were visualized as a brown nuclear deposit at sites of DNA fragmentation. In normal (unstimulated) tumours, apoptosis was rarely detected in isolated tumour cells (Figure 6.3A). Upon stimulation with TNF-α, apoptosis was observed within the tumour cells and was localized to the margin between the viable and apoptotic/necrotic tumour cells (Figure 6.3B). TNF-induced apoptosis within tumour-associated microvascular endothelial cells was limited to isolated endothelial cells within a blood vessel (Figure 6.3C). This was observed in tumour-associated microvessels within the necrotic tumour tissue close to the margin between the viable and necrotic tumour cells.

6.4. DISCUSSION

The capacity of TNF-α to induce haemorrhagic necrosis of certain tumours *in vivo* has been proposed to be mediated, at least in part, by injury to the tumour-associated microvasculature. During ILP, TNF-α and IFN-γ activate tumour-associated microvascular endothelial cells which subsequently swell and detach from the subendothelial matrix through the deactivation of $\alpha_v\beta_3$ (Renard *et al.*, 1994; Rüegg *et al.*, 1998). Selective destruction of tumour-associated microvascular endothelial cells and death of tumour cells is observed after TNF-α and IFN-γ administration to a breast cancer xenograft implanted in mice (de Kossodo *et al.*, 1995). Significantly, these responses are tumour specific since normal adjacent tissues remain unaffected by the treatment. They also support the proposal that tumour-associated microvascular endothelial cells are a target for the cytotoxic action of TNF-α (in association with IFN-γ).

Apoptosis in TNF-treated TAMECs was assessed by observing nuclear condensation and fragmentation by fluorescence microscopy and TUNEL staining. Exposure to TNF-α doses of 0–5000 U/ml for up to 4 days *in vitro* was unable to initiate apoptosis in tumour-associated microvascular endothelial cells. TAMECs induced to undergo apoptosis through serum deprivation were consistently positive.

Many factors contribute to the sensitivity of endothelial cells to TNF-mediated cytotoxicity *in vitro*. For example, endothelium in the proliferative and/or motile state has an enhanced responsiveness to and increased affinity for TNF-α (Gerlach *et al.*, 1989; Spyridopoulos *et al.*, 1998). This increased sensitivity is due to TNF-inducing G1 cell-cycle arrest and repression of the transcription factor E2F (Spyridopoulos *et al.*, 1998). There were no detectable differences in sensitivity to TNF-α between subconfluent and confluent cultures of TAMECs.

Transcriptional inhibitors, such as Act. D, are included in many *in vitro* investigations into the cytotoxic actions of TNF-α (Lucas *et al.*, 1998). However, a role for TNF-R75 in TNF-mediated endothelial cell cytotoxicity would not be detected in the presence of these inhibitors (Lucas *et al.*, 1998). This is based on the observation that microvascular endothelial cells isolated from *tnfr2*^{o} mice are normally resistant to TNF-mediated cytotoxicity but are lysed when Act. D is present (Lucas *et al.*, 1998). Transcriptional

inhibitors were not added to TNF-treated TAMECs in this current study in order to reproduce more accurately conditions occurring within the tumour microenvironment. Although TNF-α concentrations of up to 50 times higher are required in the absence of Act. D to induce direct endothelial cell killing (Lucas *et al.,* 1997b), exposure of TAMECs to TNF-α at 5000 U/ml was ineffective at initiating DNA fragmentation. In other studies, TNF-α doses from 10-2000 U/ml have induced apoptosis (22–63%) in HUVECs and bovine aortic or pulmonary artery endothelial cells using a wide variety of methods (Maier *et al.,* 1996; Polunovsky *et al.,* 1994; Robaye *et al.,* 1991; Spyridopoulos *et al.,* 1998). While it has been suggested that microvascular endothelial cells have an increased sensitivity to TNF-induced apoptosis (Sato *et al,* 1996) it may be that, as with many other features, tumour-associated microvascular endothelial cells have a differential response to this action.

As TNF-α alone was unable to initiate apoptosis *in vitro*, it may be that interactions with other molecules and/or environmental conditions within the tumour may be required. In this study culture medium containing TNF-α was not supplemented with tumour-conditioned medium due to the introduction of many other cytokines and growth factors which may stimulate or inhibit TNF-induced apoptosis. Often IFN-γ is given in combination with TNF-α as it augments endothelial cell responses (Mantovani *et al.,* 1997). IFN-γ is administered 2 days prior to ILP to upregulate endothelial TNF receptor expression (Lejeune, Rüegg and Liénard, 1998). It may be that TAMECs need to be primed with IFN-γ (or other cytokines or growth factors) prior to exposure to TNF-α to increase their sensitivity to TNF-mediated apoptosis.

In vivo, apoptosis was studied in TNF-treated tumours by morphology and TUNEL labelling. Tumour cell necrosis was observed within 4 h of TNF-α administration. This was localized to the central regions of the tumour and expanded radially as time increased. Tumour-associated microvessels within the necrotic tissue were congested with erythrocytes however the majority of vessels remained intact. In a minority of vessels, the integrity of the endothelial cell layer appeared to be compromised and this was associated with the movement of erythrocytes into the tissue. This finding was supported by TUNEL labelling in which apoptotic endothelial cells were uncommon.

As described in Chapter 5, the expression and regulation of TNF-R75 by the tumour-associated microvascular endothelial cells parallels the development of tumour necrosis. This suggests that TNF-R75 is crucial for the induction of tumour cell necrosis mediated by TNF-α. Along with sTNF-α, TNF-R75 interacts with membrane-bound TNF-α. *In vitro,* the contribution, if any, of membrane-bound TNF-α to inducing cytotoxicity would not have been detected. The specific interaction between TNF-R75 and membrane-bound TNF-α, such as that produced by tumour-associated macrophages, may play a significant role in TNF-induced apoptosis *in vivo*. In particular, the localization of tumour-associated macrophages at the margin between the viable and necrotic tumour tissue (Nilsson, 1995; Nilsson, Woods and Rofe, 1996) further supports this proposal. Tumour-associated macrophages are known to secrete a wide range of cytokines and growth factors including TNF-α, IL-1, IL-6 and TGF-β (Seljelid and Busund, 1994).

