NEOVASCULARIZATION INTO THE EPIPHYSEAL GROWTH PLATE: A Morphological Study of the Metaphyseal Vessels

By

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ABSTRACT

The metaphyseal vessels which invade the calcified cartilage of the epiphyseal growth plate were examined by a variety of techniques to determine their morphology, replicative dynamics and growth patterns as they relate to endochondral ossification. For descriptive purposes four different regions of these vessels were characterized: 1) Sprout Tips - the terminal ends of the capillary sprouts which actually impinge upon the hypertrophic chondrocytes of the growth plate. 2) Region of Extended Calcified Cartilage - those vessels deeper within the metaphysis which are surrounded by an extracellular matrix predominantly composed of extended septa of calcified cartilage. 3) Region of Bone Deposition - further still from the epiphyseal cartilage the microvessels are contained within a network of active bone deposition laid down upon a calcified cartilage framework. 4) Region of Primary Vessels - at a distance of 350-500 μm from the hypertrophic chondrocytes are dilated vessels with one or two layers of smooth muscle in their walls, that supply and drain the metaphyseal capillary plexus. Electron microscopic examination following perfusion fixation at physiological conditions of pressure and flow rate with either 2.3% glutaraldehyde alone or mixed with 2% tannic acid, demonstrated several ultrastructural features. The sprout tips of the metaphyseal capillaries are continuous blind-ended vessels lined by an attenuated fenestrated endothelium with no underlying basement membrane. Progressing deeper into the metaphysis, an abluminal basement membrane-like material is found in regions adjacent to the endothelial cell nucleus but not beneath more attenuated portions of the cell. Upon reaching the region of bone deposition, a more complete basement membrane is present and covers an

increasingly larger percentage of the abluminal cell surface; although it is never entirely continuous. Dividing endothelial cells are most frequently found in the region of bone deposition at an average of 175-200 μm behind the apicies of the growing sprout tips. Serial sections revealed that dividing endothelial cells retain junctional attachments to neighbouring cells of the capillary wall throughout mitosis. The cells also form microvillar adhesion sites between daughter cells (and adjacent cells) prior to the completion of cytokinesis, such that they are junctionally linked before dissolution of the cell bridge. Unlike many angiogenic vessels, in the metaphyseal capillaries endothelial cell division occurs at a location where circulatory flow has already been established, and it must produce the cells necessary for continued growth while maintaining an intact vascular wall. Throughout the metaphyseal sprouts the endothelial cells display many features associated with growing vessels including luminal microvilli, abluminal cellular projections, and abundant cytoplasmic organelles. Dividing pericytes are characteristically found distributed evenly between the regions of extended calcified cartilage and bone deposition (50-350 μm from the sprout tip). The capillaries and post-capillary venules which act as the parent vessels from which the metaphyseal capillaries are derived are thought to be located within the region of bone deposition near their union with the larger primary vessels (250-350 μm from the sprout apecies). The metaphyseal capillary sprouts represent a continuous unidirectional angiogenic vascular network which grows via a constant radiating elongation from a growth centre that remains a fixed distance behind the growth front.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTH - Adrenocorticotrophic Hormone

AIF - Anti-Invasive Factor

ADP - Adenosine Diphosphate

BD - Region of Bone Deposition

CAM - Chick Chorioallantoic Membrane Assay

CC - Region of Extended Calcified Cartilage

CDGF - Cartilage-Derived Growth Factor

CP - Cell Projections (Processes)

DC - Daughter Cell

DNA - Deoxyribonucleic Acid

EC - Endothelial Cell

EM - Electron Microscopy / Electron Microscopic

rER - Rough Endoplasmic Reticulum

FGF - Fibroblast Growth Factor

bFGF - Basic Fibroblast Growth Factor

GH - Growth Hormone

L - Lumen

LM - Light Microscopy / Light Microscopic

O - "Other" Cell Type (Osteoblast/clast Lineage)

OC - Osteoclast

P - Pericyte

PAS - Periodic Acid Schiff Reaction

PV - Region of Primary Vessels

RBCs - Red Blood Cells

RHT - Ruthenium Hexamine Trichloride

ST - Sprout Tips

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Cover: Scanning electron micrograph of a plastic corrosion cast of the metaphyseal capillaries as they project into lacunar spaces formerly occupied by hypertrophic chondrocytes. Each sprout tip has characteristic branching knob-like termini (Courtesy of Dr. A. L. Arsenault).

GENERAL INTRODUCTION AND OUTLINE OF THESIS OBJECTIVES

The Metaphyseal capillaries

During endochondral ossification epiphyseal growth plates undergo several developmental changes characterized by specific morphological features involving chondrocytes and their surrounding extracellular matrix. In this developmental sequence chondrocytes proliferate and synthesize increasing amounts of extracellular matrix which later becomes calcified. The cells then undergo hypertrophy, and ultimately become invaded by capillaries from larger metaphyseal blood vessels. It is known that calcification of the longitudinal septa of the extracellular matrix of the terminal chondrocytes must occur before capillary endothelial cells and their associated osteoprogenitor cells can infiltrate the zone of provisional calcification (Trueta and Amato, 1960; Trueta and Buhr, 1960). However, the mechanism of this vascular ingrowth, the systemic and local factors that control it, and even the morphology and growth pattern of the capillaries themselves are poorly understood.

Microangiography utilizing radio-opaque dyes has demonstrated that 80% of the blood vessels on the metaphyseal side of the growth plate are derived from the nutrient artery (Trueta and Morgan, 1960). The remaining 20% of the vessels that supply the periphery of the plate are end ramifications of periosteal perforating metaphyseal arteries and are morphologically indistinguishable from those originating from the nutrient artery (Trueta and Morgan, 1960). Vessels imaged in this manner are seen to approach the growth plate cartilage while dividing into finer arterioles, before all turning

back at approximately the same level and descending in vessels indistinguishable from the ascending branches. Trueta and Morgan (1960) also found that while these capillary loops closely approached the growth plate, there was no direct contact between them and the terminal hypertrophic chondrocytes. The "space" between the capillary loops and the growth plate cartilage was seen to be filled with accumulated erythrocytes, which they suggested could be the result of obstructed vascular outpouchings that failed to fill with the injected material. Further studies at the electron microscopic level determined that the space was in fact occupied by capillaries through which there was minimal circulation (Trueta and Little, 1960; Anderson and Parker, 1966; Schenk et al., 1968). Schenk and co-workers (1968) called these vessels capillary sprouts and they speculated that the circulation in this region might either be lined by a discontinuous endothelium, or be open-ended.

The use of vascular casts, produced by the injection of polymer plastics into the circulation, has helped clarify the three-dimensional appearance of the metaphyseal vessels (Draenert and Draenert, 1985; Arsenault, 1987). These studies confirm that anastomosis of the metaphyseal microvessels occurs at a distance to the growth plate cartilage and that blunt-ended capillary sprouts project up towards the hypertrophic chondrocytes from this point (see cover photograph). Arsenault (1987) has described them as closed capillary sprouts that range in length from 100 to 600 μm and possess irregular and knob-like endings. While many of the sprouts feature lateral projections or bifurcations, they do not form characteristic capillary loops, which raises interesting questions as to the circulation through them.

The only studies to actually address the ultrastructural characteristics of the metaphyseal vessels have centered around the sprout tips in an attempt to determine the blood flow through them. The major question researchers in the field attempted to address was whether or not the vessels represented a closed or open circulatory system. Not surprisingly, they have been described both as sinusoidal capillaries forming a continuous lining (Langer, 1875; Ranvier, 1875; Brookes and Landon, 1964) and as discontinuous capillaries which are open-ended in some locations (Trueta and Little, 1960; Anderson and Parker, 1966; Schenk et al., 1968). The only point on which there appears to be consensus is that the capillaries of the sprout tip have a basement membrane that is either inconspicuous or absent. The endothelial cells of the sprout tip have a well developed rough endoplasmic reticulum and Golgi complex, but they have fewer pinocytotic vesicles than endothelium in other regions of the body (Schenk et al., 1968). The endothelial wall is often so highly attenuated that it appears to be composed of little more than apposed plasma membranes in some locations (Brookes and Landon, 1964). Perivascular cells surround parts, but not all of the vascular wall, and are seen to send out projections towards the calcified cartilage matrix (Schenk et al., 1968).

The principle reason behind the study of these vessels was not an interest in the properties of the endothelium *per se*, but rather the role the vasculature played in the process of endochondral ossification. It became clear that there was an intimate relationship between calcification of the cartilage matrix and the advancement of the metaphyseal vessels. Experiments employing the selective destruction of the metaphyseal and the epiphyseal vascular systems

made it possible to speculate on the various roles the vascular system plays in the growth of long bones. Trueta and Amato (1960) demonstrated the metabolic dependence of the reproductive chondrocytes of the epiphysis, and perhaps those of all of the chondrocytes of the growth plate, on the epiphyseal vessels. If the epiphyseal vessels are destroyed, there is a rapid degeneration of the growth plate cartilage even though a rich capillary network is present on the metaphyseal side (Trueta and Amato, 1960). This has led to the conclusion that the metaphyseal vessels have a limited nutritive function in terms of supplying metabolites to the growth plate cartilage. Destruction of the metaphyseal vessels results in a rapid increase in the number of hypertrophic chondrocytes. The hypertrophic zone quickly extends to several times its normal size and the surrounding cartilage matrix fails to calcify (Trueta and Amato, 1960). These results suggest a role for the metaphyseal vessels both in the removal of hypertrophic chondrocytes and in the calcifiction of the cartilage matrix.

Interestingly, the condition observed during occlusion of the metaphyseal vessels is identical in many ways to that seen in rickets (vitamin D deficiency). In rachitic rats, the hypertrophic zone becomes exaggerated in length, the cartilage matrix fails to calcify, and the metaphyseal vessels do not invade the chondrocytes (Trueta and Buhr, 1963; Arsenault et al., 1988). Upon administration of vitamin D to rachitic animals, there is a rapid reversal of the condition as the cartilage matrix calcifies, the blood vessels invade the region, and within a short time the growth plate is returned to its original size. The same pattern is observed if, after the initial destruction of the metaphyseal vessels, new vessel growth is allowed to take place and

revascularize the area adjacent to the growth plate. From this, two significant relationships between metaphyseal microvessel growth and the process of endochondral ossification can be inferred. The first is that calcification of the cartilage matrix occurs before the blood vessels, and their accompanying perivascular cells, can enter the region and begin bone deposition. In the absence of calcification there is no vascular invasion, the hypertrophic chondrocytes continue to remain viable, and the region elongates. The second is that if the metaphyseal vessels are not present, calcification of the cartilage matrix cannot take place in the first place. When the blood vessels are experimentally removed there is obviously no vascular invasion, but there is also no calcification of the hypertrophic matrix. The same situation is also observed if the metaphyseal vessels are present, but there is insufficient vitamin D available. This produces the interesting paradox that blood vessel invasion is dependent upon calcification of the cartilage matrix, yet this calcification process requires the presence of those very same vessels. This type of interdependency must require communication between the two tissues in order to produce the synchronous series of events that allow the complex process of endochondral ossification to progress forwards.

Microvessel Growth and Angiogenic Factors

Insight as to what humoral factors may be involved in the relationship between the growing vessels and the hyaline cartilage of the growth plate came from a surprising source. Researchers working on angiogenesis, the growth and differentiation of capillaries, were uncovering a great deal of information relevent to the process of endochondral ossification, even if this was not their initial intent.

In the last ten years a great many substances have been isolated, purified, and even sequenced, that can either promote or inhibit the growth of nascent vessels in a variety of ways (Folkman and Klagsbrun, 1987). It quickly became apparent that most every tissue in the body contained various growth factors that were capable of influencing endothelial cell growth; hyaline cartilage, like that found in the epiphyseal growth plate, was no exception. Endothelial cells have been shown both in vivo and in vitro to be a fairly stable population of cells with a low mitotic rate and a turnover time amounting to several years in some tissues (Tannock and Hayashi, 1972; Hudlicka, 1984). In the absence of the proper stimulus, or perhaps the removal of inhibition, capillaries will not proliferate and extend into the surrounding tissue. The proper growth of microvessels requires the presence of angiogenic stimulators which can act at any of the several steps involved in capillary growth (Folman and Klagsbrun, 1987).

It has been recognized for many years that postnatal capillary growth is the result of the extension of new capillary sprouts from pre-existing vessels. Initial studies had been conducted using transparent tissues, such as the tadpole tail, through which blood vessel growth could be observed. In the 1920's, Sandison developed the technique of inserting a transparent chamber in the rabbit's ear which allowed for the direct study of capillaries as they grew during normal postnatal development (Sandison, 1924, 1928). He was able to describe how endothelial cells would initially project away from their vessel of origin. With time, this blind-ended sprout of endothelial cells would acquire a lumen and some blood would flow into it. If the pattern of growth was followed further, adjacent sprouts could be seen

to approach each other, to fuse, and to form a closed capillary loop through which a normal circulatory flow became established. Later still, depending upon the flow rate through the nascent vessels and other factors such as the inherent vascular pattern of the particular tissue under examination, the capillaries could be seen to develop into arterioles and venules as the need dictated. These experiments elucidated two principles of blood vessel growth still held today: (a) all postnatal capillary growth is the result of outgrowth from pre-existing vessels and (b) initial capillary growth is in the form of closed sprout tips with a minimal circulatory function, that later anastomose to form loops.

Capillary growth, regardless of the stimulus that brings it about, follows the same sequential steps. Initially, the capillary sprouts arise either from existing capillaries or from small venules that lack smooth muscle in their walls (Ausprunk and Folkman, 1977). In the presence of a growth stimulus, the endothelial cells of the vessel will begin to break down their basement membrane and to protrude through the vascular wall (Glaser et al., 1983). The endothelial cells, using many of the same secreted enzymes used in basement membrane degradation, clear a path through the tissue that they are migrating through en route to the angiogenic stimulus. The capillary sprout elongates and a lumen forms within it. The sprout continues to grow via a combination of elongation of existing cells and addition of new endothelial cells, which are proliferating at a point distal to both the vessel origin and the sprout tip (Ausprunk and Folkman, 1977; Sholley et al., 1984; Folkman, 1986). The tip of one sprout then joins with another to form a capillary loop, and new sprouts form from these loops to produce an entire microvascular

network (Zawicki et al., 1981). After a loop has been formed, the endothelial cells of the vessel begin to synthesize and secrete a basement membrane which also encloses the pericytes that have enveloped the vessel.

Folkman and Haudenschild (1980) were the first to successfully culture capillary endothelial cells. There were several important implications resulting from this discovery. Primarily it enabled researchers to routinely culture endothelial cells derived from capillaries, as opposed to endothelium derived from large vessel sources such as the human umbilical vein or the bovine aorta. Endothelial cells from different organs have distinctive properties, but also along a given vascular segment there is a structural and physiological variation in endothelium in its progression through artery, arteriole, capillary, venule, and vein (Simionescu and Simionescu, 1984). Therefore, conclusions based on studies using endothelial cells derived from large vessels were not always relevant to the situation in microvessels and it is, after all, in these vessels where neovascularization occurs. A second important finding was that a pure culture of capillary endothelial cells would form closed tubes complete with a lumen, which would anatomose to form a vascular network. This demonstrated that endothelial cells contained all the necessary properties to form a capillary network (Folkman and Haudenschild, 1980) and helped support earlier findings that only endothelial cells could give rise to other endothelial cells (Clark and Clark, 1932, 1939, 1940; Maximow and Bloom, 1948). Still another significant finding was that the capillaries would not grow unless the medium had been preconditioned by tumour cells; cells which were later found to be potent producers of angiogenic factors. This not only

established the need for a promoter of endothelial cell growth to enable successful culture, but it also provided an assay system whereby various agents could be tested to determine if they were capable of stimulating endothelial proliferation and differentiation.

The combination of understanding the principles of capillary growth and having model systems with which to study it made it possible to identify angiogenic stimulants and to determine at which step in the neovascularization process it produced its effect. As more endothelial cell growth factors became identified, it was apparent that they could act on endothelial cells by increasing their mitotic activity, by acting as a chemotactic agent towards which a capillary sprout would grow, and by triggering the synthesis and secretion of proteolytic enzymes to degrade the surrounding extracellular matrix (Folkman and Klagsbrun, 1987). Some angiogenic factors stimulated endothelial cells in all of the ways mentioned, while others would act on only one or two particular aspects of the neovascularization process.

Cartilage-Derived Growth Factor

It is likely that some angiogenic agents might be acting locally in endochondral ossification to stimulate the metaphyseal vessels to sprout and to invade the hypertrophic chondrocytes. For a long time the emphasis was on systemic hormones such as growth hormone and progesterone, which stimulate capillary growth; and also on cortisone, ACTH, and estrogen, which are all known to suppress capillary growth. Without a doubt these hormones must affect the process of endochondral ossification. In 1977, Michael Klagsbrun and co-workers isolated an intrinsic local growth factor that could also

possibly be involved. This cartilage-derived growth factor (CDGF) with a molecular weight of 16,300 D was purified from bovine scapular hyaline cartilage through a combination of gel filtration chromatography and isoelectric focussing. CDGF was shown to stimulate DNA synthesis and cell division in a dose-dependent manner in both quiescent 3T3 cells and chondrocytes in vitro (Klagsbrun et al., 1977). As the growth factor was purified further it was found to be primarily located intracellularly with a much smaller amount found paracellularly (Azizkhan and Klagsbrun, 1980). When this substance was tested for its ability to stimulate endothelial cell cultures derived from human umbilical vein it produced no detectable effect. However, when CDGF was retested on endothelial cell cultures obtained from capillaries, it was found to stimulate both the proliferation and the migration of endothelial cells at concentrations as low as 0.5 µg/ml (Klagsbrun and Smith, 1980). Chondrosarcomas have also been shown to produce large amounts of the same growth factor (Shing et al., 1984). In the normal growth of long bones, this raises the interesting question of self-regulation, as chondrocytes are capable of producing a growth factor which is able to stimulate the proliferation of more chondrocytes, while at the same time increasing endothelial cell division and migration.

Eventually, the primary amino acid sequence for CDGF was determined and found to consist of 146 amino acids that were virtually identical in sequence to those in basic fibroblast growth factor (bFGF). Even though the preceeding ten years has seen the characterization of a great number of angiogenic factors from virtually every tissue of the body, after sequencing these molecules it is now apparent that the vast majority are very similar to, or

identical to, either acidic or basic FGF (Folkman and Klagsbrun, 1987). Basic FGF has no signal sequence and is for that reason assumed to be a cytoplasmic, rather than a secretory, protein (Fiddes et al., 1987; Sommer et al., 1987). It is tempting to speculate that as the hypertrophic chondrocytes are destroyed at the metaphyseal side of the growth plate, bFGF is liberated from the cell and aids in the further propagation of capillaries into the hypertrophic zone. Both acidic and basic FGF bind strongly to heparin, which is speculated to have both a role in the modulation of angiogenesis (Folkman et al., 1983; Folkman 1985; Klagsbrun and Shing, 1985) and specific receptors on a variety of cell types (Folkman and Klagsbrun, 1987; Moscatelli et al., 1987). The fact that most every tissue possesses a form of FGF, or some other angiogenic factor, indicates that the ability to induce blood vessel growth is a fundamental property of the whole organism. An obvious partial explanation for this ubiquitous distribution is the wound healing process which requires new blood vessel growth into damaged tissue as a necessary part of the repair mechanism. Every tissue would need the ability to induce neighbouring vessels to sprout and revascularize a region in which the existing vessels had been destroyed (Folkman 1986).

Anti-Invasive Factor

The finding that chondrocytes produce bFGF, a potent stimulator of angiogenesis, may not be surprising considering the wide distribution of this molecule in various tissues of the body, but it is unusual considering that cartilage is viewed as being essentially an avascular tissue. With the exception of vascular invasion during endochondral ossification, which is very regulated in its advancement,

cartilage is devoid of a vascular network. Even during embryonic development regions of undifferentiated mesenchyme which will later become cartilage, either never become vascularized (Hudlicka and Tyler, 1986), or the blood vessels regress prior to cartilage differentiation (Hallmann et al., 1987).

Why is it then that cartilage is able to remain avascular? Kuettner and Pauli (1982) suggested that it could be due to any or all of the following: (a) the cartilage macromolecules are arranged in such a way as to prevent penetration by capillary endothelial cells, (b) components of the cartilage matrix are undegradable by the proteolytic enzymes used by capillary endothelial cells to invade tissue, and (c) endogenous substances of the cartilage matrix are specifically directed at the invasive apparatus of the endothelial cell. Regardless of the mechanism(s) employed by normal cartilagenous tissue to repel blood vessels, if calcification of the cartilage matrix occurs, blood vessel invasion can take place. Thus far, the majority of work has centered around isolating endogenous substances contained in cartilage which are directed against the invasive apparatus of growing capillaries (possibilty "c"), due to the therapeutic possibilities of such substances in the treatment of cancer.

The chick chorioallantoic membrane (CAM) assay system has been used extensively in the study of what properties of cartilage, and other avascular tissues, confers onto them resistance to vascular invasion. The CAM is an immunologically protected site in the living egg where it is possible to observe the development of blood vessels. If tissues that normally have a blood supply are explanted onto the CAM, they are rapidly invaded by blood vessels. However, hyaline

cartilage placed on the CAM is not penetrated by blood vessels, yet calcified cartilage is invaded (Eisenstein et al., 1973). If the cartilage is first extracted with 1.0 M guanidine hydrochloride, a relatively weak extractant, its resistance to invasion is reduced and blood vessel ingrowth takes place (Sorgente et al., 1975). The extract, which removes a percentage of the total macromolecular proteoglycans, was subsequently found to have antiproteolytic activity and thus might be capable of blocking the enzymatic invasive apparatus of the endothelial cells (Eisenstein et al., 1975). Researchers working on tumour neovascularization found that when a cartilage extract is placed in a rapidly growing tumour, there is little or no vascularization of the area immediately surrounding the implant. Again the isolated fraction was found to contain a protein which was capable of inhibiting protease activity (Langer et al., 1976). In fact, if the same extract is administered systemically, blood vessel formation is reduced throughout the animal and the endothelial cell mitotic index is also reduced (Kaminski et al., 1978).

Kuettner and Pauli (1983) set about to identify the family of low molecular weight proteins that could be extracted by mild salt solutions from avascular tissues such as cartilage. They collectively named these molecules anti-invasive factor (AIF) and found the extract to consist of seven major protein bands. Endothelial growth and development consists of several phases including cell migration, cell division, and endothelial digestion of the extracellular matrix by proteolytic enzymes. AIF has been demonstrated to possess inhibitory action against at least two of these three steps. As mentioned, the extract contains a variety of proteins that have an inhibitory effect against several proteinases. One acts against neutral

metalloproteases (which digest collagen type IV and V - both of which are found in the endothelial basement membrane - and other mammalian collagenases), a second acts against trypsin (as well as chymotrypsin, plasmin, proteoglycan-degrading enzymes, and other neutral proteases), while a third acts against thiol proteases such as cathepsin B and papain (Kuettner and Pauli, 1982, 1983). Other factors in AIF have a strong inhibitory effect on endothelial growth *in vitro* by displaying a lethal effect on dividing cells and by reducing the growth rate of the remaining endothelial cells by 40% (Kuettner and Pauli, 1982; Pauli and Kuettner, 1982). A second non-cytotoxic growth inhibitor has been isolated from bovine scapular cartilage that increases the doubling time of endothelial cells *in vitro* from 24 hours to 40 hours by arresting the cells in the G_1 phase of the cell cycle (Sorgente and Dorey, 1980). Yet another possible effect as it relates to endochondral ossification, is that a cartilage-derived collagenase inhibitor reversably inhibits osteoclastic activity (Horton et al., 1978). Osteoclasts and "chondroclasts" are also a necessary part of the invasion mechanism in the growth plate, so inhibiting the actions of these cells would also quell microvessel growth.

