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**Regulation of Retinal Endothelial Cells and Pericytes by
VEGF, TGF-beta1 and SPARC**

by

QI YAN

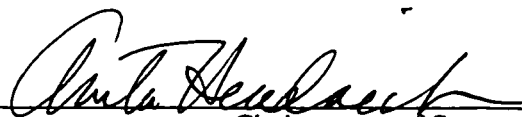
A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by



Chairperson of Supervisory Committee

Program Authorized
to Offer Degree

Biological Structure

Date

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Abstract

**Regulation of Retinal Endothelial Cells and Pericytes by
VEGF, TGF-beta1 and SPARC**

by Qi Yan, MD

Chairperson of the Supervisory Committee: Professor Anita Hendrickson
Department of Biological Structure

Ocular diseases characterized by retinal neovascularization are among the principal causes of visual loss worldwide. In an effort to understand the components of the retinal angiogenesis and its regulation by various molecules, I developed a procedure to isolate retinal microvascular endothelial cells (EC) and pericytes from the primate and bovine species. This was important because retinal capillary EC are different from EC derived from other vascular beds. Retinal EC expressed von Willebrand factor, acetylated low-density lipoprotein, CD31, and did not contain smooth muscle α -actin. Monkey retinal EC could be maintained to passage 3, whereas bovine retinal EC could be maintained to passage 8. Subconfluent cultures of monkey retinal EC secreted extracellular matrix proteins that included fibronectin, laminin, collagen types I and IV, and SPARC. VEGF and bFGF both stimulated retinal EC replication, which was also elevated under hypoxia. The synergism of VEGF and bFGF on retinal EC proliferation and cell invasion was observed in a 3-dimensional assay but not in 2-dimensional dish culture.

Unlike VEGF and bFGF, the role of transforming growth factor (TGF)- β 1 in angiogenesis has been controversial. The effects of TGF- β 1 on retinal microvascular cells are not fully defined. I have found that TGF- β 1 inhibited the proliferation of both retinal EC and pericytes in a concentration-dependent manner. Moreover, TGF- β 1 induced specifically apoptotic cell death in retinal EC but not in pericytes. Apoptosis of retinal EC mediated by TGF- β 1 was associated with a decreased level of the cyclin-dependent kinase inhibitor p21^{waf1/cip1}, compared with that observed in the apoptosis-resistant cells. In contrast, the translation product of the tumor suppressor gene p53 was increased in the TGF- β 1-treated apoptotic cells, suggesting p53 mediated retinal EC apoptosis is independent of p21^{waf1/cip1}. Thus, I have proposed that p21^{waf1/cip1} and p53 function

in distinct pathways that are protective or permissive, respectively, for the apoptotic signals mediated by TGF- β 1.

SPARC is expressed in a variety of tissues during embryogenesis and remodeling, and is believed to regulate vascular morphogenesis and cellular differentiation. Although usually limited in normal adult tissues, SPARC is expressed at significant levels in the adult bovine retina, whereas newborn calf retina showed a similar expression pattern with a greatly reduced level. Strong reactivity with anti-SPARC antibody was found in the soma of ganglion cells and their axons. SPARC was also present in retinal astrocytes but not in Mueller cells, capillaries of the inner retina but not large vessels. To determine the significance of SPARC expression in retina, I test two hypotheses. 1) SPARC has counteradhesive properties that can cause cell rounding, and would thus induce retinal EC apoptosis in vitro; 2) SPARC is one of the endogenous inhibitors that either maintain the quiescence of retinal EC under normal physiological conditions or inhibit retinal neovascularization. My conclusions are that SPARC or its peptides did not induce retinal EC apoptosis in vitro under the experimental conditions used; SPARC and its peptides were potent inhibitors of retinal EC replication, as they effectively inhibited EC proliferation induced by retinal extract. Future study will focus on testing the role of SPARC in retinal neovascularization through the use of wild-type vs. SPARC-null mice in an animal model of the retinopathy, and on delineating mechanisms whereby SPARC regulates retinal pericyte function.

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ABBREVIATIONS

bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAM	Chick chorioallantoic membrane
CD	Cluster determinant
CPM	Counts per minute
DAB	Diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis (β -aminoethyl ether)-tetraacetate
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
HEPES	4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid
kDa	kilo Dalton
LPS	Lipopolysaccharide (endotoxin)
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDS	Plasma-derived serum
QR1	Quail retina protein 1
SC1	Synaptic cleft protein 1
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cells
SPARC	Secreted protein, acidic, and rich in cysteine
TCA	Trichloroacetic acid
TGF- β	Transforming growth factor- β
Tris	Tris [hydroxymethyl] aminomethane
Triton x-100	Polyethylene glycol 4-isooctylphenyl ether
TSP	Thrombospondin
Tween 20	Polyoxyethylene (20) sorbitan monooleate
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

ACKNOWLEDGMENTS

To Anita Hendrickson, who supported and encouraged me consistently through my graduate program; to Helene Sage, who provided a great environment for me to learn and helped me in all aspects; to all the people in the Sage Lab, your friendship made my years there so enjoyable and I thank you for the help and discussions; to my husband Yi Li, who is always there for me with perfect understanding; to my little boy Kevin Li, who made my life so rich and valuable; to my parents, I always want to honor them; I thank you all, for all the good deeds - that helped me to prosper.

DEDICATION

To Yi, and Kevin, who gave me love, joy, and energy.

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1. Normal Retinal Vasculature and Abnormal Retinal Neovascularization

In humans and many other mammals, the retina has a dual blood supply. The central retinal artery forms the retinal circulation, which supplies the inner layers of the retina, but does not extend external to the outer plexiform layer. The photoreceptor layer is avascular and is nourished from the rich vascular plexus of the choroid, from which oxygen and nutrients diffuse to the retinal pigment epithelium and to the photoreceptor layer (including the outer nuclear layer) (Fig. 1.1). The intrinsic retinal vasculature consists of four planar beds, distributed within the nerve fiber layer, ganglion cell layer, and at the inner and outer borders of the inner nuclear layer (Fig. 1.1) (Gariano et al., 1994). Capillaries are totally absent from the center of the fovea and the most peripheral retinal edge.

The development of the retinal vasculature is achieved via two distinct mechanisms: vasculogenesis and angiogenesis (Ashton, 1970; Cogan et al., 1963; Michaelson, 1948). Vasculogenesis, the *de novo* formation of primitive blood vessels by differentiation of angioblasts (vascular precursor cells), occurs only during embryonic development of the retina; vessels spread from the optic disk to the margin of the retina, involving the differentiation of precursor cells into primitive capillary networks (Chan-ling et al., 1990; Gariano et al., 1996). Angiogenesis, the formation of new capillaries from pre-existing vessels, occurs during later development of the retinal vasculature and forms the plexus of vasculature at the inner retina. This process is critical for normal growth and development of retinal vasculature and it ceases once vascular development is complete. In addition, the retinal glial cells (such as Mueller cells, astrocytes) and ganglion neurons are involved in the regulation of retinal vascular development or pathological neovascularization as a consequence of their synthesis of angiogenic factors and /or extracellular matrix (ECM) proteins; or by direct contact with endothelial cells (EC).

In the normal adult retina, angiogenesis is lacking and EC have a very low labeling index (0.01%), with a turnover time of three or more years (Engerman et al., 1967). Despite their very low turnover, adult EC are not postmitotic but have the ability to form

new blood vessels. Aberrant angiogenesis occurs during the progression of retinal pathological vasoproliferative disorders such as diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, sickle cell retinopathy, and occlusion of the central retinal vein (Garner et al., 1994). All of these disorders are characterized by neovascularization, in which quiescent EC become activated and angiogenesis occurs in an uncontrolled, non-self limiting manner, and extensive proliferation of new blood vessels is directly responsible for many of the destructive events of these diseases. Leakage and bleeding, followed by organization of the clot and fibrosis, could ultimately lead to retinal detachment and irreversible damage to vision. Diabetic retinopathy represents the main cause of blindness in the working population and is the most-studied of all forms of neovascular disease. It is believed that many forms of pathological retinal neovascularization have a similar etiology that differs only in temporal rate (Aiello et al., 1995; Frank, 1995).

It seems that under normal conditions, stringent mechanisms operate to prevent microvascular EC growth, and that during the onset of disease these mechanisms are somehow "overridden", resulting in neovascularization. The extremely low rate of turnover of EC in retinal vessels and the absence of angiogenesis in the normal adult retina probably result from interactions between complex multifactorial regulatory systems which maintain a balance between stimulation and inhibition of angiogenesis. Regulation of retinal angiogenesis at the microvascular level is a complex process and is not well understood. The potential candidates for regulating retinal angiogenesis might be soluble growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)- β 1; nonstructural ECM glycoproteins such as SPARC (secreted protein, acidic, and rich in cysteine) and thrombospondin (TSP), cell-matrix interactions and interactions between EC and pericytes. In addition, other accessory cell types such as smooth muscle cells (SMC), astrocytes, Mueller cells, as well as environmental factors such as hypoxia, also play a pivotal role in the regulation of physiological and pathological angiogenesis.

Retinal angiogenesis is extremely important to retinal development, physiological functions, and pathological conditions. The loss of balance of various angiogenic factors would induce retinal neovascularization and cause blindness. Understanding the mechanisms involved in the regulation of retinal angiogenesis is important both for locating

the site of derangement in disease and also for developing inhibitors of angiogenesis as potential therapeutic agents.

1.2. Regulation of Retinal Angiogenesis

For the process of angiogenesis to begin, EC must escape the normal growth control mechanisms. In response to angiogenic stimuli, the normally quiescent capillary EC start to migrate through the surrounding ECM and proliferate to form a new capillary network (Ausprunk et al., 1977). Angiogenesis in physiological (controlled) or pathological (uncontrolled) processes involves the same basic, sequential, well-characterized steps, although there are fundamental differences between them. The sequential events of angiogenesis include the following: (1) local degradation of the basement membrane surrounding the endothelial cell tube. The degradation of the local basement membrane and the surrounding ECM is mediated by specific proteolytic enzymes, including the plasminogen-plasmin system and collagenases. (2) the migration of EC. The EC protrude through the basement membrane and assume a bipolar configuration forming a sprout off the parent vessel. The cells at the tip of the sprout migrate and elongate. The migrating EC are exposed to interstitial ECM which consists mainly of type I collagen. Chemotactic substances and growth factors are involved in the regulation of endothelial migration and elongation. (3) proliferation of EC. A major component of angiogenesis, the regulation of this step is the combination of multiple factors including growth factors and matricellular proteins. The proliferation of EC is a critical step for the formation of the new vessels. Finally (4) tube formation, which includes the formation of a new capillary lumen, deposition of new basement membrane, and recruitment of pericytes. Thus, the formation of new microvessels requires a number of interactions that must be regulated by various factors, both spatially and temporally.

1.2.1. Cytokines (VEGF, bFGF, and TGF- β 1)

VEGF: VEGF was initially identified as a highly specific mitogen for vascular EC (Leung et al., 1989). Identified by its blood vessel-permeabilizing activity, it is also known as vascular permeability factor (VPF) (Keck et al., 1989). Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa (Ferrara et al., 1992). VEGF/VPF is angiogenic in vivo; its expression as well as the expression of its cognate receptors strongly correlates with pathological angiogenesis and with vasculogenesis and angiogenesis during

embryonic development and pathological angiogenesis (Pierce et al., 1995; Takagi et al., 1996). Current research findings indicate that ischemia/hypoxia-associated ocular angiogenesis in humans or animal models is correlated with increased levels of VEGF, both in vitro and in vivo. In vitro, VEGF is secreted by retinal glial cells, vascular EC, pericytes and pigment epithelial cells and its expression is upregulated by hypoxia (Simorre-Pinatel et al., 1994; Aiello et al., 1995; Liu et al., 1995; Nomura et al., 1995; Shima et al., 1995; Hata et al., 1995). Therefore, VEGF is the most probable candidate for the 'vasoformative factor' which is released by retinal cells in response to hypoxic conditions. In vivo, VEGF is diffusible, eliciting iris and retinal neovascularization when injected into the vitreous of monkeys (Miller et al., 1994). VEGF immunoreactivity has been detected in the retinas of patients with diabetic proliferative retinopathy but not in normal retinas (Aiello et al., 1994). In situ hybridization studies in eyes removed from patients with various ocular angiogenic diseases have localized VEGF mRNA to the retinal layers expected to be ischemic (Pe'er et al., 1995; Minchenko et al., 1994). Despite the growing recognition of VEGF in angiogenesis-dependent diseases, emerging data suggest that multiple growth factors and other influences may operate together in pathologic angiogenesis. The context of VEGF with other existing cytokines in the retina such as TGF- β 1 and bFGF needs to be investigated further, especially in hypoxia.

bFGF: It is understood now that the soluble mitogenic substance bFGF acts as potent inducer of angiogenesis in both developmental and pathologic conditions. Bensaïd et al. (1989) have shown that cultured retinal EC are able to synthesize and release bFGF into culture medium. Elevated levels of bFGF have been found in the vitreous of patients suffering from diabetic retinopathy, neovascular membranes of the retina, and choroids (Baird et al., 1985; Hanneken et al., 1991). In contrast to VEGF, bFGF is a cellular, rather than a secreted protein. Thus, bFGF could be a cell-associated angiogenesis factor that is released only under special circumstances, such as cell damage. Release of bFGF from cells could occur following hypoxic injury to other cells, such as retinal pigment epithelial cells, which are sensitive to hypoxia (Khaliq et al., 1996), or cell lysis after hypoxic injury (Graeber et al., 1996). In vitro, both VEGF and bFGF stimulate cell proliferation of vascular EC from a variety of sources, including primate retinal microvascular EC (Yan et al., 1996). The demonstrated synergism between VEGF and bFGF for induction of microvessel-like structures in two different experimental models of in vitro angiogenesis

(Goto et al., 1993; Pepper et al., 1992) suggests that expression of these two growth factors may be concurrently regulated *in vivo* to optimize angiogenesis. Given the temporal and spatial colocalization of VEGF and bFGF in a variety of ocular angiogenic disorders, and the lack of documentation of the responses of retinal EC to these cytokines, the synergism between these growth factors in the process of retinal angiogenesis should be investigated.

