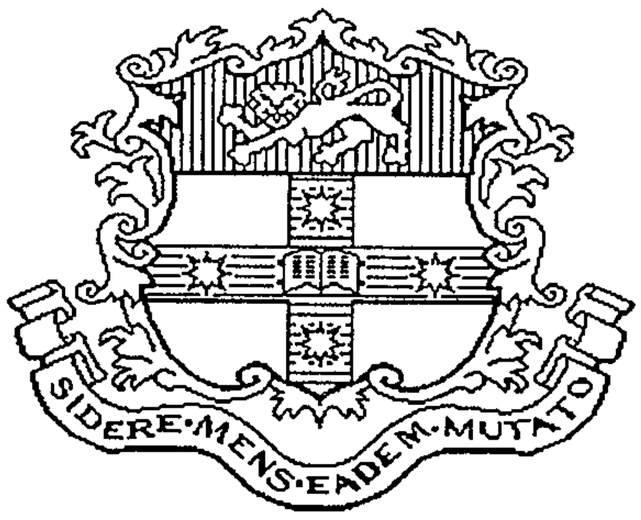
THE INFLUENCE OF BASEMENT MEMBRANE PROTEINS ON RE-VASCULARIZATION NETWORKS FORMED AFTER ACUTE INJURY



ROSEMARY LOUISE GARTON

BDS (London) LDS RCS (England)

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Dedication

To my husband

Justin for his encouragement, humour and unwavering support to undertake this Master's degree, while simultaneously achieving success in his own career.

I owe you many brownie points.

To friends and family

For their humour, sympathy, support and ideas.

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Declaration of Authorship

This is to certify that the work presented in this thesis was carried out by the author, Rosemary Louise Garton, who was a postgraduate candidate for the Master of Dental Science, in the discipline of Orthodontics, School of Dental Science, Faculty of Dentistry, University of Sydney, and that the work has not been submitted to any other university or institution for a higher degree.

31st July 1997

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Rosemary Louise Garton

Abstract

In most cases of orthodontic tooth movement the potential for vascular damage occurs as the periodontal ligament capillaries are stretched or compressed. Angiogenesis, the formation of new blood vessels from the established microvasculature, is a minimal requirement for tooth movement and for a continuing source of vascular cells. The mechanism by which angiogenesis occurs has not been well investigated. It has been proposed that basement membrane proteins may influence the formation of a re-vascularization network, after an episode of acute injury (Vracko and Benditt, 1972).

A vascular model was developed using human umbilical vein endothelial cells cultured in the presence of an extracellular matrix (Matrigel) which has the capacity to initiate vascular cell differentiation, to form capillary-like networks.

Vascular networks denuded of cells by hypotonic lysis were subsequently re-seeded using an identical number of endothelial cells. The networks were monitored at fifteen minute intervals for two hours. The original and final profiles were compared visually and by computer-assisted manipulation and superimposition. Utilising photographs of cross-sectioned samples embedded in glycol methacrylate taken at variable focal levels, the three dimensional relations were shown. Antibodies to human type IV collagen and human laminin demonstrated the relative importance of these proteins in initial formation and re-establishment of the network.

The two-dimensional network formed on the surface of the extracellular matrix, causing a significant structural effect on the gel underneath, with surface penetration occurring after a considerable period. Rapid endothelial cell death left residual basement membrane that showed a capacity to guide network regeneration. Type IV collagen appeared to be essential for this guidance while laminin was essential for tube formation.

The model proved satisfactory, with some limitations, for investigating network formation and network reinstatement after acute injury. The data support the concept that previous basement membrane proteins influence the re-vascularization network formation after simulated acute injury.

Introduction

Different tissues and organs, such as connective tissue, muscle and bone, have clearly defined and spatially arranged blood supplies, which are adapted to the metabolic requirements of the respective tissues.

In healthy persons it has been found that nailbed capillary patterns are reproducible and comparable over a three year observation period, and that each capillary could be easily recognised (Vayssairat *et al.,* 1982). This suggests that individual network patterns exist and that the maintenance of a constant vascular pattern is important.

The periodontal ligament and its microvascular supply are situated between two essentially immobile structures, cementum and alveolar bone. The unique location, structure and function of this tissue implies strict requirements concerning the anatomical arrangement of the blood vessels. The minimum requirement for tooth movement is the maintenance of an intact vascular system, to provided a constant source of cells and nutrients.

The vasculature of the periodontium can be disrupted by various stimuli including inflammatory disease, as well as tensile and pressure forces. In relation to orthodontic manipulation, the vascular network of the periodontal ligament is of particular interest, since orthodontic force can easily compress the ligament and vessels, resulting in inactivation of the cellular components of the ligament and eventually ligament cell death (Gianelly, 1971). Tensile and pressure forces produce changes in alveolar vascular activity and rate of collagen formation and degradation. The triggering agents, including cytokines and neurotransmitters, have the capacity to initiate an inflammatory response (Davidovitch *et al.*, 1988).

When cells die and drop out of this system, as a result of senescence or injury, organisms have the ability to repair the ensuing defect. If cellular relations are to be re-established as they existed prior to injury, the replacing cells and extracellular elements must be positioned in the exact spatial relations, involve the same cell types and closely approximate the original numbers of cells prior to injury.

In small wounds, closure is achieved by independent cell migration (Wong and Gottieb, 1988). Experiments on the regeneration of rat and rabbit

skeletal muscle after injury with, freezing, ischaemia or *in situ* autografting, showed that each type of injury produced complete necrosis of cells, leaving the basement membrane intact. This maintained a map of the spatial relations between the muscle fibres and capillaries, which could provide a physical substrate for the growth and orientation of any proliferating cells (Volkman, 1893).

Repopulation of the defect with new cells by growth or migration along the scaffold of *basal lamina* re-established the spatial relations as they existed prior to injury. This process occurred within 3 weeks, as long as the *basal lamina* remained physically intact (Vracko and Benditt, 1970; 1972). Spatial relations were maintained even when portions of the tissue were excised and reimplanted at right angles to the original orientation (Clark, 1946).

If, however, the *basal lamina* scaffold was destroyed by either severe crushing, cauterisation with heat or strong acids, by infection or totally removed and replaced by a fibrin clot, orderly reconstruction did not occur, resulting in the formation of a scar and loss of function.

Angiogenesis is the neovascularization or formation of new blood vessels from an established microcirculation by the sprouting from pre-existing vessels. It is a complex process mainly quiescent in adults, but it may occur rapidly in several circumstances and involves a number of steps, including:

- activation of endothelial cells and pericytes
- detachment of endothelial cells from the vascular wall of the parent vessel (typically a capillary or postcapillary venule)
- degradation and penetration of the *basal lamina* that invests the cells
- invasion of the surrounding interstitial extracellular matrix (ECM) as a vascular bud or sprout, which lengthens by migration and proliferation of endothelial cells and pericytes
- lumen formation, along with the appearance of pericytes and the development of a new basement membrane
- organisation of the network into larger microvessels (Ausprunk and Folkman, 1977; Paka and Paweletz, 1991).

In the studies reported in this thesis a model of vascular network formation *in vitro* was used to investigate the hypothesis that basement membrane proteins, remaining after detachment or death of the endothelial cells, provide a template for new network formation following re-seeding with endothelial cells. Identification of important components and evaluation of the fidelity of the model were further objectives.

Aims and Objectives

Specific Aims

1. To develop a model *in vitro* to obtain data relating to vascular pattern formation by cultured human umbilical vein endothelial cells (HUVEC).

2. Determine the relation of the cultured endothelial cell network to extracellular matrix components.

3. Determine the role of the basement membrane in re-vascularization.

4. Determine any differences in the influence of an immature versus mature basement membrane network on re-vascularization.

Hypothesis to be tested

That post-traumatic re-vascularization is guided by the presence of a previous basement membrane network.

Demonstrated by:

- re-seeding of cultures denuded of cells by hypotonic lysis will leave the previous basement membrane and result in the re-establishment of an identical vascular network in all three dimensions.

- human collagen type IV identification by the new HUVECs is essential in establishing an identical new network formation. Human laminin is essential for tube formation.

Review of Literature

An understanding of the biological principles that influence a vascular pattern or re-vascularization network developing in a particular location is essential in the clinical practice of orthodontics and for its development as an academic discipline.

Specialised vascular patterns have been identified and quantified over the last sixty years. Most of the investigations have focused on the gross patterns of arteries, arterioles, veins and venules in relation to organs and tissues of the human (Grant, 1937) and various other animals including Maccaca fuscata monkeys (Ohkuma and Ryan, 1982), rats (Hossler, Douglas and Douglas., 1986), pigs (Van den Ingh and Van der Linde-Sipman, 1986) and rabbits (Caggiati *et al.*, 1992). Krey (1975) found that choridal human eye flat had a distinct segmental capillary layer arrangement with individual lobules supplied by peripheral arteriole branches and drained by a central venule originating in the centre of a star-shaped capillary system. Individual vascular units were interconnected by several capillaries crossing interlobular arterioles. Other organs have been identified as having distinctive microvascular patterns; the metabolic demands of the tissue appear a primary determining factor of vascular density and structure.

The microstructure in various tissues has been found to vary in the presence or absence of coils and in the structure of the walls in relation to factors such as: thickness and fenestration density. The actual vessel density appears to vary over time and with disease or change in function.

There have been few studies on the individual variation of microvascular pattern. Krylova and Soboleva (1995) noticed a marked individual variability of morphometric parameters and contours of the capillary layers in human nailbeds. This supported studies on the microvasculature pattern of the human hand skin by Maricq in 1977.

There follows a review of the literature on the vascular patterns of the periodontium, including the determinants of pattern development, the effect of inflammation, vascular repair and of angiogenesis. These aspects will be examined to give a better understanding of the role of the previous vascular network in the re-vascularization occurring after an episode of acute injury.

Periodontal ligament

The periodontal ligament is unique among the connective tissues in the mammalian body with an ability to tolerate high intermittent pressures during mastication as well as continuous pressure during orthodontic treatment. The ligament also generates proprioceptive information and has an ability to remodel and allow tooth movement and eruption.

The primary role of the periodontal ligament micro-vasculature is the provision of nutrients and removal of toxic material, as well as being implicated in mechanisms for tooth support and eruption (Moxham and Berkovitz, 1982; Berkovitz, 1990)

Morphology of the periodontal ligament microvasculature

The periodontal membranes of the maxillary teeth are supplied by branches of the superior alveolar artery and the palatine vessels via the palatal mucous membrane. The mandibular teeth are supplied by the mandibular and lingual arteries. The coronal periodontal ligament vessels branch and anastomose with vessels supplying the gingival tissue providing communication between medulla of the bone and the coronal third of the periodontal ligament (Schroeder, 1986).

The structure of the capillary network appears to depend on the characteristics of the tissue supplied, rather than the origin of the supplying vessel (Kindlova, 1965).

Kindlova and Matena (1962) first discovered the vascular pattern of the periodontal membrane in dogs, using corrosive preparations and histological sections (Figure 1). The vascular network of the periodontal ligament consists principally of a venous system made up of axially aligned, post-capillary sized venules (PCV) and collecting venules (CV) arranged in palisades located adjacent to the bony wall (Weekes and Sims, 1986). Branches are given off towards the tooth and from these, capillaries arise which are arranged in a flat plexus. These vessels are located nearer to the alveolar bone than the root surface (Birn, 1966).

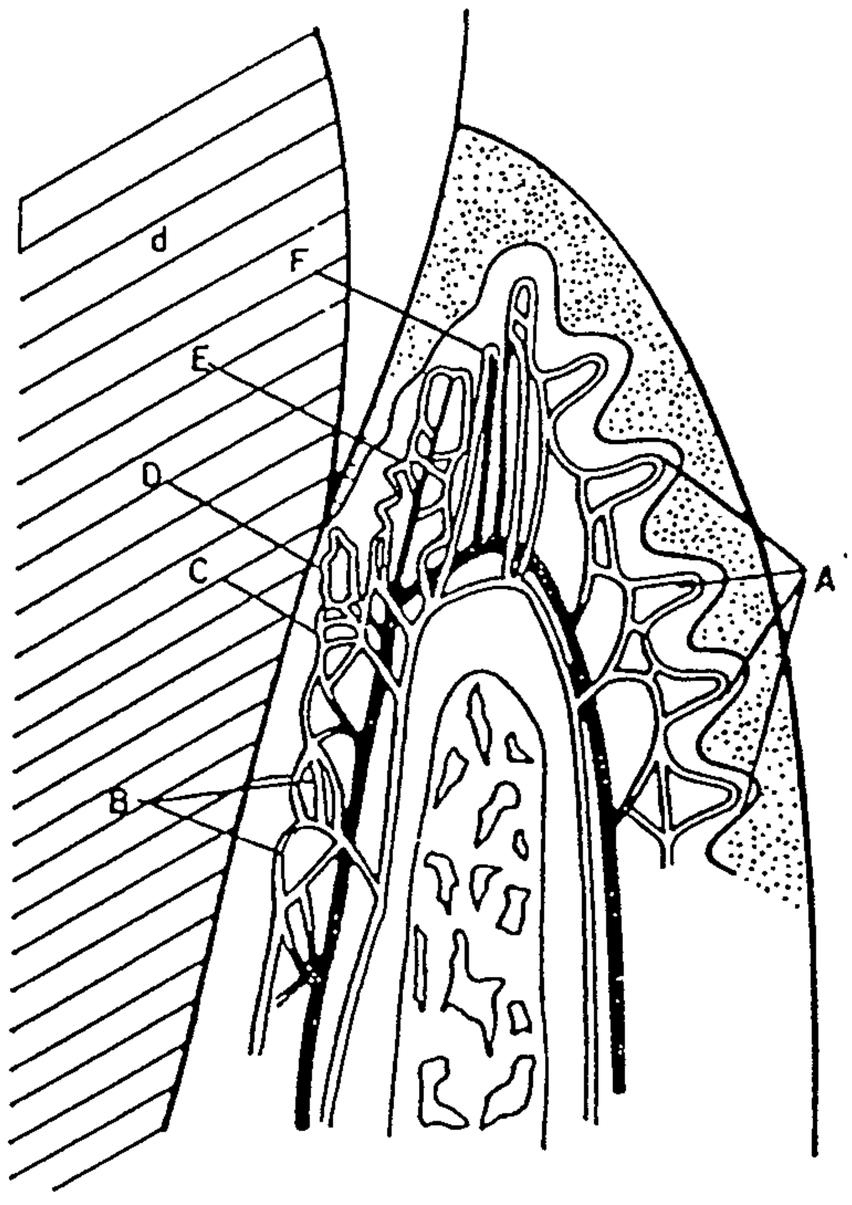


Figure 1: Schematic representation of the blood supply of the periodontal membrane and the gingival tissue.

(Kindlova 1965).

A: subepithelial network of gingiva.
B: capillary network of the periodontal membrane
C: denser capillary network in the cervical part of the periodontal membrane
D: coiled capillaries resembling glomeruli
E: capillary loops with coiled arterial part
F: simple capillary loops

In the cervical part of the periodontal membrane this pattern is modified so that the capillary vessels form a denser coronal network. From these vessels, single capillary tubes are given off which form coiled structures resembling glomeruli and then return to the originating network. More coronally still, looped capillaries with coiled arterial parts are found in the region of the epithelial attachment (Kindlova, 1965).

Blood vessels perforate the *lamina dura* and form anastomoses between the main periodontal vessels and those of the Haversian system and cancellous tissue of the alveolar bone. These vessels probably provide escape routes for blood when the ascending vessels of the periodontal membrane are compressed as a result of the pressure exerted during mastication. The vessels that perforate bone originate from branches of either the apical or gingival vessels. Larger vessels perforate the bone around the margins of the tooth sockets especially in the molar region (Kindlova, 1965).

Vascular volume as a percentage of the total periodontal ligament varies from 7% in an adult mouse molar, up to 50% near the growing base of a rat incisor (Moxham and Berkovitz, 1983). Volumes also vary with the presence or absence of eruptive tooth development. Blood vessel distribution varies according to tooth type and depth with predominance in the third next to the bone, diminishing as the tooth is approached (Rhodin, 1967). Reliable anatomical-physiological-type experiments to determine properties such as rates of blood flow have yet to be developed.

Characteristically, the vessels of the ligament are thin walled and have a large lumen volume to wall ratio. They are surrounded by loose connective tissue and pass between the clearly defined bundles of principle fibres of the periodontal membrane running partly in grooves in the alveolar bone.

Three specific microvascular patterns have been identified in rat molar periodontal ligament (Weekes and Sims, 1986).

- *Cervical :* principle vessels course occluso-apically in tracts of 3-6 vessels; each tract comprises intervening capillary networks.
- *Middle third:* characterised by vertically orientated capillary loops connecting with arterial and venous vessels.
- *Apical third:* distinct, irregular anastomosing system.

These investigations indicate for the periodontal membrane a non-homogeneous distribution of vessels, with an unusually high proportion of venous arterioles and postcapillary venules.

Classification of postcapillary vessels

Endothelial cells are a heterogeneous population and it is now recognised that large vessel endothelial cells differ from microvascular endothelial cells (Bicknell, 1993). Microvascular post-capillary vessels consist of flattened endothelial cells that lie on a *basal lamina* surrounded by an interrupted layer of pericytes which in turn are invested in an extracellular matrix.

Bennett, Luft and Hampton (1959) recommended a classification based on, the presence or absence of continuous basement membrane, presence and positions of fenestrations and presence or absence of pericytes. Later classification was based on lumen calibre, endothelial cell lining, and pattern of vessel branching and anastomoses as well as the components of the blood vessel wall. Rhodin (1968) suggested that the lumen to wall ratio should be around 1:10 for post-capillary vessels.

