Model Systems to Study Proliferative Retinal Diseases

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medicine, Dentistry and Nursing

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Contents

Abstract

Proliferative retinal diseases are a major cause of blindness in the western world and are characterised by retinal neovascularisation and/or retinal membrane formation and contraction. Although a vast amount of work has contributed to the understanding of the clinical features of the diseases our knowledge of many of the molecular mechanisms are incomplete. This is partly due to limitations of in vitro models.

The aims of this study are **a)** to assess the in vitro growth characteristics of retinal arterioles versus venules (because retinal neovascularisation almost always occurs on the venous side of capillary network); **b)** to develop an in vitro model to study retinal neovascularisation and **c)** to investigate the effect of oxygen and/or growth factors and extracellular matrix components on the contraction of retinal cell types thought to be involved in retinal membrane formation.

Cellular outgrowth was observed to occur significantly earlier from venules than arterioles in culture. This relationship was found to be largely substrate independent and was enhanced by the presence of exogenous VEGF. It was further demonstrated that the number of arterioles that presented any outgrowth was considerably less than that of venules in the presence of various extracellular matrix components (ECM) with the exception of vitronectin. These findings suggest important differences exist within the biology of the two vessel types and implicate further VEGF and vitronectin in retinal neovascular pathology.

A simple reproducible organ culture model representing several stages of retinal neovascularisation was successfully developed for the second part of the study.

This model also demonstrated directional growth of retinal vascular cells and addressed many of the problems overlooked in previous in vitro models. This reliable model can now be used as a more realistic tool for investigating the molecular events involved in new vessel formation and to test putative agents for therapeutic intervention.

Finally, the use of the collagen gel contraction model demonstrated that the retinal cell types (fibroblasts, RPE cells and pericytes) tested were capable of eliciting a contractile response. It was further demonstrated that this retinal cell mediated contraction was affected by varying oxygen concentrations with greatest contraction observed in a physiological oxygen environment. This contractile response in varying oxygen was found to be cell specific and further affected by the addition of exogenous growth factors and ECM components. The most interesting findings were as follows. **a)** A difference in contractile response was observed from different morphology RPE cells in varying oxygens. **b)** A significant increase in fibroblast mediated contraction was observed in the presence of $TGF\beta_2$ in all oxygens - particularly hypoxia. Finally, **c)** the demonstration of an oxygen-dependent decrease in pericyte mediated contraction in the presence of VEGF. This study demonstrates a potential role for oxygen in retinal membrane contraction. The results from this study provide a greater understanding of retinal membrane pathogenesis and an insight into the broader issues of retinal vascular physiology.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Thanks also to my family and friends, for being my inspiration; for their unstinting support, patience and belief in me. Thank you.

Dedication

This thesis is dedicated to the memory of my father and the strength of my mother.

CHAPTER ONE – INTRODUCTION

1.1 Overview

Proliferative retinal diseases are a leading cause of new cases of blindness in the western world. They are characterised by fibrovascular and fibrocellular proliferation into the vitreous, in, on or beneath the surface of the retina. The underlying clinical features are retinal neovascularisation and/or retinal membrane formation leading to visually debilitating conditions such as vitreous haemorrhage, retinal tears and retinal detachment. Advances in laser surgery and vitreoretinal surgical techniques have led to better treatment/management of proliferative retinal diseases but visual outcome is still relatively poor. A vast amount of information regarding neovascularisation and membrane formation is now available, but the cellular and molecular mechanisms involved are still not fully understood. This is thought to be due, in part, to the lack of suitable animal models and in vitro models being unrepresentative of the retinal situation.

Thus, the aims of this study were threefold. Firstly, to determine whether any differences exist between arterioles and venules, by comparing their growth characteristics in vitro, since neovascularisation in vivo almost always occurs from the venous side of the capillary network. Secondly, to develop a simple reproducible model to study retinal neovascularisation addressing some of the problems associated with previous in vitro models. Finally, to investigate factors that affect retinal cell contraction in the context of retinal membrane traction, paying particular attention to the possible role of oxygen, which has so far been largely ignored.

1.2 The retina

1.2.1 Embryology

The eye is a complex organ composed of multiple tissue types, but its basic structure is complete 33 days after conception (Federman and Gouras, 1994). The retina and pigment epithelium first arise as distinct anatomic features as a result of three embryological processes; the development of the optic vesicle, the invagination of the optic cup and the closure of the foetal fissure (Mann, 1964). The pigment layer is formed from the outer thinner layer of the optic cup. It is a single layer of cells that become columnar in shape and develop pigment granules within their cytoplasm (Bairati and Orzalesi, 1963).

The neural layer is formed from the inner layer of the optic cup (Duke-Elder and Cook, 1963). However, in the region of the cup that overlaps the lens, the inner layer is not differentiated into nervous tissue. This anterior one-fifth of the inner layer persists as a layer of columnar cells, which together with the pigmented epithelium of the outer layer extend forward onto the posterior surface of the developing cilliary body and iris (Mayer, 1969; Hollenberg and Spira, 1973). The posterior four-fifths of the inner layer of the optic cup undergo cellular proliferation, forming an outer nuclear zone and an inner marginal zone without any nuclei. Eventually, the cells of the nuclear zone invade the marginal zone so that the neural part of the retina is made up of inner and outer neuroblastic layers (Mann, 1950; 1964). The inner neuroblastic layer forms the ganglion cells, the amacrine cells, and the bodies of the sustentacular fibres of muller. The outer neuroblastic layer gives rise to the horizontal bipolar nerve cells and the rod and cone cells (Nilsson, 1970; Cohen, 1972). By the eighth month of foetal life all the layers of the retina can be recognised.

1.2.2 Retinal structure and function

The retina consists of an outer pigmented layer and an inner neurosensory layer that are embryologically derived from the neuroectoderm. The retina is continuous with the optic nerve posteriorly, and it extends forward to become the epithelium of the ciliary body and the iris. The outer surface of the retina is in contact with Bruch's membrane of the choroid; the inner surface is in contact with the vitreous body. At the iris, both layers of cells continue on its posterior surface and they both become pigmented. At the centre of the posterior part of the retina is an oval yellowish area, known as the macula lutea. It is the retinal area for the most distinct vision. It has a central depression, the fovea centralis. The optic disc leaves the retina about 3mm to the medial side of the optic disc. The optic disc is slightly depressed at its centre, where it is pierced by the central retinal artery and vein. At the optic disc, there is a complete absence of rods and cones; thus it is insensitive to light and is referred to as the blind spot. This is seen as a paler pink colour on examination, much paler than the rest of the retina.

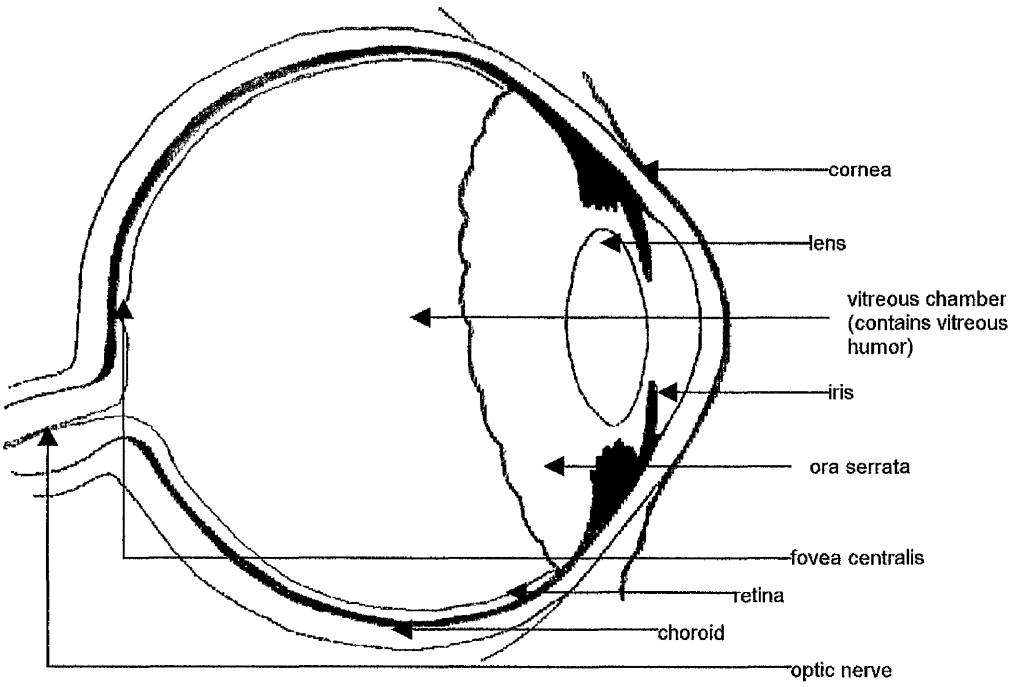


Figure 1.1 - Schematic diagram of the retina in the context of the full orbit.

1.2.2.1 The retinal pigment epithelium

The retinal pigment epithelium consists of a single layer of hexagonal-cuboidal cells. These cells are pigmented with melanin and are responsible for: stray light absorption, removal of excessive heat, supplying nutrients and oxygen to photoreceptors as well as acting upon the breakdown products of photoreceptors (Young, 1967; Mayer, 1969). The basal plasma membrane of each cell has many folds and rests on a basement membrane that forms part of the Bruch's membrane of the choroid. The apical ends of the cells have multiple projections (microvilli) that extend between and surround outer segments of rods and cones in the photoreceptor layer. The cells within the pigment epithelium are attached closely to each other by tight junctions at the basal level (zonula adherens) and in the apical region (by zonula occludens) thus leaving practically no room for any intracellular space. These tight junctions are very important in isolating the retina from the systemic circulation and thus create what is known

as the blood retinal barrier (Cohen, 1963; Dowling, 1970; Hogan et al, 1971). The barrier created by these junctions limits the flow of ions and prevents diffusion of large toxic molecules from the choroid capillaries to the photoreceptors of the neural retina. Thus the integrity of the pigment epithelium is crucial to the function of neural retina and ultimately visual ability.

1.2.2.2 The neural retina

The neural retina is the internal layer of the eye. It appears as a thin transparent membrane having a purplish-red colour in living subjects. The function of the neuroretina is to receive focused light waves and convert them into nerve impulses, these nerve impulses are transformed into visual perception by the CNS. Traditionally, based on light microscopic findings the neural retina was said to consist of nine different layers; which are from outside inwards as follows:

- the internal limiting membrane
- the nerve fibre layer
- the ganglion cell layer
- the inner plexiform layer
- the inner nuclear layer
- the outer plexiform layer
- the outer nuclear layer
- the external limiting membrane
- the photoreceptor layer

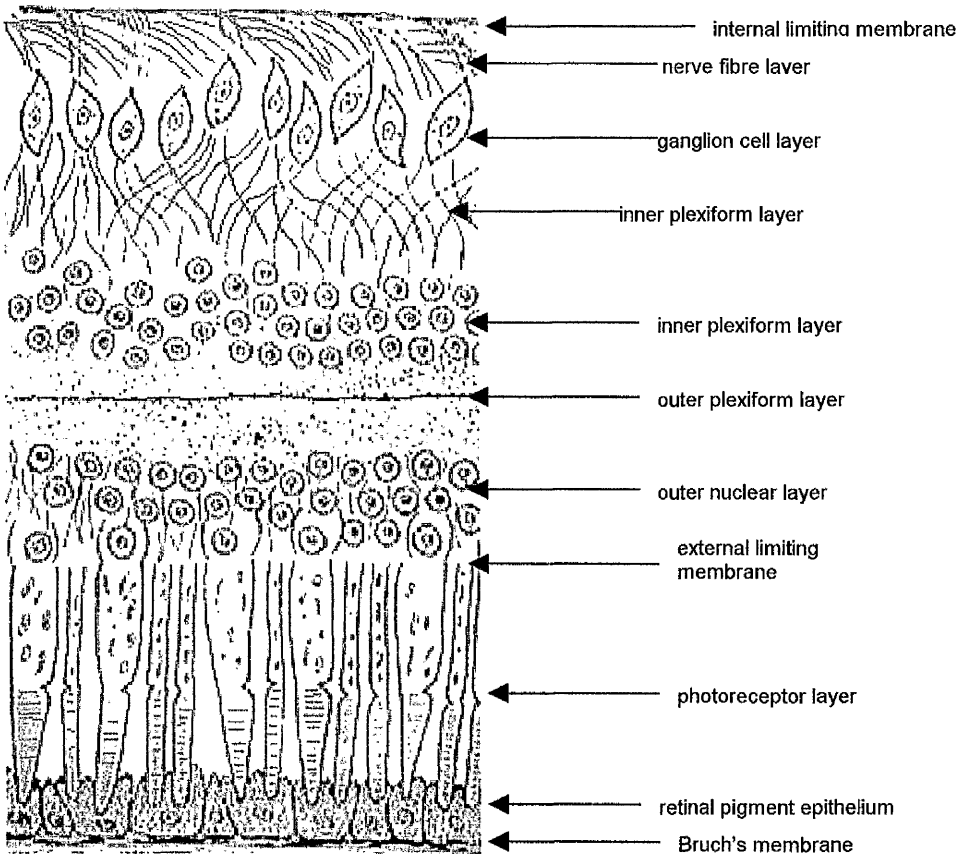


Figure 1.2 –Schematic diagram illustrating the layers of the retina (Courtesy of ME Boulton).

The internal limiting membrane: covers the inner surface of the retina forming its boundary with the vitreous. It is made up of offshoots of muller cells (Fine, 1961).

The nerve fibre layer: consists of the axons of the ganglion cells that are converging toward the optic disc (Boycott and Dowling, 1969).

The ganglion cell layer: is formed by the bodies of ganglion cells. It is about 10 cells deep near the macula reducing to single cell thickness peripherally (Dowling, 1987).

The inner plexiform layer: is made up of synaptic connections between the bipolar, amacrine and ganglion cells (Boycott and Dowling, 1969; Kolb, 1970; Boycott and Kolb, 1973).

The inner nuclear layer: consists of the nuclei of the bipolar cells, the horizontal cells, the amacrine and ganglion cells (Gallego, 1971)

The outer plexiform layer: is made up of photoreceptor axons and the synapses they form with bipolar cell dendrites. In addition there are connections between horizontal cells and the photoreceptor synapses (Raviola, 1976; Lasansky, 1976).

The outer nuclear layer: consists of photoreceptor nuclei. Cone nuclei are arranged just below the limiting membrane, while rod nuclei are further down and less regularly distributed (Boycott and Dowling, 1969).

The external limiting membrane: is a combination of junctional complexes formed by synapses and junctional complexes of rod and cone cells with bipolar and horizontal cells, thus forming a supporting network around the photoreceptors, holding them into position. The external limiting membrane is an important physical barrier to exudates and demarcates the deepest penetration of retinal capillaries (Fine, 1961).

The photoreceptor layer: photoreceptors are sensory cells that transform light into electrical energy by chemical processes. Impulses then travel along visual paths to produce perception of an image (Dowling, 1987). There are two types of photoreceptor, distinctly named to describe the shape of their outer segments. Rods (cylindrical in appearance) are mainly responsible for vision in dim light and produce images consisting of varying shades of black and white (Walls, 1942). Meanwhile the cones (conical in appearance) which adapt to bright light, are responsible for colour vision and can resolve fine detail (Latties et al, 1968; Cohen, 1972; Brown and Wald, 1963, 1964).

Supporting cells: of the neural retina are often collectively called "retinal glial cells" because they support retinal neural cells and are thought to fulfil a similar

role to glial cells proper in the central nervous system. Muller cells are the major non-neuronal cell type and are generally long and thin with long processes which can run through the whole thickness of neural retina; subsidiary branches extend out horizontally, surrounding and supporting the nerve cells. Muller cells thus fill in most of the space of the neural retina not occupied by the neurons (Cohen, 1963; Kuffler et al, 1966).

Towards the outer surface of the neural retina, electron microscopy has revealed the existence of gap junctions between the photoreceptor cells and the radial processes of the Muller cells. Tufts of microvilli project from the ends of the Muller cells in the spaces between the inner segments of the rods and cones and are thought to assist in metabolism, possibly providing nutrients (Kuffler et al, 1966).

Astrocytes are another retinal cell type found predominantly in the ganglion cell layer and the nerve fibre layer. These glial cells have short and long processes extending in all directions. Some attach themselves to nerve cells to gain footholds that assist the supporting and protecting functions of the astrocytes. These glial cells promote movement of substances from and to the vascular system, hence they are important for local nourishment (Beck et al, 1984; Janzer and Raff, 1987). Microglia are the smallest in number within the retina and behave as connective cells. Few are present in healthy tissue but they proliferate for phagocytosis when damage occurs (Jiang et al, 1994).

1.2.3 The retinal vasculature

The retinal vasculature is responsible for the constant supply of nutrients and removal of waste products within the retina, hence facilitating the maintenance of retinal transparency, neurotransmitter activity and photoreceptor metabolism (Michaelson, 1954).

1.2.3.1 Development of the retinal vasculature

In mammals the retinal vasculature is formed by the sprouting of mesenchymal cells from the hyaloid artery, into the retina (Ashton et al, 1954; Ashton, 1957). Differentiation of the mesenchymal cells into endothelial cells takes place with subsequent formation of solid cords (Ashton, 1966; 1970). This in turn is followed by the formation of lumens and intercellular junctions between adjacent endothelial cells (Noden, 1990). Gradual canalisation occurs with capillaries evolving in a polygonal fashion to form primitive vascular networks. It is from such networks that there develops a recognisable system of arteries, capillaries and veins by a process that involves atrophy of some vessels and hypertrophy of others (Beach and Jacobson, 1979; Bellhorn, 1980). A major factor influencing the proliferation and maturation of the vasculature in the later stages of embryogenesis and foetal development is local metabolic demand and this factor is thought to persist throughout life. Initial activity is concerned with cell proliferation and migration, functions facilitated by fibronectin secretion (Roth, 1977; Ashton and Tripathi, 1975). There then follows a switch to laminin production, which induces further differentiation so that a lumen is formed and cell-cell attachments are formed (Grant et al, 1989). Anchorage of the developing endothelium to the extracellular matrix is vitally important, and production of heparan sulphate proteoglycan and the cell adhesion promoting nidogen/entactin molecule is thought to encourage stability. Type IV collagen also serves to bind the various components and form a basement membrane on the abluminal side of the capillary (Navaratnam, 1991; Beach and Jacobson, 1979). There are various theories concerning the emergence of a lumen, including partial separation of adjacent cells and the formation of a transcellular channel within a single cell (Folkman and Haudenschild, 1980). The addition of

pericytes, smooth muscle cells and fibroblasts to the outer aspect of maturing vessels is less well understood but is believed to involve perivascular mesenchyme (Shakib and Oliveira, 1966).

1.2.3.2 Anatomy of the retinal vasculature

The adult retina receives its blood supply from two sources; the outer laminae, including the rods and cones and outer nuclear layer, are supplied by the choroidal capillaries; (the vessels do not enter these laminae, but tissue fluid exudes between these cells), and the inner laminae are supplied by the central retinal artery and vein. It should be emphasised that the integrity of the retina depends on both of these circulations, neither of which alone is sufficient.

The vasculature concerned with this study is derived from the central retinal artery and vein and these vessels are illustrated in the diagram below **(fig. 1.3)**.

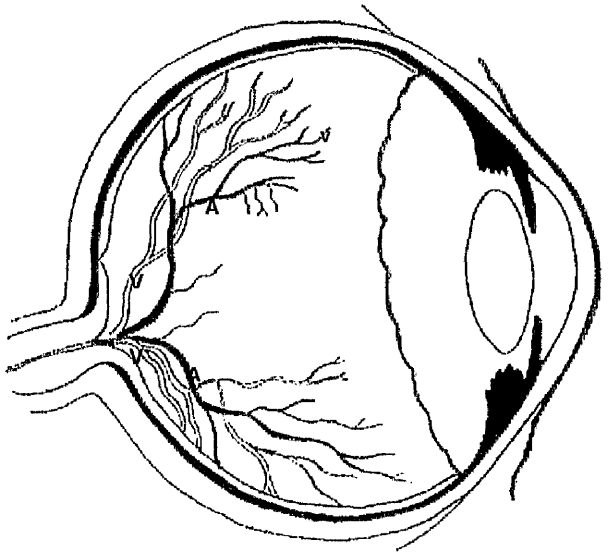


Figure 1.3 Diagram illustrating the origin of the vasculature in the neural retina. The vitreous is removed to allow visualisation of the vessels. As this is a cross-section of the globe only two of the four main arteries and veins are visible. The smaller calibre vessels and capillaries are not shown for clarity. (Shaded vessels indicate arterial system and the non-shaded vessels represent the venous system).

The central retinal artery ramifies into two upper and two lower vessels immediately as it appears at the optic disc. Thereafter the branches extend to serve the entire retina after successive bifurications (Michealson, 1954). Similarly the venous branches ramifies into two upper and two lower trunks to form the central retinal vein at the optic disc (see fig. 1.4). The four arteries each supply a quadrant of the retina; there is no overlap and there is no anastomosis between branches within a quadrant. The diameter of the vein is about one-third to one-quarter larger than that of the corresponding artery (Hogan and Feeney, 1963a). The pattern of the veins, although similar is not identical to that of arteries. The arterial branches run in the nerve fibre layer close to the internal limiting membrane and give rise to the arterioles throughout the different layers of the neural retina, reaching as far as the internal nuclear layer (Kuwabara and Cogan, 1961: Kuwabara et al, 1961). The arterioles give rise to a diffuse capillary network.

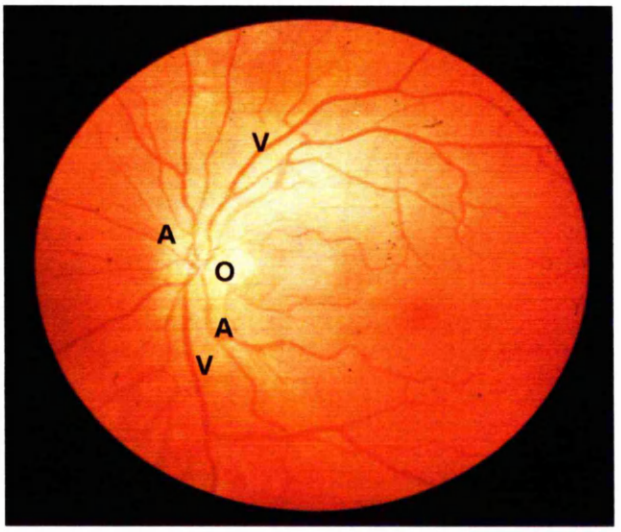


Figure 1.4 Fundus photogragh of a normal retina. (O= optic disc; A = artery; V = vein)

The retinal capillaries form superficial and deep nets but do not extend outside the inner nuclear layer. Retinal venules arise from the capillary networks and join one another to form the larger superficial retinal veins. Arterioles are thus connected through elaborate networks in which the capillaries are suspended from two arterioles situated in the superficial layers. Venules are also found in the superficial layer in the center of clusters between the two feeding arterioles (Kuwabara, 1969; Michaelson, 1954).

1.2.3.3 Structure of the general vasculature

In the general vasculature arteries are described as being composed of three layers: the tunica intima, the tunica media, and the tunica adventitia.

The tunica intima (innermost covering) has an innermost lining of endothelial cells (simple squamous epithelium), a thin subendothelial layer of fine connective tissue and an external elastic layer containing elastic fibres.

The tunica media (middle covering) is the thickest layer of arterial wall in large arteries. It is composed mainly of connective tissue, smooth muscle cells, and elastic tissue. The walls of the largest arteries (elastic arteries) have elastic tissue rather than smooth muscle. In the smaller arteries (muscular arteries), the elastic tissue in the tunica media is replaced by smooth muscle.

The tunica adventitia (outermost covering) is composed of mainly collagen fibres and elastic tissue. Occasional smooth muscle fibres run longitudinally next to the outer border of the tunica media.

Arteries decrease in caliber to become arterioles, which are also composed of the characteristic three tunicae, but to a lesser extent than their parent arteries. The lumens of arterioles are lined by a monolayer of endothelial cells. A thin sheet of elastic tissue, the internal elastic membrane separates the tunica intima and the tunica media although in some arterioles the tunica media consists of a

single layer of smooth muscle fibres that are wrapped around the arterioles' long axis. Since the arteriole is embedded within an organ, no tunica adventitia is present. Instead a thin layer of collagenous connective tissue binds the vessel to surrounding tissue.

Arterioles eventually branch out to form capillaries that connect the arterial and venous circulations. Briefly, the wall of the capillary consists of a single layer of endothelial cells on a thin basement membrane of consisting of collagen and glycoprotein.

Blood drains from the capillaries into venules. The transition from capillaries to venules occurs gradually. The immediate postcapillary venules are called pericytic venules, because contractile cells called pericytes are wrapped around them. Pericytic venules consist mainly of endothelium and a thin tunica adventitia. Postcapillary venules play an important role in the exchange of nutrients and waste products between blood and interstial fluid. Muscular venules generally accompany muscular arteries and arterioles. They have a thin wall, a continuous epithelium and no pericytes.

Venules eventually become veins. Veins become larger and less branched as they move away from the capillaries toward the heart. The walls of veins contain the same three tunicae as arterial walls, but the tunica media is much thinner. Also, venous walls contain less elastic tissue, collagen ous tissue, and smooth muscle. The smooth muscle fibres that are found in veins are arranged in either a circular or longitudinal pattern.

1.2.3.4 Structure of the retinal vasculature

Retinal arteries – the main retinal artery is classically thought to be of the medium-sized muscular type (Balliart, 1923). The endothelial cells run parallel to the axis of the vessel. The plasma membrane of adjacent cells may show

overlapping or interdigitation. These cells resemble the endothelium of other arteries except that they contain fewer pinocytotic vesicles (Kuwabara, 1962; Pease, 1962). External to these cells is a broad basement membrane continuous with that of the first layer of smooth muscle cells. The basement membranes of the endothelial and smooth muscle cells are orientated both circularly and longitudinally. The first layer of muscle was found to lie close to the endothelium, being separated from it by the combined basement membrane layer of the two cells. Each succeeding layer of muscle is described as being separated by basement membrane between the endothelium and first muscle layers (Hogan and Feeney, 1961; 1963a).

Retinal veins – the principal branches of the central retinal vein near the disc are small in comparison to other venous systems (Hogan and Feeney, 1963a). The endothelial cells are described as resembling those of the artery but possess a thinner basement membrane. This membrane was described as similar in structure to that of the arteries in the same study, containing scattered collagen fibrils and no elastin or elastic lamina (Hogan and Feeney, 1963a; Kuwabara and Cogan, 1960; kuwabara, 1969). The basement membrane was found to be continuous with that of the pericytes' and extends around to their external surfaces. In the large posterior veins the pericyte basement membrane merges with the collagennous adventitia and are thought to have collagen fibres embedded in it. In the smaller veins the wide basement membrane layer is thought to blend with that of the adjacent glial cells and contain only a few reticulin type fibres (Hogan and Feeney, 1963a). External to this the venous wall was in contact with the basement membranes of the glial and muller cells. Most importantly the equatorial and peripheral main venous trunks show progressive diminution in size and narrowing of the adventitial layer and these

retinal veins lack a muscularis (Hogan and Feeney, 1963a). The endothelium, pericytes and basement membrane have the same relationships to each other as in the capillary and venule.

Retinal arterioles –usually have a smaller caliber in the retina than in most other organs, yet its structure is comparable to that of other arterioles (Pease, 1962; Polyak, 1941, Rhodin, 1967). The lumen is lined with endothelial cells and their basement membranes resemble those of adjacent smooth muscle cells. The plasma membrane of the smooth muscle cell frequently is contiguous or interlocked with that of the endothelial cell. The smooth muscle cells are less well developed than in most arterioles (Pease, 1962). The arteriolar adventitia is well developed, consisting of collagen fibres that course longitudinally (Hogan and Feeney, 1963b). It is limited on the neural side by the basement membrane of smooth muscle cells. As the arterioles become metarterioles, the adventitia diminshes in width and finally disappears as the capillary is reached. The smooth muscle cells of the media are gradually replaced by pericytes in the same manner (Hogan and Feeney, 1963b; Maeda, 1958).

Retinal venules –are difficult to identify in comparison to arterioles because they have no smooth muscle cells and the pericytes of the capillaries continue into their walls (Kuwabara and Cogan, 1960; Hogan and Feeney, 1963b). The endothelium, basement membrane and pericyte are indistinguishable from that of the capillary. Collagen fibrils appear in the outer vessel walls when it becomes 8 microns in size. They were found to generally increase in number as the vessel enlarged (Majno and Palade, 1961; Maeda, 1958).

Retinal capillaries – The structure of the retinal capillaries place them in the class with the capillaries found in the CNS, lungs and heart as they have a continuous endothelium and basement membrane (Bennet et al, 1959; Rhodin,

1976; 1968). There are however some differences from those described in other tissues. The conjacent basement membranes of endothelial cells and pericytes are considerably thicker and the basement membrane varies in thickness and density depending on the presence of an adjacent pericyte (Reinecke et al, 1962; 1961; Maeda, 1958; Hogan and Feeney, 1963b). The endothelial cells parallel to the vessel axis and the cytoplasm are non-fenestrated. The pericytes form an integral part of the capillary wall, their axis being parallel to that of the vessel (Hogan and Feeney, 1963b; Kuwabara, 1969). Their investment by a broad basement membrane, which is in intimate relation to that of the endothelium, as well as that of the adjacent glial and muller cells, places them in the wall of the vessel rather than on its outer surface (Reinecke et al, 1962; Hogan and Feeney, 1963a, 1963b). They are closer to each other than the pericytes of other capillaries so that there is about a 1:1 ratio between endothelial cells and pericytes. They send out longitudinal and circumferential processes that envelop the wall and overlap those of adjacent cells to or overlapping each other (Kuwabara and Cogan, 1963; Cogan and Kuwabara, 1967).

1.2.3.5 Components of the retinal vasculature

All vessels are composed of a non-thrombogenic layer of endothelial cells resting on a thin extracellular matrix which lines the intimal surface of the vessel walls. The thin specialised extracellular layer beneath endothelial cells is known as a basement membrane. Vessel walls are composed of other vascular cells such as smooth muscle cells and pericytes which have their own basement membranes (Grant et al, 1991, Kleinman et al, 1993)

Endothelial cells – of the retinal vasculature are flat polygonal shaped cells with contractile filaments in the cytoplasm (Rahi and Ashton, 1978). The vascular endothelial cell is a complicated cell with many unique functions, and as a result has an important role in the development of most retinal vascular diseases (D'Amore, 1992b; Petty and Pearson, 1989). Endothelial cells have the following general functional properties (see Cines, 1998):

- maintenance of uninterrupted blood flow (i.e., preserving vascular integrity) by inducing platelet aggregation to plug injured sites and by triggering the sequence of events needed for clotting
- inhibition of inappropriate intravascular thrombosis and lysis of established clots
- regulation of vascular permeability by active and passive transport and intracellular junction complexes
- participation in the control of vascular tone
- source of growth factors involved in angiogenesis and wound repair
- inflammatory role, in terms of leukocyte binding through the expression of specific adhesion molecules
- source of basement membrane constituents and other matrix proteins e.g., fibronectin

Smooth muscle cells - reside in the tunica media of most blood vessels. The media is composed principally of smooth muscle cells, elastic components and collagen fibres. The number of smooth muscle cells present depends on whether a given vessel is muscular or elastic. Vascular smooth muscle cells are spindle shaped and have a distinctly uniform high density of contractile protein filaments (mostly in the form of a cell specific actin), which are aligned parallel to the long axis of the cells (Rhodin, 1967, 1968). Smooth muscle cells

are involved in the control of vascular tone (Dublin, 1989; Komura, 1988) and found only in the larger retinal vessels.

Pericytes - these cells lie external to the endothelial lining and have characteristic pseudopodia that envelop the capillary and are sandwiched between layers of basement membrane (Cogan and Kuwabara, 1967; Rhodin, 1968). Pericytes are particularly important to the retinal vasculature where they exist at a 1:1 ratio with endothelial cells (Hirschi and D'Amore, 1996). Such a high ratio is not found in any other capillary network and because of this, pericytes feature in retinal vascular disorders such as proliferative diabetic retinopathy. The location of pericytes invites the comparison with smooth muscle cells of larger vessels; especially because they contain smooth muscle isoforms of both actin and myosin (Skalli et al, 1989; Nehls and Drenckhahn, 1991; Herman, 1993). There are also thought to be structural differences among pericytes on the arterial and venous sides of the capillary beds. In general, pericytes are more numerous and more extensive processes on venous capillaries and post capillary venule (Meyrick and Reid, 1979; Simionescu et al, 1975). Furthermore, pericytes on larger venules compared to those of venous capillaries tend to be longer and exhibit more contact with the endothelium (Rhodin, 1968). The differences in distribution and structure among pericytes suggest that they may have vessels or tissue specific roles. Pericytes have a variety of proposed functions, including regulation of capillary blood flow; as multipotent mesenchymal cells and specific precursors to vascular smooth muscle cells; phagacytosis; and regulation of new capillary growth. The proposed role of pericytes in capillary blood flow was based on the presence of alpha smooth muscle actin, tropomyosin and muscle myosin isoforms in pericytes (Wallow and Burnside, 1980). As well as the presence in pericytes of

a cyclic-GMP dependent protein which is involved in the regulation of smooth cell contraction (Joyce et al 1985a; 1985b; De Nofrio et al, 1989). Recent studies have shown that it is the pericyte rather than the endothelial cell that influences capillary tone (Gilles and Su, 1993; Murphy and Wagner, 1994; Chakravarthy et al, 1995). Endothelial cells are known to produce the vasoconstrictor endothelin-1, and pericytes have been shown to express endothelin receptors (Masaki, 1995; Takahashi et al, 1989). Experimental evidence also exists to show that pericyte relaxation can be brought about by nitric oxide (NO) in the same way as occurs in smooth muscle cells (Busse et al, 1995; Haefliger et al, 1994; 1997).

Pericytes are also described as multipotent and a considerable amount of evidence is accumulating, demonstrating the capability of pericytes to become microglia, adipocytes, osteoblasts and phagocytes (Boya, 1976; Richardson et al, 1983; Brighton et al, 1992; Cancilla et al, 1972). Pericytes are thought to play a regulatory role in the control of capillary growth (D'Amore, 1992a). For example during wound healing, it is thought that pre-existing micro-vessels, or newly derived pericytes come into contact with endothelial cells that are forming new vessels and exert an inhibitory effect on endothelial cell proliferation (Crocker et al, 1970; Sato and Rifkin, 1989). The same type of cell-cell contact are thought to be vital to the manitenance and functioning of the quiescent vessel. In the adult vessel pericytes are thought to synthesize and secrete soluble factors which exert bi-directional effects on growth and differentiation throughout the life of the vessel (Orlidge and D'Amorel, 1989; Sato et al, 1990).

The Basement membrane – provides not only support and an adhesive surface for the endothelium, but also maintains the normal differentiated phenotype of the cell layer (Kleinman et al, 1993; Grant and Kleinman, 1997). The basement

membrane is also important in maintaining cell polarity and regulates many processes including endothelial cell adhesion, differentiation and proliferation (Kleinman et al, 1987; Liley, 1992). In the adult, blood vessels are fairly stable structures due mainly to the presence of a basement membrane. The basic component of basement membranes is type IV collagen, which acts as a structural backbone and allows other membrane components to bind (Timpl et al, 1979a). It also is described as having an inhibitory effect on endothelial cell proliferation, such that basement membrane dissolution is required prior to any new vessel formation (Roberts and Forrester, 1990). Laminin is another major constituent, relating primarily to cell attachment, whereas fibronectin has a wider range of binding ability (Timpl et al, 1979b; Engel, 1981). Proteoglycans (of which heparan sulphate proteoglycans are the overwhelming representative) are also present and by virtue of their anionic charges, are crucial to the selective barrier function of the basement membrane (Dziadek et al, 1985; Yurchenko and Schittny, 1990).

1.3 *Proliferative Retinal Diseases*

Proliferative retinal diseases are accountable for a large proportion of new cases of blindness in the developed world (Federman and Gouras, 1994). In this study the term proliferative retinal diseases is used in a general sense to collectively describe retinopathies characterised by a proliferative stage in the pathology; whether the proliferative stage is fibrous in nature (e.g., proliferative vitreoretinopathy) or has several fibrovascular stages (e.g., proliferative diabetic retinopathy, retinopathy of prematurity or Eales' disease) (Garner, 1994). Described below is the pathophysiology of the two major clinical features of proliferative retinal diseases: neovascularisation and retinal membrane formation.

1.3.1 Retinal Neovascularisation

As two vascular beds, the inner retinal network and the choriocapillaris feed the retina, proliferation of new vessels can be of three types: intraretinal, subretinal or preretinal.

Intraretinal neovascularisation occurs when areas of the inner retina become ischaemic. It is not known whether these vessels are the result of remodelling pre-existing damaged vessels or are new vessels and thus are frequently termed "intraretinal microvascular abnormalities" (Archer, 1977).

Subretinal neovascularisation is essentially a characteristic of age-related disciform and macular degeneration, but is also observed in Coats' disease and retinopathy of prematurity. New vessels beneath the retina are usually of choroidal origin, reaching the retina either through gaps in Bruchs' membrane or passing around the edge of the Bruch's membrane at the disk margin. Initially,

such proliferation is between Bruch's membrane and the pigment epithelium, but eventually spreads to the subretinal space proper.

Although intra- and subretinal neovascularisation are major features in certain proliferative diseases, it is thought that the pathobiology is similar to preretinal neovascularisation and only the new vessel origin is different. Hence, the majority of this section will concentrate on describing the pathobiology of preretinal neovascularisation.

Preretinal neovascularisation, described as new vessels in front of the retina usually occurs from or near the larger retinal veins (Henkind and Wise, 1974; Archer, 1977). Diabetes mellitus, branch retinal vein occlusion, and sickle cell anaemia are well-documented causes (see Davis, 1992; Garner, 1994). Other less frequent conditions include various types of posterior segment inflammatory disease involving the retinal vasculature (Graham et al, 1987), such as sarcoidosis (Doxanas et al, 1980), Behcet's disease, Eales' disease (Ashton, 1962) to name a few. Impaired retinal circulation appears to be the common factor in all diseases with pre-retinal neovascularisation where new vessels generally arise at the periphery of the ischaemic areas (Dobree, 1964). These new vessels can penetrate the internal limiting membrane and form a dense capillary plexus between the inner surface of the retina and the outer surface of the vitreous gel (Wise, 1956). These immature vessels are delicate and highly permeable and this 'leakiness' and 'bleeding' is thought to encourage the fibrous deposition associated with neovascularisation (Ashton, 1966). As the disease progresses larger vessels that contain a relatively denser fibrous component may appear. In the later stages of the disease the vessels may regress, leaving only lattices of avascular fibrous tissue (preretinal membrane) adhering to the retina and posterior hyaloid membrane. Problems arise from the fragility of the

immature vessels and the contractile nature of the accompanying fibrous tissue, such that neovascularisation can result in haemorrhage into the vitreous, behind the posterior vitreous face or beneath the internal limiting membrane. This causes visual loss by direct obstruction of vision (Frank, 1991). Further complications may arise from the presence of a retinal membrane which can lead to visual loss from retinal tears or traction retinal detachment.

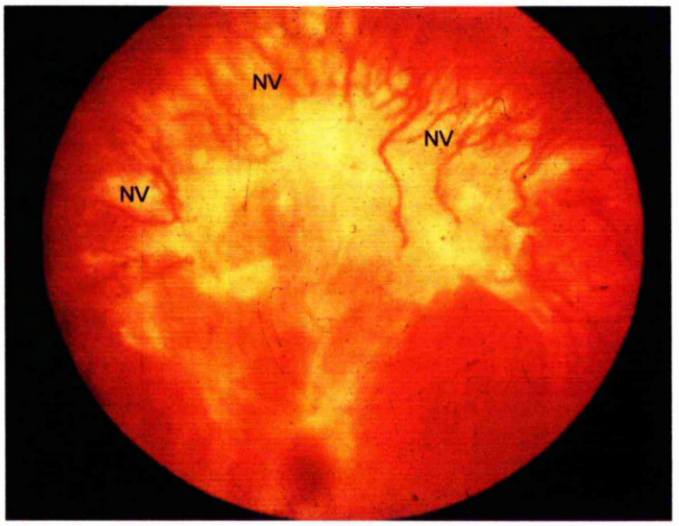
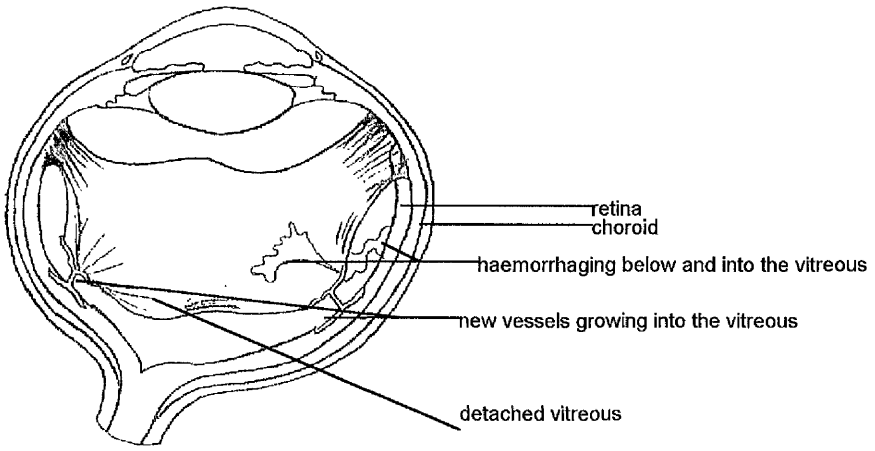


Figure 1.5 Fundus photograph of a retina with extensive neovascularisation, (NV = new vessels growing haphazardly within the neuroretina).

(a)



(b)

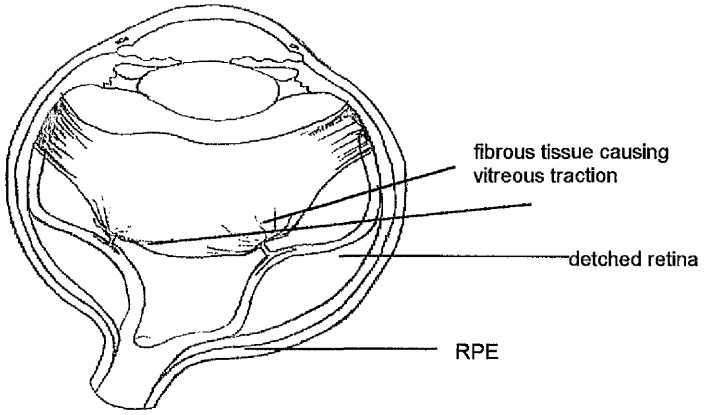


Figure 1.6 Schematic representation of some of the complications associated with retinal neovascularisation. (a) Vitreous detachment causes haemorrhaging due to the immature and fragile nature of the new vessels and (b) although the vessels have regressed the remaining fibrous tissue can lead to retinal detachment.

1.3.2 Retinal Detachment and Retinal Membrane formation

The separation of the sensory retina from the retinal pigment epithelium is termed retinal detachment, and is generally thought to occur after an alteration in normal vitreoretinal relations (Federman and Gouras, 1994). One such alteration is posterior vitreous detachment (PVD). PVD is a process occurring in most normal eyes during or after the sixth or seventh decade. The vitreous does not adhere to the retina and so PVD can occur asymptomatically in normal eyes. However, in eyes that have exaggerated vitreoretinal adhesions (for example, where vitreous fibrils have become incarcerated between clefts in between muller cells) the vitreous surface can pull away from the retina exerting tension, leading to rhegamatogenous retinal detachments. This type of detachment is mobile and convex (bullous) and may involve the whole of the retina (Hiscott and Grierson, 1994).

A retinal detachment may also be static in nature and produce a localised detachment without tearing the retina (Leaver, 1989; Bonnet, 1994). These rigid detachments are termed tractional retinal detachments and are due to the tension generated by fibrocellular or fibrous membranes with attachments to the retina and vitreous. There are a variety of conditions which can result in the formation of a retinal membrane of this type, a large proportion of which are the retinal proliferative diseases mentioned in section 1. 3.1.

Tractional membranes most frequently appear on the inner retinal surface; preretinal and subretinal membrane describe the origin of the vasoproliferation and the resultant membrane; and depending on the location there is epimacular or epipapillary membrane. However the commonest term is 'epiretinal' membrane. There are two main types of epiretinal membranes - fibrovascular and fibrocellular. Fibrovascular epiretinal membranes are present in the

pathology of ischaemic retinopathies such as during proliferative diabetic retinopathy. Fibrocellular epiretinal membranes, which consist of several different cell types (glial cells, RPE cells and inflammatory cells) in a variable amount of extracellular matrix, (structural and interstitial collagens, plasma and cellular glycoproteins) may complicate a variety of disorders (Nagasaki and Shinagawa, 1995). The commonest cause of membrane formation is rhegamatogenous retinal detachment. This is because after treatment of rhegamatogenous retinal detachment there has been shown to be a proliferation of cells on the surface of the retina in approximately 60% of eyes (Rachal and Burton, 1970; Lewis, 1994). Many of these membranes are asymptomatic and known as 'simple' epiretinal membranes. However, approximately 1 in 10 cases of retinal detachment present clinical complications (Lewis, 1994) and the presence of such 'complex' epiretinal membranes after retinal reattachment/retinal detachment surgery is a condition described as proliferative vitreoretinopathy.

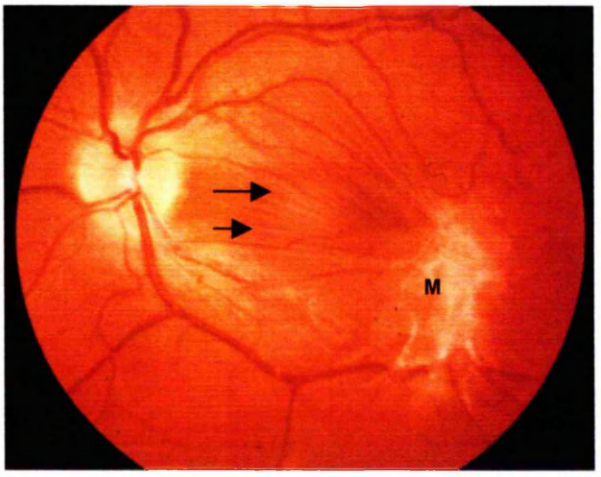


Figure 1.7 Fundus photograph of a retina with a retinal membrane (M) causing distortional folds (arrows) within the retina.

1.3.3 Proliferative Vitreoretinopathy

Proliferative vitreoretinopathy (PVR) is a clinical condition characterised by proliferation and contraction of cellular membranes on the retinal surfaces or, in/on the vitreous. This condition is particularly difficult to manage as these membranes exert traction and may cause recurrent detachment by reopening otherwise successfully treated breaks, or create new retinal breaks. The contraction of these membranes can cause partial and full thickness folds in the neuroretina i.e., focal traction detachment - from the tractional forces applied tangentially on the retinal surface (Lean, 1994; Lewis, 1994). When this process occurs on or adjacent to the macula causing it to become distorted or obscured and thus vision is severely impaired (Lean, 1994; Lewis, 1994).

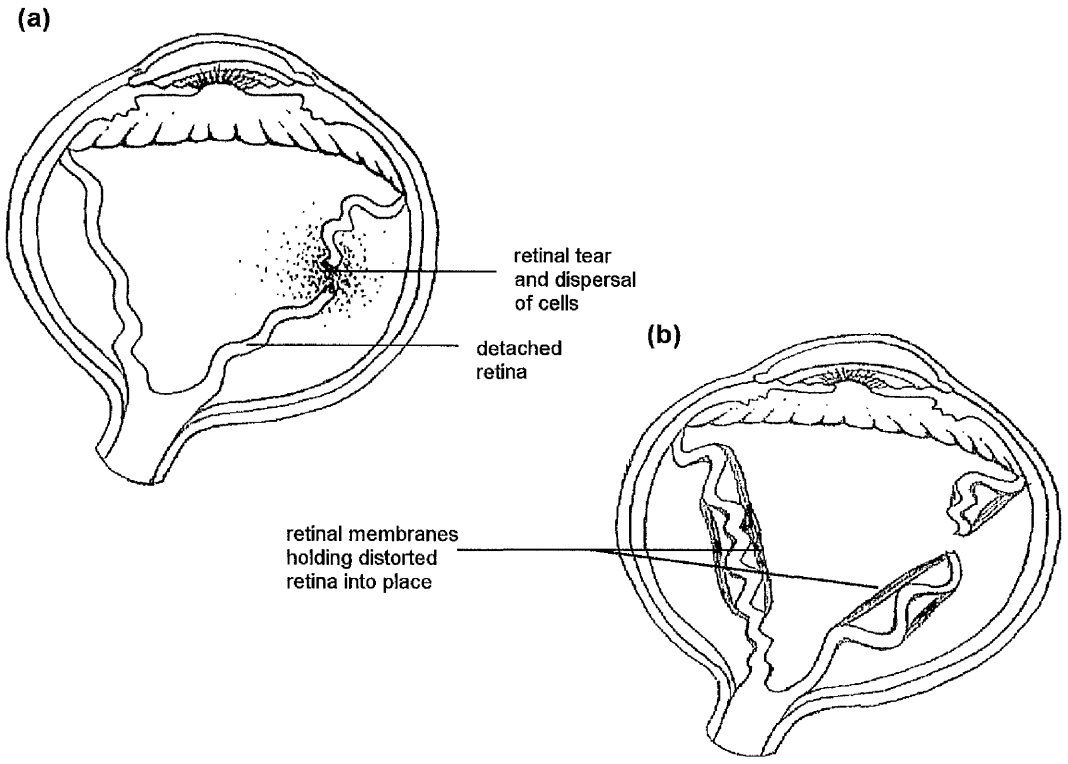


Figure 1.8 Schematic representation of PVR (a) Retinal detachment and tear leading to dispersal of retinal cells; (b) end stages of membrane formation and contraction.

1.3.4 Treatment of Proliferative Retinal Diseases

1.3.4.1 Laser Treatment

Early detection and treatment of proliferative retinopathy in any disease may prevent or significantly retard visual loss. The use of laser photocoagulation is used as a non-invasive treatment method with a relatively low complication rate and a significant degree of clinical success (Vine, 1985). The laser is used to destroy limited areas of capillary non-perfusion. However, the prognosis for untreated neovascularisation is exceedingly poor: half or more eyes that contain retinal neovascularisation are blind within 5 to 6 years, and within 2 to 3 years when the disc is involved (Federman and Gouras, 1994).

1.3.4.2 Vitreoretinal Surgery

The basic principles of the surgical treatment of complications such as retinal detachment complicated by a retinal membrane is the closure and sealing of retinal breaks; removal of retinal membranes; occasional vitrectomy and the complete release of any retinal traction (Michels, 1984; Hannekan and Michels, 1991). Techniques such as retinopexy and laser photocoagulation are used to promote chorioretinal adhesion (Charteris, 1995). Silicone oil, expansile gases and perfluorocarbon liquids are used to stabilise and flatten the retina during/after surgical manipulation particularly if vitrectomy is involved (Han et al, 1994). As vitreoretinal surgical techniques have advanced the anatomical success rate of retinal surgery in severe membrane formation has increased to around 90% in some reports (The Silicon Study Group, 1993). Visual results however are often disappointing. Justification of multiple procedures, increasing ocular morbidity and the high cost of such surgery is therefore difficult to find.

1.4 Factors Affecting Neovascularisation

1.4.1 The Mechanism of neovascularisation

Neovascularisation (angiogenesis) is the formation of new blood vessels and can occur both as a physiological and pathological process. In the adult, neovascularisation occurs infrequently with exceptions being the female reproductive system and the body's repair processes as in the healing of wounds or fractures. However, uncontrolled neovascularisation can often be pathological, for example, the growth of solid tumours depends on vascularisation and a prime concern of this study, neovascularisation of the retina. During embryonic vessel formation the vasculature originates from undifferentiated mesenchymal cells (Noden, 1990), in contrast new vessels associated with neovascularisation arise from an existing vascular bed, (Ausprunk and Folkman, 1977). The new vessels generally arise from the capillary network, at the margins of the ischaemic areas. In order for new vessel formation to occur, five major cellular events are required; capillary basement membrane breakdown, endothelial cell migration, lumen formation and vessel remodelling (Folkman and Haudenschild, 1980; Ribatti et al, 1991).

