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Isolation, culture and characterisation of murine microvascular endothelial cells

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ISOLATION, CULTURE AND CHARACTERISATION OF MURINE MICROVASCULAR ENDOTHELIAL CELLS.

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Abstract

Microvascular endothelial cells were isolated from the brain and lung tissue of C57 mice establishing a model for studies concerning the functional and behavioural diversity of these cells. Brain microvascular endothelial cells were isolated and cultured using a modification of published methods. A novel procedure was developed for the isolation and purification of lung microvascular endothelial cells. The isolation and culture techniques for lung derived cells were extensively developed in order to overcome the difficulties encountered in obtaining pure microvessel fragments. In the modified technique, the whole lung tissue was perfused *in situ* and the pleural membrane devitalised; the peripheral lung tissue was homogenised, digested with collagenase/dispase, and the isolated microvessel fragments were purified by Percoll gradient centrifugation and differential plating. The cells were successfully cultured on collagen coated culture wells in Ham's F-10 culture medium supplemented with endothelial cell growth factors, maintaining their differentiated characteristics.

Lung and brain microvessel cultures were characterised by morphological and immunocytochemical studies using light and electron microscopy. Lung microvascular endothelial cells grew as colonies of polygonal shaped cells while brain endothelial cells grew as colonies of spindle shaped cells which formed typical contact-inhibited monolayers. In order to enhance culture purity, the lung derived cell colonies were selectively cloned forming contact-inhibited monolayers. Brain and lung microvascular endothelial cells had the ability to express von Willebrand factor, accumulate fluorescently labelled acetylated low density lipoprotein (Bo-Ac-LDL) and provide a non-thrombogenic surface to platelets. In addition, lung derived cells formed capillary-like structures on Matrigel confirming their endothelial origin.

Binding studies conducted at 4°C and 37°C using an analogue of α-Melanocyte stimulating hormone, [^{125}I-Tyr^{2}, Nle^{4} D-Phe^{7}] α-MSH, indicated that a small population of melanocortin receptors was expressed on the surface of brain but not lung microvascular endothelial cells. The existence of a specific binding site for α-MSH on brain endothelial cells was confirmed by photoaffinity labelling with [^{125} I-Tyr^{2}, Nle^{4}, D-Phe^{7}, ATB-Lys^{11}] α–MSH and SDS-PAGE analysis. This study provides a valuable insight into the isolation, culture and characterisation of organ-derived microvascular endothelial cells.

To

Mum and Dad

Julie, Clayton and "Junior" Hardisty

and

Nicola and Owen Hazel

Thank you

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Abbreviations

APS	Ammonium persulphate
ACE	Angiotensin converting enzyme
Ac-LDL	Acetylated low density lipoprotein
ACTH	Adrenocorticotrophic hormone
BSA	Bovine serum albumin
BAEC	Bovine aortic endothelial cells
CPM	Counts per minute
CNS	Central nervous system
CS	Calf serum
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagles medium
DAB	3-3' Diaminobenzedine tetra hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular matrix
ECGS	Endothelial cell growth supplement
ELAM-1	Endothelial leukocyte adhesion molecule-1
aFGF	acidic Fibroblast growth factor
bFGF	basic Fibroblast growth factor

FCS	Foetal calf serum
FAB-MS	Fast atom bombardment-mass spectrometry
GS-1	Griffonia simplicifolia-1
HUVEC	Human umbilical vein endothelial cells
HBSS	Hanks balanced salts solution
HMEC-1	Human microvascular endothelial cell line-1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HBT	Hepes buffered tyrode
HPLC	High performance liquid chromatography
ICAM-1	Intercellular adhesion molecule-1
ICAM-2	Intercellular adhesion molecule-2
IL-1	Interleukin-1
Lu-ECAM-1	Lung-endothelial cell adhesion molecule-1
LDL	Low density lipoprotein
MSH	Melanocyte stimulating hormone
mRNA	messenger Ribonucleic acid
MC1-R	Melanocortin 1-receptor
MC2-R	Melanocortin 2-receptor
MC3-R	Melanocortin 3-receptor
MC4-R	Melanocortin 4-receptor
NEAA	Non essential amino acids
PAEC	Porcine aortic endothelial cells

PECAM-1	Platelet-endothelial cell adhesion molecule-1
PDS	Plasma derived serum
PPHS	Platelet poor human serum
PDGF	Platelet derived growth factor
PD-ECGF	Platelet derived-endothelial cell growth factor
POMC	Pro-opiomelanocortin
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PGI_2	$Prostaglandin_2$
RDGF	Retinal derived growth factor
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TNF	Tumour necrosis factor
TGF-β	Transforming growth factor-β
TLCK	tosyl-lysine-chloromethyl-ketone
TFA	Trifluoroacetic acid
TEM	Transmission electron microscopy
UEA-1	Ulex europeaus-1
VEGF	Vascular endothelial growth factor
v/v	volume for volume
w/v	weight for volume

Arg	Arginine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Lys	Lysine
Met	Methionine
Nle	Nor leucine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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1. INTRODUCTION

1.1. Introduction

Endothelial cells are the most widely distributed cell type in the body forming a single, continuous cell layer which lines the blood vessels of the entire vascular system. It is now widely accepted that the function of endothelial cells is not simply as an inert barrier lining. Due to its strategic position at the interface between the blood and the tissue, the endothelium plays a vital role in the maintenance of homeostasis and the control of many other events in vascular biology. Studies conducted over the past 30 years have identified a diverse range of structures and functions of the endothelial cell layer in normal activities and diseases. Differences in the functional activities of the microvessels have been shown to exist between various organs and tissues and these diverse activities are paralleled by the structural variations of the microvascular endothelium (Simionescu and Simionescu 1984; Wagner 1988; Ryan 1989).

The work in this thesis has involved the isolation, culture and characterisation of pure cultures of organ derived microvascular endothelial cells from murine tissues, and the investigation of a receptor mediated pathway for the uptake of α-melanocyte stimulating hormone on these cells. The introduction will describe the structure and functions of endothelial cells and the problems associated with isolating, maintaining and characterising pure microvascular endothelial cells in culture. The functions of the melanotropins and melanotropin receptors, and finally the aims of this project will be briefly described.

1.1.1. Distribution of Endothelial Cells

Three categories of endothelium have been determined based on variations in the continuity of the endothelium and its basement membrane: continuous; fenestrated and

discontinuous (sinusoidal) endothelium. The continuous endothelium is the most widely distributed in mammalian tissues, it occurs in large vessels, arterioles, most venules of skeletal, heart and smooth muscles, lung, brain, skin, subcutaneous tissue, sinus and mucous membranes.

Fenestrated endothelial cells are found in organs whose functions require a high rate of fluid exchange (Taylor and Granger 1984), this includes the intestinal mucosa, exocrine and endocrine glands and the glomerular and peritubular capillaries of the kidney. These cells display circular openings known as fenestrae which usually occur in clusters at irregular intervals and are commonly closed by a thin diaphragm possibly derived from the basal lamina.

The distribution of discontinuous endothelial cells is more restricted than other endothelial cell types: they are found predominantly in the liver, spleen and bone marrow and are characterised by their lack of basement membrane (Simionescu and Simionescu 1984; Malik and Siflinger-Birnboim 1993).

1.1.2. Microvascular Endothelial Cell Structure

The components of the microvascular wall are dynamic features able to facilitate many physiological activities such as permeability, transport, uptake, synthesis and metabolism of many substances. The microvessel wall is a composite of various parallel layers of different composition and function: the plasma-endothelial interface, endothelium, basal lamina and adventitia (Petrak and Goddard 1989). The unique position of the endothelial cell is such that the luminal face is directly exposed to the blood, and the abluminal face is exposed to the interstitial fluid. The abluminal face is attached to the basal lamina which is produced by the endothelial cell itself; this in turn mediates contact with the surrounding tissues (Simionescu and Simionescu 1984).

Endothelial cell surface

At the luminal face of the endothelial cell is the plasma membrane which, depending on the location within the tissue, coats open plasmalemmal vesicles, coated pits, transendothelial channels and fenestral diaphragms but is omitted from intercellular junctions. This endothelial cell coat is formed from a variety of membrane components including: plasma proteins, such as heparin; albumin and fibrin, which are adsorbed on to the luminal surface; glycoproteins; sialoglycoconjugates and proteoglycans, such as heparan sulphate. Together these membrane components constitute the plasma-endothelial interface.

The distribution of these membrane components varies in different organs as well as along the surface of the endothelium of the same organ; the plasma membrane is more or less prominent in areas such as the vesicles, transendothelial channels and fenestral diaphragms (Simionescu *et al.* 1981b; Simionescu *et al* 1982a). These membrane components may function as: 1) a sieving network; 2) a locally differentiated charge barrier generated by preferential distribution of the different components; and 3) specific binding sites and receptors for molecules undergoing endocytosis and transcytosis, with some of these components being continuously active while others require stimulus.

The endothelial cell surface, vesicles, channels and fenestrae exhibit microdomains of different charge. The preferential distribution of the sialoglycoconjugates, proteoglycans and glycoproteins is related to the distribution of these differentiated microdomains throughout the endothelium. Electrophysiology studies (Sawyer and Srinivasan 1972) and studies involving charged tracers *in situ* (Skutelsky *et al.*, 1975) and *in vitro* (Pelikan *et al.*, 1979) have shown that the endothelial cell surface has an overall negative charge although the anionic sites have a very refined distribution (Simionescu *et al.*, 1981a).

The differentiated microdomains are involved in endocytosis and transcytosis across the capillary endothelium. In addition the combination of the molecular composition of the

cell and the charge causes the endothelial cell to exhibit a non-thrombogenic surface. Concurrently, this effects the involvement of the endothelial cell surface in coagulation, anticoagulation and homeostasis (Simionescu and Simionescu 1986).

The endothelial cell surface also displays a number of receptors and binding sites including, those for histamine, lipoproteins, insulin (Hachiya *et al.*, 1988), thrombin, transferrin (Jefferies *et al.*, 1984) and albumin (Shasby and Shasby 1985). A variety of enzymes are also associated with the luminal surface of the microvascular endothelium such as angiotensin-converting enzyme (Ryan *et al.*, 1976), Factor VIII (Jones *et al.*, 1981) and prostacyclin which also contribute to the diverse functions of the endothelium (Simionescu and Simionescu 1986; Ryan 1989).

Coated pits, plasmalemmal vesicles and transendothelial channels

Coated pits (diameter: ~80-120nm) and vesicles have been located on both endothelial surfaces , however they are more numerous on the luminal surface. They are found in most endothelial cell types, but particularly in the bone marrow sinusoids and endocrine tissues. In continuous and fenestrated capillaries coated pits and vesicles have high-affinity anionic sites however, in sinusoidal capillaries, both anionic and cationic sites are expressed (Simionescu and Simionescu 1984). High affinity receptors have also been located in coated pits, demonstrating their ability to take up specific macromolecules such as low-density lipoproteins (LDL) by receptor-mediated endocytosis (Vasile *et al.*, 1983).

Microvascular endothelial cells are particularly rich in spherical, membranous plasmalemmal vesicles (diameter: ~60-80nm). Of these vesicles, 70% appear on the luminal face enabling the actual surface area to increase two-fold, while the remaining 30% of the vesicles are located within the cytoplasm. The density of these plasmalemmal vesicles depends on the organ in which they are located and the type of microvessel, the

brain microvessels display the lowest vesicle density. Plasmalemmal vesicles are involved in endocytosis and transcytosis. In addition they fuse together to form transendothelial channels, which are also involved in membrane transport. Although transendothelial channels have been located in several types endothelia the morphology of these channels is different in continuous and fenestrated capillaries (Simionescu and Simionescu 1984).

Basement membrane, smooth muscle cells and connective tissue

The endothelium is supported by a basement membrane 5-8 nm thick, known as the basal lamina. It is composed of collagens (primarily types IV and V), laminin, glycoproteins and fibronectin all of which are produced by the endothelial cells themselves. The basement membrane is uninterrupted in continuous and fenestrated capillaries but discontinuous in sinusoids, in all cases forming a negatively charged barrier (Simionescu *et al.*, 1982b).

The cells which are adjacent to the microvascular endothelium are the pericytes, they are surrounded by an endothelial cell basal lamina and are widespread in the microvasculature of most tissues (Shepro and Morel 1993). Smooth muscle cells along with the pericytes provide the contractile elements of the microvessel wall. Unlike pericytes which are found in the basal lamina, smooth muscle cells have their own basement membrane. However both of these cell types have discrete points of contact with the endothelium. The microvessel wall also contains elastic fibres, connective tissue consisting of components such as fibrils, mast cells and macrophages and nerves. These components fulfil a complex role providing varying degrees of support, production of vasoactive mediators and defence, according to the location of the microvessels.

Intercellular junctions

The endothelial cells in the microvascular endothelium are squamous endothelia, linked together by two types of intercellular junctions: occluding "tight" junctions and

communicating "gap" junctions. These junctions are considered to be important in the regulation of the permeability of ions and small metabolites, and the maintenance of cell surface polarity. The tight junctions (zonula occludens) have been shown to exhibit high-resistance and are considered to seal the intercellular spaces between cells in both *in vivo* and *in vitro* studies (Larson and Sheridan 1982). These junctions are present in several types of endothelial cells and are especially prominent in brain vascular endothelium (Rutten *et al.*, 1987). The variation of tight and gap junctional distribution and of gap junctional size (up to 6nm) throughout the microvasculature reflects the various degrees of tightness and permeability in different organs (Simionescu and Simionescu 1984; Franke *et al.*, 1988).

1.1.3. Endothelial Cell Functions

The role of the endothelium extends to many more diverse functions in normal conditions and diseases such as: regulation of membrane transport and uptake; regulation of coagulation and fibrinolysis; synthesis and release of vasoactive hormones; participation in immune reactions and tumour cell adhesion and vascularisation. It is clear that the structural variations of the endothelium can account for the distinct functions of the endothelium of various organs and of specific regions within a single organ.

Selective membrane transport and uptake

One of the primary functions of the endothelium is to act as a semipermeable membrane through which fluids and solutes are transported, thereby controlling the plasma and extracellular fluid volume (Ryan 1989). Transport of substances such as water, ions, small lipid-soluble and lipid-insoluble molecules and large lipophilic and hydrophilic proteins occurs by diffusion, passive or active transport, endocytosis and transcytosis, (Vetvicka and Fornusek 1988; Renkin and Curry 1982). Several factors control the transport of plasma molecules across the vascular endothelium including: plasma and

haemodynamic forces (hydrostatic and oncotic pressure forces); physicochemical properties of the molecules (molecular size, shape, charge and chemistry) and the properties of the endothelial cells themselves (surface charge, structure and site-specificity) (Renkin and Curry 1982; Simionescu and Simionescu 1984).

Molecules can be internalised into the cell by adsorptive or fluid-phase endocytosis. Alternatively molecules are transported across the cell to the interstitial fluid by non-specific adsorptive, fluid-phase or receptor-mediated transcytosis (Simionescu 1988). Microvascular permeability was originally studied *in vivo* or *in situ* (Crone and Levitt 1984), however more recent studies performed *in vitro* have involved endothelial cells cultured on permeable filter membranes. This provides a rapid method of assessment of endothelial permeability and drug metabolism (Del Vecchio *et al.*, 1987; Siflinger-Birnboim *et al.*, 1987; Shasby and Roberts 1987; Albelda *et al.*, 1988). Further studies investigated the specific binding sites involved in receptor mediated transcytosis for several plasma components including albumin (Shasby and Shasby 1985; Ghitescu *et al.*, 1986; Cooper *et al.*, 1987), insulin (Hachiya *et al.*, 1988), transferrin (Jefferies *et al.*, 1984; Tavassoli 1988) and lipoprotein lipase (Saxena *et al.*, 1991).

The variations in the transport and permeability throughout the endothelium can be accounted for by the differences in the morphology of the vascular endothelium (Simionescu 1988). For each type of capillary endothelium, specific pathways have been designated which play a role in capillary transport; a schematic diagram of capillary endothelial transport is shown in Figure 1. The routes of transport that are thought to exist in the continuous capillary endothelium are 1) the transcellular pathway which transports water, small lipid soluble substances and respiratory gases; 2) the vesicular pathway whereby plasmalemmal vesicles carry plasma or interstitial fluids across the cell depending on the concentration gradient and the density of vesicles; 3) small and large "pore" pathways which may provide a continuous route across the cell and 4) transendothelial channels which are formed by the opening of two or more vesicles

simultaneously (Taylor and Granger 1984; Renkin 1988; Malik and Siflinger-Birnboim 1993). Transport processes for fenestrated capillaries are the same as that of unfenestrated capillaries, in addition however, the unique features of the fenestrated capillary are also involved in transport processes. These features are open fenestrae where the basement membrane provides a restrictive barrier and diaphragmed fenestrae (Renkin 1988). Transport processes in discontinuous endothelial cells occurs via the vesicular pathway and endothelial gaps.

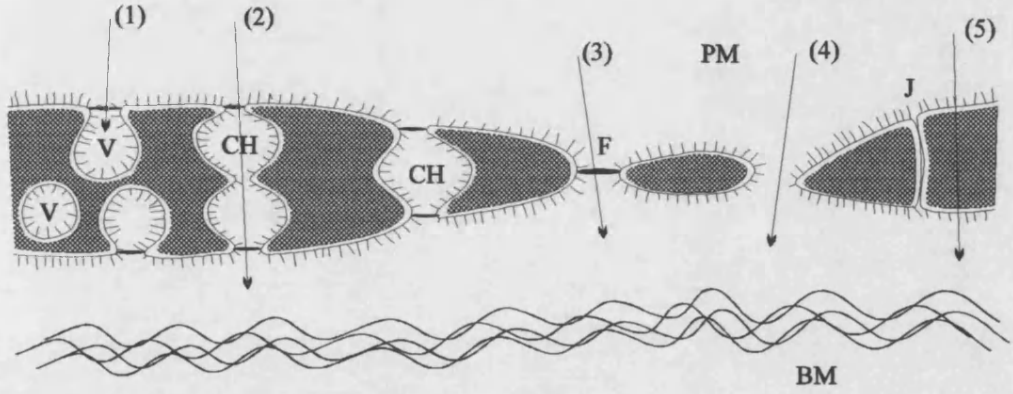

Figure 1. The endothelial cell layer: PM = plasma membrane; V = vesicle; CH = transendothelial channel; F = fenestrae, J = endothelial cell junction, BM = basemant membrane. The transport processes: (1) = the vesicular pathway; (2) = transendothelial channels; (3) = through fenestrated capillary endothelial cells; (4) = through gaps in discontinuous endothelial cell capillaries; (5) = transcellular pathway (modification of Simionescu and Simionescu 1984).

Coagulation and fibrinolysis

The endothelium plays an important role in homeostasis regulating the fluidity of the blood due to its involvement in anti-coagulation, fibrinolysis and pro-coagulation processes. Thrombomodulin (TM) is a transmembrane glycoprotein located on the endothelial cell surface, its main function is to bind the procoagulant protease thrombin changing its properties to result in the generation of anti-coagulant activity. The formation of this thrombin-TM complex activates the Protein C anti-coagulation pathway, decreasing clotting activity, Factor V activation and platelet activation.

Activation of Protein C is in turn involved in the inactivation of Factors Va and VIIIa which are responsible for the anticoagulant effect (Esmon 1988; Ryan 1989; Ford 1992). Endothelial cells also produce heparin-sulphate and other heparin-like proteoglycans which act as anti-coagulants by binding to antithrombin III resulting in the inactivation of thrombin and Factor Xa (Marcum *et. al.*, 1988).

The fibrinolytic system is primarily involved in the dissolution of blood clots. This involves components such as plasminogen activators and plasminogen activator inhibitors which effect the conversion of the inactive proenzyme plasminogen into the active form, plasmin. It is the endothelium that synthesises and secretes these components of the fibrinolytic system, in addition it is likely that the endothelial cell surface and its extracellular matrix components contribute to the regulation of vascular fibrinolysis (Curriden *et al.*, 1988).

Under normal conditions the endothelial cell provides a non-thrombogenic surface, responsible for the anti-coagulant mechanisms which prevent activation of coagulation and fibrin deposition on the vessel wall. In diseased states, however, endothelial cells become procoagulant when exposed to substances such as endotoxin, thrombin, interleukin-1 (IL-1) or tumour necrosis factor (TNF). As a result of these stimuli there is an increased synthesis and expression of thromboplastin by the endothelial cells which can initiate blood coagulation by activating Factor IXa and Xa. Factor Xa activates thrombin initiating the production of insoluble fibrin, activation of platelets and inhibition of fibrinolytic activity.

Synthesis and release of vasoactive substances.

As described previously, endothelial cells express receptors and specific binding sites for a number of vasoactive substances such as angiotensin-converting enzyme (ACE) and prostacyclin. ACE activates the vasopressor hormone angiotensin II and inactivates

bradykinin, thereby playing an important role in blood pressure regulation. The endothelial cell surface has been targeted as a site of drug therapy due to the accessibility of the blood stream to the endothelial surface receptors and binding sites.

Inflammation and immune reactions

Under normal conditions the endothelium is immunologically unreactive, however inflammatory conditions activate the vascular endothelium inducing a variety of reactions. These responses include synthesis and secretion of prostacyclin, endothelin, TNF and IL-1, expression of surface antigens and increased vascular permeability (Pober and Cotran 1990).

Endothelial cells have been shown to express a number of cell surface adhesion molecules. These molecules have a role in both inflammation and metastasis. In the event of inflammation for instance, lymphocytes specifically attach to the vascular endothelium at sites of inflammation. The lymphocytes subsequently migrate through the post-capillary venule wall into the inflamed tissue. It is the expression of cell adhesion molecules known as "homing receptors", which are activated by inflammatory cytokines such as IL-1 and TNF, that may pinpoint the position for these events to occur (Springer 1990). Several endothelial cell adhesion molecules are thought to be involved in inflammation these include: Endothelial leukocyte adhesion molecule-1 (ELAM-1), Vascular cell adhesion molecule-1 (VCAM-1) and Intercellular adhesion molecules-1 and 2 (ICAM-1 and ICAM-2). The role of these and other adhesion receptors in the immune system is reviewed by Springer (1990) and Pober and Cotran (1991). This lymphocyte trafficking has also been shown to occur around the venules in the central nervous system when immune reactions develop (Hughes *et al.*, 1988). The system involved in lymphocyte adhesion to the brain endothelium however, is thought to be peculiar to brain endothelial cells regulating the traffic of lymphocytes across the blood-brain barrier (Male *et al.*, 1990).

Tumour cell adhesion

Metastasis is the spread of tumours cells throughout the body via the blood stream. This is thought to occur through a complex cascade system: tissue invasion, detachment of malignant cells, transport through blood vessels, extravasation and proliferation at a secondary site (Hart *et al.*, 1989). Cell adhesion plays an important role in the metastatic process. Malignant tumour cells adhere to specific sites on the endothelium, and their subendothelial matrix (basement membrane). The adhesion receptors function in three ways: 1) as cell-substratum adhesion receptors present on the basement membrane; 2) cell-cell adhesion receptors that promote contact between the junctions of adjacent cells of the same type and 3) cell-cell adhesion molecules on the luminal surface of the cell which are important in tumour cell adhesion (Albelda 1991).

Tumour cell adhesion to the extracellular matrices and basement membranes has been shown to occur mainly at the site of the glycoproteins of the integrin receptors. The integrin receptors are a family of cellular receptors including fibronectin and vitronectin receptors and very late antigen (VLA) which link the cell cytoskeleton with the extracellular matrix (Haynes 1987; Albelda *et al.*, 1989). Many tumour cells express integrin-like molecules at the cell surface and it is an alteration in these integrins and the integrin receptors on the extracellular matrix that is associated with cancer progression (Hart *et al.*, 1989; Lauri *et al.*, 1991; Steeg 1992).

The tight junctions which exist between adjacent cells are mediated by specific adhesion molecules providing the characteristic permeability barrier. There are a number of cell-cell adhesion molecules which function in this way, including cadherins which provide calcium dependant adhesion, integrins (Fawcett and Harris 1992) and immunoglobulins, including platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Albelda *et al.*, 1990). Initial *in vitro* and *in vivo* studies showed that some tumour cells attach to the endothelial cells at the junctional points resulting in endothelial cell retraction and adhesion of the tumour cell to the subendothelial cell matrix (Kramer 1982).

Metastasis appears to be regulated by organ-specific adhesion molecules expressed on the luminal surface of endothelial cells. Experimental evidence to support this theory has shown that lung and liver-metastatic cells adhere preferentially to endothelial cells isolated from lung and liver respectively (Nicolson *et al.*, 1989). Since then a lung specific endothelial cell adhesion molecule (Lu-ECAM-1) has been identified (Zhu *et. al.*, 1991 and 1992). It has been the use of membrane vesicles (Johnson *et al.*, 1991) and the recent advances in microvascular endothelial cell isolation and culture which have enabled further more extensive studies to be conducted. The organ preference of metastasis has been reviewed by several investigators including Pauli *et al.* (1990) and McCarthy *et. al.* (1991).

Angiogenesis

Angiogenesis, or the formation of new blood vessels, occurs in normal conditions, such as wound healing, as well as in disease states such as atherosclerosis (Ross and Harker 1976), inflammation (Pober and Cotran 1990) and tumour growth (Folkman and Klagsburn 1987; Folkman 1990). In tumour angiogenesis, vessels sprout from the pre-existing circulatory system towards the tumour site. The first step in this process is the dissolution of the basement membrane of the post-capillary venule. Following this, the endothelial cells migrate towards the tumour and undergo cell division. Thereafter the developement of three-dimensional tube-like structures, vascular loops and a basement membrane occurs which eventually results in the development of a vascular bed (Blood and Zetter 1990).

Angiogenesis can be induced by a variety of factors including basic and acidic fibroblast growth factors (FGF's), TNF and angiogenin. FGF's and TNF modulate endothelial cell growth directly, unlike angiogenin which is produced by the tumour (Fett *et. al.*, 1985). Angiogenesis *in vitro* was initially studied by Folkman and Haudenschild (1980). They showed that capillary endothelial cells alone formed tube-like structures without the

involvement of mast cells and pericytes which are associated with the capillary endothelial cell *in vivo.* Since this original study a variety of approaches of studying angiogenesis have been investigated *in vitro,* especially those concerning the effect of the extracellular matrix and growth factors on angiogenesis (Jackson and Jenkins 1991;Vlodavsky *et al.*, 1991; Kowalczyk and McKeown-Longo 1992).

1.1.4. Large Vessel Endothelial Cell Culture

Isolation and culture

The first studies into the structural and functional properties of endothelial cells which were limited to ultrastructural analysis *in vivo* and were conducted by several investigators including Weibel and Palade (1964). This was due to the difficulties encountered in isolating, identifying and maintaining pure cultures of endothelial cells *in vitro.* The techniques involved in the isolation and culture of large vessel endothelial cells were developed by a number of investigators. Human umbilical vein endothelial cells (HUVEC) (Jaffe *et al.*, 1973a; Gimborne *et al.*, 1974) and bovine aortic endothelial cells (BAEC) (Booyse *et al.*, 1975) were isolated in these original studies.

Endothelial cells were isolated by collagenase digestion and cultured in media containing very high concentrations of foetal calf serum (~20-35%). The growth properties of the cultures were studied extensively using techniques such as DNA synthesis, [^{3}H] thymidine incorporation and cell proliferation studies in which cells were counted with a haemocytometer chamber. Culture conditions were modified in order to obtain optimum growth whilst maintaining the characteristic cell morphology in primary culture and subculture. Each group of researchers however, modified the isolation technique and culture conditions in order to perfect a method for maintaining endothelial cells in long term culture.

Characterisation

Endothelial cells were identified initially by phase contrast light microscopic studies of the cell morphology. Studies showed that endothelial cells adhered to the substratum as small clusters of polygonal cells which grew into a single cell-thick confluent monolayer where they showed a characteristic "cobblestone" morphology. Differentiation between endothelial cells and non-endothelial cell contaminants, particularly smooth muscle cells and fibroblasts, was also possible by light microscopy. Fibroblasts grew as long slender cells forming whirling patterns and overlapping layers (Jaffe *et al.*, 1973a) and smooth muscle cells grew with a multi-layered characteristic "hill and valley" effect (Ross 1971; Chamley *et al.*, 1977).

Cell morphology was examined further using scanning and transmission electron microscopy. The ultrastructure of cultured endothelial cells showed that the cytoplasm contained a large number of vesicles, prominent Golgi bodies, mitochondria, large quantities of smooth and rough endoplasmic reticulum, fine filaments and clusters of ribosomes. In addition the cytoplasm contained oval-shaped bodies first described by Weibel and Palade (1964). These Weibel-Palade bodies are peculiar to endothelial cells and were found in abundance in human umbilical vein endothelial cells (Jaffe *et al.*, 1973a; Gimborne *et al.*, 1974). Although their presence was detected in bovine aortic endothelial cells, they were not as abundant as described in human umbilical vein endothelial cells (Schwartz 1978).

Original immunofluorescent studies demonstrated the presence of blood group antigens (Jaffe *et al.*, 1973a) and Von Willebrand factor (Jaffe *et al.*, 1973b; Booyse *et al.*, 1975) exclusively on endothelial cells. Following these early studies, the isolation, culture and characterisation techniques of large vessel endothelial cells have become well established. A wide variety of studies have been conducted using cultured endothelial cells in order to elucidate the structural and functional properties of the endothelium in health and diseased states. These include the effects of altering the culture conditions (Schlor *et al.*,

1983), extracellular matrix (Young and Herman 1985) and the influence of growth factors (Vlodavsky *et al.*, 1979; Gospodarowicz 1990; Sutton *et al.*, 1991). Endothelial cells have also been used in permeability and transport studies (Albelda *et al.*, 1988; Ming-Liu *et al.*, 1993; Alexander *et al.*, 1993) and investigations concerning the role of endothelial cells in inflammation (Cozzolino *et al.*, 1990; Klein *et al.*, 1992).

Alternative large vessel endothelia

As well as BAEC's and HUVEC, large vessel endothelial cells have been isolated from several different species and tissues. Porcine aortic endothelial cells (PAEC) were first isolated and characterised by Slater and Sloan (1975). The porcine cardiovascular system has been shown to possess many similarities to that of human, therefore cells isolated from porcine tissue provide a suitable alternative to HUVEC. Porcine endothelial cells are also useful in studies connected with atherosclerosis, as older pigs develop spontaneous aortic atherosclerotic plaques, thus providing a suitable experimental model for atherosclerosis (Rosenthal and Gotlieb 1990).

Large vessel endothelial cells have also been isolated from bovine and porcine pulmonary arteries. This has provided a useful model for studying endothelial cell functions in the lung. Studies have, for example, investigated the interactions of neutrophils and pulmonary endothelial cells with respect to vascular injury (Dodek *et al.*, 1990) and pulmonary artery permeability and transport mechanisms (Shepard *et al.*, 1989; Siflinger-Birnbiom *et al* 1991).

Variations exhibited by endothelial cells

Differences have been shown to exist between aortic endothelial cells isolated from different species. For example a feature typical of porcine aortic endothelial cells is their lack of ability to exhibit Von Willebrand factor or Weibel-Palade bodies which are characteristic of BAEC and HUVEC (Rosenthal and Gotlieb 1990). As well as species

variations, differences have been reported to exist between large vessel endothelial cells isolated from various sites in the vasculature. These include the density of insulin receptors (Bar and Sandra 1988), types of intercellular junctions (Simionescu *et al.*, 1976) and production of angiotensin-converting enzyme (Johnson 1980).

Since the early 1970's the structural and functional properties of large vessel endothelial cells have been extensively investigated, as these cells are easy to obtain and can be readily isolated and maintained in long-term culture. Species and site specific variations have been shown to exist in large vessel endothelial cells, however the comparison between large and microvessel endothelial cells was impeded due to the difficulty of isolating and maintaining pure cultures of microvessel endothelial cells.

1.1.5. Microvessel Endothelial Cell Culture

Organ-derived microvascular endothelia

As shown previously, the structural and functional properties of microvascular endothelial cells are heterogeneous. The phenotypic diversity of these cells from different tissues within an organism, as well as within different vascular beds, has opened up the study of endothelial cell biology. Since the importance of the microvasculature was established a number of investigators have attempted to isolate and maintain pure microvessel cultures from a variety of sources. Not only were microvascular endothelial cells isolated from different tissues but also different species:

1). Adrenal cortex: bovine (Folkman *et al.*, 1979; Voyta *et al.*, 1984; Orlidge and D'Amore, 1987; Alessandri *et. al.*, 1992) and human (Folkman *et al.*, 1979).

2). Skin: human (Davidson and Karasek 1981; Braverman 1989; Jackson *et al.*, 1990; Beitz *et al.*, 1991).

3). Epididimal fat pads: rat (Madri *et al.*, 1983; Williams *et al.*, 1984).

4). Heart: murine (Haisch *et al.*, 1990), rat and human (Nishida *et al.*, 1993).

5). Retinal: bovine (Gitlin and D'Amore 1983; Schor and Schor 1986).

6). Hepatic sinusoidal: murine (Belloni *et al.*, 1992), rat (Soda and Tavassolli 1984), guinea pig (Shaw *et al.*, 1984).

7). Ovary: murine (Auerbach *et al.*, 1985).

8). Lung: Bovine (Chung-Welch *et al.*, 1988; Ryan and White 1986; Del Vecchio *et al.*, 1992), human (Hewett and Murray 1993) rat (Ryan and White 1986) and murine (Chopra *et al.*, 1990; Belloni *et al.*, 1992)

9). Brain: bovine (Bowman *et al.*, 1983; Audus and Borchardt, 1986; Carson and Haudenschild 1986; Beer Stolz and Jacobson, 1991), rat (Hughes and Lantos, 1986; Rupnick *et al.*, 1988; Abbott *et al.*, 1992), human (Dorovini-Zis *et al.*, 1991), porcine (Tontsch and Bauer 1989) and murine (Tontsch and Bauer 1989; Belloni *et al.*, 1992).

1.1.6. Isolation Techniques

For the isolation of organ-derived microvascular endothelial cells from different species, there is a wide range of materials and techniques involved in the isolation procedure. The isolation of microvascular endothelial cells basically requires two steps: 1) dissociation of the blood vessels from the surrounding tissue components and 2) separation of the blood microvessel fragments from large vessels and non-endothelial cell contaminants.

The techniques have been modified by the researchers in order to obtain the optimum conditions for the isolation of organ and species specific microvascular endothelial cells, that is, isolating the maximum number of microvascular endothelial cells whilst keeping non-endothelial cell contaminants to a minimum. Isolation procedures need to vary

taking into account the amount of connective tissue and the ratio of microvessels to large vessels in the particular type of tissue, however differences also exist in the isolation procedures of the same type of tissue by different groups of researchers. Although several groups of workers have reported the isolation and culture of microvascular endothelial cells from various sources, some of the techniques used suggest that contamination with a variety of non-endothelial cells is inevitable.

Pre-treatment and dissection of the tissue

Pre-treatment of the tissues *in situ* and *in vitro* has been utilised in order to remove blood cells from the tissue. When isolating murine lung microvessels Belloni *et al.*, (1992) perfused the right ventricle of the heart with heparin to remove blood cells from the pulmonary circulation, this was followed by perfusion with 0.1% collagenase/dispase to initiate separation of the blood vessels from the connective tissue. Nishida *et al.* (1993) used retrograde perfusion of rat heart tissue with oxygenated Krebs Henseleit bicarbonate buffer to remove the blood cells.

Isolation of microvascular endothelial cells from the lung and heart have the additional problem of mesothelial cell contamination. Mesothelial cells are a simple squamous cell type that covers the surfaces exposed to the inner body cavities, including the pleural membrane of the lung and the pericardium (Rheinwald 1989). These cells are not distinguishable from microvascular endothelial cells in culture using conventional characterisation methods (discussed in section 1.1.11). With lung and heart tissue from human or large animals the pleural membrane and pericardium can be removed manually (Chung-Welch *et al.*, 1988; Hewett and Murray 1993; Nishida *et al.*, 1993). However this is not physically possible in tissue obtained from small animals. Mesothelial cells can be devitalised by very brief treatment with 70% ethanol (Chopra *et al.*, 1990; Nishida *et al.*, 1993), followed by washing extensively with a buffer solution.

Careful dissection of the tissue can dramatically reduce the contamination of the culture by non-endothelial cells and large vessel endothelial cells. Removal of large vessels from the tissue is an important step in reducing contamination by large vessel endothelia (Madri *et al.*, 1983; Nishida *et al.*, 1993). With lung tissue it is important to select the peripheral section of the lung as this avoids large vessels and therefore possible contamination (Chopra *et al.*, 1990; Hewett and Murray 1993).

For the isolation of brain microvessels the meninges and superficial blood vessels should be removed and also the mid- and hind-brain, the striatum, optic nerve and white matter (Bowman *et al.*, 1983; Hughes and Lantos 1986; Audus and Borchardt 1986; Carson and Haudenschild 1986; Beer Stolz and Jacobson 1991; Abbott *et al.*, 1992). Careful dissection at this stage reduces the possibility of contamination of cells such as astrocytes, pericytes, neurons and smooth muscle cells, although other separation techniques employed later in the isolation procedure and selective culture techniques also reduces contamination by non-endothelial cells.

Mechanical disruption of the tissue

Mincing of the tissue using a scalpel, Dounce homogeniser, shearing using a glass pipette or other mechanical means are used to break up the tissue and release the microvessel fragments. This method of dissociation of the microvessels from the surrounding non-vascular tissue is usually accompanied by enzyme digestion.

Enzyme digestion

Enzyme dissociation of the capillary fragment separates the blood vessel from the basement membrane because of the high content of collagens on the microvascular basal lamina which can be digested by collagenase and other enzymes. Isolation of the capillary fragment can therefore occur by incubation with collagenase (Folkman *et al.*, 1979; Madri *et al.*, 1983; Chung-Welch *et al.*, 1988; Del Vecchio *et al.*, 1992), a

combination of collagenase with the neutral protease dispase, which has been reported to disperse fibroblasts to a single cell suspension without affecting the blood vessels themselves (Bowman *et al.*, 1983; Zetter 1984; Abbott *et al.*, 1992; Belloni *et al.*, 1992) or with trypsin and EDTA (Davidson and Karasek 1981).

Several investigators have also used a combination of more than one of the above treatments: 1) The isolation of bovine brain microvascular endothelial cells by Audus and Borchardt (1986) used two enzyme digestion steps, the first involved incubation with 0.5% dispase at 37°C for 3 hours, and the second used 1mg/ml collagenase/dispase for a further 5 hours at 37°C; 2). Nishida *et al.*, (1993) initially treated rat heart tissue with 0.2% collagenase for 30 minutes at 37°C, followed by incubation with 0.02% trypsin for a further 30 minutes at 37°C. However, they omitted the trypsinisation step when dealing with human heart. This perhaps gives some indication of the specialisation of the techniques required for isolation cells from different tissues as well as from different species.

The length of incubation with the enzyme solution, its concentration, and the temperature of incubation varies greatly between different groups of workers in the isolation of the cells from the same and different types of tissue and species. An isolation procedure which does not use enzyme digestion, or one which uses only a short digestion step, requires the endothelial cells to migrate out of the ends of a small segment of capillary fragment (Chopra *et al.*, 1990). As a result of this, it would take longer to establish the cultures and heavier contamination with pericytes is also possible due to the incomplete removal of the basement membrane.

Longer dissociation times may be beneficial in producing purer cultures by actually minimising pericyte contamination. It has been suggested that endothelial cells do not appear to be affected by long incubations with proteases due to their low rate of endocytosis; pericytes however can internalise the enzymes and become damaged

(Bowman 1990). Excessive disruption of the blood vessels with too high a concentration of collagenase or for an excessive duration, however, can result in the production of a suspension comprising primarily single microvascular endothelial cells, which generally show poor attachment and become non-viable in culture (Abbott *et al.*, 1992). The reason for the large range of incubation times, concentrations and types of enzyme used is unknown.

Isolation using "Dynabeads"

Human endothelial cells have the ability to bind selectively to the lectin *Ulex europeaus* 1 (UEA-1) via the α-L-fructose residues on the endothelial cell surface (Hormia *et al.*, 1983). This selectivity can be adapted for use in the isolation of microvascular endothelial cells by covalently linking UEA-1 to magnetic polydisperse polymer particles "Dynabeads". UEA-1 lectin is covalently bound to Tosyl-activated M-450 Dynabeads and incubated with enzyme treated microvessel tissue segments. The microvessels and single cells that attached to the beads are plated on to gelatin coated culture dishes in selective culture medium and the cells are detached from the beads by competitive binding with free fructose (Jackson *et al.*, 1990). Binding of UEA-1 lectin to Dynabeads can also be used as a method of purification of cultures contaminated with non-endothelial cells (Jackson *et al.*, 1991). There may however be a variable degree of non-specific binding in this preparation, and in some tissues there may also be a degree of cross-reactivity with mesothelial cells and some epithelial cells.

UEA-1 lectin is selective for endothelial cells from human tissue, other lectins may be useful in tissues from other species. It has been suggested that *Griffonia simplicifolia* 1 lectin (GS-1) binds specifically to alveolar capillary endothelial cells from rat lung when examined in semi-thin and thin frozen sections (Bankston *et al.*, 1991). GS-1 is thought to be selective for the vascular endothelium of the rat lung, however applying this to

tissue culture may again result in cross reactivity with mesothelial cells and other non-endothelial cell contaminants.

Microcarrier beads

Ryan and White (1986) described the isolation of endothelial cells from the pulmonary microvasculature using a non-enzymatic isolation technique involving microcarrier beads. The beads are approximately 40-80μm in size and are composed of a polystyrene core coated with a "special" coating, having an overall positive charge. The lungs of small animals are perfused *in situ* via the pulmonary artery with cold 0.02% EDTA solution containing the microcarrier beads, in order to loosen the cells. The lungs are perfused by anterograde perfusion (artery to vein) but the cells are not collected as they get trapped by the sieving action of the pulmonary capillary bed on account of their size. However the cells are collected when the direction of flow is reversed. This anterograde and retrograde perfusion technique is repeated several times until sufficient perfusate is collected.

This technique is reported to isolate pre- and post-capillary vessels without contamination with large vessel endothelial cells or capillary endothelial cells. However, when the perfusate is collected, large vessel contamination at the point of canulation is inevitable. Attempts to isolate capillary endothelial cells with 5-10μm sized beads were not successful as beads of that size are phagocytosed by the endothelial cells themselves (Ryan 1990).

1.1.7. Separation Techniques

The second step in the isolation procedure is the separation of microvascular endothelial cells from non-endothelial cell contaminants such as pericytes, smooth muscle cells and fibroblasts as well as from connective tissue debris. This involves the use of one or more

of the following separation techniques: density dependant sedimentation with BSA;, Percoll gradient centrifugation; the use of dextran gradients; cell sieving, and differential plating methods.

Velocity sedimentation

The separation of capillaries from other cell types and tissue fragments is a crucial step in preparing pure endothelial cell cultures. There are several methods of separation by centrifugation which are used on their own or in conjunction with other techniques. Centrifugation with 25% BSA provides a density dependant gradient used in the isolation of brain microvessel fragments in the early stages of the isolation procedure (Hughes and Lantos 1986; Abbott *et al.*, 1992). This method separates the heavier capillary fragments from myelin, astrocytes, neurons and other single cells which are less dense. Separation with 10% BSA has also been utilised in the isolation of microvascular endothelial cells from rat epididimal fat pads (Madri *et al.*, 1983) and murine lung tissue (Belloni *et al.*, 1992).

Density dependant centrifugation with dextran is a widely used method, also providing initial separation during the early stages of the isolation procedure. The concentration of dextran used is variable, ranging between 13% and 20%. Separation using dextran has been used for isolation of microvascular endothelial cells primarily from brain tissue (Belloni and Nicolson 1988; Audus and Borchardt 1988; Rupnick 1988; Bowman 1990; Dorovini-Zis *et al.*, 1991). This method removed myelin, and other cell debris from the crude capillary fragments.

The crude capillary fragments separated by BSA or dextran density dependant centrifugation are often treated with a second enzyme digestion step in order to further digest the basement membrane assisting the removal of the pericytes; this is followed by fractionation by Percoll gradient centrifugation (Audus and Borchardt 1986; Bowman

1990; Dorovini-Zis *et al.*, 1991; Abbott *et al.*, 1992). Separation of the capillary fragments occurs using a 45% or 50% Percoll gradient which is pre-spun at high speed in order to form the gradient and re-centrifuged at a lower speed in order to separate the endothelial cells from the debris. Alternatively, the cell suspension is layered on top of the Percoll solution and centrifuged at high speed; during this centrifugation the Percoll forms a gradient and the cell suspension separates forming the appropriate bands. This method of separation often occurs in the latter stages of the isolation procedure, and is used in the separation of the capillary fragments from pericytes, red blood cells, damaged cells and other cell debris.

Cell sieving

Selection of microvessel fragments using nylon screens of different screen size is a separation technique used in the isolation procedures of a variety of tissues. This includes capillary fragments isolated from: retina (Gitlin and D'Amore 1983), brain (Carson and Haudsenchild 1986; Belloni and Nicolson 1988; Bowman 1990), lung (Chung-Welch *et al.*, 1988; Belloni *et al.*, 1992; Hewett and Murray 1993), adrenal glands (Folkman *et al.*, 1979) and heart (Nishida *et al.*, 1993). Separation by this method is based on size and occurs individually or in conjunction with density dependant centrifugation. The cell suspensions are filtered through sterile nylon screens with a pore size of 100-160μm and the filtrate containing vessel fragments separated from the cell debris is collected and retained. Following this, a second filtration step is often employed using a mesh with a smaller pore size (approximately 50μm) where the material remaining on the screen is retained separating the capillary fragments from any single cells collected from the initial separation (Belloni and Nicolson 1988; Bowman 1990).

Differential plating

Separation by differential plating occurs in conjunction with other techniques. The isolated capillary fragments are cultured on plastic tissue culture dishes, often coated with a physiologically relevant substratum such as collagen, gelatin or fibronectin. The vessel fragments adhere more rapidly to the culture dish than single cells (Zetter 1984). By rinsing the culture dishes with fresh culture medium 1-3 hours after plating it is possible to remove contaminating single cells such as smooth muscle cells, fibroblasts and pericytes whilst retaining the adhered vessel fragments (Gitlin and D'Amore 1983; Chung-Welch *et al.*, 1988; Rupnick 1988). Smooth muscle cells and fibroblasts also adhere more rapidly and tightly to glass than endothelial cells. Therefore, following an incubation period of approximately 1 hour on glass coverslips, the non-adherent endothelial cells can be transferred on to coated plastic culture dishes (Haisch 1990).

1.1.8. Cell Culture Conditions

The growth conditions used for the culture of capillary endothelial cells varies with the species and tissue from which the cells are isolated. This includes the choice of medium, sera, extracellular matrices and growth factors. The culture conditions are modified in order to obtain the optimum conditions, that is, maximum endothelial cell growth without altering the cell morphology and keeping contaminating non-endothelial cells to an absolute minimum.