Possible Roles for these Factors in the Growth Plate

Despite all that has been done regarding factors contained in cartilage that can influence blood vessel growth, very few studies have examined the effect that such agents would have *in vivo* on the vessels that they are most likely directed against: the invading capillaries in endochondral ossification. One of the few studies to touch on this involved the use of matrix-induced endochondral

ossification. In this process, ground up bone matrix is implanted subcutaneously and it induces endochondral bone formation at that site (Urist, 1965; Reddi an Huggins, 1972). This triggers a sequential series of events in a defined time pattern as follows: initially the mesenchymal cells are converted into chondrocytes which produce cartilage (Syftestad and Caplan, 1984), the cartilage matrix then calcifies, blood vessels and osteoprogenitor cells invade the region, bone is laid down by osteoblasts on top of the calcified cartilage septa, and ultimately a marrow cavity with hematopoietic cells is formed within the bone ossicle (Reddi and Huggins, 1975; Reddi and Anderson, 1976). This system has the advantage of allowing the examination of a particular phase of endochondral bone formation as most cells in the plaque pass through the developmental stages in unison. Thus by harvesting plaques at defined time intervals after implantation, it is possible to quantitate the levels of various factors present during a particular stage of endochondral ossification. This is accomplished with greater accuracy than could be achieved by surveying the entire gradient of development, as is found in the growth plate, while utilizing a system which closely resembles the natural condition. Taking advantage of this system, Reddi and Kuettner (1981) studied the changing levels of several variables during matrix-induced endochondral ossification, including protease activity, protease-inhibitory activity, and amounts of hyaluronic acid. They observed that protease-inhibitory activity was greatest when chondrogenesis was maximal and then diminished during cartilage calcification and its subsequent vascular invasion. Protease activity on the other hand, was maximal as early as mesenchymal cell proliferation, however, it seemed to be complexed

with an enzyme inhibitor. The protease activity then disappeared during chondrogenesis (when one would assume endothelial cell inhibition to be maximal) before reappearing during cartilage calcification and vascularization. Hyaluronic acid, a component of the cartilage matrix which is also an angiogenic inhibitor due to the inability of endothelial cells to enzymaticaly digest it, is present throughout the process, but is at its lowest point during cartilage calcification. From this, it begins to become clear just what role calcification of the cartilage matrix plays in influencing the microvasculature in adjacent tissues.

If all the available data is combined from morphological studies on the growth plate and other biochemical studies on cartilage, it is possible to propose a model. Initially the chondrocytes of the growth plate produce low molecular weight molecules which serve as angiogenic inhibitors by neutralizing the invasive proteolytic enzymes used by endothelial cells during migration and by inhibiting endothelial cell division. These molecules, contained within the extracellular matrix of the cartilage, are sufficent to repel any blood vessel invasion and keep the tissue avascular. When the cartilage matrix calcifies, these molecules likely lose some or all of their effectiveness, either because calcification alters their structure, or because they become trapped within the matrix and can no longer diffuse away from the cartilage to affect the adjacent blood vessels. The simple removal of inhibition, while necessary, is not in itself sufficient to bring about vascular invasion, as angiogenesis will not take place in the absence of stimulation. The source of the initial stimulus is unclear. It could be brought about by systemic hormones, cartilage-derived growth

factor, or by other influences such as mast cell-derived growth factors, as mast cells are frequently seen in the region of vascular invasion. Regardless of the initial impetus, it would appear that once started, the vascular ingrowth would be self-perpetuating. As chondrocytes in the region of calcified cartilage were lysed, more intracellular CDGF would be liberated from within the cells and thus stimulate continued vessel growth. Also, limited flow through the newly created vessel sprouts would produce blood stasis and hypoxia in the local environment. Hypoxia and the buildup of metabolites such as ADP also provide a strong stimulus for angiogenesis. Therefore, once started, the vascular invasion would be able to continue forward as long as, and as far as, calcification of the cartilage matrix allowed. Considering that in the adolescent rat the epiphyseal growth plate grows at the rate of about 200 μm per day (Hunziker, unpublished data), it is imperative that sufficient vascular growth occur to keep pace with this.

Thesis Objectives

While it has never been demonstrated, intuitively it seems obvious that the metaphyseal capillaries are the site of ongoing angiogenesis. It would be impossible for the growth plate to advance at the constant rapid rate that it does, if vascular invasion was unable to maintain at least an equal pace. Since the size of the growth plate remains constant during growth under normal circumstances, removal of chondrocytes at the metaphyseal side must be equivalent to their addition in the proliferative zone at the epiphyseal side. Some authors have recognized this such as A.H. Reddi (1981) who wrote, "Despite its importance to osteogenesis and bone

formation we still do not understand the role played by angiogenesis." The growth plate might serve as excellent system with which to study angiogenesis, while at the same time increasing our knowledge of the process of endochondral ossification.

Given that cartilage has been shown to contain substances that both promote and inhibit neovascularization, the microvessels of the growth plate would appear to be the ideal location on which to study the interaction of these molecules in normal growth. However, before one can assess the effects of any of these substances on the metaphyseal capillaries, some fundamental properties of the vessels themselves must first be worked out. For example, it is impossible to speculate on the relative effects of promoters and inhibitors of endothelial cell proliferation on the metaphyseal vessels, until the site of endothelial cell division in these vessels is known. Simply because of the location of endothelial proliferation in the metaphyseal capillaries, it may be possible to rule out the effects of some humoral factors due to physical or physiological constraints.

It is the goal of this thesis to address the following fundamental questions: (1) Are the metaphyseal capillaries morphologically similar to other recognized angiogenic systems? (2) Where are the sites of endothelial cell proliferation in these vessels? (3) Are the capillaries of the "open-ended" and/or the "closed" variety? (4) Is there a basement membrane around the vessel that must be broken down for vascular invasion to take place? (5) What are the growth dynamics of the endothelial cells and their accompanying perivascular cells? (6) How do the endothelial cells of the metaphyseal capillaries complete cell division while maintaining the integrity of the vascular wall? In an attempt to answer the above

questions this thesis was undertaken as a morphological analysis of the metaphyseal nutrient vessels of the epiphysel growth plate. By addressing the fundamental properties inherent to these capillaries, it is anticipated that much of the evidence already accumulated would begin to come together with repect to the role of angiogenesis in the postnatal growth of long bones.

It has been the intent of this study to view the metaphyseal capillaries as growing microvessels that are likely to closely follow many of the growth patterns already well established in other angiogenic systems. With this insight, it has been possible to examine them as a vascular system first, and only after this initial understanding, is the information then applied to what is known about endochondral ossification. The little work that has been done on the metaphyseal capillaries has been carried out by investigators whose prime interest was the mineralization process and how the vessels might fit into that scheme. The purpose of this research effort was to study the vascular system *per se* within the confines of calcified cartilage and bone. Ideally, by utilizing all the information available on angiogenesis, it will be possible to identify any relationships between the metaphyseal capillary growth pattern and the pattern of enodochondral bone formation. But first, it must be shown that this system does display the features associated with an angiogenic system.

Chapter 1: ULTRASTRUCTURE OF THE GROWING TIBIAL METAPHYSEAL VESSELS DURING ENDOCHONDRAL OSSIFICATION IN THE RAT

Abstract

The metaphyseal vessels which interface the metaphyseal-epiphyseal junction of the rat proximal tibiae were perfusion fixed under physiological conditions of pressure and flow rate and examined by electron microscopy. This anastomotic network of capillaries is derived from larger parent vessels. Extending from the capillary bed are growing, close-ended sprout tips which advance towards the terminal hypertrophic chondrocytes of the overlying epiphysis. For descriptive purposes four different regions of these vessels were characterized: 1) Sprout tips - the terminal ends of the capillary sprouts which actually impinge upon the hypertrophic chondrocytes of the growth plate. 2) Region of extended calcified cartilage - progressing deeper into the metaphysis away from the epiphyseal cartilage the capillaries pass through a region where the surrounding extracellular matrix is predominantly composed of extended septa of calcified cartilage. 3) Region of bone deposition - further still from the growth plate the capillaries are contained within an area of active bone deposition laid down upon a calcified cartilage framework. 4) Region of primary vessels - finally, at a distance of 350-500 μm from the hypertrophic chondrocytes the vessels are more dilated and may contain one or two layers of smooth muscle in their walls. The sprout tips of the metaphyseal capillaries are lined by an attenuated fenestrated endothelium which remains continuous even at

its thinest points. Preventing excessive pressure and flow rate of perfusates in the vascular system during fixation left the blunt-ended capillary sprouts intact and eliminated rupturing of their terminal aspects. Ultrastructurally, the endothelial cells of the three zones closest to the growth plate cartilage feature many of the characteristics of growing vessels including abluminal cellular projections, apical microvilli, and abundant cytoplasmic rER and free ribosomes. The basement membrane, as shown by perfusion fixation with 2% tannic acid, is seen to be absent at the sprout tip, but to become increasingly prominant in regions more distal to the hypertrophic chondrocytes. Progressing deeper into the metaphysis from the sprout tip there first appears an abluminal basement membrane-like material that is found adjacent to the region of the endothelial cell nucleus, but is absent at more attenuated portions of the cell. In the region of bone deposition a more complete basement membrane begins to appear and covers an increasingly larger percentage of the abluminal surface of the endothelium. While a morphologically fully formed basement membrane eventually appears at a distance from the sprout tip, it does not form a completely continuous layer at any point along the metaphyseal capillaries.

Introduction

The metaphyseal vascular supply to the tibial epiphyseal growth plate has an important functional relationship with the proper progression of endochondral bone formation. In the absence of the metaphyseal capillaries the hypertrophic zone of the epiphysis continues to elongate and the surrounding extracellular matrix fails to calcify (Trueta and Amato, 1960). Vascular invasion of the

hypertrophic chondrocytes and the surrounding calcified cartilage by the metaphyseal capillaries is necessary for the conversion of the tissue into bone. Not only must the capillaries remove hypertrophic chondrocytes at the metaphyseal side at the same rate as chondrocyte cell division is adding cells at the epiphyseal side, but they must also provide the blood borne factors necessary for cartilage calcification and deliver nutrients required by the osteoprogenitor cells. While it is generally accepted that these vessels must be rapidly growing in order to keep pace with the bone elongation observed in adolescent animals, the organization of these vessels has not been fully elucidated. The origin of blood vessel growth in long bone formation is also unknown, however, all postnatal capillary growth is thought to be the result vascular sprouting from existing capillaries (Sandison, 1924, 1928).

Vascular corrosion cast experiments have illustrated that the vessels that impinge upon the hypertrophic chondrocytes are blind-ended sprouts and frequently display bulbous endings (Draenert and Draenert, 1985; Arsenault, 1987). Interconnecting anastomotic branches can be seen between the capillary sprouts down from the terminal endings and likely represent the most distal site in the tissue where a capillary loop is formed. This is the same pattern observed in other vessels known to be involved in angiogenesis, or the formation of new capillaries (Sholley et al., 1984; Folkman, 1986). Although the morphology of the metaphyseal capillaries has been examined several times previously (Trueta and Little, 1960; Brookes and Landon, 1964; Schenk et al., 1967), they have not been evaluated in comparison to other tissues known to be undergoing neovascularization. In the past ten years, assay systems such as

induced vascularization of the rabbit cornea, the chick chorioallantoic membrane (CAM), and the hamster cheek pouch have elucidated a variety of parameters that characterize nascent capillaries. The present study was designed to determine if the ultrastructural features of the metaphyseal capillaries were comparable to those of other angiogenic vessels. Features dealt with specifically include endothelial cell ultrastructure, the presence of pericytes, endothelial cell junctions, and especially the endothelial basement membrane.

In most microvessels the endothelium rests on a basement membrane of collagen (types IV and V), fibronectin, glycoproteins, and laminin, all produced by the endothelial cells themselves (Jaffe et al., 1976, 1978; Kefalides et al., 1976). The basal lamina in mature microvessels is continuous in continuous and fenestrated capillaries, discontinuous in sinusoidal capillaries, and splits to surround the pericytes adjacent to the vessel wall. The endothelium of the growth plate has been characterized as thin and fenestrated (Kuettner and Pauli, 1982) with an inconspicuous or frequently absent basement membrane (Brooks and Landon, 1964; Anderson and Parker, 1966; Schenk et al. 1967, 1968). In each of the studies cited, the morphological examination of the metaphyseal vessels centred around that part of the sprout that reaches the terminal chondrocytes, and did not follow the capillary further down.

During capillary growth, the basement membrane has more than simply a structural role, as it appears to exert a directive influence during angiogenesis. When most angiogenic substances are tested on endothelial cells in vitro , they are seen to stimulate cell proliferation and migration, but they are incapable of inducing the

formation of vascular sprouts, branches, and ultimately closed tubes (Alessandri et al., 1983). The cytoskeletal changes required to bring about these events can be induced however, if the endothelial cells are cultured on substrates such as amniotic basement membrane (Madri and Williams, 1983) or interstitial collagen (Montesano et al., 1983). So while it is apparent that extracellular matrix-endothelial cell interaction is important in vessel differentiation, it should also be noted that enzymatic digestion of the basement membrane and the surrounding connective tissue is also essential to endothelial cell movement during angiogenesis. In growing vessels there is a distinct pattern of basement membrane breakdown during cell migration away from the parent vessel in the early stages of angiogenesis, followed by synthesis and secretion of a new basement membrane by the newly formed vessels. The components of the basement membrane and extracellular matrix are laid down in a particular developmental sequence by the endothelial cells as the capillary sprout grows (Nicosia and Madri, 1987).

The basement membrane is particularly useful in evaluating capillary growth because it is laid down in a very characteristic pattern in growing microvessels regardless of the stimulus which brings about neovascularization (Folkman et al., 1981). The sprout tip has no basement membrane, while lower down there is a fragmented basement membrane, and near the vessel from which the sprout originated, there is a complete basement membrane (Folkman et al., 1981). Defined as such, the appearance of the basement membrane along the length of a growing capillary sprout can be used as a reference with which to correlate other aspects of the angiogenic process (particularly the location of endothelial cell division).

Ultrastructurally, growing endothelial cells display abundant rER and free ribosomes, few pinocytotic vesicles, abluminal pseudopods, luminal microvilli (Shepro and D'Amore, 1984), and less elaborate punctate appositional contacts as opposed to the occluding junctions seen in more mature capillaries (Shumko et al., 1988). Understanding the morphology of the basement membrane and the ultrastructural appearance of angiogenic endothelial cells provides a picture of neovascularization in other tissues. The appearance of the metaphyseal capillaries can then be compared and certain conclusions can be drawn. For example, following changes in the basement membrane along the length of a capillary sprout provides insight not only into the possible growth state of the microvessel in question, but also provides orientation as to which aspect of the vessel likely represents the growth front. In this way the morphology of the metaphyseal capillaries was examined with particular attention paid to those aspects which could indicate both the nature and extent of neovascularization in the tissue.

Since some features of growing capillaries are not evident until some distance from the sprout tip, it was necessary to examine the metaphyseal capillaries at a distance from the hypertrophic chondrocytes. The region observed extended from the growth plate cartilage down approximately 600 μm into the metaphysis, or up to the point at which hematopoietic cells appeared in the surrounding tissue. The present study was designed specifically to examine the ultrastructure of the metaphyseal capillaries from their origin to the sprout tip in order to advance our understanding of the role played by blood vessel invasion and its interrelationships with endochondral bone formation. Specific ultrastructural features dealt with include

cellular content, cellular projections (both luminal and abluminal), cell junctions, and the appearance of the basement membrane. The observations thus obtained would be examined for similarities, and differences, between the capillaries involved in endochondral ossification and those from other angiogenic tissues.

Materials and Methods

Transmission Electron Microscopy: Juvenile (age 28-35 days) male Sprague-Dawley rats (100-150 g) were used in this study as this age and weight corresponds to the time when long bone growth is proceeding at its maximal rate (Gallo and Weinberg, 1982). Animals were perfusion fixed using a modified method of Zinkernagel et al. (1972). Anesthetized animals were perfused intracardially. All perfusates were warmed to 37° C and flow through the cannula was carefully adjusted to 40 ml/min and carried out at a pressure of 100 mmHg to approximate physiologic flow through the aorta, and to prevent rupturing the terminal aspects of the vessels being examined. Ringer's saline was prepared with 0.025% heparin and 0.1% procaine, to prevent coagulation and aid in flow through the vessels, and adjusted to 300 mOsm and pH 7.3. The saline was perfused for about 5 minutes or until the flow through the cut external jugular veins ran clear. The flow of saline was then terminated and 2.3% glutaraldehyde in 0.05 M sodium cacodylate (300 mOsm, pH 7.3) was then perfused through the animal for 15 minutes. Proximal ends of tibiae were then excised, split longitudinally and immersed in fix for 30 minutes, and then cut into 2 mm cubes and placed in fixative for an additional 30 minutes. The specimens were then demineralized by immersion in a 4.13% solution of EDTA (300 mOsm, pH 7.3) maintained at 4°C and changed twice daily

for 2-3 days (Warshawsky and Moore, 1967). The end point of demineralization was determined by radiologic examination of the specimens. They were then post-fixed in 1% osmium tetroxide for 2 hours. The tissue was then dehydrated in successive grades of methanol followed by infiltration and embedding in Spurr resin (J.B.EM Services Inc., St. Laurent, Que.). Thin (gold) sections were then cut on a diamond knife, placed on copper grids , stained with 5% aqueous uranyl acetate and 0.5% lead citrate in 0.1 N NaOH, and examined on a Philips 300 electron microscope operated at 60 kV.

Basement Membrane Studies: For light microscopic examination, tissue was prepared by immersion fixation of excised tibiae in 2% paraformaldehyde, demineralized as above, dehydrated, and embedded in paraffin. Sections 10-20 μm thick were cut on a microtome, dried on glass slides, and deparaffinized by immersion in xylol followed by immersion in successive grades of alcohol. Some sections were then stained with periodic acid Schiff (PAS). Other blocks prepared in this manner were embedded in Spurr resin, 0.5 μm thick sections were cut on a ultramicrotome with a glass knife, and stained with 0.05 M toluidine blue.

For transmission electron micrscopic examination of the basement membrane, tissue was prepared in the same manner as that stated above for conventional electron microscopic viewing, except that in two animals 2% tannic acid was added to the perfused fixative (Singley and Solursh, 1980), and in two others 1% ruthenium hexamine trichloride (RHT) was added to the fixative, buffer, and osmium tetroxide (Hunziker et al., 1982). The tissue prepared with tannic acid was decalcified, while the RHT treated tissue was not.

Results

General Morphology:

For descriptive purposes and to make comparisons more readily between tissue obtained from different animals, the capillaries of the metaphyseal side of the epiphyseal growth plate were divided into regions based on various morphologic characteristics. The four regions, progressing from the growth plate cartilage distally as depicted in the light micrgraph in Figure 1 are: (a) sprout tips (ST) - that part of the vessel within the space formerly occupied by the terminal hypertrophic chondrocytes, (b) region of extended calcified cartilage (CC) - that aspect of the vessel extending from approximately 50 to 150 μm down from the hypertrophic chondrocytes and surrounded by a calcified cartilage matrix containing type II collagen, (c) region of bone deposition (BD) - at approximately 150 to 350 μm from the growth plate cartilage is a region typified by the deposition of osteoid with type I collagen on a calcified cartilage framework, and (d) region of primary vessels (PV) - a region found between 350 and 500 μm from the epiphyseal-metaphyseal interface which is characterized by larger vessels which display one or more layers of smooth muscle in their walls, and represent arterioles and venules that supply and drain the capillary plexus. While the actual measured distance from the growth plate and the extent to which a given zone may extend can vary between animals, all metaphyseal capillaries pass through these same regions in the same sequence, regardless of other parameters. Therefore, catagorization by morphological critera, as opposed to by measuement only, removes to some extent errrors caused by differing planes of section and errors due to size differences between animals.

Figure 1: Photomicrograph showing an overview of the epiphyseal growth plate and the metaphyseal vessels. Vessel classification zones are illustrated: ST, sprout tips; CC, capillaries within the region of extended calcified cartilage; BD, capillaries within the region of active bone deposition; PV, larger primary vessels which supply and drain the capillary plexus. Arrowheads, hematopoietic cells; bar = 15 μm. x 850.


1
ST
CC
BD
PV
PV

In perfusion fixed animals the sprout tips are seen at the electron microscopic level to be composed of highly attenuated capillaries, dilated at their ends, which may enter one or more terminal chondrocyte lacunae (Fig. 2a). The capillaries are fenestrated (Fig. 2c), possess junctional attachments to other endothelial cells of the vessel wall, perinuclear rER and free ribosomes, Weibel-Palade Bodies, and contain few transcytotic vesicles which are occassionally seen to open on both sides of the vascular wall and may be covered by a diaphragm (Figs. 2b,c). The lumen of the vessel is continuous through to its most apical extension where it is frequently so attenuated as to be little more than two apposed plasma membranes (Fig. 2c). Other regions of the apex show endothelial cell cytoplasmic extensions away from the vessel in the direction of the terminal chondrocytes and calcified cartilage septa (Figs. 2b,d; 3c). A basement membrane is absent, but some fragments of closely apposed abluminal material may exist in certain regions (Figs. 3b,d). Pericytes cover a portion, but not all of the microvascular wall, and are most noteably absent at the very apex of the vessels (Figs. 2b, 3b). Endothelial cells of the sprout tips enter the spaces vacated by the terminal chondrocytes where they occasionally appear to be the only cell type present. However, a variety of cells can also be found in this area including mast cells, osteoclastic-like cells (chondroclasts), and pericytes (Figs. 2a, 3a). Sprout apices often have small portions of their wall covered by a discontinuous one-cell thick layer whose origin is unclear, but is likely composed of either pericytes or cells involved in breaking down the cartilage matrix (Fig. 2b). The apex of the microvascular wall also exhibits rounded endothelial cells whose nuclei bulge into the lumen more prominantly

than do those of the other cells of the wall.

The closed fenestrated lining of the sprout tip features endothelial cells that are joined by junctional complexes throughout (Figs. 2c,e). While the exact nature and relative tightness of these junctions could not be determined by the methods employed in this study, the junctions are morphologically similar to adherens and occluding junctions (Farquhar and Palade, 1963; Gilula, 1974). Punctate junctional attachments were observed in the sprout tip (Fig. 2c). Morphologically more elaborate junctions were also seen in this area in tangental sections through sites of endothelial adhesion (Fig. 2e). Occassionally sites of attachment between cell processes or microvilli and the luminal vessel wall were observed. (Fig. 2e arrowheads). To address the nature of the cell junctions present a lanthanum tracer experiment using the method of Revel and Karnovsky (1967) was attempted, but was unsuccessful due to insufficient resolution of membrane structure in decalcified tissue.

The vessels found within the region of extended calcified cartilage are also exclusively capillaries and share many of the ultrastructural features of the tips that they give rise to (fenestrae, junctional attachments, transcytotic vesicles, abundant perinuclear rER, free ribosomes, Weibel-Palade bodies, and vesicles opening on both the luminal and abluminal sides which frequently have diaphragms). The perivascualar lining of pericytes is more continuous than at the sprout tips and dividing pericytes can be found in this area as well. Endothelial cells in this region often displayed microvilli that projected into the lumen of the capillary (Fig. 2f).