1.4.1.1 Capillary Membrane Basement Breakdown

Degradation of the vascular basement membrane is a necessary precedent to the initiation of a capillary sprout or bud (Gross et al,1982). This may partly be because of the physical constraints an intact basement membrane places on an active endothelium, but perhaps more important are its biochemical effects. It is postulated that it is the vascular endothelial cell that mediates the degradation of the basement membrane by the release of a cascade of proteolytic enzymes

local to the site of degradation (Klagsbrun, 1991). One group of enzymes thought to be involved is the plasminogen activator family (Moscatelli and Rifkin, 1988). Two types have been identified within mammalian systems; urokinase plasminogen activator family, and the tissue type plasminogen activator family (Pepper et al, 1987; Gross et al 1982). Other matrix proteases, which have more recently been implicated in regulating the integrity of the basement membrane, include collagenase, stromeolysin and gelatinase (collectively known as the metalloproteinases). The actions of these matrix enzymes are thought to be balanced by their inhibitors (Auerbach and Auerbach, 1994).

1.4.1.2 Endothelial Cell Migration and Proliferation

Once a gap in the basement membrane has been created migration and proliferation of endothelial cells rapidly ensues. Migrating endothelial cells accumulate at definite focal points on the parent capillary wall. The bleb thus formed develops into a bud that in turn forms a sprout and becomes the leading tip of the nascent vessel (Ausprunk and Folkman, 1977). Accompanying this migratory process is the organised formation of solid strands of cells behind the leading tip as well as extensive proliferation of endothelial cells proximal to the leading tip of the newly emerging blood vessel (Archer, 1983).

1.4.1.3 Lumen Formation

New vessel formation is completed by the development of a lumen (Navaratnam, 1991). However, how solid strands and cords of endothelial cells acquire lumina is still a matter of debate. One theory involves the formation of intercellular lumina by enlargement of intercellular spaces by neighbouring endothelial cells (Folkman and Haudenschild, 1980). An alternative theory exists where intracellular lumina are thought to be initiated by the appearance of vacuoles within the cytoplasm of endothelial cells (Glaser et al, 1994).

Subsequent enlargement and fusion of vacuoles within neighbouring endothelial cells leads to the development of a tube like structure, (Folkman & Haudenschild, 1980). Other mechanisms that have been proposed for lumina formation include; the partial withdrawal of endothelial cells from the basal lamina thus forming cavities. Alternatively, endothelial cells of the primary bleb grow with a lumen that is continuous with the lumen of the parent vessel i.e., the new vessel grows as a capillary sprouting from the parent vessel (D'Amore, 1994; Dike and Ingber, 1996))

1.4.1.4 Anastomosis

The formation of a new vascular network is completed when neighbouring sprouts or tubes fuse, i.e., anastomosis (Archer, 1983). Neighbouring sprouts from the parent blood vessels grow parallel to each other away from the parent vessel. However, at a definite distance they tend to incline towards each other at their leading tips. Endothelial cells at their leading tips meet and make primary contacts that tighten and develop into compact structures. Intercellular gaps in both tips enlarge and fuse to form a continuous lumen and hence a new blood vessel has formed (Bischoff, 1995).

1.4.1.5 Vessel Remodelling

Once a network of tubes (immature vessels) has been formed, blood flow commences. These new vessels tend to be leaky and fragile and give rise to extensive exudates and haemorrhage. As the new vessels proliferate there is a constant process of reorganisation and remodelling (involving atrophy of certain vessels, and the formation of new vascular channels) to form the new vasculature (Garner, 1994; Ingber and Folkman, 1989).

1.4.1 The role of growth factors in retinal neovascularisation

The vascular endothelium of the healthy retina is remarkably quiescent, with a turnover of thousands of days. In contrast, diseases such as diabetic retinopathy, central retinal vein occlusion and retinopathy of prematurity, feature vascular endothelial cells with a turnover as low as five days (Archer, 1983; Glaser, 1994). As early as 1948, Michaelson postulated that in disorders such as these, the ischaemic retina induces the release of a diffusible biochemical factor and hence a trigger for the radical change in endothelial cell behaviour leading to retinal neovascularisation.

The establishment of techniques to study cultured cells of the microvasculature as well as in vivo models allowed the identification and characterisation of distinct angiogenic factors. These mitogens have diverse functions within the tightly controlled angiogenic process. Some of these angiogenic factors induce endothelial cell proliferation, migration or lumen formation or a combination, while others induce basement membrane proteolysis and extracellular matrix production. It should also be emphasised that these mitogens are not all endothelial cell specific. While some may act directly on endothelial cells, others may function indirectly by mobilising host cells (macrophages, mast cells and lymphocytes); other cells of the vasculature (pericytes, smooth muscle cells); and in the case of the retina, RPE cells and glial cells to produce angiogenic factors. The presence of angiogenic inhibitors is thought to counterbalance the effect of the angiogenic stimulators and provide the delicate balance associated with the normal quiescent retinal vasculature. The angiogenic factors associated with retinal neovascularisation include vascular endothelial growth factor (VEGF) (Dvorak et al, 1995; Boulton et al, 1998; Cao

et al, 1999), basic fibroblast growth factor (bFGF) (Gospodarowicz et al, 1987; Boulton et al, 1997; Cao et al, 1999), insulin-like growth factor (IGF-1) (King et al, 1985, Meyer-Schwickerath et al, 1993; Boulton et al, 1997), platelet derived growth factor (PDGF) (Heldin and Westermark, 1989, Tanihara et al, 1997), epidermal growth factor (EGF) (Carpenter and Cohen, 1979; ; Patel et al, 1994), transforming growth factor alpha (TGFα) (Schreiber et al, 1986, Tanihara et al, 1997; Patel et al, 1994), transforming growth factor-beta (TGFβ1 andTGFβ2) (Roberts et al, 1988; Hirase et al, 1998). This study concentrates on two potent retinal angiogenic factors, bFGF and most recently VEGF.

1.4.2.1 Basic fibroblast growth factor

Basic-FGF (FGF-2) is a cationic, 18 KDa polypeptide and a prototypic member of a family of heparan-binding growth factors (Mascarelli et al, 1987). The FGF family consists of at least nine members (now known as FGF-1 ...FGF-9). The FGF family use one of four molecules in FGF-receptor family (now known as FGFR1...FGFR-4), bFGF is reported to bind to FGFR-2 (Tanihara et al, 1997). Endothelial cells synthesise substantial amounts of bFGF, which is not only cell-associated but is also associated with extracellular matrix (Baird and Ling, 1987; Vlodavsky et al, 1987). bFGF is capable of inducing endothelial cell proliferation, migration and vasculogenesis in a wide variety of systems (Kumar et al, 1999). Although strong indications that bFGF plays important physiological roles, elucidation of mechanisms of bFGF action have been hampered by the fact that bFGF lacks a signal sequence and appears, for the most part, not to be secreted (Abraham et al, 1986, Klagsbrun and D'Amore, 1991). This fact led to the suggestion that bFGF might be secreted by alternate pathways, including cell injury or cell death thereby allowing them to initiate the

healing response (Bashkin et al,1989; Adamis et al, 1991; Ku and D'Amore, 1995; Hartnett et al, 1999). Numerous reports regarding the expression of bFGF have been published since its identification. In particular bFGF has been demonstrated in retinal capillaries and the neural retina (Hanneken et al, 1989; Hageman et al, 1991; Connolly et al, 1992). Basic-FGF has been shown to alter its distribution with the progression of retinal neovascularisation and has been found in preretinal membranes, vascular basement membranes and in the vitreous of patients with advanced diabetic retinopathy (Hanneken et al, 1991; Fredj-Reygrobellet et al, 1991; Boulton et al, 1997). More importantly FGF-receptor mRNA has been reported in the normal and diseased retina (Launay et al, 1994; Torriglia et al, 1994). Most relevant to this study, was the demonstration of soluble forms of the extracellular domains of the high affinity FGF-receptors and their distribution in the extracellular matrix, and on vascular endothelial cells in the retina and human vitreous fluid (Hanneken et al, 1994; 1995a;1995b;). Convincing data also indicate that bFGF and VEGF are synergistic in their angiogenic response (Goto et al, 1993; Mandriota and Pepper, 1997). Basic-FGF is thought to be widely associated with the activity of other growth factors, cytokines and proteases including TGFβ and plasminogen activator (Schott and Morrow, 1993; Gajduset et al, 1993).

1.4.2.2 Vascular endothelial growth factor

VEGF comprises of five main isoforms produced by alternative splicing of mRNA: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$ (Ferrara and Davis-Smyth, 1997; Wei et al, 1996). VEGF was initially characterised as a heparin binding angiogenic growth factor displaying high specificity for endothelial cells as well as being a potent vasopermeablity factor (Leung et al, 1989). VEGF

molecules have a signal peptide sequence and are secreted as homodimers (Ferrara and Davis-Smyth, 1997). The mature form of VEGF is the $VEGF_{165}$ homodimer which has a molecular weight of ~45Kd (Nicosia, 1998). The bioavailability of $VEGF_{189}$ and $VEGF_{206}$ is highly dependent on their ability to bind heparin and almost completely sequestered by the extracellular matrix (Park et al, 1993). $VEGF_{121}$, which is slightly acidic because it lacks the basic amino terminus, responsible for heparin binding, is the most soluble form (Houck et al, 1992)

The three secreted VEGF splice forms $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ induce proliferation of endothelial cells in vitro and in vivo angiogenesis (Park et al, 1993; Zhang et al, 1995; Poltrak et al, 1997). Examination of expression of various splice variants was examined, it was found that most cell types produce several VEGF variants simultaneously. Usually the 121 and 165 forms predominate, but expression of the 189 form was reported in most VEGF producing cells (Bacic et al, 1995).

VEGF binds with affinity to tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). Binding of VEGF causes receptor dimerisation followed by autophosphorylation of the receptor and signal transduction (Neufeld et al, 1999; Vaisman et al, 1990; Mustonen and Alitalo, 1995). The functional differences between VEGFR-1 and VEGFR-2 and gene knock out experiments have confirmed a role for both receptors in angiogenesis (Shalaby et al, 1995; Waltenberger et al, 1994; Kroll and Waltenberger, 1997).

The most exciting aspect of VEGF's properties is the observation that its expression is influenced by local oxygen environment, making it the ideal candidate for Michaelson's ischaemia induced diffusible biochemical factor. The mechanisms that regulate VEGF production by oxygen availability have

therefore received particular attention and are thought to be similar to that of erythropoeitin (Goldberg and Schneider, 1994). Hypoxia-induced transcription of VEGF mRNA is apparently mediated, at least in part, by the binding of hypoxia-inducible factor 1 (HIF-1) to an HIF-1 binding site located in the VEGF promoter (Levy et al, 1995; Liu et al, 1995). In addition to the induction of transcription, hypoxia promotes stabilisation of the VEGF mRNA (Levy et al, 1997; 1998). The expression of VEGFR-1 and VEGFR-2 have also been reported to be affected by hypoxia, although to a lesser extent than that of VEGF. The transcription of VEGFR-1 has been shown to be enhanced by hypoxia (Gerber et al, 1997). VEGFR-2 production is also upregulated under hypoxic conditions, but the mechanism responsible appears to be post-translational (Waltenberger et al, 1996). Of relevance to this study, patients with proliferative retinopathy have been shown to have increased levels of intra-ocular VEGF, with a correlation between levels of VEGF and degree of active retinopathy (Aiello et al, 1994). Histological examination of human post mortem eyes demonstrated increased retinal VEGF levels in diabetic patients (Lutty et al, 1996). In situ hybridisation and immunohistochemical studies have demonstrated the expression of VEGF in the normal human adult retina and in retinas displaying non-proliferative diabetic retinopathy (Gerhardinger et al, 1998). Up regulation of intraocular VEGF and its receptors has been demonstrated in several animal models of retinal neovascularisation (Gilbert et al, 1998; Aiello et al, 1995a; Robinson et al, 1996). Furthermore, recent experiments have demonstrated hyperproliferation of blood vessels in transgenic mice engineered to overproduce VEGF in retinal cells (Okamato et al, 1997). VEGF stimulates retinal endothelial cell growth in vitro; and hypoxia

increases VEGF production in retinal pericytes, RPE cells, Muller cells, ganglion cells and glial cells (Thieme et al, 1995; Aiello et al, 1995a).

1.4.3 The role of hypoxia in proliferative retinal diseases

Hypoxia is used to describe the reduced oxygen levels which occur in many pathological situations including ischaemic heart disease, wound healing and tumour formation. Yet hypoxia is actually a relative term which is specific to the pathological situation in question. Thus 'hypoxia' should be defined as an oxygen tension lower than that found in a physiological environment for a given tissue organ.

Insufficient oxygen or hypoxia in the retina was causally related to retinal neovascularisation as long ago as 1953, with the Indian ink injection studies of postmortem eyes by Ashton. It was demonstrated that neovascularisation follows retinal capillary non-perfusion and inner-retinal ischaemia, but the precise role of hypoxia has yet to be elucidated. It has been postulated that neovascularisation is mediated by the release of diffusible factor(s) from a hypoxic area which induces new vessel growth; as described above these factors have a wide spectrum of biological activities and could regulate neovascularisation at multiple levels of control.

The retinal oxygen supply is derived from two sources: the choriocapillaris, which supplies 60% of the required oxygen and the inner retinal capillary network which supplies the remaining 40% (Dollery et al, 1969). Oxygen levels in the normal retina have been determined in animals using micro-electrodes. The studies demonstrated a non-uniform distribution of oxygen supply and consumption in the various retinal layers. Oxygen tensions are highest in the region of the RPE/photoreceptor complex (up to 70mmHg) while in the mid-retina values can reach as low as 17mmHg. Values at the retina-vitreous

interface appear to be intermediate between the two. There is also some topographic variation within the retina and immediately pre-retinally, oxygen tensions being slightly higher adjacent to arterioles than in intervascular areas (Cringle et al, 1992). Inconsistencies arise in the measurements of the retina before and during pre-retinal neovascularisation, with values ranging from 12.1mmHg to 31.4mmHg (Maeda et al, 1990). It was also demonstrated that experimental occlusion of the retinal blood vessels leads to a reduction in intraretinal oxygen tension and that temporary occlusion of the retinal circulation in albino rats resulted in a proliferative response with mitotic figures in both retinal capillary endothelial cells and pericytes. Thus, suggesting a relationship between retinal oxygenation and cell proliferation in-vivo (Stefansson et al, 1988).

There is now increasing evidence that a number of growth factors are selectively modulated by hypoxia. These growth factors also implicated in retinal neovascularisation include bFGF, PDGF-B, TGFβ, EGF, IGF-1 and as discussed earlier VEGF. PDGF expression has been shown to be stimulated by hypoxia in both pulmonary and aortic endothelial cells (Kourembanas et al, 1993; 1997). Human dermal fibroblasts and smooth muscle cells have been shown to increase TGFβ expression under hypoxia (Clark and Coker, 1998; Khaliq et al, 1995). In vitro studies have demonstrated that retinal microvascular endothelial cells, pericytes and RPE cells are capable of producing IGF-1 and production increases towards hypoxia (Moriarty et al, 1994). Parallel studies on the effect of hypoxia on bFGF production demonstrated endogenous levels of this growth factor decrease as oxygen concentration was reduced, yet an increase (of ~300%) of bFGF receptors was observed (Shreeniwas et al, 1991). Thus suggesting rapid internalisation and

degradation of bFGF. This is further supported by studies demonstrating the proliferative response of HRPE cells in vitro. HRPE cell proliferation was maximal at hypoxia when exposed to bFGF and EGF, receptors for both growth factors were up regulated and shifted from low to high affinity receptors (Khaliq et al, 1995).

There is now an increasing amount of evidence hypoxia is involved in the induction of retinal neovascularisation possibly through the regulation of various angiogenic growth modulators.

1.4.4 The role of ECM components on neovascularisation

In the quest for a biochemical factor – the role of extracellular matrix components on retinal neovascularisation are often overlooked. In some respects the extracellular matrix controls the responsiveness of the endothelial cells to soluble factors (Ingber and Folkman, 1989). Matrix components bind to specific cell surface receptors, which may initiate second messenger signals, e.g. via tyrosine kinase, mediated protein phosphorylation, thereby inducing specific cell responses (Howe et al, 1998). Alternatively, matrix factors may interact directly with cytoskeletal elements within the cell such as actin and talin, which then cause alteration in cell shape and state of activation (Yamada and Miyamoto, 1995). Furthermore, several growth factors are known to bind strongly to some extracellular matrix components, such that the matrix may act as a reservoir for active and inactive forms of the proteins (Hay, 1991). Immunohistochemical and in-situ hybridisation studies involving normal and pathological retina have identified a number of integrins, fibronectin, laminin, thrombospondin, collagens I II and IV, and more recently vitronectin as the extracellular matrix components implicated in retinal neovascularisation.

1.4.4.1 Fibronectin

Fibronectin is a 450KDa glycoprotein, and the first structural glycoprotein, based on its immunohistochemical and cell adhesion promoting activity, defined as an extracellular matrix component. Fibronectin consists of two identical polypeptide subunits, held together by disulphide bridges. The molecule is produced by most mesenchymal and epithelial cells (cellular fibronectin) and is present in the extracellular matrix or plasma (plasma derived fibronectin). There is a single fibronectin gene located on chromosome 2, yet fibronectins from different sources contain chains that differ in size, indicating a number of different splice variants. To date the family of fibronectins contains at least eight splice variants (Grant et al, 1998). Fibronectin contains binding domains for cells, collagens, heparan, some proteoglycans, DNA, hyaluronic acid and fibrin (Engel, 1981; Ingber, 1990; Kleinman et al, 1993). These structural domains provide the chemical basis for the diverse biological functions of fibronectin including the regulation of growth differentiation, cell shape and migration, as well as its interactions with other matrix components. A number of peptide sequences have been identified which may be involved in cell binding including an RGDS (arg-gly-asp-ser) sequence, which is common to many glycoproteins such as laminin, collagen, vitronectin and fibrinogen (Curtis et al, 1995). The RGD sequence is not only involved in cell attachment, but may also have a role to play in migration and second messenger signalling, where its interaction with the integrins have been shown to initiate cell growth and enzyme secretion (Damsky and Werb, 1992).

Fibronectin has been demonstrated in the basement membrane of the normal retinal vasculature (Essner and Lin, 1988; Das et al, 1990). It is difficult to determine whether the fibronectin is cellular or plasma in origin. This is due to

the antibodies used in most studies to detect fibronectin are cross reactive with fibronectin species and therefore immunologically indistinguishable. However in situ hybridisation studies have shown fibronectin mRNA in the retina and retinal micro-vessels, supporting the possibility of local synthesis of fibronectin in the human retina (Grant et al, 1998). Previous studies have demonstrated the presence of fibronectin in the newly formed capillaries of retinas with proliferative retinal diseases, as well as increased intra-vitreal fibronectin (Casaroli-Marano et al, 1995). There is also increased fibronectin deposition within the vessel walls of the existing vasculature in diabetic retinas (Bek and Ledet, 1996). In-situ hybridisation and immunohistochemical studies have implicated fibronectin in embryological vessel formation and pathological neovascularisation (Paulsson, 1992). Studies using angiogenic model systems, researchers have shown fibronectin presence in the early stages of vessel formation (Nicosia and Madri, 1987; Nicosia et al, 1993). Fibronectin is also thought to facilitate migration but not attachment/ proliferation of endothelial cells in vitro (M^{c}Intosh et al, 1988; Roberts and Forrester, 1990; Madri, 1997; Grant et al, 1998). Receptors for fibronectin include $\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins, which have been shown to be present on endothelial cells (Albelda et al, 1989; Conforti et al, 1989 and Argraves, 1987).

1.4.4.2 Laminin

Laminin is the major structural glycoprotein with a high molecular weight (>800KDa). Laminin has several biological functions including, cell attachment, growth promotion, self assembly (to form basement membranes) and interactions with other matrix components (Grant and Kleinman, 1997). The laminins form a large family of glycoproteins exhibiting structurally and functionally different subclasses and isoforms where each laminin consists of an α, β and γ chain (Burgeson et al, 1994). The first described and most extensively studied laminin is laminin-1, which is derived form the matrix of Engelbreth-Holm Swarm tumour and exists as a cruciform structure formed by $\alpha 1$, $\beta 1$, and $\gamma 1$ chains (Timpl et al, 1979; Grant and Kleinman, 1997).

Unlike other adhesion molecules, laminin possesses eight different binding peptide sequences, indicating that it may act as a binding protein for several cells and matrix molecules at one time while simultaneously providing a compact meshwork for this purpose. Certain laminin peptides have been shown to promote endothelial cell growth and certain laminin peptides have been shown to induce angiogenesis (Grant and Kleiman, 1997). One peptide (YIGSR, tyr-ileu-gly-ser-arg) promotes differentiation/maturation in endothelial cells, while a second peptide (RGD, arg-gly-asp) promotes adhesion (Grant et al, 1989). This latter peptide is only available to the cell after limited proteolysis, which may therefore be a prerequisite for the initiation of angiogenesis during the stage of basement membrane degradation. A third peptide sequence from laminin (IKVAV, ileu-lys-val-ala-val) has been implicated in tumour angiogenesis, possibly via activation of cell surface plasminogen activator (Kibbey et al, 1993). This finding supports the notion that laminin degradation products promote angiogenesis, it was subsequently proposed that native

laminin protein has a regulatory effect on endothelial cells inhibiting proliferation and only in a cascade of events with a laminin digestion step does endothelial cell proliferation occur. Laminin similar to fibronectin, is involved in signal transduction as well as promoting cell adhesion, and the site of activity appears to be close to the IKVAV-containing site in the molecule (Kubota et al, 1988, Paulsson, 1992).

The presence of laminin has been well documented in the normal retina, not only in the basement membrane of retinal vessels, but associated with the extracellular matrix of the ILM (Belford et al, 1987; Essner and Lin, 1988). With respect to retinal neovascularisation, laminin has been identified in newly formed retinal capillaries in diabetic retinas (Marano et al, 1995). Increased laminin production is also observed in the vessel walls of diabetic retinas in comparison to normal retinas (Bek and Ledet, 1996).

Laminin is known to bind to several cell surface receptors including a number of integrins and 67kDa non-integrin cell surface receptor. The integrins being the best studied, include $\alpha_3\beta_1$, $\alpha_1\beta_1$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which are expressed in retinal and choroidal vessels in vivo, and endothelial cells in vitro (Brem et al, 1994; Davis and Camarillo, 1995; Tarone et al, 1990). Integrins including $\alpha_v\beta_3$ and β_1 have also been demonstrated on new vessels of diabetic retinas (Marano et al, 1995). More recent studies have demonstrated that the 67-Kda laminin receptor is preferentially expressed by proliferative retinal vessels in an animal model of ischaemic retinopathy (Stitt et al, 1998).

1.4.4.3 Vitronectin

Vitronectin is an adhesive glycoprotein found in plasma and in a variety of tissues and belongs to a group of adhesion moloecules that play key roles in the attachment of cells to their surrounding matrix (Preissner, 1991; Seiffert and Schleef, 1997). Vitronectin plays a pivotal regulatory function in the complement, coagulation and fibronolytic system (Seiffert , 1997). In addition, vitronectin binds to and stabilises the biological activity of type 1 plasminogen activator inhibitor (PAI-1), the physiological inhibitor of both tissue and urinary type plasminogen activators (Seiffert and Loskutoff, 1991; van Mejjer and Pannekoek, 1995). Thus vitronectin is thought to provide a unique regulatory link between cell adhesion and proteolytic enzyme cascades (Seiffert, 1997).

Vitronectin presence has been shown in newly formed capillaries in diabetic retinas as well as increased deposition in vascular walls of diabetic retinas (Marano et al, 1995; Van Aken et, 1997; Bek and Ledet, 1996). A significant increase in vitreous and plasma vitronectin was also reported in patients only with PDR undergoing vitrectomy for a variety of vitreoretinal pathologies (Esser et al, 1994).

Vitronectin has been demonstrated to bind to specific integrin receptors. The platelet integrin glycoprotein IIb/IIa, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_8\beta_1$ have so far been identified as vitronectin binding integrins (Preissner et al, 1997a; 1997b). Vitronectin contains a single RGD-sequence, and site directed mutagenesis studies reveal that this sequence is required for cell adhesion to vitronectin and cannot be compensated for by any other parts of the molecule (Preissner, 1991; Seiffert, 1997).

In the context of this study, the vitronectin binding integrins $\alpha_v\beta_3$, and $\alpha_v\beta_5$ are increased on blood vessels associated with PDR (Robinson and Aiello, 1998).

In addition, subcutaneous injection of cyclic-peptide antagonist of the vitronectin receptor - α_v inhibits neovascularisation (Hammes et al, 1996). Microvascular endothelial cells in vitro can be induced to express α_v, α_5 and β_3 subunits in the presence of various angiogenic factors (Enenstein et al, 1992).

1.4.4.4 The collagens

Collagens are a family of highly characteristic fibrous proteins, constituting a quarter of the total protein in mammals, and are produced by many cell types (Hay, 1991). At least 12 different types of collagen have been described in mammalian tissue and these differ in structure and function and tissue distribution.

A number of studies have demonstrated varied distribution of collagen types in large and small retinal vessels. Collagen types I, III, IV, V and VI have been demonstrated in larger retinal vessels. Collagen types I, IV and V with small amounts of III and VI were present in small vessels, and types I, IV and V in capillary basement membranes (Marshall et al, 1990; Jerdan and Glaser, 1986; Kennedy et al, 1986). It has been suggested that the presence and distribution of collagen I is unique to human retinal vasculature and gives greater stability to the extracellular matrix of vessels (Marshall et al, 1993; Jerdan and Glaser, 1986). Collagens have specific binding sites for cells including the RGD sequence (Dedhar et al, 1987). Cell binding occurs via integrin-mediated and non-integrin-mediated mechanisms. The integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ have been demonstrated to bind collagens I and IV (Davis, 1992). Collagen has been shown to affect endothelial cells at different stages of the angiogenic process. Collagen I has been shown to be a good substrate for retinal endothelial cell growth in-vitro, cell migration is poor unless within a 3-

dimensional collagen matrix (Schor et al, 1983, Sage and Vernon, 1994, Roberts and Forrester, 1990). Cell migration on type IV collagen is inhibited, behaviour that is irreversible even when laminin is present. This supports the view that collagen IV is strongly anti-angiogenic and hence basement membrane collagen IV degradation is necessary for retinal neovascularisation. In contrast, type III collagen is associated with elastic vascular tissue and is found in highest concentrations in healing wounds with active neovascularisation (Madri and Basson, 1992).

1.4.4.5 Other matrix molecules

Several other molecules (thrombospondin, SPARC (secreted protein rich in cysteine), nidogen/entactin and heparan sulphate proteoglycan), have also been shown to be involved in the interaction of endothelial cells and extracellular matrix and hence the angiogenic process. Thrombospondin is a 450KDa trimeric glycoprotein secreted into the extracellular matrix by many cell types including endothelial cells. It has been shown to inhibit neovascularisation in the rat cornea model and inhibits migration of capillary endothelial cells in vitro. A 19aa peptide from the heparan-binding domain of thrombospondin can disrupt endothelial cell focal adhesions and is also the most abundant protein in platelet-α granules to which TGFβ is tightly bound (Iruela-Arispe et al, 1995; Bischoff, 1995).

Another matrix component secreted by endothelial cells is SPARC. SPARC differs from typical extracellular matrix proteins in that it is anti-adhesive; it does not promote cell attachment and can disrupt focal adhesions. The role of SPARC in angiogenesis has been studied using both in-vitro and in-vivo assays. SPARC is synthesised in the microvasculature of the murine

embryonic brain (Nicosia et al, 1991). It was suggested that the degradation of SPARC in the extracellular matrix by proteases releases angiogenic factors that then direct the reorganisation of endothelial cells into new vessels (Bischoff, 1995;). Hence, this anti-adhesive glycoprotein appears to be important in endothelial cells with the extracellular matrix and the remodelling of this matrix during angiogenesis.

It has also been shown that a low density heparan sulphate proteoglycan (HSPG) is an important basement membrane component, possibly as a reservoir for growth factors such as FGFs which are tightly bound to HSPGs (Ausprunk et al, 1981a; 1981b; Bashkin et al, 1989; Adamis, 1991).

Entactin/nidogen is a single chain that exhibits both type IV collagen and laminin binding activities, and contains RGD sequences (Nicosia et al, 1993). These are all thought to be cell attachment sites and hence this glycoprotein may be involved in facilitating the end stages of neovascularisation.

1.4.4.6 Cell adhesion and receptors

In the last decade detailed information has become available regarding the biology of cell-cell and cell-matrix interactions. The molecules that are thought to facilitate these interactions include the immunoglobulin superfamily, the cadherins, the selectins and the integrins.

Members of the immunoglobulin (Ig) and cadherin superfamilies of cell adhesion molecules have also been identified in endothelial cells. PECAM-1 (platelet derived cell adhesion molecule-1) also known as endoCAM and CD31 is expressed in large and small vessel endothelial cells as well as platelets and has been shown to reside at inter-endothelial cell junctions (Kim et al, 1998; Madri, 1997; Albelda et al, 1990). Members of the cadherin family of cell

adhesion molecules have been identified in bovine endothelial cells and postulated to function in tight junction formation and regulation of permeability .

To date the majority of work regarding neovascularisation and adhesion molecules has focused on the integrin family of receptors; although roles for selectins, immunoglobulin superfamilies and cadherins have been proposed, the mechanisms of how they exert their control over the angiogenic process needs much more investigation.

The integrins are heterodimeric transmembrane glycoproteins, made up of an alpha and a beta chain, of which there are at least 15-alpha and 8-beta molecular variants (Bosman, 1993; Luscinskas and Lawler, 1996). The ligand specificity is thought to occur through the alpha chain, all of which have a long extracellular domain which contains several cation binding regions and a small intracellular domain (Haas and Plow, 1994). The beta chains have characteristically longer intracellular domains that provide an anchor for the heterodimer to the cell cytoskeleton through interactions with viniculin and talin (Burridge et al, 1988). Several integrin families are expressed during angiogenesis. Endothelial cells in culture express a number of different itegrins, including the β_1 subfamily ($\alpha_{1\text{-}6}$ β_1) and the β_3, β_4, and β_5 family members (Brooks, 1996). Integrin expression and function has been investigated by a number of laboratories using in vitro and in vivo angiogenesis models. Previous studies have, demonstrated the presence of $\alpha_2\beta_1$ (a receptor for collagens and laminin) and $\alpha_5\beta_1$ (a fibronectin receptor) on endothelial cells in monolayer culture (Brooks, 1998). It was proposed that these receptors were involved in regulating the integrity and permeability of the monolayer. $Alpha_v beta_3$ integrin was found to be involved in calcium dependent signalling and endothelial cell migration on vitronectin, while $\alpha_2\beta_1$ mediated calcium independent signalling on

a collagen substrate (Robinson and Aiello, 1998). The integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$ have been demonstrated on microvascular endothelial cells (Cheng and Kramer, 1989). More specific to this study, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ heterodimers were demonstrated on new vessels from patients with retinal neovascularisation (Luna et al, 1996, Freidlander et al, 1996). Moreover, there was no detectable signal for $\alpha_v\beta_3$ by immunostaining in normal human retina. A number of in vivo studies have centered around the fact that $\alpha_v\beta_3$ is up regulated during angiogenesis (Horton, 1997) and the effect of integrin agonists. In several hypoxia-induced models of retinal neovascularisation, intervention with an α_v - integrin antagonist resulted in significant reduction in unwanted angiogenesis (Preissner et al, 1997; Hammes et al, 1996 and Luna et al 1996). Another study demonstrated $\alpha_v\beta_3$ and $\alpha_v\beta_5$ antagonists specifically blocked new vessel formation with no effect on established vessels in a murine model of retinal angiogenesis (Friedlander et al, 1996), thus opening avenues for possible therapeutic intervention for angiogenic therapy.

1.5 Contraction and proliferative retinal diseases

The contractility of the retinal membranes found in proliferative retinal diseases plays a crucial role in visual outcome, i.e., the contractile event can lead to retinal detachment, retinal tears and/or haemorrhage when a vascularised membrane is involved. The contractile response proceeds a series of critical events including the migration of cells through the surrounding matrix, extracellular matrix deposition and remodelling of the matrix with ultimate formation of the fibrous cellular membrane. The contractile event is now thought to be cellular in origin rather than extracellular (Porter, 1998; Cordeiro, 1997). Several theories exist as to how contraction of retinal membranes occurs. Initially it was proposed that myofibroblasts (fibroblasts with some of the characteristics of smooth muscle cells), observed in PVR membranes were responsible for the decrease in area of the membrane in a similar way these cells were thought to behave in wound contraction (Gabbianni et al, 1972). It has since become evident that it may not be the myofibroblast that is responsible for wound contraction (Ehrlich, 1990) since the majority of contraction occurs before the appearance of myofibroblast cells in a wound situation, also contraction of the membranes occurs when the proportion of such cells are small. It was hence hypothesised that cell migration and subsequent adhesion is responsible for membrane contraction (Hitchens et al, 1986). An alternative theory where the cells involved migrate and attach to nearby structures by extending lamellapodia, and then retract. The nearby structures are thought to be mainly collagen fibrils, which are reeled in by the cell and this results in a shortening of that particular structure. A repetitive sequence of retraction and extension of lamellopodia then ensues by any

number of cells present in the membrane resulting in a contractile event (Glaser et al, 1987)

1.5.1 Retinal membrane formation

Retinal membrane formation is described as a fibrocellular cascade in which various retinal and inflammatory cell types are said to proliferate, migrate and deposit extracellular matrix on the surface of the retina or beneath it (Machemer and Laqua, 1975). Each step in the cascade is encouraged by various growth factors and cytokines until the membrane contracts an event which also is affected by various factors. The fibrocellular cascade can manifest alongside a neovascular response in disorders such as proliferative diabetic retinopathy, branch retinal vein occlusion or in its own right as in conditions such as proliferative vitreoretinopathy.

1.5.1.1 Cellular composition

Immunohistochemical analysis has identified RPE cells in post-mortem tissue as well as in animal models of PVR, (Machemer et al, 1978; Hiscott et al, 1994). In vitro models have suggested that RPE cells within the eyecup may undergo metaplastic change to macrophage or fibroblast-like morphology. Furthermore, there is a consistent presence of RPE cells in the contractile membranes formed particularly in PVR, this is thought to be an important cell type and hence much work has focused on their involvement in the pathogenesis of the condition.

Glial cells have been shown to be a part of simple and complex epiretinal membranes (Morino et al, 1990). The cellular derivation of the glial component is still under much speculation, since Muller cells, astrocytes, microglia and perivascular glia all have the potential to proliferate and contribute

to epiretinal membrane formation. It has been proposed that 'simple' epiretinal membranes may form in relation to retinal breaks and holes (Foos, 1974; Clarkson et al, 1977) and these glial membranes can potentially provide a scaffold for the formation of the more complex membranes seen after retinal detachment. Immunohistochemical analysis of postmortem/postoperative donor tissue has shown that non-traction membranes were mainly glial and that other cellular components were necessary to produce a contractile response. Most studies investigating retinal membrane formation have identified cells catergorised as fibroblasts or myofibroblasts, although their origin has not as yet been established (Clarkson et al, 1977; Machemer et al, 1978). Some studies have proposed the origin of fibroblast-like cells as dedifferentiated RPE cells or in diseases such as PDR, from vascular endothelial cells. The possibility that they may originate from glia, pericytes or hyalocytes has not yet been ruled out. The breakdown of the blood-retinal barrier and the presence of an inflammatory response has led to the consistent identification of macrophages and more recently lymphocytes in retinal membranes by immunohistochemical techniques (Charteris et al, 1992, 1993; Limb, 1997). In reality, given the ability of the cells involved to change morphology and immunohistochemical characteristics in vivo and in vitro, the exact cellular origin of a retinal membrane is difficult to ascertain. Membranes produced as a result of vasoproliferative disorders (of which PDR is the most common) have cell compositions that may vary in proportion/presence of different cell types to those of nonvascular membranes (Hiscott and Grierson,1994).

1.5.1.2 Extracellular matrix

In the context of retinal membrane formation, extracellular matrix components are necessary for a diversity of functions. The presence of fibronectin has been confirmed from the early stages of membrane formation, in immunohistochemical and mRNA studies in animal models of PVR and post-mortem human tissue (Hiscott et al, 1992, Weller et al 1988 and Hiscott et al 1993). Fibronectin is thought to be chemotactic for various cell types as well as enhancing migration and facilitating proliferation of potential membrane forming cells (Hiscott et al, 1992b). There is still some debate as to the origin of glycoproteins in membranes. It is thought that initially a breakdown of the blood-retinal barrier allows plasma fibronectin in areas of eventual membrane formation (as well as various growth factors) enticing cells which are then thought to produce their own matrix components and an autocrine control mechanism ensues.

Vitronectin expression has also been demonstrated in PVR membranes, with some co-localisation with fibronectin (Weller et al 1991a). Receptors for vitronectin have also been demonstrated on pre-retinal membranes (Weller et al, 1991b). This cell spreading factor is thought to initiate cell/matrix interactions preceding contractile events.

Laminin presence has also been demonstrated in retinal membranes, particularly if a vascular component is present or if internal limiting membrane fragments are associated with the membrane (Morino et al, 1990, Casaroli-Morano et al, 1994).

Collagen types I, II, III and IV have all been identified within epiretinal membranes (Morino et al, 1990) and some studies indicate a greater collagen presence in the later stages of PVR/PDR membrane development. Collagen II

is thought to be vitreal in origin (Hiscott et al, 1985), immunohistochemical analysis has associated collagen I, II and IV with RPE and fibroblast like cells, rather than glial cells in PVR membranes

1.5.1.3 Integrins in retinal membrane formation

As described in section 1.4.4.6 integrins are necessary for a wide range of cell-cell and cell-extracellular matrix interactions. In diseases such as PVR, membrane formation also relies on such matrix communication for progression, integrin interaction has become a recent focus for therapeutic intervention. A study by Robbins et al (1994) demonstrated the presence of 9 integrin subunits of which β_1 and α_6 subunits, were prominent at the edges of most PVR and PDR membrane samples. Furthermore, pigmented cells in membrane tissue, expressed up to four more integrins than their normal counterparts, such that besides expressing α_4, they also stained positively for α_5, α_6, β_1 and β_2 (Robbins et al, 1994, Brem et al 1994). Moreover α_2 and β_1 subunits were detected in close association with each other as well as on cytokeratin positively stained cells, thus in keeping with several in vitro studies demonstrating the importance of $\alpha_2\beta_1$ in RPE cell mediated contraction (Kupper and Ferguson, 1993, Hunt et al, 1994). Kupper and Ferguson (1993) demonstrated that human cell mediated vitreous (type II collagen) contraction could be blocked by antibodies and peptides that neutralised/antagonised the function of $\alpha_2\beta_1$ integrin heterodimers. They further demonstrated that the potential for contraction could be conferred upon non-contracting cells by stable transfection of cells with α_2 CDNA. Hunt (1994) demonstrated β_1, α_2 and α_5 integrins on RPE cells, but only $\alpha_2\beta_1$ were found to participate in RPE cell mediated collagen gel contraction. This effect was also neutralised by antibodies against these integrins.

1.5.1.4 Growth factors and retinal membrane formation

The progression of any proliferative response is known to be regulated/controlled by a number of growth factors and cytokines. Thus many studies have reported the presence of a number of growth modulators in the vitreous, subretinal fluid and retinal membrane samples from postoperative and post mortem tissue with varying degrees of proliferative retinopathy.

The presence of aFGF, EGF and IGF-1 was reported in diabetic and PVR membrane tissue by Fredj – Reyrobellet and colleagues (1991) using an indirect immunofluorescent technique. The same group investigated the presence of the above growth factors as well as TGFβ-1 using intravitreal and subretinal fluid cells from patients suffering from PVR in various stages of development (Baudouin et al, 1993). Acidic FGF was in all vitreous and subretinal specimens, EGF was demonstrated in all of the intravitreal samples and all but one of the subretinal samples. IGF was demonstrated in the majority of vitreal and subretinal samples, yet the reactivity within each sample was higher than most of the aFGF samples. TGFβ-1 was found in approximately half of intravitreal and subretinal samples with lower immunoreactivity (Boudouin et al, 1993). A study by Patel, and co-workers (1994) demonstrated increased levels of EGF, TGFα as well as their receptor (EGF-R) in the diabetic retina (when compared to non-diabetic retina) and increased immunoreactivity for EGF, TGF-alpha and EGF-R was observed in the majority of PDR membranes analysed immunohistochemically. A study by Connor et al (1989) demonstrated that $TGF\beta_2$ levels were increased in eyes with PVR membranes, but not $TGF\beta_1$. A more recent study detected the presence of PDGF and bFGF in vitreous samples taken from patients with a variety of vitreoretinal disorders. Basic-FGF and PDGF concentrations were found to be elevated in PVR even in

the absence of vitreous haemorrhage, and not in patients with retinal detachment uncomplicated by PVR indicating a possible autocrine mechanism in which the cellular membrane constituents participate (Cassidy et al,1998). A number of cytokines have also been linked to the pathogenisis of PVR including IL6, IL8, INFγ and low levels of IL2 (Kenarova et al,1997; Aksunger et al, 1997; Abu-el-Asrar 1997). Although many studies have implicated a number of growth factors and cytokines by their presence, it is not clear whether a given growth factor or cytokine is responsible for encouraging membrane formation or facilitating the contractile response. The use of invitro models of RPE cell, fibroblast and glial cell proliferation, migration and contraction has allowed the investigation of putative mechanisms, whereby many of the growth modulators mentioned above may exert their control. Below is described a few examples of methods by which growth factors implicated in retinal membrane formation can be used to elucidate a mechanistic insight. This is by no means inclusive of the large amounts of information derived from many such studies in recent years.

Campochioaro and colleagues (1994) investigated the role of PDGF in retinal membrane pathogenesis. This study utilised the wounded RPE monolayer model to show increased expression of PDGF and PDGF-beta receptors in the cells along the edge of the wound. Moreover, RPE cells were shown to secrete PDGF and a neutralising antibody to PDGF significantly decreased RPE cell growth in serum free medium. These findings suggest PDGF as an autocrine stimulator of RPE cell wound repair.

Smith-Thomas et al (1996), suggested that bFGF was a stimulator of RPE cell proliferation, as well as PDGF and TGFβ_1. These mitogens were demonstrated to have an additive/synergistic effect with ECM proteins on RPE cell proliferation. A study by Schwegler et al (1997) also demonstrated the use of

cell proliferation assays as a tool for investigating the effect of aFGF and bFGF on RPE cell growth and found that bFGF stimulated RPE proliferation, an effect which was not seen in the presence of aFGF.

A popular method of investigating growth factor action is utilising the collagen gel contraction model. Raymond and Thompson (1990) demonstrated the enhancing ability of TGFβ on RPE cell mediated contraction using this model. Similarly Pena et al (1994), investigated the effect of two TGFβ isoforms, $TGF\beta_1$ and $TGF\beta_2$ which are reported to have different effects on different cell types (Clark and Coker, 1999). It was found that $TGF\beta_1$ and $TGF\beta_2$ equally enhanced fibroblast mediated contraction in a dose dependent manner. This action was neutralised with anti TGFβ antibodies (Pena et al 1994). A more recent study investigated the effect vitreous collected from patients with varying stages of membrane formation of Muller cell mediated contraction. The contributions of individual growth factors to vitreous activity were assessed by inhibition of contraction by specific neutralising antibodies. Hardwick and colleagues (1997) concluded that the vitreous of patients with retinal detachment, proliferative retinal disease and vitreous haemorrhage contain varying amounts of growth factors that stimulate traditional forces Muller cells. The majority of the activity was attributed to IGF-1 and a smaller proportion to PDGF.

1.5.1.5 The Role of matrix enzymes in proliferative retinal diseases

An aspect that has of late generated a lot of interest is the role of matrix remodelling enzymes. Sparked by the finding that a group of enzymes collectively known as the matrix metalloproteinases (MMPs) appeared to play a crucial role in the processes of cell movement within extracellular matrix remodelling with a resultant contractile event. The cellular and molecular

mechanisms associated with the induction and cessation of this enzyme activity are not yet clear. Recent studies have investigated the presence, production and roles of some MMPs in vivo and in vitro. The MMPs so far implicated in retinal membrane activity are MMP1 (interstitial collagenase), MMP2 (gelatinase), MMP3 (stromelysin) and MMP9 (type IV collagenase) (Hunt et al 1993; Kon et al, 1998). The action of these MMPs are thought to be regulated via a number of mechanisms including the involvement of tissue inhibitors of matrix metalloproteinases (TIMPs) or α_2 macroglobulin (Kon et al, 1998; Birkedal-Hansen, 1995).

1.5.6.1 The role of oxygen in proliferative diseases

As mentioned in section 1.4.3 hypoxia is an important aspect when considering proliferative retinal diseases, particularly when new vessel formation occurs. It is also well documented that low oxygen is an issue in any wound situation, especially when considering non-vascularised PVR membranes or areas of fibrosis within neovascular membranes. Studies looking at the effect of oxygen concentration on cell proliferation, the response to and production of various growth factors, as well as the regulation of receptors have demonstrated that oxygen may be important when considering a pathological wound situation. Previous work in this laboratory has suggested that oxygen modulates the release of tissue digesting enzymes and their inhibitors by RPE cells indicating a possible role for oxygen in the pathology of retinal membrane formation. Moreover, Khaliq et al (1995, 1996) demonstrated the ability of oxygen to modulate the response of RPE cells to bFGF and EGF by a mechanism involving receptor regulation. This is in agreement with an earlier study investigating a similar mechanism in fibroblasts (Wing et al, 1988). These and

several other studies indicate the importance of oxygen, particularly when considering membrane formation in the context of proliferative retinal diseases (Akeo et al, 1992; Knorr et al, 1993).

1.6 Aims

It is evident that the refinement of molecular and cell biological techniques has allowed the accumulation of an enormous amount of information regarding the mechanisms involved in the different stages of neovascularisation and a pathological wound situation, as retinal membrane formation is often viewed. However, much of the detail remains fragmentary and the information cannot always be applied to the specialised situation of the retina. This is due to the fact that many studies rely on either in-vivo vessel formation in non-retinal vascular beds or the use of unrepresentative in vitro models. Furthermore, studies investigating the mechanism of contraction in retinal membranes and the role of various growth factors/extracellular matrix have not as yet addressed the potentially important role of oxygen.

Thus the primary aims of this study are: -

a) To assess the in vitro growth characteristics of retinal arterioles versus venules (because retinal neovascularisation almost always occurs on the venous side of the capillary network),

b) To develop an in vitro culture model to study pre-retinal neovascularisation using retinal tissue,

c) To determine the effect of varying oxygen concentrations, growth and extracellular matrix components on retinal cells using the collagen gel contraction model

CHAPTER TWO - A COMPARISON OF THE GROWTH CHARACTERISTICS OF RETINAL ARTERIOLES AND VENULES IN-VITRO

2.1 Overview

New vessel formation in vasoproliferative retinopathies almost always occurs on the venous side of the capillary network (Henkind and Wise, 1974; Archer, 1983; Kuwabara and Cogan, 1969). This predilection for venous outgrowth has never been addressed at the cell biological level, but a number of possibilities have been considered. These include differences within the cells of the vasculature of each vessel type, or within the composition of the basement membrane of arterioles and venules resulting in the majority of neovascularisation in vivo occurring from or near retinal veins.

The aim of this study was to investigate whether differences exist in the biology of retinal arterioles and venules at the cellular level. This was undertaken using cell culture techniques to assess vascular cell attachment, morphology, and migration from bovine retinal arterioles and venules. The effect of extracellular matrix components and growth factors was also assessed due to their involvement in the earliest stages of retinal neovascularisation (Klagsbrun and D'Amore, 1991; Sage and Vernon, 1994).

2.2 Experimental methods and design

2.2.1 Histological characterisation of retinal vessels

Arterioles and venules were fixed after dissection from the retinas, processed and embedded in wax. The serial wax sections produced were stained either with haematoxylin and eosin or Massons' trichrome. Arterioles and venules from the same retina were compared due to variability of size from individual retinas. Arterioles and venules were characterised by the fulfilment of 3 criteria. (1) At the dissection level, on the basis of vessel position and width (venules tended to be wider than arterioles). (2) At the histological level – with arterioles containing generally more stained nuclei. Finally, (3) thicker extracellular matrix, visualised when stained with massons' trichrome.

Isolation of retinal vessels – each bovine retina was carefully removed by dissecting a globe slightly posterior to the limbus. The vitreous together with the neuroretina was gently lifted away from the RPE layer. The retina was cut off at the optic disc and both the vitreous and the posterior eyecup were discarded. The retina was given two washes in PBSA, transferred to, and floated in, a 90mm petri-dish containing 15ml PBSA. Each retina was straightened out to enable visualisation of vessels prior to dissection. Whether a vessel was an arteriole or a venule was decided on criteria such as position (exit of vessel from optic nerve) and diameter of vessel. Using a Zeiss dissecting microscope in a sterile tissue culture cabinet, arterioles and venules were micro-dissected by gently teasing the vessels away from the surrounding tissue using fine pointed forceps. Dissecting scissors were then used to cut vessels away from the retina taking care not to disturb any surrounding tissue. In this way vessels up to 20mm long were removed and placed in MEM/HEPES (Appendix II).

Processing of vessels for wax sections – freshly dissected retinal vessels fixed in 10% NBF (Appendix II) were washed two times in PBSA and dehydrated through a series of concentrations of alcohol (v/v H_2O) as follows. 25% for 10 minutes, 33% for 10 minutes, 50% for 10 minutes, 75% for 20 minutes, 90% for 20 minutes, 2 changes of 100% alcohol for 20 minutes each. Vessels were then immersed in a series of chloroform/alcohol (v/v) concentrations as follows: 25% chloroform for 15 minutes, 33% chloroform for 15 minutes, 50% chloroform for 15 minutes and 2 changes of 100% chloroform for 30 minutes each. The vessels were then embedded in wax for 20 minutes using a tissue embedding system. The wax blocks were trimmed and 7μm thick sections were cut on a microtome. The sections were placed into a warm water bath, allowed to flatten at 40°C - 50°C, and transferred onto APES coated microscope slides (Appendix III). The slides were dried on a hot plate for 3-4 hours, then placed in an oven at 57°C overnight for further drying and section adhesion.

Haematoxylin and eosin staining of wax sections - wax sections were placed in a 60-70°C oven for at least 20 minutes. The sections were de-waxed in a bath of xylene in an extractor hood for 5 minutes, and placed in two further changes of xylene for 2 minutes each. The sections were rehydrated through a decreasing series of alcohol concentrations (v/v H_20): 100% alcohol for 10 seconds (twice), 90% alcohol for 10 seconds, 75% alcohol for 10 seconds, 50% alcohol for 10 seconds, 25% for 10 seconds, and finally taken into distilled water. After washing with distilled water, the sections were stained with Harris's haematoxylin for 5 minutes, washed in cold running tap water, and then exposed to hot running tap water until sections were "blue" (about 5 minutes or less). The sections were then stained with 1% eosin for 5 minutes, washed in cold running tap water for 1-5 minutes, dehydrated in increasing concentrations

of alcohol (from 10% to 100%) and finally cleared in xylene. The stained slides were removed from the xylene and gently lowered onto coverslips spotted with practamount mounting medium (Appendix I).