Culture medium

The culture medium used for the growth of microvascular endothelial cells is very variable. The alternative types of culture medium include: Dulbecco's modified Eagle's Medium (DMEM) (Chung-Welch *et al.*, 1988; Nishida *et al.*, 1993), Medium 199 (M199) (Madri *et al.*, 1983; Hewett and Murray 1993), Minimum Eagle's Medium (MEM) (Audus and Borchardt 1986), Dulbecco's modified Eagle's with F12 nutrient mix

(DME-F12) (Belloni *et al.*, 1992; Haisch 1990), Ham's F10 (Abbott *et al.*, 1992), RPMI-1640 (Shaw *et al.*, 1984) and a 1:1 combination of fresh culture medium with medium conditioned with tumour cells (Folkman *et al.*, 1979) or endothelial cells (Beer Stoltz and Jacobson 1991).

Tumour conditioned culture medium is prepared by incubation of the required medium for 24-48 hours with tumour cells, such as mouse sarcoma 180 or B16 melanoma cells (Folkman *et al.*, 1979). Similarly endothelial cell conditioned medium is prepared from BAE cells (Gajdusek and Schwartz 1982). The use of conditioned medium has been reported to stimulate capillary endothelial cell growth (Folkman *et al.*, 1979). This is thought to be due to the release of stimulatory factors from large vessel endothelial cells and tumours which effect the production and release of basic fibroblast growth factor (bFGF) in turn stimulating endothelial cell growth (Schweigerer *et al.*, 1987). BAE cell conditioned medium may also inhibit the proliferation of smooth muscle cells (Carson and Haudenschild 1986). This is thought to be due to the secretion of a heparin-like substance from the endothelial cells which inhibits smooth muscle cell growth (Castellot *et al.*, 1981). The selection of culture medium varies depending on the species and tissue from which the cells are isolated and the ability to provide optimum culture conditions when supplemented with various growth factors and serums.

Growth serum

Sera are supplemented into the culture medium to support cell growth. The type of serum and the concentration used is selected to provide the most satisfactory conditions for cell growth. The alternative types of serum include foetal calf serum (FCS), plasma derived serum (PDS) and calf serum (CS).

Plamsa derived serum has been produced from a number of sources including bovine, human, equine and porcine. It is prepared from platelet poor plasma and has been

reported to selectively support the growth of endothelial cells in preference to non-endothelial cells. This is due to the fact that platelet poor serum does not contain platelet-derived growth factor (PDGF) which is required for growth of pericytes and smooth muscle cells (Gitlin and D'Amore 1983). Smooth muscle cells have been shown to express a receptor for PDGF. When isolated from primary culture and grown in the presence of PDGF or serum containing PDGF smooth muscle cells have also been shown to secrete PDGF (Speir *et al.*, 1991). Chung-Welch *et al.* (1988) reported that medium containing PDS with additional growth factors promoted the most effective and selective growth of microvascular endothelial cells in the early stages of primary culture and passaged cultures. The cells did however, grow equally well in medium supplemented with FCS in the latter stages of growth.

1.1.9. Growth Factors

The long-term growth of capillary endothelial cells *in vitro* requires the addition of growth factors known to stimulate the proliferation and differentiation of endothelial cells. The growth factors utilised for this purpose are extracted from a number of highly vascularised tissues and can effect the growth of cells in culture either alone or in conjunction with heparin, often producing a synergistic stimulatory effect. There is an increasing number of growth factors which have been shown to provide a positive or negative effect on the growth of endothelial cells. These growth factors include acidic and basic fibroblast growth factor (aFGF, bFGF), transforming growth factor type β (TGF-β), endothelial cell growth supplement (ECGS), retinal-derived growth factor (RDGF), vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF).

aFGF and bFGF

Acidic and basic FGF are closely related potent mitogens which stimulate the proliferation of large and capillary vessel endothelial cells in culture, and promote angiogenesis *in vivo*. Both of these proteins are isolated from a number of tissues such as retina, brain, adrenal gland, corpus luteum, as well as various tumours (Schweigerer *et al.*, 1987), and are purified on the basis of their strong affinity for the glycosaminoglycan, heparin (D'Amore 1992). FGF is required to stimulate capillary endothelial cell growth when the cells are seeded at low densities (Speir *et al.*, 1991), however, aFGF and bFGF are not equally potent as bFGF possesses 30-100 fold greater stimulatory effects on endothelial cells in culture (Gospodarowicz 1989).

The growth factor bFGF is located on the luminal surface of vascular endothelial cells and on the subendothelial extracellular matrix. Its release is thought to be effected by the presence of heparin and heparin-like molecules (Ishai-Michaeli *et al.*, 1992), and is thought to act via high affinity receptors and cell surface heparin sulphate proteoglycans, determined to be low affinity receptors (Klagsburn and Baird 1991).

In culture, heparin is known to possess an inhibitory effect on smooth muscle cells and stimulatory effect on human umbilical vein endothelial cells. In addition, heparin potentiates the mitogenic effect of aFGF making it equally as potent as bFGF to large vessel endothelial cells. For the growth of capillary endothelial cells however, the addition of heparin to culture medium in the presence of aFGF or bFGF is thought to have a potent inhibitory effect on cell growth (Gospodarowicz 1989). However, bFGF is used as a growth factor in the culture of a number of microvascular endothelial cell preparations (Karasek 1989; Braunhut and Palomares 1991).

VEGF, PD-ECGF and TGF-β.

Vascular endothelial growth factor (VEGF) is a heparin binding protein which stimulates the proliferation of endothelial cells *in vitro* but to a lesser extent than bFGF

(Bikfalvi *et al.*, 1991). VEGF is angiogenic *in vivo* and may have a role in the development of blood vessels (Jakeman *et al.*, 1992). High density binding sites for VEGF are distributed throughout various vascularised tissues, including brain, pituitary gland, kidney and ovary, suggesting further physiological roles (Ferrera *et al.*, 1992). Mouse sarcoma 180 and some smooth muscle cells have been shown to secrete a VEGF like mitogen (Ferrera *et al.*, 1992). Culture of microvascular endothelial cells with sarcoma 180 conditioned medium has been used in a number of studies to stimulate cell growth (Folkman *et al.*, 1979; Alessandri *et al.*, 1992).

Platelet-derived endothelial cell growth factor (PD-ECGF) is also an endothelial cell mitogen but unlike FGF it does not bind heparin, nor does it stimulate fibroblast or smooth muscle cell growth. The specificity of PD-ECGF and the fact that it is derived from platelets suggests that PD-ECGF may have a role in maintaining the integrity of the blood vessels (Gospodarowicz 1990; D'Amore 1992).

Transforming growth factor β (TGF-β) has an inhibitory effect on endothelial cell proliferation. Studies involving the co-culture of endothelial cells with smooth muscle cells or pericytes were found to result in the inhibition of endothelial cell growth (D'Amore 1992). Studies also indicate that the effects of TGF-β on endothelial cell proliferation depend on the cell phenotype and extracellular matrix (Sutton *et al.*, 1991).

ECGS and RDGF

Endothelial cell growth supplement is a commercially prepared supplement derived from bovine neural or pituitary tissue, probably consisting of a number of endothelial cell stimulatory growth factors. ECGS is one of the most widely used endothelial cell growth supplements, used in the culture of microvascular endothelial cells from a variety of species and tissues. In the majority of studies ECGS is used in conjunction with heparin (Rupnick *et al.*, 1988; Tontsch and Bauer 1989; Jackson *et al.*, 1990; Haisch *et*

al., 1990; Beer Stoltz and Jacobson 1991; Dorovini-Zis 1991; Belloni *et al.*, 1992; Abbott *et al.*, 1992; Hewett and Murray 1993). This combination has been shown to have a synergistic effect increasing microvascular and large vessel endothelial cell growth (Thornton *et al.*, 1983; Abbott *et al.*, 1992).

Retinal derived growth factor (RDGF) is generally prepared from bovine retinas and contains an endothelial cell mitogen. It is used in conjunction with heparin, PDS and FCS as a stimulant for endothelial cell growth, but not for the growth of pericytes (Gitlin and D'Amore 1982). Not only is RDGF used as a growth stimulant in cultures of retinal capillary cells (Gitlin and D'Amore 1982), but it is also used in cultures of adrenal cortex (Orlidge and D'Amore 1987), lung (Chung-Welch *et al.*, 1988), heart (Nishida *et al.*, 1993) and brain (Carson and Haudenschild 1986).

1.1.10. Extracellular Matrix

The basement membrane and basal lamina, described in section 1.1.2, are specialised extracellular matrices on which endothelial cells sit (Mosher *et al.*, 1992). This substratum influences cell functions such as growth, migration and differentiation. The influence of the extracellular matrix (ECM) on vascular endothelial cell growth has been widely studied *in vitro.* Original studies showed that capillary endothelial cells grown on interstitial collagens (type I and III) proliferated until they formed a typical monolayer, however cells grown on basement membrane collagens (type IV) were induced to form capillary tube-like structures (Madri and Williams 1983). Since then, the influence of the ECM and growth factors such as FGF and TGFβ on capillary morphogenesis *in vitro* have been more widely studied (Grant *et al.*, 1989; Aumailley *et al.*, 1991; Jackson and Jenkins 1991; Schnaper *et al.*, 1993), and is the subject of a short review by (Ingber and Folkman 1989).

The selection of an appropriate substratum on which to culture capillary endothelial cells is therefore important for cell proliferation. ECM such as native collagen gels or Matrigel (Lawley and Kubota 1989; Kinsella *et al.*, 1992) promote the differentiation of cells resulting in the formation of capillary tube-like structures whereas purified ECM molecules such as laminin, fibronectin, collagens and gelatin promote cell proliferation forming endothelial cell monolayers (Ingber and Folkman 1989; D'Amore 1992). Microvascular endothelial cell monolayers *in vitro* are generally cultured on plastic culture wells coated with gelatin, fibronectin or collagen. The choice of substratum depends on the species and tissue from which the cells are isolated, as well as the culture conditions. The substratum is also selected on the basis of providing the optimum conditions for cell proliferation in culture.

1.1.11. Methods of characterisation

The ability to identify and distinguish between microvascular endothelial cells and non-endothelial cell contaminants in culture is based on the structural and functional properties of endothelial cells *in vivo* and *in vitro*. Microvascular endothelial cells display a distinctive pattern of growth in culture and possess a number of typical ultrastructural features. The typical markers for identification include: angiotensin converting enzyme (ACE), prostacyclin production, expression of Von Willebrand factor and uptake of acetylated low density lipoprotein (Ac-LDL). Other properties typical of endothelial cells include their ability to undergo angiogenesis *in vitro*, provide a non-thrombogenic surface to platelets and the expression a number of specific adhesion molecules. The morphology of microvascular endothelial cells, the effect of the culture conditions on cell growth and the expression of the endothelial cell markers has been shown to be species and organ specific (Rupnick *et al.*, 1988; Belloni *et al.*, 1992; Nishida *et al.*, 1993).

Light and electron microscopy

Phase-contrast microscopy is utilised in demonstrating the characteristic growth patterns of microvascular endothelial cells in culture. Cells are seeded on to the culture wells as small clusters of cells or capillary fragments. These cell clusters and fragments grow forming small cell colonies which expand and merge to form typical contact-inhibited monolayers. The morphological characteristics of microvascular endothelial cells is distinct from non-endothelial contaminants such as fibroblasts and smooth muscle cells. The morphology of these contaminating cells is fairly distinctive and possible to identify in culture (see section 1.1.4).

Several of the ultrastructural features described previously (see section 1.1.4) are characteristic of microvascular endothelial cells. Another ultrastructural feature of endothelial cells is the existence of Weibel Palade bodies originally identified in large vessel endothelial cells (see section 1.1.4). These have been identified in some microvascular endothelial cells, but appear to be more abundant in human tissues than in bovine, murine or rat tissues (Folkman *et al.*, 1979; Zetter 1984). Electron microscopy alone cannot offer conclusive proof of endothelial identity, as organ and species differences in ultrastructure are thought to exist, and epithelial and mesothelial cells may contain similar features.

Von Willebrand factor

Factor VIII complex is composed of two components, von Willebrand factor (99%) bound to the protein Factor VIII (1%). Von Willebrand factor has two main functions *in vivo*: it facilitates platelet adhesion to the subendothelial matrix and the interaction with Factor VIII to form blood clots. Factor VIII-related antigen is the antigen for von Willebrand factor, which is measured in the assay for Factor VIII. Synthesis of von Willebrand factor / Factor VIII-related antigen is localised to endothelial cells and megakaryocytes in the tissue and in culture making it a reliable marker for endothelial

cells *in vitro* (Zimmerman *et al.*, 1983). The endothelial cell origin can be detected by indirect staining of permeabilized cultured cells (Zetter 1984), however, the staining intensity is variable (Mukai *et al.*, 1980).

Uptake of acetylated LDL

Receptor mediated uptake of low density lipoprotein occurs in cells such as endothelial cells, fibroblasts, monocytes and macrophages. The lipoproteins bind predominantly to the coated pits where the are internalised into the cell and degraded by the lysosome. The receptor mediated "scavenger cell pathway" is an alternative pathway for the metabolism of LDL which has been chemically modified by acetylation or acetoacetylation (Pitas *et al.*, 1981). The receptor in the scavenger pathway is different to the receptor for unmodified LDL in terms of specificity, regulation of cholesterol levels and biochemical properties. More importantly with respect to endothelial cells, is that acetylated LDL metabolised by the scavenger pathway in endothelial cells occurs at a much accelerated rate compared to the other cell types. Voyta *et al* (1984) applied these properties to identify endothelial cells in culture using fluorescently labelled acetylated LDL.

Angiotensin converting enzyme and prostacyclin

Angiotensin converting enzyme (ACE) is found in large and microvessel endothelial cells (Caldwell *et al.*, 1976), alveolar macrophages (Friedland *et al.*, 1977) and some epithelial cells (Ward *et al.*, 1979). Due to the variety of tissues in which this enzyme is located, ACE cannot be used as a definitive marker of endothelial cell origin, however the absence of ACE would rule out endothelial cell origin. ACE can be detected immunologically (Auerbach *et al.*, 1982) and by radioimmunoassay (Ryan and Mayfield 1986). Prostacyclin (PGI_2) is involved in platelet aggregation, and have been shown to be produced by endothelial cells *in vivo* and *in vitro*. The production of prostacyclin in

culture is stimulated by a variety of substances such as thrombin and can be used as another marker for endothelial cell origin (Zetter 1984).

Non-thrombogenic surface and angiogenesis

As described previously, the endothelium provides a non-thrombogenic surface, responsible for the prevention of platelet adhesion to the vessel wall. This phenomenon is maintained in culture and can be utilised as a method for distinguishing between endothelial cells and smooth muscle cells or fibroblasts, since platelets rapidly adhere to the surface of both smooth muscle cells and fibroblasts (Belloni *et al.*, 1992).

Angiogenesis of microvessel endothelial cells can also be demonstrated *in vitro* (see section 1.1.3) and provides a very reliable method of distinguishing between endothelial and mesothelial cells (Chung-Welch *et al.*, 1989; van Hinsbergh *et al.*, 1990).

Alternative endothelial cell markers

Cell adhesion molecules have been used recently as endothelial cell specific markers *in vitro* (Hewett and Murray 1993). A number of cell adhesion molecules have been identified such as EndoCAM, an endothelial cell adhesion molecule which is involved in cell-cell adhesion (Albelda *et al.*, 1990), PECAM-1, (Rubin, 1992) and organ specific adhesion molecules such as Lu-ECAM-1 (Zhu *et al.*, 1991 and 1992). These adhesion molecules may be used in the identification of endothelial origin.

1.1.12. Microvascular Endothelial Cell Lines

The role of large vessel endothelial cells has been widely studied *in vitro* with cells isolated from primary culture and with the use of established cell lines. However, as significant differences exist between large vessel and microvessel endothelial cells. Many of the physiological events which occur in the vasculature happen at the microvascular

level, the growth of pure cultures of microvascular endothelial cells has become very important. Due to the great difficulties of isolating and maintaining pure long term microvascular endothelial cell cultures from various species and tissues, studies involving the establishment of immortalised microvascular cell lines have been investigated (Montesano *et al.*, 1990; Dubois *et al.*, 1991;Ades *et al.*, 1992).

Ades *et al.*, (1992) transfected and immortalised human dermal microvascular endothelial cells. Microvascular endothelial cells at passage 6 were transfected with the simian virus 40 large T antigen and underwent characterisation studies in parallel with primary cultured cells for comparison. The immortalised cell line HMEC-1 assumed cobblestone morphology, and unlike primary cultures they could be passaged up to 95 times without affecting growth characteristics or morphology. HEMC-1 cells also tested positive for the expression of Von Willebrand factor, UEA-1, the uptake of Ac-LDL, and formed capillary tube-like structures when seeded on to Matrigel coated culture wells, although some differences with primary cultured cells did exist.

Murine microvascular endothelial cells are notoriously difficult to culture (Dubois *et al.*, 1991), therefore the establishment of murine microvascular endothelial cell lines has been investigated. Cell lines have been prepared from normal endothelial cells isolated from brain (bEnd.1 cells) (Montesano *et al.*, 1990), skin (sEnd.1) and thymus (tEnd.1) by infecting primary cultures of mouse brain with a hemangioma derived polyoma middle T oncogene-expressing retrovirus (Williams *et al.*, 1988 and 1989). These cell lines have been utilised in studies investigating proliferation and angiogenesis of microvascular endothelial cells *in vitro* (Montesano *et al.*, 1990; Bocchietto *et al.*, 1993). A cell line has also been derived from vascular tumours (hemangiomas) in transgenic mice (Py-4-1) (Dubois *et al.*, 1991). The angiogenic properties of these tumourigenic endothelial cells (Py-4-1) and normal endothelial cells (bEnd cells) have been compared *in vitro* (RayChaudhury *et al.*, 1994).

1.1.13. Blood-Brain Barrier

The blood-brain barrier consists of a single continuous layer of endothelial cells which lines the microvessels of the brain. These endothelial cells are bound together by tight intercellular junctions, exhibit only a few pinocytotic vesicles and lack fenestrations. Together, these unique properties provide a very selective, restrictive barrier that effectively excludes the passage of most polar molecules peptides and proteins from the blood to the brain. Some small water soluble molecules (e.g. amino acids) and neuropeptides are transported across the blood-brain barrier by specific carrier mechanisms, in addition, some water soluble drugs such as L-DOPA are transported by the neutral amino acid pathway (Partridge 1986; Audus 1990). Some reports also suggest that certain blood-borne proteins are transported across the blood-brain barrier by a vesicular mechanism (Guillot *et al.*, 1990).

Initial studies of the blood-brain barrier *in vivo* provided useful information about the regional variations of the brain and provided indirect evidence of endothelial cell function as a selective barrier. However studies involving cultures of brain microvascular endothelial cells have allowed advances in the structural and functional analysis of the barrier, in particular, the study of transport mechanisms (Shah *et al.*, 1989; Smith and Borchardt 1989; Audus and Borchardt 1986, Abbott *et al.*, 1992).

In our laboratory, the structure and function of melanotropins and melanotropin receptors have been studied extensively with a view to exploiting receptor mediated processes involved in drug targeting to melanoma cells (Ahmed *et al.*, 1992; Sahm *et al.*, 1994). This prompted an investigation into the expression of melanocortin receptors in the microvasculature as a mechanism of transport by receptor mediated transcytosis particularly across the blood-brain barrier.

1.1.14. The Melanotropins

Pro-opiomelanocortin (POMC) is a peptide hormone precursor which is processed by post transcriptional cleavage into an array of biologically active neurohumoral peptides. It is expressed primarily in the pituitary, but is also present in distinct regions of the brain and other peripheral tissues. The substances produced by the processing of POMC include: α, β and γ melanocyte stimulating hormone (α, β and γ-MSH), β-lipotropic hormone, β-endorphin and adrenocorticotropic hormone (ACTH) which is the precursor of α-MSH. These peptides possess a variety of well known physiological actions including: the regulation of adrenal glucocorticoid and aldosterone production (ACTH); control of melanocyte growth and pigment production (α-MSH, β-MSH, β-lipotropic hormone and ACTH); and analgesia (β-endorphin), as well as having actions related to central neural functions such as behaviour, memory and learning (Eberle 1988).

1.1.15. α-Melanocyte Stimulating Hormone : Structure and Functions

α-Melanocyte stimulating hormone (α-MSH) is a linear 13 amino acid peptide: Ac-Ser^{1}-Tyr^{2}-Ser^{3}-Met^{4}-Glu^{5}-His^{6}-Phe^{7}-Arg^{8}-Trp^{9}-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2. It is synthesised in the *pars intermedia* of the pituitary gland, in the brain and in peripheral tissues of mammalian species. It is located throughout the central nervous system (CNS) and in peripheral tissues including: skin; testes; ovaries; placenta; adrenal medulla and gastrointestinal tract (Eberle, 1988).

α-MSH is known to stimulate pigment dispersal in lower vertebrates and can induce melanogenesis in various mammalian cells. The activity of α-MSH on cultured murine B16 melanoma cells and human melanocytes has been widely studied (Siegrist *et al.*, 1988 and 1992; Eberle, 1991; Ahmed *et al.*, 1992; Lunec *et al.*, 1992; Hunt *et al.*, 1994; Sahm *et al.*, 1994). α-MSH has been shown to influence melanogenesis and metastatic behaviour in murine melanoma cells by binding to specific receptors on the cell

membrane. Activation of the α-MSH receptor results in increased cyclic AMP production, stimulation of tyrosinase activity and increased melanin content (Lunec *et al.*, 1992; Siegrist *et al.*, 1988; Sahm *et al.*, in press). In human melanoma cells this mechanism is not so evident, although human melanocytes do express high affinity on binding of α-MSH (Eberle, 1991; Siegrist *et al.*, 1992; Hunt *et al.*, 1994).

In addition to the effects of α-MSH on pigment cells, α-MSH also has well recognised effects in the pituitary and peripheral tissues such as effecting the release of pituitary hormones such as prolactin, influencing the immune system by modulating some of the effects of interleukin-1 and having cardiovascular and renal effects (Eberle 1988).

α-MSH in the central nervous system has a wide variety of effects suggesting a role as a neuropeptide in the CNS. There are reported effects of α-MSH in the CNS on attention, hearing and memory as well as showing behavioural; neurophysiological and thermoregulatory effects (Eberle, 1988). It is the distinct pharmacological properties of melanocortins in the nervous and immune system compared with those expressed on melanocytes and adrenal cortical cells suggested that the existence of multiple melanocortin receptors.

1.1.16. Melanocortin Receptors

α-MSH and ACTH receptors (MC1-R and MC2-R, respectively) control pigmentation and adrenal steroidogenesis in response to stimulation from POMC peptides. These receptors are a G-protein coupled receptor subfamily based upon their sequence homology which stimulates adenylyl cyclase. Isolation of the receptors has enabled the identification of some unusual structural features which make them quite distinct (Cone and Mountjoy 1993).

Studies investigating the presence of α-MSH receptors in lacrimal tissue suggested that α-MSH may function as a neurotransmitter (Leiba *et al.*, 1990). These studies were conducted using [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}]α-MSH as a radioligand. This is a superpotent analogue of α-MSH exhibiting 10 times greater biological potency than the native α-MSH (Sawyer *et al.*, 1980, Eberle 1988). The primary localisation of ACTH and α-MSH is the melanocyte and adrenal cortex, however binding sites for ACTH and α-MSH have been found throughout the brain using *in situ* hybridisation, having numerous regulatory properties. The cloning of receptors for α-MSH and ACTH (Mountjoy *et al.*, 1992) have provided probes for further examination of α-MSH and ACTH expression.

A receptor for α-MSH and ACTH peptides has been identified specifically in the hypothalamus and limbic system of the brain and is known as MC3 (Roselli-Rehfuss *et al.*, 1993) which is activated by γ-MSH peptides. The distribution of MC3-R mRNA in the neurons throughout the brain suggests the role of melanocortin peptides as neuromodulators and neurotransmitters as well as having a role in endocrine, cardiovascular and thermoregulatory functions. This MC3 receptor was also identified by Gantz *et al.*, (1993a) using the technique of polymerase chain reaction (PCR). Like MC1-R and MC2-R it is thought to be a G-protein linked cell surface receptor, however unlike MC1-R, MC3-R is thought to recognise ACTH, α, β and γ-MSH equally well which is demonstrated by cyclic AMP production. In addition to being expressed in the brain, MC3-R has also been identified in the placenta and gut but not in melanoma cells or in adrenal tissue.

A fourth member of the melanocortin receptor family is MC4-R. It has been shown to be expressed primarily in the brain with a notable absence in the adrenal cortex, placenta and melanocytes (Gantz *et al.*, 1993b). MC4-R has been shown to demonstrate an increase in cyclic AMP levels in response to α-MSH and ACTH, and is thought to possess unique pharmacological characteristics, tissue distribution and chromosomal localisation.

1.1.17. Aims and Scope

A great deal of research has been conducted in the area of endothelial cell biology, investigating the structural and functional diversity of endothelial cells from different organs and species. Many of the important functions attributable to the pulmonary vasculature occur at the microvascular level, however, most *in vitro* studies have concentrated on endothelial cells derived from large vessels, partly due to the great difficulties encountered in the isolation and culture of pure microvascular endothelial cells. Although much interest has been shown in microvascular endothelial cell biology, there are wide variations in the isolation techniques and culture conditions, and considerable variability between the properties reported for microvascular endothelial cell cultures. We now know that endothelial cells from different sites exhibit heterogeneity and clearly, there is a major need to have well characterised lung and brain derived microvascular endothelial cells in order to study their role in drug delivery.

The aim of this study is to determine the optimum conditions for the isolation, culture and characterisation of murine lung and brain derived microvascular endothelial cells, in order to assess the structural and functional diversity of these cells and their role in drug delivery.

Following the development of brain and lung derived microvascular endothelial cell cultures, it is the intention of this study to investigate the expression of receptors on the surface of the microvascular endothelia which could be involved in specific receptor mediated transcytosis. Of particular interest is the delivery of peptides to the brain, as the vascular endothelia of the blood-brain barrier provides a highly selective barrier to the passage of drugs and macromolecules. This may provide an important insight in to the mechanism of selective drug delivery involving extravasation of macromolecules from the vascular system.

2. General Materials and Methods

2.1. Cell Culture

2.1.1. Culture of Cell Lines

The three cell lines used throughout the study were bovine aortic endothelial cells (BAE-1), human umbilical vein endothelial cells (HUVEC) and fibroblasts (3T3-L1). BAE-1 were obtained from the European collection of animal cell cultures (Porton Down) at passage number 18. HUVECS were obtained from the Wellcome Foundation, Kent, U.K. at passage number 21 and the fibroblasts were obtained from the department of biochemistry, University of Bath at passage number 13.

2.1.2. Cell Culture Medium and Solutions

Water

All water used for the preparation of cell culture medium and solutions was freshly double distilled by a bi-distillation Fistreem still (Fisons Ltd) fitted with a Fistreem predioniser (Fisons Ltd) and sterilised in an autoclave (British Steriliser Co. Ltd, Swingclave type SFT-LAB) at 121°C for 15 minutes.

Balanced salt solutions

Phosphate buffered saline (PBS) was obtained in tablet form (Oxoid Ltd Basingstoke, Hants). Five tablets were dissolved in 500ml of freshly double distilled water, producing 0.02M PBS pH7.4. This was sterilised at 121°C for 15 minutes and stored at 4°C.

Hanks' balanced salts solution without calcium and magnesium (Ca/Mg-free HBSS, Gibco BRL, Paisley, Scotland), was obtained as a 10X concentrate containing phenol red

indicator. The concentrate was diluted to 1X with sterile double distilled water, adjusted to pH7.4 with 1M NaOH and stored at room temperature.

Acid and base solutions

10%$^{V}/_{V}$ hydrochloric acid (HCl), 7.5%$^{W}/_{V}$ sodium bicarbonate ($NaHCO_3$) and 1M sodium hydroxide (NaOH) were prepared from stock solutions and solids obtained from BDH chemicals. The solutions were prepared with double distilled water, sterilised and stored at room temperature.

Tissue culture medium and supplements

Dulbecco's Modified Eagle's Medium (DMEM, Gibco), RPMI 1640 (Flow laboratories, Irving, Scotland) and Medium 199 (M199, Gibco) were obtained as sterile 10X concentrates containing phenol red indicator but without L-glutamine and sodium bicarbonate. The concentrated solutions were diluted using sterile double distilled water. The medium supplements L-glutamine (200mM), penicillin (5000IU/ml) and streptomycin (5000µg/ml) (pen/strep) and non-essential amino acids (NEAA) were obtained from Flow laboratories and Gibco, respectively. Growth medium and NEAA were stored at 4°C whereas L-glutamine and penicillin/streptomycin were divided into aliquots and stored at -20°C.

Foetal calf serum was batch tested to obtain the serum which provided the most suitable cell growth. Batch testing involved supplementing different serums at various concentrations to normal growth medium, in order to determine the serum which provided maximum cell growth without effecting cell morphology. The most suitable growth occurred with serum obtained from Flow laboratories (batch numbers 9130012 and 10855) and with serum obtained from Gibco (batch number 30A0212S). The serum was divided into aliquots and stored at -20°C. New-born calf serum was obtained from Flow laboratories (batch number 056005) and stored at -20°C.

Endothelial cell growth supplement (ECGS) from bovine neural tissue was obtained from Sigma Chemical Company, Poole, Dorset. One vial contained 15mg of ECGS which was diluted as required with Hanks' balanced salts solution (HBSS) and filtered using a 0.2μm sterile filter unit (ministart NML, Sartorius) The ECGS was dispensed into aliquots and stored at -20°C.

Heparin was obtained from Sigma containing 176 units/mg, weighed out as required, dissolved in HBSS and sterile filtered. The heparin solution was divided into aliquots and stored at -20°C.

Culture medium was prepared aseptically from sterile components according to the following recipes, stored at 4°C and used within two weeks:

	DMEM	**M199**	**RPMI 1640**
10X Medium	50ml	50ml	50ml
Water	410ml	421.5ml	421.5ml
L-glutamine	5ml	5ml	5ml
Penicillin/streptomycin	5ml	5ml	5ml
NEAA	5ml	5ml	5ml
FCS	55.6ml	as required by different cell lines	as required by different cell lines

2.1.3. Equipment

All sterile tissue culture procedures were carried out using standard tissue culture techniques, in a vertical recirculating laminar flow cabinet (MDH Ltd.). Non-sterile procedures were performed at the bench.

The cells were maintained in an LEEC PF2 incubator (Laboratory and Engineering Company) and kept humidified under a constant atmosphere of 5% CO_2 gas. The thermostatic controls were adjusted to give a temperature of 37°C, which was regularly checked using a digital thermometer with a thermocouple probe (Jenway Ltd.)

Cell growth and morphology were monitored daily using an inverted biological microscope (WILD M40, Wild Heerbrugg Ltd, Switzerland). The cells were counted on a standard double grid haemocytometer (Fisons Ltd) having been stained with 0.4% trypan blue (Sigma) in PBS (see section 2.1.4).

Tissue culture flasks were obtained from Falcon (Becton Dickinson UK Ltd) in sizes: 25cm², 75cm² and 175cm². Six-well, 24-well and 96-well plates were obtained from Corning, U.K. Sterile tissue culture tubes of various sizes and 30ml screw topped sterile universal vials were obtained from Sterilin Ltd. Polypropylene 2ml ampoules for freezing cells in liquid nitrogen were obtained from J. Bibby Sciences.

Items of glassware that were re-used were rinsed immediately after use. The glassware was subsequently soaked in a 2% solution of RBS 25 (Fisons Ltd) at approximately 40°C for 30 minutes. The glassware was then washed thoroughly, rinsed in three changes of tap water and soaked in the final change for 30 minutes. This rinsing process was repeated with single distilled water and finally all items were soaked in freshly double distilled water for 1 hour. The glassware was dried in a hot air oven, capped with aluminium foil and sterilised by dry heat at 160°C (Gallencamp Sterilising Oven) for a minimum of 1 hour.

Non-glass items including plastic syringes, Gilson pipette tips, bottle tops and other re-usable plastics were also rinsed immediately after use. They were washed thoroughly by boiling in three changes of fresh distilled water, soaked in double distilled water, dried, sealed in autoclave bags (DRG Hospital Supplies, Bristol, U.K.) and sterilised at 121°C for 15 minutes.

Long glass pipettes and Pasteur pipettes were plugged with cotton wool, sealed in autoclave bags and sterilised at 121°C for 15 minutes.

2.1.4. Cell Culture Methods

Routine culture of BAE-1 and 3T3-L1 cell lines

The cell lines were routinely grown in DMEM supplemented with 7.5% $NaHCO_3$, 1%L-glutamine, 50IU/ml penicillin, 50µg/ml streptomycin, 1% NEAA and 10% foetal calf serum and adjusted to pH7.4. The cells were generally grown in 75cm² flasks in a CO_2 incubator at 37°C, allowing the cells to be buffered under an atmosphere of 5% CO_2. The medium was changed on alternate days and the cells were subcultured weekly when they had reached confluence.

To sub-culture the cells, the old medium was discarded and the cells were washed three times with PBS. The flask was then incubated with 0.25% trypsin, 0.02% ethylenediaminetetraacetic acid (0.25%w/v trypsin/0.02%w/v EDTA) for 5 minutes at 37°C. After trypsinisation the cell suspension was gently agitated to dislodge all the cells and aspirated with a sterile Pasteur pipette. From one confluent flask between 4 and 6 flasks of BAE-1 and 3T3-L1 cells were obtained depending on the experimental requirements. Each confluent 75 cm² flask contained approximately $2x10^7$cells.

Routine culture of HUVECS

Human umbilical vein endothelial cells (HUVECS) were routinely cultured in growth medium comprising a 1:1 mixture of M199 and RPMI 1640. Both M199 and RPMI 1640 were supplemented with 7.5% $NaHCO_3$, 1% L-glutamine, 50IU/ml penicillin, 50µg/ml streptomycin and 1% NEAA and adjusted to pH7.4 with 1M NaOH. The 1:1 mixture of M199 and RPMI was prepared and then supplemented with 5% new-born calf

serum, 5% foetal calf serum, 17 units/ml of heparin and 2.5μg/ml endothelial cell growth supplement (ECGS); the cells were cultured at 37°C in a CO_2 incubator. The cells were subcultured by incubation with 0.25% trypsin, 0.05% EDTA for approximately 1-2 minutes at room temperature and split in a ratio of 1:3 or 1:4 as required.

Determination of cell number

Determination of the cell density required the preparation of a suspension of subcultured cells. The cell suspension was thoroughly agitated and 0.4 ml of this suspension was removed and mixed with 0.1 ml of trypan blue dye, this suspension was loaded on to a haemocytometer chamber under a coverslip and the cell number was determined.

Each side of the haemocytometer chamber was divided into nine large squares by triple white lines, the four corner squares being further sub-divided into sixteen squares per corner. A total count of the four corner squares and the central square of the haemocytometer grid was made using the inverted microscope. Each large square had an area of $1mm^2$, when the coverslip was attached to the chamber such that interference patterns could be observed, the depth of the chamber was 0.1mm. The total volume of each square was therefore:

$$1 \times 1 \times 0.1 = 0.1mm^3 = 0.0001cm^3 = 10^{-4}ml$$

The total cell number per ml was given by $10^4 \times n$ where n is the mean of the five large squares. When the dilution of the cell suspension with the dye was accounted for, the final equation for assessing cell density became: $5 (n \times 10^4 / 4)$.

Cell viability

The cell viability was also determined during the haemocytometer count of the cell density using trypan blue exclusion. Viable cells excluded this dye whereas non-viable cells become stained dark blue.

Cell storage in liquid nitrogen

Cells which required longer term storage were frozen down routinely in liquid nitrogen vapour. A cell suspension was prepared from a confluent flask by the standard trypsinisation procedures and resuspended in 5ml of fresh medium. The cell suspension was transferred into sterile centrifuge tubes and centrifuged at 400g for 10 minutes (Jouan B3-11 bench centrifuge). The supernatant was discarded, and the cell pellet was resuspended in 1ml of growth medium in the presence of the cryoprotectant dimethyl sulphoxide (DMSO). A 10% solution of DMSO prepared in growth medium was sterilised by filtration with a 0.2μm filter prior to use. Replicate 1ml aliquots of the cell suspension were placed in 2ml polypropylene screw top ampoules and placed in a biological freezer unit plug (Type BF6, Union Carbide) in the top of a liquid nitrogen freezer (Type LR 33-10, Union Carbide). This allowed the cells to be cooled to below -70°C in liquid nitrogen vapour at a rate of approximately 1°C per minute. The ampoules were then transferred to a liquid nitrogen freezer (Type LR-40, Union Carbide) for long term storage.

This method for freezing cells was used for BAE-1 and 3T3-L1 cell lines. For HUVECS, however, samples needed to be frozen in medium which comprised 20% foetal calf serum, 10% DMSO and 70% growth medium.

Recovery from liquid nitrogen

When the cells were recovered from long term storage, the ampoule from storage was thawed out immediately in a water bath pre-set at 37°C. The contents of the ampoule

were aseptically removed, resuspended in 9ml of growth medium and centrifuged at 400g for 10 minutes. The supernatant was removed and the pellet was resuspended in fresh culture medium and transferred into a culture flask to undergo routine culture. The procedure for the recovery of cells from liquid nitrogen was the same for all cell lines.

2.2. Primary Culture

2.2.1. Primary Culture of Murine Brain and Lung Microvascular Endothelial Cells

The development of the individual techniques for the isolation and culture of murine brain and lung microvascular endothelial cells will be discussed in future chapters (see sections 3 and 4). Several of the materials and methods utilised in the finalised techniques established for isolation and culture of microvascular endothelial cells were common for both brain and lung tissue.

2.2.2. C57 Mice

Both male and female C57 mice were used for preparation of microvascular endothelial cells. The mice were obtained from Harlan Olac Ltd. Oxon, England.

2.2.3. Buffer and Stock Solutions

Preparation of HEPES buffer.

A HEPES buffer solution comprising of Ca/Mg-free HBSS (with phenol red indicator) with between 10mM-25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, Calbiochem), 100 I.U./ml penicillin and 100 μg/ml streptomycin was aseptically prepared one day prior to use. The buffer was adjusted, to pH7.4 with 1M NaOH and stored overnight at 4°C.

Immediately prior to use the buffer was oxygenated (95%O_2 / 5%CO_2) for 10 minutes under sterile conditions. In addition the buffer was supplemented with bovine serum albumin (BSA, Sigma fraction V) in order to produce a final concentration of 0.5% BSA. The final solution was readjusted to pH7.4 with 1M NaOH.

Preparation of BSA solution

A 25% BSA solution was prepared in HEPES buffer solution one day prior to use. The BSA powder was scattered on the surface of the buffer and allowed to dissolve slowly for approximately 1½ hours to avoid the BSA congealing. The BSA solution was subsequently filtered using a 0.8μm filter, thereafter sterilised using a 0.2μm filter and stored in a sterile universal vial at 4°C overnight.

Preparation of enzyme solutions

The basic enzyme digestion mixture consisted of 0.1% collagenase/dispase obtained from Boehringer Mannheim, dissolved in a solution of Ca/Mg- free HBSS containing 10 mM HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin. The enzyme solution was sterile filtered using a 0.2μm filter, aliquoted and stored at -20°C.

DNase-1 and TLCK (tosyl-lysine-chloromethyl-ketone) were both obtained from Sigma. DNase-1 (2000U/vial) was dissolved in 1ml Ca/Mg-free HBSS, dispensed into 200μl aliquots and stored at -20°C. TLCK was dissolved in freshly double distilled water (2.49mg/20ml), sterile filtered, divided into aliquots and stored at -20°C.

Before use, the enzyme digestion solution was warmed to 37°C and oxygenated under sterile conditions for 2-3 minutes. The solution was readjusted to pH 7.4 and supplemented with 20 units/ml DNase1 and 0.147 μg/ml TLCK.

2.2.4. Preparation of a Percoll Gradient

50% isotonic Percoll gradient medium was aseptically prepared and stored at 4°C until required. The Percoll gradient medium consisted of 50ml Percoll, obtained from Sigma, with the addition of 5.5ml of 10X strength HBSS containing calcium and magnesium and 45ml of 1X strength HBSS. The pH was adjusted to pH7.4 with 10% HCl.

The ultracentrifuge tubes required for the preparation of the Percoll gradient were cleaned and sterilised prior to use. The screw capped ultracentrifuge tubes (10ml size, Dupont (UK) Ltd) were cleaned by sonication in Milton sterilising fluid, filled with double distilled water and resonicated before use. The tubes were sterilised by shaking with ethanol for approximately 2-3 minutes and rinsed with buffer. Fresh buffer was added to the tubes and agitated on a shaker at 37°C for approximately 20 minutes.

The Percoll gradient medium was allowed to warm up to room temperature and the pH carefully readjusted. Under sterile conditions 7ml of the Percoll gradient medium was transferred to the ultracentrifuge tubes and the gradient was formed by ultracentrifugation at 25000g for 1 hour at 4°C (16500rpm on a Beckman L8-70M ultracentrifuge with a 70,000rpm Type70.ITI rotor head, serial number 6E 1064).

2.2.5. Preparation of Collagen Coated Plates

Collagen type 1 from calf skin (Sigma) was required as an extracellular matrix. Under sterile conditions, 50mg collagen was dissolved in a few drops of glacial acetic acid and diluted with 150ml of sterile double distilled water. The collagen solution was divided into aliquots and stored at -20°C.

Collagen coated plates were freshly prepared on the day of use. 96-well plates were incubated with 100µl of collagen solution per culture well, for 24 and 6-well plates

0.25ml and 1ml of collagen solution, respectively, was required for each well. The plates were incubated at room temperature for 1-2 hours.

Following incubation, the collagen was fixed on to the plates by exposure to ammonia vapour according to the following protocol. In a fume hood, approximately 3-5 ml of ammonia solution (BDH chemicals) was added to tissue in a plastic box. Under sterile conditions the collagen solution was carefully removed from the plates, these were then transferred to the fume hood where they were exposed to the ammonia vapour for 10 minutes. The plates were removed from the vapour and washed, under sterile conditions, with Ca/Mg-free HBSS. After three washes the plates were incubated with Ca/Mg-free HBSS at 37°C until required. This procedure could be carried out at any time during the day they were required provided the plates were not allowed to dry out at any point.

2.2.6. Equipment

Preparation of dissection equipment

All dissection instruments were washed thoroughly with double distilled water, covered with aluminium foil and sealed with autoclave tape. The instruments were sterilised in an autoclave at 121°C for 15 minutes. To maintain sterility, all instruments were soaked in 70% alcohol during the dissection procedure.

Preparation of glassware and pipettes

All items of glassware and re-usable plastics were sterilised by autoclaving (see section 2.1.3). The Pasteur pipettes, some of which were fire polished in a Bunsen flame to constrict the tips, were plugged with cotton wool sealed in autoclave bags and autoclaved at 121°C for 15 minutes.

Dissection conditions

All dissection work carried out under aseptic conditions was performed in an open fronted laminar flow cabinet (Fellclean air Ltd), swabbed with 70% alcohol. Non-sterile preparations were carried out on the bench.

2.2.7. Primary Culture Medium and Supplements

Tissue culture medium

Lung and brain microvascular endothelial cells were grown in Ham's F-10 nutrient mix obtained from Gibco as a 10X concentrated liquid without sodium bicarbonate. The medium was diluted to 1X with sterile, freshly double distilled water as required.

Tissue Culture Supplements

Stock solutions of penicillin (5000 I.U./ml) and streptomycin (5000µg/ml) (pen/strep) and L-glutamine (200mM) were divided into 5ml aliquots and stored at -20°C.

Endothelial cell growth supplement (ECGS), obtained from Sigma in a 15mg vial was dissolved in 2ml Ca/Mg-free HBSS, dispensed into 20 x 100µl aliquots and stored at -20°C.

A stock solution of heparin was prepared by dissolving 400mg (176 units/mg) in 50 ml Ca/Mg-free HBSS. This solution was sterile filtered, divided into 4-5ml aliquots and stored at -20°C.

Amphoteracin B (Fungizone) was obtained sterile from Gibco, as a 250µg/ml stock solution prepared in water. It was divided into 4-5ml aliquots and stored at -20°C.

Foetal calf serum, obtained from Flow laboratories, (batch number 10855) was divided into 20ml aliquots and stored at -20°C.

Platelet poor human serum (PPHS) was obtained from Sigma (batch number 122H9300), sterile filtered using a 0.2μm filter, divided into 10ml aliquots and stored at -20°C.

A solution of various growth supplements was prepared containing:

Double distilled water	10ml
Vitamin C (Ascorbic acid, BDH chemicals) 10mg/ml	500μl
Glutathione (reduced form, Sigma)	325mg
Insulin (from bovine pancreas Sigma)	5mg
Transferrin (bovine, iron free, Sigma)	5mg
Selenium dioxide (Sigma) 500μg/ml	10μl

The supplement solution was sterile filtered, divided into 1ml aliquots and stored at -20°C.

Hams F-10 medium was prepared aseptically, each time it was required, from sterile components according to the following recipe:

1X Hams F-10 medium	10ml
Foetal calf serum	1.0ml
L-glutamine	120μl
Penicillin/streptomycin	120μl
Heparin	120μl
Fungizone	120μl
supplements	120μl
ECGS	100μl

2.2.8. Growth of Cells on Filter Membranes

Preparation of filters

Filter culture was performed on transparent Falcon cyclopore cell culture inserts (25mm diameter) suitable for use in 6-well plates, where the pores on the membrane were 0.45μm in diameter. The filters were transferred to 6-well plates using sterile forceps, some of the filters were coated with collagen type 1 following the same method used for coating the culture well plates. The filters were prepared for seeding by applying 1.5-2.5ml of complete culture medium to each well of the 6-well plate (basolateral section) and 1.5-2.5 ml of medium to each filter chamber (apical section). The plates were placed in a humidified CO_2 incubator at 37°C to ensure that the filter was sufficiently moist whilst the cells were being prepared for seeding.

Culture on filter membranes

Cells were seeded directly from primary culture or subculture following growth on collagen coated 6-well plates, where the appropriate trypsinization procedures were used for each cell type (see sections 3.2.5 and 4.2.1). The cells were added to the apical chamber, at the required density, in complete culture medium. The medium from the apical chamber containing non-adherent cells was changed after 2-4 hours in culture, thereafter the medium from both chambers was changed on alternate days using a sterile long glass Pasteur pipette.

The growth and morphology of the cells on filter membranes was assessed at regular intervals using light and electron microscopy (see section 5.2.1).

3. PRIMARY CULTURE OF MURINE LUNG MICROVASCULAR ENDOTHELIAL CELLS

3.1. Introduction

The preliminary studies of lung microvascular endothelial cell culture were based on several published methods for large vessel and microvascular endothelial cell isolation and culture (Folkman *et al.*, 1979; Gitlin and D'Amore 1983; Zetter 1984; Haisch *et al.*, 1990; Abbott *et al.*, 1992; Belloni *et al.*, 1992; Nishida *et al.*, 1993). A wide variety of isolation techniques, extracellular matrices and culture conditions were studied in order to obtain the optimum conditions for isolation and culture of murine lung microvascular endothelial cells. These optimum conditions included the isolation of the maximum yield of endothelial cells, without excessive contamination by large vessel endothelia or non-endothelial cells. In addition, modification of the culture conditions was also required to provide optimum growth, whilst maintaining the characteristic cell morphology in primary culture and subculture.