The vessels within the region of bone deposition feature endothelial cells that more closely resemble capillaries in other

Figure 2: Morphological characteristics of the metaphyseal capillaries:

(a) Low magnification electron micrograph. Enclosed areas of interest are shown at higher magnification. Arrowhead, dividing cell in the region of extended calcified cartilage; bar = 10 μm. x 1,000.

(b) Sprout tip of invading metaphyseal capillary. Endothelial abluminal processes project into the lacuna left by a hypertrophic chondrocyte (arrowhead). No perivascular covering is evident at the extreme sprout tip. L, lumen; EC, endothelial cell; bar = 2 μm. x 4,700.

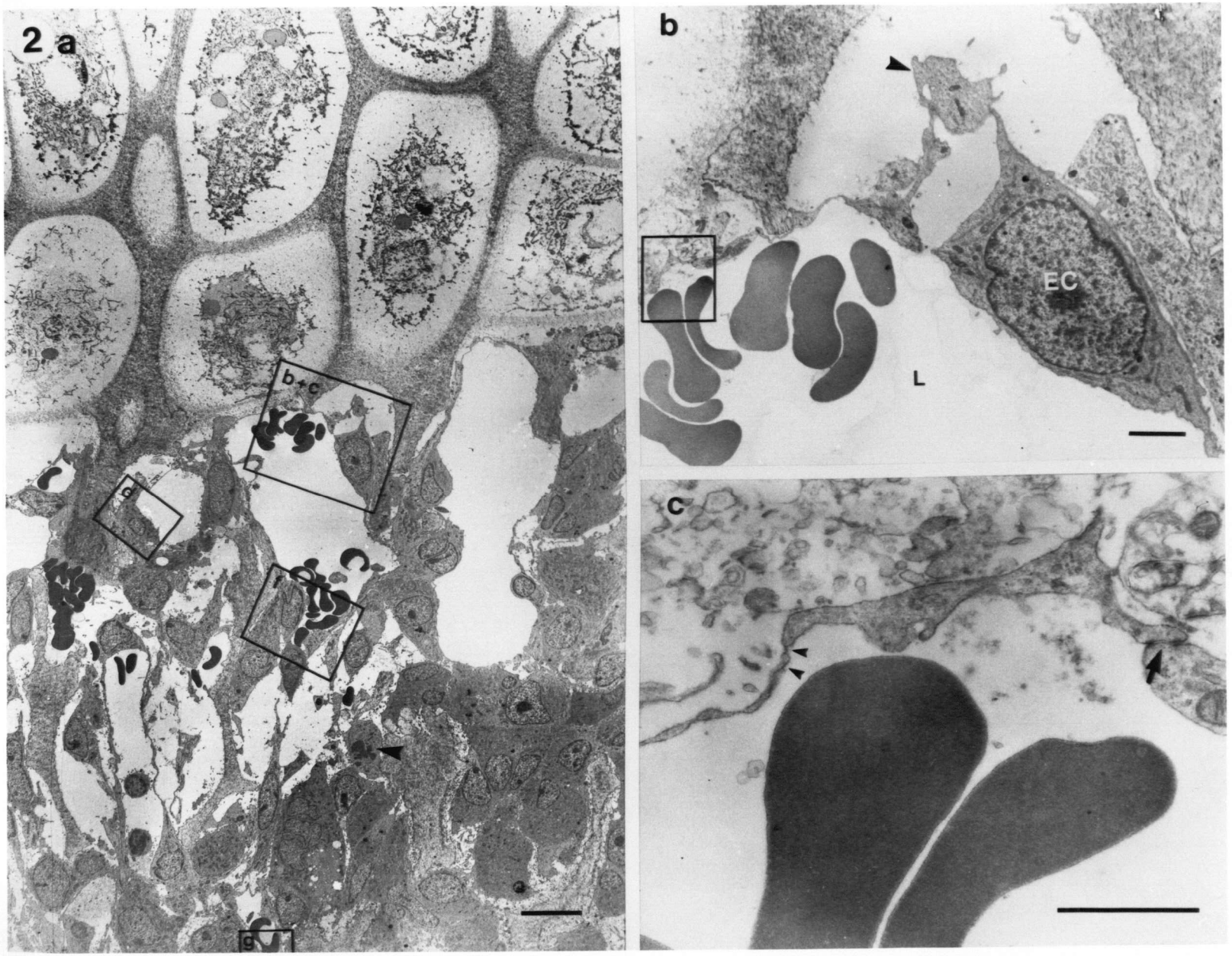
(c) Higher magnification inset of Figure 2b. The highly attenuated continuous endothelial lining has junctions between cells (arrow) and numerous fenestrae (arrowheads); bar = 1 μm. x 24,000.

(d) Endothelial cell near sprout apex with abluminal cellular projections extending towards the calcified cartilage matrix (arrowheads). L, lumen; P, pericyte; bar = 1 μm. x 8,100.

(e) Elaborate endothelial cell junctions from a tangental section through an attenuated portion of the capillary wall of a metaphyseal sprout tip (not seen in Fig. 2a). Arrowheads indicate sites of junctioning between the cell and adjacent cell processes; bar = 0.5 μm. x 40,000.

(f) Capillary wall at a distance from the sprout tip with apical luminal cellular projections (microvilli - arrowheads); bar = 1 μm. x 6,800.

(g) Within the region of extended calcified cartilage the attenuated endothelial lining features many junctions between adjacent cells (arrowheads) and is more completely lined by perivascular cells; bar = 1 μm. x 12,000.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regions of the body (Fig. 3f). The cells were attenuated and fenestrated with extensive interdigitating junctional attachments between neighbouring cells and an underlying layer of closely opposed perivascular cells (Fig. 2g). The majority of dividing endothelial cells observed were located in this region and found to be at an average of 180-200 μm from the vessel tip. The dividing cells are typically rounded and resemble other dividing cells but with two significant differences: they possess microvilli on their luminal surface and they maintain junctional attachments to neighbouring cells during division. Endothelial cells in the immediate vicinity also occasionally show microvillar processes. Dividing pericytes are also frequently found in these areas (cell division to be covered in greater detail in the following chapter).

At approximately 350-500 μm from the terminal chondrocytes are found the primary vessels which supply and drain the capillary sprouts of the epiphyseal growth plate (Figs. 1, 3a). They also have a fenestrated endothelial lining but adjacent cells are joined by more elaborate junctions and the cells have a more consistant thickness throughout the vessel wall. They have a well developed basement membrane and an external coat of 1-2 layers of smooth muscle. Located only a slight progression down from these vessels (approximately 100 μm), is an area where bone marrow cells begin to appear outside of the vessel walls (Fig. 1 arrowhead). The capillaries into which the larger vessels empty (250-350 μm from the sprout tip) have the appearance of mature capillary beds. The endothelial cells are completely covered by a perivascular lining, possess extensive cell junctions, and have no microvilli or basal projections (Fig. 3f).

Basement Membrane:

Light microscopic sections stained with PAS failed to demonstrate any significant positive reaction underlying the metaphyseal capillary sprouts. There was however, a positive reaction indicating the presence of a basement membrane under the endothelium of the larger parent vessels. An anti-laminin immunofluorescence study was undertaken, but was unsuccessful due to nonspecific binding of the secondary antibody.

At the EM level tissues prepared by conventional means, RHT and tannic acid perfusion all showed similar results. However, of the three preparations the tannic acid fixation illustrated the basement membrane most clearly and was thus the one used for demonstration purposes in Figures 3a-f. Figure 3a is a low magnification overview for orientation purposes for Figures 3b-f. In the sprout tip the basement membrane was present only as small intermittant fragments of fibrillar extracellular material (Fig. 3b). Endothelial cells in this region frequently displayed basal cell processes that projected away from the vessel unencumbered by the basement membrane (Figs. 3c, 2d). In the vessels surrounded by the region of extended calcified cartilage there first appeared areas of patchy abluminal extracellular matrix, but it does not resemble a fully formed basement membrane, and it is confined mostly to the area immediately adjacent to the endothelial cell nucleus (Figs. 3d,e). Lower still, either deeper within the regions of extended calcified cartilage or bone deposition, a more fully formed, but a largely discontinuous basement membrane was found. For the most part it was still largely confined to regions adjacent to the endothelial cell body and was infrequently found in attenuated regions of the cell. Capillaries closer to the primary

Figure 3: (a) Low magnification electron micrograph of tissue prepared with 1% tannic acid to highlight the basement membrane. Enclosed areas of interest are seen at higher magnification; bar = 10 μm. x 800.

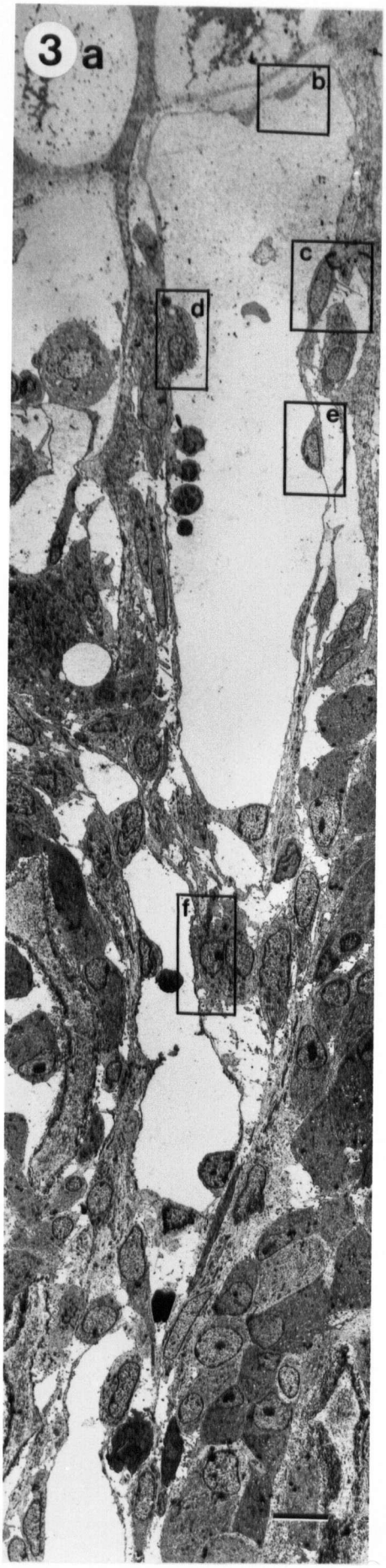
(b) Sprout tip endothelial wall with no apparent underlying basement membrane and diffuse abluminal collagen fibres (arrowheads). L, lumen; bar = 1 μm. x 11,300.

(c) Endothelial cell with prominant abluminal cellular projections (CP). L, lumen; bar = 1 μm. x 8,700.

(d) Endothelial cell with apical microvilli (large arrowheads) located in the region of extended calcified cartilage. Abluminal basement membrane is beginning to be laid down beneath the endothelial cell body (small arrowheads). L, lumen; bar = 1 μm. x 14,500.

(e) Endothelial cell from the region of extended calcified cartilage. Basement membrane that is present is confined largely to the area beneath the the cell body (arrowheads) and is not present under the attenuated portion of the adjacent endothelial cell. L, lumen; bar = 1 μm. x 14,500.

(f) Attenuated portion of an endothelial cell located in the region of active bone deposition. The basement membrane is more fully formed and is found covering large areas of the endothelium (arrowheads), although it never becomes completely continuous. L, lumen; bar = 1 μm. x 18,000. Inset: higher magnification of mature basement membrane; bar = 1 μm. x 30,000.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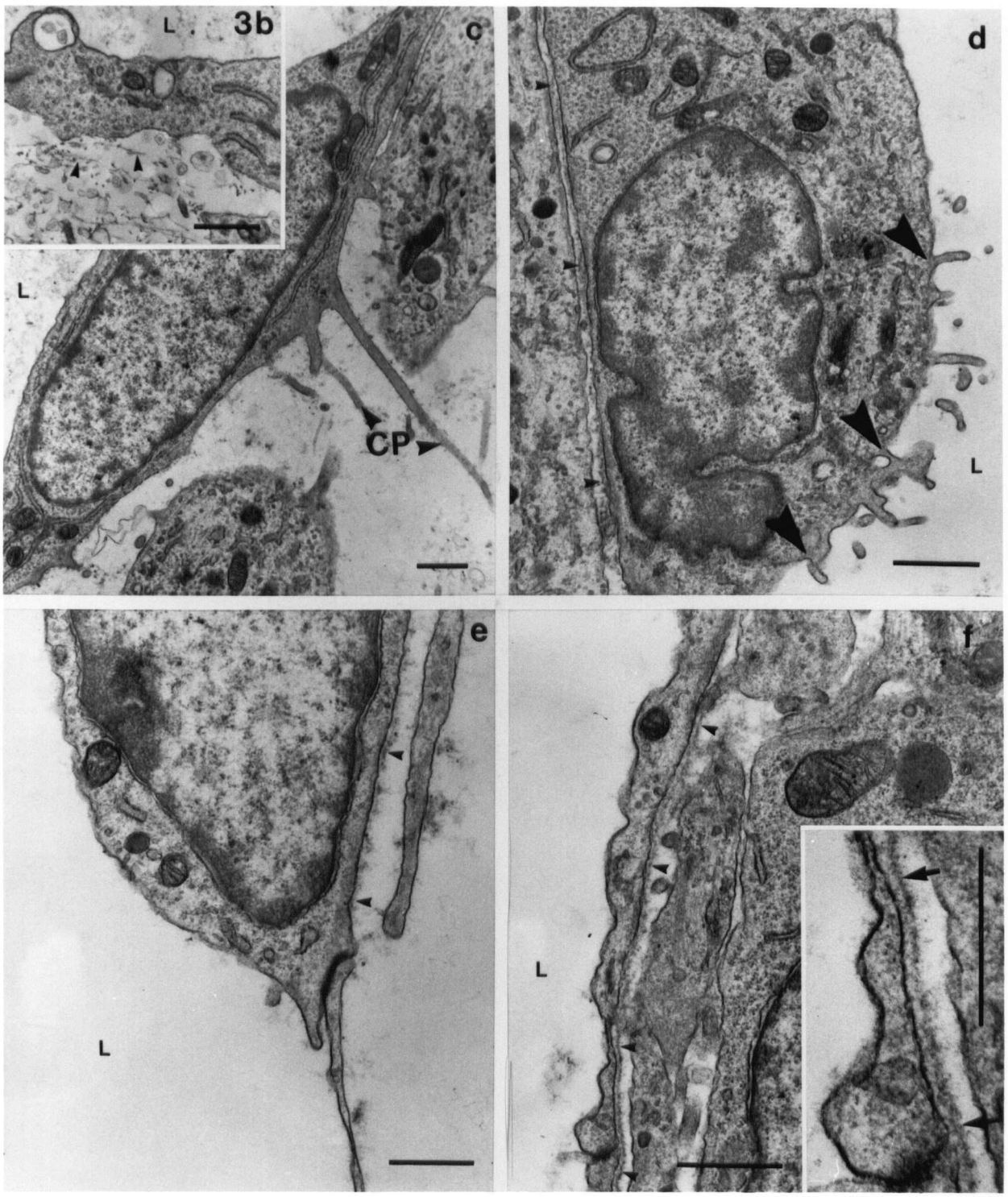
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vessels often displayed a basement membrane which was fully formed and covered a larger area of the vascular wall, although it was rarely completely continuous (Figure 3f). At this point the basement membrane was seen to split and enclose the pericytes which surround the endothelium.

The basement membrane distribution shown in Figures 3a-f is indicative of the changes seen along a metaphyseal capillary from the sprout tip down to the larger vessels. The order of the observed changes in basement membrane morphology is always constant and is not dependent upon where a transition takes place. Although the actual distance from the sprout tip to where these changes take place can vary among animals, and even within different sections of the same animal, the same progression is always observed. For example, areas of discontinuous completed basement membrane can occasionally be found underlying regions of vessels contained within calcified cartilage. However, if the same vessel is followed up towards the sprout tip, it will first show incompleted areas of basement membrane in the extracellular region beneath the endothelial cell nucleus, and finally no basement membrane at all once the sprout tip is reached.

Discussion

Ultrastructural investigations have yielded conflicting results as to whether the endothelium comprising the vessel tips is a continuous or a discontinuous lining. While it has been previously reported that the capillary sprouts have gaps in the vascular wall (Trueta and Little, 1960; Anderson and Parker, 1966; Schenk et al., 1968), the majority of these studies used immersion fixed epiphyseal growth plates, while those utilizing perfusion fixation often did not

perfuse at physiological pressure and flow rate. The thin walled nature of these terminal capillary sprouts makes them highly susceptable to rupture during perfusion, due to an excessive pressure of perfusates. For the same reason immersion fixed vessels would have a tendancy to rupture when compressed during cutting of the growth plates for tissue processing. This study used perfusion fixation at conditions closely approximating physiological levels (a pressure of 100 mmHg and a flow rate of 40 ml/min.) in order to minimize artifactual vessel damage due to these parameters. The results thus obtained indicate that the metaphyseal capillaries are closed vessels in agreement with other earlier studies (Langer, 1875; Ranvier, 1875; Brookes and Landon, 1964). The only openings observed in the vessel wall were tears in the endothelium in regions were there was tissue damage or poor fixation. In all other areas the highly attenuated vessel tips, composed of little more than apposed plasma membranes in some regions, formed closed ends that occasionally filled the entire empty lacuna. It should be noted however, that even in well preserved areas extravascular red blood cells were sometimes found.

Plastic corrosion casting has demonstrated the three-dimensional organization of the metaphyseal capillaries entering the growth plate cartilage, as being composed of blunt, rounded, bulbous ended sprouts and not capillary loops (Arsenault, 1987). The plastic casts are very similar to those seen in tumour angiogenesis and other sites of neovascularization (Sholley et al., 1984; Folkman, 1986). The site of vessel anastomosis and the formation of closed capillary loops clearly does not take place at the sprout apex and some connecting branches between adjacent sprouts can be seen at a distance from the vessel tips (Arsenault, 1987; also shown in cover

micrograph). This could in part explain the results obtained by Trueta and Morgan (1960) when they found that injected radioopaque material turned back to enter the venous system prior to reaching the terminal chondrocytes. The space in between the uppermost reaches of the dye and the terminal hypertrophic chondrocytes was observed to be filled with clustered erythrocytes. The last point at which the circulation could loop back would be at the site of the connections seen in the plastic casts. The accumulation of RBCs are likely to be part of a largely noncirculating pool trapped in the blind capillary sprout tips that impinge upon the growth plate cartilage and create a local blood stasis.

Specific morphological characteristics are associated with all growing vessels regardless of the angiogenic stimulus that induces neovascularization (Folkman et al., 1981). Several of these traits are observed in the capillary sprouts of the metaphyseal vessels. Endothelial cells of the metaphyseal capillary sprout tip often exhibit abluminal cellular projections, luminal microvilli, abundant rER and free ribosomes, few pinocytotic vesicles, and the underlying layer of pericytes is discontinuous or absent; all typically observed at the growth front of angiogenic vessels. The distribution of the basement membrane in the metaphyseal capillaries is also similar to that described by Folkman et al. (1981) and conforms to the morphology that they described. At the sprout tip the basement membrane is absent, while further down it is fragmented, and near the vessels from which the sprout originated (the parent vessels) the basement membrane is continuous. In the metaphyseal microvasculature the sprout tips are adjacent to the hypertrophic chondrocytes, the vessels within the calcified cartilage and the upper extension of the region of bone

deposition feature a fragmented incompletely formed basement membrane, and the capillaries in the lower region of bone deposition on down to the larger primary vessels have a fully formed basement membrane that covers large portion of the abluminal surface. From this, it appears possible that the parent vessels which give rise to the metaphyseal sprouts are the capillaries that are at an average of 250-350 μm from the growth plate and are supplied by the larger primary vessels located slightly further down. This is in agreement with vascular cast experiments which measure the sprouts as being up to 600 μm in length (Arsenault, 1987) and thus place the vessels of origin at some distance fromn the sprout tip. The results of this investigation are consistant with those of previous investigators who also found that the metaphyseal sprout tip endothelium lacked a basement membrane (Brookes and Landon, 1964; Anderson and Parker, 1966; Schenk et al., 1967). To this can be added the finding that a basement membrane eventually does come to lie under the capillaries if they are traced back far enough towards their origin. A possible explantion for the observed distribution of the basement membrane of the metaphyseal vessels approaching epiphyseal growth plate is as follows. The sprout tips of the metaphyseal capillaries that contact the hypertrophic chondrocytes represent the growth front of the vessels and have no basement membrane. Progressing deeper into the metaphysis, the vessels further from the tip begin to synthesize basement membrane products that are first seen as fragments adjacent to the endothelial cell body which produced them. At about 250-350 μm from the sprout tip a more fully formed basement membrane is found to cover some, but not all of the abluminal surface of the endothelium. These vessels contained within the region of bone deposition probably represent the

parent capillaries which give rise to the blind-ended sprouts of the metaphysis and feature a formed, but discontinuous basement membrane reminiscent of that found under bone marrow capillaries.

The tibial metaphyseal capillaries do differ from other growing microvessels in some respects. Many growing vessels begin as a solid endothelial sprout which initially does not have a lumen (Sandison, 1928; Nicosia and Madri, 1987). The metaphyseal vessels involved in vascular invasion always have a lumen through which blood cells are able to pass, even if flow through them is limited. Solid endothelial sprouts were not observed, indicating that once growth has begun the sprouts might be progressing more as a result of elongation of existing closed vessels rather than by de novo formation of new sprouts. A second apparent difference exists in the appearance of the junctions between the endothelial cells. In the CAM assay, early blood vessels have been described as consisting of an attenuated, but continuous endothelium with an incomplete or absent basement membrane, and punctate junctional attachments between cells (Shumko et al., 1988). Even at the sprout tip in the metaphyseal vessels the endothelium appears to be linked by well developed junctional complexes. Although punctate contacts are seen in thin regions of the vessel wall (Fig. 2c), tangential sections through junctions in the sprout tip reveal even more elaborate contacts (Fig. 2e). The probability of sectioning along the long axis of a site of attachment between adjacent endothelial cells would decrease in areas where the vessel wall is highly attenuated. This may in part account for the fact that multiple-site attachments between neighbouring endothelial cells have not been previously described in the metaphyseal sprout tip.

In summary, the results of this study demonstrate that the continuous capillaries of the metaphysis have many of the morphological features asssociated with growing capillaries elsewhere. They do, however, have some unique properties including the maintenance of an enclosed lumen and junctional attachments between cells during sprout growth. It was then undertaken to determine the location of endothelial cell division in the growing metaphyseal capillaries and ascertain whether it too was consistant with an angiogenic model for these vessels.