Along with endothelial cell apoptosis, TNF-treated tumours contained TUNEL-positive tumour cells. As determined in Chapter 5, these tumour cells constitutively express TNF-R55 while TNF-R75 is limited to isolated tumour cells. Tumour cells may contribute to the antitumour action of TNF-α through TNF-α binding to TNF-R55 and initiating the signalling cascade. Higher concentrations of TNF-α may be required to initiate this response as TNF-R75 is not expressed and therefore cannot contribute to signal transduction through ligand passing.

The observation that tumour-associated microvessels within the centre of the tumour are most sensitive to TNF-mediated cytotoxicity is in contrast to the accepted view that proliferating endothelial cells (normally those at the periphery of the tumour which are undergoing active angiogenesis) have an increased sensitivity to TNF-mediated cytotoxicity (Gerlach *et al.,* 1989). As these tumour-associated microvessels have only recently been incorporated into the tumour they may still possess features of normal host microvessels which protect them from TNF-α.

Microenvironmental conditions within the tumour also appears to play a key role in determining the sensitivity of tumour-associated microvascular endothelial cells to TNF-mediated cytotoxicity. In general, microvascular endothelial cells exposed to hypoxic conditions are resistant to TNF-induced apoptosis (Sampson and Chaplin, 1994). As

growth factor-stimulated endothelial cells are selectively sensitive to TNF-α (Schweigerer, Malerstain and Gospodarowicz, 1987), their presence within tumours may increase the sensitivity of tumour-associated microvessels to TNF-mediated cytotoxicity, even when exposed to hypoxic conditions.

As a functional vasculature is an absolute requirement for tumour growth, the data indicate that exposure to TNF-α is followed by critical vascular disturbance. This may occur by apoptosis, in which loss of only a few endothelial cells is effective or by ischaemia, either as a result of erythrocyte congestion and/or the induction of procoagulant activity. The lack of homeostatic control due to the absence of smooth muscle cells and associated structures in tumour-associated vessels may also contribute to the maintenance of blood flow disturbance (Balkwill, 1992). In the current study, tumour-associated microvessels in TNF-treated tumours contained endothelial cells undergoing apoptosis as well as vessels congested with erythrocytes. These responses directly influence the structure and function of the tumour-associated microvasculature. They also support a potential mechanism through which these vessels are more vulnerable to the induction of necrosis than endothelial cells within normal tissues.

In the present tumour model, it is clearly evident that TNF-induces haemorrhagic necrosis *in vivo*. While there appears to be some changes to the tumour-associated microvasculature, widespread vascular destruction is not evident. Nevertheless it seems plausible that even if only small numbers of endothelial cells within a vessel are affected, blood flow is compromised causing the tumour cells surrounding this vessel to undergo necrosis. Indeed, endothelial cells have an intrinsic amplification mechanism such that elimination of a single endothelial cell can inhibit the growth of up to 100 tumour cells (Denekamp, 1993). Therefore, every endothelial cell within a particular vessel may not need to be affected for a response to be observed. Additionally, as tumour-associated microvascular endothelial cells are immature. As they are not supported by accessory cells such as pericytes and a basal lamina, the loss of individual endothelial cells would result in a total breakdown in vascular integrity.

6.5 SUMMARY

TNF-α induces haemorrhagic necrosis in susceptible tumours. Tumour-associated microvascular endothelial cells have been proposed to be the primary cell type initiating this response. A panel of techniques including (fluorescence microscopy and TUNEL staining were unable to detect TNF-induced apoptosis in TAMECs despite a range of doses and time schedules being applied. There was no difference between subconfluent/proliferating and confluent TAMEC cultures. Apoptosis was readily observed in TAMECs induced to undergo apoptosis through serum deprivation. In vivo, TNF-induced haemorrhagic necrosis was initiated in the central areas and expanded radially as exposure increased. Tumour-associated microvessels within necrotic areas were filled with erythrocytes and the majority of vessels appeared to be intact. A minor proportion of vessels had erythrocytes escaping into the tissue and this was associated with a compromise in the integrity of the endothelial cell barrier. By TUNEL labelling, isolated apoptotic endothelial cells were observed in TNF-treated tumours. These findings suggest that the tumour-associated microvasculature contributes to TNF-induced haemorrhagic necrosis either through endothelial cell apoptosis or disruption to blood flow by erythrocyte congestion, thrombosis or a combination of the two.

FIGURE 6.1

Morphological observations of TNF-induced apoptosis in TAMECs

TAMECs were stimulated with TNF-α (0–5000 U/ml) for 0–4 days to induce apoptosis. Cells were either stained with acridine orange and ethidium bromide as described in Section 6.2.2.1 or by TUNEL using the ApopTag® plus peroxidase *in situ* apoptosis detection kit as described in Section 6.2.3.

A. TAMECs induced to undergo apoptosis through serum deprivation and stained with acridine orange and ethidium bromide. These cells demonstrate many features of apoptosis including condensation of their cytoplasm, formation of surface protuberances, chromatin condensation and fragmentation, ×1050.

B. TAMECs treated with TNF-α (5000 U/ml) for 4 days and stained by TUNEL. There is no evidence of TNF-induced apoptosis, ×150.

C. TAMECs induced to undergo apoptosis through serum deprivation and stained by TUNEL. Features of apoptosis including chromatin condensation and fragmentation are observed, ×1600.

D. TAMECs induced to undergo apoptosis through serum deprivation deprivation and stained by TUNEL. DNA fragmentation is observed within the nucleus of apoptotic cells as well as the formation of surface blebbing, ×1600.