3.2. Materials and Methods

3.2.1. Preliminary Isolation Technique

The original isolation technique involved the use of six C57 mice, either male or female, for each preparation (see section 2.2.2). The mice were killed by cervical dislocation, the lungs removed and transferred into a sterile universal vial containing Ca/Mg-free HBSS. Under sterile conditions the peripheral section of the lung was dissected and chopped into 1-2mm pieces. The dissected lung tissue was subsequently incubated for 1 hour at 37°C with 0.5% collagenase type II (Sigma), dissolved in high glucose DMEM (Sigma).

Following the incubation procedure the enzyme digestion mixture was centrifuged at 200g for 10 minutes and the pellet was resuspended in Ca/Mg-free HBSS. This process was repeated and the pellets were combined, and recentrifuged at 400g for 5 minutes. The supernatant was removed and the pellet was suspended in M199 supplemented with 7.5% $NaHCO_3$, 2mM L-glutamine, 50IU/ml penicillin, 50μg/ml streptomycin, 1% NEAA and 2.5μg/ml Amphoteracin B, and the cells were plated on to a gelatin coated 6-well culture dish (see section 3.2.3).

After 2-3 hours in culture the cells were washed with Ca/Mg-free HBSS and incubated with fresh medium. After 24 hours in culture the medium was removed and replaced with fresh M199 with supplements and in addition containing 100μg/ml Heparin and 75μg/ml ECGS but without Amphoteracin B. This represented the standard culture medium used in preliminary experiments.

3.2.2. Variations in the Isolation Technique.

In order to improve the isolation protocol and increase the initial yield of cells isolated from the tissue, variations in the isolation technique were investigated. Improvements of the isolation protocol were assessed daily by examination of the cultures using phase contrast light microscopy.

Enzyme digestion

Variations in the enzyme digestion technique were examined. Sigma type II collagenase concentrations were varied between 0.05% and 0.5% for digestion times of 30 minutes to 3 hours. Other types of collagenase were investigated: collagenase/dispase obtained from Sigma and collagenase/dispase from Boerhinger. The use of TLCK and DNase 1 in the enzyme digestion mixture and the effect of a second enzyme digestion step was also examined (Hughes and Lantos 1986, Audus and Borchardt 1986). Pretreatment of the lungs to cause initial breakdown of the tissue by perfusion of the lungs *in situ* with solutions of collagenase (0.05-0.1%) in Ca/Mg-free HBSS was also investigated (Belloni *et al.*, 1992).

Homogenisation

A homogenisation step using between 2 and 10 strokes of a Dounce homogeniser was examined as a method of breaking up the tissue further. This was followed by further shearing of the tissue with a long glass Pasteur pipette to ensure that the maximum yield of cells was being obtained from the lung tissue. The effect of sieving the tissue through nylon mesh to separate out fragments on the basis of size was also investigated.

Centrifugation

The effect of variation in the speed and the length of time of centrifugation was examined. Speeds of between 200g and 800g for up to 10 minutes were applied during washing steps to establish whether these variations caused any disruption of the tissue. Disintegration of the tissue to some extent during the initial washing procedure would be beneficial, however in the later stages of the isolation procedure harsh disruption of the fragments was not recommended.

Separation of the homogenised tissue by density dependent centrifugation was studied. The tissue was centrifuged on a BSA density gradient (between 10 and 25%) at 1200g for 15 minutes (Zetter, 1984). The use of a 40%/25%, discontinuous Percoll gradient (Haisch *et al.*, 1990) and a 50% preformed isotonic Percoll gradient (Abbott *et al.*, 1992) was also investigated in order to improve separation of the capillary fragment.

3.2.3. Culture Conditions: Substrata and Extracellular Matrices.

Substrata

The effect on cell growth and morphology of the density at which the cells were seeded from initial culture and subculture, and the different substrata was investigated. The cells were cultured on 96, 24, and 6-well plastic tissue culture plates (Corning), Primera culture plates (Falcon, Becton Dickinson) and 2-well chamber slides (Lab-Tek, Nunc Inc.). Cells were also grown on transparent anopore filter membranes (Nunc), Millipore CM transparent filters and Falcon transparent filter membranes (Becton Dickinson).

Extracellular matrices

A variety of extracellular matrices were tested to establish the most suitable conditions for cell growth. Tissue culture wells were coated with sterile solutions of 0.5% and 1%

gelatin (tissue culture grade, Sigma). The gelatin solution was incubated on the plates for 1 hour at room temperature and the excess solution was aspirated prior to the addition of the cells.

The effect on cell growth and morphology of collagen type 1 from calf skin and rat tail was investigated. The collagen was fixed to the plastic wells by ammonia vapour, and washed with Ca/Mg-free HBSS prior to the addition of the cells (section 2.2).

Cell growth and morphology on fibronectin coated wells was also studied. A 25µg/ml solution of fibronectin was prepared in Ca/Mg-free HBSS and filter sterilised. The fibronectin was added to the wells, and the excess was aspirated after 1 hour.

Cell growth and morphology was examined on the basement membrane Matrigel (see section 5.2.5).

3.2.4. Culture Conditions: Medium, Serums and Growth Factors

Culture medium

Several types of culture medium were studied in order to establish the most suitable conditions for microvascular endothelial cell culture. The cells were cultured in M199, DMEM, Dulbecco's Modefied Eagle's Medium with F12 nutrient mix (DME/F12), and Hams F10 (all obtained from Gibco). In each instance the medium was supplemented with L-glutamine, penicillin/streptomycin, and various concentrations of sera and growth factors.

The effect on cell culture of conditioned medium was also studied. RPMI 1640 medium was tumour conditioned by incubation for 2 days with B16 melanocytes and was combined as a 1:1 mixture with M199. Medium conditioned with DMEM from BAE-1 cells and 3T3-L1 fibroblasts (both used after 2 days incubation) was also combined 1:1

with M199. The use of endothelial cell plating medium (serum free) and endothelial cell growth medium (serum free), both obtained from Sigma, was also examined.

Growth serum

The culture medium was supplemented with between 10 and 20% foetal calf serum in order to determine the most suitable requirements for endothelial cell growth This was determined by assessing the increase in the size of the endothelial cell colonies and the extent of non-endothelial cell growth.

The effectiveness of supplementing the medium with platelet poor human serum (PPHS) in conjunction with foetal calf serum was also studied (Gitlin and D'Amore 1983, Belloni *et al.*, 1992). The cells were cultured with between 0 % and 5% PPHS in the presence of 10-20% FCS.

Growth factors and supplements

The culture medium was supplemented with 75µg/ml ECGS, 80µg/ml heparin, and the effect of the addition of the supplements glutathione, transferrin, vitamin C, selenium dioxide (see section 2.2.7) was also observed.

3.2.5. The Final Method for the Isolation and Culture of Murine Lung Microvascular Endothelial Cells

For each preparation ten C57 mice, either male or female, of approximately 30-40g weight were killed by cervical dislocation. The lungs were exposed and perfused *in situ* with 0.05% collagenase (Sigma Type II) in Ca/Mg-free HBSS which had been filter sterilised. The perfused lungs were excised and transferred into a sterile 30 ml universal vial containing HEPES buffer solution at 4°C

Under sterile conditions, the lungs were carefully immersed in 70% ethanol for 30 seconds and transferred directly into a beaker containing fresh buffer solution at 4°C. This process was carried out in order to fix the pleural membrane which contains mesothelial cells .

Only the peripheral section of each lobe of the lungs was required for the isolation procedures this selected for microvascular endothelial cells without large vessel contamination. The appropriate section from each lung was removed, chopped into 1-2mm pieces and transferred into a fresh buffer solution. The lung tissue was strained in order to remove the buffer and incubated for 1 hour at 37°C, with gentle agitation, in 10 ml of an enzyme digestion mixture consisting of 0.1% collagenase/dispase solution containing 20U/ml DNase1 and 0.147μg/ml TLCK.

The incubate was homogenised using a Dounce homogeniser (4 strokes) and sheared using a long glass Pasteur pipette for 5 minutes to break up the fibres. The suspension was centrifuged at 600g for 5 minutes (Jouan bench centrifuge), the supernatant carefully removed and the pellet resuspended in 15ml of 25% BSA (prepared in buffer). The resulting suspension was sheared again with a Pasteur pipette to ensure complete dispersal of the tissue and centrifuged at 1200g for 15 minutes.

The pellet was resuspended in buffer and the BSA solution was centrifuged again at 1200g for a further 15 minutes. The pellets were pooled together, washed with buffer, centrifuged at 600g for 5 minutes and resuspended in the remaining 5ml of the enzyme mixture for a further 3 hours at 37°C under gentle agitation.

The enzyme digest was dispersed using a Pasteur pipette and centrifuged at 600g for 5 minutes. The supernatant was removed and the pellet resuspended in 1ml of buffer. The cell suspension was layered on top of a pre-set 50% isotonic Percoll gradient and centrifuged at 1200g for 20 minutes. The capillary fragments, appearing approximately one third of the way down the gradient, were transferred into a clean universal vial,

containing buffer, and washed twice in buffer by centrifugation at 600g for 5 minutes. The pellet was resuspended in a 1:1 mixture of buffer and Hams-F10, centrifuged at 600g for 5 minutes and finally suspended in freshly prepared complete culture medium.

The fragments were plated on to 6 wells of a 35mm tissue culture dish pre-coated with collagen type I, from calf skin (see section 2.2.5). After 2-3 hours in culture the medium was removed, the cells washed with Ca/Mg-free HBSS. The cultures were incubated in a humidified CO_2 incubator at 37°C in fresh complete culture medium (see section 2.2.7), thereafter, the medium was changed every other day. After 4 days in culture the PPHS was reduced in concentration from 5% to 2%.

Subculture of the cells

Due to the presence of several contaminating cell types, especially smooth muscle cells, pericytes and fibroblasts, the endothelial cells were selectively cloned after approximately 5-7 days in culture. The cultures were washed 3 times with Ca/Mg-free HBSS. Penicylinder cloning rings (size 7mm diameter) were fixed to the surface of the dish with sterilised high density vacuum grease (Dow Corning, USA) and the selected colonies were trypsinised using 0.05% trypsin/0.02% EDTA for 2-5 minutes at 37°C.

The action of the trypsin was inhibited by the addition of culture medium and the cells were gently removed using a long glass Pasteur pipette. The cells were plated on to freshly collagen coated 6-well tissue culture dishes in complete culture medium.

3.2.6. Cell Purity

Several non-endothelial cell types were regularly found to contaminate the culture. Contaminating cells included macrophages, pericytes, smooth muscle cells, fibroblasts and possibly mesothelial cells. Non-endothelial cells in culture were identified by

morphology and through characterisation studies (see section 5). The extent of contamination was examined routinely by light microscopy and photographed using a Nikon 35mm camera. The following procedures were employed in order to reduce non-endothelial cell contamination:

1. The lungs were carefully washed with 70% ethanol in order to fix the pleural membrane containing mesothelial cells (Chopra *et al.*, 1990; Nishida *et al.*, 1993).

2. The cells were washed with Ca/Mg free HBSS 2-3 hours after plating to remove any unattached cells (Zetter 1984).

3. After 3 days in culture the cells were incubated with Ca/Mg-free HBSS for 15-20 minutes at 37°C (Abbott *et al.*, 1992).

4. The endothelial cell colonies were selectively isolated using penicylinder cloning rings.

3.3. Results

3.3.1. Preliminary Isolation and Culture

The original isolation procedure used the lungs of six C57 mice. Cells obtained by this procedure were seeded on to one 6-well tissue culture plate. The tissue was dissected selecting only the peripheral section in order to reduce the possibility of contamination from large vessel endothelial cells. Having minced the tissue, it was centrifuged at a very low speed (200g) in an attempt to remove some of the red blood cells and open the microvessels, enabling access of the enzyme solution to the microvascular endothelial cells.

The lung tissue was incubated with a 0.5% collagenase enzyme digestion solution initially for 1 hour at 37°C. This collagenase digestion initiated the disintegration of the tissue however the pieces remained almost completely intact. The cells removed by digestion with the enzyme solution were collected by gentle centrifugation (200g) of the tissue forming a pellet. This cell pellet was washed by further centrifugation and plated on to gelatin coated plates in culture medium M199.

This preliminary isolation technique was very unspecific, and produced few endothelial cells. The endothelial cells obtained by this method were isolated as single cells, which after only a few days in culture became non-viable. Alternatively, any viable cells were over-grown by faster proliferating non-endothelial cell contaminants such as fibroblasts and smooth muscle cells. Improvement of the isolation technique was required to alleviate the lack of specificity and the problem of single cell production.

As a result of the preliminary isolations, several factors were assessed as possible causes of this lack of specificity produced by the initial method. Collagenase digestion may

have been too harsh, causing complete disruption of the capillary fragments thereby increasing the likelihood of fibroblasts, smooth muscle cells and macrophages to contaminate the culture. Alternatively it was also possible that digestion of the tissue was incomplete and vessel fragments were not being released from the surrounding connective tissue.

As well as the problems posed by the isolation technique, it was also necessary to consider the effectiveness of the extracellular matrix, gelatin, to provide a suitable attachment factor for the cells and the ability of the medium to satisfy the growth requirements.

3.3.2. Development of the Isolation and Culture Techniques.

Initial variations in enzyme digestion

The initial alterations to the preliminary isolation technique was to the collagenase digestion. The concentration of collagenase was maintained at 0.5% however, the duration of collagenase digestion was increased to 1½, 2, and 3 hours. This resulted in a notable increase of contaminating cells and no obvious capillary fragments being produced, suggesting excessive non-specific disruption of the tissue. A reduction in length of collagenase digestion to 30 minutes however, reduced the quantity of contaminating cells and after 2 days in culture a few small colonies of 10-15 cells were apparent, (Figure 2) as well as several single cells which became non-viable after 2-3 days in culture. As a result of these findings the effect of a reduction in the concentration of collagenase used over a longer time interval was examined in an attempt to increase the yield of cell colonies isolated.

Isolation by collagenase digestion was studied with 0.2% and 0.1% collagenase solutions for time periods of 30 minutes up to 3 hours. Although a few colonies were isolated

using these alternative protocols, none of these alternative isolation procedures resulted in a substantial increase in the yield of cell colonies produced. Due to the relative ineffectiveness of these protocols, the use of collagenase/dispase was investigated as an alternative to the harsher collagenase. The effect of collagenase with the neutral protease dispase, which offers a more gentle enzymic digestion leaving the blood vessels intact (Zetter 1984), was therefore be assessed for an increased period of incubation. This study was accompanied with an investigation into the use of a second mild enzyme digestion step in an effort to provide a more sensitive and specific isolation procedure.

Under the culture conditions employed at the time of the initial isolation procedures, any colonies isolated by collagenase digestion did not proliferate, therefore in parallel with the manipulations in the isolation technique, improvements in the culture conditions were also investigated.

Initial variations in culture conditions

The contents of the culture medium were very important for cell growth, however, suitability of the medium may vary with the species and tissue from which the cells were isolated (Gitlin and D' Amore 1983). A wide variety of culture medium, supplements and sera have been used in the study of endothelial cell culture offering various results . In this study Medium 199 was chosen as the standard culture medium as it had provided suitable culture conditions for large vessel endothelial cells isolated by primary culture. The lack of cell growth from initial isolations however, prompted an investigation into the effectiveness of M199 as a suitable culture medium for microvascular endothelial cells.

For each isolation procedure the cells were seeded on to one 6-well plate. A direct comparison was made between the standard M199 and the alternative medium. For each

isolation protocol, 3 wells were incubated with standard culture medium M199 and 3 wells were incubated with the alternative culture medium.

Altering the culture conditions offered varying results. Conditioned medium is thought to be very useful in promoting culture of capillary endothelial cells (Folkman *et al.*, 1979; Beer Stoltz and Jacobson, 1991), however, culture with a 1:1 mixture of M199 and either tumour conditioned medium or medium conditioned with BAE-1 cells or fibroblasts did not show any significant improvement on endothelial cell growth compared with M199 alone. Culture with serum free endothelial cell plating and growth medium had no effect on cell growth. The ineffectiveness of these different types of culture medium to promote cell growth could have been effected by the sparse cell densities.

Incubation with DME/F-12 nutrient mix supplemented with increased concentrations of FCS (20%) and platelet poor human serum (5%) provided slightly more suitable culture conditions for cell growth. The endothelial cell colonies were healthy and began to grow slowly; after 3-4 days the colonies contained 20-30 cells (Figure 3). These culture conditions however, also caused rapid proliferation of fibroblast and smooth muscle cells and after 5-6 days in culture the cells became completely overgrown with non-endothelial cell contaminants. Due to the lack of improvement in the selective growth of endothelial cells from these alterations, further modifications to the isolation procedure and culture conditions were sought.

Centrifugation and the introduction of a second enzyme digestion

Variations of the centrifugation technique were investigated in order to determine the effect of the speed and duration of centrifugation on the cells during the washing procedures. Centrifugation speeds of 200-800g for 5-10 minutes altered the size of the cell pellet produced. The minimum speed and duration of centrifugation used during

washing procedures did not appreciably reduce the amount of non-endothelial cell contamination. The maximum centrifugation speeds produced a larger pellet containing some debris, however this did not attach to the culture well and contamination by non-endothelial cells was not appreciably effected. The variations in centrifuge speed and duration did not appear to cause damage to the cells and therefore a higher centrifugation speed was chosen as this provided the most satisfactory washing regime whilst ensuring that no cells were lost in the supernatant. For routine washing procedures 600g for 5 minutes was employed as standard speed and duration of centrifugation. This centrifugation regime appeared to have no damaging effect on the isolation procedure.

A modification of the isolation technique involving two gentle enzyme digestion steps was investigated. A comparative study between collagenase/dispase and collagenase both obtained from Sigma was conducted using the two step enzyme digestion protocol. The dissected lung tissue was incubated with the appropriate enzyme (0.1 %) for 45 minutes to initiate disintegration of the tissue. The incubate was then sheared with a Pasteur pipette in order to assist the break up of the connective tissue and expose the microvessels. This was followed by a second enzyme incubation for 2 hours to further digest the tissue fragments and remove any pericytes attached to the basement membrane. The use of 0.1% collagenase resulted in a large increase in non-endothelial cell contamination. Although the use of collagenase/dispase did not offer any significant improvement over collagenase alone in the isolation of endothelial cell colonies, no appreciable increase in the proportion of contaminating cells present was observed.

Introduction of a homogenisation procedure

At this point the use of a homogenisation step was employed in an attempt to disrupt the connective tissue and provide easier access of the enzyme to the microvessels. The tissue was washed by centrifugation and incubated in 0.1% collagenase/dispase (Sigma)

for one hour. The tissue was then homogenised with 2 strokes of a Dounce homogeniser and sheared using a Pasteur pipette. The resulting suspension was centrifuged and resuspended in collagenase/dispase for a second period of incubation lasting 2 hours. After the second incubation the pellet obtained by centrifugation was washed, resuspended in standard M199 culture medium and plated on to gelatin coated culture wells. The homogenisation procedure produced a large amount of tissue debris which did not attach to the wells and was removed when the medium was changed.

This homogenisation and two step enzyme digestion technique showed an improvement in the isolation of the cells, a few colonies of 10-15 cells were isolated but again did not grow. The yield of non-endothelial cells isolated did not increase significantly and therefore manipulation of this method was investigated in order to increase the yield and selectivity of the isolation technique. This homogenisation and two step digestion method was also conducted on lung tissue using to 0.1% collagenase. This resulted in a large increase in contaminating cells which masked any evidence of endothelial cell isolation.

Cell sieving and preliminary Percoll gradient centifugation

Cell sieving using nylon screens with different mesh sizes, is a technique widely employed to selectively collect vessel fragments on the basis of size without contaminating single cells. The effect of cell sieving was therefore investigated in an attempt to selectively isolate microvascular endothelial cell fragments. This procedure was ineffective and rapidly dismissed because of the gelatinous nature of the homogenised tissue and the difficulty of successfully removing all of the necessary material from the sieve.

Another attempt to selectively isolate the endothelial cells involved the use of a Percoll gradient. A discontinuous density gradient was prepared using layers of 40%, 25% and

0% Percoll and the cell suspension was centrifuged at 600g for 15 minutes (Haisch *et al* 1990). The homogenised tissue however did not show much separation under these conditions therefore an alternative Percoll gradient was required

Further modifications of the isolation technique

Pretreatment of the lungs *in situ* was investigated in order to remove the blood from the vessels and give the vessels an initial exposure to collagenase (Belloni *et al.*, 1992). 0.1% collagenase was considered to be too harsh as it resulted in excessive break up of the vessels, therefore a solution of 0.05% collagenase was employed.

Collagenase/dispase obtained from Boehringer was prepared in a solution of HEPES buffer. The pretreated tissue was incubated in collagenase/dispase for one hour with gentle agitation and homogenised with two strokes of the homogeniser. After washing with buffer and shearing with a Pasteur pipette the homogenate was resuspended in enzyme solution for a further 2 hours, washed and seeded on to a gelatin coated 6-well plate in M199.

These modifications resulted in an increased yield of capillary fragments however, non endothelial contaminants were also prevalent (Figure 4). Further modifications of these techniques were employed in an attempt to increase the purity of the cultures. The collagenase/dispase solution was supplemented with TLCK and DNase 1. The solution was supplemented with TLCK which inhibits clostripain, a non-specific proteinase contaminant of collagenase preparations which damages cells (Hefley *et al.*, 1981). DNase 1 was used to degrade DNA liberated from ruptured cells.

Following the initial preparation of the tissue and the enzyme digestion, the homogenised tissue was centrifuged using 25% BSA at 1200g for 15 minutes. This centrifugation step further purified the culture by causing the sedimentation of the more dense material which consisted principally of the capillary fragment. At this stage the capillary

fragments were not sufficiently disrupted, and were therefore incubated with the enzyme solution for a further 2 hours before seeding. This modification resulted in a slightly purer culture, however contaminating cells present especially pericytes were possibly introduced into the culture as a result of the further disruption of the fragments during the second enzyme incubation. Therefore, purification of the culture following the second incubation was required, this involved separation using 50% Percoll gradient centrifugation. Centrifugation with 10% and 15% BSA (Madri *et al.*, 1983) did not cause any separation and the final culture became overgrown with contaminating cells at a more accelerated rate.

Percoll gradient centrifugation

An increase in the incubation time of the second enzyme digestion to three hours did not appear to appreciably effect the non-endothelial cell contamination, in fact, further digestion the tissue slightly increased the number of colonies observed. Further modifications in the duration of the second incubation period however, resulted in excessive disintegration of the tissue fragments and an increase in single cell production.

Following the second incubation the cell pellet was loaded on top of a pre-spun 50% isotonic Percoll gradient and centrifuged for 20 minutes at 1200g. Percoll gradient centrifugation resulted in the formation of a distinct band of red blood cells at the bottom of the gradient and a band of single cells near the top of the gradient. The clusters of endothelial cells appeared in the centre of the gradient. This band also contained some fragments of lung tissue not completely disrupted by homogenisation or enzyme digestion; when seeded on to the wells however, this material did not attach and was therefore removed when the wells were washed and the medium changed.

Final modifications of the isolation protocol

The final isolation technique that was established involved pretreatment of the lungs *in situ* with 0.05% collagenase to initiate tissue digestion. The first one hour enzyme incubation was followed by homogenisation and shearing of the tissue to expose the microvessels. The fragments were separated by density centrifugation with 25% BSA, and incubated with the enzyme solution for a further three hours. The cell clusters that were obtained following this enzyme digestion procedure were separated by a 50% Percoll gradient centrifugation before plating on to one 6-well tissue culture dish (Figure 5).

Subsequent attempts to improve the isolation technique met with some success, resulting in an increase in the microvascular endothelial cell yield and a decrease in the non-endothelial cell contamination. The variation in the yield of the cells isolated was assessed when the tissue was homogenised with up to ten strokes of the Dounce homogeniser. This study showed that four or five strokes of the homogeniser caused sufficient disruption to release the microvessels from the connective tissue but further homogenisation resulted in excessive disruption of the tissue fragments and therefore increased isolation of single cells and contaminants.

An important alteration which was made to the isolation technique involved treatment of the lungs immediately after they were excised. The lungs were treated with 70% ethanol in order to devitalise the pleural membrane which contains mesothelial cells (Chopra *et al.*, 1990, Nishida *et al.*, 1993). These are not immediately distinguishable from endothelial cells therefore removal of the pleural membrane was essential. As the pleural membrane of murine lungs is too small to remove manually therefore the method of fixing the membrane with ethanol was employed. The ability to distinguish between endothelial cells and mesothelial cells by conventional characterisation techniques was very difficult. Mesothelial cells can be distinguished from endothelial cells in culture, as microvascular endothelial cells can be induced *in vitro* to undergo angiogenesis (Chung-Welch *et al.*, 1989). This is demonstrated by characterisation studies (section 5).

Following the limited success of the initial variations in the culture conditions, further modifications to the growth conditions were investigated. This occurred in parallel with the modifications to the isolation technique described previously, and with the modifications of the extracellular matrix.

Alternative attachment factors and culture medium

Gelatin did not appear to be the most suitable attachment factor, as when the cells were disturbed, for example during washing, some of the cells had a tendency to detach from the surface of the well. As an alternative to gelatin, collagen type 1 from calf skin was used. This provided more suitable attachment for the cells and therefore a more satisfactory base for cell growth (Figure 6).

As an alternative to M199, cell growth in Hams F-10 was investigated as this been shown to provide suitable culture conditions for brain microvascular endothelial cells (Abbott, *et al.* 1992). The medium was supplemented with concentrations of 10-20% FCS and 5% PPHS as was used with DME/F-12. Unlike cell growth in M199, in Hams F-10 the cells were able to form relatively large colonies. In the presence of 10% FCS and 5% PPHS the cells isolated seemed to grow successfully without being rapidly overgrown by non-endothelial cells. After the initial 3-4 days in culture the concentration of PPHS was reduced to 2% with no notable effect on cell growth being observed. 5% PPHS was required on first plating the cells as it appeared to stimulate initial endothelial cell growth without accentuating the growth of contaminating cell types. Cells could be grown for up to 21 days in culture however after this time contaminating cells were extensively incorporated into endothelial cell colonies therefore, purification by subculture prior to this time was required.

In addition to high concentrations of sera, the medium was supplemented with heparin which inhibits the growth of smooth muscle cells, and endothelial cell growth

supplement. When used together heparin and ECGS have a synergistic effect increasing endothelial cell growth (Thornton *et al.*, 1983; Abbott *et al.*, 1992). Other supplements consisting of glutathione, selenium dioxide, transferrin, vitamin C and insulin were also beneficial in endothelial cell culture (Haisch *et al.*, 1990; Abbott *et al.*, 1992). Cell growth in Hams F10 medium in the presence of PPHS, ECGS and heparin offered the most suitable for endothelial cell proliferation and viability in culture. Figures 7, 8, 9 & 10 depict the growth of lung microvascular endothelial cell colonies from day 1 to day 14 in culture.

3.3.3. Lung Microvascular Endothelial Cell Growth and Morphology

When first plated out the capillary fragments isolated formed small colonies of 5-10 cells (Figure 5) and after 2-3 days in culture the fragments started to grow and divide as small colonies of polygonal shaped cells (Figure 7). However many of the single endothelial cells that were isolated became non-viable after approximately 3 days in culture. The cells remained in contact with the original colony and divided from the colony perimeter until they began to merge together after approximately 7 days in culture (Figure 11).

Cell purity

The contaminating cells that were observed in the cultures included fibroblasts, macrophages pericytes and smooth muscle cells (Figures 12, 13 & 14). The cultures were washed initially, 2-3 hours after plating; the clusters of endothelial cells adhered to the wells more rapidly than single cells, therefore this washing procedure considerably reduced contamination by non-endothelial cells. Treatment of the pleural membrane of the lungs with 70% ethanol was an important step in the elimination of contamination with mesothelial cells.

Incubation of the cultures for 15 minutes at 37°C with Ca/Mg-free HBSS during the initial 2-3 days of culture also appeared to reduce the extent of non-endothelial contamination without affecting the endothelial cells themselves (Abbott *et al.*, 1992). The endothelial cells show stronger adherence to the collagen coated surface of the wells and therefore incubation of the cells in a calcium free environment causes non-endothelial cells to detach more rapidly than the endothelial cells.

PPHS was used to supplement the medium as, unlike FCS, it does not contain the platelet factors released during clotting which are known to promote proliferation of non-endothelial cells such as fibroblasts, smooth muscle cells and pericytes (Gitlin and D' Amore, 1983). The presence of non-endothelial cells may however, contain some growth factors required for endothelial cell proliferation therefore a compromise between cell purity and cell proliferation may be necessary, as cells cultured in medium supplemented with PPHS alone did not appear to grow as rapidly as cells cultured with both PPHS and FCS.

Subculture

The procedure established for isolation of lung microvascular endothelial cells was modified in order to maximise the yield of endothelial cells while reducing non-endothelial cells to a minimum. The inability of the method to completely eliminate the presence of contaminating cells meant that the colonies formed needed to be subcultured by penicylinder ring cloning. Cultures allowed to grow to confluence from primary culture appeared heavily contaminated with non-endothelial cells. After approximately 7 days in culture the colonies were cloned and seeded on to freshly collagen coated wells. These subcultured cells tended to grow and form colonies in a similar manner to those in primary culture (Figure 15). Subcultured cells reached confluence after approximately 20 days in culture (Figure 16).

Substrata and seeding densities

Cells were seeded on to 96, 24 and 6-well plates in order to determine the effect of the seeding density on cell growth. Attempts to grow cells on 96 and 24-well plates from the initial isolation were unsuccessful, as cultures which were contaminated with non-endothelial cells were overgrown rapidly and due to the size of the wells were difficult to purify . Attempts to grow cells on Primera 6-well plates designed specifically for primary culture (Falcon, Becton Dickenson) were unsuccessful as very few cells attached to the well. Cells obtained from the initial isolation were therefore seeded on to one 6-well plate, thus enabling cloning and purification techniques to be performed. Colonies isolated by the cloning technique were seeded on to 6-well plates. The cells obtained from this procedure were seeded at high density, as seeding cells in insufficient numbers resulted in a greatly reduced growth rate. Cells grown from subculture on 24-well plates were observed to grow more slowly even at the same relative seeding density, than cells grown on 6-well plates .

Cells for fluorescence microscopy were originally seeded on to glass coverslips coated with gelatin however although these cells were able to attach as single cells but did not grow. The uptake of fluorescent compounds by cells seeded on glass coverslips was identified by fluorescent microscopy, however in subsequent experiments involving fluorescence, cells were grown on plastic non-autofluorescent 2-well chamber slides as this demonstrated the fluorescent uptake by the cells under more suitable culture conditions.

For electron microscopy studies, cells were seeded on to collagen coated transparent Falcon filters and were exposed to medium from both the apical and basolateral chambers. The cells grew as colonies and appeared to have the same morphology under the light microscope as cells grown on plastic culture plates. The image obtained by light microscopy however was speckled in appearance due to shadows cast by the pores and the composition of the filters, making detailed examination of the cell culture and

identification more difficult (Figure 17 & 18). Cell attachment on Nunc anopore transparent filters and Millipore CM transparent filters was poor regardless of being coated with gelatin or collagen.

Extracellular matrices

After establishing the isolation technique and culture conditions modifications of the attachment factors were investigated. Several attachment factors were assessed including gelatin, fibronectin and collagen. The treatment of the culture wells with collagen type 1 from calf skin provided the most suitable substrate for cell attachment, even in preference to type 1 collagen from rat tail. This was demonstrated in a study conducted in parallel with collagen from both sources. Gelatin (0.5% and 1%) showed some attachment however, cells that attached originally began to detach during the washing procedure. Fibronectin proved to be a suitable substrate for cell attachment and growth but showed no significant advantages over collagen. Cells seeded on to thin layer Matrigel coated wells resulted in the formation of tube-like structures characteristic of lung microvascluar endothelial cells (Figure 19).

Light, fluorescence and electron microscopy

Details of the materials and methods involved in these microscopy studies are shown in section 5.2.1

Figure 2. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells after 2 days in culture. The cells were isolated from murine lung following the preliminary isolation technique. Magnification X400

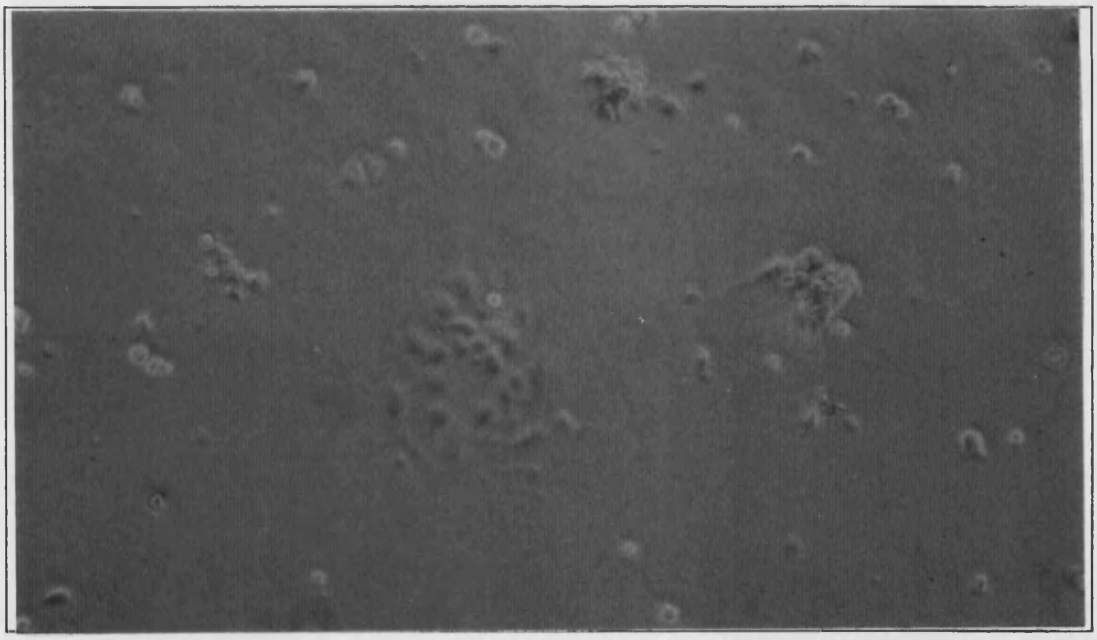

Figure 3. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells after 3 days in culture. The cells were isolated by incubation with 0.1% collagenase for 3 hours and cultured in DME-F12 medium. Magnification X200

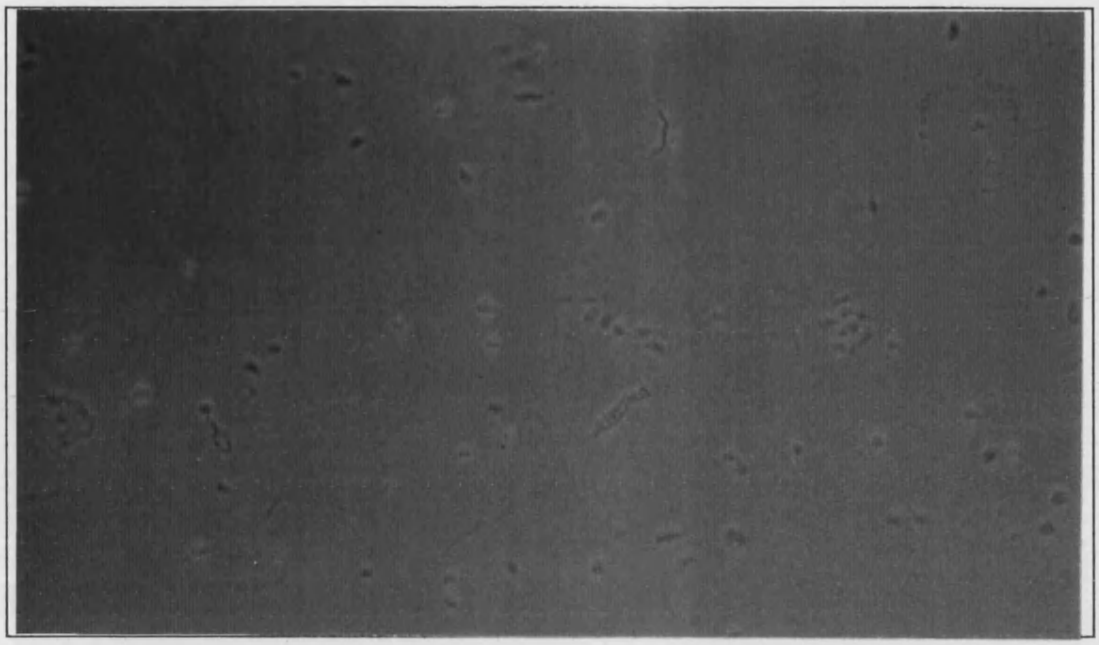

Figure 4. A phase contrast photomicrograph showing microvascular endothelial cell clusters 1 day after plating. The cells were isolated using a modification of the preliminary isolation technique, which resulted in an increase in contaminating non-endothelial cells. Magnification X200

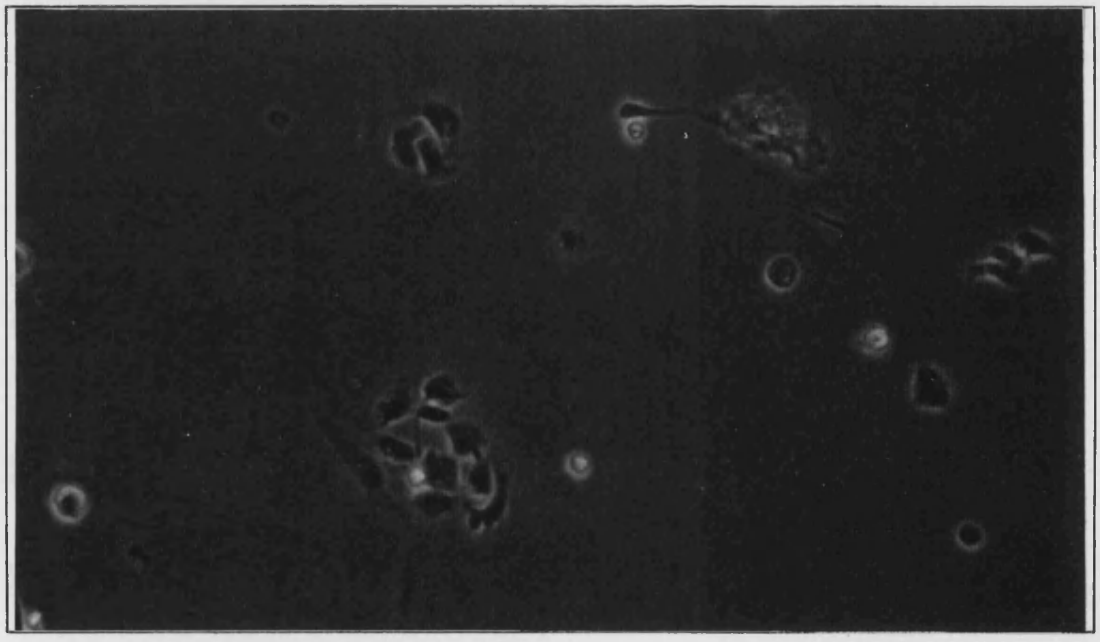

Figure 5. A phase contrast photomicrograph showing microvascular endothelial cell clusters 24 hours after plating, where the cell clusters were separated by 50% Percoll gradient centrifugation. Magnification X400

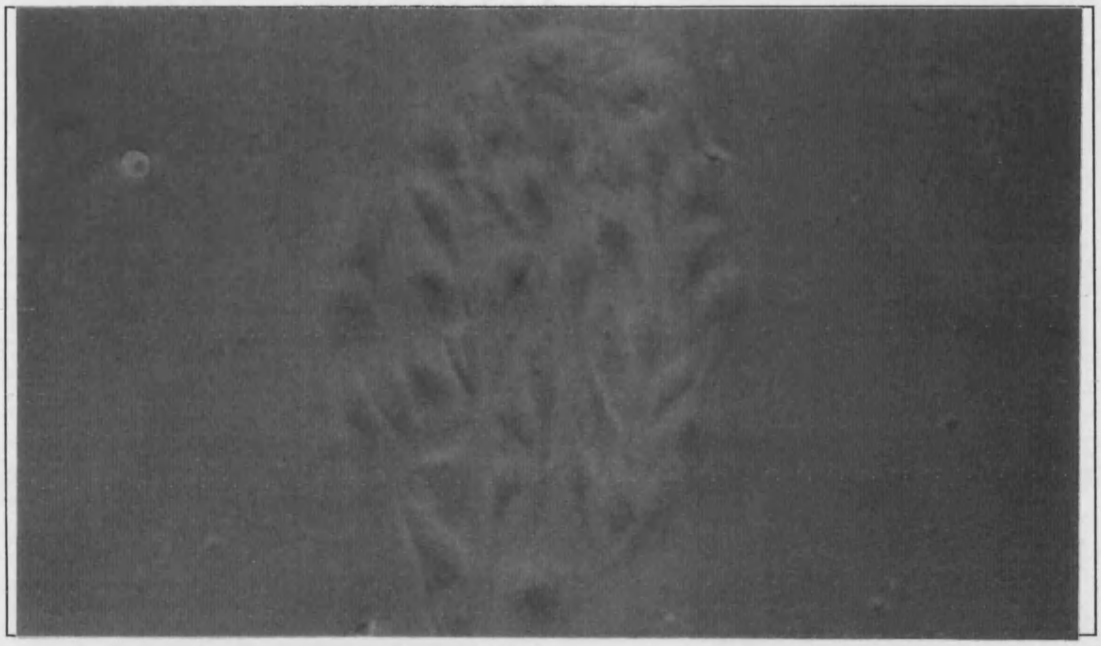

Figure 6. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells grown on a collagen coated culture well. Magnification X400.