Chapter 2: CELL PROLIFERATION IN THE METAPHYSEAL BLOOD VESSELS AND SURROUNDING TISSUES

Abstract

Over 100 thin sections from 45 separate tissue blocks obtained from five different animals were examined ultrastructurally to determine the location of endothelial, pericyte, and osteoprogenitor cell division in the metaphyseal vessels and surrounding calcified tissue of the epiphyseal growth plate. Dividing endothelial cells were most frequently found in metaphyseal capillaries located an average of 175-200 μm from the sprout tip-hypertrophic chondrocytes interface. This corresponds morphologically to an area where the capillaries are typically surrounded by a region of active bone deposition. Functionally the region of bone deposition can be subdivided into an upper proliferative half in which dividing endothelial and perivascular cells are found (150-250 μm from the sprout tip) and a lower half that contains the maturing capillaries and post-capillary venules from which the metaphysel sprouts are derived (250-350 μm from the sprout tip). Dividing pericytes are also found at a distance to the sprout tip, but are found over a wider distribution than are the endothelial cells. They are characteristically found across an area that ranges from 50-250 μm from the metaphyseal border of the growth plate cartilage and unlike dividing endothelial cells, which are found almost exclusively in the region of bone deposition, dividing pericytes are found with almost equal frequency in both the region of bone deposition and the region of extended calcified cartilage. Light microscopic autoradiography

using ^{3}H-thymidine demonstrated that label is incorporated in the vascular wall in a region distal to both the sprout tip and the larger arterioles and venules that supply the metaphyseal capillaries. Within 24 hours labelled endothelial cells are seen in the region of the sprout tip indicating that newly formed cells are capable of migrating approximately 200 μm in one day (from the region of bone deposition to the sprout tip). This study demonstrates the relationship between the proliferation of pericytes and endothelial cells in an actively growing vascular system during endochondral ossification.

Introduction

As described in Chapter 1, growth of the metaphyseal capillaries must occur at a rapid rate in order to produce the network of microvessels that invades the growth plate cartilage as part of its replacement by bone. The continuous advancement of capillary sprouts into the calcified cartilage of the hypertrophic zone could not take place without the constant production of new endothelial cells to line the nascent vessels. While a great deal of work has been done regarding the intrinsic factors of hyaline cartilage that both inhibit and promote endothelial cell division, the anatomical location of endothelial proliferation in the growth plate has yet to be firmly established.

In most tissues the turnover rate of endothelial cells is extremely low and therefore endothelial mitotic figures are not frequently observed (Engerman et al., 1967). The endothelial mitotic index increases in growing tissues, but in the growth plate where the vessels are thought to be advancing rapidly, few proliferating

endothelial cells have been demonstrated. Schenk et al. (1968) observed endothelial mitotic figures very infrequently in capillaries adjacent to the sprout tip less than 100 μm from the hypertrophic chondrocytes. They suggested that growth of the metaphyseal vessels was the result of endothelial cell division in regions where the sprouts arise from underlying capillary loops. However, the exact location of the vessels from which the capillary sprouts originated was not determined.

Ultrastructurally the endothelial cells of the metaphyseal capillaries display many features common to growing microvessels including apical microvilli, abluminal cellular projections, abundant cytoplasmic rER, and the appearance of a basal lamina along the length of the capillary sprout. If the metaphyseal capillaries are also similar to other growing vessels with respect to their site of endothelial cell division, where would one expect to find this site in the metaphysis? In growing capillaries the site of endothelial cell division has been shown to be at a distance both to the growing front of the vessel and to the vessel from which the sprout is derived (Folkman et al., 1981). Folkman et al. (1981) demonstrated that the cells at the leading tip of a growing capillary do not incorporate ^{3}H-thymidine, but that the cells behind them do and then divide. Endothelial cell division also takes place at a distance from the capillaries that give rise to the sprouts, as the cells near the origin remain crowded and do not incorporate label. The area of endothelial cell division in a typical growing capillary sprout is seen to be in a region approximately half way between the sprout tip and the vessel of its origin, and at a point where the underlying basement membrane is fragmented and discontinuous (Cavallo, 1973).

Therefore, if the vessels involved in endochondral bone formation have a similar site of endothelial cell division, it is likely that proliferation would take place at a distance from the sprout tip in an area characterized by a fragmented basement membrane.

As illustrated in the previous chapter, the metaphyseal capillaries have no basement membrane at the sprout tip, but do demonstrate a fragmented basement membrane beginning at approximately 100 μm from the metaphyseal border of the growth plate cartilage. The basement membrane continues in this state for another 150 μm, or until about 250 μm from the growth plate, at which point it becomes more continuous and covers a larger percentage of the abluminal surface. It would be in this area, which is at a distance to both the sprout tip and the suspected vessels of origin, where the morphology of the metaphyseal capillaries most closely resembles that described above for the site of endothelial cell division in other growing capillaries.

Few studies have dealt with endothelial cell division in the metaphyseal capillaries and those that have (Schenk et al., 1968) observed endothelial cell division very infrequently. This could be explained if the site of endothelial cell division is located at a distance from the sprout tip and, therefore, was not examined in studies of the growth plate-metaphyseal vessel interface. The present study surveyed the metaphyseal capillaries deep into the metaphysis until the point where the vessels became larger and haematopoetic cells were found in the surrounding tissue. Dividing cell counts from electron microscopic sections and light microscopic autoradiography with ^{3}H-thymidine were undertaken to determine the site of endothelial cell division in the metaphyseal vessels. The dividing cells were

then correlated with respect to the morphological region of the vessel (described in the previous chapter) in which they were contained and their distance from the hypertrophic chondrocytes. The high resolution of the electron microscope enabled the accurate localization of dividing cells and the clear distinction of endothelial cells from pericytes. It was thus possible to separately evaluate the proliferative responses of endothelial cells and pericytes to the angiogenic stimuli responsible for metaphyseal capillary growth.

Materials and Methods:

Cell Division Studies: Tissue was perfusion fixed and prepared for conventional transmission electron microscopic studies in the manner described in Chapter 1. From each of the five animals studied, three blocks were randomly chosen, and from each block three separate regions (ie. various depths into the block) were examined; thus a total of 45 separate regions were surveyed. Several sections (5-10) were taken from each region and placed on 75-mesh copper grids. Dividing cells were then catagorized by location and cell type and for dividing endothelial cells, the distance from the sprout tip was also determined.

Autoradiography: Thirty 28-day-old male Wistar rats (100-125g body weight) were given a single injection of ^{3}H-thimidine egual to 0.5 μCi per gram of body weight, while 6 others were given an equivalent injection of saline in order to act as controls. The animals were divided into 6 equal groups. One group of 6 animals (5 experimental and 1 control per group), was killed at each of the

following times: 1, 3, 6, 12, 18, or 24 hours postinjection. The animals were given a lethal injection of sodium pentobarbital and their tibias were excised prior to death. The excised bones were then split longitudinally and placed immediately in a 2.3% solution of glutaraldehyde. The tissue was then prepared into tissue blocks in the same manner as that described in Chapter 1 for electron microscopic studies. Sections from these blocks were then cut and prepared for light microscopic high speed scintillation autoradiography (Durie and Salmon, 1975; Goldgefter and Toder, 1976).

Microscope slides were first soaked in a solution of 35 ml saturated sodium bichromate solution in 1,000 ml of concentrated sulfuric acid followed by an overnight wash in tap water at room temperature. They were then washed for 1 hour in distilled water, stirring constantly, and subsequently dipped into 2 changes of 80% ethanol. Sections 1.0 μm thick were floated on a water bath containing gelatin and then heat dried on glass slides. The slides were then dipped in Kodak NTB Nuclear Track Emulsion in total darkness at 42°C for 10 seconds and dried at 22°C for 1 hour (Baserga and Malamud, 1969). The specimens were then dipped for 5 minutes in "Aquasol" scintillator solution at 22° C. The coated slides were placed in Bakelite slideboxes in which there was also a small amount of desicant packed in gauze, and stored in a refrigerator at -70°C. The autoradiographs were then developed on progressive days to determine the best exposure time, which turned out to be 28 days. Development was carried out for 5 minutes in Kodak D-19 Developer at 18° C with occasional agitation, followed by a brief rinse (10 seconds) in water, and 8 minutes in Kodak Rapid Fix also with occasional agitation. The tissue was then stained at 20°C without interuption in

1.5% toluidine blue. The slides were then rinsed in distilled water and the excess emulsion removed from the back of the slide with a razor blade. Sections from control animals were also prepared in the same manner to determine if the chemical nature of the tissue was exposing silver grains in the absence of radioactivity.

Results:

Dividing Cell Counts:

A total of over 100 sections from 45 separate tissue blocks of growth plate capillaries were examined and the total number of cells observed undergoing cell division was recorded. Each mitotic figure was categorized by its position in relation to the four regions previously defined (ie. sprout tip, vessels within extended calcified cartilage, vessels within bone deposition, and primary vessels). The cell type was then determined as being either a mitotic endothelial cell, pericyte, or other undetermined cell type (Fig. 4a). Cells were classified as dividing endothelial cells only if they possessed a luminal surface and adherens-type junctional attachments to other recognizable endothelial cells. All dividing endothelial cells observed possessed luminal microvilli and retained junctional attachments to adjacent endothelial cells during mitosis. Cells were designated as pericytes if they were immediately adjacent to the endothelium on the abluminal side, but did not form adherens-type junctions with endothelial cells. Containment within the same basement membrane as endothelial cells could not be used as a criterion because the basal lamina was not always present. All mitotic figures found outside of the microvascular wall were grouped into an "other" category, and while their exact identity could not be ascertained, most were likely either of the osteoblast or osteoclast

lineage.

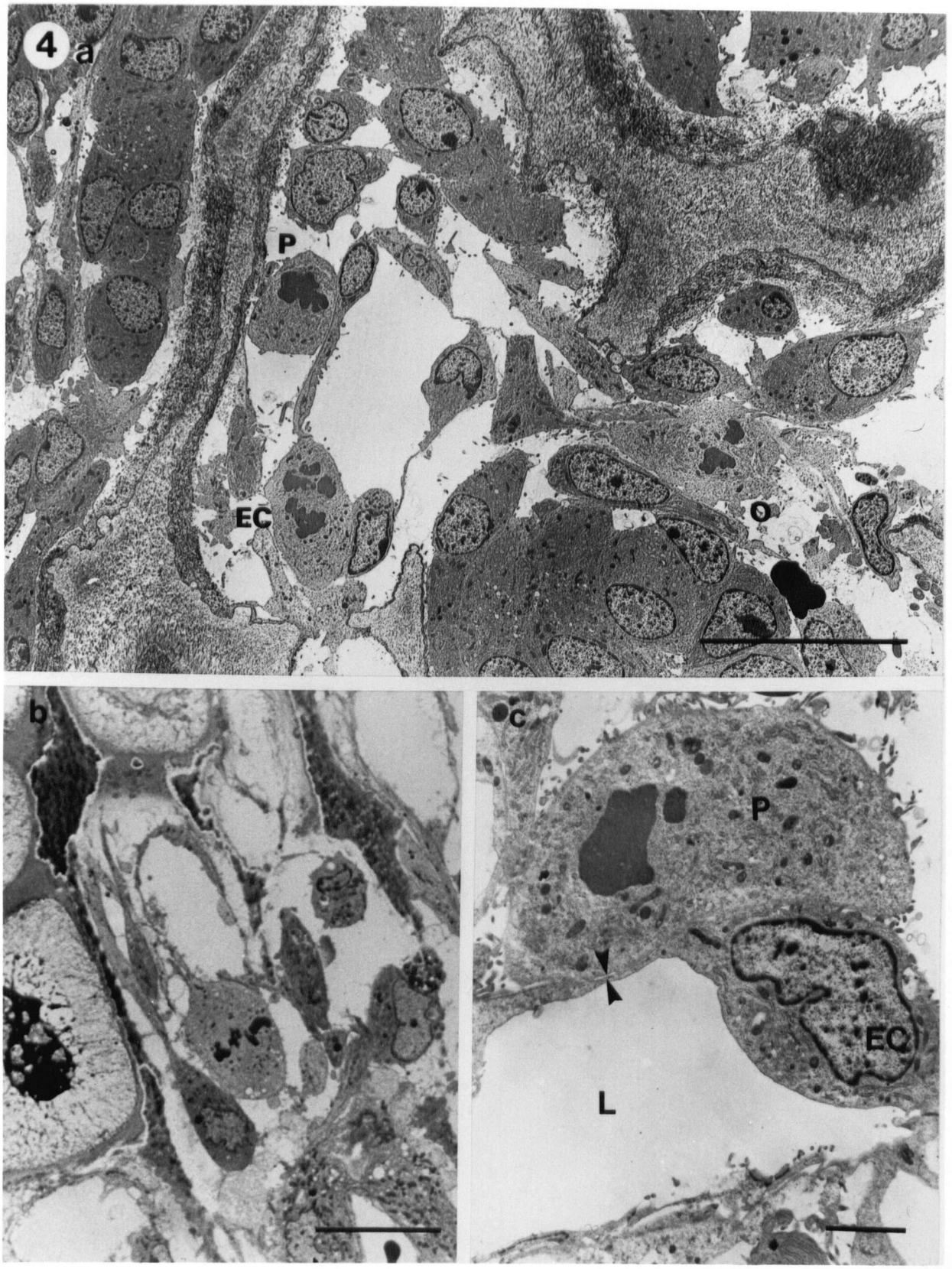
This cell count study was done at the EM level to enable the accurate distinction of pericytes from endothelial cells which could not have been achieved at the light microscopic level. Figure 4b shows a high magnification light micrograph of a vascular cell in metaphase in the sprout tip near the hypertrophic chondrocytes. It is difficult at this magnification to discern whether this cell is an endothelial cell or a pericyte lying immediately adjacent to the vascular wall. Often pericytes are so closely opposed to endothelial cells (Fig. 4c) that the space between them can only be seen at the higher magnification afforded by the electron microscope. At this level it is also possible to utilize other criteria, such as the presence or absence of adherence junctions between cells, for differentiation purposes.

Dividing endothelial cells made up 12.6% of the total number of mitotic figures, while dividing pericytes accounted for 19.5% (Fig. 6). Combined, these two vascular cell types account for over 32% of the mitotic figures observed between bone marrow and the growth plate cartilage and therefore make up a significant percentage of the total proliferative activity in the growing metaphysis. As expected, cells thought to be primarily involved in bone formation (making up the "other" catagory) were the most frequently observed dividing cells. It has been suggested that the population of extravascular cells found near bone surfaces is largely composed of immediate precursors to both osteoblasts and osteoclasts in the long bones of growing rats (Young, 1962; Owen, 1970, 1980). Fully differentiated osteoclasts and osteoblasts are generally nonmitotic (Owen, 1980) and are derived from precursor cells of the monocyte and nonmigratory connective-tissue

Figure 4: (a) Low magnification electron micrograph demonstraing the 3 dividing cell types counted: EC, mitotic endothelial cell junctionally attached to the adjacent endothelium; P, mitotic pericyte on the abluminal side of the endothelium; O, "other" mitotic figures not immediately related to the microvascular wall (comprised mostly of preosteoblasts and preosteoclasts); bar = 10 μm. x 3,500.

(b) Light micrograph of the metaphyseal sprout tip showing a dividing cell in the centre of the field. Positive identification of the cell as either endothelial or perivascular is impossible; bar = 5 μm. x 4,300.

(c) Electron micrograph of dividing pericyte (P) closely opposed to the abluminal surface of an endothelial cell (EC). An attenuated space is seen between the two cells (arrowheads) and no junctional attachments are found between them. L, lumen; bar = 2 μm. x 6,600.


4 a
P
EC
O
b
c
P
EC
L

cell lines respectively (Young, 1962). For this reason, the data listed in the "other" catagory is thought to represent, for the most part, mitotic precursors of the osteoclast and osteoblast cell lines.

The majority of dividing endothelial cells were located at a distance from the sprout tip, in that part of the vessel which is surrounded by cells involved in active bone deposition (Fig. 5). The data shown in Figure 6 illustrates the distribution of mitotic figures by both cell type and location. More than twice as many endothelial mitotic figures were located in the region of bone deposition than in the other three areas combined. Only rarely are dividing endothelial cells found in the upper reaches of the metaphyseal capillaries (the sprout tip and the region of extended calcified cartilage) or lower down in the parent and primary vessels. The parent vessels are fully formed mature capillaries or post-capillary venules (located 250-350 μm from the sprout tip) that are thought to give rise to the capillary sprouts (note: in Figure 6 the heading "parent vessels" should read "primary vessels"). Dividing pericytes (Fig. 5) are not often seen in the sprout tips or parent vessels, but are observed in the regions of extended calcified cartilage and bone deposition with about the same frequency (Fig. 6). Other mitotic figures, likely preosteoblasts and preosteoclasts, are found in large numbers in the three lower areas where bone deposition is initiated (region of extended calcified cartilage) and continued (regions of bone deposition and primary vessels).

The distribution of dividing cells was analyzed using the Chi-sqaure statistical analysis to test the null hypothesis that the mitotoic figures were evenly distributed throughout the metaphysis. To calculate this, and to correct for differences in size between the

Figure 5: Low power electron micrograph showing the location of a dividing endothelial cell in the region of bone deposition relative to the sprout apicies of the metaphyseal capillaries (connected arrows). Measurement of this distance (eg. 234 μm) is taken as a straight line connecting the dividing cell to the most closely related sprout tip apex. Three of the zones are indicated, ST, sprout tip; CC, region of extended calcified cartilage; BD, region of bone deposition; white box, area seen at higher magnification in Figure 4a; arrowheads, dividing pericytes in both the regions of extended calcified cartilage and bone deposition; bar = 25 μm. x 630.

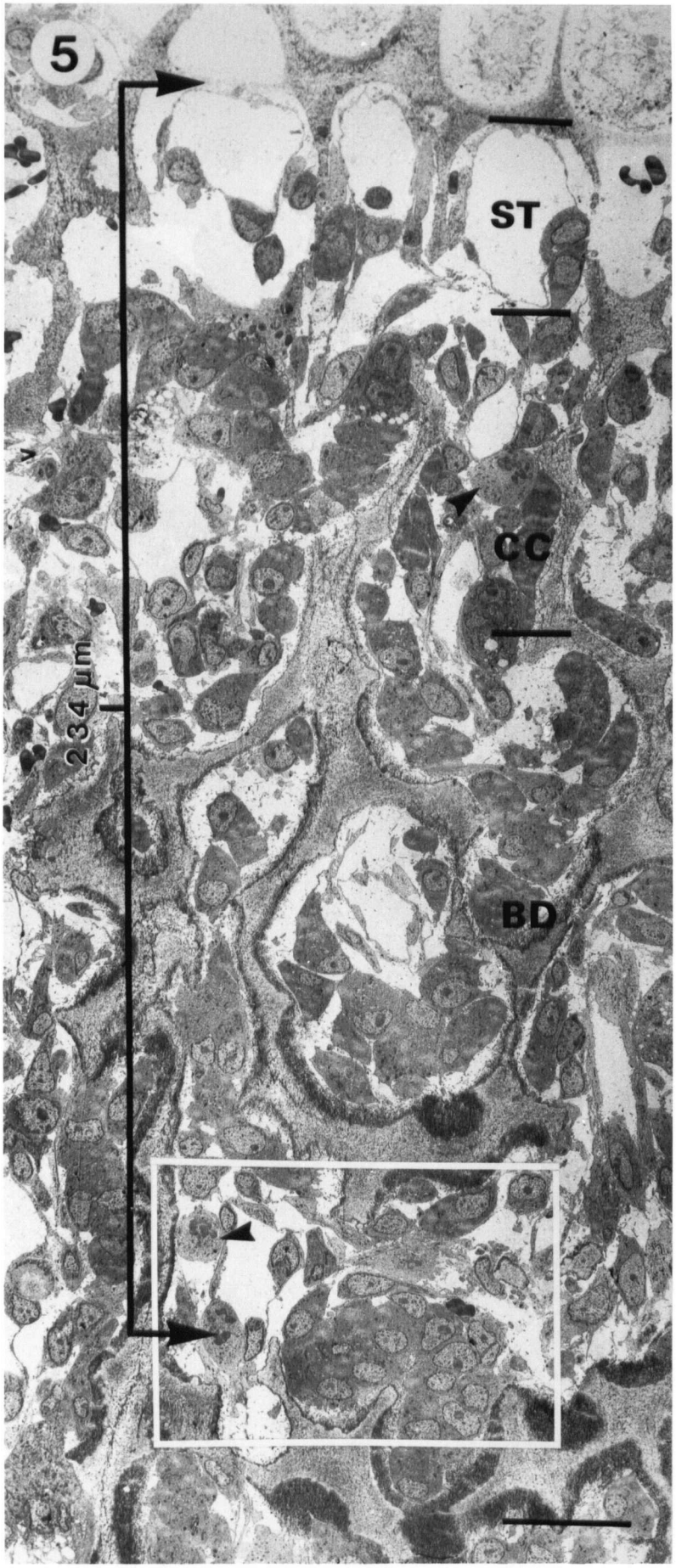
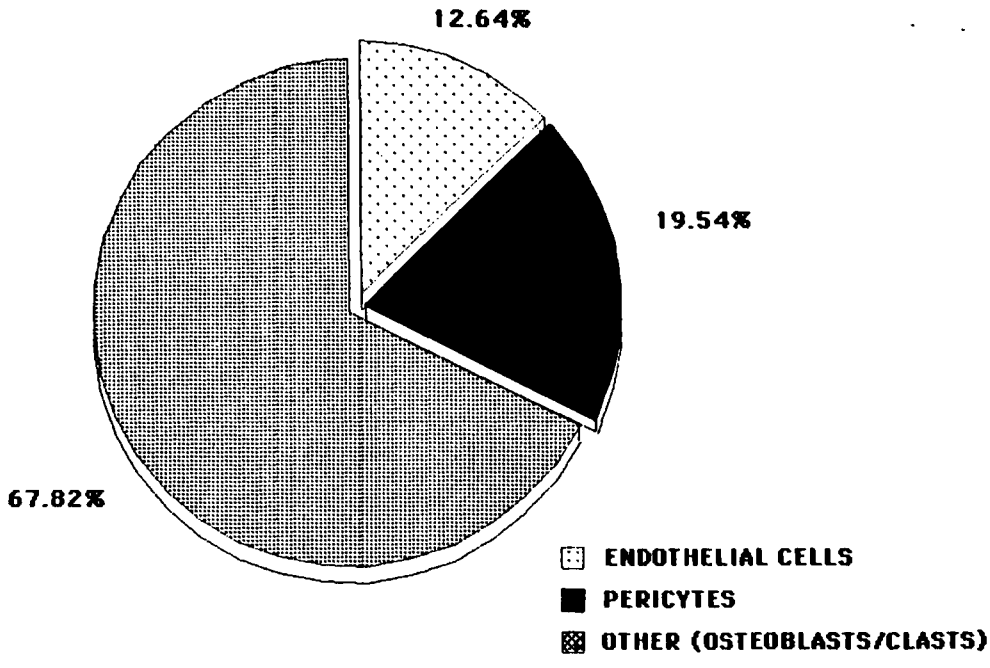

5
ST
CC
BD
234 µm

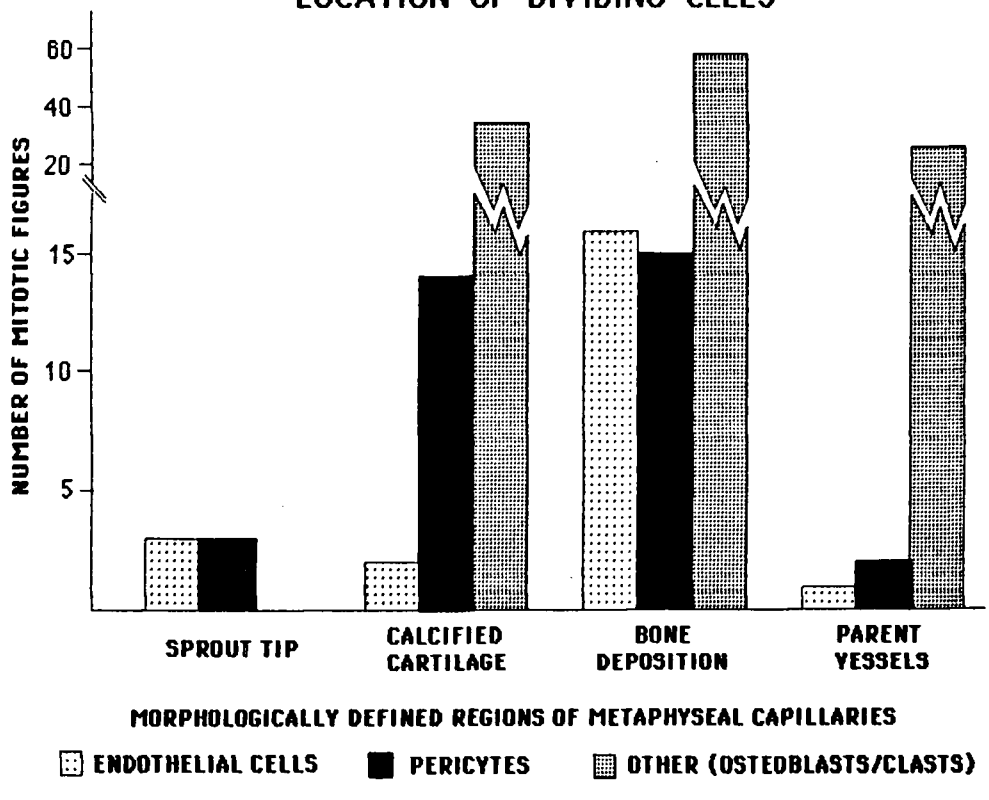
Figure 6: Percent Dividing Cell Types - a pie graph illustrating the breakdown of total mitotic figures by cell type. Location of Dividing Cells - a breakdown by location of the 3 dividing cell types counted. Note that the majority of dividing endothelial cells are located in the region of bone deposition, while mitotic pericytes are predominantly found spread over the regions of extended calcified cartilage and bone deposition. Preosteoblasts/clasts are found in the interstitium surrounding the metaphyseal capillaries in the lower 3 regions of the vessels.