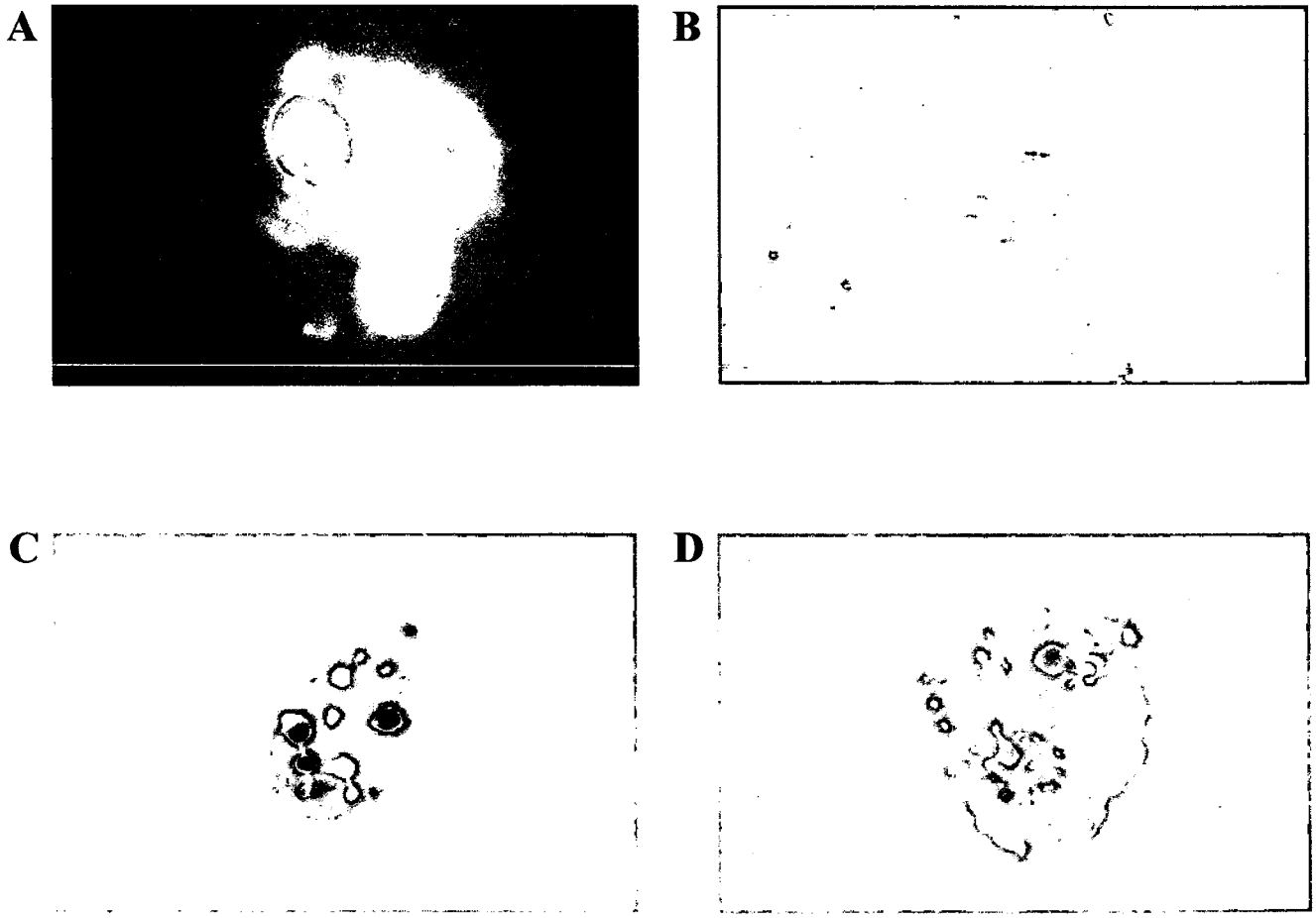

A
B
C
D

FIGURE 6.2

Morphological observations of TNF-induced apoptosis in tumour tissues

The morphology of TNF-induced tumour apoptosis and necrosis was observed in formalin-fixed, paraffin-embedded tissues. Sections were stained with H&E.

A. Untreated tumour. Nests of tumour cells are supported by a vascular stroma, ×240.

B. Tumour treated with TNF-α for 4 h *in vivo*. Areas of tumour cell necrosis (N) are observed as well as pockets of viable tumour cells, ×120.

C. Tumour treated with TNF-α for 24 h *in vivo*. Tumour-associated microvessels congested with erythrocytes are surrounded by necrotic tumour cells. In addition, areas of erythrocyte extravasation from the tumour-associated microvessels are observed, ×220.

D. Tumour treated with TNF-α for 24 h *in vivo*. This tumour-associated microvessel and surrounding tumour cells are undergoing necrosis/apoptosis. Microvascular endothelial cells lining the blood vessel lumen are not observed and there is a disruption to the integrity of the vessel lining, ×720.