Sims (1983) reported that 70 % of post-capillary-sized venules had no pericytic cellular investment. Freezer and Sims (1987) devised a classification for microvessels of mouse periodontal ligament, based on the vessel diameter and the appearance of pericytes.

Endothelial identification

Endothelial cells have prominent nucleoli, a mitochondria-rich perinuclear region, endoplasmic reticulum, Golgi complexes and attenuated peripheral cytoplasm containing free ribosomes and micropinocytosis vesicles. The peripheral cytoplasm often contains abundant microfilaments. The chief characteristic is the presence of a large number of Weibel-Palade bodies (WPBs), which are membrane-bound, rod shaped granules containing parallel arrays of 6-26 tubular structures. These bodies produce and store von Willebrand factor (VWF) and the cell adhesion molecule P-selectin (also known as PADGEM, GMP-140 and CD62) (Koedam *et al.*, 1992). Stimulation of endothelial cells causes simultaneous translocation of the granule membrane protein P-selectin and rapid fusion of WPBs with the cell surface membrane, resulting in the exocytic release of VWF (Osborn, 1990; Pober and Cotran, 1990).

The VWF marker provides characteristic identification of endothelial cells (Weibel and Palade, 1964) resulting in the whole cell (except the sprout tips) being stained. If TRITC-phalloidin stain is used, strong bonds to the total endothelial cell body, including the margins and distal extensions, may provide a complete view of the actin networks (Nehls, Denzer and Drenchkhahn, 1992).

Endothelial cell components

Endothelial cells line the wall of blood vessels usually contacting the extracellular matrix of the basement membrane and contain collagen IV and V. The endothelial basement membranes are composed of collagen IV, heparan sulfate proteoglycan and glycoproteins laminin and nidogen / entactin. Collagen has adhesion-promoting activity, while laminin has been demonstrated to have potent actions on cells by stimulating adhesion, growth, differentiation and migration (Grant, Heathcote and Orkins, 1981; Kleinman *et al.*, 1985).

Functional aspects of the periodontal ligament circulation

The primary role of the blood circulation in the periodontal attachment is to provide exchange of nutrients and metabolites between the blood and the periodontal tissue, including dentine. It is believed that the blood circulation also has a role in proprioception, tooth eruption and tooth support (Berkovitz and Moxham, 1995).

An in-depth knowledge of junctions, vesicles, fenestrae and tissue channels would allow an understanding of the role of the vasculature and the mechanism by which the structure facilitates the rapid transfer of fluid between vessels and the extravascular matrix (Casley-Smith, 1983).

The different arrangements of the capillary systems in the periodontal membrane and gingivae are probably due to the different metabolic demands of these tissues and the distance from capillary to tissue over which oxygen must diffuse (Henquell *et al.,* 1977).

Wedl (1881) suggested that the tightly coiled capillaries in the connective tissue adjacent to the coronal aspect of the periodontal membrane provide a resilient cushion which counteracts masticatory pressure. However, as Boehl (1954) noted, the thick collagen fibres of the periodontal ligament are far more resilient to compression and tension than the thin walled capillary loops, and so are more likely to resist this pressure.

Basement membranes

All microvessels have a basement membrane, composed of an amorphous laminar structure in immediate contact with various cell types. The capillary basement membranes interphase the cell layer and the underlying connective tissue (Vracko, 1974).

Basement membranes were originally identified by staining for glycoproteins (periodic acid-Schiff reaction). Later, electron microscopy revealed a three-layered structure, one of which is an electron-lucent *lamina rara* (or *lucida*) with a thickness of 10-50 nm closely apposed to the cell plasma membrane. Chan and Inoue (1994), suggested that this layer was really an artefact of conventional tissue preparation. This layer is in close contact with the *basal lamina* (*lamina densa* or basement membrane) proper, which has a thickness of 30-300 nm and is composed of fine filaments and globules. The region underlying the *basal lamina* is the *lamina fibroreticularis,* which is poorly defined and contains many fibrils, providing contact to the underlying connective tissue.

Basement membranes are highly insoluble and are composed of approximately 90% protein (containing equal amounts of collagen-like and non-collagen proteins, including various glycoproteins) and 8% carbohydrate, with little or no lipid content. Despite research using large quantities of basement membrane produced during subcutaneous propagation by tumour models, such as mouse Engelbreth-Holm-Swarm tumour, the full analysis of a typical membrane is still unknown, but up to 50 different components have been discovered up to date (Orkin *et al.*, 1977).

Basement membranes are relatively stable structures in adult organisms, with a turnover ranging from 40 days in the colon to 2 years in the skin. Situations of rapid remodelling are well known, and may be due to increased cellular activity and/or loss of a stabilising extracellular environment (Bernfield, 1981). The role of basement membrane in angiogenesis will be discussed later.

The thickness of basement membrane is uniform for a given structure, but differs between structures and species. The membrane appears thinner in distended capillaries than constricted ones. When a basement membrane is denuded of cells, it becomes a pliable sheet which crumples readily, forming folds and pleats with some degree of elasticity (Vracko, 1970).

Basement membrane functions include aiding cell adhesion, cell proliferation and differentiation, tissue morphogenesis, guidance in regeneration and repair by controlling cell migration and invasion (Vracko, 1974), as well as maintaining tissue architecture and forming a filtration barrier for the blood vessels (Timpl and Dziadek, 1986).

The function of the basement membrane as a scaffold for orderly cell positioning appears to have an important role in the pathogenesis of disorders associated with so-called thickening of the basement membrane. Almost invariably, increased investment with basement membrane is composed of multiple layers of the membrane, rather than of a continuous and homogeneous widening of a single basement membrane layer. Examples includes, aging and diabetes mellitus (Ashton, 1974) and the response to skeletal muscle injury in nondiabetic animals (Vracko and Benditt, 1972; Timpl and Dziadek, 1986).

The initial distance between the plasma membrane of the newly formed cell and the old basement membrane determines whether a new basement membrane is laid down. If the plasma membrane is immediately apposed to the old basement membrane, then a new basement membrane is not formed and the old basement membrane layer becomes the basement membrane for the new cells. Alternatively, the old basement membrane may be separated from the plasma membrane of the new cell by some distance, due to cell debris or the old basement membrane being thrown into folds and the plasma membrane of the new cell touching only the innermost surfaces of the basement membrane folds and ridges. In this case, the new cells develop a new layer of basement membrane. Wherever this occurs, the cell is lined by two or more layers of basement membrane with any cell debris encased between the two layers, as commonly occurs in regenerating capillaries of skeletal muscle (Vracko and Benditt, 1972).

The original basement membrane of skeletal muscle fibres remains intact long enough to provide the template for the reconstruction of the damaged muscle. Removal of old redundant basement membrane layers appears to be mediated by interstitial fibroblast-like cells applied to the outer surfaces of skeletal muscle fibres. This removal is more effective in skeletal muscle fibres than in skeletal muscle capillaries, resulting in fibres generally having a single basement membrane layer and capillaries having two layers one month after healing is completed (Vracko and Benditt, 1972).

Collagen IV

Type IV collagen is a major constituent of basement membranes, together with laminin and heparan sulphate proteoglycans (Martinez-Hernandez, and Amenta, 1983). It consists of a triple helix 340 nm long, which is highly cross-linked by disulfide bridges and non-reducible lysine-derived bonds. These numerous bonds contribute to the mechanical stability of the basement membrane. Brief digestion with pepsin or bacterial collagenases causes the collagen to become soluble (Timpl *et al.*, 1978; Kuhn *et al.*, 1981).

The high flexibility of type IV collagen contributes to the formation and elasticity of basement membrane. There may be tissue-specific heterogeneity in the organization of collagen aggregates in basal laminae. This has been demonstrated in recent work with the restricted localisation of new laminin-like glycoproteins, such as S-laminin in basal laminae (Kino *et al.*, 1991).

Laminin

Laminin is a high molecular weight glycoprotein present in all basement membranes and, like collagen IV, it possesses a multi-domain structure apparently capable of fulfilling many biological functions (Yamada, 1983, Kleinman *et al.*, 1985; Nicosia *et al.*, 1994). Laminin provides two different mechanisms for cell adhesion, one forming bonds directly to fibroblasts and, via laminin receptors, to other membrane components (Timpl and Dziadek, 1986). Laminin promotes adhesion, attachment, migration, proliferation and differentiation of endothelial cell phenotype in neovascularization (Form *et al.,* 1986; Kubota *et al.*, 1988; Nicosia *et al.,* 1994).

Laminin has an asymmetrical cross-shaped structure, with one long rod-like arm terminating in a globular domain and three shorter arms each containing two globules in terminal and central positions. The precise arrangement of the constituent chains and inter-chain disulfide bridges is not yet known. Some regions active in cell attachment have been sequenced (Graf *et al.*, 1987).

Studies *in vitro* indicate that newly forming vessels deposit the basement membrane components laminin and type IV collagen independently (Yurchenco, Cheng and Colognato, 1992). Initial secretion of laminin is

followed by type IV collagen at the same time as lumen formation occurs (Kramer *et al.*, 1984; Niscosia and Madri, 1987).

Fibronectin

Fibronectin is one of the abundant extracellular matrix large glycoproteins with similar biological properties to laminin (Yamada, 1983). It consists of two very similar polypeptide chains connected to each other by disulfide bonds at the C-terminal ends.

Cultured endothelial cells synthesise fibronectin (Schoefl, 1963; Jaffe and Mosher, 1978; Birdwell, Gospodarowicz, and Nicolson, 1978) and endothelial cells produce fibronectin *in situ,* promoting elongation of the capillary tips (Clark *et al.*, 1982a; Nicosia and Madri, 1987; Nicosia, Bonanna and Smith, 1993). During endothelial cell growth, the fibronectin may mediate endothelial cell adherence (Clark, 1985) and chemotaxis (Yamada, 1983). Fibroblasts also synthesise fibronectin (Martinez-Hernandez and Amenta, 1983).

Collagenase

The enzyme collagenase is the most important enzyme involved in the degradation of connective tissue matrix. There is evidence that the major portion of collagenase is derived from infiltrating leukocytes, especially neutrophils and macrophages. Fibroblasts, when stimulated by a factor produced by macrophages, become active producers of collagenase and proteoglycan-degrading neutral proteinases (Rygh *et al.*, 1986).

Vascular changes in injury

Acute tissue injury can result in haemorrhage and subsequent replacement of the blood clot by granulation tissue. A prominent feature of reparative granulation tissue is the large number of new blood vessels and the lack of order or orientation. Maturation of the vascular and collagenous components to form scar tissue occurs by poorly understood mechanisms.

Vascular response to orthodontic forces

Reitan (1975) described the vascular response to orthodontic tooth movement resulting from a light and continuous load. It took an average of 5 days for the periodontal tissues to be compressed to the point where there is a cessation of tooth movement. This period is reduced markedly in the presence of excessive force.

Acute tooth displacement in response to external loading depends on an immediate redistribution of vascular volumes within the periodontal ligament. Gaengler and Merte (1983) provided evidence that blood is shunted, during loading, from compression zones to tension zones or out into the alveolus. Even relatively low pressures are capable of producing damage. Gianelly (1969) showed that loads over 50g resulted in reduced vascular flow in dog periodontal ligament, but most vessels still remained patent. He also showed that a force of 75g over seven days did not significantly impair the structural integrity of the periodontal ligament, but that loads of 75g to 125g compressed the periodontal ligament vasculature. However, Kvinnsland, Heyeraas and Ofjord, (1989) measured blood flow after five days of orthodontic force application and reported an increase in blood flow in all experimental teeth.

External forces of a physiological nature impinge upon a tooth mainly in the axial (intrusive and extrusive) or horizontal plane. Much work has been done by Picton (reviewed by Moxham and Berkovitz, 1982) on the responses to physiological and orthodontic loading, relating them to the amount of tooth support in the presence of periodontal pathology. Extrusion effects on post-capillary-sized venules were found to be only transitory (Parlange and Sims, 1993).

In clinical orthodontics, in the initial or re-activation phase, over-compression and over-stretching of the periodontal ligament capillaries is

common. Using vital microscopic techniques, Gaengler and Merte (1983) showed that both compression and tension in the periodontal ligament led to ischaemic areas, beginning in the venules, extending to capillaries and eventually involving arteries. An adequate vascular supply is essential for a constant supply of nutrients and oxygen to support the increased demand of the multiplying cells and differentiation of specialised cells.

Vascular breakdown allows whole blood to be extruded into the periodontal ligament, thus stimulating the release of various cytokines including interleukin-1 and growth factors which enhance chemotaxis, cell proliferation and bone remodelling (Davidovitch, 1995).

Using scanning electron microscopy on vascular casts, Burger *et al.*, (1983) recorded the early changes in periodontal ligament microvasculature following orthodontically-induced pressure. During the initial hours of the inflammatory response, preformed capillaries and post-capillary venules rapidly became widely distended and tortuous, this change intensifying progressively. The connective tissue became oedematous and endothelial cell intercellular contacts were opened (Ausprunk and Folkman, 1977, Paka and Paweletz, 1991). Recently Rygh (1995) showed dilation of the blood vessels did not occur and that microvascular reorganisation within 27 hours is limited to capillary sprouting (Burger *et al.*, 1983).

Acute inflammation as an initial response to orthodontic loads

The response of the periodontal ligament cells during light force stimulation, tension or extrusion, is an increased production of cAMP and interleukin-1α (IL-1α) and interleukin-1β (IL-1β) between 1-24 hours and increased prostaglandin-E production between 24-48 hours (Saito *et al.*, 1991; Yousefian *et al.*, 1992). This results in an initial stage of inflammation and vasodilation, (Rygh, 1972; Gaengler and Merte, 1983), which causes an increased blood flow (Rygh *et al.*, 1986; Kvinnsland *et al.*, 1989) (disputing Gaengler and Merte, 1993), distension and increased vascular permeability via gap junctions (Iida, Warita and Kurihara., 1992). A resultant stasis will only occur if the inflammatory process continues and intensifies.

Within hours of acute injury, intravascular accumulation of platelets and polymorphonuclear leukocytes (PMNs) occurs, with early diapedesis of leukocytes across the endothelium. This involvement of inflammatory cells

in angiogenesis is well recognised (Clark and Clark, 1939). Before and during vascular sprouting, inflammatory cells are observed adhering to the endothelium of the parent vessels, as well as passing through the endothelial junctions and the pericyte-endothelial space.

Between one to six hours after stimulation, the number of PMNs decrease markedly. The monocytes, either individually or in small clusters of two or three, simultaneously appear trapped between the endothelial cells and the pericytes within the *basal lamina* .

Macrophages are key regulators of wound neovascularization. Activated macrophages or culture supernatants from the cells, induce new capillary growth *in vitro* and *in vivo* (Koch, Polverini and Leibovich, 1986; Folkman and Klagsbrun, 1987; Sunderkotter *et al.*, 1991; 1994). They also induce regression once tissue regeneration is complete.

Leukocytes although not essential for the initiation and continuation of angiogenesis, facilitate or augment vascularization (Sholley, Gimbrone and Cotran, 1978). Fibrin material, erthyrocytes, macrophages and dividing fibroblasts appear progressively in the interstitium. Angiogenesis is accompanied by variable fibroblast proliferation.

Under heavy force, with gross compression, increased permeability is followed by stasis and endothelial cell death. Simultaneously the interrupted blood flow to the area provides an angiogenic stimulus, promoting the ingrowth of new endothelial cells required to re-establish vascular continuity.

Orthodontic tooth movement may stimulate the nervous as well as the immune system (Davidovitch *et al.*, 1988; Kvinnsland and Kvinnsland, 1990) causing an increase in cAMP and cGMP in periodontal cells. This facilitates the binding of neurotransmitters (e.g. substance P [SP]) to specific cellular receptors on the endothelial cells, resulting in a rapid vasodilation and migration of lymphocytes, monocytes, proteins and fluid into the extracellular space. Cytokines such as tumour necrosis factor-α (TNF-α) induce IL-1 production by monocytes, enhance PGE and collagenase production and increase osteoclast numbers. The IL-1 in turn attracts leukocytes, stimulates fibroblast proliferation and enhances bone resorption. Lymphocytes produce gamma interferon which inhibits bone resorption induced by IL-1, and favours tissue repair.

To date prostaglandins have been the only chemical mediator of orthodontic tooth movement to have been used clinically. Lipoxogenase products may have a similar role. Cytokine production may account for many cellular effects associated with orthodontic movement. The phosphatidylinositol pathway is likely to account for a number of cellular events seen in mechanically deformed tissues.

Histamine has been shown to cause variability in vascular permeability during angiogenesis (Schoefl, 1963; Yamagami, 1970). Extracellular fibrin is deposited during inflammation and wound healing (Vernon and Pratt, 1988). In fact, fibrin gel induces neovascularization *in vitro* (Nicosia, Tchao and Leighton, 1982) and *in vivo* (Dvorak *et al.*, 1987).

Angiogenesis

Angiogenesis is a multistep complex process which has been considered in several studies under various pathological conditions (Schoefl, 1963; Yamagami, 1970; Ausprunk and Folkman, 1977; Sholley *et al.*, 1984; Dvorak *et al.*, 1987; Wakui, 1988; Paka and Paweletz, 1991). It is intimately associated with a variety of biological processes, including growth and development, wound repair, increases in body mass, the menstrual cycle and pregnancy. It is also associated with pathological processes including neoplasia, inflammation, wound repair and collaterisation in response to ischaemic stimuli (Schor and Schor, 1983; D'Amore and Thompson, 1987).

The target vessels for angiogenesis factors are the post-capillary venules and small terminal venules. The morphogenetic processes require endothelial cells to exhibit a special set of complex behaviours (angiogenic phenotype Folkman and Shing, 1992) that include migration, proliferation, intercellular alignment, adhesion, and lumen formation.