⑥

PERCENT OF DIVIDING CELL TYPES



LOCATION OF DIVIDING CELLS



four zones, average measurements were used. The average sizes were: sprout tip - 50 μm (from the hypertrophic chondrocyte-blood vessel interface extending down 50 μm); zone of calcified cartilage - 100 μm (50-150 μm from the sprout tip); region of bone deposition - 200 μm (150-350 μm from the sprout tip); and primary vessels (350-500 μm from the sprout tip). For all three cell types counted (endothelial cells, pericytes, and others) the probability that the mitotic figures were evenly distributed was less than 0.05. This analysis indicates that the individual centres of mitosis for the three cell types are confined to distinct morphological regions of the metaphysis.

When a dividing endothelial cell was located, it was photographed at low magnification and measured for its approximate distance behind the sprout tip. This length was taken as being the distance following a straight line from the centre of the dividing cell to the apex of the sprout tip most directly above it (Fig. 5). While this figure is subject to certain limitations because a vessel can rarely be followed with certainty from the mitotic figure to the sprout tip in thin sections, it does have certain advantages. The vascular growth front is always known in the metaphyseal vessels because the capillaries are always progressing towards the growth plate cartilage. Also, this removes uncertainty as to the exact location of the sprout tips because the unidirectional growth of the capillaries cannot progress beyond the limits delineated by the hypertrophic chondrocytes. Since the forward front of the vessel is "fixed" in this manner, measurements from this point have a consistant origin. This makes it easier to find the approximate distance from the sprout tip to the dividing endothelial cells than would be possible in a less restricted three-dimensional capillary plexus. It

Table I : Distance From Dividing Endothelial Cells to the Sprout Tip

Sprout Tip	Extended Calc. Cart.	Bone Deposition	Primary Vessels
26 um 24 um 24 um	74 um 105 um	156 um 234 um 190 um 192 um 191 um 136 um 344 um 190 um 232 um 234 um 176 um 260 um 181 um 166 um 116 um 107 um	479 um
Average: 24.7 um	89.5 um	194.1 um	479 um

could be expected however, that in most cases this measurement procedure underestimates the actual distance as it does not account for an increase in length due to the curved path of the vessels.

Table 1 shows the measurements obtained for the dividing endothelial cells in the metaphyseal vessels. The overall average distance that dividing endothelial cells were located behind the sprout tip was 174 μm. Of the endothelial mitotic figures located in the region of bone deposition, all but one were found between 100 and 275 μm from the sprout tip with an average distance of 194 μm. This corresponds to a region where the metaphyseal capillaries lie on top of a fragmented basement membrane. Angiogenic vessels typically feature dividing endothelial cells behind the sprout tip in a region characterized by an incomplete basement membrane (Folkamn et al. 1981).

Autoradiography:

The autoradiographic study attempted to provide some information as to the growth dynamics of the metaphyseal vascular cells by tracing their fate following the incorporation of a single dose of ^{3}H-thymidine at various time intervals over a 24 hour period. Autoradiographic experiments confirmed the location of endothelial cell division, as label was first seen (1 hour after injection) at a distance from the sprout tip in the region of bone deposition (Fig. 7a). The 3 hour interval did not show any significant changes from the 1 hour sample, but by 6 hours labelled cells were seen to reach the region of extended calcified cartilage (fig. 7b). By 12 hours labelled endothelial cells were seen to reach higher within the region of extended calcified cartilage (Fig. 7c), but a smaller number of labelled endothelial cells were still seen within the region of bone

Figure 7: Light microscopic autoradiographs demonstrating endothelial cell migration during vessel growth.

(a) Labelled endothelial cells in the region of bone deposition 1 hour after injection with H-thymidine (arrowheads); bar = 10 μm. x 2,800.

(b) Labelled endothelial cell (large arrowhead) and a labelled vascular cell nearby (small arrowhead) which is either an endothelial cell or a pericyte at 6 hours post-injection; bar = 10 μm. x 2,400.

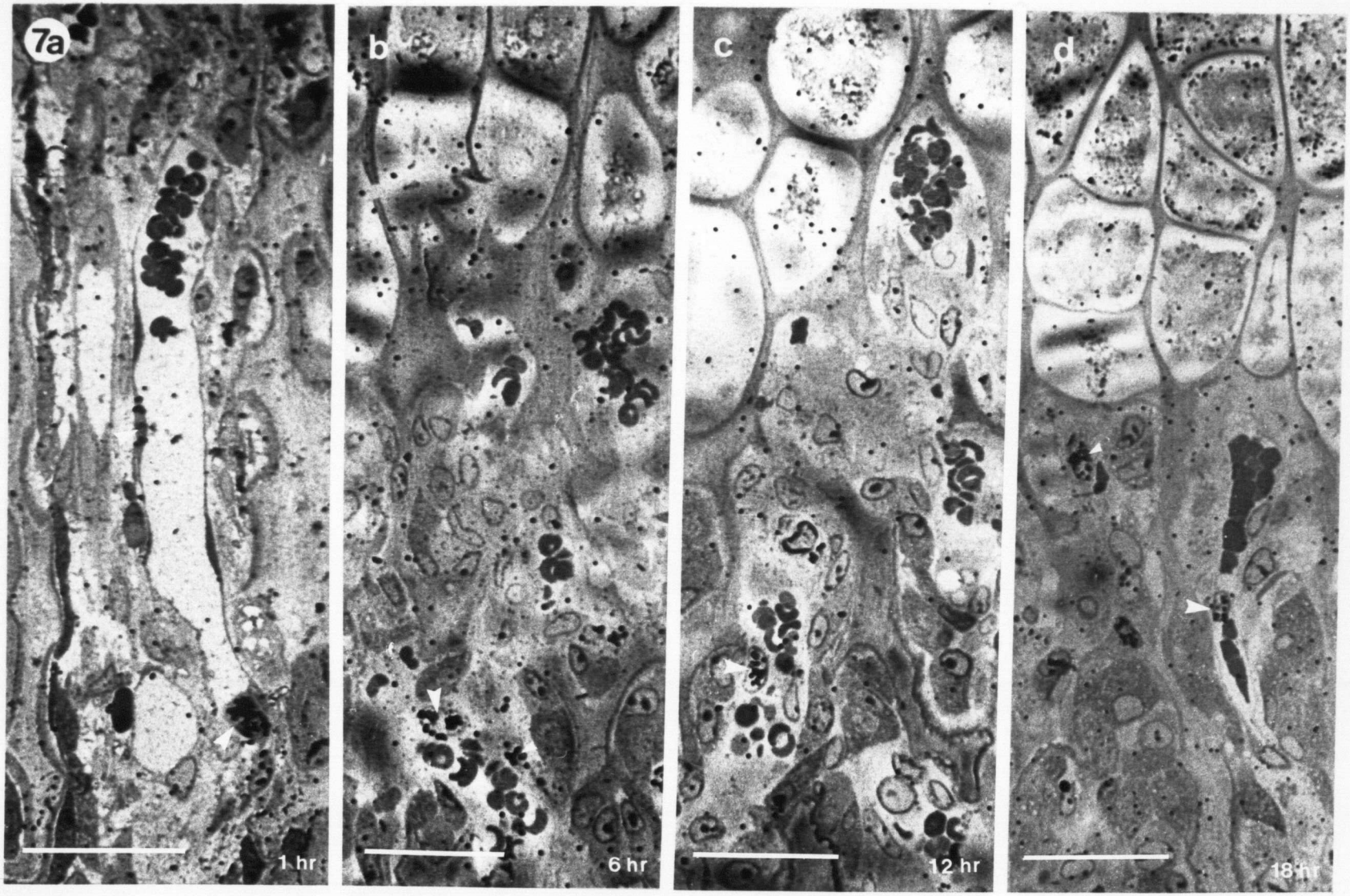
(c) Labelled endothelial cell in the zone of extended calcified cartilage (arrowhead) at 12 hours post-injection; bar = 10 μm. x 2,500.

(d) At 18 hours post-injection labelled endothelial cells are seen close to the sprout tip (large arrowhead) while some labelled cells are seen within the sprout tip (small arrowhead); bar = 10 μm. x 2,500.

(e) By 24 hours labelled endothelial cells are quite prevalent close to the sprout tip (arrowheads); bar = 10 μm. x 1,500.

(f) Also at 24 hours post-injection labelled endothelial cells are found within the sprout tip (arrowhead) invading the hypertrophic chondrocytes; bar = 10 μm. x 2,400.

(g) Control: a section of the epiphyseal growth plate and metaphyseal vessels which was processed for autoradiography but not previously injected with H-thymidine; bar = 10 μm. x 1,600.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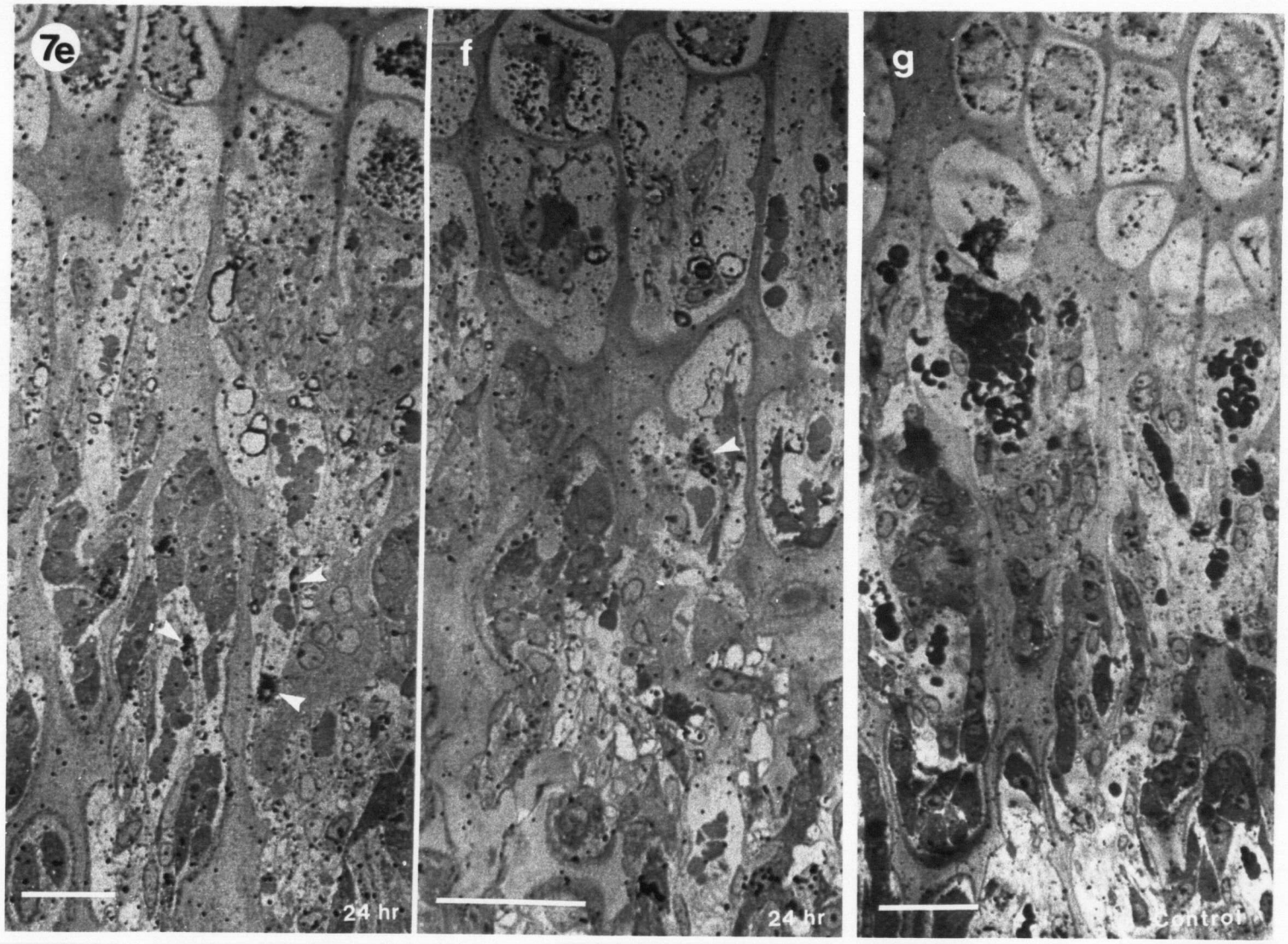
7e
24 hr
f
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control

deposition. Very few cells in the sprout tip exhibited label in the early time intervals (1, 3, 6, and 12 hours post-injection). By 18 hours post-injection labelled endothelial cells could be seen to be approaching the sprout tip (Fig. 7d large arrowhead). Some labelled cells were found in the sprout tip at 18 hours (Fig. 7d small arrowhead) and labelled endothelial cells appeared in the sprout tip at 24 hours (Fig. 7f) while many more labelled vascular cells were seen in the immediate vicinity (Fig. 7e). At no point in time were all the endothelial cells of a particular region of the capillary completely labelled as only a percentage of the cells incorporated label, however, the site of peak labelling could be followed with time. Therefore, although there is a defined area where cell division takes place and cells are capable of reaching the growth front within 24 hours, the growth pattern of the vessels over a 1-day period is not due solely to endothelial cell division and must involve other factors such as cell migration and cell spreading.

Discussion:

Chapter One illustrated the ultrastructural similarities between the endothelial cells of the metaphyseal sprouts and those in other growing vessels, particularly with respect to the basement membrane. Like the vessels described by Auersprunk and Folkman (1977) the metaphyseal sprouts have no basement membrane at the sprout tip, a fragmented basement membrane further down, and an almost complete basement membrane in the capillaries approximately 300 μm from the hypertrophic chondrocyte-blood vessel interface. In this study the metaphyseal capillaries were examined for the site of endothelial cell proliferation and the majority of dividing cells were found at an average distance of approximately 200 μm from the sprout tip in a

region of active bone deposition. This corresponds to an area where the metaphyseal capillary basement membrane is typically fragmented and only partially covers the microvascular wall. Autoradiography also confirmed this pattern as the endothelial cells of the sprout tips and the vessels of origin did not initially incorporate label, but the cells in between these two areas did.

Folkman et al. (1981) described the morphology of a typical capillary sprout during angiogenesis as consisting of three distinctive areas. The leading edge of the sprout tip is composed of migrating nondividing cells that have no underlying basement membrane. Behind this is a second zone featuring nonmigrating dividing endothelial cells resting on a fragmented basement membrane. Farther still from the sprout tip, the vessel consists of maturing endothelial cells which neither migrate or divide, but rest crowded on an intact basement membrane. In the tumour angiogenic system used by Ausprunk and Folkman (1977) the endothelial cells of the capillary sprout remained out of contact with each other, except for the cells located immediately adjacent to the vessel from which the sprout was derived. The advancing vascular front in many growing capillaries is a solid, blind-ended, endothelial sprout which initially has no lumen and no circulatory flow through it (Sandison, 1924, 1928).

Despite the many similarities, the metaphyseal vessels do appear to have some unique features not seen in other growing capillary sprouts. The capillaries maintain a closed lumen through which blood cells can pass and adjacent endothelial cells remain junctionally attached throughout the length of the capillary sprout. For this reason it appears doubtful that growth of the metaphyseal capillaries is due to continual sprouting because solid endothelial

sprouts without a lumen are never seen. Vessel growth can occur as a result of elongation of existing microvessels to expand a capillary bed where the highest mitotic activity is seen to be in the vessels proximal to the growth front (Chalkley et al., 1946; Schoefl, 1963). In fact, capillaries have the capacity to grow significant distances in the absence of cell division through migration and cellular extension of existing endothelial cells (Sholley et al., 1984). By tracing the fate of the metaphyseal endothelial cells it was possible to locate their site of origin and trace their migration towards the sprout tip with time. The intermingling of labelled and unlabelled cells throughout this progression indicates that these vessels too grow via a complex combination of division, elongation, and migration.

It is possible to hypothesize a growth mechanism for the metaphyseal capillary sprouts. Typically during angiogenesis, a solid endothelial sprout is formed by cells breaking through the basement membrane and migrating away from a parent vessel, which is either a capillary or a post-capillary venule. Eventually a lumen forms in the blind-ended sprout as it elongates and two such sprouts can meet and join to form a capillary loop, or a "generation" of vascular growth. If further growth is required a second generation may sprout from the newly formed loop and the plexus will thus progress forward generation after generation until growth is completed (Sandison 1924, 1928). The sprouts of the metaphysis may in part utilize such a growth system, but modified so as to account for the maintenance of an intact lumen throughout the process. Therefore, although outgrowth from an existing vessel may produce the initial sprout, once formed, the sprout continues to grow by elongation as opposed to forming a capillary loop from which a second generation of sprouts evolves. The

site of endothelial cell division remains behind the sprout tip within the region of bone deposition and provides the new cells necessary for the forward progression of the vessel. The region of bone deposition can be functionally subdivided into an upper prolifertive half containing dividing endothelial and perivascular cells (150-250 μm from the sprout tip) and a lower maturing vessel half that contains the capillaries and post-capillary venules that are the true parent vessels of the metaphyseal sprouts (250-350 μm from the sprout tip). It is interesting that dividing endothelial cells are found at an average distance of 200 μm behind the sprout tip as this is the same length new capillaries have been observed to grow in one day (Ausprunk and Folkman, 1977). The autoradiographic data in this experiment indicates that endothelial cells are capable of reaching the sprout tip wihin 24 hours, although many unlabelled cells are also seen here. Also, the growth plate itself has been seen to advance at approximately 200 μm per day in rapidly growing rats (Hunziker, in press). This sets up the possibility that dividing endothelial cells in the metaphysis are found one day's growth behind the advancing sprout tip and that the rate of capillary growth is the limiting factor in the progression of endochondral bone formation.

Corrosion casting of the metaphyseal vascular system has demonstrated that almost all of the terminal sprout tips bifurcate or display small lateral projections, while at slightly deeper levels there are extended bifurcating branches (Arsenault, 1987). It is possible that the formation of closed capillary loops is through anastomoses of these lateral projections rather than the sprout tips themselves. This would allow continued elongation of the vessel tip apically while still producing the architecture necessary to close off

the vasculature and produce capillary loops. Connections between neighbouring sprouts are seen further still from the sprout tip (Arsenault, 1987) and likely represent the extreme apex of the closed aspect of the circulatory system in the metaphysis as described previously (Trueta and Morgan, 1960). Unlike some angiogenic vessels in which blind-ended sprouts are derived by outward growth from a capillary loop, in the metaphyseal vessels it seems probable that the formation of closed loops is the result of lateral outgrowths from the capillary sprouts.

It has been suggested that pericytes may have a function in the regulation of microvascular growth through cell-to-cell interactions with the endothelium. When endothelial cells and pericytes are co-cultured and allowed to come into contact with each other, capillary endothelial cell growth is inhibited (D'Amore and Orlidge, 1987). The presence or absence of pericytes at the growth front of a capillary sprout may also be important in the regulation of angiogenesis in vivo. For example, in retinal neovascularization during diabetic retinopathy pericytes degenerate and are lost in the surrounding vessels prior to vascular ingrowth (Speiser et al., 1968). Also, during wound repair there is a correlation between the appearance of pericytes in the vessel wall and the cessation of vascular growth (Crocker et al., 1970). In the present study the proliferative response of pericytes to vascular growth was found to be similar to that of endothelial cells, with the principle site of pericyte cell division behind the sprout tip, but at a distance to the vessel of origin (Fig. 5). The location of pericyte cell division is not however, as spatially confined as the growth centre for endothelial cells and spreads across the regions of calcified

cartilage and bone deposition (from about 50-250 μm from the sprout tip). Although dividing pericytes are found more frequently than dividing endothelial cells (Fig. 6), very few are found near the vessels of origin and the sprout tips. In fact, pericytes are typically absent from the sprout tip of the metaphyseal capillaries (Chapter 1). The deeper primary vessels of the metaphysis appear to possess a mature, continuous, nondividing population of pericytes, the capillaries in the regions of exended calcified cartilage and bone deposition have a population of dividing discontinuous pericytes, and the sprout tips have few pericytes and none at the extreme tip of the vessel. This raises the interesting possibility that the production of pericytes, which are thought to be one of the factors influencing the inhibition and cessation of vessel growth, is itself stimulated by those factors which promote angiogenesis. Therefore, when a vessel is stimulated to grow, at least one factor which will ultimately be involved in the termintion of that growth, is stimulated also.

In summary, this study demonstrated that the site of endothelial cell division in the metaphyseal capillaries is found at a distance from the sprout tip (an average of 175-200 μm) in a region of active bone deposition. Dividing endothelial cells are found in a location where the capillaries typically display a fragmented basement membrane; a pattern which is observed in many growing vessels. The morphology of the vessels suggests that the principle mechanism of growth in these capillaries is through elongation of closed luminal sprouts which form capillary loops via anastomosis of lateral branches. Dividing pericytes are also found behind the sprout tip, but have a wider distribution than do proliferating endothelial cells.

Chapter 3: THE FORMATION OF JUNCTIONAL ATTACHMENTS BETWEEN DAUGHTER CELLS DURING ENDOTHELIAL CELL DIVISION

Abstract

The endothelium of the metaphyseal capillary sprouts forms a continuous attenuated squamous lining. During endochondral bone formation these growing vessels possess a region of endothelial cell division which is located behind the sprout tip in an area where the microvascular wall consists of the endothelium and a discontinuous layer of perivascular cells. Electron microscopic examination of serial sections of this region showed that during cytokinesis the endothelial cells establish junctional attachments between daughter cells even before cell separation is complete; thus the integrity of the vascular wall is never compromised during cell division. Junctions with adjacent endothelial cells are also formed along the cleavage plane prior to the completion of cytokinesis. Numerous microvilli from both the daughter cells and adjacent endothelial cells often make contact and form junctions with the plasma membrane of the dividing cells. A model for endothelial junction formation between daughter cells during cytokinesis and the role that microvilli play in the process is proposed.