Masson's trichrome staining of wax sections – dewaxing was carried out by immersing the sections in 2 changes of xylene, each for 3 to 5 minutes. Rehydration followed through a descending series of alcohols from absolute through to 50% alcohol (absolute 2 changes, 90%, 70% and 50%, 10 seconds in each), and finally the slides were rinsed with water. The slides were stained in Harris haematoxylin for three and a half minutes and washed with running water for 3 minutes. The slides were differentiated with 1% picric acid in 70% alcohol for 3 minutes and washed in tap water for 1 minute. The slides were then stained in 0.5% Biebrich scarlet in 1% acetic acid for 2 minutes followed by a brief rinse in water. Subsequently the slides were treated with a 1:1 mixture of 5% phosphotungstic acid and 5% phosphomolybidic acid (mordant) for 3 minutes and the excess solution was drained. The slides were stained in 2.5% fast green in 2.5% acetic acid for 20 minutes. The slides were washed in running water followed by dehydration through an ascending series of alcohols from 50%, 70%, 90%, absolute (2 changes) for 30 seconds in each and cleared in xylene (2 changes) for 3 to 5 minutes in each change. Finally the slides were mounted in practamount media and left in a 37°C oven overnight to dry. This modification of Masson's trichrome stains cell nuclei blue, basement membrane green, and elastin pale red.

2.2.2 A comparison of growth characteristics of retinal arterioles and venules in vitro

The growth characteristics of arterioles and venules were determined using a number of different parameters. These parameters were:

(I) attachment efficiency – over a period of 14 days,

(II) presence of cellular outgrowth,

(iii) time taken for cellular outgrowth to appear and

(iv) type and nature of cellular outgrowth.

Attachment was assessed on whether part or the entire vessel was floating and the transparency of the vessel (the transparency of the vessel indicated that the vessel had survived the trauma of dissection). Since good attachment was essential for outgrowth, cultures, that had floating vessels, were discarded and those with partially attached vessels were monitored closely and discarded if no outgrowth occurred after 2 weeks. Presence of cellular outgrowth was recorded since good attachment did not necessarily lead to cellular outgrowth. Time taken for cellular outgrowth to appear was recorded since a clear and consistent difference was observed in time taken for outgrowth to appear between arteriolar and venous cultures. The type and nature of outgrowth described the cell type, which appeared as initial outgrowth i.e., endothelial cells, pericytes, smooth muscle cells (cells of the vasculature) and fibroblasts, glial cells or RPE cells (non-vascular retinal cell types). Furthermore whether individual cells were present or as actively dividing colonies.

Culture and maintenance of retinal vessels - arterioles and venules were cut into fragments between 5 and 10mm long and each placed some distance apart onto 35mm culture dishes. Two to three vessels were placed in each dish as quickly as possible after removal to prevent drying out of the vessels. A drop of

growth medium (DMEM + 20% serum, Appendix II) was placed onto the vessels that were then covered with a sterile glass coverslip. A small amount of pressure was applied to force out any trapped air bubbles, 2ml of growth medium were then added carefully and the culture placed in a standard CO_2 incubator at 37°C within a humidified atmosphere containing 5% CO_2 and 95% air. The culture was not disturbed for at least 36 hours to allow for attachment. The growth medium was removed and replaced with fresh growth medium every 2 days. The cultures were maintained for up to 8 weeks in culture and monitored by inverted microscopy and photography.

2.2.3 Effect of post mortem time on outgrowth from retinal arterioles and venules

This study was undertaken to optimise the retinal vessel culture system. Arterioles and Venules were dissected from retinas removed from eyes either, less than five hours post-mortem time or, longer than 20 hours post-mortem time. Eyes longer than 20 hours post mortem were stored at 4°C until used.

2.2.4 Comparison of growth media on retinal vessel outgrowth

In order to maximise growth and to select for endothelial cell growth in the culture system, two growth media were compared; DMEM + 20% serum, (growth medium for retinal pericytes) and ESFM + 10% PPP, (growth medium for retinal capillary endothelial cells). Test cultures were set up as described in section 2.2.2 and monitored for time of outgrowth, attachment and presence of endothelial cell only cultures.

2.2.5 Effect of substrate on retinal vessel outgrowth

The parameters described in section 2.2.2 were investigated in the presence of various extracellular matrix components. This study was undertaken to determine the effect of growth substrates on retinal vessel growth, and included: collagen I, denatured collagen (gelatin), fibronectin, laminin and vitronectin. Collagen I was chosen as it present in retinal vascular membranes to a higher degree than in the general vasculature, and described as having a positive effect on vascular integrity (Marshall et al, 1993; Jerdan and Glaser, 1986). Gelatin was chosen because of numerous studies describing this substrate as highly effective at encouraging endothelial growth in vitro (Captenades and Gerritsen, 1990; Schor and Schor, 1986). The fibronectin and vitronectin used in this study were plasma derived, as the increase of these components in proliferative retinal diseases are thought, initially to be plasma derived. The laminin isoform used in this study was laminin-1, although several laminin isoforms are known to exist in the vascular basement membranes, laminin-1 not only contains many of the binding site required for endothelial cell activity – it is also present in the interstitial matrix.

Coating culture dishes with extracellular matrix components - extracellular matrix molecules were made up at the following concentrations. Gelatin - 0.1% (w/v); collagen I - 0.2mg/ml; laminin - 20μg/ml; fibronectin - 20μg/ml and vitronectin - 0.5μg/ml under sterile conditions in double distilled water with the exception of gelatin which was filter sterilised after allowing the gelatin to dissolve into solution in a 37^0C water bath. The concentrations of the extracellular matrix components used were based on a combination of previous studies and preliminary studies in this laboratory (Wong et al, 1988; Su and Gilles, 1992; Capetandes and Gerritsen, 1990; McIntosh et al, 1988; Roberts

and Forrester, 1990). Two or five millilitre aliquots were pippetted into 35mm culture dishes or 25cm^2 tissue culture flasks respectively and left to stand for one hour. The excess solution was removed and the dishes/flasks left to air dry in a sterile tissue culture cabinet for at least 4 hours. To allow direct comparability arterioles and venules from the same retina were used for each test substrate as well as the control, which consisted of arterioles and venules with no growth substrate in identical conditions. Each experiment with all test substrates and control were set up in duplicate on three separate occasions.

2.2.6 Effect of growth factors on retinal vessel outgrowth

The study to determine the effect of growth factors on retinal vessel outgrowth was set up as in 2.2.2, except the growth medium was supplemented with 5ng/ml of either bFGF or VEGF. The growth factors investigated in this study were chosen because they are not only heavily implicated in proliferative retinal diseases, they have been shown to be potently mitogenic and chemotactic for vascular endothelial cells and pericytes in vitro (Nicosia, 1998; Kumar et al,1999; Hartnett et al, 1999). The concentrations of the growth factors used were based on previous studies and product information from the supplier (Yoshida et al, 1996; Aiello et al, 1995; Khaliq et al, 1995). The medium was changed every 48 hours and fresh growth factor was added each time. To maximise comparability, arterioles and venules from the same retina were used for each test substance and the control. Each experiment in each test conditions was set up in triplicate on four separate occasions. All statistical analysis was undertaken using SIMFIT and Microsoft® Excel statistics software. Statistical analysis involved Mann-Whitney-U, Kolmogorov-Smirnov-D paired and unpaired t-tests for significance.

2.2.7 Characterisation of retinal vessel outgrowth

Cellular outgrowth was monitored using inverted microscopy, with photomicrographs being used to record a journal of outgrowth. Initial characterisation was based on morphological analysis of cultures. Cultures chosen at random were immunostained using the method below to identify cell type. Thus a semi-quantitative criteria was used for identification of outgrowth. Anti-von Willebrand's factor (vWbF) was used as an endothelial cell marker and α-smooth muscle actin (αsma) was used as a pericyte marker. Positive controls used were retinal vessels. Negative controls included omission of primary and / or secondary antibodies on retinal vessels, retinal vessel cultures. All cultures were dual stained to achieve maximum information form vessel cultures.

Immunostaining of retinal vessels in culture - cultures were fixed in 10% NBF (Appendix III) for at least 24 hours. Cultures were prepared with three x1 hour washes to remove any excess NBF and unattached debris. Cultures were permeabilised with triton-X 100 solution (Appendix III) for 30 minutes and then washed 3 times with PBSA to remove any remaining triton. Cultures were then incubated in 20% serum (v/v PBSA) of relevant species for 1 hour at room temperature to block non-specific immunoglobulin binding sites. The serum was removed and replaced with a solution containing the primary antibody in 0.2% serum solution. The cultures were then given 3 thorough washes with PBSA to remove any excess/unbound antibody and incubated for 1 hour with a fluorescent-labelled secondary antibody. The cultures were given three final washes with PBSA before mounting with gelvatol mountant (Appendix III). All staining was examined using conventional fluorescence microscopy (Olympus

vanox AHB53)and photographs (using Elite 400 Kodak film) were taken of representative staining of random samples.

Table 2.1 – Summary of immunohistochemical reagents

Primary antibody (species)	Secondary antibody (species)	Blocking serum (species)
anti-Von Willebrand factor (rabbit)	anti-rabbit FITC (goat)	goat
anti-α smooth muscle actin (mouse)	anti-mouse TRITC (goat)	goat

2.2.8 Sub-culture of retinal arteriolar and venous endothelial cells

In order to determine whether endothelial cells had different proliferative capabilities when cultured from different vessel types, pure cultures of retinal arteriolar and venous endothelial cells were sub-cultured. Cultures of retinal vessels were set up as above using 35mm dishes precoated with 0.1% gelatin. The growth medium (ESFM + 10%PPP) was removed and replaced with fresh medium every 48hr. Once colonies of endothelial cells were established they were monitored by inverted microscopy for contaminating cell types. Cultures with dividing pericytes and glial cells were discarded after one week. As endothelial cell colonies appeared to reach confluence, the coverslip was removed using sterile forceps and the medium was decanted. The cultures were washed twice with PBSA and 0.2ml trypsin in Puck's Minimal media (Appendix I) was added for approximately 30 seconds at room temperature. The trypsin solution was neutralised with 3ml growth medium and the cell suspension placed into a 35mm culture dish pre-coated with 0.1% gelatin. Cells were left to attach for 36-48hr after which the medium was removed and 2ml of fresh growth medium added.

2.2 Results

2.2.1 Histological characterisation of retinal vessels

Retinal arterioles could be differentiated from venules at the dissecting microscope level based on vessel diameter. Venules were usually wider than arterioles (fig. 2.1), in some bovine retinas this difference was not always apparent and these retinas were discarded.

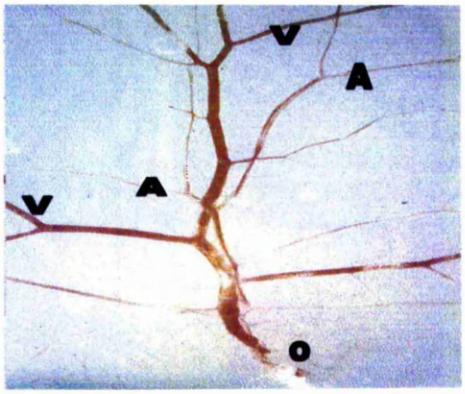


Figure 2.1 - Photograph (x4) of part of a bovine retina, demonstrating an obvious difference in width between arterioles and venules. O designates the point at which the retina was dissected from the optic disk. 'V' – represents venous branches; 'A' – represents the arteriolar branches.

Confirmation that venules and arterioles had been successfully isolated was obtained by assessing the morphology and staining distribution of the different vessels. Arterioles demonstrated many stained nuclei, tightly packed between layers of extracellular tissue (fig. 2.2a). The stained nuclei in arteriolar sections made up several cell layers enclosing a lumen much smaller in diameter than that of venous sections (fig. 2.2b). Venous sections displayed much fewer stained nuclei interspersed between extracellular tissue generally only one cell layer thick, surrounding a large lumen. Staining with Masson's trichrome confirmed presence of collagen by dense green staining in arteriolar sections, the collagen fibrils were found organised in irregular bundles around the cells in the vessel wall (fig. 2.3a). Venous sections stained with Massons' trichrome displayed less distribution and dense stain than that observed for arterioles (fig. 2.3b).

A total of 433 vessels were included in the study, of which 77 were for histological examination. All vessel types were successfully identified using the above criteria by two independent observers. All vessel data is summarised in appendix V.

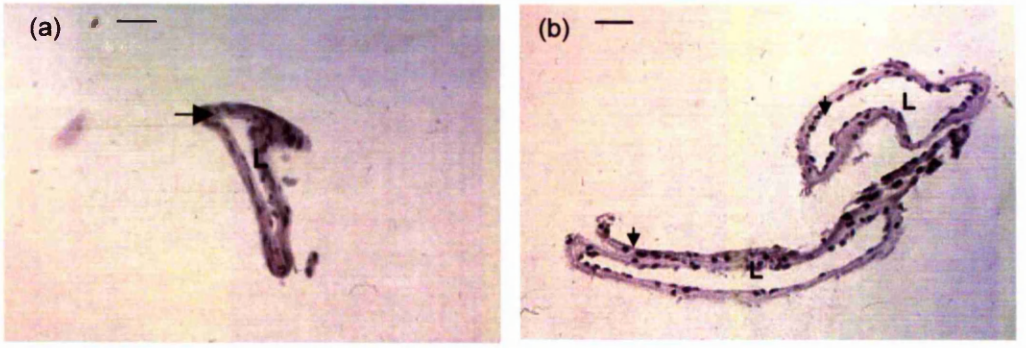


Figure 2.2 Photomicrographs displaying typical examples of (a) haematoxylin and eosin stained arteriolar and (b) venous sections, note the dense staining of the extracellular matrix in the arteriolar section in comparison to the venous section. 'L' indicates vessel lumen; arrows indicate cell nuclei (scale bar =500μm).

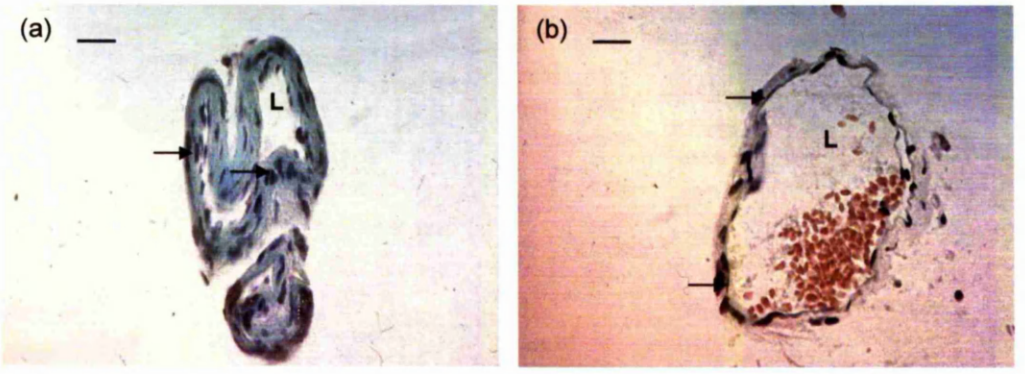
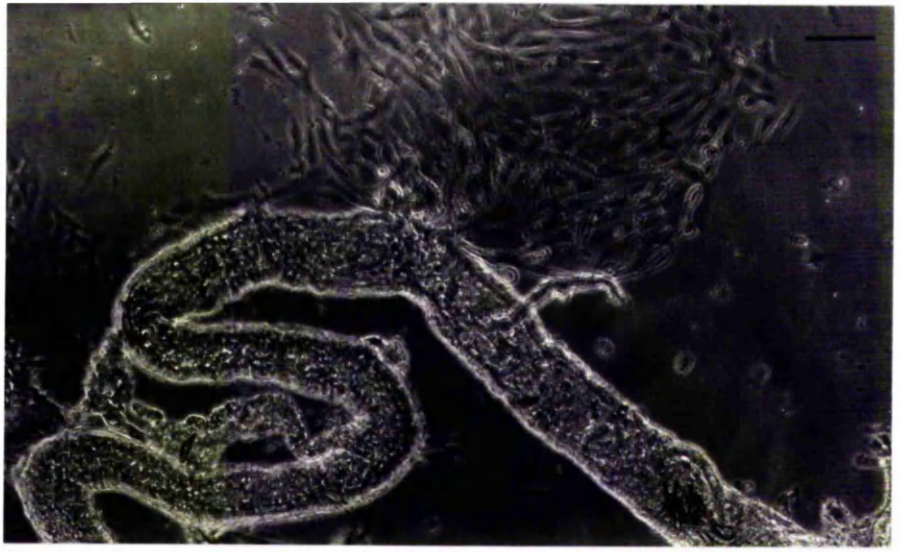


Figure 2.3 – Masson's trichrome stained (a) arteriolar and (b) venous sections, note the green/blue trichrome stain having a wider and more dense staining pattern in the arteriolar section in comparison to the venous section. The venous section also demonstrates a large lumen containing yellow/orange stained erythrocytes. 'L' indicates vessel lumen; arrows indicate cell nuclei (scale bar = 1000μm).

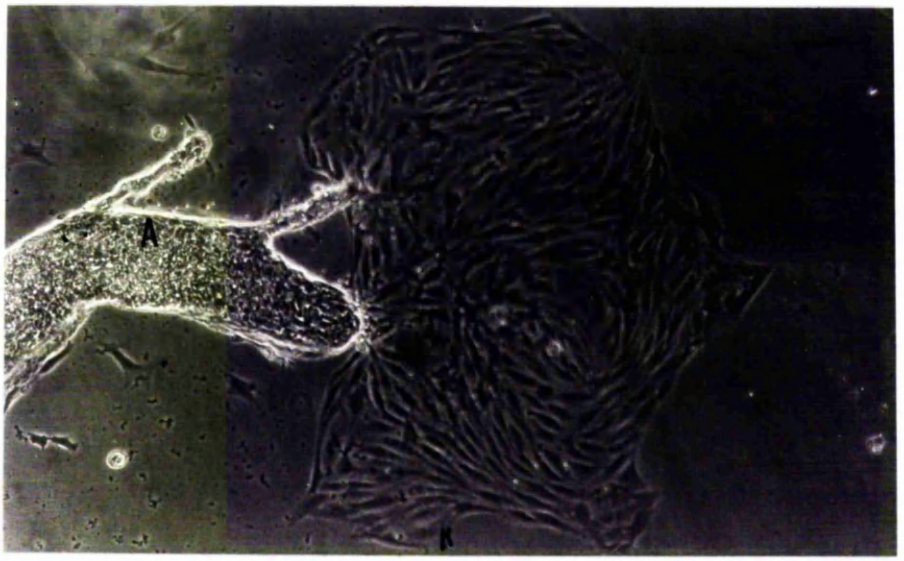
2.3.2 Comparison of growth characteristics of retinal arterioles and venules in vitro

Initial studies comparing arteriolar and venous growth in vitro used less than 5hr post mortem time tissue cultured in serum containing medium. Endothelial cells were observed to be the first cell type growing out of approximately 30% of arteriolar and venous cultures. In general cells only grew out from tips or capillary branch sites from arterioles, whereas no consistent point of outgrowth was observed for venules. Cells grew out as polygonal shaped cells in the characteristic cobblestone colonies of endothelial cells in culture, (fig 2.4a and 2.4b) and initially covered areas of the dish adjacent to the vessel. Within 1-2 days of observing large colonies of endothelial cells, pericyte-like cells were observed growing out as individual cells from all areas of arterioles and venules (fig. 2.4c). Pericyte-like cells had an irregular shape, with many cellular projections and obvious cytoplasmic stress fibres. In some arteriolar and venous cultures only a few cells of this type would appear and did not increase in number. However in the majority of cultures these cells once visible would become the major cell type within 3 to 4 days, overlapping and overgrowing the endothelial cell colonies. After 4 weeks in those cultures that still displayed endothelial cells, the vessel would become dissociated and endothelial cells at the centre of colonies detached leaving gaps. The remaining endothelial cells surrounded these gaps by extending processes to adjacent cells such that each gap was surrounded by at least one endothelial cell (see fig 2.4d and 2.4e). From these initial studies, it was apparent that attachment, outgrowth and the early stages of cell reorganisation/mobilisation occurred within the first 20 days of culture; hence further studies maintained cultures for approximately 3 weeks.

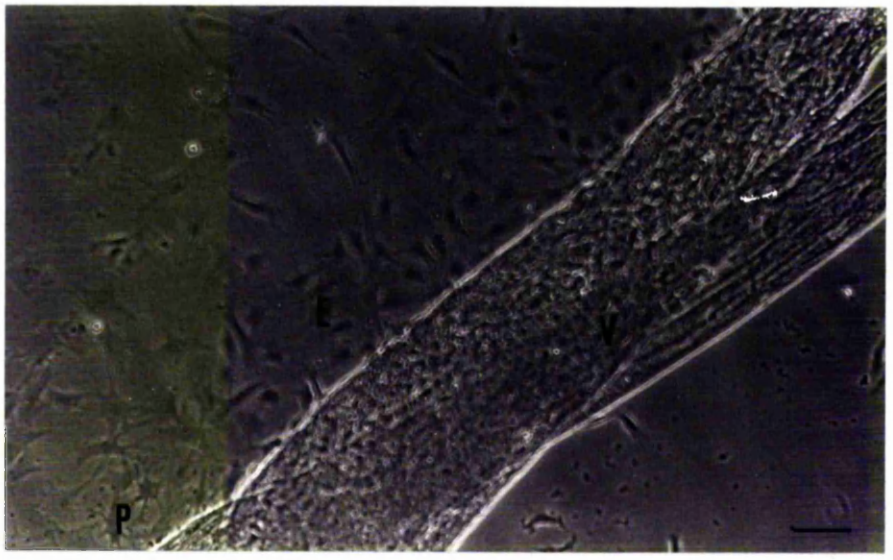
2.4 a.



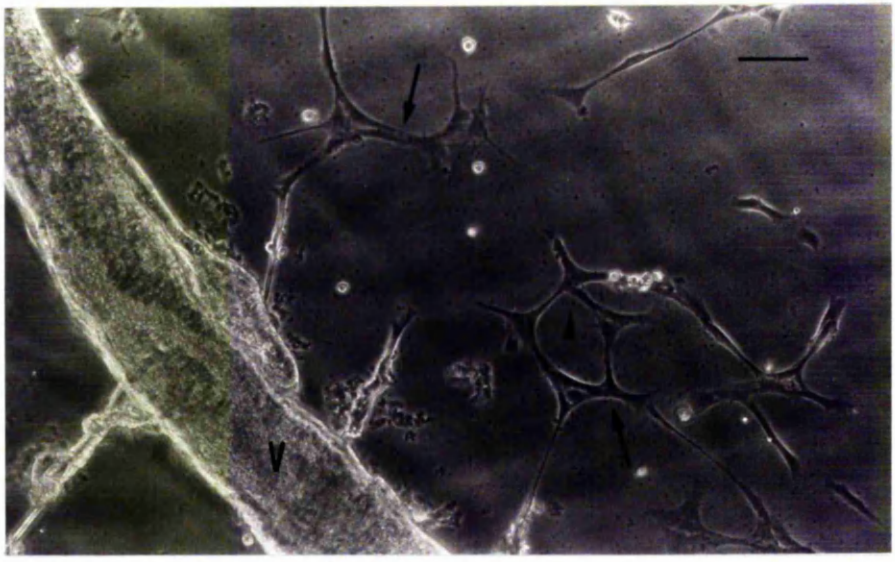
b.



c.



d.



e.

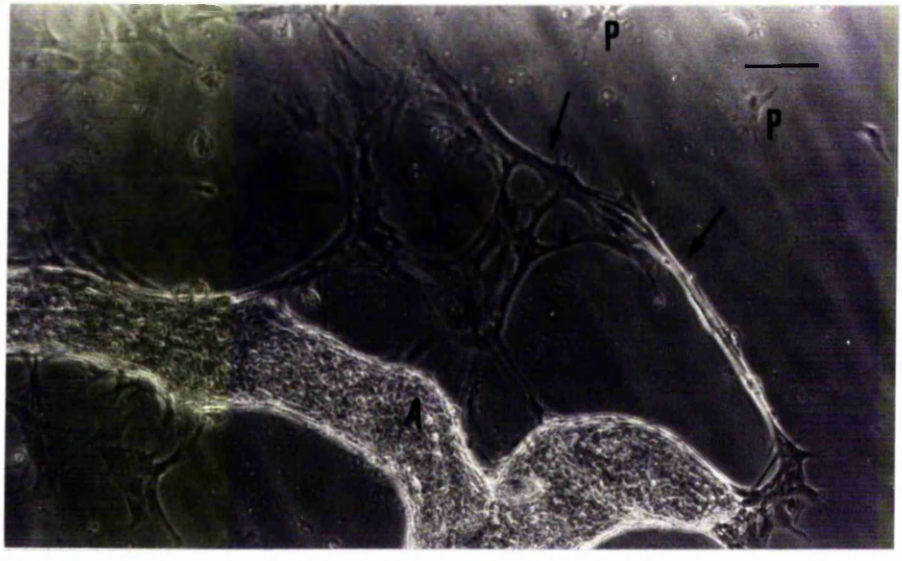


Figure 2.4 – Photomicrographs demonstrating typical outgrowth observed from (a) a venous culture at day 12, (b) an arteriolar culture after 18 days, (c) a venous culture after 20 days where pericyte-like cells are observed growing out as single cells at the edge of an endothelial cell colony, (d) a venous culture after 25 days demonstrating cell-cell contacts and (e) an arteriolar culture after 35 days demonstrating similar cell networks. 'V' and 'A' indicates venule and arteriole respectively; 'E' indicates endothelial cell-like colony; 'P' indicates pericyte-like cells. Arrows indicate cell-cell contacts and arrow heads indicate where endothelial cells have detached from the culture dish, (Scale bar = 500μm).

2.3.3. Effect of post mortem time on outgrowth from retinal arterioles and venules

The percentage of vessels displaying attachment was approximately 70% for both vessel types. Using longer post-mortem eyes increased venous attachment to 83.33% and arteriolar attachment was increased by more than 20% to 91.3%. Only a third of all venules and 43.3% of arterioles from fresh retina displayed outgrowth; in comparison to 70.83% of venules and 69.56% of arterioles displaying outgrowth when longer post mortem time retina were used.

Table 2.2 - Comparison of post-mortem time of tissue on retinal vessel outgrowth:

Experiment details	No. of Vessels	% Vessels Displaying Attachment.	% Vessels Displaying Outgrowth.	Average day of First Outgrowth (± S.D).	P value (MWU,KSD tests)
Venules < 5hr pm	18	72.22	33.33	11.83 (±1.17)	0.203
Arterioles < 5hr pm	16	68.75	43.75	14.14 (±2.79)	0.434
Venules >20hr pm	24	83.33	70.83	8.76 (±2.86)	0.021
Arterioles >20hr pm	23	91.30	69.56	10.94 (±2.84)	0.025

(MWU = Mann-Whitney-U; KSD = Kolmogorov-Smirnov-D tests; p values are for comparison of day of first outgrowth of arteriolar versus venous cultures, $p<0.05$ was regarded as significant.)

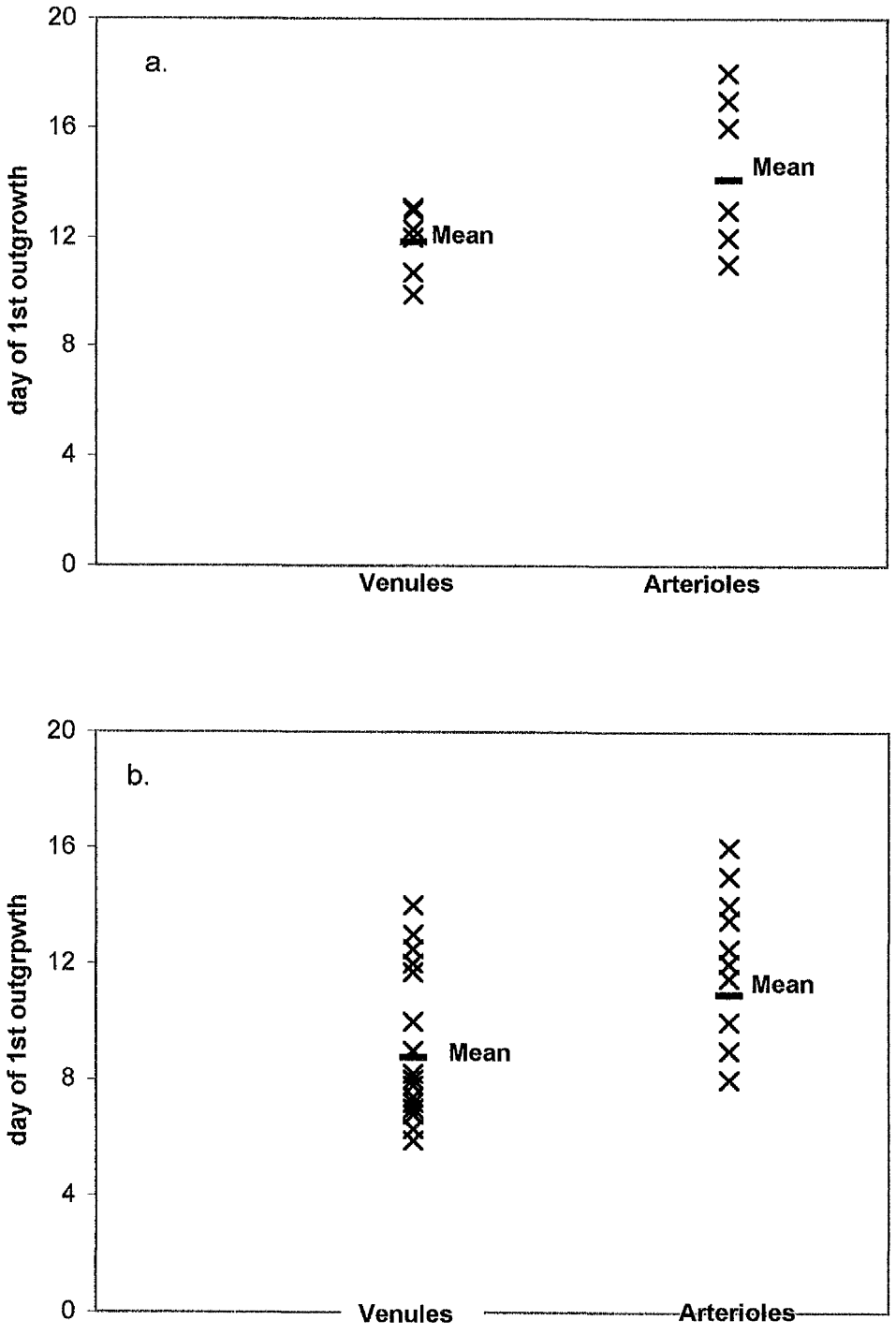


Figure 2.5 – Graphical representation of the day first outgrowth was observed from (a) vessels obtained from less than 5hr post mortem time tissue (n=6,7 for venules and arterioles respectively) and (b) vessels obtained from greater than 20 hr post mortem time tissue (n=17, 16 for venules and arterioles respectively). The horizontal bars in each data set represent the mean day of first outgrowth.

Cellular outgrowth was observed on average to occur earlier from venous cultures than arteriolar cultures whether fresh or longer post mortem tissue was used. This was found to be significant when longer post mortem tissue used ($p<0.05$) (fig 2.5). Furthermore, the time taken for outgrowth to appear from venous and arteriolar cultures obtained from longer post mortem tissue was significantly earlier than that of fresh tissue ($p< 0.05$), based on this finding subsequent studies used tissue, which was at least 20 hours post mortem.

Inverted microscopy demonstrated that outgrowth from arteriolar and venous cultures from older post mortem time retinas occurred similar to that described above for vessels obtained from fresh eyes although in these studies cultures were maintained for a maximum of 4 weeks. Endothelial-like cells were the first cell type to grow out in 30% of arteriolar and venous cultures from fresh retinas, approximately at the same time as for pericytes in 15% of cultures and pericyte like cells were the first cell type observed in approximately 50% of arteriolar and venous cultures. When longer post-mortem time tissue was used, endothelial-like cells were the first cell type to appear in approximately 40% of venous cultures and approximately 25% of arteriolar cultures. Endothelial-like cells appeared at the same time as pericyte-like cells in approximately 20% of venous and arteriolar cultures. Pericyte-like cells were the cell type to appear in approximately 40%to appear in approximately 40% of venous cultures and 60% of arteriolar cultures. Pericytes were the dominant cell type observed in the majority of cultures of fresh and longer post mortem time tissue. All of the data regarding cell type and time of outgrowth can be found summarised for each vessel culture in appendix V.

2.3.4 Comparison of growth medium on retinal vessel outgrowth

When retinal vessels were cultured using ESFM +10%PPP, 97.56% of venules and 93.18% of arterioles attached. When vessels were cultured using DMEM + 20% serum venous attachment was decreased to 82.93% of vessels displaying attachment yet arteriolar attachment was similar with 91.3% of arterioles attaching. The percentage number of venules displaying outgrowth was 82.93 and 70.83 when cultured in ESFM + 10% PPP and DMEM + 20% serum respectively. The percentage number of arterioles displaying outgrowth was 36.63 and 69.56 when cultured in ESFM + 10%PPP and DMEM + 20% serum respectively. Thus the number of arterioles and venules displaying outgrowth was similar in the presence of serum but the number of arterioles displaying outgrowth was considerably reduced in plasma containing medium. On average, outgrowth occurred earlier for venules in comparison to arterioles in both growth media, which was found to be significant in serum containing media $P<0.05$ and more so in plasma containing media (fig 2.6).

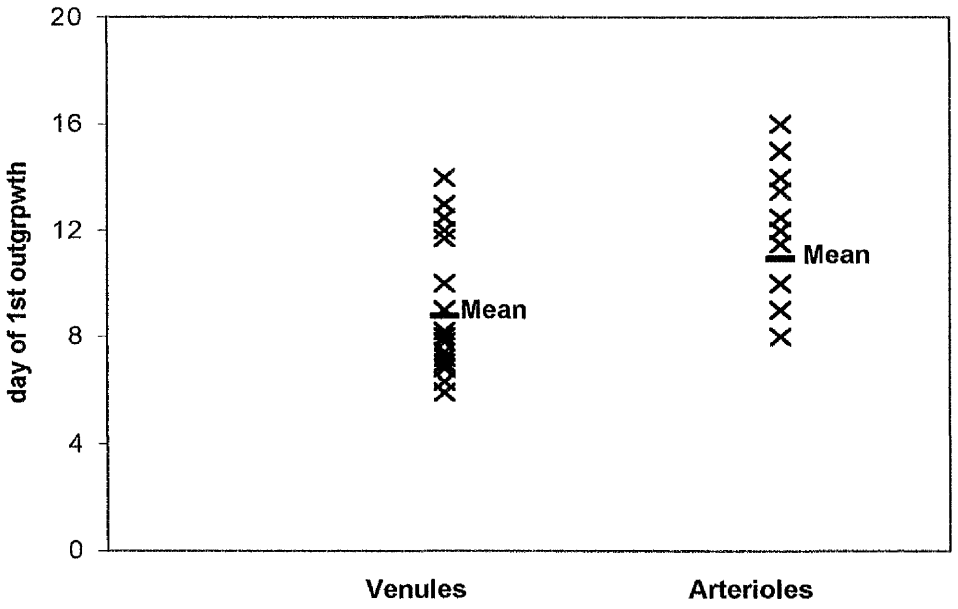
Table 2.3: Comparison of Growth medium on retinal vessel outgrowth.

Experiment Details	No. of Vessels	% Vessels Displaying Attachment.	% No. Vessels Displaying Outgrowth.	Average day of First Outgrowth (± St. Dev.)	P value (MWU,KSD tests)
Venules ESFM +Plasma	41	97.56	82.93	7.74 (±2.56)	0.0001
Arterioles ESFM +Plasma	44	93.18	36.63	11.71 (±3.64)	0.011
Venules DMEM +Serum	24	83.33	70.83	8.76 (±2.86)	0.021
Arterioles DMEM +Serum	23	91.30	69.56	10.94 (±2.84)	0.025

(MWU = Mann-Whitney-U; KSD = Kolmogorov-Smirnov-D tests; p values are for comparison of day of first outgrowth of arteriolar versus venous cultures, $p<0.05$ was regarded as significant.)

Morphological and immunohistochemical analysis demonstrated that when ESFM + 10%PPP was used as the growth medium the proportion of cultures with mainly endothelial cells was much higher than observed in serum containing medium. Initial outgrowth consisted of endothelial cell colonies in approximately 60% and 50% of venous and arteriolar cultures respectively (see Appendix V). Thus due to the combination of ESFM+10%PPP producing increased attachment and more pure endothelial cell colonies it was chosen as the medium to culture vessels in the presence of various growth factors and substrates.

a.



b.

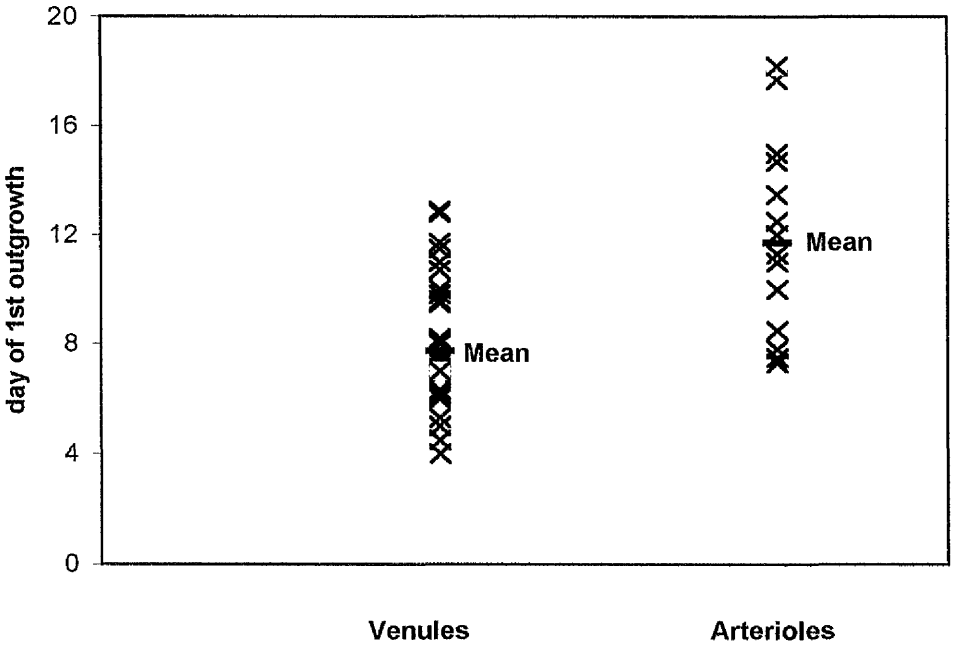


Figure 2.6 –Graphical representation of the day of first outgrowth observed when retinal cells were cultured in (a) serum containing medium (n=17, 16 for venules and arterioles respectively) and (b) plasma containing medium (n=34, 20 for venules and arterioles respectively). The horizontal bar in each data set represents the mean day of first outgrowth.

2.3.5 Effect of substrate on retinal vessel outgrowth

Gelatin decreased venous attachment and the number of venules displaying outgrowth in comparison to growth on plastic, but increased the time taken for growth to appear above that of the control ($p<0.05$). Gelatin did not affect the attachment efficiency of arterioles but increased the number of vessels producing outgrowth and the day of first of outgrowth significantly ($p<0.05$) above that of the control. Again venules displayed outgrowth significantly earlier than arterioles ($P<0.001$). On gelatin coated plates, endothelial-like cells were the first cell type to appear in approximately 90% and 70% of venous and arteriolar cultures. These endothelial-like cells remained the dominant cell type in approximately 75% and 40% of venous and arteriolar cultures. This is much higher than when non-coated dishes were used with either serum or plasma containing medium where pericyte-like cells were the dominant cell type present when the cultures were stopped.

Venous cultures demonstrated less efficient attachment on collagen than on plastic culture dishes, although arteriolar cultures exhibited attachment similar to that on plastic in the presence of collagen. The number of venous cultures producing outgrowth on collagen coated dishes was considerably less than was observed from arteriolar cultures and in the absence of collagen. However, the number of arteriolar cultures producing outgrowth on collagen coated dishes was increased in the presence of collagen. There was no difference in the time outgrowth appeared between arteriolar and venous cultures on collagen coated dishes yet, both produced outgrowth earlier than on plastic. First outgrowth was observed to be endothelial-like cells in 100% and 75% of venous cultures respectively. Very small numbers of pericyte-like cells were observed in either

arteriolar or venous cultures. Endothelial-like cells remained the dominant cell type in all venous cultures and 50% of arteriolar cultures.

Table 2.4: Effect of various growth substrates on retinal vessel outgrowth.

	No. of Vessels		% Vessels Displaying Attachment		% No. Vessels Displaying Outgrowth		Average Day of First Outgrowth (± St. Dev.)		P value (MWU, KSD tests)
Substrate	V	A	V	A	V	A	V	A	
Plastic	17	16	97.56	87.50	82.93	43.75	6.65 (±1.77)	9.57 (±3.16)	0.004 0.008
Gelatin	13	17	88.68	84.21	77.36	66.66	5.61 (±3.25)	8.40 (±3.81)	0.0003 0.0012
Collagen	9	7	77.78	85.71	44.44	57.14	6.25 (±1.71)	6.25 (±1.71)	1.00 1.00
Fibronectin	14	14	85.71	78.57	64.29	50.00	8.89 (±3.21)	13.86 (±1.13)	0.386 0.573
Laminin	14	13	85.71	78.57	92.31	46.15	7.81 (±2.35)	7.17 (±3.38)	0.308 0.974
Vitronectin	14	14	100.0	100.0	100.0	100.0	7.14 (±1.61)	8.71 (±2.27)	0.107 0.044

(MWU = Mann-Whitney-U; KSD = Kolmogorov-Smirnov-D tests; p values are for comparison of day of first outgrowth of arteriolar versus venous culures, $p<0.05$ was regarded as significant.)

Fibronectin was found to be the least effective substrate, in that attachment was less efficient in both vessel types and the number of vessels producing outgrowth was decreased compared to that on plastic in venous cultures. Time taken for outgrowth to occur on fibronectin coated was significantly increased for both venules and arterioles ($P<0.05$) in comparison to plastic dishes. Morphological analysis demonstrated that less than 20% of outgrowth from both vessel types consisted of endothelial-like cells. Pericyte like cells were the first cell type observed in approximately 70% and 50% of venous and arteriolar

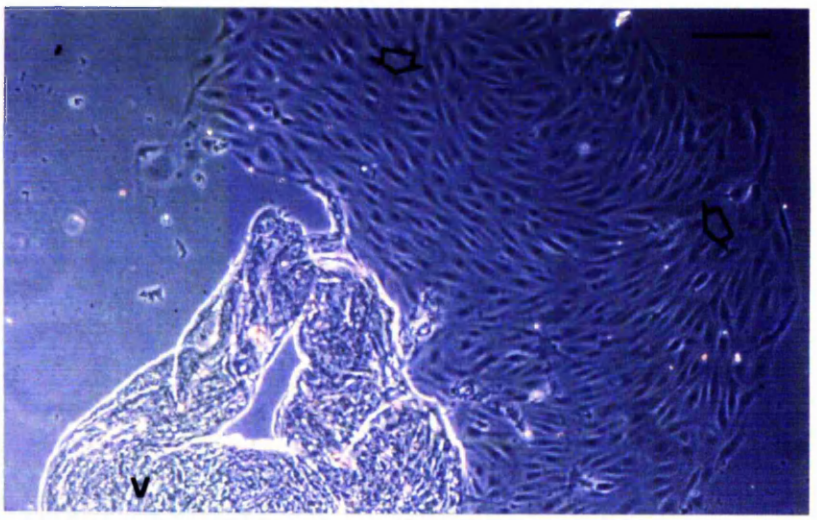
cultures respectively and remained the dominant cell type in approximately 60% of both arteriolar and venous cultures. In a number of arteriolar cultures smooth muscle like cells were also observed, these cells initially displayed features of early passage fibroblasts in monolayer culture, but in the later stages of culture became long and spindle shaped cells with obvious cytoplasmic stress cables. These cells were only observed in arteriolar cultures and were not observed on any other substrates.

Laminin did not affect attachment or number of vessels displaying outgrowth in arteriolar or venous cultures, nor did day of first outgrowth occur earlier than either control. Laminin was however the only substrate where outgrowth from arterioles was on average earlier than from venules, but this was not found to be statistically significant. Endothelial-like cells were the first cell type observed in less than 20% of venous cultures and approximately 30% of arteriolar cultures. Pericyte-like cells were observed as the first cell type in approximately 40% of venous cultures and 70% of arteriolar cultures. Yet pericyte and endothelial-like cells were observed in equal numbers in approximately 70% of venous cultures and 50% of arteriolar cultures when the cultures were stopped. When endothelial cells were present (more often in arteriolar cultures) however, they appeared to be actively dividing colonies.

Vitronectin was found to be overall the most effective growth substrate increasing attachment and percentage number of vessels producing outgrowth to a maximum 100% for both arterioles and venules (i.e., all the vessels cultured produced outgrowth). Outgrowth still occurred significantly earlier from venules than from arteriolar cultures. Outgrowth on vitronectin coated dishes consisted of endothelial-like cells in approximately 60% of both venous and arteriolar cultures. However when cultures were stopped both cell types

appeared to be present in equal numbers in approximately 65% of both venous and arteriolar cultures. Colonies of endothelial-like cells appeared to be actively dividing and cells covered large areas of the culture dish in a short amount of time (fig 2.7a).

a.



b.

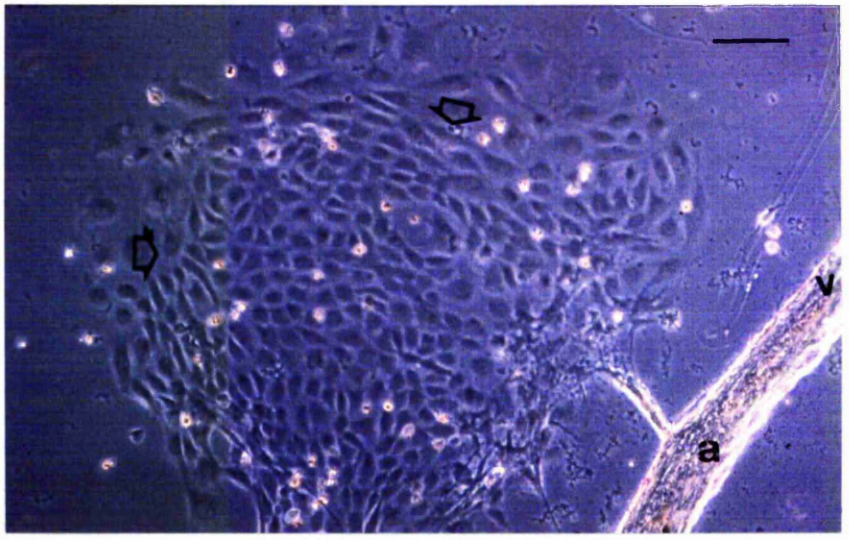


Figure 2.7 –Photomicrographs displaying typical cellular outgrowth observed from (a) a venous culture after 5 days on a vitronectin coated dish and (b) on an arteriolar culture 10 days after in the presence of VEGF containing growth medium. 'V' indicates vessel, arrows indicate endothelial cell colonies (scale bar = 1000μm).

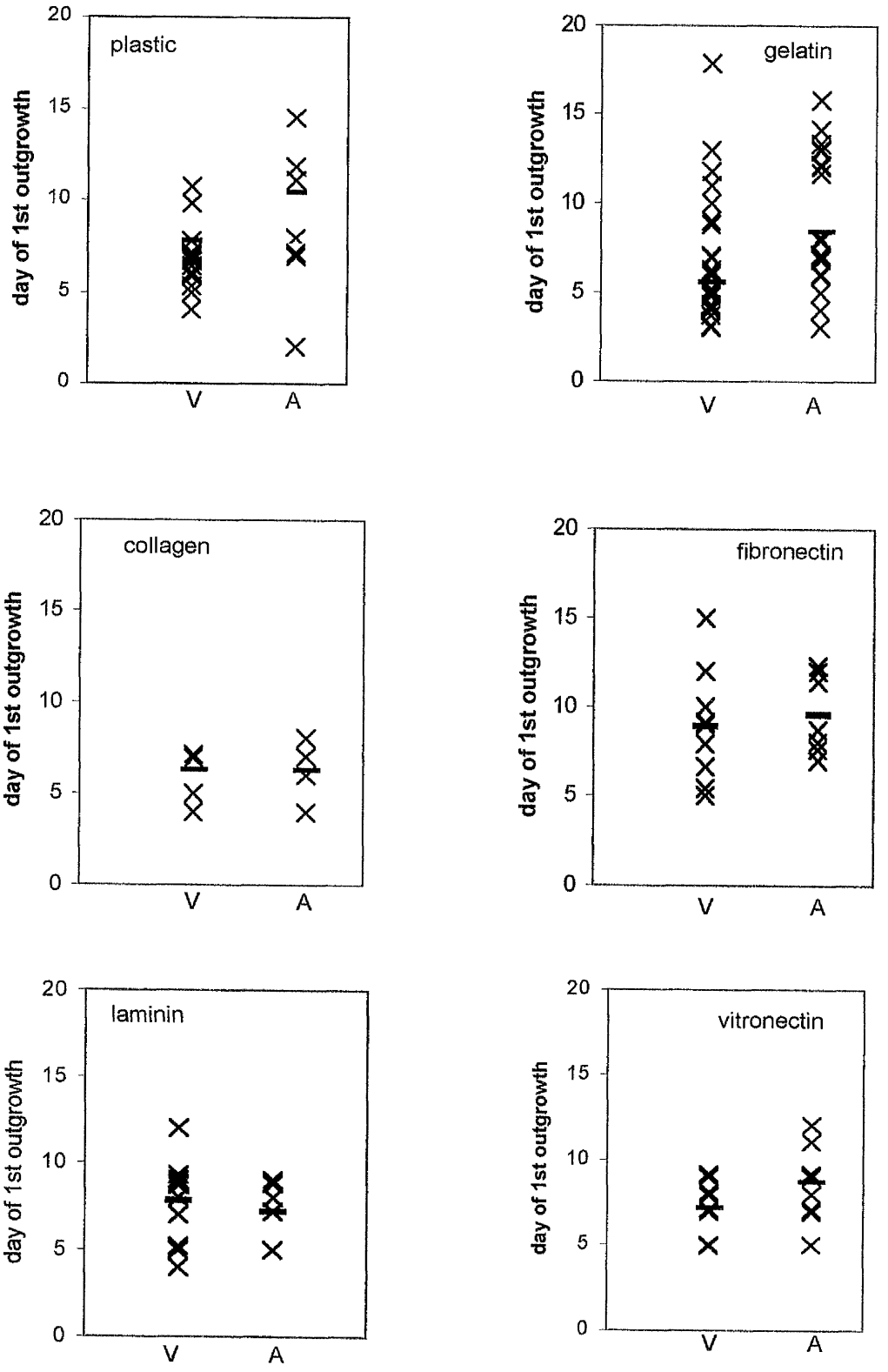


Figure 2.8 – Graphical representations of the day of first outgrowth observed for arterioles (A) and venules (V) on various growth substrates, (n=17, 7 for V, A on plastic; n=41, 38 for V,A on gelatin; n=4,4 for V, A on collagen; n=9,7 for V,A on fibronectin; n=11, 6 for V, A on laminin; n=14, 14 for V, A on vitronectin). The horizontal bar in each data set represents the mean day of first outgrowth for each data set.

