Adhesion and Invasion Studies of Uveal Melanoma

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Julia Keren Lynda Woodward

Institute for Cancer Studies and
Department of Ophthalmology and Orthoptics
University of Sheffield, UK

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I hereby declare that no part of this thesis
has been previously submitted
in support of any other degree, or qualification,
at this, or any university, or institute of learning.

This thesis is dedicated to my husband, David,
for his love, friendship and support during my PhD.

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Contents

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Abbreviations

α1β1	alpha 1 beta 1 integrin
α2β1	alpha 2 beta 1 integrin
α3β1	alpha 3 beta 1 integrin
α4β1	alpha 4 beta 1 integrin
α4β7	alpha 4 beta 7 integrin
α5β1	alpha 5 beta 1 integrin
α6β1	alpha 6 beta 1 integrin
α6β4	alpha 6 beta 4 integrin
αvβ3	alpha v beta 3 integrin
αvβ5	alpha v beta 5 integrin
ANOVA	Analysis of variance
APES	3-aminopropyl-triethoxysilane solution
β1	beta 1 integrin
BSA	Bovine serum albumen
CC	Cultured cells
CEA	Carcinoembryonic antigen
CFDA-SE	Carboxy-fluorescein diacetate, succinimidyl ester
CM	Conditioned media
DMSO	Dimethyl sulphoxide
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
FACS	Flow assisted cell sorting
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
Fn	Fibronectin
FS	Frozen sections

GRO	Growth related oncogene
HDMECAs	Human dermal microvascular endothelial cells
HGF	Hepatocyte growth factor
HULECs	Human liver endothelial cells
ICAM	Intercellular adhesion molecules
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
MFI	Median fluorescent intensity
MIP	Macrophage inhibitory protein
MHC	Major histocompatibility complex
MMP	Martrix metalloproteinase
PAI	Plasminogen activator inhibitor
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PS	Paraffin sections
RANTES	Regulated on activation, normal T-cell expressed and secreted
RPMI-1640	Roswell Park Memorial Institute-1640
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error mean
SNARF	SNARF-1 carboxylic acid, acetate, succinimidyl ester
SOM	Sheffield Ocular Melanoma
STC	Short-term cultures
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
u-PA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VCAM	Vascular cell adhesion molecules
VEGF	Vascular endothelial cell growth factor

Publications

(J.K.L. Woodward; nee J.K.L. Baker)

Abstracts

Baker, J.K.L., Elshaw, S.R., Mathewman, G.E., Nichols, C.E., Murray, A.K., Parsons, M.A., Rennie, I.G., Sisley, K. Ubiquitous expression of degradative enzymes and their inhibitors in primary posterior uveal melanoma, and differential expression of integrins as a response to culture. *Clin Exp Metastasis* 1999; **17(9):** 764. Poster presentation at the VIII International Congress of the Metastasis Reseach Society (September 1999).

Baker, J.K.L., Rennie, I.G., Murray, A.M., Sisley, K. An *in vitro* model of metastasis for uveal melanoma. *Invest Ophthalmol Vis Sci* 2001; 42(4): S219. Poster presentation at the Association for Research in Vision and Ophthalmology 2001 Annual Meeting (April 2001).

Baker, J.K.L., Rennie, I.G., Murray, A.M., Sisley, K. Study of uveal melanoma metastasis and the use of an *in vitro* model. Oral presentation at the Xth International Congress of Ocular Oncology (June 2001).

Rennie, I.G., Baker, J.K.L., Elshaw, S.R., Murray, A.K., Nichols, C.E., Sisley, K. HGF, GRO and MIP-1β stimulate uveal melanoma migration whilst IL-1α and TGF-β inhibit migration. *Invest Ophthalmol Vis Sci* 2002 (in press). Poster presentation at the Association for Research in Vision and Ophthalmology 2002 Annual Meeting (May 2002).

Publications

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Woodward, J.K.L., Nichols, C.E., Rennie, I.G., Parsons, M.A., Murray, A.M., Sisley, K. An *in vitro* assay to assess uveal melanoma invasion across endothelial and basement membrane barriers. *Invest Ophthalmol Vis Sci* 2002 **43(6):** 1708-1714.

Woodward, J.K.L., Elshaw, S.R., Murray, A.K., Nichols, C.E., Cross, N., Rennie, I.G., Sisley, K. Uveal melanoma invasion is stimulated by HGF and GRO whilst IL-1α and TGF-β are inhibitory. *Invest Ophthalmol Vis Sci* 2002 (in press).

Summary

Posterior uveal melanoma is the most common intraocular malignancy in adults. Metastasis occurs in approximately 50% of all cases, and spread is primarily to the liver. Very little is known about the factors that regulate uveal melanoma metastasis, and it possible that this tumour may follow Paget's 'seed and soil' hypothesis. Identification of further indicators associated with the metastatic phenotype, may aid in detecting patients most at risk. Using a series of short-term cultures, this study aimed to further identify factors that may be involved with uveal melanoma metastasis to the liver.

Detachment of cells from the primary tumour mass requires changes to adhesive and degradative interactions. Uveal melanomas were shown to ubiquitously express some integrins, degradative enzymes and inhibitors, whilst expression of others (α1, α2, α4, and α6) are more variable, and may in part be related to the *in vitro* stimulation. Once migrated through the surrounding matrix, and basement membrane, migration through the endothelium is essential. Using an *in vitro* model of transendothelial invasion, results indicated that for uveal melanoma, invasion seemed to correlate with a wider range of prognostic indicators, than through the basement membrane alone.

Uveal melanoma cells may disseminate widely, but primarily initiate metastatic growth at the liver alone. Factors associated with uveal melanomas have been reported to assist in liver targeting, and similarly most uveal melanomas in this study responded positively to factors secreted by cells of hepatic origin. Cells derived from dermal tissues nevertheless also stimulated invasion. After co-culture of uveal melanoma cells with dermal or hepatic endothelial cells, notable changes were however only observed through interactions with hepatic endothelial cells, suggesting that despite attraction to other sites, uveal melanomas may only arrest in the liver. In particular, an increase in uveal melanoma α4 expression, accompanied by an increase in hepatic endothelial VCAM-1 expression may be involved in liver targeting.

Chapter 1

Introduction

Introduction

Whilst uveal melanomas are rare in occurrence, they are associated with a poor prognosis and may invade and metastasise rapidly, characteristically forming secondary tumours in the liver. This feature raises a fundamental question of why tumour cells arrest within the vasculature of specific organs. This process of tumour seeding is known to involve changes to the mechanism by which primary tumour cells break free of their connective tissue, how they migrate into adjacent vasculature, and then how they adhere and invade into organs that support formation of secondary metastasis. Identifying the molecules essential to uveal melanoma metastasis will underpin the development of improved therapy, targeted at the processes involved in tumour spread.

Cancer as a cellular disease

"Every cell is born from another cell"
Virchow

Deaths from infectious diseases, such as Small Pox, Influenza and Tuberculosis have declined in the last century due to great advances in treatment and prevention of these diseases. Today, malignant neoplasms may develop in 25% of the population (reviewed by Underwood, 2000). One of the definitions used in the Collins English Dictionary, illustrates cancer as 'an evil influence that spreads dangerously', however, despite progress in diagnosis and treatment, many factors important in the development of cancers still remain to be discovered.

Pictorial and written evidence from early civilisations document the effects of cancer, often attributing the cause to various gods. In 400BC, Hippocrates first associated the origin of the disease with natural causes and described cancer as an imbalance of bodily fluids. Further historical evidence was provided by the Greek physician Galen, who first identified breast cancer, observing swollen pincer-like outgrowths surrounding the cancer in its later stages, that seized upon adjacent tissue (reviewed by Hecht, 1987). This observation ultimately led to the current terminology, where the word cancer is derived from the Greek word *Karinos* related to carcinoma and from the Latin word for crab. Sir Percival Pott made one of the most notable observations in 1775, noticing that young men,

who had been chimney sweeps in their teenage years, had a high rate of death due to cancer of the scrotum (reviewed by Calman and Paul, 1978). Pott further identified a putative carcinogen, observing that the disease may develop years after exposure to such an agent. Later in the nineteenth century, with the development of the microscope, cancer was further recognised as a cellular disease. More recently, over the past decades research including aspects such as biochemistry, genetics, molecular and cellular biology and immunology has sought a greater understanding of the processes controlling normal and malignant cellular growth, and collectively, rapid progress has been made towards identifying and understanding the underlying mechanisms.

In normal tissue, homeostasis requires careful regulation of the rates of cell proliferation and death. For a cell to become neoplastic, through an accumulation of mutations in specific genes, the cell reproduces uncontrollably, in defiance of the normal restraints and may further invade and colonise tissues away from the site of origin (reviewed by Underwood, 2000). The number of mutations and specific genes involved further determines the degree of progression. In broad terms, two classes of genes are involved: proto-oncogenes and tumour suppressor genes (reviewed by Underwood, 2000). Under normal circumstances, these genes are involved in regulating cell proliferation, aging and death, differentiation, behaviour, and integrity of the genome but when mutations arise dysfunction may result.

If neoplastic cells remain clustered as a single mass, and only grow locally without invasion of the basement membrane, the tumour is known as 'benign' and through surgical removal, no detrimental effect may result (reviewed by Underwood, 2000). However, if tumour cells divide uncontrollably, growing into and destroying the surrounding tissue, the tumour is known as 'malignant' and may be fatal for the patient (reviewed by Underwood, 2000). Invasion of tumours cells into the lymphatic system or blood stream allows transport around the body to distant sites, and potentially further proliferation in tissues normally reserved for other cells (reviewed by Underwood, 2000). With regard to patient survival, one of the most important features of malignancy is this acquisition of a migratory and invasive phenotype, causing the tumour to become harder to eradicate (reviewed by Underwood, 2000). This process is known as 'metastasis' and for uveal melanoma, will be the major focus of this investigation.

A. Tumour Invasion and Metastasis

Despite unprecedented advances in tumour therapy, the main cause of treatment failure and death from cancer is metastasis (reviewed by Sugarbaker and Ketcham, 1977; reviewed by Underwood, 2000). Metastasis can be simplified as the detachment of malignant cells from the primary tumour mass, followed by dissemination and establishment at a distant site. In a more detailed description by Marshall and Hart (1996), the process was described as 'altered cell growth associated with a series of adhesion and de-adhesion events which are coupled with regulated tissue degradation'. During the progression of the disease, tumour cells undergo a series of changes and may acquire a permanent, irreversible, and malignant phenotype. Early work in this field using the B16 cutaneous melanoma cell line identified a propensity of specific clones to form colonies in the lung, concluding that cells with a highly metastatic potential exist within primary tumours (Fidler, 1973; Fidler and Kripe, 1977). With time, it has become evident that the metastatic process is selective for sub-populations of cells within a heterogeneous malignant neoplasm, and is not a random process (Fidler and Hart, 1982).

For a tumour to establish secondary colonies, metastatic clones must undergo a complex sequence of interrelated steps, with each potentially being both dependent on the host responses and intrinsic properties of the tumour cell (Fidler and Hart, 1982). Figure 1.a illustrates these sequential steps. Each stage will be discussed in further detail in the proceeding sections (1.1-1.6). Due to the hostility of these processes, only a very small number of circulating tumour cells initiate metastatic colonies (Fidler, 1970).

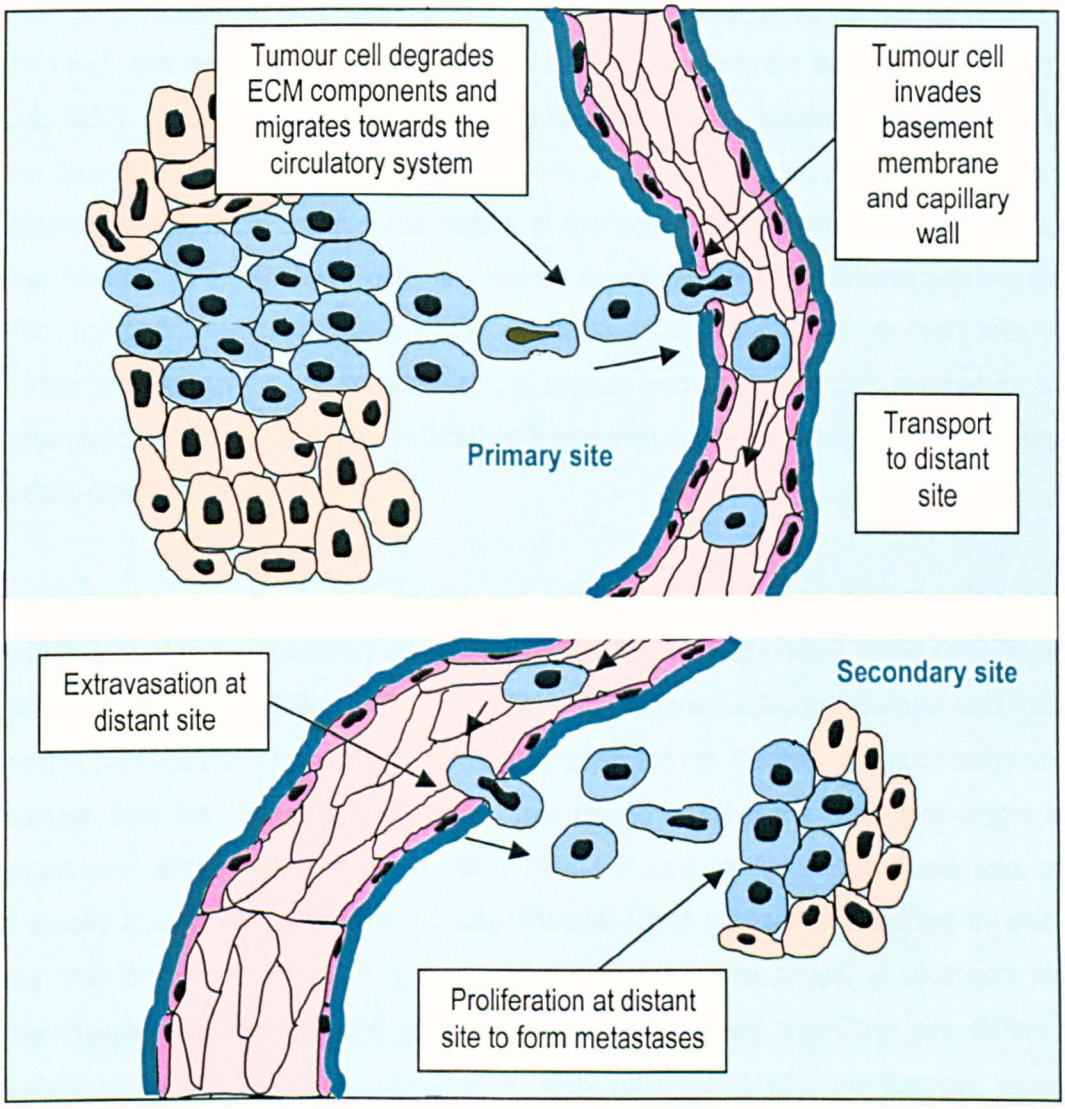


Figure 1.a. Schematic diagram of metastatic spread (normal tissue is represented by the light blue areas; tumour cells are shown in blue; basement membrane is shown in green; normal cells are shown in beige and endothelial cells are shown in pink). Tumour cells from the primary tumour mass have been illustrated migrating and invading through the ECM, the basement membrane, and the endothelial cell wall. Cells then circulate in the blood stream before under-going to reverse process at the secondary site. Metastatic tumour cells further proliferate to form metastases.

Theories of metastasis

Tumour cells often appear to exhibit a degree of site-specific metastasis, disseminating to preferred organs (e.g. Auerbach, 1988; Muller *et al.*, 2001). Despite spread being facilitated commonly via haematogenous, lymphatic or transcoelomic routes, particular organs are frequently involved in metastatic growth, relating to the tumour type (e.g. Hart and Fidler, 1980; Muller *et al.*, 2001). In

1889, Paget proposed the 'seed and soil' hypothesis after it was observed that certain organs, such as the heart, skin and skeletal muscles, despite ample blood flow, are rarely sites of metastases (Paget, 1889). This explanation suggested that for each primary tumour there is a colonisation pattern determined by the conditions in the endothelium of the microvasculature and the functional environment of the target organ. The validity of this concept is becoming increasingly evident. Tumour cells are potentially likely to act as 'seeds', expressing particular determinants required for specific metastatic progression seen in many cancers. The target organ, or 'soil', may attract circulating tumour cells through secreted factors, express necessary receptors required for tumour cell attachment, and once cells have established themselves in the secondary environment, promote metastatic growth.

Early advances were made when it was observed that B16 melanoma cells show specific patterns of metastatic spread to the pulmonary and ovarian tissue, but not to the renal tissue or at the site of surgical stress (Hart and Fidler, 1980). In a later study, different sub-populations of cells from two melanoma cell lines and a fibrosarcoma cell line were injected into the internal carotid artery of mice, metastases from the different melanoma cell lines formed in either the brain parenchyma or the meninges and ventricles (Schackert and Fidler, 1988). In contrast, fibrosarcoma cells were able to form lesions throughout the brain and it was concluded that the microenvironment in which the tumour cells arrest also appeared to play a significant role in the growth of secondary lesions. Further research identified that the surroundings of the secondary organ can also dictate other phenotypic effects on metastases (Horak *et al.*, 1986; Nicholson, 1986). For example, amelanotic melanoma cell lines can produce melanotic brain metastases in syngeneic mice (Radinsky *et al.*, 1995). The authors of this study concluded that the microenvironment of the brain might be inducing melanocyte-stimulating hormone receptor (MSH-R) expression on the melanoma cells, allowing cells to become receptive to the effects of alpha-MSH, and stimulating amongst other effects, cell proliferation and melanin production.

In 1928, a second opposing theory for metastasis was proposed, known as the 'mechanical entrapment' theory (Ewing, 1928). This hypothesis suggested that tumour cells arrest in the first capillary bed visited in the passage away from the tumour. The first organ therefore encountered by circulating venous blood (the lungs) has a high incidence of secondary tumour growth. This premise

would seem feasible, as tumour cells are often large in size and consequently, as diameters of the tumour cells will often be greater than those of the capillaries, entrapment may result. This theory is supported by recent evidence in which using intravital microscopy, colon adenocarcinoma cells were shown to arrest in the liver microcirculation, physically lodging in the narrow entrance of the sinusoid (Ding *et al.*, 2001). Similar results were however not seen with lymphoma cells, which showed a more specific pattern of metastasis, suggesting that simple entrapment is not necessarily the only explanation. Pre-treatment of mice with IL-1α has also been shown to facilitate arrest of B16F1 melanoma cells at the presinusoidal portal vein branches (Scherbarth and Orr, 1997). Without IL-1α treatment, melanoma cells adhered at a different site on the sinusoidal endothelium, implicating an effect of IL-1α on the site of attachment.

It is now thought that these theories are not mutually exclusive and each may be partially correct; ultimately non-random distribution of metastases may be dependent upon the tumour phenotype (Ding *et al.*, 2001). Key dissemination sites are primarily dictated by anatomical conditions but 'seed and soil' factors of the site play an important part also. Some tumours disseminate widely and the first capillary bed encountered by the circulating tumour cells will be the most common site for metastatic growth. Other tumours are far more selective and metastatic growth will only result in particular organs, often by-passing several more proximal organs. Both mechanisms nevertheless vary with the site of the primary tumour, stage of disease and the metastatic potential of the neoplasm under study, and tumour cells circulating in the blood may not inevitably result in metastatic growth. There is also recent evidence to suggest that other factors additionally influence metastasis, including the time taken for the tumour cells to reach and attach at the site of extravasation (Paku *et al.*, 2001) whilst other reports suggest that tumour emboli are more successful at generating metastases than single cells (Al-Mehdi *et al.*, 2001).

1.1. Changes in tumour cell adhesion and cohesion

For successful invasion, tumour cells must undergo a series of adhesion and de-adhesion steps, to facilitate detachment from the primary tumour mass, and penetration through the extra cellular matrix (ECM), basement membrane and endothelial cell layer at both the primary and secondary sites (Figures 1.i and 1.3.i) (reviewed by Orr *et al.*, 2000). During migration through the ECM, the leading edge of the cell adheres to new substrates, requiring altered cell adhesion molecule expression,

guiding the cells, and providing the necessary traction required to pull the cell forward. The trailing edge of the cell retracts from the matrix proteins, involving dissociation of cell adhesion molecules from their ligands. Such external movements are all regulated by internal signals. Cell adhesion molecules facilitating these interactions include the integrin family, the selectin family, the immunoglobulin (Ig) family and the cadherin family, affecting processes including cell activation, proliferation and apoptosis. To enable a potentially metastatic tumour cell to escape from the confines of primary tumour mass, the adhesion molecule profile of the cell must suitably alter to allow both escape from the confines of the primary tumour, whilst also promoting attachment to the surrounding stroma. Typically cadherin molecules, facilitating homophilic cell-cell interactions, are involved in these initial stages allowing escape of the tumour cells from the primary site, with loss of E-cadherin expression often being correlated with the metastatic phenotype of many epithelial tumours (Sommers *et al.*, 1991; Murant *et al.*, 1997). In contrast however, N-cadherin molecules may be involved in hetereotypic interactions with cells including both fibroblasts and endothelial cells (Sandig *et al.*, 1997; Voura *et al.*, 1998b; Li *et al.*, 2001). Once detached from the primary tumour mass, tumour cells must then attach to, and degrade the host ECM and basement membrane before adhering to and migrating through, the local endothelium. Tumour cell attachment to the endothelium at another site may be further assisted by adhesion molecules that may either be constitutively expressed by the target endothelium, expressed in response to tumour and host cell stimuli, or may be organ-specific (reviewed by Orr *et al.*, 2000; e.g. Vidal-Vanaclocha *et al.*, 2000; Abdel-Ghany *et al.*, 2001).

Integrins

The integrin family of adhesion molecules primarily facilitates tumour cell migration and invasion through the ECM and basement membrane. In common with inflammatory responses, attachment to the endothelium and transendothelial migration may also utilise these receptors, in conjunction with other families of adhesion molecules. Integrins form a family of transmembrane cell-cell and cell-matrix adhesion molecules (reviewed by Hynes, 1987). To date, at least 25 members of the family have been identified (reviewed by Humphries, 2000). Each is a heterodimeric complex of an α- and β-subunit, requiring divalent cation binding for activation (reviewed by Hynes, 1987). In mammals, 19 α-chains (120-180kD) and eight β-chains (90-110kD) are to date, known to exist (reviewed by Humphries, 2000). Theoretically, numerous permutations could exist, but only certain structures are

possible as many α-chains can only associate with particular β-chains. Classification is based accordingly upon the β-subunits and both chains are required for interaction with the cellular cytoskeleton and ECM. Upon binding, the carboxyl-terminal of the β-chain binds a variety of intra-cellular complexes, initiating a cascade of responses (e.g. Burridge *et al.*, 1992; Schaller *et al.*, 1992). During attachment of leucocytes and tumour cells to the endothelium, once cells have initially docked (and in some case, rolled) on the endothelium, integrins have also been shown to be responsible for tight interactions prior to extravasation and are involved in the transendothelial migration process (e.g. Wagner *et al.*, 1996; Price *et al.*, 1996).

Individual integrins will often bind more than one ligand (reviewed by Hynes, 1987). Each ECM component potentially is recognised by more than one receptor. Of particular importance in cell-matrix binding are the β1- and β3- subfamilies; the former are generally involved in adhesion to connective tissue macromolecules such as fibronectin, laminin, and collagen, whereas β3-integrins bind vascular ligands such as fibrinogen, von Willebrands factor (Factor VIII), thromospondin, and vitronectin (reviewed by Hynes, 1992). In addition, ligand binding is often via particular consensus sequences. For example, the first and most common sequence to be elucidated was the Arg-Gly-Asp (RGD) sequence present in fibrinogen, vitronectin, and other adhesive proteins (Humphries, 1990).

Differences in integrin expression between normal and malignant tissue have been well documented for many cancers. Loss of laminin and collagen receptors have commonly been reported in lung, breast, colon, and pancreatic cancer, suggesting detachment and escape from the basement membrane, which is composed principally of these components (e.g. Hall *et al.*, 1991; Koukoulis *et al.*, 1991; Pignatelli *et al.*, 1990). Increased expression of these and other integrins has nevertheless, also been reported during the metastatic process, allowing adhesion, during migration, to substrates not previously encountered, including up-regulation of αvβ3 expression during melanoma progression (Albeda *et al.*, 1990; Voura *et al.*, 2001b).

Selectins

Initial 'loose' interactions of tumour cells to the endothelium are commonly mediated by selectins, before firm adhesive interactions are established (Chen *et al.*, 1997). The selectins are a small family

of intercellular adhesion molecules with three members: E-, P- and L-selectin and are differentially expressed by endothelial cells and leucocytes (reviewed by Brown, 1997). Of particular importance in invasion and metastasis is E-selectin, being transiently expressed on endothelial cells following stimulation by inflammatory cytokines such as IL-1, TNFα or bacterial endotoxins (e.g. Ye *et al.*, 1995; Moss *et al.*, 2000). Much evidence exists implicating a role for E-selectin in neutrophil, monocyte, and T lymphocyte rolling during inflammatory responses, facilitating initial weak interactions with the endothelium (reviewed by Springer, 1994). Upon endothelial cell stimulation, E-selectin is synthesized *de novo* and translocated to the cell surface, with levels peaking one to four hours after the initial stimulus (Ye *et al.*, 1995).

Ligands for selectins are generally sialylated, glycosylated, or sulphated glycans on glycoproteins, glycolipids, or proteoglycans, such as sialyl Lewis X (sLe^x) and sialyl Lewis A (sLe^a). These ligands have been identified on leucocytes and a number of tumour cells. For example, colorectal tumour cell expression of sLe^x has been correlated with increased metastasis to the liver and such tumour cells have been shown to adhere to liver sinusoidal cells via an E-selectin-glycosylated mucin interaction (Hoff *et al*, 1989; Bresalier *et al.*, 1998).

Immunoglobulin (Ig) superfamily

Another family of adhesion molecules implicated in a number of processes of the metastatic cascade is the immunoglobulin (Ig) superfamily. The Ig family is the largest group of adhesion molecules, and members include the intercellular adhesion molecules (ICAMs), vascular-cell adhesion molecules (VCAMs), platelet-endothelial-cell adhesion molecule (PECAM-1), and neural-cell adhesion molecule (NCAM). Typically, ligands of this family include ECM components, integrins and other Ig superfamily members and interactions may facilitate amongst other functions, flattening of leucocytes onto the blood vessel wall prior to extravasation (reviewed by Brown, 1997). Many members may be involved in metastatic progression, but ICAM and VCAM are of notable interest with respect to tumour and endothelial cell interactions (e.g. Tamaki *et al.*, 1995; Vidal-Vanaclocha *et al.*, 2000).

ICAM-1 is widely expressed by many cell types and soluble ICAM-1, secreted by both tumour and endothelial cells has been implicated in tumour cell evasion of the host immune responses (Fonsatti *et al.*, 1999; Zhang and Adachi, 1999). Under normal conditions, resting endothelial cells express

low levels of both ICAM-1 and VCAM-1 but expression is increased upon stimulation by inflammatory cytokines such as IL-1, IFN-γ and TNF-α (reviewed by Meyer and Hart, 1998; e.g. Vidal-Vanaclocha *et al.*, 2000). In addition to the association of ICAM and VCAM with metastasis, the involvement of endothelial PECAM-1 has also been suggested, binding to αvβ3 (Buckley *et al.*, 1996). Recent reports have however indicated a redistribution of this molecule away from the endothelial junction during transendothelial cell migration, and thus its precise relevance in invasion remains unclear (Voura *et al.*, 2001a).

1.2. Release of proteolytic enzymes and invasion of local host stroma

Surrounding all cells is a complex network of a variety of proteins and polysaccharides, secreted locally, primarily by fibroblasts, and assembled into an organised meshwork known as the ECM (Eyre, 1980; Kleinman *et al.*, 1981; Hay, 1982; Hedman *et al.*, 1982). The basement membrane is a similar structure, residing at the interface between the epithelial or endothelial layer and connective tissue (Kleinman *et al.*, 1986). For a potentially metastatic tumour cell to invade through the surrounding matrix and basement membrane, degradation of the individual components is essential. The ligands for many adhesion molecules include ECM and basement membrane proteins, either native to the host, or subsequently produced as a result of tumour cell invasion.

Under normal conditions there is a highly regulated balance between synthesis and degradation of the ECM and basement membrane. Net degradation is required for a number of physiological and pathological processes, such as tumour cell invasion, leucocyte migration in response to injury or infection, and embryogenesis (reviewed by Nagase and Woessner, 1999). For tumour cell migration and invasion, degradation of ECM and basement membrane at both the primary and secondary sites is therefore imperative. Interactions between tumour and host cells induce the release of proteolytic enzymes by both cell groups, facilitating ECM breakdown (e.g. Kataoka *et al.*, 1993; Noel *et al.*, 1994). The involvement of three families of proteases has been identified in tumour invasion: metallo-, seryl- and cysteine-proteases. Of these classes, metalloproteinases and plasminogen activators (seryl-proteases) have been most widely implicated in tumour invasion (e.g. Monteagudo *et al.*, 1990). ECM breakdown is focused at the invasive front, confining the enzyme cascade to the invading pseudopodia ('invadopodia'), and by chemotaxis, migration of the tumour cell will be directed through the modified matrix. Much of the degradation initiated at the invasive front, is further

orchestrated by the host cells and not the tumour mass (Poulsom *et al.*, 1993; Wolf *et al.*, 1993). This process is repeated until the vessel wall may be intravasated and the direction of tumour cell migration and invasion will be determined by both locally produced chemoattractants and the preferred adhesion pathway (reviewed by Varner and Cheresh, 1996). Proteolytic degradation itself can also enhance the process by creating fragments which act as chemoattractants, whilst also exposing adhesion binding sites enabling cellular attachment (reviewed by Liotta and Kohn, 2001).

Extra Cellular Matrix (ECM) composition

Two main classes of ECM macromolecules exist, which interact to form the network surrounding all cells (Eyre, 1980; Kleinman *et al.*, 1981; Hay, 1982). The first group comprises of polysaccharide chains, known as glycosaminoglycans, which are often covalently linked to proteins, forming proteoglycans. Glycosaminoglycans form hydrated gels within the extra cellular space; proteoglycans may also been found on the cell surface as cellular receptors for, for example, cell-matrix and growth factor binding (reviewed by Alberts *et al.*, 1994). The second group includes fibrous and structural proteins, such as collagen and elastin, and adhesive proteins, such as fibronectin and laminin. Fibrous proteins are embedded in the hydrated gel-like substance formed by the glycosaminoglycans (Eyre, 1980; Kleinman *et al.*, 1981; Hay, 1982). Due to the hydrated state of the ECM, diffusion of a variety of substances including nutrients and hormones is facilitated between the blood and tissues. Collagen fibres give tissue an extent of tensile strength, whereas elastin fibres give a degree of elasticity (Eyre, 1980). Fibronectin enhances the attachment of fibroblasts and other cells to the ECM, whereas laminin promotes adhesion of epithelial cells to the basement membrane (Hay, 1982; reviewed by Alberts *et al.*, 1994).

Basement membrane composition

Both the epithelial and endothelial cell layers are separated from the underlying stroma by basement membranes (reviewed by Yurchenco and Schittny, 1990). The basement membrane is secreted and assembled largely by the cells that rest on it. The composition can vary between different tissue types (Kleinman *et al.*, 1986), and from the ECM and in particular the presence of type IV collagen is specific to basement membranes, having a more flexible structure than fibrillar collagens. Other components of the basement membrane, such as heparan sulphate proteoglycans, entactin and laminin, bind to type IV collagen (Timpl *et al.*, 1979; Durkin *et al.*, 1988). These components

collectively provide a selective barrier for the movement of specific cells, and in damaged tissue provide a structure along which regenerating cells can migrate.

1.2.1. Matrix Metalloproteinases (MMPs)

The involvement of MMPs in ECM and basement membrane breakdown has been widely implicated in metastasis. MMPs are a family of extra cellular endopeptidases, with a physiologically optimum pH, and are dependent upon the Zn^{2+} ion for activity (Van Wart and Birkedal-Hansen, 1990; reviewed by Nagase and Woessner, 1999). Together, the MMP family (including collagenases, gelatinases, stromelysins and membrane-type MMPs [MT-MMPs]) is capable of degrading most of the ECM proteins, successfully disrupting the structural integrity of the connective tissue and consequently allowing cellular motility through the surrounding stroma. Each member is secreted as a pro-enzyme and possesses a conserved sequence in a pro-domain, maintaining latency (Van Wart and Birkedal-Hansen, 1990). Regulation of these enzymes is controlled by a number of factors including transcriptional control by growth factors, inflammatory cytokines and oncogenes, post-translational control by activation from the latent form and inhibition by endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (Werb *et al.*, 1977; Murphy *et al.*, 1989; reviewed by Gomez *et al.*, 1997). While most of the MMPs are secreted in a soluble form, MT-MMPs remain bound to the cell membrane and these enzymes have been shown to be involved in pro-MMP activation (Sato *et al.*, 1994; Hofmann *et al.*, 2000c).

As described previously, MMP expression is not restricted to tumour cells. Stromal cell expression (fibroblasts, inflammatory cells and endothelial cells) of these enzymes is becoming increasingly evident (e.g. Poulsom *et al.*, 1993; Wolf *et al.*, 1993; Ito *et al.*, 1995). The precise mechanism by which tumour cells activate stromal cell expression is still unclear, but the involvement of direct cell contact and the secretion of tumour cell soluble factors have been described (Hofmann *et al.*, 2000b). For example, infiltrating endothelial and inflammatory cells produce MMP-2 and MMP-9, aiding both tumour and endothelial cell migration (Heppner *et al.*, 1996). MMP-12 expression has also been detected in infiltrating macrophages, possibly due to an inflammatory response to the tumour (Heppner *et al.*, 1996). Tumour cells may further be responsible for activating secreted stromal cell proenzymes. For example, in breast carcinoma, fibroblasts have been shown to express a range of MMPs, including MMP-1, -2, -3, -11 and a MT-MMP family member, assisting tumour cell migration

(Heppner *et al.*, 1996). MT-MMP mRNA has been identified in fibroblasts, yet the protein is localised at the invasive front of the tumour cell, focusing stromal gelatinase A activation by MT-MMP to the tumour cell invadopodia (Heppner *et al.*, 1996; Nakahara *et al.*, 1997).

There is now much evidence implicating increased MMP expression with more advanced stages of the disease. For example, *in vitro* cell lines established from advanced melanomas are almost all positive for MMP-9 expression, whereas cell lines derived from early, non-invasive melanomas are invariably MMP-9 negative (MacDougall *et al.*, 1995). *In vivo*, in breast carcinoma and cutaneous melanoma, MMP-2 activation rather than expression, has been correlated with disease progression, as activation may be localised to the invading pseudopodia of the tumour cells (Azzam *et al.*, 1993; Heppner *et al.*, 1996; Nakahara *et al.*, 1997).

1.2.2. Tissue Inhibitors of Metalloproteinases (TIMPs)

Endogenous inhibition of MMPs resides at the level of TIMPs; a net loss in TIMP activity may result in enhanced MMP activity (Hicks *et al.*, 1984; Tananka *et al.*, 1995). To date, four members of the family have been identified and each interacts with their appropriate proenzyme at the region flanking the substrate-binding site, blocking activity (e.g. Golberg *et al.*, 1989; Goldberg *et al.*, 1992; reviewed by Gomez *et al.*, 1997). TIMP-1 preferentially forms a 1:1 complex with activated MMP-1 and –3 and with both pro- and active forms of MMP-9; interaction with latent forms of the enzyme prevents proenzyme cleavage whereas interaction with the active form causes protease inhibition (Goldberg *et al.*, 1992). TIMP-2 forms a 1:1 complex with both latent and active forms of MMP-2, but also has an inhibitory effect against other MMP family members (Butler *et al.*, 1998). These proteins may be secreted in an unbound form or complexed to pro-MMP-2 (Goldberg *et al.*, 1989). Unlike other TIMPs, TIMP-3 remains insoluble after secretion, remaining in the stroma and binding to ECM components (Leco *et al.*, 1994). TIMP-4 has been most recently isolated and has been found to inhibit both invasion and metastasis in breast carcinoma cells (Wang *et al.*, 1997). TIMP expression may further be regulated by a number of factors including IL-1β, TGF-β and EGF (e.g. Ganser *et al.*, 1991; Overall *et al.*, 1991; reviewed by Gomez *et al.*, 1997).

Conflicting evidence exists regarding TIMP involvement in the metastatic process and their actions often seem paradoxical. TIMP-2 over-expression in metastatic melanoma cells has been seen to

decrease invasion, whilst also inhibiting endothelial cell migration (Valente *et al.*, 1998). Conversely, in breast carcinoma, TIMP-1 expression has been associated with shortened survival (Ree *et al.*, 1997), whilst in colon carcinoma, increased TIMP-1 expression has been correlated with tumour progression (Hewitt *et al.*, 2000). In malignant melanomas, over-expression of MMP-1 and MMP-3 has also been shown to be accompanied by an over-expression of TIMP-1 and TIMP-3, again inferring a dual function of these regulators in metastasis (Airola *et al.*, 1999). In addition, TIMP-1 and TIMP-2 have shown to also exhibit growth factor-like functions, and inhibit angiogenesis (Bertaux *et al.*, 1991).

1.2.3. Plasminogen Activators (PAs) and their Inhibitors

In addition to MMPs, plasminogen activators (PAs) have been shown to play a vital role in the metastasis of a number of tumours. These enzymes are responsible for breakdown of plasminogen into plasmin. Once in this form, plasmin degrades fibrin clots, activates MMPs and elastases and degrades other ECM proteins, such as laminin and fibronectin (e.g. Werb *et al.*, 1977; reviewed by Andreasen *et al.*, 2000). Two types of PA have been identified; urokinase PA (u-PA) and tissue-type PA (t-PA). Degradation of the ECM has commonly involved u-PA, whilst the action of t-PA has been associated with thrombolysis (reviewed by Andreasen *et al.*, 1997). Stationary cells express a receptor for both the pro- and active form of u-PA at focal contacts; when stimulated to migrate, expression of the u-PA receptor, u-PAR is focused primarily at the leading invasive edge, targeting u-PA activity to this region (Carriero *et al.*, 1994). u-PAR also binds vitronectin and interacts laterally with several β-chains, coordinating the focus of u-PA activity and paradoxically promotes cell adhesion to this substrate (reviewed by Andreasen *et al.*, 2000). Whilst bound, u-PA activates cell-associated plasminogen, initiating the proteolytic cascade required for local degradation, degrading surrounding proteins and also catalysing the conversion of some MMPs from the pro- to the active form. Expression of u-PA has consequently been commonly associated with cell invasion and metastasis (e.g. Pyke *et al.*, 1991; Bajou *et al.*, 1998).

To date, four endogenous inhibitors of PAs have been identified, PA inhibitors (PAIs)-1, -2 and –3, and protease nexin 1 regulating PA activation (reviewed by Meyer and Hart, 1998). Both tumour and endothelial cells express high levels of PAI-1, which appears to be a necessity for optimising tumour cell invasion; absence of host PAI-1 expression has been shown to inhibit tumour cell invasion and

vascularisation (Bajou *et al.*, 1998). PAI-1 has also been shown to bind vitronectin, thus preventing u-PAR-expressing cellular adhesion, whilst aiding tumour cell detachment and increasing tumour cell invasion (Kanse *et al.*, 1996; Deng *et al.*, 1996). In addition, PAI-1 disrupts u-PAR-vitronectin binding, again promoting cell detachment and increasing migration (Kanse *et al.*, 1996; Deng *et al.*, 1996). It is therefore not surprising that concurrent increases in u-PAR and PAI-1 expression have been reported (Liu *et al.*, 1995).

1.3. Tumour cell dissemination

Once migrated through the ECM and the basement membrane, tumour cells must further migrate through the vessel wall, facilitating dissemination. In order to successfully establish secondary colonies, transendothelial cell migration or invasion, must be undergone at both the primary (intravasation) and secondary sites (extravasation). These two processes share common features, and thus when describing extravasation in this section, a similar (but reverse) process may be undergone during intravasation. When considering the former, this process also bears many similarities with leucocyte transendothelial migration during inflammatory responses (Voura *et al.*, 1998a and b). In addition, dissemination is also aided by the process of angiogenesis, which is commonly associated with malignant tumour progression, supporting growth of the tumour by establishing a blood supply (Folkman *et al.*, 1989).

1.3.1. Transendothelial cell migration

Figure 1.3.1.a illustrates the process of extravasation of a tumour cell at the secondary site. Once strong adhesive interactions have been made with endothelial cell, tumour cell migration through the endothelium is facilitated.

In common with metastasis, during an inflammatory response, leucocytes must successfully navigate the endothelial cell barrier to affect the required response at the site of inflammation (Muller, 1995). As with leucocytes, tumour cells may form a series of adhesive interactions with the endothelium prior to extravasation (Voura *et al.*, 1998a). For leucocytes, initial contact with the vessel wall is in part a random event, perhaps enhanced by local changes in flow characteristics; loose interactions are formed mediated by selectins, integrins and CD44 expressed on the leucocyte surface, allowing 'rolling' along the endothelium (reviewed by Springer, 1994; Degrendele *et al.*, 1996; Chen *et al.*,

1997). These interactions, together with soluble chemoattractants associated with the endothelium or the local tissue, trigger the up-regulation of specific leucocyte integrins and induce conformational changes in integrin heterodimers, resulting in greater affinity with the vasculature (reviewed by Springer, 1994; e.g. Simon *et al.*, 1995; Tsang *et al.*, 1997). Once adhered to the luminal side of the endothelium, it only takes a few minutes to reach the sub-endothelial basement membrane. Adherent leucocytes initially move towards endothelial cell junctions, followed by diapedesis between endothelial cells and finally migration through the sub-endothelial matrix potentially aided by specific chemoattractants (Tsang *et al.*, 1997). To date, there is no direct evidence to suggest that leucocytes utilise degradative enzymes to migrate, but instead pass along predefined lines of weakness between capillary endothelium.

In contrast, unlike leucocyte migration, the time taken for the extravasation of tumour cells is much greater (Voura *et al.*, 1998a). The precise mechanisms by which tumour cells migrate through normal vascular endothelium remain unclear, but evidence has suggested that tumour cells penetrate the endothelial junction (Voura *et al.*, 1998a and 1998b). *In vivo* studies have also concluded that the time taken for these processes and the mechanisms may further vary between tumour cell types and the site of extravasation (Paku *et al.*, 2001). *In vitro* studies of cutaneous melanoma adhesion to the endothelium have identified specific cellular changes occurring in both cell types (Voura *et al.*, 1998a and 1998b). In these studies, after initial adhesion melanoma cells were seen to bleb on the basolateral surfaces, whilst endothelial cells showed an abundance of microfilaments at contact regions. In response, adhesion molecules at endothelial cell junctions redistributed, causing local dissolution, allowing melanoma cell pseudopodia to penetrate the endothelial cell layer. Attachment of melanoma cells to the underlying matrix facilitated spreading, prior to endothelial cell processes reforming the endothelial junction. Interestingly in these studies, unlike leucocytes and other tumour cell types, melanoma cells were not seen to roll along the endothelium after initial attachment. More recently, as described previously, these authors have also reported a redistribution of PECAM-1 away from the endothelial junctions during melanoma cell transendothelial cell migration and a vital role for melanoma N-cadherin and $\alpha v\beta 3$ expression during this process (Sandig *et al.*, 1997; Voura *et al.*, 1998b; Voura *et al.*, 2001a and b).