Introduction

During endochondral ossification the growing vessels of the metaphysis invade the calcified cartilage of the epiphyseal growth plate. These closed, blind-ended, continuous capillary sprouts have

dividing endothelial cells in their walls at an average of about 200 μm behind the sprout tip in a region of active bone deposition (Chapter 2). All dividing endothelial cells found in this area of cell proliferation were seen to possess luminal microvilli and to retain junctional attachments to neighbouring endothelial cells throughout mitosis. Cells of the microvascular wall immediately surrounding dividing endothelial cells also often exhibit luminal microvilli. In the metaphyseal capillaries an intact lumen complete with limited blood flow through the vessel, is maintained throughout the growth process and requires that all endothelial cells, including those in mitosis, remain junctionally linked.

The endothelial cells of a continuous endothelium are linked by intercellular junctions that consist of two basic types: occluding (tight) junctions and communicating (gap) junctions (Simionescu et al., 1974, 1975, 1976). The occluding junctions form a physical link between adjacent endothelial cells as well as forming a barrier that prevents the passage of large macromolecules between cells (Simionescu and Simionescu, 1984). *In vitro* studies of angiogenic vessels place the site of endothelial cell division at a location behind the tip of the growing sprout in a region where adjacent cells of the endothelium are not joined to each other (Ausprunk and Folkman, 1977). The endothelial cells progress through the early stages of sprout formation separated from one another and only later do they form occluding junctions between them. The mechanism by which the cells contact each other and initiate junctional attachments is unclear.

The metaphyseal endothelial cells are an *in vivo* angiogenic system with blood flow through them and as such, have some physiologic constraints that *in vitro* systems do not. In this

closed, continuous and growing microvascular wall, dividing endothelial cells must progress through cell division while maintaining the integrity of the barrier. During mitosis, it is therefore imperative that endothelial cells retain their attachments to neighbouring cells and form junctional attachments between daughter cells prior to the completion of cytokinesis to prevent temporary gaps from occurring in the endothelium. The present study addressed this problem through serial sectioning of a large number of growing metaphyseal vessels in the area where endothelial cell division takes place. Endothelial cells were observed to form junctions between daughter cells even before the cells were completely separated. A role for microvilli in the initiation of cell contact leading to junctional formation is suggested. This may represent a mechanism that could be used in the formation of endothelial attachments during angiogenesis in other systems.

Materials and Methods

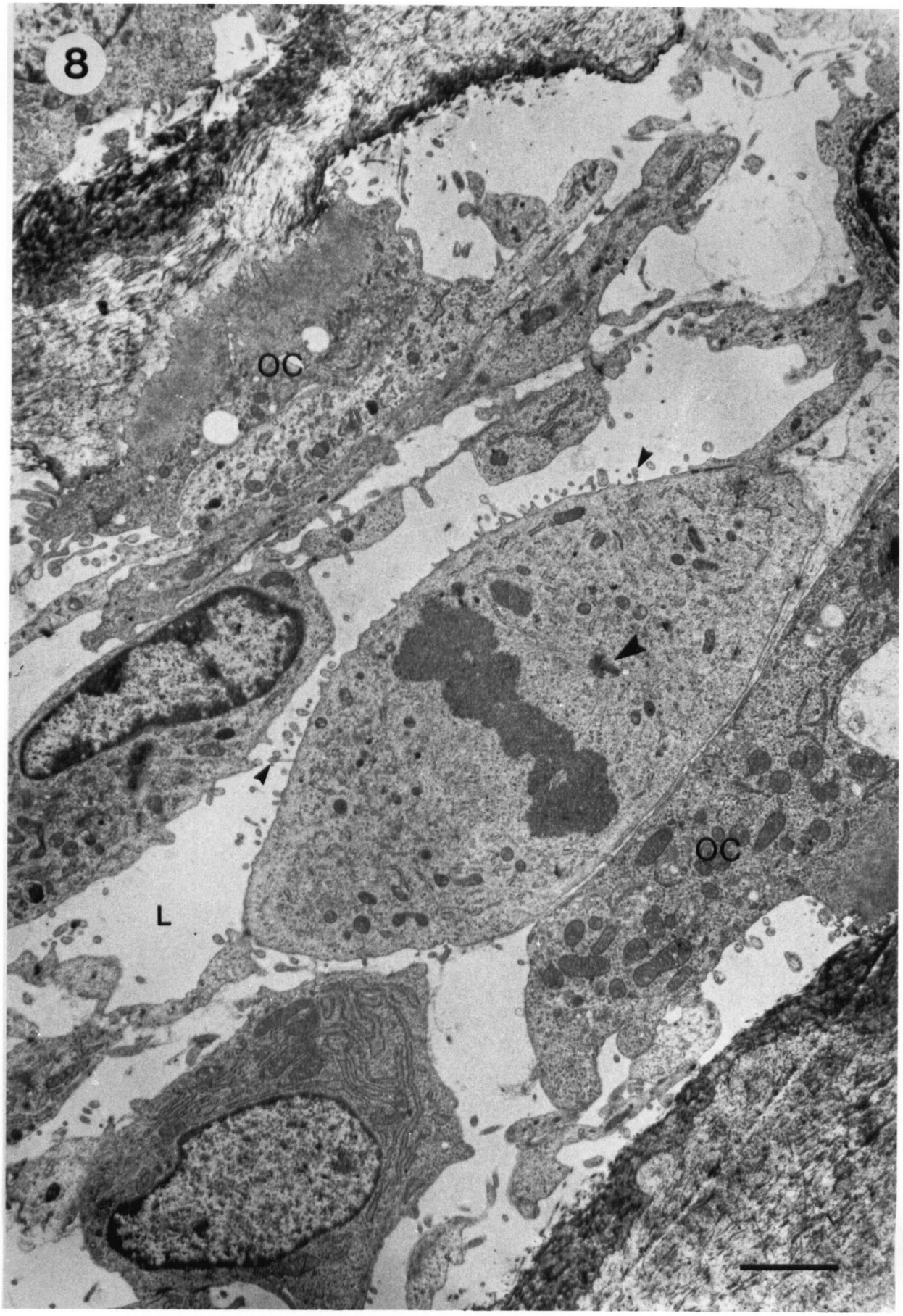
Juvenile male Sprague-Dawley rats (100-150 g) were perfusion fixed, their tibiae excised, decalcified, and prepared for electron microscopy as described in Chapter 1. Thin sections (about 60 nm in thickness) of the tibial epiphyseal growth plate were cut on a diamond knife. A total of 45 separate tissue block faces were obtained from 5 separate animals (9 tissue blocks from each animal). Between 10 and 40 serial sections were collected in order from each block face and placed on 75-mesh grids, stained with 5% uranyl acetate and 0.5% lead citrate, and viewed on a Phillips 300 electron microscope.

Results

With the exception of a discontinuous layer of perivascular cells, the microvascular wall in the metaphyseal capillaries consists of a single squamous layer of highly attenuated endothelial cells. All endothelial cells observed in mitosis possessed luminal microvilli and retained junctional attachments to adjacent cells of the vascular wall (Fig. 8). Although the cells round up during cell division, they do not dissociate from the cells around them and the microvascular wall is not compromised by gaps between cells.

A problem exists during cytokinesis, when the newly formed daughter cells must separate from each other while simultaneously forming junctions with adjacent cells. A daughter cell must also form attachments to the cell from which it is separating along the newly-formed boundary created in the cleavage plane. As the cleavage furrow invaginates during cytokinesis, the endothelial daughter cells remain joined by a cytoplasmic bridge that contains the remnants of the mitotic spindle (Figs. 9b,f). Serial sections through cells at this point in cytokinesis showed that lateral to the cytoplasmic bridge were sites of junctional attachment on the newly forming edge of the cell along the cleavage furrow (Figs. 9a,e). It could not be ascertained whether the attachments seen were between the daughter cells themselves, or the processes of intervening adjacent endothelial cells which were joined to both daughter cells. Regardless of this, it was clear that the endothelial daughter cells were forming cellular attachments to other cells of the vascular wall along the invaginating cleavage furrow, even though the cells still remained in cytoplasmic continuity at the cellular bridge. In sections of the same cells peripheral to the cell bridge, the daughter cells appear separated due

Figure 8: Electron micrograph of a dividing endothelial cell in metaphase. Note the apical microvilli (small arrowheads) in this cell and its junctions with adjacent endothelial cells. Large arrowhead indicates centriole and mitotic spindle,; OC, osteoclast; L, lumen of capillary; bar = 2 μm. x 6,500.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OC
OC
L

Figure 9: Series of electron micrographs of serial sections through an endothelial cell undergoing cytokinesis. The luminal erythrocyte is useful as an indicator of depth between sections.

(a) Cell attachments are present in the region of the cleavage plane between the two daughter cells; bar = 2 μm. x 3,600.

(b) In the central pole between the two daughter cells they remain joined by a cellular bridge; bar = 2 μm. x 3,600.

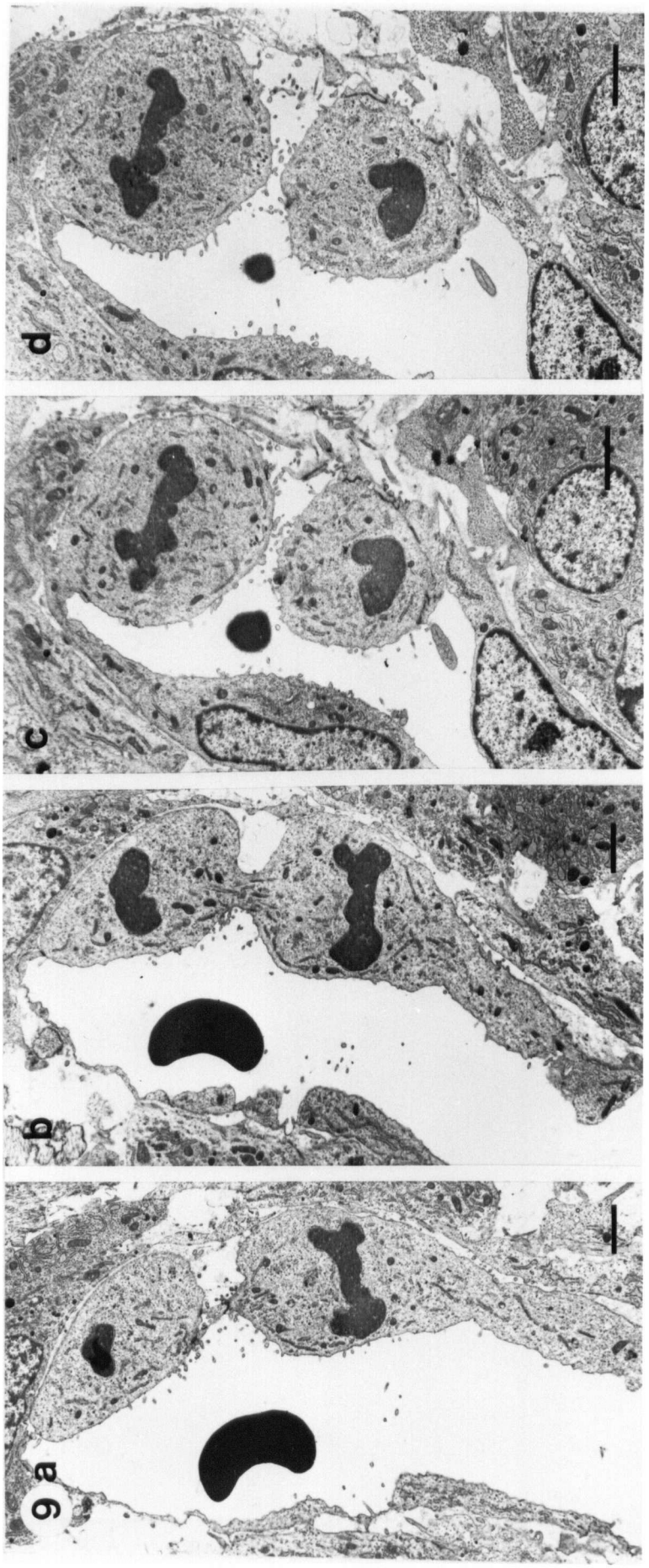
(c) Peripheral to the cell bridge (on the opposite side of the cell bridge as Fig. 9a) a "space" is seen between the daughter cells which contains numerous microvilli; bar = 2 μm. x 4,700.

(d) Further still from the cell bridge many profiles of microvilli are observed in the region of the cleavage furrow between the daughter cells; bar = 2 μm. x 4,500.

(e) Higher magnification of Figure 9a showing numerous junctional sites on the newly forming face between daughter cells (black arrowheads). No direct attachments between daughter cells are seen, but an intervening cell process (likely from an adjacent endothelial cell) attaches to both cells. Microvillar profiles contact and adhere to the cell process; bar = 0.5 μm. x 29,000. Inset: A -37.1° tilt demonstrating the central junction (white arrowheads); bar = 0.2 μm. x 67,000.

(f) Higher magnification of Figure 9b illustating the cell bridge between the daughter cells and the microtubular remnants of the mitotic spindle (arrowheads); bar = 0.5 μm. x 26,500.

(g) Higher magnification of Figure 9c. Microvilli intervene between the daughter cells which appear separated due to the plane of section. Arrowheads indicate sites of junctional attachment between the fine cell processes; bar = 0.5 μm. x 12,500.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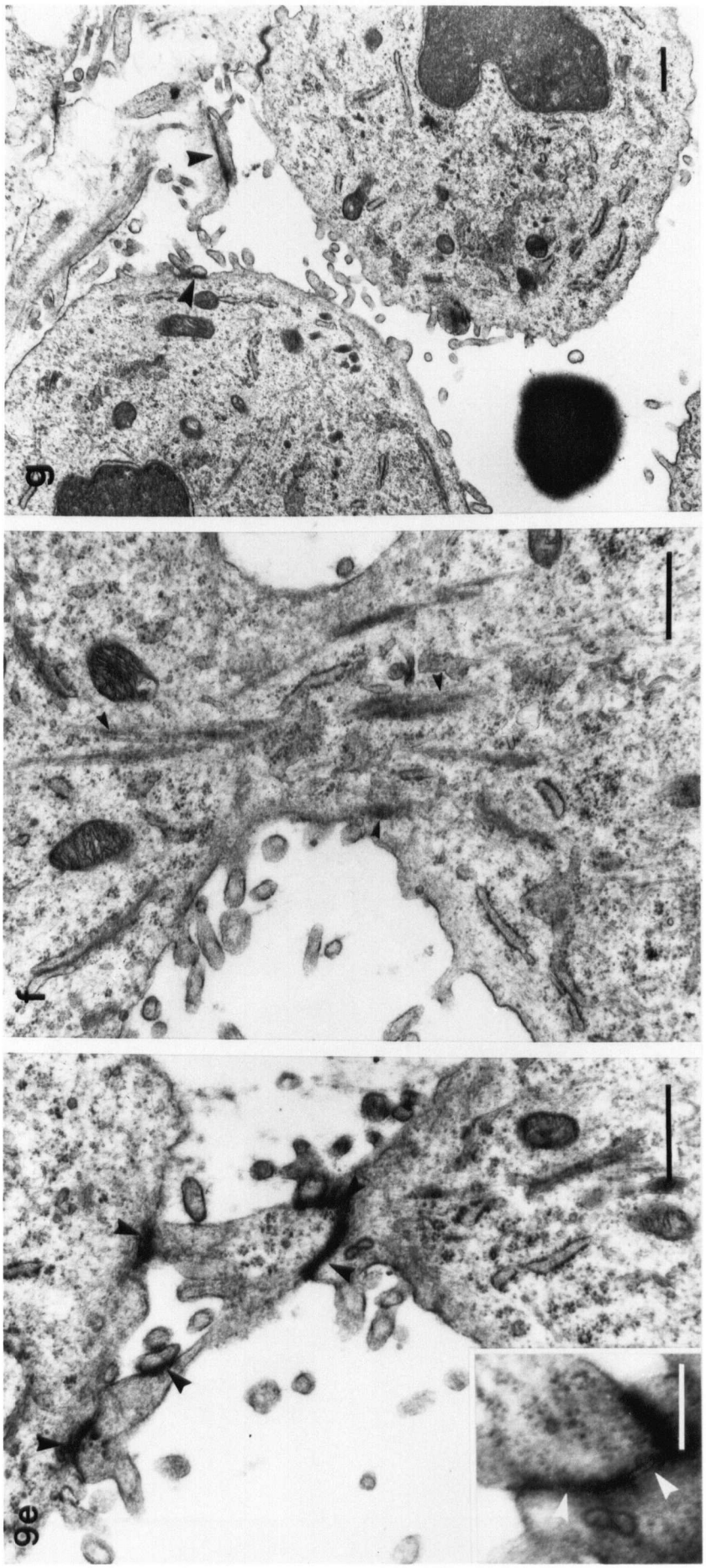
g
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9e

to the plane of section and microvilli can be seen projecting from both cells into the space between them (Figs. 9c,d,g). Microvilli were always present on the luminal surface of separating endothelial cells and often appeared to contact and form cell junctions on the cell surfaces between the daughter cells (Figs. 9e,g).

Occasionally microvilli from the two daughter cells were seen to intermingle prior to cell separation. Figure 10 shows two endothelial cells that are still joined by a cell bridge between them that is abundant with the microtubules which are remnants of the mitotic spindle. Numerous microvilli and an amorphous electron dense material were found in the area between the two cells bounded by the spindle remnants on one side and the underlying basement membrane on the other. Some microvilli were in close contact with each other and intertwined together such that it appeared as though there were sites of attachment between them (Fig. 10, black arrowheads). Microvillar projections were seen to project from virtually every surface of the cell except its abluminal side, including: the cell membrane between the separating daughter cells, the surface of the cell bridge which connects the two cells, and the luminal surfaces of both cells (Fig. 10). Also in Figure 10 numerous caveolae were present on the surface of one of the daughter cells and the surface of the cell bridge in the region of their adjoinment (white arrowheads).

As the process of cytokinesis continues, the daughter cells begin to separate completely and the cytoplasmic bridge which used to connect them is no longer complete. However, microtubules of the disintegrating mitotic spindle are still present and enable identification of the region where the two cells were formerly joined. Figure 11 shows serial sections through two daughter cells which have

Figure 10: Electron micrograph of the area around the cell bridge joining two daughter cells during cytokinesis. The capillary lumen is seen above the cell bridge and the basement membrane is seen below it. Note the numerous intertwining microvilli in the region beneath the cell bridge which appear to contact and adhere to each other (black arrowheads) and the electron-dense extracellular material also seen in the vicinity. Microtubules that comprised the mitotic spindle are found within the cell bridge (arrows) and several caveolae are present at the point where the bridge adjoins to one of the daughter cells (white arrowheads); bar = 0.5 μm. x 43,000.

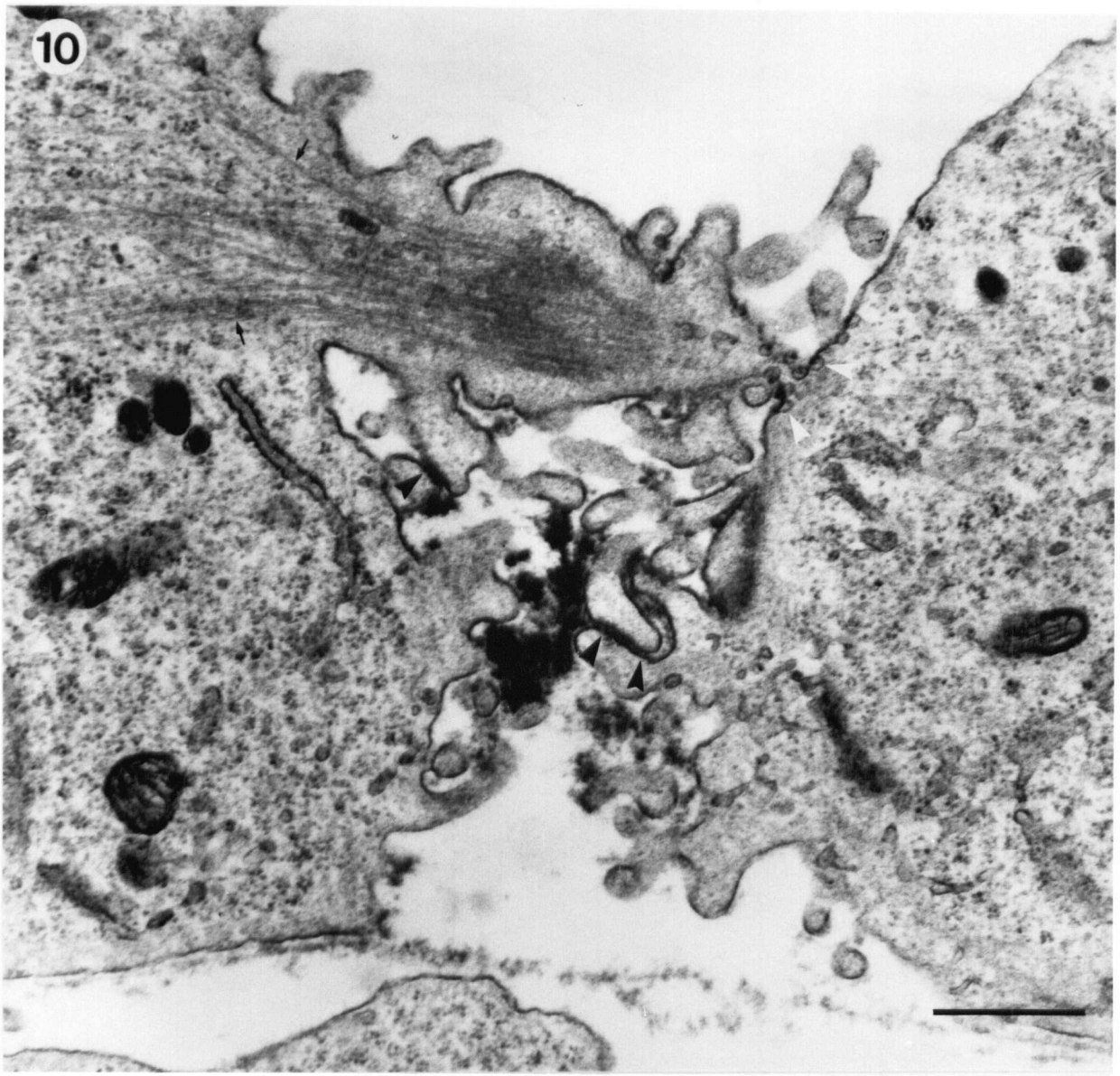
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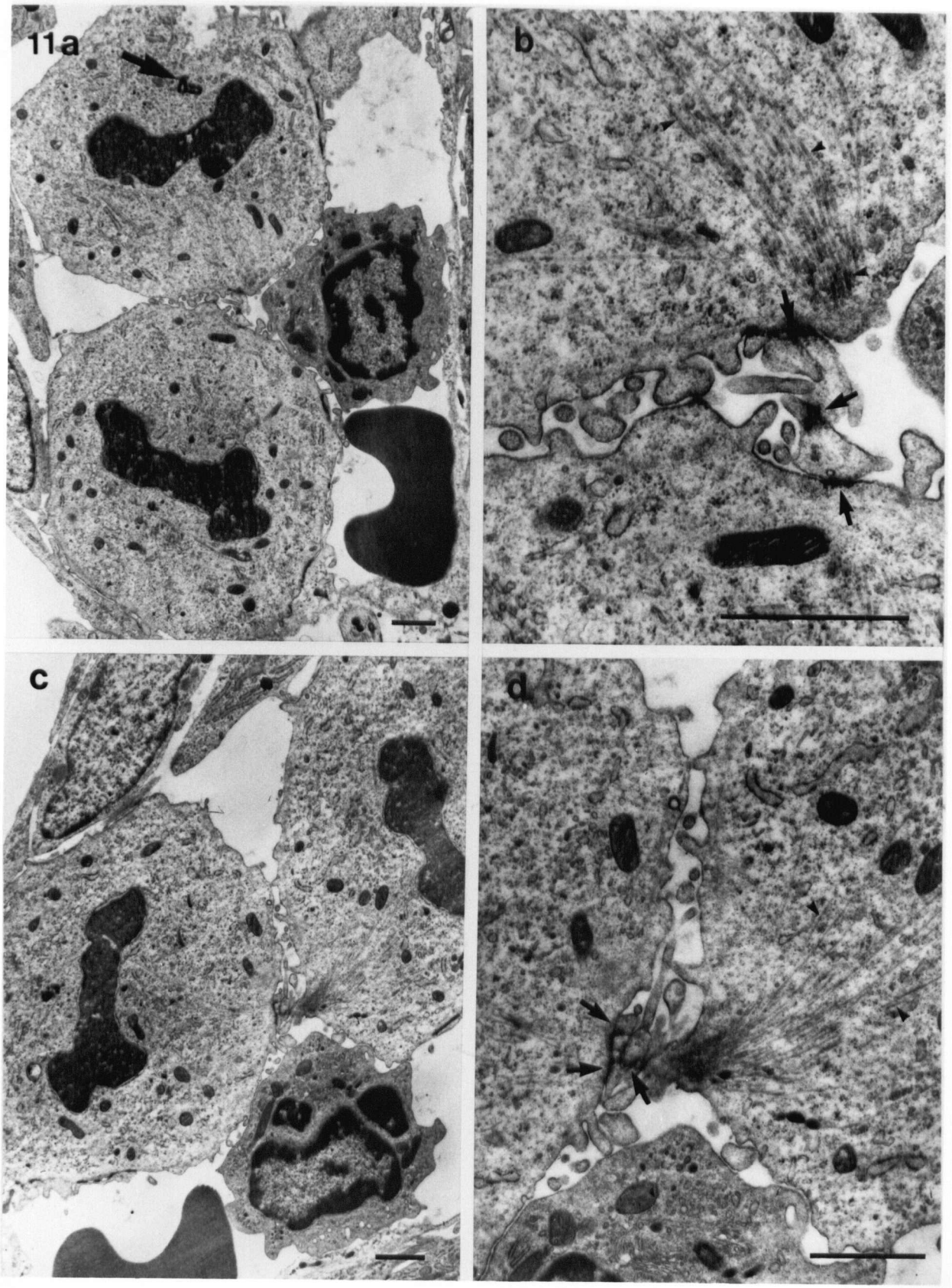
Figure 11: Electron micrographs of serial sections through two daughter cells nearing the completion of cytokinesis.