TGF- β 1: TGF- β is a homodimeric protein with a molecular weight of 25 kDa and is a multifunctional regulator of cellular activities. TGF- β 1 has been implicated in the positive regulation of angiogenesis *in vivo* in the CAM (chick chorioallantoic membrane) and cornea (Yang et al., 1990; Folkman, 1987). TGF- β is a strongly chemotactic for macrophages *in vitro*, and it is possible that the release of angiogenic factors from macrophages accounts for its angiogenic capacity in the CAM and cornea experiments. In contrast to the angiogenic effect *in vivo*, it has been reported by many investigators that TGF- β inhibits the proliferation of EC *in vitro* (Heimark et al., 1986; Muller et al., 1987; Takehara et al., 1987). There are reports of a bifunctional effect of TGF- β on EC proliferation, stimulating growth at low doses (0.02-0.1 ng/ml) and inhibiting at higher concentrations (>0.5 ng/ml) (Myoken et al., 1990). It is reported that many of the cellular and biochemical processes affected by TGF- β are linked to differentiation. It is therefore possible that growth inhibition of EC by TGF- β (if it is true) primes them for differentiation and / or is critical for the maintenance of a differentiated state. Choi and Ballermann reported that TGF- β 1 induced apoptosis and associated capillary formation in EC, demonstrating that capillary morphogenesis *in vitro* is associated with apoptosis, and interference with TGF- β 1 receptor signaling inhibits this process in capillary EC (Choi et al., 1995).

Unlike VEGF and bFGF, which are well-characterized positive angiogenic factors, the role of TGF- β 1 in angiogenesis is more complicated. Apparently conflicting results have been obtained: while it has been reported that TGF- β 1 promotes the organization of single EC embedded in collagen gels into tube-like structures (Madri et al., 1988), it has also been demonstrated that TGF- β 1 inhibits EC invasion and tube formation (Muller et al., 1987). In addition, there is increasing evidence that the nature of the cellular response elicited by a specific factor depends on the presence of other regulatory molecules (Sporn et al., 1988). Recent studies indicate that TGF- β 1 down-regulates VEGF receptor 2/flk-1

expression in vascular EC, implying that TGF- β 1 is a major regulator of the VEGF/flk-1 signal transduction pathway in EC (Mandriota et al., 1996). More information is needed to define, the role of TGF- β in retinal EC and pericytes.

1.2.2. Hypoxia

The retina is the most metabolically active tissue in the body and therefore exhibits a high rate of oxygen consumption. EC line the vascular lumen and are primary sensors and responders to alterations in blood oxygen tension. Observations of EC exposed to hypoxic conditions *in vitro* yield contradictory results about the effect of hypoxia on the proliferation of non-retinal EC. In bovine aortic or capillary EC incubated under hypoxic conditions, cell growth was slowed in a dose-dependent manner at lower oxygen concentrations, and progression into S phase from G1 was inhibited, concomitant with decreased thymidine kinase activity, while other reports suggest, however, that hypoxia stimulates proliferation and migration of EC (Shreeniwas et al., 1991; Meininger et al., 1988; Fanburg et al., 1987). Responses to hypoxia may vary depending on the origin of EC (Koroma et al., 1995; Thieme et al., 1995). Thus, specific studies with retinal EC and pericytes *in vitro* are necessary to determine the mechanisms that mediate and influence the stimulatory effect of hypoxia on retinal neovascularization.

In retinopathy of prematurity, premature infants were placed into high oxygen environments for a period of time and then returned to room air. Retinal neovascularization of the infant has been proposed to be a response to hypoxia brought on by withdrawal of supplemental oxygen (Patz, 1982). The EC proliferation that occurs in diabetic retinopathy has been attributed to local hypoxia (Olk et al., 1993, Garner, 1994). Hypoxia appears to be the common precursor which induces retinal neovascularization (Pierce et al., 1995, Aiello et al., 1995). Presumably, as the retina becomes hypoxic, it elaborates factors that induce retinal neovascularization.

Recent research data suggest that hypoxia increases VEGF expression in retinal cells (e.g., astrocytes, pericytes, pigment epithelial cells, Mueller cells, and EC), which promotes proliferation of retinal EC (Stone et al., 1996; Aiello et al., 1995, Simorre-Pinatel et al., 1994). VEGF mRNA expression is rapidly and reversibly induced by exposure to low oxygen in a variety of cultured cells. It has been shown that a similarity exists between the mechanisms leading to hypoxic regulation of VEGF and erythropoietin (Epo) (Goldberg et al., 1994). The expression of both genes is significantly enhanced by cobalt

chloride. The hypoxic induction of both VEGF and Epo genes is inhibited by carbon monoxide, suggesting the involvement of a heme protein in the process of sensing oxygen levels (Goldberg et al., 1994). Furthermore, hypoxia inducibility is conferred to both genes by sequences in 5' and 3' untranslated regions that behave like classical transcriptional enhancers (Madan 1993). In addition, investigators also reported that hypoxia upregulates the expression of VEGF receptors (Brogi et al., 1996); however, information about how retinal capillary cells responded to bFGF is lacking.

1.2.3. Pericytes

Capillaries are composed of two types of cells, EC and pericytes (also named Rouget, or mural cells). Pericyte is a distinct, polymorphic cell of mesenchymal origin that contains multiple, branching cytoplasmic processes that encircle capillaries and postcapillaries. Pericytes are intimately associated with EC, share a common basement membrane with the EC (Rhodin, 1968), and form a single layer that covers a variable amount of the abluminal EC surface (Fig. 1. 2). The frequent sites of contact between EC and pericytes have been characterized (Tilton et al., 1979; Mazanet and Franzini-Armstrong, 1982). Pericytes are found in the microvasculature of connective tissue, nervous tissue, muscle, and lung. The degree of EC coverage by pericytes appears to be tissue-specific. The pericyte to endothelial ratio is 1: 1 for the retina, approximately 1:10 in the lung, and 1:100 in striated muscle (Shepro and Morel, 1993).

Interactions between EC and pericytes are known to be important in the maintenance of vascular integrity, including growth control (Sato et al., 1988; Antonelli-Orlidge et al., 1989); studies also indicate that the presence of pericytes is correlated with EC quiescence (Crocker et al., 1970; Ausprunk and Folkman, 1977). The functions of pericytes are thought to influence directly retinal EC and further, retinal vasculature. For example, lack of pericytes leads to ruptured capillaries (Lindahl et al., 1997). It is important therefore to elucidate the responses of pericytes to hypoxia and to several critical cytokines in the retina.

1.2.4. Apoptosis

Apoptosis is a normal physiological cell death process by which multicellular organisms maintain cellular homeostasis. It is an active process of gene-directed cellular destruction. Apoptosis has an important biological significance in morphogenesis and

embryogenesis. The term apoptosis has been used to distinguish active cell death, whereas cells participate in their own demise from passive necrotic death, in which severe environmental situations lead to cell membrane damage, loss of osmoregulation, and cell lysis. Typically, apoptosis is characterized by the condensation of chromatin around the nuclear periphery and the cleavage of DNA into oligonucleosome-sized fragments by an endogenous endonuclease (Raff et al., 1993).

Angiogenesis and vascular restructuring occur throughout growth and development and involve both proliferation and apoptosis of vascular EC. Although many factors inducing EC proliferation and differentiation are known (Folkman, 1995), little is known about regulation of EC apoptosis. Deprivation of serum or matrix adhesion induce apoptosis in human umbilical vein EC (HUVEC) (Araki et al., 1990; Meredith et al., 1993). FGF and phorbol esters reduce the apoptosis of serum deprived HUVEC (Araki et al., 1990). An important role for $\alpha v \beta 3$ integrins in inhibition of apoptosis in newly formed blood vessels has been demonstrated by Brooks et al. (1994). Regression of capillaries by selective endothelial cell apoptosis has been demonstrated as a mechanism of capillary network in the retina after down-regulation of VEGF expression (Alon et al., 1995). A recent study demonstrated that capillary morphogenesis in vitro is associated with apoptosis (Choi et al., 1995). This study raises the intriguing possibility that apoptosis is a phenomenon necessary in the process of capillary morphogenesis.

1.2.5. Matricellular Protein SPARC

SPARC (also known as osteonectin, BM-40/43K) is an acidic, cysteine-rich component of the extracellular milieu that displays a high degree of interspecies sequence conservation (Villarreal et al., 1989). SPARC is associated with proliferation, morphogenesis, remodeling, and cellular migration. It is a Ca^{++} -binding glycoprotein secreted by many cells, e.g., EC, fibroblasts, osteoblasts, and platelets (Kasugai et al., 1991). SPARC is also expressed by cultured bovine aortic EC and is increased further when these cells form cords and /or tubes (Iruela-Arispe et al., 1991). During the development of the CAM, SPARC is expressed by EC in newly-formed blood vessels (Iruela-Arispe et al., 1995). It is anti-adhesive for EC and fibroblasts in vitro (Sage et al., 1992). In cultures of subconfluent proliferating EC, it has been shown to induce changes in cell shape (Lane and Sage, 1990), delay entry into the S-phase of the cell cycle (Funk et al., 1991), and increase the production of endothelial type plasminogen activator inhibitor-1

(PAI-1) (Hasselaar et al., 1991). As an important regulator of plasminogen activity, PAI-1 controls locally many morphogenetic processes that require proteolysis. Hasselaar et al (1992) also found that the basal level of migration of bovine aortic EC was unaffected by SPARC; however, SPARC inhibited cellular migration induced by bFGF in a dose-dependent manner. SPARC binds several divalent cations that include Cu^{++} , and both Cu^{++} and Cu^{++} -binding proteins/peptides have been shown to stimulate angiogenesis in vivo (Raju et al., 1982). SPARC might also modulate the interaction of cells with growth factors. For example, it binds to the B-chain of PDGF and inhibits the binding of PDGF to its receptors (Raines et al., 1992). SPARC also interacts with VEGF and neutralizes the mitogenic effect of VEGF on bovine and human EC in vitro (Kupprion et al., unpublished data) These and other studies collectively indicate that SPARC is important in the regulation of endothelial functions, and an important mediator of cell-matrix interactions during angiogenesis.

Is SPARC expressed in retina? Is it involved in regulating retinal vascular morphogenesis and angiogenesis? These questions are addressed in the chapter 6.

1.2.6. Objectives of the Research

The experiments described in the following chapters were designed to investigate the regulation of retinal EC and pericyte behavior by the growth factors VEGF, bFGF, and TGF- β 1 and by the matricellular protein SPARC. VEGF, bFGF and TGF- β 1 are believed to regulate retinal angiogenesis; however, the effect of these cytokines on specific functions of retinal capillary cells have not been well documented, especially in the context of hypoxia. Because differences exist among EC derived from different vascular beds or organs, it is important to use retinal microvascular EC and pericytes for studies of retinal vasculature. Chapter 2 describes the methods for isolation and culture of retinal microvascular EC. Studies described in Chapter 3 addresses the effects of VEGF and bFGF on retinal EC and pericytes under conditions of hypoxia and normoxia. Chapter 4 describes TGF- β 1-induced apoptosis on retinal EC but not pericytes.

The second part of this research (Chapters 5 & 6) is designed to investigate the potential role of SPARC in retinal angiogenesis. SPARC is an important modulator of vascular morphogenesis and angiogenesis in vitro (Iruela-Arispe et al., 1995; Sage and Vernon, 1994); however, virtually nothing is known about the function of SPARC in retinal angiogenesis. In Chapter 5, the expression and localization of SPARC in bovine

retina is reported. In Chapter 6, I describe a potential role of SPARC in regulating the functions of retinal EC and propose future studies for further examining the role of SPARC in retinal neovascularization.

Figure 1.1. The vascular architecture of the normal mammalian retina.

A is a diagram of retinal vascular distribution. Muller cell is yellow; four vascular beds are red, astrocytes are blue, and ganglion cells are green. PE, pigment epithelium; PR, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; and NFL, nerve fiber layer.

B is a monkey retina section with capillaries reacted for ATPase. Intrinsic vessels are in NFL, GCL, and at the inner and outer borders of INL (Gariano et al., Invest Ophthalmol Vis Sci. 1996;37:98).