Tumour cell extravasation potentially also requires endothelial retraction to enable the larger cells to migrate through (Voura *et al.*, 1998a). This again differs from leucocyte extravasation, in which cells extravasate without much disruption to the endothelial layer due to their smaller size and flexibility. Studies on the human pancreatic cancer cell line PSN-1 have identified a soluble, tumour cell-derived factor, known as endothelial cell retraction factor (Kusama *et al.*, 1995). This factor stimulated endothelial cell retraction, thereby inducing breakdown of intercellular junctions, increasing vascular permeability and exposing sub-endothelial matrix. Further studies have correlated these effects on endothelial cell retraction with the down-regulation of endothelial cell focal adhesion kinase $pp125^{FAK}$ (Okamoto *et al.*, 1998). Alternative investigations have however shown the involvement of other factors such as a lipoxygenase metabolite of arachidonic acid (12[S]-HETE) (Honn *et al.*, 1994) and sublethal concentrations of H_2O_2 (Bradley *et al.*, 1995). H_2O_2 production by tumour cells, as a result of endothelial cell damage may also lead to tumour cell attachment to the underlying matrix (Offner *et al.*, 1996).

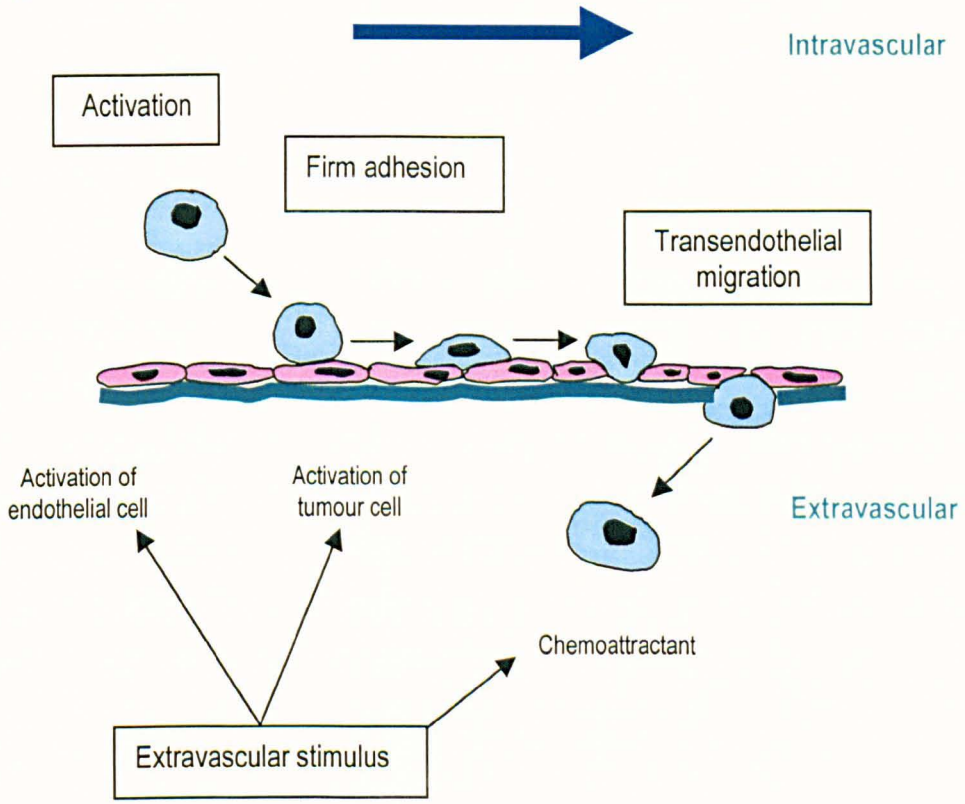


Figure 1.3.1.a. Diagrammatic representation of transendothelial cell invasion during extravasation. Tumour cells (blue), adhere to the endothelium (pink), migrate between endothelial cells, adhere to and degrade underlying basement membrane (green) ECM components and migrate towards a stimulus. The dark blue arrow indicates the direction of blood flow.

In vivo evidence has however, concluded that there are several other mechanisms by which tumour cells may extravasate (Paku *et al.*, 2001). Firstly endothelial cell retraction may be followed by fragmentation of the basement membrane by cellular processes, ultimately causing the breakdown of the capillary. Alternatively, endothelial cells may completely cover the tumour cells prior to penetration of the basement membrane. Finally, destruction of the capillary may be a result of intra-capillary proliferation. The precise mechanism utilised is therefore potentially dependent upon the site of extravasation, and properties of the tumour cells, determining the response of the endothelium (Paku *et al.*, 2001).

1.3.2. Tumour angiogenesis

Tumour cell dissemination is further assisted by the development of a new blood supply to the tumour. In the absence of an adequate blood supply, the diffusion of oxygen and nutrients across numerous cell layers limits the size of the tumour. To grow beyond 1-2mm^3 most tumours must successfully initiate the development of a new vascular bed from pre-existing vessels, which will support the expanding malignant cell population (Folkman *et al.*, 1989). This process, known as angiogenesis, involves the degradation of the ECM around each vessel and then the migration, proliferation and differentiation of endothelial cells to form tubes, and eventual new vessels (reviewed by Folkman, 1995). Through the development of a new vascular network, tumour growth is stimulated whilst also providing a route for extravasation. The walls of the new vessels often have fragmented basement membranes, additionally allowing passive tumour cell leakage (Nagy *et al.*, 1989). Endothelial cells at the growing tip degrade the surrounding ECM, also passively assisting the escape of tumour cells into the tumour neovasculature (Fisher *et al.*, 1994). In many tumours, a significant association has been found between vascular density and poor prognosis, both in terms of disease free and overall survival. For example, microvessel counts at the margin of the primary tumour in breast cancers have been proven to be a predictive marker of metastasis, increasing the probability of extravasation and dissemination (Weidner *et al.*, 1991).

The switch to the angiogenic phenotype depends upon a change in the net balance of positive and negative angiogenic factors, released by both the tumour and host cell populations (Folkman and Klagsbrun, 1987). Properties of the tumour mass, including hypoxia and hypoglycaemia can induce this transition by activating pro-angiogenic factors (Tuder *et al.*, 1995). In response, growth of the

tumour will result through an influx of blood through the new vessels and through paracrine stimulation of tumour cells by numerous growth factors and matrix proteins produced by, and transported via the new capillary endothelium. Recently the presence of 'mosaic vessels' in tumours, in which the vessel wall is lined by a heterogeneous layer of endothelial and tumour cells, have also been discovered, raising the possibility of tumour cells mimicking endothelial cells (Chang *et al.*, 2001).

The angiogenic process

To date, many pro-angiogenic growth factors have been identified, including VEGF, FGF, IGF-1, TGF-β1, TNF-α, and HGF (e.g. Roberts *et al.*, 1986; Frater-Schroder *et al.*, 1987; Connolly *et al.*, 1989; reviewed by Carmeliet and Jain, 2000). In response, endothelial cells alter their morphology, producing finger-like protrusions on the cell surface adjacent to the basal lamina, intercellular gaps appear and the endothelial cells retract. Subsequent proteolytic degradation of the basal lamina and surrounding ECM permits endothelial cells from post capillary parent venules to migrate and form sprouts, employing a variety of proteolytic enzymes, including both MMPs and PAs, moving chemotactically towards the aforementioned growth factors (Fisher *et al.*, 1994; reviewed by Folkman, 1995). Degradation of the surrounding matrix may cause further activation of additional angiogenic growth factors (bFGF, VEGF and IGF-1) bound within the ECM. During migration, as gaps between endothelial cells increase, neighbouring endothelial cells follow from the parent vessel. Endothelial cells initially travel as solid cords but subsequently develop inter- and intra-cellular spaces that form lumen (Folkman and Haundenschild, 1980). Proliferation of endothelial cells allows the length of the sprout to increase and blood will flow when the two hollow sprouts join at their tips to form a loop (Folkman and Klagsbrun, 1987).

Angiogenesis, micrometastasis and dormancy

Angiogeneis is therefore a vital stage of both primary and metastatic tumour development. For metastatic colonies to therefore grow to any considerable size at a distant site, further neovascularisation is required. Until this occurs the micrometastases are said to be dormant and may remain asymptomatic and clinically undetectable for months or years (Demicheli *et al.*, 1994). Suppression of angiogenesis retains a balanced, high rate of proliferation and apoptosis in the micrometastases (Holmgreen *et al.*, 1995). In more than 50% of cancer patients, metastasis has

already occurred at the time of initial diagnosis and when the primary tumour is removed, many die within months (reviewed by Fidler and Ellis, 1994). It has therefore been theorised that removal of the primary tumour might be responsible for removing circulating angiogenic inhibitors and hence initiating angiogenesis of the micrometastases. This principle has been used as the basis for the development of anti-angiogenic drugs, inhibiting microvascular proliferation with a consequent inhibition of angiogenesis-dependent growth (O'Reilly *et al.*, 1994; O'Reilly, *et al.*, 1997). Of notable interest has been the identification of a group of endogenous proteins that inhibit endothelial cell proliferation, suppressing growth of metastases *in vivo*. Among these proteins, two peptides known as angiostatin and endostatin have been shown to be potent angiogenesis inhibitors. These are the breakdown products of plasminogen and collagen type XVII, respectively which may be facilitated by enzymatic activity accompanying tumour growth, hence possibly explaining induction of metastatic growth after the surgical removal of the primary tumour (O'Reilly *et al.*, 1994; O'Reilly, *et al.*, 1997).

1.4. Tumour cell survival during dissemination

Once intravasation has occurred, tumour cells disseminate to the secondary site. As described earlier, the pattern of dissemination is likely to be determined by both anatomical conditions and the microenvironment of the target organ. Spread is commonly facilitated by either the haematogenous or lymphatic system. For a cell to successfully disseminate, the tumour cell must therefore survive against host immune responses during the transportation.

Originally it was hypothesised that tumour cells are capable of evading the host immune responses, through resisting immune recognition and attack by host inflammatory cells (reviewed by Pardoll, 2001). As such, tumour cells would still be seen as foreign, but the immune response is in some way avoided. This hypothesis would provide an explanation for the inactivation of tumour cell major histocompatibility complex (MHC) molecules often observed, as this may prevent T cell recognition (Travers *et al.*, 1982). Opposing this theory, it has also been suggested that the host immune system instead recognises tumour cells as self and not foreign (Ochsenbein *et al.*, 2001; Shankaran *et al.*, 2001). In this explanation, the location and properties of the tumour cells during development may determine whether an immune response is invoked or tolerated. Tumour cell antigen expression may therefore be determined during the early stages of tumour progression such as degradation of the surrounding stroma and angiogenic development, which may be sufficiently disruptive to provoke a

pro-inflammatory response (reviewed by Fuchs and Matzinger, 1996). In such cases, immune responses may be initiated, facilitating attack by host cells. Alternatively, if an inflammatory response is not induced during this period, tumour cell survival may result. Secretion of soluble ICAM-1 by both tumour and endothelial cells has also been reported for a number of cancers, and suggested as a mechanism by which tumour cells may escape immune surveillance (Fonsatti *et al.*, 1999; Zhang and Adachi, 1999). In these studies, tumour and host inflammatory mediators such as IL-1α and IFNγ induced secretion, and it is thought that soluble ICAM-1 may further block the LFA-1 receptor, thereby inhibiting T and natural killer cell attachment and providing a means by which these cells prevent immune attack.

1.5. Arrest at the target organ

If tumour cells survive dissemination in the circulation, the next step of the metastatic process involves arrest at the target organ. Mechanical arrest of single cells in capillaries can account for a proportion of these adhesive events during metastasis; whereas more directed adhesive interactions of tumour emboli may occur in larger vessels (Scherbarth and Orr, 1997). As predicted by Paget's model, evidence exists showing antigenic differences between parts of vascular beds in different organs, but it is possible that this is not the only determining factor (Paget, 1889; Auerbach *et al.*, 1987).

As briefly described earlier (1.1 and 1.3.1), upon initial contact of the tumour cells with the endothelium, weak or transient interactions are formed (a process known as 'docking') facilitated primarily by selectins, inducing activation of both cell types through cytokines, growth factor, free radicals and bioactive lipids (Chen *et al.*, 1997). To reinforce the interaction, firm attachments are made via expression of inducible adhesion molecules (principally integrins). These firm interactions consequently initiate a cascade of intracellular responses, ultimately inducing endothelial cell retraction, tumour cell motility and transendothelial cell migration (Figure 1.3.1.i) (reviewed by Haier and Nicholson, 2001). The influence of fluid flow and local shear forces are however becoming increasingly evident (e.g. Mogiri *et al.*, 1995; Yoshida *et al.*, 1999). Once endothelial cells have retracted, tumour cells then adhere with stronger interactions to the underlying basement membrane.

For the degree of metastatic site-specificity commonly seen, regulators and receptors involved in the process may be specifically expressed at the secondary sites promoting tumour cell adhesion, migration and invasion (reviewed by Liotta, 2001; e.g. Abdel-Ghany *et al.*, 2001; Muller *et al.*, 2001). The importance of the host and tumour-derived growth factors, cytokines and chemokines in tumour progression is becoming ever more apparent, affecting various stages including tumour cell migration (chemotaxis), leucocyte infiltration, cell adhesion molecule expression, tumour cell proliferation and angiogenesis (e.g. Leek *et al.*, 1998; reviewed by Balkwill and Mantovani, 2001; e.g. Muller *et al.* 2001). Following Paget's original hypothesis, to affect the required response the tumour cells involved must therefore express the necessary receptors (Paget, 1889; Nicolson, 1993; e.g. Wang *et al.*, 1998b). Recent advances have reported that both breast cancer and malignant melanoma cells, express distinct and non-random patterns of chemokine receptors which may play a critical role in determining the metastatic destination of these tumour cells (Muller *et al.*, 2001). Blocking these receptors subsequently prevented metastasis, inferring that in general terms, target organs may produce soluble factors, which chemotactically attract circulating tumour cells expressing the relevant receptors.

Inflammatory mediators may also enhance adhesive interactions between tumour cells and the endothelium. For example, as described previously, B16 melanoma cells have been shown to arrest primarily within large pre-sinusoidal hepatic portal vessels after IL-1α treatment (Scherbarth and Orr, 1997). Without IL-1α treatment, melanoma cells were seen to attach to sinusoidal vessels, suggesting that IL-1α may potentially facilitate adhesion to the endothelium. In agreement with this theory, *in vivo* and *in vitro* studies similarly using B16 melanoma cells, have shown that adhesion to the hepatic sinusoidal endothelium stimulated secretion of endothelial TNF-α, IL-1β and IL-18, which ultimately increased melanoma adhesion to the endothelium (Vidal-Vanaclocha *et al.*, 2000).

Site-specific endothelial adhesion molecules on the target endothelium may also promote attachment of specific tumour cell types. For example, LuECAM has been localised to endothelium of lung vessels only, promoting specific attachment of B16 melanoma cells (Zhu *et al.*, 1991). More recently, hCLCA2, a Ca^{2+}-sensitive calcium chloride channel protein, has been found to be expressed on the endothelial luminal surface of pulmonary arteries, arterioles, and venules, mediating adhesion of breast cancer cells via $\alpha6\beta4$ (Abdel-Ghany *et al.*, 2001).

1.6. Extravasation into organ parenchyma and growth in the target organ

Once arrested at the secondary site, tumour cells must extravasate through the vessel wall and degrade the underlying basement membrane and ECM by the processes described earlier (1.1-1.3). As detailed previously (1.3.1), the mechanisms underlying extravasation have been shown to vary between tumour cell types and target organs (Paku *et al.*, 2001) and controversy exists regarding the specific mechanisms underlying these processes. The authors of this study further concluded that the time taken for extravasation and the precise involvement of the endothelium in the process differs between different target organs and may be determined by properties of the tumour cells. In organs such as the adrenals and the brain, tumour cells were seen to induce endothelial retraction and penetrate the basement membrane. In contrast in the liver and lung, the endothelium covered tumour cells prior to penetration of the basement membrane.

It has however also been observed that, using a mouse model, upon intraportal injection of B16F1 melanoma cells, the majority of tumour cells survive and extravasate at the liver (Luzzi *et al.*, 1998). In contrast to other reports suggesting that few circulating tumour cells ever form metastases (Fidler, 1970), these authors suggested that lack of metastatic development is determined by the failure of initial growth or the development of microscopic tumour into macroscopic tumours. Further contrasting evidence relating to metastatic development was also published recently, in which it was reported that tumour cells, once attached to the endothelium, might proliferate intravascularly. Metastatic foci consequently developed within the capillary, without the need for extravasation, ultimately leading to capillary destruction (Al-Mehdi *et al.*, 2001).

Infiltrating lymphocytes and local host cells are additionally considered to play a pivotal role in producing the inflammatory microenvironment required to support tumour growth, both at the primary and secondary sites. Studies on prostate and breast cancer have described the pertinent effect of host stromal cells on metastatic development, presenting evidence for the effect on tumour cell growth and migration upon interaction with such cells (Brooks *et al.*, 1997; Lang *et al.*, 1998; Lang *et al.*, 1999; Lang *et al.*, 2000; Hall *et al.*, 2002). In breast carcinoma, direct interactions between tumour cells and host fibroblasts have been shown to enhance fibroblast MMP synthesis, further assisting tumour cell invasion (Ito *et al.*, 1995), whilst using a sophisticated co-culture system, Hall *et*

al., (2002) have more recently shown that the consequence of the stromal cells on prostate carcinoma morphology and migration was in part determined by the stromal cell origin, describing differential effects of host cells derived from benign or malignant tissue. The host tissue therefore potentially plays a vital function in metastatic development at both the primary and secondary sites, whereby malignant tumours may further influence host tissue behaviour.

To conclude, no one mechanism of metastasis may therefore explain the spread of tumours from one site in the body to another. The process is highly complex and is likely to vary from one tumour type to another. Advances are rapidly being made in the cancer field, and hopefully with time, a wealth of targets for prevention and treatment will become evident for all tumour types.

B. Posterior Uveal Melanoma

Ocular melanomas may develop from a variety of ocular tissues, including the uveal tract (iris, ciliary body and choroid), conjunctiva, eyelid, orbit and nasolacrimal duct (McCartney, 1995). The tissue from which the tumour has been derived may have important implications on the biological behaviour and prognosis of the tumour (Rennie, 1991). Some of these major sites are illustrated in Figure 1.a. Although ocular melanomas can occur at a number of sites, the most common site is the uveal tract. Of these, ciliary body and choroidal tumours are most frequently observed and readily disseminate. Conversely iris melanomas are less common and very rarely metastasise (McCartney, 1995; reviewed by Singh *et al.*, 2001).

Posterior uveal melanomas are the most common intra-ocular neoplasms in adults, comprising about 79% of all non-cutaneous melanomas (Scotto *et al.*, 1976). They still however remain rare in comparison with other malignancies, representing an annual incidence of 5-7 cases per million of Western populations (Egan *et al.*, 1988). Despite the successful treatment of uveal melanomas by various techniques, including enucleation, radiotherapy and local resection, the overall mortality rate remains unchanged, with secondary tumours appearing in between 19-35% of patients within five years (reviewed by Pyrhonen, 1998). Recurrences have however been recorded up to 42 years after the primary tumour was detected (Egan *et al.*, 1988). In such cases, it is therefore possible that tumour cells access the bloodstream in the early stages of tumour growth to invade secondary organs, where they remain in a dormant state for many years before they progress into metastatic disease. Median survival following detection of metastases is 4-6 months, and the liver is the principle site of metastasis (Char, 1978; Egan *et al.*, 1988).

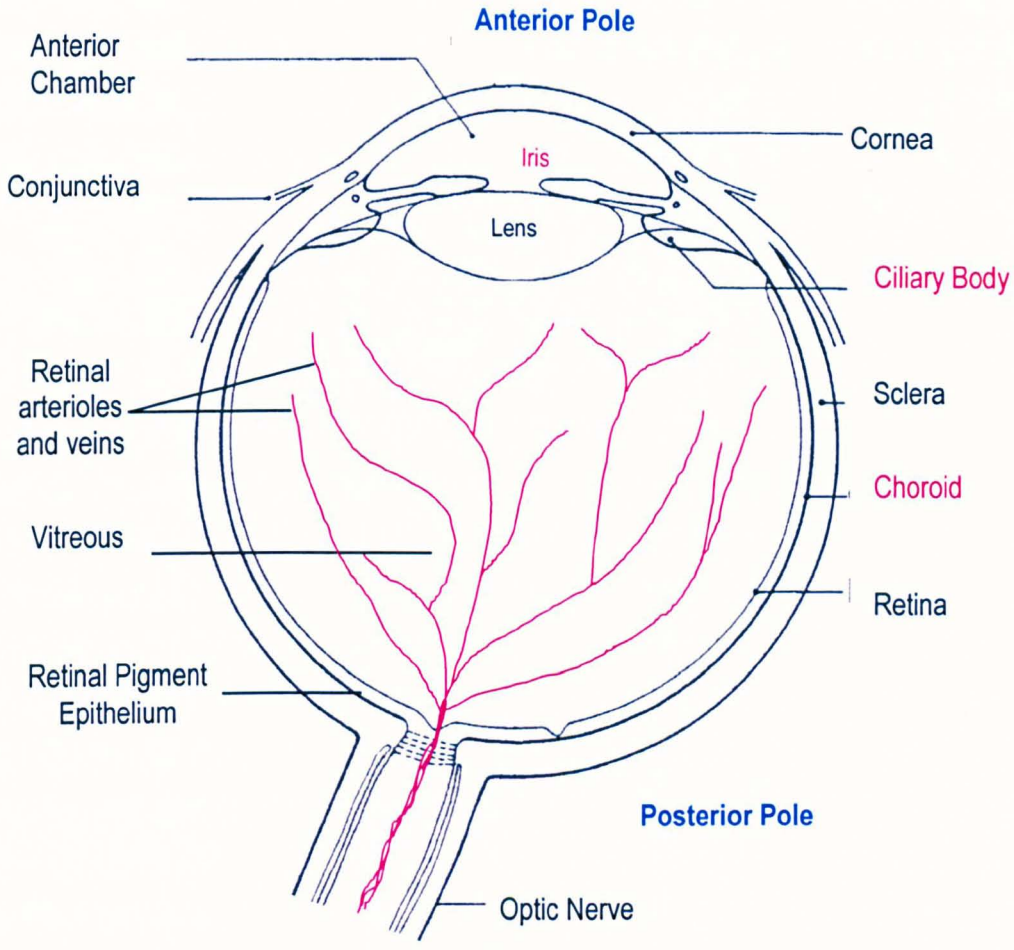


Figure 1.a. Diagrammatic representation of the cross section through the human eye, adapted from Bron *et al.*, 1997. Labels indicating common sites for uveal melanomas are shown in red.

1.1. Aetiology of Uveal melanoma

1.1.1. Age and patient gender

The age incidence of posterior uveal melanoma varies, but commonly the median age at diagnosis is approximately 55 years (Egan *et al.*, 1988). Diagnosis is rare in children whilst after 70 years of age; incidence appears to decline for both sexes (Blair-Smith and Egan, 1980; Egan *et al.*, 1988; Rosenbaum *et al.*, 1988). Distribution patterns have however been shown to vary between the sexes, with some studies showing a slight male predilection for uveal melanoma (Scotto *et al.*, 1976; Albert *et al*, 1980). Females have also shown a slight increase between the ages of 20 and 39 years,

possibly being related to hormonal patterns, whilst women who have given birth have a lower incidence than nulliparous women (Holly *et al.*, 1991). This would indicate the involvement of an ovarian hormone, but as yet, studies have found no correlations between incidence, oestrogen levels and oestrogen receptor expression (Hartge *et al.*, 1989). More recently a study has been published in which no correlation was made between patient gender and melanoma-specific mortality (Kroll *et al.*, 1998).

1.1.2. Incidence

Although few factors have been proven to predispose to uveal melanoma, the most outstanding observation is that of the low incidence in non-whites. Several studies have all shown a dramatically lower incidence in the black population in comparison to the white population (Scotto *et al.*, 1976; Blair-Smith and Egan, 1980; Egan *et al.*, 1988) and as with cutaneous melanoma, fair-skinned, blue-eyed individuals appear to be at an increased risk (Gallagher *et al.*, 1985; Egan *et al.*, 1988; Casswell *et al.*, 1989). In a recent population-based study, it was however reported that eye colour is the strongest independent predictor of uveal melanoma, where individuals with grey, hazel and blue eyes being at the greatest risk (Vajdic *et al.*, 2001).

The lower incidence of uveal melanoma in individuals with dark skin and eyes, would suggest that this may be related to the protective influence of pigmentation, filtering ultraviolet light (Egan *et al.*, 1988). Unlike cutaneous melanoma, the incidence of uveal melanoma has nevertheless been relatively constant over the past decades and there appears to be no correlation between incidence and latitudinal location (Egan *et al.*, 1988). Sufficient evidence does however exist to suggest an involvement of sunlight in the aetiology of uveal melanoma (Holly *et al*, 1990; Seddon *et al.*, 1990).

Recent evidence has also documented an association between radiofrequency radiation and uveal melanoma, suggesting an increased risk of development of uveal melanoma with higher mobile phone usage (Stang *et al.*, 2001). At present, reports are limited in this area and ongoing research could ascertain the importance of this finding.

1.2. Treatment and Prognosis

1.2.1. Prognostic factors

A number of clinical, histo-pathological and cytological prognostic indicators have been determined for patients with uveal melanoma, including tumour size and location, cell type, vascularity and cytogenetic abnormalities.

Tumour size

The single most important prognostic factor with regard to patient survival is possibly tumour size (Shammas and Blodi, 1977). Most pathologists classify these tumours according to their largest dimension: small (less than or equal to 10mm), medium (11-15mm) and large (greater than 15mm) (reviewed by Parsons and Sahel, 2000). Prognosis has been ascertained by a combined study of eight investigations, in which the authors observed five-year mortality rates following enucleation (Diener-West *et al.*, 1992). In summary, mortality rates of 16% for small tumours, 32% for medium tumours and 53% for large tumours were seen and to date, these figures have remained unchanged (Diener-West *et al.*, 1992; reviewed by Singh *et al.*, 2001).

Tumour location

Uveal melanomas can arise in the iris, ciliary body and choroid (Figure 1.1.a). Iris melanomas are by far the least aggressive of all uveal melanomas, rarely metastasising and comprising 3% of all uveal melanomas (reviewed by Singh *et al.*, 2001). Tumours of the iris are also often relatively small upon diagnosis and have the lowest mortality rate of about 3-5% in 10 years (reviewed by Shields *et al.*, 2001; reviewed by Singh *et al.*, 2001). In contrast, melanomas of the choroid and ciliary body are far more aggressive and patients have an increased risk of metastasis (McCartney, 1995; reviewed by Gragoudas and Egan, 2000). Choroidal melanomas are the most common seen comprising around 85% of all uveal melanomas; ciliary body tumours are less common, occurring in 10% of patients (reviewed by Parsons and Sahel, 2000). In an early study, patients with ciliary body melanomas, treated by enucleation were shown to have a five-year mortality rate of 53%; in contrast, again after enucleation, patients with choroidal melanomas had a five-year mortality rate of 14% (Seddon *et al.*, 1983).

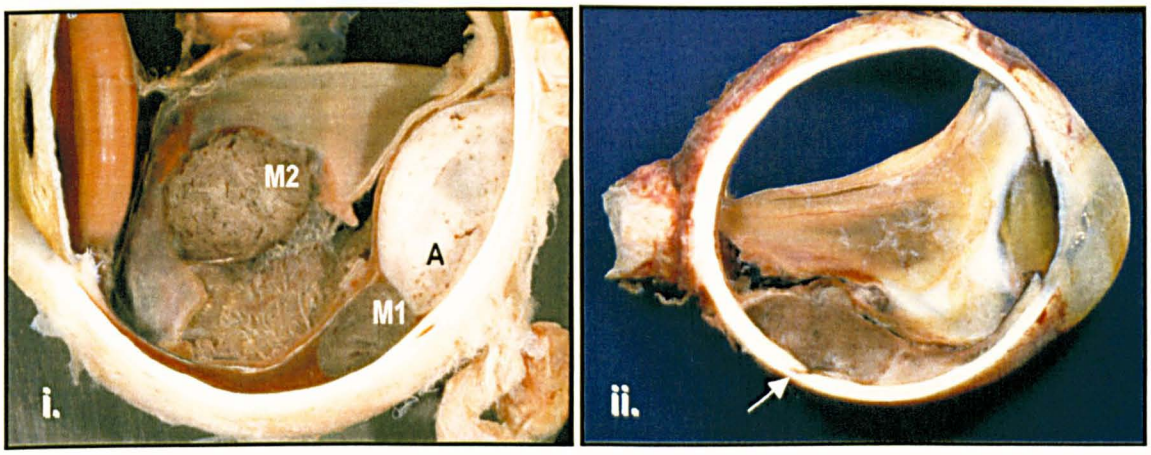


Figure 1.1.a. Examples of choroidal malignant melanomas. i) An amelanotic (A) and a melanotic component (M1) have remained beneath the retina, whilst a second melanotic (M2) lesion has penetrated the retina (taken from Parsons and Sahel, 2000). ii) The choroidal melanoma has caused complete retinal detachment, and can be seen invading through the sclera (indicated by the white arrow).

Cell type

Uveal melanoma can morphologically be classified into subtypes, determined by their cell type (spindle and epithelioid), which can further be correlated with mortality (McLean *et al.*, 1983). Two types of spindle cells may be observed; spindle A cells, which are long and slender with elongated nuclei (Figure 1.1.b.ii) and spindle B cells, which are more oval with a prominent nucleolus. In contrast, epithelioid cells are larger, with more cytoplasm containing fewer intermediate filaments than spindle cells, large rounded nuclei and prominent large nucleoli Figure 1.1.b.i). Tumours with spindle A cells were found to have similar prognosis as those with spindle B cells; the generic term 'spindle cell' is consequently commonly used (reviewed by Parsons and Sahel, 2000). In general terms, tumours are therefore classified as spindle cell, epithelioid cell, and mixed cell (comprised of both spindle and epithelioid cells) (Spencer, 1986; reviewed by Parsons and Sahel, 2000). Disagreement nevertheless exists between pathologists, regarding the permitted number of spindle cells in epithelioid tumours and therefore in reality, uveal melanomas containing epithelioid cells are classified as 'mixed' (reviewed by Parsons and Sahel, 2000).

When considering prognosis, tumours composed of purely spindle cells have a greater survival rate than those of a mixed cell population, which in turn have a greater survival rate than purely epithelioid tumours (McLean *et al.*, 1983). Increases in the epithelioid content of the tumours have further been

correlated with higher mortality rates (Seddon *et al.*, 1983) and an early study of choroidal and ciliary body tumours, observed that 81.2% of patients with purely spindle tumours had a 15 year survival rate, contrasting with 28% for patients with purely epithelioid tumours (Paul *et al.*, 1962). As cell characterisation may however often be subjective, other prognostic indicators must always been taken into account.

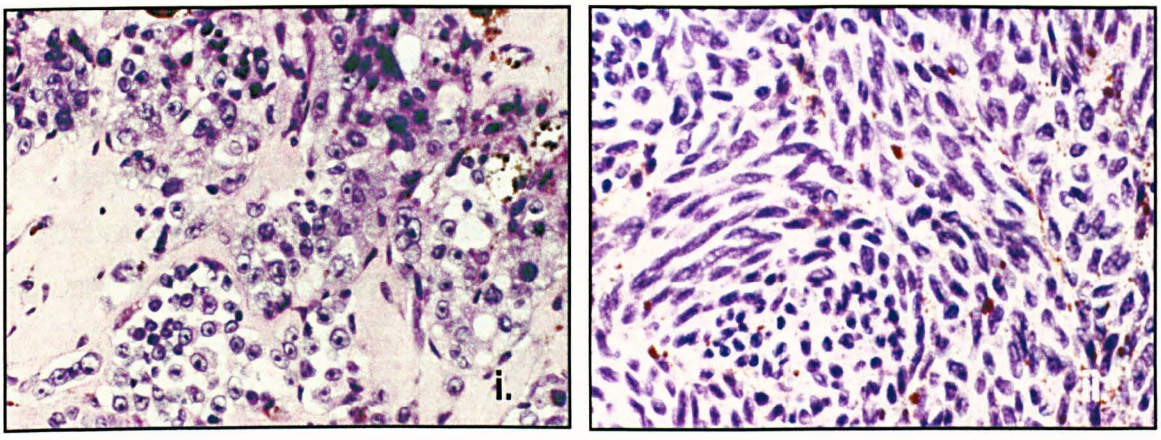


Figure 1.1.b. Histological sections showing epithelioid (i) and spindle A (ii) cells (Parsons and Sahel, 2000). Epithelioid cells are larger, rounded cells with prominent nucleoli. Spindle A cells are longitudinal with slender nuclei.

Microcirculation architecture

The specific architecture of the vascular networks observed in uveal melanomas has also been associated with prognosis. Studies investigating tumour vascularity in uveal melanomas have identified a correlation between the presence of closed vascular loops in choroidal and ciliary body melanomas and poor survival (Folberg *et al.*, 1993; Rummelt *et al.*, 1995). Such vascular patterns may be more significant when considering patient mortality from metastatic disease, than tumour location, size and cell type (Folberg *et al.*, 1993; Rummelt *et al.*, 1995).

Cytogenetics

Over the past decade, specific genetic alterations have been observed in uveal melanomas, and associations have been made with prognosis. Cytogenetic analysis of ciliary body and choroidal melanomas has shown abnormalities of chromosomes 3, 6 and 8 (Sisley *et al.*, 1997; White *et al.*, 1998). Ciliary body tumours have preferentially presented with chromosomal abnormalities of monosomy 3 and an additional 8q and this has further been correlated with a worse prognosis (Sisley

et al., 1997; White *et al.*, 1998). In contrast, abnormalities of chromosome 6 have been more commonly associated with choroidal melanomas (Sisley *et al.*, 1997).

Other prognostic indicators

Other notable prognostic indicators include age of the patient (patients below 60 years of age have a better prognosis), extrascleral spread, extent of lymphocytic infiltration, integrity of Bruch's membrane and cellular proliferation (reviewed by Singh *et al.*, 2001). With regard to the latter, expression of two uveal melanoma associated-antigens (Ki-67 and PCNA) involved in proliferation has been identified and decreased cell cycling times has been associated with a worse prognosis (Mooy and de Jong, 1996; Seregard *et al.*, 1998). More recently, weak expression of ICAM-1 has also been correlated with an increased risk of metastatic disease (Anastassiou *et al.*, 2000b).

1.2.2. Treatment

A range of techniques are now in practice for the treatment of uveal melanomas, and often the preferred treatment will be dependent upon how the patient has presented. These broadly include enucleation, surgical resection, radiotherapy, transpupillary thermotherapy, and photocoagulation (reviewed by Singh *et al.*, 2001). No one modality is superior for all tumours and often a combination of these modalities may be used. Factors that may influence the choice of treatment include patient preference, size and location of the tumour, age of the patient and state of the fellow eye. For example, small tumours are responsive to transpupillary thermotherapy with fewer complications (reviewed by Singh *et al.*, 2001). Brachytherapy employs the use of ruthenium-106 and iodine-125 episcleral plaques and despite the general lack of sensitivity of melanomas to radiotherapy, have been found to be successful for treating small tumours in viable locations (reviewed by Rennie, 1997). The argon laser has also been valuable for treating small choroidal tumours, but is only suitable for tumours less than 3.5-4mm in thickness (Rennie, 1991).

Despite apparent effective removal, or irradiation of the primary tumour, the survival rate of 15 years, still remains unchanged at about 50%, due to the high rate of metastasis (Egan *et al.*, 1988; reviewed by Rennie, 1997). The mortality rates after treatment by enucleation, plaque radiotherapy, proton beam radiotherapy and helium ion therapy appear to be consistent (reviewed by Singh *et al.*, 2001). Only 2-3% of patients present with metastases at the time of initial diagnosis, yet within two to three

years, many of these surrender to metastatic disease (Pach and Robertson, 1986). Some clinicians have suggested that surgical manipulation of the globe at enucleation promotes dissemination of malignant cells and adversely affects survival (Zimmerman *et al.*, 1978). Despite arguments supporting this hypothesis, due to the complexity of the metastatic process, it seems unlikely that any cells released from the primary tumour at the time of surgery would be able to efficiently metastasise to a distant site. At present, due to the nature of the secondary disease, treatment of metastases has had little success (reviewed by Pyrhonen, 1998; reviewed by Gragoudas and Egan, 2000), and the effectiveness of liver screening tests at the time of diagnosis still remains uncertain. Novel therapies for early detection of potentially metastatic tumours are therefore urgently required.

1.3. Invasion and metastasis of uveal melanoma

Uveal melanoma most commonly metastasises to the liver (Char, 1978); studies have shown that 95% of patients with metastasis will develop liver involvement (reviewed by Gragoudas and Egan, 2000), appearing as multi-foci metastases in 19-35% of patients within five years (reviewed by Pyrhonen, 1998). Others sites less frequently colonised include the subcutaneous tissue, bone and pulmonary tissue (Char, 1978). Once liver metastases have been observed, prognosis is poor and patients usually die within six months (Gragoudas *et al.*, 1991). As metastases still develop after adequate treatment of the primary tumour it therefore seems feasible that micrometastases dispersed throughout the liver, may have established before any treatment can be offered (Shields, *et al.*, 1985).

Despite much evidence implicating the liver as the primary target for metastatic uveal melanoma explanations determining this pattern of spread are still unclear. Due to the anatomical structure of the liver, it would seem possible that mechanical entrapment of tumour cells is feasible. During transportation from the eye, a number of other capillary beds may be encountered; yet secondary tumours are rarely seen in these sites (Char, 1978). Unlike, for example colorectal carcinoma for which secondary tumours present as a single focus in the liver, as uveal melanoma metastases develop as multi foci, this may further imply that the spread is more related to Paget's 'seed and soil' hypothesis (Paget, 1889), than simply due to physical arrest.

1.3.1. Adhesion molecule and degradative enzyme expression

It is therefore possible that specific characteristics of malignant uveal melanoma cells may promote invasion and metastasis. There is extensive evidence suggesting the involvement of adhesion molecules in cutaneous melanoma progression, yet knowledge is limited regarding their profiles in uveal melanoma. Certain reports have suggested that unlike cutaneous melanoma, expression of αvβ3 may not be an absolute requirement for uveal melanoma metastasis (Ten Berge *et al.*, 1993; Marshall *et al.*, 1998; Seftor *et al*, 1999). Expression of αvβ5 by primary uveal melanomas has also been reported (Natali *et al.*, 1997; Marshall *et al.*, 1998), and loss of αvβ1 and α5β1 has also been correlated with increased invasiveness *in vitro,* and the aggressive phenotype, respectively (Marshall *et al.*, 1998; Beliveau *et al.*, 2000; Beliveau *et al.*, 2001). Gain of α6β1 expression has additionally been associated with an epithelioid cell type and consequently poor prognosis (Rohrbach *et al.*, 1994; Elshaw *et al.*, 2001). ICAM-1 expression in uveal melanomas has also been documented (Natali *et al.*, 1990; Creyghton *et al.*, 1995) and loss of this receptor has now been seen to be associated with an increased risk of metastasis within the first five years after diagnosis (Anastassiou *et al.*, 2000b).

Type IV collagenases have been widely implicated in many types of tumour cell invasion and metastasis. Ubiquitous expression of a 72kD gelatinase (MMP-2) has been reported in uveal melanoma, whereas expression of a 92kD gelatinase (MMP-9) has been more varied (Cottam *et al.*, 1992; Beliveau *et al.*, 2000; Elshaw *et al.*, 2001). MMP-2 expression has been further correlated with poor prognosis and a higher incidence of metastatic disease in uveal melanoma (Vaisanen *et al.*, 1998; El-Shabrawi *et al.*, 2001) whilst secretion of a 117kD MMP has also been associated with cells of an epithelioid morphology and an aggressive phenotype *in vitro* (Beliveau *et al.*, 2000). Besides MMPs, reports of the involvement of the PA/plasmin system in ECM degradation are scarcer in uveal melanoma (De Vries *et al.*, 1995), but TGF-β2, present in the uvea, has been shown to activate t-PA secretion from epithelioid cell lines derived from intraocular tumours, yet inhibits secretion and activity of u-PA from cell lines derived from orbital tumours (Park *et al.*, 1996). In addition to the degradative enzymes, expression of the PA inhibitor PAI-1 has also been reported in the majority of uveal melanoma metastases (Ma *et al.*, 1997).

1.3.2. Uveal melanoma dissemination

Once in the circulation, uveal melanoma cells therefore principally target the liver. Recent research has potentially identified the involvement of intermediate filaments in hepatic uveal melanoma metastasis, but only limited research has been carried out in this area. A small number of pro-angiogenic factors have also been reported in uveal melanoma, but again evidence is limited. Recently, the potential importance of other vascular networks has been described.

Intermediate filaments

Diagnosis of early melanomas originally relied upon the presence of specific intermediate filament markers such as vimentin (a mesenchymal marker). As malignancy progresses, other cytokeratins (characteristic of an epithelial phenotype) have been identified; the co-expression of which suggests a 'dedifferentiated' or 'inter-converted' phenotype (Hendrix *et al.*, 1996). In addition to their roles in determining cell shape and spreading, intermediate filaments have been postulated to play a role in a number of signal transduction pathways, linking the ECM with the nucleus. Co-transfection of a vimentin-positive human melanoma cell line (with a low invasive ability) with cDNAs for keratins 8 and 18, resulted in a two- to three-fold increase in invasiveness *in vitro* and an increase in focal adhesions between the cytoskeleton and ECM. This suggested that co-expression of vimentin and keratin contributed to a more invasive phenotype (Chu *et al.*, 1996). A study using human uveal melanoma cell lines demonstrated that cell populations co-expressing vimentin and keratins 8 and 18 were six-fold more invasive than uveal melanoma cells expressing vimentin only, and eight- to thirteen-fold more invasive than normal uveal melanocytes. This work has proposed an association between intermediate filament co-expression and uveal melanoma metastasis (Hendrix *et al.*, 1998a). Expression of the c-met proto-oncogene (hepatocyte growth factor (HGF) receptor) has further been correlated with the inter-converted phenotype and increased invasive ability *in vitro*, by uveal melanoma cell lines (Hendrix *et al.*, 1998b). Liver metastases were HGF-positive and hence it was postulated the regulation of the inter-converted phenotype by HGF might be involved in spread (Hendrix *et al.*, 1998b) of c-met-expressing uveal melanoma cells.