(a) The two daughter cells do not have a cell bridge remaining between them, but can be recognized by the appearance of their nuclei and the presence of remnants of the mitotic spindle. Arrowhead, centriole; bar = 1 μm. x 7,500.

(b) Higher magnification of Figure 11a demonstrating junctional attachments between the plasma membranes of the daughter cells and other endothelial cell processes of unknown origin (arrows) close to the area where the cell bridge used to span across the two cells, as indicated by the spindle remnants (arrowheads); bar = 1 μm. x 32,500.

(c) A second section taken close to the point where the cell bridge used to connect the two daughter cells; bar = 1 μm. x 8,600.

(d) Higher magnification of Figure 11c showing many cell junctions left behind in the region where the cell brige has detached (arrows). The former cell bridge is identified by a plethora of microtubules (arrowheads) and appears to separate unequally between the daughter cells; bar = 1 μm. x 20,000.


11a
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d

separated completely since although vestiges of the spindle apparatus can be seen, none of the serial sections displayed a point at which a microtubule-containing cytoplasmic bridge spanned continuously between the two cells. At the site where the daughter cells were previously continuous at the cell bridge numerous junctions can be seen. Several adherens contacts are seen on the adjoining plasma membranes of the daughter cells as well as multiple intercellular attachments with intervening cell processes of undetermined origin (Figs. 11b,d). Numerous microvilli are observed in the region between the two cells and some of them appear to form attachments with the daughter cell surface and with other microvilli. This morphological picture suggests that as the cell bridge containing the remains of the spindle is broken between the daughter cells, it is replaced by several adherens-like cell junctions. These junctions are apparently due to multiple attachments between daughter cell processes, the surface of the retracting cell bridge, intervening cell processes from adjacent endothelial cells, and the cell bodies of the daughter cells.

In endothelial cells observed in the final stages of cytokinesis the dissolution of the cell bridge appears to occur on the surface of one of the daughter cells. Rather than being divided equally between the two new cells, one of the daughter cells appears to acquire most of the cell bridge while the other cell retains little of it (Fig. 11d). The detachment of the cell bridge from one of the daughter cells may be the result of caveolae in the region coalescing to sever the connection (Fig. 10).

When cytokinesis is complete, the individual daughter cells have extensive adherens-like junctions between them as well as attachments to adjacent endothelial cells on their newly formed

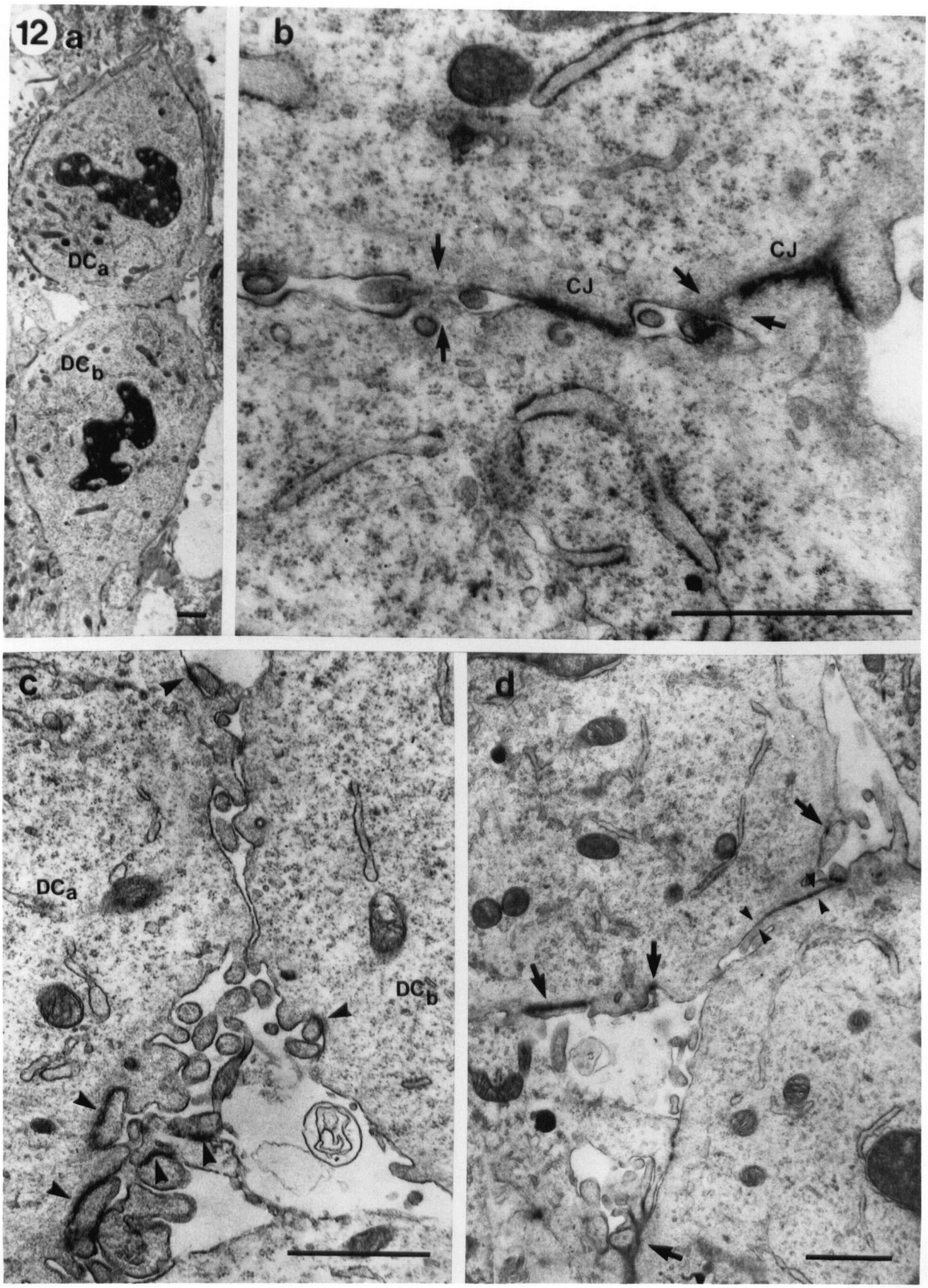
Figure 12: Electron micrographs of serial sections through two daughter cells after complete dissolution of the cell bridge.

(a) Low magnification micrograph showing characteristic chromatin pattern of post-mitotic cells and the diamond pattern of the endothelial lining. Note the extensive junctions between the recently separated daughter cells and between the daughter cells and the adjacent endothelial cells. DCa, daughter cell "a"; DCb, daughter cell "b"; bar = 1 μm. x 4,800.

(b) Higher magnification of Figure 12a showing cell junctions between the two daughter cells (CJ) along their intervening surface (previously the cleavage furrow). It is possible that the daughter cells have not yet fully completed cell separation as sites of cytoplasmic continuity between them may still exist (arrows); bar = 1 μm. x 42,000.

(c) In a second section through the cells they appear separated due to the plane of section and numerous microvilli are present in the cleft between them, many of which contact and adhere to the daughter cell plasma membrane (arrowheads). DCa, daughter cell "a"; DCb, daughter cell "b"; bar = 1 μm. x 25,000.

(d) Extensive junctions are present between the daughter cells (arrowheads) while other endothelial cell processes contact and adhere to the daughter cells along their newly formed surface (arroheads); bar = 1 μm. x 15,000.


12 a
DC a
DC b
b
CJ
CJ
c
DC a
DC b
d

boundaries. Cells at this point in division can be recognized, even though spindle remnants are no longer present, by the cell nuclei which are condensed, irregularly shaped, and heterochromatic (Fig. 12a). The junctions between daughter cells extend over a larger percentage of the area where the cells are opposed to each other (Fig. 12b,d) than was the case at previous times in the separation process. At points along the boundary between the daughter cells there appear to be isolated points where cytoplasmic continuity still exists between the cells even though they have otherwise already separated and formed junctional attachments (Fig. 12b). Other cell junctions can be seen on the newly formed cell boundaries and are thought to be the site of daughter cell attachment to neighbouring endothelial cells (Fig. 12c,d). In serial sections that do not pass through junctions between daughter cells, microvillar processes from both cells can be seen in the intervening space (Fig. 12c). Also at several points on the cell membrane there are cell junctions that appear to be the result of microvilli contacting and adhering the daughter cells (Fig. 12c).

Discussion

The squamous endothelial lining of capillaries must, among their many other functions, provide a continuous non-thrombogenic surface that permits blood cells to freely flow along their luminal surface. In the metaphyseal capillaries, endothelial cell division takes place in a region where the microvascular wall consists of only endothelial cells and a discontiuous lining of pericytes. A fragmented basement membrane also covers some portions of the abluminal side of the endothelium. A problem exists when the

endothelial cells in these growing capillaries undergo cell division because they must still maintain the integrity of the capillary lumen and allow normal blood circulation to continue. Dividing daughter cells must be able to complete cytokinesis without leaving spaces in the vascular wall that could cause leakage into the surrounding tissue or initiate thrombus formation.

Examination of serial sections through dividing endothelial cells has provided insight into how cell junctions are formed between daughter cells during cytokinesis and prior to cell separation to keep the endothelium intact. Many epithelial cells (particularly in stratified epithelium) round up and remain separated from each other during mitosis and only form cellular attachments after the completion of cell division. Endothelial cells in vitro have also been observed to complete cell division in a region of the capillary sprout where adjacent endothelial cells are not joined to one other (Ausprunk and Folkman, 1977). However, during growth of the metaphyseal capillaries the endothelial cells divide in an aspect of the capillary sprout that has a continuous endothelium with some blood flow through it. As shown in this study, throughout mitosis the dividing endothelial cells retain junctional attachments with the endothelial cells immediately adjacent to them. In addition, it has been shown that endothelial cells are capable of forming cellular attachments on the newly formed face along the cleavage furrow even before the cytoplasmic bridge connecting them is broken. Therefore, the endothelial daughter cells form adhering junctions with both the cell from which they are separating and the neighbouring endothelial cells prior to the completion of cytokinesis, thus keeping the microvascular wall intact.

Numerous microvilli, from both the daughter cells and adjacent endothelial cells, are found in the region of the cleavage plane and appear to be involved in the formation of junctional attachments on the surface of the separating cells. Many of these microvilli contact the cell membrane of the separating endothelial cells during cytokinesis and form an attachment site that is morphologically similar to intermediate cell junctions. It is likely that the initiation of contact and the formation of adhering junctions during cytokinesis is at least partially due to membrane interactions resulting from microvillar contacts. Cell junctions are also seen to be left behind between daughter cells in the area where the cell bridge spanning the two cells becomes disconnected.

From the morphological data provided by this work it is possible to propose a model as to how endothelial cells establish the new junctions necessary to keep the microvascular wall intact during cell division. As shown in the schematic (Fig. 13) the endothelial cells of the microvasculature are arranged in a diamond pattern. When an endothelial cell divides the cleavage furrow forms along its central pole. Therefore, when the resultant new cell boundaries are created, each daughter cell must form occluding junctions with three cells around it: the other daughter cell from which it is separating and two adjacent endothelial cells. The microvilli that are seen to contact the daughter cells along the cleavage furrow are largely derived from cell processes of the adjacent endothelial cells which project into the potential space between the separating cells. Ultimately it is the adjacent endothelial cells which will form the most extensive junctional attachments with the daughter cells. The daughter cells themselves will remain linked to each other only at a

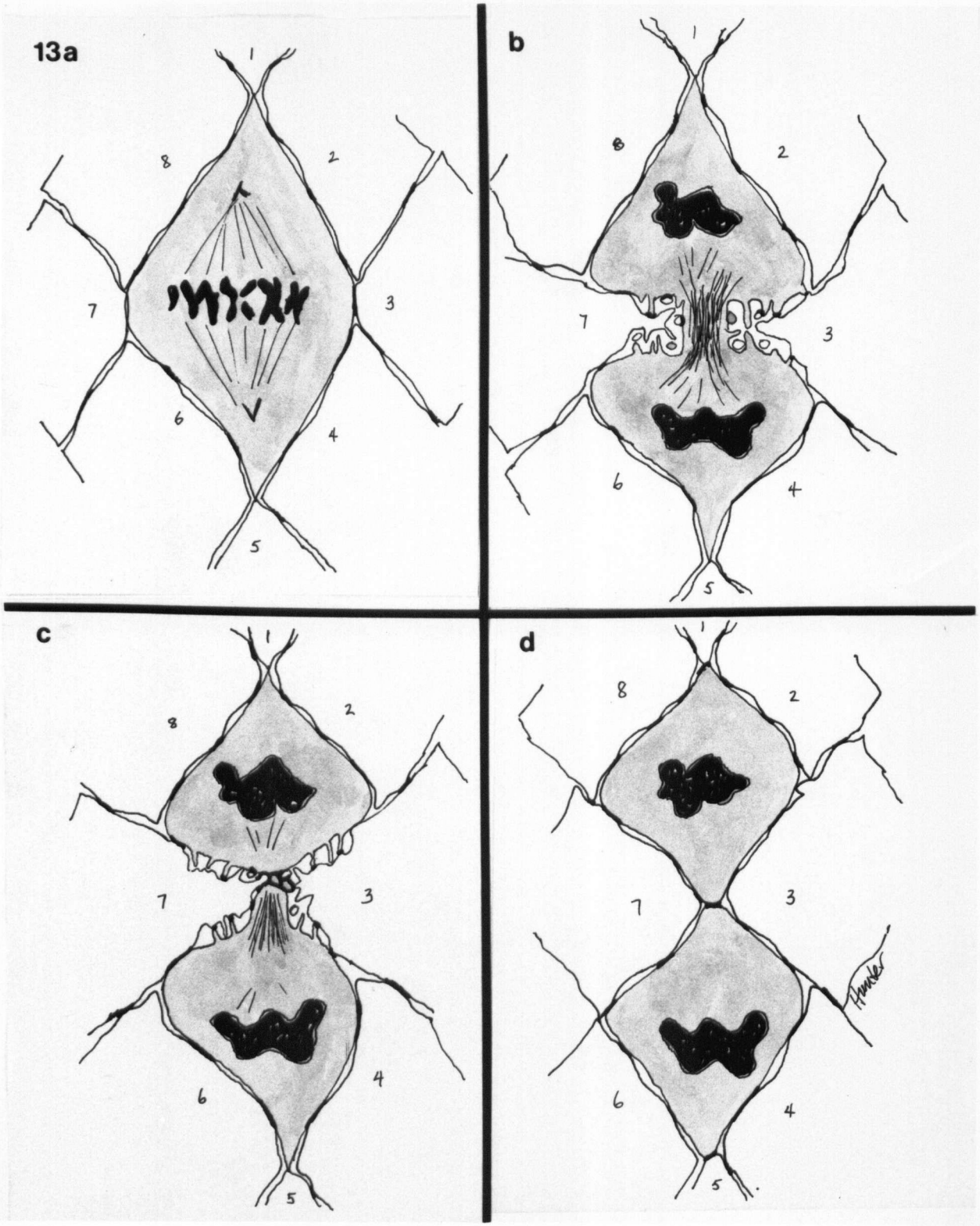
Figure 13: Schematic drawings showing the process of cytokinesis in a continuous endothelial cell wall (flattened into a monolayer for presentation purposes).

(a) A dividing endothelial cell retains its attachments to adjacent endothelial cells during mitosis. Due to the "diamond-shaped" pattern of the endothelium each cell is typically associated with 8 others.

(b) While the daughter cells still retain cytoplasmic continuity at the cell bridge, neighbouring endothelial cells (numbers 3 and 7) move into the region of the cleavage furrow and establish sites of cellular attachment via microvillar processes.

(c) The dissolution of the cell bridge typically occurs at the surface of one of the daughter cells leaving one cell with a greater percentage of its remnants. At the site where the bridge detaches numerous cellular attachments are left in its place.

(d) At the completion of cytokinesis the daughter cells remain joined to each other at a relatively small site, but they have formed extensive junctions with the endothelial cells which filled in the potential space between them along the cleavage furrow (numbers 3 and 7). Completion of the entire process requires the re-arrangement of cell junctions in neighbouring cells and may explain in part the finding that microvilli are frequently present on the surfaces of cells adjacent to dividing endothelial cells.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relatively small site (the "points" of the two new diamonds). These attachments are probably the result of microvillar contacts between the two cells and adherence points which are left behind in the region where the cell bridge breaks between the daughter cells.

In summary, the endothelial lining of the metaphyseal capillaries remains continuous during endothelial cell division due to two features of the proliferating cells: (a) the dividing cells remain attached to adjacent endothelial cells during mitosis and (b) during cytokinesis the separating daughter cells are capable of forming adhering junctions with each other and with adjacent endothelial cells prior to cell separation. The initial formation of the cell contacts required for this to take place are probably the result of microvillar processes which establish attachments with the plasma membranes of surrounding cells. As a result the microvascular lining remains closed throughout endothelial mitosis and growth of the metaphyseal capillaries. The same cell division pattern may also be observed in other growing vessels which also have blood flow through them and during normal turnover of endothelial cells in other blood vessels.

CONCLUSIONS AND FUTURE DIRECTIONS

This study was undertaken to provide a better understanding of the metaphyseal capillaries in terms of their morphology, growth pattern, and role in endochondral bone formation (the results are summarized in Figure 14 and Table II). In order for long bone growth to occur these vessels must continuously invade the hypertrophic chondrocytes of the epiphyseal growth plate. This requires a substantial amount of constant blood vessel growth, or angiogenesis, and these capillaries display many of the morphological characteristics described in other growing vessels, both *in vitro* and *in vivo*, including:

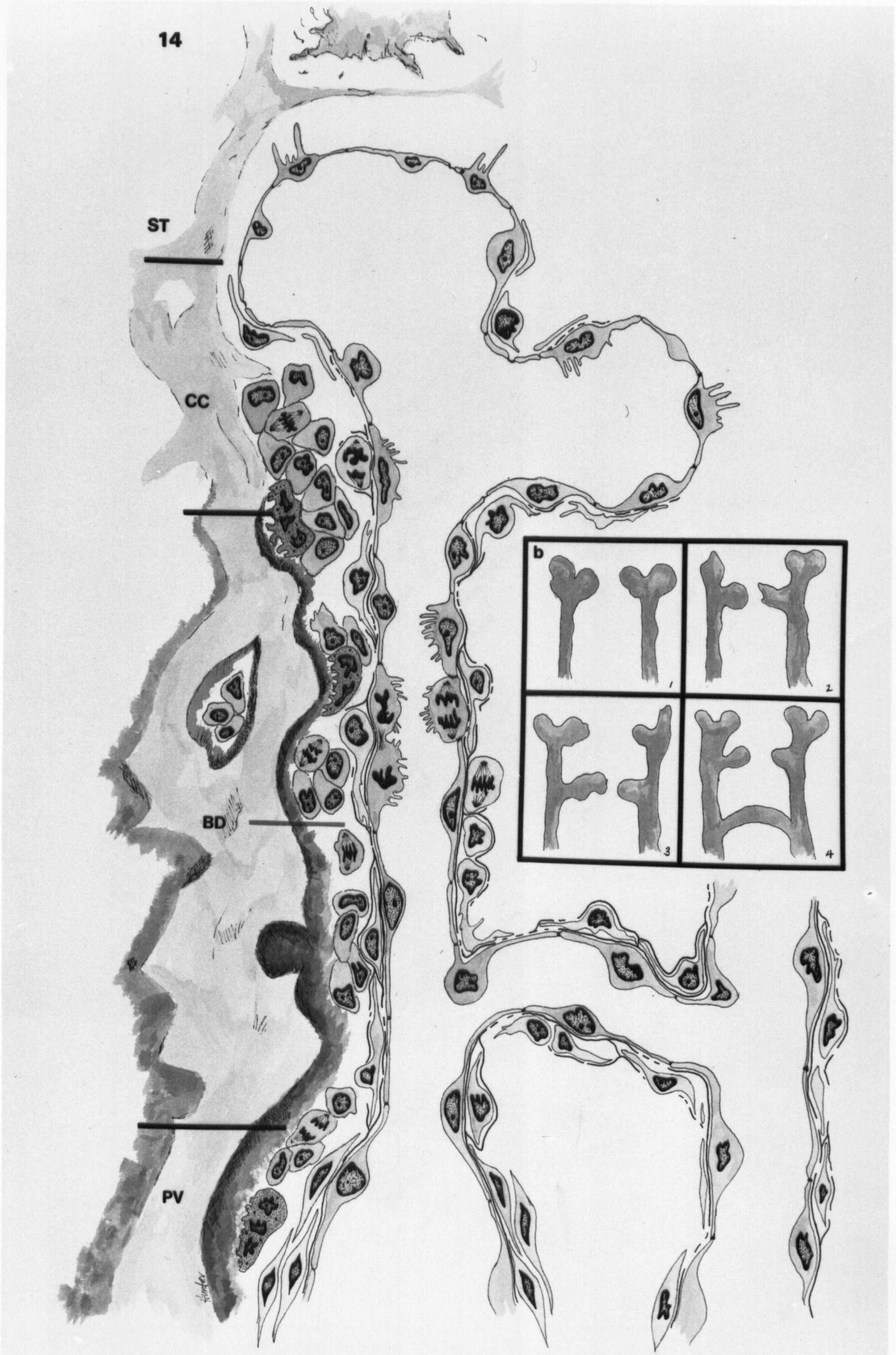
- the growth front of the vessel is blind-ended and highly attenuated with fenestrations, apical microvilli, adluminal cellular projections, and abundant cytoplasmic rER and free ribosomes. The endothelium of the sprout tip has no underlying basement membrane and perivascular cells are noticeably absent at the extreme tip of the vessel.
- progressing further from the growth front and deeper into the metaphysis, a basement membrane begins to be laid down beneath the endothelium and the perivascular cells form a more continuous adluminal lining.
- dividing endothelial cells are most frequently found approximately 200 μm from the sprout tip-hypertrophic chondrocyte interface in an area where the surrounding tissue is involved in active bone deposition. This area of cell division provides the necessary cells for vascular bed expansion and is located at a distance to not only the sprout tip, but also the parent vessels of the metaphyseal sprouts.