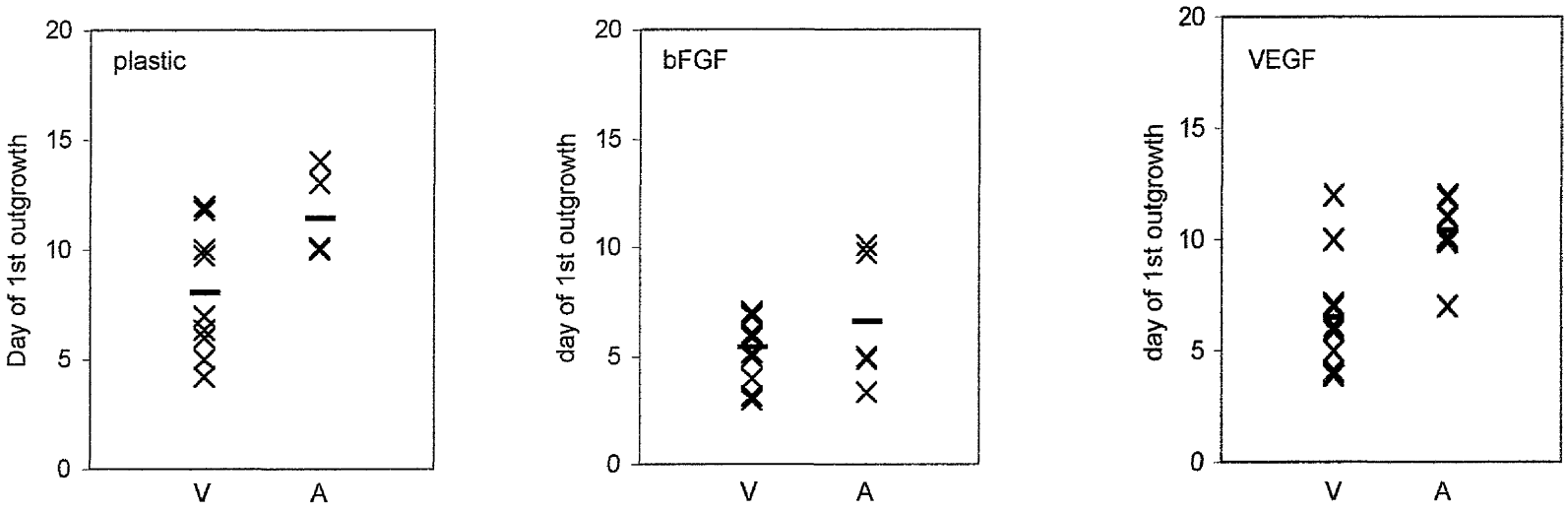


Figure 2.9 – Graphical representation of the day of first outgrowth of venules (V) and arterioles (A) in the presence of bFGF or VEGF. The y-axis represents the day of first outgrowth (n=9,5 for V, A on plastic; n=12,5 for V, A cultured in bFGF containing medium, n=12, 9 for V, A cultured in VEGF containing medium). The horizontal bar represents the mean day of first outgrowth for each data set.

2.36 Effect of growth factors on retinal vessel outgrowth

When retinal vessels were cultured in the presence of bFGF all the arterioles and venules cultured attached. Outgrowth occurred significantly earlier than in the absence of bFGF ($p<0.05$) for both arterioles and venules. All venules produced outgrowth in the presence of bFGF but the number of arterioles producing outgrowth remained identical to the control. Initial outgrowth consisted of endothelial-like cells in approximately 80% of arteriolar cultures, but pericyte-like cells appeared soon after and cultures consisted of equal numbers of both cell types when they were stopped. Endothelial-like cells were the first cell type to appear in approximately 30% of venous cultures and pericyte-like cells were the first cell type to appear in approximately 60% of cultures. However, similar to arteriolar cultures both cell types were observed equally in the majority of cultures when they were stopped.

VEGF increased attachment and proportion of venules producing cellular outgrowth to a maximum 100%, but did not affect arteriolar attachment while significantly increasing the number of arterioles displaying outgrowth. Outgrowth from venous cultures was significantly earlier than from arteriolar cultures ($p< 0.001$) in the presence of VEGF. VEGF decreased time taken for venules and arterioles to produce outgrowth in comparison to the control, but this was not statistically significant. Outgrowth of endothelial and pericyte-like cells appeared simultaneously in approximately 75% of both venous and arteriolar cultures and neither cell type appeared to be the dominant cell type in any cultures when they were stopped (fig 2.7b).

Table 2.5: The effect of growth factors on retinal vessel outgrowth

Experiment Details	No. of Vessels	% Vessels Displaying Attachment.	% No. Vessels Displaying Outgrowth.	Average day of First Outgrowth (± ST. DEV.)	P value (MWU,KSD tests)
Venules Control	10	90.00	90.00	8.00 (±3.04)	0.119 0.0451
Arterioles Control	12	98.50	41.67	11.40 (±1.95)	
Venules bFGF [5ng/ml]	12	100.00	100.00	5.42 (±1.44)	0.696 0.530
Arterioles bFGF [5ng/ml]	12	100.00	41.67	6.60 (±3.21)	
Venules VEGF [5ng/ml]	12	100.00	100.00	6.50 (±2.43)	0.0017 0.0016
Arterioles VEGF[5ng/ml]	12	91.67	75.00	10.44 (±1.59)	

(MWU = Mann-Whitney-U; KSD = Kolmogorov-Smirnov-D tests; p values are for comparison of day of first outgrowth of arteriolar versus venous cultures, $p<0.05$ was regarded as significant.)

2.3.7 Characterisation of retinal vessel outgrowth

Of the 433 vessel cultures included in the study, a total of 101 were randomly selected for immunohistochemical analysis. The staining density and distribution of the vessels selected are summarised in Appendix V.

In general fluorescent green, perinuclear staining indicated the presence of von Willebrands factor. The staining was granular in appearance and was unevenly distributed in the retinal vessels, which also served as a positive control (fig 2.10).

Fluorescent red stained microfibrils within the cytoplasm of irregular shaped cells indicated the presence of α-smooth muscle actin, a positive marker for pericytes. A similar staining pattern was observed along vessels with a more organised distribution of cytoplasmic fibrils was observed and also served as a positive control.

All cultures tested confirmed morphology observed and recorded using inverted microscopy, although staining intensity varied between vessels and cultures. Staining in all test cultures was comparable to the positive control. No staining comparable to the positive controls were observed in the negative controls.

2.3.8 Sub-culture of Retinal Arteriolar and Venous Endothelial Cells.

Cultures of endothelial cells originating from both arterioles and venules were obtained successfully using the technique described in section 2.2.8. The cells displayed attachment after 2 to 3 days, post seeding with very low attachment efficiency in that approximately 5-10% of cells would attach. Only 20% of cultures successfully sub-cultured produced the cobblestone monolayer of cells characteristic of dividing endothelial cells (fig. 2.13). The remaining cultures did not appear to divide and the few cells that did display attachment would become larger and present large vacuoles before detaching from the dish. When confluence was achieved, the majority of cultures acquired pericyte features, whereby the regular epithelial shape was replaced with membrane projections and the cell-cell contact decreased, and obvious stress fibres were visible.

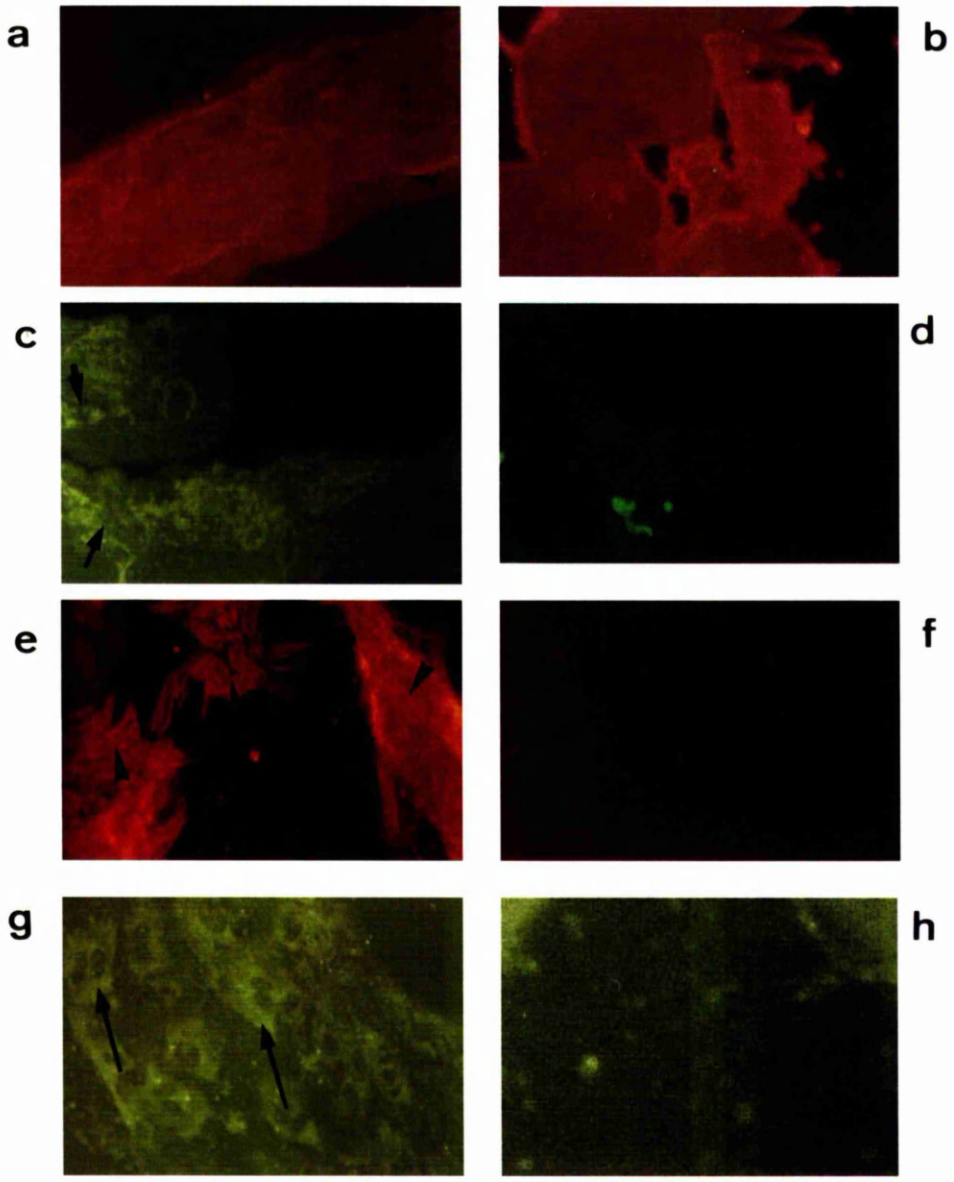


Figure 2.9 Fluoresecence photomicrographs of α-smooth muscle actin distribution in (a) retinal venule, (b) negative control (omission of primary antibody); vWbf distribution in (c) a retinal vessel and (d) negative control (omission of primary antibody). Example of typical distribution of α-smooth muscle actin in cellular outgrowth in (e) of pericytes (f) endothelial cells and example vWbf distribution in (g) endothelial cell outgrowth and (h) pericyte outgrowth. Note the fine granular fluorescence observed in endothelial cells within the vessel wall in outgrowth (arrows). The specific pattern of fibrillar red fluorescence is presented in both pericytes within the vessel and in outgrowth (arrowheads). Although some autofluorescemce in controls (b, d, f, g) no comparable fibrillar or granular staining is observed. (Scale bar = 500μm)

2.4 Discussion

Although the processing of dissected retinal vessels was initially problematic due to their small size (most of the vessels processed for this study were the size of a human eyelash) a technique was developed whereby very gradual dehydration and omission of the vacuum oven stage gave improved sections. Haematoxylin and eosin staining confirmed vessel type in the majority of vessels but this was only definite in those vessels that were taken from the same retina and were of the same calibre. The major differences between arterioles and venules are as follows. The extracellular matrix of arterioles is greater than in venules as they have a higher collagen/elastin content. Secondly, the diameter of lumen differs. Venules have larger lumens when compared to similar calibre arterioles. Finally, arterioles can have several layers of endothelial cells with larger vessels also possessing smooth muscle cells (Hogan and Feeney, 1963a; 1963b).

It was difficult to distinguish a larger venous section from a smaller arteriolar section on the basis of cellularity and extracellular matrix thickness alone. Occasionally, the only separating feature was the vessel lumen. This, however, was not always successfully preserved due to the lack of physical support from surrounding tissue following dissection. The use of Masson's trichrome staining (which stains collagen fibrils a blue/green colour), clarified extracelllular matrix arrangement and allowed the successful identification of all arteriolar and venous sections.

The growth measuring techniques in this study involved assessing attachment of vessels, presence of cellular outgrowth, time taken for cellular outgrowth to appear and immunohistochemical analysis of cellular outgrowth. Many

alternative methods of growth measurement/assessment exist including: measuring the area covered by cells, counting the number of cells present in any given area at specific time points, assessing cell density (Pepper et al, 1990; Auerbach et al, 1991). The difficulties of these techniques is that they were largely developed for cultures of single populations of cells, and do not adapt to situations which involve several cell types, particularly in this culture system where different cell types overlap and overgrow. Methods involving adhesion beads with electrical receptors quantifying electrical resistance as a measure of cell attachment have also been reported (Noiri et al, 1998), yet the cost effectiveness for the little more attainable information on vessel attachment available in this study would be low. More sophisticated methods such as 'still mode video recording' with computerised image analysis are also available, but again the time and cost effectiveness of such techniques when used on a large number of cultures was difficult to justify. Time dependence of outgrowth was chosen as a major factor when investigating growth characteristics between the two vessel types because clear differences in time of outgrowth were observed in preliminary cultures. Monitoring time of outgrowth was a method of recording a growth characteristic that was a discrete observation and therefore had very small room for error, such that measurements were accurate and consistent. Thus monitoring vessel attachment, cell morphology and presence and time of first outgrowth were found to be an efficient way of recording and comparing growth characteristics of a large number of vessel cultures in a number of different conditions for this study.

The major drawback of this culturing system was that only the presence of two antigens could be investigated in each vessel culture, thus limiting the amount of immunohistochemical information available.

Initial studies comparing the in vitro growth characteristics of retinal arterioles and venules demonstrated that both vessel types were capable of producing cellular outgrowth. Initial outgrowth was confirmed to be endothelial in origin by the presence of von Willebrand factor antigen. After a number of weeks in culture most of the visible cells were pericytes. Pericytes are described as microvascular cells that surround the endothelium of capillaries and venules (Rhodin, 1968; Sims 1986) but this description seldom includes arterioles (as arterioles are more often reported to possess smooth muscle cells (Henkind and Oliveira, 1968; Rhodin 1967). Thus, the fact that the arteriolar cultures contained pericyte-like cells and those cells stained positive for α-smooth muscle actin, with the fibrils orientated in a fashion identical to those in venous cultures raises questions as to the origin of these pericytes. There are a number of possible explanations including, pericytes may well have a contaminating cell type when the arteriolar cultures were set up. Although possible, the pericytes did not usually appear in arteriolar cultures until 14 to 20 days. Another explanation could be that these cells are indeed smooth muscle cells, given that α-smooth muscle actin expression is a positive marker of both smooth cells and pericytes (Skalli et al 1987; Herman 1993, Villaschi et al 1994). However, these cells displayed typical irregular pericyte morphology and not spindle shaped smooth muscle cell morphology (Orlidge and D'Amore 1987). An alternative theory is that smooth muscle cells become pericytes during in vitro angiogenesis as reported by Nicosia and Villaschi, (1995). This study used the aortic ring model of in vitro angiogenesis (Nicosia and Ottinetti 1990; Nicosia et al 1992) to demonstrate smooth muscle cell differentiation to pericytes that contributed to newly formed in vitro capillaries. Although further studies are required, this theory of smooth muscle cell involvement not only

offers a possible explanation for the presence of pericytes in this culture system but the presence of pericytes in embryonic and pathological new vessel formation (Navaratnam, 1991; Archer, 1983; Ashton 1970).

It is also interesting to note that the small number of venous and arteriolar cultures that were not overgrown with pericytes did display the features of endothelial cell network formation and the characteristics of in vitro vessel formation. This phenomenon has previously been described in a number of studies (Montesano 1992, Folkman and Haudenschild, 1980; Grant and Kleinman, 1997). However, this study was not specifically concerned with in vitro vessel formation (which is discussed in depth in chapter 3).

Although attempts were made at investigating the proliferative capabilities of arteriolar and venous endothelial cells (section 2.3.8) no immunohistochemical investigation was undertaken. Although a further valuable insight and another method of quantifying growth would have been available, proliferation was not focused on in this study.

In order to improve growth conditions, as well as to direct growth towards endothelial cells, the effect of different post-mortem time tissue and varying growth media were investigated.

The observation that longer post-mortem time increased attachment of both venules and arterioles suggests that these vessels possibly had more protruding cell processes to facilitate attachment. This may have been caused by autolysis of the basement membrane during the longer storage period. Also some physical damage was more likely to occur during dissection and transfer of vessels from longer post-mortem tissue. The fact that more outgrowth was observed from longer post-mortem tissue and that more of the cells were endothelial in origin suggests that many of the pericytes could have been lost

during the storage procedure leaving gaps for endothelial cell migration. This is in keeping with the theory that pericytes can contact-inhibit endothelial cell growth (Orlidge and D'Amore 1987; D'Amore, 1992) since endothelial outgrowth appeared to be inhibited from vessels from fresh retina and not from vessels from longer post mortem tissue.

Attachment of retinal arterioles and venules was further increased when cultured with ESFM + 10% PPP in comparison to DMEM + 20% serum and although the percentage number of arterioles displaying outgrowth was lowered all outgrowth from venules was endothelial in origin. This is possibly because pericytes require more complex medium with additional factors that are present in the medium (Hirschi and D'Amore, 1996; Schor et al, 1995; Shepro and Morel, 1993).

The finding that venules produced outgrowth on average earlier than arterioles is interesting when considering the in vitro situation of neovascularisation almost always occurring on the venous side (Henkind and Wise, 1974; Cogan et al, 1961; Ashton, 1951). This relationship was found to exist whether serum or plasma supplemented media was used and statistically significant when analysed using Mann-Whitney-U and Kolmogorov-Smirnov-D paired tests. Variables which exist in a culture system such as this, include between donor variation, the size of the vessel, origin in fundus and trauma during dissection and care was taken to reduce all variables when possible. This was not always a feasible option due to the limited number of vessels available from a single retina. However, the observation that venules produced outgrowth on average earlier than retinal arterioles suggests that both vessels contained the "information" required to produce cellular outgrowth but for some reason this

"information" was more readily available to the venous cells. In this culture system arterioles and venules were cultured with no other retinal cell types. Other retinal cell types such as glial or RPE cells may be responsible for the production of angiogenic stimuli in the pathological situation predisposing to neovascularisation. There was also no representation of the potential influence or constraints of the interstitial matrix. Thus studies investigating the effect of various ECM components and growth factors within this culture system were undertaken to increase our understanding of the in vivo process.

In order to reduce variability and allow comparability these studies used vessels from the same retina in each of the culture conditions. Such that together with the experimental repeats the numbers of vessels were adequate and the variability was reduced as much as was feasibly possible allowing statistical analysis.

Studies investigating the effect of various ECM components on retinal arteriolar and venous growth characteristics found that vitronectin was the most effective growth substrate. This was because all venous and arteriolar cultures displayed excellent attachment and produced outgrowth on vitronectin coated dishes. Complete or 100% attachment or outgrowth efficiency was not observed for either vessel on any other substrate. This finding is in keeping with a proposed role for vitronectin in the earliest stages of new vessel formation and its localisation in newly formed retinal capillaries (Reilly and Nash, 1988; Casaroli-Marano et al, 1995). Vitronectin is thought to encourage endothelial cell migration due to its cell stabilising and adhesive properties (Preissner, 1991; Seiffert, 1997). It was also observed that even though vitronectin was the most effective substrate, outgrowth still occurred significantly

earlier from venules in comparison to arterioles. A possible explanation for this may lie at the level of cell signalling or, more specifically, cell-matrix interaction induced signalling. It is known that endothelial cells are capable of expressing several integrin receptors including the vitronectin receptor $\alpha_V\beta_3$ (Cheresh, 1987; Dejana et al, 1988; Charo et al, 1990). It may be that integrin receptors such as this one are more directly accessible to venous endothelial cells, possibly due to less physical constraints, thus allowing rapid signal transduction to convert quiescent endothelial cells into a more migratory morphology.

Gelatin was found to be the next most effective substrate with two-thirds of arterioles producing cellular outgrowth, in comparison to all other substrates (with the exception of vitronectin) where only approximately half of arterioles produced outgrowth. Outgrowth was again observed significantly earlier in venules than in arterioles on a gelatin substrate and both vessel types produced outgrowth significantly earlier on gelatin than on plastic. This is, surprisingly, in contrast to the observations of growth characteristics on a collagen type-I substrate. This is because gelatin is described as a denatured or fragmented form of collagen and as such some similarity in growth characteristics were expected (Schor et al, 1980). It is possible, however, that the denatured form of the interstitial protein acted as a chemoattractant. This may be due to the unmasking of receptor sites facilitating endothelial cell growth on the changed conformation of gelatin, which is also described as fragmented collagen. In support of this theory is a study by Davis (1992) who discovered that melanoma cells would bind to denatured collagen through a different receptor than native collagen type-I. Interestingly, this cell type bound through RGD sites via $\alpha_V\beta_3$ integrin to denatured collagen, as opposed to $\alpha_2\beta_1$ or $\alpha_1\beta_1$, the usually reported

collagen recognition sites (Howe et al, 1998; Preissner et al, 1997; Brooks, 1998). Recent studies have demonstrated the presence of this integrin heterodimer on newly formed capillaries with no expression in normal vessels (Brooks et al, 1994 and Preissner et al, 1997). Parallels in this study include the effectiveness of vitronectin and gelatin in inducing endothelial cell mobilisation - the first obligatory step of angiogenesis (Zetter, 1987). Thus, it may be through this recognition site that the cascade of neovascular events begins, on the venous side of the capillary network.

The evidence in this study is, although promising, circumstantial and further requires identification and amount of functionality of the specific integrins involved using techniques such as affinity chromatography and neutralising antibodies/integrin antagonists (Casaroli-Morano et al, 1995; Dejana et al, 1988; Cheng and Kramer, 1989).

The fact that type-I collagen lowered venous attachment and growth, further implicates the role of extracellular matrix behaving as a regulator of endothelial growth (Sage and Vernon, 1994; Zetter, 1990; Madri, 1997). This apparent inhibitory effect may be because collagen I is a major interstitial collagen and contact with endothelial cells would be limited until invasion of the interstitial matrix occurs (Jackson et al, 1992; Vernon et al 1995). It should be noted, however, that when arterioles or venules did produce outgrowth there was no difference in time taken for it to appear. This suggests that an alternative mechanism of endothelial mobilisation exists here, different to that observed on vitronectin or gelatin. The only other substrate where outgrowth from arterioles and venules was on average on the same day was laminin.

Laminin is known to be a large basement membrane glycoprotein distributed in the normal and pathological retina (Belford et al, 1987; Essner and Lin, 1988)

and is vital for endothelial adhesion, differentiation and stabilisation (Kleinman et al, 1993; Grant and Kleinman, 1997). Laminin appeared favourable to both venous and arteriolar attachment and there was no difference in time taken for outgrowth to appear between the two vessel types, yet more than half of the arterioles cultured produced no cellular outgrowth in comparison to more than 90% of venules cultured producing cellular outgrowth. It is difficult to explain why laminin should have different effects on these vessel types and again raises questions to arteriolar endothelial cell accessibility. Native laminin is reported to have a regulatory effect on endothelial cell growth, this is thought to be due several of its much studied active sites. It is possible that the laminin sites required for attachment were available to both venous and arteriolar cells, but the sites required for subsequent migration were less accessible to arteriolar cells. Further studies investigating combinations of the various laminin peptides on retinal vessel outgrowth may give a valuable insight into the early stages of retinal neovascularisation (Malinda et al, 1999; Grant and Kleinman, 1997; Ingber and Folkman, 1989; Madri and Williams, 1983 and Grant et al, 1989).

In contrast, fibronectin was a poor substrate for both arteriolar and venous outgrowth. This is at variance with other studies (Ingber and Folkman, 1989; McIntosh et al, 1988; Bowman et al, 1982; Roberts and Forrester, 1990, Grant et al, 1998) that reported that fibronectin was an excellent substrate for endothelial cell growth and migration. Reasons for the discordant findings could be the lack of serum in this system, such that the positive response to fibronectin in some systems could be due to the high level of fibronectin already present in the system and/or additional factors present in the serum. Differing concentrations of fibronectin has been shown to alter endothelial cell phenotype

(Ingber and Folkman (1989) demonstrated a high level of fibronectin promoted well-spread cells that proliferated whereas lower fibronectin concentrations led to cell rounding and loss of viability. Intermediate fibronectin concentrations promoted the formation of capillary tubes. It has also been shown that in vivo fibronectin is present at endothelial and pericyte junctions (Essner and Lin, 1988). It is possible in a system such as this where both cell types are interactive, another level of control is exerted, which is not observed when only endothelial cells or pericytes are studied.

When exogenous bFGF was added to this culture system all arterioles and venules displayed excellent attachment. However, all venules and less than half of the arterioles displayed outgrowth. This was surprising since bFGF is known to be a potent stimulator of endothelial cell growth (Burgess and Maciag, 1989; Broadley et al, 1989; Lindner et al, 1990; Vlodavsky et al, 1987; Yoshida et al, 1996) yet it failed to stimulate arteriolar outgrowth above that of the control. Reasons for this observation are difficult to explain partly because an understanding of bFGF's mechanism of action is incomplete (D'Amore, 1992; Hartnett et al, 1998). Although bFGF in vitro is capable of acting directly on endothelial cells (Thomas et al, 1987; Gospodarowicz et al, 1987; Esch et al, 1985) it is not known whether bFGF is acting directly on the endothelial cells or acting with other cell types (pericytes, smooth muscle cells) or to ECM components. It is, however, clear that the pathway available to bFGF to initiate venous endothelial cell outgrowth is less available in arterioles. This may well be representative of the in vivo situation and requires further investigation.
Addition of VEGF increased attachment of all venous cultures - all of which produced outgrowth. The number of arterioles that produced outgrowth was

raised significantly to 75%. This is in contrast to the findings after the addition of bFGF and suggests that VEGF had greater accessibility to arteriolar cells. .. The VEGF isoform chosen for this study - $VEGF_{165}$ - is normally secreted from VEGF-producing cells (Leung et al, 1989; Ferrera et al, 1991) and encourages endothelial cell proliferation and migration in vitro and in vivo (Neufield et al, 1999; Aiello et al, 1995 and Klagsbrun and Soker, 1993). The fact that VEGF increased arteriolar outgrowth (albeit less than venous outgrowth) may be explained by the fact that unlike bFGF, VEGF does not lose its mitogenic activity if it is not bound to cell surface associated heparin-like molecules (Houck et al, 1992). Thus, if the basement membrane/extracellular matrix of arteriolar endothelial cells does behave as a barrier to exogenous VEGF alternative mechanisms exist whereby endothelial cell outgrowth can be encouraged. However, even in the presence of VEGF venous outgrowth occurred significantly earlier than that observed in arteriolar cultures. This observation is again reflective of the in vivo situation and suggestive of arteriolar endothelial growth inhibition even in the presence of potent endothelial growth stimulators.

In summary this culture system allowed the assessment of growth characteristics of two vessel types, from the same tissue in vitro.

Previous studies utilise vascular endothelial cells or pericytes obtained from whole retinas, pooling sub-populations of cells and diluting potential important information from one sub-population of cells (Aiello et al, 1995b; Canfield and Schor, 1994).

Many studies have also used endothelial cells from non-retinal vascular beds such as bovine aortic endothelial cells or more commonly human umbilical vein

endothelial cells, which may not be representative of the specialised retinal situation.

Studies investigating growth characteristics such as attachment and migration, using vascular cells in monolayer culture do not account for the intimate relationship of these cells with each other and the extracellular matrix (Form et al, 1986; Hoying and Williams, 1996; Vernon and Sage, 1999). Co-culture studies although useful for assessing some affects conferred upon one cell type to another do not always take into account the importance of the orientation that exists for example between endothelial cells and pericytes in vivo. The culture system employed in this study allowed the measurement of growth characteristics of vascular cells in the presence of various growth modulators without removing any of the cell-cell interactions that exist in vivo.

2.5 Conclusion and future work

This study demonstrated that outgrowth from retinal venules occurred on average earlier than retinal arterioles. This relationship was significant when vessels were cultured on plastic (in the presence of serum or plasma), vitronectin, gelatin and in the presence of VEGF. Furthermore, it was apparent from growth on some substrates that good attachment was not a prerequisite for arterioles producing outgrowth. Thus, this was the first study to demonstrate significant differences at the cell biological level in the growth characteristics of the cells originating from retinal arterioles and venules and allowed the elucidation of the direct effects of some of the key regulators of the angiogenic process in vivo.

Further studies are now required to explain why endothelial cells of different origin should display different growth characteristics in vitro. Investigations at the transcriptional control level using techniques such as differential display on endothelial cell mRNA from venules and arterioles would increase our knowledge of the earliest stages of neovascularisation.

CHAPTER THREE – DEVELOPMENT OF AN IN-VITRO MODEL TO STUDY RETINAL NEOVASCULARISATION.

3.1 Overview

Retinal neovascularisation is a potentially blinding condition that is associated with many ocular disorders concerning the retinal vasculature. In recent years knowledge of the molecular and cellular events involved in this complicated multistage process of neovascularisation has increased exponentially (D'Amore, 1994). Yet there are still no suitable preventive or pharmaceutical methods of intervention to halt the progression of the neovascular process. The stages of endothelial cell proliferation and migration from a mature, quiescent, retinal capillary network have often been addressed in vitro using actively proliferating non-retinal endothelial cells (Auerbach et al, 1991). Furthermore, in vitro models rarely represent the unique situation within the retina. Hence the objective of this study was to develop an in vitro model system of new vessel formation using retinal tissue, with the ultimate aim of being to utilise the model system to test putative angiogenic and anti-angiogenic substances.

3.2 Experimental methods

3.2.1 Preparation of an in vitro model system to study retinal neovascularisation.

Extraction of collagen – rat tail tendon collagen (type I) was extracted using the method described by Schor et al (1980). Ten rat-tails were fractured from the tip and collagen fibres pulled out (3 or 4 fractures per tail were usually required). The fibres were placed in 200ml of sterile 70% alcohol (v/v in distilled water) at 4°C for 1 hour. The alcohol was then decanted and replaced with 500ml of sterile 3% acetic acid (v/v in distilled water) and left stirring at 4°C for 24hr. The resultant mixture was then centrifuged at 3000 g for 2 hours at 4°C. The supernatant was dialysed against double distilled water (approximately 100 times volume of collagen) at 4°C over 48 hours with 8 changes of water. The dialysed collagen was finally centrifuged at 3000 g at 4°C for 2 hours. Absorbance was measured at 230nm to determine concentration of collagen. The collagen was diluted with sterile double distilled water to give a final concentration of 2mg/ml. The collagen stock solution was supplemented with 0.5% penicillin and streptomycin solution (Appendix II) and stored at 4°C until required.

Preparation of retinal vessels and explants - bovine retinal vessels were obtained as described in section 2.2 and placed in MEM/HEPES. Retinal explants were obtained by floating a retina in 15ml of PBSA in a 90mm petri dish. Each retina was cut into small polygonal pieces 4-5mm in diameter. The explants were taken from within a 15mm radius of the optic nerve and all contained obvious vessels.

Preparation of 3-dimensional collagen matrices –the matrices were prepared by mixing 8.5ml of the collagen stock solution, 1ml 10X DMEM and 0.5ml sodium bicarbonate solution (7.5% w/v in double distilled water) all at 4°C to prevent collagen thickening before use. The collagen solution was divided into five 2ml aliquots in 35mm culture dishes and allowed to gel in a standard cell culture incubator at 37°C for 2-3 hours prior to use.

Culture of retinal vessels and explants in a 3-dimensional collagen matrix – in order to represent a 3-dimensional environment a collagen matrix was used as the basic structure for the in vitro model system. Retinal vessels or explants were placed either in between two collagen layers or within a rapidly polymerising collagen solution.

Method 1 - Bovine retinal vessels and explants were prepared as described above. The collagen solution was prepared as described above; where 1ml aliquots of collagen solution were placed in 35mm cell culture dishes and allowed to gel. Two to three hours later a retinal vessel and/or explant was placed on the collagen matrices. The vessels and/or explants were then gently overlaid with a further 1ml of collagen solution and left to set overnight at 37^0C after which 1ml of growth medium was added. The growth medium (DMEM + 20% serum - unless otherwise stated) was removed and replaced with fresh medium every 2-3 days by gently pipetting off the old medium so as not to disturb the fragile collagen matrix. Outgrowth was monitored using inverted microscopy and photography.

Method 2 - Bovine retinal vessels and explants were isolated as described above. The collagen solution was prepared as described earlier in this section after which 2ml aliquots were each placed directly in 35mm culture dishes.

Immediately after aliquotting, retinal vessels and explants were carefully immersed into the rapidly polymerising gel and orientated into the desired position/distance apart. (N.B. if the vessel/explant were not placed in matrix quickly enough, it would set on the surface of the matrix and would generally float off when overlaid with media). The matrix was allowed to set for at least 3-4 hours and overlaid with 1ml growth medium, which was removed and replaced with fresh medium every 2-3 days.

3.2.2 Comparison of growth medium on model system to study retinal neovascularisation

In order to attain efficient vascular outgrowth, the effect of several growth media was compared on the model system by observing retinal vessel and explant growth. Retinal vessel and explants were set up as described above and the ability of DMEM, DMEM + 5%, 10%, 20% serum, ESFM and ESF + 10% PPP to encourage cellular outgrowth, migration and cell-cell network/contact formation of endothelial cell were assessed.

3.2.3 Culture of retinal vessels treated with collagenase

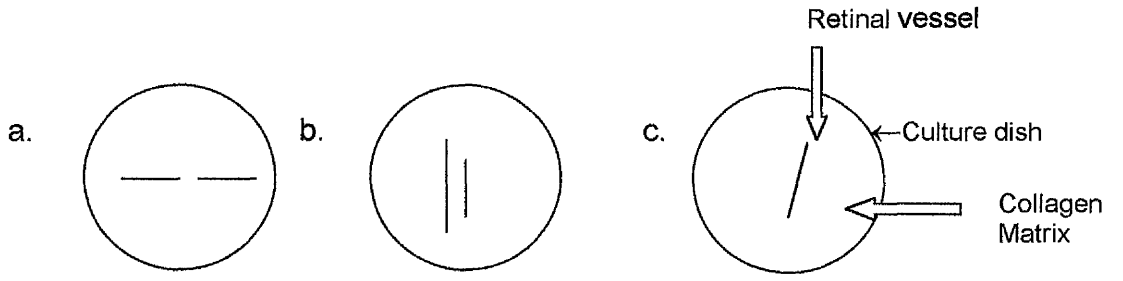
This study was undertaken to determine whether enzyme pre-treatment had an effect on type and nature of outgrowth (i.e. accelerating endothelial cell outgrowth). Retinal vessel cultures were set up as described above except that prior to placing the vessels on the matrix the vessels were subjected to collagenase treatment. The freshly isolated vessels were placed in a range of collagenase concentrations ranging from 0.5mg/ml to 10ng/ml enzyme in MEM/hepes for varying periods i.e., from 1 to 24 hours. After enzyme digestion, vessels were washed thoroughly with PBSA 3 times.

3.2.4 Culture of retinal vessels and explants with potential growth stimulators

Retinal vessels in 3-dimensional cultures were set up to assess whether potential growth stimulators could encourage directional growth from cells of the vasculature. These studies involved using retinal vessels, explants, a slow release polymer containing a growth factor and a suture soaked in growth factor as potential growth stimulators.

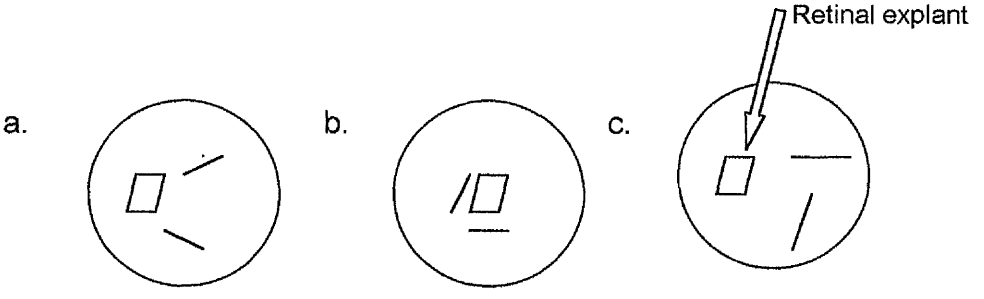
Retinal Vessels and explants as Potential Growth Stimulators - 3-dimensional cultures of retinal vessels were set up as described in section 3.2.1. Retinal arterioles and venules were placed individually on/in collagen matrices in various configurations (see fig. 3.1below). As outgrowth from arterioles almost always occurred from the vessel tips or the capillary branch points and no consistent point of outgrowth was observed for venules, vessels were placed in various orientations to determine whether growth could be encouraged from other areas in arteriolar and limited in venous cultures. Such that vessels were either a) placed in close proximity (between 1-10mm apart at the tips of each vessel); b) placed in close proximity to each other (between 1-10mm apart – lying parallel to each other along the length of the vessels) or c) were placed individually in matrices.

Fig. 3.1 Diagrams to illustrate model system where retinal vessel used as directional stimulus:-



A study was undertaken to determine whether retinal explants could act as directional stimulus for growth from retinal vessels in the 3-dimensional system. Cultures were set up as described in section 3.2.1. Vessels were placed in different orientations and distances from explants (see fig. 3.2). Vessels were placed: a) in close proximity to each explant such that each vessel tip was between 1-5mm away: b) the vessel was parallel to one edge of the explant and c) greater than 5mm away from the explant in any orientation.

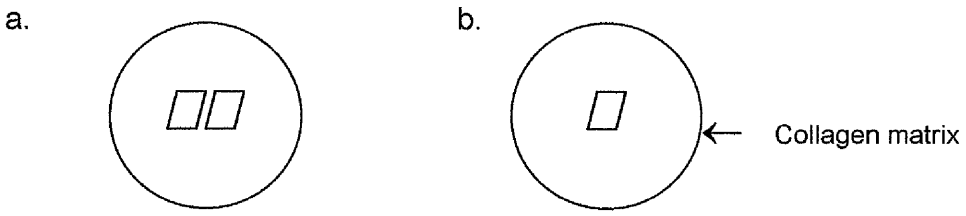
Fig. 3.2 Diagrams to Illustrate model system where retinal explants used as directional stimulus:-



Retinal explants were also used as potential growth stimulators for the vascular cells when still enclosed within retinal tissue (fig. 3.3). This was achieved by 3-dimensional retinal explant cultures being set up as described above except two

retinal explants were used and placed at a) varying distances from one another as well as individually in collagen matrices.

Fig.3.3 - Diagrams to illustrate model System where retinal explants were used as directional stimulus:-



A Slow release polymer containing bFGF as a potential growth stimulator-

Preliminary studies were undertaken to determine whether a specific angiogenic substance would encourage directional growth from cells of retinal vessels in the 3-dimensional model system. A growth modulator (bFGF) delivery system (slow release polymer) previously used in the lab was used as a starting point. The slow release polymer consisted of methylcellulose (1% - w/v in DMEM) and 10ng of basic fibroblast growth factor (bFGF). The methylcellulose solution was initially made up to 2% w/v in double distilled water. The mixture was then microwaved for 15 seconds (to help dissolve the methylcellulose) and then fully dissolved by placing on a magnetic stirrer overnight. The 2% methylcellulose solution was centrifuged at 1000g for 2 hours to remove any undissolved fragments. A 2μg/ml bFGF solution was made in 2 x DMEM, which was added to the 2% methylcellulose in 1:1 ratio to give the correct final concentration of 1μg/ml bFGF in the slow release polymer solution. Ten micro-litres of the slow release polymer solution (containing 10ng bFGF) was pipetted onto a teflon block and allowed to dry for 1 to 2 hours at room temperature. Retinal vessels

were placed on collagen matrices as described earlier and a polymer was placed at varying distances (between 1 and 10mm) from each vessel. A second layer of collagen was placed carefully over the polymer and vessel and left overnight in the incubator to set. One milli-litre of growth medium was then added to the matrix and replaced with fresh medium every 3 to 4 days. Polymer with no growth factor was used as the control.

Retinal vessels with sutures soaked in growth factor as a stimulus for directional growth – this study was undertaken to investigate whether growth factors placed within the matrix in a specific pattern (created by suture) would encourage direction and/or orientation of growth from retinal vessels or explants. Collagen matrices containing retinal explants or vessels were set up as described in section 3.2.1 (method 2). The collagen matrix was left overnight to ensure it had thoroughly gelled. A 7mm curved silk suture (pre-soaked overnight in 100ng/ml VEGF or 100ng/ml bFGF) was pulled through the matrix using fine forceps. The suture was placed in an adjacent area, as close to the vessel as possible without disturbing attachment of the vessel. The cultures were left overnight and then overlaid with 1ml growth medium, being careful not to disturb the gel at the suture entry or exit point.

3.2.5 Characterisation of the model system to study retinal neovascularisation

Initial characterisation was carried out on the basis of the 3-d morphology and origin of a cell type. This was undertaken using a Zeiss inverted microscope and typical examples of growth were recorded using a contax 400 camera.

Immunostaining of retinal vessels and explants 3-dimensional cultures – immunostaining was undertaken to identify the cell types present in the model system. Cultures were fixed in 10% neutral buffered formalin for at least 48 hours. Cultures underwent 3 gentle 1 hour washes with PBSA on the horizontal shaker. The cells from the vessel/explants were permeabilised with 0.1% triton (v/v PBSA) for 1 hour. The cultures were then incubated for 3 hours with 20% serum (V/V PBSA) of the required species (refer to table 1; section 4.2) to block any non-specific immunoglobulin binding sites. The serum was decanted and the culture was incubated with the primary antibody in 0.2% serum (v/v PBSA) overnight. The cultures received 3 X 1 hour washes with PBSA to remove any non-bound primary antibody prior to incubating overnight with a fluorescent-tagged secondary antibody at room temperature. The cultures were finally given 3x1 hour washes with PBSA and viewed using fluorescent inverted microscopy. Positive controls included staining retinal vessels and cells (refer to section 2.2). Negative controls included omission of primary or secondary antibody.

Histology of model system –several methods of fixation and processing for wax embedding were utilised for the model system. Initial methods used a standard alcohol and chloroform concentration for dehydration (see section 2.2). Other methods included the use of low temperature wax and no use of the vacuum oven. Yet, tremendous shrinkage still occurred resulting in a complete loss of cell/vessel orientation. This in turn led to the use of vibratome sectioning of the model system.

Preparation of model system for vibratome sectioning – the tissue samples were prepared for vibratome sectioning using an agarose embedding technique.

A small section of vessel containing matrix was removed from the model system using a 7mm trephine. This sample was then trimmed further using a scalpel, to reduce the size as much as possible.

The 0.2% agarose solution (w/v in PBSA) was made and dissolved by heating for 15 to 30 seconds in the microwave. Two ml of agarose solution was placed in a mould and allowed to cool down. The sample was then placed in the agarose and positioned to achieve the required orientation for sectioning and allowed to set for at least one hour. The agarose embedded tissue were removed from the mould and sectioned on the vibratome to produce 200μm sections or stored at 4°C in PBSA until ready for use.

Vibratome sections were used for immunostaining to confirm presence of cell types. The protocol used was the same as for flat mount staining.

3.3 Results

3.3.1 Development of an in vitro model system to study retinal neovascularisation.

Retinal vessels cultured in a 3-dimensional collagen gel – There was no difference observed from the time of first outgrowth between arterioles and venules, since outgrowth occurred from all vessels within 4 to 7 days in culture. Venules almost always initially exhibited outgrowth from along the length of the vessel whereas growth from the arterioles almost always occurred from the tips (fig 3.4). Initial outgrowth consisted of cells displaying typical elongated, bipolar endothelial cell morphology typical in a 3-dimensional environment. These endothelial-like cells tended to grow out radially from the edge of the vessel into all planes of the matrix by 10 days in culture. In some cultures the cellular outgrowth would appear organised as if the cells were growing along the collagen fibrils (fig 3.5a). After 10 to 12 days in culture, pericyte-like cells would appear as single cells in areas densely populated with endothelial cells (fig 3.5b). Pericyte like cells did not undergo any obvious change in morphology once a 3-dimensional shape (a dense cell body with multiple pseudopodia) was adopted. Endothelial-like cells would continually migrate and invade further areas of the gel and then extend processes towards neighbouring cells (fig 3.5c). In this way organised networks of cells would appear where straight cellular extensions would be joining two cells or two cell masses – often described as capillary tube formation. Within 3 to 4 weeks, the pericyte like cells would increase in numbers and overlap areas of cell-cell networks and appeared to be following the 'tracks' of the endothelial-like cells

and eventually stopping their growth, such that networks of endothelial-like cells would be surrounded by pericyte-like cells. The pericyte-like cells would appear to increase in numbers and size by week 5.

a.

b.

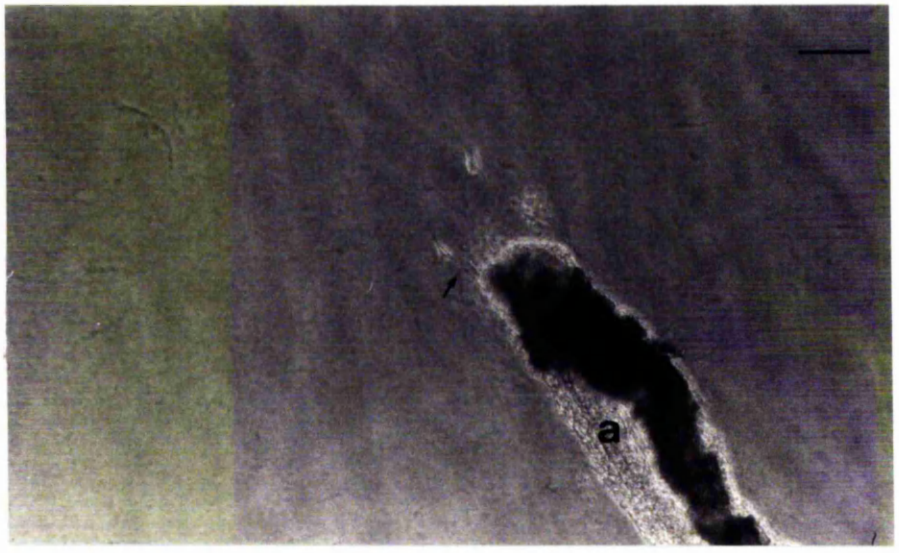
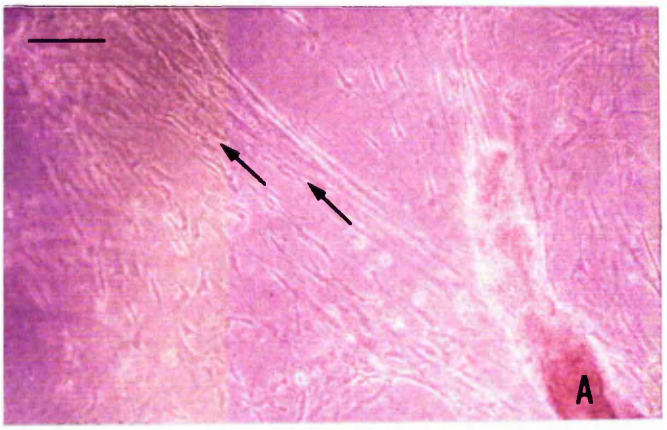
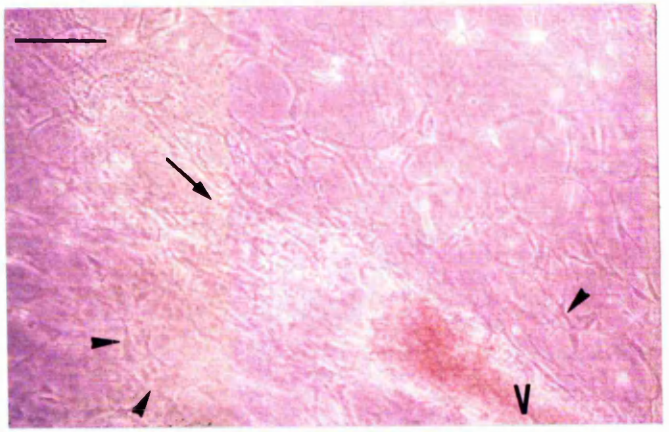


Figure 3.4 - (a) Photomicrographs of a typical (a) venule after 5 days in 3 dimensional culture, cell processes can be seen extending from the edge of the vessel wall, and (b) of an arteriole after 5 days in culture, cell processes can be seen extending from the tip the of vessel. 'V' indicates venule, 'A' indicates arteriole; arrows indicate cell processes (scale bar in (a) = 500μm and in (b) = 250μm).

a.



b.



c.

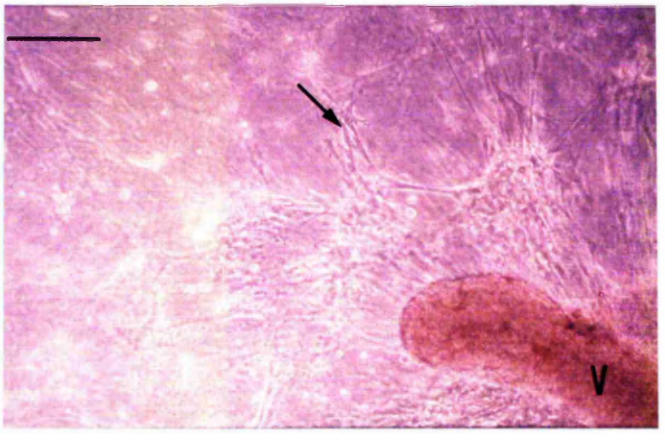


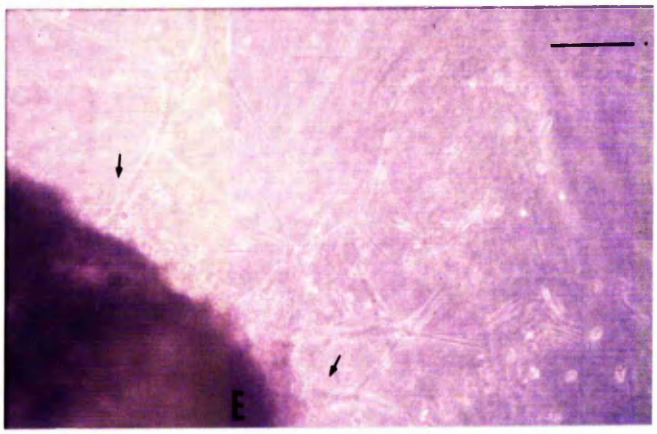
Figure 3.5 - Photomicrographs of a typical (a) arteriolar culture after 10 days; cells can be seen growing along collagen fibrils, (b) venous culture after15 days and (c) 20 days. Pericyte-like cells can be seen at the edges of endothelial-like cell networks. Pericyte-like cells were visualised when focusing through a 3-dimensional matrix using inverted microscopy (due to this cell type displaying multiple cell projections in all directions), this is less visible in 2-dimensional photomicrographs. 'V' indicates venule, 'A' indicates arteriole, arrows indicate endothelial-like cells, arrow-heads indicate pericyte-like cells, (scale bar=250μm).

Retinal explants in a 3-dimensional culture – retinal explants were cultured individually in a collagen matrix. Explants exhibited poor outgrowth in comparison to arterioles and venules with approximately 50% producing any outgrowth within two weeks of culture. Outgrowth from explants was considerably slower than that of vessels in culture, such that outgrowth was never observed before 10 days in culture. During days 10 to 14 in culture cells were visible growing from the edges of the explant (fig 3.6a), and many cells appeared to be rounding up and dissociating throughout all the retinal layers. Cells once visible would appear to migrate out as single and occasionally as sheets of associated cells, radially in all planes of the matrix, not only parallel to the explant. Various cell types could be observed in all layers of the matrix (fig 3.6b). Within 18-21 days of culture significant cell damage and extensive dissociation had occurred with the retinal explant but the vasculature remained intact in nearly 80% of cultures (fig 3.6c). Some endothelial-like cells could be seen migrating out of the vascular scaffolding at this stage, although very few developed cell-cell networks.