Angiogenesis

In common with other tumours, microvessel configuration has been associated with poor prognosis in choroidal and ciliary melanomas (Lane *et al*, 1997; Folberg *et al.*, 1997) and due to the lack of lymphatic vessels in the eye; haematogenous metastasis is the prime route of spread for uveal melanoma. The switch to the angiogenic phenotype depends upon the net balance of positive and negative angiogenic factors released by both the tumour cell and host cell populations. VEGF and bFGF expression has been observed in uveal melanoma, supporting endothelial growth (Boyd *et al.*, 2002). Expression of the former has further been shown, in conjunction with the expression angiopoietin-2 and absence of angiopoieten-1, and consequently it has postulated that this may be responsible for the typical vascular patterns associated with poor prognosis (Ijland *et al.*, 1999). Angiopoetin-1 and –2 are involved in regulating maturation and stabilisation of newly formed vessels; the latter blocks the function of the former, thus inhibiting the early maturation and stabilization of the new vessels, rendering the vessels more prone to a proliferative response to VEGF (Ijland *et al.*, 1999). Contradictory reports have however correlated VEGF expression by ciliochoroidal melanomas with the presence of necrosis but not with metastasis or angiogenesis (Sheidow *et al.*, 2000).

Once disseminated, uveal melanoma metastases may remain dormant as micrometastases for many years before neovascularisation is induced, allowing growth to a clinically detectable size (Shields *et al.*, 1985). It is possible that for uveal melanoma, tumour emboli disseminate at the time of enucleation and that by removing the primary tumour, anti-angiogenic peptide levels fall, permitting endothelial cell proliferation and subsequent metastatic growth. Evidence supporting this theory is however scarce, and recent observations have reported variable expression of angiostatin by uveal melanoma cell lines (Ijland *et al.*, 1999; Apte *et al.*, 2001).

Of further importance has been the possible involvement of 'vasculogenic mimcry' in uveal melanoma. Whilst angiogenesis involves the recruitment of new vessels into the tumour from pre-existing vessels, highly aggressive and metastasis uveal melanoma cells (yet not non-aggressive tumour cells) are capable of forming highly patterned vascular channels *in vitro* (Folberg *et al.*, 2000). These channels are devoid of endothelial cells and surround tumour emboli *in vitro and in vivo*,

mimicking the patterned networks seen in aggressive tumours, correlating with poor prognosis (Maniotis *et al.*, 1999), but their relevance in *in vivo* metastasis still remains unclear.

1.3.4. Uveal melanoma metastatic development in the liver

Once transported, uveal melanoma cells therefore arrest principally in the hepatic microvasculature, although as described earlier, other organs such as the subcutaneous tissue, bone and pulmonary tissue may occasionally be involved (Char, 1978).

Liver structure and secondary disease

The liver is the primary site of metastasis for some of the most common human malignancies, including the entire gastrointestinal tract, the lung and the breast and metastases are frequently untreatable and associated with poor prognosis (reviewed by Underwood, 2000; e.g. Miyagawa *et al.*, 2002; Wu *et al.*, 2002). For many of these malignancies, metastatic tumour cells follow a direct route via the hepatic portal vein and establish themselves in the first target organ encountered and often metastases are well defined and form solid tumours. In contrast, for uveal melanoma, an explanation for the hepatic nature of metastases still remains unclear and micro-metastases are commonly dispersed throughout the organ, remaining undetected for many years.

The human liver is primarily composed of hepatocytes, arranged into structures known as lobules; the sub-structure of which allows maximum contact of hepatocytes with blood flowing through the liver (reviewed by Underwood, 2000). In cross section, lobules are roughly hexagonal, with larger branches of the hepatic portal vein and hepatic artery, running along the angles of the lobular margins. From these larger vessels, blood is transported towards a central vein of each lobule via sinusoids, and thus, due to the thickness of each hepatocyte plate, each hepatocyte is bathed by blood on at least two sides. Each sinusoid is lined by cells analogous to endothelial cells, and as such creates the initial barrier to which tumour cells must interact prior to intravasation (reviewed by Underwood, 2000). Phagocytic Kupffer cells also line the sinusoids, performing a macrophage-like function, removing depleted erythrocytes and other debris from the circulation (reviewed by Underwood, 2000). There is much evidence to suggest that Kupffer cells are responsible for the secretion of many local cytokines, which may subsequently be required for the metastatic process. For example, in response to stimulation, hepatocytes and Kupffer cells of the liver have been also

shown to produce a number of factors, including IL-8 and TNF-α (Mawet *et al.*, 1996; Rowell *et al.*, 1997). Stellate cells are also present close to the sinusoids and hepatic venules, and are involved in collagen synthesis aiding hepatic fibrinolysis (reviewed by Underwood, 2000).

The initial site of tumour cell arrest is predominantly the liver sinusoids (Vidal-Vanaclocha *et al.*, 2000). As described previously, pre-treatment of mice with IL-1α has been shown to affect the site of B16F1 melanoma cell arrest at the sinusoidal endothelium, promoting adhesion to the pre-capillary vessels (Scherbarth and Orr, 1997). More recent evidence has suggested that arrested cells suffer extreme deformations, suggesting that tumour cells do not roll on the endothelium and instead arrest in the capillary bed (Paku *et al.*, 2001). Due to the intricate nature of the hepatic capillary bed, this would seem a feasible explanation (Ding *et al.*, 2001). Whichever mechanism is implicated, extravasation involves tumour cell interaction with the sinusoidal endothelium, the subendothelial basement membrane and hepatocytes, before proliferating to form secondary colonies. There is still nevertheless evidence to suggest that the specificity of spread seen in many cancers cannot be explained purely by mechanical arrest and may depend upon the tumour type. For example, a component of the sinusoidal basement membrane, heparan sulphate proteoglycan has been shown to play a critical role in liver colonisation of lung carcinoma cells (Tovari *et al.*, 1997). In a more recent study by this group, as detailed earlier (Section A, 1.3.1) cells preferentially metastasising to the liver and lung, were shown to migrate to these organs in a shorter period of time, when compared with other organs less commonly colonised (Paku *et al.*, 2001). The mechanism utilised by these cells to extravasate in these organs, involved tumour cells becoming covered by the endothelium prior to penetration of the basement membrane, differing from extravasation at other sites. Due to an ill-defined basement membrane in the liver between the microvilli of the hepatocytes, tumour cells were further observed to 'sink' into the hepatic bed leading to a disappearance of the hepatic microvilli.

Further insight into the factors involved in the targeting of uveal melanomas to the liver may therefore be of vital importance for elucidating potential sites for therapeutic targets, with an ultimate aim of preventing these tumours from establishing secondary disease.

C. Study background and aims of the investigation

1.1. Background

The use of primary short-term cultures

Previous reports on uveal melanoma have commonly used established cell lines that may differ both genotypcially and phenotypically from the original tumour. Ultimately, the use of primary cultures should nevertheless provide a closer representation of responses *in vivo*. This present study was to be carried out in conjunction with the Academic Unit of Ophthalmology and Orthoptics and the Institute for Cancer Studies. Using tumour samples collected directly from theatre, this laboratory provided the expertise for culturing solid uveal melanoma tumour samples. By culturing solid tumours as short-term cultures, effects of long-term culturing would hopefully be avoided, thus more closely resembling properties of the tumour *in vivo*. As no two tumours behave in an identical manner, regarding individual tumours, it may also be possible to further correlate the behaviour *in vitro* with known histo-pathological data. As cells were to be cultured directly from solid tumours, cell numbers could therefore be a limiting factor, together with the uncertainty of establishment in culture. More established uveal melanoma cell lines were available for study also, when high cell numbers were required, allowing experimental repetition, and as such could be used to develop methods in which short-term cultures could be assessed later. Although uveal melanoma samples typically contain a low number of infiltrating cells (de Waard-Siebinga *et al.*, 1995), it would nevertheless still be necessary to ascertain that melanoma cells are actually the cultured cell type selected for.

In vitro models of tumour cell migration and invasion

Most studies of uveal melanoma invasion have utilised animal models (e.g. Patz *et al.*, 1959; Bradl *et al.*, 1991; Grossniklaus *et al.*, 1995), and as yet, few *in vitro* assays have been reported. *In vitro* studies of cell migration and invasion of other tumour types have commonly employed methods, such as the Boyden chamber and Transwells, with upper and lower compartments separated by a porous membrane, often coated with an ECM substrate or a reconstituted basement membrane (Falk *et al.* 1980; Albini *et al.*, 1987). Alternatively, other methods of studying invasion have involved gel invasion assays in which cells attach to and invade into three-dimensional matrices over a longer period of time (Andresen *et al.*, 2000) whilst for the study of cutaneous melanoma, the use of a

sophisticated three-dimensional reconstructed skin model has also recently been reported (Eves *et al.*, 2000). Many of these studies have nevertheless, involved the use of established cell lines. Previous research by this laboratory, has investigated invasion of uveal melanoma short-term cultures through a basic invasion assay *in vitro*, with the inclusion of a minimal fibronectin barrier alone (Elshaw *et al.*, 2001). However as yet, the study of primary uveal melanoma cell invasion in a more realistic system has not been studied.

In vitro models for studying tumour cell adhesion to the endothelium

Tumour cell adhesion to the endothelium is a vital step during metastasis. *In vitro* assays to study these processes have commonly involved adhering tumour cells to a confluent layer of endothelial cells, allowing further investigation into the molecules that facilitate and effect these interactions (Teti *et al.*, 1997; Scott *et al.*, 2001). There have been few reports as yet, investigating the adhesive interactions between uveal melanoma cells and the endothelium. As uveal melanomas primarily target the liver, there is a possibility that site-specific determinants on the liver endothelium, may assist in this process.

1.2. Aims of the investigation

Limited knowledge exists regarding the mechanisms by which uveal melanomas metastasise to the liver. This tumour might potentially follow the theory underlying Paget's 'seed and soil' hypothesis suggesting that metastatic uveal melanoma cells express a distinct genotypic and phenotypic profile that facilitates arrest and subsequent growth in the liver. Using short-term uveal melanoma cultures, the overall aims of this study were to investigate differences between cells with varying invasive abilities *in vitro* and further correlate this information with known histo-pathological data. This may provide further information defining the aggressive nature of some uveal melanomas. As little is known relating to uveal melanoma invasion and metastasis to the liver, the specific aims of this study are described below.

- As previous studies have commonly used cell lines, differences between *in vitro* and *in vivo* expression of adhesion molecules, degradative enzymes and their respective inhibitors by uveal melanomas, potentially involved in metastasis was assessed.

- Past studies have employed basic *in vitro* invasion molecules to study uveal melanoma invasion, and consequently a potentially more representative *in vitro* model of transendothelial uveal melanoma cell invasion was developed.
- As uveal melanomas primarily target the liver, the effects of hepatic-derived cells and secreted factors on uveal melanoma invasion were investigated.
- As organ-specific adhesion molecules may be involved in site-specific metastasis, potential differences in adhesion of uveal melanoma cells to endothelial cells derived from hepatic and dermal sites were studied.

Chapter 2

Materials and Methods

A. Materials

Details of the addresses of all suppliers are listed in Appendix I.

2.1. General laboratory equipment and reagents

Water

All solutions were prepared using water purified by a Purite Select Analyst HP system and the pH was measured using a Jenway 3020 pH meter.

Sterilisation of solutions and equipment

Where required, solutions and equipment were autoclaved prior to use at 20lb/sq inch for 20 minutes (Boxer Laboratory Equipment). Autoclave tape was obtained from Rexam Medical Packaging.

Centrifugation

With the exception of endothelial cell, unless otherwise stated, cells for tissue culture procedures were pelleted by centrifugation at 200g (1000rpm) at 25°C (Heraeus Sepatech Megafuge 1.0 R centifuge) for 5 minutes. Endothelial cells were pelleted at 228g (1100rpm) at 25°C for 5 minutes.

Safety cabinet

Standard aseptic technique was applied to all tissue culture procedures in a laminar flow class II microbiological safety cabinet (B.S.5726) (Walker Safety Cabinets Ltd.). Fifteen minutes before use, the cabinet was switched on and swabbed with 1% (v/v) Virkon (50% (v/v) potassium peroxoxmonosulphate, 5% (v/v) sulphamic acid, 15% (v/v) sodium alkyl benzene sulphonate) (Antec Interantional Ltd.) and Azowipes (70% (v/v) isopropyl alcohol) (Vernon carus). All equipment and reagent containers were cleansed in a similar way prior to use. Where possible, work with different cell lines was kept separate and the cabinet sanitised between different cell lines.

FACSort

Samples were analysed using a Beckon Dickenson FACSort Vantage flow cytometer, using logarithmic scale for fluorescence intensity, with technical assistance from Mrs Olivia Smith. The data were collected and analysed using Cell Quest software (Beckon Dickinson).

Incubation

All cell cultures were maintained at 37°C, 100% humidity and 5% carbon dioxide at ambient oxygen concentrations (Sanyo CO_2, Heto-Holten Cellhouse 170, water jacket CO_2 incubators).

Plate reader

Plates were analysed using a Wellfluor microplate fluorescence reader (Denley). The data were collected and analysed using Wellfluor Data Capture software (Denley).

Microscopes

Cell confluency was monitored on a daily basis using an inverted phase contrast microscope (Olympus CK2). For fluorescence studies, cells were visualised using an Olympus BX50 and an inverted Leica DM IRB fluorescent microscope. For scanning electron microscopy, a Phillips SEM501 scanning electron microscope was used, with the assistance of Mr Ian Palmer (Department of Pathology, Royal Hallamshire Hospital, Sheffield, UK).

Liquid Nitrogen

BOC gases supplied liquid nitrogen.

Ice machine

Ice was prepared using an ice machine from Scotsman Ice Systems Ltd.

Water bath

For maintaining set temperatures, a Grant water bath was used.

Glassware and non-disposable equipment

Pyrex glassware was used and cleaned by washing in a dishwasher, rinsing in distilled water and air-drying. Volumes of less than and including 1ml were transferred using Gilson pipettes (Anachem Ltd.) with appropriate tips (Sarstedt). For volumes above this, a Pipetboy-ACU (Scientific Laboratory Supplies) electronic pipette pump with gamma irradiated disposable plastic pipettes was used. For disposal of media, glass Pasteur pipettes (150mm or 230mm) (SLS) were used with a standard aspirator (Genetic Research Instrumentation). Glass microscopes slides (25x75mm) and coverslips (22x50mm and 13mm diameter) were obtained from BDH. Cell density was estimated by counting using an Improved Neubauer haemocytometer slide (Merck/BDH).

Plastic and disposable laboratory equipment

All tissue culture flasks (25cm^2 and 75cm^2) were obtained from Nalge NUNC International. Nalgene supplied cryovials (1.2ml) and media filters. Bibby Sterilin supplied plastic centrifuge tubes (13ml), petri dishes (60x15 mm and 90x15 mm), universal tubes (20ml) and pipettes (10ml and 25ml). 50ml centrifuge tubes, FACS analysis tubes (12mm), 24-well and 96-well cell culture cluster plates were obtained from Corning-Costar. Syringes and microlance needles were acquired from Beckton

Dickenson. Plastic Pasteur pipettes (1ml) were from SLS, plastic pipette tips and eppendorf tubes (1ml and 2ml) were from Starstedt, and stainless steal surgical blades were from Swann Morton.

Reagents

General chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich or Merck/BDH unless otherwise stated, and stored according to manufacturer's instructions. All quantities were weighed using a Sartorius Basic electronic balance.

2.2. Biological samples

Details of all tumours and cell lines used in this study are summarised in Appendices II and III.

2.2.1. Ocular melanoma samples and cells

Fresh posterior uveal melanoma samples were collected from surgery at the Royal Hallamshire Hospital (Sheffield, UK) after enucleation. Ethical approval was obtained prior to study and informed patient consent was acquired before collection of tumour samples. Removal was in collaboration with an ophthalmic pathologist. For tissue culture, samples were placed into sterile PBS. For immunohistochemistry tumour samples were either placed in 10% buffered formalin and subsequently processed using a Citadel 2000 Processor (Shandon), or were snap frozen in liquid OCT in iso-pentane, and stored at -70°C or in a liquid nitrogen vessel. For classification purposes, tumours are identified numerically, and are known as Sheffield Ocular Melanomas (SOM). All histo-pathological data can be found in Appendix II.

2.2.2. Endothelial cells

HDMECAs

Normal human dermal microvascular endothelial cells (adult) (HDMECA) (TCS CellWorks Ltd.) were used as representative endothelial cells for these studies in most experiments. These cells are derived from human adult dermis and have a finite useful lifespan of approximately 15 population doublings. Generally cells were used up to passage ten.

HULECs

Human liver endothelial cells (HULECs) were a gift from Dr Lance Burns (Department of Surgery and Anaesthesia, Royal Hallamshire Hospital, Sheffield, UK). Cells were extracted from liver resections

for colonic metastases. These cells grow as a monolayer and attain the characteristic cobblestone morphology of endothelial cells (Burns *et al.*, 1999). Cells were used within ten passages.

2.2.3. HepG2 cells

HepG2 cells (a human hepatoma cell line) were used to represent hepatocytes in these studies and were obtained from the European Collection of Animal Cell Cultures (ECACC) (catalogue number 85011430). Cells were derived from a 15-year-old male Caucasian, are of epithelial morphology and produce a wide range of proteins, including factor VIII, prothrombin and antithrombin III. Cells are viable for up to 90 passages.

2.2.4. A375 cells

A375 cells (a cutaneous melanoma cell line) were used when a comparison with cutaneous melanoma was required and were obtained from the European Collection of Animal Cell Cultures (ECACC) (catalogue number 88113005). The cells were derived from a 54-year-old female with malignant melanoma, and are of epithelial morphology.

2.2.5. Other cell types

Alternative cell types used in these studies included A549, JFF and NAK 3.2 and were used in certain experiments as controls.

A549

A549 cells (a human lung carcinoma cell line) were obtained from the European Collection of Animal Cell Cultures (ECACC) (catalogue number 86012804) and are derived from a 58-year-old Caucasian male.

JFF

JFF cells are primary human dermal fibroblasts and were a gift from Dr D Donovan (Department of Pathology, Royal Hallamshire Hospital, Sheffield, UK).

NAK 3.2

NAK 3.2 cells are primary human dermal keratinocytes and were a gift from Dr S St-John Smith (Department of Dermatology, Royal Hallamshire Hospital, Sheffield, UK).

2.3. Tissue culture reagents and solutions

All tissue culture reagents were purchased from Sigma-Aldrich, unless otherwise stated and stored according to manufacturer's instructions. Where required, solutions were autoclaved, as previously described.

2.3.1. General reagents and solutions

Phosphate-buffered saline (PBS) and phosphate-buffered saline EDTA (PBSe)

PBS was prepared by dissolving one tablet (Oxoid Ltd.) in 100ml distilled water and was autoclaved prior to use. For PBSe 0.2g of ethylenediaminetetra-acetic acid (EDTA) was added per litre of PBS.

Trypsin

Trypsin was prepared at 1:20 2.5% trypsin in 550μM EDTA in sterile PBS and stored at 2-8°C. All cells were dissociated from tissue culture flasks using trypsin unless otherwise stated.

Cell dissociation solution

For adhesion and FACS assays, cell dissociation solution was used according to the manufacturer's instructions.

Di-methyl sulphoxide (DMSO)

For freezing cells, DMSO at 10% (v/v) sterile dimethyl sulphoxide (DMSO) was prepared and used immediately.

Trypan blue

Cell viability was monitored using sterile trypan blue, prepared at 0.5% w/v in PBS. Viable cells were then assessed using a haemocytometer.

Bovine serum albumen (BSA)

For preparation of serum free media and buffers for flow cytometry, BSA was added at a concentration of 0.1% and 0.5%, respectively.

Foetal bovine serum (FBS)

FBS was obtained from Helena Biosciences, and used at the concentrations noted. Prior to use, FBS was stored at -20°C.

Gill's haematoxylin

Gill's haematoxylin was prepared by mixing distilled water (750ml) with ethylene glycol (250ml), haematoxylin (2g), and sodium iodide (0.2g). After mixing well, glacial acetic acid (20ml) was added and the solution was stirred for 1 hour at room temperature.

2.3.2. Growth media

Ocular melanoma Growth Media

All ocular melanoma cultures were maintained in Roswell Park Memorial Institute (RPMI) 1640 growth medium (Invitrogen) supplemented with 20% (v/v) FBS, penicillin (100U/ml), streptomycin (100μg/ml), fungizone (5μg/ml), glucose solution (0.2%) and epidermal growth factor (EGF) (0.2μg/ml).

Endothelial cell Growth Media

Endothelial cells were grown in microvascular endothelial cell growth media containing growth supplement (heparin, hydrocortisone, human epidermal growth factor, human fibroblast growth factor, dibutyryl cyclic AMP and 5% (v/v) FBS) and antibiotic supplement (gentamicin (50μg/ml), amphotericin (50μg/ml)) (TCS CellWorks Ltd.). Prior to seeding cells, tissue culture flasks were coated with 3ml of attachment factor (TCS CellWorks Ltd.).

HepG2 Growth Media

HepG2 cells were maintained in RPMI 1640 containing 10% (v/v) FBS, penicillin (100U/ml), streptomycin (100μg/ml), fungizone (5μg/ml), glucose solution (0.2%).

A375 Growth Media

A375 cells were grown in Dulbecco's minimal medium containing 10% (v/v) FBS, penicillin (100U/ml), streptomycin (100μg/ml), fungizone (5μg/ml).

A549 and JFF Growth Media

A549 and JFF cells were grown in Dulbecco's minimal medium (Invitrogen) containing 10% (v/v) FBS, penicillin (100U/ml), streptomycin (100μg/ml), fungizone (5μg/ml).

NAK 3.2 Growth Media

NAK 3.2 cells were maintained in Keratinocyte serum free medium (Invitrogen), supplemented with EGF and bovine pituitary extract (25mg) (Invitrogen).

2.4. Reagents, solutions and equipment used for visualising cells

2.4.1. Immunohistochemistry

APES coating of slides

Glass slides were coated with 3% (v/v) 3-aminopropyl-triethoxysilane (APES) solution in acetone (APES) to aid adherence of sections to the slides.

Cutting frozen tissue

Frozen sections were cut using a Kryostat 1720 digital cryostat (Leitz) onto APES-coated glass slides.

Preparation of and cutting paraffin sections

Tumour samples from surgery were immediately fixed in formalin, processed and further embedded in liquid paraffin wax (Merck/BDH). Sections of paraffin-embedded tissue were prepared using a Reichert-Jung 2030 microtome.

Staining cells

Prior to staining, prepared sections or areas of cells grown on slides were circled using a wax pen (Dako Corporation). For all protocols, a standard three-stage immunoperoxidase technique (Vectastain Elite ABC kit) (Vector Laboratories Ltd.) was used. Details and dilutions of the primary antibodies used in these studies are listed in Table 2.4.1.a. The stain was further visualised with a peroxidase substrate solution; 3-amino-9-ethyl carbazole (AEC) (Vector Laboratories Ltd., Peterborough, UK), prepared according to the manufacturer's instructions. When desired, cells were counterstained with Gill's haematoxylin, and specimens were then cover-slipped using DAKO aqueous mounting medium (DAKO Corporation, CA, USA).

Antigen	Antibody code	Nature of antibody	IgG concentration	Working dilution			Supplier
				CC	FS	PS	
α1	MCA1133	Mouse monoclonal	20μg/ml	1:50	1:50	N/A	Serotec
α2	NCL-CD49b	Mouse monoclonal	Not given	1:100	1:100	N/A	Novocastra
α3	NCL-CD49c	Mouse monoclonal	Not given	1:100	1:100	N/A	Novocastra
α4	P4G9	Mouse monoclonal	81μg/ml	1:100	1:100	N/A	Dako Corporation
α5	P1D6	Mouse monoclonal	21μg/ml	1:50	1:50	N/A	Dako Corporation

α6	NCL-CD49f	Mouse monoclonal	Not given	1:100	1:100	N/A	Novocastra
αvβ3	MCA757G	Mouse monoclonal	1mg/ml	1:150	1:150	N/A	Serotec
β1	MCA532	Mouse monoclonal	1mg/ml	1:100	1:100	N/A	Serotec
MMP-2	42-5D11	Mouse monoclonal	2mg/ml	1:500	1:50	1:1000	Chemicon International Inc.
MMP-9	56-2A4	Mouse monoclonal	3mg/ml	1:2000	1:100	1:500	Chemicon International Inc.
TIMP-2	67-4H11	Mouse monoclonal	2mg/ml	1:2000	1:150	1:1000	Chemicon International Inc.
u-PA	N/A	Goat polyclonal	Not given	1:200	1:200	1:1000	Chemicon International Inc.
PAI-1	N/A	Sheep polyclonal	Not given	1:500	1:500	1:500	Chemicon International Inc.
PAI-2	N/A	Sheep polyclonal	Not given	$1:10^4$	$1:10^3$	1:1000	Chemicon International Inc.
E-selectin	P2H3	Mouse monoclonal	1mg/ml	1:50	N/A	N/A	Chemicon International Inc.
VCAM-1	P3C4	Mouse monoclonal	1mg/ml	1:50	N/A	N/A	Chemicon International Inc.
ICAM-1	P2A4	Mouse monoclonal	1mg/ml	1:250	N/A	N/A	Chemicon International Inc.
S-100	NCL-S100	Mouse monoclonal	Not given	1:30	N/A	N/A	Novocastra
Melan-A	NCL-MelanA	Mouse monoclonal	Not given	1:25	N/A	N/A	Novocastra
HMB45	NCL-HMB45	Mouse monoclonal	Not given	1:45	N/A	N/A	Novocastra
Mouse IgG_1 negative control	N/A	Mouse monoclonal	Not given	As appropriate	As appropriate	As appropriate	Dako Corporation

Table 2.4.1.a. Details of primary antibodies used in immunohistochemical studies. N/A; not applicable, CC; cultured cells, FS; frozen sections, PS; paraffin sections.

2.4.2. Flow cytometry

FACS buffer was prepared using PBS with 0.5% BSA and refrigerated until required. Details and dilutions of the primary and secondary FITC-labelled antibodies used in these studies are listed in Table 2.4.2.a.

Antigen	Antibody code	Nature of antibody	IgG concentration	Working dilution	Supplier
α1	MAB1973	Mouse monoclonal	1mg/ml	1:100	Chemicon International Inc., CA, USA
α2	MAB1998	Mouse monoclonal	1mg/ml	1:100	Chemicon International Inc., CA, USA
α3	MAB2056	Mouse monoclonal	1mg/ml	1:100	Chemicon International Inc., CA, USA
α4	MAB1698 3	Mouse monoclonal	1mg/ml	1:50	Chemicon International Inc., CA, USA
α5	MAB1986	Mouse monoclonal	0.2mg/ml	1:20	Chemicon International Inc., CA, USA
α6	MAB1378	Rat monoclonal	1mg/ml	1:100	Chemicon International Inc., CA, USA
β4	MAB2058	Rat monoclonal	1mg/ml	1:100	Chemicon International Inc., CA, USA
E-selectin	P2H3	Mouse monoclonal	1mg/ml	1:50	Chemicon International Inc., CA, USA
VCAM-1	P3C4	Mouse monoclonal	1mg/ml	1:50	Chemicon International Inc., CA, USA
ICAM-1	P2A4	Mouse monoclonal	1mg/ml	1:100	Chemicon International Inc., CA, USA
FITC-labelled goat anti-mouse IgG	AP124F	N/A	N/A	1: 100	Chemicon International Inc., CA, USA
FITC-labelled mouse	AP136F	N/A	N/A	1: 100	Chemicon International Inc., CA, USA

anti-rat IgG					
Mouse IgG_1 negative control	N/A	Mouse monoclonal	N/A	As appropriate	Dako Corporation, CA, USA

Table 2.4.2.a. Details and optimum antibody working dilutions of primary antibodies used FACS analysis. N/A; not applicable.

2.4.3. Labelling cells with succinimidyl esters

For cellular detection and tracking, cells were labelled with succinimidyl esters (Molecular Probes, Inc.) prior to use. In all cases, tumour cells were labelled with carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE) and endothelial cells were labelled with SNARF-1 carboxylic acid, acetate, succinimidyl ester (SNARF-1). For FACS analysis and invasion assays, endothelial cells were labelled at 2μM and 20μM, respectively. For adhesion assays, tumour cells were labelled at 5μM. These reagents passively diffuse into the cells and are colourless and non-fluorescent until their acetate groups are cleaved by intra-cellular esterases to yield highly fluorescent compounds. After reacting with intracellular amines, the fluorescent conjugates become stable in the cell and can be fixed with aldehyde fixatives. CFDA-SE emits light at a 492-517nm and SNARF-1 emits light at 580-640nm.

2.4.4. Scanning electron microscopy

Mr Ian Palmer carried out all processing for electron microscopy. All reagents and consumables used can be obtained from TAAB Laboratories Equipment Limited. For critical point drying, a Polaron 300 critical point dryer was used, and an Edwards Sputter coater was used for sputter coating.

2.5. Reagents, solutions and equipment used in Invasion Assays

To study invasion, Transwell inserts (6.5mm diameter) (Corning/Costar) were used. The base of the insert is a polycarbonate membrane, with 8μM pores, unless otherwise noted. Each insert was coated with an artificial basement membrane of Matrigel (1.5mg/ml) (Beckton Dickenson), unless

otherwise stated. Matrigel was made up according to manufacturer's instructions in serum free media (RPMI 1640). For optimisation of the transendothelial cell invasion assay, an extra cellular matrix (ECM) solution was also used, made up with equal volumes of fibronectin (15μg/ml), collagen type IV (15μg/ml) and laminin (15μg/ml), diluted in sterile PBS. Invasion through alternative pore sizes of 3μM and 5μM was assessed. For removal of non-invaded cells, disposable cotton buds were obtained from Boots Company PLC. Other equipment, solutions and reagents required for the invasion assays have been previously described (2.1-2.4).

2.6. Reagents, solutions and equipment used in Adhesion Assays

Hoescht (33342) for used for counterstaining cells in the adhesion assays. Plates were analysed using a Wellfluor microplate fluorescence reader (Denley). The data were collected and analysed using Wellfluor Data Capture software (Denley). Other equipment, solutions and reagents required for the adhesion assays have been previously described (2.1-2.4).

B. Methods

2.1. General cell culture

Passaging of cell lines and short term cultures (STCs)

Cell confluency was monitored on a daily basis and cultures were passaged towards the end of the log phase of cell growth (between 60-80% confluency). Cells were grown in either 25cm^2 or 75cm^2 tissue culture flasks and were seeded in volumes of 5 or 10ml of culture medium, respectively. All cell lines and STCs used grew in an anchorage dependent manner.

Sub-culturing

Growth medium was aspirated from the tissue culture flask using sterile glass Pasteur pipettes and a standard aspirator. Attached monolayers were washed in 2ml of PBS. 2ml of trypsin-EDTA was added to the flask and the flask incubated at 37°C for 3 minutes. The cells were resuspended by gentle flushing in 10ml of culture medium containing FBS to neutralise enzyme activity. Cells were transferred to a clean 13ml centrifuge tube and pelleted by centrifugation. The supernatant was discarded and the pellet resuspended in pre-equilibrated culture medium before transferring to fresh flasks at an appropriate dilution. Prior to seeding endothelial cells, culture flasks were coated with 3ml of attachment factor and incubated for 30 minutes at 37°C. Residual attachment factor was discarded before flasks were used. Cultures were monitored regularly and the media replaced with fresh pre-equilibrated media every 3 days, to retain cell viability.

Preparation of frozen cell cultures

Frozen stocks of all cell lines and STCs were prepared from sub-confluent cell cultures where cells were in the log phase of growth. A suspension of cells was prepared and pelleted by centrifugation. Cells were resuspended in the appropriate growth medium supplemented with 10% (v/v) sterile DMSO. 1ml aliquots of this cell suspension were rapidly transferred to 1.2ml cryovials and frozen, in a polystyrene rack, surrounded in cotton wool, at -70°C for 24 hours, before transfer to a liquid nitrogen vessel for long term storage.

Revival of frozen cell cultures

Cryovials were removed from liquid nitrogen and thawed rapidly in a water bath at 37°C. Cell suspensions were transferred to a 13ml centrifuge tube, diluted in 10ml of the appropriate medium and pelleted by centrifugation. The supernatant was discarded and the pellet resuspended in 10ml of the appropriate medium. Cells were allowed to recover and plate down for 24 hours, before the

medium was removed and replaced with fresh medium. Cells were then cultured and passaged as described before. Cells were passaged at least twice before use in assays, to allow full recovery.

Estimation of cell number and cell viability by trypan blue exclusion

Cell density for seeding fresh flasks was estimated by counting using a haemocytometer slide. Where appropriate, cell viability was tested using trypan blue exclusion. 50μl of dilute cell suspension was added to 50μl of trypan blue mixed and left to stand for 2 minutes. 10μl of this suspension was then added to a clean counting chamber and examined under a phase contrast inverted microscope. Four grids of sixteen squares were counted containing a volume of $1mm^2$ each and cell number was counted using the following equation:

$$\text{Number of cells/ml} = \frac{\text{cell count x 10,000}}{4} \times \text{dilution factor}$$

Preparation of STCs

Fresh posterior uveal melanoma samples were collected in surgery and placed into sterile PBS in a 25ml Universal container. Each sample was chopped finely with a scalpel in a plastic petri dish (60 x 15 mm), washed in PBS and transferred to a clean 13ml centrifuge tube using a glass Pasteur pipette. Cells were pelleted by centrifugation at 200g (1000 rpm) for 10 minutes, the supernatant was discarded and the cells resuspended in appropriate growth medium for ocular melanomas before transferring to $25cm^2$ tissue culture flasks. Cultures were carefully monitored daily and the media changed every 3-5 days, with fresh pre-equilibrated media. Where possible, STCs were used for studies between passages 1 and 6. Once STCs appeared to be growing in culture indefinitely, they were considered to be more stable cell lines. To establish techniques and for standardisation of assays, an invasive (SOM 196B) and a non-invasive uveal melanoma cell line (SOM 157d [Shaif-Muthana *et al.*, 2000]) were used. Both cell lines were originally established in this laboratory, and are of mixed cell morphology. SOM 196B was used between passages 25 and 33 and SOM 157d was used between passages 95 and 105 in all assays.

Characterisation of primary melanoma cultures

To confirm that STCs were melanoma in origin, cells were grown on sterile glass slides, and immunohistochemistry was used to investigate the expression of the melanoma markers S-100, HMB45 and Melan-A (2.1.1).

2.2. Visualisation of cells

2.2.1. Immunohistochemistry

Growing cells on slides

To study the expression of specific proteins by cells grown in culture, cultured cells were grown on sterile glass slides (25x75mm) for 24 hours. Once cells had reached confluency in a 75cm^2 tissue culture flask, a cell suspension was prepared and cells were pelleted by centrifugation. Cells were then resuspended in appropriate growth medium. Glass slides were placed in a clean petri dish (90x15mm) using sterile forceps and 1ml of cell suspension was pipetted onto each slide. Cells were allowed to plate down (for approximately 1 hour) before 10ml of appropriate growth medium was added to the petri dish to cover the glass slides. Slides were incubated for 24 hours. Prior to fixation, slides were washed in PBS and allowed to air dry. Cells were fixed in 100% acetone for 10 minutes before transferring to a –20°C freezer for storage. For immunohistochemial studies, slides were removed from storage 16 hours before use.

Preparation of glass slides for use with frozen and paraffin sections

Glass slides were coated with APES solution to aid adherence of sections to the slides. Prior to coating, slides were washed thoroughly in detergent and distilled water before air-drying. Slides were then coated in APES solution for 30 seconds before being rinsed in acetone and distilled water twice and allowed air-drying.

Preparation of frozen tissue

Fresh tumour samples were collected in surgery at the Royal Hallamshire Hospital, after enucleation and immediately placed into the liquid OCT compound and snap frozen in iso-pentane, covered in further OCT compound and stored at -70°C or in a liquid nitrogen vessel.

Cutting sections from frozen tissue

8μM sections of frozen tissue were mounted on APES-coated glass slides and allowed to thoroughly dry-out overnight at room temperature prior to fixation. To fix sections, slides were placed in 100% acetone for 10 minutes. Slides were stored at –20°C until required. Sections were removed from storage for 10 minutes before use.

Processing tissue to paraffin wax

Tumour samples from surgery were immediately fixed in formalin prior to processing. Samples were dehydrated through up-graded ethyl alcohols and then cleared in xylene before being embedded in liquid paraffin wax.

Cutting paraffin-embedded sections

5μM sections were cut and mounted onto APES-coated glass slides to help adherence of sections, racked and dried overnight in an oven at 60°C. When required, sections were de-waxed through two changes of xylene at 5 minute intervals, and re-hydrated through a series of graded alcohol solutions. Initially this involved clearing the sections of xylene by immersing in 100% ethanol for 5 minutes. This was followed by 5 minutes in a trough of fresh 100% ethanol, and finally partially re-hydrated in 70% ethanol for 5 minutes before. Sections were then blocked for endogenous peroxidase activity, by immersing sections in 3% hydrogen peroxide solution in methanol for 20 minutes. Sections were next rinsed in tap water before antigen retrieval methodology was used. A trough containing 200ml of 0.1% (w/v) calcium chloride was allowed to equilibrate to 37°C in an incubator, before dissolving trypsin in the solution to prepare a 0.1% (w/v) solution. Slides were transferred to the trough of enzyme solution and maintained at 37°C before rinsing in water at room temperature, to inhibit enzyme action by diluting the enzyme and lowering the temperature. When high levels of melanin were present in the tumour, sections were bleached which involved submerging sections in 0.25% (v/v) potassium permanganate solution for 2 hours. Sections were then washed in 1% (v/v) oxalic acid for 1 minute and rinsed in PBS.

Immunohistochemical staining protocols

Prior to staining, prepared sections or areas of cells grown on slides were circled using a wax pen to retain solutions on the sections or cells during the procedures. All antibody and enzyme incubations were performed in a humidity chamber at room temperature, and the solutions were diluted in PBS. For all protocols, a standard three-stage immunoperoxidase technique was used. The slides were initially rinsed in PBS before incubating with 1.5% normal (blocking) horse or rabbit serum (dependent on the origin of the primary antibody) for 30 minutes to prevent non-specific binding. Normal serum was then removed from the slides, and the primary antibody applied by pipette, at an appropriate dilution. Slides were incubated with the primary antibodies for 2 hours at room temperature. The primary antibodies were removed from the slides using a pipette before washing slides in PBS for 5 minutes. A 0.5% biotinylated secondary link antibody was next applied (diluted in

PBS with 1.5% normal serum), prepared from the same species in which the preferred serum for blocking is made, and slides were incubated for 30 minutes. Slides were rinsed thoroughly in PBS for 5 minutes, before incubating with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex for 30 minutes. At the end of this period, slides were washed in buffer and finally the stain was visualised with AEC, prepared to the manufacture's instructions. After incubating with AEC for 30 minutes, a red reaction product developed. Excess AEC was rinsed from slides using distilled water. At this point, a counter-stain of Gill's haematoxylin was applied if desired, and then 'blued' in tap water. Sections or cells were then cover-slipped with aqueous mounting medium. IgG isotype control antibodies were included in each experiment. For use as positive controls and titration experiments, antibodies were screened on alternative cell types, to check for specific binding. For assessment of integrin antibodies, A375 cells, keratinocytes and fibroblasts were chosen. Breast cancer cells (MCF-7) and sections were used for antibodies against degradative enzymes and inhibitors, whilst A375 cells were used for melanoma marker antibodies.

Analysis

Two independent observers scored the intensity of positive staining semi-quantitatively, as described by McCarty *et al.*, 1985 and Vora *et al.*, 1997. The percentage of cells staining was determined and staining was considered homogenous if 75% of cells stained and heterogeneous if the percentage of cells stained was between 5% and 75%. Staining intensity was scored as + weak, ++ medium, or +++ strong.

2.2.2. Indirect Immunofluorescence Staining of Melanoma and Endothelial Cells and Detection of Staining by Flow Cytometry (FACS assay)

To analyse expression of surface antigens by cultured cells, FACS analysis was also used.

Staining cells for FACS analysis

Cells were harvested using cell dissociation solution and resuspended at a concentration of $5x10^5$ cells/ml in appropriate growth media. When pelleting cells for FACS analysis, cells were centrifuged at 4°C. For each antibody to be tested, 200µl of cell suspension was pipetted into 13ml centrifuge tubes (100,000 cells) and 5ml of appropriate growth media was added to each tube. Cells were further pelleted, the supernatant was discarded and the cells were resuspended in the remaining volume of media. Cells were washed twice by adding 5ml ice-cold FACS buffer to each tube and pelleted. After the final centrifugation, the buffer was removed and the cells were resuspended in the

remaining volume. The primary antibody was diluted in the FACS buffer and 50μl of the diluted antibody was added to the suspended cells (Section A, Table 2.4.2.a). Cells were incubated on ice for 30 minutes. After this incubation period, 5ml buffer was added to each tube and the cells were pelleted, before washing twice in FACS buffer. After the final centrifugation, the buffer was removed and the cells were resuspended in the remaining volume. An appropriate FITC-labelled secondary antibody, dependent upon the primary antibody, was prepared on ice at a dilution of 1:100 with buffer and 100μl was added to each sample. Cells were incubated on ice for a further 30 minutes. After this time, cells were again washed twice and resuspended in 300μl FACS buffer. Samples were transferred to 12mm FACS tubes for flow cytometric analysis on the same day. In all cases, test samples were run against a control sample, labelling cells with an appropriate IgG_1 isotype control antibody. Results were expressed as the relative median fluorescent intensity (MFI), comparing test with control samples. Relative MFI values of greater than two were taken as positive, as expression levels were considered to have doubled. Experiments were repeated three times and a mean calculated.

Incubation of endothelial cells with TNF-α or IL-1β

To stimulate adhesion molecule expression on endothelial cells, cells were pre-incubated with either TNF-α (1ng/ml) or IL-1β (1ng/ml) for a period of 24 hours. After this period, cells were analysed for adhesion molecule expression by FACS analysis.

Generation of tumour cell conditioned media (CM) and its effect on endothelial cell surface antigen expression

To assess the effect of CM from tumour cells on endothelial cell adhesion molecule expression, the media was first aspirated from T75 flasks of semi-confluent tumour cells. Cells were washed with PBS and 10ml endothelial cell growth media was replaced in the flask. Cells were incubated for 24 hours. After this period, the media was again aspirated from flasks of endothelial cells, replaced with tumour cell CM and incubated for a further 24 hours. FACS analysis was then carried out to assess and changes in adhesion molecule expression.