Prior to and during angiogenesis *in vivo* there is an accompanying inflammatory response, along with capillary growth involving vascular sprout formation from pre-existing capillary and small venule walls lacking smooth muscle cells (Ausprunk and Folkman, 1977; McCracken, Burger and Klintworth, 1979; Phillips, Whitehead and Knighton, 1991; Diaz-Flores, Gutierrez and Vareia, 1994). Venules were thought to be the predominant location for angiogenesis, mainly because of the larger surface area of the venular plexus in the areas most explored (Burger *et al.*, 1983). Recently, vessels of greater calibre in the venous circulation, have been shown to be capable of intense angiogenesis, with vascular sprouts arising from the endothelial cells in the intima of the vein (rat femoral vein) after a single application of prostaglandins E1 and E2 in triacetin solution into soft surrounding tissue (Diaz-Flores *et al.,* 1994).

The essential events involved in angiogenesis are shown in Figure 2. The process commences with the disruption of focal contacts between adjacent endothelial cells, pericytes and smooth muscle cells (Ausprunk and Folkman, 1977; Phillips *et al.*, 1991). This is followed by the activation of the endothelial cells and pericytes to prepare for protein synthesis.

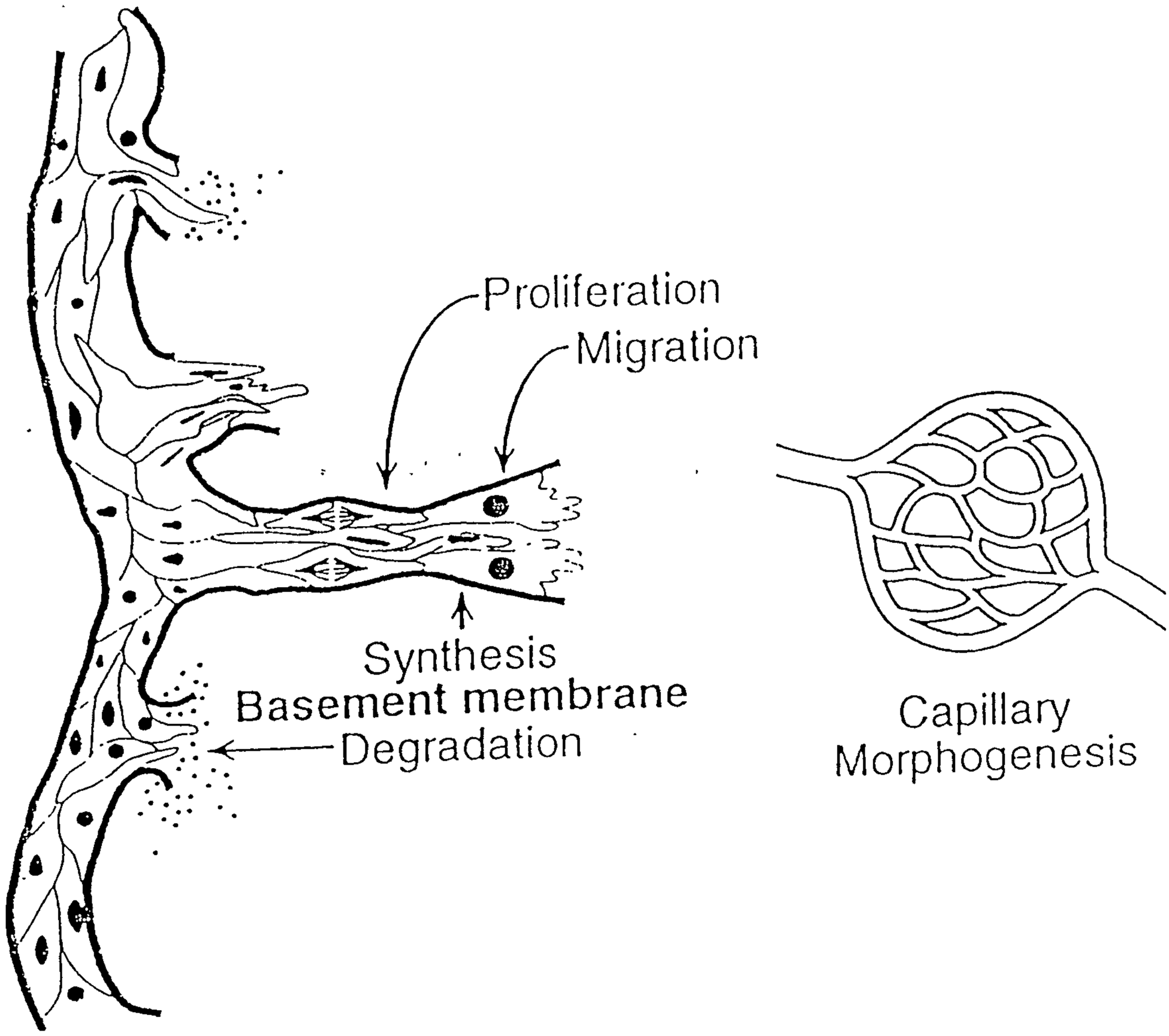


Figure 2 Diagram depicting several of the key steps in the angiogenic cascade.

(Polverini 1995)

Proteolytic destruction of the pre-existing vessel *basal lamina* exposes the vascular endothelium to the interstitial connective tissue (Rifkin *et al.,* 1982; Montesano *et al.,* 1986). The endothelial cells respond by migrating into the interstitium towards the angiogenic stimulus, and the perictyes follow. The distal cells are believed to proliferate rapidly and supply new cells, while the migrating endothelial cells simultaneously secrete new matrix components, resulting in the ultimate formation of a new basement membrane, lumen formation and capillary loop generation. These are later modified and organised into larger microvessels. After between three and five days, the number of sprouts increases to produce a rich anastomosing plexus with the capillary loops lengthening. On maturation of the plexus there is a return to a quiescent state (Form *et al.,* 1986).

Inflammatory phenomena associated with angiogenesis

An inflammatory response may precede and accompany angiogenesis. This involves vascular dilation, increased vascular permeability and diapedesis of leukocytes, immunological activity, debridement and fibroplasia (Clark and Clark, 1939; McCracken *et al.*, 1979).

Endothelial cells and pericyte activation

Prior to migration and proliferation, endothelial cells undergo phenotypic alteration, becoming plump with intracellular organelles (Schoefl, 1963; Yamagami, 1970; Ausprunk and Folkman, 1977; McCracken *et al.*, 1979; Nicosia *et al.,* 1982; D'Amore and Thompson, 1987). This is typical of cells involved in increased DNA-synthesis and those preparing for the secretion of proteases such as metalloproteases and plasminogen activators (Burger *et al.*, 1983; Pepper *et al.*, 1990). This hypertrophy results in the cells bulging into the vascular lumen.

The pericytes enlarge, shorten their process and increase the number of cytoplasmic polyribosomes (McCracken *et al.*, 1979; Diaz-Flores *et al.*, 1992).

Basal lamina degradation

Fragmentation and disintegration of the basement membrane is caused by the activated endothelial cells synthesising and secreting proteolytic enzymes, including plasminogen activator and collagenase (Montesano *et al.*, 1986). This exposes the vascular endothelium to the interstitial connective tissue, allowing endothelial cell migration from the parent vessel (Schoefl, 1963; Ausprunk and Folkman, 1977; Folkman and Shing, 1992).

Subtle alterations appear throughout the parent vessel but there is complete disintegration on the side closest to the angiogenic stimulus, where the endothelial sprouts begin. The endothelial cell sprouts have no basement membrane, but are surrounded by a homogeneous provisional substratum with altered proteoglycans (Clark *et al.*, 1982a). During this process microscopic bleeding can occur.

Endothelial cell migration

Activated endothelial cells alter the expression of cell-cell and cell-matrix adhesion molecules, exhibit re-organization of cytoskeletal elements, secrete components of extracellular matrix and express cell surface adhesion molecules such as integrins, members of the immunoglobulin supergene family and selectins (Madri, Pratt and Tucker, 1988; Ingber and Folkman, 1988; Gamble *et al.*, 1993).

Endothelial cell migration in response to the extracellular matrix, depends on the intregin family of cell adhesion receptors (Zutter and Santoro, 1990; Leavesley *et al.*, 1993) while the spreading and migration of the endothelial cells is mediated by integrins $\alpha_2\beta_1$ and $\alpha_4\beta_3$ (Languino *et al.*, 1989; Leavesley *et al.*, 1993). The $\alpha_2\beta_2$ integrins appear to be a major receptor for laminin on endothelial cells (Languino *et al.*, 1989).

The endothelial cells protrude through the vascular wall by sending out pseudopodia, and migrate into the interstitial matrix towards the angiogenic stimulus (Ausprunk and Folkman, 1977; Rifkin *et al.*, 1982; Pepper *et al.*, 1992). The chemotactic behaviour is facilitated by the secretion of plasminogen activator and collagenase (Moscatelli, Gross and Rifkin, 1981) with endothelial cell mitosis as a secondary event (Yamagami, 1970; Sholley, Gimbrone and Cotran, 1977; McCracken *et al.*, 1979).

Endothelial cell migration toward the angiogenic stimulus begins in two forms (Paka and Paweletz, 1991). In the linear form, a single endothelial cell projection and/or pseudopod migrates into the surrounding connective tissue from the parent capillary (Ausprunk and Folkman, 1977; Folkman, 1986). In the second formation two or more cellular processes from the endothelial cells form nearly parallel processes toward the angiogenic stimulus (bipolar configuration) (Burger *et al.*, 1983; Folkman, 1984; Wakui, 1988). The tips of these processes or sprouts, contain giant dense bodies and are connected via intercellular junctions composed of numerous free polyribosomes and intermediate filaments. As these processes extend, the filaments become embodied within the parent vessel wall. These processes form the endothelial sprout with a narrow slit-like lumen (Wakui, 1988).

In both cases of migration, the endothelial cells align with each other to create sprouts which may be solid, or may have intercellular slit-like lumena. The elongation and proliferation of the endothelial cell progressively lengthens the sprout by telescoping (Sholley *et al.*, 1984).

The presence of abundant contractile intermediate filaments in the endothelium of the sprouts could be important for the extension and migration of these sprouts (Wakui, 1988). The loosening or disruption of cell junctions may release endothelial cells from contact inhibition and allow proliferation, while increasing vascular permeability (Ausprunk and Folkman, 1977). Other researchers have never observed opened intercellular contacts in the new capillaries (Hudlicka, Brown and Eggington, 1992) and suggest that pre-existing intercellular junctions contribute to a parallel movement of the endothelial cell and preserve the inside-outside polarity of the sprouting endothelial cell (Paka and Paweletz, 1991).

Endothelial cell proliferation

Mature endothelial cells are generally quiescent, with an extremely slow turnover rate in the healthy adult organism, but the endothelial cells can quickly convert to a proliferative state during angiogenesis (Folkman, 1984). However endothelial cell proliferation has been shown to be non-essential (Sholley *et al.*, 1984).

Endothelial DNA synthesis in the parent vessel occurs before sprouting, and within 6 to 8 hours after an angiogenic stimulus is applied. The time

and exact site of division is controversial. Clark and Clark (1939), suggested that proliferation occurred in the new capillary tip on commencement of the sprout budding, with endothelial proliferation taking place in the cells following the "leader endothelial cell". Sholley *et al.* (1984), disputed this theory and indicated that endothelial cell mitosis began, close to the parent vessel in the newly formed vessels or in the parent vessel, soon after migration had commenced (Ausprunk and Folkman, 1977; Folkman, 1982; Clark, 1985; Folkman, 1986).

The capacity of angiogenic stimuli to induce replication in confluent endothelial cells may be dependent on the disruption of the cell-cell contacts, and on the modulation of cytoskeletal organization including destabilisation of the microtubules to effect changes in cell shape. Collagen in the interstitium also seems to influence endothelial cell proliferation (Madri and Stenn, 1982; Schor, Schor and Allen, 1983).

Formation of new capillary vessel lumens

The exact moment at which the lumen of the new capillary connects with the lumen of the parent vessel has not been clarified. It could be formed at the beginning or early phases of sprout formation, while the sprouting endothelial cells are in the wall, or while they are budding off from the wall of the parent vessel (Yamagami, 1970; Wakui, 1988; Paka and Paweletz, 1991). The lumen is formed by increasing the curvature of the endothelial cell between the adjacent endothelial processes (Wakui, 1988).

Others believe the lumen of the new vessel appears first in the sprout, merging later with the parent vessel (Ausprunk and Folkman, 1977; Folkman and Haudenschild, 1980; Folkman, 1984.), indicating that the lumen develops by transversal division of the endothelial cell (Nicosia *et al.*, 1982).

Pericytes in angiogenesis

During the initial phase of angiogenesis, pericytes of the parent vessel undergo hypertrophy and characteristic changes in preparation for increased DNA synthesis and intense proliferation as described earlier for endothelial cells (Schoefl, 1963; Sholley *et al.*, 1977; Diaz-Flores and Dominguez, 1985; Diaz-Flores *et al.*, 1992). Decreased surface contact between pericyte and endothelium, disruption and fragmentation of the pericyte *basal lamina* in some areas and pericytic projection into the extravascular space have been observed. Pericytic numbers increase and endothelial cells undergo mitosis when in close association with these pericytes (Sholley *et al.*, 1977; Diaz-Flores *et al.*, 1992).

Most investigators consider pericyte involvement to be pronounced at the end of the proliferative capillary sprouting stage, following lumen formation (Folkman and Haudenschild, 1980; D'Amore and Thompson, 1987; Paka and Paweletz, 1991) and that the pericyte presence is unrelated to development and organization of the *basal lamina* .

Recently pericytes have been found associated with the tip or in front of the advancing tips of endothelial sprouts during angiogenesis. Pericytic processes appear to bridge the gaps between opposing endothelial sprouts (Hudlicka *et al.*, 1996). This suggests that pericytes may serve as guiding structures for endothelial cell outgrowth (Nehls *et al.*, 1992). Sato and Rifkin (1989) attributed the presence of pericytes at the tips of migrating endothelial cells to the stimulation of endothelial cell mitosis, while the presence of pericytes in older regions of the growing capillaries may actually inhibit endothelial cell proliferation (Figure 3).

Changes in extracellular matrix, formation of new *basal lamina*

In quiescent microvasculature, laminin, fibronectin, entactin, heparan sulphate proteoglycan and collagen types IV and V are present in the basement membrane (Foidart *et al.*, 1980; Pepper *et al.*, 1990). During angiogenesis, local fragmentation of the basement membrane of the parent vessel and subsequent degradation of the interstitial matrix are observed (Rifkin *et al.*, 1982; Kalebic *et al.*, 1983; Madri and Williams, 1983), presumably by the action of proteolytic enzymes including plasminogen activator and collagenase (Folkman, 1982).

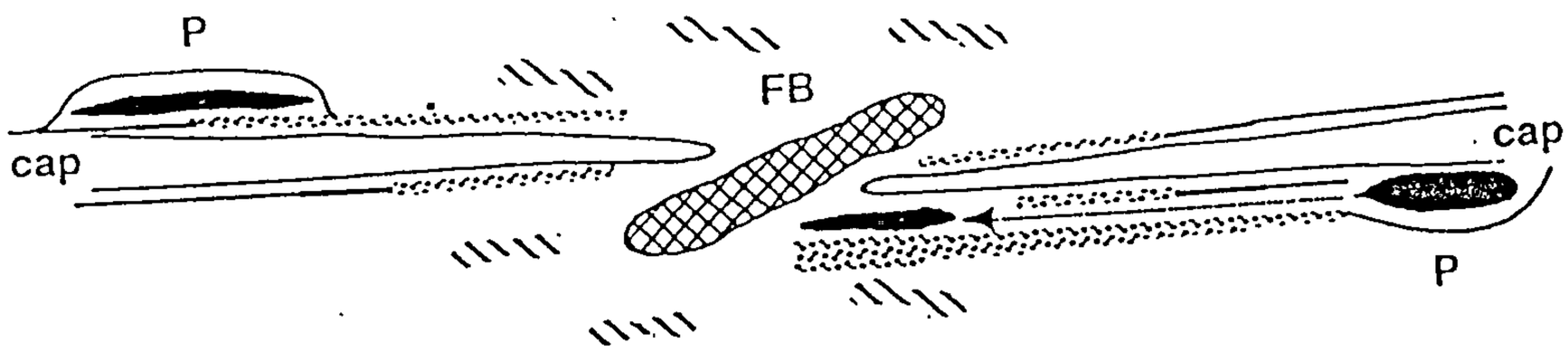


Figure 3: Hypothetical relations between capillary sprouts, pericytes and interstitial cells.

P= pericyte; FB = fibroblast; cap = capillary endothelial cell. Changes in basement membrane integrity/ composition are designated by transition between solid and stippled layer. Collagen bundles are represented by striated elongated structures.