A

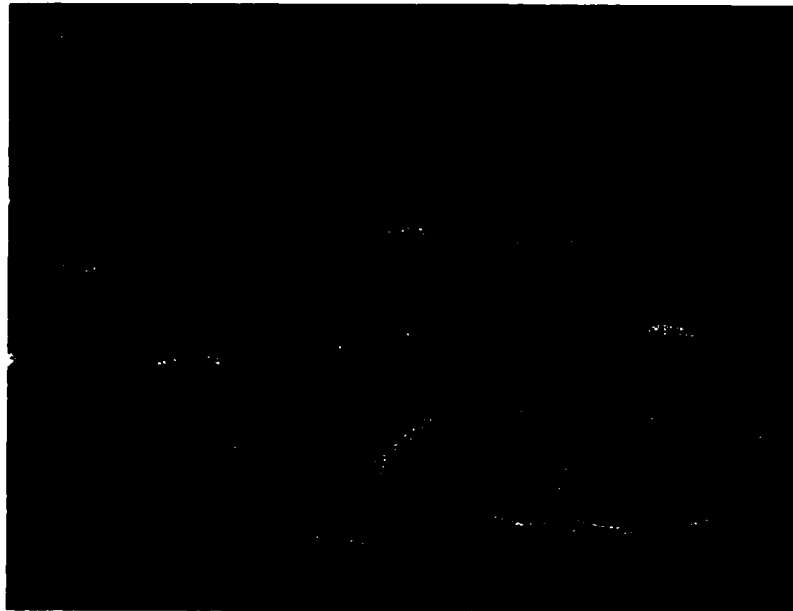
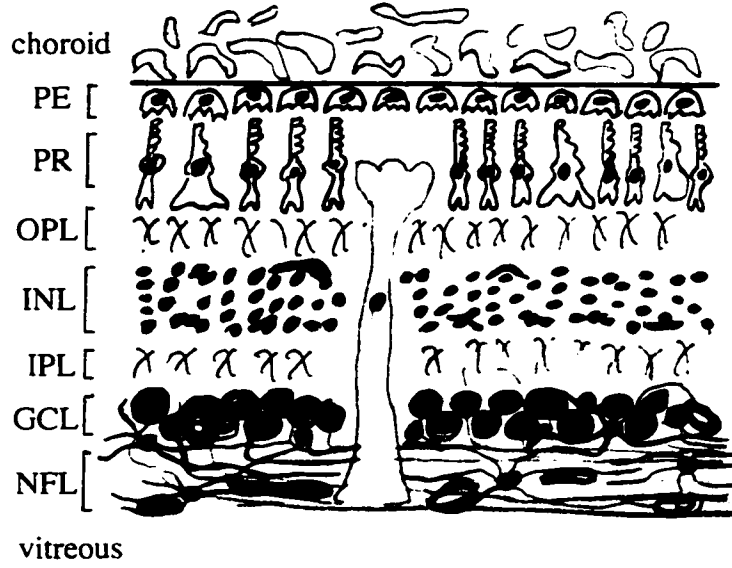
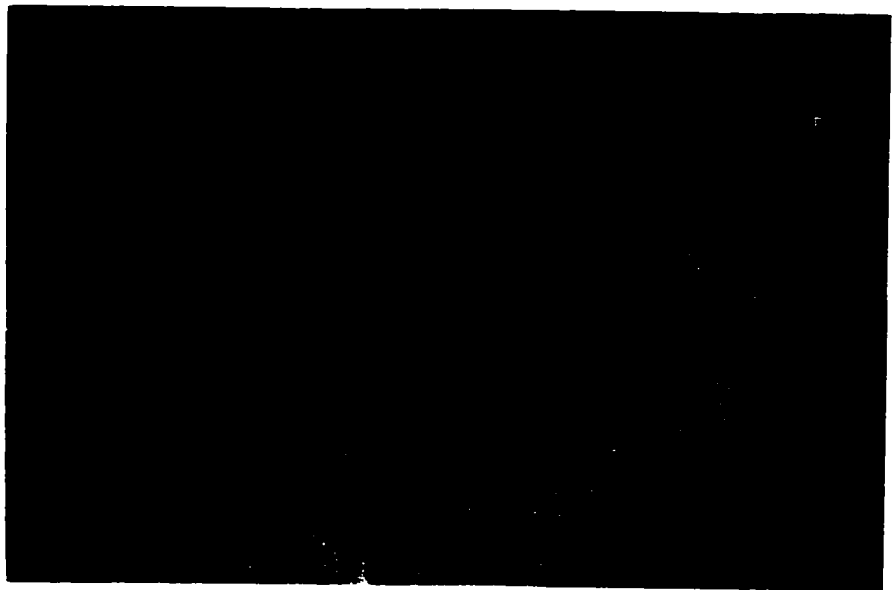


Figure 1.2. Scanning electron micrograph of pericytes covering the surface of a capillary.

N, nuclear area of a pericyte; 1° primary process of the pericyte; 2° secondary processes encircling capillary; 3° tertiary processes extending from the 2°. The surface of an underlying endothelial cell is indicated by arrowheads. (Shepro and Morel, FASEB J. 1993, 7:1032).



CHAPTER 2: PRIMARY CULTURE AND CHARACTERIZATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM MACACA MONKEY RETINA

2.1. Summary of Chapter 2

The purpose of this chapter is to develop methods for the culture of microvascular EC from mammalian retinas and to investigate their propagation and survival *in vitro*. The availability of retinal EC will allow me to test the effects of various angiogenic factors on these cells and the properties of these cells. The animal used in this chapter is *Macaca* monkey, chosen for the following reasons: 1) *Macaca* monkey retina is a widely accepted model for the human retina, although cultures of EC from primates have not been available; 2) monkey has been used to develop retinal neovascularization models; in addition, there are a number of human diseases that are best modeled in *Macaca* (e.g. SIV as a model of HIV infection). It is therefore important to develop a methodology which allow investigators for primate endothelial studies *in vitro* related to these disorders.

EC from *Macaca* retinal capillary fragments were cultured on fibronectin-coated dishes in QB-58 serum-free medium containing 20 μ l/ml bovine retinal extract, 90 μ g/ml heparin, 10% fetal bovine serum, and 10% monkey serum. Under these culture conditions, migrating cells emerged from capillary fragments after 1 to 2 days and formed large colonies by 1 week. Cells exhibited a mean doubling time of 44.5 hours during the first 3-5 days of culture, and 23 hours at 6-8 days in culture, and formed a confluent monolayer by 12-14 days. These cells demonstrated uptake of ac-LDL (acetylated-low density lipoprotein), expressed von Willebrand factor and the cell adhesion protein CD31, and did not contain smooth muscle α -actin. Prior to purification, 92% of the cells in primary cultures were identified as EC. The EC could be maintained *in vitro* more than one month without addition of growth factors; however, bFGF and VEGF each stimulated cell replication. Secreted extracellular proteins included fibronectin, collagen types I and IV, laminin, and SPARC.

This study is the first description of the culture and propagation of purified retinal EC from *Macaca* monkey, a widely-accepted model for the human retina. These cultures will be highly relevant to studies of abnormal vascular disease in the human eye.

2.2. Introduction to Chapter 2

It is now appreciated that retinal microvascular endothelium is an integral component in the development of diabetic retinopathy and retinopathy of prematurity, which are major causes of blindness worldwide. Although EC have a common topography throughout the vasculature, fundamental differences are found among EC derived from different species and from different organs or vascular beds (Sage et al., 1981; Zetter, 1988; Kumar et al., 1987; Page et al., 1992; Rymaszewski et al., 1992). *Macaca* monkey retina is a widely-accepted model for the human retina (Wassle and Boycott, 1991; Curcio and Hendrickson, 1991), and the monkey has been used to develop a model of retinal neovascularization in vivo (Miller et al., 1994) that might be more relevant to human disease than the extant rat or mouse models (Reynaud and Dorecy, 1994; Pierce et al., 1995). A dependable method for the isolation and culture of primate retinal microvascular EC would improve our understanding of the factors regulating the retinal EC, particularly because cultures of retinal EC from various animals have demonstrated that significant differences exist among species (Schor and Schor, 1986; Rymaszewski et al., 1991; Greenwood, 1992)

The availability of cultured monkey retinal microvascular EC provides a potentially important tool in the study of retinal neovascularization and the underlying mechanisms controlling EC growth in a primate. In this report we provide the first detailed description of the isolation and culture of *Macaca* monkey EC and analyze some of the factors and parameters of their propagation.

2.3. Materials and Methods

Isolation and Culture of Retinal EC

Macaca eyes were obtained from the Tissue Program of the Regional Primate Research Center at the University of Washington in accordance with the approval protocols consistent with the ARVO Statement on Animal Experimentation. For each cell preparation, both eyes were removed from one deeply anaesthetized *Macaca nemestrina M. fascicularis* (2.5 to 8 years old) and were placed in ice-cold calcium- and magnesium-free Hank's balanced salt solution (HBSS) containing 10 mM Hepes (pH 7.4), 0.1% w/v bovine serum albumin, 100 U/ml penicillin G, and 100mg/ml streptomycin SO₄ (preparation buffer). With the aid of a dissecting microscope, an incision was made in each eye posterior to the ciliary body and was extended circumferentially. The anterior half of the eye and the lens

were discarded. The vitreous was removed and the retina was gently pulled away from the retinal pigment epithelial cell layer. The retina was washed in preparation buffer and was minced finely with ophthalmic scissors. Subsequently, the tissue was transferred to a 15 ml centrifuge tube, washed by a brief centrifugation, and dissociated into tissue suspension by flushing through a glass pipette for 10-20 minutes. The tissue fragments were washed twice with preparation buffer and were resuspended in 10 ml of 0.3% collagenase/dispase (Boehringer Mannheim Biochemica, Indianapolis, IN) containing 20 U/ml DNase I (Sigma Chemical Co., St. Louis, MO) at 37°C with gentle, constant agitation for 2-3 hours. The fragments were washed twice with 10 ml Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and were filtered through a 100µm nylon sieve (Becton Dickinson, Franklin Lakes, NJ). The filtrate was subsequently washed twice with the same medium, resuspended in 10 ml DMEM containing 20% FBS, and plated in 2 ml aliquots at 37°C onto 100 mm culture dishes precoated with 25 µg/ml bovine fibronectin (Sigma). Capillary segments and EC aggregates adhered to the dishes within 2-3 hours. The medium subsequently was aspirated and the dishes were rinsed 2-4 times with DMEM containing 10% FBS to remove non-capillary fragments, debris, and unattached cells. A complete culture medium was added that consisted of serum-free QB-58 medium (Quality Biological, Inc., Gaithersburg, MD) containing 10% FBS, 10% adult monkey serum, 20 µg/ml retinal extract (Glaser et al., 1980), 90 µg/ml heparin (Sigma), 100 U/ml penicillin G, and 100µg/ml streptomycin SO₄. The cultures were maintained at 37°C in 5% CO₂ and were provided with fresh media every 3 days. The following techniques were performed to obtain pure populations of EC:

a) *Weeding the non-endothelial cells.* At day 5, the cells arising from capillary segments formed colonies consisting of spindle-shaped cells, which appeared to be EC by morphology and by labeling with specific molecular markers (see below). The non-endothelial cell colonies exhibiting morphology distinct from that of EC were removed by a sterile-sharpened wood applicator under a phase-contrast microscope. Cultures were monitored every day and the weeding procedure was repeated if non-EC were present. By day 12-14, the EC colonies contacted each other, and 90% of the dish was covered by confluent EC. At this point, the medium was removed and dishes were rinsed with calcium- and magnesium- free phosphate buffered saline (PBS) and 0.02M ethylenediaminetetraacetate (EDTA). A nonenzymatic cell dissociation solution (Sigma) was used to remove the cells, which were suspended in DMEM with 20% FBS and were

transferred to fibronectin-coated dishes without centrifugation. The plating density was approximately $1.8 \times 10^4/\text{cm}^2$. Cells adhered to the dishes within 2 hours, and the complete culture medium was replaced. Cultures were passaged at a split ratio of 1:2.

b) Cloning. The EC colonies were isolated and dissociated with trypsin in cloning cylinders. Suspended cells were plated at a density of 1000 cells/35 mm dish or 1-5 cells/well in 96 well plates precoated with fibronectin in complete culture medium. Basic fibroblastic growth factor (bFGF, Biomedical Technologies, Inc., Stoughton, MA) (25 ng/ml) was added to some culture wells.

c) Fluorescence-activated cell sorting (FACS). Cells were prelabeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine-tagged perchlorate-acetylated low-density lipoprotein (DiI-ac-LDL) (Biomedical Technologies, Inc.) at $2 \mu\text{g}/\text{ml}$ in culture medium by incubation overnight. Cells were washed in calcium- and magnesium-free PBS and 0.02 M EDTA, and were incubated with nonenzymatic cell dissociation solution. The cells were washed in DMEM containing 10% FBS and were resuspended in the same medium at 10^6 cells/ml. Sorting was carried out at an excitation wavelength of 514 nm and an emission wavelength above 550 nm (FACStar, Becton Dickinson).