Co-culturing endothelial and tumour cells for FACS analysis

The consequence of co-culturing tumour and endothelial cells was also investigated. Endothelial cells from confluent flasks (approximately $1.5x10^6$cells) were pre-labelled with SNARF-1 as described below (2.2.3). Cells were re-seeded in a T75 flask, pre-coated with attachment factor and allowed to plate down for 1 hour. Cells were then checked to ensure coverage of the flask. After this period,

$5x10^5$ tumour cells were washed in PBS, resuspended in endothelial cell growth media and added to the flask. Flasks were incubated for 24 hours. By incubating co-cultures with 3ml cell dissociation solution at 37°C, cell types were dissociated selectively from the flask. Tumour cells were incubated for 15 minutes with cell dissociation solution before removal and were placed on ice until required. Fresh cell dissociation solution was replaced and flasks were incubated for a further 45-60 minutes, to remove endothelial cells. FACS analysis was then performed to analyse any changes in adhesion molecule expression.

2.2.3. Labelling cells with succinimidyl esters

To label cells, cells were dissociated from their culture flasks and resuspended at a concentration of $5x10^5$ml in serum free media. Cells were pelleted and resuspended in sterile PBS containing the probe at the required concentration. Cells were incubated in a water bath at 37°C for 15 minutes. Once labelled, cells were pelleted and resuspended in serum-free media and incubated in a water bath for 30 minutes at 37°C to ensure complete modification of the probe. Cells were further washed in sterile PBS before resuspending in the required volume of serum-free media, supplemented with 0.1% BSA for each assay.

2.2.4. Scanning electron microscopy

Mr Ian Palmer (Department of Pathology, Royal Hallamshire Hospital) carried out all processing for electron microscopy. To assess transendothelial cell invasion, co-cultures of tumour and endothelial cells were set up as described below (2.3.2). Non-invaded cells were not removed before fixation, whilst membranes were similarly not removed from plastic inserts prior to fixation. For assessing adhesion of tumour cells to the endothelium, endothelial cells were grown to confluence ($5x10^5$/ml) for 24 hours on 13mm glass coverslips, pre-coated with attachment factor in the base of a 24-well plate, in endothelial cell growth media. Media was removed by aspiration; monolayers were rinsed twice with PBS before tumour cells ($2x10^5$/ml) were seeded onto the endothelial cell layer. Co-cultures were incubated for 24 hours.

Membranes and coverslips were washed in 0.1M sodium cacodylate buffer for 1 minute before fixing using 2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 30 minutes at

room temperature. After fixation, samples were washed in 0.1M sodium cacodylate buffer for 5 minutes before covering with 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 30 minutes at room temperature. Samples were further washed in 50% ethanol for 5 minutes. For 'en-bloc' staining, samples were covered with 2% uranyl acetate in 50% ethanol for 30 minutes before further rinsing in 50% ethanol for 5 minutes and dehydrating through graded ethanols.

For preparation of the membranes for scanning electron microscopy, samples were removed from the plastic insert with a scalpel. Membranes and coverslips were then transferred from 100% ethanol into 100% acetone through a graded series. Cells were critical point dried on cover slips using liquid carbon dioxide for 30 minutes. Samples were mounted on aluminium scanning electron microscopy stubs using double-sided carbon adhesive disks before sputter coating using a gold cathode for 1 minute at 50mA. Cells could then be observed under scanning electron microscopy.

2.3. Invasion assays

To assess the invasive capacities of cell lines and STCs, a modified Boyden Chamber system was employed.

2.3.1. Standard Invasion Assays

Coating Transwell inserts with Matrigel

Prior to use, Matrigel was diluted in RPMI 1640, to a concentration of 1.5mg/ml (according to manufacturer's instructions), and frozen at –20°C in 100μl aliquots. All manipulations were performed on ice and plastic ware chilled, to prevent Matrigel polymerisation. For each assay, a fresh aliquot of Matrigel was thawed. Each membrane was evenly coated with 10μl Matrigel and allowed to air dry for 30 minutes in a sterile environment. When alternative substrates have been used to coat membranes, these have been noted in the relevant chapter.

Preparation of cells for invasion assay

Cell lines or STCs were dissociated from their culture flasks and after centrifugation, were resuspended in 10ml PBS, to wash the cells thoroughly. After further centrifugation, cells were resuspended in an assay medium of RPMI 1640 medium, supplemented with 0.1% BSA at a concentration of $5x10^5$/ml. 200μl of cell suspension was pipetted into the upper chamber of the

Transwell insert ($1x10^5$/well) whilst 600µl of assay medium was pipetted into the lower chamber. Cells were incubated at 37°C for 24 hours, unless otherwise noted.

Removal of invaded cells, fixation and staining of membranes

After 24 hours, cells in suspension were removed from the upper chamber of the well. Non-invaded cells attached to the upper surface of the membrane were removed by gently scraping the membrane with a disposable cotton bud. Invaded cells on the under side of the membrane were fixed in 100% ethanol for 5 minutes. To stain the nuclei of the cells, membranes were placed in Gill's haematoxylin for 10 minutes. Membranes were finally 'blued' in tap water for 3 minutes. To mount membranes, a scalpel was used to carefully detach the membrane from the insert. Membranes were mounted and cover-slipped using aqueous mounting medium, ensuring that the invaded side was uppermost.

Counting invaded cells

Invaded cells were counted under phase contrast microscope at x400 magnification. For each membrane, ten random fields of view were counted. All experiments were set up in triplicate and a mean value was calculated. Where possible, experiments were also repeated three times and a mean value was again calculated.

Inclusion of cells and CM in the lower chamber of the invasion assay

To investigate the effects of different cell types on invasion, cells were grown to confluence ($5x10^5$ cells) in appropriate media in a 24-well plate and were then used in Transwell assays. Prior to the assay, media was replaced with RPMI 1640 supplemented with 0.1% BSA. Transwell filters pre-coated with Matrigel were placed in 24-well plates, thus including the different cell types in the lower chamber as potential source of chemoattractants. Tumour cells were dissociated from flasks and invasion assays were carried out as described above. Alternatively to assess the effect of CM on invasion, CM was collected after incubating each cell type to be assessed with RPMI with 0.1% BSA for 24 hours. By using CM and by growing the cells on the lower surface of the well of the Transwell chamber, it was possible to assess whether factors secreted by cells were still effective in the absence of the cellular source. CM was produced as previously described (2.2.2). CM was then stored at -20°C until required for further use. Prior to use, CM and was filter sterilised (0.2µM pore filter), and added to the lower chambers of test wells. Negative controls were included in which neither cells or CM was added to the lower chamber. Assays were incubated at 37°C for 24 hours and levels of invasion were assessed as previously described. Each experiment was performed in triplicate and where possible, was repeated three times and a mean calculated.

2.3.2. Transendothelial Cell Invasion

To study transendothelial cell invasion, the basic invasion assay method was used, with the addition of an endothelial cell monolayer on the membrane. HDMECAs were used for this purpose, unless otherwise noted. HDMECAs were stripped from their culture flasks as previously described and resuspended in microvascular endothelial cell growth media at a concentration of $5x10^5$/ml. 200μl of cell suspension was pipetted onto each membrane, previously coated with Matrigel ($1x10^5$/well). Cells were incubated for 24 hours at 37°C, to allow cells to plate down and form a monolayer across the membrane. Before tumour cells were added to the assay, the growth medium was removed from the upper chamber of the insert, and the cells were washed with PBS, to remove any traces of FBS. Tumour cells were added to the upper chamber at the concentration previously described. The assay was subsequently set up as described above and incubated for a period of 24 hours (Fig. 2.3.2.a). Each experiment included appropriate positive and negative controls set up in triplicate, and where possible was repeated three times. Negative control wells were endothelial cells alone. Tumour cell invasion through basement membrane components only ('standard invasion levels'), acted as positive controls for each culture. Levels of transendothelial cell invasion were compared with positive controls. Endothelial cells and non-invaded cells were removed as previously described and analysis was carried out as described before (2.3.1).

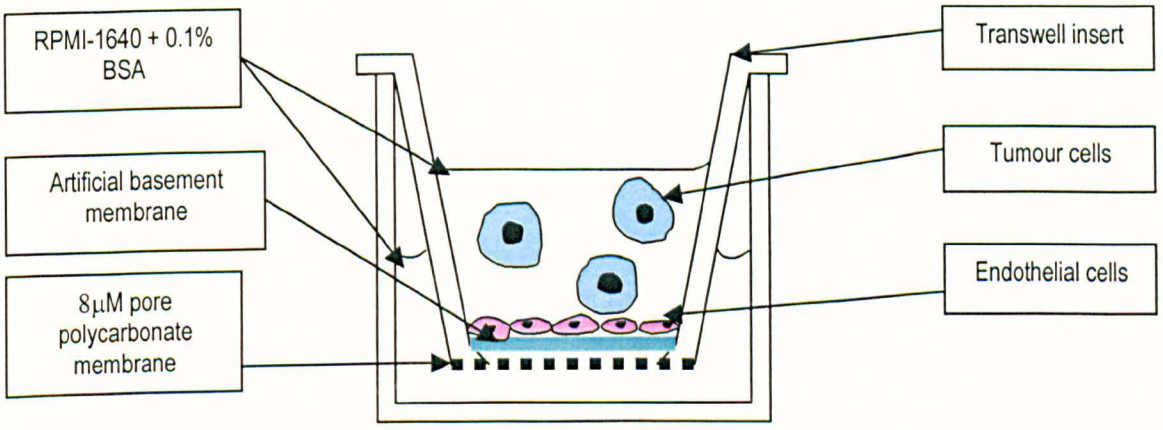


Figure 2.3.2.a. Schematic Diagram of the Transendothelial Cell Invasion Assay. Endothelial cells (shown in pink) are grown to confluence on a Matrigel-coated (shown in green) 8μM pore Transwell membrane. Tumour cells (shown in blue) in the upper chamber then invade through the endothelial cell layer, artificial basement membrane (Matrigel), and 8μM pore membrane, into the lower chamber.

Tracking of tumour and endothelial cells in the transendothelial cells assay

For tracing endothelial and tumour cell movement in the transendothelial cell invasion assay, cells were labelled with succinimidyl esters prior to use, as described previously (2.2.3) at the noted concentrations and in triplicate. Labelled cells were used as before in the assay. Wells were observed under fluorescence before and after removal of endothelial and non-invaded cells, to allow both assessment of the endothelial cell layer and cellular interactions between the two cell types, and the invaded cells.

2.4. Adhesion assays

To study the adhesive interactions between endothelial and tumour cells, HDMECAs or HULECs were dissociated from their culture flasks and resuspended in endothelial cell growth medium at a concentration of $2x10^5$/ml. 100ml of cell suspension was then pipetted into each well of a sterile 96-well plate, pre-coated with attachment factor ($2x10^4$/well). For each experiment, each condition was repeated in six wells. Cells were incubated for 24 hours to allow growth to confluence in the bottom of each well. Prior to setting up the adhesion assay, the growth medium was removed from the wells and the cells were washed twice with PBS, to remove any traces of FBS.

Tumour cells were dissociated from their culture flask, using sterile 550μM EDTA in PBS. Using the method previously described, tumour cells were labelled with CFDA-SE (2.2.3) and resuspended at a concentration of $5x10^5$/ml in RPMI 1640 medium supplemented with 0.1% BSA. Prior to adding tumour cells to the endothelium, to ascertain that the cell suspension was made up of single cells rather than clumps of cells, a drop of the cell suspension was placed on a glass slide, and observed under the microscope. Ensuring that the suspension was well dispersed, 50μl of the cell suspension ($25x10^3$/well) was carefully pipetted into each well containing the endothelial cell layer. Plates were incubated at 37°C for 4 hours and were laid separately on the incubator shelf when multiple plates were used (ensuring that plates acclimatised to the required temperature at the same time). During the incubation period, plates were rotated to overcome any vibrational effects of the incubator. Five minutes prior to the end of the incubation period, endothelial cells were counterstained with Hoescht (10μM) and replaced in the incubator. Positive and negative controls were included in each assay. For positive controls, as uveal melanoma cells preferentially adhere to fibronectin (Wagner *et al.*,

1997), tumour cell adhesion to fibronectin-coated wells (15μg/ml) was assessed; for negative controls, no tumour cells were added to endothelial cell monolayers. After the incubation period, plates were removed from the incubator and any non-adherent cells were removed by a process of 'inverting and blotting' the plate and washing with PBS, repeating this three times. Cells were fixed with 4% formaldehyde/PBS for 15 minutes, before rinsing in distilled water and allowing air-drying overnight in a dark, dry atmosphere before analysing using a microplate fluorescence reader. Six wells were analysed for each condition, and a mean calculated. Experiments were further repeated three times and a mean of the three experiments calculated.

2.5. Statistical analysis

The Analysis of Variance (ANOVA) test was used throughout these investigations to compare population means, assessing treatment values against an appropriate control. The test calculates the total amount of variation in a set of data, and the degree of variation associated with possible causes. In each instance, the underlying assumptions were assessed to check randomness, independence, additivity of the treatment effect, homogeneity of variance and normality of the residuals. For FACS data, the one-way ANOVA test was used, as the data was non-normally distributed. For two sets of data (transendothelial cell invasion and FACS data), due to the presence of low counts, the variance was found to be heterogeneous, and consequently a square root transformation was applied ($\sqrt{(x+0.5)}$). In all cases p values of less than 0.05 were taken as significant.

C. Health and Safety

All procedures were carried out in accordance with health and safety requirements by COSHH (Control of Substances Hazardous to Health). Laboratory coats and disposable latex gloves (Ansell Medical) were worn at all times when handling biological specimens.

Chapter 3

Expression of melanoma-associated antigens, integrins, degradative enzymes and inhibitors in uveal melanoma

3.1. Introduction

As highlighted in the Introduction (Section B, 1.3.1), previous investigations have reported expression of a number of integrins, and degradative enzymes in uveal melanoma (Cottam *et al.*, 1992; Ten Berge *et al.*, 1993; Creyghton *et al.*, 1995; Vaisanen *et al.*, 1999; Elshaw *et al.*, 2001; El-Shabrawi *et al.*, 2001). These studies have however, used a selection of tissue samples, and as yet, no study has looked at expression of a wide range antigens by the same tumour sample. The effect of the *in vitro* environment on expression by uveal melanoma cells in culture when compared with expression *in vivo*, has furthermore, only been investigated in a limited fashion (Marshall *et al.*, 1998).

Aims of the study

Primarily, the aim of this current study was therefore to assess expression of a panel of integrins, degradative enzymes and inhibitors in a series of ten primary uveal melanomas, with the aim of relating expression to known prognostic indicators. Immunohistochemistry was used to compare differences in expression between STCs, and frozen and paraffin-embedded sections, and results were related to known histo-pathological data (Appendix II).

To clarify that cultured cells were malignant uveal melanoma in origin, a series of 23 STCs and two cell lines were also stained for expression of a panel of melanoma-associated antigens (Melan-A, S-100 and HMB-45), where sufficient cells were available.

3.2. Results

3.2.1. Immunohistochemical analysis of melanoma marker expression in uveal melanoma short-term cultures

Three melanoma-associated antigens were chosen for assessment of melanoma classification; all of which are routinely used in cutaneous melanoma diagnostics (Orchard, 2000) and their application has been previously reported in uveal melanoma classification (de Vries *et al.*, 1998; Heegaard *et al.*, 2000). As choroidal melanomas of mixed cell populations are not 100% sensitive for one antibody alone, three different antibodies were used in the hope of maximising the frequency of positive results. In most cases cultures were also analysed by cytogenetics by colleagues in this laboratory, as characteristic abnormalities in chromosomes 3, 6 and 8 have previously been described in uveal melanomas (reviewed by Singh *et al.*, 2001).

A panel of 23 STCs and two cell lines (SOM 157d and SOM 196B) were assessed for melanoma marker expression; all tumours were derived from either the choroid or ciliary body (Appendix II). Cells were grown on glass slides and stained by immunohistochemistry, as previously described (Methods 2.2.1). Tumours were only assessed where sufficient numbers of cells were available surplus to experimental requirements, and therefore not all tumours used for further studies were screened. Tumours assessed included SOM 200, 238, 239, 250, 253, 256, 267, 275, 277, 282, 290, 295, 296, 301, 305, 306, and 307. Tumours that were not investigated for melanoma marker expression were SOM 260, 262, 263, 269, 272, 280, and 281. Results are summarised in Table 3.2.1.a and examples of staining are shown in Figure 3.2.1.a. A375 cells acted as positive controls for these studies (Figures 3.2.1.a.iv and 3.2.2.a.iii and iv).

S-100 was most ubiquitously expressed, with cytoplasmic and nuclear staining in 23 of the 25 cultures (Figure 3.2.1.a.ii). The antibody used detected both S-100A and S-100B proteins, and has been reported to be less specific than antibodies against both Melan-A and HMB-45 (Heegaard *et al.*, 2000) (potentially reflected by these results). All ciliary body melanomas expressed S-100 (SOM 253, 282, 295, 296, 305 and 311) whilst expression was not ubiquitous in the choroidal melanomas (Appendix II). No association could be made between epithelioid, mixed or spindle cell tumours and S-100 expression (Appendix II).

SOM	Passage	Melan-A	S-100	HMB-45
157d	101	+ 25%	+++ 35%	+++ 55%
196B	25	+++	+/++	++
200	3	++	++	+
238	<5	-	+++	-
239	14	-	+++	-
250	2	-	+++	-
253	4	-	+++	-
256	1	+++	+++	+++
266	11	-	++	-
267	7	-	-	-
274	<5	+	+++	-
275	<5	+++	+	+++
277	2	-	+	+
282	4	-	++	-
290	5	-	-	-
295	5	-	+++	-
296	4	-	+ 50%	-
301	3	+++	+	+++
302	4	++	++	++
304	1	+++	++	+++ 80%
305	1	-	++	-
306	3	+++	++	+++
307	3	-	+++	+++ 5%
308	2	+++	++	+++
311	1	+++ 40%	+++	++/+++

Table 3.2.1.a. Expression of melanoma-associated antigens in a panel of uveal melanoma short-term cultures and cell lines. Uveal melanomas are classified numerically and known as Sheffield Ocular Melanomas (SOM). The percentage of cells staining was determined and staining was considered homogenous if 75% of cells stained and heterogeneous if the percentage of cells stained were between 5% and 75%. Staining intensity was scored as + weak, ++ medium, or +++ strong. With the exception of two tumours (SOM 267 and 290), all tumours expressed at least one melanoma-associated antigen. <5, indicates that the passage number was below five, but was unknown.

In contrast, Melan-A and HMB-45 were expressed in fewer cultures, with 12 out of 25 and 13 out of 25 cultures expressing these proteins, respectively (Figure 3.2.1.a.i and iii). Nine tumours expressing S-100, did not express Melan-A or HMB-45. No association could be made between tumour location and Melan-A or HMB-45 expression (Appendix II). For HMB-45, high levels of expression were more commonly seen in spindle cell tumours (SOM 266, 274, 275, 301 and 304), with mixed and epithelioid tumours showing lower levels of expression, whilst Melan-A stained showed no

association with cell type (Appendix II). Two tumours did not express any of the antigens studied (SOM 267 and SOM 290).

With the exception of three STCs (SOM 239, 266 and 267) and the two cell lines (SOM 157d and SOM 196B), all cultures were assessed before passage five. No direct association could be made between passage number and expression of any of the antigens studied. Excluding the two cell lines however, it was however generally observed that tumours of a higher passage were more likely to lose Melan-A and HMB-45 expression (SOM 238, 239, 250, 253, 266, 267, 277, 282, 290, 295, 296, and 305), when compared with cultures of a low passage (SOM 200, 256, 274, 275, 301, 302, 304, 306, 307, 308, and 311).

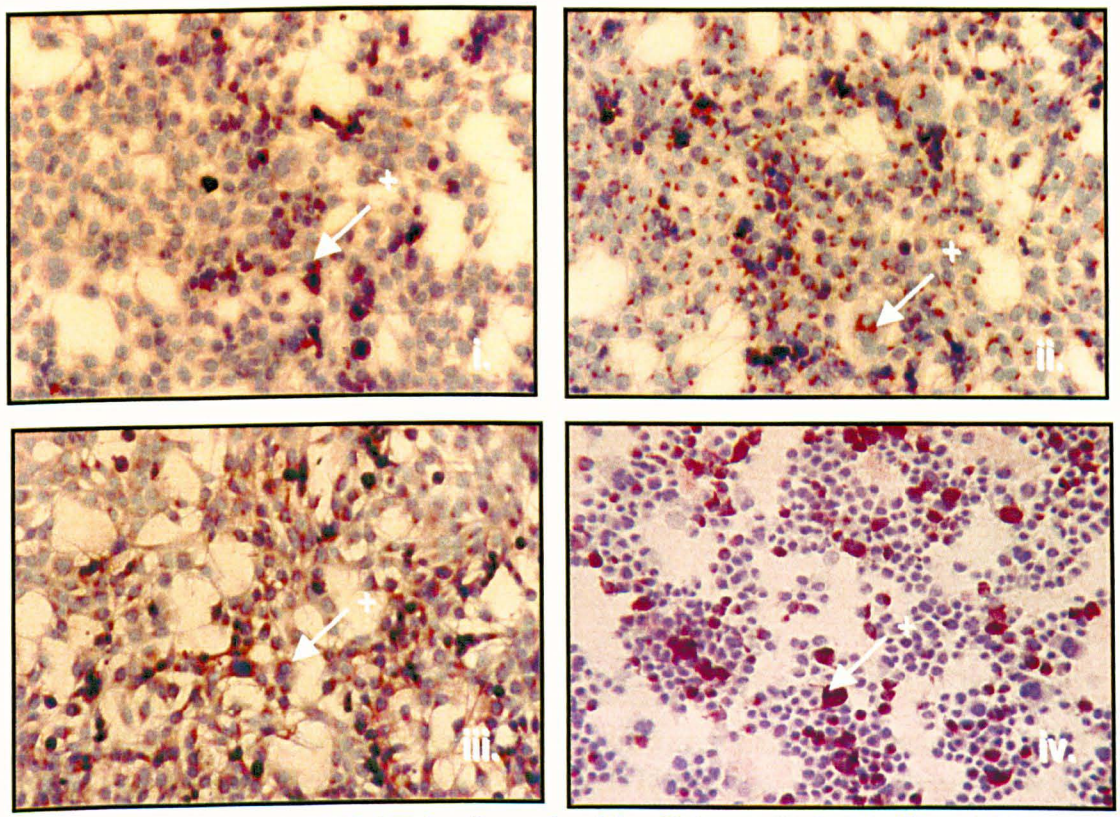


Figure 3.2.1.a. Cultured SOM 157d cells, stained for Melan-A (i), S-100 (ii) and HMB-45 (iii). A375 cells, stained for HMB-45 acted as positive controls (iv). SOM 157d cells are of epithelioid morphology, and were stained by the standard ABC immunohistochemistry method with an end substrate of AEC (visible as a bright magenta stain) (see 2.2.1). The '+' indicates examples of cells staining positive. Nuclei were counter-stained with Gill's haematoxylin (visible as a purple stain), and cells were viewed under x100 magnification. Melan-A and HMB-45 are both cytoplasmic proteins whereas S-100 proteins are present in both the cytoplasm and nucleus. Staining was heterogeneous, with the percentage of positive SOM cells graded as 25-30% (+) for Melan A (i), 30-40% (+++) for S-100 (ii), and 50-60% (+++) for HMB-45 (iii). A375 cells were graded as 30% (+++) for HMB-45 (iv).

3.2.2. Immunohistochemical analysis of integrin expression in cultured cells and frozen sections of posterior uveal melanoma

The results of the immunohistochemical studies of integrin expression are summarised in Tables 3.2.2.a and b and examples of staining are shown in Fig. 3.2.2.a. Melanin pigmentation in paraffin sections was bleached, but this was not feasible for frozen sections; black pigmentation in frozen sections did not however interfere with the magenta colour of the AEC substrate. All frozen and paraffin sections were stained by J.K.L. Woodward, and were counter-stained with Gill's haematoxylin (Figure 3.2.2.a.ii). STCs were stained by Ms Shona Elshaw to assist speed of analysis, and counter-staining was omitted due to personal preference for techniques, but this did not effect the true immunological staining (Figures 3.2.2.a.iii and iv). Integrin expression was restricted to cultured cells and frozen sections as antibodies used were unsuitable for formalin-fixed tissue.

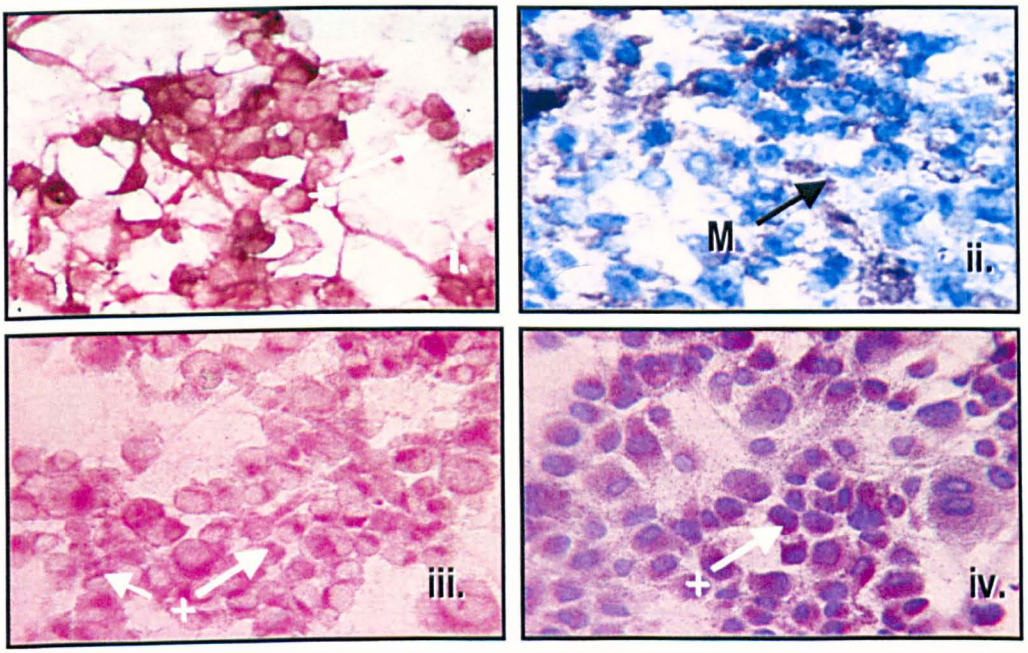


Figure 3.2.2.a. Cultured uveal melanoma cells (i) and frozen sections (ii) stained for $\alpha 1\beta 1$ and $\alpha 2\beta 1$. A375 cells were included as positive controls (iii and iv). Cells were stained by the standard ABC immunohistochemistry method with the end substrate being AEC (magenta in colour). Nuclei in ii) and iv) were counterstained with Gill's haematoxylin (visible as a purple stain), and cells were viewed under the magnification noted below. The '+' indicates examples of cells staining positive. Areas of black staining in plate ii are melanin granules (M) present in the tumour and for sections staining positively, did not obscure observation of positive staining. i) Positive heterogeneous staining for $\alpha 1\beta 1$ in cultured cells of SOM 210 (x200) (no counter-stain). 50-60% of cells stained positively (++) for $\alpha 1\beta 1$. ii) Negative staining for $\alpha 1\beta 1$ in a frozen section of SOM 210 (x400) (counter-stained with Gill's haematoxylin). iii) Positive staining for $\alpha 1\beta 1$ by cultured A375 cells (x400) (no counter-stain) (positive control). iv) Positive staining for $\alpha 1\beta 1$ by cultured A375 cells (x400) (counter-stained with Gill's haematoxylin) (positive control). Counter-staining did not effect positive staining.

Variation in integrin expression between frozen sections and STCs

Expression specifically targeted to the α-subunit showed some variation, with primarily differences in levels of expression of α1, α2, α4, and α6 found between frozen tissue sections and cultured cells from the same tumours (Table 3.2.2.a). Seven of the ten uveal melanomas lacked α1 expression, yet cultures established from the same tumours were found to express this integrin (Figures 3.2.2.a.i and ii). Similarly, four of the ten tumours (SOM 200, 239, 248, 256) did not express α2 but the respective STCs stained positively. In contrast frozen sections from three tumours (SOM 250, 253 and 256) stained positively for α6, whilst corresponding STCs did not. Inconsistencies in α4 expression were also seen; for two tumours (SOM 238 and 239), expression was present or absent in the cultured cells, with the reverse pattern seen in the frozen sections. No associations could be made between expression in frozen sections or cultured cells with tumour cell type, location or patient survival (Appendix II). In particular, patients SOM 250 and SOM 255 are known to have developed metastases, yet no specific associations could be made between integrin expression in these patients and the development of secondary disease.

		SOM									
Antibody		**200**	**210**	**238**	**239**	**248**	**250**	**253**	**255**	**256**	**260**
α1	FS	-	-	++	-	-	+++ 50%	-	-	-	+++ 10%
	STC	+/++ 50%	++ 50-60%	+	+	+ 50%	++	+ 50%	+ 70%	+/++	++ 50%
α2	FS	-	+++	+	-	-	+	+/++	+	-	+++
	STC	+++	+/++	++	++/+++	+++	++/+++	+++	+++	++	+++
α4	FS	+ 20%	+ 60%	-	+/++ 70%	+ 20%	+++ 60%	+ 50%	++ 10%	+ 10%	+/++ 40%
	STC	+ 5-10%	++ 40%	+	-	+++	+ 50%	+/++ 60%	+	+	+
α6	FS	+/++	+	+	+/++	++	-	-	++/+++	-	++
	STC	+ 50%	++/+++	-	-	+ >50%	+ 5-10%	+/++ 60%	+/++ 60%	+	+

Table 3.2.2.a. Expression of integrins in posterior uveal melanoma (Sheffield Ocular Melanomas (SOM)) in which variation was seen between frozen sections (FS) and short-term cultures (STC), detected by Immunohistochemistry. The percentage of cells staining was determined and staining was considered homogenous if 75% of cells stained and heterogeneous if the percentage of cells stained were between 5% and 75%. Staining intensity was scored as + weak, ++ medium, or +++ strong. Variation in expression of α1, α2, α4, and α6 was shown between cultured cells and frozen sections.

Integrins showing no variation in expression between frozen sections and STCs

Staining for the α3, α5, and β1-subunits and αvβ3 was consistent between frozen tissue and STCs (Table 3.2.2.b). Although frozen sections and STCs of all ten tumours were positive for αvβ3 expression, stronger expression was however observed in cultured cells in nine out of ten cases, but it was not possible to quantify these observations. No associations could be made between expression in frozen sections or cultured cells with tumour cell type, location or patient survival (Appendix II).

		SOM									
Antibody		200	210	238	239	248	250	253	255	256	260
α3	FS	++	+++	++/+++	+++	+++	++/+++	++	+++	+++ 30%	+++
	STC	++	++	++	++	+++	+++	+++	+++	+++	+++
α5	FS	+/++	++	+	++	+	++	++	++/+++	+	++/+++
	STC	++	+/++	++	++	+++	+++	+++	++	++/+++	+++
β1	FS	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	STC	+++	++	++	+++	++	++/+++	+++	++	+++	+++
αvβ3	FS	+ 5%	+/++	+ 5%	+ 50%	+ 10%	+	+ 10 %	++	+ 10%	+/++
	STC	+/++	++	++/+++	+	++	++	+++	++/+++	+/++	++/+++

Table 3.2.2.b. Expression of integrins in posterior uveal melanoma (Sheffield Ocular Melanomas (SOM)) in which variation was not seen between frozen sections (FS) and short-term cultures (STC), detected by immunohistochemistry. Staining was determined as for Table 3.2.2.a. No variation in expression of α3, α5, β1 and αvβ3 was shown between cultured cells and frozen sections.

3.2.3. Immunohistochemical analysis of degradative enzyme expression and the expression of their inhibitors in cultured cells, frozen and paraffin-embedded sections of posterior uveal melanoma

The results of the immunohistochemical studies of MMP-2, MMP-9, TIMP-2, u-PA, PAI-1 and PAI-2 expression are summarised in Tables 3.2.3.a and b. TIMP-1 expression was not investigated, as an appropriate antibody was not available at the time of study. Tissue samples (both frozen and paraffin-embedded) and cultured cells from all ten uveal melanomas were found to express MMP-2, TIMP-2, u-PA, PAI-1 and PAI-2. Variation was only observed in MMP-9 expression. Expression of MMP-9 was detected in all STCs and sections of frozen tissue, but only seven out of the ten paraffin-embedded samples stained positively for MMP-9 (SOM 200, 210, 239, 248, 250, 256 and 260) suggesting that the fixation process maybe preventing positive staining in these instances. Slight increases in expression of MMP-9 were also seen in cultured cells from three tumours, when compared with respective paraffin and frozen sections (SOM 239, 248 and 250). Again, no correlation could be made between expression patterns, prognostic indicators, metastatic disease and patient survival (Appendix II).

		SOM									
Antibody		200	210	238	239	248	250	253	255	256	260
MMP-2	FS	+	++	++	+++ 50%	++ 10%	++	+	+	+	+ 50%
	PS	++/+++	++	++/+++	++/+++	+/++	++	++	++	++	++
	STC	++	++	++	++	+++	+++	+++	++	+ 50%	++ 50%
MMP-9	FS	+ 5%	++	+++	+/++	+	+ 50%	+/++	+	+	+/++
	PS	++	++	-	++/+++	++/+++	+/++	-	-	++	+++
	STC	+++	++	+++	++	+++	++	++/+++	++	+++	+++
TIMP-2	FS	+++	+++	+++	+++	+++	+++	++/+++	+++	+++	++/+++
	PS	++	++	+++	+++	++	+++	+	++	+	+
	STC	+/++	+/++	+/++	+/++	+/++	+/++	+/++	+/++	+	++

Table 3.2.3.a. Expression of MMP-2, MMP-9 and TIMP-2 in frozen and paraffin sections (FS and PS, respectively) and short-term cultures (STC) in posterior uveal melanoma (Sheffield Ocular Melanoma (SOM)), detected by immunohistochemistry. The percentage of cells staining was determined and staining was considered homogenous if 75% of cells stained and heterogeneous if the percentage of cells stained was between 5% and 75%. Staining intensity was scored as + weak, ++ medium, or +++ strong. Slight variation in expression of MMP-9 expression was shown between paraffin sections and frozen sections and cultured cells. No variation in MMP-2 and TIMP-2 was seen.

Antibody		SOM 200	210	238	239	248	250	253	255	256	260
u-PA	FS	+++	+++	+++	+++	+++	+++	+++	+++	++/+++	+++
	PS	+++	+++	+	+++	++/+++	++	+++	+	+	+
	STC	+++	+++	++/+++	+++	+++	+++	++/+++	++/+++	+++	++
PAI-1	FS	+++	+++	+++	++/+++	+++	+++	+++	+++	++/+++	++/+++
	PS	++	++	+	+++	+++	++	+++	+++	++	+++
	STC	+++	++/+++	+++	+++	++	+++	++/+++	++/+++	+++	+++
PAI-2	FS	+++	+++	+++	++/+++	+++	+++	++	+++	++	+++
	PS	+++	+++	+/++	+++	+++	++	+++	+++	++/+++	+/++
	STC	++	+++ 50%	++	+	+/++	+/++	+/++	+/++	+++	+++

Table 3.2.3.b. Expression of u-PA, PAI-1 and PAI-2 in frozen and paraffin sections (FS and PS, respectively) and short-term cultures (STC) in posterior uveal melanoma (Sheffield Ocular Melanoma (SOM)), detected by immunohistochemistry. The percentage of cells staining was determined as for Table 3.2.3.a. No variation in expression of u-PA, PAI-1 or PAI-2 was seen.

3.3. Discussion

Cell culture has been widely used over a number of years, and provides an excellent system for the study of cellular responses and behaviour, offering advantages including providing a readily available supply of cells with similar phenotypes, often with high proliferation rates. For cells to become immortalised as cell lines, genetic alterations may however have occurred to facilitate continuous growth, and consequently cells do not fully represent the tissue of origin. When considering *in vitro* results in relation to clinical details of individual patients, the use of cell lines is therefore often inappropriate due to the drift from the original phenotype. In support, serial passage of kidney glomerular mesangial cells has been shown to result in altered expression of a number of ECM components, degradative enzymes and inhibitors (Schnaper *et al.*, 1996).

3.3.1. Confirmation of STC melanoma status

Melan-A (MART-1)

The Melan-A protein is the product of the MART-1 gene and is expressed in the cytoplasm during melanocyte differentiation (Chen *et al.*, 1996). The antibody is particularly useful in diagnostics due to its high sensitivity and specificity for melanocytic lesions (Chen *et al.*, 1996; Heegaard *et al.*, 2000) and high expression in uveal melanomas is well documented (Mulcahy *et al.*, 1996; de Vries *et al.*, 1998; Heegaard *et al.*, 2000). No definition has however been made between benign and malignant lesions or between spindle and epithelioid cells (Nicotra *et al.*, 1997; Orosz, 1999; Heegaard *et al.*, 2000). In this present study, only approximately half of the cultures expressed Melan-A (Table 3.2.1.a) and in agreement with previous reports (Nicotra *et al.*, 1997; Orosz, 1999; Heegaard *et al.*, 2000) no association could be made between expression and cell type or tumour location.

S-100

The S-100 proteins are a family of calcium-binding proteins, including S-100A and S-100B that differ in the composition of the alpha and beta chains. Both are expressed in melanocytes and melanoma cells in the nucleus and cytoplasm in both benign and malignant melanoma cells (Orosz, 1999; de Vries *et al.*, 2001). In this current study the majority of the cultures expressed the S-100 antigen and in particular all ciliary body tumours studied expressed the S-100 protein (Table 3.2.1.a). Conjunctival and iris melanomas have however also been shown to express S-100, together with pigmented and non-pigmented epithelium of the iris and ciliary body, and RPE cells (Heegaard *et al.*,

2000). Due to the high levels of expression in this present study, detection of other cell types at this stage therefore had to be taken into consideration. As all of the tumours studied were either choroidal or ciliary body melanomas, culture of conjunctival or iris melanomas seemed unlikely.

HMB-45 (gp100)

The HMB-45 antibody recognises the cytoplasmic gp100 protein (Adema *et al.*, 1993; de Vries *et al.*, 2001). The gp100 protein is associated with melanocyte differentiation throughout melanoma progression and the HMB-45 antibody has been classically used, together with the S-100 antibody, in melanoma diagnostics (de Vries *et al.*, 2001). This antibody has nevertheless reputedly been reported to be less sensitive than antibodies against other melanoma-associated markers yet, is highly specific (Heegaard *et al.*, 2000; de Vries *et al.*, 2001; Xu *et al.*, 2002). This was reflected in this current study where only approximately 50% of the STCs studied, expressed gp100 (Table 3.2.1.a). Tissue and cell lines from both benign and malignant choroidal uveal melanomas have been shown to stain positively for gp100, whilst non-melanocytic cells, including RPE cells have not (Steuhl *et al.*, 1993; Luyten *et al.*, 1996; Mulcahy *et al.*, 1996; Luyten *et al.*, 1998; de Vries *et al.*, 1998; Heegaard *et al.*, 2000). When considering uveal melanoma cell types, most studies have described strong expression in spindle, mixed cell type and epithelioid melanomas (Steuhl *et al.*, 1993; de Vries *et al.*, 1998; Heegaard *et al.*, 2000) and this was reflected in this current study. As all cell types did not however constitutively express this antigen, no direct conclusions could be drawn. No associations could also be made between tumour location and gp100 expression.

In conclusion, tumours expressing S-100 and either Melan-A or gp100, were most likely to be uveal melanoma in origin, due to the high specificity of the latter two antibodies. Nine cultures however stained positively for S-100, but did not express Melan-A or gp100 and no association could be made between this staining pattern and cell type or tumour location (Table 3.2.1.a). Uveal melanomas are relatively free from infiltrating cells (de Waard-Siebinga *et al.*, 1995), and the behaviour of these tumours in subsequent assays was not significantly different from other uveal melanomas studied, and thus it is probable that these were uveal melanoma in origin. It is entirely possible that the lack of staining reflects time in culture, as loss of the differentiation markers (Melan A, gp100, and S-100), was more likely at higher passage. Also previous analysis of the long-term cell line SOM 157d has shown differences in staining response at earlier and later passage numbers. A number of

characteristic chromosomal abnormalities of SOM 157d cells have however remained consistent. Cytogenetic analysis of the original tumour sample from SOM 267 detected characteristic markers associated with uveal melanomas, whilst the behaviour of both tumours in *in vitro* assays was again not significantly different from other tumours studied. Analysis of further melanoma-associated antigens, such as tyrosinase (Mulcahy *et al.*, 1996; de Vries *et al.*, 1998) could be of further use in classification of these cultures in which S-100, Melan-A and gp100 expression was absent.

3.3.2. Differences between the *in vitro* and *in vivo* expression of integrins, degradative enzymes and their inhibitors.

3.3.2.1. Integrin expression

Expression in response to culture

β1-subunit expression was ubiquitous throughout frozen sections and cultured cells of all tumours studied (Table 3.2.2.b). The β1-subunit is known to associate with all of the α-subunits chosen for investigation (reviewed by Gonzalez-Amaro and Sanchez-Madrid, 1999), whilst some of the α-subunits studied could have also associated with alternative β-chains. In agreement with published data, consistent α3- and α5-subunit and αvβ3 expression was also seen in all frozen sections and cultured cells of the tumours studied where minimal variation occurred between specimen types (Ten Berge *et al.*, 1993; Elshaw *et al.*, 2001).

Differences in expression of α1, α2, α4, and α6 were however observed between *in vitro* and *in vivo* (Table 3.2.2.a), with expression of α1 and α2 being most susceptible to *in vitro* up-regulation. In a previous study examining epithelial and endothelial derived tissues, a reverse pattern has been reported for α1β1, whereby expression was not observed in cultured cells yet this receptor was expressed in the corresponding tissue (reviewed by Albeda and Buck, 1990). Differences in the cell type studied could however account for the variation observed between studies. It is of interest that EGF, a factor present in uveal melanoma growth media, has been shown to enhance expression of α1β1 in PC12 cells (Danker *et al.*, 2000), and α2β1 in squamous cell, and vulvar carcinoma, lung adneocarcinoma and corneal epithelial cells (Waleh *et al.*, 1994; Krensel and Lictner, 1999; Smida Rezgui *et al.*, 2000; Song *et al.*, 2001), and therefore could have been responsible for the apparent up-regulation in certain cases. In addition, TGF-β and PDGF-BB up-regulate mesangial cell α1β1

expression (Kagami *et al.*, 1999) whilst TNFα, IL-1β, TGF-β and FGF-2 increase α2- and β1-subunit expression *in vitro* (Nejjari *et al.*, 1999; Kawashima *et al.*, 2001); each of which could have been secreted by the melanoma cells in culture, or present as an undefined constituents of the FBS. Observations from this present study would also provide an explanation for contradictory evidence in the literature, where α2β1 expression was reported absent in frozen uveal melanoma samples (Ten Berge *et al.*, 1993), whilst cultured uveal melanoma cells expressed the α2-subunit (Creyghton *et al.*, 1995; Elshaw *et al.*, 2001).