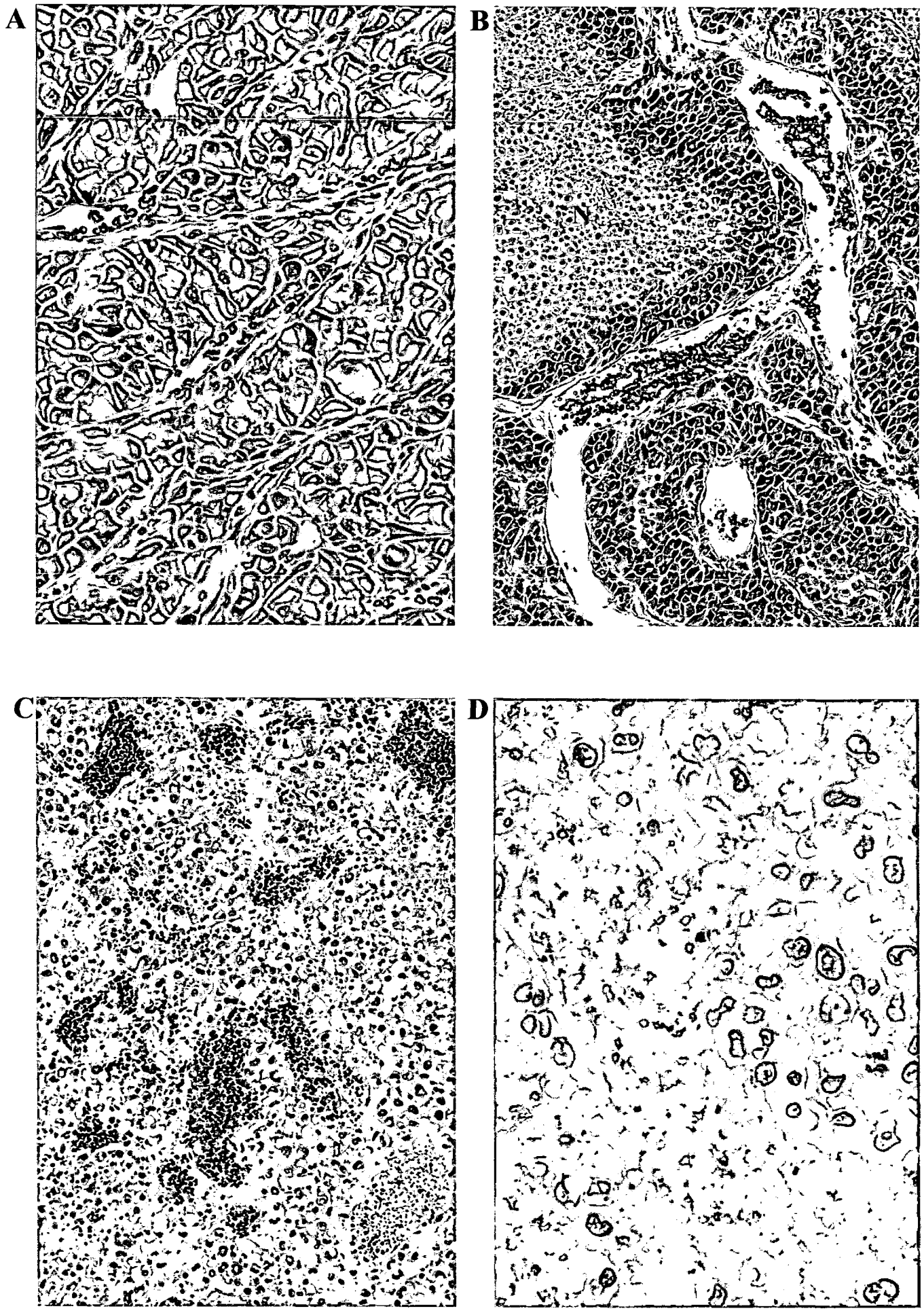

A
B
C
D

FIGURE 6.3

In situ detection of apoptosis by TUNEL labelling

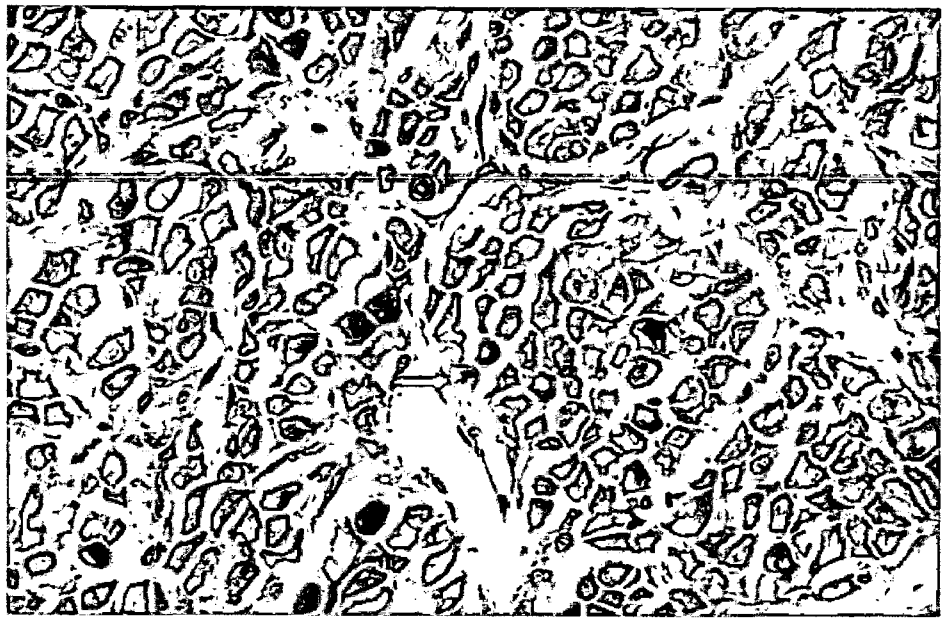
In situ apoptosis was observed in TNF-treated formalin-fixed, paraffin-embedded tumour tissues. DNA fragmentation was detected using the ApopTag® plus peroxidase *in situ* apoptosis detection kit as described in Chapter 6, Section 6.2.3.

A. Untreated tumour. Tumour cell apoptosis is evident (arrow) within individual tumour cells within the tumour nests, ×460.

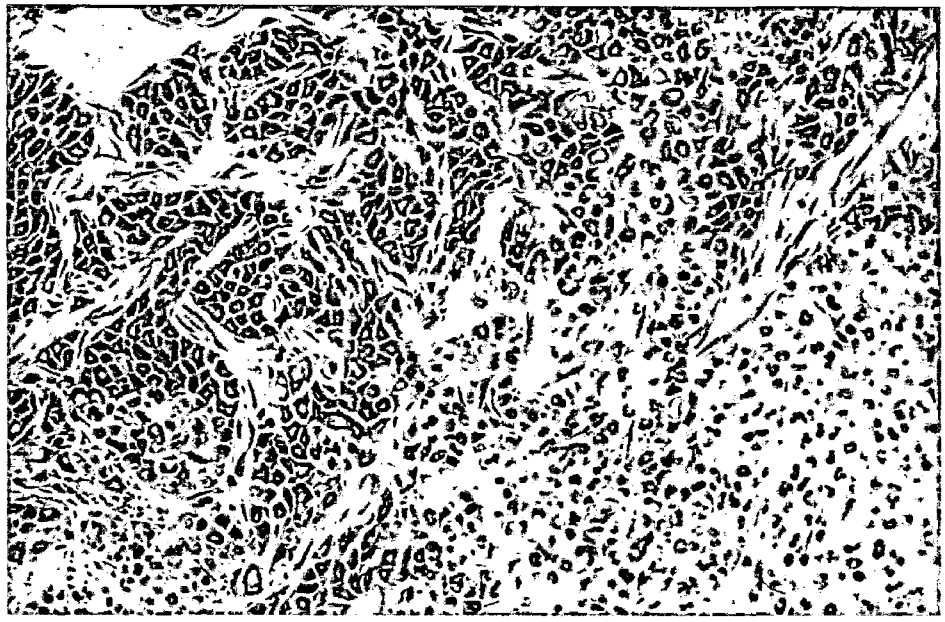
B. Tumour treated with TNF-α for 4 h *in vivo*. Apoptotic tumour cells are visible at the margin between the viable and necrotic/apoptotic tumour tissue, ×220.