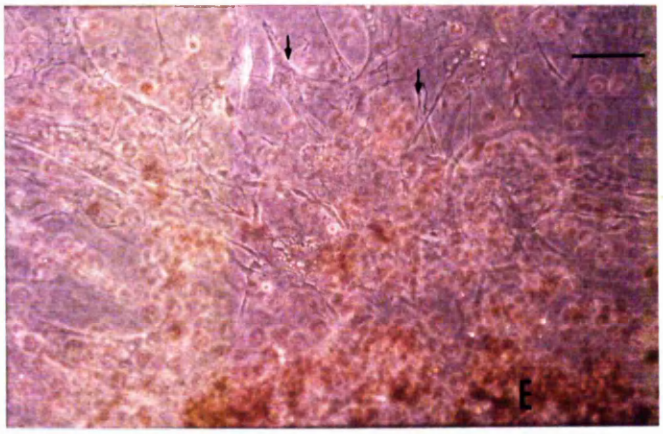
3.3.2 Comparison of various growth medium on the model system

Effect of medium on retinal vessel model system – retinal vessel outgrowth in a 3-dimensional environment was dependent on the presence of serum. When the amount of serum was varied, outgrowth as well as vessel integrity was affected (fig 3.7). With no serum, outgrowth did not occur and the vessel was completely dissociated within 10 days in culture. The same number of vessels successfully adhered and produced outgrowth in all serum containing cultures.

a.



b.



c.

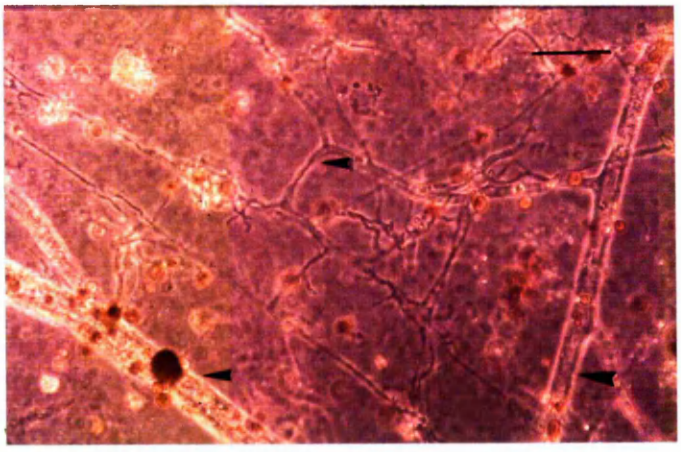


Figure 3.6 - Photomicrographs demonstrating typical cultures of retinal explants (a) after 12 days in culture, cells can be seen migrating from the edge of the explant into the surrounding matrix, (b) after 18 days in culture, many rounded-up and dissociated cells can be observed and (c) after 25 days in culture, where only the vasculature remains. 'E' indicates explants, arrows indicate cellular outgrowth and arrowheads indicate the intact vasculature (scale bar = 250μm).

When DMEM+5% or DMEM +10% serum was used outgrowth was similar in nature yet and slower in comparison to DMEM +20% serum (fig 3.8). However, outgrowth of endothelial-like cells was much lower and hence less cells formed the networks of connecting cell processes observed in DMEM + 20% serum. Overgrowth of pericyte-like cells was much more extensive in lower serum level growth medium which subsequently appeared to contact inhibit endothelial-like cells much earlier (fig 3.9). When retinal vessels in the model system were cultured in the presence of ESFM, vessels became dissociated within 10 – 14 days and no outgrowth was observed. When retinal vessels were cultured with ESFM supplemented with 10% human platelet poor plasma (PPP), although cell damage / death occurred outgrowth was observed in approximately 20% of cultures. Outgrowth occurred as endothelial-like single cells, that seemed unable to migrate any distance from the vessel, nor did there appear to be any increase in numbers. There were very few pericyte-like cells observed in these cultures but the vessels were extensively dissociated by day 20. Thus these cultures were not maintained beyond 20 days and hence very few displayed cell-cell networks observed with endothelial-like cells.

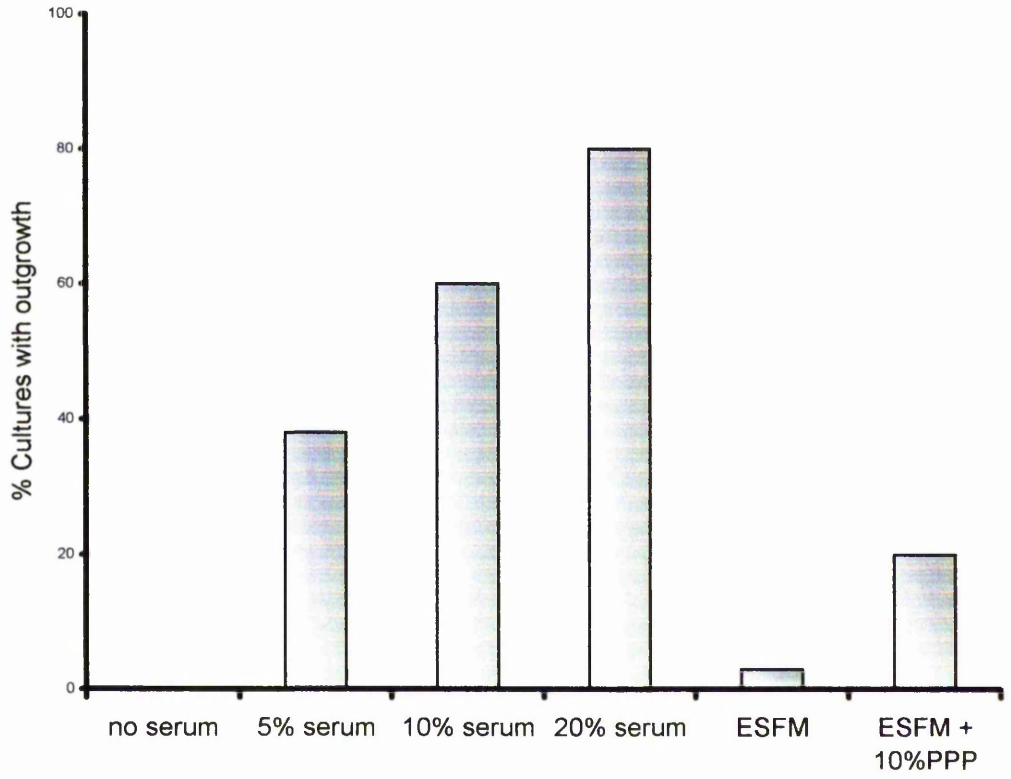


Figure 3.7 – Bar chart representing the percentage of vessel cultures producing outgrowth with the different growth media tested after 21 days. Highlighted here is the observation that growth was not observed without serum. Growth was observed in the presence of ESFM + 10% PPP but it failed to support growth to the extent of any of the serum supplemented media.

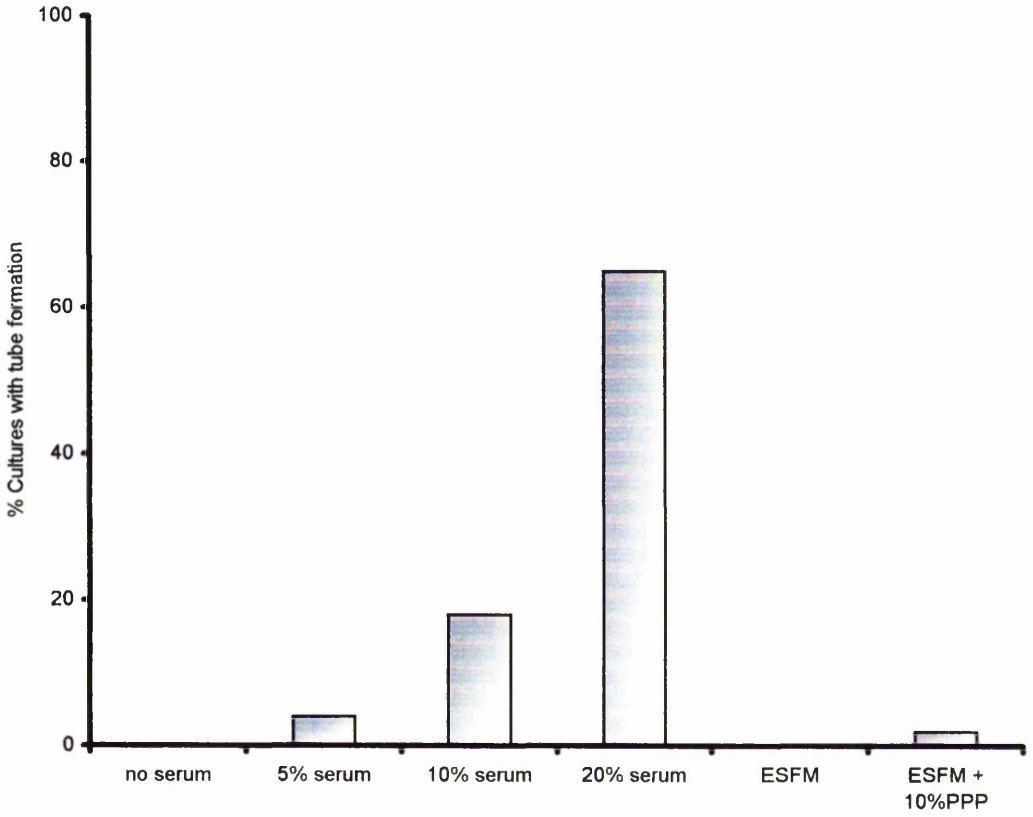


Figure 3.8 – Bar chart representing the percentage of cultures containing cell-cell networks after 21 days in the various growth media tested. This graph highlights the requirement of serum for the formation of cell-cell contacts

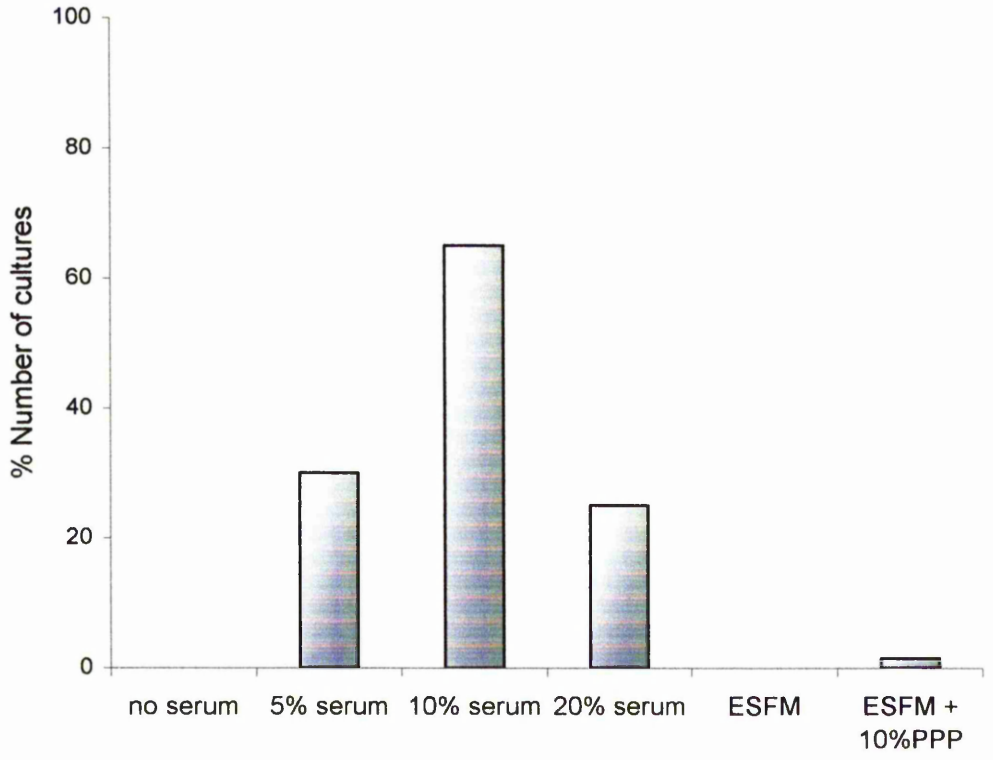


Figure 3.9 – Bar chart demonstrating the percentage of vessel cultures where cells displaying pericyte-like morphology were the dominant cell type after 21 days in the various growth medium tested.

The effect of various growth media on retinal explant culture – no cellular outgrowth was observed when DMEM or ESFM were used as the growth medium. The explant degenerated within 72 hours of culture as cells were observed rounding up and dissociating by 36 hours.

When the growth medium used was DMEM + 5% serum, outgrowth was occasionally observed as single cells but they were difficult to distinguish from the large numbers of dissociated, rounded up cells. By day 15 most explants were completely degraded. When DMEM + 10% serum was used outgrowth was observed in a similar nature to DMEM + 20% serum. The cells observed were mainly neuronal in appearance and migrated out as single cells.

When ESFM supplemented with 10% PPP was used as the growth medium, outgrowth occurred in fewer explant cultures. The outgrowth consisted of few endothelial-like cells and very few other cell types. By day 14 in plasma supplemented cultures the explants displayed extensive cell damage and cellular outgrowth was difficult to visualise.

The most effective growth medium was DMEM + 20% serum although the percentage number of explants displaying outgrowth was approximately half that of retinal vessels in identical conditions.

3.3.3 The effect of enzyme pre-treatment on retinal vessel outgrowth

Vessels that had been pre-digested with collagenase were placed in 3-dimensional collagen matrices as described in section 3.2.1. Concentrations of collagenase from 0.5mg/ml (W/V in DMEM) to 10ng/ml were used over various time intervals ranging from 20 minutes to 4 hours. However, the vessels were degraded/damaged irreversibly and no growth was observed. A slower

digestion technique with lower concentrations of collagenase (5ng/ml over 24 hours), damaged the vessels to a lesser extent but the enzyme associated with the vessels led to the slow digestion of the collagen matrices. Extensive washing with buffer to remove enzyme caused further damage to the vessels and that the matrices were still dissociated within 4-8 days. Thus final studies conducted used collagenase at a concentration of 5ng/ml with 3X1 hour washes in buffer. Very little growth was observed in only 15% of cultures, as single cells usually after 12 to 14 days. It was difficult to determine the origin of cells as the vessel was very degraded by this stage (fig.3.10a). Growth was not observed in the majority of cultures due to the lack of adhesion of vessels.

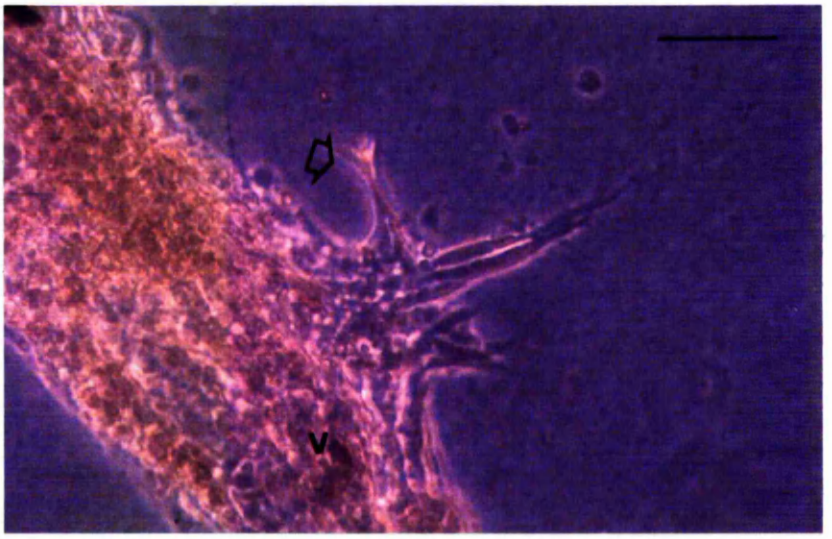


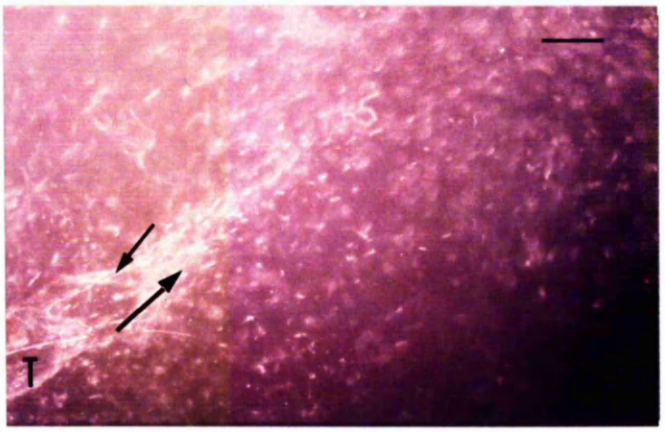
Figure 3.10 - Photomicrograph demonstrating a degraded retinal venule 5 days after collagenase treatment. Cells can be observed extending processes from damaged areas of the vessel. 'V' indicates venule, arrows indicate cell projections (scale bar = 500μm).

Figure 3.11- Photomicrograph showing a typical retinal venules after 15 days in culture displaying outgrowth into all areas of the surrounding matrix. 'V' indicates venule, arrow-heads indicate cells and direction of growth (scale bar = 100μm)

3.3.4. The effect of potential growth stimulators on model systems to study retinal neovascularisation

Retinal vessels as potential growth stimulators for retinal vessels - retinal vessels were set up as described in section 3.2.4. It was observed that any initial outgrowth occurred earlier when more than one vessel was placed in each matrix. The cellular outgrowth, primarily of endothelial-like cell morphology was observed by day 5 as small bipolar cells migrating out of the vessel edges into the collagen matrix. The cells grew out in all directions (fig 3.11), but once a certain cell density was reached in an area, the majority of cells would migrate towards the neighbouring vessel (fig 3.12a). As the cells were directing growth towards the neighbouring vessel, some cells would also be forming networks and establishing contacts with adjacent cells (fig 3.12b). By day 15 pericyte-like cells would also appear, but this cell type did not seem to follow this pattern of directional growth. This phenomenon could be seen with inverted phase contrast microscopy, and could be observed in all planes of the 3-dimensional matrix not just those of the vessels. Not all cultures displayed this directional growth, it was observed that cells only migrated towards a neighbouring vessel if the vessels were in very close proximity to (ordinarily, no more than 3mm away). Also outgrowth from both vessels did not occur to the same extent, such that one vessels outgrowth may be directed to the nearest vessel, this outgrowth was not always reciprocated.

a.



b.

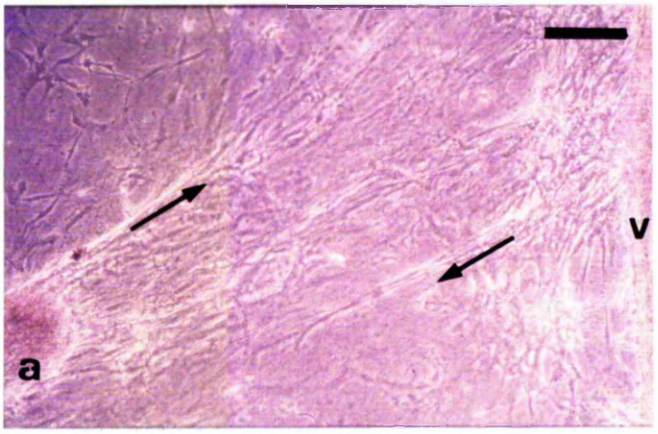


Figure 3.12 - Photomicrographs demonstrating typical growth observed when (a) two arterioles were cultured in the same matrix after 10 days, a mass of cells can be observed growing towards another arteriole, the tip of which is just visible (indicated by 'T'), arrows indicate cells and their direction of growth; (b) an arteriole (indicated by 'A') and a venule (indicated by 'V') are cultured within the same matrix after 10 days. (Scale bar = 100μm in (a) and 400μm in (b)).

Retinal explants as potential growth stimulators for retinal vessels – retinal vessels were placed in varying orientations and distances away from retinal explants. Initial outgrowth was always observed from the vessel first, within approximately 5 days of culture. Outgrowth did not occur from any specific area of the vessel initially. Vessels with tips nearest to an explant displayed outgrowth along the whole length of vessel by day 10, outgrowth was observed in all directions and planes, as did vessels orientated parallel to explants (fig 3.13a). During days 10 to 14 some explants had cells appearing at edges all round the explant, although in some cultures (approximately 40%) the cells would appear to outgrow nearest to the vessel. It was also at this point that the mass of cells emerging from adjacent vessels would direct their growth towards the explant (fig 3.13b). Again, not all vessels displayed outgrowth that was directional and vessels more than 5mm away displayed outgrowth as in section 3.3.1

The cells growing out from vessels were again initially endothelial-like cells forming cell-cell contacts and networking, followed by pericyte-like cells. Cells outgrowing from explants also included a variety of cell types with vascular cells being in general the last cell type to appear, by this time visualisation and accurate assessment of growth was difficult due to the deterioration of the retinal tissue.

a.

b.

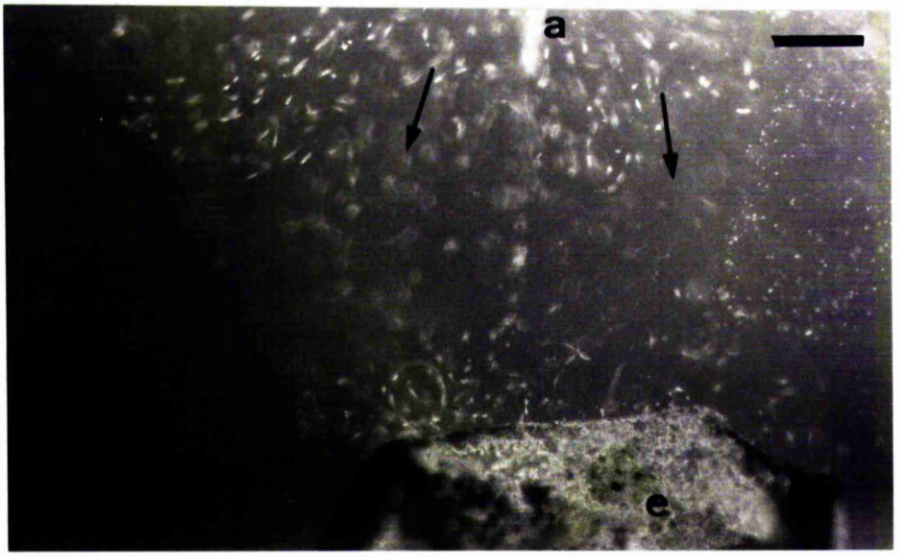


Figure 3.13- Photomicrographs demonstrating examples of directional growth, (a) shows a venule (indicated by 'V') displaying the majority of outgrowth directed towards a retinal explant (indicated by 'E') after 8 days in culture and (b) displays growth from an arteriole (indicated by 'A') after 10 days in culture with a retinal explant (indicated by 'E'). Arrows indicate cells and their direction of growth (scale bar = 50μm).

Retinal explants as potential growth stimulators for retinal explants – retinal explants were placed in collagen matrices as described in section 3.2.4, where explants were placed at varying distances apart. It was observed that poor or no outgrowth was exhibited in explants cultured more than 5mm apart, i.e., growth was similar to that described in section 3.3.1. Better outgrowth was observed when explants were cultured within 3mm of each other. Cells initially appeared of unknown morphology. These cells were observed to grow out radially in all levels of the collagen matrix by day 20. By this time cell death/damage was obvious but not as extensive as when explants were cultured individually. In these cultures it was also observed that the capillary network was preserved intact and did not appear to be deteriorating. By day 25 vascular cells were observed growing out from the preserved vasculature and increasing in number. Directional growth was not observed from retinal explant outgrowth although in a small number of cultures some cells did grow towards the adjacent explant.

A slow release polymer containing growth factor as a potential growth stimulator - slow release polymers containing bFGF were placed at different distances from retinal vessels /explants in collagen matrices. No cellular outgrowth was observed in these vessels/explants after 7 days, after which time the vessels and explants began to degrade.

Model system with sutures soaked in bFGF and VEGF as potential growth simulators – retinal vessels and explants were set up as described in section 3.2.5. Very little outgrowth was observed in only 10% of retinal vessel suture

cultures and no outgrowth was observed in explant suture cultures. The vessels that displayed outgrowth did so within day 3 of culture i.e., prior to the first change of medium. After this, no increase in outgrowth or cell-cell network formation was observed. In the remaining 90% of cultures, the matrix had collapsed to varying degrees and vessel/explant attachment was disturbed preventing any continuation of growth.

3.3.4 Characterisation of model system to study retinal neovascularisation

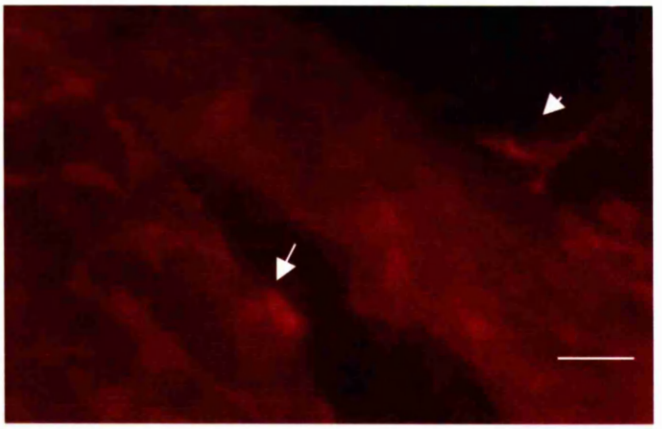
Initial flat mount staining with anti von Willebrands factor (vWBf) and anti α-smooth muscle actin identified endothelial cells (cells with dense, peri-nuclear staining) and pericytes (cells with cytoplasmic fibril staining) within the model system. Endothelial cells were also identified as the cell type forming cell-cell networks in all cultures tested. However a major shortcoming of this technique, was that the thickness of the gel meant that it was not suitable for high-resolution photographs using the Vannox fluorescent microscope.

The next stage of characterisation involved production of wax sections. Several methods of processing did not improve the quality of sections. Massive shrinkage due to the collagen gel having a high water content meant that dehydration was not a viable option.

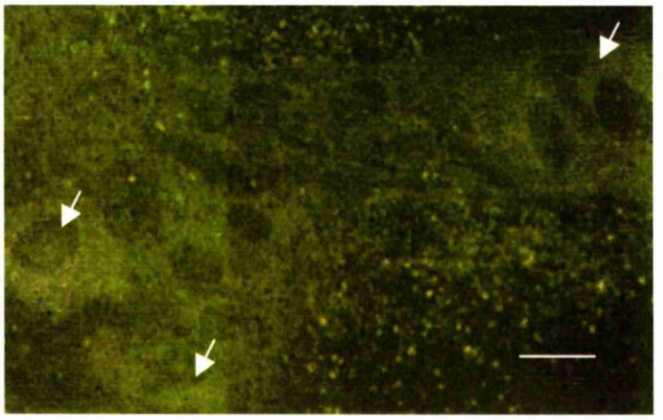
An alternative method of agarose embedding followed by vibratome sectioning allowed the production of much improved sections. This sectioning method relied upon the development of method 2 for preparing a 3 D matrix (section 3.2.1) which prevented the separation of the two layers and hence loss of orientation. Immunostaining using the vibratome sections allowed excellent

preservation of orientation and integrity of model system. However, a major drawback of this system was that although at 200 microns it was a lot thinner, than when flat mount staining was used; the majority of the fluorescent photographs appear out of focus due to the fluorescence from the underlying layers of cells.

a.



b.



c.

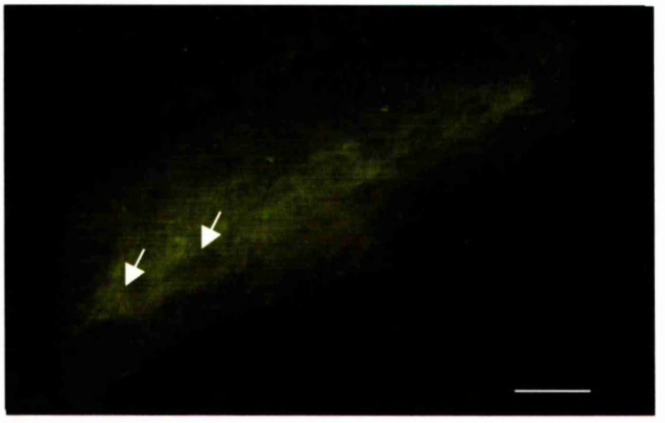


Figure 3.13 – Photomicrographs displaying examples of (a) α-smooth muscle actin distribution in a flat mount stained venous culture, (b) von Willebrand factor in a flat mount stained arteriolar culture and (c) von Willebrand factor distribution in a vibratome section of an arteriole 3-dimensional culture. Note the strong but blurred fluorescence observed in (a) and the weaker, blurred fluorescence observed in (b). Arrows indicate positively identified cells, which appear clear when focusing trough the inverted microscope. Much clearer fluorescence is observed in (c) where dense green perinuclear staining of endothelial cells can be seen, indicated by arrow-heads (scale bar = 500μm for(a) and (b) and 250μm for(c)

3.4 Discussion

The initial stages of this study involved the development of an in vitro model to represent the early stages of retinal neovascularisation. In an attempt to address the problems associated with previous in vitro models of neovascularisation, retinal arterioles and venules were microdissected and placed in a 3-dimensional collagen matrix. The choice of bovine retinal vessels as the starting tissue within a 3-dimensional matrix had several advantages: -

a) The vascular cells are quiescent and retinal in origin thus representative of the situation in vivo,
b) The use of actual retinal vessels as the source of endothelial cells rather than monolayer cultures of endothelial cells; requires the proteolysis/degradation of the basement membrane thus representing the first step in retinal angiogenesis,
c) The use of a 3-dimensional collagen I, (a major component of the retinal vascular ECM, (Marshall et al, 1993) matrix requires digestion of the surrounding matrix to enable any vascular cell invasion or migration, thus representing the obligatory step of endothelial cell migration of any neovascular response.

The observation that time of outgrowth from arterioles and venules was not different in a 3-dimensional matrix was consistent with the findings in chapter 2, where arterioles and venules cultured on a collagen I substrate had identical time of first outgrowth. This may be because collagen I is an interstitial collagen and abundantly expressed in almost all tissues as well as the retinal vasculature and hence affect endothelial cells from both vessel types in the same way (Hay, 1991; Montesano, 1992; Marshall et al, 1993).

The finding that venules almost always initially exhibited outgrowth along the length of the vessel was in contrast to arterioles in which outgrowth almost always occurred from the tips of the vessel, suggests a possible difference in the biology of the vessel types. Venules may be more fragile than arterioles and thus more susceptible to mechanical damage during the trauma of dissection. This may have allowed endothelial migration from localised areas of basement membrane damage. An alternative explanation may be that venous endothelial cells have a greater capacity to upregulate expression of matrix degrading enzymes to respond to an angiogenic stimulus (Pepper et al, 1988; Vaheri et al, 1990; Mandriota and Pepper, 1997). This pathway may be unavailable or inhibited for arteriolar endothelial cells, such that those arteriolar vessels only displayed growth at the dissected ends of the vessels. Comparison of arteriolar and venous culture medium for production of matrix degrading enzymes using techniques such as zymography or western blotting, or the investigation of mRNA expression during the various stages of vascular cell growth are now required (Kon et al, 1998; Haas et al, 1998).

Endothelial cell presence was confirmed both with flat mount staining and in vibratome sections. These cells were capable of invading the matrix, but only when displaying bipolar morphology suggesting a change in morphology is required for normally quiescent endothelial cells to become active and migrate/invade their surrounding matrix. This has been reported in a number of other studies, with endothelial cells from the macro- and microvasculature (Madri and Pratt, 1986; Jackson and Jenkins, 1991; Canfield and Schor, 1994).

As demonstrated in fig 3.5 some cultures displayed vascular cells, organised as if the cells were growing along the collagen fibrils. This is suggestive of the

endothelial/vascular cells recognising and binding to specialised receptor sites within the collagen I matrix, this is a mechanism implied in several other in vitro studies (Feder et al, 1983; Iruela-Arispe et al, 1991; Sage and Vernon, 1994; Grant et al, 1991). It is possible that endothelial cells are binding via $\alpha_2\beta_1$to 'RGD' sequences within collagen I fibrils. Thus a stable matrix scaffold may be a prerequisite for new vessel formation and is now widely accepted as important level of control for in vivo neovascularisation (Madri and Basson, 1992; Vernon et al, 1995). More information on matrix control within retinal neovascularisation can be obtained using this model by adding additional matrix components into the collagen solution prior to polymerisation and confirmed by using many of the matrix receptor antagonists or neutralising antibodies (Hammes et al, 1996; Luna et al, 1996).

The presence of pericytes were confirmed by flat mount staining and immunohistochemical analysis of vibratome sections. The finding that pericytes were not observed until day 10 to 12 suggests that many of the pericytes were lost due to the storage of retinae at 4°C for 24 hours. This was also observed in primary culture of pericytes where a low yield of pericytes is obtained when longer post mortem retina are used, this is in contrast to endothelial cells, this cell type demonstrates an equal and occasional higher yield when isolated from longer post mortem tissue. The loss of pericytes does however make the model system more representative of the in vivo situation particularly in the context of diseases such as PDR, where pericyte dropout is the earliest sign of retinopathy (Davies, 1992). The loss of pericytes not only leaves gaps where endothelial cells may sprout and the contact inhibition conferred upon endothelial cells by pericytes is also lost.

The formation of organised networks of endothelial cells has been well documented in other in vitro studies and some similarities exist in this system. The majority of these studies however, use actively proliferating endothelial cells taken from monolayer culture (Folkman and Haudenschild, 1980; Maciag et al, 1982; Ingber et al, 1987; Saunders and D'Amore 1992; Canfield and Schor, 1994; Zimrin et al, 1995; Zimrin and Williams, 1995). A further consideration is that many of these studies only observe the behaviour of one cell type within a matrix, and do not address the possibility of other cell types inhibiting or contributing to the formation of an organised network. In this study endothelial cells were seen to continually migrate and invade further areas of the matrix before extensions between one or more cells were observed. However, with time pericytes would surround these networks and appear to inhibit any further endothelial cell growth. A study by Montesano and colleagues (1993) demonstrated the positive effect of transformed fibroblasts on in vitro tube formation and Nicosia and co-workers (1994) demonstrated the ability of rat tail fibroblasts to stabilise tube formation of aortic endothelial cells as well as the ability of smooth muscle to participate in in vitro angiogenesis by becoming pericytes (Nicosia and Villaschi, 1995). However, this is the first demonstration of another retinal vascular cell type participating in endothelial network formation in vitro.

Retinal explants were cultured in a similar fashion to retinal vessels, in order to address the issue of other non-vascular retinal cell types contributing to the neovascular process. Again explants were chosen rather than monolayer cultures of retinal cell types to mimic the in vivo orientations and interactions of quiescent retinal cells with the vasculature. Although some cellular outgrowth

was observed it was difficult to determine the exact origin due to the large amount of cell death/damage within the explant. Some of these findings are consistent with the reports of Forrester et al, 1990, who cultured retinal explants within collagen matrices as a model of PDR. Yet, Forrester and colleagues reported that the majority of the cellular outgrowth observed was endothelial in origin, this was not found to be the case in this study. Initial vascular cell outgrowth was limited although the actual vasculature was preserved for the duration of cultures in most explants, with possible cells growing out at the end but by this time cell debris of the disintegrated retina made visualisation even after immunostaining, unfeasible. The presence of cells that did not stain positive for cytokeratin, factor VIII or α-smooth muscle actin occurred only in a small number of cultures. In these cultures it should be noted that the retinal vasculature was highly preserved. A possible explanation for this is that this (possibly neuronal) cell type had a modulatory effect on cells of the vasculature, possibly by the production of certain factor/s. Alternatively, endothelial cells may well have been inhibited by the presence of the full complement of pericytes intact in the fresh retinal explant (Orlidge and D'Amore, 1987; D'Amore, 1992a; 1992b).

Outgrowth from retinal vessels was found to be dependent on the presence of serum. This is consistent with other observations in other in vitro models of angiogenesis/neovascularisation (Feder et al, 1983; Madri and Pratt, 1986; Forrester et al, 1990), and suggestive of factors within serum participating in the initial stages of basement membrane degradation/endothelial cell activation. Without serum, outgrowth did not occur from venules or arterioles.

The amount of serum present in the medium appeared to direct the nature of growth observed in the model. Although attachment of the vessel to the matrix and subsequent outgrowth occurred in the presence of growth medium supplemented with 5 or 10% serum, outgrowth was slower in comparison to when the medium was supplemented with 20% serum.

ESFM did not improve cellular outgrowth above that observed in serum. Even though endothelial serum free medium contains many endothelial cell mitogens, such as bFGF and insulin (Gorfien et al, 1992) and was developed as a medium sufficient to nourish both large and small vessel endothelial cells. Sufficient cell numbers of endothelial cells or pericytes were never achieved in this environment so that cell-cell contacts and network formation were not established, indicating the requirement of additional factors. When ESFM was supplemented with 10%PPP, culture growth was still not significantly improved. Although outgrowth was observed in 80% of vessels – these cells failed to migrate any distance and were not supported by this medium. This is in contrast to what was observed on a 2-dimensional collagen I substrate. Hence, plasma supplemented ESFM was sufficient to encourage possible basement membrane breakdown followed by endothelial sprouting, but was not enough to encourage endothelial cell migration and subsequent 3-dimensional matrix remodelling. These findings demonstrate the importance of multiple factors involved in in-vitro vessel formation and the correlation with the in vivo situation.

Without serum, the explant degenerated rapidly; even the vasculature was not preserved. Serum improved the amount of cellular outgrowth, with high variability between retinal samples, making the deduction of any trends between serum concentration and any outgrowth difficult. This variability could be

reflective of the in vivo situation where for example, not all cases with background retinopathy go on to produce retinal neovascularisation (Kohner, 1989; Davies, 1992). ESFM or plasma supplemented ESFM did not encourage outgrowth nor did it encourage preservation of the vasculature, suggesting again that this medium was not sufficient to support endothelial cell outgrowth. This may be because platelet poor plasma is lacking in many platelet-associated growth factors that may be necessary to support retinal explant growth. However the increased amount of cell death/damage impaired visualisation of the model system.

Enzyme pre-treatment was used as a possible method of a) removing any remaining pericytes from the dissected vessels, which inhibited endothelial cell network formation in vitro in the later stages of culture and b) to facilitate endothelial cell migration by digesting both arteriolar and venous vessels to the same extent.

Several methods of enzyme pre-treatment failed to increase endothelial cell outgrowth, in fact cellular outgrowth was not observed in 85% of the test cultures. This was thought initially to be due to high enzyme concentrations degrading the vessels to an unfavourable extent; and as discussed earlier, integrity of the parent vessel appears to be important to subsequent cell outgrowth. Even when lower concentrations of enzyme were used, the vessels ability to adhere to the matrix was impaired indicating a disruption of cell matrix interactions which may be necessary for stability. Thus collagenase pre-treatment was not found to a viable option within this culture system, as improved yield of endothelial cells was not observed.

The observation that cellular outgrowth occurred earlier when more than one vessel was present in the matrix suggests that vessels/cells within the vessels may have the ability to exert a regulatory effect on each other. When retinal vessels were cultured in close proximity to one another, a different pattern of outgrowth was observed to when cultured individually. The cellular outgrowth from one vessel would direct their growth to a neighbouring vessel when a certain cell density was reached. The fact that cells were observed to migrate towards a neighbouring vessel suggests that factor/s released into the surrounding matrix by neighbouring vessels are chemoattractants for the cells. This directional effect on growth is only seen when vessels were less than approximately 3mm apart, indicating that either the factor/s have a short half-life in this environment or become matrix bound and are unavailable. It should also be noted that even when cellular growth was directional, the cells continued to form cell-cell contacts and form cellular networks. This phenomenon is possibly reflective of in vivo situations during vessel remodelling where new capillary growth has been observed to be directed towards pre-existing vessels or even cells of the vasculature (Ashton, 1951; Archer, 1983).

This directional growth was further demonstrated when retinal vessels were cultured in various orientations and close proximity to retinal explants. The retinal vessels would produce directional growth when cells were visibly migrating from the retinal tissue. This suggests that the cells of the retina also possess the ability to modulate vascular cell outgrowth in this model. In these cultures, vascular cell networking and tube formation was also demonstrated and suggests that the retinal cells or factors they produced encouraged this directional growth and hence stages of in vitro vessel formation. It may be that vascular mitogens such as IGF-1 and bFGF, known to be consitutively

expressed in many retinal cell types (McAvoy and Chamberlain, 1990) are upregulated in ischaemic tissue (Fredj-Reygrobellet et al, 1991; Hannekan et al, 1991) may be acting as the stimuli.

It was also observed that when two explants were cultured within close proximity, better growth was observed from the explants and less cell death/damage was apparent. No directional growth was observed from cells growing from the explants, indicating that directional growth was vascular cell specific in this culture system or the modulator/s produced were not chemotactic for other retinal cells types in these conditions.

To ascertain whether the direction of growth observed above was due to the action of specific growth factors the model system was adapted such that growth factors could be incorporated and allow quantitative analysis. Slow release polymers consisting of methylcellulose, (an inert biodegradable polymer originally used during cataract surgery), growth medium and bFGF were initially used to deliver small amounts of growth factor in the system. Initial results were disappointing, with no cellular outgrowth detected in any of the test dishes. It is a possibility that the polymer was toxic within this culture system although no adverse effects were observed when placed in monolayer cultures. Alternatively, it is not known how much bFGF was released into the medium at any time. It may be possible that a large dose of the growth factor inhibited vascular cell outgrowth, although this is unlikely as bFGF has been shown to encourage endothelial cell migration and proliferation in a number of in vitro/vivo studies (see Klagsbrun and D'Amore, 1991). Further studies are required to quantify bFGF release and also to find an alternative polymer as the protein carrier.

Utilising sutures soaked in bFGF and VEGF as potential growth stimulators attempted to develop the model system so that it would be possible to encourage vessel formation in vitro. Initial studies displayed very poor outgrowth from vessels and no measurable outgrowth from explants. The main reason for this lack of growth was thought to be due to the disruption of the matrix by the suture. The loss of stable scaffolding prohibited vascular cell growth to any distance. The requirement of a stable matrix for new vessel formation is reflective of the situation in vivo; where new vessels arise on, in or beneath the retina as well as intra-vitreally (Archer, 1983).

A problem with this study was the difficulty in characterising in detail the cellular outgrowth and any deposition of matrix. However after the employment of a number of techniques, vibratome sectioning allowed excellent preservation of vessel and cell matrix orientation. High resolution inverted microscopy demonstrated the positive/negative immunostaining of cell specific antigens. A major shortcoming of this technique was the problem in recording of immunostaining results. The thickness of the vibratome sections (200 μm) meant the photographs would appear out of focus due to the background fluorescence of underlying cell layers. Further studies are thus required using a technique such as confocal microscopy, which would allow full visual analysis through the depth of the thick sections without compromising any quality of the resolution.

3.5 Conclusions

The culture system developed in this study is a highly reproducible in vitro model representing several stages of retinal neovascularisation. It addresses the stages of basement membrane degradation, invasion of the surrounding matrix by migrating vascular cells as well as cell-cell network formation. The use of quiescent retinal vessels as a starting tissue and the demonstration of retinal explants (i.e. non-perfused retinal tissue) behaving as a directional stimulus for retinal vascular cell growth, is an accurate yet simple representation of the in vivo situation. This model can be further manipulated with ease, to investigate the molecular mechanisms involved in the earliest stages of retinal neovascularisation and hence increase our understanding of the disease as well as determine the best point of therapeutic intervention.

CHAPTER FOUR - THE EFFECT OF GROWTH FACTORS, EXTRACELLULAR MATRIX COMPONENTS AND/OR OXYGEN ON COLLAGEN GEL CONTRACTION BY RETINAL CELLS

4.1 Overview

Cell mediated contraction has been shown to play a critical role in the pathogenesis of most proliferative retinal diseases. Visual loss occurs due to complications arising from the formation of a fibro-cellular membrane on the surface of the retina (Charteris, 1995). These retinal membranes, which are highly contractile, are thought to be formed by the migration and proliferation of various retinal cells with subsequent deposition of extracellular matrix components (Machemer and Laqua, 1975). It is believed that a greater understanding of the contractile response may lead to new therapeutic intervention in proliferative retinal diseases. One of the most popular models for studying cell-mediated contraction is the collagen gel contraction model (Bell et al, 1979). This model has been used to study the contractile response of a diversity of cell types, which has led to the accumulation of a great deal of data regarding contraction. One aspect that has largely been ignored is local oxygen environment. It has been demonstrated that standard cell culture conditions maintain cells in a hyperoxic environment, and that oxygen is a potent modulator of cell proliferation, growth factor production and protein synthesis (Boulton, 1992; Khaliq et al, 1995; Stefansson, 1990). Cellular contraction in a more physiological oxygen environment has also yet to be investigated. Furthermore, in the pathological retina, a local area of hypoxia can occur in the avascular fibrous membrane that may be involved in the promotion or the trigger of the contractile response (Stefansson, 1990).

It was therefore hypothesised that oxygen has a role to play in retinal cell mediated contraction. To investigate this, retinal cells were seeded in collagen

gels and the effect of oxygen, growth factors and extracellular matrix components were assessed.

The cells assessed in this study were: human retinal pigment epithelial cells (HRPE), because they are heavily implicated in PVR; bovine tenon's capsule fibroblasts (BTCF), because of the presence of 'fibroblastic cells' in retinal membranes; bovine retinal capillary pericytes (BRCP), these cells are known to be contractile but their presence has yet to be confirmed in retinal membranes (Clarkson et al, 1977; Hiscott et al, 1985; Machemer et al, 1975).

The oxygen concentrations used for this study were previously set up in the lab to represent both physiological and pathological oxygen concentrations. The oxygen concentrations in the retina are thought to range from 70-90mm Hg in the retinal pigment epithelium, due to the rich choroidal blood supply (Pournaras et al, 1989; 1995). This is reduced to 30-40mm Hg at the vitreoretinal interface and decreased to as low as 12-17mm Hg mid-vitreous (Maeda et al, 1990). The effect of this oxygen gradient has not yet been investigated in terms of the movement of cells (particularly RPE cells) through the retina in pathological conditions or in terms of cell mediated contraction. Furthermore all previous studies report the contractile ability of various retinal cell types using standard cell culture incubators which demonstrated an oxygen tension of approximately 135mm Hg i.e., hyperoxic for most cell types. Thus the oxygen environments used in this study were 10mmHg (representing hypoxia) 30mm Hg, 70mm Hg (representing physiological oxygen conditions) and 135mm Hg (standard or hyperoxic conditions) (Rosen et al, 1991; Khaliq et al, 1995).

The effects of varying oxygen concentrations on cell mediated collagen contraction were investigated in the presence of the following three growth factors: TGFβ2, VEGF and bFGF.

The effect of TGFβ2 on cell mediated collagen contraction was investigated because of TGFβ2 (and its receptors) documented presence in the normal retina and PVR/PDR membranes (Clark and Coker, 1998; Anderson et al, 1995; Obata et al, 1996). The effect of this particular isoform in the transforming growth factor – beta family has also been extensively studied in the context of the contractile response of a variety of ocular fibroblasts (Tanihara et al, 1997). However, this effect has yet to be demonstrated in an oxygen environment other than that of standard cell culture conditions.

The effect of VEGF on cell mediated contraction was investigated on all the cell types studied. The presence of VEGF is well documented in proliferative retinal membranes, particularly the isoform used in this study $VEGF_{165}$.(Aiello et al, 1994; 1995; Schneeberger et al, 1997). $VEGF_{165}$ is capable of binding to both VEGFR-1 and VEGFR-2 and these high affinity binding sites have been demonstrated on RPE cells, pericytes and on retinal membranes (Takagi et al, 1996; Gilbert et al, 1998; Wen et al, 1998; Gerhardinger et al, 1998). Furthermore, VEGF is known to be up regulated in a hypoxic environment, such low oxygen environments may well exist in avascular of retinal membranes. Yet the effect of VEGF on cell mediated contraction has never been investigated in standard or hypoxic oxygen conditions.

The presence of bFGF and its receptors have been demonstrated in both the physiological and pathological retina, but its precise role in retinal membrane formation or contraction remains unclear (Hanneken et al, 1995; Cassidy et al, 1998; Kon et al, 1999). The effect of bFGF on cell mediated contraction, is somewhat conflicting in the literature (Smith-Thomas et al, 1996; Kurosaka et al, 1995). BFGF is also largely present in ischaemic tissue or sites of tissue injury, yet its effect on cell mediated contraction in any oxygen environment other than

standard has not been reported. Thus the effect of bFGF on the contractile response of all the cell types tested in this study was investigated.

As well as investigating the effect of growth factors in varying oxygen environments on the contractile response of retinal cells, the effect of extracellular matrix components was investigated on fibroblast mediated contraction. An enormous number of previous studies have documented the presence, upregulated expression and redistribution of various extracellular matrix components and their receptors with respect to retinal membrane formation and contraction (Asaga et al, 1991; Agrez et al 1991; Hiscott et al, 1993; Ohsato et al, 1994; Nagasaki and Shinagawa, 1995). Evidence in the literature documents the positive effect of fibronectin on fibroblast mediated contraction, yet few studies have examined the role of laminin or vitronectin in the context of cell contraction (Smith-Thomas et al, 1996). Furthermore, very limited information on the role of oxygen in this context is available, thus this study includes preliminary experiments investigating the effect of extracellular matrix components and oxygen on cell mediated contraction.

4.2 Experimental methods and design

4.2.1 Primary isolation of retinal cells

Bovine Tenon's capsule fibroblasts (BTCF) - bovine eyes were obtained from a local abattoir and used within 24hr post mortem. Fibroblast primary cell cultures were obtained using the method described by Boulton et al (1992). The conjunctiva above the Tenon's capsule was washed with copious amounts of sterile PBSA. An area of epithelium was removed and a conjunctival flap was raised using forceps. Approximately 4mm^2 segments were removed using sharp dissecting scissors and washed by immersing several times in sterile PBSA. The tissue explants were then transferred to a sterile 25cm^2 tissue culture flask which had its surface pre-scratched with a hypodermic needle (to facilitate attachment of the explant). The flask was incubated (inverted) at 37°C for 45 minutes after which the flask was reverted and 3mls of DMEM + 20% FCS (appendix II) medium was added. Proliferation of fibroblasts was observed within one week of isolation of explants, after which the explants were removed and the cells allowed to grow to confluence. Thereafter the cells were sub-cultured and maintained as described in section 4.2.2.

Human retinal pigment epithelial cells (HRPE's) - were obtained using the method described by Boulton et al (1983). Human donor tissue was used within 48hr post-mortem and each eye was dissected slightly posterior to the limbus, the vitreous and the neuroretina removed gently from the RPE layer. The retina was cut at the optic disc and both the retina and vitreous were discarded. The posterior eyecup was washed with PBSA (appendix II) and treated with 0.25% trypsin solution (appendix II) for 1hr at 37°C (to allow the majority of the RPE cells to detach from Bruch's membrane). Thereafter, the suspension was collected from

the eye cup after gently aspirating up and down and pipetted into a centrifuge tube with F10 + 20% FCS (appendix II). The cell suspension was centrifuged at 70g for 5min. The pellet was resuspended in growth medium (F10 + 20% serum) and plated into 1 or 2 wells of a 24 well plate, which were maintained at 37°C in a standard CO_2 incubator. Upon reaching confluence, the cells were sub-cultured and maintained as described in section 4.2.2.

Bovine retinal capillary pericytes (BRCP) - Method 1 (Wong et al, 1987).

Twenty bovine eyes were enucleated within two hours post-mortem and transported on ice from the local abattoir to the laboratory. The eyes were dissected 5mm posterior to the limbus and the retinae removed and placed in 20 ml of MEM/HEPES (appendix II). The retinae were washed twice with cold MEM/HEPES to remove any adherent RPE cells and loose rod outer segments followed by homogenisation in cold MEM/HEPES (six up and down strokes of a rotary Teflon-glass homogeniser driven at approximately 250 rpm). The homogenate was centrifuged at 300g for 10 minutes and the resultant pellet resuspended in 10 ml of cold MEM/HEPES. Microvessels were trapped on a 85μm nylon mesh and transferred to a sterile petri-dish containing 15 ml PBSA (appendix II) plus 500μg/ml collagenase, 200μg/ml pronase and 200μg/ml deoxyribonuclease. The enzyme cocktail and microvessels were incubated at 37°C on a rotary shaker. The enzymatic digestion process was monitored by light microscopy to determine the end point of digestion i.e. where most of the vessels were devoid of non-vascular retinal cells, but the majority of the pericytes remained attached (this was usually 15-20 minutes). The treated microvessels were trapped on a 53μm nylon mesh, washed in cold MEM/HEPES and centrifuged at 125 g for 5 minutes. The resultant pellet was resuspended in 10ml

DMEM + 20% FCS (appendix II). The suspension was plated into two 25cm^2 tissue culture flasks, which were maintained at 37°C in a CO_2 incubator. The growth medium (DMEM + 20% FCS) was changed every 3-4 days and cultures were subcultured at a ratio of 1:2 upon reaching confluence.