For α6, the potential of both up- and down-regulation *in vitro* was evident. This could be supported by reports in which EGF has enhanced expression of β1, β4, and α6 subunits and the α6β4 integrin in squamous cell carcinoma, corneal epithelial cells, and colon cancer cells (Waleh *et al.*, 1994; Pouliot *et al.*, 2000; Mariotti *et al.*, 2001; Song *et al.*, 2001). Furthermore TNFα, IL-1β, TGF-β, FGF-2 and IL-12 have also been shown to induce the expression of α6 and β1 in hepatocarcinoma and T helper cells (Colantonio *et al.*, 1999; Nejjari *et al.*, 1999). Conversely, TGF-β, TNFα and IL-1β have been reported to down-regulate α6 expression in cultured endothelial and melanoma cells (Defilippi *et al.*, 1992; Moretti *et al.*, 1997; Teti *et al.*, 1997) and similarly could have affected the responses seen in this present study. With regard to α4 expression, for eight out of the ten tumours, expression was seen, both *in vitro* and *in vivo*. For the remaining two tumours, a *vice versa* scenario was seen, and it was therefore difficult to draw reliable conclusions.

Differences in expression may therefore occur in various tissues not only as a result of tissue-associated phenomena, but also because of cultural conditions (reviewed by Albeda and Buck, 1990). Two basic mechanisms of integrin activation have been described as signalling can be either intracellular ('inside-out signalling') or extracellular ('outside-in signalling') (reviewed by Hynes, 1992). For example, EGFR activation causes phosphorylation of α6β4 via the tyrosine kinase Fyn, inducing cellular migration and invasion (Mariotti *et al.*, 2001). In this current study, exogenous production or addition to the growth media of cytokines, growth factors and other matrix proteins is likely to have affected integrin expression via such mechanisms, whilst activation of one integrin type could also have a significant effect of the expression of a second integrin type.

Association of expression with prognosis

As described previously, differences in expression of α1, α2, α4, and α6 were observed between tumours, but few associations could be made between expression of all integrin subunits studied, known prognostic indicators and patient survival (Appendix II). In agreement, Anasatassiou and co-workers (2000a) have also shown that expression of α2, α3, and αvβ3 in paraffin sections of uveal melanomas could not be correlated with prognosis. In the study by Elshaw and colleagues (2001), cultured uveal melanoma cells, whilst ubiquitously expressing a number of integrins, variably expressed α4β1 and α6β1, and the latter was only seen in cultures with epithelioid morphologies. As cultured uveal melanocytes lacked α1β1, α4β1, and α6β1 expression, it was consequently hypothesised that in particular, α6β1 expression is associated with worse prognosis. This published data is also consistent with the report that pre-metastatic uveal melanoma cells do not express α6β1 (Rohrbach *et al.*, 1994). The results of this present study do not however reflect these findings and no association was made between α6 expression and cell type.

Expression of the fibronectin receptor α5β1 has been correlated with a less aggressive phenotype; highly invasive epithelioid cells lacked expression, whilst primary melanocytes and less invasive cultures were positive for α5β1 (Beliveau *et al.*, 2000). Results in this present study were again contradictory as no specific association could be made with α5 expression and epithelioid and spindle morphology. Finally, most evidence for cutaneous and uveal melanoma has suggested that decreased expression of αvβ3 is associated with metastatic development (Ten Berge *et al.*, 1993; Danen *et al.*, 1995; Seftor *et al.*, 1999), but results shown here indicated ubiquitous expression in all ten primary tumours studied. As αvβ3 expression has been reported to be important during early stages of malignant uveal melanoma progression, but is reduced during later stages of metastasis (Danen *et al.*, 1995; Seftor *et al.*, 1999) it is possible that tumours assessed in this present study were resected during the early stages of development. This theory would hold true for eight of the patients in which no metastatic disease has, as yet, been discovered.

3.3.2.2. Degradative enzyme and inhibitor expression

Expression in response to culture

Ubiquitous expression of MMP-2, TIMP-2, u-PA, PAI-1 and PAI-2 was shown in both paraffin and frozen sections and cultured cells (Tables 3.2.3.a and b). Variation was only seen in paraffin sections stained for MMP-9 (Table 3.2.3.a). Such differences are likely to be a result of the processing procedure masking the epitope and effectively denaturing the antigens. Previous work using in part, other methods of detection, has however found similar variability in MMP-9 expression between individual cases (Cottam *et al.*, 1992; Elshaw *et al.*, 2001; El-Shabrawi *et al.*, 2001). In this present study, there was some variation between *in vitro* and *in vivo* expression, but this could not be quantified, and more sensitive methods would be required. However, in support of *in vitro* MMP stimulation, in a similar comparative investigation on a series of nine brain tumours, using zymography, immunohistochemistry and immunocytochemistry, local micro-environmental conditions *in vitro* were responsible for stimulating MMP expression, including MMP-9 (Rooprai *et al.*, 1998).

Association of expression with prognosis

As consistent expression of MMP-2, TIMP-2, u-PA, PAI-1 and PAI-2 was observed, with slight variation in MMP-9 most likely to be related to processing, few conclusions relating to histo-pathological data (Appendix II), prognosis and survival could be drawn. MMP-2 and MMP-9 expression has been previously associated with extra scleral spread and poor prognosis in uveal melanoma (Cottam *et al.*, 1992; Vaisanen *et al.*, 1999; El-Shabrawi *et al.*, 2001) but *in vitro* studies using zymography and invasion assays, have not been able to correlate MMP-2 expression with levels of uveal melanoma invasion or prognosis (Elshaw *et al.*, 2001). As no differences were seen between the two metastatic tumours and the remaining tumours in which metastatic disease has not been diagnosed, no correlation with invasive behaviour, patient survival and prognostic indicators could be made.

To date, there is little evidence regarding TIMP expression in uveal melanoma. As the antibody used would detect bound and unbound forms of this inhibitor, it is feasible that TIMP-2 detected was bound to MMP-2, potentially inhibiting its action. TIMP-2 overexpression has however been associated with increased cutaneous melanoma invasion (Valente *et al.*, 1998), and thus for the two metastatic tumours studied, TIMP-2 could have promoted invasion rather than inhibited it. With regard to the PA

system, u-PA and PAI-1 have both been found to be expressed at a higher level in the metastases of uveal melanomas in comparison to the primary tumour, whilst uveal melanocytes only weakly express u-PA (Tripathi *et al.*, 1990; De Vries *et al.*, 1995; Ma *et al.*, 1997). No association could however be made between expression and the metastatic tumours assessed in this study.

In light of the results presented here and previous studies, differences in expression between *in vivo* and *in vitro* conditions should be taken into consideration. More sensitive and quantitative methods should be enlisted to identify significant changes in response to culture. For example, with regard to protease secretion, more definite conclusion may be drawn using functional methods such as zymography, or quantitative approaches such as ELISAs. mRNA studies by Northern blots, RT-PCR or *in situ* hybridisation would also be informative for detection of any of the regulators studied in this investigation.

In summary, these results have highlighted that uveal melanomas ubiquitously express a number of integrins, degradative enzymes and their inhibitors, but expression of the $\alpha 1$, $\alpha 2$, $\alpha 4$, and $\alpha 6$ integrins is more variable, and may in part, be due to *in vitro* regulation.

Chapter 4

Transendothelial cell invasion of uveal melanoma

4.1. Introduction

Various attempts have been made to develop a suitable animal model to more closely study the metastasis of uveal melanoma (reviewed by Grossniklaus *et al.*, 2000). Cases have been reported occurring naturally in cats and dogs, but they are infrequent and the relationship to human disease is less apparent (Dubielzig, 1990). Induced models share some similarities with human uveal melanoma metastasis but each has unique advantages and disadvantages. Initial problems may arise when inducing chemically, or with radiation, as malignant pigmented neoplasms may not be melanocytic in origin (Patz *et al.*, 1959; reviewed by Grossniklaus *et al.*, 2000). Murine models have also been used, but metastases induced using the cutaneous B16 melanoma cell line can be pulmonary, and not hepatic (Grossniklaus *et al.*, 1995). Rabbit models are possibly more suitable for study, due to the larger size of the rabbit eye and their longer life span, but again the development of hepatic metastases is dependent on the specific model used. In addition, successful implantation often requires immune suppression. Transgenic mice have also been utilised, but the origin of the tumours is again often unclear and the resulting metastatic spread is not hepatic (Bradl *et al.*, 1991). Despite advances using animal models of uveal melanoma metastasis, the pattern of spread is therefore often dissimilar from that of human uveal melanoma and such models may not accurately represent the human situation *in vivo*.

Aims of the study

Due to the limitations associated with animal models of uveal melanoma, the purpose of this study was to develop a modified *in vitro* assay to assess uveal melanoma invasion across endothelial and basement membrane barriers, that is perhaps, more realistic than previous *in vitro* invasion models (Elshaw *et al.*, 2001). In this model a microvascular endothelial cell monolayer has been included, in addition to an artificial basement membrane, and has been used to mimic invasion for a series of 16 primary uveal melanomas and one cell line (SOM 196B). Levels of invasion were correlated with histo-pathological markers of prognosis. Cellular invasion through endothelial cells and basement membrane was also compared with invasion through the basement membrane alone. For one culture (SOM 196B), scanning electron microscopy was performed. In addition, for a small number of cultures, both cell populations were pre-labelled with fluorescent probes, to trace cell movement through the invasion assay.

4.2. Results

4.2.1. Optimisation of the transendothelial cell model of invasion

Prior to assessment of uveal melanoma STC transendothelial cell invasion, experimental parameters relating to the model had to be established. For this purpose, the cell line SOM 196B was used, due to its known invasive characteristics through basement membrane barriers alone and continued availability of cellular material; most STCs die within ten passages or fail to produce adequate material.

Membrane pore size

To optimise the correct pore size for the model, maximising tumour cell migration, whilst minimising endothelial cell migration, SOM 196B cells and HDMECAs were independently tested for their invasion through Transwell membranes with pore sizes of 3, 5 or 8μM, pre-coated with Matrigel. Results are illustrated in Figure 4.2.1.a. Movement of HDMECAs was minimal through all pore sizes, with a maximum of 2-3 cells/well (x400 magnification) only migrating. In these cases, it is probable that only passive migration of endothelial cells is occurring, as opposed to active invasion. In contrast, SOM 196B cells invaded through all pore sizes tested. To therefore maximise tumour cell invasion, and in particular for low invading tumours, a pore size of 8μM was chosen. Figure 4.2.1.b.i shows an example of invaded cells on the underside of an 8μM pore membrane (all non-invaded cells had previously been removed from the top side of the membrane and thus all visible cells have invaded through the Matrigel-coated membrane); nuclei have been stained with Gill's haematoxylin. Invaded cells were also seen to be viable (Figure 4.2.1.b.ii; in this instance, cells had been pre-labelled with CFDA-SE and observed under fluorescence).

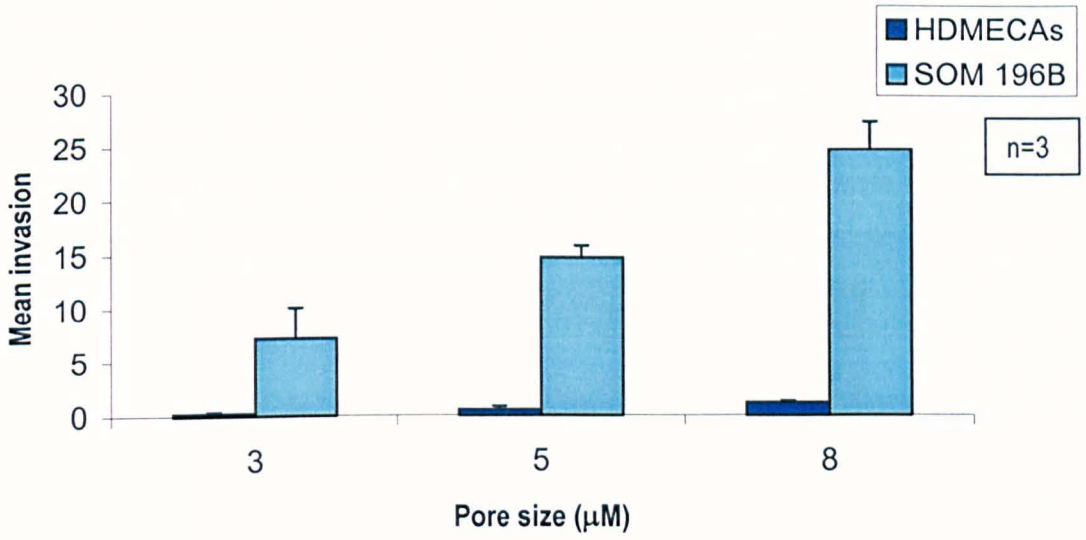


Figure 4.2.1.a. Invasion of SOM 196B and HDMECAs, separately through Transwell membranes of varying pore size. Expressed as mean counts per field of view at x400 magnification. Mean (± SEM) invasion through 3, 5 and 8μM pores. The graph represents the mean of three experiments. The x-axis shows the membrane pore size and the y-axis shows the mean cell invasion per field through Matrigel under x400 magnification.

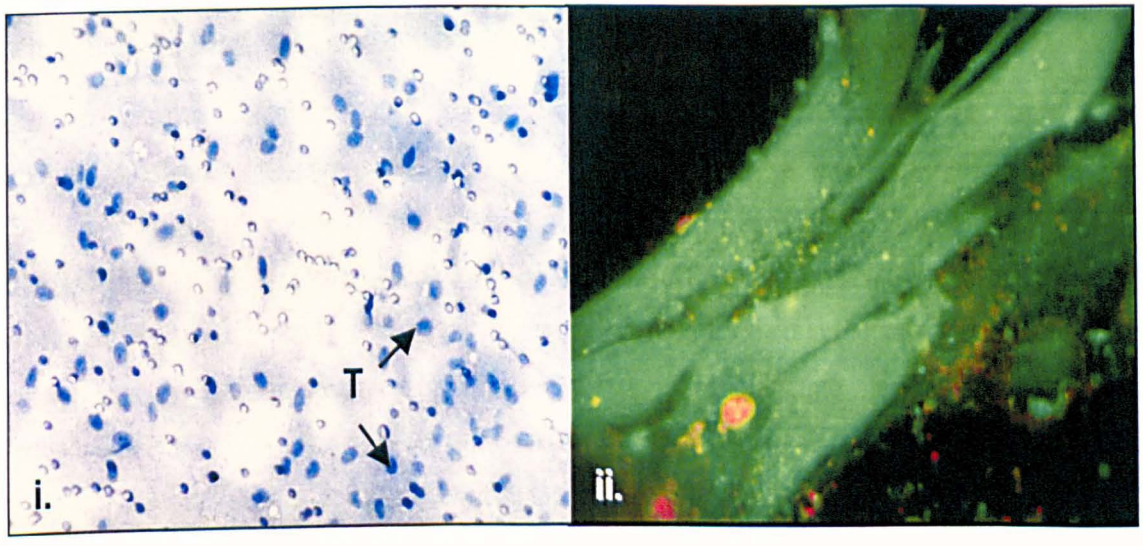


Figure 4.2.1.b.i. Invaded tumour cells (T) (SOM 277) on the underside of the Transwell membrane (8μM pores) (x200 magnification). All non-invaded cells from the topside of the membrane have been removed. Nuclei are stained with Gill's haematoxylin, and an example of an invaded cell is indicated. **ii. Viable Invaded tumour cells (SOM 196B) on the underside of the Transwell membrane (x1000 magnification).** Cells are labelled with CFDA-SE (green), and were observed under fluorescence (492-517nm); areas of red staining are artefacts of the labelling process.

Endothelial cell seeding density

To ensure that endothelial cells were forming a confluent monolayer, HDMECAs were seeded at densities of 20 000, 50 000, 100 000 and 150 000 cells/well and incubated for either 7 or 24 hours. When seeded at 100 000 cells/well for 24 hours, a confluent monolayer was achieved (Figure 4.2.1.c) where few gaps between cells were seen, and cells did not form layers of more than one cell thick. Passive tumour cell migration between endothelial cells would therefore be reduced to a minimum.

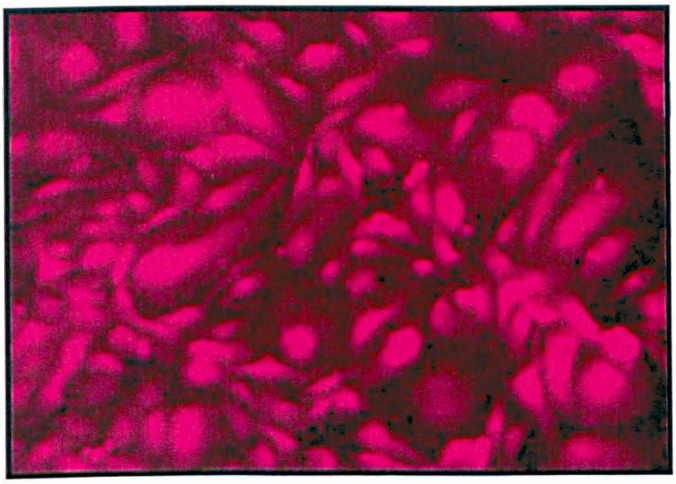


Figure 4.2.1.c. HDMECAs, seeded at 1X10^5, labelled with SNARF-1 and grown to confluence on the topside of an 8μM pore membrane, pre-coated with an artificial basement membrane (x100 magnification). Cells were observed under fluorescence at 580-640nm.

Time course experiments

In order to establish an appropriate incubation time for the assay, SOM 196B and HDMECAs were independently tested for invasion through 8μM pore membranes, pre-coated with Matrigel. This was also repeated for transendothelial cell invasion of SOM 196B through HDMECAs. Invasion levels were monitored over a period of 48 hours and results are represented in Figure 4.2.1.d. Subsequent to 24 hours, negligible increases in invasion of SOM 196B cells through Matrigel alone were seen. Transendothelial cell invasion of SOM 196B was minimal at all time points. HDMECA migration was also negligible at all time points. An incubation time of 24 hours was consequently chosen for transendothelial cell invasion. Time points subsequent to 24 hours were avoided, as tumour cell doubling times are likely to occur after this point. It was not feasible to assess growth characteristics of each individual culture and hence by choosing a time point of 24 hours, direct comparison of invasion between cultures could be made. To ascertain whether any cells may have detached from

the membrane and further settled on the lower surface of the well, media from the lower chambers was observed and lower well surfaces were trypsinised; no cells were found to be present.

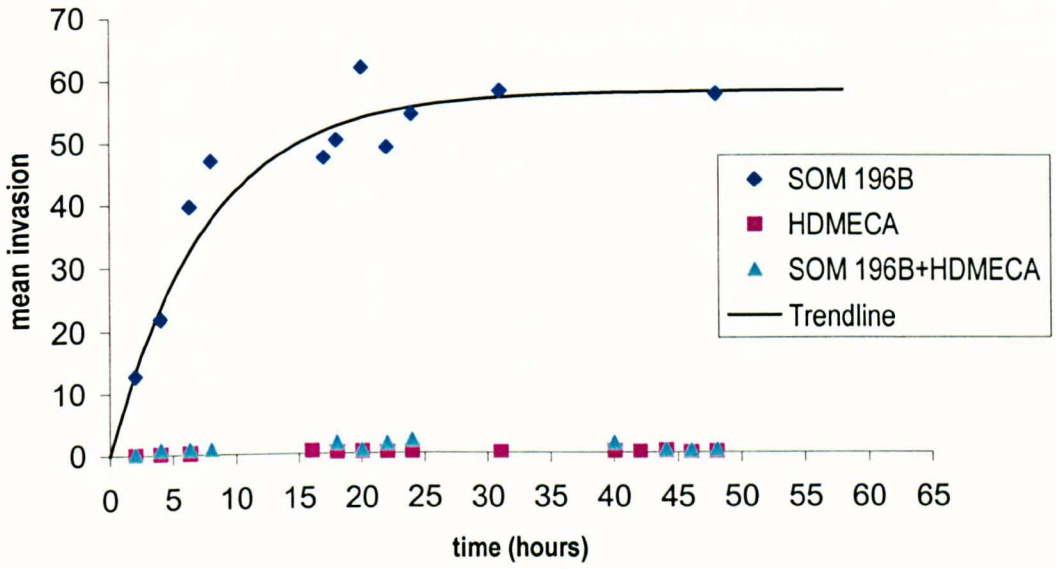


Figure 4.2.1.d. Time courses of independent invasion of SOM 196B and HDMECAs, and transendothelial cell invasion of SOM 196B through HDMECAs through 8μM pores. Counts are expressed as mean counts per field of view under x400 magnification, and represent the mean of one experiment, set up in triplicate. For each well, ten fields of view were counted and a mean calculated. The x-axis shows the time in hours and the y-axis shows the mean invasion through Matrigel under x400 magnification.

Comparing transendothelial cell invasion through HDMECAs and HULECs

HDMECAs were used in all assays with STCs. As uveal melanomas primarily target the liver, microvascular endothelial cells derived from the liver would be appropriate for these studies, as differences potentially exist between dermal and liver endothelial cells. To validate the use of dermal endothelial cells, and to investigate this point, transendothelial cell invasion of SOM 196B was assessed using both HULECs and HDMECAs. HULECs were freshly extracted and not commercially available making their use in all assays impractical. After repeating the experiment three times, no significant difference in levels of invasion through the two types of endothelium was observed ($P>0.05$). Levels of transendothelial cell invasion of SOM 196B cells were 5.45 (± 0.123) and 6.53 (± 1.20) cells per field under x400 magnification, for invasion through HDMECAs and HULECs,

respectively. As dermal endothelial cells were more readily available the remaining assays were undertaken using these cells.

Comparing Matrigel with an ECM solution

Matrigel was used throughout the course of these studies, due to its commercial availability, ease of reproducibility and wide acceptance as an artificial basement membrane. The concentration at which it was used ensured that a thin barrier could be observed across the membrane, blocking the filter's pores. Matrigel is however a mouse derivative. The Humane Research Trust provided the funding for this investigation and the aim of this charity is to promote the reduction of the use of animals and animal products in experimentation. At the end of this investigation, with an aim of producing a comparable equivalent to Matrigel for future use, invasion of SOM 196B through membranes pre-coated with Matrigel or a freshly prepared ECM solution was compared (without an endothelial cell layer). The ECM solution was made up using equal concentrations of type IV collagen, fibronectin, and laminin. Comparable levels of invasion were seen between Matrigel and ECM-coated membranes (35.7 (± 2.92) and 34.6 (± 1.80) cells/field at x400 magnification, respectively) and no significant differences were found ($P>0.05$). Due to time constraints this assay was only repeated once, but another colleague in the laboratory (Ms Shona Elshaw) was able to reproduce similar results.

4.2.2. Transendothelial cell invasion of uveal melanoma STCs

Once all parameters had been established using the cell line SOM 196B, transendothelial cell invasion of a further 16 STCs was investigated. A description of the precise methodology is given in Methods 2.3.2. All STCs were used within five passages of being established in culture and assays were repeated once only due to insufficient cell numbers. The results of the transendothelial cell invasion studies on STCs are summarised in Figure 4.2.2.a and represent the mean invasion for tumours with and without the inclusion of a dermal endothelial barrier. Data is expressed in terms of mean number of cells invading per field of view at x400 magnification, after counting ten fields of view for triplicate wells. In all assays, appropriate positive and negative controls were included. Tumour cell invasion through basement membrane components only acted as positive controls ('standard invasion levels'), whilst endothelial cells alone acted as negative controls.

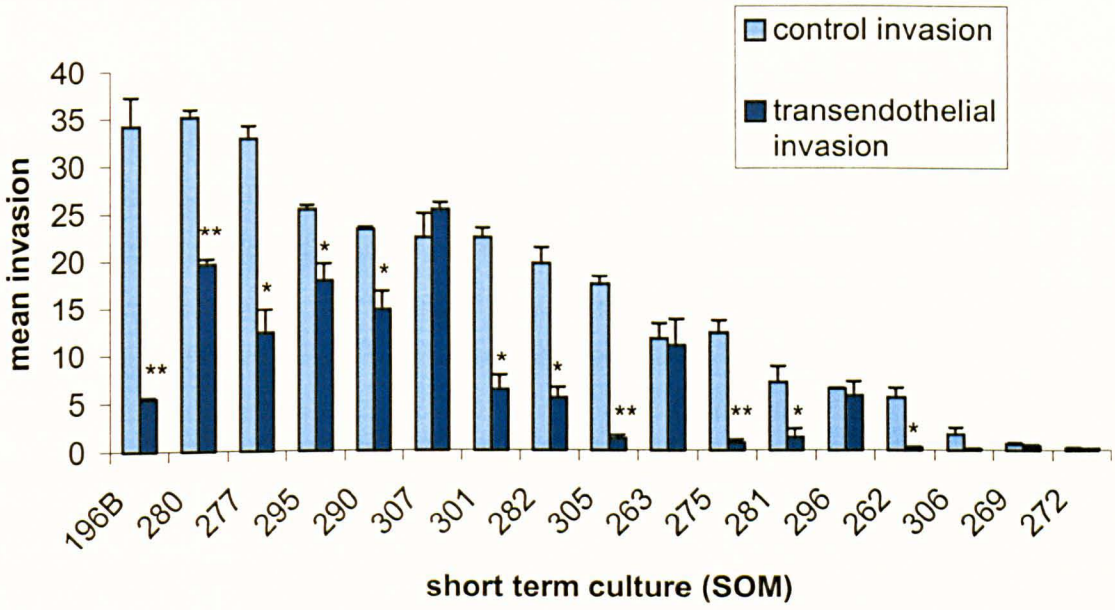


Figure 4.2.2.a. Invasion of primary uveal melanoma cultures through the transendothelial cell assay. Expressed as mean counts per field of view under x400 magnification after counting ten fields of view for triplicate wells. Mean (± SEM) invasion 8μM pores after 24 hours. For SOM 196B, counts represent the mean of three experiments; for STCs, counts represent the mean of one experiment, counting triplicate wells. The x-axis shows the short-term culture (SOM) and the y-axis shows the mean invasion under x400 magnification. *, P<0.05 and **, P<0.001 when compared to positive controls (tumour cells alone).

In wells with endothelial cells alone, maximum endothelial cell invasion was 2-3 cells/well and was considered negative. Using the ANOVA statistical test, inclusion of an endothelial layer resulted in a significant reduction in invasion (P<0.05) of SOM 196B, 262, 275, 277, 280, 281, 282, 290, 295, 301 and 305 when compared with invasion through the basement membrane alone. In relative terms, the invasive tumour cell populations of these cultures might therefore potentially be less capable of overcoming the endothelial cell barrier. Conversely, invasion of cultures that was not significantly reduced by the addition of an endothelial cell barrier (P>0.05) (SOM 263, 269, 272, 296, and 307) may infer the presence of invasive tumour cell populations that are more efficient at overcoming such barriers. Three tumours (SOM 269, 272 and 306) were considered non-invasive in both assays. For SOM 307 a higher number of cells invaded in the transendothelial cell assay, when compared with the number seen after invasion through the basement membrane only (25.3 ± 0.76 SEM and 22.4 ± 2.53 SEM, respectively). This raised the possibility that both cell types could have been co-migrating

and to further assess this, a selection of tumours (SOM 196B, 295, 301, 306 and 307) were labeled with fluorescent probes and tracked. The results from this are presented in 4.2.3.2.

Most tumours had only recently been excised, so it was therefore not possible to correlate behaviour *in vitro* with the clinical course of the disease. When the 'control' invasion of cultures through the basement membrane alone was however compared with histo-pathological data (Appendix II), it was apparent that no correlation could be made between prognostic indicators and the relatively high invading melanomas (with invasion levels of over 20 cell counts per field [SOM 196B, 277, 280, 282, 290, 295, 301, 305 and 307]). However, when comparing invasion of all STCs through the transendothelial cell assay with the histo-pathological data, a closer correlation could be made with prognostic indicators. For example some of the invasive tumours, for which invasion levels were over 50% of the control levels (SOM 280, 295, and 296), were on average larger tumours than the most invasive tumours through the control basement membrane only assays. These tumours were also more likely to be of a mixed cell type and with ciliary body involvement. Conversely other tumours (SOM 263, 290, and 307) reflecting these invasion patterns were nonetheless very small in volume, and one (SOM 295) was of spindle morphology; factors associated with good prognosis. The test sample was therefore too small to make reliable correlations.

4.2.3. Monitoring tumour cell invasion

4.2.3.1. Scanning Electron Microscopy

To identity the invaded cells, scanning electron microscopy was initially used to visualise the process of transendothelial cell invasion process using SOM 196B and HDMECAs. Co-cultures were set up as previously described (Methods 2.3.2) but endothelial and non-invaded tumour cells were not removed prior to processing. Observations of the upper side of the membrane (non-invaded cells), in wells with endothelial cells alone or with co-cultures, confirmed the presence of a confluent layer of cells covering the surface on the membrane, uniformly coated with Matrigel, covering membrane pores. On the lower side of the membrane (invaded surface), occasional invaded cells were visible, but due to gross phenotypic similarities of tumour and endothelial cells (such as physical size), it was not possible to determine the nature of these cells. Figure 4.2.3.a illustrates a cell migrating from the upper surface towards the under side of the membrane.

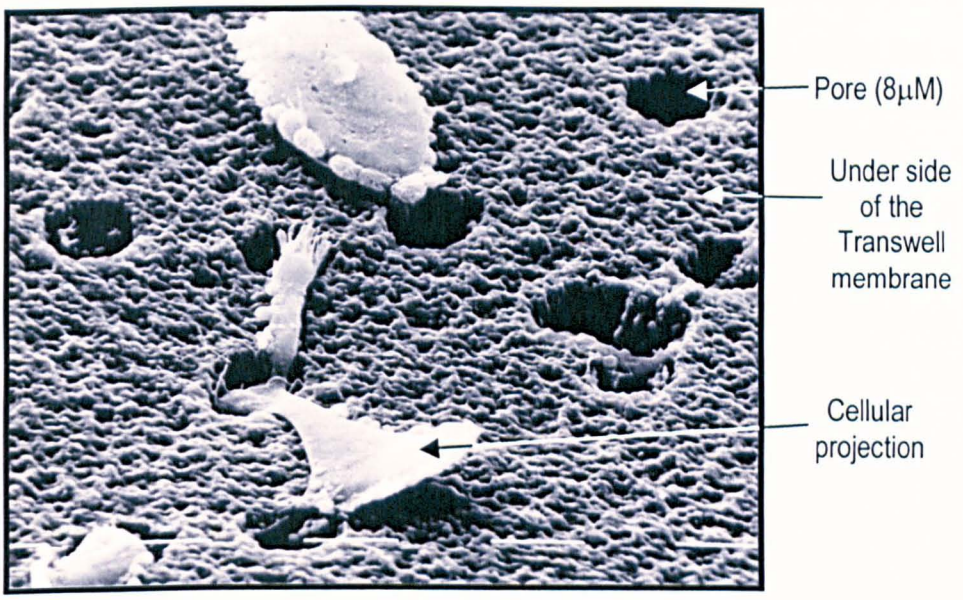


Figure 4.2.3.a. Scanning electron micrograph from a co-cultured well. The under (invaded)-side of the Transwell membrane can be seen, with cellular projections through the 8μM pores (x1250 magnification).

4.2.3.2. Tracing cell movement

In order to trace movement of both cell types in the transendothelial cell assay, in all cases tumour and endothelial cells were independently labelled with CFDA-SE and SNARF-1. This procedure was carried out on SOM 196B, and four STCs (SOM 295, 301, 306 and 307), and compared with the cutaneous melanoma cell line, A375 and the lung carcinoma cell line, A549. Due to insufficient cell numbers in most STCs, only four cultures were pre-labelled. Experiments were set up in triplicate with appropriate controls (tumour cells invading the basement membrane alone or endothelial cells only) and non-invaded cells were again removed. By observing wells prior to the removal of non-invaded cells, endothelial cell confluence could again by confirmed (Figure 4.2.1.c), whilst also allowing observation of tumour cell interactions with the endothelium (Figures 4.2.3.b.i and ii). As this procedure was only used to identify invaded cell populations, no quantitative measurements were made.

Cell lines

When comparing SOM 196B with A375 it was evident from previous studies that levels of invasion through basement membranes alone were comparable (34.2 ± 3.08 SEM and 35.9 ± 3.49 SEM,

respectively). In the transendothelial cell assay, numbers of invaded cells were however 5.45 ± 0.12 SEM and 33.3 ± 0.75 SEM (Figure 4.2.3.b.iii), respectively. By labelling cells, it was evident that a small number of endothelial cells were present on the invaded side of the membrane in the SOM 196B assay (Figure 4.2.3.b.v). In contrast no endothelial cells were seen in the A375 assay, and A375 cells alone had invaded through the endothelial cell barrier (Figure 4.2.3.b.iii). Using A549 in this assay, it became clear that these cells are comparatively less invasive as few tumour cells were observed on the lower side of the membrane in both the standard and transendothelial cell invasion assays. Furthermore, no endothelial cell migration was seen when A549 cells were used in the transendothelial cell assay.

STCs

To substantiate the results using cell lines, four STCs were also pre-labelled and cell movement traced. In agreement with the pattern shown by A549, SOM 306 was non-invasive, with negligible numbers of tumour cells migrating (0.1 ± 0.1) and no endothelial cell movement. Phenotypically, SOM 306 varied from most cultures as cells appeared to lack any cellular projections and consequently few interactions with endothelial cells were evident (Figure 4.2.3.b.i). SOM 301 was invasive through basement membrane barriers alone, yet invasion was significantly reduced in the transendothelial cell assay ($P<0.05$) and no endothelial cell co-migration was observed. Both SOM 295 and SOM 307 were considered invasive in the transendothelial cell assay, but similar to SOM 196B, a small percentage of endothelial cells were seen to co-migrate (Figures 4.2.3.b.iv and vi). With regard to SOM 307, co-migration of tumour and endothelial cells might therefore explain the increased invaded cell number observed in the previous transendothelial cell assay in which no distinction was made between invaded cell types (4.2.2).

Figure 4.2.3.b. Photographic images of uveal melanoma and endothelial cells in the transendothelial cell invasion assay (for figure, see following page). Tumour cells were labelled with CFDA-SE (green) and endothelial cells were labelled with SNARF-1 (red) and observed under fluorescence at 492-517nm and 580-640nm, respectively.

i) Tumour cell interactions (SOM 301) with the endothelium on the non-invaded (upper) side of the membrane (x100 magnification).

ii) Tumour cell interactions (SOM 306) with the endothelium on the non-invaded (upper) side of the membrane (x100 magnification).

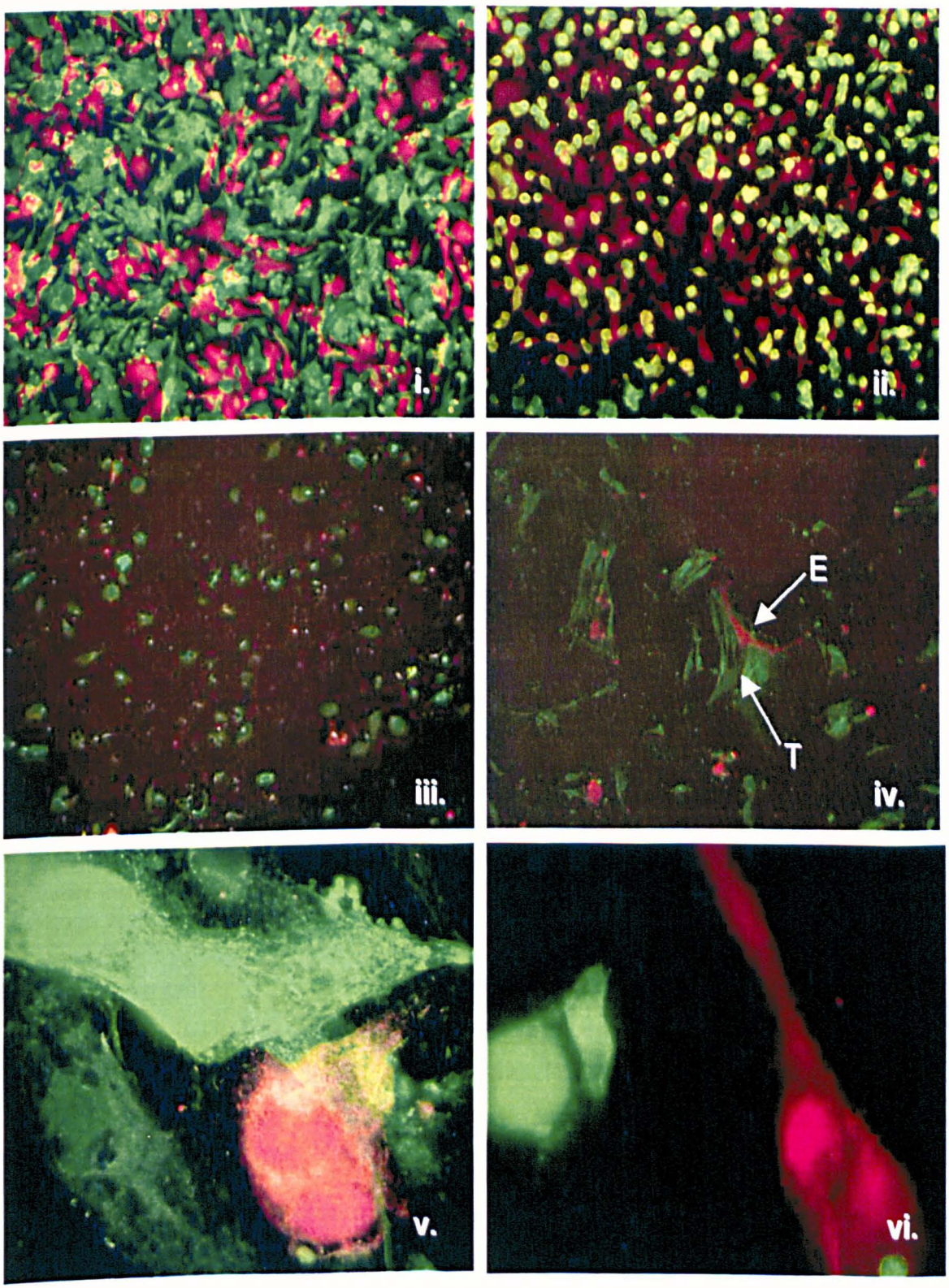
iii) A375 cells on the invaded (under) side of the membrane after invasion through endothelial and basement membrane barriers, showing tumour cell invasion only (x100 magnification). Endothelial cells and non-invaded tumour cells had been previously removed from the non-invaded surface.

iv) SOM 295 cells on the invaded (under) side of the membrane after invasion through endothelial and basement membrane barriers, showing co-migration of tumour (T) and endothelial cells (E) (x100 magnification) (indicated by the white arrows). Endothelial cells and non-invaded tumour cells had been previously removed from the non-invaded surface.

v) SOM 196B cells on the invaded (under) side of the membrane after invasion through endothelial and basement membrane barriers, showing co-migration of tumour and endothelial cells (x1000 magnification). Endothelial cells and non-invaded tumour cells had been previously removed from the non-invaded surface.

vi) SOM 307 cells on the invaded (under) side of the membrane after invasion through endothelial and basement membrane barriers, showing co-migration of tumour and endothelial cells (x1000 magnification). Endothelial cells and non-invaded tumour cells had been previously removed from the non-invaded surface.

Figure 4.2.3.b. For figure legend, see previous page. Tumour cells (T) are labelled green and endothelial cells (E) are labelled red.



4.3. Discussion

4.3.1. The transendothelial cell model of invasion

Figure 4.3.1.a illustrates how the *in vitro* model of transendothelial cell invasion mimics the process *in vivo*. In its true sense the model is a model of extravasation, but mimicking intravasation also, would be impracticable in this system, as addition of a basement membrane layer over the endothelium would be difficult to manipulate.

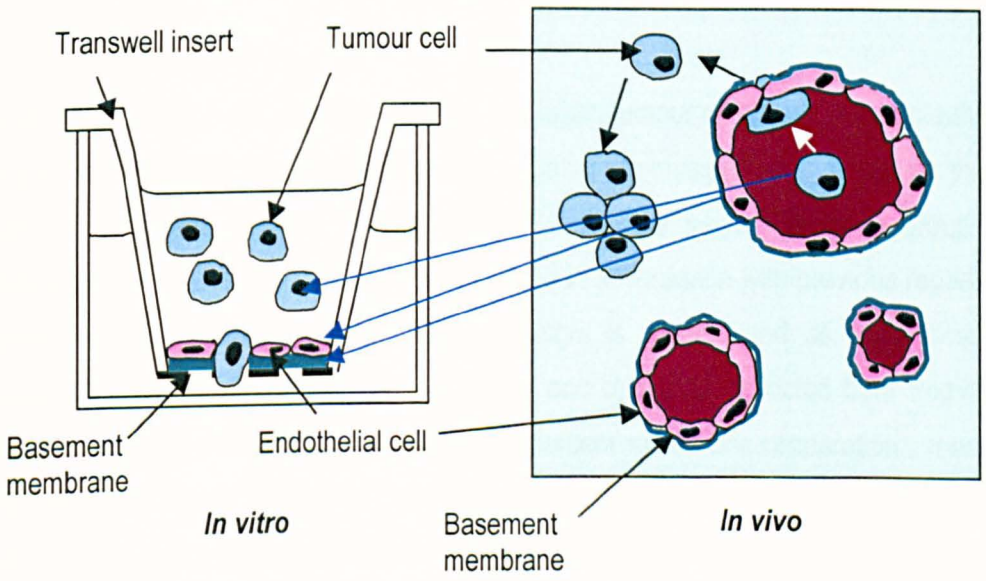


Figure 4.3.1.a. ***In vitro*** **Transwell representation of transendothelial cell invasion** ***in vivo*****.** During extravastion, tumour cells must migrate between endothelial cells and invade through the underlying basement membrane. Tumour cells are shown in blue. Endothelial cell are shown in pink. A green line represents basement membranes. The light blue areas represent normal tissue.

Membrane pore size

To model *in vivo* transendothelial cell invasion, tumour cells must invade through an intact endothelial cell barrier. Similar models of transendothelial migration for leucocytes have used membranes with 3μM pores (Mohle *et al.*, 1997). As endothelial cells are physically too large to migrate through pores of 3μM, when seeded at the correct density endothelial cells form a confluent monolayer and are unable to migrate. Leucocytes are smaller in size and thus are able to migrate through both the endothelial cell layer and the 3μM pores. Results from this current study have however shown that

generally tumour cells are considerably larger in diameter and thus are unable to migrate through the 3μM pores in any great number (Figure 4.2.1.a). In agreement with this study, other transendothelial cell models of tumour invasion have also used 8μM pore membranes (Li and Zhu, 1999; Laferriere *et al.*, 2001). The obvious disadvantage with such models is the possibility of endothelial cell migration through pores of this size, and previous studies have often not considered this problem. In this study by tracing movement of labelled tumour and endothelial cells, co-migration of the two cell types was occasionally observed. This phenomenon of co-migration must therefore be taken into account in *in vitro* assays and will be discussed in further depth below.