Immunocytochemistry

The EC cultures were fixed in 100% cold methanol for 10 min at 4°C , washed with PBS, incubated with 70% methanol containing 3% H_2O_2 for 30 minutes to inactivate endogenous peroxidases, and subsequently blocked with 10% normal goat serum for 1 hour. The primary antibody was incubated in a humid chamber for 2 hours at room temperature. The following antibodies were used: rabbit anti-human von Willebrand factor (1:200 dilution of a 5.4 g/L stock solution; Dako, Carpinteria, CA), monoclonal mouse anti-human endothelial cell protein CD31 (1:40 dilution of a 15.7 g/L stock solution; Dako), rat anti-mouse CD34 (1:50 dilution of a 0.5 mg/ml stock solution; Pharmingen, San Diego, CA), rat anti-mouse VCAM-1 (1:50 dilution of a 0.5 mg/ml stock solution; Southern Biotechnology Associates Inc., Birmingham, AL), and monoclonal mouse anti-smooth muscle actin (1:500 dilution of a 8.1 mg/ml stock solution; Sigma). After washes with PBS (3x5 minutes), cells were incubated 1 hour with biotinylated species-appropriate secondary antibodies at a dilution of 1:200. A complex of avidin-biotin-peroxidase was reacted for 30 minutes and was subsequently visualized by exposure of the cells to a solution of 3,3'-diaminobenzidine-4-HCL containing 0.02% H_2O_2 for 2-5 minutes. The cells were rinsed with tap water and were photographed. The cultures were also stained by

single-or double-label immunofluorescence. The cells were incubated with one or two primary antibodies as described above, followed by the appropriate secondary antibodies conjugated with Texas red or fluorescein isothiocyanate (FITC) at a dilution of 1:200, mounted in 50% glycerol, and photographed with a Zeiss fluorescence microscope. For the uptake of Dil-Ac-LDL, cells were incubated 4 hours or overnight at 37°C in culture medium containing 10 µg/ml Dil-Ac-LDL, fixed for 15 minutes at room temperature in 3% paraformaldehyde (pH 7.4), rinsed in distilled water for 5 seconds, mounted in glycerol, and photographed under the fluorescence microscope. For lectin binding, cells were fixed in 3% paraformaldehyde and were incubated with *Ulex europaeus* lectin conjugated to FITC (1:50 dilution of a 1 mg/ml stock solution; Sigma) for 1 hour in PBS.

Assay for ³H-Thymidine Incorporation

The effects of different culture media and growth factors on EC were studied by ³H-thymidine incorporation. EC were seeded in 24-well plates at a density of 5000 cells/well in complete culture medium and were grown for 20 hours. Cultures were rinsed several times with DMEM without serum and were incubated in DMEM with 2% FBS for 16 hours. The medium was changed as described in the figure legends. Control cultures were grown in DMEM containing 2% FBS. The cultures were incubated for 48 hours, exposed to 2 µCi/ml [methyl-³H]-thymidine (81.3 Ci/mmol, 1mCi/ml; New England Nuclear, Boston, MA) for the last four hours. The cultures were subsequently washed three times with PBS, incubated in ice-cold 10% trichloroacetic acid (TCA) for 1 hour, washed 2 times with anhydrous ethanol at -20°C, and air-dried. 300 µl of NaOH (0.2N) was added to each well for 30-40 minutes at 68°C. The cpm incorporated into DNA were quantified by liquid scintillation counting in 3 ml Ecolume (ICN, Irvine, CA). Data were expressed as means ± S.D. of three experiments performed in triplicate.

Metabolic Labeling and Analysis of Secreted Proteins

EC cultured on fibronectin-coated dishes were radiolabeled at approximately 90% confluence. The cells were rinsed five times with serum-free DMEM and were incubated for 1 hour in serum-free DMEM supplemented with 50µg/ml sodium ascorbate and 64 µg/ml β-aminopropionitrile fumarate. The medium was aspirated, and cultures were incubated for 20 hours in the same DMEM containing 20 µCi/ml L-[2,3,4,5-³H] proline (100 Ci/mol) (Amersham, Arlington Heights, IL). At the end of the incubation, the medium was collected and was centrifuged briefly to remove cells and debris. The following

proteinase inhibitors were added to the final concentrations indicated: 0.2mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 2.5 mM EDTA, and 0.5 µg/ml pepstatin A (Peninsula Laboratories, San Carlos, CA). The medium was dialyzed against 0.1 N acetic acid and was finally lyophilized.

Lyophilized proteins were solubilized in 2x SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) sample buffer (Laemmli, 1970), and incorporated cpm were measured by scintillation counting. Samples equivalent to 30,000 cpm were reduced with 50 mM dithiothreitol and were denatured by heating at 100°C for 3-5 minutes in sample buffer. Proteins were examined by discontinuous SDS-PAGE on horizontal slab gels. Protein molecular-weight standards included myosin (200 kD), phosphorylase b (97.4 kD), bovine serum albumin (68 kD), ovalbumin (43 kD), α-chymotrypsinogen (27.5 kD), and lysozyme (14.3 kD). Proteins were stained with Coomassie brilliant blue R-250, destained, and incubated in amplifier (DuPont, Boston, MA). Dried gels were exposed to RP X-Omat film (Kodak, Rochester, NY) at -70°C.

Western Blot Analysis

Culture media proteins prepared as described above but without radiolabel were subjected to SDS-PAGE and were transferred to 0.4µm nitrocellulose membranes at 100 V for 1 hour at 4°C. The membranes were stained with amido black to assess the efficiency of transfer and were subsequently blocked in 5% nonfat dried milk and 0.05% Tween-20 for 2 hours prior to incubation with antibodies for 16 hours at 4°C. Primary antibodies were mouse anti-human cellular fibronectin (Sigma), rabbit anti-mouse collagen IV (1:5000 dilution of a 5 mg/ml stock solution; Collaborative Biomedical Products, Bedford, MA), rabbit anti-mouse laminin (1:1000 dilution of a 1 mg/ml stock solution; Collaborative Biomedical Products), rabbit anti-mouse SPARC IgG (directed against a synthetic C-terminal peptide of SPARC) (Lane and Sage, 1990) at 9 µg/ml, and an antiserum prepared in guinea pig against rat type I collagen prepared in our laboratory. The titer and specificity of the anti-type I collagen IgG antibody were determined by enzyme-linked immunoadsorbent assays and Western blot analyses of both purified collagen and conditioned media from cultured fibroblasts (Iruela-Arispe et al., 1991) The blots were washed in PBS and were incubated in ¹²⁵I-protein A at 0.5 µCi/ml (NEN, Boston, MA). After several washes, the blots were exposed to X-ray film.

2.4. Results

In this study, we observed the growth of monkey endothelium derived from retinal microvasculature. The microvascular fragments composed of 3-10 cells each adhered to fibronectin-coated dishes after 2-3 hours (Fig. 2.1A). Migration of cells from these fragments began 1-2 days after plating (Fig. 2.1B), and after 4 days in culture, the cells formed contiguous islands of spindle-shaped cells that were closely apposed to one another (Fig. 2.1C). After 1 week a marked proliferation occurred that resulted in the formation of large colonies (Fig. 2.1D). We confirmed that these colonies were composed of EC through the use of EC markers (see below). The major contaminating cells were smooth muscle cells, which grew into dense colonies of multilayered, small, elongated cells that could be readily distinguished from endothelial colonies under phase-contrast microscopy (Fig. 2.1E). In addition, these cells stained for smooth muscle α -actin, did not contain von Willebrand factor or CD31, and did not bind DiI-Ac-LDL (data not shown). Although there is no unique marker to date for the identification of pericytes, these cells can be recognized by characteristic morphologic features (Wong et al., 1987; Gitlin et al., 1983; Buzney et al., 1983) and were occasionally seen among endothelial colonies. The non-EC were removed from cultures as described in Methods. After 12-14 days in culture, EC colonies contacted each other and formed a confluent monolayer (Fig. 2.1F). At confluence, proliferation ceased and the cells did not overgrow into multilayered aggregates. Some cells cultured at saturation density for 2-3 weeks maintained a uniform monolayer configuration. In contrast, some of the EC colonies produced a sprouting pattern after the monolayer was formed (Fig. 2.1G). The sprouting cells were elongated and associated with other EC to form long, cord-like structures (Fig. 2.1H).

To confirm the identity of the cultured EC, we used several EC markers. Monkey EC produced von Willebrand factor (Fig. 2.2A). Punctuate fluorescence was observed after incubation in DiI-ac-LDL (Fig. 2.2B). Antibody against CD31, a protein highly specific to EC, stained the cell surface, especially regions of intercellular contacts (Fig. 2.2C). These cells did not express smooth muscle α -actin (data not shown). The *Ulex europaeus*- agglutinin-1 (UEA-1) binds to α -L-fucosyl residues of glycoproteins present on the surface of human EC (Pereira et al., 1978; Hormia et al., 1983) and has been used as a marker for human capillary EC. Figure 2.2 also shows double immunofluorescence labeling for CD31 (C) and UEA-1 (D). The arrow in Fig. 2. 2D indicates a cell that was labeled by UEA at a significantly higher level than the adjacent EC (shown in C as reactive with anti-CD-31 antibody). UEA is therefore not a specific marker for monkey EC *in vitro*.

VCAM-1 was not detected in unstimulated retinal monkey EC, and staining with anti-CD34 antibody was also negative (data not shown).

To assess the purity of primary cultures, we performed flow cytometry on cells labeled with DiI-Ac-LDL. Cultured retinal vascular smooth muscle cells served as a negative control to set the range of fluorescence intensity for positive cells, as indicated by the bars in Fig. 2.3. Primary cultures contained approximately 90% EC (Fig. 2.3B). After fluorescence-activated cell sorting of the labeled cells, approximately 99% EC were obtained (Fig. 2.3C).

Plasma-derived serum (PDS) has been used for the selective growth of retinal EC in culture (Greenwood, 1992; Gitlin and D'Amore, 1983; Bowman et al., 1982; Grant and Colleen, 1991). In our system, PDS did not inhibit selectively the growth of purportedly non-EC, and more importantly, it did not support the growth of monkey retinal EC. Cultures grown in PDS initially exhibited a colony size similar to that of EC grown in FBS. However, after 1 week in PDS, the cells failed to proliferate and became enlarged and attenuated, lost characteristic endothelial morphology, and often deteriorated after 14 days (Fig. 2.4). After testing different culture conditions for the growth of monkey retinal EC, we arrived at an optimal culture medium, as described in this report, for long-term propagation of EC. EC incubated in QBSF-58/complete culture medium gave rise to larger and denser colonies than the EC in DMEM/complete culture medium. Potential differences in the proliferation of cells grown in DMEM *versus* QBSF media, both of which contained FBS, retinal extract, and heparin at the same concentrations, were assessed by the incorporation of ³H-thymidine (Fig. 2.5). Results substantiated the microscopic observations: QBSF culture medium was superior to DMEM for stimulation of endothelial cell proliferation ($p < 0.01$). In addition, retinal extract was superior to endothelial cell growth supplement (Sigma) for the proliferation of monkey EC (data not shown).

By counting the cells growing within colonies, we ascertained that EC grew in complete culture medium with a mean doubling time of 44.5 hours from days 3-5; the doubling time decreased to 23 hours from days 6-8. After 1 week, the size of the endothelial colonies enlarged rapidly, colonies contacted each other, and a uniform population of EC formed a monolayer by 12-14 days in culture. Monkey EC could be passaged only at high density. When we cloned the EC at low density, most cells did not attach; the few attached EC were spread but vacuolated, failed to divide, and eventually detached. A few attached EC grew slowly into a small colony in the presence of high doses of bFGF (25 ng/ml). The EC sorted by FACS grew poorly on fibronectin-coated dishes,

and by one week EC exhibited signs of senescence or degeneration, e.g., the cells were large, flattened, and vacuolated. Eventually, most of the cells lifted off the substrate and died. We found that the standard procedure of trypsin treatment and centrifugation decreased the plating efficiency significantly. With nonenzymatic cell dissociation and no centrifugation, 95% of the EC adhered and grew on fibronectin-coated substrate. Passaged EC could be maintained in culture for more than one month and could be cultured to passage four in complete culture medium without the addition of growth factors such as bFGF or VEGF. After one month, EC grew slowly and required additional growth factors to traverse the cell cycle.

To achieve quiescence, we seeded EC in 2% FBS in DMEM. The addition of VEGF and bFGF improved DNA synthesis (Fig. 2.6). The maximum stimulation of thymidine incorporation was 30 ng/ml of VEGF and 10 ng/ml bFGF in 2% FBS/DMEM. Although the simultaneous addition of bFGF and VEGF resulted in an enhanced mitogenic effect (Fig. 2.6C), synergy between these two growth factors was not observed.

For analysis of proteins secreted by monkey EC, subconfluent cultures were metabolically labeled with ^3H -proline, and newly-synthesized proteins secreted into the culture medium were characterized by SDS-PAGE (Fig. 2.7). ^3H -proline-labeled proteins secreted by subconfluent, non-sprouting retinal primary or passage 3 EC were visualized by autoradiography (Fig. 2.7, lanes 1-2). Fibronectin, SPARC, laminin, and collagen types I and IV could be identified as prominent secretory products of both primary and passaged subconfluent cultures. The identity of these proteins was confirmed by Western blotting (Fig. 2.7, lanes 3-7).