Sato and Rifkin 1989

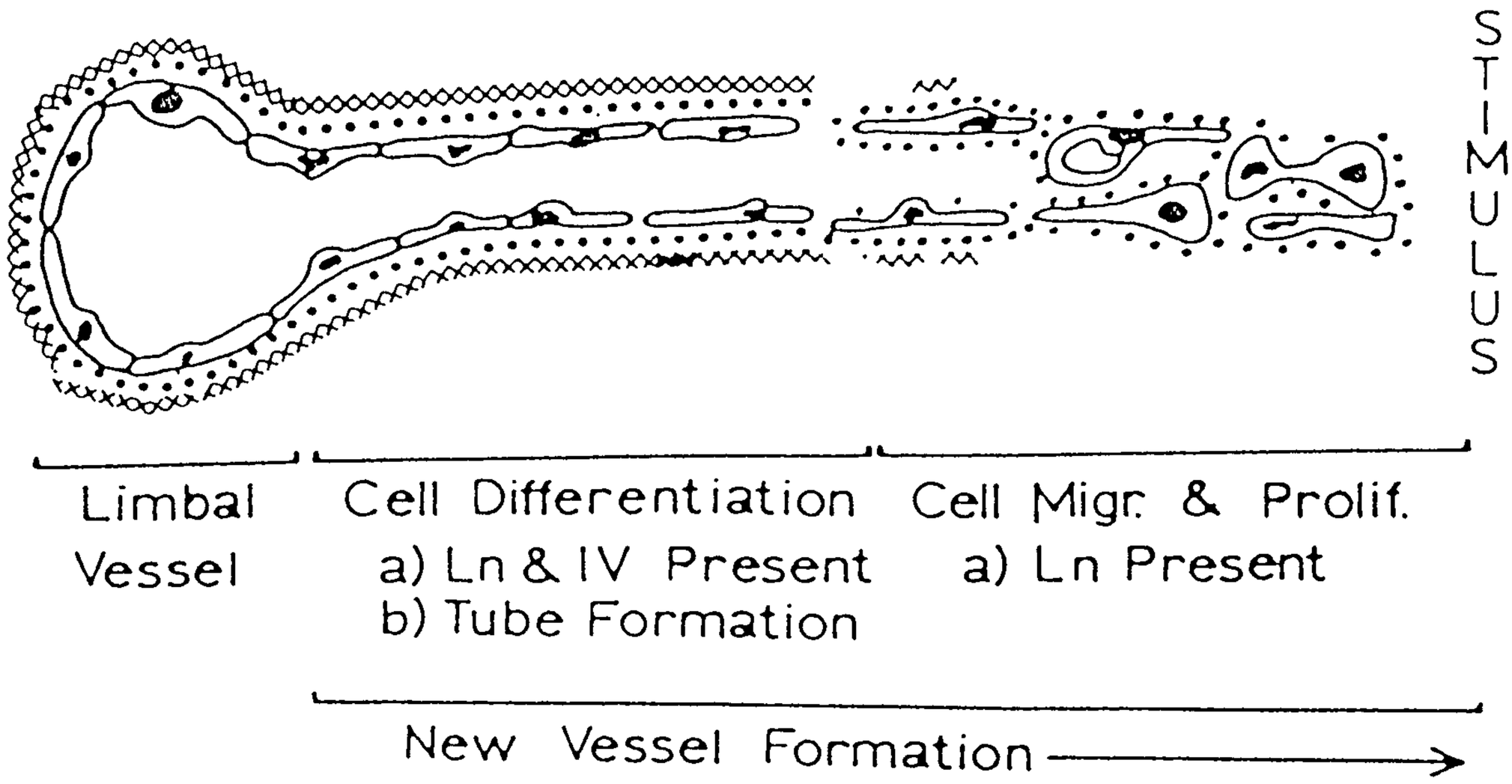


Figure 4: Systematic representation of the angiogenic response noted in the murine cornea.

After stimulation, the endothelial cells lining the Limbal vessel degrade the investing basement membrane and migrate into the stroma towards the injury site in response to a variety of factors. After the initial migratory response, the endothelial cells undergo a proliferative response. Migration and proliferation occur at and near the distal tip of the neovascular response, whereas endothelial cells further back, near the limbal vessels, exhibit a lower proliferative rate and organise into tube-like structures with definite lumena and cell-cell and cell-matrix interactions. Endothelial cells in the more distal area secrete laminin, but do not form morphologically identifiable basement membrane. Endothelial cells further back secrete both laminin and type IV collagen, and form morphologically identifiable basement membranes. The temporally staggered appearance of laminin and type IV collagen correlates with the rates of endothelial cell proliferation noted *in situ* during angiogenesis, and the *in vitro* effects of the basement membrane components.

key: Ln = laminin; IV = type IV collagen; Migr = migration; Profil = Proliferation

Nicosia and Madri (1987), using immunofluorescence (Figure 4) found that the extracellular matrix of solid endothelial sprouts consisted of a delicate fibrillar network of fibronectin, fibrils of type V collagen and patchy amorphous deposits of laminin and type IV collagen. Fibronectin stained intensely and appeared to be the predominant component of the provisional sub-endothelial matrix (McKinney and Panner, 1972).

When endothelial cells underwent tube formation and extension (Folkman and Haudenschild, 1980; Folkman, 1986), the deposition of fibronectin decreased, becoming discontinuous, while laminin and type IV collagen increased. Laminin occurs throughout the newly formed vessels, as well as in individual cells at the migrating, proliferating tips (Form *et al.*, 1986). The presence of Type IV collagen has been associated with lumen formation *in vitro*; this component tends to accumulate to form a continuous feltwork in the sub-endothelial space, but not at vessel tips (Haralabopoulos *et al.*, 1994). In the late stages of angiogenesis, increased amounts of type I and III collagen are observed in the perivascular space.

Capillary loop formation

A short distance away from the parent vessel, the capillary sprouts containing migrating endothelial cells, begin to branch and join other tips to form capillary loops. Other capillary sprouts then appear from these loops to form a plexus. Pericytes have been implicated in guidance of this stage (Figure 5).

Early changes; persistence, involution and differentiation

Immature vessels seem to require angiogenic stimuli to persist (Ausprunk, Falterman, and Folkman, 1978). During angiogenesis, a substantial number of newly formed vessels regress (Clark and Clark, 1939). Once the new vessels reach the source of stimuli, the blood flow becomes sluggish, initially in the smallest distal capillary branches, and in the less advanced vessels which then tend to regress (Ausprunk *et al.*, 1978; Auerbach, Auerbach and Polakowski, 1991).

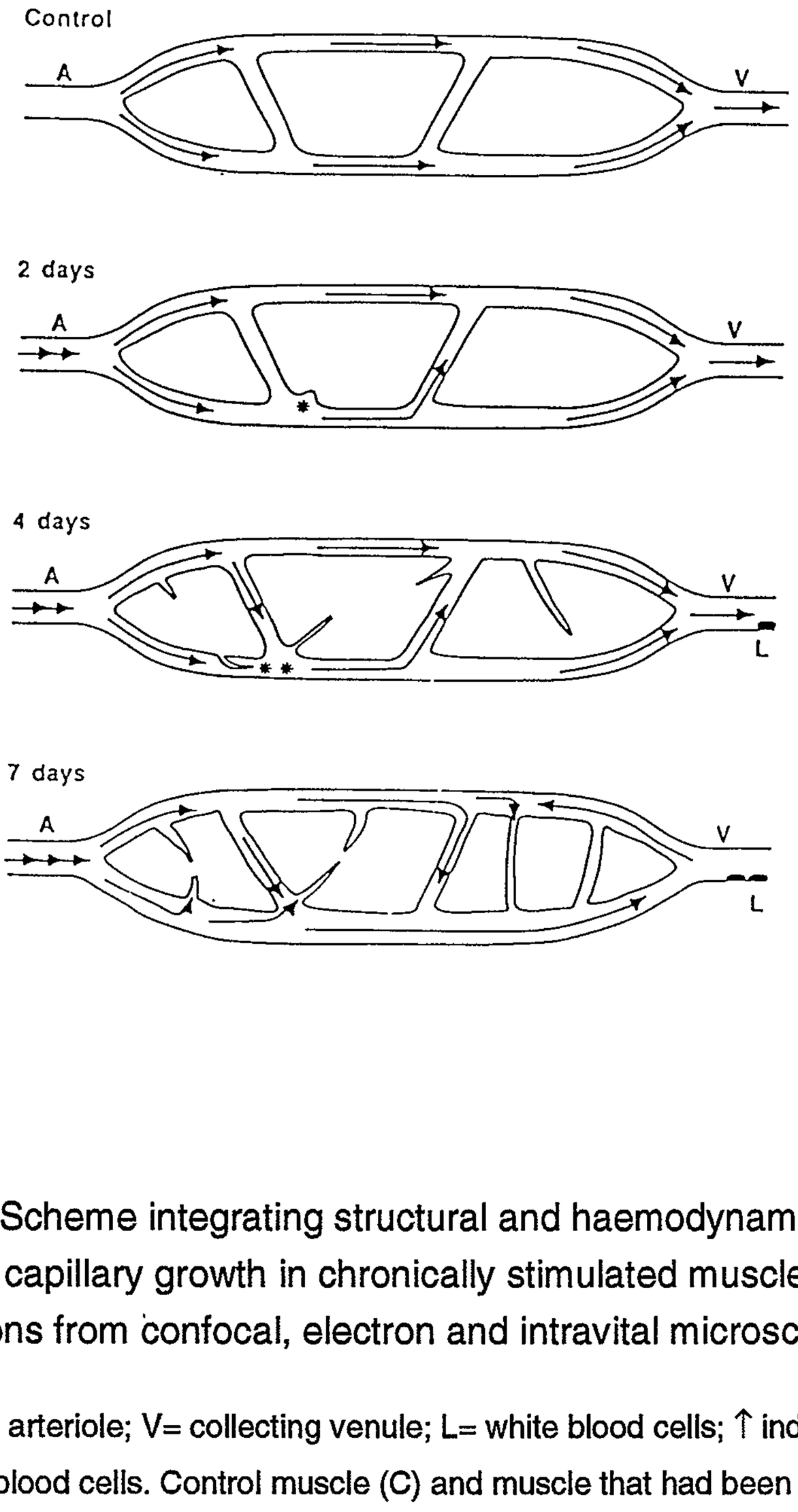


Figure 5: Scheme integrating structural and haemodynamic changes leading to capillary growth in chronically stimulated muscles based on observations from confocal, electron and intravital microscopy.

A= terminal arteriole; V= collecting venule; L= white blood cells; ↑ indicate the direction of flow of red blood cells. Control muscle (C) and muscle that had been stimulated for 2, 4, and 7 days. * = bulging of the endothelial cells in 2-day-stimulated muscle; * = sprouting into lumen in 4-day-stimulated muscle.

There are two main sequences of events in the vessel regression:

The first involves the aggregation of platelets, fibrin polymerisation, stasis of blood and vessel occlusion by erythrocytes. Endothelial cells thin and fenestrate, the organelles in the distal tips of the capillaries swell, vacuolization occurs, the plasma membrane is disrupted and cytolysis is followed by deposition of granular material into the interstitium. Finally, degeneration of vessel wall cells occurs and mononuclear cells remove the vascular debris (Ausprunk *et al.*, 1978).

The second type of vascular regression involves endothelial deletion. Individual endothelial cells protrude into the capillary lumen and form adherent junctions with the vessel walls. Later the nuclear and cytoplasmic compartments condense, along with cellular and nuclear lobulation and fragmentation. There is disruption of cell organelles, loss of plasma membrane integrity and reduced cytoplasmic density. The degenerate cell fragments are engulfed by viable mural endothelial cells (Azmi and O'Shea, 1984).

Capillary network formation and organization

A functional circulation is established post-natally, by anastomoses of the newly formed capillaries with larger calibre pre-existing venous vessels (Diaz-Flores *et al.*, 1994). In the arterial circulation, ingrowing capillaries join the arterial wall (Diaz-Flores *et al.*, 1990a). Some signals for capillary regression have been identified, providing evidence that families of down-regulating angiogenic mediators may function by initiating events leading to apoptotic death of endothelial cells (Re *et al.*, 1994).

Angiogenesis in the formation of neovascular collateral vessels

Although quiescence is normal in adult organs, an alternative route of blood supply may arise from preformed or neovascular collateral vessels in response to ischaemic stimuli. For example; re-vascularization in ischaemic heart conditions and coronary artery collateral development in experimental coronary artery occlusion can be accelerated using heparin treatment. This new growth is rapid but limited.

Control of angiogenesis

Factors controlling angiogenesis are either stimulators or inhibitors (Folkman and Klagsbrun, 1987, Klagsbrun and D'Amore, 1991). Although the majority of stimulatory molecules are proteins, many are growth factors, which induce endothelial cells to divide, migrate towards the inducing stimulus and then differentiate into tubular structures. These factors are secreted by a variety of local cells, including the endothelium and function in an autocrine and/or paracrine manner, in response to exogenous or endogenous stimuli.

Direct stimulation through endothelial cell surface receptors may induce endothelial proliferation and/or migration *in vivo* and *in vitro*. When the *in vitro* action fails or is inhibited, the angiogenic factor is considered to be indirect, by attracting and activating accessory cells (inflammatory macrophages) to produce angiogenic mediators, such as heparin, or by stabilising and/or enhancing the function of stimulatory molecules normally present in the extracellular matrix.

The inhibitors almost all influence the ability of cells to produce, interact with, or degrade the extracellular matrix (Maragoudakis, Sarmonika and Panoutsacopoulou, 1988; Madri *et al.*, 1988; Ingber and Folkman, 1989). The use of many *in vitro* and *in vivo* systems has facilitated the following assessment of angiogenesis control:

- direct angiogenic growth factors include: a and b fibroblast growth factors (aFGF and bFGF) (Folkman and Klagsbrun, 1987); vascular endothelial growth factors (VEGF); platelet-derived endothelial growth factor (PD-EGF).

- indirect angiogenic growth factors include: Transforming growth factors-α and β; Epidermal growth factor-α; Tumour necrosis factor-α; Platelet-derived growth factor; E series prostaglandins; angiogenin; monobutyrin; adenosine; okadoic acid; hydrooxyeicosatrienoic acid; some copper complexes; hyaluronic acid degradation products; age-associated glycosyation end-products.

- Angiogenesis antagonists include: cartilage derived angiogenic inhibitor, thrombspondin, protamine, platelet factor-4, interferon, angiostatic antibiotics and steroids. Heparin and heparin sulphate play a key role in these mechanisms.

Models to study re-vascularization patterns

Organ culture systems

As far back as 1865, Waldader ascribed the role of guiding regenerating muscle cells to sarcolemmal tubes (Sarolemma= plasma membrane and basement membrane of skeletal muscle fibres). In 1893 Volkman showed that injured skeletal muscle will completely "regenerate" and become functional only if the "*Basal Lamina*" remains intact. When the basement membrane was destroyed by either crushing or cauterisation with heat or strong acid or an excised piece was removed and replaced with a fibrin clot, scar formation and loss of function resulted.

In 1946 Clark demonstrated that "regeneration" of mammalian skeletal muscle fibres occurred in the direction of the old fibres, even if an excised piece of muscle was reimplanted at right angles to the original orientation. More recent experiments in the 1970's by Vracko and Benditt reported on regeneration of rat and rabbit skeletal muscle after injury with freezing, ischaemia or *in situ* autografting. Each injury type produced complete necrosis of cells leaving the basement membrane intact and so maintaining a template of spatial relations between the muscle fibres and capillaries. Repopulation of the defect occurred with the new cells able to grow along the inside surface of the existing basement membrane, with full re-established of the network within three weeks (Vracko, 1972; Vracko and Benditt, 1972).

The replacement cells of muscle fibres and capillaries frequently form a new layer of basement membrane which is of the usual thickness and is deposited primarily along the outer surfaces of plasma membranes, in locations in which the new cells are separated from the old basement membrane. Removal of redundant basement membrane layers is mediated by interstitial fibroblast-like cells applied to the outer surfaces of skeletal muscle fibres. This mechanism is more effective in skeletal muscle than in muscle capillaries resulting in skeletal muscle having a single layer and the capillaries often having two layers remaining by one month post-injury (Vracko and Benditt, 1972).

Reconstruction of skeletal muscle occurs even if excised portions are minced into 1mm^2 pieces and reimplanted into the defect. In the early stages of repair the newly proliferating immature muscle cells orientate in

the direction of implanted muscle fragments (Carlson, 1968). In the later stages, the muscle fibres are orientated in an irregular and disorderly fashion (Kasper, 1971). These observations suggest that proliferating muscle cells are "threading" through the short, randomly orientated fragments of basement membrane tubes, resulting in a partially reconstituted and nonfunctional skeletal muscle.

Cells can only identify basement membrane of their own cell type, as demonstrated by experiments using skeletal muscle cells and renal medulla intact basement membrane scaffold. Despite the similarity to muscle basement membrane the skeletal muscles did not recognise the renal product (Vracko, 1974).

Probably the first organ used for investigating the importance of basement membrane was the kidney. Oliver (1953) reported that re-epithelization of renal tubules after acute inflammation occurred from neighbouring healthy cells migrating along the residual basement membrane scaffold. If the basement membrane was damaged, the regenerating tubular epithelial cells did not have the capacity to bridge large gaps. However, when the gaps were small, epithelial and mesenchymal tissues interacted to reform the wall of the tubule.

The layering of new basement membrane was first shown in rat kidney experiments using silver nitrate uptake for 10 months prior to necrosis caused by freezing, followed by a period of repair with no silver nitrate uptake. Histological investigations showed the old basement membrane labelled with silver and new unlabelled cells were seen growing inside the old basement membrane tube.

Similarly, dog lung tissue was killed with oleic acid, leaving the basement membrane intact. Three days later, reconstruction with epithelial, endothelial and septal cells began from adjacent uninjured parenchyma each using their own original basement membrane and without formation of new membrane. Within three weeks total re-construction had occurred. In the presence of lung abscesses and caseating granulomas, healing occurred with scar formation (Vracko 1970). Other experiments utilising the pancreas showed similar results following injury (Vracko, 1974).

Repair of injured nerve fibres relies on the presence or absence of an intact basement membrane. Axons of peripheral nerves regrow along tracts of injured nerves and regain full function, while nerves in the brain and spinal cord heal with scar formation and reduced function (O'Daly and Imaed, 1967). The difference between these types of nerve fibres is that peripheral nerves have a basement membrane while spinal and brain nerves do not.