Artificial basement membranes

Prior to invasion through the vessel endothelial cell layer, tumour cells must overcome the basement membrane, together with the surrounding ECM. Certain confusion exists regarding the distinction between migration and invasion assays with the definitions varying between groups. For the purposes of these studies, the difference was defined in accordance with previous reports relating to the barrier imposed (Youngs *et al.*, 1997). Matrigel is widely used as an artificial basement membrane (Scott *et al.*, 2001; Voura *et al.*, 2001a and b) and is extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma as a solubilised basement membrane preparation. It was originally demonstrated the levels of tumour cell invasion *in vitro*, were inversely proportional to the concentration of Matrigel used as a barrier (Albini *et al.*, 1987). However, other contradictory reports have demonstrated that invasion *in vitro* through Matrigel could not be correlated with invasion *in vivo* (Simon *et al.*, 1992).

Advantages of the use of Matrigel nevertheless include its ease of reproducibility and commercial availability. The concentration recommended by the manufacturers instructions ensured that the Matrigel layer formed a physical barrier for invasion coating the porous membrane, and uniformly covering the filter's pores. Matrigel does however have its disadvantages. For example, various growth factors have been identified in Matrigel (Taub *et al.*, 1990; Vlodavsky *et al.*, 1996) which are likely to differ from human basement membranes, and could have attracted invading tumour cells. To address this, the use of growth factor-reduced Matrigel may provide a more realistic representation. In addition, unlike human basement membranes, which have a high type IV collagen content, the major substrate in Matrigel is laminin (Kleinman *et al.*, 1986; McGuire and Seeds, 1989). Matrigel

also does not possess cross-linked collagens nor does it have the ordered structure of the basement membrane.

Due to the funding for this project, to establish a system less reliant on animal resources, an alternative ECM was constructed composed of equal concentrations of type IV collagen, fibronectin, and laminin. The advantages of such an ECM would be defined constituent concentration more in keeping with the *in vivo* state (Kleinman *et al.*, 1986), and as human components were used, animal use was reduced. Using SOM 196B, no significant differences were observed between the invasion assays using Matrigel or ECM solution ($P>0.05$), therefore validating its use for these purposes. There are however still limitations with this model, as *in vivo* the proportions of these substrates is unlikely to be 1:1:1 and other critical structural proteins and soluble factors are likely to be present. The ECM solution can nevertheless be further refined by the inclusion of additional substrates.

As an alternative to Matrigel, invasion assays have been reported employing the use of intact human amniotic membranes (Gehlsen *et al.*, 1992; Siegel *et al.*, 1993; Singh *et al.*, 1996), and some correlation has been made with invasiveness *in vivo* (Yagel *et al.*, 1989; Gehlsen *et al.*, 1992). Basement membranes are nevertheless known to vary in their composition between tissues, and even within different structures of the same tissue, and are also likely to differ between malignant and non-malignant tissue (Lang *et al.*, 1999; Lang *et al.*, 2000). With regard to uveal melanoma invasion, even human amniotic membranes will therefore not fully represent the situation *in vivo*, besides which, such membranes would also be difficult to acquire.

Endothelial cell layer

Inclusion of an endothelial cell layer would make *in vitro* invasion assays more realistic and as no endothelial cell migration was seen in wells with endothelial cells alone, it was expected that cells would form a barrier through which tumour cells actively penetrated. Confluent endothelial cells would have also secreted additional basement membrane proteins, thus increasing the obstacle for the invading cells (Sage and Bornstein, 1982). A recent study reported the use of a similar transendothelial cell invasion system with bovine aortic endothelial cells (large vessel endothelial cells), assessing transendothelial migration of seven human malignant and non-malignant cell lines (Li and Zhu, 1999). The authors however described the formation of confluent endothelial cell

monolayers on both the upper and lower surfaces of the membrane. This phenomenon was not observed in the *in vitro* model presented here (confirmed by fluorescent labelling), possibly due to the use of endothelial cells derived from a different source. In addition, comparable levels of transendothelial invasion through dermal and hepatic endothelial cells was observed ($P>0.05$), and thus a model using dermal endothelial cells as a barrier to uveal melanoma cells may therefore still be representative of the situation *in vivo*. Further data assessing potential differences between dermal and liver endothelial cells used in this study, is presented in Chapter 6.

Monitoring cellular movement

During angiogenesis, endothelial cells are known to migrate (reviewed by Folkman, 1995), and this had to be considered in the transendothelial model. Reports are scarce in which authors have assessed the possibility of endothelial co-migration, but limited evidence suggests that this phenomenon is not occurring in such assays (Okada *et al.*, 1994). In this present study, due to few phenotypic differences between the two cell types, no conclusions could be drawn regarding the nature of the invaded cells using scanning electron microscopy (Figure 4.2.3.a). Labelling cells with fluorescent probes however identified a degree of endothelial co-migration, but this was dependent upon the tumour type, and specific culture (Figure 4.3.2.b). These probes have been previously shown to have no effect on cellular adhesion to endothelial cells (Davenport *et al.*, 1995) and should thus have had little influence on the transmigration process. It is feasible that the co-migration of endothelial cells in these cases is a passive process, in which endothelial cells follow invading tumour cells through spaces in the basement membrane formed through tumour cell degradation. Alternatively, tumour cells could stimulate endothelial cell migration, involving either secreted tumour-derived factors, or cell-cell interactions between aggressive tumour and endothelial cells, reflecting inherent properties of the particular invasive uveal melanoma cells. Due to time restrictions it was not possible to explore this further, but it would be of interest to study this phenomenon with a wider range of uveal melanoma STCs and other cell types. As only a relatively small degree of endothelial co-migration was observed limited associations could still be drawn.

4.3.2. Transendothelial cell invasion of uveal melanoma STCs

In general STC invasion in the transendothelial cell model, seemed to correlate more closely with a wider range of known prognostic indicators than invasion through the basement membrane alone (Elshaw *et al.*, 2001). There were however some exceptions, which may reflect behaviour seen *in vivo* since not all tumours behave in accordance with prognostic indicators (Mooy *et al.*, 1996). Long-term follow-up is however required to draw more definite conclusions, as for most tumours studied, survival data was limited to an average of 12 months and correlation with long-term survival for tumours was not possible. In agreement with published data, suggesting a correlation between transendothelial invasion *in vitro*, with metastatic potential *in vivo* (Li and Zhu, 1999), it is nevertheless of interest that one tumour (SOM 196B), was resected 41 months previously, and the patient is known to still be alive and disease free. Once in culture, this tumour invaded well without the inclusion of an endothelial cell layer. Yet invasion was significantly reduced ($P<0.05$) in the transendothelial cell invasion assay.

It is widely understood that the metastatic process is highly inefficient (reviewed by Weiss, 1990). Only a small percentage of cells in the primary neoplasm will acquire the necessary phenotype required to facilitate successful extravasation. Once tumour cells have disseminated, only a fraction will reach the secondary site, and continue growth to become eventual metastatic foci. *In vivo* studies have shown that only 0.01% of tumour cells injected into the circulation will progress to form metastatic colonies (Fidler, 1970). In previous invasion assays, tumour cells have been considered to be invasive if they are capable of traversing basement membrane barriers alone. In contrast, in the transendothelial model, cells have been considered to be invasive if they are able to overcome both the endothelial cell and basement membrane barriers together. Evidence from this study would suggest that tumours that are more effective at transendothelial invasion than others (for example SOM 280 and SOM 295) would equate more closely with highly aggressive tumours in which percentages of greater than 0.01% have the propensities to metastasise.

The *in vitro* model itself has limitations. *In vivo* during intravasation, tumour cells are separated from the endothelium with a basement membrane, whereas in this *in vitro* model, there is no segregation. Many factors also influence the establishment of metastases and thus although tumour cells may be capable of overcoming both barriers, aspects such as the effect of the host stroma, survival in the

bloodstream and proliferation in the target organ cannot be assessed by such a model. For example, surrounding stromal cells effect invasion of cutaneous melanoma and prostate cancer cells, as shown in recent reports using alternative *in vitro* invasion models (Eves *et al.*, 2000; Hall *et al.*, 2002).

It has now been reported that tumour cells might not simply migrate through the endothelial cell lining of blood vessels. In colon cancer a small sub-population of tumour cells are present in newly formed blood vessels (Chang *et al.*, 2000). The authors of this study suggest that these 'mosaic vessels' are formed during neovascularisation, where unstable junctions are formed between new endothelial cells allowing tumour cells to participate in the vessel wall formation. Tumour vascularisation is a dynamic process with ongoing migration and formation of capillaries that allows some tumour cells to take up temporary residence in the vessel wall prior to their transit into the lumen (reviewed by Folkman, 2001). The wider relevance of these 'mosaic vessels' in metastasis of other tumours remains to be established.

As yet, mosaic vessels have not been reported in uveal melanoma but vascular channels conducting blood that are entirely lined by tumour cells have been recently reported (Timar and Toth, 2000); a phenomenon known as vasculogenic mimicry which is distinct from the significance of mosaic vessels (Maniotis *et al.*, 1999). These authors concluded that aggressive uveal melanomas *in vitro* and *in vivo*, acquired the capability to form vascular channels that are not lined by endothelial cells providing a blood supply for growth and metastasis, functioning independently from angiogenic vessels. This evidence is however controversial and alternative reports have suggested that even in aggressive uveal melanomas, the majority of blood flow occurs via endothelial-lined vessels as opposed to vessels comprised of tumour cells (McDonald and Foss, 2000). It is nevertheless still feasible that uveal melanoma metastasis is more complex than originally thought, potentially utlising a number of mechanisms to successfully establish metastases.

In conclusion, although a small number of uveal melanomas have been studied, invasion through the transendothelial model appears to correlate more closely with a wider range of prognostic indicators than invasion through the basement membrane alone. Long-term follow-up of the patients studied will elicit more relevant information.

Chapter 5

Tissue targeting of metastatic uveal melanoma cells

5.1. Introduction

The pattern of metastasis commonly observed in uveal melanoma raises the possibility that the hepatic environment attracts metastatic tumour cells, promoting secondary development. Conversely, such cells might not be attracted to other sites and their arrest may even be inhibited. In the rare instances where cells are seen to metastasise to organs other than the liver, a specific phenotype might allow successful arrest and growth at these sites. Alternatively, other organs could attract metastatic tumour cells, but instead tumour cells are less adept at growing elsewhere, and although the liver is commonly associated with secondary disease, it remains unclear as to whether metastatic uveal melanomas, given sufficient time, could also eventually grow elsewhere.

The liver is the largest organ in the body fulfilling a variety of essential functions, including the metabolism of nutrients and drugs and the synthesis of a number of metabolites and proteins. Despite being the targets for many regulatory factors, cells of the liver have also been found to secrete a range of cytokines, growth factors, and chemokines, which may be imperative for metastatic development. Kupffer cells, stellate cells and infiltrating leukocytes have commonly been reported to be the major source of these, including IL-1, IL-8, and TNFα (reviewed by Ramadori and Armbrust, 2001) and have been associated with secondary growth of malignancies such as colorectal carcinomas (Gangopadhyay *et al.*, 1996; Minami *et al.*, 2001). Secretion of factors including HGF, IGF-I, and IGF-II, by the hepatic sinusoidal endothelium has also been reported (Rak *et al.*, 1996), which whilst attracting tumour cells, could also ultimately promote attachment. Hepatocytes, which constitute the major cell type of the liver have generally been associated as target cells for regulators secreted by other cell types in the liver but evidence has nevertheless been published reporting synthesis of factors such as IGF-I, IGF-II, IL-8, GRO, RANTES and TNF-α (Rowell *et al.*, 1997; Long *et al.*, 1998a and b). . The liver therefore represents an excellent 'soil' for many secondary cancers.

Many inflammatory mediators regulate directional migration and invasion of both leucocytes and tumour cells (McKenzie, *et al.*, 1994; Krasagakis, *et al.*, 1995; Luan, *et al.*, 1997; Kunz, *et al.*, 1999). For example, both HGF and its receptor c-met, have been linked with cutaneous melanoma progression (Hamoen *et al.*, 2001), whilst expression of specific chemokine receptors by both cutaneous melanoma and breast carcinoma cells has been shown to be potentially responsible for cells targeting specific sites (Muller *et al.*, 2001). For uveal melanoma, both HGF and EGF increase

invasion (Hendrix *et al.*, 1998a and b; Ma and Niederkorn, 1998), and expression of EGF and IGF-I receptors has been correlated with death from metastatic liver disease (Monique *et al.*, 2000; Ericsson *et al.*, 2002). As metastatic uveal melanoma cells therefore appear to target the liver in most cases, further investigation into a wider range of factors that promote and inhibit uveal melanoma migration and invasion is essential for the understanding of uveal melanoma metastasis.

Aims of the study

To determine if the liver favourably contributes to this specific pattern of metastasis through the production of stimulatory regulators, invasion of a series of uveal melanoma cultures was investigated in response to soluble factors secreted by cells derived from the liver. Results were compared with cells from sites less frequently colonised, to assess whether factors secreted by other tissues would have a similar effect on invasion.

5.2. Results

5.2.1. The effect of HepG2 cells and CM on the invasion of uveal melanoma cell lines and STCs

As metastatic uveal melanoma cells primarily target the liver, to assess the consequence of soluble factors secreted by cells derived from hepatocytes, the effect of HepG2 cells and CM on the invasion of SOM 196B, SOM 157d, and a series of seven STCs was investigated. SOM 196B had already been established as an invasive cell line in standard assays through Matrigel barriers, whilst SOM 157d was poorly invasive. Results of initial studies on the effect of HepG2 cells and CM on STC invasion are represented in Figure 5.2.1.a. Basal levels of invasion *in vitro* (without any stimulation) through Matrigel barriers alone varied between STC. Cultures were broadly classified as 'non-invasive' (SOM 157d, 269, 272 and 280); 'weakly invasive' (SOM 263 and 275) and 'highly invasive' (SOM 196B, 267 and 277).

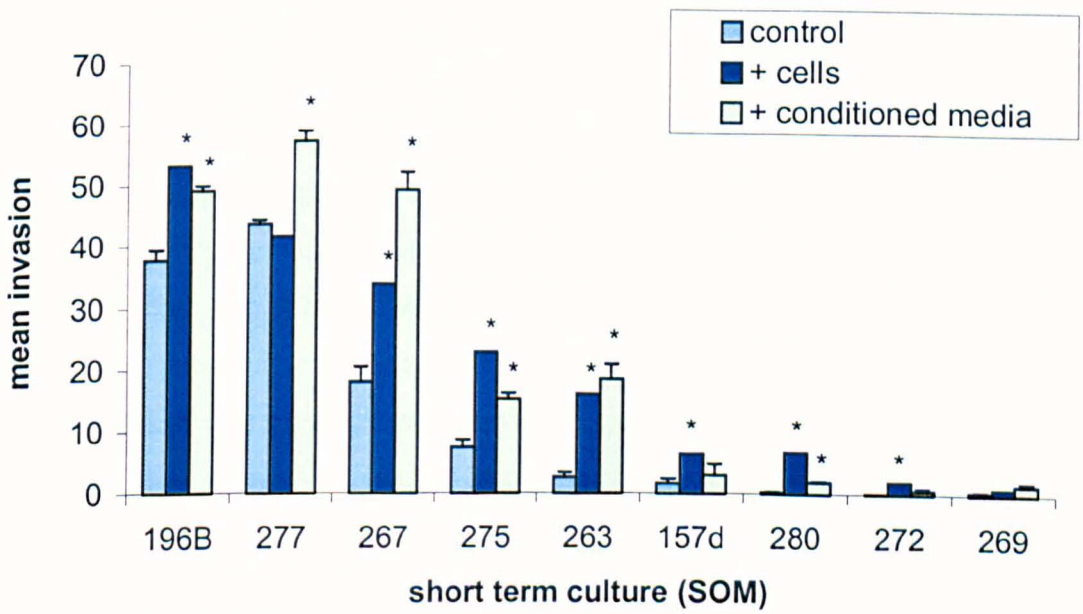


Figure 5.2.1.a. Effect of HepG2 cells and CM on STC invasion. Expressed as mean counts per field of view under x400 magnification. Mean (± SEM) invasion 8μM pores after 24 hours. Results with cell lines (SOM 157d and SOM 196B) represent the mean of three experiments; experiments with short-term cultures represent the mean of ten fields of view for triplicate wells from one experiment. The x-axis shows the Sheffield Ocular Melanoma (SOM) and the y-axis shows the mean invasion through Matrigel under x400 magnification. *, $P<0.05$ when compared with control invasion (without stimulation of invasion). SOM 196B, 267, and 277 were considered 'highly invasive'; SOM 263 and SOM 275 were considered 'weakly invasive'; SOM 157d, 269, 272, and 280 were considered 'non-invasive'.

Highly invasive cultures

Both HepG2 cells and CM significantly increased invasion of SOM 196B and SOM 267 ($P<0.05$) whilst HepG2 CM only significantly increased invasion of SOM 277 ($P<0.05$). Instead, invasion levels of SOM 277 decreased with the addition of HepG2 cells to the assay. The decrease seen was nevertheless negligible ($P>0.05$) when compared with control invasion of tumour cells without any stimulation and may have been a result of experimental error. Figure 5.2.1.b shows photographic images of the effect of HepG2 cells and CM on the invasion of SOM 267.

Non- and weakly invasive cultures

HepG2 cells but not CM significantly increased invasion of SOM 157d. This pattern was also observed for the non-invasive culture SOM 272, whilst for SOM 263, 275, and 280, both HepG2 cells and CM significantly increased invasion ($P<0.05$) (Figure 5.2.1.a). Neither HepG2 cells nor CM significantly increased invasion of SOM 269 ($P>0.05$) and under all experimental conditions, cells were still only weakly invasive (increasing from 0.433 ± 0.120 to $0.9 \pm 5.75 \times 10^{-5}$ and 1.5 ± 0.451, respectively).

With the exception of SOM 269, non- and weakly invasive cultures (SOM 157d, 263, 272, 275, and 280) presented proportionally higher responses, when compared with highly invasive cultures (SOM 196B, 267 and 277). For example, when comparing the outcome observed with the two cell lines, the proportional effect seen of HepG2 cells on invasion levels of SOM 157d was nevertheless greater than for the invasive culture SOM 196B (increasing invasion from 1.64 ± 0.754 to 6.32 ± 0.00167, and 37.8 ± 1.66 to 53.2 ± 0.00306, respectively). This was in agreement with a comparable study by Ms Shona Elshaw, in which the effect of defined factors on invasion was investigated. Stimulatory factors were shown to cause a far greater response on non-invasive cells when compared with invasive cells. With regard to all of the non- and weakly invasive cultures described in this current study, even upon stimulation, only small numbers of cells were nevertheless seen to invade. In addition, no true associations could be made between these results, histo-pathological data, and clinical outcome (Appendix II).

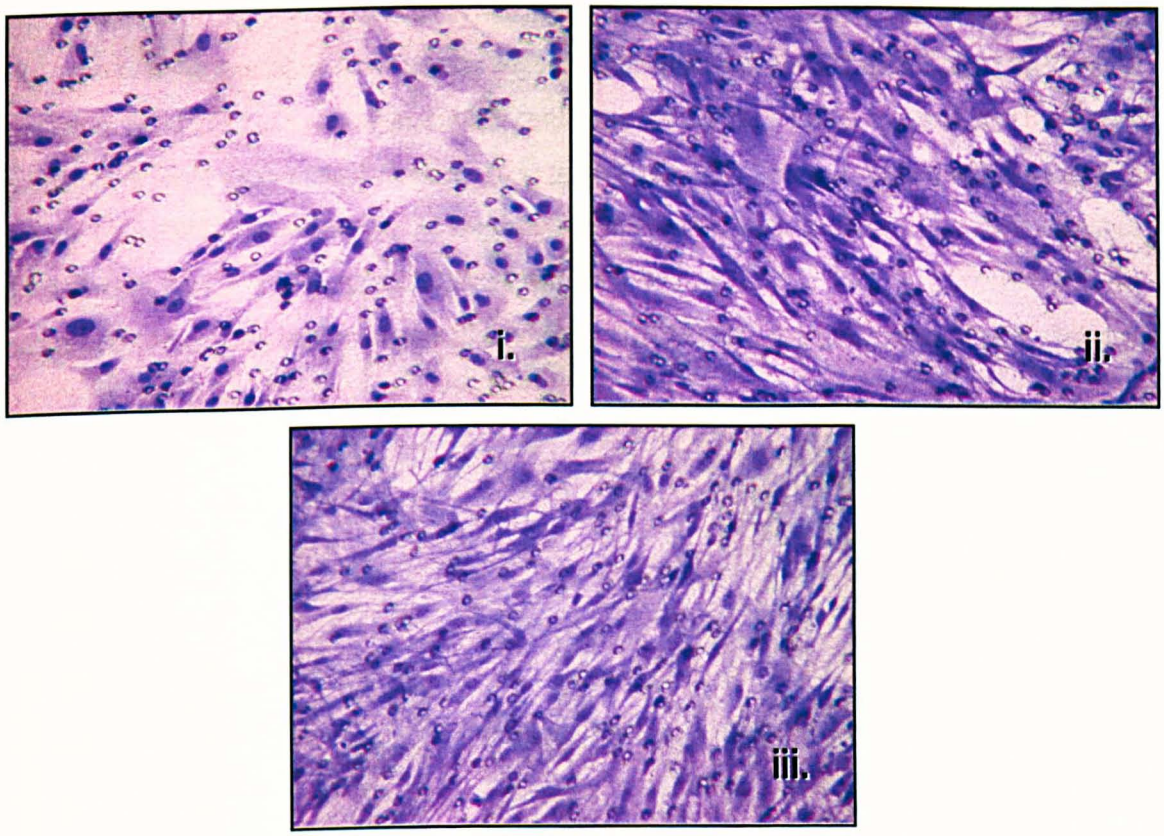


Figure 5.2.1.b. Photomicrographs of the invasion of SOM 267 without stimulation (i), and after stimulation with HepG2 cells (ii), or CM (iii). Both HepG2 cells and CM stimulated SOM 267 invasion. All images show invaded cells on the underside of the 8mM pore Transwell membrane (x100 magnification). Nuclei are stained with Gill's haematoxylin, and all non-invaded cells have been removed.

Comparing the effect of HepG2 and A495 cells and CM on STC invasion

To briefly assess whether the effect of HepG2 cells and CM on STC invasion was specific to cells of hepatic origin, the effect of cells from a site not commonly colonised by uveal melanoma cells was also assessed. For this purpose the consequence of A549 cells (a lung carcinoma cell line) and CM on the invasion of two STCs (SOM 277 and SOM 280) was investigated and the effect compared with that of HepG2 cells and CM (Figure 5.2.1.c). Both STCs were used at passage four to enable a direct comparison to be made and were chosen due to their invasive abilities *in vitro*. The invasive nature of SOM 280 appeared to vary with increasing passage and this would account for differences in levels of invasion seen between this and the previous study (4.2.2).

Invasion levels of SOM 277 significantly increased in response to HepG2 CM ($P<0.05$) (Figures 5.2.1.a and c). As described previously, in contrast, in response to HepG2 cells a slight decrease in invasion of SOM 277 cells was seen, but this result was not significant ($P>0.05$), and may have been as a result of slight experimental variation. This pattern was nevertheless reflected with the inclusion of A549 cells and CM in the assay, but the decrease in invasion seen by SOM 277 cells in response to A549 cells was highly significant, decreasing from 40.6 ± 1.07 to 26.5 ± 2.00 ($P<0.05$). In comparison, A549 CM slightly increased invasion of SOM 277, but the increase was negligible ($P>0.05$) (Figure 5.2.1.c).

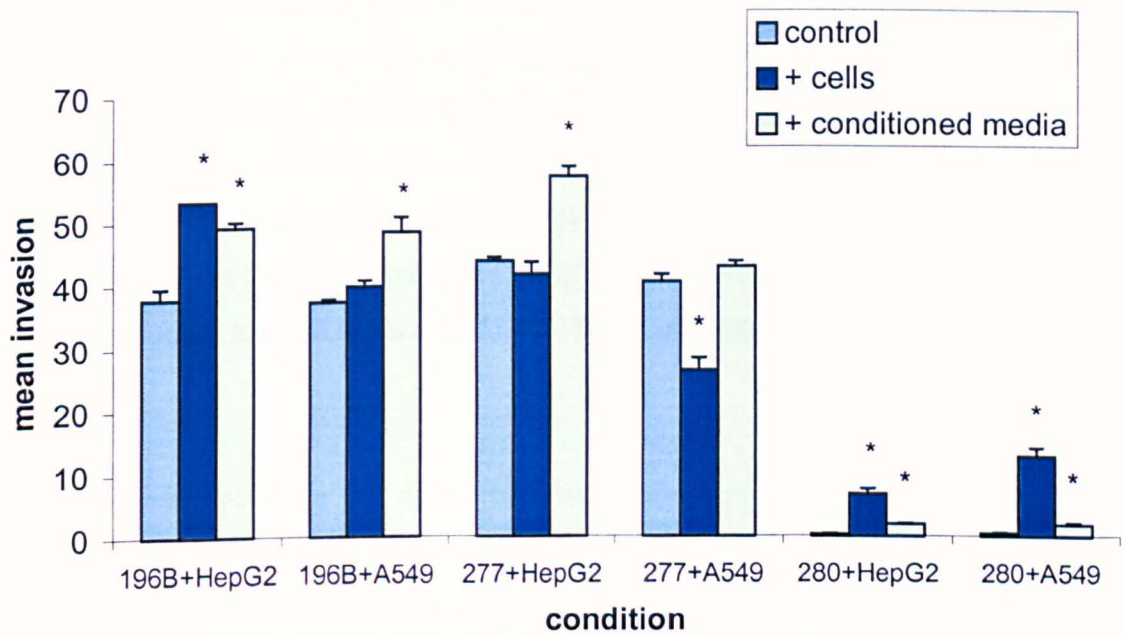


Figure 5.2.1.c. Effect of HepG2/A549 cells and CM on the invasion of SOM 196B, 277 and 280. Expressed as mean counts per field of view under x400 magnification. Mean (± SEM) invasion 8μM pores after 24 hours. Results with SOM 196B represent the mean of three experiments, and are included as positive controls; experiments with SOM 277 and 280 represent the mean of triplicate wells from one experiment. The x-axis shows the short-term culture (SOM) used with either HepG2 or A549 cells or CM. The y-axis shows the mean invasion through Matrigel under x400 magnification. *, $P<0.05$ when compared with control invasion (without stimulation of invasion).

For SOM 280, a non-invasive culture *in vitro*, CM from and the cells of both HepG2 and A549 cells, significantly increased invasion ($P<0.05$) (Figures 5.2.1.a and c). In contrast to SOM 277, for both HepG2 and A549, responses seen by the inclusion of cells in the lower chamber of the assays, were considerably greater when compared with the inclusion of CM. Similar to other STCs, responses of the non-invasive culture SOM 280 were proportionally higher, when compared with the invasive

culture SOM 277. With regard to histo-pathological data (Appendix II), both patients presented with tumours with similar characteristics, and thus no association could be made between the behaviour *in vitro* in response to HepG2 or A549 cells or CM, and prognostic indicators.

5.2.2. The effect of cells and CM from alternative cell types on the invasion of SOM 196B

In addition to hepatocytes, alternative cells of the liver such as hepatic endothelial cells may also influence invasion. As primary liver endothelial cells (HULECs) were obtainable for study, such cells were chosen. Other cell types present in the liver, such as Kupffer cells, were not used due to a lack of an available source. To therefore establish if chemoattractants specifically associated with the endothelium of the liver may contribute to the specific pattern of spread seen in uveal melanoma, the study was extended to assess the effect of the HULECs and the respective CM on invasion of SOM 196B. As cell numbers were limited with STCs, invasion of this cell line only was assessed at this stage. Results of this study are represented in Figure 5.2.2.a and represent the mean data of three experiments. Similar to HepG2 cells and CM, HULECs significantly increased invasion of SOM 196B ($P<0.05$).

As it is a feasible possibility that cells from other tissues less commonly associated with uveal melanoma metastasis could also stimulate invasion, the effects of cells and CM from primary dermal keratinocytes (NAK 3.2) and fibroblasts (JFF), and the lung carcinoma cell line A549 on SOM 196B invasion was assessed. Uveal melanomas have occasionally been seen to metastasise to sub-cutaneous and pulmonary tissue (Char, 1978), and thus cells derived from these tissues were chosen for study. In addition, to investigate potential autocrine responses, the effects of SOM 196B cells and CM on the invasion of the same cell type was also studied. With the exception of A549 cells, all other cell types, together with respective CM significantly increased invasion of SOM 196B ($P<0.05$) (Figure 5.2.2.a). A549 cells stimulated a slight increase in invasion ($P>0.05$) whilst A549 CM significantly increased invasion ($P<0.05$). Cells derived from primary cultures (NAK 3.2, SOM 196B, JFF, and HULECs), were also generally seen to have a stronger stimulatory effect on invasion than the established cell lines HepG2 and A549. The effect of SOM 196B cells and CM on invasion of the same cell type, further implied possible autocrine stimulation.

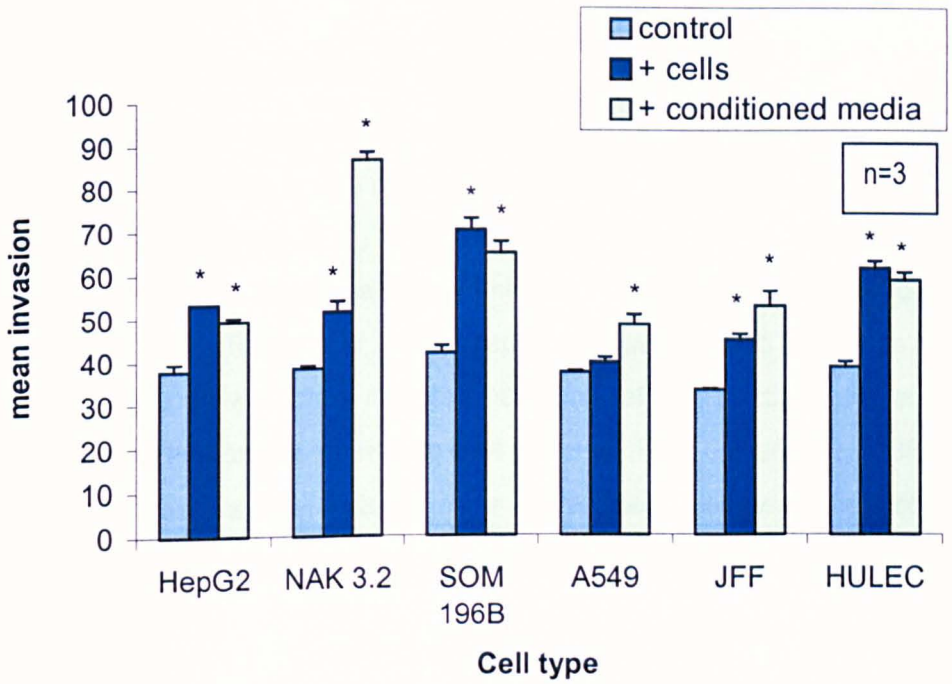


Figure 5.2.2.a. Effect of cells and CM from a series of cell types on invasion of SOM 196B. Expressed as mean counts per field of view under x400 magnification. Mean (± SEM) invasion 8μM pores after 24 hours. Results represent the mean of three experiments. The x-axis shows the different cell types used to assess the effect on invasion and the y-axis shows the mean invasion through Matrigel under x400 magnification. *, $P<0.05$ when compared with control invasion (without stimulation of invasion).

5.3. Discussion

5.3.1. Paracrine stimulation of invasion by HepG2 cells

As the liver is largely composed of hepatocytes, the effects of cells derived from hepatocytes on uveal melanoma invasion *in vitro* were initially assessed. Hepatocytes produce a number of factors (Rak *et al.*, 1996; Rowell *et al.*, 1997; Long *et al.*, 1998a and b), which may potentially attract circulating tumour cells through paracrine stimulation. Due to the constraints of long-term culturing of human hepatocytes (Runge *et al.*, 1999), HepG2 cells were used to provide an approximation. As these cells were derived from a malignant hepatoma cell line, functional differences between such cells and native hepatocytes are likely to exist (Lin *et al.*, 1997; Stonans *et al.*, 1999; Liu *et al.*, 2000). Cells should have however retained some characteristic features of hepatocytes providing an indication of whether uveal melanoma cells are favourably attracted to the liver in preference for other sites.

In this present study, HepG2 cells and CM nevertheless increased uveal melanoma STC invasion and the extent of the effect appeared to depend upon the individual culture (Figure 5.2.1.a). In support, in the comparable study by Ms Elshaw, stimulatory factors, and in particular HGF, had the most overt effect on non-invasive cultures. HepG2 cells may have thus produced amongst other factors HGF to cause the responses seen, but as yet there is no published evidence to support this. As tumours were surgically resected, all cases were already classified as large tumours and thus associated with a poor prognosis (reviewed by Singh *et al.*, 2001). With the exception of SOM 269, no correlation could be made with other prognostic indicators. The non-invasive culture SOM 269 showed little response to either HepG2 cells or CM, and morphologically was composed of spindle B cells, and as such, associated with a better prognosis. Other tumours that responded positively to HepG2 cells and CM were however of a similar morphology and thus no connections can realistically be made. The number of tumours studied was therefore too small to draw reliable conclusions and could have been biased towards the specific tumours studied.

With the exception of SOM 157d and SOM 196B, all tumours had been recently excised and thus no association could be made with the clinical course of the disease. Most reports of established uveal melanoma cell lines indicate that they do not possess the characteristic chromosomal abnormalities of chromosomes 3 and 8 associated with primary uveal melanoma tumours (Aubert *et al.*, 1993; De

Waard-Siebinga *et al.*, 1995; Blom *et al.*, 1997; Sisley *et al.*, 1997; Guan *et al.*, 1998; White *et al.*, 1998). As detailed in Chapter 3 (3.3.1), this has similarly been shown for SOM 157d and SOM 196B, yet samples from the original tumours displayed these characteristic changes. Other chromosomal markers have however been consistently retained, suggesting that these cell lines are still representative of the original tumour. Although the cell line SOM 157d was classified as non-invasive, the patient from whom the tumour originated has died of metastatic disease, indicating that the tumour itself was highly invasive; thus suggesting a degree of pre-selection in culture. High levels of invasion in response to HepG2 CM could therefore have been as a result of specific metastatic characteristics that have been retained from the original tumour. By using STCs within five passages, it was consequently hoped that cells more closely resemble the original tumour *in vivo*, and close follow-up of these patients will elicit more information regarding responses *in vitro* and *in vivo*.

5.3.2. Paracrine stimulation of invasion by hepatic endothelial cells

As the liver is comprised of a number of cell types, including hepatocytes, sinusoidal endothelial and Kupffer cells and infiltrating leucocytes, cells in addition to hepatocytes are also likely to attract tumour cells. Brief assessment of the paracrine effects of primary hepatic endothelial cells indicated that these cells could also stimulate invasion of SOM 196B (Figure 5.2.2.a). Endothelial cells have been reported to produce a number of factors including HGF, IGF-I, IGF-II, TGF-β, GRO, RANTES and IL-1 (Matsumoto *et al.*, 1992; Rak *et al.*, 1996; Krishnaswamy *et al.*, 1999); each of which could have stimulated invasion. Similarly, capillaries have been shown to attract cutaneous melanoma cells (Rak *et al.*, 1996; von Bulow *et al.*, 2001). Murine lung endothelial cells, but not hepatic sinsusoidal endothelial cells, have furthermore been shown to release MCP-1 which stimulated the migration of lung-metastasising RAW117-L17 cells *in vitro* (Wakabayashi *et al.*, 1995). This raises the possibility that factors secreted by hepatic endothelial cells may contribute to attracting circulating uveal melanoma cells, but assessment of a wider range of tumour and endothelial cell types is required to confirm this hypothesis.

5.3.3. Paracrine stimulation of invasion by cells from tissues of different origins

As uveal melanomas occasionally metastasise to sites other than the liver (Char, 1978), study of the stimulatory effects of primary keratinocytes (NAK 3.2) and dermal fibroblasts (JFF), and a lung

carcinoma cell line (A549), also identified that with the exception of A549, factors secreted by these cells could also stimulate invasion of SOM 196B ($P<0.05$) (Figure 5.2.2.a). The effect of A459 cells and CM was however more variable, and after analysis of two further STCs, it became evident that that the effect was dependent upon the individual culture, either stimulating or inhibiting invasion (Figure 5.2.1.b). Throughout all experiments in this investigation, exact effects on stimulation (or inhibition) were seen to vary between inclusion of cells or CM in assays (Figures 5.2.1.a and b, and 5.2.2.a). As variation was not large, experimental error could explain the apparent differences, whilst production of CM may show more inherent variability between individual flasks of cells than incorporation of cells in the assay. An alternative explanation could however relate to differences in concentrations or activities of factors secreted by the cells or present in the CM.

These results nevertheless raise the question as to why uveal melanoma cells are not frequently seen to metastasise to alternative sites. It could be speculated that although other tissues may attract uveal melanoma cells, as patients rapidly die as a result of liver metastases, secondary disease may have established elsewhere, but to lesser degree than that in the liver. Metastasis might as a consequence, be primarily associated with the liver and not other sites where disease is less evident. Alternatively, as metastatic development involves a number of stages, despite other tissues attracting metastatic uveal melanoma cells, subsequent metastatic development may not be supported, relating to both the microenvironment of non-hepatic sites, and inherent properties of the tumour, resulting in the cells being inefficient at metastasising elsewhere. The liver may therefore be the main organ providing the optimum micro-environmental conditions to promote all stages of uveal melanoma metastatic development. For those tumours that are seen to metastasise elsewhere, these cells could be more effective 'metastasisers' than those disseminating to the liver alone. As however, this aspect was carried out on SOM 196B alone, results will be specific for this culture, and thus by assessing the effect on a wider range of uveal melanoma cultures more precise conclusions could be drawn.

5.3.4. Autocrine stimulation of invasion

During tumour progression, in addition to paracrine stimulation from host cells, autocrine stimulation is often also evident, and this was reflected for SOM 196B (Figure 5.2.2.a). Similar observations have been seen previously, using metastatic variants of RAW117 large-cell lymphoma cells

(Wakabayashi *et al.*, 1994). In further support, cutaneous melanoma cells secrete a number of autocrine growth factors (Lazar-Molnar *et al.*, 2000; reviewed by Ruiter *et al.*, 2002), whilst autocrine expression of high levels of IL-8 by metastatic cells associates with increased *in vitro* invasion through MMP-2 up-regulation (Luca *et al.*, 1997; Wang *et al.*, 1998a). As this present investigation was similarly only carried out on one culture, results could again have been biased towards these cells, and analysis of a wider range of cultures would elicit more precise results.

Growth factors produced by melanoma cells have however also been shown to have paracrine effects on other cell types in the surrounding stroma (Lazar-Molnar *et al.*, 2000; reviewed by Ruiter *et al.*, 2002). In response, endothelial cells, fibroblasts, keratinocytes, monocytes, lymphocytes and granulocytes secrete either activating or inhibitory factors affecting processes such as tumour growth, angiogenesis, adhesion and migration. In this current investigation cell types were initially separated with an artificial basement membrane, thus allowing diffusion of soluble mediators, but direct cell-cell interactions between the two cell types were theoretically not possible. Cross talk between the co-cultures could therefore have had an effect on invasion, which was not present in wells with CM alone, whereby soluble factors secreted by tumour cells in the upper chamber diffused into the lower chamber, stimulating cells grown in the chamber to secrete further paracrine factors. In support of this premise, primary uveal melanomas, co-cultured with endothelial cells in a similar experimental system, have been shown to produce bFGF and VEGF, which subsequently supported endothelial cell growth (Boyd *et al.*, 2002).

When considering the results presented in this chapter, as checkerboard analysis was not carried out, it was not possible to distinguish between intrinsic motility and chemotactic responses to autocrine and paracrine factors. To fully validate this data, it would therefore be necessary to assess the effect of the addition of CM or cells to upper well only, the lower well only, or to both wells. Chemotactic responses could be confirmed if movement occurred only in the presence of a concentration gradient. This is nevertheless impracticable when considering the effect of viable cells, and would only be of use to assess CM. Blocking tumour cell receptors in the invasion assays, and using procedures such as ELISAs and mRNA studies such as RT-PCR or *in situ* hybridisation, could aid to identify the specific regulatory factors produced by these cells.

In summary, these results confirm that cells associated with the liver are capable of stimulating uveal melanoma invasion. As alternative cell types also stimulated invasion of one culture, it is possible that other sites similarly attract uveal melanomas, but it remains unclear as to whether secondary disease could develop at these sites. As cells of pulmonary origin had diverse effects on uveal melanoma cultures, in some instances, arrest may be inhibited in such tissues.

Chapter 6

Adhesion of uveal melanoma cells to the target endothelium

6.1. Introduction

As detailed in the Introduction (Section A), following Paget's theory, inherent properties of the microvasculature of the target organ could play a pivotal role in determining the pattern of spread seen for many tumours (Paget, 1889). In support, endothelial cells from different organs are known to display diverse structural and biochemical characteristics (Rymaszewski *et al.*, 1992), but cells used *in vitro* may not always reflect their *in vivo* heterogeneity (Swerlick *et al.*, 1992; reviewed by Thorin and Shreeve, 1998). A number of endothelial cell molecules are constitutively expressed including PECAM-1 (CD31) and the factor VIII-related antigen, allowing identification of most endothelial cell types (reviewed by Garlanda and Dejana, 1997). As again highlighted in the Introduction (Section A, 1.5), a number of specialised endothelial cell molecules have also been identified in specific vascular regions, which might further aid site-specific metastasis for some tumours (Zhu *et al.*, 1991; Abdel-Ghany *et al.*, 2001). Expression of other endothelial cell molecules such as E-selectin, ICAM and VCAM, are further activated by inflammatory cytokines or growth factors in the local environment, and the relevance of these markers has been widely reported in tumour cell adhesion to the endothelium (Scherbarth and Orr, 1997; Vidal-Vanaclocha *et al.*, 2000; Minami *et al.*, 2001).

Aims of the study

For uveal melanoma, it is possible that characteristics of the adhesion molecule profiles of the microvascular endothelial cells of the liver influence the metastatic spread of uveal melanoma. In Chapter 4 (4.2.1), in a preliminary experiment, no significant differences in the level of invasion were seen between the transendothelial invasion through dermal and liver endothelium for one uveal melanoma culture (SOM 196B) ($P>0.05$) ($n=3$). As differences are nevertheless likely to exist between these endothelial cell types, the purpose of this investigation was to further examine potential variation between HDMECAs and HULECs *in vitro*. For this study, adhesion of uveal melanoma cells to both endothelial cell types was assessed. In addition flow cytometric analysis of the two-endothelial cell types before and after culture with cytokines, or uveal melanoma cells and their conditioned media (CM), was investigated.