2.5. Discussion

To the best of our knowledge, this is the first description of the culture and characterization of monkey retinal EC. Numerous techniques and culture conditions have been developed for bovine retinal EC (Schor and Schor, 1986; Wong et al., 1987; Gitlin and D'Amore, 1983; Buzney et al., 1983; Bowman et al., 1982; Grant and Colleen, 1991; Capetandes and Gerritsen, 1990), and pure human retinal EC have been obtained by cell sorting (Rymaszewski et al., 1991). However, previously published methods could not be adapted readily to monkey EC. By using modified EC culture techniques, we obtained pure monkey retinal EC cultures that retained many of their phenotypic properties. These monkey EC cultures should be valuable for experiments designed to probe the intrinsic properties of primate retinal EC.

Many lines of evidence support the identification of these cultures as EC. At confluence, the cultures showed contact-inhibition of growth, a characteristic of vascular endothelium. Immunocytochemical studies provided further evidence for the endothelial origin of these cultures. CD31, a highly-specific EC marker which is not present on any other cell type except leukocytes (Kuzu et al., 1992), might be involved in homotypic intercellular interactions. Staining for CD31 showed that monkey EC deposited this antigen at points of cell-cell contact. The monkey cultures also produced von Willebrand factor, a widely accepted marker for EC (Jaffe, 1988), and exhibited uptake of DiI-ac-LDL, a property used for EC identification and isolation (Voyta et al., 1984). Although UEA has been described as a marker of human EC *in vitro* and *in vivo* (Potzsch et al., 1990; Walker, 1985), and UEA-coated Dynabeads have been utilized for the isolation of human EC (Jackson et al., 1990; Hewett and Murray, 1993), this lectin was not a specific ligand for monkey EC *in vitro*. Similar results have been described *in vivo* (Gariano et al., 1996). In fact, in our mixed cultures, UEA reacted preferentially with non-endothelial cells. Human cerebral EC in primary culture constitutively expressed low levels of VCAM-1, an EC membrane glycoprotein, which was increased after treatment with inflammatory mediators (Wong and Dorovini-Zis, 1995; Orledge et al., 1989). However, monkey retinal EC did not express detectible levels of VCAM-1 under basal conditions *in vitro*. It is also possible that the lack of immunostaining with anti-VCAM-1 and anti-CD34 was due to species incompatibility of the antibodies as opposed to the absence of *Macaca* antigen.

A critical step for the isolation of large numbers of EC was minimization of the growth of other cell types. The microvessel isolation procedure was designed to limit the number of non-EC plated on the dish. A high density of 92% EC was generated in the primary cultures, a higher percentage than reported for human retinal EC (Rymaszewski et al., 1991). Cell sorting produced a higher purity of 99%, but this procedure shortened the life-span of the EC. Although the manual removal of contaminants surrounding EC colonies is tedious and time-consuming, mechanical weeding during early days of culture isolated pure EC colonies and ensured that a uniform population of EC in the cultures was available for subsequent analysis. In the impure cultures, the progressive increase in the number of smooth muscle cells and pericytes coincided with the arrest of EC proliferation. This arrest might be mediated by the activation of transforming growth factor- β (Orledge et al., 1989).

Monkey retinal EC were sensitive to factors in their culture medium. PDS, which lacks platelet-derived products, has been used for the growth of retinal EC from other

species (Greenwood, 1992; Bowman et al., 1982; Grant and Collee, 1991). However, the value of PDS as a selective agent for capillary EC has been brought into question with the discovery that microvascular EC derived from human adipose tissue express 30,000-40,000 platelet-derived growth factor (PDGF) receptors per cell and proliferate in response to PDGF (Beitz et al., 1991). PDGF might also be required for monkey retinal EC, because we found that EC proliferation was significantly reduced in PDS, compared to that in whole serum. In contrast, fetal bovine serum produced apparently higher rates of proliferation than adult bovine whole serum or adult monkey serum; QBSF medium was also important for the optimal culture of retinal EC. When QBSF medium was replaced by DMEM, the incorporation of ³H-thymidine into EC DNA was significantly reduced (Fig. 2.5). A critical ingredient was crude retinal extract, which contains a growth stimulant for EC but not for pericytes (Glaser et al., 1980; D'Amore et al., 1981).

Unlike bovine retinal EC, which grew rapidly, survived repeated passages, and could be cloned, monkey retinal EC were much less hardy. We did not succeed in cloning these cells. They could be passaged for more than one month, but thereafter exhibited a reduction in growth rate. A plating efficiency of <50% was obtained when trypsin was used to isolate cells for passage. Treatment of cells with trypsin has been shown to cause a loss of membrane lipid, inhibit angiotensin-converting enzyme, and suppress bradykinin receptors (Kirkpatrick et al., 1985; Sung et al., 1989). Nonenzymatically-treated monkey EC survived freezing, storage, and thawing.

Previous studies of bovine EC have indicated that these cells synthesized and secreted growth factors such as bFGF (Brooks et al., 1991) and VEGF (Simorre-Pinatel et al., 1994) that were involved in the autocrine regulation of EC growth. Exogenous VEGF and bFGF each induced the proliferation of monkey EC. The mitogenic effects of these growth factors on EC were tested in 2% rather than 10% serum (Simorre-Pinatel et al., 1994; Bensaid et al., 1989) to minimize the mitogenic effects of serum on EC and to ensure a low basal level of DNA synthesis. Our data show that bFGF is a more potent mitogen than VEGF. The effect of VEGF on retinal EC growth stimulation measured by DNA synthesis was at best 2-fold; however, the mitogenic effect of VEGF on retinal EC was elevated to 3 to 4-fold under hypoxic conditions (Q. Yan, unpublished data). Although Goto et al.(Goto et al., 1993) observed a synergistic effect of VEGF and bFGF on the proliferation of bovine capillary EC within collagen gels, we were unable to confirm this result with monkey EC in the two-dimensional culture system used in our study.

A dynamic and reciprocal relationship exists between capillary EC and the extracellular matrix that can influence cellular phenotype, particularly several properties relevant to angiogenesis (Sage and Vernon, 1994). Our laboratory has demonstrated that angiogenesis *in vitro* is associated with changes in several matrix proteins, including SPARC and type I collagen in bovine aortic EC (Sage and Vernon, 1994; Iruela-Arispe et al., 1991; Vernon et al., 1995). In our experiments with monkey retinal EC, some of the contact-inhibited monolayers subsequently displayed a “sprouting” growth pattern that resulted in cord-like or protocapillary structures; monkey retinal microvascular EC therefore have the capacity to form capillary-like structures *in vitro* (Folkman and Haudenschild, 1980). This property could be advantageous for studies on the regulation of growth factors and the synthesis and degradation of matrix proteins during primate angiogenesis *in vitro*. In this study, we examined the synthesis of secreted proteins by cultured non-sprouting EC and showed that fibronectin, SPARC, laminin, and collagen types I and IV were the principal products. The changes in matrix protein biosynthesis when these cells undergo angiogenesis *in vitro* (as shown in Fig. 2.1H) are currently under investigation.

In summary, we have shown that monkey retinal EC can be isolated, cultured, and characterized. The availability of these cells allows an in-depth analysis of their growth parameters. This approach should provide important new information on EC properties that could lead to a better understanding of defects or alterations in the processes underlying pathological retinal neovascularization.

Figure 2.1. Phase contrast photomicrographs of monkey retinal EC in culture. (A) Capillary fragment 3 hours after initial plating; no cells have grown out; (B) Capillary fragment after 48 hours in culture with two small colonies of emerging cells; (C) EC forming a colony of spindle-shaped cells after 4 days in culture; (D) A large colony composed of EC after 7 days in culture; (E) A large, dense smooth muscle cell colony (arrow) is adjacent to a small EC colony; (F) A confluent, contact-inhibited monolayer of EC after 12 days in culture; (G) A monolayer of EC showing a sprouting phenotype after confluence; (H) An EC culture forming cord-like structures (arrows) 6 days after confluence.

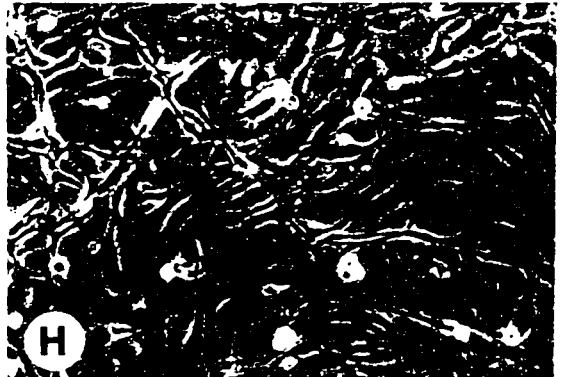
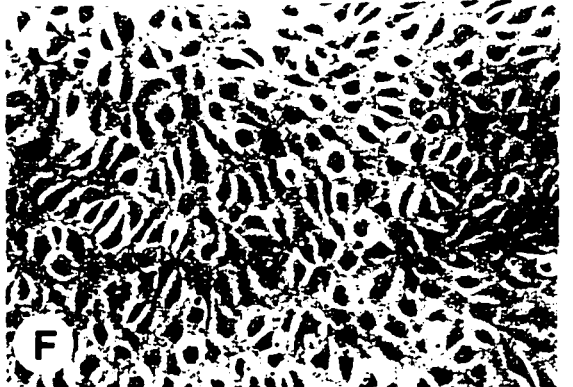
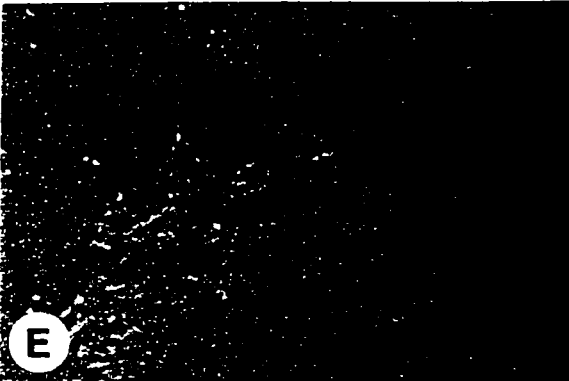
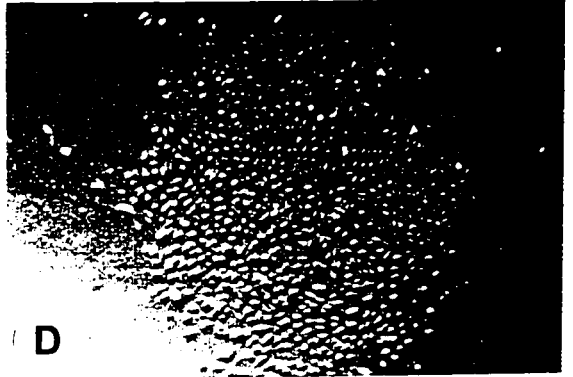
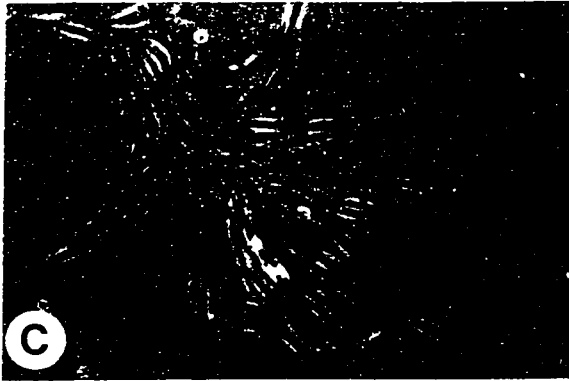
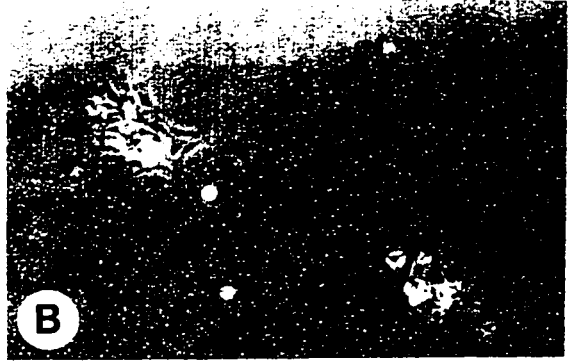


Figure 2.2. Characterization of EC cultured from monkey retinal microvasculature.

(A) Monolayer of EC immunocytochemically labeled for von Willebrand factor by the avidin-biotin-peroxidase technique. The cytoplasm is stained but not the nucleus; (B) Accumulation of DiI-ac-LDL (10 μ g/ml) by cultured EC after a 12 hour incubation; (C, D) Double-labeling immunofluorescence of cultured microvascular cells with anti-CD31 antibody and UEA-1 lectin. Staining by anti-CD31 antibody conjugated to Texas red (C) is localized to EC cell membranes, especially at intercellular junctions. An unlabeled cell is indicated by an arrow. UEA-1 conjugated to FITC (D) labeled both the non-endothelial cell (arrow) and EC.