The speed and quality of skin repair depends mostly on the depth of the injury incurred in burns and skin graft sites (Converse and Robb-Smith, 1944). If the basement membrane remains intact, full function returns whereas, if the membrane is damaged scar tissue formation occurs.

Re-vascularization of autografts has demonstrated that pre-existing graft vessels fuse with the host's invading capillaries from the surrounding connective tissue (Sasaki *et al.,* 1991).

Natural occurrence of re-vascularization in pathology

In skin conditions such as pemphigus vulgaris and epidermolysis bullosa simplex where a dermal inflammatory reaction is followed by subepithelial vesicles or bulla formation just above the basement membrane leaving the basement membrane intact, repair is complete. But in epidermolysis bullosa dystophica (recessive type) the basement membrane is part of the blister roof and healing results in scarring. It appears that the most critical factor for complete repair is contact guidance for new cells along the previous basement membrane template.

The function of basement membrane as a scaffold for orderly cell positioning appears important in the pathogenesis of disorders associated with thickening of the basement membrane.

In diabetes mellitus and in experiments relating to skeletal muscle injury in nondiabetic animals, the excess basement membrane appears to be of normal thickness but multiple layered, deposited sequentially by a new generation of cells. Indirect immunofluescence and immunoperoxidase studies, using affinity-purified antibodies, support this idea in the early stages of the disease, but show reduced staining in severe, late stage cases (Falk *et al.*, 1979). The response to injury of peripheral nerves, renal tubules and regenerating glomerulus as described earlier, support this concept.

Several causes have been indicated for the excessive accumulation of basement membrane in pathological conditions such as diabetes mellitus. The first suggest that although cells ordinarily make a single "normal" complement of basement membrane, under appropriate circumstances these cells are activated to produce new uninterrupted, uncontrolled layers (Vracko and Benditt, 1970) (there is no evidence for the theory).

The second suggestion indicates that the formation of basement membrane is normal, but the removal of the old basement membrane is disrupted, leaving it present for longer than normal or leaving it permanently. Removal is normally immediate in skeletal muscle fibres after an episode of acute injury, but generally takes months in the capillaries of skeletal muscle and in injured peripheral nerves and renal tubules (Vracko and Benditt, 1970; 1972).

The last suggestion with the most evidence, indicates that cells only synthesis a single "normal" complement of basement membrane in their life-cycle, but where an accelerated rate of cell death occurs, the new cell generation deposits a new layer of basement membrane and an accumulation of membrane forms (Vracko and Benditt, 1974b).

More than 50% of patients with juvenile or maturity-onset diabetes mellitis develop a generalised microangiopathy (Williamson and Kilo, 1977) within 10-20 years following onset. Thickening usually occurs with a single inner basement membrane layer but occasionally involves repeated duplications into multilaminated structures (Vracko, 1974).

Quite a few changes found in diabetic basement membrane are also age-related phenomena. These include gradual thickening of capillary basement membrane with age (except females in the age period 25-50 years) (Williamson and Kilo, 1977), with a two fold increase in collagen IV and loss of proteoglycans.

Models to study vascular patterns

Different human tissues, such as connective tissue, muscle and bone, clearly have a characteristically organised blood supply (Gray's anatomy book) with only limited individual variation. The different arrangements are possibly due to different metabolic demands. The distance over which oxygen must diffuse from capillary to tissue is critical for tissue oxygenation and aerobic metabolism.

Anatomical investigations into the characteristic vascular arrangement of different organs have concentrated on animals such as: rat, pigs; dogs and frogs, the majority of this literature is written in Russian, Japanese and German, is still untranslated and unobtainable by the author.

Many investigations concentrate on the variations of wall structure, including the diameter, thickness, presence or absence and location of fenestrations in varying physiological conditions. Investigations have included the density, intercapillary distance and type of capillary patterns formed, whether these patterns are random, ordered with constant intervals or in discrete clusters. Henquell *et al.,* (1977) suggested that the maximal distance over which oxygen must diffuse to tissues is more critical to aerobic metabolic capacity than the average distance. Kayer *et al.*, (1982) suggested that quantifying the median diffusion distance to an arrangement of capillary vessels identified as random, ordered or clustered was more meaningful than the distance to the closest-individual capillary vessel .

There have been few investigations into individual microcirculation patterns. Most studies using corrosion casts, immunofluorescence or more recently, scanning electron microscopy and computer-assisted image analysis have been on animals including fish, rats, frogs, dogs and monkeys. Organs investigated include the thymus (Miodonski *et al.,* 1995); bone marrow (De Bruyn, Breen and Thomas, 1970); eyes (Hayereh, 1974; Krey ,1975); skeletal muscle (Plyley, Sutherland and Groom, 1976); skin (Vico and Cartilier, 1993) and gingiva (Callender, 1972).

Using fluorescein Krey (1975) demonstrated that in all choroidal flat mounts of rhesus monkeys and man (Hayreh 1974), there is a distinct segmental arrangement of the capillary layer. Individual lobules were supplied by

peripherally located arteriolar branches and drained by a central venule originating in the centre of a star-shaped capillary system.

Models to study maintenance of individual vascular patterns

There are very few studies on the maintenance of a particular individual vascular pattern over any period of time. The tissue most commonly investigated has been the human nailbed capillary network. Kenik, Maricq and Bole, (1981) used nailbed capillary microscopy to show the unchanging reproducibility in the normal individual, of their own unique vascular pattern over a period of three years and Krylova and Soboleva (1995) found that each capillary in an individual nailbed was easily re-recognised as unique.

Individual variation in vascular patterns

The identification of pathology such as Raynaurd's syndrome, by the presence of tortuous capillary patterns in human nailbed vascular systems, has been disputed by Vayssairat *et al.,* (1982) who demonstrated that normal subjects may have up to 10% of their nailbed capillaries as tortuous vessels. Fahrig *et al.*, (1995) compared the ten digits of individuals and found an average of 4.5 capillary branchings per person in 78% of normal subjects, with the most occurring in the IV and V digits of both hands. Meanderings were found in 94% of normal subjects, 64% had tortuous loops, 25% had haemorrhagic extravasations and 19% had apical dilatations of the capillaries. Hence the majority of so called pathological diagnostic nailbed findings were well within normal variation, thus indicating a need to consider greater deviations in numbers of branching or haemorrhagic extravasations being present before any medical condition can be accurately diagnosed. For example, in systemic lupus endothelium, there is a significant increase in the length of the capillary loops up to 700μm, compared to 350μm in healthy normal subjects.

Effect of function can be shown as an increased capillarity in adult skeletal muscle as a result of endurance training (Hudlicka *et al.,* 1992) and after chronic experimental indirect electrical stimulation, where capillary numbers per muscle doubled (Brown *et al.,* 1976).

Adaptation of arterioles to moving capillaries

The capillary network of the enamel organ in a continuously growing and erupting rat mandibular incisor is supplied by a series of arteries which, after penetrating the bone, are disposed in a row in the periodontium along the tooth and linked together by anastomoses. The branches from these arteries are subjected to adaptive changes consistent with a forward movement of the capillaries in relation to the arteries.

The mechanism on which this constant movement relies, is that each artery in the row supplies a section of the passing plexus by coupling and uncoupling to short-lived arterioles, which in turn go through a cycle of proliferation, elongation and degeneration. Proliferation takes place at the posterior end of the section, where new arterioles replace arterioles discarded from the preceding artery. By growing in length, the arterioles keep pace with the migrating capillary network. When capillaries are within reach of the next artery, the arterioles disconnect, become obliterated and die in the intermediate zone of the periodontium, in which they have gradually been displaced by proliferation of the inner-tooth-related layer of the periodontal connective tissue (Moe, 1981).

When a tooth moves through bone under orthodontic therapy a similar phenomenon must occur for maintenance of a blood supply. This brings into question the importance of the re-construction of an identical network on re-vascularization following orthodontically-induced endothelial necrosis.

Models of angiogenesis.

In vivo model of angiogenesis

Many neovascularization experimental models use lesions, tumours or immunological reactions in various animals including, rabbit, rat, mouse and guinea pig cornea. The cornea is normally avascular and any neovascularization can be easily distinguished from any parent vessel of the limbus (Ausprunk and Folkman, 1977). Use of immunologically privileged tissues including; chorio-allantoic membrane, hamster cheek pouch, the anterior eye chamber, or yolk sac of the early chick embryo, allows the investigation into the growth of xenogeneic grafts (Klagsbrun, Knighton and Folkman, 1976), but has the disadvantage of creating an unnatural environment from which findings have to be extrapolated to a normal situation.

With the introduction of fibrin or gelatin implants impregnated with test substances such as angiogenic inhibitors (Dvorak *et al.*, 1987) and more recently, with the development of inert, biocompatible slow-release polymer pellets, which permit a sustained release of angiogenic or anti-angiogenic factors, much interesting information has come to light.

Animal models have clearly demonstrated many important biochemical changes associated with re-vascularization. However, the process of vascular growth is difficult to monitor and to manipulate experimentally *in vivo,* along with the continuing problem of determining the individual responses of different cell types. The use of immunolocalization techniques has partially overcome this problem, but identification of cell types is still dependent on empirical morphological methods.

Therefore investigators have devised developmental models in which endothelial cells form multicellular cords or tube-like structures *in vitro* that resemble microvascular sprouts or networks.

In vitro models of angiogenisis

Morphological aspects of the components of the angiogenic sequence have been investigated in a number of *in vitro* systems (Fryer, Birnhaum and Luttrell, 1966; Ingber, Madri and Folkman, 1986; Ingber and Folkman, 1988). The majority of models used, generate capillary-like structures from monotypic cultures of macro-vascular or micro-vascular endothelial cells.

In vitro culture systems can be simple, simulating spontaneous angiogenesis, by allowing spontaneous cellular cord network formation from confluent monolayers of endothelial cells, grown on unmodified tissue culture plastic (Folkman and Haudenschild, 1980; Ingber and Folkman, 1989; Vernon *et al.*, 1995). Other methods include the inducement of endothelial cells to sprout directly from explanted sectioned rings of macro-vasculature rat aorta (Nicosia, Tchao and Leighton, 1983). This approach has helped to eliminate the host response and simplify the number of interactions present.

Endothelial cell culture techniques have advanced substantially, with the maintenance of cultures possible for many passages (Jaffe *et al.*, 1972; Gimbrone, Cotran and Folkman, 1974). Endothelial cells isolated from both small and large vessels including, human umbilical veins (Maciag *et al.*, 1982), bovine aortas and capillaries and rat capillaries, are capable of forming random networks of capillary-like tubes *"in vitro"*, when grown under appropriate culture conditions (Folkman and Haudenschild, 1980; Maciag *et al.*, 1982; Montesano and Orci, 1985; Montesano, Mouron and Orci, 1985 and Kubota *et al.*, 1988).

The recovery rates of denuded surfaces in confluent endothelial cell monolayers have produced quantitative assessments of the angiogenic response, endothelial cell locomotion (chemokinesis) and directionality (chemotaxis) (D'Amore and Thompson, 1987; Auerbach *et al.*, 1991). The rate of endothelial cell proliferation in culture in response to test factors is determined by DNA synthesis, nuclear staining and DNA content. The changes in endothelial cell function during angiogenesis, such as modulation of the production of cytokines, proteinase release, fibrinolytic activity and synthesis of basement membrane components can also be determined.

Role of the changes in extracellular matrix

The mechanism used by individual endothelial cells to generate spatial information that directs the cells to form complex structures during the angiogenic programs, is still not completely known. It is reasonable to assume that the nucleus, cytoplasm and cell structure of endothelial cells are sites of action for gene products relevant to vascular morphogenesis. In addition to these cellular compartments, the extracellular matrix seems to play an important role in translation of uni-dimensional gene regulation to three-dimensional vascular structure.

Endothelial cells remodel the structural organization and composition of the surrounding extracellular matrix during vascular sprouting, principally by the secretion of enzymes which digest the pre-existing basement membrane (Kalebic *et al.*, 1983; Montesano, Orci and Vasalli, 1983) and by the synthesis of glycoproteins, proteoglycans and collagens (Iruela-Arispe, Diglio and Sage, 1991).

Changes in the extracellular matrix have important regulatory effects on the various stages of microvascular morphogenesis by modifying the organization, morphological features, function and behaviour of endothelial cells. These include cellular phenotype, migration and proliferation (Folkman and Haudenschild, 1980; Delvos *et al.*, 1982; Madri *et al.*, 1983; Tseng, Gospodarowicz and Stern, 1983; Montesano *et al.*, 1983; Schor *et al.*, 1983; Form *et al.*, 1986; Madri and Pratt, 1986; Nicosia and Madri, 1987; Ingber and Folkman, 1989).

Laminin and collagen IV proteins isolated from basement membrane, have been shown to facilitate the differentiation of endothelial cells into tube-like structures within six hours with little or no proliferation (Lawley and Kubota, 1989), while interstitial collagens only stimulate endothelial cell migration and proliferation (Madri and Williams, 1983; Montesano *et al.*, 1983; Kubota *et al.* 1988).

The rate of tube formation in cell culture is related to the composition of the extracellular matrix (Maciag *et al.*, 1982; Madri and Pratt, 1986; Nicosia and Ottinetti, 1990). Spontaneous angiogenesis occurs on two dimensional collagen IV gel or collagen type I (Greenburg and Hay, 1982), or on plastic alone (Kubota *et al.*, 1988). This is an inconvenient model due to its

unpredictability and long period of cultivation, taking up to six weeks to occur (Folkman and Haudenschild, 1980).

Angiogenesis-like behaviours are expressed more rapidly *in vitro* among a great variety of extracellular matrices for example; clotted fibrin, basement membrane matrix, serum fibronectin and type I collagen gels either in sandwiched three dimensional form (Delvos *et al.*, 1982; Montesano *et al.*, 1983), or when combined with laminin at ratios of 1:1 or 2:1, or with heparan sulfate or α-phorbol 12-myristate 13-acetate (PMA) (Montesano *et al.,* 1983; Jackson *et al.,* 1994). The sulfation appears to be vital for collagen-induced tube formation (Kinsella *et al.*, 1992). Fibrinonectin, laminin, collagen type I and collagen type IV extracellular matrices all individually promote attachment, but only when they are combined do they promote differentiation.

Network formation is determined by gel viscosities which influence the surface tension to be overcome prior to tubing, in both thin films and thick gels (Nicosia *et al.*, 1983; Nehls, Schuchardt and Drenckhahn, 1994; Ingber and Folkman, 1989; Kubota *et al.*, 1988; Gamble *et al.*, 1993). Reduction of the surface tension by introducing collagen-coated micro-carrier beads to the apical surface of type I collagen matrix aids rapid network formation (Inger and Folkman 1988).

Tube formation *In vitro*

When cultured human umbilical vein endothelial cells (HUVECs) are placed onto Matrigel, a laminin-rich reconstituted basement membrane extract derived from the murine Engelbroth-Holm Swarm tumour line, the cells display high motility, little or no proliferation, enhanced cell-cell communication and tend to align to form networks that subsequently give rise to capillary-like patent tubes within eighteen hours (Kubota *et al.*, 1988; Grant *et al.*, 1994).

Collagen type IV and Laminin in angiogenesis

In collagen type IV models, it takes days for the endothelial cells to synthesis and secrete sufficient quantities of laminin and collagen type IV for tube formation (Jackson *et al.,* 1994). When synthesis and degradation of basement membrane components are disturbed by using monoclonal anti-human collagen type IV, anti-human laminin, anti-entactin or cycloheximide, a protein synthesis inhibitor (Grant *et al.,* 1991), despite the abundance of basement membrane components in Matrigel, vascular formation is inhibited (Kubota *et al.*, 1980; Ingber and Folkman, 1988; Grant *et al.,* 1989). This indicates that *de novo* synthesis of basement components is essential in the development of active vessels *in vivo* and *in vitro* (Maciag *et al.*, 1982; Form *et al.*, 1986; Nicosia and Madri, 1987; Ingber and Folkman, 1988; Haralabopoulos *et al.*, 1994). The use of ascorbic acid, a promoter of collagen synthesis, enhances tube formation (Grant *et al.,* 1991).

Addition of anti-human laminin or laminin B1 chain synthetic peptides, (which bind to laminin cell surface receptor and incorporate the peptide in the gels), to a seeded model, allows cell attachment but completely inhibits capillary tube formation (Graf *et al.,* 1987; Kubota *et al.*, 1988; Lawley and Kubota, 1989). This suggests that laminin is an important regulatory agent in angiogenesis (Grant *et al.*, 1994) causing endothelial cell differentiation and inhibiting angiogenesis. Using antibodies of defined specificity, three sequences on the laminin molecule have been linked to the control of three different stages of angiogenesis *in vitro.* The YIGSR-containing sequence controls morphological changes, the RGD-containing sequence controls cell attachment (Grant *et al.*, 1989) and the SIKVAV sequence appears to enhance several morphological processes important in endothelial cell differentiation, by initiating branching and the formation of new capillaries from parent vessels and invasion into the extracellular matrix (Grant *et al.*, 1992; Schnaper, Kleinman and Grant, 1993). Monoclonal anti-α_2 integrin attaches to the laminin receptor $\alpha_2\beta_1$ integrin and inhibits attachment of endothelial cells (Languino *et al*, 1989).

Anti-collagen type IV has less of an inhibitory effect when used at a titre of 1:4-1:10 (Kubota *et al*, 1988; Lawley and Kubota 1989) but has been shown to promote attachment and spreading. It also acts as a non-thombogenic surface preventing platelet aggregation (Madri *et al.*, 1980a). Platelets are

activated by collagen IV, but not by laminin or heparin sulfate proteoglygan (Tryggvason, Gehron-Roby and Martin, 1980).