Table II : Summary of Morphological Data

	Sprout Tip	Extended Calc. Cartil.	Bone Deposition Cell Div.	Bone Deposition Parent V.	Primary V.
Microvilli	+	+	+	-	-
Basal Cell Projections	+	+	-	-	-
Fenestra	+	+	few	few	-
Basement Membrane	-	-	some	discon-tinuous	discon-tinuous
Dividing ECs	-	-	+	-	-
Dividing Pericytes	-	+	+	-	-
Dividing Osteoprog.	-	+	+	+	+
Migrating Cells	+	+	+	-	-

Figure 14: (over) Summary drawing of the features of the metaphyseal capillaries. See above table and text for details.

(b) Model of mechanism of anastomosis of lateral microvascular projections leading to the formation of capillary loops connecting adjacent sprouts.


14
ST
CC
BD
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3
4

- endothelial cells are capable of migrating from their site of origin up to the sprout tip within 24 hours.
- at approximately 250-350 μm behind the sprout tip is a capillary bed with a fully formed, but discontinuous, basement membrane. These microvessels consist mostly of capillaries and post-capillary venules and probably represent the vessels from which the metaphyseal sprouts are derived; the parent vessels of the plexus. Therefore, the region of bone deposition can be functionally divided into an upper proliferative zone (150-250 μm from the sprout tip) and a lower parent vessel zone (250-350 μm from the sprout tip).
- between 350-500 μm behind the growth front are the primary vessels of the plexus (larger arterioles and venules) that supply and drain the capillary bed.

However the metaphyseal capillaries have some unique features that have not been described in other angiogenic sytems:

- perfusion fixation at conditions similar to physiological parameters of vascular pressure, flow rate, temperature, and osmolarity demonstrated that the metaphyseal capillaries are a closed vascular system. Even in the most highly attenuated regions of the sprout tips, the endothelial cells are junctionally linked together and form a continuous wall around a central lumen. This is in contrast to angiogenic systems described previously which grow via the formation of solid endothelial sprouts, with gaps between adjacent cells, that secondarily develop a lumen as growth progresses.
- these results when combined with information from vascular cast experiments, suggest that the metaphyseal sprouts grow by elongation of the vessel tips. The formation of capillary loops appears to be

due to the lateral spreading of endothelial cells to form vascular projections which anastomose with those of adjacent sprouts, and is not directly related to endothelial cell division (which takes place at a distance to the anastomoses). This is opposite to the pattern observed in other growing vascular beds, where a capillary loop is formed via the anastomosis of two sprout tips. The capillary loop so formed can then serve as the parent vessel for a subsequent generation of sprout outgrowth. The vascular arrangement observed in the metaphysis could be due to confinement of the vessels within columns of calcified cartilage which results in a more linearly directed growth pattern.

- this study also examined the site of pericyte mitosis in a growing vascular system in vivo . Given the suspected role of these vessels in terminating the angiogenic process, it is significant that pericytes, like endothelial cells, are stimulated to divide in an area which is both distal to the sprout tip and the parent vessels of the plexus. However, dividing pericytes are found over a slightly wider region of distribution than are dividing endothelial cells.

- as a result of the metaphyseal capillaries retaining a functional lumen throughout the growth process, the endothelial cells are required to complete cell division while still maintaining the integrity of the microvascular wall. The endothelial cells accomplish this by sustaining junctional attachments with adjacent cells throuhgout mitosis. Secondly, daughter cells form junctions along the developing cleavage plane with both the cell from which they are separating and adjacent cells prior to the completion of cytokinesis. This appears to be largely through contacts initiated and established by microvillar processes from both the daughter cells and adjacent

endothelial cells.

This study was able to work out a number of details relating to the development of the metaphyseal capillaries. It was the first one to examine the vessels in detail and to follow them for some distance from the epiphyseal growth plate and was thus able to add some information about their general morphology. Although some information about the growth dynamics and cell proliferation of the microvascular wall was gained, the exact mechanism by which the final vascular pattern is achieved remains to be determined. The method by which endothelial cells are able to complete cell division while still within the confines of a continuous vascular wall is an intriguing, but still incomplete, finding. The morphological findings in this study could provide background for others working on junctional formation and on mechanisms of endothelial cell turnover.

Continued work along these lines could provide some truly interesting findings as to the biochemical interactions involved in angiogenesis. The growth plate microvasculature seems to be the ideal place to examine the effects of angiogenic growth factors and angiogenic inhibitors in vivo. It is hoped that the findings here can provide an insight into such studies by helping to define certain aspects of vessel growth - such as basement membrane synthesis, endothelial cell proliferation, and pericyte proliferation - in the metaphyseal capillaries. The metaphyseal sprouts offer a distinct advantage for those working on long-term studies of vascular growth because endochondral bone formation occurs throughout the life of rats, and the direction of microvascular advancement is always known to be towards the hypertrophic chondrocytes. These factors combine to

make this microvascular bed an ideal, and as yet undiscovered system with which to study a great number of aspects of the angiogenic process.

REFERENCES:

Anderson, C.E., and J. Parker (1966) Invasion and resorption in endochondral ossification. J. Bone Jt. Surg., **48A**:899-914.

Ausprunk, D.H., and J. Folkman (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumour angiogenesis. Microvas. Res., **14**:53-65.

Arsenault, A.L. (1987) Microvascular organization at the epiphyseal-metaphyseal junction of growing rats. J. Bone Mineral Res., **2**:143-149.

Arsenault, A.L., W.L. Hunter, and A.B. Hodsman (1988) Microvascular organization at the epiphyseal-metaphyseal junction of normal and rachitic rats. In: Behaviour of the Growth Plate. H.K. Uhthoff and J.J. Wiley eds. Raven Press, New York, pp. 309-316.

Azizkhan, J.C., and M. Klagsbrun (1980) Chondrocytes contain a growth factor that is localized in the nucleus and is associated with chromatin. Proc. Natl. Acad. Sci. USA, **77**:2762-2766.

Baserga, R., and D. Malamud (1969) Modern Methods in Experimental Pathology: Autoradiography, Techniques and Aplications. Harper and Row, New York, New York.

Brookes, M., and D.N. Landon (1964) The juxta-epiphyseal vessels in the long bones of foetal rats. J. Bone Jt. Surg., **46B**:336-345.

Bundgaard, M. (1984) The three-dimensional organization of tight junctions in a capillary endothelium revealed by serial-section electron microscopy. J. Ultrastruct. Res., **88**:1-17.

Cavallo, T. (1973) Ultrastructural autoradiographic studies of the early vasoproliferative response in tumour angiogenesis. Am. J. Pathol., **70**:345-362.

Chalkley, H.W., G.H. Algive, and H.P. Morris (1946) Effect of the level of dietary protein on vascular repair in wounds. J. Natl. Cancer Inst., **6**:363-372.

Clark, E.R., and E.L. Clark (1932) Observations on living preformed blood vessels as seen in a transparent chamber inserted into the rabbit's ear. Am. J. Anat., **49**:441-477.

Clark, E.R., and E.L. Clark (1939) Microscopic observations on the growth of blood capillaries in the living mammal. Am. J. Anat., **64**:251-299.

Clark, E.R., and E.L. Clark (1940) Microscopic observations on the extraendothelial cells of living mammalian blood vessels. Am. J. Anat., **66**:1-49.

Crocker, D.J., T.M. Murad, and J.C. Geer (1970) Role of the pericyte in wound healing. Exp. Mol. Pathol., **13**:51-57.

D'Amore, P.A., and A. Orlidge (1987) The role of the pericyte in microvascular growth control. In: Current Communications in Molecular Biology: Angiogenesis, Mechanisms and Pathobiology. D.B. Rifkin and M. Klagsbrun eds. Cold Spring Harbor Laboratory, pp.125-130.

Draenert, K., and Y. Draenert (1985) The role of the vessels in the growth plate: morphological examination. Scan. E.M., 1:339-344.

Durie, B.G.M., and S.E. Salmon (1975) High speed scintillation autoradiography. Science, 190:1093-1095.

Eisenstein, R., K.E. Kuettner, C. Neapolitan, L.W. Soble, and N. Sorgente (1975) The resistance of certain tissues to invasion. III. Cartilage extracts inhibit the growth of fibroblasts and endothelial cells in culture. Am. J. Pathol., 81:337-346.

Eisenstein, R., N. Sorgente, L.W. Soble, A. Miller, and K.E. Kuettner (1973) The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme. Am J. Pathol., 73:765-774.

Engerman, R.L., D. Pfaffenbach, and M.D. Davis (1967) Cell turnover of capillaries. Lab. Invest., 17:738-743.

Farquhar, M.G., and G.E. Palade (1963) Junctional complexes in various epithelia. J. Cell Biol., 17:375-412.

Fiddes, J.C., J.L. Whang, A. Mergia, A. Tumolo, and J.A. Abraham (1987) Genes for the angiogenic growth factors: basic and acidic fibroblast growth factors. In: Current Communications in Molecular Biology: Angiogenesis, Mechanisms and Pathobiology. D.B. Rifkin and M. Klagsbrun eds. Cold Spring Harbor Laboratory, pp. 24-31.

Folkman, J. (1985) Regulation of angiogenesis: a new function of heparin. Biochem. Pharm., 34:905-909.

Folkman, J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? Cancer Res., 46:467-473.

Folkman, J., D. Ausprunk, and R. Langer (1981) Connective tissue: small blood vessels and capillaries. In: Textbook of Rheumatology. W.N. Kelley, E.D Harris Jr., S. Ruddy, and C.B Sledge eds. W.B Saunders, Philadelphia, Vol. I, pp. 210-220.

Folkman, J., and C. Haudenschild (1980) Angiogenesis **in vitro**. Nature, 288:551-556.

Folkman, J., and M. Klagsbrun (1987) Angiogenic factors. Science, 235:442-447.

Folkman, J., and A. Moscona (1978) Role of cell shape in growth control. Nature, 273:345-349.

Folkman J., S. Taylor, and C. Spillberg (1983) The role of heparin in angiogenesis. In: Development of the vascular system. Pitman Books, London (Ciba Foundation Symposium 100). pp. 132-149.

Fraser, R.A., and J.G. Simpson (1983) Role of mast cells in experimental tumour angiogenesis. In: Development of the Vascular System. Pitman books, London (Ciba Foundation Symposium 100), pp. 120-131.

Gallo, P.V., and J. Weinberg (1982) Neuromotor development and response inhibition following prenatal ethanol exposure. Neurobehav. Toxicol. Teratol. 4:505-513.

Gilula, N.B. (1974) Junctions between cells. In: Cell Communication. R.P Cox, ed. Wiley, New York, pp. 1-29.

Glaser, B.M., T. Kalebic, S. Garbisa, T.B. Connor and L. Liotta (1983) Degradation of basement membrane components by vascular endothelial cells: role in neovascularization. In: Development of the Vascular System. Pitman Books, London (Ciba Foundation Symposium 100), pp. 150-162.

Goldgefter, L., and V. Toder (1976) Scintillator distribution in high speed autoradiography. Science, **195**:208.

Hallmann, R., R.N. Feinberg, C.H. Latker, J. Sasse, and W. Risau (1987) Regression of blood vessels precedes cartilage differentiation during chick limb development. Differentation, **34**:98-105.

Horton, J.E., F.H. Wezeman, and K.E. Kuettner (1978) Inhibition of bone resorption **in vitro** by a cartilage-derived anticollagenase factor. Science, **199**:1342-1345.

Hudlicka, O. (1984) Development of microcirculation: capillary growth and adaptation. In: The Handbook of Physiology. Section 2: The Cardiovascular System. E.M. Renkin and C.C. Michel, eds. American Physiological Society, Bethesda. Vol IV, Part 1, pp. 165-216.

Hudlicka, O., and K.R. Tyler (1986) Angiogenesis: The Growth of the Vascular System. Academic Press, London.

Hunziker, E.B., W. Herrmann, and R.K Schenk (1982) Improved cartilage fixation by ruthenium hexammine trichloride: a prerequisite for morphometry in growth cartilage. J. Ultrastruct. Res., **81**:1-12.

Jaffe, E.A., C.R. Minik, B. Adelman, C.G. Becker, and R. Nachman (1976) Synthesis of basement membrane collagen by cultured human endothelial cells. J. Exp. Med., **144**:209-225.

Jaffe, E.A., and D.F. Mosher (1978) Synthesis of fibronectin by cultured human endothelial cells. J. Exp. Med. **147**:1779-1791.

Kaminski, M., G. Kaminska, and S. Majewski (1978) Inhibition of new blood vessel formation in mice by systemic administration of human rib cartilage extract. Experientia, **34**:490-491.

Kefalides, N.A., J.D. Cameron, E.A. Tomichek, and M. Yanoff (1976) Biosynthesis of basement membrane collagen by rabbit corneal endothelium **in vitro**. J. Biol. Chem., **251**:730-733.

Klagsbrun, M., R. Langer, R. Levenson, S. Smith, and C. Lillehei (1977) The stimulation of DNA synthesis and cell division in chondrocytes and 3T3 cells by a growth factor isolated from cartilage. Exp. Cell Res., **105**:99-108.

Klagsbrun, M., and Y. Shing (1985) Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. Proc. Natl. Acad. Sci. USA, **82**:805-809.

Klagsbrun, M., and S. Smith (1980) Purification of a cartilage-derived growth factor. J. Biol. Chem., **255**:10859-10866.

Kuettner, K.E., and B.U. Pauli (1982) Vascularity of cartilage. In: Cartilage. B.K. Hall ed. Academic Press, New York, 1:281-312.

Kuettner, K.E., and B.U. Pauli (1983) Inhibition of neovascularization by a cartilage factor. In: Development of the Vascular System. Pitman Books, London (Ciba Foundation Symposium 100), pp. 163-173.

Langer, K. (1875) Uber das gefasssystem der rohrenknochen, mit beitragen zur kenntniss des baues und der entwickelung des knochengewebes. Separatabdruck aus dem XXXVI bande der Denkschriften der Mathematisch-Naturwissenschaftlichen Classe der Kaiserlichen Akademie der Wissenschaft, Wein.

Langer, R., H. Brem, K. Falterman, M. Klein, and J. Folkman (1976) Isolation of a cartilage factor that inhibits tumour neovascularization. Science, **193**:70-72.

Madri, J.A., and S.K. Williams (1983) Capillary endothelial cell cultures: phenotypic modulation by matrix components. J. Cell Biol., **97**:153-165.

Marx, J.L. (1987) Angiogenesis research comes of age. Science, **237**:23-24.

Maximow, A.A., and W. Bloom (1948) In: A Textbook of Histology. 5th Ed. W.B. Saunders, Philadelphia. pp. 253-255.

Montesano, R., L. Orci, and P. Vassalli (1983) **In vitro** rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J. Cell Biol., **97**:1648-1652.

Moscatelli, D., M. Presta, J. Joseph-silverstein, and D.B. Rifkin (1987) Presence of basic fibroblast growth factor in a variety of cells and its binding to cells. In: Current Communications in Molecular Biology: Angiogenesis, Mechanisms and Pathobiology. D.B. Rifkin and M. Klagsbrun eds. Cold Spring Harbor Laboratory, pp. 47-51.

Nicosia, R.F., and J.A. Madri (1987) The microvascular extracellular matrix: developmental changes during angiogenesis in the aortic ring-plasma clot model. Am. J. Path., **128**:78-90.

Owen, M. (1970) The origin of bone cells. Int. Rev. Cytol., 28:213-238.

Owen, M. (1980) The origin of bone cells in the postnatal organism. Arthr. Rheum., 23:1073-1080.

Pauli, B.U., and K.E. Kuettner (1982) The regulation of invasion by a cartilage-derived anti-invasive factor. In: Tumour Invasion and Metastasis. L.A. Liotta and I.R. Hart eds. Martinus Nijhoff, The Hague, pp. 267-290.

Porter, G.A., and P.W Bankston (1987) Myocardial capillaries in the fetal and the neonatal rat: a morphometric analysis of the maturing myocardial capillary bed. Am. J. Anat., 179:108-115.

Ranvier, L. (1875) Traite technique d'histologie. Paris: F. Savy.

Reddi, A.H. (1981) Cell biology and biochemistry of endochondral bone development. Coll. Res., 1:209-226.

Reddi, A.H., and W.A. Anderson (1976) Collagenous bone matrix-induced endochondral ossification and hemopoiesis. J. Cell Biol., 69:557-572.

Reddi, A.H., and C.B. Huggins (1972) Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc. Nat. Acad. Sci. USA, 69:1601-1605.

Reddi, A.H., and C.B. Huggins (1975) Formation of bone marrow in fibroblast-transformation ossicles. Proc. Nat. Acad. Sci. USA, 72:2212-2216.

Reddi, A.H., and K.E. Kuettner (1981) Vascular invasion of cartilage: correlation of morphology with lysozyme, glycosaminoglycans, protease, and protease-inhibitory activity during endochondralbone development. Dev. Biol., 82:217-223.

Revel, J.P., and M.J. Karnovsky (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J. Cell Biol., 33:C7-C12.

Sandison, J.C. (1924) A new method for the microscopic study of living growing tissues by the introduction of a transparent chamber in the rabbit's ear. Anat. Rec., 28:281-287.

Sandison, J.C. (1928) Observations on the growth of blood vessels as seen in the transparent chamber introduced into the rabbit's ear. Am. J. Anat., 41:475-496.

Schenk, R.K, D. Spiro, and J. Wiener (1967) Cartilage resorption in the tibial epiphyseal plate of growing rats. J. Cell Biol. 34:275-291.

Schenk, R.K., J. Wiener, and D. Spiro (1968) Fine structural aspects of vascular invasion of the tibial epiphyseal plate of growing rats. Acta Anat., 69:1-17.

Schoefl, G.I. (1963) Studies on inflamation. III. Growing capillaries: their structure and permeability. Virchows Arch. Pathol. Anat., 337:97-141.

Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun (1984) Heparin affinity: purification of a tumour-derived capillary endothelial cell growth factor. Science, 223:1296-1299.

Sholley, M.M., G.P Ferguson, H.R. Seibel, J.L. Montour and J.D. Wilson (1984) Mechanisms of neovascularization. Lab. Invest., 51:624-634.

Simionescu, M., and N. Simionescu (1984) Ultrastructure of the microvascular wall: functional correlations. In: The Handbook of Physiology. Section 2: The Cardiovascular System. E.M. Renkin and C.C. Michel, eds. American Physiological Society, Bethesda. Volume IV, Part 1, pp. 41-102.

Simionescu, M., N. Simionescu, and G.E. Palade (1974) Characteristic endothelial junctions in sequential segments of the microvasculature. J. Cell Biol. 63:316a.

Simionescu, M., N. Simionescu, and G.E. Palade (1975) Segmental differentiations of cell junctions in the vascular endothelium. The microvasculature. J. Cell Biol. 67:863-885.

Simionescu, M., N. Simionescu, and G.E. Palade (1976) Segmental differentiations of cell junctions in the vascular endothelium. Arteries and veins. J. Cell Biol. 68:705-723.

Singley, C.T., and M. Solursh (1980) The use of tannic acid for the ultrastructural visualization of hyaluronic acid. Histochem., 65:93-102.

Sommer, A., M.T. Brewer, R.C. Thompson, D. Moscatelli, M. Presta, and D.B. Rifkin (1987) Primary structure of a human fibroblast growth factor derived from protein and cDNA sequencing. In: Current Communications in Molecular Biology: Angiogenesis, Mechanisms and Pathobiology. D.B. Rifkin and M. Klagsbrun eds. Cold Spring Harbor Laboratory. pp. 33-36.

Sorgente, N., and C.K. Dorey (1980) Inhibition of endothelial cell growth by a factor isolated from cartilage. Exp. Cell Res., 128:63-71.

Sorgente, N., K.E. Kuettner, L.W. Soble, R. Eisenstein (1975) The resistance of certain tissues to invasion. II. Evidence for extractable factors in cartilage which inhibit invasion by vascularized mesenchyme. Lab. Invest., 32:217-222.

Speiser, P., A.M. Gittelsohn, and A. Patz (1968) Studies on diabetic retinopathy. Arch. Opthalmol., 80:332-337.

Syftestad, G.T., and A.I. Caplan (1984) A fraction from extracts of demineralized adult bone stimulates the conversion of mesenchymal

cells into chondrocytes. Dev. Biol., **104**:348-356.

Tannock, I.F., and S. Hayashi (1972) The proliferation of capillary endothelial cells. Cancer Res., **32**:77-82.

Trueta, J., and V.P. Amato (1960) The vascular contribution to osteogenesis. III. Changes in the growth cartilage caused by experimentally induced ischaemia. J. Bone Jt. Surg., **42B**:571-587.

Trueta, J., and A.J Buhr (1963) The vascular contribution to osteogenesis. V. The vasculature supplying the epiphyseal cartilage in rachitic rats. J. Bone Jt. Surg., **42B**:572-581.

Trueta, J., and K. Little (1960) The vascular contribution to osteogenesis. II. Studies with the electron microscope. J. Bone Jt. Surg., **42B**:367-376.

Trueta, J., and J.D. Morgan (1960) The vascular contribution to osteogenesis. I. Studies by the injection method. J. Bone Jt. Surg., **42B**:97-101.

Urist, M.R. (1965) Bone: formation by autoinduction. Science, **150**:893-899.

Warshawsky, H., and G. Moore (1967) J. Histochem. Cytochem. **15**:542-549.

Young, R.W. (1962) Cell proliferation and specialization during endochondral osteogenesis in young rats. J. Cell Biol., **14**:357-370.

Zawicki, D.F., R.K. Jain, G.W. Schmid-Schoenbein, and S. Chien (1981) Dynamics of neovascularization in normal tissue. Microvasc. Res., **21**:27-47.

Zinkernagel, R., U.N. Riede, R.K. Schenk (1972) Ultrastrukturelle untersuchungen der juxtaepiphysaren kapillaren nach perfusionsfixation. Experientia, **28**:1205-1206.