C. Tumour treated with TNF-α for 24 h *in vivo*. A number of cells lining the blood vessel are positive for DNA fragmentation (arrows). Neighbouring tumour cells are also TUNEL positive, ×750.

A



B



C



CHAPTER 7. CONCLUDING REMARKS

As an integral part of the tumour microenvironment, the vasculature is critical for neoplastic growth. Tumour-associated vasculature is structurally and functionally different from that in normal tissues, however, the significance of these changes is not completely understood nor is the mechanism whereby these changes are induced by the tumour microenvironment.

The principal aim was this study is to test the hypothesis that the anti-tumour actions of TNF-α are mediated through tumour-associated microvascular endothelial cells. More specifically, the following questions were addressed:

a) Is it possible to isolate and culture microvascular endothelial cells from solid tumour tissue and are these isolates a representative *in vitro* model of the tumour-associated microvasculature?
b) Are the endothelial cell adhesion molecules E-selectin, ICAM-1 and PECAM-1 upregulated by TNF-α?
c) Are one or both of the TNF receptors expressed by tumour-associated microvascular endothelial cells and does their expression and/or regulation mediate the anti-tumour action of TNF-α? And is the expression of one or both TNF receptors linked to the expression of adhesion molecules?
d) Is TNF-α directly cytotoxic to tumour-associated microvascular endothelial cells *in vivo* and *in vitro*?

These questions have been addressed through a number of experiments examining the expression of TNF-inducible molecules at the gene and protein level. The study focussed on tumour-associated microvascular endothelial cells which were established *in vitro* by the development of a novel method to isolate and culture these cells and comparison with established *in vivo* techniques.

With the focus on the tumour-associated microvasculature as a therapeutic target, a more complete understanding of the biology of these cells particularly in terms of responses to antitumour agents is required. Endothelial cell cultures are a simple tool which can be used to screen for, and investigate the mechanisms of action, of antivascular agents. Despite the knowledge of endothelial cell heterogeneity, *in vitro* investigations are generally being

performed on macrovascular endothelial cells or microvascular cells isolated from normal tissue sources. Although tumour-associated microvascular endothelial cells originate from normal host vessels, they demonstrate many distinct functional and phenotypic differences from normal microvascular endothelial cells. To reflect accurately the responses occurring within the vascular component of tumours, these studies should be performed on endothelial cells isolated from neoplastic specimens.

In the current study, a method was developed to isolated and culture tumour-associated microvascular endothelial cells from solid tumour tissue. TAMECs were separated from a mammary adenocarcinoma passaged in Dark Agouti rats. Pure cultures of tumour-associated microvascular endothelial cells were obtained by mechanical and enzymatic digestion prior to selection through their preferential adhesion to plastic. The isolation techniques and selective growth media used reduced contamination by tumour cells, fibroblasts and smooth muscle cells. This was found to be crucial in producing pure cultures of TAMECs. While selection through the preferential adhesion to plastic may have reduced the yield of endothelial cells, this step was required to remove contaminating cells such as tumour cells, fibroblasts and smooth muscle cells. Their presence during the first days of culture would soon lead to overgrowth of contaminating cells as they proliferate *in vitro* at an accelerated rate compared to microvascular endothelial cells. To decrease the proliferation of any minor contaminants, PPHS and heparin were supplemented in the culture medium. To support the proliferation of TAMECs and attempt to reproduce the tumour microenvironment, culture medium contained tumour-conditioned medium and ECGS. Using these isolation and culture techniques, pure population of TAMECs were routinely achieved.

To minimize potential phenotypic drift from the tumour-specific environment, only primary and passage one TAMECs were characterized. The endothelial identity of cultures was established using morphological, ultrastructural, immunohistochemical and metabolic techniques. TAMECs formed a confluent, contact-inhibited monolayer of typical cobblestone appearance and retained many of the features of tumour-associated microvascular endothelial cells *in vivo*. They expressed a number of endothelial cell markers including ACE, vWF, (TNF-stimulated) E-selectin and PECAM-1 however immunostaining for RECA-1, *Bandeiraea simplicifolia* BS-1 isolectin B_4 and ICAM-1

(after exposure to TNF-α) were not detected. These markers were also absent in tumour tissues (*in vivo*) and support the proposition that the phenotype exhibited by tumour-associated microvascular endothelial cells is a product of the unique environment within the tumour. It also demonstrates that TAMECs in early passages retain many features of the tumour microenvironment *in vitro* and can be used as a culture model of the tumour-associated microvasculature.

While these studies were performed on isolates from a tumour passaged in rats, a model which allowed direct correlation of events *in vivo* and *in vitro,* the method developed to segregate and culture TAMECs is applicable to neoplastic specimens in general. Comparison of cultures and tissue sections showed that the potential for loss of tumour-specific features *in vitro* did not occur in primary and passage one isolates. However, the reliance on primary and passage one cultures does mean that a continual, albeit minimal, source of tumour tissue is required. Establishing microvascular endothelial cells cultures from human tumours will still require confirmation of the endothelial phenotype *in vitro* by comparing to sections of the source of the tumour tissue.