Microstructure of endothelial cells in tube formation *in vitro*

Integrins are cell surface molecules that mediate the cell adhesion to neighbouring cells or to the extracellular matrix. Anti-integrin antibodies directed at major integrin receptors, enhance capillary formation, converting endothelial cells from a proliferative phenotype towards differentiation. The VLA-2 integrin receptor helps in the induction of tube formation by collagen I, with possible protein kinase C activation (Jackson *et al.*, 1994).

Electron microscopy has revealed that in early tube development the lumens are filled partly with collagen fibrils, partly with membranous and amorphous material originating from the endothelial cell. The surrounding cells have typical endothelial junctional complexes and a perforated structure, with some resemblance to a basement membrane in direct contact with the gelatin substratum (Kubota *et al.,* 1988).

Endothelial cells distort malleable substrates *in vitro* as they move over them by a process referred to as traction (Vernon *et al.*, 1992; Scnaper *et al.*, 1993; Davis and Camarillo, 1995). This phenomenon can be seen when endothelial cells are seeded on Matrigel; the gel becomes distorted and the cells appear to pull the gel towards them. It is thought that traction may be responsible for collagen cable formation, which in turn may guide network formation.

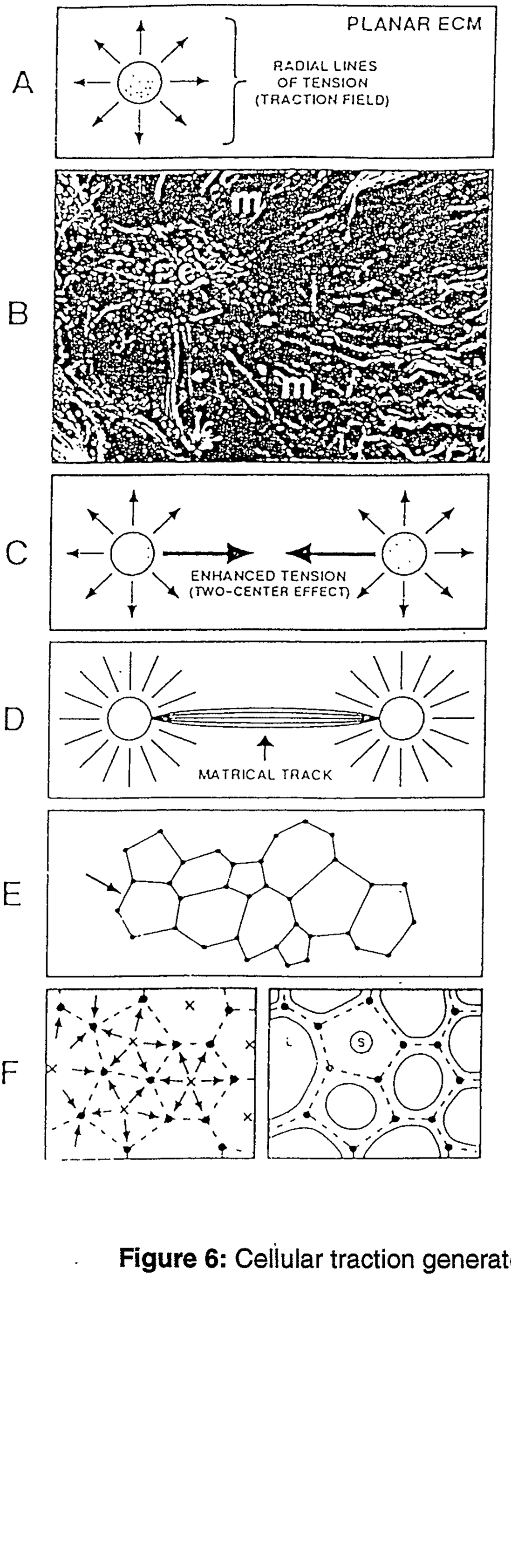
Tube formation in Matrigel models

Traction, tension and tessellation

The phenomenon of traction can be visualised by the culturing cells on thin films of polymerised silicone. The pull of each cell generates tension wrinkles and a pleated array of compression folds in the sheet that emanate from beneath each cell body (Harris, Wild and Stopak, 1980; Vernon *et al.*, 1992). Monolayers of subconfluent endothelial cells exert traction on the extracellular matrix in a similar manner, organising a planar substrate of extracellular matrix *in vitro* into complex patterns (eg. gels of Matrigel or type I collagen).

Each endothelial cell or aggregate of cells acts as a traction centre by continual pulling of extracellular matrix, eventually generating radiating traction fields. Where adjacent traction fields overlap, tension within the extracellular matrix is enhanced, causing "fibres" of extracellular matrix to align into narrow tracks that connect the traction centres. A planar field of traction centres will become connected by multiple two-centre effects that form a tessellated ie, network-like, pattern within the layer of extracellular matrix. When the layer is highly malleable, the stresses generated by these traction fields in the extracellular matrix will eventually perforate the sheet of extracellular matrix and reorganise the matrix into a web of cables (Vernon and Sage, 1992) [Figure 6].

The evidence indicates that it is via the conversion of extracellular matrix from sheets to webs that networks of type I collagen are generated by confluent monolayers of endothelial cells during spontaneous angiogenesis *in vitro*. Planar networks of endothelial cells typically arise *in vitro* in intimate association with extracellular matrix. Therefore, it is likely that traction-mediated tessellation of extracellular matrix is typical of models of vascular development that generate flat networks of endothelial cells.



A: viewed from above, a cellular traction centre (shaded circle) in contact with malleable, planar ECM (rectangle) generates radial lines of tension (arrows) in the ECM that constitute a traction field.

B: An EC cultured on a layer of Matrigel is seen by scanning electron microscopy (x2400 at 60°). Ribbons of Matrigel (M) that comprise a traction field radiate from beneath the EC. Long microvilli (arrows) extend from the edge of the EC and contact the ECM.

C: Tension in ECM between adjacent traction centres is enhanced (large arrow) as a consequence of the two-centred effect.

D: Fibres of ECM (black arrows) align along the direction of principle stress. Fibres influenced by the two-centred effect align to form a matrical track that connects the traction centres.

E: Traction centres (Small black dots) arranged in a field on planar ECM become connected by matrical tracks (eg. Arrows) that form a network.

F: left panel: where planar ECM (shaded) is highly malleable, centripedal movement (arrows) of ECM to traction centres (black dots) results in clearance of ECM from the central areas(X) that are bordered by two-centred effects (dotted lines)

F: right panel; clearance process diagrammed in left panel is manifested as perforations (white arrows) in the sheet of ECM (Shaded). Perforations are small (s) initially but enlarge with time (L). ECM aligned by two-centred effects (dotted lines) between traction centres (black dots) is resistant to cell-generated stresses. (Diagrams adapted from Vernon *et al.*, 1995)

Figure 6: Cellular traction generates patterns in planar ECM models.

Traction and morphogenesis in vitro

Vernon *et al.*, (1992) showed that cellular elongation and progressive motility across the surface of the gel was restricted to the tracks of aligned matrix and did not occur until the tracks appeared. The morphological changes were inhibited by microfilament-disrupting agents cytochalasin D, or microtubule inhibitor, colchicine (Grant *et al.,* 1991). This suggests that changes in Matrigel result from the basement membrane components remodelling causing the formation of extracellular matrix cables, which act as templates to direct the formation of complex cellular patterns and individual cellular cords (Vernon *et al*, 1992).

Large amounts of amorphous and granular debris are clearly visible in the channel area of the tube (Maciag *et al.*, 1982) suggesting that aligned HUVEC's use the cell-surface debris and neighbouring cells as a scaffolding, such that podia are extended over the debris to form single cell junctions (Folkman and Haudenschild, 1980).

Traction-mediated contraction of type I collagen gels by endothelial cells *in vitro* is stimulated by basic fibroblast growth factor (Vernon and Sage, 1995) which also enhances angiogenic sprouting *in vivo* (Olivo *et al.*, 1992) and promotes the invasion of collagen by endothelial cells *in vitro*. The function of traction in matrical invasion during angiogenesis is unclear, but it might contribute directly to the propulsion of endothelial cells through the extracellular matrix and/or facilitate the reorganisation or clearance of extracellular matrix immediately ahead of the invading endothelial cells.

High traction forces by sprouting endothelial cells might align extracellular matrix over greater distances and thereby form pathways for cellular migration, consistent with the "follow-the-leader" behaviour exhibited by endothelial cells that sprout *in vivo* and *in vitro*. Matrical pathways could also facilitate the development of anastomoses between vascular sprouts and endothelial cells, as the tips of adjacent sprouts would align extracellular matrix between them by a traction-mediated two-centred effect, approach one another via a matrical pathway and fuse to establish a common lumen (Figure 7).

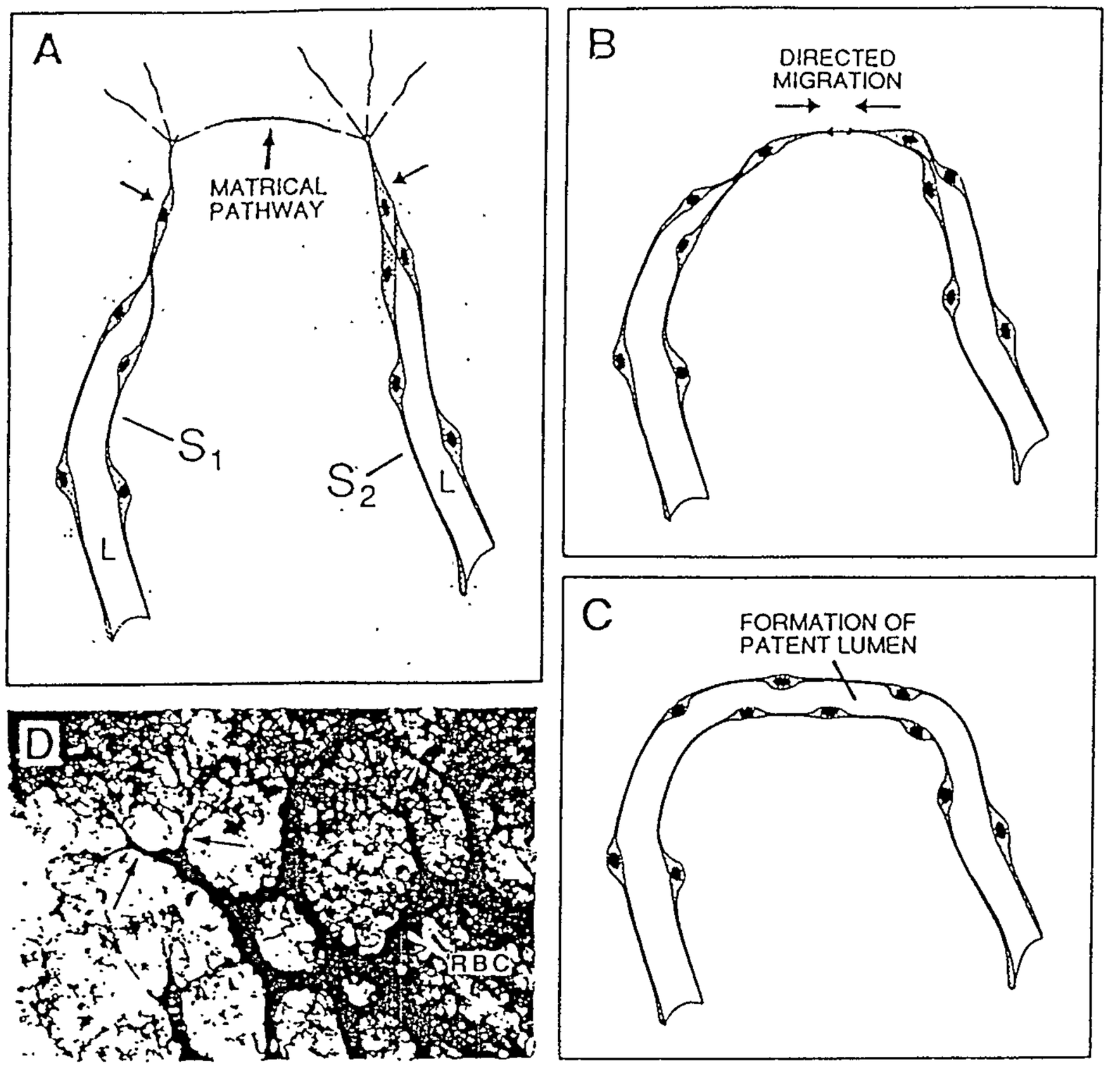


Figure 7: Hypothetical role of matrical pathways in the anastomoses of angiogenic sprouts *in vivo.*

A: migratory: ECs (small arrows) at tips of adjacent sprouts (S1 S2) create a connecting pathway of aligned ECM (matrical pathway) as a consequence of traction-mediated two centre effect. Lumens (L) of sprouts are indicated.

B: Migratory ECs approach one another (arrows) via the matrical pathway.

C; The vascular loop is completed as ECs meet, adhere, and interact to form a patent lumen.

D: Advancing front of the planar capillary network within the nerve fibre of fetal Macaca monkey retina (day 105 of gestation). Capillary sprouts with patent lumens that contain red blood cells (RBC) are labelled darkly with an immunoperoxidase reaction for the cell adhesion molecule CD31, a marker for ECs. ECs at tips of sprouts(arrows) extend filamentous, cytoplasmic processes. Matrical pathways might facilitate the formation of cytoplasmic bridges (arrowheads) that initiate anastomoses (X250).
Adapted from Vernon and Sage 1995

Control of cell morphology may be due to an equilibrium between traction generating elements of the cytoskeleton and the mechanical properties of the extracellular matrix, or by coupling via transmembrane cell surface molecules between the force-generating elements of the cytoskeleton and the plasma membrane.

Alteration of the mechanical properties of the extracellular matrix has a significant effect in its response to cellular traction. Decreased thickness over rigid support inhibits alignment of extracellular matrix by cells and prevents the development of cellular networks (Vernon *et al.*, 1992). Cell-mediated reorganisation of gelled fibrillar type I collagen *in vitro* is suppressed as the concentration of collagen in the gel is increased. Cultures lacking collagen do not generate networks of extracellular matrix, while the secretion of collagen increases the tensile strength of the matrix.

Inhibition of endogenous metalloproteinases blocks angiogenesis within the chick chorio-allantoic membrane by suppressing the invasion of type I collagen gels and by inhibiting the organization of endothelial cells into networks on Matrigel *in vitro*. Proteolysis reduces the mechanical resistance of the extracellular matrix to penetration and reorganisation by the migratory endothelial cells. The angiogenic stimulators basic fibroblast growth factor and vascular endothelial growth factor both promote degradation of extracellular matrix by endothelial cells through increased synthesis of plasminogen activator, collagenase and other proteases.

Objectives of the present investigation

The formation of initial networks on extracellular matrices relies on traction centres by the endothelial cells forming fields of traction. When neighbouring traction centres coincide the extracellular matrix becomes modified into collagen fibrils and these fibrils act as templates for cell migration and eventual tube formation (Jackson and Jenkins, 1991).

If the endothelial cells utilise a template of collagen "fibres" for initial network formation, a further extension of this idea could be that an episode of acute injury which caused the complete lysis of a previous network, leaves the previous basement membrane intact. Future angiogenesis could utilise the basement membrane of the pre-existing network (Jackson and Jenkins, 1991) or the organisation of the extracellular matrix for the re-establishment of a vascular network. The relative importance of each could be investigated.

As outlined previously the aims of this investigation were to:

1. Develop a vascular model *in vitro* that mimics angiogenesis by using cultured human umbilical endothelial vein cells.
2. Determine the relation of the cultured endothelial cell network to extracellular matrix components.
3. Determine the role of the basement membrane in re-vascularization.
    - In this model, rapid endothelial cell death caused by hypotonic shock would leave the previous basement membrane intact and leave the extracellular matrix still capable of further network formation by re-seeding human umbilical vein endothelial cells.
    - Determination of the relations of the re-vascularization network to the original network would involve the investigation of the relative importance of the previous basement membrane and matrical collagen fibrils in these relations.
4. Determine any differences in the influence of an immature versus mature basement membrane on re-vascularization by establishing the relative importance of collagen type IV and laminin in identification of the original template and to determine the three dimensional relations of the cultured cells to the reconstituted basement membrane proteins in this model system.

Materials and Methods

Experimental approach

1. Endothelial cells were cultured on 24 well Matrigel-coated culture dishes to form capillary-like networks. The networks were allowed to mature and then killed with water and re-seeded with an identical number of cells. Comparison of networks formed was made by taking photographs of the same field using an inverted phase-contrast photo-microscope.

2. By using embedded samples and sectioning, the relation of the endothelial cells to the Matrigel was shown at 6 hours and 18 hours.

3. By taking photographs of a chosen microscopic field, the relations of the new network to the old was investigated.

4. Transverse embedded sections of re-seeded samples demonstrated the relations of old to new cells.

5. Antibodies specific to human type IV collagen and laminin were used to investigate the importance of identification of the basement membrane components layed down by the first network for establishment of an identical network on re-seeding.

6. Embedded samples were studied by transmission electron microscopy and analysed for the relations of living and dead cells.