6.2. Results

6.2.1. Adhesion of tumour cells to HDMECAs and HULECs

Figure 6.2.1.a represents the levels of adhesion of melanoma cell lines to fibronectin, HDMECAs and HULECs. Figure 6.2.1.b represents the levels of melanoma cell adhesion to HDMECAs and HULECs, expressed as a percentage of the total cell number adhering to fibronectin. By assessing both sets of data, the efficiency of attachment to the fibronectin and endothelial cells could be compared between tumour cell types. This was reflected in this investigation for all melanoma types, in which the highest levels of adhesion were seen to fibronectin (Figure 6.2.1.a). When comparing the ability of individual cell lines to adhere to either HDMECAs or HULECs, no significant difference was found in levels of adhesion compared to that of fibronectin ($P>0.05$).

There were however significant differences in adhesion levels between invasive and non-invasive cell lines. When comparing adhesion of SOM 196B, SOM 157d and A375 to each endothelial cell type and fibronectin, highly significant variation was seen between the two uveal melanoma cell lines to both HDMECAs and HULECs ($P<0.001$) (Figures 6.2.1.a and b). SOM 196B cells adhered at the highest levels to both endothelial cell types and fibronectin, whilst A375 and SOM 157d cells adhered less well, with SOM 157d cells adhering in the lowest numbers. Significant variation between levels of adhesion of A375 cells and uveal melanoma cells to either endothelial cell type or to fibronectin was only seen when comparing attachment of SOM 196B and A375 cells to HDMECAs ($P<0.05$). It would thus appear that relative adhesion to fibronectin, HDMECAs and HULECs bears some relationship to the invasive properties of the cells *in vitro* through Matrigel barriers.

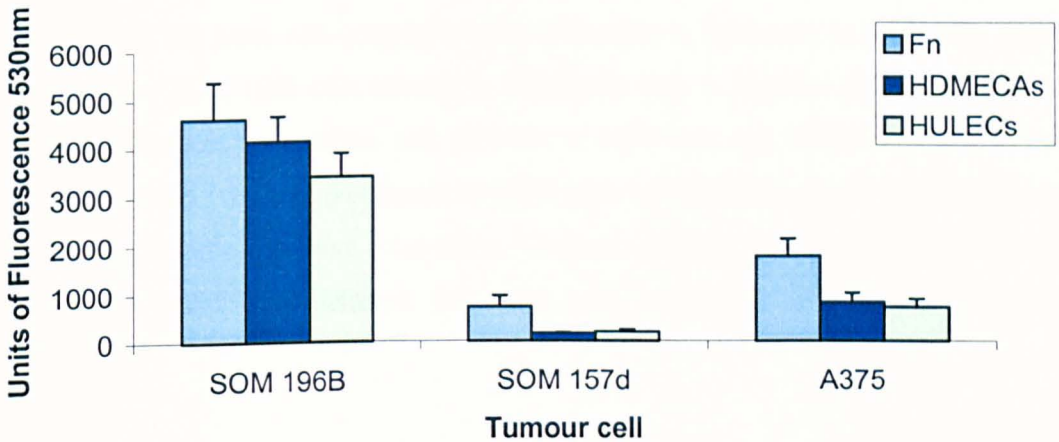


Figure 6.2.1.a. Adhesion of the uveal melanoma cell lines SOM 196B and SOM 157d and the cutaneous melanoma cell line A375 to fibronectin (Fn), HDMECAs and HULECs. Mean (± SEM) number of tumour cells adhering to Fn or different endothelial cell types. Results represent the mean of three experiments. Units of fluorescence at 530nm are measured on the y-axis. No significant differences in adhesion of SOM 196B, SOM 157d and A375 cells to fibronectin were observed ($P>0.05$) when compared with adhesion to either HDMECAs or HULECs. When comparing adhesion to either endothelial cell types between tumour cell types, significantly higher levels of SOM 196B cells adhered to both HDMECAs and HULECs, when compared with SOM 157d ($P<0.001$).

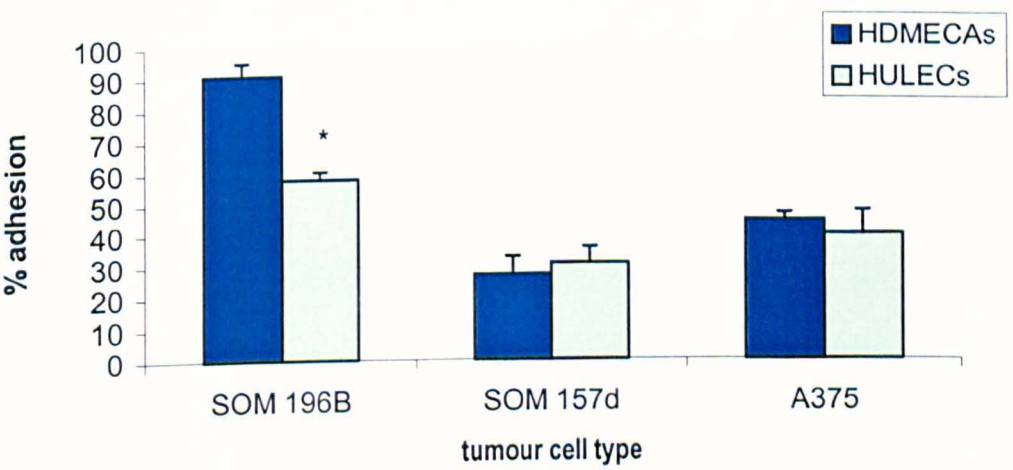


Figure 6.2.1.b. Relative adhesion of SOM 196B, SOM 157d and A375 to HDMECAs and HULECs, expressed as a percentage of the total cell number adhering to Fn. Mean (± SEM) number of tumour cells adhering to the different endothelial cell types. Results represent the mean of three experiments. The x-axis shows the tumour cell types and the y-axis shows the % of tumour cells adhering. *, $P<0.05$ when comparing adhesion of SOM 196B cells to HDMECAs and HULECs.

Although no significant difference was found in the adhesion of SOM 196B to endothelial cells when compared with attachment to fibronectin ($P>0.05$), when the relative adhesion of SOM 196B to the two endothelial cell types was compared, using adherence to fibronectin as a baseline, significantly higher levels of SOM 196B cells adhered to HDMECAs than to HULECs (Figure 6.2.1.b) ($P<0.05$). Negligible differences were shown with adhesion of A375 cells and SOM 157d to HDMECAs and HULECs ($P>0.05$). A slight preference by A375 cells for HDMECAs and by SOM 157d cells for HULECs was nevertheless seen. In all cases, it must however be taken into consideration that some the adherent tumour cells detected, may have been adhering to one another and not to the endothelium.

As tumour-endothelial interactions could occur at several locations, to briefly assess which sites might be implicated; scanning electron microscopy was performed on a co-culture of SOM 196B cells and HDMECAs. Figure 6.2.1.c shows a scanning electron micrograph of SOM 196B cells adhering to HDMECAs. From this image it was not possible to identify precise locations of adhesive interactions, but SOM 196B cells did appear to attach to the upper surface of the HDMECA cells, as opposed to docking at intercellular junctions. The SOM 196B cell indicated can be seen to be projecting pseudopodia between the endothelial cells.

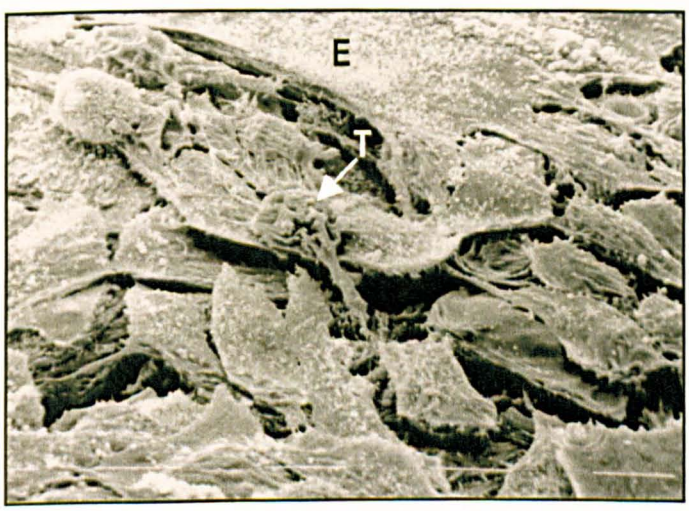


Figure 6.2.1.c. A scanning electron micrograph of SOM 196B cells (T) adhering to HDMECAs (E) (x640 magnification). SOM 196B cells can be seen adhering to the upper surface of the HDMECA cells and projecting pseudopodia between the endothelial cells.

6.2.2. FACS analysis of adhesion molecule expression by HDMECAs and HULECs

When grown in culture no gross morphological differences could be seen between HDMECAs and HULECs and both cell types exhibited characteristic cobblestone morphology (Figure 6.2.2.a).

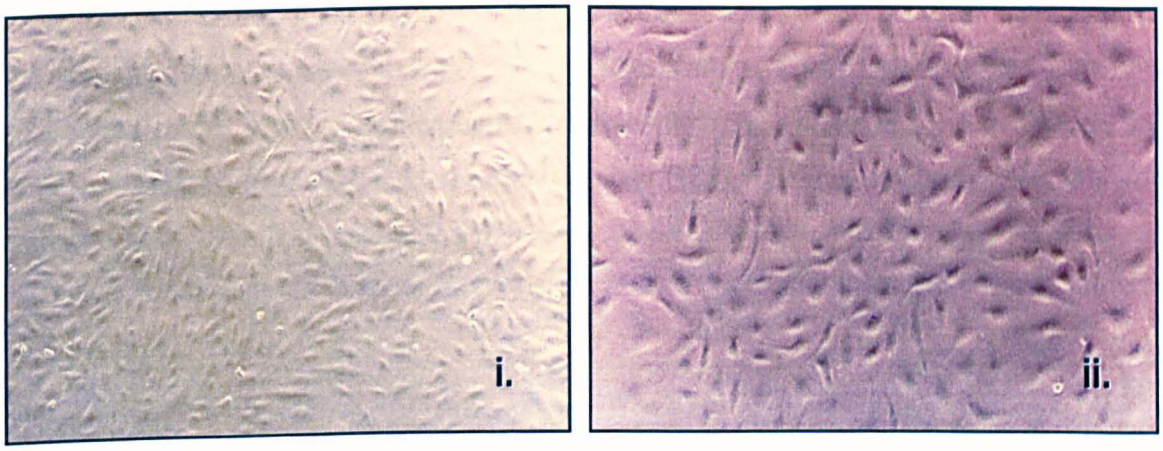


Figure 6.2.2.a. Photographic images of HDMECAs (i) and HULECs (ii) grown in culture, taken by light microscope, under phase-contrast (x100 and x200, respectively), illustrating similar morphological features in culture.

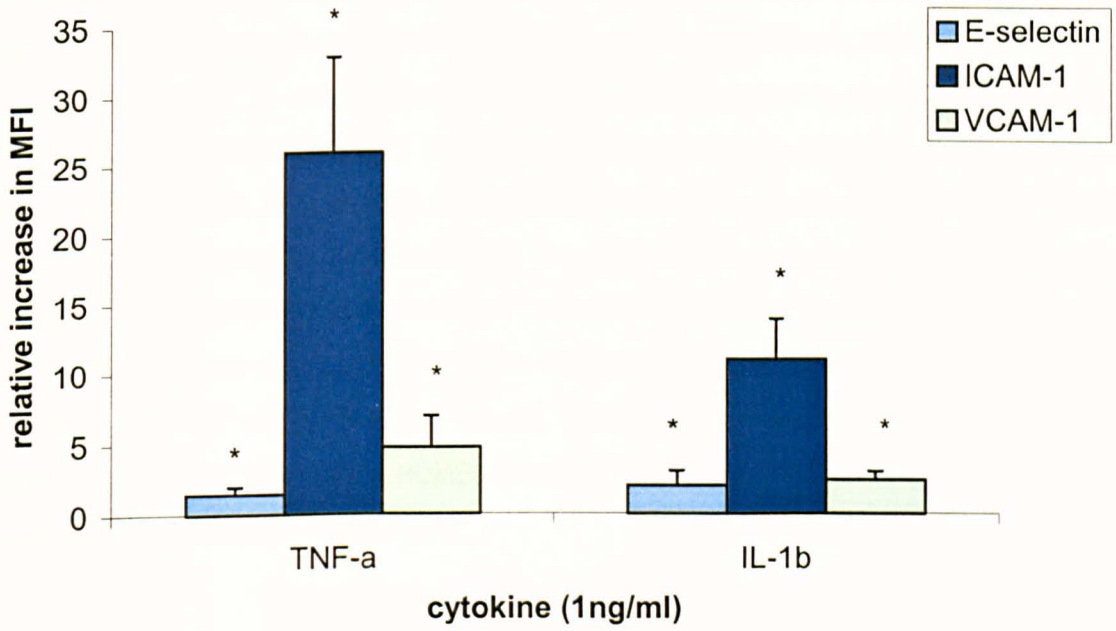
Adhesion molecule expression of HDMECAs and HULECs

Initial studies to characterise HDMECA and HULEC expression of a selection of adhesion molecules using FACS, identified that both cell types without stimulation, expressed ICAM-1, α1, α2, α3, and α5, and no significant differences were shown in the levels of expression between the two endothelial cell types ($P>0.05$). The only significant difference was shown for α4 ($P<0.05$); HDMECAs expressed this subunit at significantly higher levels than HULECs, which were considered negative for α4. With regard to adhesion molecules typically involved in tumour cell adhesion, both HDMECAs and HULECs therefore expressed high levels of ICAM-1, but did not express E-selectin or VCAM-1.

6.2.2.1. Effect of cytokine modulation (TNFα and IL-1β) on endothelial adhesion molecule expression

As previously mentioned, inflammatory mediators secreted by either tumour or host cells up-regulate E-selectin, ICAM, and VCAM expression (Tamaki *et al.*, 1995; Steinbach *et al.*, 1996; Vidal-Vanaclocha *et al.*, 2000; Minami *et al.*, 2001), and therefore to initially assess whether this could be reproduced in HDMECAs and HULECs, increases in expression of these adhesion molecules was assessed by stimulating cells with TNFα or IL-1β. Both cytokines significantly stimulated expression of E-selectin and VCAM-1 on both endothelial cell types ($P<0.05$), whilst also significantly increasing ICAM-1 expression ($P<0.05$) (Figure 6.2.2.b and Tables 6.2.2.a-c). The stimulatory effect was also greatest for ICAM-1 (Figure 6.2.2.b and Tables 6.2.2.a-c). There was however, no significant difference in the increase in levels of expression of these adhesion molecules between the two-endothelial cell types ($P>0.05$).

i. HDMECAs



ii. HULECs

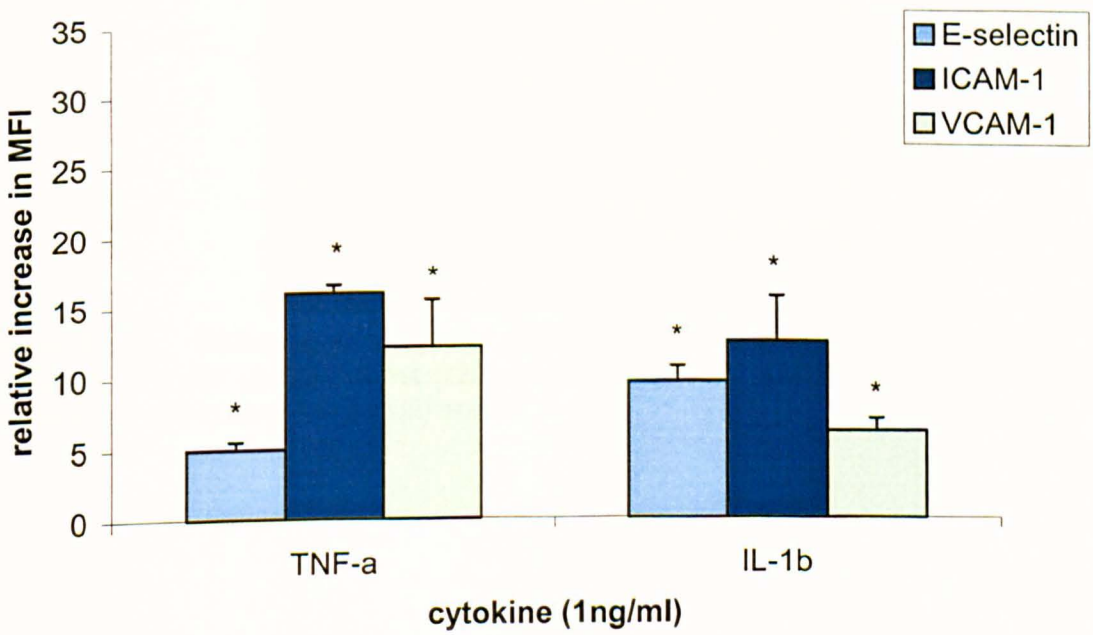


Figure 6.2.2.b. Relative increase in expression of E-selectin, VCAM-1 and ICAM-1 by i) HDMECAs and ii) HULECs, after treatment with TNF-α or IL-β at 1ng/ml for 24 hours, assessed by FACS analysis. Mean (± SEM) relative increase in expression. Results represent the mean of three experiments. The x-axis shows the experimental conditions and the y-axis shows the relative increase in expression when comparing with cells cultured alone. *, $P<0.05$ when comparing adhesion molecule expression after cytokine treatment with control levels of expression (untreated cells).

6.2.2.2. Ability of tumour cells to stimulate endothelial cell adhesion molecule expression

As tumour cells may therefore directly effect endothelial adhesion molecule expression, the study was extended to investigate the effect of uveal melanoma cells on HDMECA and HULEC adhesion molecule expression. As this could be in response to soluble secreted factors or direct cell contact, HDMECAs and HULECs were grown in tumour cell CM or co-cultured with tumour cells for 24 hours. In all cases, the effect of SOM 196B and SOM 157d cells were assessed, and compared with the effect of A375. Results from these studies are presented in sections below under the sub-headings 'changes to endothelial E-selectin', 'changes to endothelial VCAM-1' and 'changes to endothelial ICAM-1' and are summarised in Figure 6.2.2.e and Tables 6.2.2.a-c. Data regarding stimulation of expression by TNFα and IL-1β is also included in these tables to illustrate levels of up-regulation. Figure 6.2.2.c shows an image of HDMECAs cultured with SOM 157d, taken by light microscope, under phase-contrast (x200 magnification).

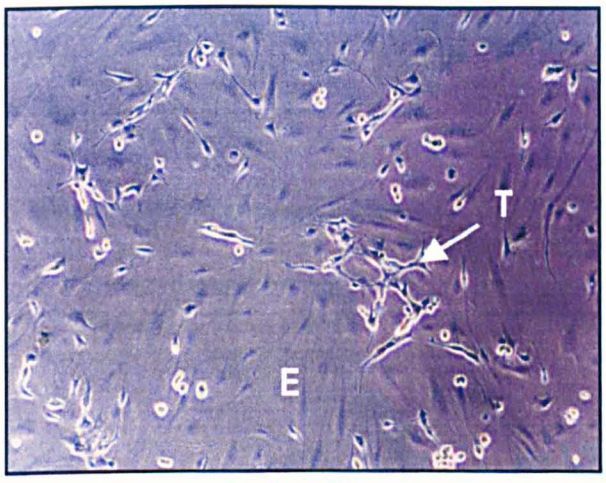


Figure 6.2.2.c. Photographic images of HDMECAs cultured with SOM 157d taken by light microscope, under phase-contrast (x200 magnification). A spindle SOM 157d cell (T) can be visualised adhering to the endothelium (E) beneath.

Changes to endothelial E-selectin

Results regarding E-selectin expression are summarised in Table 6.2.2.a and Figure 6.2.2.e. No significant increases in E-selectin expression by either endothelial cell type were shown after culture with CM from each of the melanoma cell lines ($P>0.05$). In contrast, co-culturing HULECs with SOM 196B cells resulted in a significant increase in expression of E-selectin, when compared with corresponding control endothelial cells (control cells) ($P<0.05$), (an increase from 1.22 ±0.0441 MFI to 1.62 ±0.0715 MFI). This result was not reflected when culturing HULECs with either SOM 157d or A375. Similarly culturing HDMECAs with each melanoma cell type did not result in a significant increase in expression of E-selectin ($P>0.05$).

	TNFα	IL-1β	SOM 196B (invasive cell line)		SOM 157d (non-invasive cell line)		A375	
			CM	Co-culture	CM	Co-culture	CM	Co-culture
HDMECAs	1.49 ±0.538	2.02 ±1.00	0.901 ±0.103	0.986 ±0.0219	1.05 ±0.0107	0.875 ±0.0228	0.940 ±0.109	1.19 ±0.0709
HULECs	4.97 ±0.556	9.61 ±1.04	0.868 ±0.0618	1.33 ±0.0355	1.01 ±0.0438	1.12 ±0.0576	0.948 ±0.0306	0.954 ±0.0766

Table 6.2.2.a. Mean (± SEM) relative increase in expression of E-selectin by HDMECAs and HULECs after treatment with TNF-α, IL-1β, tumour-cell conditioned media (CM) and after culture with tumour cells. Results represent the mean of three experiments. A significant increase in expression was shown by HDMECAs and HULECs after culturing with TNFα and IL-1β and by HULECs are culturing with SOM 196B for 24 hours ($P<0.05$) and these results in highlighted in blue.

Changes to endothelial VCAM-1

Results regarding VCAM-1 expression are summarised in Table 6.2.2.b and Figure 6.2.2.e. Growing either HDMECAs or HULECs in the CM produced by all of the melanoma cell lines did not significantly increase VCAM-1 expression, when compared with corresponding control endothelial cells (without CM) ($P>0.05$). Similarly co-culturing HDMECAs or HULECs with each melanoma cell line did not significantly increase VCAM-1 expression ($P>0.05$). Culturing HULECs with SOM 196B nevertheless increased expression of VCAM-1 (an increase from 1.36 ±0.0333 MFI to 1.94 ±0.217 MFI), whilst culturing HDMECAs with A375 increased VCAM-1 expression (an increase from 0.983 ±0.198 MFI to 1.35 ±0.0903 MFI) but neither of these results was significant ($P>0.05$). In these instances, experiments would need to be repeated further to draw more exact conclusions. Limited conclusions were nonetheless possible.

	TNFα	IL-1β	SOM 196B		SOM 157d		A375	
			CM	Co-culture	CM	Co-culture	CM	Co-culture
HDMECAs	4.76 ±2.21	2.40 ±0.578	0.972 ±0.117	1.158 ±0.205	0.853 ±0.062	0.963 ±0.080	0.886 ±0.204	1.477 ±0.265
HULECs	12.2 ±3.33	6.12 ±0.853	0.780 ±0.0913	1.416 ±0.131	0.937 ±0.0465	1.10 ±0.0456	0.903 ±0.0237	0.941 ±0.129

Table 6.2.2.b. Mean (± SEM) relative increase in expression of VCAM-1 by HDMECAs and HULECs after treatment with TNF-α, IL-1β, tumour-cell conditioned media (CM) and after culture with tumour cells. Results represent the mean of three experiments. A significant increase in expression was shown by HDMECAs and HULECs after culturing with TNFα and IL-1β for 24 hours ($P<0.05$) and these results are highlighted in blue. Increases were observed after co-culture of SOM 196B with HULECs, and with A375 and HDMECAs, but these results were not significant ($P>0.05$) and are shown in red.

Changes to endothelial ICAM-1

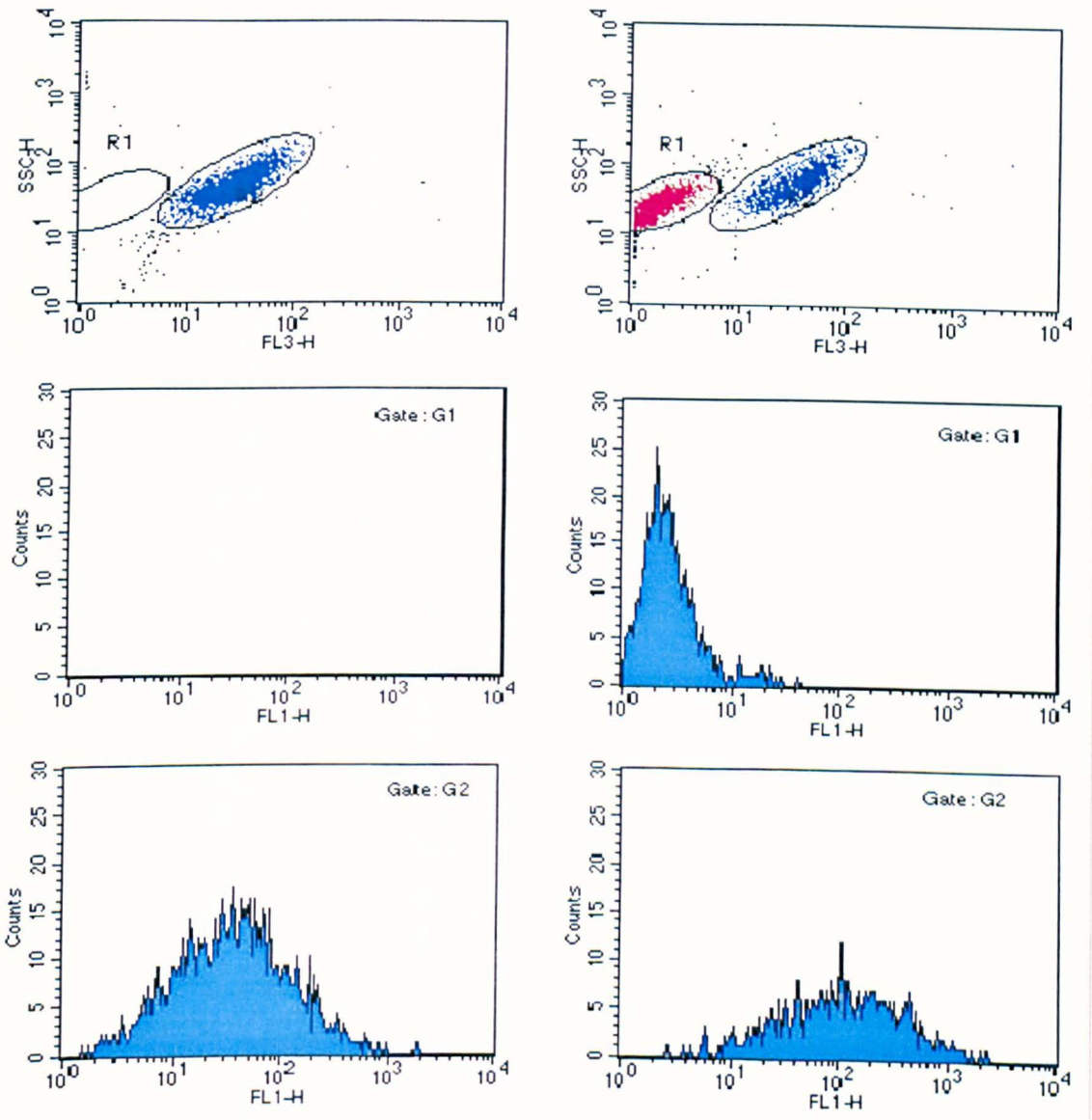
Results regarding ICAM-1 expression are summarised in Table 6.2.2.c and Figure 6.2.2.e. CM from each melanoma line did not significantly increase ICAM-1 expression by HDMECAs or HULECs, when compared with corresponding control endothelial cells (without CM) ($P>0.05$). However, CM from SOM 157d increased ICAM-1 expression levels by both endothelial cell types. For HDMECAs, an increase from 14.9 ±2.09 MFI to 23.7 ±2.44 MFI was seen whilst for HULECs, an increase from 4.63 ±0.270 MFI to 7.74 ±2.78 MFI was seen. CM from SOM 196B also increased HDMECA ICAM-1 expression but this increase was to a lesser extent (from 7.36 ±1.55 MFI to 10.1 ±2.81 MFI).

	TNFα	IL-1β	SOM 196B		SOM 157d		A375	
			CM	Co-culture	CM	Co-culture	CM	Co-culture
HDMECAs	25.9 ±6.95	11.0 ±2.92	1.38 ±0.191	0.941 ±0.231	1.70 ±0.403	2.19 ±0.241	0.993 ±0.289	2.46 ±0.389
HULECs	16.0 ±0.560	12.5 ±3.23	0.800 ±0.141	3.25 ±0.454	1.62 ±0.485	1.58 ±0.141	1.14 ±0.124	0.871 ±0.151

Table 6.2.2.c. Mean (± SEM) relative increase in expression of ICAM-1 by HDMECAs and HULECs after treatment with TNF-α, IL-1β, tumour-cell conditioned media (CM) and after culture with tumour cells. Results represent the mean of three experiments. Significant increases in ICAM-1 expression were seen by HDMECAs and HULECs after culture with TNFα, IL-1β and SOM 157d cells for 24 hours ($P<0.05$) and these results are highlighted in blue. Increases were observed after culture of HDMECAs with SOM 196B CM, co-culturing SOM 196B cells with HULECs, culturing HULECs and HDMECAs with SOM 157d CM, and after co-culturing A375 cells with HDMECAs, but these results were not significant ($P>0.05$) and are shown in red.

Significant increases in ICAM-1 expression by HDMECAs and HULECs were shown when cells were cultured with SOM 157d cells for 24 hours when compared with corresponding control endothelial cells (control cells) ($P<0.05$). Levels increased from 13.2 ±0.484 MFI to 28.7 ±2.34 MFI and 13.4 ±0.669 MFI to 21.0 ±1.10 MFI, respectively. Flow cytometry dot plots and histograms illustrating ICAM-1 expression by HDMECAs cultured alone (controls) and after co-culture with SOM 157d are shown in Figure 6.2.2.d. As illustrated, cell populations could be easily distinguished due to differences in fluorescence between SNARF-1 and FITC. Culture of both endothelial cell types with SOM 196B and A375 did not significantly increase ICAM-1 expression ($P>0.05$). Increases were nevertheless still shown after culturing HULECs with SOM 196B cells (from 12.6 ±3.96 MFI to 44.3 ±20.1 MFI) and HDMECAs with A375 cells (from 7.18 ±1.81 MFI to 16.3 ±2.38 MFI). In these

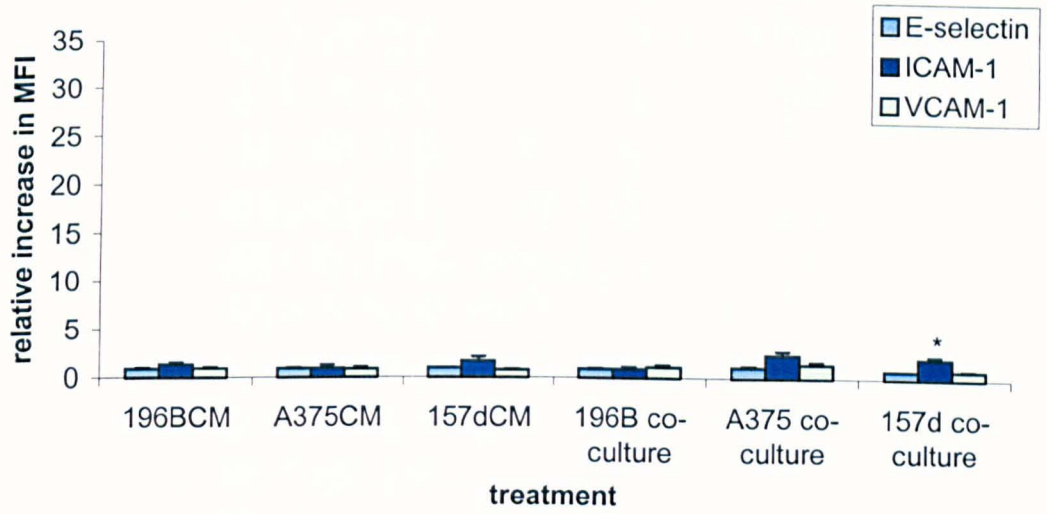
instances, the experiments would again need to be repeated further to draw more precise conclusions. Similarly, limited conclusions have nevertheless been described.



i. HDMECAs cultured alone *ii. HDMECAs cultured with SOM 157d*

Figure 6.2.2.d. Flow cytometry dot plots and histograms illustrating ICAM-1 expression by (i) HDMECAs cultured alone (controls) and (ii) after co-culture with SOM 157d. HDMECA ICAM-1 expression has been increased after culture with SOM 157d cells. Endothelial cells were labelled with SNARF-1 prior to co-culturing with SOM 157d cells for 24 hours and are shown in blue. Tumour cells were selectively disaggregated from the endothelial cells and are shown in red. Cells were then stained with avidin-FITC conjugates and analysed with flow cytometry. Gate G1 refers to cells in the region R1 of the dot plot (SOM 157d cells) and gate G2 refers to cells in the region R2 (HDMECAs).

i. HDMECAs



ii. HULECs

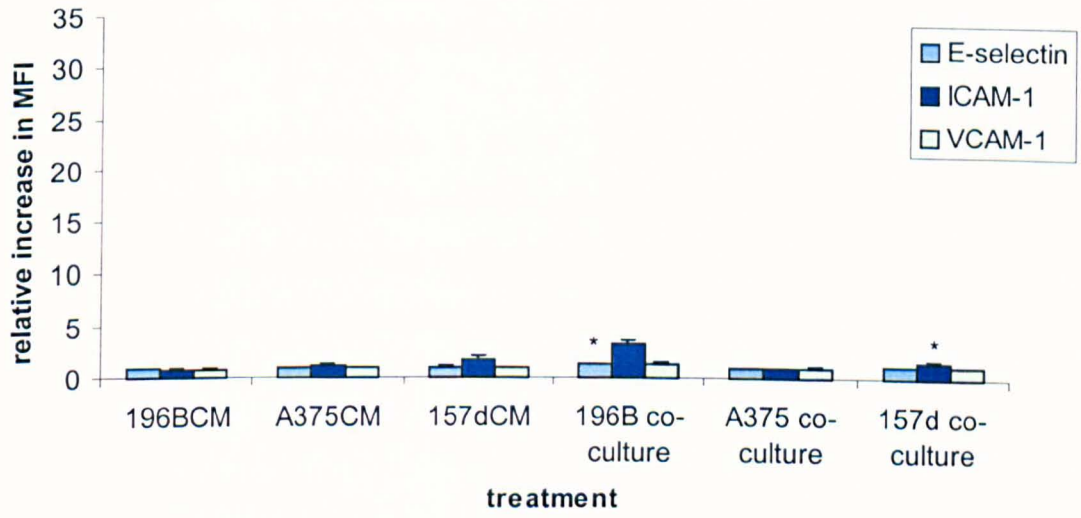


Figure 6.2.2.e. Relative increase in expression of E-selectin, VCAM-1 and ICAM-1 by i) HDMECAs and ii) HULECs, after treatment with conditioned media from uveal melanoma cell lines SOM 196B and SOM 157d and the cutaneous melanoma cell line A375 and after co-culture with SOM 196B, SOM 157d and A375, assessed by FACS analysis. Mean (± SEM) relative increase in expression. Results represent the mean of three experiments. The x-axis shows the experimental conditions and the y-axis shows the relative increase in expression when comparing with cells cultured alone. Scale of the y-axis is comparable with that of Figure 6.2.2.b. *, $P<0.05$ when comparing adhesion molecule expression after treatment with control levels of expression (untreated cells).

6.2.3. FACS analysis of adhesion molecule expression by tumour cells

6.2.3.1. Ability of endothelial cells to stimulate tumour adhesion molecule expression

As described previously, tumour cell α4 (the VCAM-1 ligand) is commonly involved in tumour-endothelial interactions. As expression of α4 had been previously seen to vary between uveal melanoma cultures (3.2.2), it is possible that this integrin plays a role in uveal melanoma metastasis. After co-culture with endothelial cells, expression of this subunit was assessed in tumour cell samples selectively disaggregated from the co-cultures, for the three-melanoma cell lines. In addition, as other β1-integrins and ICAM molecules have been reported to be involved in tumour-endothelial interactions (Price *et al.*, 1996; Papadimitriou *et al.*, 1999), for one co-culture arrangement, the effect on a wider range of tumour cell adhesion molecules was also investigated. Due to time restrictions, this could not be extended to all co-culture systems. Prior to co-culture, FACS analysis revealed that SOM 196B and A375 cells expressed α1, α2, α3, α5, and α6. A375 cells also strongly expressed α4 and ICAM-1, whilst SOM 196B cells only weakly expressed α4 and were negative for ICAM-1. SOM 157d cells similarly expressed α2, α3, α5, and α6 and like SOM 196B, only weakly expressed α4 and did not express ICAM-1. None of the cells studied expressed the β4 subunit.

Changes to tumour cell α4 expression

α4 may be associated with either the β1 or β7 subunits. Due to the specificity of the antibody for the α4-subunit alone, no distinction could be made between α4β1 and α4β7 expression. Figure 6.2.3.a and Table 6.2.3.a represent the increase in expression of the α4-subunit by SOM 196B, SOM 157d and A375, after co-culture with HDMECAs or HULECs, when compared with control samples (tumour cells cultured alone). As time was a limiting factor, the effect of endothelial CM on tumour cell α4 expression was not assessed.

When cultured alone, both SOM 196B and SOM 157d weakly expressed the α4-subunit whilst A375 cells expressed the subunit at higher levels. Culturing SOM 196B and SOM 157d cells with HULECs resulted in a significant increase in α4 expression by SOM 196B and SOM 157d cells, when compared with control cells ($P<0.05$). This result was however not reflected for A375. Meanwhile culturing each melanoma line with HDMECAs did not result in significant increases in α4 expression ($P>0.05$). The highest increase in expression of α4 after culture with HDMECAs was nevertheless

shown after culturing with A375 (from 3.7 ±0.0710 MFI to 4.93 ±0.606 MFI). Figure 6.2.3.b shows flow cytometry dot plots and histograms illustrating α4-subunit expression by SOM 196B when cultured alone or after co-culture with HULECs.

	HDMECA	HULEC
SOM 196B	1.18 ±0.172	1.47 ±0.0149
SOM 157d	1.07 ±0.0313	1.13 ±0.0114
A375	1.34 ±0.185	0.900 ±0.105

Table 6.2.3.a. Mean (± SEM) relative increase in expression of α4 by SOM 196B, SOM 157d and A375 after culture with HDMECAs and HULECs, assessed by FACS analysis. Results represent the mean of three experiments. A significant increase in α4 expression by SOM 196B and SOM 157d cells was shown after culture with HULECs for 24 hours ($P<0.05$) and this result is highlighted in blue. Increases in α4 expression by A375 cells was also seen after co-culture with HDMECAs, but this result was not significant, and is highlighted in red.

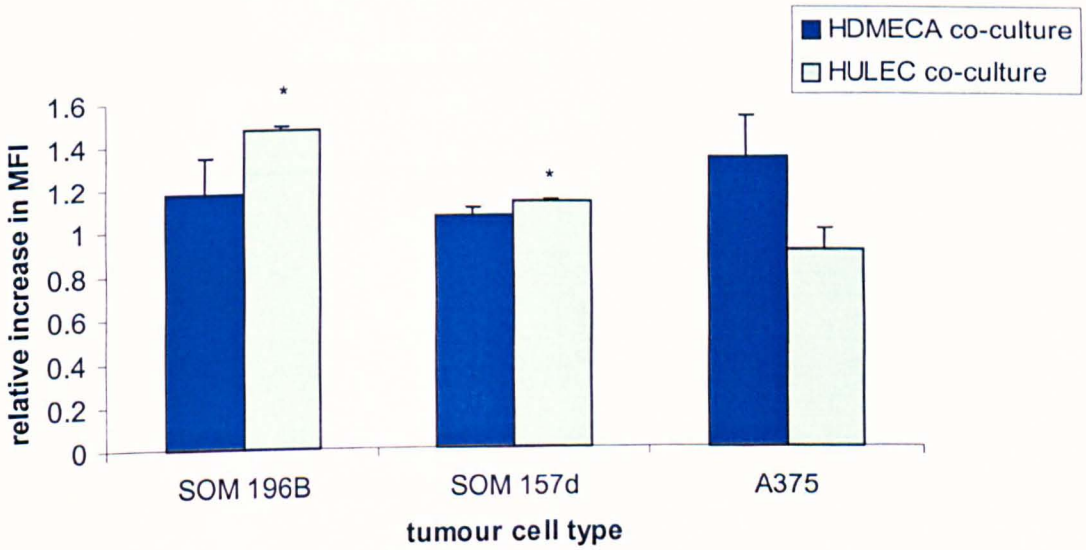
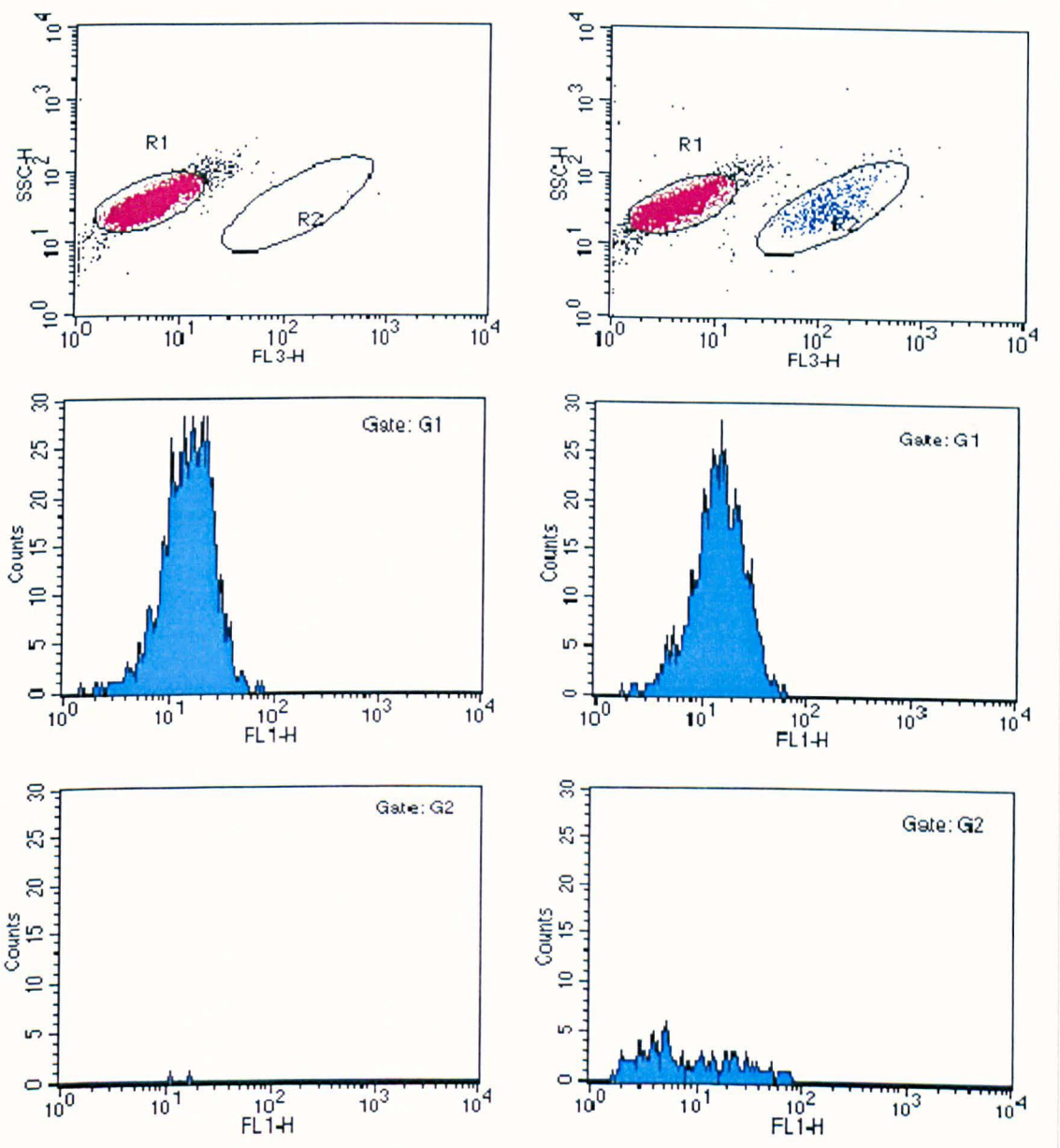


Figure 6.2.3.a Relative increase in expression of α4 by SOM 196B, SOM 157d and A375, after co-culture with HDMECAs and HULECs, assessed by FACS analysis. The x-axis shows the tumour cell type and the y-axis shows the increase in MFI after co-culture, when compared with tumour cells cultured alone. Mean (± SEM) relative increase in expression. Results represent the mean of three experiments; the * indicates a significant ($p<0.05$) difference in α4-subunit expression by SOM 196B and SOM 157d after culture with HULECs.



i. SOM 196B cultured alone *ii. SOM 196B after co-culture with HULECs*

Figure 6.2.3.b. Flow cytometry dot plots and histograms illustrating α4-subunit expression by SOM 196B when cultured alone (i) or after co-culture with HULECs (ii). SOM 196B α4 expression has been increased after culture with HULECs. Endothelial cells were labelled with SNARF-1 prior to co-culturing with SOM 196B cells for 24 hours and are shown in blue. SOM 196B cells were selectively disaggregated from the endothelial cells and are shown in red. Cells were then stained with avidin-FITC conjugates and analysed with flow cytometry. Gate G1 refers to cells in the region R1 of the dot plot (SOM 196B cells) and gate G2 refers to cells in the region R2 (HULECs).