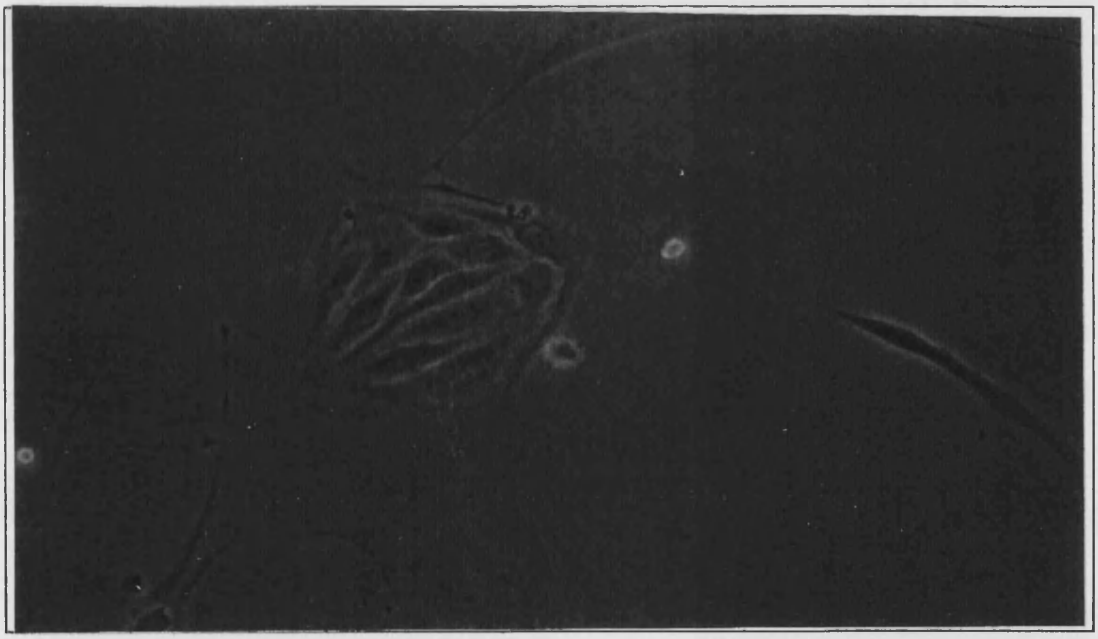

Figure 7. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells after 2 days in culture. Magnification X400

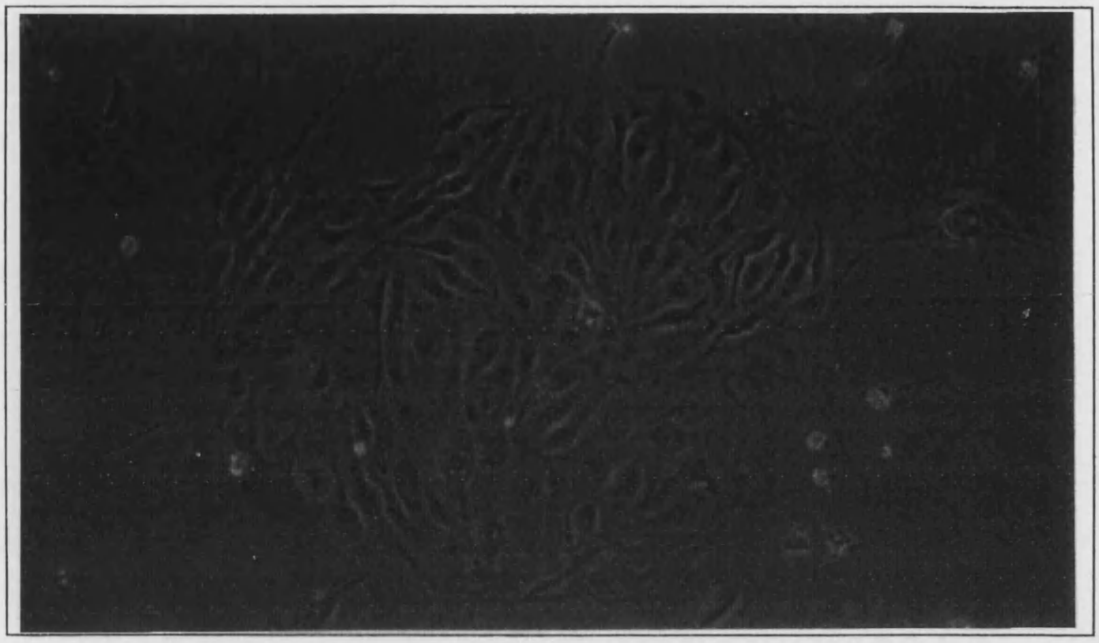

Figure 8. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells after 7 days in culture. Magnification X200

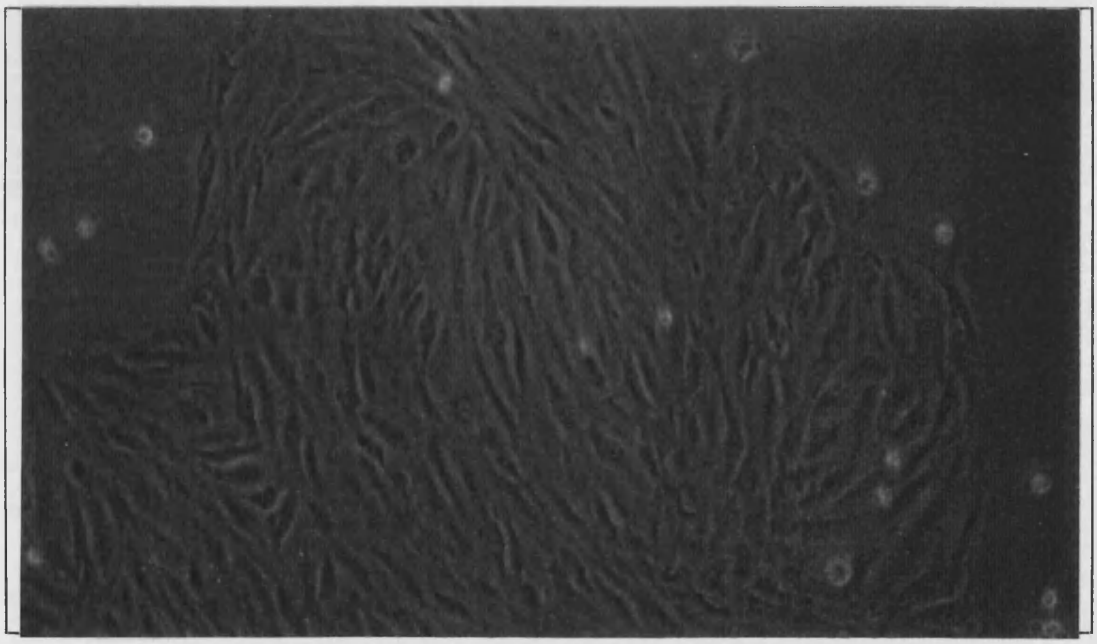

Figure 9. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells after 10 days in culture. Magnification X200

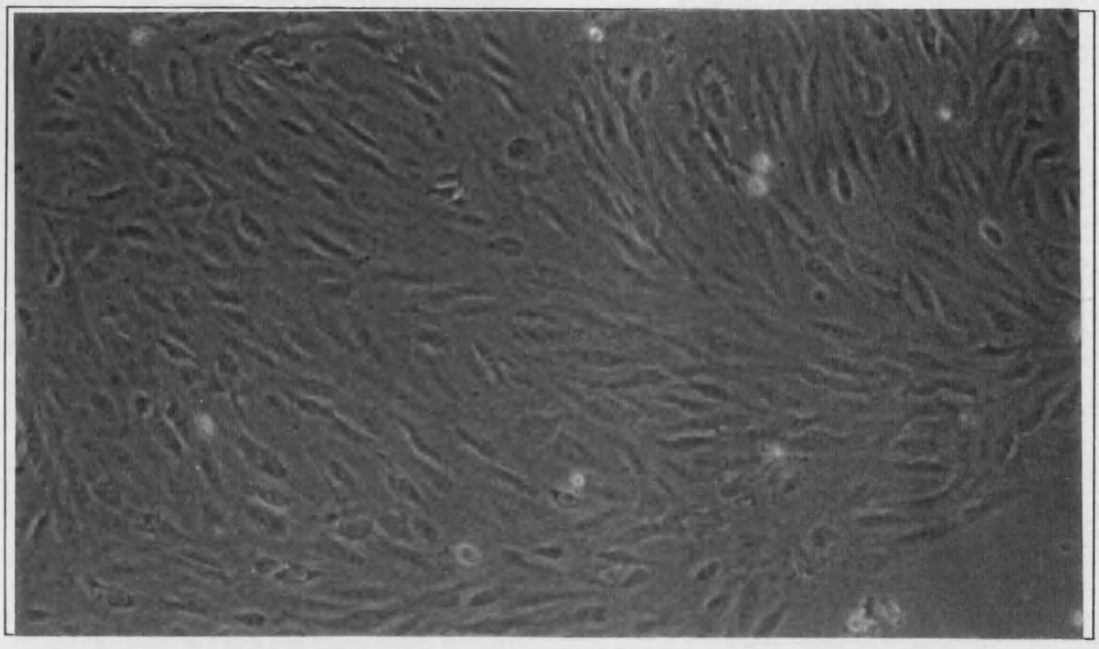

Figure 10. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells after 14 days in culture. Magnification X200

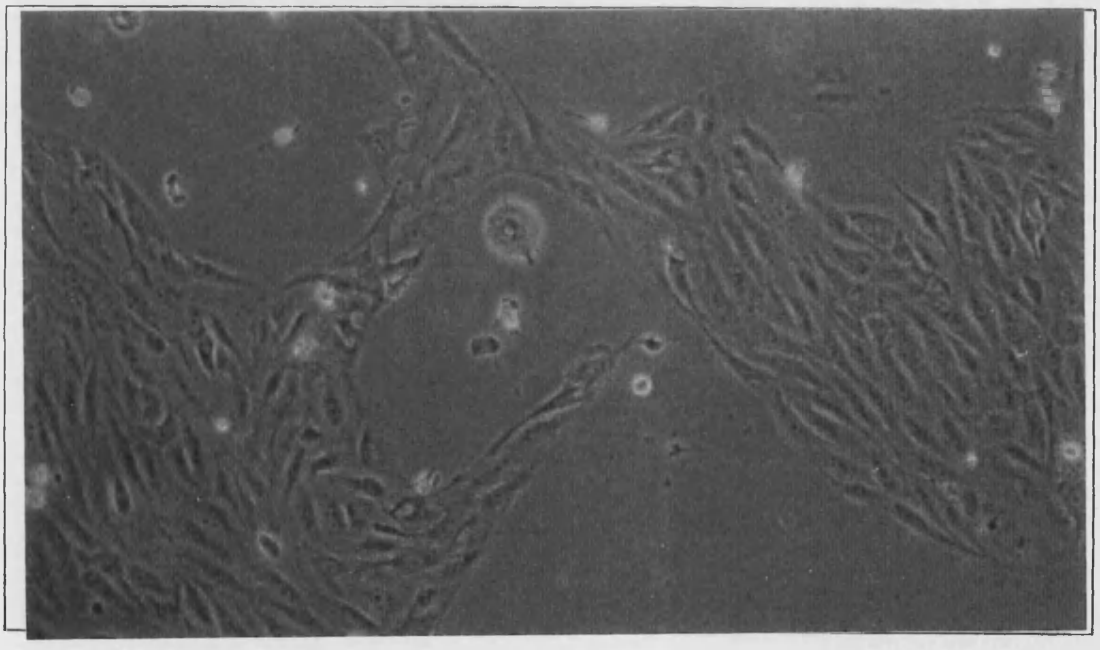

Figure 11. A phase contrast photomicrograph showing 2 colonies of microvascular endothelial cells merging together after 7 days in culture. Magnification X200

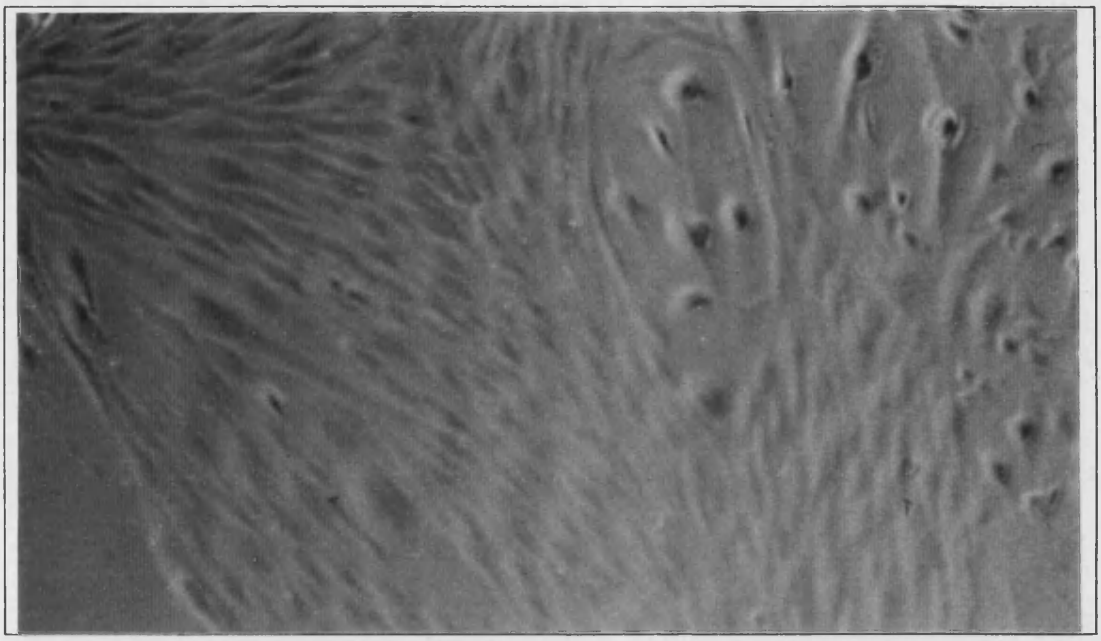

Figure 12. A phase contrast photomicrograph showing a small colony of microvascular endothelial cells and contaminating non-endothelial cells, probably pericytes and macrophages. Magnification X200

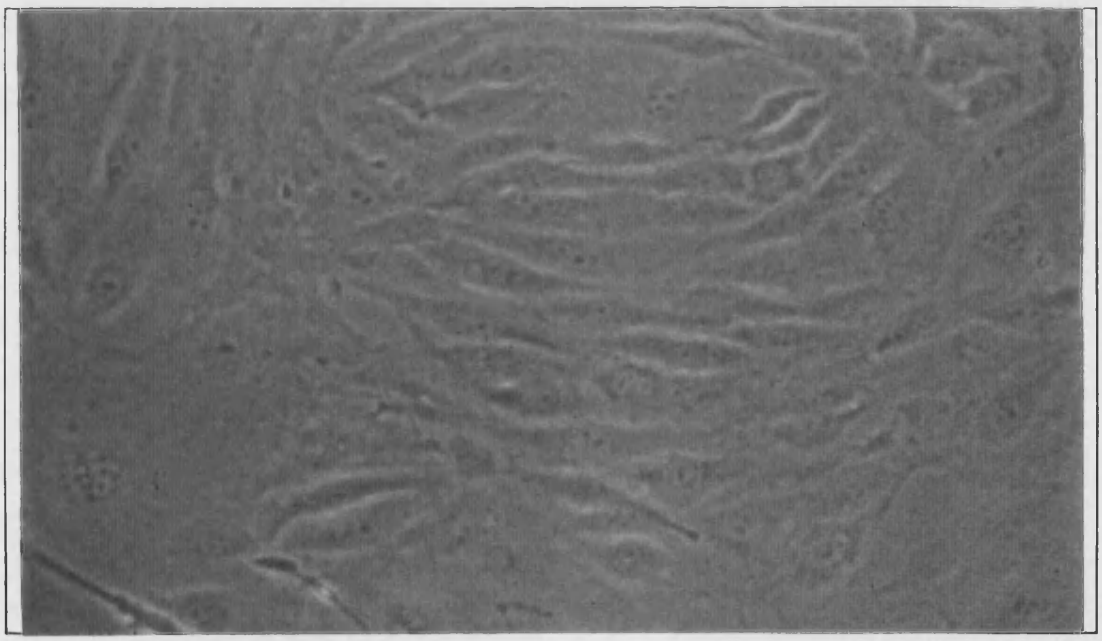

Figure 13. A phase contrast photomicrograph showing a colony of microvascular endothelial cells and contaminating non-endothelial cells, probably fibroblasts. Magnification X400

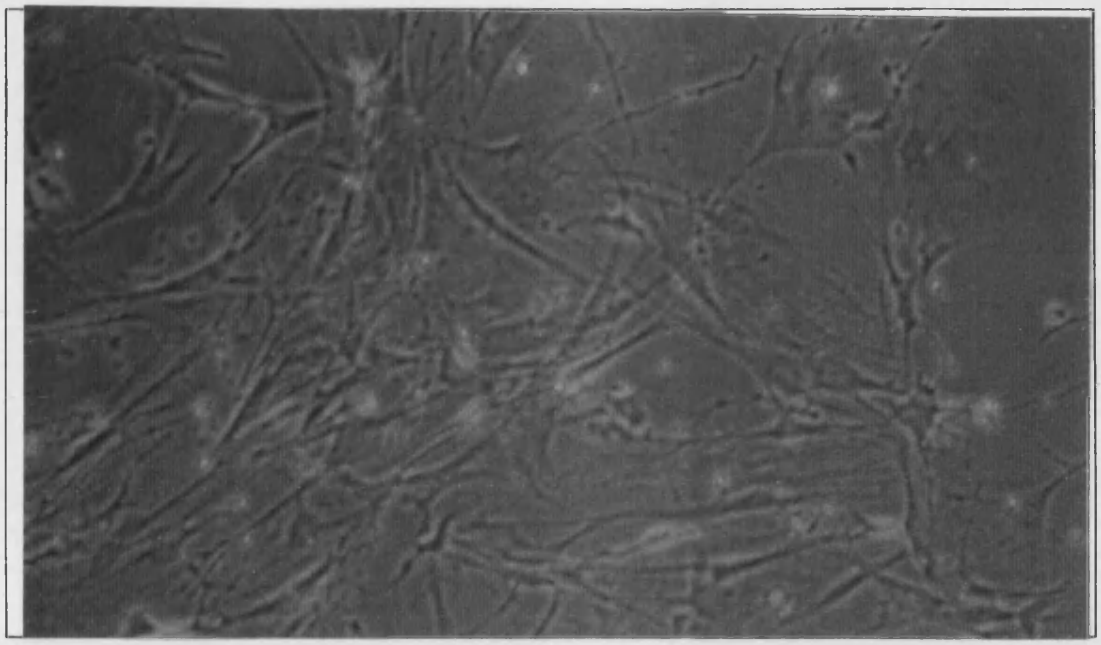

Figure 14. A phase contrast photomicrograph showing non-endothelial cell contaminants probably smooth muscle cells and pericytes. Magnification X200

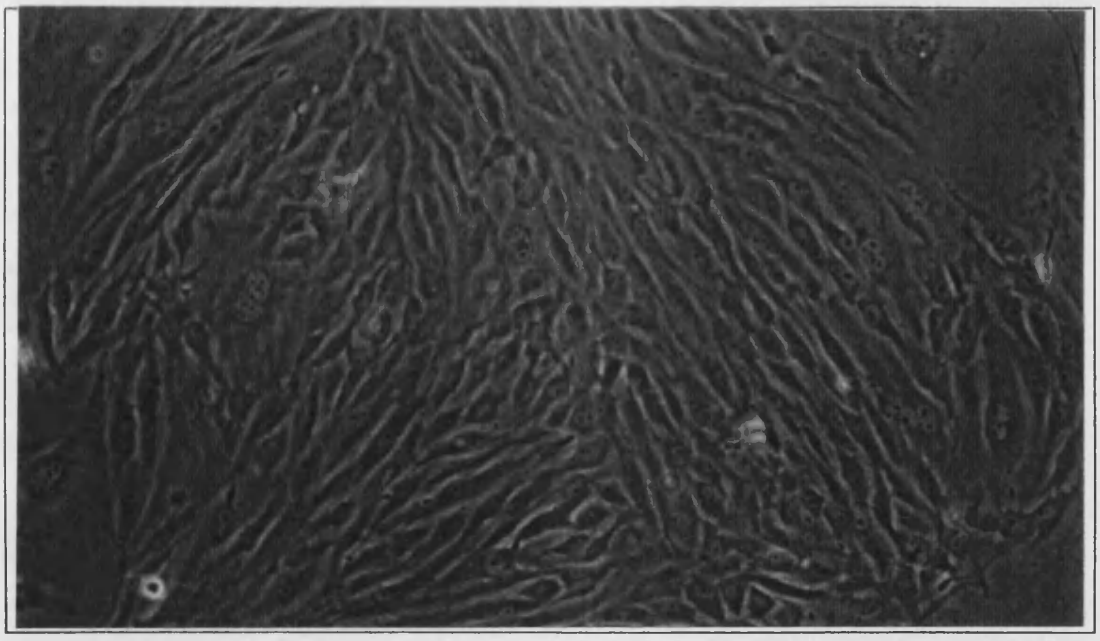

Figure 15. A phase contrast photomicrograph showing a colony of microvascular endothelial cells, passage 1, 7 days after plating. Magnification X200

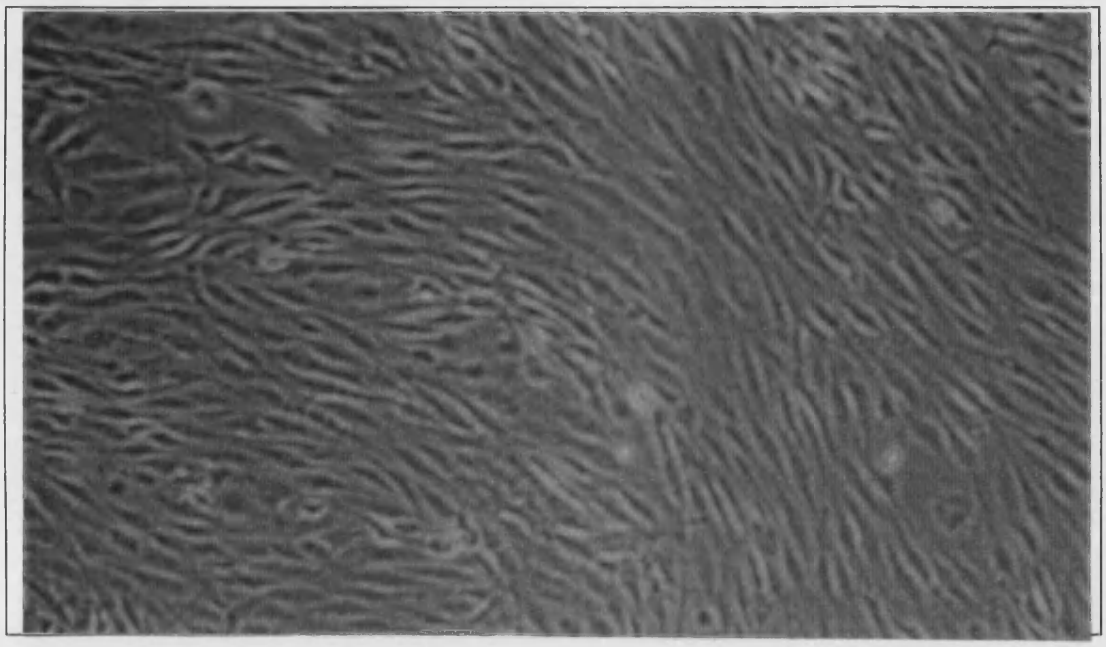

Figure 16. A phase contrast photomicrograph showing a confluent monolayer of microvascular endothelial cells, passage 1, after 20 days in culture. Magnification X200

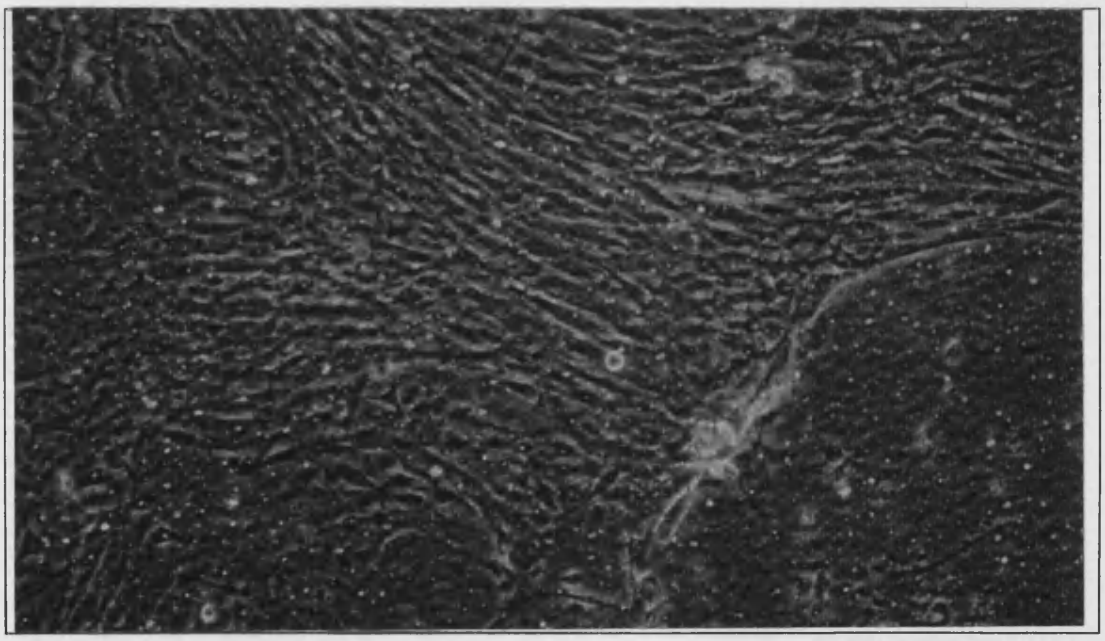

Figure 17. A phase contrast photomicrograph showing a colony of microvascular endothelial cells grown on filter membranes from primary culture. Magnification X200

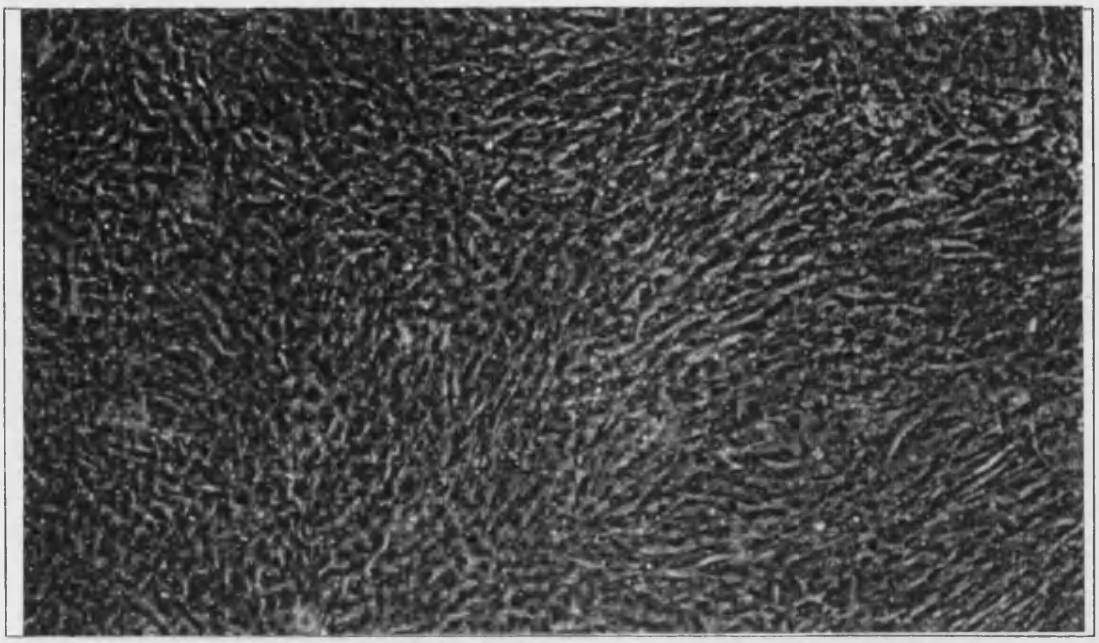

Figure 18. A phase contrast photomicrograph showing a confluent monolayer of microvascular endothelial cells grown initially on collagen coated culture wells, subcultured and seeded onto filter membranes. Magnification X200

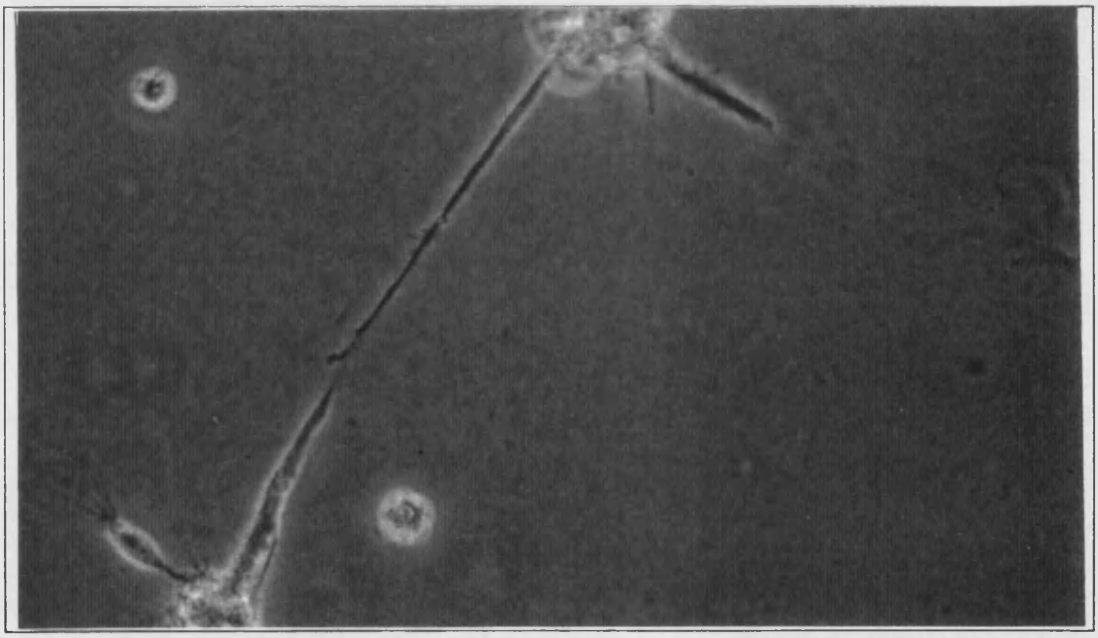

Figure 19. A phase contrast photomicrograph showing microvascular endothelial cells grown on Matrigel coated culture wells. Magnification X400

3.4. Discussion

Isolation techniques

When developing this method for the isolation of lung microvascular endothelial cells the primary objective was to overcome the lack of specificity of some other reported techniques. Primary cultures of lung microvascular endothelial cells are highly likely to be contaminated with large vessel endothelial cells and non-endothelial cells such as pericytes, smooth muscle cells and mesothelial cells. This contamination, however, can be minimised initially at the level of cell isolation. The structure of the microvessel is such that pericytes and smooth muscle cells lie very close to the endothelium having discrete points of contact (Shepro and Morel 1993); thus detachment of endothelial cells from the surrounding tissue completely avoiding contaminants is a very difficult task.

The type of enzyme, concentration of enzyme solution, the duration and temperature of incubation can vary depending on the organ and species from which the cells are isolated. Many of the methods of isolation of lung microvascular endothelial cells described in the literature have modified these factors in order to optimise the isolation procedure (Chung-Welch *et al.*, 1988; Chopra *et al.*, 1990; Del Vecchio *et al.*, 1992; Hewett and Murray 1993). Modifications the original isolation techniques including those described by Folkman *et al.*, (1979) and Zetter (1984), have produced a large variety of isolation procedures. In this study, each time the isolation techniques is adapted, it is an attempt to improve the isolation method to maximise the number of endothelial cells without increasing the non-endothelial cell contaminants.

Although the preliminary isolation technique described previously (section 3.2.1) was unspecific, it provided an indication of the requirements needed when establishing the final method designed to isolate lung microvascular endothelial cells. With lung tissue,

the micovessels are surrounded with large quantities of elastic connective tissue which impedes their release. Therefore, modifications of the preliminary isolation technique involved mechanical disruption of the tissue by homogenisation and dispersal using a Pasteur pipette. In addition, optimisation of the collagenase digestion technique produced a greater yield of microvessel endothelial cells, isolated as small clusters of cells. Isolation of the microvessel fragment by enzyme digestion is a widely used technique involving the use of collagenase (Chung-Welch *et al.*, 1988; Del Vecchio *et al.*, 1992), collagenase/dispase (Belloni *et al.*, 1992) and other enzymes. In this study, after extensive investigations, the optimum conditions for collagenase digestion of the tissue were established where the use of collagenase/dispase was preferred. Collagenase with the neutral protease dispase offered a more gentle enzymic digestion leaving the blood vessels intact (Zetter 1984). The use of a second enzyme digestion step was employed in order to remove pericytes from the basement membrane.

Many of the modifications of the basic isolation technique reported here proved effective in purifying the cultures by reducing the large vessel endothelial cell and non-endothelial cell contamination. Pre-treatment of the lungs *in situ* removed blood from the tissue and initiate the release of the microvessels. Fixation of the pleural membrane devitalised mesothelial cells (Chopra *et al.*, 1990), and density gradient and Percoll gradient centrifugation was effective in reducing contamination with fibroblast, pericyte, smooth muscle cell and macrophage impurities. The cultures were monitored daily by phase contrast microscopy to assess the extent of growth and contamination by non-endothelial cells.

Culture purity

By paying particular attention to the isolation technique, the extent of contamination by large vessel and non-endothelial cells was reduced. In culture, some of these contaminating cells are easily distinguishable, however they have a tendency to outgrow

the endothelial cells, if they are present in sufficient quantities. In these studies, excessive contamination with smooth muscle cells for example showed a typical "hill and valley" effect achieved by compacting layers of cells on top of each other (Patton *et al.*, 1990). In addition, pericytes grew as large cells which spread out in an irregular form, having the ability to retract upon themselves forming nodules; while fibroblasts were seen as long spindle shaped cells which have extended filopodia (Shepro and Morel 1993). Mesothelial cells are indistinguishable from microvascular endothelial cells by phase contrast microscopy.

In this study, the majority of contaminating cells not removed in the isolation procedure could be removed by washing the cultures 2-3 hours after plating, treatment with Ca/Mg-free HBSS 2-3 days after plating and selective subculture with cloning penicylinders. In addition selective growth medium was used to provide suitable conditions for endothelial cell growth without stimulating excessive proliferation of the non-endothelial cell contaminants.

Culture conditions

Not only is there a wide variation between isolation techniques reported in the literature, but there is also great diversity in the types of culture medium used, growth factors, growth serum and extracellular matrices. Investigators have cultured lung microvascular endothelial cells in a variety of media including a 1:1 mixture of RPMI 1640 with endothelial cell conditioned medium (Del Vecchio *et al.*, 1992), DMEM (Chung-Welch *et al.*, 1988; Minuteeau-Hanschke *et al.*, 1990), M199 (Hewett and Murray 1993) and DME-F12 (Belloni *et al.*, 1992). Cultures have been supplemented with various concentrations of plasma-derived serum (PDS) prepared from platelet poor plasma, foetal calf serum (FCS) and growth factors.

The growth factor which has been used most frequently to supplement lung microvascular endothelial cell cultures is endothelial cell growth supplement (ECGS) with the addition of heparin (Chung-Welch *et al.*, 1988; Minuteeau-Hanschke *et al.*, 1990; Belloni *et al.*, 1992; Hewett and Murray 1993). Alternatively, retinal derived growth factor with the addition of heparin (Del Vecchio *et al.*, 1992) is also used as a growth supplement in the culture of lung microvessel endothelial cells.

In this study, the lack of significant improvement in microvascular endothelial cell growth in the initial studies investigating alternative culture conditions was possibly due a number of factors; the inability to provide adequate growth factors and constituents for selective murine lung microvascular endothelial cell growth and also the inadequate endothelial cell number obtained from the preliminary isolations. Having investigated several combinations of culture media, growth factors and sera, the most suitable conditions for culture of endothelial cells without greatly increased proliferation of non-endothelial cell contaminants proved to be Hams F-10 supplemented with both ECGS and heparin and a combination of platelet poor human serum (PPHS) and FCS. FCS contains a large number of endothelial cells growth promoting substances including platelet-derived endothelial cell growth factor (PD-ECGF) as well as inhibitory factors such as platelet derived growth factor (PDGF). High concentrations of FCS therefore, provided accelerated growth rates of both endothelial cells and non-endothelial cell contaminants. However a reduction in the concentration of FCS with the addition of PPHS provided a reasonable compromise between the cell growth rate and the purity of the culture. The addition of ECGS and heparin has been shown by Thornton *et al.*, (1983) and Abbott *et al.*, (1992) to synergistically increase endothelial cell growth. In addition, the cultures reported here were supplemented with selenium dioxide, glutathione, vitamin C and transferrin. The presence of these additives has been shown to be beneficial for cell growth (Schor *et al.*, 1983; Haisch *et al.*, 1990; Abbott *et al.*, 1992).

Growth characteristics

In this study, phase contrast light microscopy was used routinely as a method of assessing growth characteristics under the various conditions. This assessment included the effect on cell morphology, homogeneity and the growth rate of the cultures. The cells were seeded on to calf skin collagen (type I) coated culture wells which provided the preferred conditions for attachment and culture. It is possible that the preference of the cells for collagen as a substratum reflects the high content of collagens present in the connective tissues *in vivo*. In addition, specific binding sites for collagen may exist similar to those which have been shown to exist for fibronectin (Kowalczyk and McKeown-Longo 1992) and laminin (Aumailley *et al.*, 1991). Treatment of culture wells with gelatin, fibronectin or other sources of collagen did not offer any significant advantage over calf skin collagen.

Cells isolated by the final method described in this study grew successfully showing the typical cobblestone morphology expected with a contact-inhibited culture (see section 5.3.1). The cells isolated as small clusters of 5-10 cells grew and divided into distinct colonies. These colonies were selectively subcultured in order to avoid any non-endothelial cell contaminants and were seeded on to fresh collagen coated wells where they grew to confluence providing a relatively pure and reliable supply of murine lung microvascular endothelial cells. Passage 1 cells had a slightly more spindle shaped morphology than primary cultured cells which could be due to the effects of subculturing and the seeding density. All cultures were positive for all the characterisation studies (see section 5).

4. PRIMARY CULTURE OF MURINE BRAIN MICROVASCULAR ENDOTHELIAL CELLS

4.1. Introduction

Studies involving cultures of brain microvascular endothelial cells have allowed advances in the structural and functional analysis of the blood-brain barrier, particularly the study of transport pathways (Audus and Borchardt 1986, Shah *et al.*, 1989; Smith and Borchardt 1989; Abbott *et al.*, 1992). Several methods of isolating brain microvascular endothelial cells have been described (Hughes and Lantos 1986; Bowman, *et al.*, 1988; Tontsch and Bauer, 1989; Abbott, *et al.*, 1992; Belloni, *et al.*, 1992). In these and other studies there are wide variations in the starting material and isolation techniques, as well as considerable variability between the culture conditions. In this study microvascular endothelial cells were isolated and cultured using a modification of the method of Abbott *et al.*, (1992), in order to produce a reliable and relatively pure supply of murine brain microvascular endothelial cells.

4.2. Materials and Methods

4.2.1. Isolation and culture of murine brain microvascular endothelial cells.

Murine brain microvascular endothelial cells were isolated using a modification of the methods of Hughes and Lantos (1986) and Abbott *et al* (1992). Each preparation involved ten C57 mice, either male or female, weighing between 30 and 40g. The animals were killed by cervical dislocation and decapitated. The brains were very carefully removed whole and put into a sterile 30ml universal vial, on ice, containing HEPES buffer.

The brains were dissected under sterile conditions using dissection instruments which had been sterilised and allowed to stand in a beaker of 70% alcohol during this procedure in order to maintain sterility. Each brain was dissected as follows: The cerebellum was removed using a scalpel and the brain was cut in half longitudinally. An attempt was made to remove the meninges using fine forceps however, as the brains were so small this was a very difficult procedure. The mid and hind brain was removed with curved forceps and the brain was then opened up and the white matter and striatum removed. The remaining grey matter was chopped into 1-2 mm pieces and transferred in to a sterile buffer solution on ice.

This procedure was repeated for each brain and the dissected material was centrifuged using a Jouan bench centrifuge at 1500 rpm (600g) for 5 minutes. The resulting pellet was resuspended in 10ml of a prepared enzyme digestion mixture containing 0.1% collagenase dispase, 0.147µg/ml TLCK and 20U/ml DNase1, which was then incubated, gently agitating, for 1 hour at 37°C.

After incubation the suspension was sheared using a long glass Pasteur pipette until no resistance was felt, in order to disperse the grey matter. This procedure was repeated using a long glass Pasteur pipette which had been fire-polished in order to narrow the tip of the pipette and therefore further disperse the grey matter. The suspension was then centrifuged at 1500 rpm for 5 minutes, and the supernatant was carefully removed with a pipette. The pellet was resuspended in 15ml of 25% BSA (prepared in buffer) and spun at 3000 rpm (1200g) for 15 minutes.

The upper layer of the gradient containing myelin, astrocytes and neurons was carefully extracted ensuring the complete removal of all the myelin. The pellet containing the capillary fragments was resuspended in buffer and the upper layer containing myelin, astrocytes, neurons and the remaining capillary fragments was re-centrifuged to ensure the maximum removal of capillary fragments. The capillary fragments were pooled into a clean universal vial, to ensure that there was no myelin contamination and washed in buffer by centrifugation at 600g for 5 minutes. The pellet was resuspended in the remaining 5ml of enzyme digest and incubated with gentle agitation for a further 3 hours at 37°C.

During the incubation periods several preparations were made. The Percoll gradient was prepared by pre-centrifuging a 50% isotonic Percoll solution at 16500 rpm for 1 hour at 4°C (see section 2.2.4). The culture plates were collagen coated and the growth medium was freshly prepared (see section 2.2).

After incubation, the enzyme digest was dispersed using a long glass Pasteur pipette and centrifuged at 600g for 15 minutes. The pellet was resuspended in buffer and layered on top of the Percoll gradient. After centrifuging at 1200g for 20 minutes the capillary fragments formed a distinct band about 4/5 ths of the way down the gradient. The fragments were carefully removed with a Pasteur pipette and transferred into a clean

universal containing buffer. The fragments were washed primarily in buffer and subsequently in a 1:1 mixture of buffer and Hams F-10.

The pellet containing capillary fragments was resuspended in complete culture medium comprising Hams F10 medium containing 10% foetal calf serum, 5% platelet poor human serum, 2mM L-glutamine, 100 IU/ml penicillin and 100μg /ml streptomycin, 80 μg /ml heparin, 75 μg /ml endothelial cell growth supplement, and 4% supplements. The fragments were plated into 6 wells of a 35 mm tissue culture dish pre-coated with collagen and incubated in a humidified CO_2 incubator at 37°C. Fragments were also plated into 96 and 24-well plates according to experimental requirements. The medium was changed every other day and after 4 days in culture the PPHS was reduced in concentration from 5% to 2%.

Subculture of cells

The cells were subcultured on reaching confluence after approximately 10-12 days in culture. For subculture the cells were washed gently with Ca/Mg-free HBSS three times and then incubated for 20 minutes in Ca/Mg-free HBSS containing 0.02% EDTA. The cells were then treated with 0.05% trypsin/ 0.02% EDTA in Ca/Mg-free HBSS for 5 minutes at 37°C. The action of the trypsin was inhibited by the addition of complete culture medium and the cell suspension was then plated into freshly coated 6 well dishes at a ratio of 1:2 - 1:4 depending on experimental requirements.

4.2.2. Cell purity

The extent of contamination of the cultures by non-endothelial cells was examined routinely by phase contrast light microscopy and photographed using a Nikon 35mm camera. Contaminating cells included fibroblasts, astrocytes and pericytes. Two methods for purifying cultures were employed:

1. The cultures were washed 2-3 hours after plating as capillary fragments adhere more rapidly to the surface of the well than single cells (Zetter 1984), therefore this provided an initial method of purification.

2. The cells were washed and incubated with Ca/Mg-Free HBSS at 37°C for 15-20 minutes 3 days after plating.

4.2.3. Growth rates

The extent of growth of the cultures was routinely studied by observing the variation in the density of the cultures during growth. The cultures were photographed regularly under various conditions.

Extracellular matrices

Growth of the cultures on different extracellular matrices was observed:

1. The growth of cultures on collagen type 1 from calf skin (Sigma), and collagen type 1 from rat tail (Boehringer Mannheim) was compared. The collagen was fixed to the wells by wet exposure to ammonia vapour at a concentration of 0.33mg/ml (see section 2.2.5).

2. The growth of the cells on gelatin coated wells was assessed. Gelatin (tissue culture grade) was obtained from Sigma was prepared as a 1% solution and sterilised by autoclaving at 121°C for 15 minutes. The gelatin solution was incubated on the plates for 1 hour at room temperature. The excess solution was aspirated the plates were washed with Ca/Mg-Free HBSS and the cells were added.

3. The effect of fibronectin coated wells on cell culture was also examined. Fibronectin (Sigma) was prepared at a concentration of 25μg/ml and added to the wells. After 1 hour the excess fibronectin was aspirated and the cells were seeded on to the wells.

Effect of sera on growth

The effect of variation of serum concentration on the growth of the cultures was observed. The concentration of foetal calf serum varied from 10-20%, and the concentration of platelet poor human serum varied from 0-5%.

4.3. Results

4.3.1. Brain Microvascular Endothelial Cell Isolation and Culture

Isolation technique

The isolation technique was adapted from a method established for isolation and culture of rat brain microvascular endothelial cells by Abbott *et al* (1992) which was based on the technique of Hughes and Lantos (1986). Ten murine brains were used for each isolation in order to seed one 6-well plate. Each brain was very carefully dissected as the selection of only the grey matter reduced contamination by non-endothelial cells. Careful dissection, in conjunction with BSA density dependent centrifugation and Percoll gradient centrifugation improved the purity of the culture. By following these preparations, variation in purity between different cultures was kept to a minimum.

The enzyme digestion solution used in the isolation procedure produced capillary fragments (Figure 20) from which the cell colonies were formed. The choice of the enzyme required for digestion of the tissue and isolation of the capillary fragment was important, as the use of 0.1% collagenase obtained from Sigma compared to 0.1% collagenase/dispase obtained from Boehringer Mannheim caused excessive disintegration of the capillary fragments producing a single cell suspension. The single cells isolated by digestion with collagenase became non-viable after the initial culture and were removed during the washing procedure, whereas digestion with collagenase/dispase resulted in the isolation of capillary fragments, which went on to form confluent microvascular endothelial cell monolayers.

Excessive agitation of the tissue during incubation or prolonged incubation of the tissue with 0.1% collagenase/dispase also resulted in an increase of single cells in culture. On the other hand, insufficient incubation or agitation of the tissue resulted in inadequate disintegration of the capillary fragments. Fragments which were too large did not adhere to the surface of the wells and were therefore removed when the medium was changed.

Unlike the isolation of lung microvascular endothelial cells this technique did not require a homogenisation step, as the brain tissue was easily broken up by shearing with a long glass Pasteur pipette. The additional shearing of the tissue with a fire-polished pipette was essential in the isolation procedure in order to produce sufficient disintegration of the tissue. Omission of this step resulted in a much reduced yield of capillary fragments.

Culture conditions

The capillary fragments obtained from one isolation were seeded on to one or two 6-well culture dishes freshly coated with collagen type 1 from calf skin resulting in the cells reaching confluence in 10-12 days. The composition of the culture medium was important for cell growth. Cells were cultured with Hams F10 medium in the presence of PPHS, ECGS, heparin and growth supplements which provided the most suitable conditions for endothelial cell proliferation and viability in culture.

4.3.2. Brain Microvascular Endothelial Cell Growth and Morphology

Cell growth and morphology

When first plated out the capillary fragments appeared as small clusters of cells (Figure 20) which after 2-3 days in culture started to grow and divide as small colonies of spindle shaped cells (Figure 21). The majority of single cells isolated became non-viable shortly after the initial culture. The cells remained in contact with the original colony and

divided from the colony perimeter with colonies expanding radially forming typical swirling patterns as they merged to form a confluent monolayer (Figures 22, 23&24).

Subculture

When the cells reached confluence 10-12 days after plating they were either utilised for experimental purposes or were subcultured. The cells which were subcultured on to freshly coated 6-well plates grew as colonies (Figure 25) in a very similar manner to the cells isolated from primary culture until they reached confluence (Figure 26). The time taken to reach confluence was dependent on seeding densities. Subcultured cells retained their spindle-shaped morphology similar to that of primary cultured cells, however the subcultured cells did not form such densely packed monolayers The cells were only subcultured once before they were used for experimental purposes as further subculturing of cells was unsatisfactory due to poor cell attachment and growth. In addition, the cells had a more granular appearance.

Cell purity

Some of the cultures were observed to contain contaminating cells including fibroblasts, smooth muscle cells, and pericytes (Figures 27 & 28) The degree of purity varied slightly between cultures, although the steps taken to improve the purity of the culture both at the time of isolation and culture were reasonably effective. Replacing the medium after 2-3 hours in culture removed any unattached cells and as the capillary fragments adhered more rapidly than single cells (approximately 2 hours) the unattached cells removed tended to be non-endothelial.

Further purification occurred in the early stages of culture: incubation with Ca/Mg-free HBSS for 15-20 minutes after 2-3 days in culture reduced the extent of contamination without affecting the endothelial cells themselves as they showed stronger adherence to the culture well than did the contaminants. Foetal calf serum concentrations of between

10 and 20% and concentrations of between 0-5% platelet poor human serum were studied in order to assess the effect on culture purity. The extent of proliferation of non-endothelial and endothelial cells was observed by light microscopy. The introduction of increased concentrations of FCS were observed to correspond with increases in contaminating cells in culture, especially pericytes (Figure 29).

PPHS, a plasma derived serum was used as a supplement in conjunction with the FCS, as PPHS does not contain the platelet derived factors which are able to promote the proliferation of contaminating cells. Initial concentrations of 5% PPHS and 10% FCS were required for expanding individual endothelial cell colonies, thereafter 2% PPHS and 10% FCS were used for routine culture. These serum concentrations were considered to be the most suitable for endothelial cell growth whilst not causing excessive proliferation of the contaminating cells.

Extracellular matrices

An important factor for cell growth was the treatment of the culture wells with collagen type 1 from calf skin. The attachment factors, gelatin, fibronectin and collagen were assessed by light microscopy on the basis of cell homogeneity and growth rate. Collagen type 1 from calf skin gave the preferred cell attachment, even in preference to type 1 collagen from rat tail. Parallel studies involving calf skin and rat tail collagen showed that rat tail collagen did not offer the same extent of attachment as calf skin collagen and as a result the cells took longer to reach confluence.

Gelatin resulted in initial attachment of cells, however many cells appeared to detach during the washing procedure. Cell attachment and growth on fibronectin was satisfactory but showed no significant improvement over collagen.

Growth on various substrata

Brain microvascular endothelial cells were seeded on to 24 and 6-well plates. Cells were also seeded on to 2 well chamber slides for use with fluorescent studies, Falcon transparent filter membranes and Nunc anopore filters. Cells for binding and photoaffinity labelling studies cells were grown routinely on 6-well plates. Routine culture on 6-well plates appeared to be most suitable for cell growth.

Cells were also grown on collagen coated filters for use in electron microscopy studies. Nunc filters did not prove to be suitable for cell attachment, very few cells attached to coated or uncoated filters and therefore produced unsatisfactory growth. Falcon filters provided a suitable substrate for cell growth. Cells seeded on to these filters directly from primary culture reached confluence in 10-12 days (Figure 30). Examination and identification of the cells under the light microscope was impeded due to the speckled appearance of the cells produced by the filters.

Seeding density

From the initial isolation, the cells were routinely seeded on to one 6-well tissue culture dish, which reached confluence after 10-12 days in culture. Cells seeded on to two or four 6-well plates reached confluence after approximately 18 and 24 days respectively. Cells passaged 1:2 reached confluence after approximately 7 days. Cells seeded on to one 24-well plate reached confluence after 14-16 days in culture.