Human endothelial cell culture

Human endothelial cells were routinely isolated from fresh human umbilical cords obtained from Dr. Christopher Jackson Sutton Laboratories at the Royal North Shore Hospital. Transportation in sterile cord buffer solution maintained on ice at 4°C until perfusion, helped to minimise any deterioration of the cord. The umbilical cord was perfused with Hank's Balanced Salts Solution until clean, then perfused with collagenase to digest the lumen of the cannulated umbilical veins, according to the method of Jaffe *et al.*, (1973). The endothelial cells were flushed from the vein and cells collected by centrifugation and resuspended in endothelial cell medium (Appendix 1). Cells were grown in gelatin-coated cell culture flasks as a homogeneous population. The low vigour of the endothelial cells meant that non-homogenous cultures would be over-grown by contaminating fibroblasts and smooth muscle cells making contamination easily detectable. Endothelial status was confirmed by immunochemical staining for factor VIII-associated antigen and *Ulex* agglutinin binding, which is more reliable than factor VIII (Little *et al.*, 1986). During the logarithmic phase of cell growth, cell doubling occurred within 48 hours. Cultured human endothelial cells grew as a monolayer of closely apposed, polygonal large cells (Jaffe *et al.*, 1973).

Subculturing endothelial cells

Confluent endothelial cell cultures were passaged by initially rinsing with Dulbecco phosphate buffered saline (PBS) to remove floating cells, and dispersed at 37°C in trypsin-EDTA (Ethylene Diaminetetraacetic Acid) solution. After cells became free, the trypsin-EDTA was neutralised with medium containing foetal calf serum. The resultant suspension was immediately centrifuged to isolate the cells after the removal of the supernatant. Minimal time of cell exposure to trypsin is important to minimise cell death. The cells were resuspended in fresh endothelial cell media and split during passaging in a one to two ratio and placed in new gelatin-coated cell culture flasks (Appendix 1).

Cell freezing

Excess cells were stored frozen in liquid nitrogen after normal cell collection and centrifuged to form cell pellets. The pellets were resuspended in a freezing mix containing M199 medium, 40% foetal calf serum and 10 % dimethyl sulfoxide (DMSO). Cells were frozen at 10^6 / ml in cryo-vials. This is equivalent to a confluent 75 cm^2 flask. Suspensions were cooled gradually to -70°C then stored in liquid nitrogen.

Cell thawing

Cells were thawed in a water bath at 37°C and immediately diluted with 10 ml of medium, mixed thoroughly and centrifuged at 1000 rpm for 10 minutes. The supernatant was removed, so removing the dimethyl sulfoxide which is toxic, but essential to stop ice crystal formation during the freezing process. Cell suspensions were diluted in 10 ml of medium and placed in 25cm^2 culture flasks and incubated. After 24 hours the medium was changed and cells reached confluence within 3-4 days.

Materials

Endothelial cell growth medium

The medium has been developed over a number of years and contains a number of vital components consisting of: Medium 199 which is a combination of vitamins, amino acids, and other factors which support growth of explanted tissue. However for long term cell culture and propagation, the addition of a serum supplement, for example foetal calf serum, which contains undefined substances and an endothelial cell growth supplement, which is an extract of bovine neural tissue, containing growth promoting factors for human endothelial cells, are essential (Maciag, Hoover and Weinstein, 1982). All propagation medium also contains Heparin sodium which acts as an anticoagulant and a sodium pyruvate buffer. Antibiotics such as penicillin and streptomycin along with a fungicide, fungizone, were present to suppress any infection during the caesarean birth (Appendix 1).

Endothelial growth supplement was supplied (Sigma-Aldrich, Castle Hill, NSW) as a sterile lyophilised powder. The optimal working concentration

for the growth of vascular endothelial cells ranges from 75 µg/ml to 300 µg/ml.

Matrigel

Matrigel basement membrane matrix (Becton Dickinson Labware, Collaborative Research, Two Oaks Park, Bedford, MA) is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumour rich in extracellular matrix proteins. The major component is laminin, followed by collagen IV, heparin sulphate proteoglycans, entactin and nidogen. Matrigel gels rapidly at 22°C - 35°C and has a working temperature of 4°C (Appendix 1).

Matrigel stored at -20°C was warmed to 0°C on ice and a volume to form a 0.5 mm thick gel was spread on pre-cooled 24 well culture trays (Nunclon, Delta Nunc Inter Med) or chamber slides (Nunc 4804, Lab Tek, Nunc Inc. Naperville USA). The matrix was allowed to gel at 37°C for 30 minutes prior to seeding.

Experimental procedure

Cell concentration

The ideal cell concentration was considered to be approximately 85,000 cells/ml and 100 µg/ml of ECGS resulted in optimal formation of capillary networks within six to eight hours that persisted for at least 48 hours.

The cell concentration was calculated using a haemocytometer. Two grids each comprising five boxes of twenty five squares added together are equal to a cell count in 0.001 ml. Due to capillary action the volume of fluid for this surface area is constant. Cells were only counted in a particular square if they were wholly in the square or touching the top or right borders. The total cell count from this slide multiplied by 1000 gives the cell count/ml.

Seeding Matrigel for optimal tubing

Preliminary studies indicated that when suspensions of human umbilical vein endothelial cells were pipetted onto Matrigel, they attached to the gel almost immediately, underwent morphological differentiation, lined up, and began to form vascular-like networks within 6 hours. Networks were defined as straight cellular extensions joining two cell masses or branch points.

Preliminary studies in the laboratory indicated that cell seeding density and the endothelial cell growth supplement (ECGS) concentration were critical determinants for the formation and persistence of networks on Matrigel.

Seeding numbers of 70,000 cells per 1.9 cm^2 well and 65,000 cells per 1/4 chamber-slide produced ideal networks. The area of the wells was sufficient to minimise edge effects and provide a large viewing area of uniform vascular networks. These were then incubated and checked for tubing at 2 hours. At 6 hours a network had established with thin intercellular connections. High seeding (120,00 cells/ml) results in slower and limited formation of thick networks making observation more difficult. Withdrawal of ECGS induces a disruption of pre-formed vascular networks.

Passage 4-8 endothelial cells were used in the following investigation with more than one source of cells used for each of the experimental protocol.

Hypotonic shock and instant cell death solution leaving the gel intact

Dispite numerous chemical cocktails used in this present study, the most efficient procedure and least destructive to the Matrigel was sterile hypotonic ultra-pure water (Milli-Q system) at 18 Meg Ω (Milli-pore LTD Sydney) which caused instant death of the cells leaving the basement membrane intact.

Maintenance of identical temperatures of all solutions and graded changes in osmolarity proved essential to avoid irreversible damage or distortion of the gel, since even minor temperature variations resulted in instant Matrigel degeneration. Hence water at 37°C was added for 2 minutes to each well to dilute the medium to a 50% concentration, this was removed and replaced twice with 100% water each time for 2 minutes. The water was subsequently displaced using PBS at a 50% concentration for 2 minutes and replaced with 100% PBS for 2 minutes. Eventually this was replaced with 100% medium ready for re-seeding. At each change of solution care to avoid direct rinsing of the gel avoided disruption of the gel.

Establishment of a second network

On re-establishment of normal osmolarity, the Matrigel was inspected for damage or gross distortion in the preselected microscopic field. Often, due to the osmolarity changes, the initially chosen region, no longer had optimal light diffraction and visualization of the network was impaired. If this proved too severe or damaged gel was present, the experiment was abandoned. If the network appeared intact and a good image maintained, re-seeding took place using identical numbers and technique to the initial seeding.

Photographic recording

The networks were photographed at 15 minute intervals, at previously recorded stage co-ordinates using a template secured to the microscope stage, with the Zeiss camera mounted on a inverted phase-contrast microscope (Axiovert 10, Zeiss Germany). Exposure, duration and winding functions were all controlled from a Zeiss photographic control unit attached to the camera (MCO80, Zeiss Germany). All photographs had to be taken individually due to the necessity for minor adjustment to the focus. Photographs were taken using a 10x objective due to problems with light dispersion at lower powers. The camera and microscope were installed completely inside an CO_2 incubator (I R Sensor, supplied by Quantum Scientific PTY LTD Aust.) and maintained at 37°C and 5% CO_2 throughout the experimental period.

Comparison of first and second networks in two dimensions.

Photographs of the original 6 hour networks prior to hypotonic treatment, at treatment and at time of re-seeding, were compared with photographs taken every fifteen minutes for the following 2 hours, at 6 hours and some at 18 hours from re-seeding. The experiment was repeated and recorded at least three times for each experimental condition. Thirty runs of the main experiment were undertaken each with concurrent controls, to ensure good technique and consistency of the experimental procedures.

Computer-assisted analysis was undertaken, using images scanned into a Power Macintosh computer and manipulated utilising Adobe Photoshop Version 3.5 Macintosh software (Adobe Systems Incorporated). The original images were “cleaned” and superimposed on the re-seeded networks, and both networks were “cleaned” and superimposed.

Controls

Appropriate controls were included and recorded on three consecutive experiments for each stage of the protocol. The controls ensured that exposure to water did not irreversibly affect the Matrigel. Observation of a six hour network over the two hour experimental period ensured that there was no significant modification of the network over this period and controls using initial networks exposed to water returned to normal osmolarity, ensured that the cells were killed and did not re-establish the network over the subsequent two hour period.

Controls were run concurrent with each experiment recorded to ensure consistent technique and effectiveness of the procedures used.

Three controls were used:

Control 1: Matrigel was laid down and allowed to gel over a 30 minute period in the same incubator used for the experiments, then 0.5 ml of medium was placed over the gel in each well and left in the incubator for 6 hours. At which time, the gel was then exposed to the process of osmotic changes identical in time and concentrations to the main experiment. Once normal osmolarity was re-established the wells were seeded at the optimum cell concentration of 70,000 cells per well and photographed at 30 minute intervals for 2 hours unmoved after the initial photograph.

Control 2: Matrigel was allowed to gel as above for 30 minutes, then seeded at time 0 with 70,000 cells per well and allowed to network in the incubator for 6 hours. Photographs were taken at pre-arranged co-ordinates at 30 minute intervals over the next 2 hours, covering the period of experimentation.

Control 3: Matrigel was allowed to gel as above for 30 minutes and seeded with 70,000 cell per well at time 0. The cells were incubated for 6 hours, good networks were identified and then exposed to the osmotic changes for identical times and concentration gradients as the main experiment. A microscopic field was then identified and photographed at 30 minute intervals over the next 2 hours.

Established primary network re-seeded

To investigate the influence of an established network, since the majority of the literature suggested that patent vascular tubes only form after 18 hours, the experiment was repeated including the controls, but extending the original incubation period to 18 hours instead of 6 hours and photographs again taken at pre-determined stage co-ordinates.

Three dimensional study of the original network at 6 hours and 18 hours

Matrigel was gelled on 4 well chamber slides, (Nunc, Medos Co Pty Ltd, Lidcombe Australia) as described before. Because of the reduced surface area of 1.8 cm^2 a cell concentration of 66,000 +/- 500 per well was used. The chambers were allowed to network for 6 hours, then exposed to the identical osmotic changes for identical time periods as in the previous experiment. Re-seeding took place using 66,000 cells per well and the slides were returned to the incubator for 2 hours.

At two hours the chambers were viewed to check for cell density and the progress of the second network. Then 2 hours, 4 hours and 6 hours after re-seeding two samples of each time period were fixed with Karnovsky's fixative (Appendix 1) at 37°C to ensure no detrimental change in the Matrigel while fixing and utilising an externally ventilated hood due to the toxicity of the fixative vapour at this temperature. The samples were fixed in Karnovsky's solution for 5 minutes, then rinsed with PBS three times to remove any remaining fixative.

Dehydration was achieved using graded concentrations of ethanol for 10 minutes at each concentration, starting at a 30% concentration, progressing to 50%, 70%, 90% and then 100% ethanol.

After dehydration in 100% ethanol, the samples were immersed in catalysed solution A for 1 hour at room temperature. The embedding acrylic was mixed (L R White TAAB Laboratories equipment LTD Berks UK) and immediately placed in the chamber wells. These were then placed in a cold room at 4°C for at least 4 hours to prevent the plastic of the chamber-slides melting due to the exothermic reaction of the resin. Once set, the glass slide and plastic walls were gently removed from the acrylic block.

The blocks were trimmed, to fit the mounting moulds and reorientated at right angles to the expected cutting edge. New acrylic was mixed and these moulds were filled thus embedding the networked blocks, ready for slicing.

Sections of 2 μm thickness were cut using a glass knife in a Sorvall JB4 microtome and mounted on slides, dried on a hot plate then stained with Toluidine Blue. These were then rinsed and cover-slipped using mounting media (Permount). Observations were made and photographed through the microscope.

Transmission electron microscopy (TEM)

Fixing of the chamber-slide networks took place as before, and stored in PBS. After transportation to Westmead Hospital, these were washed with MOPS Buffer and under-went a second fixation using 0.2% osmium tetroxide in cacodylate buffer for three hours. The cells were flushed with distilled water for 5 minutes and any excess water removed. Then exposure to 2% uranyl acetate for 1 hour, dehydrated using alcohol at increasing concentration from 50%, to 70%, to 95% until dehydrated twice with 100% concentration. The fixed and dehydrated cells were then soaked in a 50% solution of low viscosity resin (Spurr) and alcohol for 1 hour, then pure resin for three rinses for 10 minutes each at 70°C. Then fresh resin was placed and polymerised at 70°C for 10 hours. After which time the slide was directly placed in liquid nitrogen and the differential contraction allowed the easy removal of the slide and walls of the chambers from the resin.

These blocks were sawn into small pieces and re-mounted in correct orientation. Semi-thin sections were stained using 1% Toluidine Blue dissolved in 1% Borax on a hot plate. Blocks were trimmed to the area of interest and thin sections were produced with a diamond knife mounted on a Reichert Ultra-microtome. Sections on copper grids were stained in lead citrate, examined and photographed in a Philips CM10 electron microscope at the electron Microscopy Unit at Westmead Hospital.

Studies using antibodies to human type IV collagen and laminin

To determine the significance of identification of original basement membrane proteins, two antibodies were incubated with the lysed networks for 30 minutes prior to re-seeding. The wells were then rinsed with PBS at least twice to remove any antibody before re-seeding; further rinses were too destructive to the gel. The re-establishment of the second network was observed over the next two hours and compared to the main experiment.

Both antibodies used were specific for human tissue (Sigma BioSciences St Louis USA). Firstly monoclonal anti-laminin mouse ascites fluid clone Lam-89 (IgGI isotype) localises specifically to the basal membrane of humans, pig and cat with not to mouse basal membrane (as found in Matrigel). The product shows no reaction with collagen type IV, fibronectin, vitronectin or chrondroitin sulfates type A, B or C. Working dilutions of 1:1000 were advised. Secondly monoclonal anti-collagen type IV mouse ascites fluid clone Col-94 (IgGI isotype), recognises an epitope located on the α1 and/or α2 chains of human collagen type IV, with no other reactivity. Working dilutions of 1:500 were advised.

These antibodies were dialyzed with six changes of PBS at 4°C for 48 hours, to remove the sodium azide used in storage. The resultant antibody was diluted to a stock concentration of 1:10 and final working concentration of 1:100 were used. The maximum dilution of the culture medium was 10%. As each antibody preparation was an IgGI isotype monoclonal antibody they were used as controls one against the other.

Controls were run at least three times:

Control 1: Cultures were initially seeded in the presence of the respective antibodies. The wells were observed at 2 hours, 6 hours and 12 hours for tube formation.

Control 2: Cultures were initially seeded with medium diluted with 20% concentration of PBS and allowed to network for 6 hours. This was to see any effect of diluting the media on tube formation.

Control 3: Six hour cultures were then exposed to antibodies at a 1/100 concentration and observed for 2 hours to see any effect on established networks.

Results

Establishment of the model

Morphological aspects

The endothelial cells were plated on 24 well plates or microscope chamber slides. Over the course of the experimental period over 100 separate plates were cultured. To reduce the variation between cultures, all the Matrigel used came from the same batch number (lot # 909795). The Matrigel was aliquoted at 4°C on a bed of ice (inside a UV hood to prevent contamination), into pre-cooled sterile containers, which were also stored on ice. The Matrigel was then stored at -20°C.

To ensure the lowest possible viscosity and consistency of texture, it was necessary to thaw the Matrigel at 4°C for at least 12 hours prior to use. To maximise the evenness of the coatings, the sterile 24 well plates and chamber slides were also stored at 4°C for 12 hours prior to use. For the initial 6 hour experiments a 0.5 mm thick layer of Matrigel was used, while due to lifting of the gel off the base at 18 hours twice as much Matrigel was used for these experiments (1 mm layer). Under these conditions, the Matrigel flowed and there was sufficient working time to gain an even film of gel. After coating, the dishes were placed in an incubator at 37°C for thirty minutes prior to seeding following the manufacturer's instructions. This allowed complete gelation of the material before the initial seeding.

Initially, late passages of vascular umbilical endothelial cells were acquired from frozen stocks at Westmead hospital. Variation between early and late passage cultures was noticeable especially in the speed of network formation and general vitality. To reduce this variation, cells were isolated from umbilical veins obtained at caesarean section at the Royal North Shore and Westmead hospitals. Transport of the cords in ice boxes ensured a fresh vein for extraction of endothelial cells.

This allowed the experimentation to be completed between passages 4-8 and cells from 2-3 different cords were used, for any series of experiments, to eliminate abnormal individual cell culture line responses.

Some considerable time was taken to optimise the conditions for cell extraction. Problems of fungal infections occurring due to fungal

contamination at the time of the caesarean birth, were controlled by using Fungizone 250µmg/100 ml medium in the early passages.

To prevent other sources of contamination, all the other mixtures used were aliquoted into small sterile single use containers. This also ensured that re-entry into containers was minimal and that the containers were heated to 37°C only once before exposure to the cells. This reduced any denaturing of the trypsin / EDTA and maintained a quick lifting off of the endothelial cell during passaging. Prior to passaging, the endothelial cells were rinsed with PBS to remove unattached cells thereby ensuring recovery of only viable cells.