Changes to tumour cell integrins and ICAM-1 expression

The effect on expression of tumour cell adhesion molecules previously assessed was additionally investigated after SOM 196B cells were cultured with HDMECAs (Figure 6.2.3.c). As increased ICAM-1 expression by invasive cutaneous melanomas is related to poor prognosis (Johnson *et al.*, 1989; Natali *et al.*, 1997; Johnson, 1999), the invasive uveal melanoma cell line SOM 196B was chosen for study. As SOM 196B had been previously shown to preferentially adhere to HDMECAs (6.2.1), co-culture with these endothelial cells was chosen for investigation.

After culture with HDMECAs for 24 hours, no significant changes in expression of any of the integrin subunits or ICAM-1 were shown when compared with control cells ($p>0.05$). Of note however, $\alpha 3$ expression increased by 1.67 ±0.908 MFI, whilst $\alpha 6$ expression decreased proportionally by 0.441 ±0.0212 MFI.

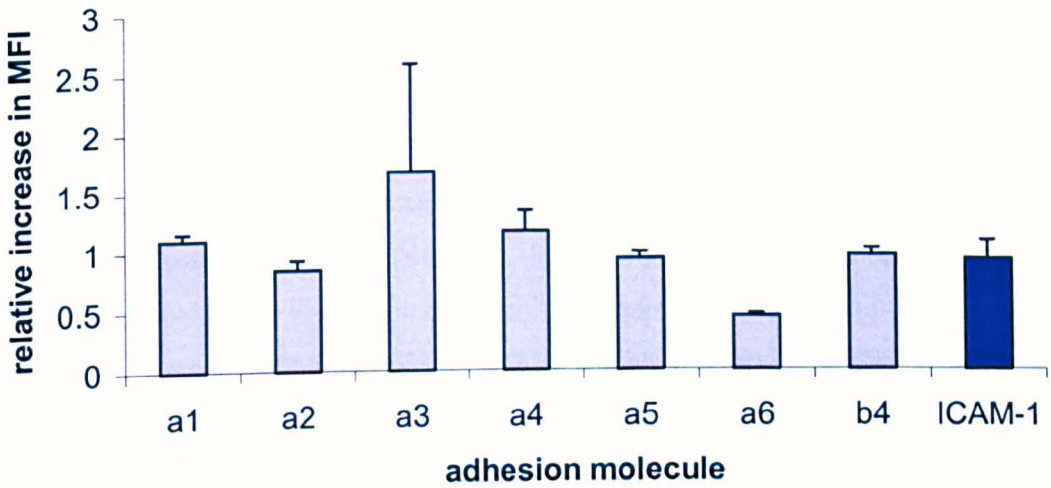


Figure 6.2.3.c. Relative increase in expression of integrin subunits and ICAM-1 by SOM 196B, after co-culture with HDMECAs when compared with expression by cells cultured alone, assessed by FACS analysis. Mean (± SEM) relative increase in expression. Results represent the mean of three experiments. The x-axis shows the adhesion molecule or subunit and the y-axis shows the increase in expression after co-culture. No significant changes in expression were shown ($P>0.05$). Increased $\alpha 3$ expression was observed whilst $\alpha 6$ expression was reduced, but these results were not significant ($P>0.05$).

6.3. Discussion

6.3.1. Adhesion of tumour cells to HDMECAs and HULECs

Despite evidence presenting alternative mechanisms of arrest and extravasation (Al-Mehdi *et al.*, 2001), there are still many published reports to suggest that different types of adhesion molecules are involved in the site-specificity of adhesive interactions between circulating tumour cells and the target endothelium. For uveal melanoma this may be of relevance in liver targeting. To investigate this further, interactions of invasive and non-invasive uveal melanoma cells with hepatic and dermal endothelial cells were studied. As described in Chapter 5 (5.3.1), invasive characteristics were defined upon the behaviour of the selected clones *in vitro*, which could not necessarily be related to the corresponding behaviour in the patient. This had to be taken into consideration when speculating conclusions.

Adhesion levels of prostate carcinoma cells have been shown to differ between primary bone marrow endothelium and HUVECs, reflecting preferential binding to the former (Scott *et al.*, 2001). In this present study, invasive cell lines (SOM 196B and A375) adhered in greater numbers to both endothelial cell types, when compared with the non-invasive cell line (SOM 157d). In support of these results, highly metastatic lymphoma cells adhere to endothelial cells in greater numbers than their poorly metastatic counterparts (Yun *et al.*, 1997). Uveal melanoma cells did not however show a significant preference for HULECs and instead the invasive uveal melanoma cell line, SOM 196B preferentially adhered to HDMECAs (Figures 6.2.1.a and b). A direct explanation for this preference remains uncertain but did indicate differential attachment to the two-endothelial cell types.

When observing adhesion, a degree of tumour cell clumping could be seen, and despite attempts to overcome this, tumour cells still appeared to bind to one another easily, inferring an inherent cohesive property of these cells (analogous to 'piggy backing'). When assays were analysed, cell clumping therefore had to be taken into consideration, and could provide an explanation for the higher levels of adhesion of SOM 196B to HDMECAs when compared with HULECs. Washing less adherent cells from the endothelial layer might have also removed loosely adhered cells and thus only tumour cells tightly bound to the endothelial cells would have been assessed.

The assay system has additional limitations, primarily as it is static, and therefore cannot be expected to reflect all aspects of the continuously changing hydrodynamic conditions *in vivo,* whilst the input of host cells cannot also be assessed. As adhesion molecules involved in tumour-endothelial interactions can vary depending upon static or hydrodynamic conditions *in vivo* (Mogiri *et al.*, 1995; Yoshida *et al.*, 1999), using a more sophisticated system under flow conditions would provide a closer approximation.

6.3.2. The effect of TNFα and IL-1β on endothelial adhesion molecule expression

As described in Chapter 5 (5.1), In the liver, Kupffer cells are one of the major sources of cytokine production, including both TNFα and IL-1β (Gangopadhyay *et al.*, 1996). Both of these cytokines up-regulate endothelial E-selectin, VCAM and ICAM expression, increasing tumour cell adhesion (Tamaki *et al.*, 1995; Steinbach *et al.*, 1996; Vidal-Vanaclocha *et al.*, 2000; Minami *et al.*, 2001), whilst promoting α4-expressing B16 melanoma cell metastasis to the lung and liver via a VCAM-1-mediated process (Garofalo *et al.*, 1995; Vidal-Vanaclocha *et al.*, 1996). Similarly in this present study, both TNF-α and IL-1β up-regulated expression of E-selectin, ICAM-1 and VCAM-1, but no significant differences in the increases in expression were seen between endothelial cell types (Figure 6.2.2.b).

6.3.3. The effect of culturing with tumour cells and tumour cell CM on adhesion molecule expression

Although direct functions of the endothelial adhesion molecules typically involved in metastasis (E-selectin, VCAM-1 and ICAM-1) were not assessed with regard to adhesion, the different patterns of up-regulation seen after culture with tumour cell types and CM studied, would infer dissimilar mechanisms of binding. The levels of up-regulation were however far less than those seen after exogenous stimulation with TNFα and IL-1β (Figures 6.2.2.b and e), suggesting either different mechanisms of up-regulation, that tumour cells were releasing factors at lower concentrations, or that factors were secreted over a different time scale. When directly comparing results from adhesion and flow cytometry experiments, it must also be taken into consideration that adhesion assays were performed over a period of four hours, whereas the flow cytometry experiments were carried out over

24 hours. To draw more accurate conclusions, it would be necessary to run assays under the same experimental parameters.

Analysis of both endothelial cell types identified differences in the expression of the α4-subunit only, which could be of relevance when considering uveal melanoma metastasis to the liver. Similarly, co-up-regulation of invasive tumour cell (SOM 196B) α4 expression and its ligand VCAM-1 on the endothelial cells after culture with hepatic endothelial cells may further imply that this integrin subunit is involved in liver targeting (Tables 6.2.2.b and 6.2.3.a and Figures 6.2.2.e and 6.2.3.a and b). In support of this premise, α4β1 and α4β7 play a role in tumour cell and lymphocyte trafficking to specific sites (Hamann *et al.*, 1994; Matsuura *et al.*, 1996; Holzmann *et al.*, 1998), whilst the VCAM-1-α4 interaction is involved in liver metastasis of other tumour types including B16 cutaneous melanoma cells, lymphoma and myeloma (Papadimitriou *et al.*, 1999; Okada *et al.*, 1999; Vidal-Vanaclocha *et al.*, 2000; Langley *et al.*, 2001). Furthermore as uveal melanocytes do not express α4β1 (Elshaw *et al.*, 2001), whilst uveal melanomas variably express this integrin (3.2.2), it is possible that this receptor is involved in malignant progression. As the non-invasive cell line SOM 157d showed an increase in tumour cell α4 after culture with HULECs, without corresponding increases in endothelial VCAM-1, the α4-VCAM-1 interaction might further be required for invasion through the endothelium. As up-regulation by SOM 196B cells was specific to HULECs, with regard to endothelial α4-expression, it could be speculated that upon direct cell contact, the VCAM-1-α4 interaction is required, but is only be stimulated in the absence of endothelial α4 expression. Conversely as dermal endothelial cells express α4, by negative feedback, up-regulation of VCAM-1 by SOM 196B is not permitted.

Cutaneous melanomas metastasise to a wider range of organs than uveal melanomas. This could be reflected by the observation of similar co-up-regulation of both VCAM-1 and α4, after co-culture of A375 with dermal but not hepatic endothelial cells (Tables 6.2.2.b and 6.2.3.a and Figures 6.2.2.e and 6.2.3.a). With regard to the possible relevance of endothelial α4 in this process, it is possible that HDMECA α4 plays a lesser important role in endothelial VCAM regulation by A375 cells.

Comparable increases in hepatic endothelial E-selectin expression after culture with SOM 196B cells (Table 6.2.2.a and Figure 6.2.2.e), suggested that this interaction is also being utilised by the invasive uveal melanoma cells, possibly increasing attachment. In support, strong evidence exists regarding the importance of the E-selectin in colon and breast cancer metastasis (Renkonen *et al.*, 1997; Kitayama, *et al.*, 2000; Moss *et al.*, 1999; Moss *et al.*, 2000; Minami *et al.*, 2001) and the colonisation of Lewis lung carcinoma cells to the liver (Brodt *et al.*, 1997; Khatib *et al.*, 1999). Incubation with SOM 196B CM did not affect the same result, and similar increases were not seen with other co-culture systems, suggesting that similar to the VCAM-α4 interaction, direct cell contact between SOM 196B and HULECs was required to influence the apparent up-regulation. As unlike VCAM-1, increases in E-selectin were specifically induced by SOM 196B cells, it is possible that this receptor is not utilised by SOM 157d or A375 cells in adhesion to the endothelium. Blocking these receptors in adhesion assays would provide further information regarding the precise interactions occurring.

For the non-invasive uveal melanoma cell line (SOM 157d), increased endothelial ICAM-1 in response to potentially soluble tumour-derived factors was most evident (Table 6.2.2.c and Figures 6.2.2.d and e). As lack of endothelial ICAM-1 has been associated with increased B16 melanoma liver metastasis (Marvin *et al.*, 1998), up-regulation of endothelial ICAM-1 by SOM 157d cells may therefore associate with a less invasive phenotype. As both dermal and liver endothelial cells were affected, the effect was less specific for the individual endothelial cell type. Similar increases were not seen with E-selectin and VCAM-1, and it could therefore be speculated that an ICAM-1-mediated but not an E-selectin or VCAM-1 interaction may be assisting SOM 157d binding to these endothelial cell types. Non-metastatic breast carcinoma cells have been shown to bind to the endothelium using an ICAM-1 mechanism whereas metastatic cells utilised E-selectin- and VCAM-mediated interactions (Moss *et al.*, 1999; Moss *et al.*, 2000), supporting results for both SOM 157d and 196B. Such interactions for SOM 196B could potentate a stronger adhesive interaction with the endothelium, as reflected by adhesion assays (Figures 6.2.1.a and b).

Conflicting evidence exists regarding uveal melanoma ICAM-1 expression (Natali *et al.*, 1997; Lawry *et al.*, 1999), but a recent report has correlated loss of tumour cell ICAM-1 expression with increased risk of metastasis within the first five years (Anastassiou *et al.*, 2000b). In this present study, A375 cells expressed high levels of ICAM-1, whilst both uveal melanoma cell types did not. As detailed

previously in Chapters 4 and 5 (4.3 and 5.3.1), patient SOM 157d died of metastatic disease, whereas patient SOM 196B is alive to date, and thus expression of ICAM-1 represents an example whereby responses seen *in vitro*, are not fully representative of the tumour *in vivo*. Adhesion to the endothelium nevertheless only represents one stage of the metastatic process. Therefore with regard to SOM 196B, despite cells being adept at adhering to the endothelium, tumour cells may not be effective at completing all other processes of the metastatic cascade (as reflected by the inefficiency of these cells at traversing the endothelium, as presented in Chapter 4 (4.3)).

Previous evidence for uveal melanoma has reported a potential association between expression of $\alpha 6\beta 1$ and poor prognosis (Rohrbach *et al.*, 1994; Elshaw *et al.*, 2001), but this could not be supported by results from Chapter 3 (3.3.2.1). Although data was not significant, a decrease in $\alpha 6$ expression by SOM 196B cells after culture with dermal endothelial cells (Figure 6.2.3.c) could therefore relate to the lack of secondary disease currently seen in this patient. Stimulated decreases in tumour $\alpha 6$ expression by dermal endothelial cells could therefore inhibit uveal melanoma extravasation at dermal sites. From study of only one culture system, it is however not possible to draw reliable conclusions.

Alternative endothelial adhesion molecules should also be considered for uveal melanoma adhesion to the endothelium. For example uveal melanoma cell lines express CD44 (Creyghton *et al.*, 1995), and expression by colon cancer cells plays a pivotal role in increasing adhesion to endothelium and transendothelial cell migration (Fujisaki *et al.*, 1999). In cutaneous melanoma, expression of N-cadherin has also been shown to assist adhesion to the endothelium, whilst similarly being involved in transendothelial migration (Sandig *et al.*, 1997; Voura *et al.*, 1998b; Li *et al.*, 2001). Adhesion is therefore likely to utilise a far greater range of adhesion molecules than those studied in this investigation.

In summary, invasive and non-invasive uveal melanoma cells varied in their levels of adhesion to the endothelium, but no preference for hepatic endothelial cells was shown. The different patterns of adhesion molecule up-regulation after co-culture would further suggest that different systems are involved in adhesion of these tumour cell types to the endothelial cells studied, which could be implicated in site-specific metastasis. As cell lines have been used in this study, the use of STCs

may provide a closer approximation to interactions occurring *in vivo*, allowing potential correlation with clinical details. Due to time constraints and a lack of sufficient cell numbers, this could not be undertaken at this stage.

Chapter 7

Final Discussion and Conclusions

7.1. Uveal melanoma and liver targeting

Figure 7.1.a illustrates the processes studied by this investigation, and summarises brief conclusions made.

An efficient method for culturing primary uveal melanomas has been established within the Academic Unit of Ophthalmology and Orthoptics, and as such allowed analysis of a wide range of primary tumours with potentially different metastatic properties. As all of the primary tumours studied were obtained by surgical means, they were of necessity, a biased sample, as their large size precluded the use of more conservative treatments. On this basis, all tumours could be classified as having a poor prognosis. Since most tumours were only recently resected, in the majority of cases metastases have, as yet not been detected. It therefore remained unclear whether cells from these primary tumours would remain confined within the boundaries of the eye (i.e. behave as relatively benign tumours, similar to iris melanomas), or invade into the circulation, potentially metastasising to a secondary tissue. Iris involvement was however limited to one tumour alone, and thus the samples studied were further prejudiced towards tumours originating either in the choroid or ciliary body. Although cell lines alone were investigated for most aspects of these studies, by assessing STCs and relating patterns of expression and responses to histo-pathological data, the results suggest that certain observations are valid.

For five of the patients of the studied (SOM 157d, 250, 255, 262, and 272) hepatic metastases had been detected, suggesting that cells had disseminated rapidly and successfully established secondary foci. Factors associated with these cells may therefore have been associated with a poorer prognosis, allowing certain conclusions to be drawn relating to the metastatic phenotype. Consistent with classic prognostic indicators, these tumours were all considerably larger than other tumours studied, and were more likely to have ciliary body involvement and characteristic chromosomal abnormalities, and an epithelioid or mixed morphology; all factors associated with a worse prognosis.

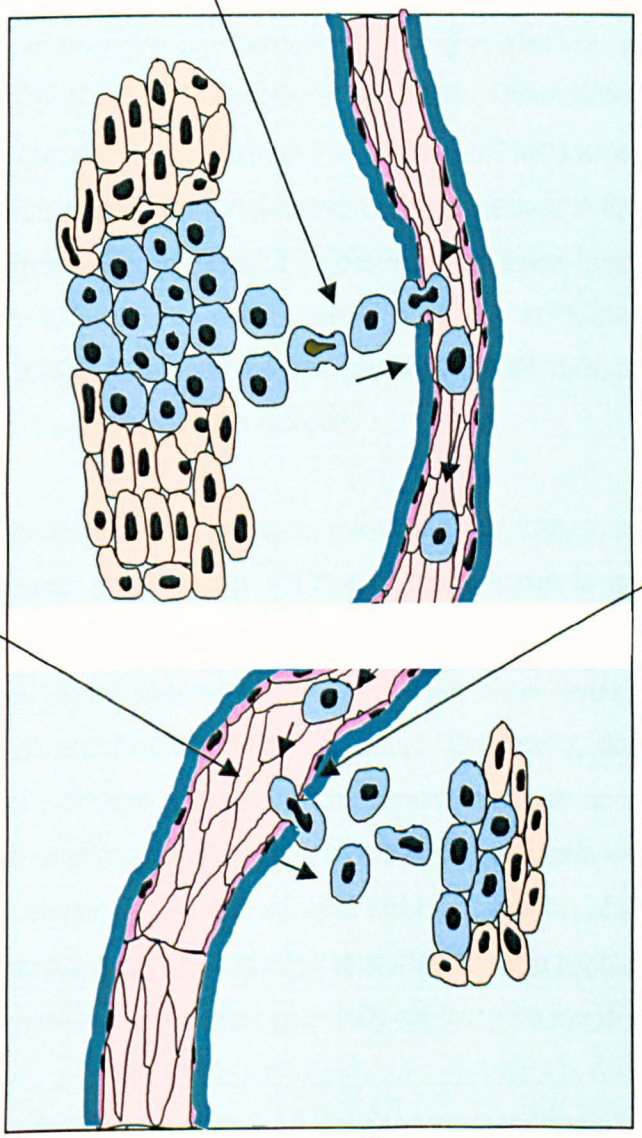
Chapter 3

The ability of tumour cells to invade through the ECM is important in metastasis, and required at both the primary and secondary sites. For tumours to accomplish this, a number of adhesive and degradative interactions are undertaken. Results from this study demonstrate that uveal melanomas ubiquitously express some integrins and degradative enzymes associated with these processes, whilst for others (α1, α2, α4, and α6), expression is more variable, potentially affected by *in vitro* stimulation.

Chapter 4

Once invaded through the ECM and BM, tumour cells must migrate through the endothelium into the circulation. Many *in vitro* studies of invasion have employed models assessing invasion through the BM alone. In this present study, a model of transendothelial migration was established, and results showed that for uveal melanoma, invasion seemed to correlate with a wider range of prognostic indicators than through the BM alone.

Primary site



Chapter 5

Once in the circulation, tumour cells are transported to the target site. For some tumours this may be the first vascular bed encountered, whilst others show some site-specificity. Results have indicated that factors secreted by cells derived from hepatocytes and the hepatic endothelium stimulate invasion, whilst invasion may be inhibited by cells derived from the lung, but the effect is dependent upon the individual tumour.

Chapter 6

At the target site, tumour cells must attach to the endothelium. Adhesion molecules expressed by the endothelial and tumour cells may further determine the site of arrest. Hepatic endothelial cells in this study differed from dermal endothelial cells in their expression of α4 alone and differences in levels of adhesion between invasive and non-invasive cells were seen. Invasive tumour cell lines, lacked ICAM-1, and co-cultures stimulated increases in hepatic endothelial ICAM-1, VCAM-1, and E-selectin, and tumour α4, and this may have been regulated by an absence of endothelial α4 expression. Non-invasive cell lines lacked expression of ICAM-1 and co-cultures stimulated increases in endothelial ICAM-1 only.

Secondary site

Figure 7.1.a. Diagrammatic representation of the metastatic cascade, identifying the processes studied in this investigation and brief conclusions drawn (adapted from Introduction A Figure 1.a). Representative normal cells are shown in beige and the light blue areas represent surrounding tissue. Metastatic tumour cells are shown in blue, the basement membrane is shown in green, and the endothelium is shown in pink.

From this present study, those aspects potentially associated with more advanced malignancies may therefore hold true for these cases. For example, with regard to adhesion molecule expression, results from previous investigations have associated α6 expression, involved in laminin binding (and hence primarily basement membrane adhesion) with the invasive epithelioid phenotype and worse prognosis (Rohrbach *et al.*, 1994; Elshaw *et al.*, 2001). In support of these observations, SOM 157d, 250 and 255 all expressed this integrin, and correspondingly were more likely to be of an epithelioid cell type. In addition, these tumours also expressed the α4 integrin subunit, known to be involved with liver metastasis of a number of other tumour types (Papadimitriou *et al.*, 1999; Okada *et al.*, 1999; Vidal-Vanaclocha *et al.*, 2000; Langley *et al.*, 2001). Conclusions drawn from this present study have similarly speculated that expression of α4 by uveal melanoma cells (in conjunction with increases hepatic endothelial VCAM-1 expression) could be involved in liver targeting, and as these tumours have disseminated to the liver, it is possible that these tumours reflect this. Of the metastatic tumours, ICAM-1 expression, also previously associated with poor prognosis (Anasatassiou *et al.*, 2000b), was additionally investigated for SOM 157d, and in support of published data, this tumour lacked expression of this receptor.

Once migrated through the ECM and basement membrane, the ability to traverse the endothelium is a further important aspect of metastasis. Of those tumours known to have already metastasised, transendothelial invasion was only assessed for SOM 262 and SOM 272 (Figure 4.2.2.a). Although SOM 262 invaded well through basement membrane barriers alone, levels were significantly reduced by the inclusion of an endothelial layer to the assay. Conversely, despite SOM 272 behaving relatively non-invasively through both the basement membrane only and transendothelial invasion assays, it is however of interest, that a high proportion of these cells were able to overcome the additional endothelial barrier. As such, these cells which possess the ability to invade through both the basement membrane and endothelium might reflect the invasive population of cells, which *in vivo* have the ability to traverse the endothelium, potentially validating the model's use.

In contrast to those patients known to have developed secondary disease, for the majority of patients studied, metastases had however as yet not been detected. It is feasible that a number of these tumours will remain benign and never invade from the primary site. Contrary to those properties associated with a poor prognosis, an absence of such factors would therefore correlate with a better

prognosis. For example, with regard to adhesion molecules, if expression of α4 and α6 is be involved in liver metastasis and poor prognosis, conversely, lack of expression may be correlated with a better prognosis. Consistent with this hypothesis, SOM 239 lacked expression of both of these subunits, and originated in the choroid, a location associated with a better prognosis (Table 3.2.2.a).

In the remaining eight of these nine cases studied for adhesion molecule expression, in which metastases were absent, expression of the α4 and α6 subunits was observed. Following the theories regarding association of α4 and α6 expression with secondary disease and poor prognosis, such tumours could therefore ultimately have the propensity to metastasise. As uveal melanomas have been detected up to 42 years after initial diagnosis (Egan *et al.*, 1988), and due to the time scale of this investigation, it seems possible that cells from these tumours could have already disseminated but as yet secondary metastatic growth has not been stimulated. No correlation could however be made between expression of α4 and α6 by these tumours and known prognostic indicators to support this proposal.

A further selection of these tumours were analysed for their ability to overcome the basement membrane and endothelium. Those tumours more likely to metastasise would therefore, by necessity, be required to overcome both of these barriers. In support of this, those tumours that were more proficient at invading through both basement membrane and endothelial cell barriers were on average more likely to be larger tumours with mixed cell morphology and ciliary body involvement (factors all associated with poor prognosis) (SOM 280, 290 and 296) (Figure 4.2.2.a). Similarly tumours that were less efficient at overcoming both of these barriers were in general of choroidal origin, a factor linked with better prognosis (SOM 196B, 262, 275, 277, 281, 301, and 306). In further support, SOM 196B, the only tumour resected over four years ago, has as yet not developed metastases. This tumour invaded well through basement membrane barriers alone, yet was poorly invasive through the transendothelial cell assay. Similar to metastatic SOM 272, the behaviour of SOM 196B in this model could therefore confirms its use, identifying those tumours more likely to disseminate.

The inclusion of an endothelial layer in the invasion assay nevertheless reduced invasion for most tumours studied (Figure 4.2.2.a). It is therefore plausible that uveal melanomas are in general,

relatively inefficient at crossing the endothelium. In support of this speculation, detection of low levels of uveal melanocytes and melanoma cells in the circulation has been reported (Tobal *et al.*, 1993; Foss *et al.*, 1995; Hanekom *et al.*, 1999). Authors however concluded that the presence of circulating cells is not a predictive indicator of metastatic disease, and leads to the possibility that although tumours cells are circulated around the body, metastatic disease might not be a consequence. In agreement with this, micrometastatic uveal melanoma cells have been detected in the bone marrow of 33.9% of patients (Eide *et al.*, unpublished data), yet metastases rarely grow at this site.

The two theories of metastasis may therefore each be partially correct for uveal melanoma. Following the mechanical entrapment theory, uveal melanoma cells could therefore circulate widely, becoming physically trapped at a variety of sites. Once lodged in capillaries, cells may consequently grow to a minimal size, or alternatively reside at certain locations until they die. The work by Eide *et al.*, (1999) would further support this hypothesis, as micrometastases may develop in locations such as the bone marrow, but without an angiogenic stimulus, cells would remain dormant. Metastatic progression at sites such as the liver would therefore only be a consequence once the balance between angiogenic inhibitors and stimulators, favours promotion of the latter, ultimately stimulating secondary growth. Successful metastatic development subsequently only results due to specific properties of both the tumour and microenvironment of the liver, promoting secondary disease, essentially following Paget's theory also (Paget, 1889). In further support of this, expression of the receptors for HGF, EGF, and IGF-I have all been previously associated with liver targeting and metastasis of uveal melanoma (Hendrix *et al.*, 1998a and b; Ma and Niederkorn, 1998; Ericsson *et al.*, 2002). A combination of factors produced by hepatic and inflammatory cells and a possible lack of angiogenic inhibitors would therefore assist in creating the favourable environment required to promote metastatic growth.

In this present study, most tumours studied responded significantly to factors secreted by cells derived from hepatocytes (Figure 5.2.1.a), but no direct association with known prognostic indicators could be made. Conversely, invasion of one of these tumours (SOM 277), whilst being attracted by hepatic cells, was in contrast, inhibited by cells of pulmonary origin, further supporting an argument for attraction to the liver, in preference to other organs. It is of interest however, that one tumour (SOM 269) did not react positively to factors secreted by the HepG2 cells. This tumour was

considerably larger than most studied, and was also of ciliary body origin, and as such associated with a worse prognosis. It could therefore be speculated that this tumour instead is capable of autocrine regulation, not necessarily requiring the same degree of support from other cell types for survival. In support of this hypothesis, SOM 196B also responded significantly to factors secreted by the same cell type (Figure 5.2.2.a), inferring that these cells have the propensity to produce a number of autocrine factors.

In contrast to cells derived from the lung, keratinocytes and fibroblasts were however also seen to stimulate invasion of SOM 196B (Figure 5.2.2.a), supporting theories suggesting that uveal melanoma cells circulate widely. It is nevertheless likely that sites elsewhere do not provide the ideal 'soil' requirements to promote metastatic growth. Consistent with this argument, after culture of SOM 196B cells with dermal or hepatic endothelial cells, notable changes in adhesion molecule expression only resulted after interactions between tumour and hepatic endothelial cells (Figures 6.2.2.e and 6.2.3.a and b, and Tables 6.2.2.a-c and 6.2.3.a). Dermal endothelial cells further expressed α4, whilst co-culture with uveal melanoma cells did not up-regulate tumour α4 expression. As it has been hypothesised from this study that α4 expression is involved with liver targeting, it is feasible that such patterns may therefore not promote arrest at dermal sites. In addition, dermal endothelial cells were observed to down-regulate SOM 196B α6 expression, which following association of α6 expression with poor prognosis (Rohrbach *et al.*, 1994; Elshaw *et al.*, 2001) could aid in preventing attachment and extravasation. As a consequence, despite attraction to other organs, successful adhesion to the endothelium would potentially only be initiated at the liver. Investigation of the regulation of tumour α6 expression by hepatic endothelial cells will provide additional information regarding the importance of this observation.

In most instances, metastatic disease may therefore only result if circulating uveal melanoma cells arrest in the hepatic vasculature, successfully extravasate, and ultimately are promoted to develop into secondary metastases. In reflection of this, invasive SOM 196B cells adhered to endothelial cells in significantly higher levels than non-invasive SOM 157d cells (Figures 6.2.1.a and b). Hepatic endothelial cells correspondingly also attracted SOM 196B cells in invasion assays (Figure 5.2.2.a). In contrast to dermal endothelial cells, hepatic endothelial cells (lacking α4 expression), up-regulated invasive SOM 196B α4 expression. This factor might be particularly important in view of the fact that

the ligand for α4, VCAM-1 was shown to be up-regulated concurrently. By extrapolation, this may suggest that increased tumour α4 was directly related to increased endothelial VCAM, ultimately effecting increases in adhesion. In addition, as these uveal melanoma cells also stimulated an increase in hepatic endothelial E-selectin expression, it seems feasible that this receptor was utilised in the adhesive interaction. Such patterns were not a consequence after culture of the non-invasive cell line SOM 157d with either endothelial cell type, and could in part infer that different interactions were being utilised for adhesion.

Although this aspect only assessed changes in response to two uveal melanoma cultures, it could be hypothesised that as, in particular the VCAM-1-α4 interaction has been associated with liver metastases of a number of other tumour types (Papadimitriou *et al.*, 1999; Okada *et al.*, 1999; Vidal-Vanaclocha *et al.*, 2000; Langley *et al.*, 2001), this interaction is primarily implicated in hepatic metastases of uveal melanoma also. This may further be regulated by an absence of endothelial α4 expression, whilst an E-selectin-mediated process might further strengthen this interaction. As endothelial ICAM-1 was up-regulated by both invasive and non-invasive cells, the involvement of this receptor might be less relevant with regard to the invasive phenotype. As however loss of ICAM-1 by uveal melanoma cells has been associated with poor prognosis (Anasatassiou *et al.*, 2000b), and due to a similar lack of expression by the uveal melanoma cells studied, a combination of all of these factors would ultimately be required for determining adhesion to the hepatic endothelium. To further confirm these speculations, it would be necessary to expand the study to a wider range of uveal melanoma cultures, whilst in light of the apparent differences in endothelial α4 expression, analysis of expression of ligands for this subunit by the uveal melanoma cultures studied will elicit further information regarding the precise involvement in adhesion to the endothelium. Additional study should also focus of the factors potentially regulating changes in expression of these adhesion molecules. Aspects of this work however also focused on SOM 196B cells. In culture these cells behaved invasively, whilst *in vivo*, metastases have yet to be detected. The observations *in vitro* therefore reflect an invasive capacity, but as metastatic disease has not yet been observed, the behaviour *in vivo*, may ultimately relate more closely to the poor ability of the cells at traversing the endothelium. More accurate conclusions could therefore be drawn through study of a highly invasive and metastatic uveal melanoma. It is still nevertheless possible that SOM 196B cells *in vivo* will

eventually successfully metastasise, and thus close observation of this patient is required to elicit more long-term conclusions.

In conclusion, if we are to consider the two theories of metastatic spread ('mechanical dissemination' and the 'seed and soil' hypothesis), the work of this current investigation has in some way, clarified aspects of the particular targeting of the liver by uveal melanomas. Despite physical entrapment of circulating uveal melanoma cells in the liver assisting metastasis, ultimately more specific 'seed and soil' factors, may play a more vital role. Factors determined by both the hepatic vasculature and microenvironment, together with the properties of the tumour cells themselves, might thus facilitate the ultimate fate of the malignancy. Only tumour cells expressing the required phenotype may eventually accomplish all of the necessary processes required to establish secondary growth. The investigation has nevertheless only examined a certain number of processes involved in metastasis for a limited number of tumours. In particular, although cells have been shown to traverse the hepatic endothelium, it is still ultimately unknown as to whether these cells may survive and proliferate in the hepatic tissue. To build a more complete picture of the process in its entirety, further research into factors promoting metastatic development for a wider range of uveal melanomas, is therefore imperative in conjunction with on-going cytogenetic analysis.

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Appendix I

Names and Addresses of Suppliers

Anachem Limited,
Anachem House, 20 Charles Street, Luton, Bedfordshire, LU2 0EB.

Ansell Medical,
Ansell House, 119 Ewell Road, Surbiton, Surrey, KT6 6AY.

Antec International Limited,
Windham Road, Sudbury, Suffolk.

Beckton Dickenson (BD),
Between Towers Road, Cowley, Oxford, OX4 3LY.

Bibby Sterilin Limited,
Tilling Drive, Stone, Staffordshire, ST15 0SA.

Boots Company PLC,
Nottingham.

British Oxygen Company (BOC),
10 Priestly Road, Guildford, Surrey.

Chemicon International, Limited,
2 Admirals House, Cardinal Way, Harrow, HA3 5UT.

Corning Incorporated,
HP-AB-03, Corning, NY 14831, USA.

Costar (UK) Limited,
10 The Valley Centre, Gordon Road, High Wycombe, Buckinghamshire, HP13 6EQ.

DAKO Limited,
Denmark House, Angel Drive, Ely, Cambridgeshire, CB7 4ET.

Denley,
Stevenage, London

ECACC,
Division of Biologies, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG.

Gelman Sciences/ Walker Limited,
Brackmills Business Park, Caswell Road, Northamptonshire, NN4 7EZ.

Genetic Research Instrumentation,
Gene House, Dunmow Road, Felsted, Dunmow, Essex, CM6 3LD.

Grant Instruments (Cambridge) Limited,
Cambridge, CB2 5QZ.

Helena Biosciences,
Colima Avenue, Sunderland Enterprise Parl, High Wycombe, Buckinghamshire, HP13 7DL.

Heraeus Equipment Limited,
Unit 9, Wates Way, Brentwood, Essex, CM15 9TB.

Invitrogen (formerly Life Technologies),
3 Fountain Drive, Inchinnan Business Park, Paisley, PA 4 9RF.

Merck/BDH,
Merck House, Poole, Dorset, BH15 1TD.

Millipore UK Limited,
The Boulevard, Blackmore Lane, Watford, Hertfordshire, WD1 8TW.

Molecular Probes Europe,
Cambridge BioScience, 24-25 Signet Court, Newmarket Road, Cambridge, CB5 8LA.

Nalge Nunc Company,
Rochester, NY-14602-0365, USA.

Nalgene,
75 Panorama Creek Drive, PO Box 20365m, Rochester, New York, 14602-0365, USA.

Novocastra Laboratories,
Balliol Business Park West, Benton Lane, Newcastle upon Tyne, NE12 8EW.

Olympus Optical Company Limited,
2-8 Honduras Street, London, EC17 0TX.

Purite Limited,
Bandet Way, Thame, Oxon, OX9 3SJ.

R&D Systems Europe Limited,
4-10 The Quadrant, Barton Lane, Abingdon, Oxon, OX14 3YS.

Sanyo,
Sanyo Scientific, 600 Suprime Drive, Bensenville, IL 60106, USA.

Scientific Laboratory Supplies Limited (SLS),
Wilford Industrial Estate, Nottingham, NG11 7eP.

Scotsman Ice Systems Limited,
20010 Bettolino di Pogliano, Milan, Italy.

Serotec Limited,
22 Bankside, Station Approach, Kidlington, Oxford, OX5 1JE.

Sigma-Aldrich Company Limited,
Fancy Road, Poole, Dorset, BH12 4QH.

Starstedt Limited,
68 Bosteon Road, Beaumont Leys, Leicester, LE4 1AW.

Swann Morton Limited,
Owlerton Green, Sheffield, S6 2BJ.

TAAB Laboratories Equipment Limited,
Aldermaston, Berks, RG7 8NA.

TCS CellWorks Limited,
Botolph Claydon, Buckingham, MK18 2LR.

Unipath Limited,
Wade Road, Basigstoke, Hants, RG24 0PW.

Vector Laboratories Limited,
Peterborough.

Vernon carus,
Preston, Lancs.

Appendix II

Histo-pathological details of patients treated for posterior uveal melanoma by enucleation. (Volume = $\pi/6$ x length x height x width)

Short-term culture (STC)	Age	Sex	Location	Metastatic disease	Volume/ mm^3	Cell type	Status
S.O.M 157d	73	M	Choroid	Yes	3647	Epithelioid	Dead
S.O.M. 196B	80	M	Choroid	No	885	Mixed	Alive
S.O.M. 200	42	M	Choroid	No	922	Mixed	Alive
S.O.M. 210	64	M	Ciliary Body	No	974	Mixed	Alive
S.O.M. 238	23	F	Choroid	No	754	Spindle	Alive
S.O.M. 239	44	F	Choroid	No	1016	Mixed	Alive
S.O.M. 248	78	M	Ciliary Body	No	1705	Mixed	Alive
S.O.M. 250	61	F	Choroid	Yes	2205	Mixed	Dead (liver metastases) (26-5-00)
S.O.M. 253	51	M	Ciliary Body/ Choroid	No	847	Spindle B	Alive
S.O.M. 255	63	F	Ciliary Body/ Choroid	Yes	1138	Spindle B	Terminally ill (liver metastases)
S.O.M. 256	80	F	Choroid	No	481	Spindle B	Alive
S.O.M. 260	58	F	Choroid	-	-	Spindle A and B	Alive
S.O.M. 262	73	M	Choroid	Yes	Too large to scale	Mixed (50% epithelioid)	Alive
S.O.M. 263	71	M	Choroid	No	725	Mixed	Alive
S.O.M. 266	62	F	Choroid	No	-	Spindle B	Alive
S.O.M. 267	50	F	Choroid	No	1395	Spindle	Alive
S.O.M. 269	72	M	Ciliary Body	No	2273	Spindle B	Alive
S.O.M. 272	71	F	Choroid	Yes	1795	Spindle B	Alive
S.O.M. 273	70	F	(Iris) Ciliary Body	No	-	Mixed (50% epithelioid)	Alive
S.O.M. 274	82	F	Choroid	-	511	Spindle	Alive
S.O.M. 275	71	M	Choroid	No	1070	Spindle B	Alive
S.O.M. 277	74	M	Choroid	No	1476	-	Alive
S.O.M. 279	72	M	Choroid	No	1263	Spindle B	Alive
S.O.M. 280	85	M	Choroid	No	2621	Mixed	Alive
S.O.M. 281	54	M	Choroid	No	1273	Spindle B	Alive
S.O.M. 282	64	M	Ciliary Body	No	1141	Mixed	Alive
S.O.M. 286	79	F	Choroid/ Ciliary Body	No	677	Spindle B	Dead (unknown cause)
S.O.M. 288	59	M	Choroid/ Ciliary Body	No	1158	Mixed	Alive
S.O.M. 289	88	F	Choroid	No	1477	Mixed	Alive
S.O.M.290	53	M	Choroid	No	754	Mixed	Alive

S.O.M. 295	13	F	Choroid/ Ciliary Body	No	Too large to scale	Spindle B	Alive
S.O.M. 296	55	M	Ciliary Body	No	1270	Mixed	Alive
S.O.M. 301	28	M	Choroid	No	1829	Spindle B	Alive
S.O.M. 302	66	M	Choroid	-	272	Mixed	Alive
S.O.M. 304	70	M	Choroid	-	171	Spindle B	Alive (lung primary tumour also)
S.O.M. 305	67	M	Ciliary Body	No	1166	Spindle B	Alive
S.0.M. 306	81	M	Choroid	No	730	Mixed (10% epithelioid)	Alive
S.O.M 307	65	M	Choroid	No	764	Spindle B	Alive
S.O.M. 308	72	M	Choroid	-	-	Spindle B	Alive
S.O.M. 311	60	F	Ciliary Body	-	-	Spindle A and B	Alive