Growth rates

The effects of different substrata, extracellular matrices, medium and seeding densities on the cell morphology and growth rates were routinely examined by light microscopy The rate of cell growth was constantly estimated by the increased size of the colonies and the time taken for the cultures to reach confluence. An initial lag phase is typical of endothelial cell growth (Rupnick *et al.*, 1988). From the original isolation the cells are

slow to grow and divide, however, as the cell number increases so the growth rate increases. This observation was important when selecting the seeding densities for primary culture and passaging cells, the cells also appear healthier when seeded at a high cell density.

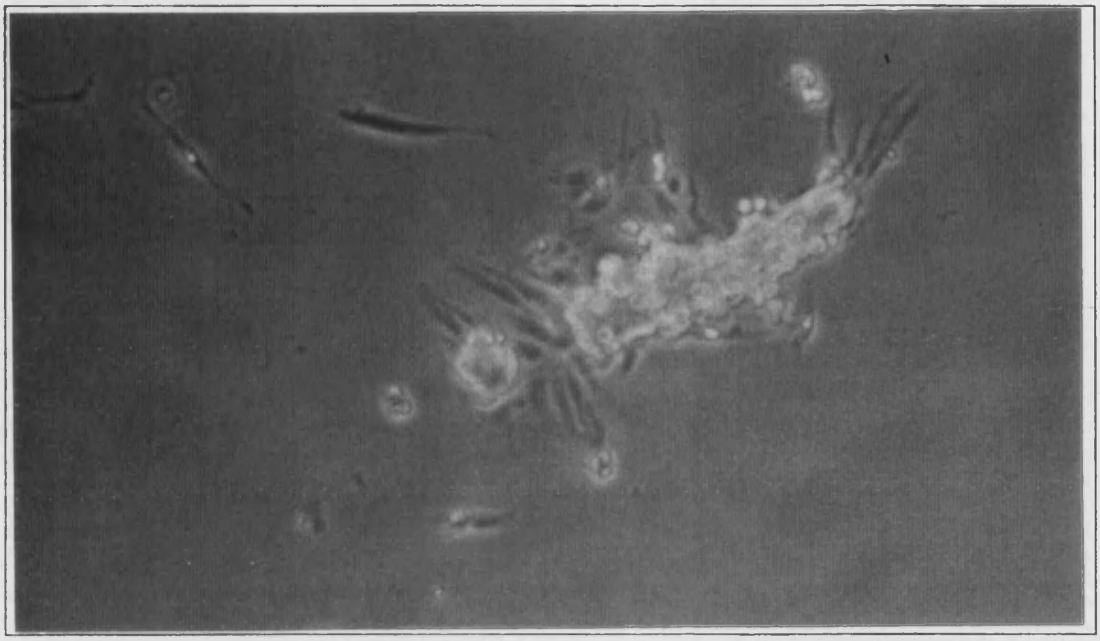

Figure 20. A phase contrast photomicrograph showing a capillary fragment isolated from murine brain, with some cell outgrowth 12 hours after plating. Magnification X400

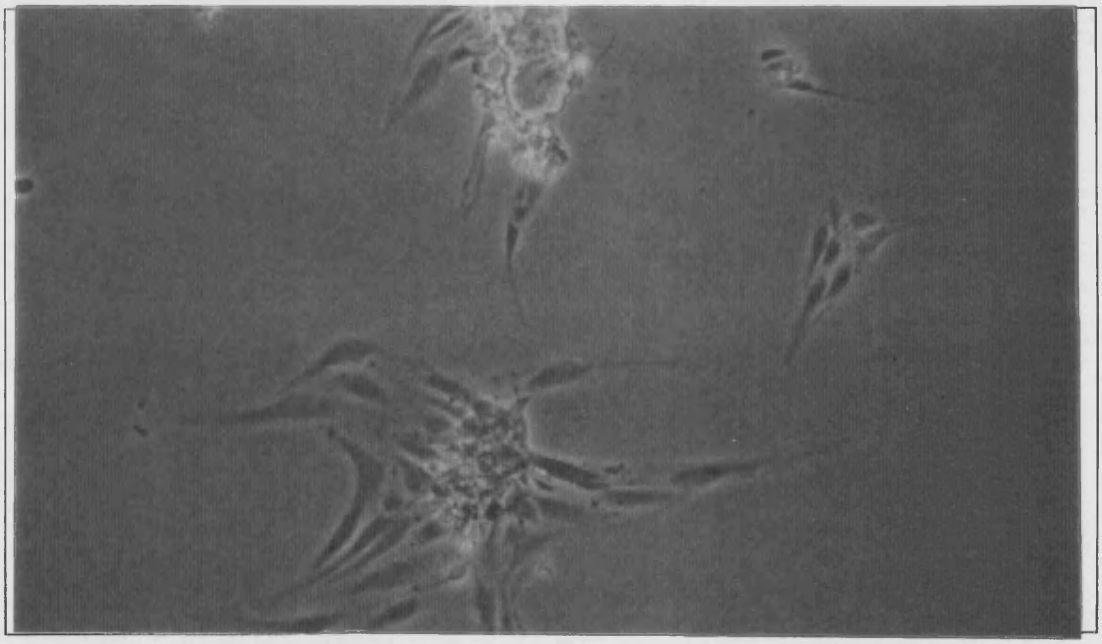

Figure 21. A phase contrast photomicrograph showing small colonies of spindle shaped cells 2 days after plating, where the capillary fragment is still prominent. Magnification X400.

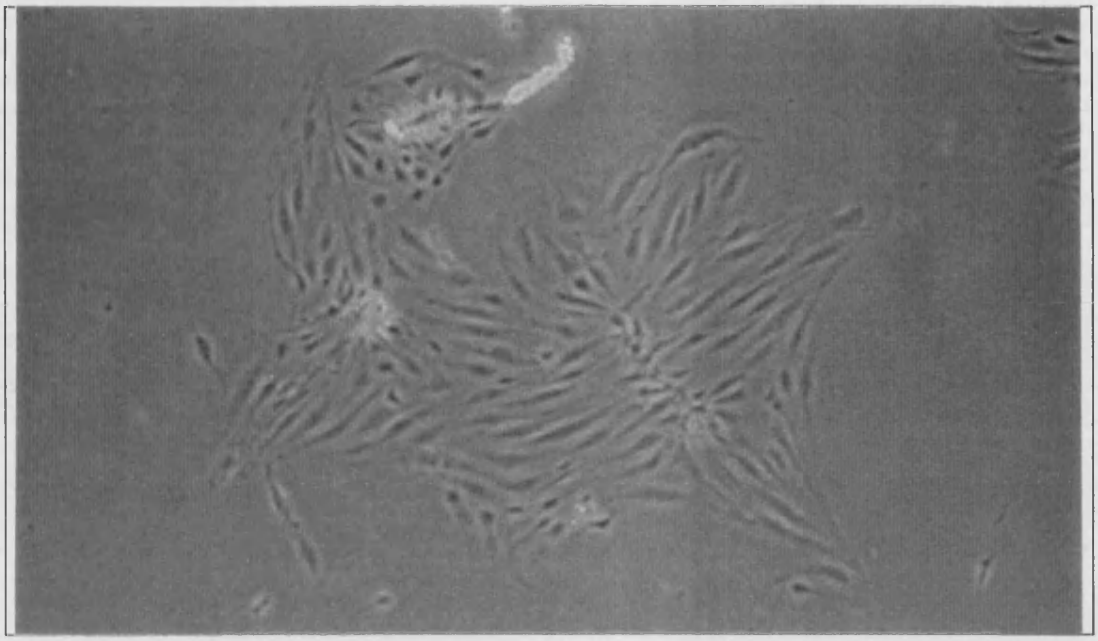

Figure 22. A phase contrast photomicrograph showing typical colonies of microvascular endothelial cells continuing to grow from the vessel fragments 3 days after plating. Magnification X200.

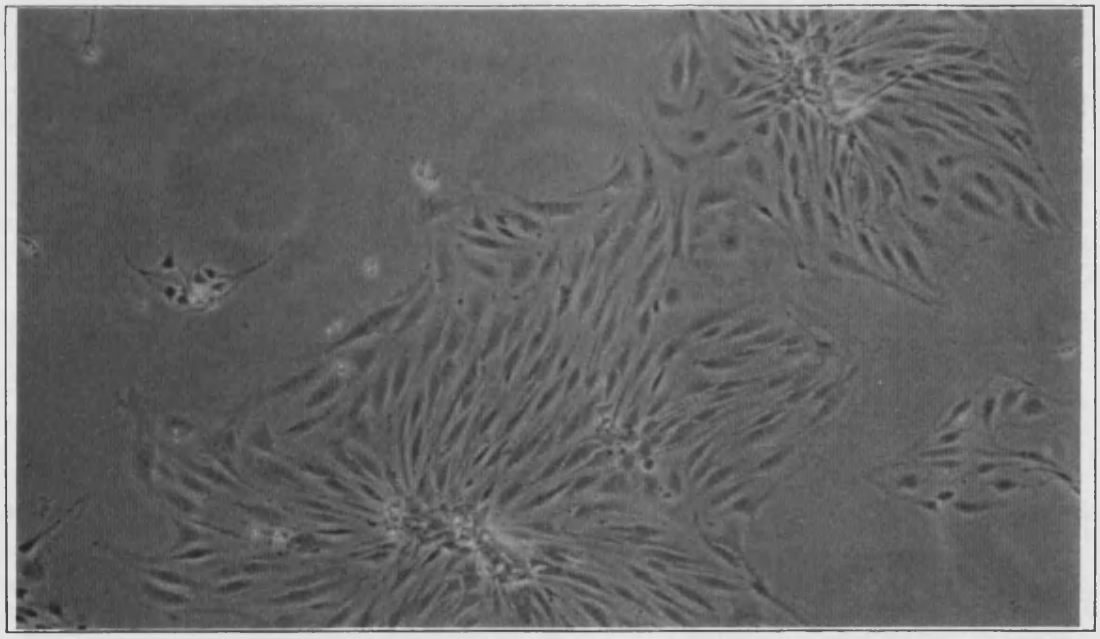

Figure 23. A phase contrast photomicrograph showing two distinct colonies of brain microvascular endothelial cells merging together after 4 days in culture. Magnification X200.

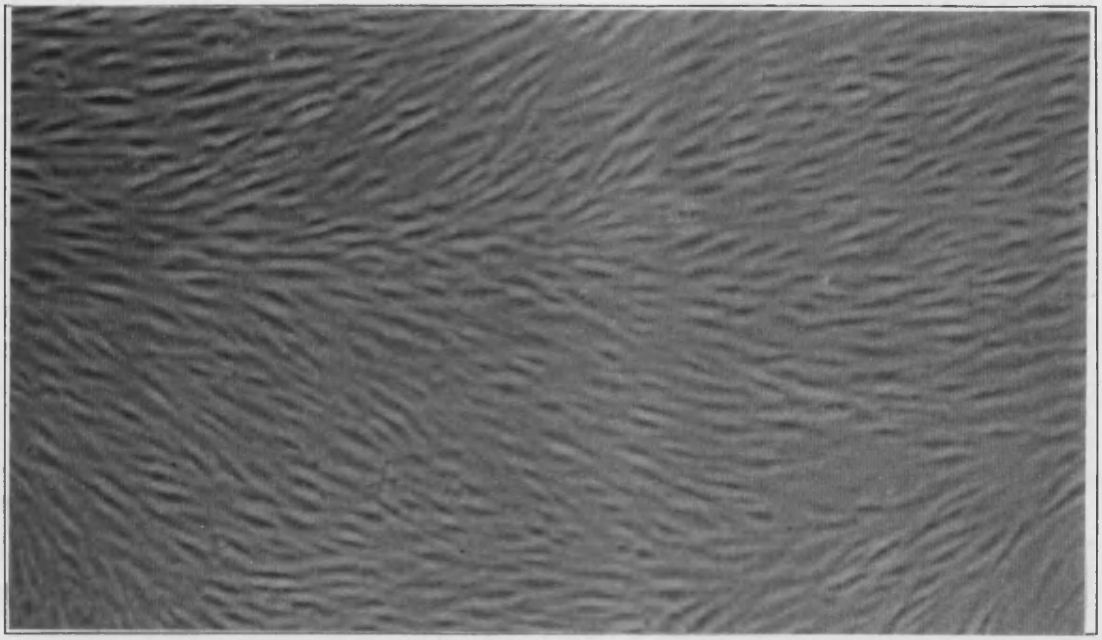

Figure 24. A phase contrast photomicrograph showing a relatively pure confluent monolayer 10 days after plating. Magnification X200.

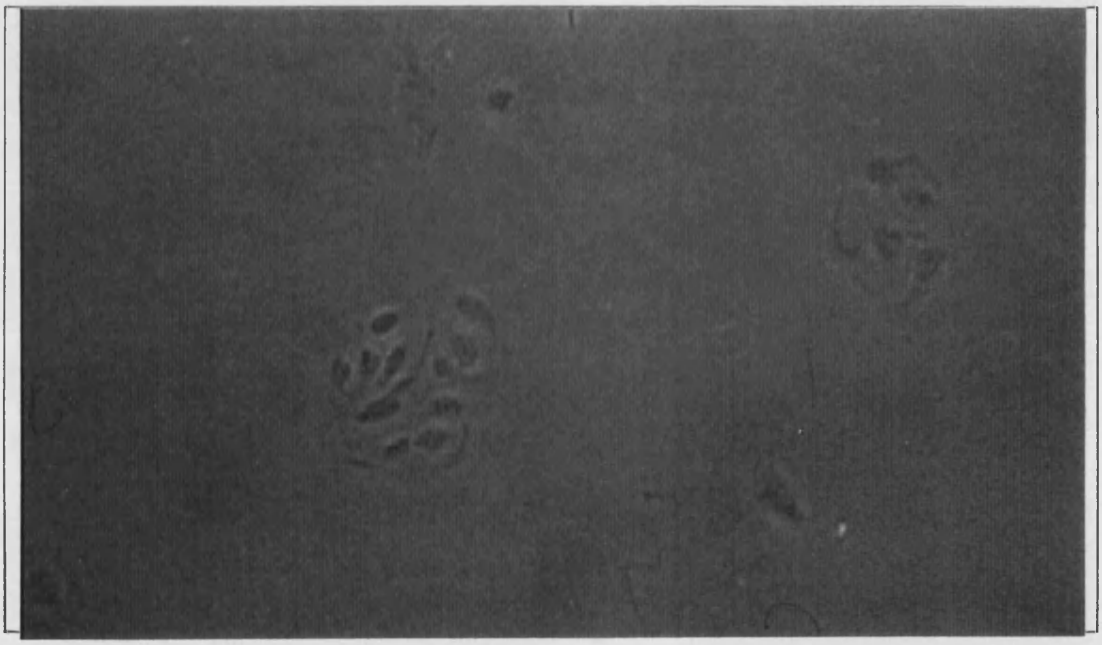

Figure 25. A phase contrast photomicrograph showing small colonies of cells 2 days after subculture (passage 1) onto a fresh collagen coated 6-well plate. Magnification X400.

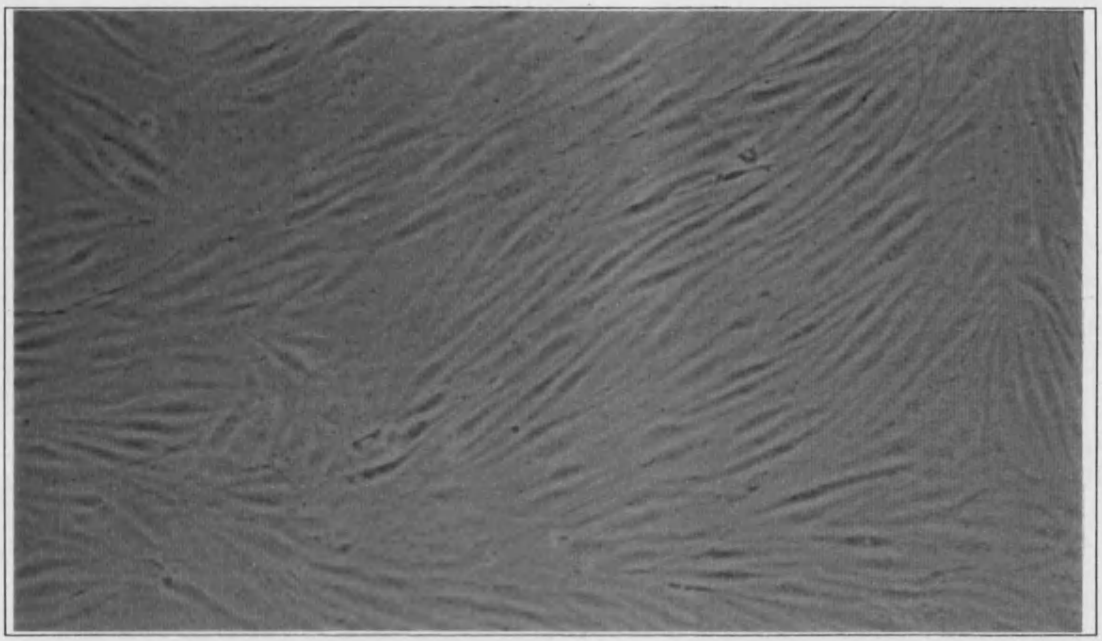

Figure 26. A phase contrast photomicrograph showing a confluent monolayer of subcultured cells (passage 1) where the cells seem to have retained the spindle shaped morphology. Magnification X200.

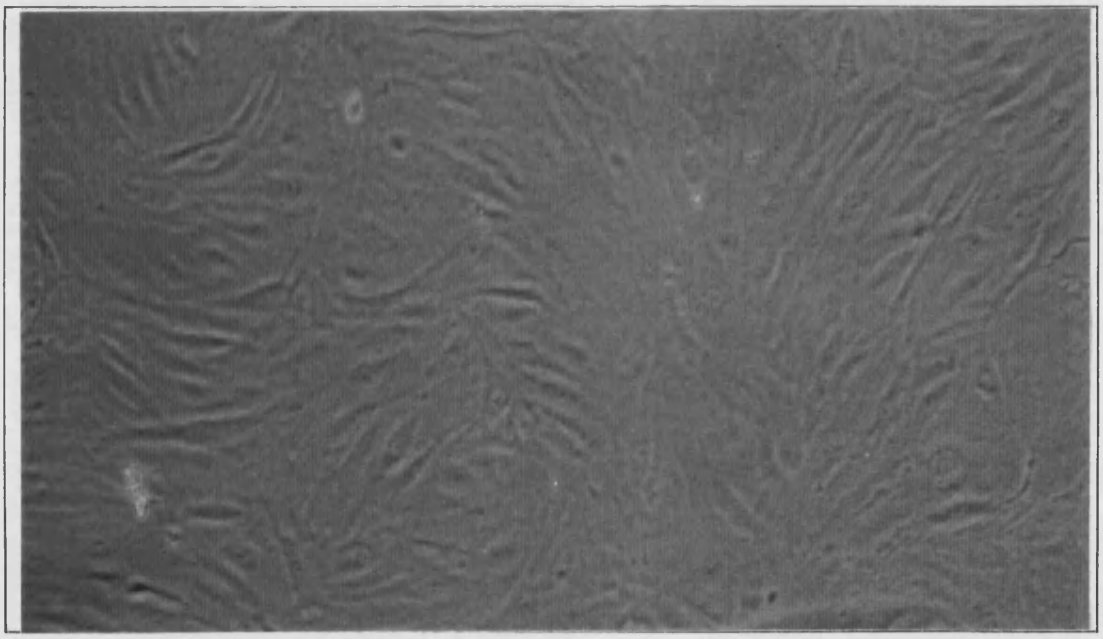

Figure 27. A phase contrast photomicrograph showing contaminating non-endothelial cells probably fibroblasts and pericytes. Magnification X200.

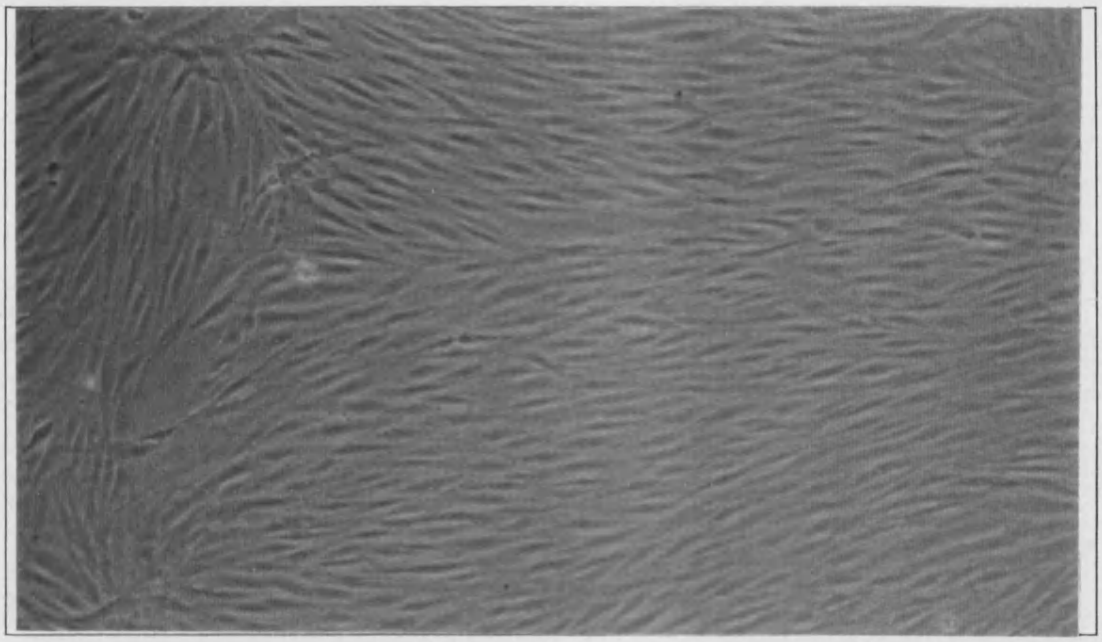

Figure 28. A phase contrast photomicrograph showing non-endothelial cell contaminants within a confluent monolayer, probably pericytes. Magnification X200.

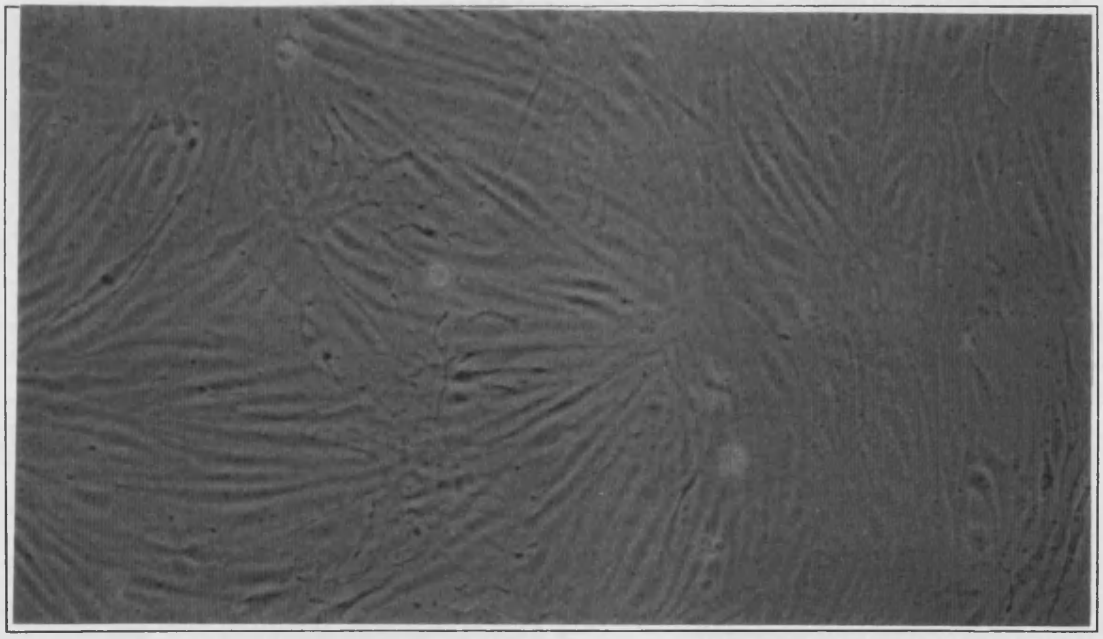

Figure 29. A phase contrast photomicrograph showing non-endothelial cell contaminants probably pericytes or glial cells. Magnification X200.

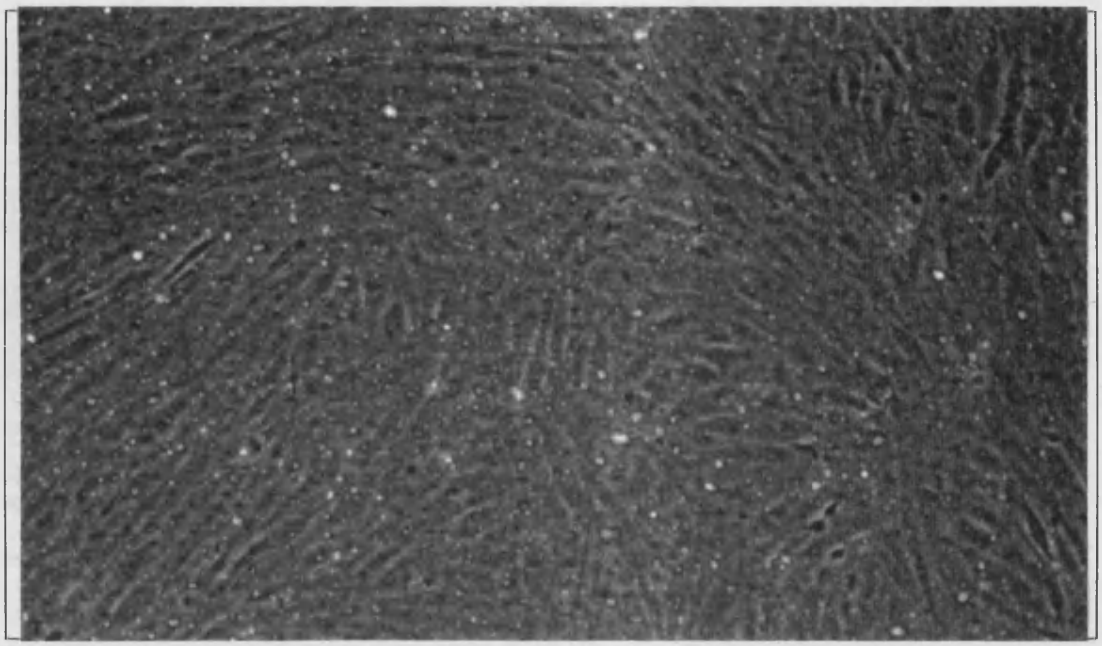

Figure 30. A phase contrast photomicrograph showing a confluent monolayer of brain microvascular endothelial cells seeded onto a collagen coated filter membrane. Magnification X200.

4.4. Discussion

Isolation technique

This isolation technique involved careful dissection selecting only the grey matter thereby restricting the endothelial cells isolated to the microvasculature. In addition, separation of microvessel fragments from single cells and other contaminants occurred by BSA density dependant centrifugation and Percoll gradient centrifugation. These steps were important for purity of the culture as the removal of all unwanted tissue resulted in reduced contamination by smooth muscle cells, astrocytes and pericytes. The methods described in the literature for the enzyme digestion step in the isolation of brain microvascular endothelial cells from the same and different species are very diverse (Bowman *et al.*, 1983; Audus and Borchardt 1986; Rupnick *et al.*, 1988; Tontsch and Bauer 1989; Dorovini-Zis *et al.*, 1991; Abbott *et al.*, 1992). It involves various types of enzymes, concentration, temperature and duration of incubation of the enzyme solution (Bowman *et al.*, 1990). The reason why these diverse techniques are successful is unknown. The techniques employed for the isolation and culture of microvascular endothelial cells from murine brain by the method described here provided a reliable and relatively pure supply of cells.

Culture purity

In this study, the majority of contaminating non-endothelial cells were removed at the time of isolation. Unlike some techniques (Folkman *et al.*, 1979; Carson and Haudenschild 1986; Beer Stoltz and Jacobson 1991), this method did not require weeding, selective cloning or FACS sorting to improve the purity of the cultures. Complete removal of the meninges, the white matter and any noticeable large vessels was an important factor in the purity of the final culture. However due to the size of the

brains removal of the meninges and white matter was very intricate and therefore slight variation in the purity of the cultures did occur.

Both the isolation technique and the culture conditions were important in maintaining a pure culture of microvascular endothelial cells. As with the cultures of lung microvascular endothelial cells, treatment of the cultures 2-3 hours after plating removed any unattached single cells improving culture purity. This technique of differential plating has been used in this and other studies to reduce contamination by single cells and non-endothelial cell contaminants in various organ derived microvessel cultures (Gitlin and D'Amore 1983; Rupnick *et al.*, 1988; Chung-Welch *et al.*, 1988). In contrast, Carson and Haudenschild (1986) have suggested that this treatment at such an early stage in the culture does not significantly reduce non-endothelial cell contamination but actually reduces the microvascular endothelial cell yield.

In agreement with Abbott *et al.* (1992), further treatment with Ca/Mg-free HBSS 2-3 days after plating also improved culture purity. Growth of these cells in selective culture medium provided preferential conditions for endothelial cell growth without immoderate proliferation of non-endothelial cell contaminants.

Culture conditions

As with lung microvascular endothelial cells, brain microvascular endothelial cells were routinely cultured in Hams F-10 media supplemented with the growth factor ECGS, insulin, vitamin C, selenium dioxide, transferrin, glutathione and heparin which provided improved cell growth (Thornton *et al.*, 1983; Schor *et al.*, 1983; Haisch *et al.*, 1990; Abbott *et al.*, 1992). The cultures reported here were supplemented with a combination of FCS and platelet poor human serum (PPHS) which provided a reasonable compromise between the cell growth rate and the purity of the culture (see section 3.4). The presence of some contaminating cells may provide growth factors which support

endothelial cell growth during the initial stages of culture and, in addition, may contribute to some of the endothelial cell functions *in vitro* as they do *in vivo* (Rupnick *et al.*, 1988).

Growth characteristics

Cultures monitored daily by phase contrast light microscopy were assessed for variability in growth characteristics including cell morphology, homogeneity and growth rate. The cells were routinely cultured on calf skin collagen coated culture wells which provided the most suitable substrata for cell attachment and growth.

Microvascular endothelial cells isolated in this study were morphologically consistent with those described in other reports (Bowman *et al.*, 1983; Carson and Haudenschild 1986; Dorovini-Zis *et al.*, 1991; Abbott *et al.*, 1992). They showed a typical pattern of growth in culture from isolation as capillary fragments through the formation of large colonies of cells which merged to form monolayers of spindle shaped cells. Occasionally phenotypic variations were seen within these cultures. Rupnick, *et al.* (1988) suggested that phenotypic variations displayed within a culture could be due to differences in the microanatomical source of the cells. Colonies expressing phenotypic variation may be selectively cloned and grown to form confluent monolayers which display cobblestone morphology (Rupnick, *et al.*, 1988; Belloni *et al.*, 1992).

The growth rate was assessed qualitatively by microscopic means. The cell density was an important factor in growth rate. Sparsely seeded cell fragments formed small clusters of cells but grew at a reduced rate in comparison with cells seeded at a higher density. However, in agreement with other studies, after this initial lag in cell growth the growth rate improved until the cultures reached confluence (Carson and Haudenschild 1986; Rupnick *et al.*, 1988; Abbott *et al.*, 1992). The time taken to reach confluence therefore varied depending on the seeding density which may be due to a release of endothelial cell

growth factors from the cells themselves. This may stimulate endothelial cell proliferation similar to the way that endothelial cell conditioned medium is used to promote microvascular endothelial cell growth.

The isolation and culture of murine brain microvascular endothelial cells by the method described here has provided a reliable and relatively pure supply of cells which retain their endothelial characteristics *in vitro* (see section 5). As a result, these cultures have proved to be suitable to permit the study of receptor mediated uptake (see section 6).

5. CHARACTERISATION STUDIES

5.1. Introduction

Characterisation studies provided a method of determining the microvascular endothelial origin of the cells and distinguished them from non-endothelial cell contaminants. These studies were based upon the properties of endothelial cells *in vivo* and *in vitro,* however some organ and species specific differences are thought to exist (Rupnick *et al.*, 1988; Belloni *et al.*, 1992; Nishida *et al.*, 1993). In this study, identification of endothelial cell origin was determined by light and electron microscopy, the uptake of fluorescently labelled acetylated low density lipoprotein (Bo-Ac-LDL), expression of Von Willebrand factor, the ability to provide a non-thrombogenic surface to platelets and the ability to undergo *in vitro* angiogenesis. In addition, the existence of organ specific differences between brain and lung microvascular endothelial cells in culture was also investigated.

5.2. Materials and Methods

5.2.1. Light and Electron Microscopy

Light microscopy

General cell growth and morphology was observed daily, using an inverted biological microscope (WILD, M40). More detailed cell morphology was also examined using a phase contrast microscope (diaphot-TMD, Nikon, U.K. Ltd.), and photographed (F301, Nikon, U.K. Ltd.) routinely in order to estimate cell growth and the extent of contamination by non-endothelial cells.

Preparation of samples for transmission electron microscopy

Transmission electron microscopy (TEM) studies were performed on cells that were grown on filter membranes (Cyclopore, Falcon) to a near-confluent state (see section 2.2.8). The cells were washed three times in 0.1M phosphate buffer adjusted to pH7.4. The samples were carefully removed from the plastic holders using a scalpel blade and were subsequently cut into quarters and prefixed with 2.5% gluteraldehyde (BDH chemicals) in 0.1M phosphate buffer (pH 7.4) at 37°C for 20 minutes.

The samples were washed in 0.1M phosphate buffer for 15 minutes with at least three changes of buffer, and then were post-fixed at room temperature for 30 minutes using 1% osmium tetroxide (OsO_4, BDH chemicals) in phosphate buffer; this procedure also stained the cells brown. The cells were washed again for 15 minutes and the samples were dehydrated using the different concentrations of ethanol and propylene oxide (BDH) shown below:

Dehydrating medium	Time (minutes)
75 %V/v ethanol	15
80 %V/v ethanol	15
95 %V/v ethanol	15
100 %V/v ethanol (dry)	2 x 15
100 % V/v propylene oxide	2 x 15

The samples were gradually exposed to resin by application of the following embedding solutions: 1) a 1:3 mixture of resin (Taab premix medium embedding resin, Taab Laboratories, Reading): propylene oxide for 1 hour; 2) a 1:1 mixture for 2 hours; 3) a 3:1 mixture for 1 hour; and finally 4) 100% resin for 24 hours with at least 3 changes of resin. After this procedure the samples were placed in separate embedding moulds (Taab), topped up with resin and oven dried at 50°C for 48 -72 hours.

A microtome (C. Reichert OM U3) fitted with a diamond knife was used to prepare 90 nm thin sections. The sections were placed on copper grids and were subsequently stained, in the dark, with 2% uranyl acetate for 7 minutes, washed 4 times with distilled water and stained for a further 7 minutes with Reynolds Lead Citrate in a petri dish lined with filter paper soaked in 1M NaOH. The sections were washed 4 times with distilled water, dried and viewed in a Jeol 1200 Ex Transmission Electron Microscope

Preparation of samples for scanning electron microscopy

For Scanning Electron Microscopy (SEM), cells were also grown on Falcon Cyclopore filters to reach confluence. The cell samples were washed for 15 minutes at 37°C with 0.1M sodium cacodylate buffer containing 5% sucrose, removed from the plastic holders and cut into small pieces. The cells were prefixed with 2.5% glutaraldehyde, 5% sucrose

in 0.1M sodium cacodylate buffer pH7.4 for 45 minutes at 37°C and washed for 15 minutes with 3 changes.

The samples were post-fixed using 1% osmium tetroxide in 0.1M sodium cacodylate buffer containing 5%^{W}/v sucrose for 15 minutes at room temperature and washed again for 15 minutes with 3 changes. The cells were dehydrated in graded acetone as shown below:

%v/v Acetone	Time (minutes)
50	10
75	10
95	10
100	2 x 10
100 dry	2 x 10

The cells were critical-point dried and sputter coated with a thin layer of gold (Edwards sputter coater S150B). The small sections of cell coated filter were fixed to 10mm x 10mm stubs (Jeol) with conductive carbon cement (Leit C) and examined using a Jeol T330 Scanning Electron Microscope.

5.2.2. Uptake of Acetylated Low Density Lipoprotein

The ability of endothelial cells to incorporate Ac-LDL was demonstrated with the fluorescent probe Bodipy (Molecular Probes, Inc). Bodipy is a neutral dye which is covalently bound to the apoprotein portion of the LDL complex and is therefore not removed during cell manipulations. Cell cultures grown on plastic 2-well chamber slides (Labtek, Gibco) or glass coverslips, both coated with collagen (see section 2.2.5). The cells were incubated with 10 μg/ml Bo-Ac-LDL in complete Hams F10 medium for 4

hours at 37°C. Following incubation the cells were washed several times with Ca/Mg-free HBSS and mounted with a fluorescence protecting mounting medium (Fluorsave, Calbiochem). Fluorescence was observed using a Nikon Optiphot-2 fluorescence microscope at an excitation wavelength of 450-490 nm.

Positive and negative controls

Positive and negative control studies were performed concomitantly with the samples requiring characterisation. BAE-1 cells and HUVECS were used to provide positive comparisons, whilst 3T3-L1 fibroblasts were used to provide negative comparisons. Mixed populations of endothelial and non-endothelial cells from primary culture were also examined.

5.2.3. Expression of Von Willebrand factor

Preparation of reagents

A buffer was prepared using PBS containing 0.05% BSA at pH7.4 for dilution's and initial washing procedures.

0.05M Tris/saline buffer was prepared from 0.79g Tris and 0.9g NaCl in 100ml distilled water and adjusted to pH5.6 with 10% HCl.

A 2units/ml solution of thrombin (Sigma) was prepared from a 2mg/10ml (10 units/ml) stock solution.

0.01M Imidazole (Sigma) was prepared from 17mg in 2.5ml Tris/saline.

The DAB stain (3-3' Diaminobenzedine tetra hydrochloride, Sigma) was prepared from several reagents. 3.75mg DAB was dissolved in 4.5ml tris/saline, to this was added

0.5ml of 0.01M imidazole, this solution was stored in the dark, on ice, until required Prior to use 167µl of the mixture was removed and 167µl of 30% hydrogen peroxide was added.

The Von Willebrand factor used was a peroxide conjugated Rabbit Anti-Human von Willebrand Factor obtained from Dakopatts, which was diluted with PBS- 0.05% BSA to give a 1:40 dilution.

Cell fixation and staining procedure

The presence of Von Willebrand factor was assessed by using an antibody against human Von Willebrand factor. The cells used for Factor VIII staining were grown on standard 6-well plates until they reached semi-confluence, they were then washed 3-4 times with PBS-0.05%BSA and stimulated with 2units/ml thrombin for 15 minutes at 37°C. The cells were again washed 3-4 times with PBS-0.05% BSA and fixed with 10% formalin (Sigma) for 1 hour at 4°C.

After fixation and further washing with PBS-0.05%BSA, 250µl/well of the specific monoclonal antibody, peroxide conjugated rabbit anti-human von Willebrand factor was added to the wells and incubated for 45 minutes at room temperature. The cells were washed firstly with PBS-0.05%BSA and then twice with tris/saline buffer pH5.6. The samples were incubated with 250 µg/well of the freshly prepared DAB reagent for 3-5 minutes, and washed 3 times with tris/saline buffer. The cells were counter stained with 250µl of hematoxylin (Harris type, Sigma) which was removed immediately by washing with buffer 3-4 times and the cells were kept in buffer to be viewed using the Nikon Diaphot microscope.

Positive and negative controls

Characterisation studies were performed on primary cultured cells which were isolated and cultured to provide a mixed population of endothelial and non-endothelial cells. Controls were incubated with 0.5ml of normal rabbit serum (Sigma), diluted with PBS-0.05% BSA to give a 1:16 solution.

5.2.4. Platelet Adhesion

Preparation of platelets

18ml of rabbit blood was collected in a syringe containing 2ml of tri-sodium citrate (3.2%). Platelet-rich plasma was prepared by centrifugation at 1200 rpm (480g) for 10 minutes. The supernatant was carefully transferred into a clean tube and supplemented with 75µl of prostacyclin (40mg/ml). The platelets were pelleted by centrifugation at 2400 rpm (960g) for 15 minutes. The pellet was washed in 10ml of Hepes buffered tyrode (HBT) solution pH7.4 containing 50µl of prostacyclin. The HBT solution was prepared according to the following recipe:

Hepes	10mM
NaCl	145mM
KCl	5mM
$MgCl_2$	1mM
NaH_2PO_4	0.5mM
Glucose	5.5mM
BSA	0.25%

The pellet was washed again and the cell number determined. The final platelet suspension was adjusted to 5 x 10^8 cells/ml and left in HBT for 1 hour, after this time the platelets were ready for use.

Platelet adhesion assay

The cells were grown, as normal, on collagen coated 6-well plates until they reached semi-confluence. One hour prior to the assay, the cell cultures were washed and incubated at 37°C with Hams F-10 medium (without supplements) containing 25mM HEPES and 5mg/ml BSA in order to reduce serum components bound to the cell surface.

To each culture well 1 x 10^8 platelets were added and incubated at 37°C for 30 minutes. Non-adherent platelets were removed by washing 10 times with Hams F-10-BSA. Platelet adherence was determined by phase contrast microscopy.

Mixed populations of endothelial and non-endothelial cells were studied in order to ascertain whether a difference existed. Adhesion to collagen coated and uncoated plates was also assessed.

5.2.5. Growth of Lung Microvascular Endothelial Cells on Matrigel

Preparation of Matrigel coated plates

Matrigel, a basement membrane matrix, was obtained from Collaborative Biomedical Products (Becton Dickinson). A thin gel method was required for plating cells on top of the gel. The Matrigel was thawed out overnight at 4°C in order to keep the Matrigel from forming an irreversible gelled compound, which occurs at 22-35°C. The gel was mixed to homogeneity with pre-cooled pipettes and aliquoted on to the wells, which

were kept on ice, at a concentration of 50µl/cm^2. The plates were placed at 37°C for 30 minutes before use.

Cell culture on Matrigel coated wells

The cells were cultured, as normal, on collagen coated plates until they formed large colonies. The cells were removed from the wells at this time by the standard trypsinization procedure for lung microvascular endothelial cells and plated on to Matrigel coated wells in complete Hams F-10 medium. The cells were allowed to grow on these plates for up to 3 days.

5.3. Results

5.3.1. Light Microscopy

Phase-contrast light microscopy was used extensively in the study of cell morphology and growth of lung and brain microvascular endothelial cells. Comparative studies of growth and morphology were performed in order to investigate the existence of organ-specific variations between brain and lung microvascular endothelial cells. The identification of non-endothelial cell contaminants and the extent of this contamination throughout the cultures was also routinely investigated.

Cell growth

The cell growth patterns were distinctive for microvascular endothelial cells. The capillary fragments isolated from the grey matter of the brain adhered to the surface of the culture dishes; thereafter the cells sprouted from the vessel fragments forming small colonies of cells. Initially, the cells grew as individual colonies with the capillary fragments remaining visible in the centre of the colony, (Figure 31), growth continued until the colonies merged to form a confluent monolayer.

In comparison, fragments isolated from lung tissue adhered to the surface of the culture dish as small clusters of four or five cells. Vessel fragments similar to those isolated from brain tissue were difficult to obtain, due to the large quantity of connective tissue present in the lung. Due to the presence of contaminating cells, lung microvascular endothelial cell colonies were not allowed to produce a confluent monolayer from primary culture. Instead, colonies were cloned before extensive growth occurred. When

the cells were subcultured, both brain and lung microvascular endothelial cells grew as individual colonies before merging to form confluent monolayers.

Cell morphology

Microvascular endothelial cells derived from murine brain and lung tissue had quite distinctive morphologies. During the initial stages of primary culture, endothelial cells isolated from brain tissue formed spindle-shaped colonies (Figure 31) which grew to confluence forming some swirling patterns. Cells isolated from lung tissue however formed colonies with a more cobblestone morphology (Figure 32).

Confluent cultures of lung microvascular endothelial cells were obtained with passage 1 cells. These cells formed very dense contact-inhibited monolayers, but lost some of the distinctive cobblestone morphology observed with the colonies in primary culture. Microvascular endothelial cells isolated from the brain also formed these very dense contact inhibited monolayers on reaching confluence. The characteristic cell morphology was retained when confluent monolayers were formed with both primary cultured and passage 1 cells.

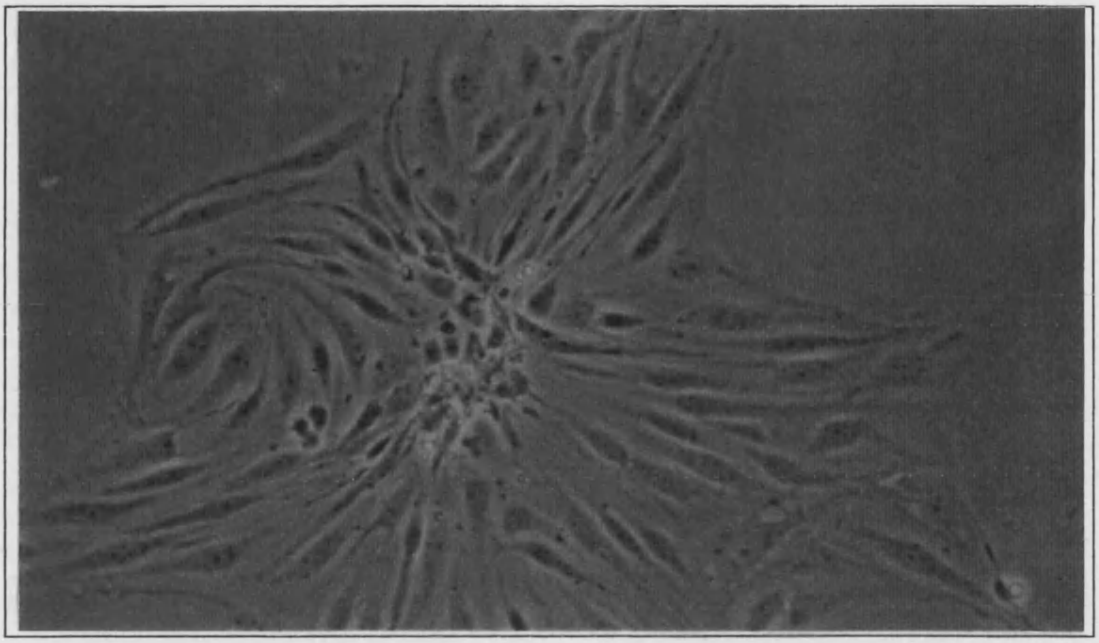

Figure 31. A phase contrast photomicrograph showing a colony of brain microvascular endothelial cells 3 days after plating, where the original capillary fragment remains noticeable. Magnification X400.