The development of a method to isolate and culture microvascular endothelial cells provides a valuable tool to investigate tumour-associated microvascular endothelial cells *in vitro*. It is a much stronger model than 'normal' microvascular endothelial cells or HUVECs which are still being used in the majority studies characterizing the tumour-associated microvasculature. Simply adding tumour-conditioned medium and/or angiogenic growth factors is not sufficient to reproduce and induce the tumour-specific phenotype of tumour-associated microvascular endothelial cells. In addition, when responses are not clearly ascribed to the tumour cells or microvascular endothelial cells, analysis using this single cell population will provide further evidence for their involvement. The establishment of an *in vitro* model of tumour-associated microvascular endothelial cells addresses the first question of the hypothesis

As the tumour microenvironment is active in controlling the expression of molecules within the tumour, the gene and protein expression of TNF-regulated molecules were examined throughout this study. This allowed an insight into regulation of these proteins and their role in tumour progression or mediating the anti-tumour actions of TNF-α.

mRNA expression was assessed by Northern blot analysis and *in situ* hybridization *in vitro* and *in vivo*, respectively. The translated protein was detected in TAMECs by cell-based ELISA and within paraffin-embedded tumour tissue by immunohistochemistry. Direct comparison between microvascular endothelial cells *in vitro* and *in vivo* as well as mRNA and protein expression provided strong evidence for the changes occuring within the tumour in response to TNF-α. It also provides the first study to directly examine the tumour-associated microvasculature *in vivo* and *in vitro* and to examine gene and protein expression Histological examination allowed the cell types expressing the genes and proteins of interest to be identified and their role in mediating the effects of TNF-α to be further understood. In addition, the comparable observations made *in vitro* and *in vivo* between TAMECs and those obtained for the tumour-associated microvasculature supports the validity of TAMECs as an *in vitro* model for assessing the actions of TNF-α.

Microvascular endothelium is an important target for the actions of cytokines including TNF-α. For TNF-α to transmit a signal and hence exert an effect, endothelial cells must express one or both TNF receptors, TNF-R55 and TNF-R75. Within tumours, many cell types express one or both receptors species, however, the role of each receptor in tumour cells and the tumour-associated microvasculature in mediating the responses observed has not been clearly defined.

Within the tumour studied, TNF-R55 was constitutively expressed by tumour-associated microvascular endothelial cells *in vitro* and *in vivo* and was not upregulated by TNF-α. It was also localized to tumour cells and tumour-associated leukocytes and demonstrates that the majority of cell types within the tumour express TNF-R55. In contrast, TNF-R75 was not constitutively expressed and was only induced in tumour-associated microvascular endothelial cells following exposure to TNF-α. The restricted expression of TNF-R75 to the tumour-associated microvasculature after cytokine stimulation appears to suggest that its expression may play a key role in signalling the effects of TNF-α.

The expression of both TNF receptors within the tumour confirms that there are cells present which can bind and signal the effects of TNF-α. While TNF-R55 is constitutively and widely expressed by most tumour cell types this does not indicate that it is these cells which are mediating the actions of TNF-α. The restricted expression of TNF-R75 to

activated microvascular endothelial cells suggests that this receptor is only expressed under certain conditions and may play a more important role in the cytotoxic effects of TNF-α. The contribution of each receptor can only be accurately elucidated through the construction of mice deficient for one or both TNF receptors. The demonstration that tumour-associated microvascular endothelial cells express functional TNF receptors *in vitro* and *in vivo* has addressed another component of the hypothesis formulated for this thesis. This demonstrates the tumour-associated microvascular endothelial cells can be a target cell within the tumour for the actions of TNF-α.

The expression of both TNF receptors by tumour-associated microvascular endothelial cells provides evidence that these cells are a major target for TNF-α. In response to TNF-α, endothelial cells proliferate, release cytokines and express cell adhesion molecules. Within tumours, endothelial cell adhesion molecule expression mediates exposure to leukocytes and is integral to a host-derived immune response. E-selectin expression was rapidly induced on tumour-associated microvascular endothelial cells by TNF-α. ICAM-1 immunostaining on TNF-stimulated tumour-associated microvascular endothelial cells *in vivo* and *in vitro* was undetectable. This was unique to the tumour-associated microvasculature since normal host microvessels at the tumour periphery were consistently positive for ICAM-1 protein. In contrast, TNF-stimulated BDECs and normal (paraffin-embedded) tissues expressed immunodetectable ICAM-1. As ICAM-1 mRNA is being produced and regulated by TNF-α, this suggests that the lack of translation is not related to deficiencies in expression and/or signalling by the TNF receptors and that it is tumour-specific posttranscriptional mechanisms which are controlling the production of ICAM-1 protein. The mechanisms involved in this novel finding are unclear but this evidently represents a means whereby the tumour can evade the immune response by decreasing the infiltration of leukocytes. The examination of endothelial cell adhesion molecule expression by TNF-treated tumour-associated microvascular endothelial cells has addressed another of the questions formulated in the hypothesis. Although these cells express TNF receptors there are further microenvironmental factors which influence their expression and regulation.

A key component of an anti-tumour response is the infiltration of cytotoxic leukocytes into tumour tissues. Due the absence of ICAM-1 protein expression, this mechanism is

severely restricted. While E-selectin (and possibly other endothelial cell adhesion molecules not examined in this study) is expressed ICAM-1 is expressed at the later stages of an inflammatory response and sustains the recruitment of leukocytes. E-selectin is the first adhesion molecule expressed and expression rapidly decreases within 6-12 hours after the stimulus. Therefore, these cells may enter in the early stages however they will not be supported by new leukocytes later in the inflammatory response. It is at this time when anti-tumour responses would be occurring.

Despite the absence of ICAM-1, the appearance of tumour-infiltrating macrophages has been observed in this tumour model (Nilsson, 1995; Nilsson, Woods and Rofe, 1996). Tumour-bearing rats were injected (i.p.) with LPS (to activate macrophages to release TNF-α) and the expression of ICAM-1 protein and macrophage infiltration examined *in vivo* by immunohistochemistry. ICAM-1 was not immunodetectable on the tumour-associated microvascular endothelial cells at any time points studied however significant macrophage infiltration was observed. This suggests that other adhesion molecules supporting monocyte/macrophage infiltration are expressed or that these cells are entering the tumour by other means. One possible way is following the vascular destruction mediated by TNF-α within the tumour.

As well as activating endothelial cells, TNF-α can induce cytotoxic responses in susceptible cells. In particular, it has been proposed that TNF-induced necrosis in tumours is primarily mediated by disruption of the tumour-associated microvasculature through endothelial cell apoptosis. The involvement of the tumour-associated microvasculature in TNF-induced haemorrhagic necrosis was confirmed by the presence of apoptosis-positive endothelial cells by TUNEL labelling. This was limited to isolated endothelial cells within the tumour and within the margin between viable and necrotic tumour tissue. While extensive vascular destruction was not observed, when combined with TNF-induced vascular disturbance through erythrocyte congestion and thrombosis, even scattered endothelial cell apoptosis has the potential to produce significant disruption to the structure and function of the tumour-associated microvasculature. Due to the absolute reliance on a functional vasculature for tumour growth, these vascular-mediated responses play a key part in TNF-induced tumour necrosis.