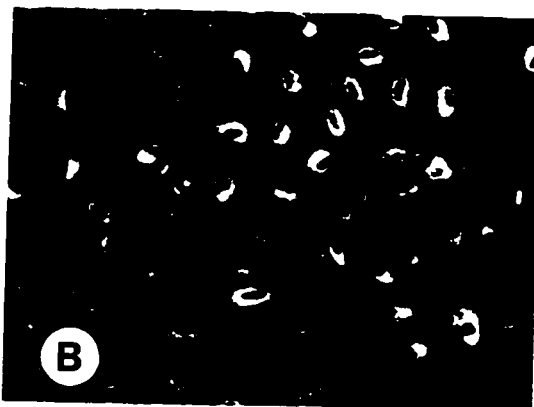
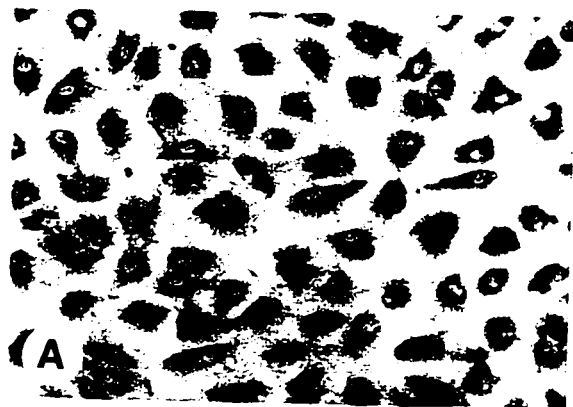
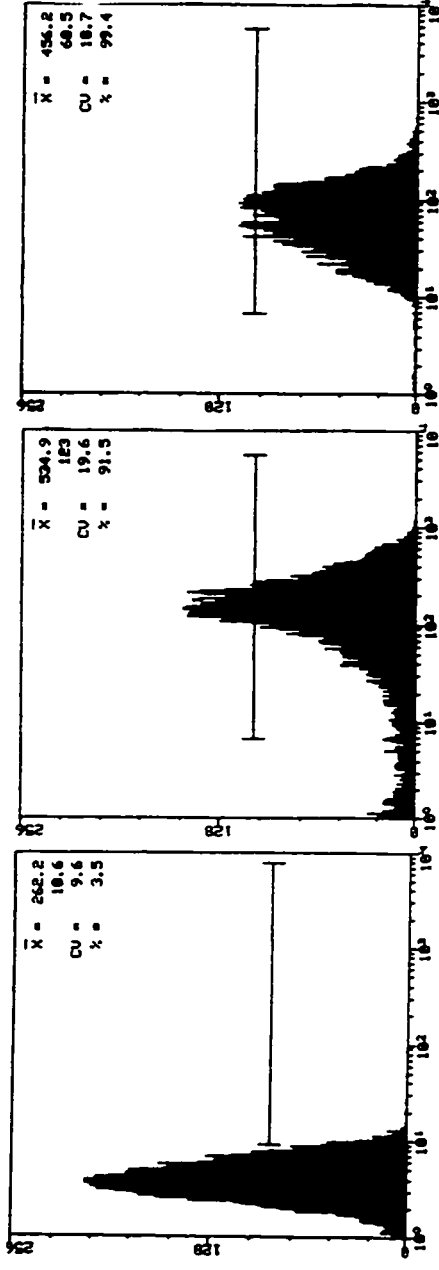


Figure 2.3. Fluorescence-activated cell sorting analysis of cultured monkey retinal EC labeled by DiI-ac-LDL.

Fluorescence-activated cell sorting analysis of cultured monkey retinal EC labeled by overnight incubation in 2 $\mu\text{g/ml}$ DiI-ac-LDL. **(A)** Monkey smooth muscle cells incubated with DiI-ac-LDL; **(B)** Day 12 primary cultures that were not subjected to the weeding procedure. The yield of DiI-ac-LDL-labeled cells was 91.5% of the total culture; **(C)** After sorting, 99.4% cells stained positively for DiI-ac-LDL.



A B C

log fluorescence intensity

Figure 2.4. Phase contrast photomicrographs of monkey retinal EC cultured in QBSF medium containing 20% PDS, heparin, and retinal extract.

(A) 4 days in culture; (B) 12 days in culture. The EC became enlarged, attenuated, and, in some instances, necrotic.

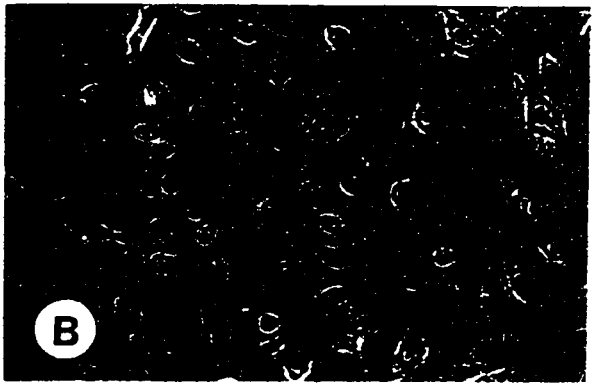
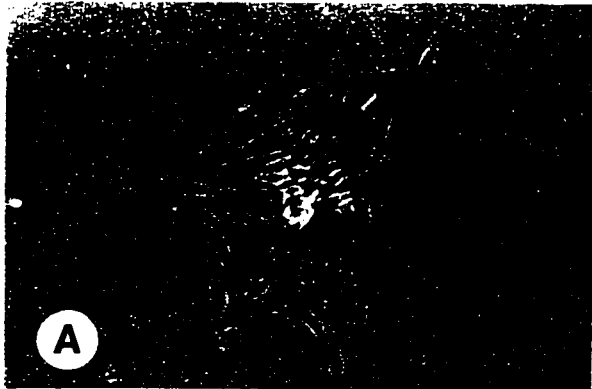


Figure 2.5. Comparative effects of DMEM and QBSF-58 on the incorporation of ^3H -thymidine by cultured EC.

Cells were plated at 5000 cells/well and were grown for 20 hours in complete culture medium; subsequently, the cells were cultured in DMEM containing 2% FBS for 16 hours, after which the medium was changed to (1) 2% FBS in DMEM; (2) DMEM containing 10% FBS and 10% monkey serum, retinal extract, and heparin; (3) QBSF medium containing 10% FBS and 10% monkey serum, retinal extract, and heparin. The cultures were incubated for 48 hours and were pulsed during the last 4 hours with ^3H -thymidine at 2 $\mu\text{Ci/ml}$. Bars represent the mean \pm S.D.; values were normalized to cpm incorporated by control cultures that were treated with 2% FBS in DMEM.

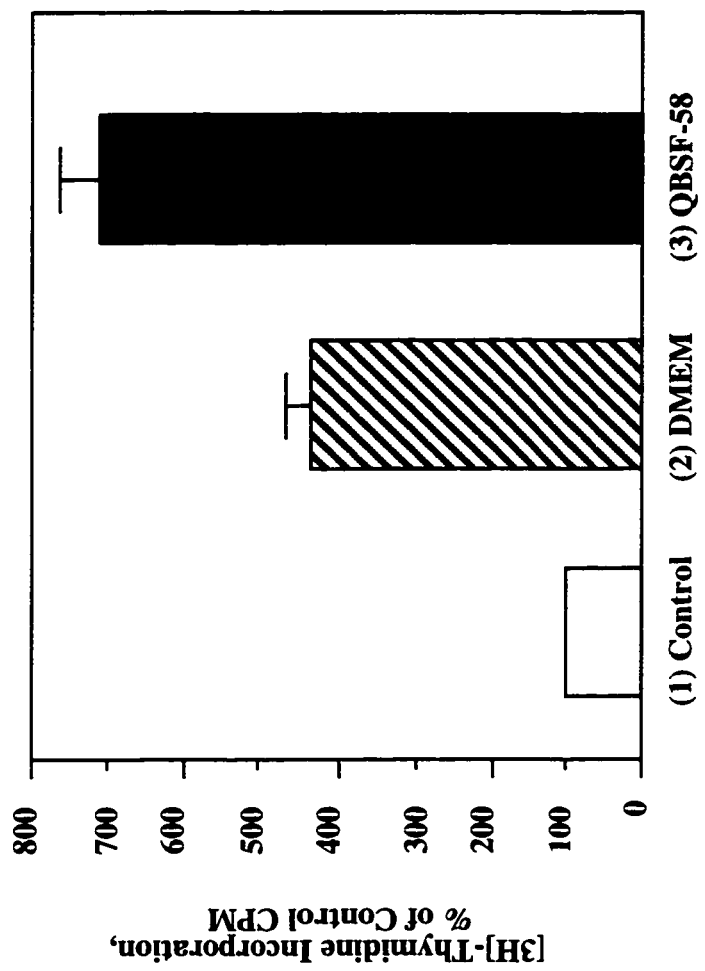


Figure 2.6. Mitogenic effects of (A) bFGF, (B) VEGF, and (C) a combination of bFGF & VEGF on the incorporation of ^3H -thymidine by retinal EC.

Cells were plated at 5000 cells/well and were grown for 20 hours in complete culture medium; subsequently, the cells were cultured in DMEM containing 2% FBS for 16 hours, after which the medium was changed to DMEM containing 2% FBS and (A) bFGF (0, 2, 5, 10, 20 ng/ml); (B) VEGF (0, 5, 10, 30, 50 ng/ml); or (C) bFGF + VEGF (0, 2+10, 5+20, 10+30 ng/ml, respectively). The cultures were incubated with the growth factors for 48 hours and were pulsed during the last 4 hours with ^3H -thymidine at 2 $\mu\text{Ci/ml}$. Data are the mean \pm S.D. of triplicate experiments; values were normalized to cpm incorporated by controls cultured in 2% FBS in DMEM.

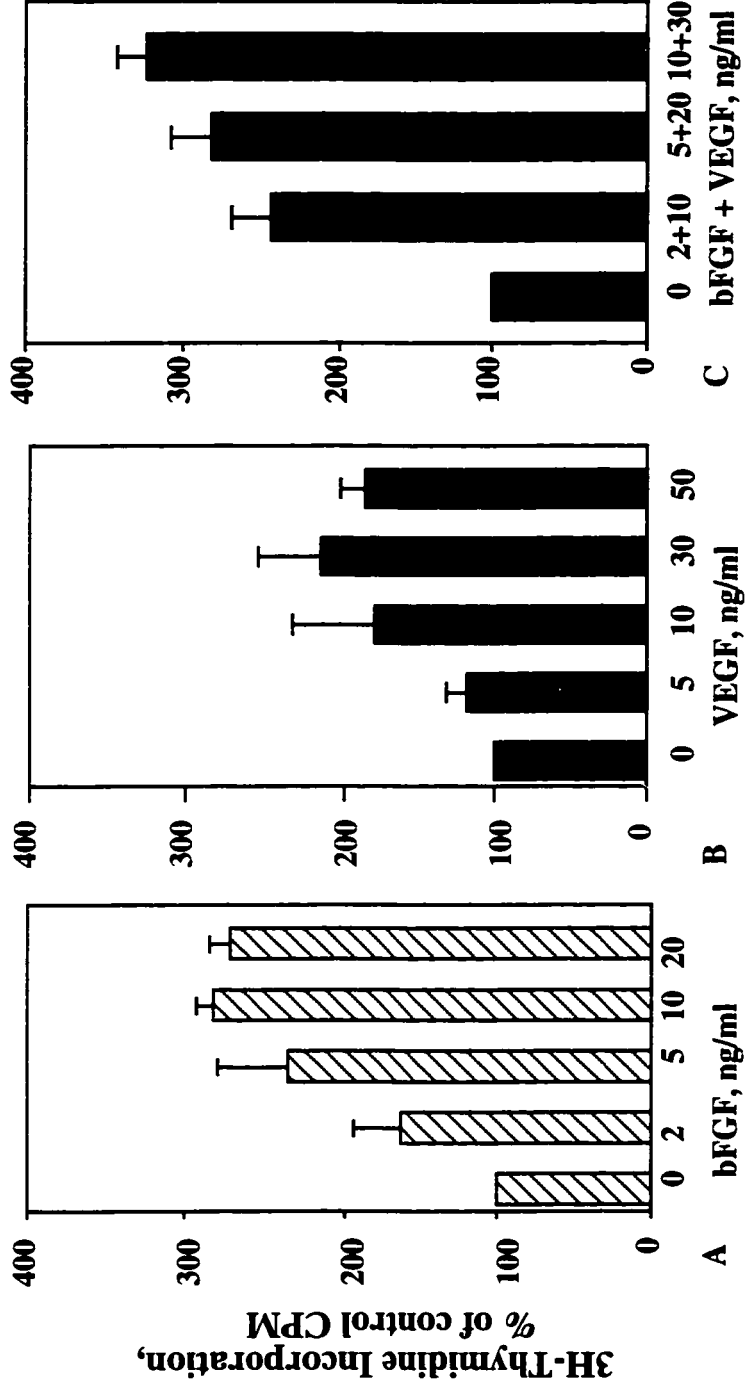
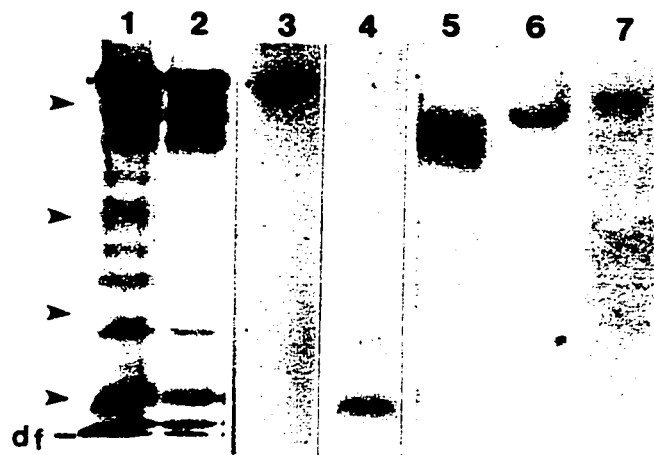


Figure 2.7. Proteins secreted by cultured monkey retinal EC. Cultures were incubated with ^3H -proline for 20 hours as described in Methods.