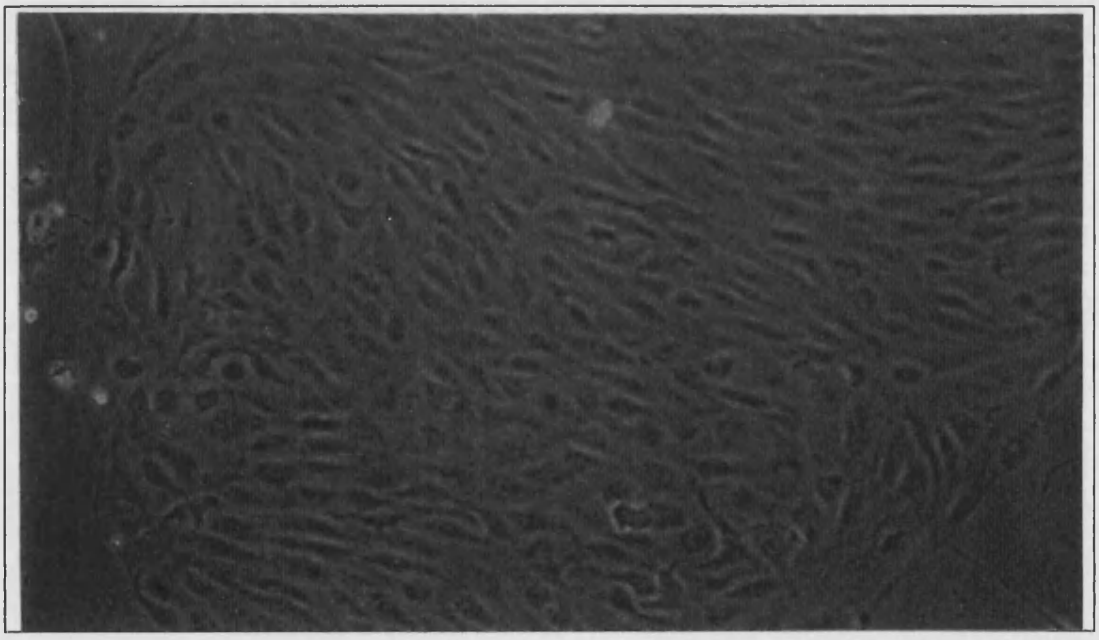

Figure 32. A phase contrast photomicrograph showing a large colony of lung microvascular endothelial cells displaying the typical cobblestone morphology, 10 days after plating. Magnification X200.

5.3.2. Electron Microscopy

Transmission electron microscopy

Electron microscopy was used in conjunction with several other characterisation techniques as a method of identifying endothelial cells, as the ultrastructure of these cells offer several distinguishing features. Transmission micrographs revealed the endothelia to be very thin and flat, with tight junctions at points of cell-cell contact, this is shown clearly in Figures 33 & 34 where the tight junctions and points of contact have a blurred appearance.

Several plasmalemma vesicles were found on the apical membrane (Figure 35), and coated pits (Figure 36) were seen at the apical and basolateral membranes, where some of the coated pits appeared to have a thin membrane covering the opening (Figure 37). The formation of the vesicles can also be seen in Figure 38. Figure 39 is a high magnification electron micrograph clearly showing mitochondrion and rough endoplasmic reticulum in the cytoplasm.

Von Willibrand factor is located in the Weibel-Palade (W-P) bodies. The presence of W-P bodies indicated positive identification of endothelial cells, this is demonstrated in Figure 37, however inability to demonstrate W-P bodies does not rule out endothelial cell origin. The appearance of W-P bodies is variable in endothelial cells while they are still positive for factor VIII related antigen, and in these cells although W-P bodies were observed, although they were not present in abundance.

Scanning electron microscopy

The cells required for scanning electron microscopy were grown to confluence on transparent filter membranes, where the cells were exposed to medium on the apical and basolateral surfaces. The endothelial cell monolayer was examined by both light and electron microscopy. Light micrographs (magnification X200) and transmission electron micrographs indicated that the monolayer consisted of a high density of cells with formed tight cell-cell contacts, this was apparent for both brain and lung endothelial cells (Figure 40 & 42).

Scanning electron microscopy demonstrated that the cells did not form a complete monolayer with every cell-cell contact forming a tight junction. This may be due to the dehydration procedure used in the preparation of the samples, as several breaks in the cells themselves were apparent. However some processes were observed which linked the cells together (Figure 41 & 43).

The transmission electron micrographs did demonstrate that tight junctions existed between the cells. However, although TEM and SEM studies were conducted in parallel, the buffers used and the dehydration procedures were different and this may explain the absence of tight junctions in the SEM preparation but not the TEM preparation. SEM showed the cells to be long and thin, and although tight junctions are not apparent some may exist between the cells which are not readily visible by scanning electron microscopy.

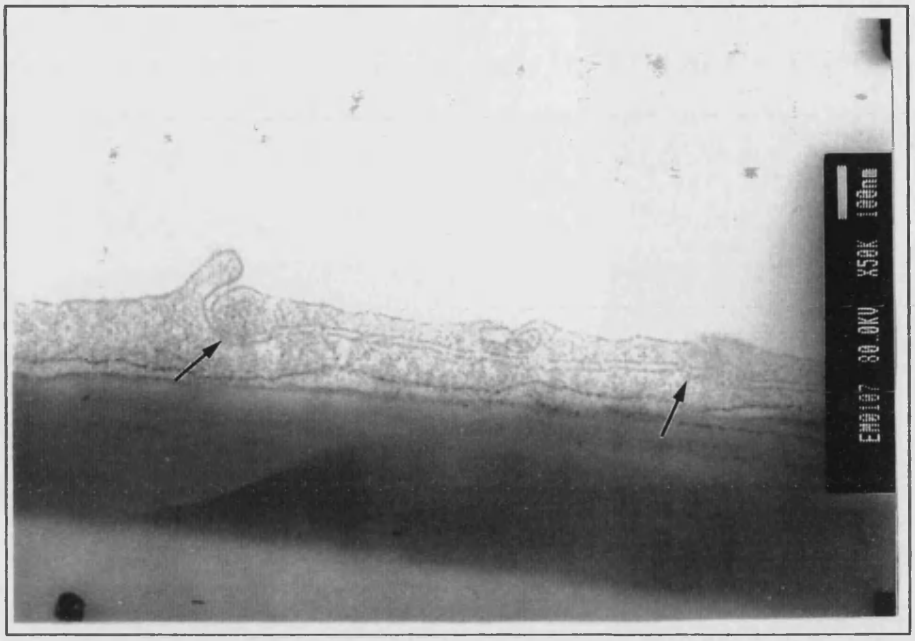

Figure 33. A Transmission electron micrograph showing a brain microvascular endothelial cell junction. The arrows indicate the points of contact between two cells which form the tight junction.

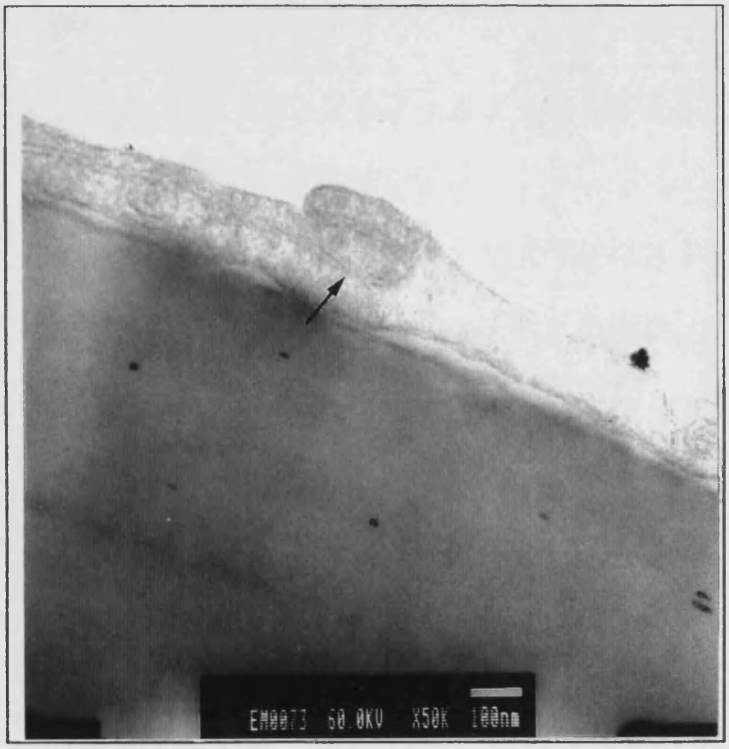

Figure 34. A Transmission electron micrograph showing a lung microvascular endothelial cell junction, where the arrow indicates the junction between two cells.

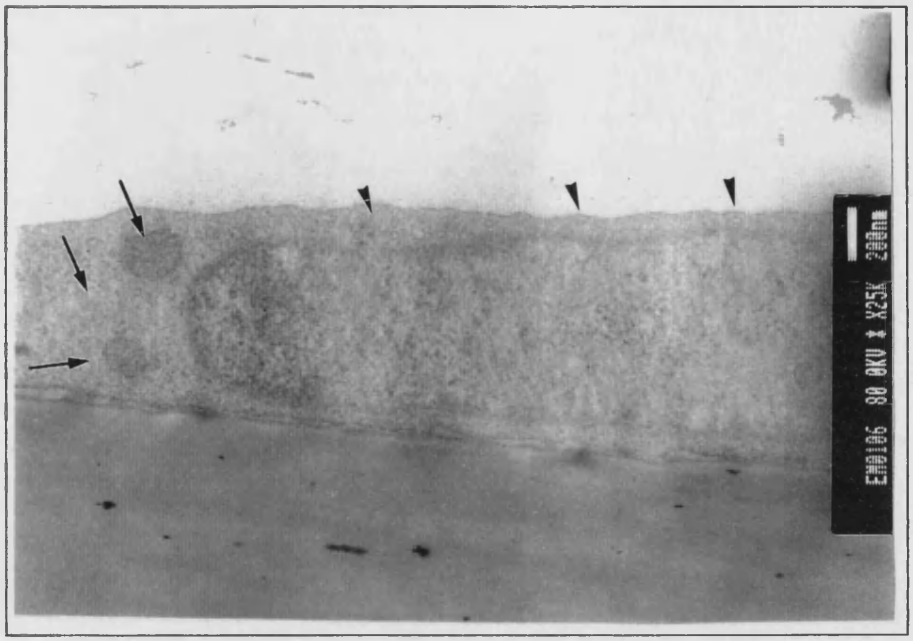

Figure 35. A Transmission electron micrograph showing plasmalemma vesicles on the apical membrane of the lung cell (*arrowheads*). Mitochondrion and rough endoplasmic reticulum can also be seen in the cell cytoplasm (*arrows*).

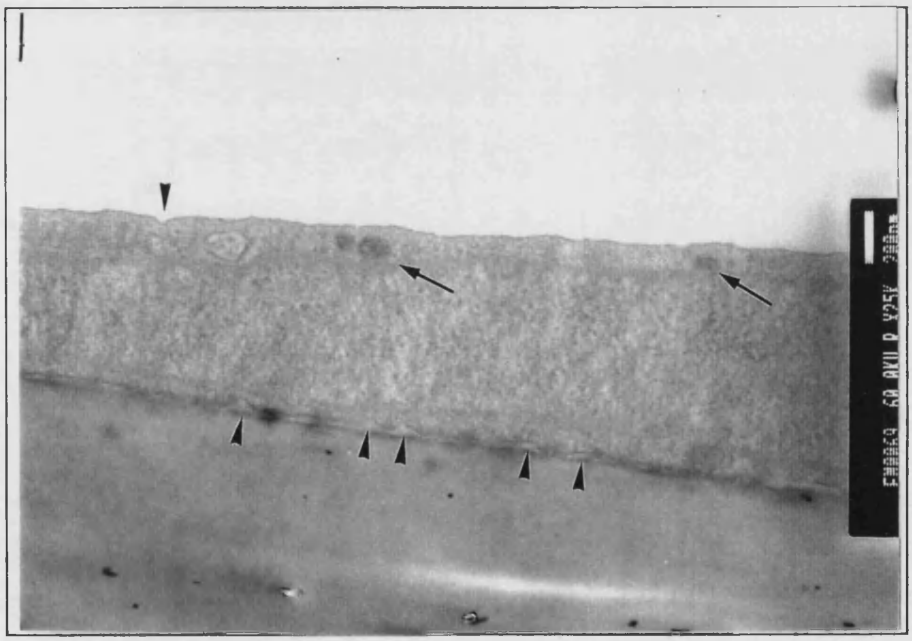

Figure 36. A Transmission electron micrograph showing coated pits at the apical and basolateral membranes of the brain cell (*arrowheads*). The presence of Weibel-Palade bodies is also shown (*arrows*).

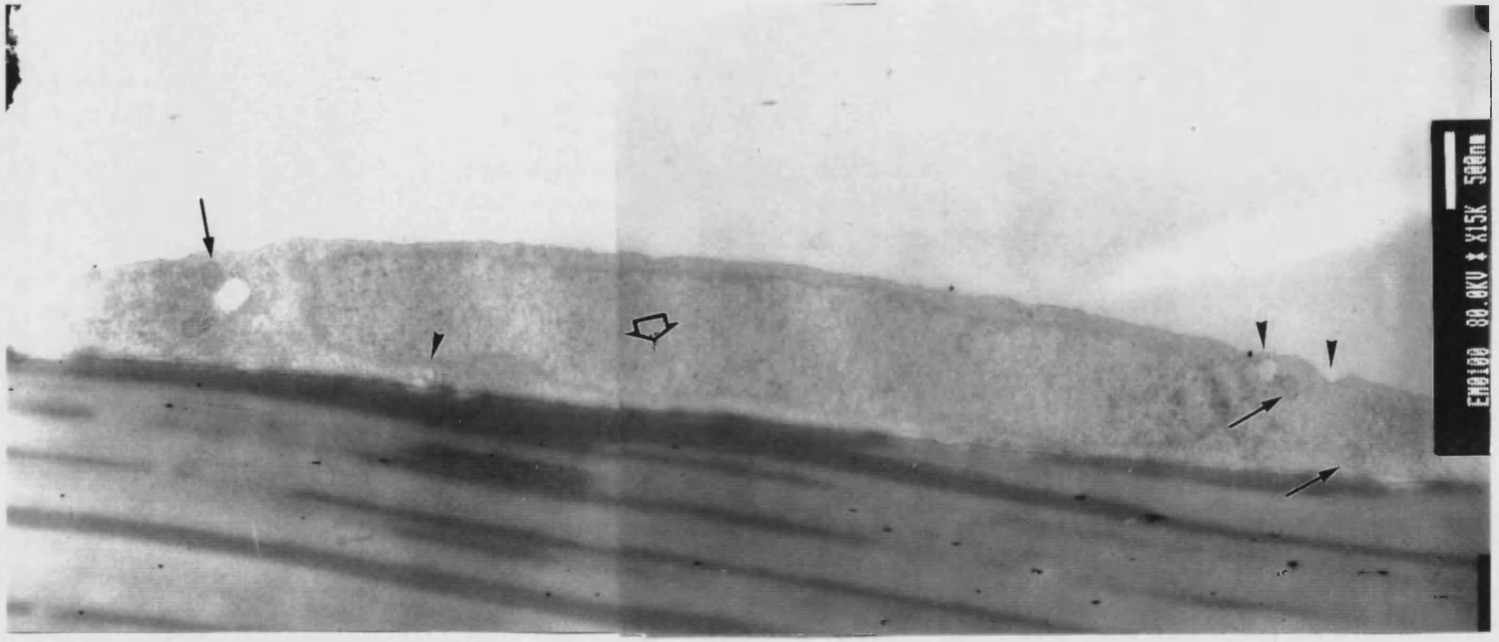

Figure 37. A Transmission electron micrograph showing coated pits some of which appear to have a thin membrane covering the opening (*arrowheads*). The open arrow indicates the cell nucleus. Mitochondrion can also been seen in the cell cytoplasm (*arrows*).

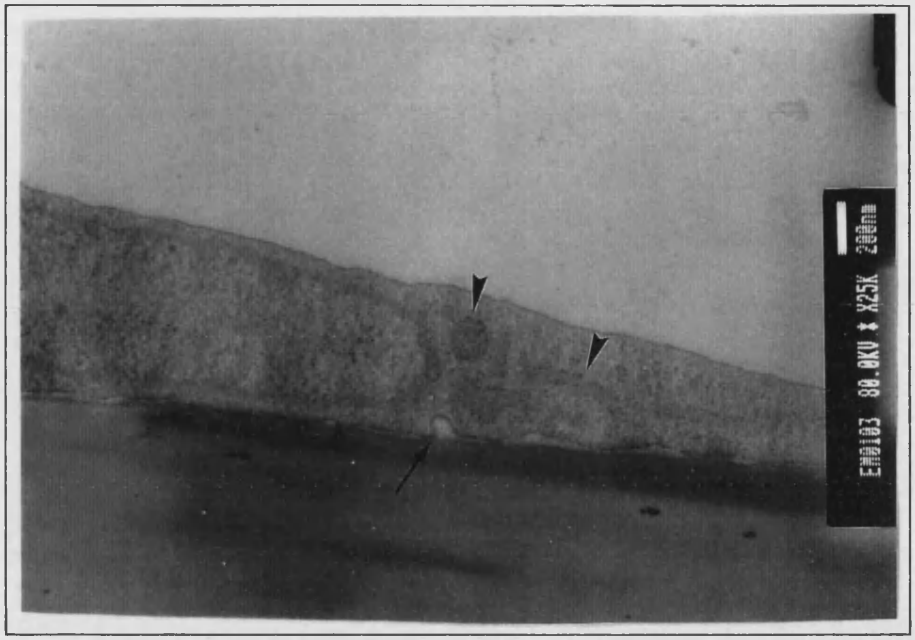

Figure 38. A Transmission electron micrograph showing vesicle formation on the basolateralmembrane of a lung microvascular endothelial cell (*arrow*). The mitochindrion and rough endoplasmic reticulum can also be clearly seen (*arrowheads*).

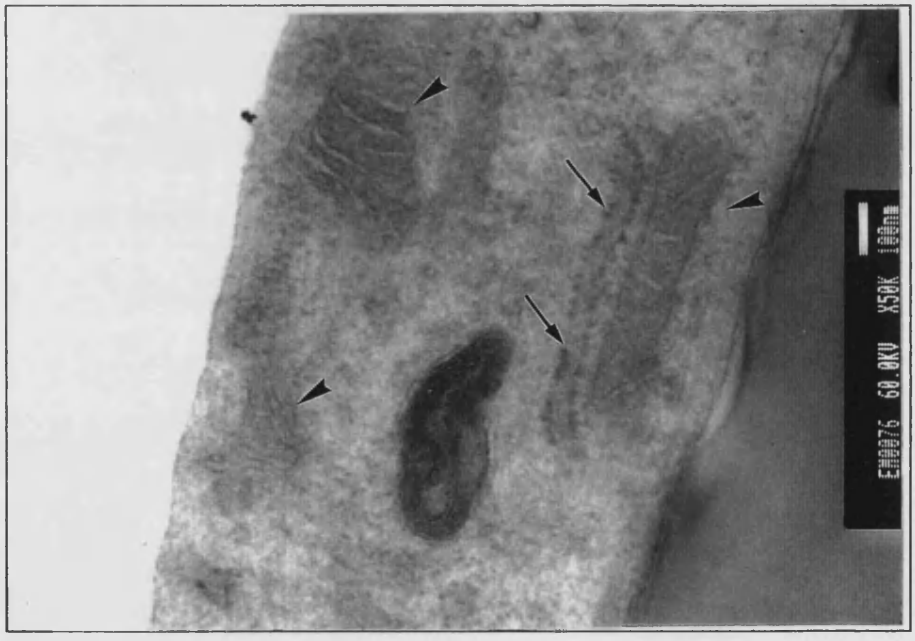

Figure 39. A very high magnification transmission electron micrograph showing mitochondrion (*arrowheads*) and rough endoplasmic reticulum (*arrows*) in the cell cytoplasm.

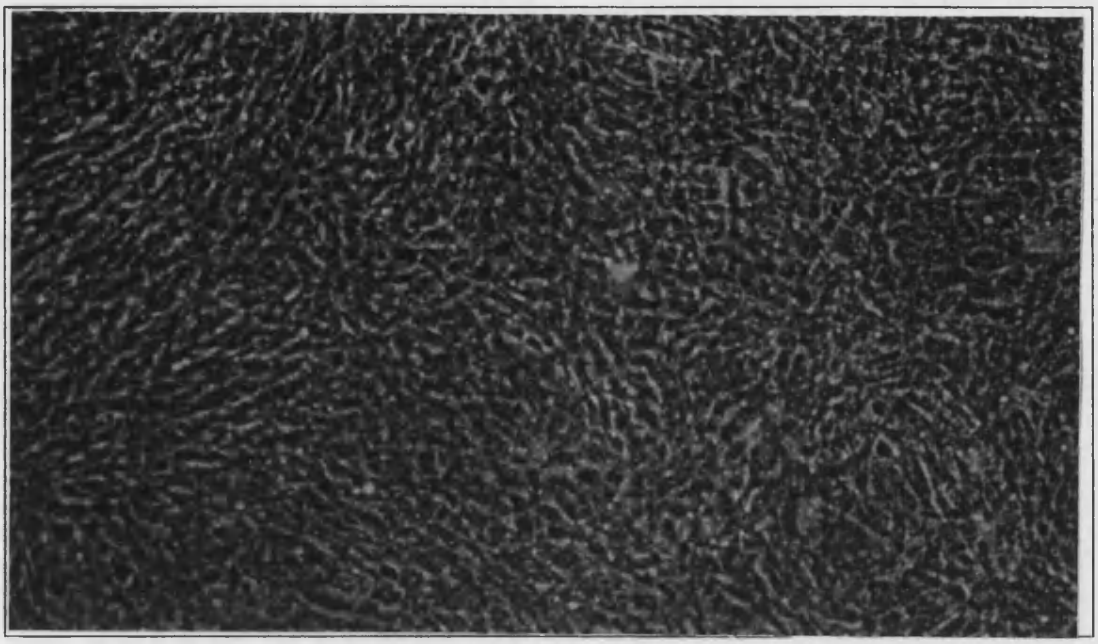

Figure 40. A phase contrast photomicrograph showing a confluent monolayer of lung microvascular endothelial cells grown on a collagen coated filter membrane prior to treatment for electron microscopy studies. Magnification X200.

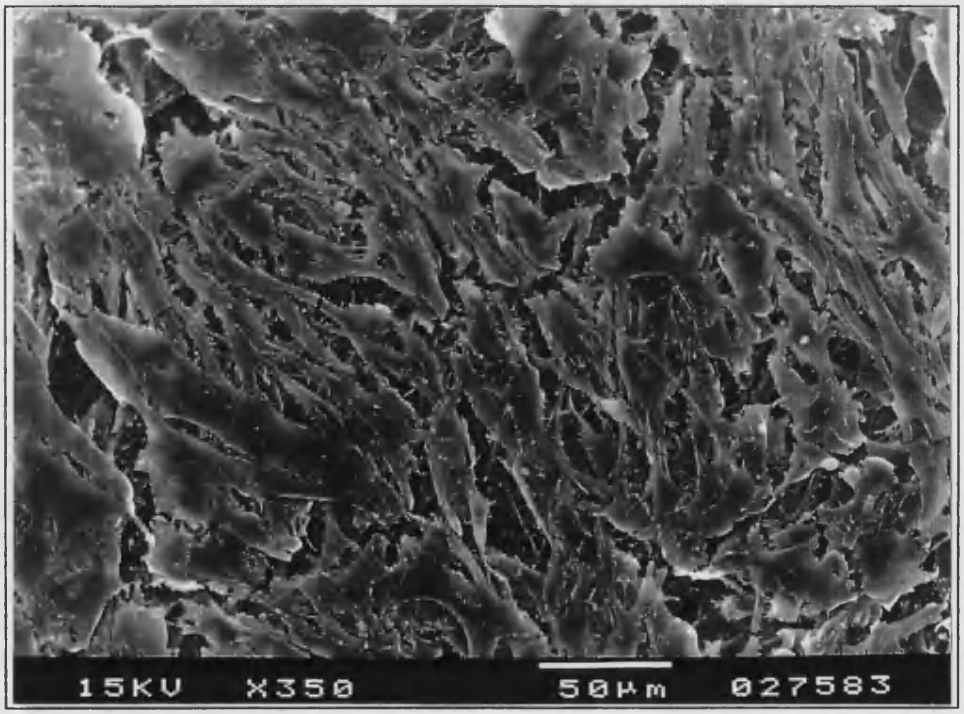

Figure 41. A scanning electron micrograph of lung microvascular endothelial cells.

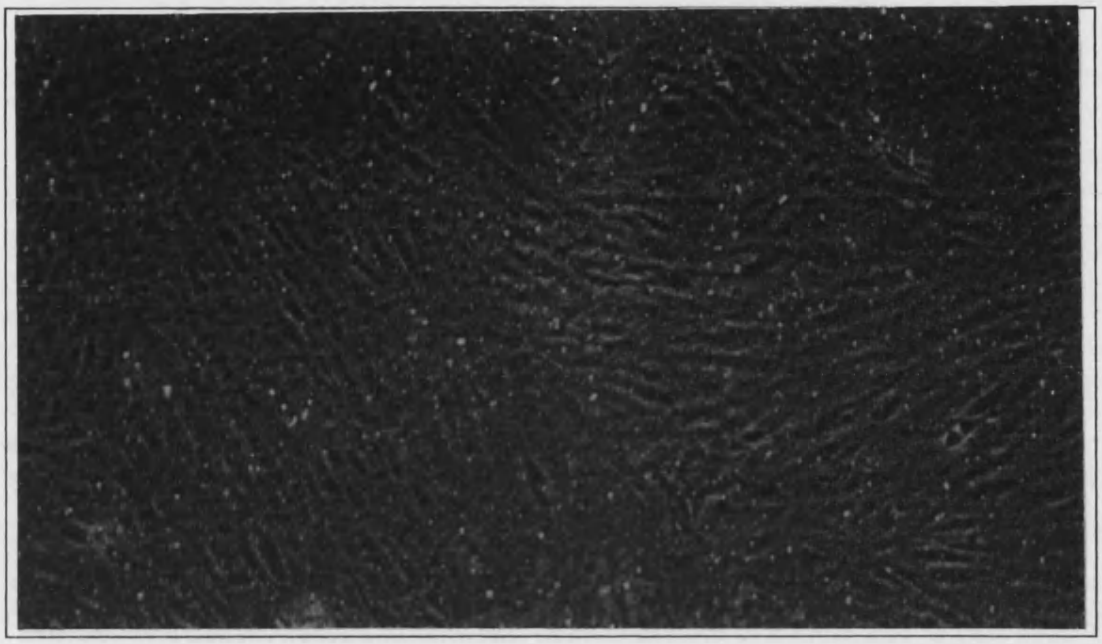

Figure 42. A phase contrast photomicrograph showing a confluent monolayer of brain microvascular endothelial cells grown on a collagen coated filter membrane prior to treatment for electron microscopy studies. Magnification X200.

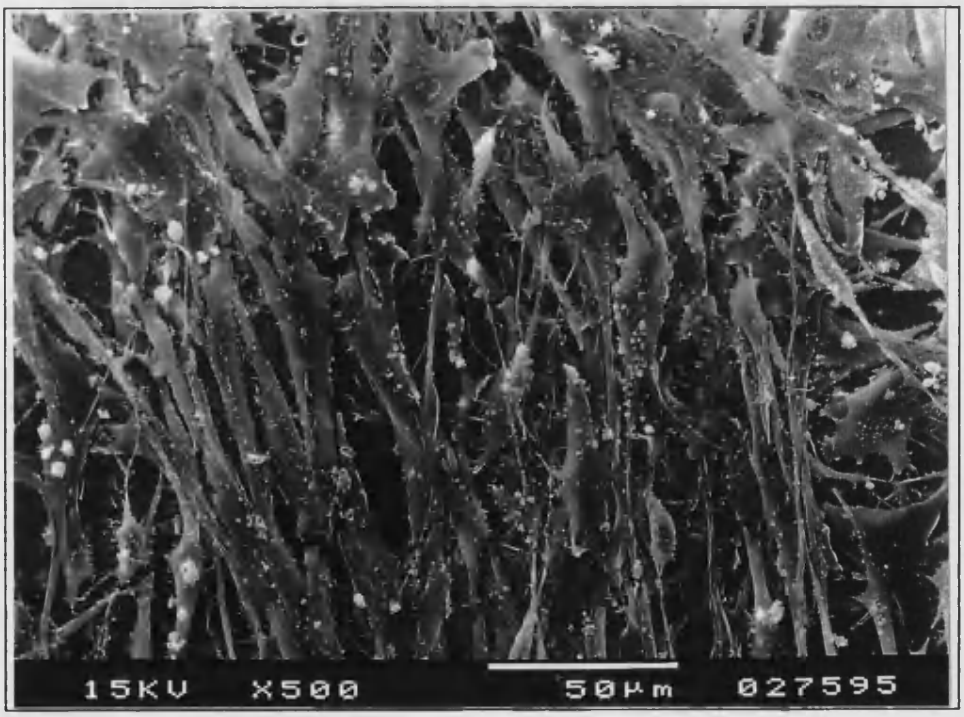

Figure 43. A scanning electron micrograph of brain microvascular endothelial cells.

5.3.3. Acetylated LDL Uptake

The fluorescent probe, Bodipy, was incorporated into the Ac-LDL, in order to allow fluorescent identification of the endothelial cells. After incubation of the cells for 4 hours at 37°C the extent of uptake of the fluorescent probe (Bo-Ac-LDL) was assessed using fluorescent microscopy. The positive and negative controls, BAE-1 cells and fibroblasts respectively, demonstrated that Bo-Ac-LDL was extensively incorporated into the BAE-1 cells. In contrast, only trace amounts of the fluorescent probe were observed in fibroblasts and other types of contaminating cells. Figures 44 & 45 compare visible and fluorescent photographs that demonstrate the extensive incorporation of Bo-Ac-LDL into BAE-1 cells that were seeded on to glass coverslips.

Both lung and brain microvascular endothelial cells grown on chamber slides demonstrated extensive uptake of Bo-Ac-LDL (Figure 46 & 47). Mixed cultures of endothelial cells and non-endothelial cell contaminants obtained from primary culture were seeded on to collagen coated glass coverslips and incubated with Bo-Ac-LDL, in order to directly compare the extent of incorporation of Bo-Ac-LDL in endothelial and non-endothelial cells. Figures 48 & 49 show visible and ultraviolet photographs respectively, which clearly demonstrate the variation in uptake of the fluorescent probe by endothelial and non-endothelial cells. The extent of uptake of Bo-Ac-LDL therefore provided another tool to enable the identification of endothelial cells.

Figure 44. A phase contrast photomicrograph showing Bovine aortic endothelial cells (BAE-1) a positive control for the uptake of Bo-Ac-LDL. Magnification X800.

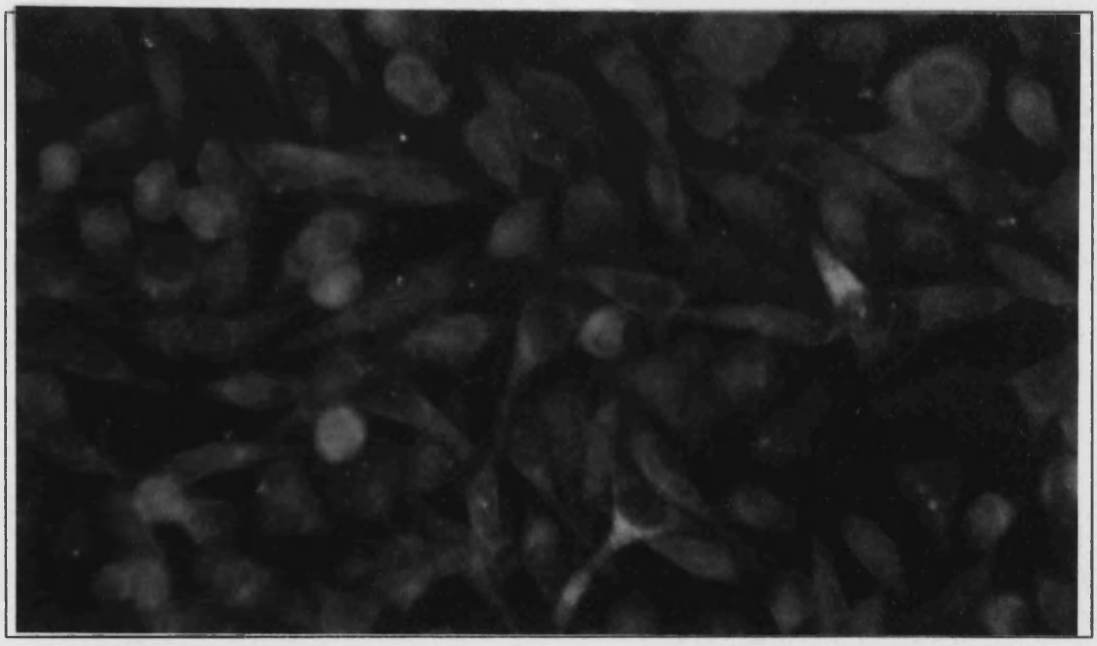

Figure 45. A fluorescence micrograph showing the uptake of Bo-Ac-LDL into BAE-1 cells. Magnification X800

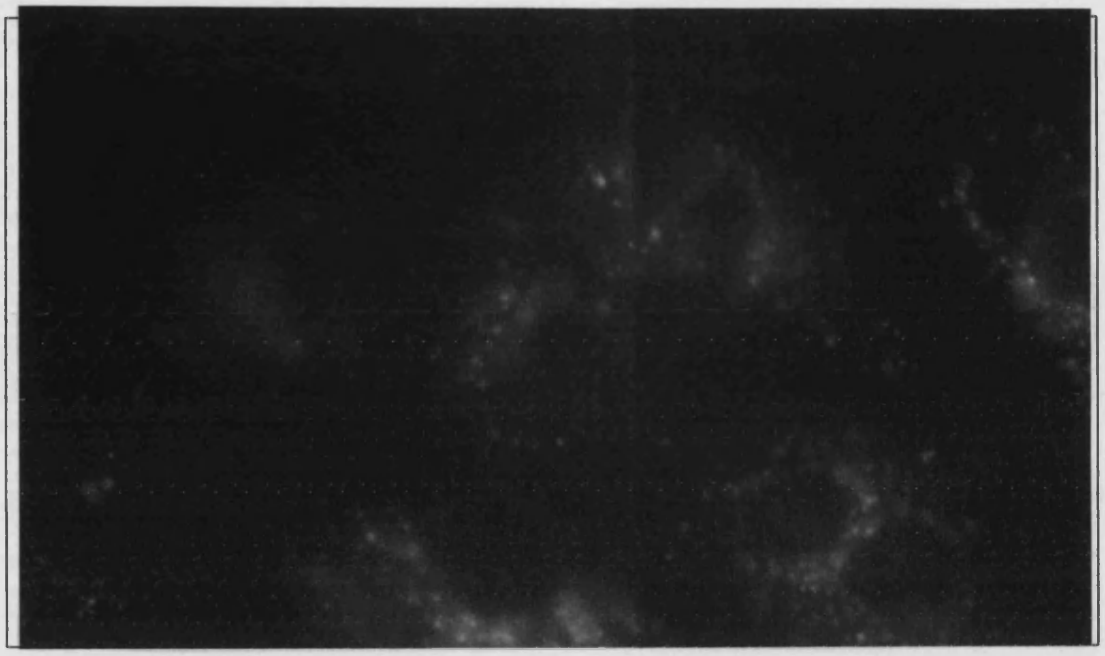

Figure 46. A fluorescence micrograph showing the uptake of Bo-Ac-LDL into lung microvascular endothelial cells. Magnification X1200

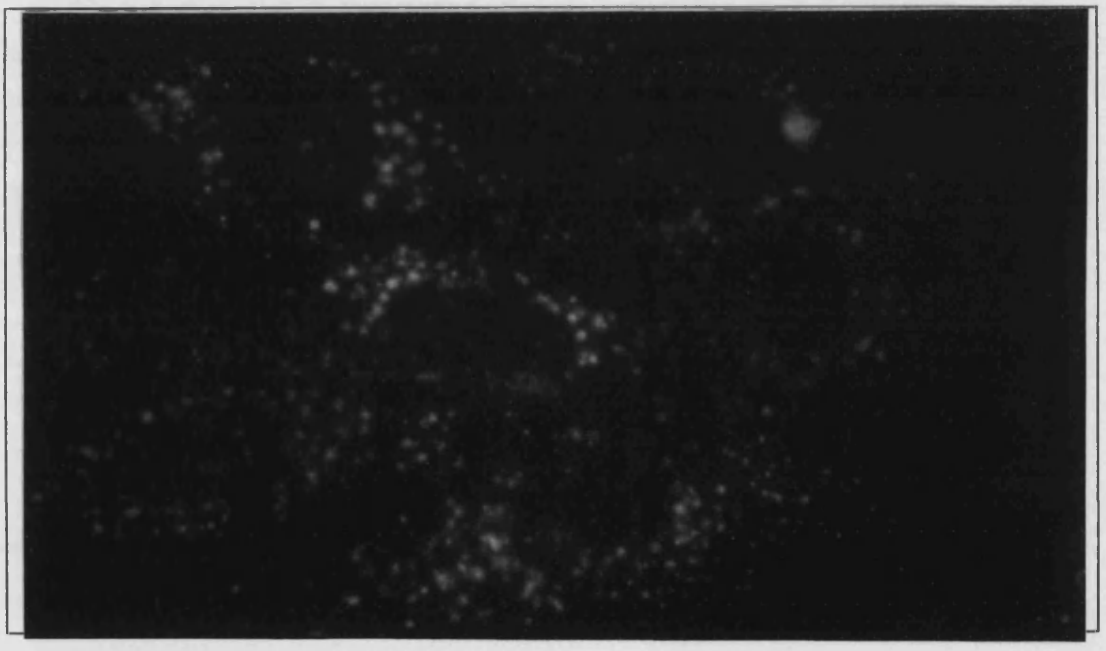

Figure 47. A fluorescence micrograph showing the uptake of Bo-Ac-LDL into brain microvascular endothelial cells. Magnification X1200

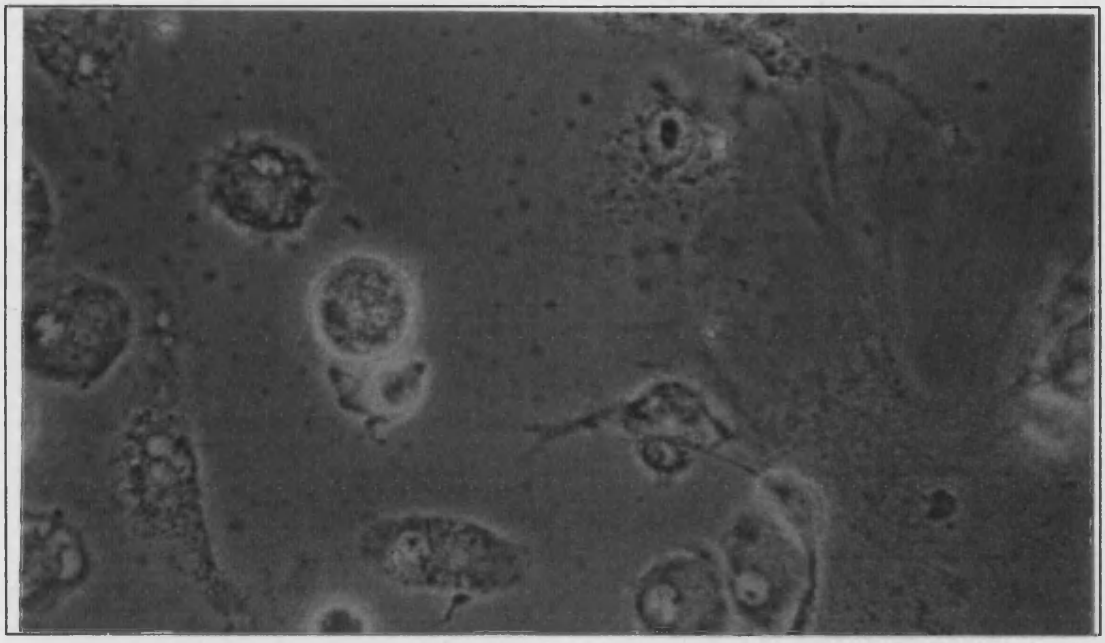

Figure 48. A phase contrast photomicrograph showing a mixed population of lung microvascular endothelial cells and non-endothelial cell contaminants prepared for uptake of Bo-AC-LDL. Magnification X800.

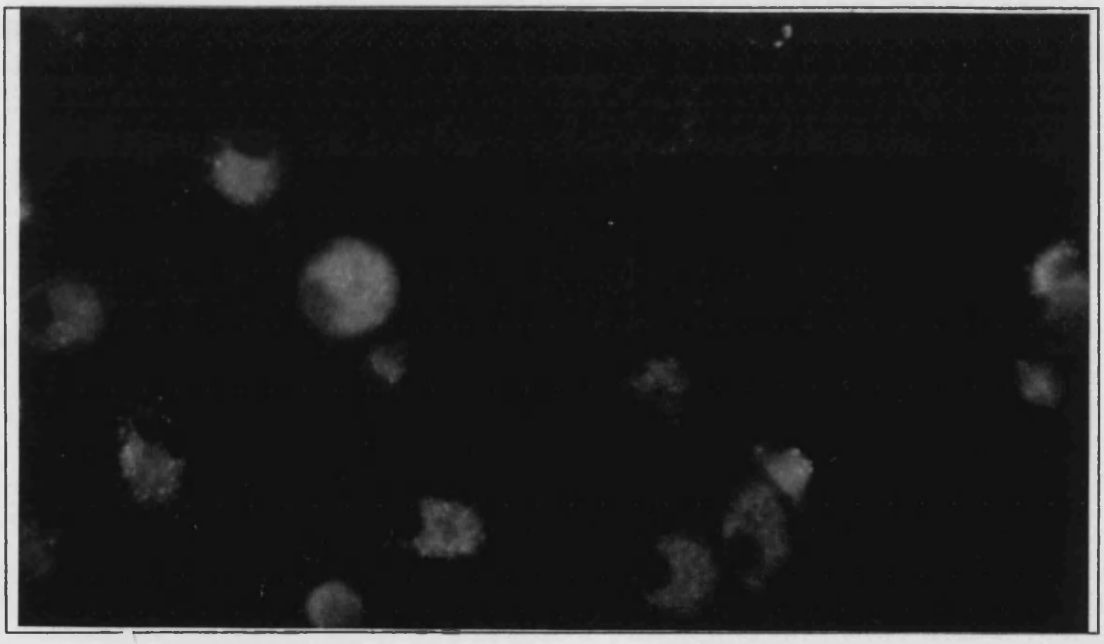

Figure 49. A fluorescence micrograph showing the selective uptake of Bo-Ac-LDL into lung microvascular endothelial cells but not into the non-endothelial cells. Magnification X800.

5.3.4. Expression of Von Willebrand factor

Von Willebrand factor (Factor VIII-related antigen) is used extensively as a highly specific marker for endothelial cells. Unlike most von Willebrand factor assays which use fluorescent conjugated antibodies and fluorescence microscopy, the method utilised in the present study involved the use of a peroxide conjugated von Willebrand factor which was stained with a DAB reagent. The cells required for this assay were grown under routine culture conditions and assessed for expression of von Willebrand factor by phase contrast microscopy.

Incubation of the cells with thrombin stimulated production of von Willebrand factor. The cells were fixed with 10% formalin, incubated with the peroxide conjugated antibody, and then stained with freshly prepared DAB reagent; the cells were counter stained with haematoxylin before being viewed by phase contrast microscopy. Positive specific staining was present in cultures of lung and brain microvascular endothelial cells (Figure 50 & 51). Mixed cultures containing endothelial cells and contaminating smooth muscle cells showed a distinct difference in the intensity of staining. The endothelial cells appeared to stain dark purple/brown in colour whereas the smooth muscle cells had a pale purple appearance which was probably due to the presence of the haematoxylin solution (Figures 52 & 53). Controls showed that intense staining was not present when cultures were exposed to normal rabbit serum.

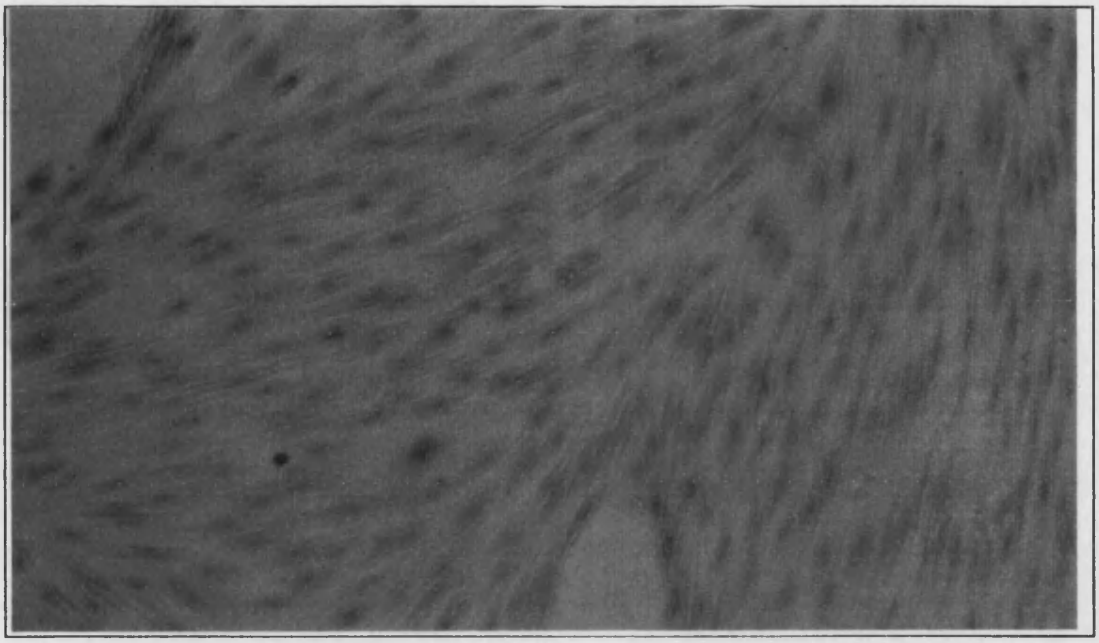

Figure 50. A phase contrast photomicrograph showing the expression of Von Willebrand factor in lung microvascular endothelial cells as an intense brown stain. Magnification X400.