The development of TNF-induced necrosis within tumour tissues followed a similar induction profile as the expression of TNF-R75 protein on the tumour-associated microvasculature. This suggests that the upregulation of TNF-R75 by tumour-associated microvascular endothelial cells is an important step in the initiation of tumour tissue necrosis. This suggests that while TNF-R55 is constitutively expressed and by most tumour cell types it may not be the prinicipla TNF receptor mediating the effects of TNF-α. The contribution of TNF-R75 to this process may be through TNF-α capture and 'ligand passing' to TNF-R55, the receptor primarily responsible for signalling and cytotoxicity.

Resistance of TAMECs (*in vitro*) to TNF-induced apoptosis suggests that the increased sensitivity of tumour-associated microvascular endothelial cells to TNF-α *in vivo* is a function of the tumour microenvironment. Exposure to angiogenic growth factors, cytokines as well as hypoxic conditions can enhance TNF-mediated endothelial cell cytotoxicity. As TAMECs express TNF receptors and TNF-α is effective at activating these cells, interactions with tumour-specific factors appear to be required to sensitize endothelial cells to undergo apoptosis. These interactions were not studied in the thesis but may provide further information regarding the importance of the tumour microenvironment in contributing to the responses of the tumour-associated microvasculature. This study has addressed the final question posed as part of the hypothesis and illustrates that tumour-associated microvascular endothelial cells are a target for the anti-tumour actions of TNF-α. This was not by the development of extensive endothelial cell apoptosis but occurred in association with vascular disturbance and a breakdown in the integrity of the tumour-associated microvessels.

This study has made a contribution to the understanding of the anti-tumour actions of TNF-α and its interaction with the tumour-associated microvasculature. The first achievement was made with the establishment of a method to isolated and culture microvascular endothelial cells from solid tumours. These cells formed an *in vitro* model to further examine the vascular actions of TNF-α. Examination of the expression and regulation of endothelial cell adhesion molecules revealed that tumour-associated microvascular endothelial cells respond to TNF-α stimulation by expressing E-selectin however ICAM-1 mRNA, detected by Northern blot and *in situ* hybridization, was not translated into immunodetectable protein. This was observed *in vivo* and *in vitro* and

suggested that factors within the tumour microenvironment were suppressing ICAM-1 translation. The effects of TNF-α are mediated by TNF receptors which were expressed by tumour-associated microvascular endothelial cells, however, their expression followed a different profile. Their contribution to TNF-mediated cytotoxicity was realized through the association between the appearance of vascular TNF-R75 expression which preceded the development of haemorrhagic necrosis.

A number of issues raised in this study clearly point to the need for further investigation. In particular, elucidating the mechanisms controlling the suppression of ICAM-1 protein on tumour-associated microvascular endothelial cells should provide valuable clues to enhancing leukocyte infiltration. Ascertaining the roles of angiogenic growth factors and microenvironmental influences such as hypoxia on ICAM-1 expression will also contribute to this understanding. The role of soluble cell adhesion molecules which have the capacity to block leukocyte attachment also needs to be clarified.

To date much effort has been directed towards understanding the actions of TNF-α and its cell-based receptors. The functions of membrane-bound TNF-α and sTNF receptors in malignant diseases is much less clear. In some tumours, the levels of sTNF receptors is plasma does rise although the relationship between this observation and tumour development is unknown. The direct contribution of each receptor can only be examined in mice and/or tumour-associated microvascular endothelial cells which the genes for one or both TNF receptors has been deleted.

In terms of TNF-α as a tumour therapy, the induction of TNF-R75 on the tumour-associated microvasculature appears to be a key requirement in mediating tumour-specific necrosis. The major problems encountered in clinical trials with TNF-α have been in dose limiting toxicities occurring before anti-tumour concentrations could be achieved. ILP has overcome this problem and is producing promising antitumour responses. Although ILP is technically demanding, the administration of TNF-α systemically in the future will require supplementary therapies to be coadministered to decrease nonspecific signalling and to enhance tumour-specific responses. Blocking antibodies (against TNF-R55) may allow the dose of TNF-α to be lowered and therefore reduce any dose-limiting toxicities mediated through TNF-R55 activation. TNF-α administration will induce microvascular TNF-R75

expression support ligand passing to the reduced level of TNF-R55 expressed. Binding to TNF-R75 and signalling through TNF-R55 will initiate tumour cell death through endothelial cell apoptosis and microvascular disturbance. Administration of EMAP-II prior to TNF-α may also be a mechanism through which to decrease the concentration of TNF-α delivered. As EMAP-II will increase the sensitivity of tumour-associated microvascular endothelial cells to TNF-α, anti-tumour responses may be achieved without adverse side effects.

In conclusion, this study has contributed to our understanding of the actions of TNF-α within tumours, in particular on the tumour-associated microvasculature. Microenvironmental factors play a key role in modifying responses to TNF-α. Translation of ICAM-1 mRNA into expressed protein is suppressed and this feature is unique to the tumour-associated vessels. TNF-mediated cytotoxicity is also specific to the tumour and appears to be related to an increased sensitivity of tumour-associated microvascular endothelial cells to the cytokine. The induction of vascular TNF-R75 expression is intimately linked to the development of haemorrhagic necrosis. Despite the difficulties associated with TNF-α therapy, with an increasing awareness of the properties and actions of this cytokine, for example through the development of receptor-specific mutants, the use of TNF-α as an antitumour agent may finally fulfil the promises of experimental models.