Method 2 (Schor et al 1995) - Day 1: five bovine eyes were enucleated within two hours post-mortem and transported on ice from a local abattoir. The eyes were dissected 5mm posterior to the limbus and the retinae removed and placed in 7ml of 2 X MEM (appendix II). The retinae were then given four washes in 2 X MEM, to remove any adherent RPE cells and loose rod outer segments. The medium was removed and the retinae chopped into fine pieces with sterile dissecting scissors. A 7ml aliquot of 0.5mg/ml collagenase in 2 x MEM (w/v) was added to retinal fragments and incubated for 4 hours at 37°C in a 25cm^2 culture flask. The digested retinae were centrifuged for 5 minutes at 100g to remove any debris. The supernatant was discarded and the pellet placed in 7ml of trypsin /EDTA in Pucks minimal medium (appendix II) and incubated at 37°C for 15 minutes in the culture flask used for collagenase digestion. 7ml of MEM+ 20% FCS (appendix II) was added to the retinae and enzyme solution to neutralise the trypsin and then centrifuged for 10 minutes at 100g. A 5ml aliquot of growth medium was added to the 'original' culture flask (since many cells had attached during the four-hour incubation period) which was placed in the incubator and labelled flask 1. The pellet of retinal cells was resuspended in 5ml of MEM + 20% FCS and placed in a 25cm^2 culture flask labelled flask 2.

Day 2 - The culture medium containing unattached cells from flask 2 were pooled along with several medium washes of the same flask and centrifuged for 5 minutes at 100 g. The supernatant was discarded and the pellet was resuspended in 5ml of MEM + 20% FCS and placed in a 25cm^2 culture flask labelled flask 3. Flask 2

(attached cells) was given two washes with PBSA (appendix I) and 5ml of growth medium added. This step was repeated with flask 1.

Day 3 - The culture medium containing unattached cells from flask 3 was pooled along with several medium washes of the same flask and centrifuged for 5 minutes at 100 g. The supernatant was discarded and the pellet was resuspended in 5ml of MEM + 20% FCS and placed in a culture flask labelled flask 4. Flask 3 (attached cells) was given two washes with PBSA and 5ml of growth media added.

Day 4 - Culture medium from culture flask 4 was discarded, the flask washed twice with PBSA and 5 ml of growth medium added.

Thus after three days of the initial isolation, four flasks labelled 1-4 respectively were generated.

Upon reaching confluence the cells were sub-cultured at a ratio of 1:2 and maintained in growth medium that was changed every 2 to 3 days.

Bovine retinal glial cells (BRG's) - Method 1-. 10 Bovine eyes were obtained from the local abattoir and each retina was removed as described for the pericyte isolation. The dissected retinas were maintained at 4^0C for 24hours and then washed in three changes of PBSA, to remove adherent retinal pigment epithelial cells. The washed retinas were cut into small pieces of no more than 10mm in diameter and floated in 25cm^2 tissue culture flasks (pre-coated with 0.1% gelatin) containing 15ml MEM + 20% serum (see Appendix II). The medium was replaced with fresh medium once some cell attachment had occurred.

Method 2 - (Savage et al, 1988). Bovine eyes were obtained from the local abattoir and 5 retinae were dissected and washed with PBSA. The retinae were cut into small pieces and homogenised with a glass-Teflon homogeniser and the

homogenate spun at 800g for 10 minutes, the supernatant was discarded and the pellet resuspended in MEM+HEPES. The cell pellet solution was passed through a 53μm mesh. The cell suspension was centrifuged at 800g for 5 minutes and the pellet resuspended in 10ml growth medium (MEM + 10%NCS) which was divided into two 25cm^2 tissue culture flasks (pre-coated in 0.1% gelatin). The cells were maintained in a standard CO_2 incubator at 37°C.

4.2.2 Maintenance and subculture of retinal cells

Retinal cells were maintained in their respective growth medium for two days after which the medium was changed to fresh medium (pre-warmed to 37°C in a water bath). This process was repeated every 2-3 days until the cells became confluent. Upon reaching confluence the cell monolayers were sub-cultured. Removing the growth medium and washing the cell monolayer twice with sterile PBSA to remove any remaining serum did this. Trypsin/EDTA solution was then added (2mls of trypsin/EDTA solution for 75cm^2 flask, 1ml for a 25cm^2 flask and 0.2 ml for a 24 well plate) monolayer cultures. The monolayer cultures were incubated in the trypsin/EDTA solution at 37°C until the cells had detached from the tissue culture flask (usually 2-3 minutes). Following complete detachment of cells 1ml of serum containing growth medium was added to the cell suspension (to inhibit further action of trypsin). The cell suspension was transferred to a centrifuge tube and centrifuged at 70g for 7 minutes. The supernatant was discarded and the cells were resuspended in fresh growth medium. All the cell cultures except BRCP were transferred to 75cm^2 tissue culture flasks from 25cm^2 once the primary isolation had reached confluence and at subsequent passages were plated at a split ratio of 3:1. BRCP were maintained in 25cm^2 tissue culture flasks during the first two passages, after which pericyte cultures were split at a ratio of 1:2. All cell

cultures were maintained at 37°C in CO_2 incubators within a humidified atmosphere containing 5% CO_2 and 95% air, unless otherwise stated.

4.2.3 The effect of cell density, cell type and passage number on cell mediated contraction

In order to develop the collagen contraction system a number of experiments were undertaken to establish and refine the ideal conditions for cell mediated contraction. Hence experiments were set up to determine the effect of cell type, cell density as well as the effect of culture passage number on the contractile event.

Preparation of cell populated collagen matrices - Method 1– initial studies relied on a method described by Schor et al (1980). Cells used in these studies included BTCF, HRPE's and BRCP. Several 75cm^2 flasks containing cells at confluence were trypsinised as described in section 4.2.2. The cell number was determined using a haemocytometer and diluted to the required cell density in a 1ml volume of FCS. The cell suspension was added to 10mls of collagen gel solution as described in section 3.2. The cell /collagen mixture was rapidly aliquotted as accurately as possible into five 35mm cell culture dishes using a 10ml sterile syringe. The cell containing matrix were left overnight to gel. The matrix was then detached from the edge of the dish by dragging a sterile spatula between the edge of the matrix and the dish. Two ml of appropriate growth medium was the added to each cell-containing matrix. The growth medium used for each cell type used was the same growth medium used for culturing cells in monolayer culture. Such that the collagen gel solution used for HRPE populated matrices contained 10X Ham's F10 and the growth medium used was F10 + 20% FCS (appendix II); the collagen gel solution used for BTCF and BRCP contained 10X DMEM and the

growth medium used was DMEM + 20% FCS. The medium was removed and replaced with fresh medium every 2-3 days unless otherwise stated.

Method 2 – The above method was modified as described by Raymond and Thompson (1990) to allow large scale studies, using cells, collagen and time more economically. Cells were trypsinised and counted on a haemocytometer as described above and the required cell number was aliquotted into sterile bijou tubes in the relevant media in a volume of no more than 100µl. Two ml of collagen solution was made as in section 3.2.1 (i.e., 1.7ml collagen I solution, 0.2ml 10X DMEM and 0.1ml 7.5% sodium bicarbonate solution (w/v in double distilled water)), added to cells and rapidly mixed. The cell/collagen mixture was immediately transferred to 4 wells of a 24 well plate in 0.5ml aliquots. The cell-populated matrices were allowed to polymerise for up to four hours, after which a sterile needle was used to detach the gel by inserting the needle around the edge of the gel. Two ml of the appropriate growth medium was added and then placed in the standard cell culture incubator at 37°C. Removing from the incubator, decanting the medium and washing with buffer twice stopped the experiment. The gels were then photographed on a Zeiss dissecting microscope with a Contax camera to provide negatives for image analysis (see section 4.2.8).

Cell type studies - In order to determine the most effective cell type at producing contraction; BTCF, HRPE's and BRCP populated matrices were set up as described in method 1 above. Cell density of $1x10^5$ cells per ml collagen was used and contraction was observed over a period of 10 days. Each experiment was set up in quadruplicate and repeated on at least 2 separate occasions. The gels were photographed every 24 – 48 hours after washing in sterile conditions, fresh growth medium was added before gels were placed back into the cell culture incubator.

Effect of cell passage on cell mediated contraction – In order to determine whether the passage number of the cells used affected the contractile response of the cell types studied. Studies were undertaken using method 2 described above where passage 3, 5, 6 and 9 HRPE cells and BTCF and passage 2,3,4,and 5 BRCP were used. Each experiment was set up in triplicate and repeated on three separate occasions.

Cell density studies – In order to determine the ideal cell number for contraction to occur, a study was undertaken using BTCF, HRPE cells and BRCP at cell numbers between $1x10^3$ and $4x10^6$ cells per ml of collagen. Each experiment was set up in quadruplicate and repeated with each collagen batch used.

4.2.4 Investigating the effect of oxygen on cell mediated contraction

In order to elucidate the effect of oxygen on cell mediated contraction a study was undertaken using the collagen contraction system in varying oxygen environments.

Manipulation of oxygen tension within the culture system – standard cell culture incubators with varied oxygen tensions previously set up in the lab were used. Briefly, standard cell culture incubators with a humidified atmosphere consisting of 5% CO_2 and 95% air were used with nitrogen substituting the air to vary the concentration of oxygen. The media oxygen tensions achieved were 10mm Hg oxygen, 30mm Hg oxygen and 70mm Hg oxygen as well as the standard cell culture incubator- 135mm Hg. The oxygen concentrations were determined using an oxygen sensor situated in the incubator (connected to the nitrogen supply) set at 37°C. An oxygen sensor was calibrated and the control unit was set to monitor the required oxygen concentration for each of the three incubators. The oxygen concentration inside each incubator was constant so long as it remained closed and sealed. The oxygen concentration was monitored during each experiment

and the sensor from each incubator was recalibrated at regular intervals. Such that the oxygen environment was consistent and reproduced for all oxygen experiments.

Preparation of cell populated matrices for oxygen environment studies – BTCF, HRPE cells and BRCP were seeded in collagen matrices as described in method 2 above. Cell densities between 0.5 and $2x10^5$ cells per gel were used depending on the collagen batch used. Each experiment was set up in quadruplicate and repeated on at least 3 separate occasions. The gels were placed in each incubator after being detached from the rim and overlaid with 2 ml of the appropriate growth medium. The gels were left undisturbed in each of the different oxygen incubators for a maximum of five days.

4.2.5 Determination of cell proliferation within cell populated collagen matrices

A study was undertaken to determine whether the contractile response observed in the varying oxygen environments was an artefact of increased proliferation or indeed a response to oxygen. This was done in two stages, firstly by determining the cell number of a cell populated matrix after the contractile episode and secondly, by determining the amount of tritiated thymidine incorporated by the cells in a collagen matrix during contraction.

Cell number determination of cell populated collagen matrices – Cell numbers in collagen gels were determined using an enzyme digestion technique to confirm that the difference in contraction produced by cells in the different oxygen environments was not due a difference in the cell number. Collagen gels were set up as described above, after five days the test gels were removed from each oxygen incubator, washed twice with PBSA (appendix II) and incubated overnight in 200μl collagenase solution (appendix II) at 4^0C. The digested cell/collagen

solution was aspirated using a hypodermic needle and 2ml syringe to disintegrate any remaining clumps of cells. The volume was made up to 1ml with serum containing media and the cells counted on a haemacytometer.

Tritiated thymidine incorporation of cell populated collagen matrices – BTCF, HRPE and BRCP populated collagen matrices at cell densities of $2x10^5$ cells per ml collagen were set up as described in method 2 above and placed in each of the "oxygen" incubators, control gels with no cells were also set up. On day 5, the test gels, were removed from their respective cell culture incubators, the media was discarded and then washed with PBSA. The test gels were then placed back into their incubators after the addition of 2ml of the appropriate growth medium containing tritiated thymidine (2μCi/ml for DMEM and 5μCi/ml for F10 due to F10's high thymidine content) per gel for eight hours. The gels were washed 3 times in PBSA (to thoroughly remove all excess thymidine) and incubated overnight in 200μl collagenase solution (appendix II) at 4°C. The cell / digested collagen suspension was transferred to a scintillation tube and inverted after the addition of 2.5ml of scintillation fluid (appendix II). Thymidine incorporation of the cells was measured by estimating β-radiation emission using a scintillation counter (appendix I). Gels with no cells were used as controls and were incubated with tritiated thymidine, washed and digested as described above. Each cell type was set up in quadruplicate in each oxygen environment, on at least three separate occasions.

4.2.6 Investigating the effect of growth factors and oxygen on cell mediated collagen contraction

The effect of 3 growth factors (TGFβ2, bFGF and VEGF) on cell mediated contraction in varying oxygen environments was assessed using the collagen contraction system. BTCF, HRPE cell and BRCP populated collagen matrices

were set up as described in method 2 above at a cell density of $2X10^5$ cells per ml collagen. After detaching the gels 2mls of growth medium supplemented 0.1ng/ml, 1ng/ml or 10ng/ml of TGFβ2, bFGF or VEGF was added and the gels placed in each incubator for a maximum of five days. Control gels with no growth factor were set up and placed in identical conditions for each cell type and growth factor.

BTCF and HRPE cell populated gels were incubated with all the growth factors; BRCP were incubated with bFGF or VEGF only.

The concentrations of the growth factors used in this study were based on the product information from the supplier and those cited in the literature (Raymond and Thompson, 1990; Hunt et al, 1994; Kurosaka et al, 1995; Smith-Thomas et al, 1996; Yoshida et al, 1996).

4.2.7 Investigating the effect of extracellular matrix components and oxygen on fibroblast mediated collagen gel contraction

In order to elucidate the effect of extracellular matrix components and oxygen on cell mediated contraction BTCF populated collagen matrices were set up at a cell density of $1x10^5$ cells per gel as described in method 2 above. Each gel was detached from the rim of the well and 2ml of growth medium supplemented with 10μg/ml fibronectin, 10μg/ml laminin or 0.5μg/ml vitronectin. These initial experiments used concentrations based on previous studies in the literature and took into account the high amount of these matrix components in serum which is required in this contraction system (Asaga et al, 1991; Smith-Thomas et al, 1996; Seiffert, 1997).

The fibronectin and vitronectin used in this study were plasma in origin to represent the increase in matrix products after a retinal break and dispersal of

cells (Nagasaki and Shingawa, 1995). The laminin isoform used for this study was EHS laminin (laminin-1), the most widely used for in vitro studies (Grant and Kleinman, 1997).

Test gels were incubated for a maximum of five days. Control gels identical to the test gels were set up with no medium supplements. Each experiment was set up in quadruplicate on at least 4 separate occasions.

4.2.8 Image Analysis and Statistical Data Collection.

Image analysis was carried out using the negatives of the whole gels from photographing the gels during and at the end of each experiment using a MOPs image analysis system. The final gel area was used to calculate a ratio of final gel area to original gel area (i.e., area of culture dish/well). In this way % original area and % decrease in area (or amount of contraction) was calculated. These ratios were used as the comparative values for analysis. The statistical tests carried out included Mann-Whitney-U and Kolmogorov-Smirnoff-D tests of variance and significance using Microsoft® Excel and Simfit statistics package.

4.2.9 Histology of contracted cell populated collagen gels

The contracted cell populated collagen gels were fixed in 10%NBF for at least 48 hours. Prior to processing for wax embedding, the gels were quartered to minimise shrinkage. The contracted collagen gel pieces were washed three times in PBSA. They were then dehydrated through a series of alcohol concentrations (v/v in distilled water) as follows. 10% for 10 minutes, 20% for 10 minutes, 35% for 10 minutes, 50% for 10 minutes, 60% for 10 minutes, 75% for 10 minutes, 90% for 20 minutes and 2 changes of 100% alcohol for 20 minutes each. The matrix pieces were the taken through a series of chloroform/alcohol (v/v) concentrations

as follows: 25% chloroform for 15 minutes, 33% chloroform for 10 minutes, 50% chloroform for 10 minutes and 2 changes of 100% chloroform for 20 minutes each. The matrix pieces were then embedded in wax for 20 minutes using a tissue embedding system. The wax blocks were trimmed and 7μm thick sections were cut using a microtome. The sections were placed into a water bath, allowed to flatten and transferred onto microscope slides. The slides were dried on a hot plate at 37°C for 3 to 4 hours, then placed in an oven at 56°C overnight for further drying and section adhesion. Heamatoxylin and eosin staining were as in section 2.2.1.

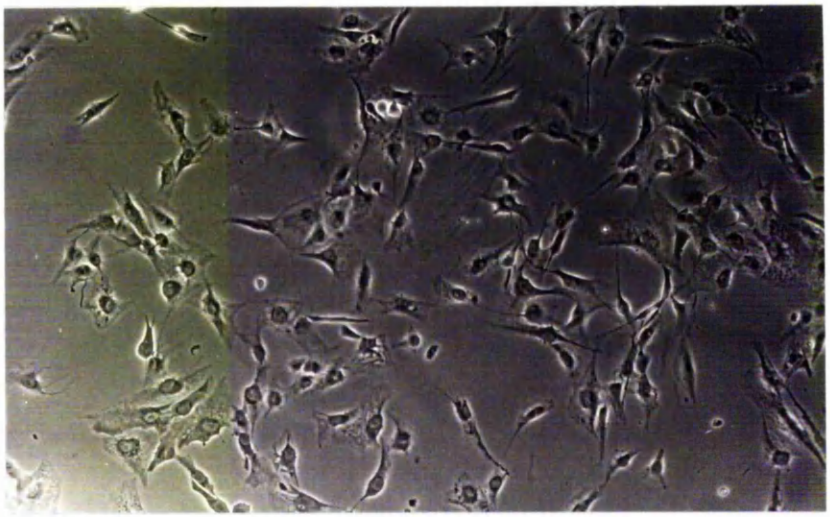
4.3 Results

4.3.1 Growth characteristics of cells in culture.

Bovine Tenon's capsule fibroblasts (BTCF) - outgrowth of fibroblasts occurred within 2-3 days following explant isolation and confluence was usually reached by 7 days (this was dependent on number of explants plated). The cells were regularly shaped with a dense nucleus at preconfluence (fig 4.1) but appeared more spindle-shaped and uniform at confluence. Cells were passaged at a ratio of 1:3 and confluence was reached within 5 days. Cultures were routinely passaged up to 12 times.

Human retinal pigment epithelial cells (HRPE's) - primary cells were isolated using the method described by Boulton and Marshall (1985). Cell attachment was largely dependent on donor age and varied between 5-10 days post plating. At confluence cultures consisted of a monolayer epithelial morphology cells which were hexagonal shaped. Cells were passaged at a ratio of 1:3 and confluence reached 7-12 days later. Purity of cultures was confirmed by positive cytokeratin 18 staining and could routinely be passaged at least 8 times. RPE cells in monolayer culture gradually changed morphology with passage, becoming more fibroblastic in appearance with the presence of stress fibres in later passages.

a.



b.

c.

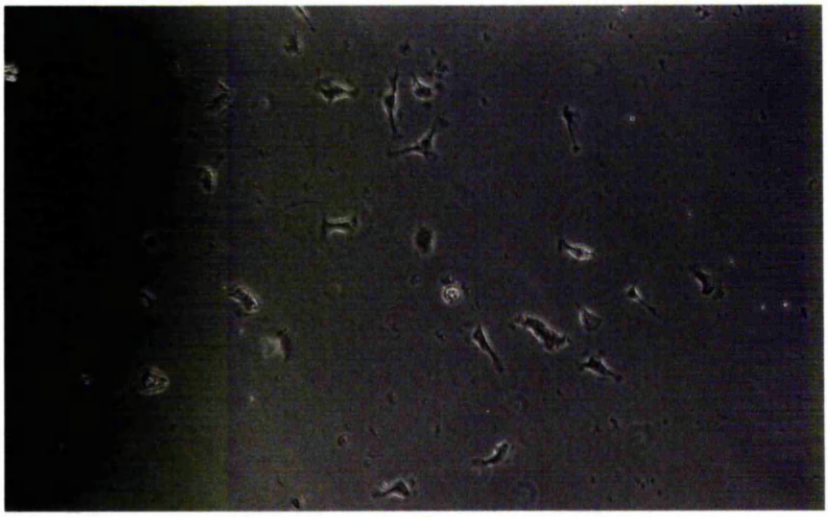
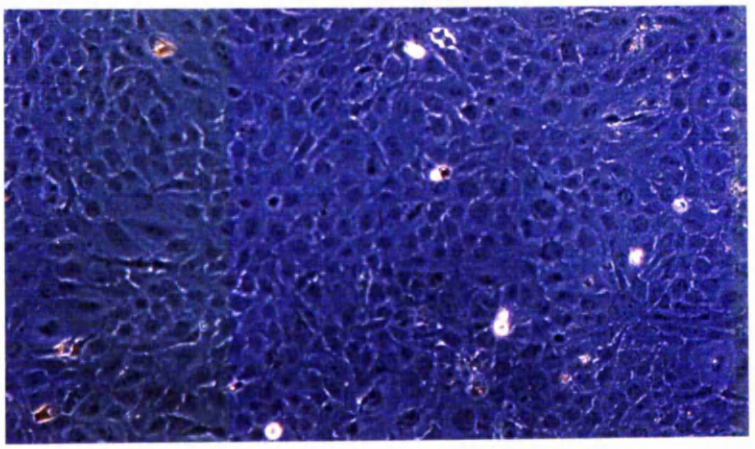
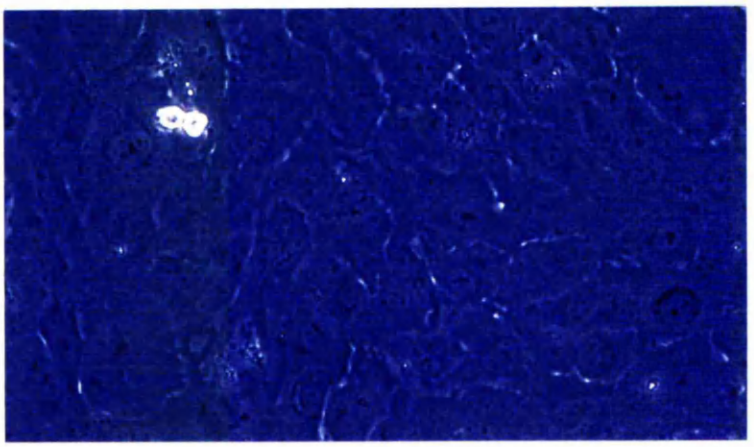


Figure 4.1 Photomicrographs of (a) P3 bovine Tenon's capsule fibroblasts at preconfluence, (b) P3 bovine retinal capillary pericytes displaying typical morphology of routinely cultured cells and (c) a rare primary culture of bovine retinal glial cells. Photomicrographs all same magnification (x100).

a.



b.



c.

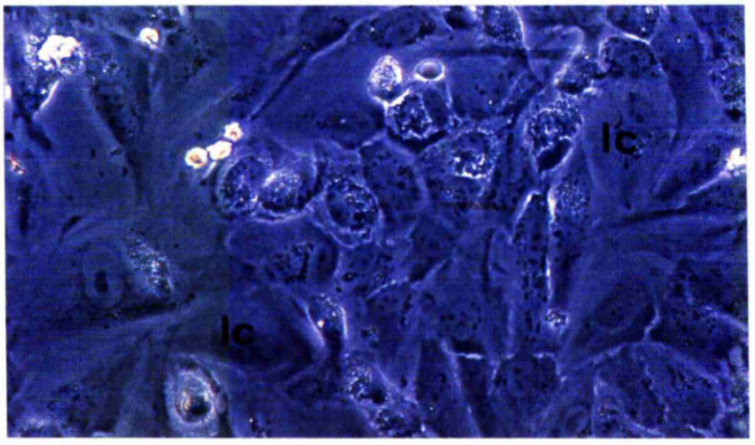


Figure 4.2 Photomicrographs of (a) P3 HRPE cells approaching confluence displaying a uniform cobblestone monolayer, (b) the same P3 HRPE cells at higher magnification and (c) P6 HRPE cells at confluence, the uniform monolayer is less evident and 'LC' indicates the large cells visible in these cultures. Magnification of (a) x100; (b) and (c) x320.

Bovine retinal capillary pericytes (BRCP) - two methods were used to isolate BRCP. The first required extensive pre-treatment of isolated vessels with a concentrated enzyme cocktail. A total of twenty retinas usually provided material for only 2 x 25cm^2 flasks. The isolated retinal vessel fragments attached to the flask within 3 days post plating and outgrowth was seen by 7 days. Cells exhibited an irregular shape and contained prominent stress fibres. Due to the limited attachment of the vessel fragments and poor growth, 14 - 20 days were required to reach confluence. Thereafter cultures were passaged and reached confluence by 14 days. The second method that required a less intensive enzyme pre-treatment of the isolated retinal vessels, resulted in a far greater yield of pericytes. 5 retinas generated 4 x 25cm^2 culture flasks of material. Attachment of vessel fragments was far greater than in the first method and outgrowth was observed within 3-4 days. Primary cultures reached confluence by 7-10 days and were thereafter passaged at a 1:2 ratio with confluence being reached by 5-7 days. The characteristics of the cells were the same as those observed in method 1.

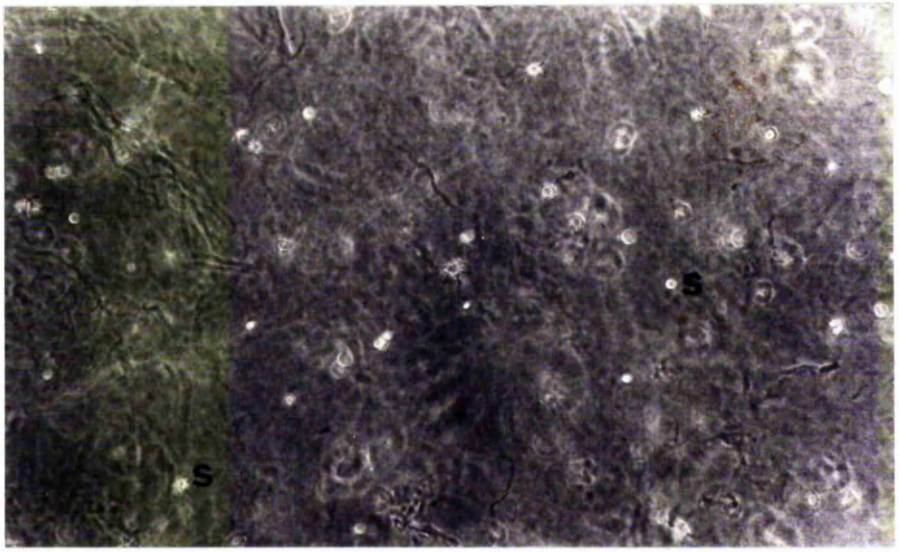
The morphology of the cells produced by either method was the same; cells were irregularly shaped and demonstrated obvious stress fibres . The cells became larger with each successive passage and even in the pre-confluent state, the cells were observed to form "heaped up" multi-layered "islands". These pericytes were α-smooth muscle actin positive, factor VIII and GFAP negative. These cells were passaged no more than 5 times, as the cells would begin to loose typical pericyte morphology and characteristics. Due to the increased yield and less laborious isolation procedure observed for method two, this method was chosen for the isolation of pericytes for all subsequent studies.

Bovine retinal glial cells (BRG'S) - were cultured using the methods described by Savage et al 1988. Very little or no yield was obtained by either method, although in some isolations some cells did appear to attach they did not increase in number (fig 4.1). The method was modified in several ways including, using collagen coated dishes and enzyme digestion steps and cells obtained were GFAP and Carbonic anhydrase II negative but α smooth muscle actin positive.

4.3.2 Cell morphology and distribution within the collagen gel.

Upon polymerisation of the gel the cells would appear evenly distributed and spherical as if in suspension. Within one hour pseudopodia were visible in the surrounding matrix (fig. 4.3). All cell types established numerous pseudopodia protruding into the matrix in all directions by 6-8 hours (fig 4.3). However, after 36-48 hours RPE cells and fibroblasts would become elongated and more spindle shaped, only pericytes retained the multi-pseuodopod morphology. As the gel reduced in size, there were visible areas of collagen aggregation. Within the next 36 hours networks of cells would appear where cell-cell contacts were apparent (fig 4.4). Most cells would be seen to have contacts with several surrounding cells in all directions. It was difficult to say whether contact was made with actual cellular structure or whether via collagen fibres. Once most cells had formed networks, the majority of cells could be seen at the edge of the gel that would as a result become thicker and darker. When histological sections were viewed layers of cells could be seen that were tightly packed on the outer surface of the contracted gel (fig 4.4). Control collagen gels set up with no cells did not produce a contractile response after the same length of time, although after 7 to 10 days the matrix would reduce in size, possibly due to the degradation of the collagen.

a.



b.

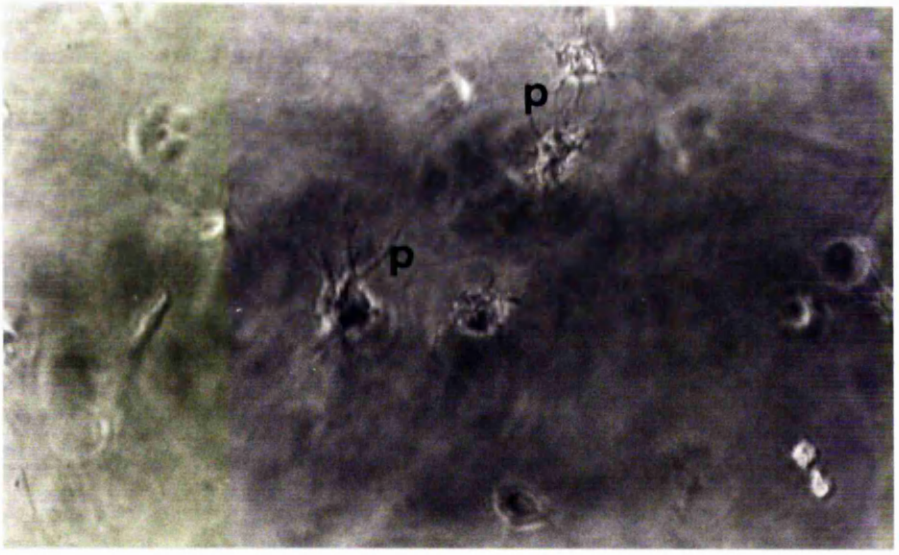
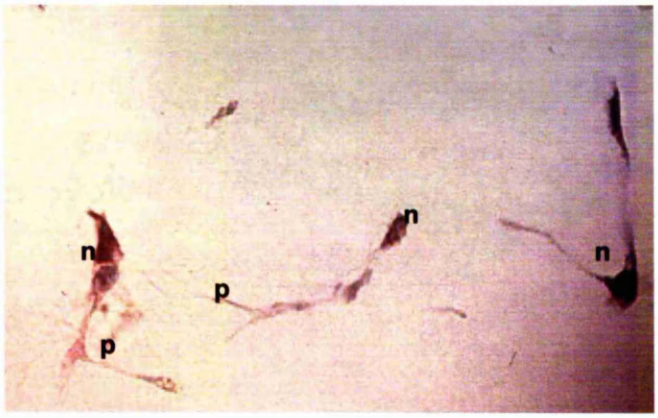


Figure 4.3 Photomicrographs of (a) fibroblasts after 1 hour within a polymerising collagen matrix. 'S' indicates spherical cells. 'P' indicates the processes already visible from a number of cells and (b) HRPE cells 6 hours after placing within a collagen gel matrix. The cells are losing their spherical morphology and processes can be seen extending in all directions into the surrounding collagen matrix. Magnification of (a) is x100 and (b) is x320.

a.



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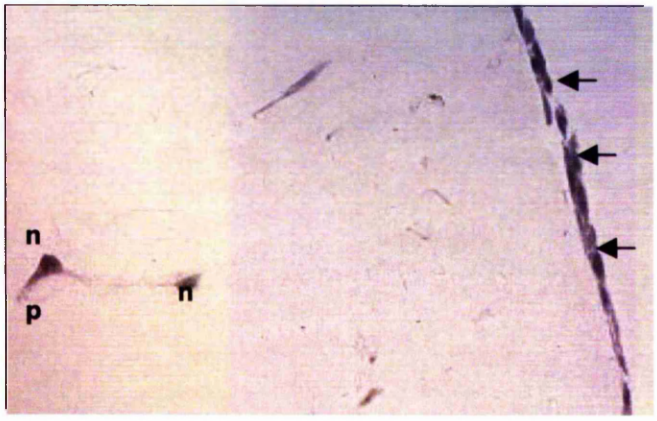


Figure 4.4 Photomicrographs of haematoxylin and eosin stained wax sections of fibroblast populated collagen matrices. (a) Cell processes can be seen contacting neighbouring cells from several cells, 'n' indicates the cell nucleus and 'p' indicates the cell processes. Processes from cells in underlying layers are also visible. (b) After 5 days in culture, cells are visible at the edge of the gel forming a monolayer of tightly packed cells, indicated by arrows. Photomicrographs all same magnification (x400).

4.3.3 The effect of cell type and cell density on collagen gel contraction

Cell type - each cell type tested displayed a time dependent increase in contraction. Fig 4.5 shows an example of the data produced, where BRCP' s produced the most efficient contractile response ($p < 0.05$), reducing the gel to almost 50% of its original area within 24hr. BTCF' s were observed to be more contractile than HRPE cells although this difference was not found to be statistically significant. The time taken for any cell type to reduce a gel to 50% of its original area varied between collagen batches, but the same trend was always observed, with pericytes taking the least time, fibroblasts being intermediate and RPE cells requiring the most time.

Passage number - HRPE cells displayed a unique characteristic in that the extent of the contractile response was dependent on the cell morphology displayed in monolayer culture, which was generally equivalent to passage number. Such that, low passage HRPE cells with epithelial morphology were significantly less contractile ($p<0.05$) than higher passage 'fibroblast-like' HRPE cells over the same period of time as represented by fig 4.6. Furthermore RPE cells which were visibly-stressed (usually higher passage and/or seeded at low density in monolayer culture) were significantly less contractile ($p<0.01$) than 'fibroblast-like' RPE cells. This trend was observed whether cells were used from the same primary isolation or different isolations and was reproduced in different collagen batches. It should be noted, however, cultures of RPE cells used for most contraction studies contained a mixture of epithelial and 'fibroblast-like' cells, since pure cultures of fibroblast-like cells were difficult to maintain and would generally become visibly stressed (i.e., demonstrate obvious cytoplasmic stress fibres) within 48-72 hours. All cells used were cytokeratin 18 positive and morphology of cultures were designated blindly by 2 independent observers.

Passage number did not affect fibroblast contraction and cultures of between P3 and P12 were used.

Pericyte cultures between passage 2 and 4 were used since later passages would contain cells that had lost pericyte characteristics and were no longer comparable.

Cell density - Cell densities of between $1x10^4$ and $4x10^5$ per ml collagen were used for all cell types. Fig. 4.7 displays the combined data of several cell density studies, again the same shaped curve was produced for all collagen batches however there was some variance with the initial cell density required to produce a contractile response. On average a cell density of between $1x10^4$ and $5x10^4$ per ml collagen was enough to induce a contractile response in pericytes which was significantly less ($p<0.05$) than that required for RPE cells and fibroblasts. RPE cells and fibroblasts produced a negligible response until cell densities of $7\text{-}8x10^4$ per ml collagen were reached. At higher cell densities of approximately $2x10^5$ cells per ml collagen, pericytes produced a 90% decrease in area of the gel, significantly more than RPE cells and fibroblasts that produced only a 65% and 70% decrease in gel area respectively. Furthermore, a doubling of cell density to $4x10^5$ cells per ml collagen did not increase the contractile response significantly, i.e., the gel had become saturated.

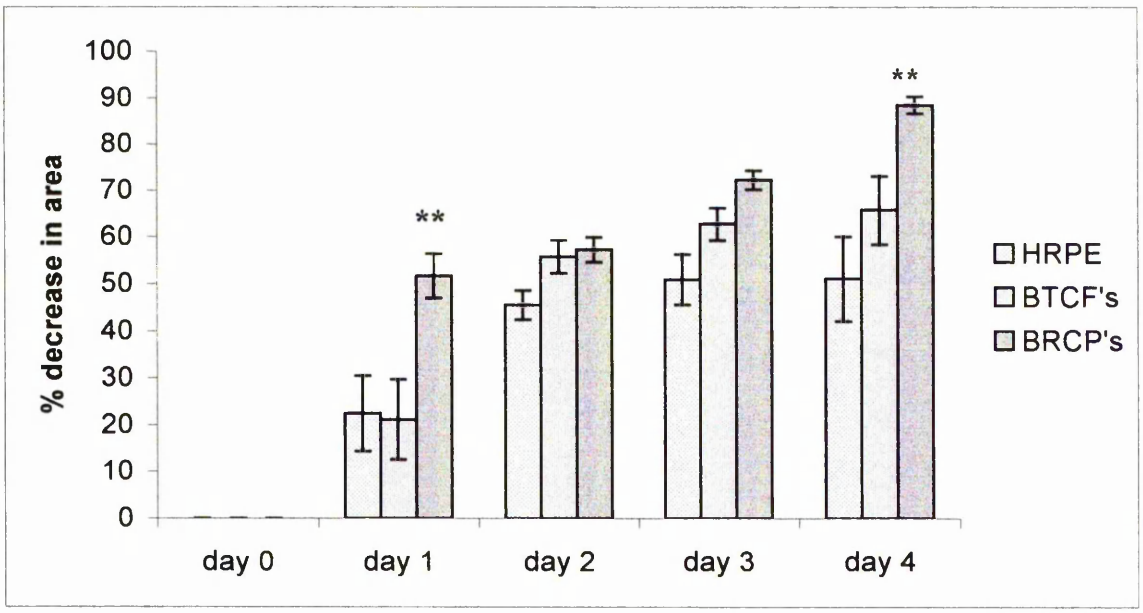


Figure 4.5.The histogram demonstrates a typical example of the data obtained for P6 HRPE, P6 BTCF and P3 BRCP (all seeded at $1x10^5$ cells/ml collagen) over 4 days. The vertical axis represents the % decrease in area of a cell-populated matrix and hence is used to represent contraction. Each bar represents the statistical mean ± standard deviation, n=4 (indicates BRCP were significantly more contractile at these time points, $p<0.05$).**

Fig. 4.6a

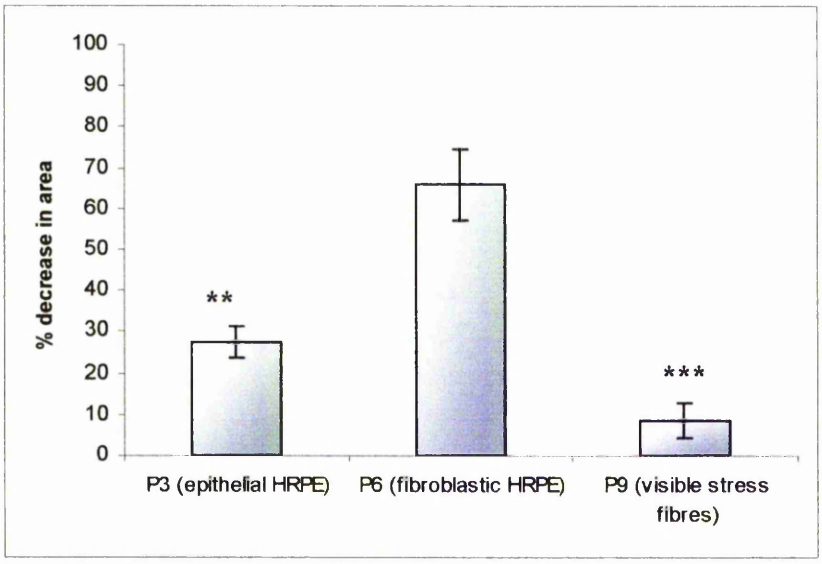


Fig. 4.6b

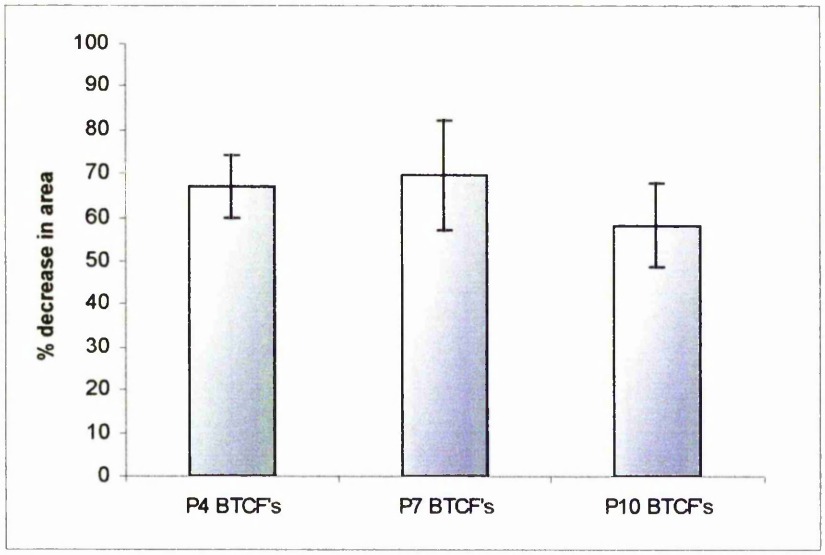


Fig. 4.6c

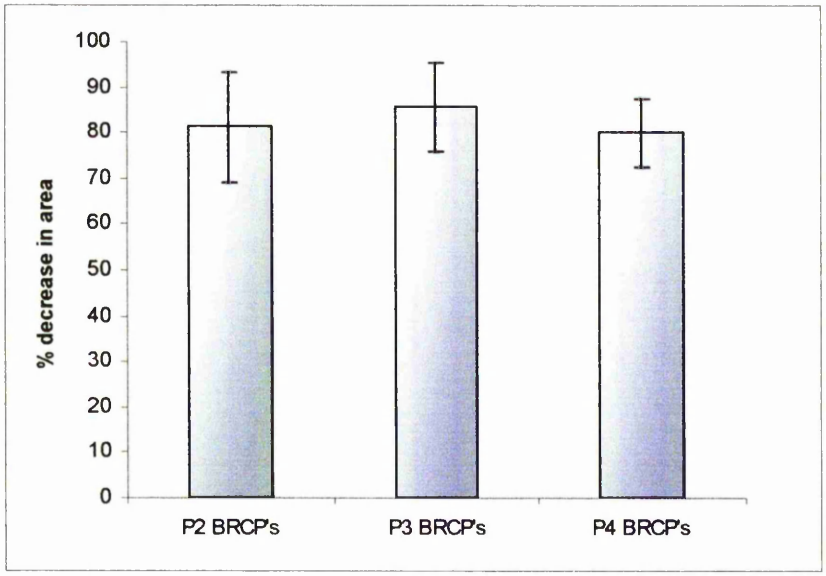
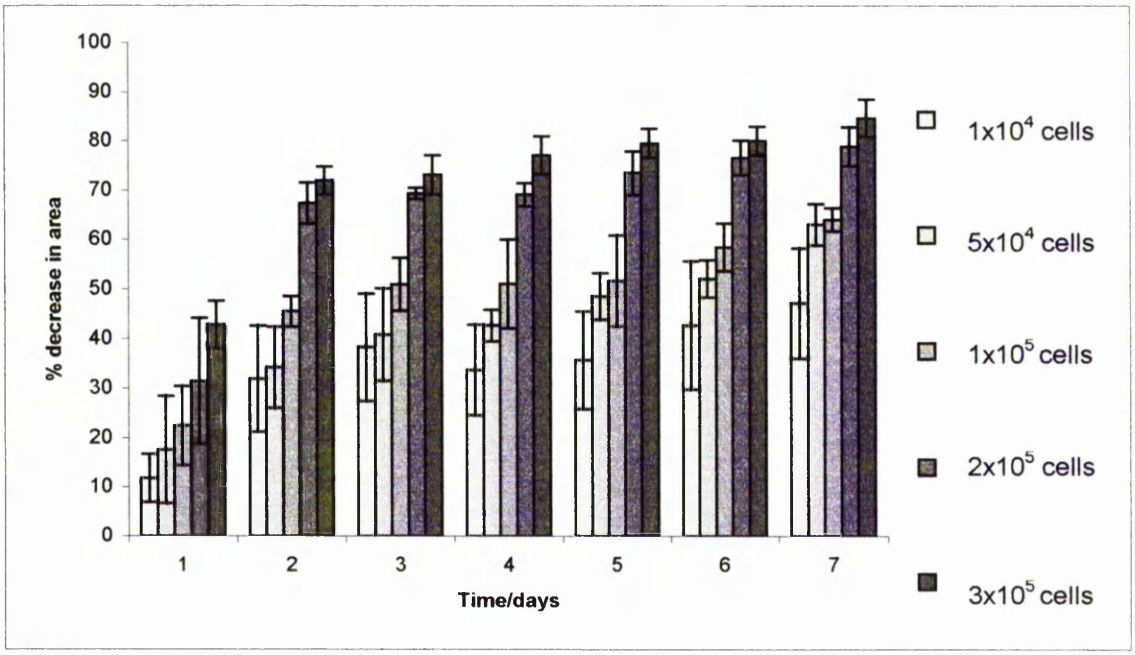


Figure 4.6 Histograms displaying examples of typical contraction data obtained for different passage (a) HRPE cells, (b) BTCF and (c) BRCP. The x-axis represents passage number. The y-axis represents % decrease in area of a cell-populated matrix (i.e., contraction). Each bar represents the statistical mean ± standard deviation, n=10. (, *** indicates low and high passage HRPE cells displaying significantly less contraction than higher passage cells, $p<0.05$ and $p<0.01$ respectively).**

Fig. 4.7 (a)


100
90
80
70
60
50
40
30
20
10
0
% decrease in area
1
2
3
4
5
6
7
Time/days
1x10^4 cells
5x10^4 cells
1x10^5 cells
2x10^5 cells
3x10^5 cells

(b)

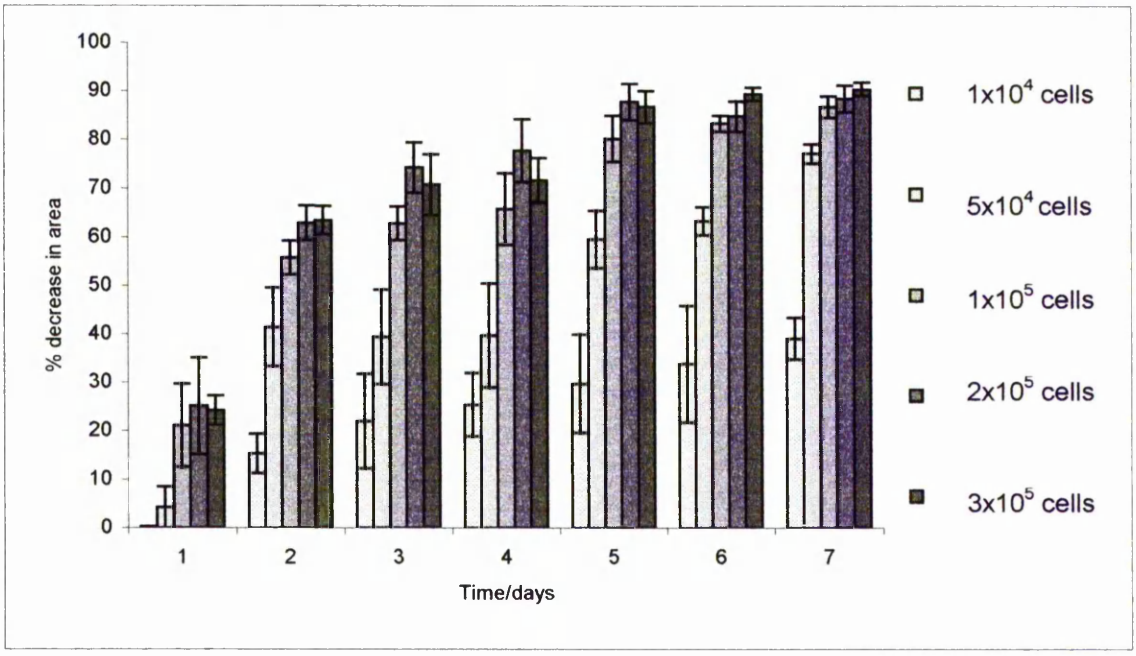

100
90
80
70
60
50
40
30
20
10
0
% decrease in area
1
2
3
4
5
6
7
Time/days
1x10^4 cells
5x10^4 cells
1x10^5 cells
2x10^5 cells
3x10^5 cells

Fig. 4.7(c)

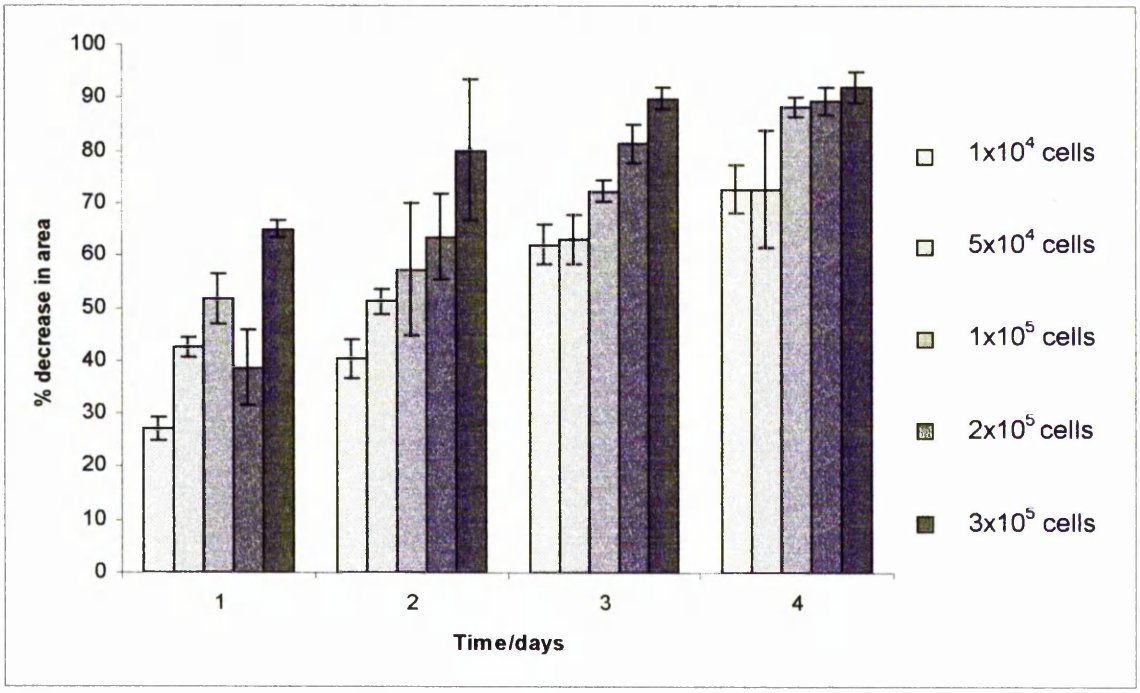
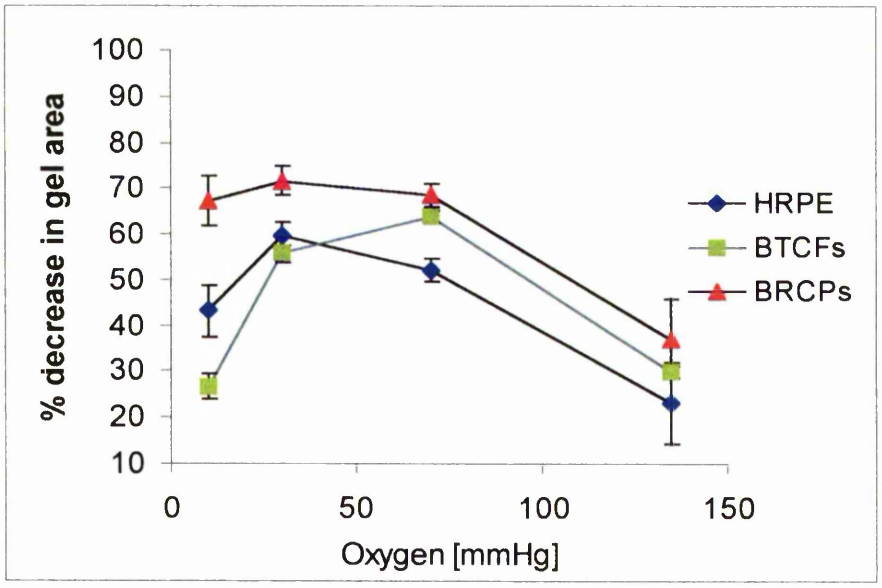


Figure 4.7 Histograms demonstrating typical data obtained for cell density studies for (a)P6 HRPE cells, (b)P6 BTCF and (c)P3 BRCP over 7 and 4 days. The x-axis represents time in days and the y-axis represents contraction. Each bar represents the statistical mean ± standard deviation, n = 4.