Culture medium was mixed in 100 ml batches when needed. The use of components previously aliquoted into volumes suitable for 100ml of medium, ensured quick and easy mixing when necessary. After mixing the medium was sterile filtered and aliquoted into 20 ml containers, then stored at 4°C. The small volumes again minimised the number of re-entries and repeated heatings. At each use, the pH was corrected using 3M hydrochloric acid or potassium hydroxide respectively for optimal conditions for network formation during each experiment.

The monotypic nature of the cultures was verified by binding of endothelial specific marker *Ulex europaeus* Lectin Type 1. The binding was determined by staining using immunoperoxidase technique. None of the cords perfused generated smooth muscle cells.

The cultures were checked daily for bacterial or fungal infection or any sign of abnormal cell distribution caused by a suspected contamination. If so, the culture was discarded. The cells were fed every second day by rinsing with sterile PBS and new pre-warmed culture medium administered.

Figure 8: Shows the duplication rate of human umbilical vein endothelial cells and the stage of cell detachment from the flask when exposed to trypsin/EDTA.

Figure "A" shows a typical cell density achieved in a 75 cm^2 flask 12 hours after thawing from storage as described in Materials and Methods. The cells are sparse and clumped into separate islands with individual cells spindle shaped. Within 24 hours the cell density has doubled, with larger clumps of elongated cells (Figure B). Cell doubling occurs in the next 24 hours, with a more constant cell layer, with only small areas remaining uncovered. Individual cells are still elongated (Figure C). 12 hours later the layer is of consistent density throughout the flask and cells are rounded giving a cobbled-stone appearance (Figure D). On exposure to trypsin -EDTA the cells immediately appear more spherical and within 60 seconds start to lift of the base, by 90 seconds all cells are floating (Figure E-G).

A: 12 hours after thawed cells were passaged into a 75 cm^2 cell culture flask

B: 24 hours later than A, the cells numbers have doubled

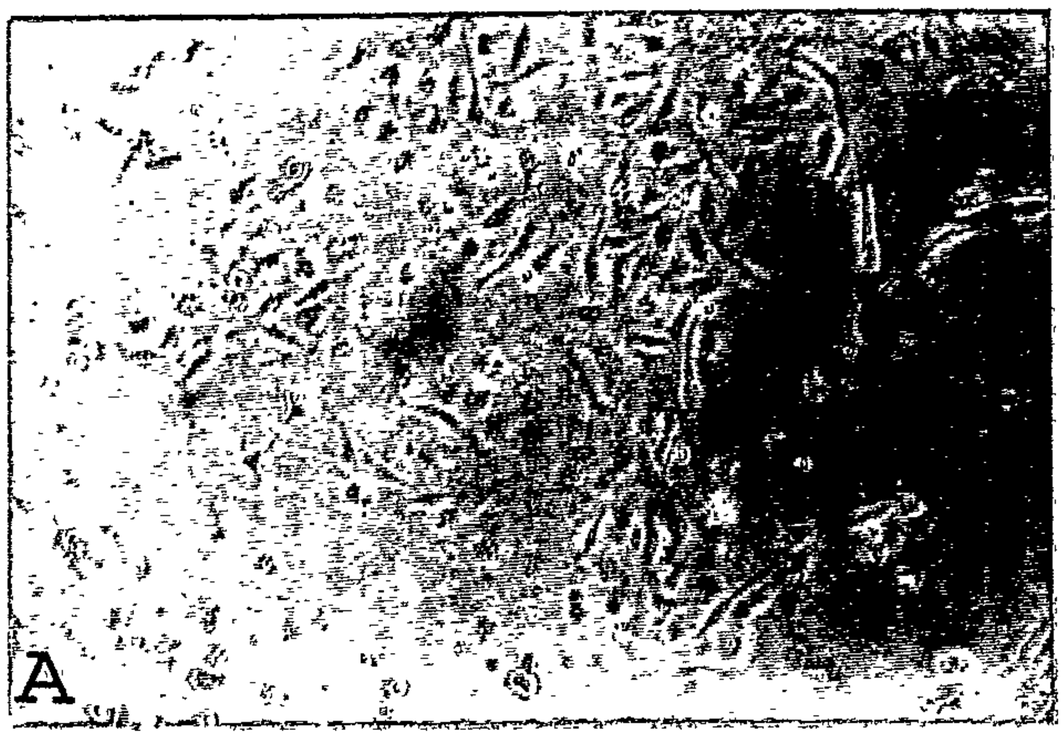
C: 24 hours later than B, the cells numbers have doubled again

D: 12 hours later true confluent layer has been achieved

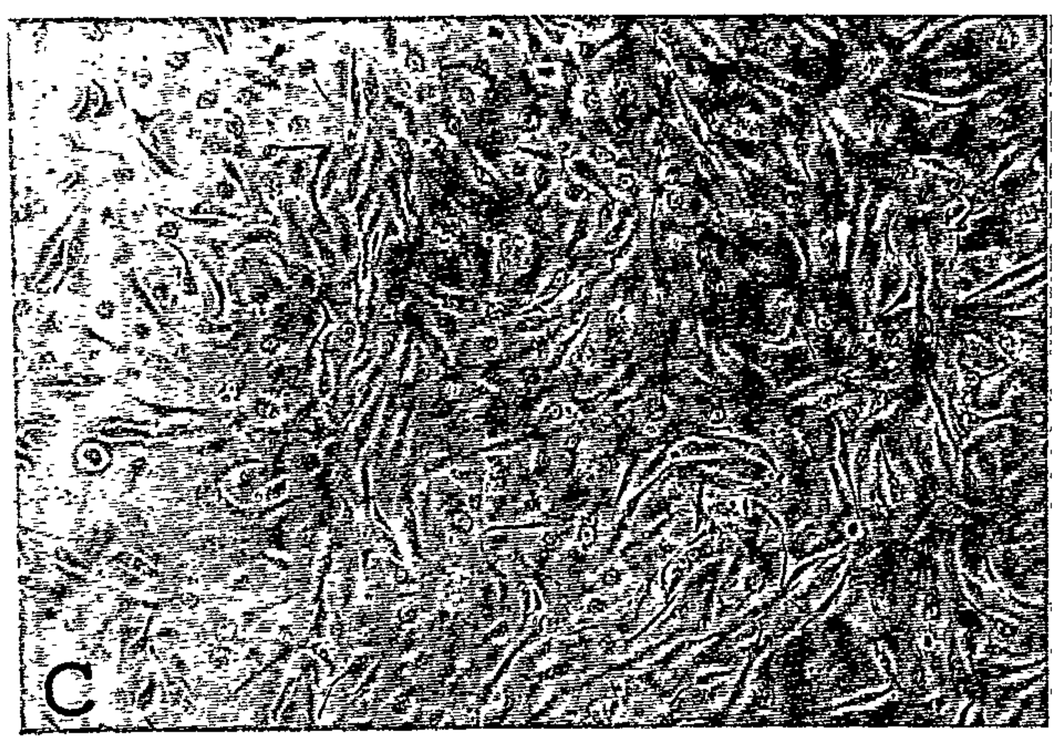
E: Confluent flask at time of initial exposure to trypsin-EDTA

F: Cells exposed for 60 seconds of trypsin-EDTA. Some cells have lifted off the flask surface.

G: Cells exposed for 90 seconds of trypsin-EDTA. All cells have lifted of the flask base.


A


B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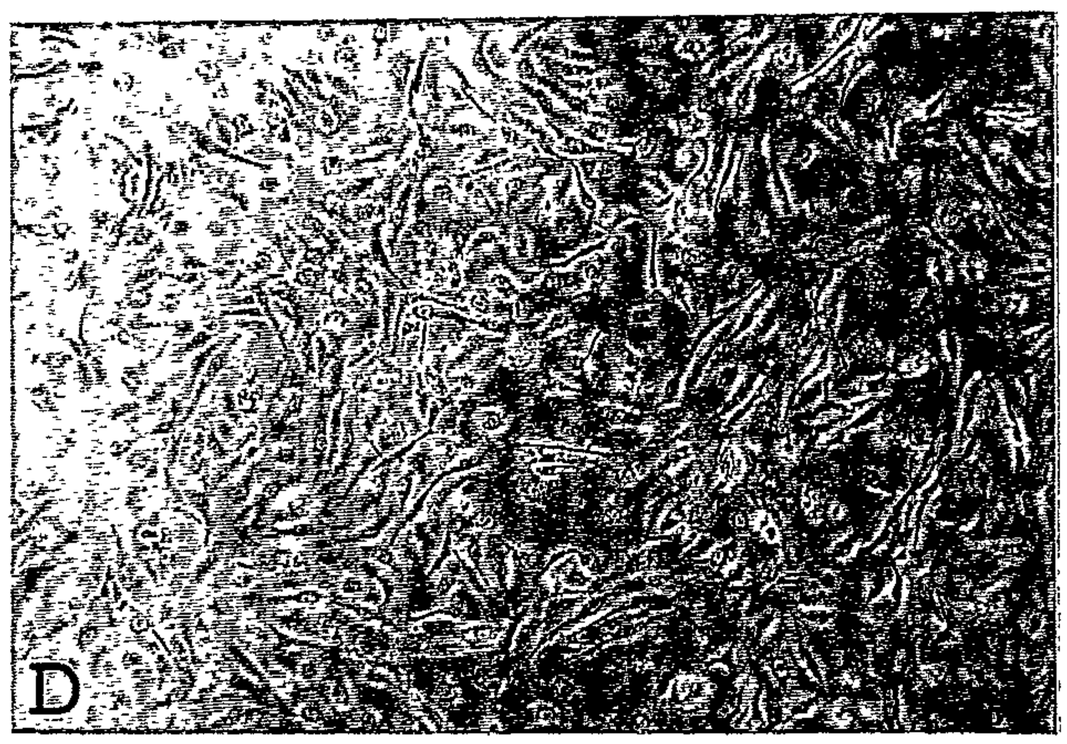
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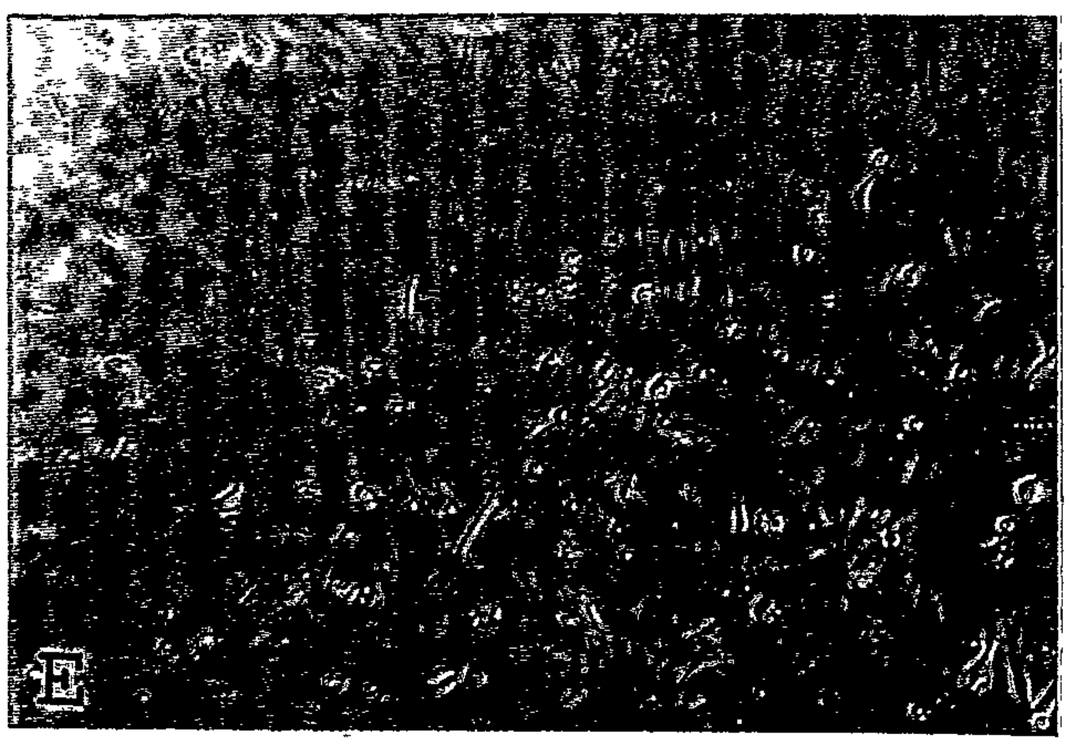
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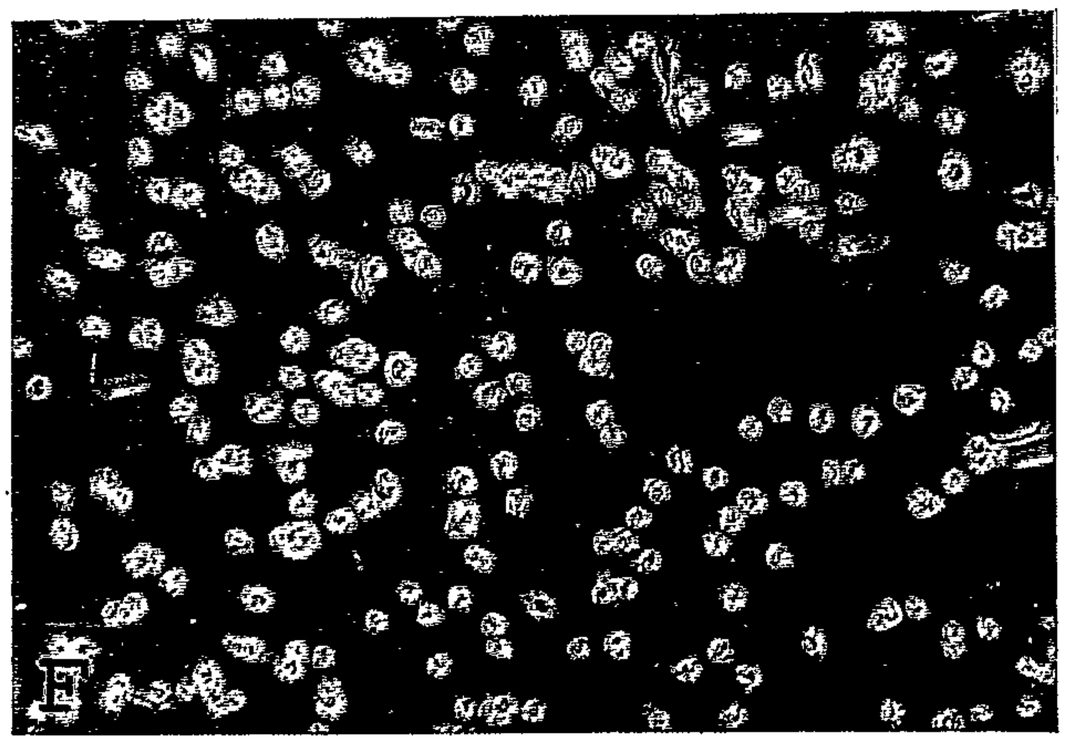
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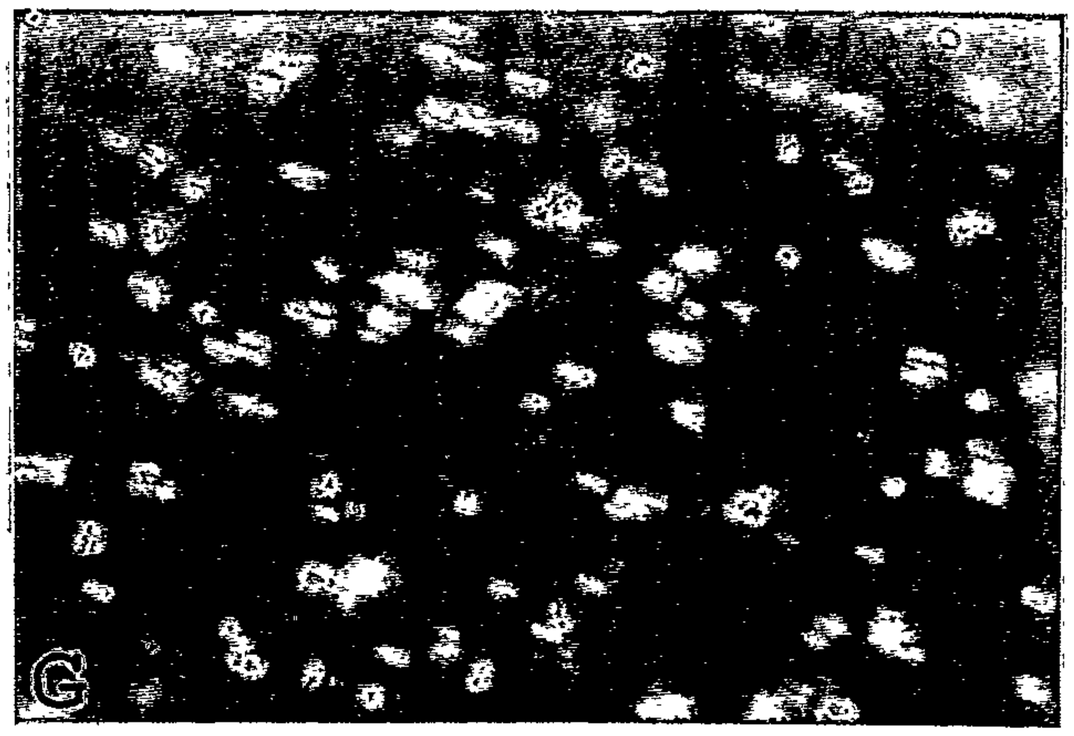
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