Proteins were analyzed by SDS-PAGE on 4%/7% acrylamide slab gels in the presence of 50 mM DTT. ^3H -proline labeled-proteins secreted by subconfluent primary EC (lane 1) or passage 3 EC (lane 2) were visualized by autoradiography. Western blots of proteins synthesized by subconfluent EC were incubated with antibodies against fibronectin (lane 3), SPARC (lane 4), type I collagen (lane 5), laminin (lane 6), or type IV collagen (lane 7). Molecular weight standards are indicated by the arrowheads on the left: 200 kD, 97.4 kD, 68 kD, and 43 kD. df, Dye front.



CHAPTER 3: REGULATION OF RETINAL ENDOTHELIAL CELLS AND PERICYTES BY VEGF, bFGF AND HYPOXIA

3.1. Summary of Chapter 3

The isolation of retinal EC from *Macaca* monkey and characterization of these cells indicated that the retinal EC are different from EC derived from other organs or vascular beds. In vivo they are surrounded by a complicated environment, regulated by multiple factors and several types of cells (these factors contributed to the difficulty of growing these cells in vitro). The limited passages for propagation of monkey retinal EC and the limited tissue supply for each preparation prompted me to switch species. Bovine EC have been widely used in vascular biological research, and bovine eyeballs can be obtained in the local slaughterhouse. Therefore, I used the methods and techniques which I had developed from the monkey EC for the culture of bovine retinal EC. Bovine EC can be maintained to passage 8 and can be frozen for later use.

This chapter reports on the isolation and characterization of bovine retinal EC and pericytes. Furthermore, the effects of hypoxia, VEGF and bFGF on the proliferation of retinal microvascular EC and pericytes have been investigated, and the effects of growth factors and hypoxia on the secretion of proteins by EC are described.

3.2. Introduction to Chapter 3

Hypoxia, the common precursor of various retinal vasoproliferative disorders, is an important environmental factor which could directly influence the angiogenic process. Hypoxia may alter the normal dynamic relationship between regulators and dispose retinal microvascular EC and pericytes toward an activated state of neovascularization.

The formation of new capillaries from an established microvasculature involves the stimulation of endothelial proliferation, migration, and organization. Endothelial proliferation has been considered as a major component of this angiogenic process. Studies of EC exposed to hypoxia in vitro have yielded contradictory observations about the effect of hypoxia on the proliferation of non-retinal EC (Shreeniwas et al., 1991; Meininger et al., 1988; Fanburg et al., 1987). To understand the effect of hypoxia on the proliferation of

retinal EC becomes critical to elucidate the mechanism of neovascularization. Retinal neovascularization is directly affected by angiogenic factors, which include bFGF and VEGF. In vitro, both factors have been shown to stimulate the proliferation of vascular EC from a variety of sources. In vivo, both factors have been shown to be upregulated in neovascular retinopathy. The response of capillary cells to these mitogens under hypoxic conditions and whether the two mitogens interact synergistically in retinal vascular cells are two important areas of investigation.

In addition, the stage prior to the proliferative phase in diabetic retinopathy is associated with the loss of pericytes (the phenomenon has been termed 'pericyte drop-out'. However, Nomura et al. (1995) reported that pericytes proliferation increased significantly under hypoxic conditions, and flt 1 mRNA and VEGF were detected in pericytes under hypoxia. They suggested that it is likely hypoxia would stimulate pericytes to be mitogenic and to secrete factors that would promote growth of EC. under physiological conditions, pericytes are considered to inhibit EC growth and maintain the quiescence of retinal EC. To understand the effect of hypoxia on the behavior of pericytes in vitro may improve our understanding of ocular angiogenic disorders.

Responses to hypoxia may vary depending on the origin of EC (Koroma et al., 1995); therefore, specific studies with retinal EC and retinal pericytes are necessary to determine the mechanisms that mediate and influence the stimulatory effect of hypoxia on the development of retinal neovascularization. In this chapter, I ask: 1) What are the effects of hypoxia on the proliferation of retinal EC and pericytes? 2) What are the growth responses of EC and pericytes to VEGF and bFGF under hypoxic conditions ? 3) Do VEGF and bFGF have a synergistic effect on the proliferation of retinal EC or on the formation of capillary-like tubes in 3-dimensional culture? 4) Can VEGF or bFGF regulate proteins secreted by retinal EC under hypoxia and normoxia?

3.3. Materials and Methods

Preparation of Cells

Bovine retinal capillary EC were isolated and cultured essentially according to our previously described methods, with some modifications for primary cultures (Yan et al., 1996). Briefly, the minced tissue fragments were digested in 0.3% collagenase/dispase (Boehringer Mannheim Biochemical, Indianapolis, IN) containing 10 U/ml DNase I (Sigma) at 37° C with gentle agitation for 30 min to 1 hr. The fragments were washed with

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and were filtered through a 40 μm nylon sieve (Becton Dickinson, Franklin Lakes, NJ). The fragments on the sieve were collected, resuspended in DMEM with 10% FBS, and plated onto dishes precoated with 20 $\mu\text{g}/\text{ml}$ bovine fibronectin (Sigma) for 2-3 hours to allow attachment of capillary fragments to the dishes. The medium was subsequently aspirated, and the dishes were rinsed gently with DMEM containing 10% FBS to remove debris, unattached cells, and noncapillary fragments. A complete culture medium was added, and EC were grown in DMEM containing 10% FBS, 10 $\mu\text{l}/\text{ml}$ retinal extract, 75 $\mu\text{g}/\text{ml}$ heparin, 100 U/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin SO_4 (complete culture medium). The dishes were washed again with 10% FBS/DMEM to remove the debris after an incubation overnight (this medium was subsequently filtered through a 0.2 μm filter and was added to the cultures). The cultures were maintained at 37°C in 5% CO_2 and were provided with fresh media every 4-6 days. Some scattered pericytes were removed by weeding (Yan et al., 1996) before day 5 in culture. Early passage cells were frozen for later use. Cells from passages 3-6 were used for experiments.

For isolation of bovine retinal pericytes, the digestion with 0.3% collagenase/dispase was increased to 2-3 hours, and the tissue fragments were filtered through a 40 μm nylon sieve. The filtrate (containing released cells) was collected, washed twice with DMEM containing 10% FBS, resuspended in 10% FBS/DMEM, and plated onto plastic dishes for an incubation overnight. Dishes were rinsed several times to remove debris and unattached cells; cell growth was achieved in 10% FBS in DMEM. No capillary fragments were observed in these preparations, as only individual cells attached to the dishes. After 1.5-2 weeks in culture, primary cultures of pure pericytes were apparent. Occasionally, a few smooth muscle cell colonies appeared in the pericyte cultures; these cells were removed by weeding (Yan et al., 1996). By 2-3 weeks, confluent pericytes were harvested and frozen for later use.

Immunocytochemistry

The EC and pericyte cultures were fixed in 100% methanol for 10 min at 4°C, washed with PBS, and incubated with 10% normal goat serum for 2 hr. Subsequently, they were exposed to primary antibodies in a humid chamber for 2 hr at room temperature: rabbit anti-human von Willebrand factor (1:200 dilution of a 5.4 g/l stock solution; DAKO, Carpinteria, CA), monoclonal mouse anti- α -smooth muscle actin (1:800 dilution of an 8.1

mg/ml stock solution; Sigma), polyclonal rabbit anti-human glial fibrillary acidic protein (GFAP; 1:1000 dilution of an 8.7 mg/ml stock solution; BioMaker, Rehovot, Israel). Negative controls included replacement of primary antibodies by normal mouse IgG1 or PBS. After several washes in PBS, we added secondary antibodies conjugated with Texas red or fluorescein isothiocyanate at a dilution of 1:200 (Sigma). Slides were mounted in 80% glycerol and were photographed with a Nikon fluorescence microscope. For the uptake of fluoresceinated, acetylated low-density lipoprotein (DiI-ac-LDL) (Biomedical Technologies, Stoughton, MA), cells were incubated 4 hr at 37°C in culture medium containing 5 µg/ml DiI-Ac-LDL, fixed in 2% paraformaldehyde, and mounted in glycerol prior to photography.

DNA Synthesis Assay and Cell Counts

Cells were plated in 24-well plates at a density of 1×10^4 cells /well in 10% FBS in DMEM and were grown for 20 hr prior to the treatments. The culture medium was changed to 5% FBS in DMEM for EC, or 0, 2% FBS/DMEM for pericytes, and cells were placed in 21% or 5% O₂ incubators for 2-4 days in the presence or absence of growth factors (see figure legends for detailed doses of growth factors). After incubation, cells were trypsinized and counted by hemacytometer, or cells were exposed to 2 µCi/ml [methyl-³H]-thymidine (81.3 Ci/mmol, 1 mCi/ml; New England Nuclear, Boston, MA) for 2-4 hr. They were washed three times with PBS, incubated in ice-cold 10% trichloroacetic acid for 1 hr, washed twice with anhydrous ethanol at -20°C, and air-dried. 300 µl of NaOH (0.2N) was added to each well for 30 minutes at 68°C. The cpm incorporated into DNA were quantified by liquid scintillation counting in 3 ml Ecolume (ICN, Irvine, CA).

BrdU Labeling and Anti-BrdU Immunostaining

BrdU was used to label the proliferation of cells in culture. BrdU was dissolved in PBS to make a 10 µM solution and added into cultures for 1-2 hr. After the incubation, cells grown on 35-mm dish were fixed in 70% ethanol and prepared for anti-BrdU staining as follow: Cells were pretreated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase. Subsequently, they were digested with pepsin (Sigma), hydrolyzed in 1.5 N HCL for 15 min at 37°C to denature DNA, and then neutralized with 0.1 M sodium borate (pH 8.5). Cells were blocked with 10% normal goat serum (Sigma), following incubation with mouse monoclonal anti-BrdU antibody (Becton-Dickinson, San Jose, CA). The

dishes were incubated consecutively with biotin-conjugated goat anti-mouse IgG1 (Vector Labs, Burlingame, CA), avidin-biotin-peroxidase complex (DAKO, Carpinteria, CA), and 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The dishes were dehydrated and coverslipped with Permount (Fisher Inc., Fair Lawn, NJ).

Preparation of 3-Dimensional Collagen Gels

Gels were prepared by raising the pH and ionic strength of rat tail collagen (Collaborative Research). Fibrillogenesis of the collagen was achieved by combining the collagen solution with 10x MEM (Gibco) and sodium bicarbonate (11.76 mg/ml) on ice to yield a final concentration of collagen of 2.1 mg/ml, pH 7.4. The prepared collagen was dispensed into 12-well plates for 0.5 ml/well and was placed in a 37° C incubator for 10 min. Cells were placed on the top of gel at a density of 5×10^4 /ml in basic growth medium (DMEM containing 10% FBS and 5 μ l/ml retinal extract).

Cells in the gels were fixed in situ with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer after 3 days in culture. Randomly-selected fields measuring 1 x 1.4 mm were photographed in each well by phase contrast microscopy at a single focal plane beneath the surface monolayer.

Metabolic Labeling and Analysis of Secreted Proteins

The method is basically the same as described in Chapter 2. However, the cells were treated with VEGF (20 ng/ml) or bFGF (10 ng/ml) in a 21% O₂ or 5% O₂ incubator for 3 days prior to ³H-proline labeling. The cells were labeled for 24 hr with ³H-proline at 5 μ Ci/ml. For SDS-PAGE analysis, samples equivalent to 2×10^5 cells /per lane were loaded.

3.4. Results

Characterization of Cultured Retinal EC and Pericytes

The morphology and phenotypic characteristics of the retinal EC and pericytes used in this study are shown in Figure 3.1. Retinal EC exhibited a cobblestone-like, contact-inhibited monolayer (Fig. 3. 1A). These cells were identified as endothelial by the uptake of DiI-ac-LDL, by reactivity with an antibody against von Willebrand factor (vWF), and by the absence of labeling with anti-smooth muscle alpha-actin IgG (Fig. 3. 1B, C, D, respectively). In contrast, pericytes exhibited an irregular morphology (Fig. 3. 1E),

characterized by a multilayered growth pattern without the "hill and valley" overgrowth typical of vascular smooth muscle cells. Moreover, these cells expressed smooth muscle alpha-actin (Fig. 3. 1F) but neither GFAP (a marker for astrocytes) nor the EC markers noted above (data not shown).

Effects of Hypoxia on the Proliferation of Retinal EC and Pericytes

Exposure to retinal EC or pericytes to low atmospheric concentrations of oxygen for 4 days led to an increase in cell growth, as reflected by an increase in ^3H -thymidine incorporation (Fig. 3. 2A) and cell counts (Fig. 3. 2B). At the 5% O_2 (pO_2 , 35 mmHg) examined, the cell number was approximately 1.5 fold of that of the normoxic controls after 4 days of incubation. For the pericytes, serum-free or 2% serum conditions were examined. Both the cell numbers and ^3H -thymidine incorporation increased under hypoxic condition in comparison to values obtained under normoxia (Fig. 3. 2C, D).