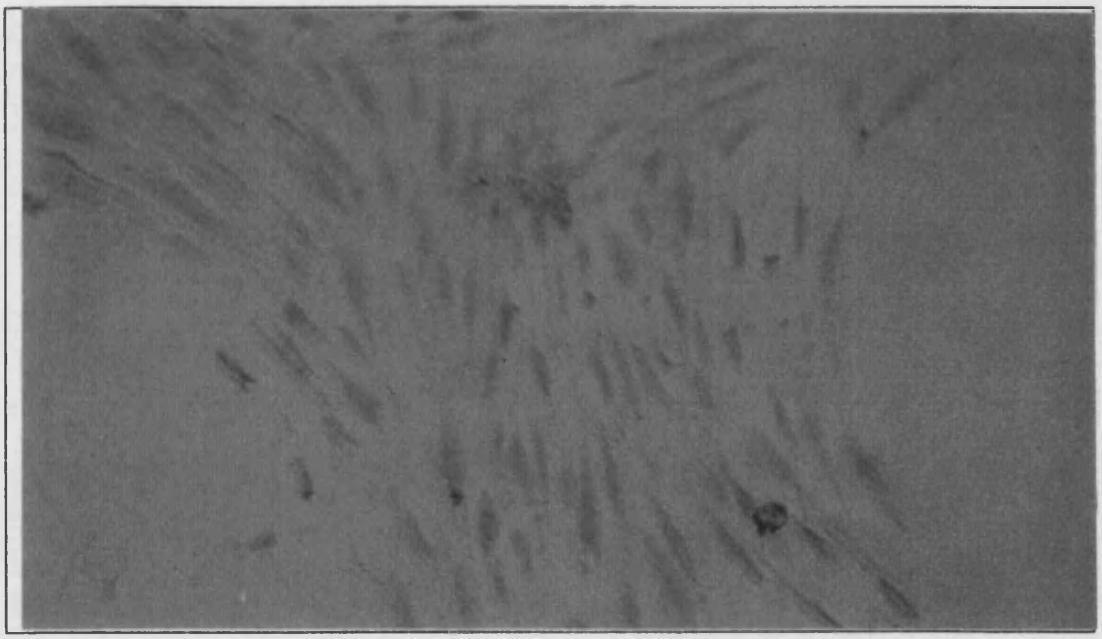

Figure 51. A phase contrast photomicrograph showing the expression of Von Willebrand factor in brain microvascular endothelial cells as an intense brown stain. Magnification X200.

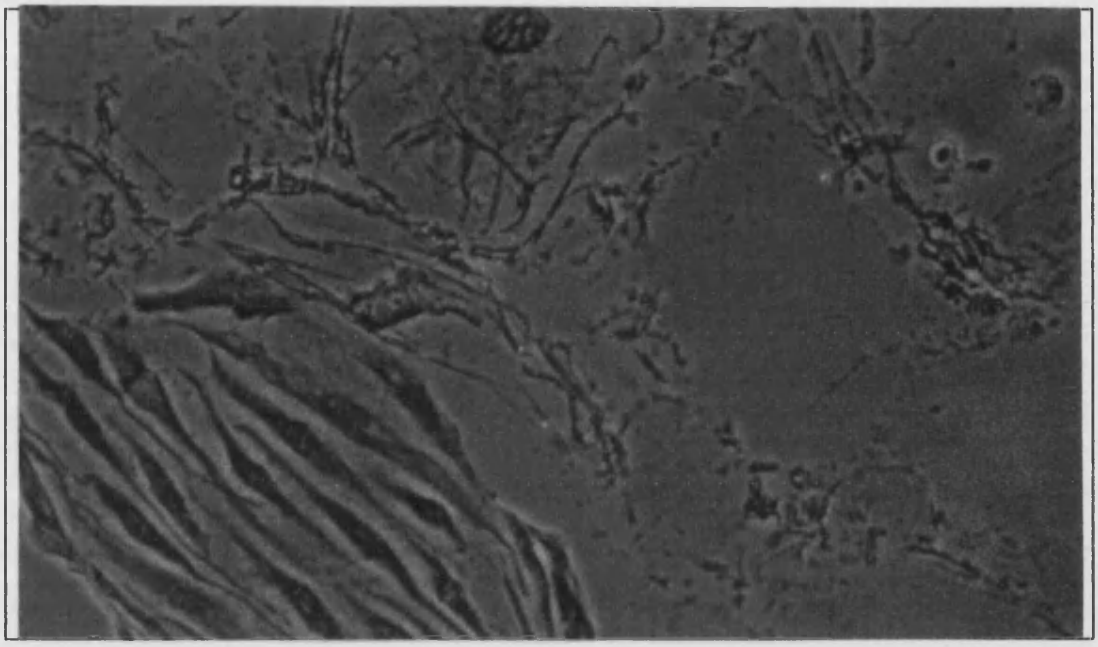

Figure 52. A phase contrast photomicrograph showing the expression of Von Willebrand factor in a mixed culture of lung microvascular endothelial cells and non-endothelial cell contaminants. Magnification X400.

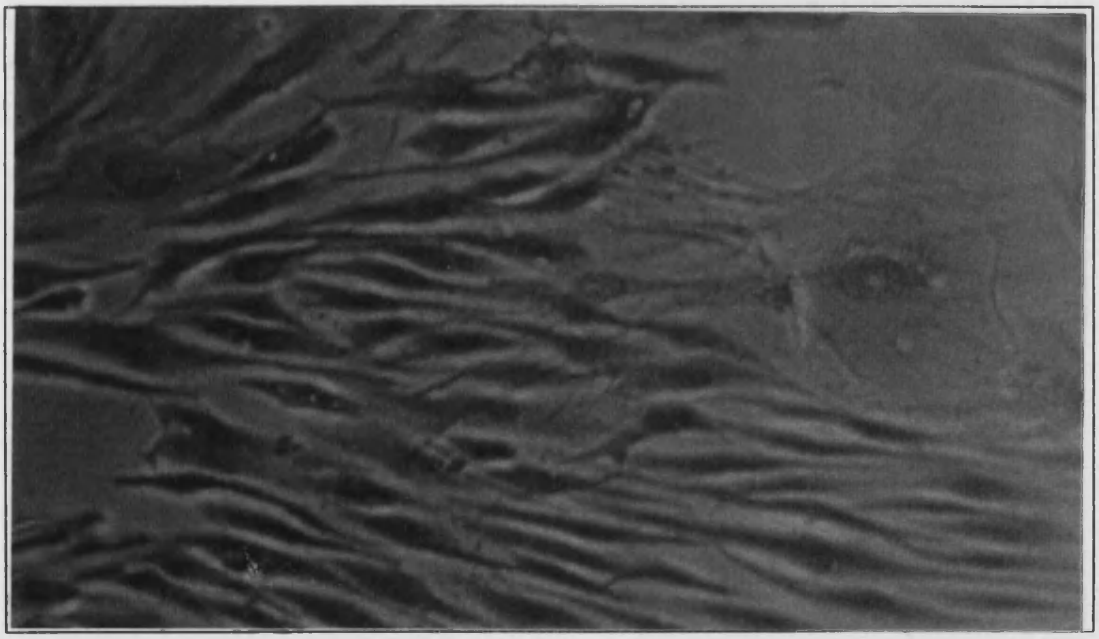

Figure 53. A phase contrast photomicrograph showing the expression of Von Willebrand factor in a mixed culture of brain microvascular endothelial cells and non-endothelial cell contaminants. Magnification X400.

5.3.5. Platelet Adhesion Studies

Endothelial cells were found to demonstrate a characteristic non-thrombogenic, non-adhesive surface in platelet adhesion assays which was not shown with smooth muscle cells, fibroblasts and pericytes. Therefore assessment of the distribution of platelet adherence provided a rapid method of identification of endothelial cells and non-endothelial cell contaminants. The adherence of platelets to the collagen coated culture wells also provided a method of determination of the cell-cell contacts exhibited by the cell colonies.

Figure 54 is a photograph of a culture of non-endothelial cell contaminants, including pericytes and smooth muscle cells, which shows platelet adherence to the apical surface of the cells as well as the culture well. Figure 55 shows the edge of a colony of brain microvascular endothelial cells, where the platelets did not adhere to the surface of the cells but adhered to the surface of the well, and a contaminating cell that was present in the culture.

Figures 56 & 57 depict cultures of lung microvascular endothelial cells. Figure 56 shows a colony of cells where the platelets did not adhere to the cell surface but adhered to the surface of the well, with no obvious gaps existing between the cells. Figure 57 shows the edge of a colony of cells which encompassed non-endothelial cells as well as exhibiting them at the colony perimeter. Platelets were clearly demonstrated to adhere to the non-endothelial cells as well as being fairly widespread between cells, indicating the lack of tight cell-cell contacts within this colony, this was probably due to the presence of these contaminating cells throughout the colony.

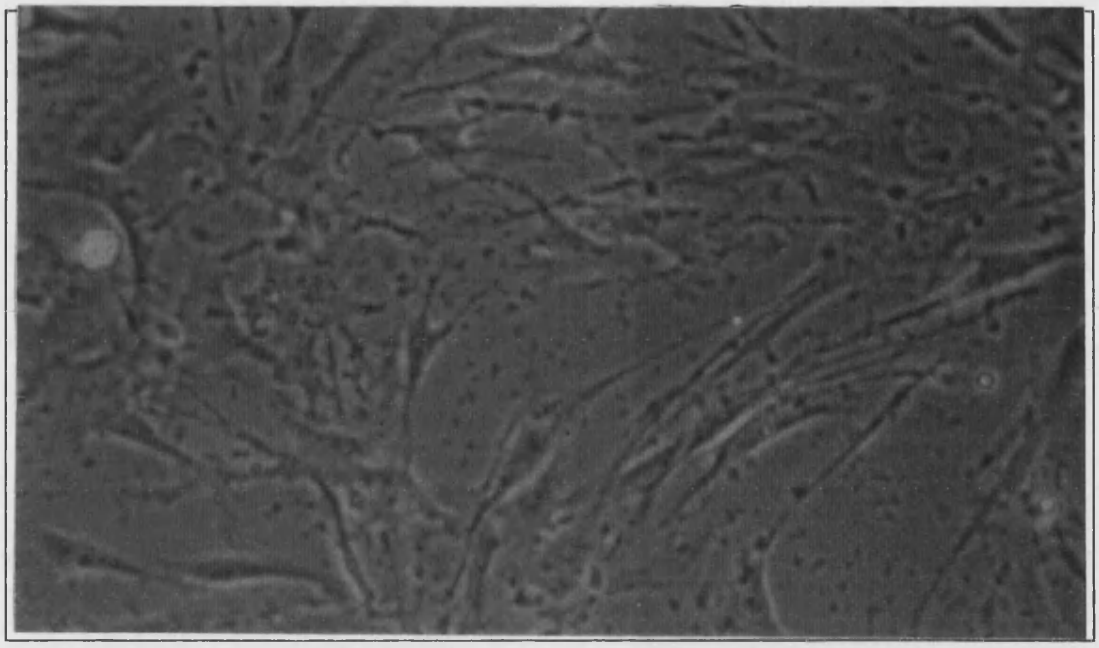

Figure 54. A phase contrast photomicrograph showing platelet adhesion to non-endothelial cells. Magnification X200.

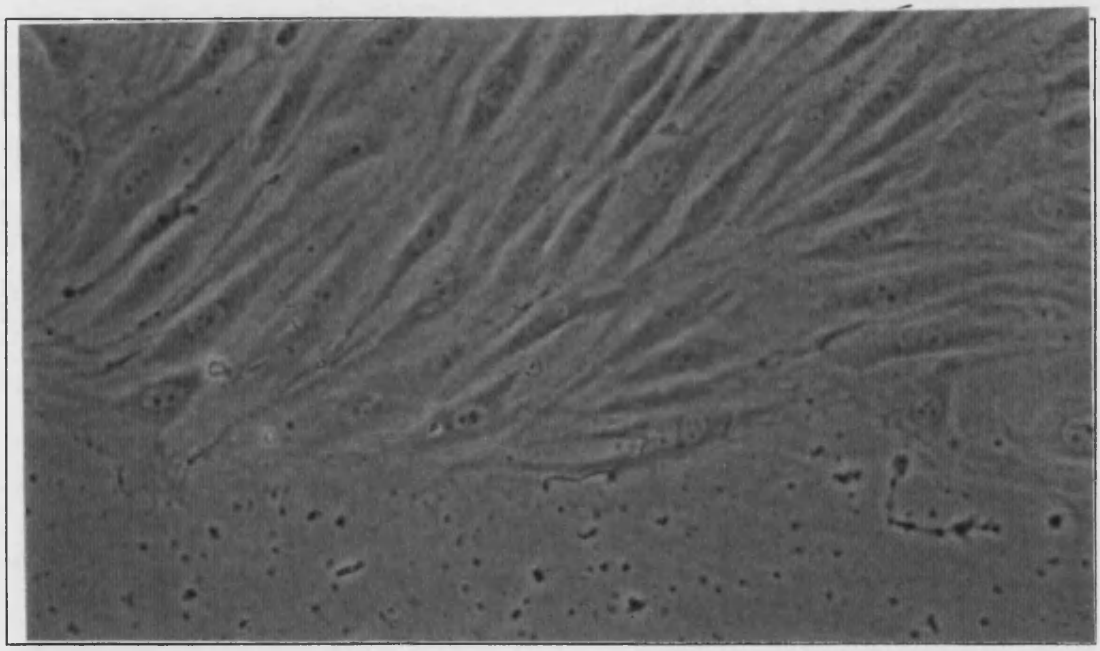

Figure 55. A phase contrast photomicrograph showing the edge of a brain microvascular endothelial cell colony where platelets adhere to the collagen coated well only. Magnification X400.

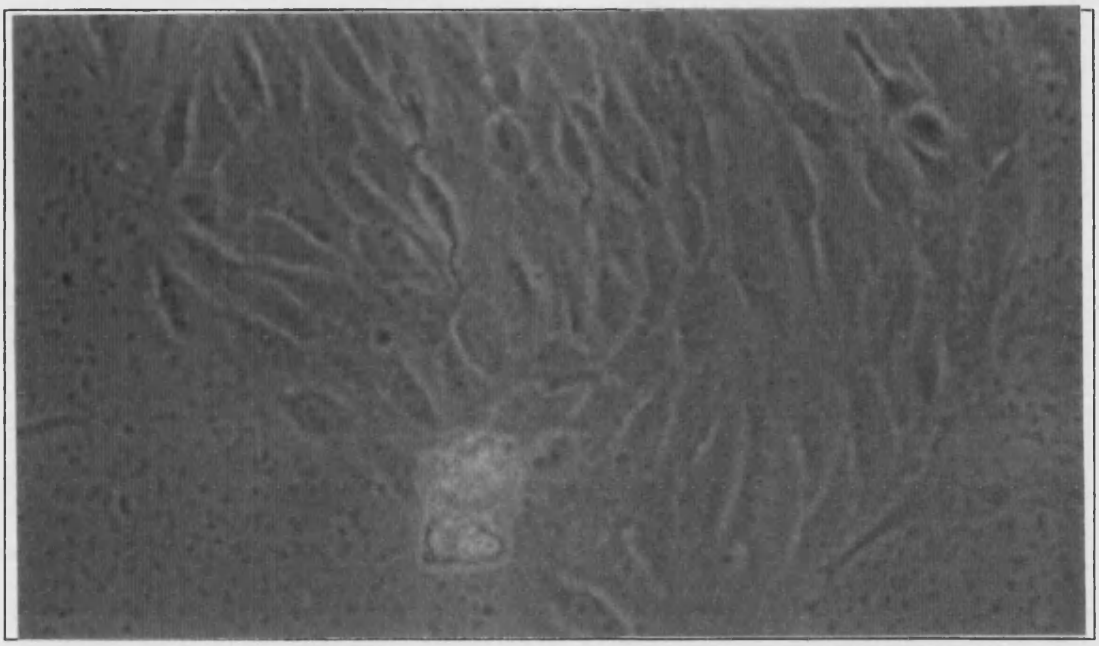

Figure 56. A phase contrast photomicrograph showing the edge of a lung microvascular endothelial cell colony where platelets adhere to the collagen coated well only. Magnification X400.

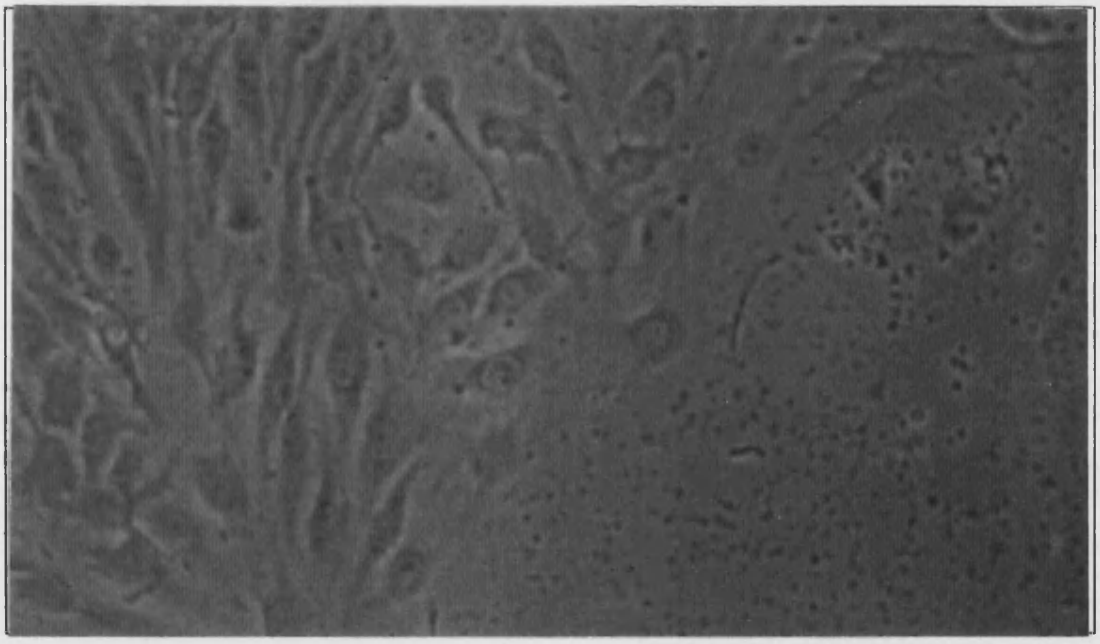

Figure 57. A phase contrast photomicrograph showing the edge of a lung microvascular endothelial cell colony interspersed with non-endothelial cells. Platelets adhere to the non-endothelial cells, gaps between cells and the surface of the well plate. Magnification X400.

5.3.6. *In vitro* Angiogenesis by Lung Microvascular Endothelial Cells Grown on Matrigel

Microvascular endothelial cells in culture are capable of undergoing morphological differentiation to form capillary tube-like structures, providing a method of differentiating between endothelial and mesothelial cells. Unlike endothelial cells, mesothelial cells do not have the ability to undergo angiogenesis *in vitro* (Chung-Welch *et al.*, 1989) when grown on the substrate Matrigel, a basement membrane matrix extracted from mouse sarcoma. Differentiation between microvessel and large vessel endothelial cells can also be established by this method and, although primary cultures of large vessel endothelial cells have been shown to differentiate into capillary-like structures *in vitro,* this occurs after a minimum of 12 hours in culture whereas microvascular endothelial cells differentiate after 4 hours in culture (Nishida *et al.*, 1993).

Figure 58 shows a colony of lung microvascular endothelial cells immediately prior to seeding on to Matrigel coated culture wells and is a typical representation of lung endothelial cells isolated in primary culture. Figure 59 shows the capillary like structures formed after a 4 hour incubation at 37 °C, on Matrigel coated culture wells, and Figure 60 shows the more extensive tube formation after 12 hours in culture.

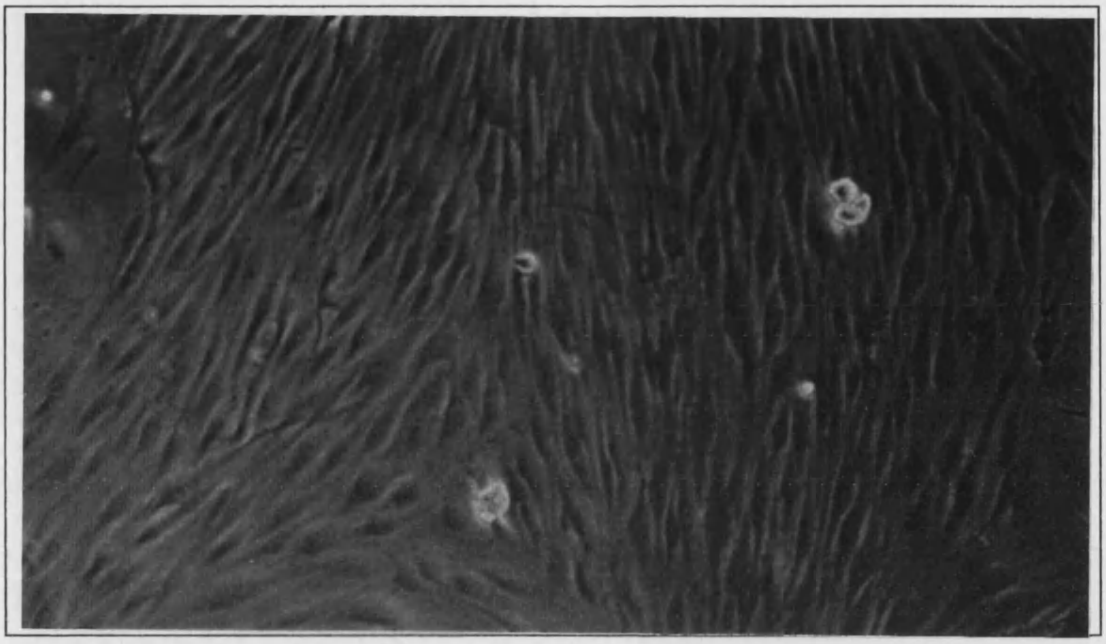

Figure 58. A phase contrast photomicrograph showing lung microvascular endothelial cells prior to seeding on Matrigel coated culture wells. Magnification X400.

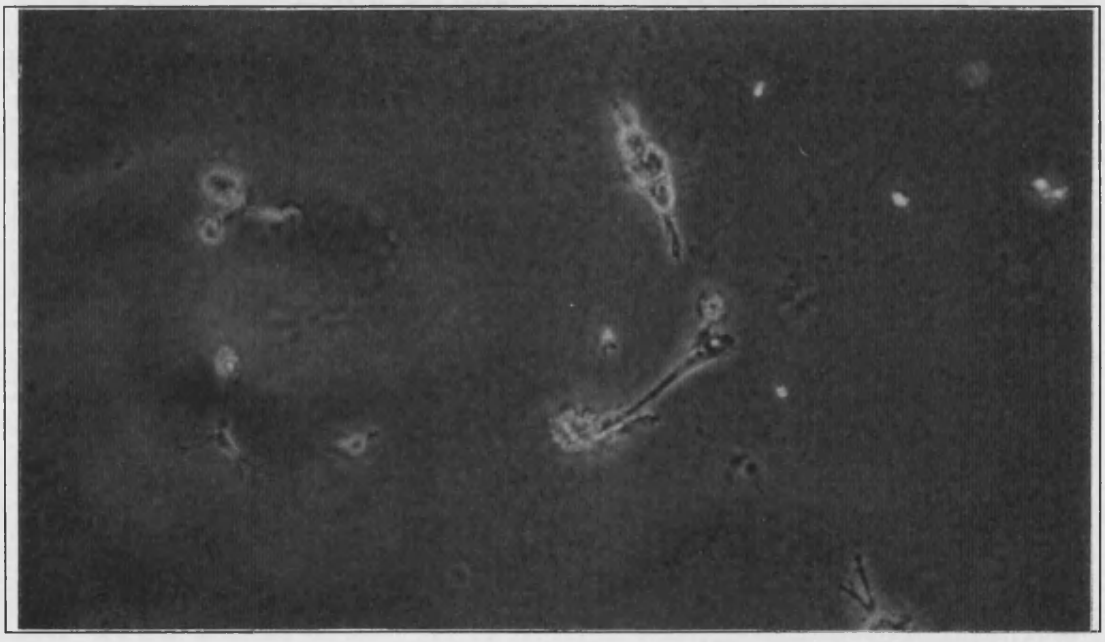

Figure 59. A phase contrast photomicrograph showing tube formation by lung microvascular endothelial cells 4 hours after seeding on to Matrigel coated culture wells. Magnification X200.

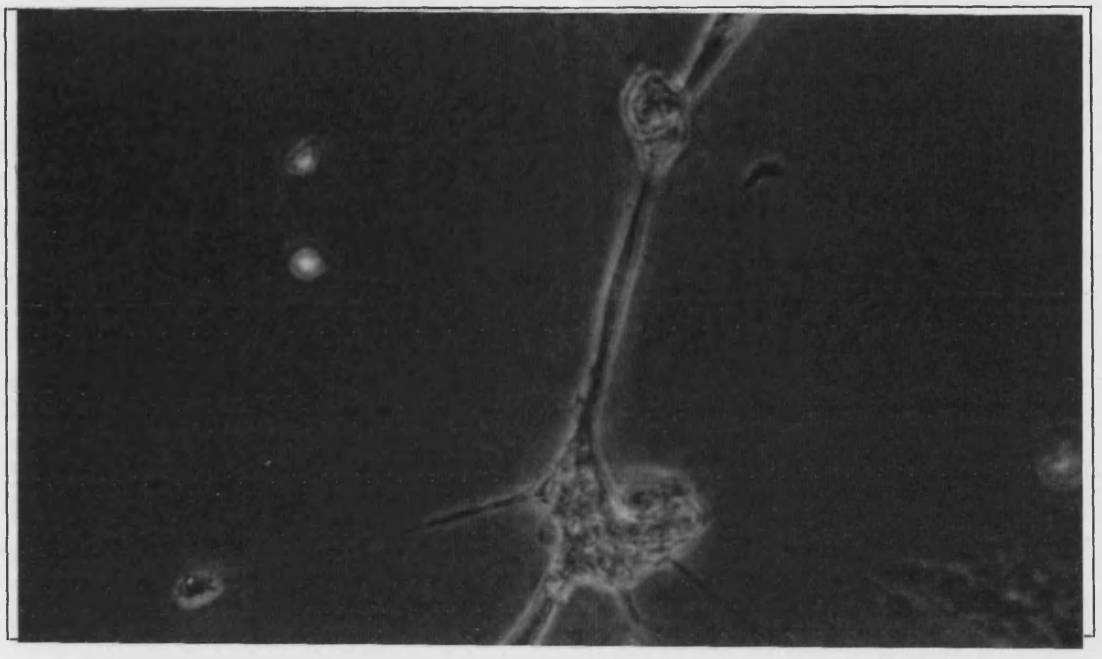

Figure 60. A phase contrast photomicrograph showing more extensive tube formation by lung microvascular endothelial cells 12 hours after seeding on to Matrigel coated culture wells. Magnification X200.

5.4. Discussion

Light microscopy

Microvascular endothelial cell growth and morphology was distinctive where cells grew in a characteristic manner forming contact-inhibited monolayers. Some of the contaminating cells present in these cultures such as fibroblasts, pericytes and smooth muscle cells could be distinguished from endothelial cells by examination of the cell morphology (see section 3.4).

The dominant cell type in primary cultures of brain microvascular endothelial cell cultures showed a spindle shaped morphology. This morphology is consistent with brain microvascular endothelial cells from other sources (Carson and Hausenchild 1986; Dorovini-Zis *et al.*, 1991; Beer Stoltz and Jacobson 1991; Abbott *et al.*, 1992), although phenotypic variations in cultured brain microvascular endothelial cells have been shown to exist (Rupnick *et al.*, 1988).

In contrast, the predominant cell type in primary cultures of lung microvascular endothelial cells showed a typical cobblestone morphology. This is consistent with lung microvascular endothelial cells from other sources (Ryan 1990; Belloni *et al.*, 1992; Hewett and Murray 1993). In this study, the appearance of elongated cells in a culture of lung endothelial cells, forming a similar pattern to that observed with brain cells, could indicate the presence of contaminating mesothelial cells (Hewett and Murray 1993), although mesothelial cells often display cobblestone morphology (Chung-Welch *et al.*, 1989).

Microvessel endothelial cells have been shown to express a number of organ specific differences as well as differences within various vascular beds. The phenotypic variations

reported here between brain and lung endothelial cells could be due to organ specific differences or differences in the microanatomical source of the cells with in the tissue. Cell morphology alone, however, was insufficient to characterise endothelial cell origin.

Transmission and scanning electron microscopy

Many of the ultrastructural features found in the transmission electron micrographs are consistent with an endothelial cell phenotype. This includes the presence of plasmalemmal vesicles and coated pits on the apical and basolateral membranes and tight junctions at the point of cell contact. Although these features are common to endothelial cells they are not exclusive (Gerritsen *et al.*, 1988). The identification of a Weibel-Palade body (Figure 37) can be used as proof of endothelial cell origin as they have not been reported to be present in other cell types. Lack of Weibel-Palade bodies does not rule out endothelial origin, as the frequency is variable between species and they are thought to be less abundant in murine tissues (Folkman *et al.*, 1979).

The ultrastructural properties of the endothelial cell *in vivo* are well characterised and display distinct organ specific features (Gerritsen *et al.*, 1988). Beer Stoltz and Jacobson (1991) however, suggested that many of the distinctive variations of these features were lost *in vitro*, possibly due to the effect of proteolytic enzymes in the isolation procedure. In the present study, no significant organ specific variations were seen between the ultrastructure of brain and lung microvascular endothelial cells. This could therefore be due to the isolation procedure and culture conditions.

Scanning electron microscopy offered no indication that a contact-inhibited monolayer was formed. This, however, was probably due to damage caused to the cells during the dehydration procedure. Given more time modifications of the dehydration process would have been attempted.

Uptake of acetylated LDL

Stein and Stein (1980) demonstrated that endothelial cells, macrophages, smooth muscle cells and fibroblasts take up and degrade acetylated LDL (Ac-LDL) in preference to LDL. Acetylated LDL is taken into the cell via specific "scavenger" receptors. This uptake and degradation occurs 30 times faster in endothelial cells than in other cells and therefore Voyta *et al* (1984) used the specific uptake of Dil-Ac-LDL as a marker for endothelial cell identification.

Primary cultures of microvascular endothelial cells from both murine lung and brain tissue stained positive for the uptake of Bodipy acetylated LDL showing punctate fluorescence, localised cytoplasmically (Figures 46 & 47). Bo-Ac-LDL was taken up by endothelial cells at a faster rate than macrophages, smooth muscle cells or fibroblasts thus confirming their endothelial cell origin. No significant difference in the extent of uptake between brain and lung microvascular endothelial cells was observed.

With microvascular endothelial cells isolated from the lung there was also the possibility of mesothelial cell contamination. Mesothelial cells have been shown to take up Dil-Ac-LDL in less than 4 hours (Chung-Welch *et al.*, 1989), and are therefore indistinguishable from microvascular endothelial cells by this means. However, taking into account the pretreatment of the lungs prior to isolation in this study and the *in vitro* angiogenesis studies described later, it is possible to confirm that these cells are indeed of endothelial origin.

Expression of Von Willebrand factor

Von Willebrand factor is localised to endothelial cells and megakaryocytes (Zimmerman *et al.*, 1983), and is used extensively as reliable, highly specific marker for endothelial cells. Both lung and brain microvascular endothelial cells in this study were found to express von Willebrand factor. A slight variation in the intensity of staining between

lung and brain cells was observed (Figures 50 & 51). This could be due to organ specific variations or alternatively due to variability in the staining technique.

Although von Willebrand factor is thought to represent an endothelial cell specific marker, mesothelial cells have also been shown to express von Willebrand factor in culture. The staining intensity for mesothelial cells, however, is faint and more diffuse than for endothelial cells (van Hinsbergh *et al.*, 1990). In addition, a negative result for von Willebrand factor does not rule out endothelial cell origin. Lymphatic endothelia (Mukai 1980) and endothelial cells isolated from porcine tissues (Rosenthal and Gotleib 1990), for example, do not express Von Willebrand factor.

Platelet adhesion

The endothelium demonstrates a non-thrombogenic, non-adhesive surface to platelets *in vivo* and *in vitro* (Zetter 1984; Belloni *et al*, 1992). Platelet adhesion assays reported here supported the other characterisation studies in demonstrating endothelial cell origin of both brain and lung cells (Figures 55 & 56). The platelets were seen to adhere to the collagen coated surface of the culture dish, smooth muscle cells and other contaminants at the edge of the colonies as well as within colonies. In some cases, adhesion was also shown between cells where tight cell-cell contacts were not apparent. The lack of tight cell-cell junctions could be due to the presence of contaminating non-endothelial cells within the colony.

In vitro Angiogenesis by lung microvascular endothelial cells

A number of features, including those related to the extracellular matrix have been shown to play an important role in the formation of capillary "tube-like" structures by microvascular endothelial cells in culture (Ingber and Folkman 1989; Lawley and Kubota 1989; Vlodavsky *et al.*, 1991; Kinsella *et al.*, 1992). Conventional characterisation techniques are not sufficient to conclusively distinguish between endothelial and

mesothelial cells in culture; therefore, *in vitro* angiogenesis is the only functional test which can qualitatively distinguish between these cells (Chung-Welch *et al.*, 1989). In addition, smooth muscle cells, fibroblasts and pericytes do not undergo *in vitro* angiogenesis providing another method of differentiating them from endothelial cells.

When grown Matrigel, the cells isolated from lung tissue in this study formed the capillary structures confirming endothelial cell origin (Figure 60). Although, under some conditions, epithelial cells can also form tubular structures *in vitro* (Montesano *et al.*, 1991), the combination of conventional characterisation studies and *in vitro* angiogenic studies provided sufficient evidence to confirm the endothelial origin of these cells.

6. BINDING OF [^{125}I-TYR^{2}, NLE^{4}, D-PHE^{7}] α-MSH TO MURINE BRAIN MICROVASCULAR ENDOTHELIAL CELLS

6.1. Introduction

Melanocortin peptides are neuropeptides which have a wide variety of influences on the nervous system including attention, learning, memory, behavioural and neuromuscular effects (Eberle, 1988). A number of melanocortin receptors have been identified in the brain (Mountjoy *et al.*, 1993; Gantz *et al.*, 1993a and b; Chhanjlani *et al.*, 1993). It is conceivable that endocrine hormones, including α-melanocyte stimulating hormone (α-MSH), might be transported by receptor-mediated transcytosis making use of receptors expressed at the surface of the microvascular endothelia. In this study, the radio-iodinated derivative of [Nle^4, D-Phe^7] α-MSH, the superpotent analogue of α-MSH, has been used to examine the possibility that a receptor for MSH is expressed by murine brain microvascular endothelial cells, and to determine the binding characteristics at 4°C and 37°C.

6.2. Materials and Methods

6.2.1. Synthesis of [Nle^4, D-Phe^7] α-MSH

The peptide [Nle^4, D-Phe^7] α-MSH, was synthesised by Dr G. W. J Olivier at the University of Bath (Ahmed *et al.*, 1992). The synthetic procedure, therefore, will only be briefly described. A solid-phase synthesis protocol was used to prepare the peptide according to the Fmoc strategy developed by Atherton and Sheppard (1989). Pepsin K resin was used as the solid phase and the amino acids were sequentially linked to form the peptide. The peptide was then purified by semi-preparative HPLC whilst being continually monitored by UV spectrophotometry at 217nm. Fractions were collected and tested by analytical scale HPLC and their identity was confirmed by co-chromatography with standard [Nle^4, D-Phe^7] α-MSH obtained from Sigma and by FAB mass spectrometry. Mass spectrometry was carried out at Swansea University SERC MS Centre. Before use, the peptide was dissolved in sterile 10mM HCl to produce a 1mg/ml stock solution which was stored in a sterile plastic screw topped eppendorf tube at 4°C.

6.2.2. Iodination of [Nle^4, D-Phe^7] α-MSH

Iodination of the peptide [Nle^4, D-Phe^7] α-MSH occurred at the Tyr^2, using a protocol based on the chloramine T procedure described by Eberle (1988).

Preparation of stock solutions

The following stock solutions were prepared and stored at 4°C:

0.25M Na_2HPO_4 in distilled water.

0.25M NaH_2PO_4 in distilled water

1% TFA (trifluoroacetic acid, Aldrich chemicals) in distilled water

50, 60, and 80% methanol (HPLC grade) with 1% TFA.

Preparation of solutions

The following solutions were prepared freshly prior to the iodination:

1) 0.25M Phosphate buffer pH7.4, prepared from the stock solutions Na_2HPO_4 and NaH_2PO_4

2) 0.25% BSA (0.0125g) in 1ml 0.25M phosphate buffer and 4ml distilled water.

3) 1% Polypep (0.05g, Sigma) in 1ml 0.25M phosphate buffer and 4ml distilled water.

4) 0.1% Chloramine T (0.01g, BDH chemicals) in 10ml distilled water dissolved immediately before use.

5) 1.5µl of [Nle^4, D-Phe^7] α-MSH from the 1mg/ml stock solution in 25µl 0.25M phosphate buffer, prepared in a 1.5ml plastic eppendorf tube.

Preparation of the column

Purification of the freshly iodinated peptide [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH was carried out on mini, disposable C18 reverse-phase column with Spherisorb ODS 'Bond Elut' (Analytical international, MFG codeOH53). The column was prepared and conditioned for the purification according to the following procedure:

prewash X3 with 1%TFA in water

wash X3 with 1% TFA in 80% methanol

wash X1 with 1ml polypep

wash X3 with 1% TFA in 80% methanol

wash X3 with 1% TFA in water.

Iodination of [Nle^4, D-Phe^7] α-MSH

The iodination procedure was performed in a radioactive fume hood. 10mCi of sodium iodide (^{125}INa, ICN Biomedicals, inc.) was added to the Eppendorf containing 1.5µl peptide in 25µl phosphate buffer. The reaction was initiated by the addition of 10µl of freshly prepared chloramine T. After exactly 30 seconds the reaction was stopped by the addition of 0.6ml of 0.25% BSA.

Purification of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH

The freshly iodinated peptide was loaded on to the pre-conditioned C18 column and the initial elutent was discarded. The column was washed twice with 0.8ml of phosphate buffer, again discarding the elutent. The column was then washed X4 with 1ml of 50% methanol/1% TFA in water, followed by washing X2 with 1ml of 60% methanol/1% TFA in water. The elutent from these washing procedures was pooled together and saved.

The peptide was further purified by HPLC using a C18 reverse-phase wide pore (300Å) column, 25cm length and 4.6mm diameter. The washes retained from the initial purification were loaded on to the column (1ml/minute, normal flow rate) and run with an exponential gradient starting at 95% A (1%TFA in water), 5% B (70% acetonitrile/1% TFA) and reaching 40% A, 60% B after 50 minutes before returning to the original proportions. The procedure was monitored by UV detection at 217nm. Fifteen 1ml fractions were collected 27 minutes from the start of the gradient and assayed on a gamma counter (LKB wallac 1277 gammamaster). The mono-iodinated peptide required was contained in only one or two fractions separated from the more lipophilic di-iodinated peptide which eluted later, and the free ^{125}INa which came off earlier. The iodinated peptide, [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH was stored at -20°C for up to 2 weeks.

6.2.3. Binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH at 4°C

Binding studies were performed using a Millipore Multi Screen Assay System (Roessler *et al.*, 1993). The cells were grown to confluence on 6-well plates and removed from the culture wells using the trypsinisation method described previously (see section 4.2.1). The cells were counted using a haemocytometer chamber and found to be greater than 95% viable by trypan exclusion (see section 2.1.4). The cells were diluted to ensure that 100µl of cells were plated on to 96 well membrane filtration units (multiscreen-HV) at a density of between 1 x 10^5 cells /well and 5 x 10^5 cells /well in complete culture media.

After incubation for up to 1 hour at 37°C, the medium was removed using a vacuum manifold supplied with the system and washed 3 times with PBS at 4°C. The plates were incubated overnight at 4°C with the appropriate concentration of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH diluted in freshly prepared binding buffer (serum-free RPMI 1640 medium, with 25mM HEPES and 0.2% BSA) in the presence and absence of a 1000-fold excess unlabelled [Nle^4, D-Phe^7] α-MSH. Preliminary binding assays were performed using 1 x 10^5 cells /well with two concentrations of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH. Binding isotherms were performed using 2 x 10^5 cells /well and 5 x 10^5 cells /well with increasing concentrations of ligand.

After incubation the cells were washed 3 times with PBS at 4°C and the membrane disks were punched out, using an 8-well punch system with disposable tips, directly into gamma tubes (LP4, Western Laboratories U.K.). Radioactivity was counted on a gamma counter. Between three and six replicates were assayed for each concentration.

6.2.4. Binding and Uptake of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH at 37°C

Binding isotherms at 37°C were performed using one concentration of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH incubated over various time intervals, between 15 minutes and 4 hours. The cells were plated on to the 96 well membrane filtration units at a density of between

1 x 10^5 cells/well and 5 x 10^5 cells/well in complete culture medium. A separate plate was required for each time point.

The plates were incubated at 37°C for up to 1 hour and the medium was then removed using the vacuum system. The cells were washed 3 times with PBS, pre-warmed to 37°C prior to use, and incubated with 1 x 10^{-10} M, [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH in binding buffer, in the presence and absence of 1000-fold excess unlabelled [Nle^4, D-Phe^7] α-MSH, at 37°C. The plates were incubated at 37°C for various time intervals.

After the appropriate time interval the plate was removed from the incubator and washed 3 times with pre-warmed PBS. At this point some of the cells received an acid wash in order to determine the extent of uptake of the peptide. These cells were exposed to [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH with no cold ligand present. The acid wash required a 5 minute incubation with pre-warmed citrate buffer pH2.5 consisting of 21.01g citric acid.H_2O, 5.43g NaCl and 23.44ml of 1M NaOH, made up to 1litre with distilled water. The cells were washed a further twice with PBS, and the membrane discs were punched out into the gamma tubes and counted on the gamma counter.

This procedure was repeated for each time point, with between three and six replicates being assayed.

6.3. Results

6.3.1. Iodination of [Nle^4, D-Phe^7] α-MSH

The identity of the peptide [Nle^4, D-Phe^7] α-MSH was confirmed by FAB mass spectrometry: $M+H^+$ calculated weight 1646.8, determined weight 1647. Following the iodination procedure the peptide was purified using a pre-conditioned C18 reverse phase column and further purified by HPLC. Figure 61 shows a characteristic HPLC elution profile which produced three peaks corresponding to non-iodinated, mono-iodinated and di-iodinated peptide. Sixteen 1ml fractions were collected from the column at 25 to 40 minutes after injection The activity of the fractions containing the iodinated peptide, which was measured on the gamma counter corresponded with the latter two peaks on the HPLC trace. The first peak showed no significant activity.

6.3.2. Binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH at 4°C

Preliminary binding studies were performed on murine brain microvascular endothelial cells (MBMVEC) in order to investigate the possibility that melanocortin receptors were present on the surface of brain endothelial cells. Prior to the incubation with [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH, the cells were incubated in complete culture medium in order to ensure that the cells had recovered from the trypsinisation procedure. Initial experiments were performed using two single concentrations of ligand, 1×10^{-10} M and 5×10^{-10} M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH and incubated with 1×10^5 cells/well at 4°C overnight (approximately 18 hours) in an attempt to ensure that equilibrium was reached.

Each preliminary study confirmed the existence of some specific binding (Figure 62). Total binding was expressed as counts per minute (CPM) of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-

MSH and non-specific binding was expressed in CPM and determined in the presence of a 1000-fold excess of unlabelled [Nle^4, D-Phe^7] α-MSH. The specific binding was obtained by subtracting non-specific binding from total binding. Preliminary binding studies were also performed on lung microvascular endothelial cells, however these studies showed no evidence of specific binding (Figure 63).

Although preliminary binding studies with brain microvascular endothelial cells demonstrated specific binding, it was relatively low. This low specific binding could be due to a low number of receptors being expressed per cell or, alternatively, it could be as a result of a low binding affinity. The preliminary binding assays also exhibited a relatively high amount of non-specific binding. This high non-specific binding was shown even in the presence of 0.2% BSA in the binding medium which was required to coat the plastic well and the filter, and therefore decrease the extent of non-specific binding. Similar binding studies involving human melanoma cells have also showed high non-specific binding (Eberle *et al.*, 1991).

The presence of an increased number of cells on the filter may lower the non-specific binding, as the area of the filter exposed to the ligand would be decreased. In an attempt to improve the specificity of the study and reduce the extent of non-specific binding to the plastic well and the filter, binding isotherms were performed where the number of cells per well was increased to 2 x 10^5 cells/well (Figure 64) and 5 x 10^5 cells/well (Figure 65).

Cell Density	1 x 10^{-10}M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH	5 x 10^{-10} M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH
1 x 10^5	304.44 ± 45.7	1771.62 ± 121.68
2 x 10^5	398.82 ±13.48	1357.32 ± 94.5
5 x 10^5	**	1658.08 ± 72.6

Table 1. The effect of increasing cell density on the non-specific binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH, in the presence of 1000-fold excess unlabelled [Nle^4, D-Phe^7] α-MSH, to MBMVEC. Each data point is expressed in CPM and represents the mean and standard deviation of five samples.

Table 1 shows the proportion of binding designated as non-specific binding for concentrations of 1 x 10^{-10} M and 5 x 10^{-10} M in the presence of 1000-fold excess of the unlabelled ligand. The extent of non-specific binding was variable however, it did not show an overall significant decrease as the cell number increased. With 1 x 10^{-10} M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH there was no significant difference between 1 x 10^5 cells/well and 2 x 10^5cells/well however, at 5 x 10^{-10} M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH, the extent of non-specific binding decreased significantly between 1 x 10^5 cells/well and 2 x 10^5cells/well, but not between 2 x 10^5 cells/well and 5 x 10^5cells/well. This was determined using a two sample t-test and pooled variences after checking with an F-test. The non-specific binding therefore, may still be attributed to binding to the plastic and the filter however there may also be some non-specific binding to the cell surface.

Figures 62 and 64 showed that the specific binding in the presence of 2 x 10^5cells per well was significantly greater than that determined in the presence of 1 x 10^5 cells per well in the preliminary studies, at concentrations of 1 x 10^{-10}M and 5 x 10^{-10} M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH (Table 2). This was determined using a non-parametric test to calculate to confidence intervals for the difference in medians. The difference in specific binding obtained with studies using 5 x 10^5 cells per well (Table 2) however, was not significantly greater than the specific binding obtained with studies using 2 x 10^5 cells per well. It should be remembered that it is necessary to carry out these experiments using cells obtained from different isolations and therefore a degree of variability in the receptor number might be expected. With B16 cells, the number of receptors per cell has been shown to be variable (Sahm *et al.*, 1994).

Cell Density	**1 x 10^{-10}M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH**	**5 x 10^{-10} M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH**
1 x 10^5	107.36	645.44
2 x 10^5	297.02	1119.86
5 x 10^5	**	1398.1

Table 2. The effect of increasing cell density on the specific binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH to MBMVEC. Each data point is expressed in CPM and was calculated by subtracting non-specific binding from total binding.

The saturation binding data was analysed by MINSQ, a non-linear regression analysis computer program, in order to estimate the number of receptors per cell and the binding

affinity. The formation of ligand-receptor complexes are governed by the Langmuir isotherm model. The relationship between the amount of ligand bound to the receptor and the ligand concentration can be expressed as:

$$Ka = \frac{[HR]}{[H](n-[HR])}$$

where:

[H] = concentration of free hormone

[HR] = concentration of ligand-receptor complex

[R] = concentration of free receptor

Ka = association constant ([HR]/[H][R])

n = total number of receptors

Kd = dissociation constant (1/Ka)

One approach to determining the total number of binding sites, n, and the affinity constant, Ka is to construct a Scatchard plot showing bound/free against bound. However in this study binding isotherms did not start to plateau and therefore although a fit was obtained, any analysis of the data from the Scatchard plot is not conclusive (Figure 66). An estimation of the binding affinity and cell number from the isotherm shown in Figure 64 using MINSQ suggested a dissociation constant (Kd) of 3.1×10^{-8}M with the number of receptors per cell being approximately 120, but again this is not conclusive. The outcome of these results will be discussed in section 6.4.