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APPENDIX A. ADDRESSES OF SUPPLIERS OF REAGENTS AND EQUIPMENT

Supplier	Address
Agfa-Gevaert N.V.	Mortsel, Belgium
Ajax Chemicals	Sydney, NSW., Australia
Amersham Pharmacia Biotech Pty. Ltd.	Piscataway, NJ., USA.
BDH	Poole, England, UK.
Becton Dickinson Labware	Franklin Lakes, NJ., USA.
Biomedical Technologies	Stoughton, MA., USA.
Bio-Rad	Hercules, CA., USA.
Commonwealth Serum Laboratories	Parkville, Vic., Australia
DAKO Corporation	Glostrup, Denmark
Department of Pathology and Immunology, Monash University Medical School	Monash, Vic., Australia
DIFCO Laboratories	Detroit, MI., USA.
Eastman Kodak Company	Rochester, N.Y., USA.
EC Apparatus Corp.	St. Petersburg, FL., USA.
Email Westinghouse Pty. Ltd.	Regents Park, NSW., Australia
Flow Laboratories Australasia Pty. Ltd.	North Ryde, NSW., Australia
Fluka Chemika-BioChemika	Buchs, Switzerland
Geneworks Pty. Ltd.	Adelaide, SA., Australia
Genzyme Diagnostics	Cambridge, MA., USA.
Graticules Ltd.	London, England, UK.
Institute of Medical and Veterinary Science	Adelaide, SA., Australia
Intergen Company	Purchase, NY., USA.
Johnson Murray Chemicals Ltd	London, England, UK.
Life Technologies Inc.	Gaithersburg, MD., USA.
LKB Produkter AB	Bromma, Sweden
Microm Laborgeräte GmbH	Walldorf, Germany
Micron Separations Inc.	Westborough, MA., USA.
Milling Industries	Adelaide, SA, Australia
Millipore Pty. Ltd	Lane Cove, NSW., Australia
Nalge Nunc International	Naperville, IL., USA.

Supplier	**Address**
Olympus Optical Co. Ltd.	Tokyo, Japan
Oxford Molecular Limited	Oxford, England, UK.
Panasonic Australia Pty. Ltd.	Belrose, NSW., Australia
Parkard Instrument Company	Meriden, CT., USA.
Perkin Elmer	Norwalk, CT., USA.
Philips Electron Optics	Eindhoven, The Netherlands
Progen Industries Ltd.	Darra, Qld., Australia
Promega Corporation	Madison, WI., USA.
QIAGEN Pty. Ltd.	Clifton Hill, Vic., Australia
Reichert	Wein, Austria
Roche Molecular Biochemicals	Castle Hill, NSW., Australia
Sakura Finetechnical Co. Ltd.	Tokyo, Japan
Santa Cruz Biotechnology, Inc.	Santa Cruz, CA., USA.
Sarstedt	Newton, NC., USA.
Sartorius AG.	Göttingen, Germany
Serotec	Oxford, England, UK.
Sigma Chemical Co.	St. Louis, MO., USA.
Sony	Tokyo, Japan
South Australian Government Department of Agriculture	Gilles Plains, SA., Australia
TAAB Laboratories Equipment Limited	Reading, England, UK.
Trace Biosciences	Castle Hill, NSW., Australia
Ulead Systems Inc.	Torrance, CA., USA.
UltraLum Inc.	Paramount, CA., USA.
Vector Laboratories	Burlingame, CA., USA.
Weber	England, UK.
Wheaton Scientific	Milville, NJ., USA.
Zeneca Ltd	Macclesfield Chesire, England, UK.
Zymed Laboratories, Inc.	S. San Francisco, CA., USA.

APPENDIX B. PRIMARY ANTIBODIES

Monoclonal anti-angiotensin converting enzyme (ACE)

(A kind gift from Dr R Auerbach, Laboratory of Developmental Biology, University of Wisconsin, Madison, WI., USA.; Auerbach *et al.,* 1982).

Isotype: IgM (mouse)

Monoclonal anti-human alpha smooth muscle actin

(DAKO, Glostrup, Denmark; Code No. M 851)

Isotype: IgG2a, kappa

Specificity: DAKO-smooth muscle actin, IA4 reacts with the alpha-smooth muscle isoform of actin as indicated by immunoblotting and ELISA. The antibody does not react with actin from fibroblasts (beta- and gamma-cytoplasmic), striated muscle (alpha-sarcomeric) and myocardium (alpha-myocardial).

Polyclonal anti mouse and rat-E-selectin (CD62E)

(Santa Cruz; Y-18)

Isotype: IgG (goat)

Specificity: Epitope corresponding to an amino acid sequence mapping to the amino terminus of the precursor form of E-selectin of mouse origin. Specific for E-selectin; non cross-reactive with P-selectin or L-selectin.

Polyclonal anti-rat intercellular adhesion molecule-1 (CD54)

(Serotec; Code No. MCA773)

Isotype: IgG1 (mouse)

Specificity: Recognises the rat ICAM-1 molecule which has an important role in various cell-cell interactions in the immune system.

Polyclonal anti-mouse platelet/endothelial cell adhesion molecule-1 (CD31)

(Santa Cruz, M-20)

Isotype: IgG (goat)

Specificity: PECAM-1 reacts with PECAM-1 of mouse, rat and human origin; non cross-reactive with other cell adhesion molecules.

Polyclonal anti-rat endothelial cell antigens-1 (RECA-1)

(Serotec; Code No. MCA970; Duijvestijn *et al.*, 1994)

Isotype: IgG1 (mouse)

Specificity: Pan endothelium (RECA-1 antigen)

Polyclonal anti-mouse and rat TNF-R55

(Santa Cruz; G-20)

Isotype: IgG (goat)

Specificity: TNF-R1 (G-20) reacts with TNF-R1 p55 of mouse and rat origin by Western blotting and immunohistochemistry; non cross-reactive with TNF-R2 p75.

Polyclonal anti-mouse and rat TNF-R75

(Santa Cruz; L-20)

Isotype: IgG (goat)

Specificity: TNF-R2 (L-20) reacts with TNF-R2 p75 of mouse and rat origin by Western blotting and immunohistochemistry; non cross-reactive with TNF-R1 p55.

Polyclonal anti-human von Willebrand factor (vWF)

(DAKO; Code No. A 0082)

Isotype: IgG (rabbit)

Specificity: The antibody reacts with human von Willebrand factor. Traces of contaminating antibodies have been removed by solid-phase absorption with human plasma proteins.