4.3.4 The effect of varying the oxygen environment on collagen gel contraction

All cell types tested exhibited a similar contractile response to oxygen: contraction was greatest at a concentration of 30 to 70mm Hg oxygen and lowest at 135 and 10mm Hg oxygen. This trend was observed in all experiments irrespective of cell line or collagen batch (fig 4.8a). The overall contractility varied by 10-15% between experiments. To overcome minor differences between experiments, particularly at the extremes of oxygen concentration the data of 5 experiments were pooled (fig. 4.8b). The combined data demonstrates that contraction was greatest 30-70mmHg for HRPE cells and BRCP ($p<0.05$), while for fibroblasts maximal contraction was apparent over a narrower range focusing at 70mmHg ($p<0.01$). Fibroblast mediated contraction was significantly lower in an oxygen environment of 10mmHg when compared to contraction in all other oxygen concentrations ($p<0.01$). In contrast HRPE and BRCP mediated contraction tended to be lower in an environment of 135mmHg oxygen. The data produced also confirmed that pericytes were the most contractile cell type being more contractile than either BTCF or RPE cells at all oxygen concentrations tested ($p<0.01$).

(a)



(b)

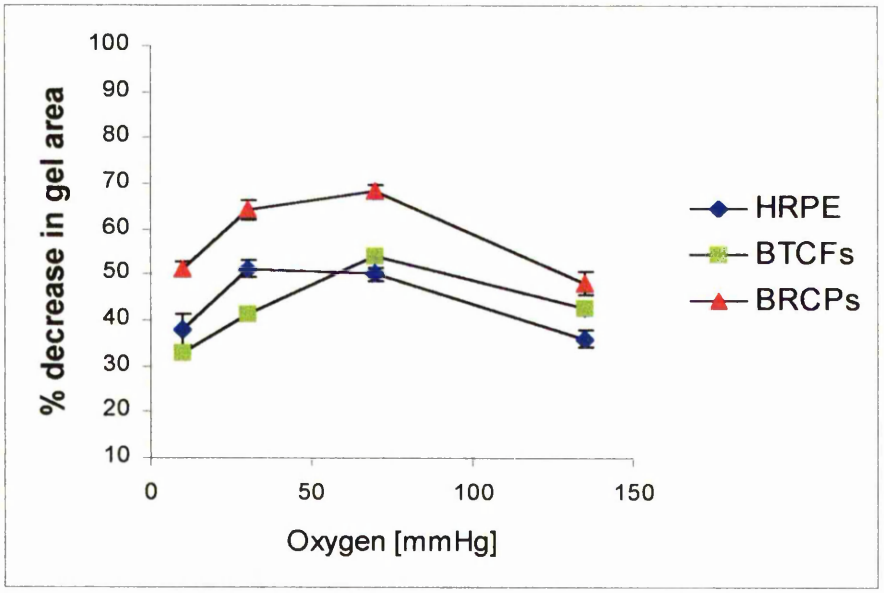


Figure 4.8 Graphical representation of the contraction observed for HRPE cells, BTCF and BRCP in varied oxygen environments. The y-axis represents contraction and the x-axis represents increasing oxygen concentration. (a) Is the data obtained from a typical experiment where n=6 for each point and (b) is the pooled data of five different experiments where n=30. Each point represents the statistical mean ± standard deviation. Both graphs highlight the different contractile profile observed of the cell types tested in each of the oxygen environments.

4.3.5 The effect of varying oxygen environment on different passage HRPE cells

Low passage HRPE cells displaying epithelial morphology produced a different curve to HRPE cells used in experiments above as well as compared to BTCF and BRCP. Contraction was greater in an oxygen environment of 10mmHg ($p<0.05$) than at 30mmHg or 70mmHg. This was in contrast to the curve produced by higher passage, 'fibroblast - like' HRPE cells which showed significantly more contraction between 30mmHg and 70mmHg oxygen than in other oxygen concentrations (fig. 4.9).

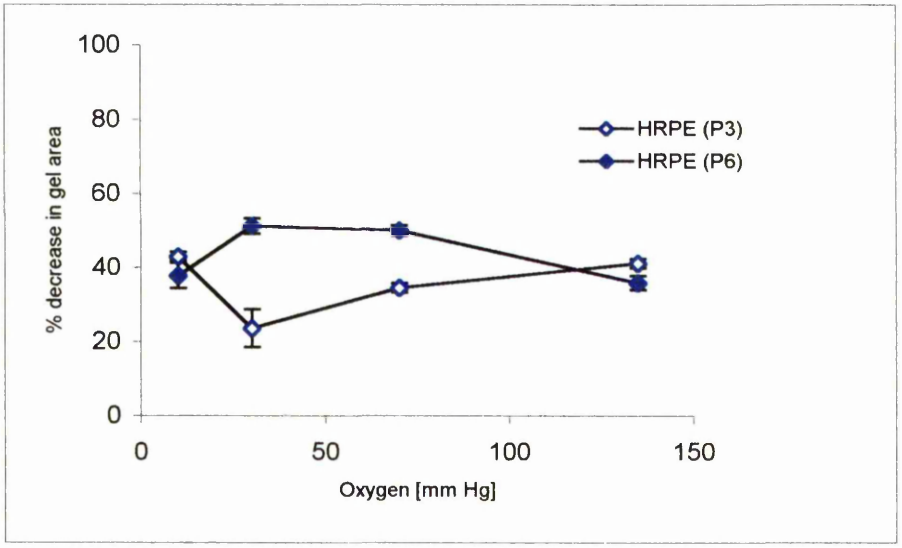


Figure 4.9 Graphical representation of the contraction observed for different passage (and morphology) HRPE cells in varied oxygen environments. The y-axis represents contraction and the x-axis displays oxygen concentration. Each point represents the statistical mean ± standard deviation, n=12.

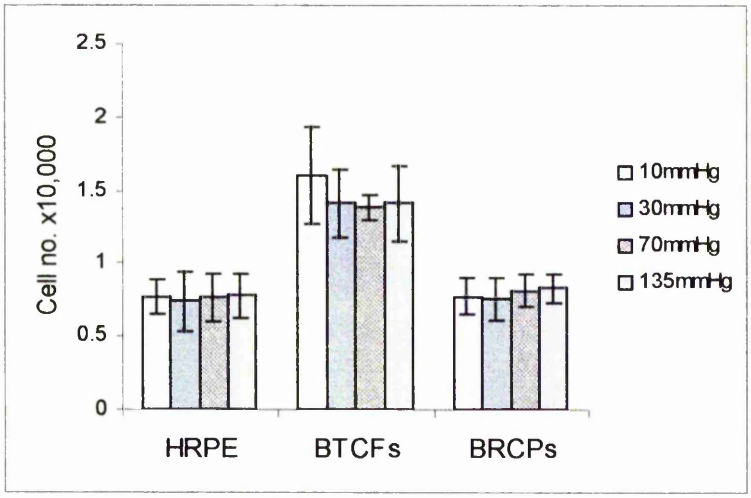
4.3.6 Determination of cell numbers and proliferation within cell populated collagen matrices

Cell number determination - cell numbers within collagen gels were determined using an enzyme digestion technique to confirm that the increase in contraction in the different oxygen environments was not a result of an increase in cell numbers. Collagenase and a combination of collagenase and pronase followed by trypsination of remaining cells were tried to improve yield and integrity of retrieved cells. The technique was associated with several problems in that, although cells were retrieved a lot clumping of cells and collagen fibres were observed. The technique was also prone to over digestion with a subsequent loss of cells (up to a 50% loss of cells).

The cell 'clumping' problem was overcome using a longer digestion period and by counting cells on a haemacytometer as opposed to a Coulter counter. The haemacytometer enabled a higher degree of accuracy to be achieved since clumps were visible and could be accounted for, which was not possible when a Coulter counter was used. Furthermore, the digestion was improved using a lower concentration of collagenase at a much lower temperature (4°C) overnight. The modified technique allowed cell loss to be reduced to less than 25% in all subsequent studies.

Cell numbers were determined for all cell types over a time course in standard oxygen conditions and no increase in cell numbers was observed. Cell numbers were determined after the gels were placed in the different oxygen environments and no significant differences were found between the cell numbers in the gels contracting in any of the oxygen environments (fig. 4.10).

(a).



(b)

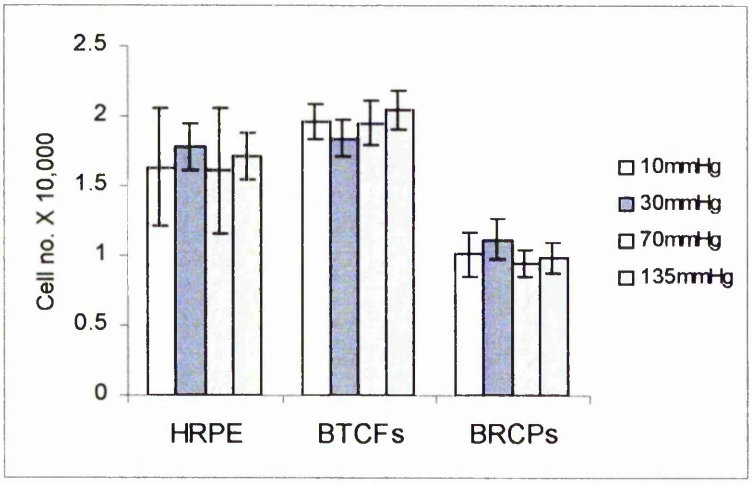
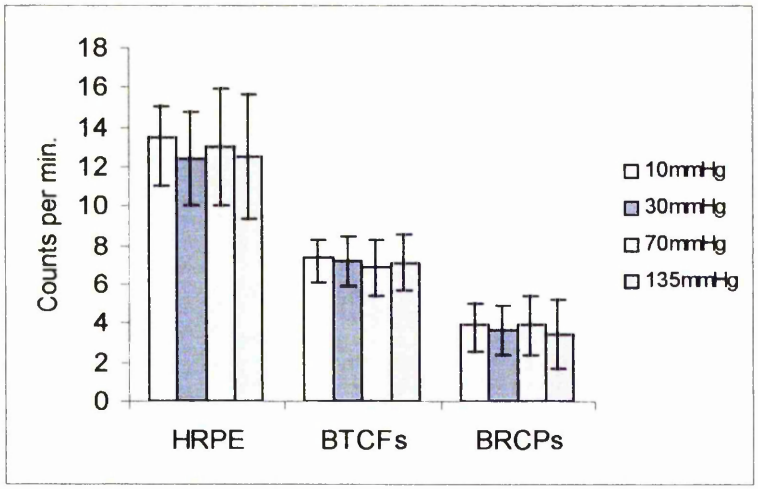


Figure 4.10 Histograms displaying two examples of the data obtained from cell determination studies for HRPE cells, BTCF and BRCP in varied oxygen environments. The y-axis represents cell number (x10,000) and the x-axis displays the oxygen concentrations for each cell type studied. Each bar represents the statistical mean ± standard deviation. Cell numbers at day 0 for (a) were $1x10^5$ cells/ml collagen for BRCP and HRPE populated matrices and $1.5x10^5$ cells/ml collagen for BTCF populated matrices, n=4. Cell numbers at day 0 for (b) were $2x10^5$ cells/ml collagen for HRPE and BTCF populated matrices and $1x10^5$ cells/ml collagen for BRCP populated matrices, n=5. These graphs highlight the observation that no increase in cell numbers was observed for any cell types in any of the oxygen environments tested.

(a)



(b)

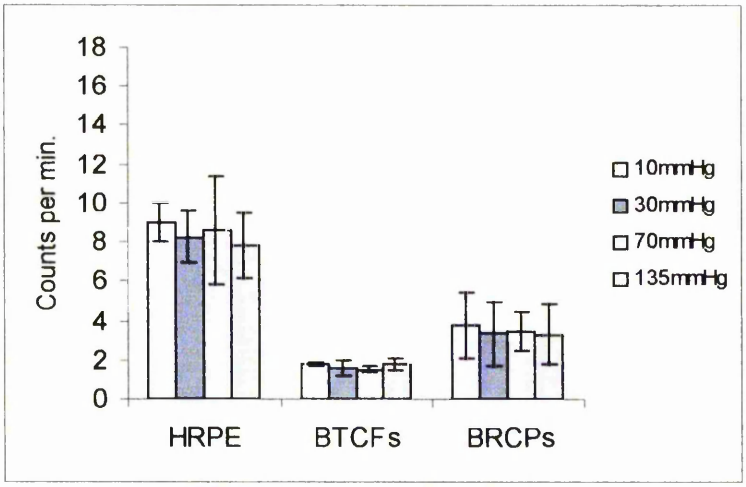


Figure 4.11 Histograms displaying the data obtained when tritiated thymidine incorporation was used as an indication of cell proliferation within cell populated matrices after 5 days incubation in varying oxygen environments. (a) HRPE cells, BTCF and BRCP were seeded at a cell density of $2x10^5$ cells/ml collagen and for (b) HRPE cells, BTCF and BRCP were seeded at a cell density of $1x10^5$ cells/ml collagen. Each bar represents the statistical mean ± standard deviation, n=4. The histograms highlight the observation that no increase in thymidine incorporation was observed when comparing all oxygen environments. It should be noted that HRPE counts appear to be higher than BTCF and BRCP cell counts. This was due to a high concentration of radioactive thymidine placed in the growth medium for incubation (to compensate for the thymidine rich growth medium required by HRPE cells) and does not correspond to a higher degree of thymidine incorporation.

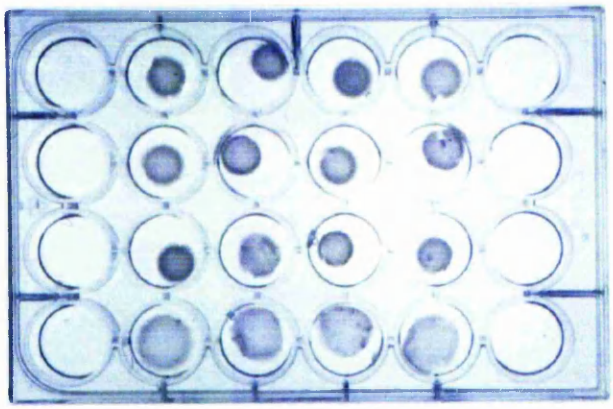
Detection of cell proliferation within cell populated collagen gels - tritiated thymidine incorporation was used as an indication of proliferation within collagen matrices to confirm the results obtained from the determination of cell numbers. This study showed no significant difference in the amount thymidine incorporated between the cell-populated gels in the different oxygen environments and the control gels containing no cells in the different oxygen environments. There was a slight increase in incorporation above the control observed in gels incubated in a 10mmHg oxygen environment, however this was not found to be significant (see fig. 4.11).

4.3.7 The effect of growth factors and oxygen on cell mediated contraction

The effect of TGFβ2, bFGF or VEGF and oxygen on fibroblast mediated collagen gel contraction - the addition of TGFβ2 increased the amount of contraction significantly in all oxygen environments, ($p<0.05$) compared to fibroblast containing gels with no growth factor added. The contractile response was greatest in environments of 30mmHg and 70mmHg oxygen ($p<0.01$) where the amount of contraction doubled after the addition of 10ng/ml TGFβ2 (fig 4.12). The contractile response produced by the addition of bFGF was subtler, in that the amount of contraction produced was only significantly greater than that of the control in an environment of 30mmHg ($p<0.05$). The same basic trend was observed for each growth factor (where TGFβ2 shifted the curve higher up at all oxygen concentrations and bFGF shifted the curve higher at the lower oxygen concentrations) when repeated on 3 separate occasions and all the data pooled to produce graph in (fig 4.13).

VEGF did not affect fibroblast mediated collagen contraction, such that no significant contraction was observed above that of the control (fig. 4.14).

(a) 10ng/ml TGFβ2.



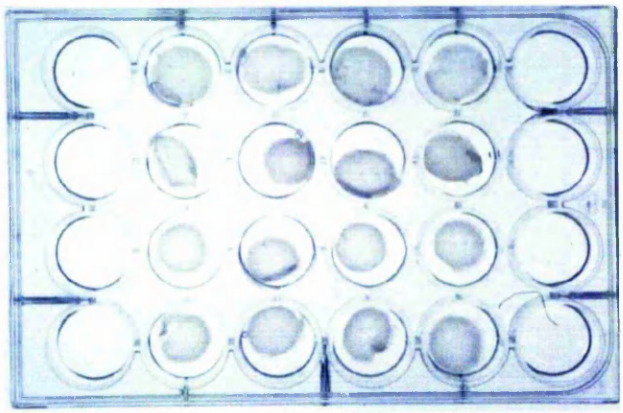
10 mm Hg

30 mm Hg

70 mm Hg

135 mm Hg

(b) 10 ng/ml bFGF



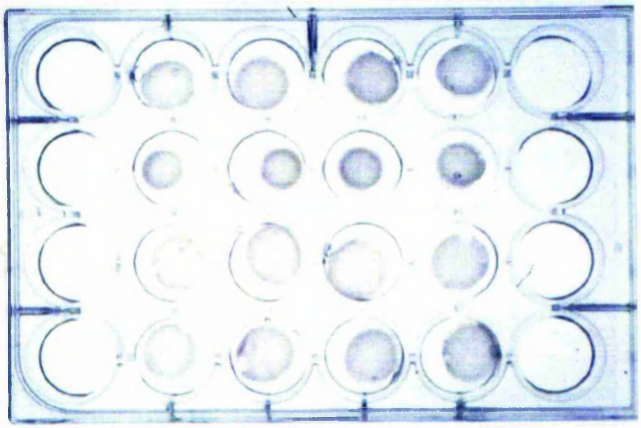
10 mm Hg

30 mm Hg

70 mm Hg

135 mm Hg

(c) control



10 mm Hg

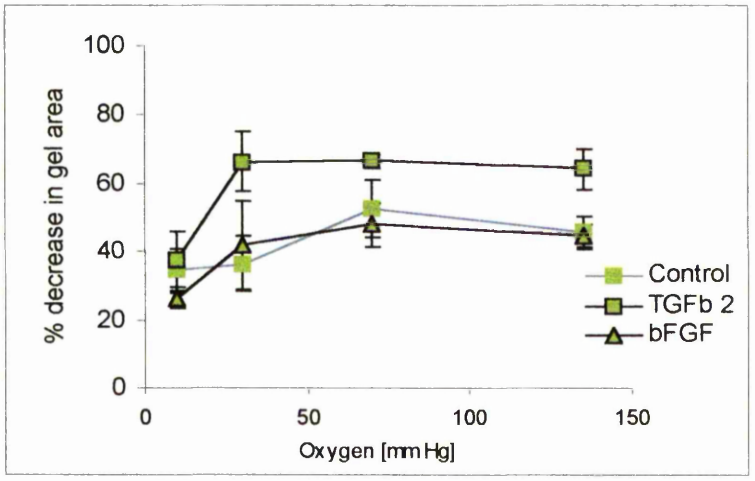
30 mm Hg

70 mm Hg

135 mm Hg

Figure 4.12 Photographs of a typical contraction experiment shows fibroblast- populated matrices at day 5 after the addition of (a) 10ng/ml TGFβ2 or (b) 10ng/ml bFGF in comparison to (c) the control. All matrices were seeded with $1x10^6$ cells/ml collagen.

4.13 (a)



(b)

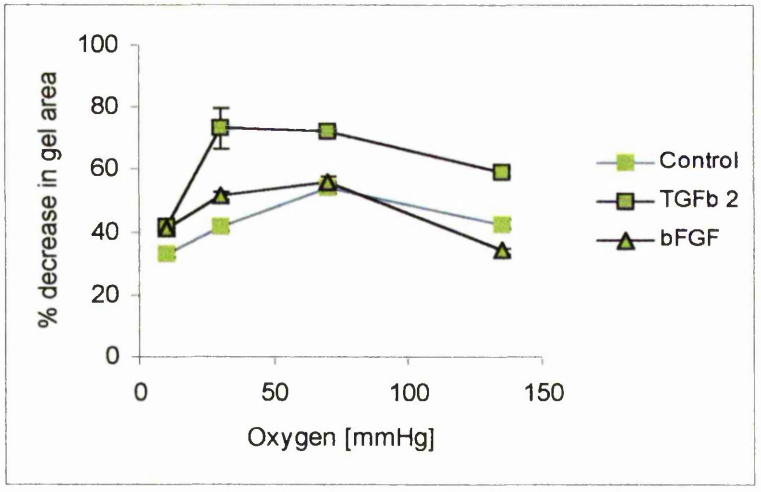
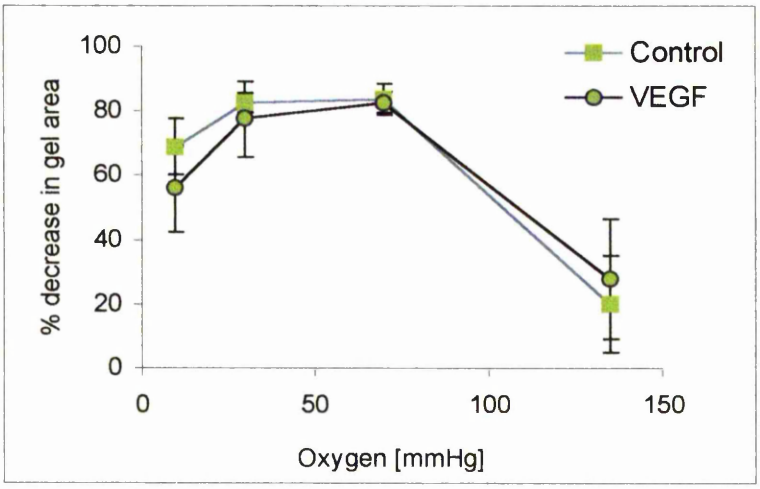


Figure 4.13 (a) graphical representation of a typical fibroblast mediated contractile response after the addition of 10ng/ml TGFβ2 or bFGF. Control gels represent fibroblast populated gels with no growth factors, n=4. (b) Represents pooled data (n=12) of fibroblast mediated contraction after the addition of TGFβ2 or bFGF after 5 days in varying oxygen environments. Each point is the statistical mean ± standard deviation. These graphs highlight the observation that TGFβ2 increased fibroblast mediated collagen gel contraction in all oxygen environments tested.

4.14(a)



(b)

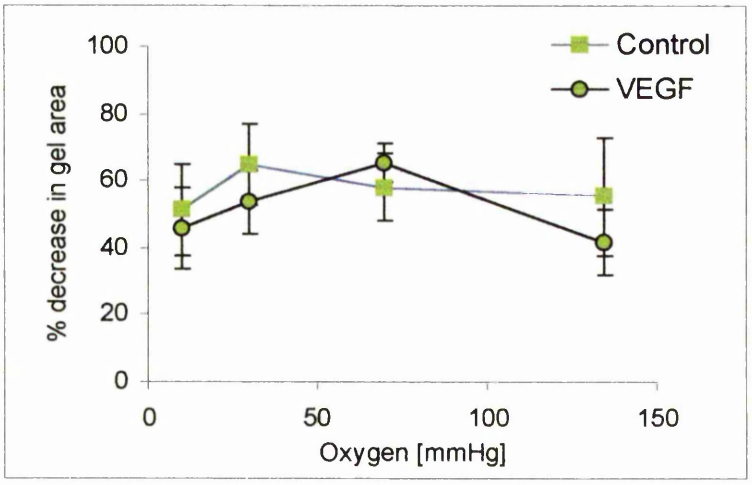


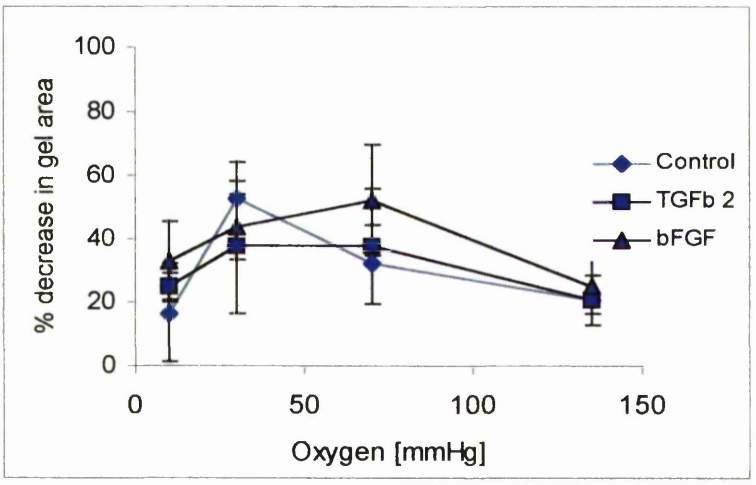
Figure 4.14 (a) is a graphical representation of the typical data obtained for fibroblast mediated collagen gel contraction after the addition of 10ng/ml VEGF in varying oxygen environments, n=4. (b) Is the pooled data of three experiments in the same conditions, (n=12). Each point is the statistical mean ± standard deviation. Both graphs demonstrate the observation that VEGF had no effect on fibroblast mediated contraction in any oxygen environment tested.

The effect of TGFβ2, bFGF or VEGF and oxygen on HRPE cell mediated collagen gel contraction - The addition of TGFβ2 to HRPE cell populated collagen matrices displayed no difference in the amount contraction at the lower oxygen concentrations in comparison to the control. However there was a decrease (or inhibition) of contraction after the addition of TGFβ2 at higher oxygen concentrations, in comparison to the control, but this decrease was not statistically significant (fig. 4.15). The addition of bFGF however, caused a change in the contractile profile at the lower end of the oxygen concentrations. The degree of contraction was significantly greater than that of the control in 10mmHg oxygen ($p<0.05$) but significantly less than that of the control of the control in 30mmHg oxygen ($p<0.05$). VEGF supplemented into the growth medium did not affect the contractile response of HRPE cells. There was no significant difference in the amount of contraction observed between the test and control gels (fig 4.16).

The effect of bFGF or VEGF and oxygen on pericyte mediated collagen gel contraction - With the exception of 10mmHg, bFGF at either 1 or 10ng/ml did not alter contraction compared to control (fig. 4.17). However at 10mmHg gel contraction was 20% greater than the control at both bFGF concentrations. Thus the greatest increase in the amount of contraction above the control was in an environment of 10mmHg after the addition of bFGF ($p< 0.05$).

The addition of VEGF on pericyte mediated gel contraction resulted in an oxygen dependent decrease in contraction. The addition of both 1 or 10ng/ml VEGF caused a 25-30% decrease in the amount of contraction observed at all oxygen environments with exception of 10mmHg. Thus significantly less contraction ($p<0.05$) was observed between 30 and 70mmHg after the addition of VEGF.

4.15 (a)



(b)

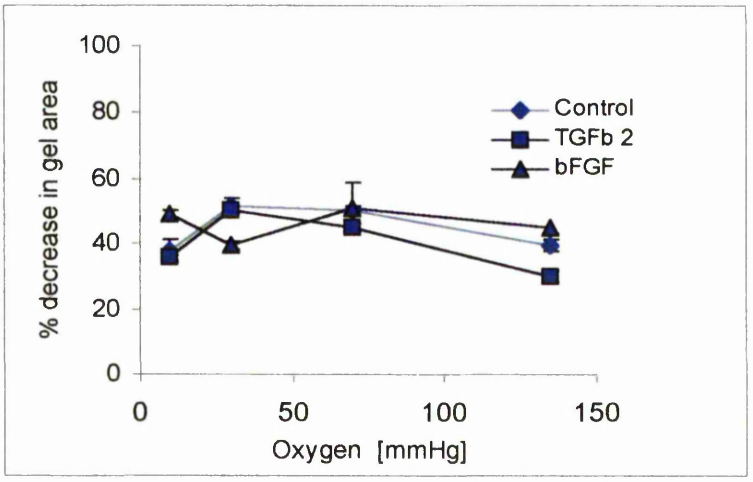
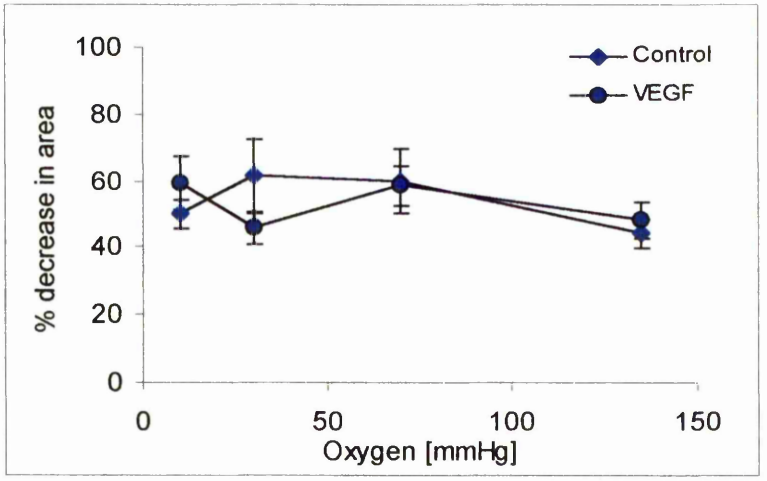


Figure 4.15 (a) graphical representation of a typical HRPE cell mediated contractile response after the addition of 10ng/ml TGFβ2 or bFGF. Control gels represent HRPE cell populated gels with no growth factors, n=4. (b) Represents pooled data (n=12) of HRPE cell mediated contraction after the addition of TGFβ2 or bFGF after 5 days in varying oxygen environments. Each point is the statistical mean ± standard deviation.

4.16 (a)



(b)

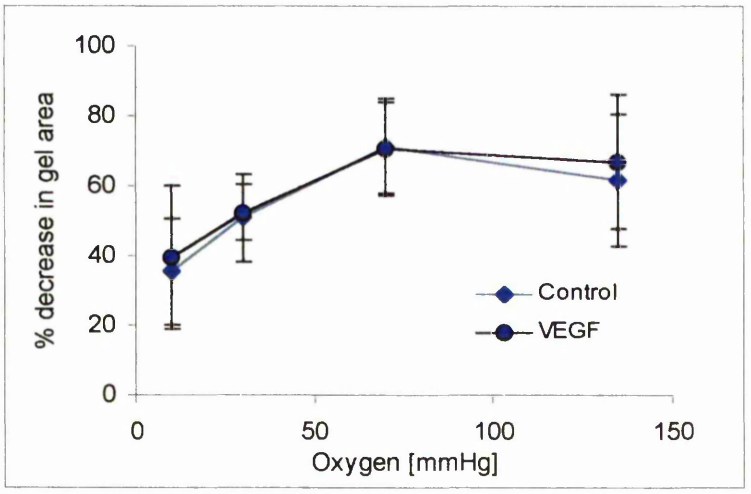
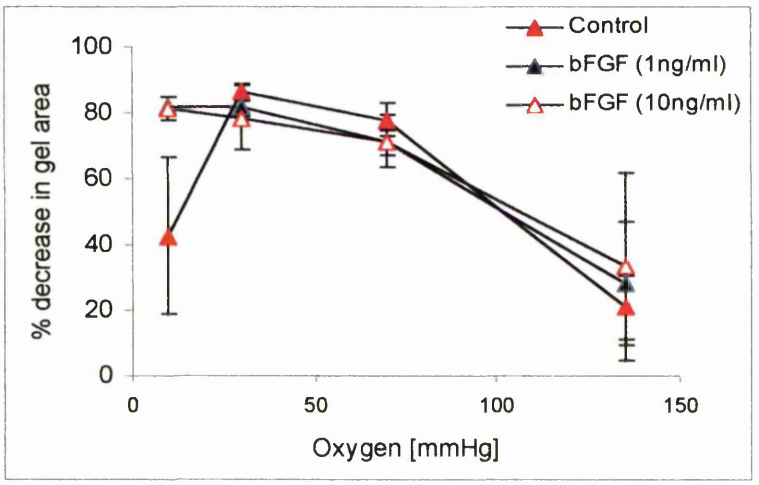


Figure 4.16 (a) is a graphical representation of the typical data obtained for HRPE cell mediated collagen gel contraction after the addition of 10ng/ml VEGF in varying oxygen environments, n=4. (b) Is the pooled data of three experiments in the same conditions, (n=12). Each point is the statistical mean ± standard deviation. Both graphs demonstrate the observation that VEGF had no effect on HRPE cell mediated contraction in any oxygen environment tested.

Fig. 4.17(a)



(b)

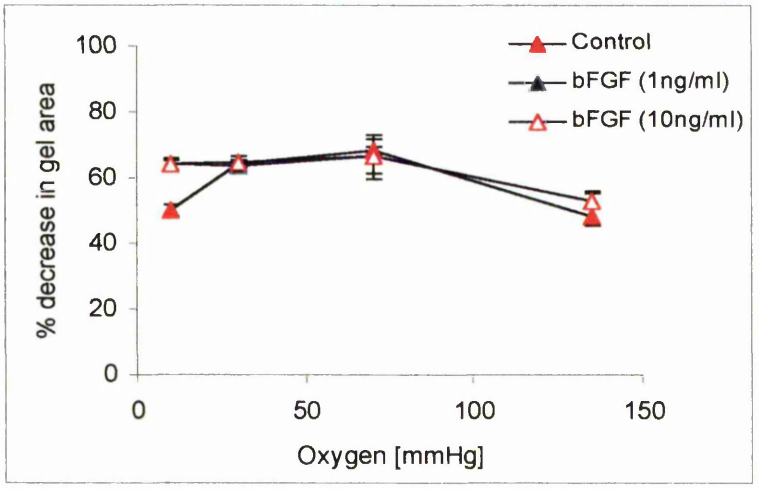
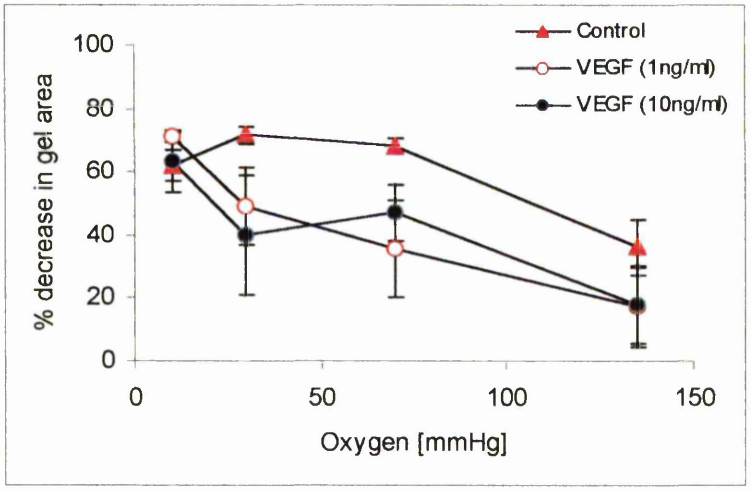


Figure 4.17 (a) is a graphical representation of the typical data obtained for pericyte mediated cell contraction in the presence of 1 or 10ng/ml bFGF in varying oxygen environments; n=4. (b) Is the pooled data of three experiments in the same conditions; n=15. Each point represents the statistical mean ± standard deviation. Both graphs highlight the observation that bFGF increased pericyte mediated contraction in an oxygen environment of 10 mm Hg.

Fig. 4.18 (a)



(b)

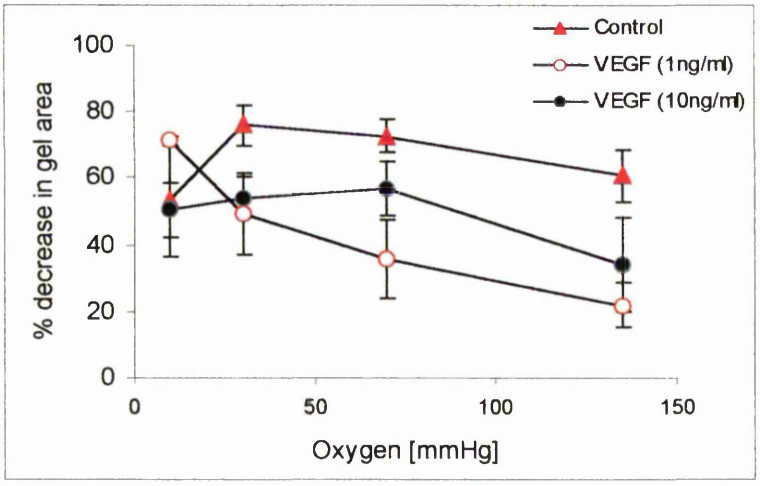


Figure 4.18 (a) is a graphical representation of the typical data obtained for pericyte mediated cell contraction in the presence of 1 or 10ng/ml VEGF in varying oxygen environments; n=4. (b) Is the pooled data of three experiments in the same conditions; n=12. Each point represents the statistical mean ± standard deviation. Both graphs highlight the observation that VEGF caused an oxygen dependent decrease in pericyte mediated contraction.

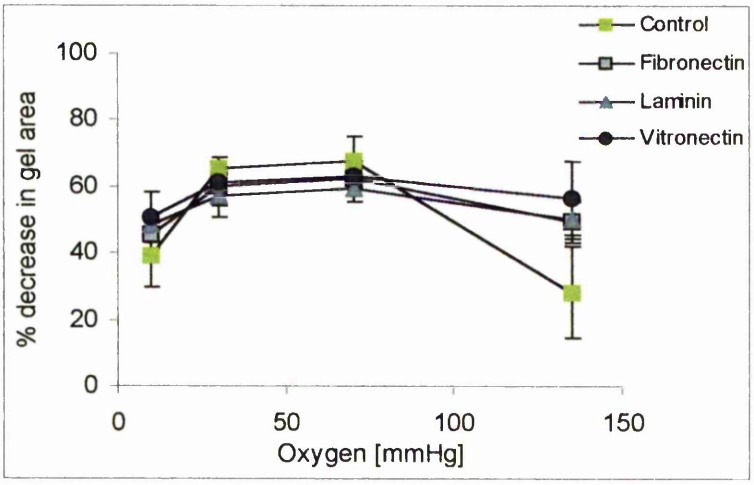
4.3.8 The effect of extracellular matrix components and oxygen on cell mediated collagen gel contraction

The effect of fibronectin, laminin or vitronectin and oxygen on fibroblast mediated collagen gel contraction - addition of fibronectin significantly increased the contractile response of fibroblasts in an oxygen environment of 10mm Hg ($p<0.005$) but significantly decreased (inhibited) contraction in 30mm Hg ($p<0.005$) and 70mm Hg ($p<0.05$) oxygen environments.

Addition of laminin significantly increased the amount of fibroblast mediated contraction observed in an oxygen environment of 10mmHg ($p<0.005$) and 135mm Hg ($p<0.01$). Contraction was significantly decreased in an oxygen environment of 70mm Hg ($p<0.005$) after laminin addition and contraction in an oxygen environment of 30mm Hg remained similar to the control.

Addition of vitronectin to fibroblast containing gels also significantly increased contraction in an oxygen environment of 10mmHg ($p<0.005$). Contraction was lower than in comparison to the control in both 30 and 70mm Hg oxygen environments, but was only found to be statistically significant in 70mm Hg oxygen ($p<0.01$). Contraction was lower than in an environment of 135mm Hg after addition of vitronectin in comparison to the control (fig. 4.19).

Fig.419 (a)



(b)

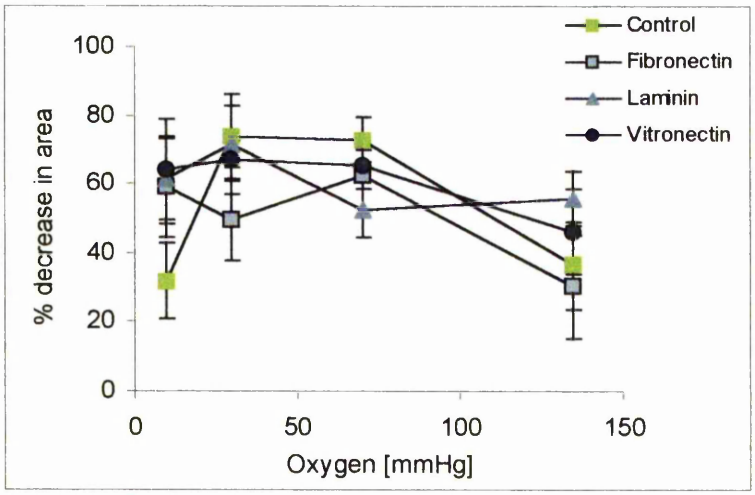


Figure 4.19 (a) is a graphical representation of the typical data observed after the addition of 10μg/ml fibronectin, 10μg/ml laminin and 0.5μg/ml vitronectin to fibroblast populated collagen matrices in varying oxygen environments; n=4. (b) is the pooled data of several experiments, n=12. Each point represents the statistical mean ± standard deviation.

4.4 Discussion

The growth characteristics of fibroblasts, pericytes and HRPE cells in monolayer culture were similar to those reported by many groups routinely culturing these cell types for numerous in vitro studies (Bell et al, 1979; Guidry and Grinnell, 1985; Gitlin and D'Amore 1983; Schor and Schor, 1986; Albert et al, 1972; Aronson, 1983).

Only low passage pericytes (P2 to P4) were used for contraction studies. This was due to the tendency of pericytes to change morphology by retracting and forming multicellular nodules in later passage cultures (Schor et al, 1990; 1995).

The observation that HRPE cells displayed morphological differences in serial passages has been previously reported and attempts have been made to overcome the apparent loss of epithelial morphology and associated in vivo characteristics (Aronson, 1983; Song and Lui, 1990). Retinal pigment epithelial cells in vivo possess strong intercellular contacts with adjacent cells and display a cobblestone-patterned morphology (Zinn and Benjamin-Henkind, 1979). Primary cultures, seeded at high cell density appear to preserve these highly differentiated characteristics. However, low density culture conditions and serial passaging requiring rapid proliferation results in subsequent loss of the typical epithelial characteristics such as cell-cell contacts and pigment granules (Nicolaisson et al, 1986; Boulton and Marshall, 1985). This present study utilised mainly passage 5 to 7 HRPE cells for contraction studies. These cell cultures still retained mostly epithelial morphology with some large cells displaying obvious stress fibres.

Early researchers have often described the in vivo epithelial morphology becoming mesenchymal in vitro as an artefact of cell culture. More recent studies have defined this mesenchymal (fibroblastic) cell type as a representation of RPE cells in a pathological situation e.g., PVR – where RPE cells have lost contact inhibition

and react to their new external environment (Opas, 1989; Grisanti and Guidry, 1995). The consequences of the varying morphology on contraction are discussed later on.

Attempts to obtain confluent monolayer cultures of glial cells were unsuccessful and hence their contractile ability was not assessed in this study. Although small numbers of cells were produced they were never cultivated beyond the first passage due to the lack of sufficient cell numbers. The most recognised method for bovine retinal glial cell culture appeared to be that described by Savage et al, (1988); although some cells were obtained, the major problem with the techniques used appeared to be lack of cell attachment and hence lack of subsequent division. This was addressed using type I collagen coated culture dishes as described by Guidry (1996) and Trachtenberg and Packey (1983). Other problems included contamination by other retinal cell types, mainly pericytes and occasionally RPE cells. The cells which were obtained did not stain positively for either of the glial cell markers tested (anti-carbonic anhydrase and anti-GFAP). There may be several explanations for the lack of staining. Firstly, it is well documented that GFAP staining is variable between cultures and indeed cultures beyond passage 5 do not express GFAP (Savage et al, 1988). Secondly carbonic anhydrase is described as a Muller cell marker rather than for astrocytes (Savage et al, 1988, Guidry, 1996); if the small number of cells that did attach were not Muller cells in origin then the immunostaining would be negative. Finally, the alpha-smooth muscle actin positive cells could still be glial in origin as glial cells have been reported to express this marker, but only in monolayer culture rather than in retinal sections (Guidry, 1996). Other methods of glial cell culture were investigated, mainly utilising human tissue (Ikeda and Puro, 1995; Puro et al, 1990; de Juan et al, 1989). However, the large cell numbers required for this

study would have been impossible to achieve with the limited amount of fresh/viable tissue. Other alternatives included the use of glial cell lines or brain derived glia (Jiang et al, 1994), but this would leave the results largely incomparable to the other retinal cell types tested. A recent method described by Guidry (1996), utilises porcine glial cells, where the retinas were dissociated using trituration of the tissue and optimal purification achieved by sequential centrifugation through a percoll gradient. Although this appears to be a promising technique for obtaining porcine glial cells, the lengthy dissociation procedure and the relatively low cell yield meant it was again impractical for this study.

The collagen gel contraction model is a well documented method of studying cell mediated contraction as an in vitro event (Bell et al, 1979; Schor, 1980; Bellows et al, 1981; Allen and Schor, 1983; Guidry and Grinnell, 1985; Montesano and Orci, 1988; Raymond and Thompson, 1990; Mazure and Grierson, 1992; Guidry and Hardwick, 1994). Originally developed as a method of studying tissue contraction in the healing wound, many researchers have applied it to the pathological wound healing situation in the retina represented by membrane formation (de Juan et al, 1989; Raymond and Thompson, 1990; Mazure and Grierson, 1992; Guidry et al 1992; Hunt et al, 1994; Grisanti and Guidry, 1995;).

This model of cell mediated contraction offers a highly representative approach to the proposed sequential steps involved in membrane contraction. These include cell migration, adhesion and subsequent matrix remodeling. These events essentially involve the formation, breakdown and reformation of cell-substratum and cell-cell contacts.

The findings of the initial cell type and density studies were in general agreement to that of published data utilising the collagen gel contraction assay, such that the

extent of contraction was proportional to the cell density (until saturation levels were reached) and increased with time - with the most contraction being observed in the earliest stages (<48 hours) post seeding (Schor, 1980, Bell et al, 1979; Guidry and Grinnell, 1985; Souren and Wijk, 1993; Eastwood et al, 1996).

The greater contraction observed in the early stages of the assay is thought to be associated with attachment of cells to the collagen fibrils with subsequent cytoskeleton changes (Grinnell, 1994; Brown et al, 1996; Eastwood et al, 1996). The ensuing migration of cells with altered 3-dimensional morphology is thought to be responsible for the remaining contraction observed (Grinnell, 1994; Eastwood et al, 1996).

Evidence for cell adhesion and locomotion being related to the contractile response has become increasingly available as more accurate/precise methods of assessing contraction are developed. One example is the work of Eastwood and colleagues, where the amount of force generated by cells seeded in a collagen gel was quantifiable. Using this culture force monitor they were able to calculate exactly when the cellular traction is produced in relation to cell morphology, cytoskeletal arrangement and distribution within a collagen matrix.

These researchers demonstrated that three distinct phases of contraction of cell populated collagen gels could be identified within the time span of the assay. Furthermore, most of the force generated by human dermal fibroblasts was produced during phase I which correlated cell attachment and associated changes in cell shape and the appearance of cell processes (Eastwood et al, 1994; 1998; Brown et al, 1996). The findings although supportive of this and other studies are not consistent with the idea that cell differentiation into specific forms of contractile fibroblasts – such as myofibroblasts are responsible for the contractile response (Gabianni et al, 1972).

The observation that pericytes were the most effective at eliciting a contractile response in comparison to HRPE and fibroblasts is not surprising when considering their situation in vivo.

Pericytes are described as cells which are continuous with vascular smooth muscle cells of arteries and veins and distinctly shaped with many cytoplasmic processes that encircle capillaries (Hirschi and D'Amore, 1996). Each pericyte possesses a cell body with a prominent nucleus and a small amount of surrounding cytoplasm. Protruding from the cell body are long processes not only capable of aligning the long axis of the capillary but capable of reaching other capillaries in certain capillary beds (Epling, 1966; Weibel, 1974; Sims, 1986). Experiments investigating the growth inhibitory effects of pericytes on endothelial cells in coculture, demonstrated the ability of some pericytes to contact up to five adjacent endothelial cells via cellular processes (Orlidge and D'Amore, 1987). Thus in vivo and in vitro morphology and the intrinsic ability of pericytes to make cell-matrix associations and cell-cell contacts, may explain why these cells are so efficient at demonstrating a contractile response (Kelley et al, 1987; 1988).

The observation that fibroblasts and RPE cells did not display significantly different contractile responses are at first glance contradictory to other. Guidry and co-workers (1992) reported that glial cells were the most contractile cell type, closely followed by fibroblasts, yet RPE cells produced a negligible contractile response in this system. Mazure and Grierson (1992), however reported the effectiveness of fibroblasts at producing a contractile response, followed by RPE cells and glial cells produced no visible contraction when plated at the same cell density. Some of these apparent contradictions may be explained by recent reports relating to cell morphology and exact origin of the cell in vivo as well as conditions of monolayer

culture affecting cell mediated collagen gel contraction (Eastwood et al, 1998; Brown et al, 1996; Eastwood et al, 1996; Grisanti and Guidry, 1995; Souren and van Wijk, 1993; Masur et al, 1996).

The effect of plating density in monolayer culture has been addressed with respect to fibroblasts and RPE cells by Masur et al (1996) and Grisanti and Guidry (1995) respectively. Both studies emphasise that low plating density in monolayer culture with subsequent high split ratio during routine passaging increases the cells' ability to produce a contractile response. In fibroblasts this was reported to be associated with loss of cell-cell contact, increased smooth muscle actin expression and the production of TGF-β. In RPE cells it is associated with the loss of their highly differentiated epithelial monolayer, less cell-cell contact and increased smooth muscle actin expression. The results obtained in this present study regarding the contractile ability of low passage HRPE cells are in agreement with the observations of Guidry et al, (1992) and Grisanti and Guidry (1995), whereas the findings of Mazure and Grierson (1992) are comparable to the response observed in this study for P6 HRPE cells.

Furthermore, comparison of the contraction profiles of fibroblasts extracted from different tissue within the same species (tendon and articular cartilage) and extracted by different means from the same tissue displayed different patterns (Eastwood et al, 1996; Brown et al, 1996). These researchers reported that fibroblasts obtained from explants were much more efficient at eliciting a contractile response than fibroblasts obtained by full enzymatic digestion of an identical tissue explant. This was proposed to be because fibroblasts isolated from explant culture were selected in that the cells with an efficient and rapid migratory response were obtained. Yet full enzymatic digestion of the tissue produced cultures of cells containing some cells less efficient in eliciting a

contractile response, with a slow migratory phenotype. Thus sub-populations of fibroblasts within tissue are said to exist and so care must be taken when comparing the contractile response of fibroblasts from the same species and tissue. Other factors that have been shown to affect cell mediated contraction include collagen concentration (Bell et al, 1979; Schor, 1980) and type of serum used since foetal and postnatal sera have been shown to modulate fibroblast contraction (Moulin et al, 1997).

The oxygen environments used in this study were 10mmHg (representing hypoxia) (Maeda and Tano, 1996) 30mmHg, 70mmHg (representing physiological oxygen conditions) and 135mmHg (standard or hyperoxic conditions) (Rosen et al, 1991; Khaliq et al, 1995).