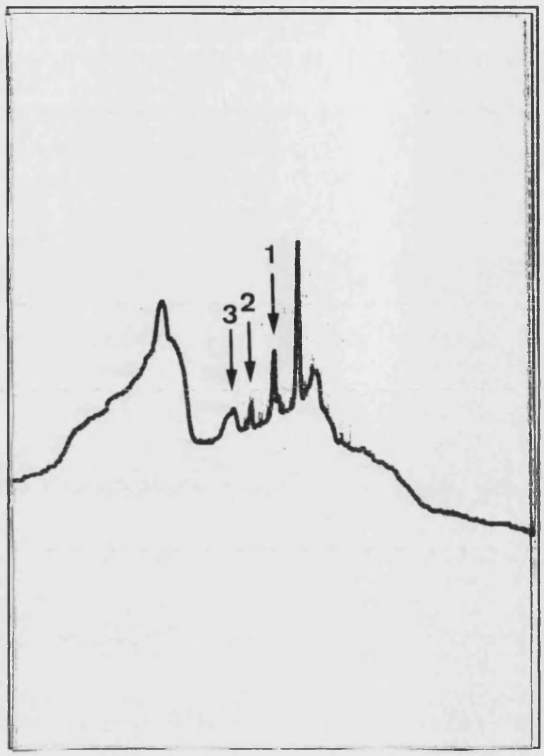

Figure 61. A characteristic HPLC trace from the purification of the reaction products from the iodination of [Nle^4, D-Phe^7] α-MSH as measured by UV absorption. Peaks 1, 2 and 3 represent non-labelled, mono-iodinated and di-iodinated peptide respectively.

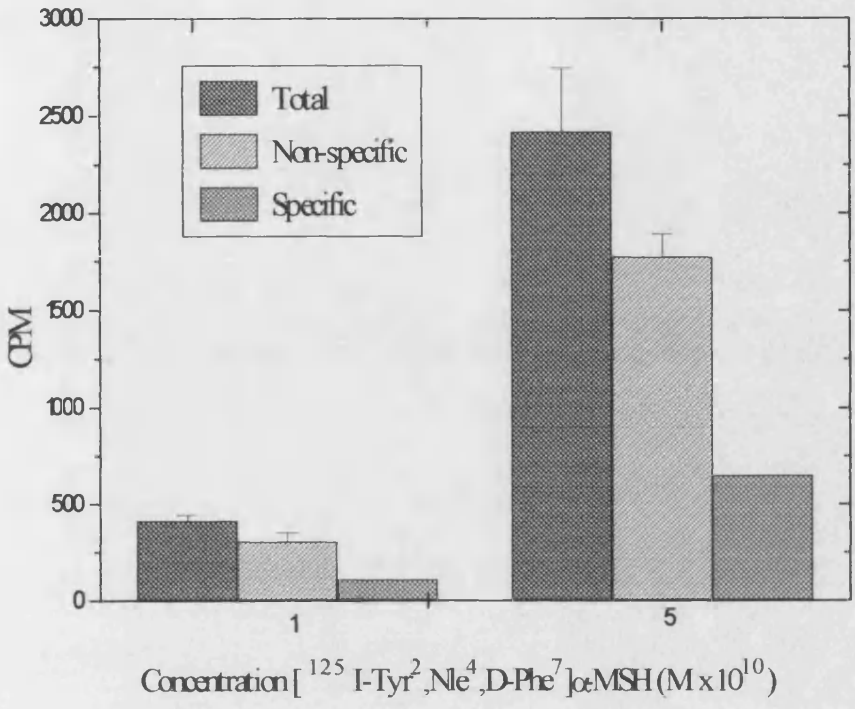

Figure 62. A preliminary study showing the binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH to murine brain microvascular endothelial cells (1 x 10^5 cells per well) at 4°C, using two different concentrations of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH. Each data point represents the mean and standard deviation of five wells.

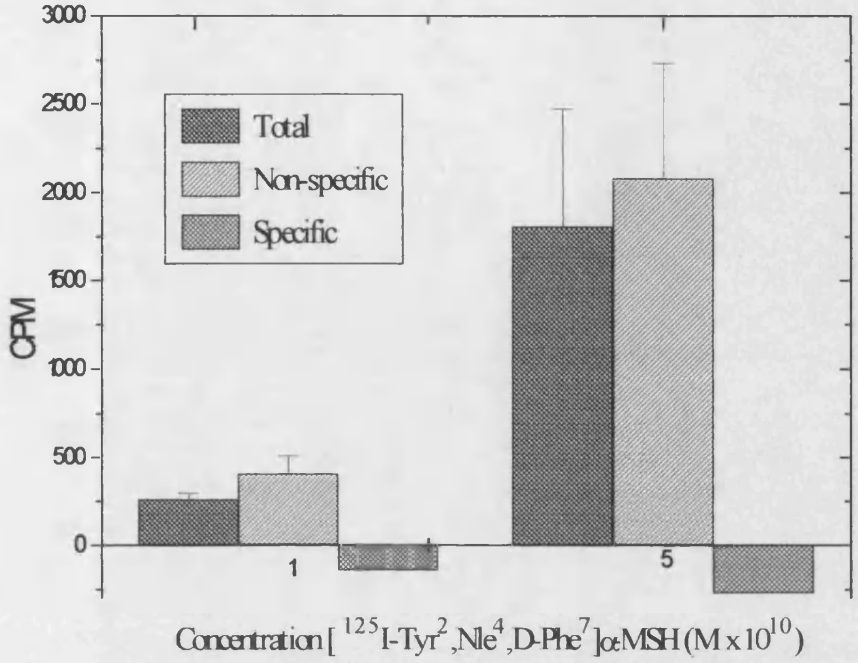

Figure 63. A preliminary binding study of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH to murine lung microvascular endothelial cells (1 x 10^5 cells per well) at 4°C, using two different concentrations of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH. Each data point represents the mean and standard deviation of five wells.

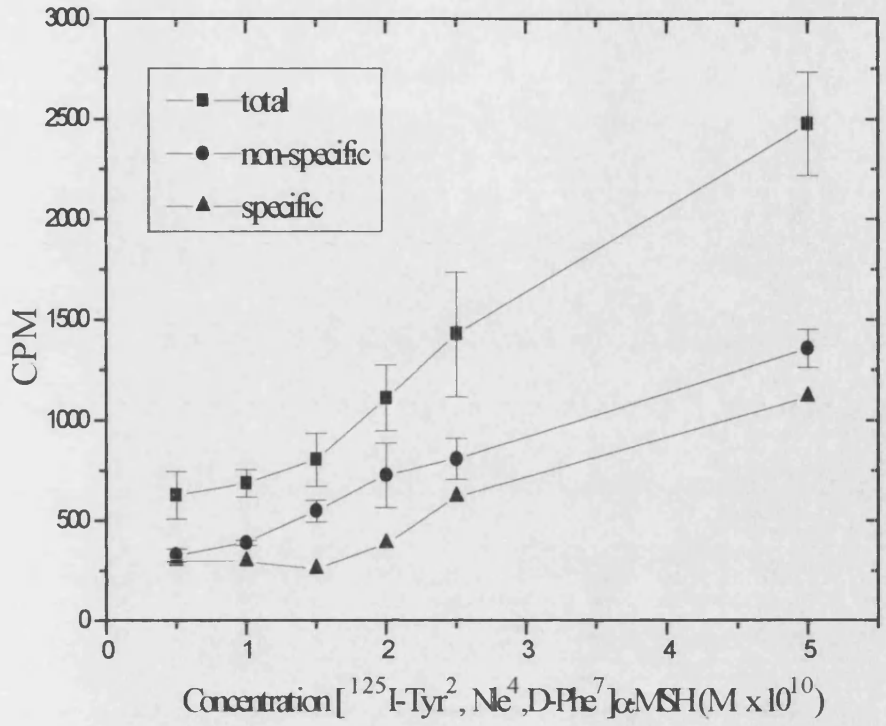

Figure 64. A binding isotherm of [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH to MBMVEC (2 x 10^{5}cells/well) at 4°C. Each data point represents the mean and standard deviation of five wells.

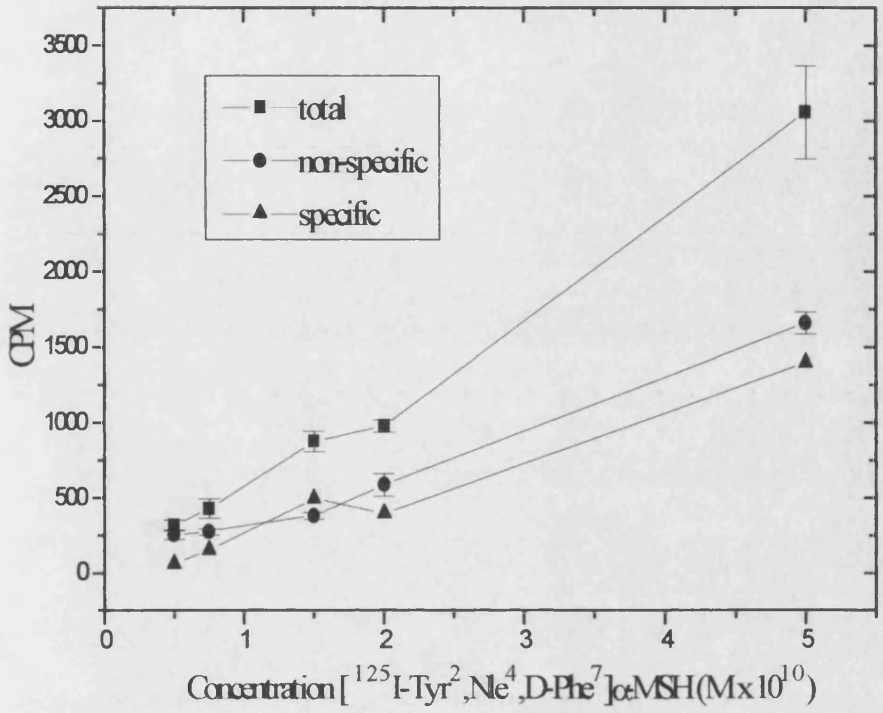

Figure 65. A binding isotherm of [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH to MBMVEC (5 x 10^{5}cells/well) at 4°C. Each data point represents the mean and standard deviation of five wells.

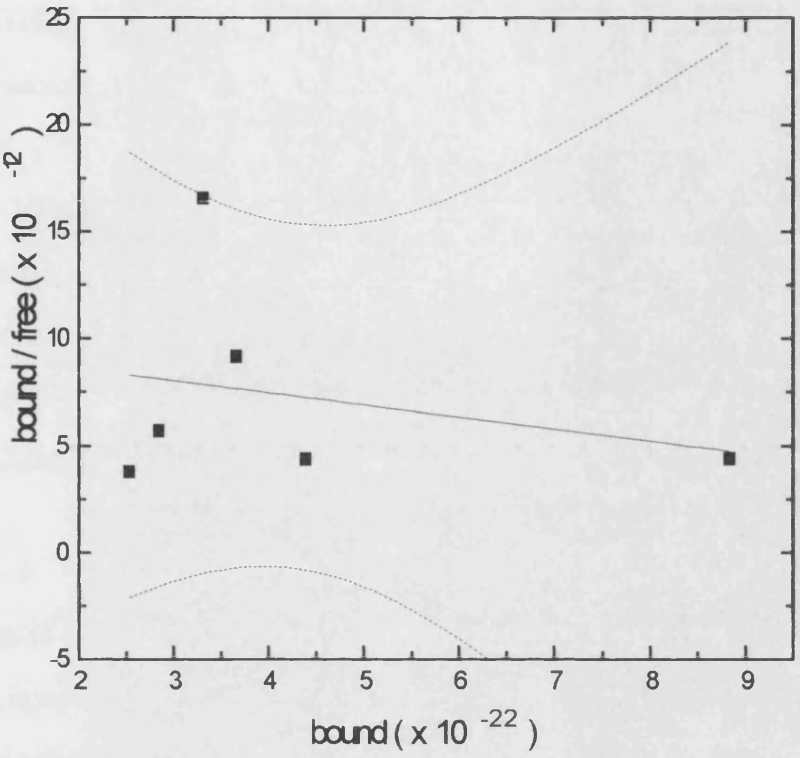

Figure 66. A Scatchard Plot analysis of the binding isotherm shown in Figure 64, showing bound [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH/free [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH (moles / cell) against bound [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH (moles / cell) with 95% confidence limits.

6.3.3. Binding and Uptake of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH at 37°C

The binding and uptake of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH by murine brain microvascular endothelial cells was studied at 37° C. The cells were incubated with 1 x 10^{-10}M [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH for between 15 minutes and 4 hours before being washed with PBS. After this time some cells were exposed to an acid-wash treatment in order to remove the extracellular bound ligand. This treatment provided a method of determining the extent of uptake of the iodinated peptide into the cells, because any remaining radioactivity associated with the cells would have to have been internalised (Richards 1992).

Figure 67 demonstrates the binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH to brain endothelial cells at 37° C. At 37° C, the specific binding represents with amount of ligand associated with the cell. This includes the amount of ligand bound to the cell surface and the amount of ligand internalised by the cell. The specific binding, expressed in counts per minute (CPM), increased rapidly during the initial 30 minute incubation. The specific binding increased further over the subsequent 30 minutes reaching a maximum of 163.6 CPM. After this time no further increase in the specific binding was demonstrated, a plateau was reached and a very gradual decrease was observed (Table 3).

Initially, the non-specific binding was found to be lower than the specific binding. The non-specific binding was however found to increase gradually during the incubation period until the specific and non-specific binding were the same (Table 3). This non-specific binding is possibly associated with the filter, although a proportion of it could be associated with non-specific binding to the cell surface.

The cells were exposed to an acid wash treatment in order to remove any extracellular bound ligand be it by specific or non-specific binding, therefore the values obtained represent the internalised peptide (Table 3). The data from the acid washed cells

displayed similar characteristics to the specific binding curve, that is, during the initial incubation period an increase in radiolabelled peptide was observed. This reached a plateau after 30 minutes incubation. As expected, the amount of radioactivity associated with the acid washed cells was less than that associated with specific binding, as specific binding represented the amount of peptide bound plus the amount internalised, whereas acid washed data represents peptide internalised only. This suggests that at 37°C [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH was internalised by brain microvascular endothelial cells, but unlike B16 cells (Richards 1992) it did not appear to be degraded.

Incubation Time	**Total binding (CPM)**	**Non-specific (CPM)**	**Acid-wash (CPM)**	**Specific (CPM)**
30 minutes	199.5 ± 49.2	78 ± 13.8	100.1 ± 13	121.5
60 minutes	265 ± 29.4	102.7 ± 4.1	103.5 ± 4.17	163.6
180 minutes	355 ± 114.8	168.7 ± 14.2	152.2 ± 6.8	161.8

Table 3. Incubation of murine brain microvascular endothelial cells with [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSHat 37° C.

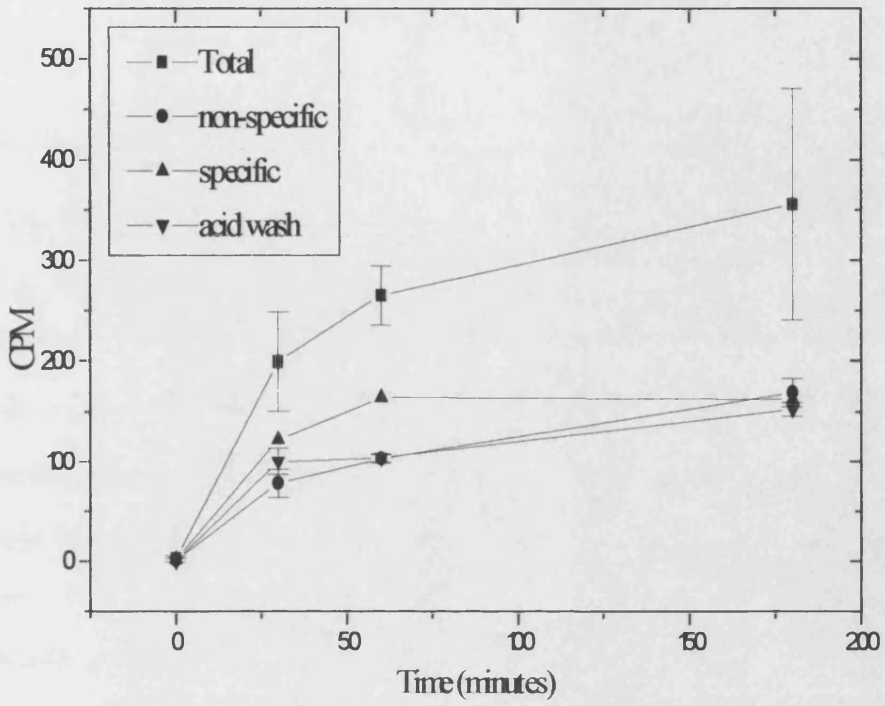

Figure 67. A binding isotherm of 1 x 10^{-10}M [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH to MBMVEC (5 x 10^{5}cells/well) at 37° C. Each data point represents the mean and standard deviation of five wells.

6.4. Discussion

Iodination of [Nle^4, D-Phe^7] α-MSH

Following the iodination of [Nle^4, D-Phe^7] α-MSH, the iodinated products were purified by HPLC, revealing three peaks of which two were identifed as having high amounts of radioactivity. This elution profile correlates with that described by Eberle (1988). The first peak represented the non-iodinated peptide, the second and third peaks represent the mono-iodinated and di-iodinated peptides respectively. The mono-iodinated peptide being less lipophilic is eluted before the di-iodinated peptide. Iodination and purification of [Nle^4, D-Phe^7] α-MSH by the methods described in this study produced a reproducible yield of mono-iodinated peptide.

Binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH at 4°C

[^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH is a super potent analogue of α-MSH due to prolonged action on reptilian/amphibian bioassays which has been shown to possess a binding affinity and biological activity ten times that of α-MSH (Eberle 1988). It has been widely used in the study of the isolation and activity of the MSH receptors as its high activity allows the detection of low receptor populations (Sahm *et al.*, 1994; Dyer *et al.*, 1993; Rosselli-Rehfuss *et al.*, 1993; Gantz *et al.*, 1993a; Ahmed *et al.*, 1992; Mountjoy *et al.*, 1992; Lunec *et al.*, 1992; Leiba *et al.*, 1990).

The concentrations of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH were selected for this study on the basis of the binding affinity of the peptide to the α-MSH receptor in B16 cells. Although increasing the concentration of the peptide has been shown to give a more reliable binding affinity it also increases the non-specific binding therefore a compromise concentration was reached (Erskine 1993). At 4°C binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7]

α-MSH is limited to the cell membrane, it is not internalised, (Richards 1992) and the receptor ligand complex is not degraded (Siegrist *et al.*, 1992). The binding isotherms conducted with 2 x 10^5cells per well and 5 x 10^5cells per well indicated that the extent of specific binding is very low and that there was also a high proportion of non-specific binding. This is not unusual, as some human melanoma cells have been shown to exhibit low specific binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH (Eberle *et al.*, 1991) and the analogue ^{125}I-[Nle^4] α-MSH (Eberle *et al.*, 1991; Siegrist *et al.*, 1992). In both cases, the specific binding is less than the non-specific binding.

No specific binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH was observed with murine lung microvascular endothelial cells suggesting that microvascular endothelial cells are heterogeneous with respect to organ specificity.

The theory of Scatchard analysis of a binding isotherm is that it provides a method of determining the number of receptor populations and estimating the binding affinity and number of binding sites. Several assumptions must be made when using Scatchard analysis (Bolander 1989):

1. The labelled hormone is biologically identical to the native hormone.
2. The labelled hormone is homogeneous.
3. The receptor is homogeneous.
4. The receptor acts independently.
5. The receptor is unoccupied prior to the incubation.
6. The reaction is at equilibrium.
7. There is no specific non-receptor binding.

In this study, Scatchard analysis was inconclusive. Although the points lie within the 95% confidence limits and a line can be fitted which suggests one population of

receptors, the fit is not satisfactory. This is typical of experimental data prepared with cells with low numbers of receptors and high non-specific binding.

The value for the dissociation constant Kd obtained using MINSQ was estimated at 31nM. This value can be compared to the values of Kd obtained for binding to the melanocortin receptor expressed in B16 melanoma cells (MC1-R) (0.48±0.08nM, Sahm *et al.*, 1994), rat hypothalamus (MC3-R) (1.66nM, Sahm *et al.*, 1993) and to extraorbital rat lacrimal gland tissue (2.2±0.2nM, Leiba *et al.*, 1990). This may indicate that the binding affinity of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH for the receptor expressed on brain microvascular endothelial cells is less than that obtained for other melanocortin receptors. In comparison, the dissociation constant obtained for the binding of transferrin to receptors expressed on liver endothelial cells has been demonstrated to be 0.189μM (Tavassoli 1988) indicating a weak binding affinity. [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH recognises various melanocortin receptors with different binding affinities therefore, the expression of an alternative melanocortin receptor on brain endothelial cells may account for the different binding affinity.

If the binding affinity of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH for the receptor on brain endothelial cells is relatively low in comparison with other melanocortin receptors therefore, the low amount of specific activity could be due in part to the binding affinity but it may also be due to the very low receptor number. The estimated receptor number is 120 receptors per cell, this is low in comparison to D10 human melanoma cells which have an estimated 966 receptors per cell (Siegrist *et al.*, 1992) and B16 cells where the number of receptors per cell has been shown to vary from 5,000 to 20,000 (Sahm *et al.*, 1994). The variability in the specific binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH to brain microvascular endothelial cells with different cell densities (Table 2) could be as a result of the variability in the number of receptors expressed per cell, as shown with B16 cells, or due to the experimental variability associated with the low affinity or low receptor number.

Binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH at 37°C

At 37°C, where the metabolic processes are functioning, binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH reached a maximum after 60 minutes, thereafter specific binding reached a steady-state plateau. This specific binding represents the surface bound and internalized ligand. The proportion of specific binding that was associated with the internalised ligand was determined by acid wash at each time point during the incubation at 37°C; this follows a similar pattern to the specific binding.

When comparing the binding of brain endothelial cells at 37°C with other cell types, the incubation of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH with B16 cells at 37°C shows a large increase in the specific binding, reaching a maximum after 30 minutes and decreasing thereafter. The reason for this decrease is unknown, however, it could be explained by intracellular degradation of the ligand or dissociation of the ligand from the receptor. The measure of internalisation by acid wash, demonstrated a gradual increase reaching a maximum after 60 minutes declining gradually thereafter (Richards 1992). A similar pattern of uptake at 37°C was demonstrated with the binding of radio-iodinated transferrin to rat liver endothelial cells (Kd 0.189μM and 1.8 x 10^6 receptors per cell). The amount of internalised ligand increased rapidly for the initial 15 minutes declining gradually thereafter; this was explained as being due to the rate of externalisation exceeding the internalisation as a result of down regulation of the receptors (Tavassoli 1988).

In the present study, this pattern of uptake was not demonstrated during the binding of [^{125}I-Tyr^2, Nle^4, D-Phe^7] α-MSH with brain microvascular endothelial cells at 37°C. This was possibly due to the low receptor number which would not be able to take up the ligand in sufficient quantities to produce this large initial increase. The pattern of uptake demonstrated in this study could suggest internalisation and transport across the cell where the receptor is recycled and the ligand is externalised intact as a steady state is reached.

The transport processes associated with large vessel and microvessel endothelial cells are heterogeneous (see section 1.1.3). In addition, the microvascular endothelial cells of the blood-brain barrier possess several unique features which provide a very selective barrier to macromolecules (see section 1.1.13). Transcytosis through the blood-brain barrier occurs by intracellular routes: fluid-phase endocytosis, adsorptive transcytosis and receptor-mediated transcytosis.

In this study, the identification of a receptor expressed on the surface of brain microvascular endothelial cells which specifically binds [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH, is thought to internalise the receptor-ligand complex which may be transported across the cell by receptor mediated transcytosis. It is also possible that a proportion of the internalised ligand may be taken up by fluid-phase endocytosis or non-specific adsorptive transcytosis. This could be estimated using the fluid-phase marker horse-radish peroxidase (HRP) or a molecule taken up by adsorptive endocytosis such as wheat germ agglutinin (WGA), a lectin which binds to carbohydrate molecules on the cell surface (Broadwell 1993).

7. PHOTOAFFINITY LABELLING OF MURINE BRAIN MICROVASCULAR ENDOTHELIAL CELLS.

7.1. Introduction

Photoaffinity labelling is an important technique in the identification, isolation and characterisation of hormone receptor proteins (Eberle 1988). Previously, it has been used to demonstrate the existence of α-MSH receptors on various murine and human melanoma cells (Ahmed *et al.*, 1992; Siegrist *et al.*, 1992) and primary cultures of rat Schwann cells (Dyer *et al.*, 1993). In this study the mono-iodinated photoaffinity probe [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}, ATB-Lys^{11}] α–MSH was used to label the receptor proteins on murine brain microvascular endothelial cells using the method of Ahmed *et al.*, (1992) which described the photoaffinity labelling of B16 melanoma cells. This was followed by SDS-PAGE analysis and autoradiography.

7.2. Materials and Methods

7.2.1. Synthesis of [Nle^4, D-Phe^7, ATB-Lys^{11}] α–MSH

The photoaffinity peptide [Nle^4, D-Phe^7, ATB-Lys^{11}] α–MSH was synthesised by Dr G. W. J. Olivier at the University of Bath. A detailed protocol of the peptide synthesis is described by Ahmed *et al* (1992).

7.2.2. Iodination of [Nle^4, D-Phe^7, ATB-Lys^{11}] α–MSH

The peptide [Nle^4, D-Phe^7, ATB-Lys^{11}] α–MSH was iodinated at the Tyr^2 by Dr G. W. J. Olivier using the method of Eberle *et al* (1988) detailed in section 6.2.2. The mono-iodinated peptide was used in all experiments. All synthesis and iodination procedures were performed in a dark room under red safety light.

7.2.3. Photoaffinity Labelling of Brain microvascular Endothelial Cells

The following procedure described for the photoaffinity labelling of murine brain microvascular endothelial cells was adapted from the procedure described by Ahmed *et al* (1992) for the labelling of B16 melanoma cells.

Tissue culture

The cells used for photoaffinity labelling were grown on 6-well plates and subcultured using the standard trypsinisation procedure for brain microvascular endothelial cells (see section 4.2.1). Four confluent 6-well plates at passage 1, grown under routine conditions, were used for the photoaffinity labelling studies.

Preparation of stock solutions.

0.5M Tris-HCl buffer pH6.8 was prepared by dissolving 12.1g of tris base (Bio-Rad Labs) in 150ml of distilled water. The pH was adjusted to 6.8 with 6M HCl and made up to a final volume of 200ml with distilled water.

1.5M Tris-HCl buffer pH8.8 was prepared by dissolving 36.3g of tris base in 150ml of distilled water. The pH was adjusted with 6M HCl and made up to 200ml with distilled water.

A solubilization buffer was prepared as follows:

10mM Tris-HCL, pH 7.4.
1.5% Triton X-100, (Sigma)
1mM phenylmethanesulphonyl fluoride (PMSF,Sigma),
1mM EDTA
1μg/ml leupeptin (Sigma)

20% (w/v) sodium dodecylsulphate SDS (BDH chemicals) was prepared by dissolving 20g SDS in distilled water with gentle stirring. The SDS solution was made up to 100ml with distilled water and stored at room temperature.
The Acrylamide/Bis (Bio-Rad Labs) solution was prepared by dissolving 29.2g Acrylamine and 0.8g N'N'-bis-methylene-acrylamide in 100ml distilled water. The final solution was filtered and stored at 4°C in the dark.

An SDS sample buffer was prepared as follows:

62.5mMTris-HCL pH6.8,
2%(w/v) SDS,
10% (v/v) glycerol,(Fisons Ltd.)

0.001% Bromophenol Blue,(Fisons Ltd.)

5% (v/v) 2-mercaptoethanol (Sigma)

The electrode (running) buffer was prepared as a 5X concentrated solution consisting of 25mM Tris, 192mM glycine and 1% SDS at pH8.3 and diluted with distilled water for each run.

0.1% Coomassie Brilliant Blue staining solution was prepared from 0.5g Coomassie blue with the addition of 40% (200ml) methanol, 10% (50ml) acetic acid and 50% (250ml) distilled water.

The de-staining solution was made up of 40% methanol, 10% acetic acid and 50% distilled water.

Photoaffinity labelling

All manipulations with the photoaffinity probe prior to UV irradiation were conducted in the dark. The cell samples were washed 3 times in freshly prepared binding buffer (serum-free RPMI 1640 medium, with 25mM HEPES and 0.2% BSA), and the cells were incubated, at 4°C, with the radiolabelled photoaffinity probe [^{125} I-Tyr^{2}, Nle^{4}, D-Phe^{7}, ATB-Lys^{11}] α–MSH at a concentration of 2 x 10^{-10}M. The cells were incubated in the presence and absence of 1000-fold excess of the unlabelled [Nle^{4} D-Phe^{7},] α–MSH.

After a 4 hour incubation the cells were washed and then irradiated for 5 minutes on ice, with UV light of wavelength 320nm, thereafter all procedures were performed at the bench under normal light conditions After UV treatment the cells were scraped from the wells, suspended in PBS and washed by centrifugation at 1600g for 10 minutes to remove any unbound ligand.

The cells were resuspended in solubilization buffer at 4°C for 1 hour and ultracentrifuged at 30 000 g for 1 hour to remove any non-solubilised material. The solubilised receptor

complexes were stored at -20°C until required for SDS/polyacrylamide gel electrophoresis.

SDS/polyacrylamide gel electrophoresis.

SDS/PAGE was performed using the Laemmli discontinuous buffer system (1970) using 10% acrylamide monomer in the separating gel and 4% in the stacking gel. The gels were prepared according to the following protocol:

	Separating Gel	**Stacking Gel**
Distilled water	20.1ml	6.1ml
1.5M Tris-HCl	12.5ml	-
0.5M Tris-HCl	-	2.5ml
20% SDS	0.25ml	0.1ml
Acrylamide/Bis	16.65ml	1.3ml
10% ammonium persulphate	0.5ml	0.1ml
TEMED	25µl	10µl.

The 10% ammonium persulphate (APS) was freshly prepared in distilled water on the day of use. The APS and TEMED (Bio-Rad) cause cross linking of the acrylamide and therefore were added to the solutions just prior to casting the gels.

The gel electrophoresis apparatus was set up according to the manufacturers instructions (PROTEAN II apparatus, Bio Rad) to give a 0.75mm thick gel. The separating gel was cast and allowed to set for 1 hour, thereafter the stacking gel was prepared, cast and allowed to set.

SDS sample buffer was added to the samples and left to solubilise at room temperature for approximately 30 minutes. The samples were loaded on to the gel and

electrophoresed in parallel with ^{14}C pre-labelled molecular mass markers, in order to determine the apparent molecular mass of the samples.

The markers used were myosin (200kDa), phosphorylase B (97.4kDa), BSA (69kDa), ovalbumin (46kDa), carbonic anhydrase (30kDa) and lysosyme (14.3kDa). Each of these markers was stained with Coomassie blue and heated for a minute before loading on to the gel.

Following electrophoresis, the gel was stained with Coomassie blue for 30 minutes, de-stained and dried in a gel drier (Bio-Rad) for 1 hour. The gel was exposed on a pre-flashed X-ray film (Hyperfilm MP, Amersham) with an intensifying screen (Hyperscreen, Amersham) for 3-5 days at -70°C.

7.3. Results

Photoaffinity labelling studies were performed on murine brain microvascular endothelial cells using the photoaffinity probe [^{125}I-Tyr^{2} Nle^{4} D-Phe^{7} ATB-lys^{11}] αMSH which has been shown to bind specifically to B16 melanoma cells (Ahmed *et al.*, 1992). These studies were carried out in the presence and absence of an excess of a 1000-fold excess of the unlabelled peptide [Nle^{4}, D-Phe^{7}] α–MSH, in order to demonstrate specific binding. SDS-PAGE analysis of the solubilized membrane proteins in the presence of the radioiodinated, photoaffinity labelled peptide only, resulted in the identification of three sharp bands (Figure 68). With two of these bands, labelling was reduced but not completely abolished with an excess of unlabelled peptide, however with one of these bands labelling was not present when the cells were incubated with an excess of unlabelled peptide. This latter band appeared at an apparent molecular mass of approximately 45kD, which is similar to the molecular mass for the receptor on B16 and other melanoma cells (Ahmed *et al.*, 1992) and for primary rat Schwann cells which are of non-melanocyte origin (Dyer *et al.*, 1993).

In Figure 68, the single lane 8 on the right of the gel shows the molecular mass markers. Lanes 1 and 2 respectively in the SDS-PAGE analysis shows the presence of proteins crosslinked with the radioiodinated, photoaffinity labelled peptide in the presence and absence of 1000-fold excess unlabelled peptide. Lanes 5 and 6 are replicates of lanes 1 and 2.

The two other strong bands had apparently higher molecular masses of approximately 57kDa and 90kDa. These bands may be due to non-specific labelling of proteins or non-specific labelling of multiple aggregates of the receptor which could be explained by the mild conditions of solubilization used (Ahmed *et al.*, 1992).

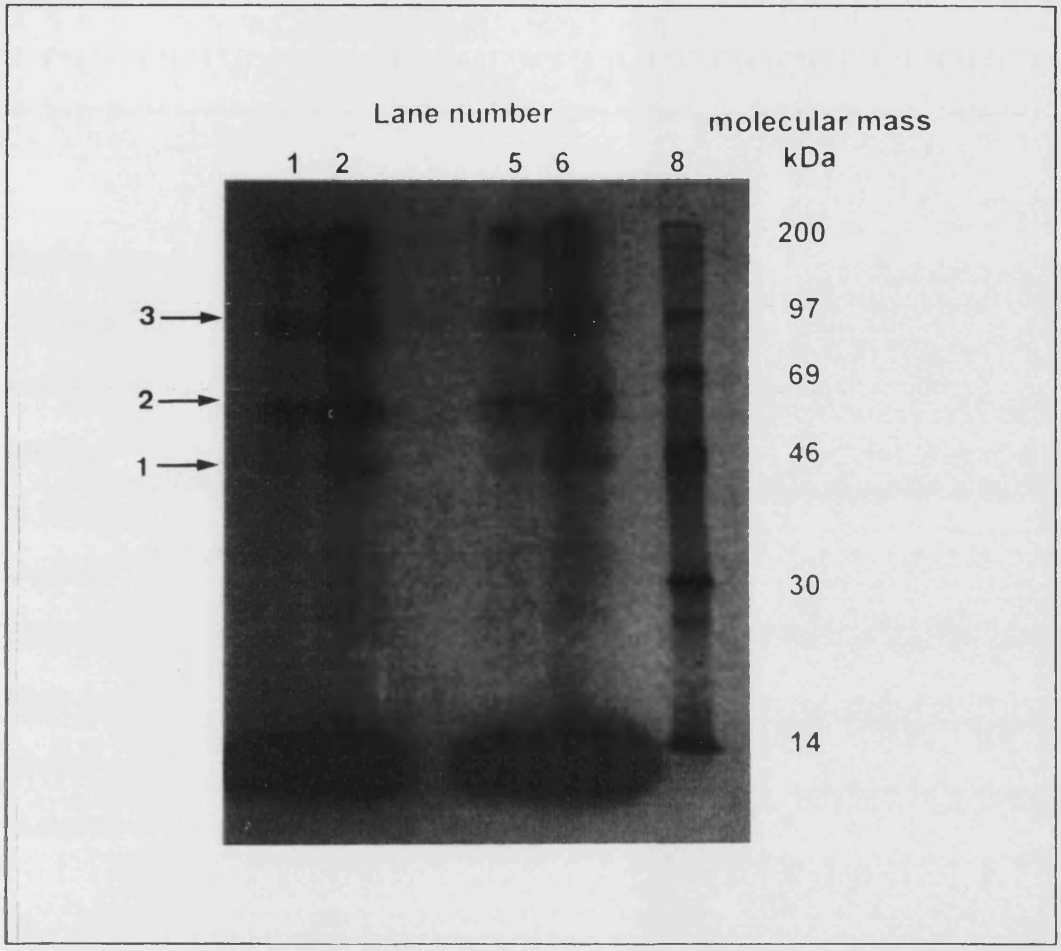

Figure 68. SDS-PAGE analysis of murine brain microvascular endothelial cells. Lanes 1 and 2 respectively represent the photoaffinity labelled proteins in the presence and absence of 1000-fold excess unlabelled peptide. Lanes 5 and 6 are replicates of lanes 1 and 2. Arrow 1 indicates the specific labelling of a protein with an apparent molecular mass of 45 kDa. Arrows 2 and 3 represent non-specific labelling of membrane proteins or multiple aggregates of the receptor.

7.4. Discussion

Further investigations into the expression of the receptors on the endothelial cells were conducted in the form of photoaffinity labelling. This is a useful tool for identification and isolation of receptors. These studies support the initial evidence from the binding analysis that a receptor for α-MSH was expressed. A distinct band, which was not seen in the presence of excess unlabelled peptide, at an apparent molecular weight of 45 kD suggests the expression of a specific receptor with a similar molecular weight to that observed with B16 melanoma cells and primary rat Schwann cells (Ahmed *et al.*, 1992; Dyer *et al.*, 1993). The assumption is made that the radio-iodinated, photoaffinity labelled peptide [^{125}I-Tyr^{2} Nle^{4} D-Phe^{7} ATB-lys^{11}] αMSH has the same affinity for the receptor as the unlabelled peptide [Nle^{4} D-Phe^{7}] α–MSH.

The presence of a higher molecular weight band at 57kDa thought to be due to non-specific binding has also been identified by SDS-PAGE analysis of B16 cells using the same photoaffinity probe (Ahmed *et al.*, 1992). The 90kDa band however, could represent a multiple aggregate of the receptor as this would possess a molecular mass of approximately 88kDa, that is twice the molecular mass of the receptor (43kDa) together with the molecular mass of the peptide (2kDa).

It is possible that a family of neural melanocortin receptors exists which could account for the diverse pattern of MSH binding exhibited throughout various regions of the brain and the diverse functions of the melanocortin peptides (Eberle 1988). The physiological role of a receptor in the brain microvascular endothelium is unknown. It is possible that unlike in B16 melanoma cells, in the case of endothelial cells the peptide is taken into the endothelial cell, not degraded but transported across the cell by receptor mediated transcytosis. The indication that multiple melanocortin receptors exist has been supported by cloning of the genes which encode the melanocortin receptors (see section

1.1.16). This work has identified that although the receptors have distinct activities, they all belong to the G-protein family of receptors (Mountjoy *et al.*, 1992; Chhajlani *et al.*, 1993; Cone and Mountjoy 1993; Gantz *et al.*, 1993 a and b). Purification and cloning of the α-MSH receptor on brain microvascular endothelial cells would therefore provide a further insight into the structure and functions of this receptor.

8. GENERAL DISCUSSION

Endothelial cell heterogeneity has been widely documented in the literature for *in vivo*, *in vitro* and *in situ* studies. Recent advances in the isolation techniques and culture conditions for organ-specific large vessel and microvessel endothelial cells has resulted in the examination of the diverse functions and structural features of endothelial cells. In this study, the isolation and growth of pure cultures of microvascular endothelial cells from the lungs of C57 mice provides the prospect of developing an *in vitro* model for the study of the endothelium with regard to extravasation of macromolecules and the role of the endothelium in metastasis of melanoma. Endothelial cells from murine tissues, however, are reported to be more sensitive to culture conditions than cells isolated from other species (Folkman *et al.*, 1979).

The lung has the potential to provide a vast quantity of microvascular endothelial cells, however, due to its intricate structure and diversity of cell types, isolation of pure cultures of microvascular endothelial cells has proved to be an elaborate and time consuming exercise. The isolation of microvascular endothelial cells required initial detachment of the microvessels from the surrounding connective tissue followed by purification of the cultures, ensuring separation from large vessel endothelia and non-endothelial cell contaminants. The final technique established for the isolation and culture of murine lung microvascular endothelial cells was modified from several preliminary studies based on studies shown in the literature for isolation and culture of microvascular endothelial cells from various organs (Abbott *et al.*, 1992; Nishida *et al.*, 1993; Belloni *et al.*, 1992; Folkman *et al.*, 1979; Zetter, 1984). Modification of the isolation technique and culture conditions was required in order to establish the optimum isolation and culture conditions.

The isolation and culture of brain microvascular endothelial cells provide a valuable model system for *in vitro* studies of the blood-brain barrier. Many investigations have been conducted into the structure and function of the blood-brain barrier due to its unique properties, including the regulation of the transport of nutrients, peptides and

drugs. A wide variety of techniques have been established for the isolation and culture of brain microvascular endothelial cells from a number of different species (Bowman *et al.*, 1983; Audus and Borchardt 1986; Rupnick *et al.*, 1988; Abbott *et al.*, 1992; Dorovini-Zis *et al.*, 1991; Tontsch and Bauer 1989). These methods involve a number of isolation techniques including homogenisation, density gradient centrifugation and cell sieving, as well as using a great variety of culture conditions and supplements. The aim of this and other studies is to determine the most suitable isolation and culture conditions which result in the production of high yields of pure microvascular endothelial cells in both primary culture and subcultivation which retain the properties of the brain endothelium *in vivo*.

The isolation technique and culture conditions are important in establishing pure cultures of microvascular endothelial cells. In this study, a similar procedure has been utilised for the isolation of brain and lung microvascular endothelial cells although several modifications have been employed in order to adapt to the differences in tissue composition. The culture conditions used satisfied the growth requirements for both cell types providing selective conditions for endothelial cell growth whilst maintaining cell morphology, homogeneity and several organ specific functions. The endothelial origin of the cells isolated from both brain and lung tissue was determined using a number of characterisation studies. Expression of Von Willebrand factor, uptake of acetylated LDL, electron microscopy and platelet adhesion studies supported the endothelial cell origin but did not indicate any organ specific differences. In contrast, differences in the morphology of brain and lung microvascular endothelial cells was apparent.

Morphological heterogeneity has been well documented *in vivo* and through *in situ* ultrastructural studies, however, contrasting opinions exist on the morphological heterogeneity of organ-derived microvascular endothelial cells in culture. Phenotypic variability was identified in cultures of cerebral microvascular endothelial cells by Rupnick *et al.* (1988). In contrast, Beer Stoltz and Jacobson (1991) found no

morphological heterogeneity between microvascular endothelial cells isolated from bovine brain and adrenal medulla. Belloni *et al.* (1992) also found no morphological heterogeneity between microvascular endothelial cells isolated from murine brain, lung and liver. In this study, morphological heterogeneity was demonstrated between murine brain and lung microvascular endothelial cells. The use of the alternative culture conditions and extracellular matrices however, can have a marked effect on cell morphology. Therefore, optimisation of the growth conditions must be investigated in order to mimic microvascular cell morphology *in vivo*.

Biochemical heterogeneity between organ-specific microvascular endothelial cells has been well documented. A number of cell surface components have been identified such as cell specific glycoproteins (Belloni and Nicolson 1988; Beer Stoltz and Jacobson 1991; Del Vecchio *et al.*, 1992) and cell surface antigens (Auerbach *et al.*, 1985) and receptors (Ryan 1989) which confirm the organ specificity of microvascular endothelial cells in culture. Due to the strategic position of the endothelium, these cell surface receptors could be exploited in drug targetting (Ryan 1989). These organ-specific differences can also be recognised by tumour cells which selectively adhere to the vascular endothelium in metastasis (Pauli *et al.*, 1990; Zhu *et al.*, 1991 and 1992). In this study the biochemical heterogeneity of organ-derived microvascular endothelial cells was demonstrated by the expression of an α-MSH receptor on brain microvascular endothelial cells which was absent on lung microvascular endothelial cells.

The expression of an α-MSH receptor on brain microvascular endothelial cells was initially identified using binding studies at 4°C. This indicated the existence of a very small population of MSH receptors where [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH displayed an estimated specific binding affinity (Kd) of 3.1 x 10^{-8}M. Due to the low receptor number, a high proportion of non-specific binding was observed. This could be due to non-specific binding to the plastic well and to proteins on the cell surface

The existence of a specific binding site for α-MSH was confirmed by photoaffinity labelling. This identified the presence of a specific receptor with an apparent molecular mass of approximately 45kDa.

Brain microvascular endothelial cells have been shown to express a number of receptors such as insulin, low density lipoprotein and transferrin (Jeffries *et al.*, 1984; Raub and Newton 1991). The receptor has the ability to bind and internalise the specific ligand through coated pits which exist on the plamsa membrane. Depending on the nature of the receptor-ligand complex, it follows one of four routes (Basu 1990): 1) the receptors recycled and ligands degraded (e.g. low density lipoprotein); 2) the receptors and ligands are both recycled (e.g. transferrin); 3) the receptors and ligands are degraded (e.g. epidermal growth factor) and 4) the receptors are degraded and the ligands are transported across the cell (e.g. immunoglobulin A and M). The receptor for α-MSH expressed on murine brain microvascular endothelial cells could have a similar fate to the transferrin receptors whereby the ligand is transported across the cell and the receptor is recycled. This could account for the binding profile of [^{125}I-Tyr^{2}, Nle^{4}, D-Phe^{7}] α-MSH to brain microvascular endothelial cells at 37°C where the increase in internalised ligand reaches a steady-state.

The physiological role of an α-MSH receptor on brain microvascular endothelial cells is unknown. *In vivo* studies have indicated that intravenously injected α-MSH reduces the regional permeability of the blood-brain barrier. These studies have suggested therefore, that the behavioural response of the melanocortin peptides may be partly as the result of selective actions on the blood-brain barrier. However, 0.01% of a quantity of peripherally injected α-MSH has been shown to reach the brain, since the biotransformation of the hormone correlates with behavioural activity. This suggests that small quantities of peptide can induce notable behavioural effects (Eberle 1988). As the *in vivo* uptake of α-MSH across the blood-brain barrier is small, it has been assumed that uptake is passive, however from the evidence presented in this study, it is possible

that uptake occurs via receptor mediated transcytosis by a small specific population of receptors on the microvascular endothelium.

Endothelial cell biology has been associated with a number of other areas of research including cancer research, drug targeting and immunology. The endothelium provides a highly specific anatomical barrier possessing specific cell surface receptors which could be exploited in drug delivery systems by selective transport of macromolecules throughout the vasculature. The pulmonary microvasculature for example, represents the majority of endothelial cells in the lung where many of the important functions attributable to the pulmonary vasculature occur. The blood-brain barrier in particular, provides a highly selective barrier limiting the access of macromolecules into the brain, and many studies have been conducted which attempt to overcome this barrier and selectively deliver therapeutic concentrations of drug. In addition, tumour angiogenesis and metastasis may also be attributable to the individual characteristics of organ-specific endothelial cells such as growth factor responses and expression of cell adhesion molecules.

The investigation of *in vitro* systems which mimic the organ specific endothelium have the potential to provide an insight into the role of the endothelium in metastasis of malignant tumours such as melanoma and in the regulation of specific carrier systems in drug delivery.

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