Contraction was found to be highest in a physiological oxygen environment (30-70mmHg) for all the cell types tested.

The observation that pericytes were highly contractile in all oxygen conditions (i.e., above that observed for all other cell types in all oxygen environments) is likely to be reflective of their natural contractile ability and not a full indication of their situation in vivo as it is probable that other regulatory mechanisms are in operation in vivo. Thus a fluctuation of the local oxygen concentration is also likely to cause the production of other regulatory factor/s from adjacent endothelial cells such as in the case of the endothelin-I/nitric oxide system, which may further control the contractile response of pericytes (Masaki, 1995).

The contractile response of fibroblasts to varying oxygen concentration was perhaps the most characteristic of all the cells tested, in that fibroblasts were most sensitive to particularly the lower extremes of oxygen concentration. The observations of the extremely significant increase in contraction in the higher

physiological oxygen environment, may seem at first to conflict with the idea of contraction being a pathological event and would not be expected to increase in a physiological oxygen environment. However, when examined closely the contractile response of fibroblasts displayed in vitro is actually reflective of their cell-matrix attachment and cell-cell formation ability in vivo, particularly considering the current school of thought regarding the mechanism of contraction (Grisanti and Guidry, 1995; Eastwood, 1996).

Thus in the physiological oxygen environment of a typical fibroblast in vivo, cell-matrix adhesion and cell-cell contact is at a maximum, necessary for the efficient response to external stimuli as well as signal transduction events. Therefore it should follow that these events are slower/less efficient in a hypoxic (pathological) oxygen environment. It should also be noted that in an in vitro system such as this, the constraints of the surrounding matrix do not exist and the bound/unbound reservoir of growth factors/cytokines are likely to participate. Thus the importance of this finding is not specifically the amount of contraction observed in a physiological or hypoxic oxygen environment but the fact that oxygen has the ability to direct the cellular responses that encompass contraction.

The contractile response of HRPE cells was interesting because it was dependent on passage number. P3 HRPE cells produced the most contraction in a hypoxic oxygen environment and produced the least contraction in a physiological oxygen environment. Yet P6 HRPE cells produced a contractile response similar to that of fibroblasts with contraction highest in the physiological range of 30-70mmHg oxygen. These results are in agreement with the findings of Grisanti and Guidry (1995) who report the ability of RPE cells to elicit a contractile response is dependent upon their morphology. It is proposed that epithelial morphology RPE with highly differentiated features exert contraction in a different way to their

transdifferentiated counterparts, such that epithelial-morphology RPE cells retain their polarity and cell-cell contacts and exert traction as sheets of cells, this is less efficient because of lower scope for attachment to the collagen substratum. This low contractile ability however is increased by reducing the oxygen concentration, and may be a mechanism, which induces RPE transdifferentiation, in vivo.

The determination of cell numbers and cell proliferation confirmed that the contractile response for each cell type was not an artefact of proliferation. Since previous studies in this and other labs have demonstrated that reducing the oxygen concentration to a more physiological level from standard (hyperoxic), increases cell proliferation of many cell types (Storch and Talley, 1988; Moriarty et al, 1991; Boulton et al, 1992). However previous studies utilising the collagen gel assay demonstrate the inhibitory effect of a 3-dimensional matrix on cell growth (Allen and Schor, 1983; Bell et al, 1979; Bellows et al, 1981). This reduction in DNA synthesis has been shown to be due to the increased proximity cell-cell contacts in contracted gels leading to contact inhibition of growth as observed in post-confluent cultures (Schor, 1980; Montesano and Orci, 1988).

Previous studies in this and other laboratories have demonstrated that local oxygen environment is capable of affecting the response of cells in monolayer culture to various exogenous growth factors/cytokines (Wing et al, 1988; Storch and Talley, 1988; Khaliq et al, 1995).

Studies utilising the collagen gel contraction assay have also demonstrated that in vitro contraction can be enhanced/inhibited after the addition of various growth factors (Raymond and Thompson, 1990; Pena et al, 1994; Smith-Thomas et al,

1996; Hardwick et al 1997). Yet to date the potentially important effect of growth factors and cytokines on retinal cell mediated contraction in varying oxygen conditions has not been addressed.

TGFβ has previously been demonstrated to increase contraction of fibroblast and RPE populated collagen gels and this effect was found to be inhibited in the presence of TGFβ neutralising antibodies (Raymond and Thompson, 1990; Pena et al, 1994; Kurosaka et al, 1996; 1998). In this present study TGFβ2 increased fibroblast-mediated contraction significantly in all oxygen environments. This effect was particularly noticeable in an environment of 30mmHg oxygen where the amount of contraction observed was more than twice that observed in control gels. There are two interesting points to be raised here. Although an increase in fibroblast mediated contractile response was observed in standard (hyperoxic) conditions; this was not representative of the extent of increase in all other oxygen environments, i.e., in situations which may represent the physiological or pathological situation in vivo. Thus previous studies may have misleading data when relating their results to an in vivo situation. Secondly the oxygen concentration most representative of fibroblasts in vivo is 30mmHg and this may be why an increased response is observed here (Maeda et al, 1990; Boulton et al, 1992).

The observation that RPE cell mediated contraction was not increased by TGFβ is at variance with several other studies which report the enhancement of RPE cell mediated contraction in the presence of exogenous TGFβ (Raymond and Thopmson, 1990; Smith-Thomas et al, 1996). It may also be that the HRPE cells in this study are already at their contractile maximum in their respective oxygen environments and additional growth factors would not display any additional effect. Furthermore, the dosage of TGFβ used may have been inadequate to elicit an in

vitro response. Considerations discussed earlier are also likely to apply, as the contraction and the responsiveness of RPE cells to external stimuli may well be affected by plating density and monolayer morphology prior to seeding in collagen (Grisanti and Guidry, 1995; Opas, 1989). It is however, evident that RPE cell mediated contraction is a highly controlled event regulated by a delicate balance of external factors and inherent properties of this complex cell type; many of which have yet to be defined.

There is some debate in the literature as to the role (if any) of bFGF in the production of a contractile response. Some reports report inhibitory effects of bFGF in their systems while others observed increases in contraction with low significance in comparison to the potent effect of TGFβ (Kurosaka et al, 1995; Smith-Thomas et al, 1996).

The addition of bFGF to fibroblast populated collagen gels resulted in a significant increase in amount of contraction in an oxygen environment of 30mmHg. In contrast bFGF caused a statistically significant decrease or inhibition of RPE mediated collagen gel contraction in an oxygen environment of 30mmHg in comparison to the control. This suggests that an oxygen environment of 30mmHg is the archetypal environment required for a cellular response to bFGF in vitro and hence may reflect bFGF's course of action in vivo.

The fact that both fibroblasts and RPE cells present a different contractile response in varied oxygen concentrations; and that the extent of this response was further affected by exogenous growth factors suggests that oxygen is not the sole factor governing the contractile responsiveness of cells.

Studies investigating the proliferative response of retinal and other cell types in varying oxygen have suggested that the activity of a stable intermediate is altered by reduced oxygen (Wing et al, 1988; Storch and Talley, 1988). More specifically,

several studies have shown that growth factor receptor up-regulation occurs in reduced oxygen (Khaliq et al, 1996; Shreeniwas et al, 1991; Laderoute et al, 1992) and this may be why an increased proliferative effect is observed in lower oxygen concentration.

Further studies investigating mRNA levels and half-life times for various growth factor receptors would give a better indication as to whether receptor up regulation is a direct result of oxygen reduction in the production of a contractile response.

In order to further study external stimuli – the effect of various extracellular matrix components on fibroblast mediated contraction was investigated.

Previous studies have demonstrated the positive effect of fibronectin on collagen gel contraction and described its role as a link molecule between cells and collagen fibrils. The effects of laminin and vitronectin have been the subject of less investigation using the collagen gel contraction model although their presence and their integrin receptors have been well documented in PVR/PDR membranes and pathological wound healing situations (Asaga et al, 1991;Agrez, 1991).

In this study contraction was significantly increased in an environment of 10mmHg (hypoxia) in the presence of all extracellular matrix components tested and the amount of contraction observed in a 70mmHg (physiological) oxygen environment was significantly decreased. It is clear from this study that a level of control exerted by extracellular matrix on the contractile event can be paralleled to both the physiological and pathological retinal situation. Thus it is possible that cell matrix interactions and subsequent cell-cell interactions are a response to the external stimuli, principally oxygen which can be further modified by growth factors.

The findings of this study also reported the effectiveness of pericyte contractility. bFGF and VEGF affected the contractile profile of pericytes in an oxygen

dependent manner. bFGF significantly increased contraction in a hypoxic environment, but had no effect at all other oxygen concentrations. Conversely VEGF caused a significant decrease in the amount of contraction observed in all oxygen environments with the exception of hypoxia.

Pericyte contractility is discussed separately from the other cell types tested as they are naturally contractile so the relevance of their response is physiologically important as opposed to only pathologically.

The two potent vascular mitogens used in this study have rarely been associated with vascular cell contraction, although the potential for such a mechanism exists. VEGF is constitutively expressed in many tissues and receptors for VEGF have been confirmed on pericytes and smooth muscle cells (Wen et al, 1998; Chen et al, 1997; Pueyo et al, 1998). bFGF is present in most tissues. It is well established that the endothelin/nitric oxide system is active within the retinal vascular network. So it may be that VEGF's regulation of contraction is a back up plan or even involved in a synergistic mechanism with other regulators. It is also possible that the hypoxia-inducible and stable VEGF is not present in adequate quantities in a hyperoxic oxygen environment such that the fact that contraction is inhibited in this environment may be clinically irrelevant. However, at the cell biological level these findings demonstrate an oxygen sensing mechanism within separate from the endothelin/NO pathway) pericytes exists. It is equally plausible that the contraction observed in pericytes is the response to the local environment of increased cell attachment and migration.

4.4 Conclusions and further work

It is clear from the findings of this study that oxygen ha a role to play in cell mediated contraction. The fact that the contractile response of the cell types tested were varied in extent in different oxygen environments has far reaching implications. Firstly, the finding that contraction was far more efficient in a physiological oxygen environment than in standard incubating conditions indicates that the mechanisms involved in contraction may have a level of control previously unaccounted for. Secondly, the fact that the addition of extracellular matrix components reversed the contractile profile of fibroblasts to that suggestive of a pathological situation suggests that oxygen may well exert its effect indirectly via cell signalling cascades involving cell-matrix interactions. This finding warrants further investigation of cell-matrix interactions, as previous studies have concentrated on growth factors and binding to their receptors as a possible level of control. Finally, the finding that VEGF is capable of causing an oxygen dependent decrease in the contractile response of pericytes, is the first demonstration that VEGF may have a dual purpose in controlling microvascular tone. Further studies are now required investigating the mode of action of VEGF in this context, possibly determining whether the pericyte specific contractile profile is a direct effect of VEGF or the activation/inhibition of other growth factors/cytokines in serum.

CHAPTER FIVE- GENERAL SUMMARY

Currently, there are no in vitro models that completely mimic the complex cellular and biochemical processes that occur during retinal neovascularisation or membrane formation/contraction. However, many in vitro assays/models have been developed which may approximate and therefore allow investigation of certain cellular events during angiogenesis and/or pathological wound healing (Auerbach et al, 1991; Bischoff, 1995; Sage and Vernon, 1994; Grant and Kleinman, 1997; Ehrlich and Rajaratnam, 1990; Kamei et al, 1996; Montesano, 1992, Madri and Pratt, 1986). These assays are not always representative of the retinal situation; thus a major focus of this study was to use and develop in vitro systems which appreciated the highly specialised nature of the retina.

The processes involved in retinal neovascularisation include: -

- basement membrane degradation
- cell migration and proliferation
- lumen formation and anastomosis
- vessel remodelling

Similarly the events leading to membrane contraction include: -

- cell migration and proliferation
- cell adhesion and attachment
- extracellular matrix remodelling and deposition

Thus the cellular processes pivotal to both features of proliferative retinal diseases involve cell migration, formation of cell-cell and cell-matrix contacts as well as matrix reorganisation (Zetter, 1987; McCarthy and Turley, 1993; Cordeiro et al, 1997). The findings reported in this study further demonstrate the importance of a migratory response in both in vitro assays used and the

model system developed. For example, the findings in chapter 2 demonstrated the 'migratory' response of venous cells occurring significantly earlier than in arteriolar cells. The findings reported in chapter 3 demonstrated the migratory response of both venous and arteriolar cells and could be directional in nature. Finally, the use of the collagen gel contraction model demonstrated that the extent of a migratory response varied between cell type and morphology.

It is well appreciated that there exists a complexity of regulatory factors that drive quiescent cells to migrate, form cell-cell contacts and reorganise their surrounding matrix (Robinson and Aiello, 1998; Brooks, 1998; Boulton et al, 1996; Grierson et al, 1996; Bennet and Schultz, 1993). A substantial part of this study involved investigating the effects of various growth factors/extracellular matrix components on these cellular processes in the context of the assays and model system developed.

The use of an in vitro retinal vessel culture system demonstrated that cellular outgrowth was observed significantly earlier from venous cultures than arteriolar cultures. The addition of various growth factors and substrates (including collagen, gelatin, laminin, fibronectin, vitronectin, VEGF and bFGF) to this culture system showed this relationship to be largely substrate independent and enhanced by VEGF. It was also demonstrated that with the exception of vitronectin, the number of arterioles that presented any outgrowth at all was considerably less than that observed for venules in any condition tested, even when attachment efficiency was comparable in both vessel types. As described earlier the processes represented by this culture system involve the initial stages of new vessel formation, which include degradation of the basement

membrane with subsequent cell migration. A requirement at the cellular level for these processes to occur is the production of a proteolytic cascade for both dissolution of the basement membrane and cell migration which in turn involves the cyclic formation and dissolution of cell-cell/ cell-substratum contacts. Numerous studies have implicated VEGF in the induction of proteolytic activity in endothelial cells (Pepper et al, 1998; Mandriota et al, 1995). Several studies have also demonstrated the interaction of endothelial cells with vitronectin allowing the formation of adhesive contacts in a proteolysis independent manner (Preissner et al, 1997). The observation that arterioles only produced outgrowth in 75% of cultures in the presence of VEGF and produced outgrowth in a 100% of cultures in the presence of vitronectin suggests alternative migratory pathways exist. This however does not explain why in the presence of either VEGF or vitronectin outgrowth was observed significantly earlier in venous cultures in comparison to arteriolar cultures.

The use of the model system described in chapter three did however provide a further insight into the possible levels of control that may exist in the early stages of retinal neovascularisation.

The findings in chapter three focus on the development of a simple, reproducible organ culture model of retinal vascular outgrowth. This model not only addressed the initial stages of vessel formation (basement membrane degradation and endothelial cell migration) but also represented vascular cell invasion of the surrounding matrix, formation of cell-cell contacts/networks (the cellular events required for lumen formation and anastomosis) (Ashton, 1957; Cogan et al, 1961; Archer, 1983; Frank, 1990; D'Amore, 1994).

Parallels with the findings in chapter 2 were observed in retinal vascular growth in a 3-dimensional matrix. Firstly, outgrowth from venules did not occur

significantly earlier than from arterioles, this was consistent with the findings in chapter 2, where no difference was found in the time taken for outgrowth to occur in arteriolar or venous cultures when collagen I was used as a growth substrate. Secondly, initial outgrowth from arterioles was only observed from the vessel tips or capillary branch points, whereas venous outgrowth was observed from all points along the vessel. This was also observed in the retinal vessel culture system described in chapter 2 – suggesting different mechanisms of outgrowth may exist in the two vessel types.

The model described in chapter 3 addressed some of the limitations associated with other in vitro models, these include: the use of retinal tissue and vessels as well as the demonstration of retinal vascular cell directional growth.

It is widely accepted that during the formation of new blood vessels, endothelial cells migrate away from a previously formed vessel, in response to stimulation (Folkman, 1979; Fan et al, 1995). That migration includes both an increased motility of the stimulated endothelial cells, as well as their migration towards the site from which angiogenic factors are released. The simple measurement of cell migration in the presence of various growth substrates/factors (chemokinesis) as in chapter 2, is a useful assay of vascular cell reactivity.

However chapter 3 demonstrates not only cell movement but directionality of that movement (chemotaxis) (Zetter, 1987; Auerbach, 1991). The factors released from retinal cells appeared to cause the directional movement, and further studies characterising the factor/s responsible for growth/migration at the molecular level will enable a better understanding of the early events required for retinal neovascularisation.

The importance of, cell-matrix adhesion, cell-cell contact and network formation was reinforced by the observation that endothelial cell contact formation

occurred regardless of directional movement in both 2-dimensional assay and 3-dimensional model systems. Cell-cell contact/communication appeared to be a necessary sequential step for retinal vessel endothelial cells subsequent to migration. The cellular events required include – cell locomotion, formation of cell contacts through extension of one or more cellular projections (i.e., change in cell shape/morphology), cell-substratum contacts. The formation of cell-cell networks in vitro is thought to represent endothelial cell reorganisation prior to lumen formation in vivo (Vernon and Sage, 1999; Saunders and D'Amore, 1992). Similarities in these cellular events and those possibly required for retinal membrane contraction were observed also in the collagen gel model of cell--mediated contraction utilised for the series of experiments reported in chapter four.

As described in chapter one the later stages of proliferative retinal diseases involve the formation of a highly contractile retinal membrane. The cellular events that lead to retinal membrane contraction are thought to be represented in part by the collagen gel contraction model. These include the formation, breakdown and reformation of cell-cell and cell-substratum contacts (Brown et al, 1996; Cordeira et al, 1997).

The findings in chapter 4 were the first demonstration of oxygen affecting the cell mediated contraction of various ocular cell types. The observation that contraction was greatest in a physiological oxygen environment and lower in either hypoxia or hyperoxia has several implications. Firstly, the efficient contractile response of all cell types in a physiological oxygen environment may be interpreted as cellular events such as cell-cell and cell-matrix attachment are at a maximum. This would be logical as cell-cell/matrix contact formation, may be decreased in less favourable environments such as high/low oxygen. This

may also be the case with cell-cell/matrix interactions observed in the model system described in chapter 3. Further studies observing cell outgrowth in the in-vitro model of retinal neovascularisation in varying oxygen environments are now required.

The observation that the contractile response was also effected by various growth factors suggests that many levels of control exist. This was emphasised by the finding that VEGF was capable of affecting pericyte contractility – a previously undemonstrated role for a potent cell mitogen.

The observation that the contractile profile of fibroblasts was increased in a hypoxic environment in the presence of fibronectin, laminin and vitronectin indicates parallels with the in vivo situation. Further studies investigating a combination of growth factors and extracellular matrix components in the presence of varied oxygen concentrations are now required.

How oxygen is involved in the contractile response of these three distinct cell types is difficult to explain as few, if any parallel studies exist. A number of possibilities exist, these include receptor regulation, matrix remodelling enzyme upregulation, production of a stable intermediate or more likely a combination of all these. Further studies are now required investigating the transcriptional and translational activity of cells eliciting a contractile response in the varying oxygen environments.

In summary, the findings reported in this study reinforce the complexity of regulatory factors that drive cells to initiate or participate in retinal proliferative diseases. The models and assays developed herein, provide a excellent starting point to investigate some of these cell mediated processes.

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Appendix I: Chemical Reagents and Suppliers

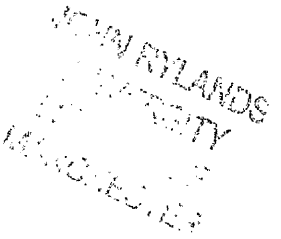
Acetic Acid	BDH
Acetone	BDH
Agarose	BDH
Amino-Propyltriethoxysilone (APE)	BDH
Basic-Fibroblast Growth Factor	British Biotechnology
Benzyl-Penicillin	Sigma
Calcium Chloride 2-Hydrate	BDH
Chloroform	BDH
Collagenase	Sigma
Deoxyribonuclease	Sigma
Disodium Hydrogen Orthophosphate	BDH
Dulbecco's Modified Eagles Medium (DMEM)	Gibco
Eagle's Mimimal Essential Medium (MEM)	Gibco
EDTA	BDH
Endothelial Serum Free Medium	Gibco
Ethanol	BDH
Fibronectin	Sigma
Foetal Calf Serum (FCS)	Gibco
Gelatin	Sigma
Gelvatol (Vinyl Alcohol 25)	Fisons Scientific Equipment
Glucose	BDH
Glutamine	Sigma
Glycerol	Sigma
Ham's FIO Medium	Gibco
Hepes Buffer	Gibco

Kanomycin Sulphate	Sigma
Laminin	Sigma
New Calf Serum	Gibco
Non-Essential Amino Acids	Gibco
Plasma	Manchester Blood Bank
Potassium Chloride	BDH
Potassium Dihydrogen Orthophosphate	BDH
Practamount	Asco Laboratories
Pronase	BDH
Sodium Bicarbonate	BDH
Sodium Chloride	BDH
Sodium Pyruvate Solution	Gibco
Streptomycin Sulphate	Sigma
Transforming Growth Factor β (TGFβ)	R&D Systems
Triton X-100	Sigma
Trypsin	Sigma
Trypsin In Puck's Minimal Media	Gibco
Vascular Endothelial Growth Factor (VEGF)	R&D Systems
Vitronectin	Sigma
Xytene	BDH

Appendix II: Cell Culture Media and Solutions

Antibiotics and Glutamine (Ab+g)

1g	Streptomycin Sulphate
1g	100μg Kanomycin Sulphate
600mg	Benzyl-penicillin
1.46g	L-Glutamine
100ml	ddH_2O



Antibiotic Solution (for collagen shock)

0.5g	Benzyl-penicillin
0.5g	Streptomycin Sulphate
100ml	ddH_2O

Collagen Solution

8.5ml	Collagen (2mg/ml)
1.0ml	10xMEM, DMEM or FIO
0.5ml	Sodium Bicarbonate Solution (7.5% w/v in ddH_2O)

DMEM + 20% Serum

20ml	10xDMEM
40ml	Foetal Calf Serum
10ml	Sodium Bicarbonate Solution (7.4% w/v in ddH_2O)
4ml	Ab+g
126ml	ddH_2O

ESFM + 10% PPP

190ml	Endothelial Serum Free Medium
10ml	Platelet Poor Plasma

F10 + 20% Serum

20ml	10xHam's F10
40ml	Foetal Calf Serum
10ml	Sodium Bicarbonate Solution (2.4% w/v in water)
10ml	Glucose Solution (8% w/v in water)
4ml	Ab+g
116ml	ddH_2O

MEM/Hepes

20ml	10xMEM
6ml	Hepes Solution
4ml	Ab+g
170ml	ddH_2O

MEM + 20% Serum

20ml	10xMEM
40ml	Foetal Calf Serum
6ml	Sodium Bicarbonate Solution (7.4% w/v in water)
2ml	Non-Essential Amino Acids Solution
2ml	Sodium Pyruvate Solution
4ml	Ab+g
126ml	ddH_2O

MEM + 10% NCS

20ml	10XMEM
20ml	New Calf Serum
6ml	Sodium Bicarbonate Solution
4ml	Ab+g
150ml	ddH_2O

2xMEM

40ml	10xMEM
6ml	Sodium Bicarbonate Solution (7.4% w/v in water)
4ml	Ab+g
150ml	ddH_2O

Collagenase Solution (for collagen gel digestion)

5mg	Collagenase
10ml	2xMEM

Collagenase Solution (for retinal vessel treatment)

100ng to 5mg	Collagenase
10ml	MEM/Hepes

Phosphate Buffered Saline (PBSA)

40g	Sodium Chloride
1g	Potassium Chloride
5.75g	Disodium Hydrogen Orthophosphate
1g	Potassium Dihydrogen Orthophosphate
500ml	ddH_2O

Trypsin Solution

0.25%	w/v Trypsin
0.002%	w/v EDTA
100ml	PBSA

Enzyme Cocktail (for Pericyte Isolation M1)

5mg	Collagenase
2mg	Pronase
2mg	Deoxyribonuclease
19ml	MEM/Hepes

Appendix III: Preparation of Solutions for Histology and Immunostaining

Primary Antibodies

Anti-Carbonic Anhydrase II (Sheep)	R&D Sytems
Anti-GFAP (Rabbit)	Eurodiagnostica
Anti-α-Smooth Muscle Actin (Mouse)	Sigma Immunochemicals
Anti-Von Willebrand's Factor (Rabbit)	Dako

All were used in a 1 in 100 dilution of antibody in 0.2% serum (v/v PBSA) unless otherwise stated.

Secondary Antibodies

Anti-Mouse TRITC Conjugate (Goat Immunoglobulin)	Sigma
Anti-Rabbit FITC Conjugate (Goat Immunoglobulin)	Sigma
Anti-Sheep FITC Conjugate (Rabbit Immunoglobulin)	Sigma

All were used in a 1 in 50 dilution of antibody in 0.2% serum

Serum

Goat Serum	Sigma
Rabbit Serum	Sigma

All blocking of non-specific antigens utilised 20% serum (v/v PBSA)

10% Neutral Buffered Formalin

4g NaH_2PO_4

6.5g Na_2HPO_4

100ml 40% Formaldehyde (v/v in water)

900ml ddH_2O

0.2% Triton X-100

0.2ml Triton X-100

100ml PBS

Preparation of APES-Coated Slides for Paraffin Section Adhesion

1. Slides immersed in 500ml 1% APES (v/v in acetone) for 2-3 minutes.
2. Slides rinsed in acetone for 2-3 minutes.
3. Slides washed in ddH_2O.
4. Slides placed in 37°C oven overnight.

Preparation of Gelvatol Mountant

1. 0.08g $NaHPO_4$ and 0.03g KH_2PO_4 added to 40ml ddH_2O in 250ml beaker and pH adjusted to 7.2.
2. 0.327g NaCl, 0.024g sodium azide and 0.6g DABCO added to the solution. The beaker was covered with foil.
3. 10g gelvatol added and the solution stirred overnight.
4. 20ml glycerol added. Solution stirred and pH adjusted to 6-7.
5. Solution spun at 5000g for 15 minutes and 10000g for 25 minutes.
6. The solution was stored in the dark at 4°C until use.

Appendix IV: Materials and Equipment Supplier

24-Well Plate	Cosvar
25cm^2 Tissue Culture Flask	Falcon
35mm Culture Dishes	Nunclon
75cm^2 Tissue Culture Flask	Falcon
90 Mm Petri Dishes	Sigma
Acrodisc Filters	Gelman
Centrifuge	Burkard
Centrifuge Tubes (Sterile)	Falcon
Co_2 Incubator	Leec
Coverslips	Chance Propper Ltd. Uk
Dialysis Tubing	Med. Int. Ltd.
Dissecting Microscope	Zeiss
Glass Microscope Slides	Chance Propper Ltd. Uk
Inverted Microscope	Zeiss
Kodak 160t Colour Film	Kodak Uk Ltd.
Kodak Elite 400 Film	Kodak Uk Ltd.
Kodak Tmax 100 Film	Kodak Uk Ltd.
Sterile Bijou Tubes	Nunclon
Sterile Filters (0.2μM)	Millipore
Sterile Tissue Culture Cabinet	Gelair
Vanox High Resolution Microscope	Zeiss
Wax	Bdk
Whatman Filter Paper	Whatman International

Appendix V: Summary of data from cultured arterioles and venules

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 1	20% DMEM 5hrs PM	Venules	Y	Y	12	endo	endo		+++/ -	+++++/ +++
			Y	Y	12	endo/peri	endo/peri			0 / 0
			Y	N						
			Y	Y	13	peri	endo/peri		+++++/ -	+++++/ +++
			Y	N				V		
			Y	N				V		
			N	N						
		Arterioles	Y	Y	18	endo	endo/peri			0 / 0
			Y	Y	17	peri	endo/peri		++/ -	+++/ +++
			Y	N						
			Y	N				A		
			N	N						
Retina 2	20% DMEM 3hrs PM	Venules	Y	N						
			Y	Y	11	peri	peri			
			Y	N						
			Y	N						
			N	N						
			N	N						
		Arterioles	Y	N						
			Y	Y	12	endo	peri			
			Y	Y	12	peri	peri			
			Y	Y	13	peri	peri			
			Y	N						
			N	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 3	20% DMEM 20 hrs PM	Venules	Y	Y	12	endo	endo/peri	V		
			Y	Y	13	endo/peri	peri	V		
			Y	Y	14	peri	peri			
			Y	N						
			N	N						
			N	N						
		Arterioles	Y	Y	16	peri	peri			
			Y	Y	16	Peri	peri	A		
			Y	N						
			N	N						
Retina 4	20% DMEM 3hrs PM	Venules	Y	Y	10	endo/peri	peri			
			Y	N						
			N	N						
		Arterioles	Y	Y	11	endo/peri	peri			
			N	N						
Retina 5	20% DMEM 5 hrs PM	Venules	Y	Y	13	peri	endo/peri	V		
			N	N						
		Arterioles	Y	Y	16	peri	peri			
			N	N						
			N	N				A		

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 6	ESFM + PPP 24 hrs PM	Venules	Y	Y	8	endo	endo			
			Y	N						
			Y	N						
			Y	Y	13	peri	peri		++++/ -	+++/++++
		Arterioles	Y	Y	15	peri	peri		++++/ -	+++/++++
			Y	N						
			Y	N						
			Y	N						
Retina 7	ESFM + PPP 24 hrs PM	Venules	Y	Y	13	peri	Peri			
			Y	N						
			Y	Y	8	endo	Endo/peri			
			Y	N						
		Arterioles	Y	Y	15	Endo	endo/peri			
			Y	N						
			Y	N						
			Y	N						
Retina 8	ESFM + PPP 24 hrs PM	Venules	Y	N						
			Y	N						
		Arterioles	Y	Y	11	Peri	endo/peri			
			Y	Y	12	Endo	peri			
			Y	N						
			N	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 9	20% DMEM 23 hrs PM	Venules	Y	Y	6	Endo	endo/peri	V		
			Y	Y	7	Endo/peri	endo/peri	V		
			Y	Y	7	Endo	endo/peri	V		
			Y	Y	5	Peri	peri			
		Arterioles	Y	Y	14	Peri	peri	A		
			Y	Y	13	Peri	peri	A		
			Y	Y	9	Endo	endo/peri			
			Y	N				A		
	ESFM + PPP 23 hrs PM	Venules	Y	Y	11	Endo	endo			
			Y	Y	10	Endo	endo/peri			
		Arterioles	Y	N						
			Y	Y	19	Endo/peri	peri			
Retina 10	20% DMEM 23 hrs PM	Venules	Y	Y	8	Peri	endo/peri			
			Y	Y	9	Peri	endo/peri			
			Y	Y	7	endo	endo/peri			
			Y	Y	8	endo	peri			
		Arterioles	Y	Y	10	endo/peri	peri			
			Y	Y	12	peri	peri			
			Y	Y	8	endo	peri			
			Y	Y	8	endo	endo/peri			
	ESFM + PPP 23 hrs PM	Venules	Y	Y	8	endo/peri	endo/peri			
			Y	Y	7	endo	endo			
		Arterioles	Y	N						
			Y	Y	18	Endo/peri	Peri			

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 20	20% DMEM 25hrs PM	Venules	Y	Y	7	peri	Peri			
			Y	Y	8	endo/peri	Peri			
			N	N						
		Arterioles	Y	Y	8	endo/peri	Peri			
			Y	Y	12	peri	Peri			
			Y	Y	12	endo/peri	Peri			
Retina 21	20% DMEM 25hrs PM	Venules	Y	Y	6	endo	Peri			
			Y	Y	7	endo	Endo/peri			
		Arterioles	Y	Y	8	peri	Peri			
			Y	Y	8	peri	Endo/peri			
			Y	Y	9	endo	Endo/peri			
Retina 30	20% DMEM 25 hrs PM	Venules	Y	Y	13	peri	Peri			
			N	N				V		
		Arterioles	Y	N						
			Y	N						
	ESFM + PPP 25 hrs PM	Venules	Y	Y	7	endo	Endo	A		
			Y	Y	7	endo	Endo/peri			
		Arterioles	Y	Y	15	endo/peri	Peri			
			Y	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 31	20% DMEM 25 hrs PM	Venules	Y	Y	12	peri	Peri			
			Y	N						
			Y	N						
		Arterioles	Y	Y	12	peri	Peri			
			Y	N						
	ESFM + PPP 25 hrs PM	Venules	Y	Y	6	endo	endo/peri			
			Y	Y	6	endo	endo/peri			
		Arterioles	Y	N						
			Y	N						
Retina 32	ESFM + PPP 24 hrs PM	Venules	Y	Y	5	endo	endo			
			Y	Y	6	endo/peri	endo/peri			
			Y	Y	6	endo	endo/peri			
			Y	Y	7	endo	peri			
			Y	Y	7	endo/peri	peri			
		Arterioles	Y	Y	11	endo/peri	peri			
			Y	N						
			N	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 33	ESFM + PPP 24 hrs PM	Venules	Y	Y	4	endo	peri			
			Y	Y	5	endo	endo			
			Y	Y	5	endo	endo/peri			
			Y	Y	6	endo/peri	endo/peri			
		Arterioles	Y	Y	7	endo	endo/peri			
			Y	Y	8	Endo	endo/peri			
			Y	N						
Retina 34	ESFM + PPP 24 hrs PM	Venules	Y	Y	7	Endo	endo/peri			
			Y	Y	7	Endo	peri			
		Arterioles	Y	Y	7	Endo	endo/peri			
			Y	Y	7	Peri	peri			
			Y	N						
Retina 39	ESFM + PPP 24 hrs PM	Venules	Y	Y	11	Peri	peri	V		
			Y	Y	10	Peri	peri			
		Arterioles	Y	Y	12	Endo/peri	peri	A		
			Y	N						
			N	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 44	ESFM + PPP 24 hrs PM	Venules	Y	Y	5	Endo	endo/peri	V		
			Y	Y	6	Endo/peri	peri	V		
		Arterioles	Y	Y	10	Peri	peri	A		
			Y	N				A		
			Y	N						
Retina 45	ESFM + PPP 24 hrs PM	Venules	Y	Y	10	Endo/peri	endo/peri			
			N	N						
		Arterioles	Y	Y	7	Endo	endo/peri			
			Y	Y	7	Endo	endo/peri			
			Y	N						
Retina 48	ESFM + PPP 24 hrs PM	Venules	Y	Y	10	Peri	peri	V		
			Y	Y	12	Peri	endo/peri	V		
			Y	Y	12	Endo/peri	endo/peri	V		
		Arterioles	Y	Y	10	Endo	endo/peri	A		
			Y	N				A		
			Y	N				A		

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 49	ESFM + PPP 24 hrs PM	Venules (V1con)	Y	Y	7	endo/peri	endo/peri	V		
			Y	Y	4	Endo	endo	V		
			Y	Y	6	endo/peri	peri			
		Arterioles (A3con)	Y	Y	10	Endo	endo/peri	A		
			Y	N				A		
			Y	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 11	ESFM + 10% PPP 0.1% gel	Venules	Y	Y	10	endo	endo			++++/ 0
			Y	Y	7	endo	endo/peri			+++/ 0
		Arterioles	Y	Y	7	peri	endo/peri			+++ / 0
			Y	Y	8	endo	endo			+++ / 0
			Y	Y	8	endo	endo			+++ / 0
			Y	Y	7	endo	endo/peri			+++ / 0
			N	N						
Retina 12	Esfm + 10% PPP 0.1% gel 26 hrs PM	Venule	Y	Y	4	endo	endo			0 / ++++
			Y	Y	9	endo	endo			0 / ++++
		Arteriole	Y	Y	16	endo	peri			0 / ++++
			N	N						0 / ++
Retina 13	Esfm + 10% PPP 0.1% gel 26 hrs PM	Venule	Y	Y	9	endo	endo			
			Y	Y	13	endo	endo/peri			
		Arteriole	Y	Y	14	peri	peri			
			Y	Y	13	endo/peri	endo/peri			
Retina 14	Esfm + 10% PPP 0.1% gel 26 hrs PM	Venule	Y	N				V		
		Arteriole	Y	N				A		
			Y	N				A		
Retina 15	Esfm + 10% PPP 0.1% gel 26 hrs PM	Venule	Y	Y	18	peri	peri			
			Y	N						
		Arteriole	Y	Y	13	endo	endo/peri			
			N	N						

Sample	Conditions	Vessel type at dissection	Vessel No.	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 16	Esfm + 10% PPP 0.1% gel 24 hrs PM	Venule	151	Y	Y	7	endo	endo	V		
			152	Y	N						
		Arteriole	153	Y	Y	4	endo	endo	A		
			154	Y	Y	6	endo	endo			
Retina 17	Esfm + 10% PPP 0.1% gel 24 hrs PM	Venule	156	Y	Y	4	endo	endo			
			157	Y	Y	6	peri	endo/peri	V		
		Arteriole	158	Y	Y	6	endo	endo	A		
			159	N	N						
Retina 18	Esfm PPP 0.1% gel 24 hrs PM	Venule	160	Y	Y	4	endo	endo	V		
		Arteriole	161						A		
Retina 19	Esfm PPP 0.1% gel 24 hrs PM	Venule	162	Y	Y	4	endo	endo	V		
		Arteriole	163						A		
Retina 22	20% DMEM 0.1% gel 25 hrs PM	Venule	175	Y	Y	3	peri	endo			0 / 0
			176	Y	Y	4	endo	endo			0 / 0
		Arteriole	177	Y	Y	12	peri	peri			0 / 0
			178	N	N						0 / 0
Retina 23	20% DMEM 0.1% gel 25 hrs PM	Venule	179	Y	Y	3	endo	endo		- / +++	++++/+++
			180	Y	Y	3	endo	endo		- / ++++	+++/++
		Arteriole	181	Y	Y	5	endo	endo/peri		+++ / ++++	++/++
			182	Y	N					- / -	++/++

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 24	Esfm PPP 0.1% gel 25 hrs PM	Venule	Y	Y	6	endo	endo	V		
			Y	Y	5	endo	endo			
			N	N						
		Arteriole	Y	Y	12	endo	endo/peri			
			Y	Y	13	peri	peri	A		
			N	N						
Retina 25	Esfm PPP 0.1% gel 25 hrs PM	Venule	Y	N					- / -	++++/++++
		Arteriole	Y	Y	12	endo	endo/peri		++++/+	++++/++++
			Y	Y	12	peri	peri		+/++	+++/+++
			Y	Y	12	endo	endo		++/++	++++/+++
			Y	Y	13	endo	endo		++/++	+++/+++
Retina 26	Esfm PPP 0.1% gel 25 hrs PM	Venule	Y	Y	12	endo	endo	V		
			Y	Y	11	endo	endo/peri			
			N	N						
		Arteriole	Y	Y	13	endo	endo/peri			
			Y	Y	12	peri	endo/peri	A		
			Y	Y	12	peri	peri			
Retina 27	ESFM + 10%PPP 0.1% gel 24 hr PM	Venule	Y	N				V		
			N	N						
		Arteriole	Y	Y	8	endo	endo			
			Y	N						
Retina 28	ESFM + 10%PPP 0.1% gel 24 hr PM	Venule	Y	Y	6	peri	endo/peri	V		
			Y	Y	5	endo	endo/peri			
		Arteriole	Y	Y	5	endo	endo	A		
			Y	Y	6	peri	peri			

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 29	ESFM + 10%PPP 0.1% gel 24 hr PM	Venule	Y	Y	5	endo	endo	V		
			Y	Y	4	endo	endo			
			Y	Y	5	endo	endo	V		
			Y	Y	6	endo	endo			
		Arteriole	Y	Y	6	endo	endo/peri			
			Y	N						
Retina 35	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	4	endo	endo			0 / 0
			Y	Y	5	endo	endo/peri			0 / 0
			Y	N						0 / 0
		Arteriole	Y	Y	7	endo	endo/peri			0 / 0
			Y	Y	6	endo	endo			0 / 0
			N	N						0 / 0
Retina 36	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	4	endo	endo		++/++++	+++/+++
			Y	Y	5	endo	endo/peri		++++/++++	++/+
			Y	Y	4	endo	endo		+++/+++	+++/+++
		Arteriole	Y	N					- / -	++++/++++
			Y	N					- / -	++++/++++
			N	N						
Retina 37	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	endo			
			Y	Y	5	unknown	peri			
			Y	Y	4	endo	endo			
		Arteriole	Y	Y	5	endo	endo/peri			
			Y	Y	7	endo	peri			
			Y	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 40	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	endo	V		
			Y	Y	4	endo	endo			
		Arteriole	Y	Y	3	endo	endo	A		
			Y	Y	5	peri	endo/peri			
			Y	N						
Retina 41	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	peri	V		
			N	N						
		Arteriole	Y	Y	5	endo	endo/peri	A		
			Y	Y	4	endo	endo			
Retina 42	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	endo			
			Y	Y	3	endo	endo			
		Arteriole	Y	Y	3	endo	endo			
			Y	Y	3	endo	endo			
Retina 43	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	endo			
			Y	Y	4	endo	endo			
		Arteriole	Y	Y	3	endo	endo/peri			
			Y	Y	7					
Retina 46	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	endo	V		
			Y	Y	5	endo	endo/peri	V		
			Y	Y	3	endo	endo			
		Arteriole	Y	Y	13	peri	peri	A		
			Y	N						
			N	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina	ESFM + 10%PPP 0.1% gel 24 hrs PM	Venule	Y	Y	3	endo	endo			
			Y	N						
			N	N						
		Arteriole	Y	Y	10	peri	peri			
			Y	N						
			N	N				A		
Retina 4	20% DMEM 0.1% collagen 3hrsPM	Venules	Y	Y	4	endo	Endo			
			N	N						
			N	N						
		Arterioles	Y	Y	7	endo	Endo/peri			
			Y	N						
Retina 5	20% DMEM 0.1% collagen 5hrsPM	Venules	Y	N						
			Y	Y	5	endo	Endo			
		Arterioles	Y	N						
			Y	Y	8	Endo	endo/peri			
			N							
Retina 11	Esfm +PPP 0.1% collagen 24hrsPM	Venules	Y	Y	7	Endo	endo		+++++++	+++/+++
			Y	Y	7	Endo	endo		+/++++	++++/++
			Y	N						
			Y	N						
		Arterioles	Y	Y	4	Endo	endo		+/++++	++++/++
			Y	Y	6	Endo	endo		- /++++	+++/+
			N	N						+++/++

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 30	Esfm +PPP 40ug/ml Fn 25hrs PM	Venules	Y	Y	9	Endo	peri			
			Y	N						
		Arterioles	Y	Y	12	Endo	peri			
			N	N						
Retina 31	Esfm +PPP 40ug/ml Fn 25hrs PM	Venules	Y	Y	15	Peri	peri			0 / 0
			N	N						0 / 0
		Arterioles	Y	Y	9	Peri	unknown			0 / 0
			Y	N						0 / 0
Retina 32	Esfm +PPP 40ug/ml Fn 25hrs PM	Venules	Y	Y	10	Peri	endo/peri		+++++/++	+++/+++
			Y	Y	12	Peri	endo/peri		+++++/++	+++/+++
			Y	N				V		
		Arterioles	Y	Y	7	Endo	endo/peri		+++++/+++	+++/+++
			Y	Y	7	endo/peri	peri		+++++/++	+++/+++
			N	N				A		
Retina 33	Esfm +PPP 40ug/ml Fn 25hrs PM	Venules	Y	Y	9	Peri	peri			
			Y	Y	8	Peri	peri			
			N	N						
		Arterioles	Y	Y	8	Peri	peri			
			N	N						
Retina 38	Esfm +PPP 40ug/ml Fn 26hrs PM	Venules	Y	Y	5	Peri	endo/peri		+++/+++	+++/+++
			Y	Y	7	Peri	peri/ unknown		+++++/+	+++/+++
		Arterioles	Y	Y	12	Peri	unknown		+++++/ -	+++/+++
			Y	Y	12	Peri	endo/peri		+++/+++	+++/+++
			Y	N					-/ -	+++/+++

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 39	Esfm +PPP 40ug/ml Fn 26hrs PM	Venules	Y	Y	5	Unknown	peri			
			Y	N						
		Arterioles	Y	Y	12	Unknown	peri			
			Y	N						
Retina 30	Esfm +PPP 20ug/ml Ln 25hrs PM	Venules	Y	Y	5	Endo	endo/peri			
			Y	Y	4	endo/peri	endo/peri			
		Arterioles	Y	Y	7	Peri	endo/peri			
			Y	N						
Retina 31	Esfm +PPP 20ug/ml Ln 25hrs PM	Venules	Y	Y	9	Peri	peri			
			Y	N						
		Arterioles	Y	Y	9	Peri	endo/peri			
			Y	N						
Retina 32	Esfm +PPP 20ug/ml Ln 25hrs PM	Venules	Y	Y	9	endo/peri	peri			
			Y	Y	9	endo/peri	endo/peri			
			N	N						
		Arterioles	Y	N						
			Y	Y	9	peri	Peri			
			N	N						
Retina 33	Esfm +PPP 20ug/ml Ln 25hrs PM	Venules	Y	Y	9	peri	Endo/peri			0 / 0
			Y	Y	9	peri	Endo/peri			0 / 0
			N	N						
		Arterioles	Y	Y	5	endo	Peri			0 / 0
			Y	N						

Sample	Conditions	Vessel type at dissection	Vessel No.	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 38	Esfm +PPP 20ug/ml Ln 26hrs PM	Venules	339	Y	Y	8	peri	Endo/peri		+++/+	+++/+++
			340	Y	Y	12	endo	Endo		+/+++	+++/+++
		Arterioles	341	Y	Y	5	endo	Endo		+/+++	+++/+++
			342	Y	Y	8	endo/peri	Endo/peri		+++/+++	+++/+++
Retina 39	Esfm +PPP 20ug/ml Ln 26hrs PM	Venules	356	Y	Y	5	unknown	Endo/peri		++++/++	+++/+++
			357	Y	Y	7	unknown	Endo/peri		++++/+++	+++/+++
		Arterioles	358	Y	N						
			359	Y	N						
Retina 30	Esfm +PPP 0.5ug/ml Vn 25hr PM	Venules	230	Y	Y	5	endo	Endo/peri		+++/++	+++/+++
			231	Y	Y	5	endo	Endo/peri		+++/++	+++/+++
		Arterioles	232	Y	Y	5	endo	Endo/peri		+++/+++	+++/+++
			233	Y	Y	7	endo	Endo/peri		+++/+++	+++/+++
Retina 31	Esfm +PPP 0.5ug/ml Vn 25hr PM	Venules	248	Y	Y	9	endo/peri	Endo/peri			0 / 0
			249	Y	Y	9	endo/peri	peri			0 / 0
		Arterioles	250	Y	Y	9	endo/peri	peri			0 / 0
			251	Y	Y	9	endo	endo/peri			0 / 0
Retina 32	Esfm +PPP 0.5ug/ml Vn 25hr PM	Venules	281	Y	Y	7	endo	endo/peri		+++/+++	+++/++++
			282	Y	Y	7	endo	endo/peri		++/++++	+++/+
			283	Y	Y	7	endo	endo		+++/+++	+++/+++
		Arterioles	284	Y	Y	7	endo/peri	endo/peri		++/+++	+++/++
			285	Y	Y	9	endo	endo		-/+++	+++/+++
			286	Y	Y	9	endo	endo/peri		++++/++	+++/++

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 33	Esfm +PPP 0.5ug/ml Vn 25hr PM	Venules	Y	Y	7	endo	endo/peri			0 / 0
			Y	Y	9	endo	endo			0 / 0
			Y	Y	9	endo	peri			0 / 0
		Arterioles	Y	Y	7	endo	endo/peri			0 / 0
			Y	Y	7	endo	endo/peri			0 / 0
			Y	Y	7	endo	endo/peri			0 / 0
Retina 38	Esfm +PPP 0.5ug/ml Vn 25hr PM	Venules	Y	Y	8	endo	endo		+++/+++	+++/+++
			Y	Y	8	endo/peri	endo/peri		+++/+++	++++/+++
		Arterioles	Y	Y	8	endo/peri	endo/peri		+++/+++	+++/++++
			Y	Y	12	endo/peri	peri		+++/+	++/+++
Retina 39	Esfm +PPP 0.5ug/ml Vn 25hr PM	Venules	Y	Y	5	endo/peri	endo/peri		++/++	+++/+++
			Y	Y	5	endo/peri	endo/peri		++/++	+++/+++
		Arterioles	Y	Y	12	peri	endo/peri		+++/+++	+++/+++
			Y	Y	11	endo/peri	peri		++++/++	++++/++++
Retina 44	Esfm +PPP BFGF 5ng/ml	Venules	Y	Y	6	peri	endo/peri		V	
			Y	Y	3	peri	endo/peri		V	
			Y	Y	5	peri	endo/peri			
		Arterioles	Y	Y	5	endo	endo/peri		A	
			Y	Y	10	peri	endo/peri		A	
			Y	N						
Retina 45	Esfm +PPP BFGF 5ng/ml	Venules	Y	Y	3	endo	endo/peri			
			Y	Y	5	peri	Endo/peri			
			Y	Y	6	endo/peri	endo/peri			
		Arterioles	Y	Y	3	endo	endo/peri			
			Y	N						
			Y	N						

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 48	Esfm +PPP BFGF 5ng/ml	Venules	Y	Y	4	endo	Endo/peri		V	
			Y	Y	6	endo	Endo/peri		V	
			Y	Y	7	Peri	Endo/peri		V	
		Arterioles	Y	Y	5	endo	Endo/peri		A	
			Y	N					A	
			Y	N					A	
Retina 49	Esfm +PPP BFGF 5ng/ml	Venules	Y	Y	7	peri	Endo/peri			
			Y	Y	6	endo	Endo/peri			
			Y	Y	7	peri	Peri			
		Arterioles	Y	Y	10	endo	Endo/peri			
			Y	N						
			Y	N						
Retina 44	Esfm +PPP VEGF 5ng/ml	Venules	Y	Y	7	endo/peri	endo/peri		+++/+++	+++/+++
			Y	Y	6	endo/peri	Peri		+++/+	+++/++++
			Y	Y	7	endo/peri	endo/peri		++/++	+++/+++
		Arterioles	Y	Y	10	endo/peri	endo/peri		+/++++	+++/+++
			Y	Y	10	endo/peri	endo/peri		+++/+++	+++/+++
			Y	Y	12	endo/peri	endo/peri		+++/+++	+++/+++
Retina 45	Esfm +PPP VEGF 5ng/ml	Venules	Y	Y	6	endo/peri	endo/peri			
			Y	Y	6	endo/peri	endo/peri			
			Y	Y	7	endo/peri	endo/peri			
		Arterioles	Y	N						
			Y	Y	11	endo/peri	endo/peri			
			Y	Y	12	peri	endo/peri			

Sample	Conditions	Vessel type at dissection	Attached (Y/N)?	Outgrowth (Y/N)?	Time 1st outgrowth observed (days)	Morphology of cell type of 1st outgrowth	Morphology of cell types at end of culture	Histology (A/V)?	Immunostaining results.	Controls
Retina 46	Esfm +PPP VEGF 5ng/ml	Venules	Y	Y	4	Endo	endo/peri	V		
			Y	Y	5	Peri	endo/peri			
			Y	Y	4	endo/peri	endo/peri			
		Arterioles	Y	Y	10	Endo.peri	Endo/peri	A		
			Y	Y	12	peri	Endo/peri			
			Y	N						
Retina 47 0	Esfm +PPP VEGF 5ng/ml	Venules	Y	Y	4	Endo	endo/peri	V		
			Y	Y	10	endo/peri	endo/peri			
			Y	Y	12	endo/peri	endo/peri			
		Arterioles	Y	Y	7	endo/peri	endo/peri	A		
			Y	Y	10	endo/peri	endo/peri			
			Y	N	12					

Key: - endo = endothelial cell-like morphology; peri = pericyte-like morphology; V= venule, A= arteriole confirmed by histological charaterisation.

+ = detection of α- smooth muscle actin antigen; + = detection of von Willebrand factor antigen (the number of +'s indicates staining intensity)
- / - indicates no staining detected in test cultures; 0 / 0 indicates no fluorescence detected in controls; controls used include omission of primary and secondary antibody.