BrdU, an artificial thymidine analog, has also been used for measuring the synthesis of DNA. Cell nuclei that have incorporated the compound are recognized by staining with anti-BrdU antibody. One of the advantages of BrdU labeling over ^3H -thymidine incorporation is that S-phase cells can be seen in the context of the non-dividing cells. Fig. 3.3 shows an example of BrdU labeling data. Consistent with ^3H -thymidine incorporation experiments, DNA synthesis increased progressively when the cells were subjected to hypoxia in comparison to normoxia (Fig. 3. 3). The presence of increased S-phase cells (Fig. 3. 3B) is evidence of enhanced cell proliferation occurring in response to hypoxic treatment.

Effects of VEGF/bFGF on the Proliferation of Retinal EC and Pericytes Cultured under Hypoxic vs. Normoxic Conditions

Retinal EC exposed for 2 days to VEGF or bFGF exhibited an increased rate of cell proliferation under both hypoxia and normoxia (Fig. 3. 4A, B). However, the proliferative response to the two mitogens was increased under hypoxia relative to normoxia; the differential response between hypoxia and normoxia was larger with VEGF than with bFGF (Fig. 3. 4). ^3H -thymidine incorporation data are consistent with cell counts (not shown). Figure 3. 4C shows the mitogenic effect of a combination of bFGF and VEGF on the incorporation of ^3H -thymidine by retinal EC. The simultaneous addition of VEGF and

bFGF resulted in an enhanced mitogenic effect, however, synergy between these two growth factors was not observed in this assay (Fig. 3. 4C).

Retinal pericytes were exposed for 4 days to VEGF or bFGF under hypoxia or normoxia. Pericytes responded poorly to VEGF under both hypoxia and normoxia; however, bFGF stimulated pericytes to divide, particularly under hypoxic conditions (Fig. 3. 5A, B). Pericytes cultured at this condition showed low cell cycle and a low labeling index with ^3H -thymidine.

3-Dimensional Cultures

Retinal EC cultured on top of collagen gels grew to confluence and subsequently invaded the gels in the presence of either VEGF (10 ng/ml), bFGF (10 ng/ml) or the combination of both after 2 days incubation. In basic growth medium without VEGF or bFGF, EC formed a confluent monolayer and did not invade the collagen (Fig. 3. 6B). After the addition of VEGF or bFGF (Fig. 3. 6C, D, and E), some cells started differing in shape and orientation, and invaded the collagen gels. Figure 3. 6C is an enlarged phase-contrast view of cell invasion within a collagen gel. The plane of focus is beneath the surface monolayer (m). A capillary sprout (s) is indicated by an arrow. Cells of the monolayer have acquired a spindle shape and exhibit some degree of alignment in response to growth medium. bFGF, one of the best characterized angiogenic polypeptides (Folkman and Klagsbrun, 1986), is a potent angiogenic inducer. At 10 ng/ml, it caused numerous EC to invade the underlying collagen matrix (Fig. 3. 6D). However, the EC invasion induced by bFGF was minimal. It was noted that cells induced by VEGF migrated further, but were less numerous in comparison to those induced by bFGF. Exposure of 3-D cultures to combined VEGF and bFGF generated a significantly increased numbers of invasive cells and their cord-like structures (Fig. 3. 6F).

Analysis of Proteins Secreted by Retinal EC in the Presence or Absence of VEGF or bFGF

To compare the proteins secreted by retinal EC with or without stimulation by growth factors, I metabolically labeled the cells in culture as described in Methods. Figure 3.7 shows ^3H -proline-labeled proteins secreted into the culture media by bovine retinal EC. The biosynthetic profiles are similar between the growth factor-stimulated cells and control cells. The major ECM proteins are fibronectin, SPARC, collagen type I and collagen type

IV. However, the quantity of secreted proteins is enhanced in the growth factor-stimulated cells (Fig. 3. 7, lane 2, 4, and 5) relative to their controls. In addition, the cells cultured in hypoxic atmosphere secreted slightly more ECM proteins in comparison to those under normoxic conditions (Fig. 3. 7, lane 1 and 3). This study indicates that although these mitogens stimulate the cell cycle significantly, they do not change the phenotype of secreted ECM proteins. However, the quantities of these proteins appear to be increased.

3.5. Discussion

In this study, I have focused on how the proliferation of retinal EC and pericytes is affected by hypoxia - the leading cause of pathological retinal angiogenesis - and by VEGF and bFGF in the context of hypoxia.

The data have confirmed that hypoxia accelerates the proliferation of both retinal EC and pericytes. These results are consistent with similar studies performed with human umbilical vein EC (Kan et al., 1985; Nomura et al., 1995). In the experimental conditions used in the present study, serum in the culture medium was minimal (2-5 %FBS). The increased DNA synthesis and cell proliferation under low oxygen tension are likely a consequence of autocrine or paracrine stimulation by growth factors released from EC and pericytes. The most probable mitogenic factor in this case is VEGF, since VEGF expression is inducible in EC by low oxygen tension (Namiki et al., 1995; Liu et al., 1995; Aiello et al., 1995). bFGF expression has been shown not to be influenced by hypoxia (Kourembanas et al., 1990). Interestingly, the proliferation of pericytes also increased significantly under hypoxia, suggesting that pericytes may secrete elevated VEGF in an hypoxic environment. In fact, pericytes were demonstrated to express VEGF and *fms*-like tyrosine kinase 1 (*flt1*) in response to hypoxia (Nomura et al., 1995). Normally, pericytes maintain microvessels in a quiescent state. It is possible that hypoxia could turn pericytes from negative into positive modulators of angiogenesis by inducing them to secrete EC mitogens such as VEGF.

The FGFs are EC mitogens that have been shown to be distributed widely among tissues and cells (Folkman and Klagsbrun, 1987). Under normal conditions FGFs are inaccessible to the endothelium and remain cell-associated or sequestered in the basement membrane (Vlodavsky et al., 1987; Folkman et al., 1988). It is believed that growth factors like bFGF, acidic FGF, and retinal-derived growth factor are produced from ischemic or injured tissues. Because of the colocalization of both VEGF and bFGF in

ocular angiogenic disorders, it is expected that these mitogens are present in the vicinity of an angiogenic response in the retina. To address the issue of whether these two different angiogenic factors are acting synergistically upon retinal vascular EC under hypoxic conditions becomes important. VEGF or bFGF stimulates retinal endothelial cell proliferation, as shown in Fig. 3. 4. The cellular proliferative responses to the same cytokine increased significantly under hypoxia, due to the elevated expression of VEGF receptors (Thieme et al., 1995; Brogi et al., 1996) and increased expression for VEGF in retinal EC (Aiello et al., 1995). Although bFGF expression is not influenced by hypoxia, and information about the regulation of the FGF receptor by hypoxia is lacking, the increased VEGF and its receptors could in part explain the enhanced DNA synthesis in hypoxic EC stimulated by bFGF (Fig. 3. 4B). When these two cytokines were added together to EC, a higher ^3H -thymidine incorporation was observed under both normoxia and hypoxia (Fig. 3. 4C), but synergism was not seen, consistent with my studies on *Macaca* monkey retinal EC (Yan et al., 1996) and others (Yoshida et al., 1996).

However, the proliferative assays utilized 2-dimensional systems, in which the ECM component was initially deposited on the surface of cell culture polystyrene dishes, followed by the seeding of vascular cells on the surface. The use of angiogenic factors with a 3-dimensional assay of vessel growth and tube formation in vitro would be more similar to a situation in vivo (Madri et al., 1988). In fact, synergistic effects of VEGF and bFGF on proliferation and cord formation of bovine adrenal cortex capillary EC within collagen gels have been reported (Goto et al., 1993; Pepper et al., 1992). Therefore, an experiment was designed to test whether synergism exists between VEGF and bFGF applied to retinal EC in 3-dimensional culture. Figure 3. 6 showed that either VEGF or bFGF alone is able to stimulate retinal EC to invade a collagen gel. It was noted that VEGF-induced EC were located deeper in the collagen gel in comparison to those induced by bFGF, possibly because the migration stimulated by VEGF was greater than that by bFGF at the concentration used. In contrast, bFGF-induced proliferation is more effective than that stimulated by VEGF (Yoshida et al., 1996). When both molecules were added together, the cord/tube-like structures were much more numerous than structures generated by the factors singly. However, this synergistic effect on retinal EC in a collagen gel is not as great as that observed for non-retinal EC by other groups (Goto et al., 1993; Pepper et al., 1992).

ECM is an important regulatory effector of EC behavior during angiogenesis. Previous studies have identified that SPARC, type I collagen, and type VIII collagen are upregulated in EC undergoing angiogenesis in vitro (Luisa-Arispe et al, 1991a; 1991b). Because of the effects of VEGF and bFGF on retinal EC during angiogenesis, I investigated whether or not these growth factors could regulate ECM proteins under normoxia or hypoxia. The data in Fig. 3. 7 show increased levels of secreted ECM proteins after stimulation of cells with growth factors (lane 4 and 5). However, the profile of the secreted proteins was unchanged. SPARC was a dominant protein secreted by retinal EC (43 kDa), but was not regulated by either bFGF or VEGF (Fig. 3. 7). The specific set of stress proteins induced by hypoxia in cultured aortic EC was not observed in retinal cells (Zimmerman et al., 1991).

In summary, this study confirmed that hypoxia increased the DNA synthesis and cell proliferation of both retinal EC and pericytes. This effect was related in part to the increased expression of VEGF and VEGF receptors in these cells under hypoxia. Furthermore, VEGF and/or bFGF increased ³H-thymidine incorporation by retinal EC and pericytes, and this effect was more pronounced in an hypoxic environment. bFGF was a more effective stimulator for pericytes than was VEGF. In contrast, VEGF-induced endothelial migration in collagen gels was more effective than that induced by bFGF. Finally, these growth factors apparently did affect the synthesis of ECM proteins in retinal EC. Interestingly, the synergism of VEGF and bFGF on cell proliferation and cell invasion was readily apparent in the 3-dimensional culture assay but was not observed in the 2-dimensional assay.

Figure 3.1. Characterization of cultured bovine retinal capillary EC and pericytes. **(A)** A confluent, contact-inhibited monolayer of EC at passage 3 (phase-contrast photograph). **(B)** Binding and internalization of DiI-ac-LDL by cultured EC (4 hr incubation). **(C)** Monolayer of EC labeled for vWF by anti-vWF IgG in conjunction with the avidin-biotin-peroxidase technique. **(D)** Retinal EC incubated with anti-smooth muscle α -actin IgG show no reactivity, whereas pericytes exhibited smooth muscle α -actin fibers throughout the cytoplasm (arrow). **(E)** Pericytes in subconfluent culture exhibit irregular morphology at passage 2 (phase-contrast photograph). **(F)** Pericytes express smooth muscle α -actin IgG.

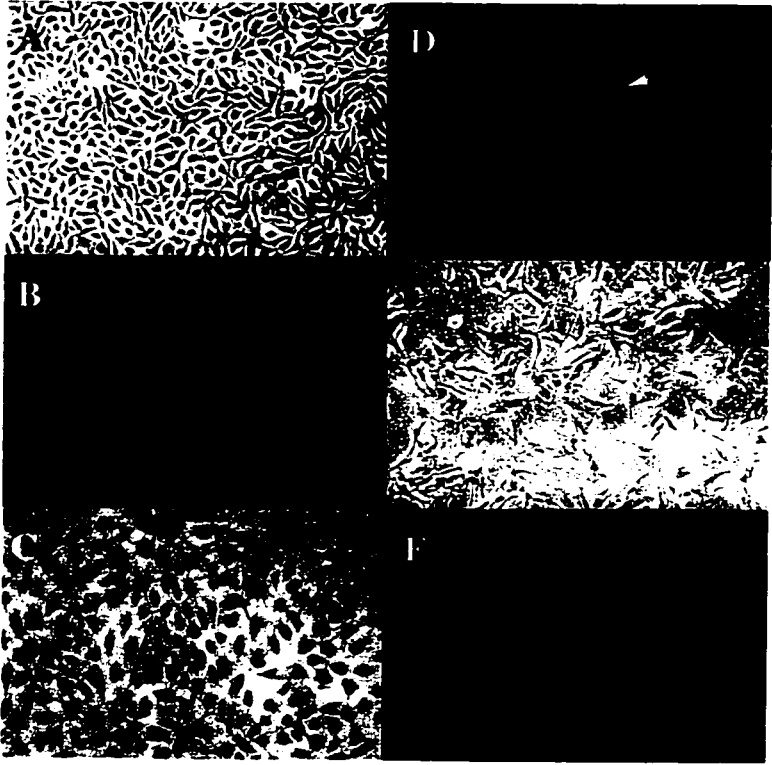


Figure 3.2. Comparative effects of hypoxia and normoxia on the proliferation of retinal EC and pericytes in culture.

Retinal EC and pericytes were prepared and plated as described in the Methods and allowed to grow for 20 hr prior to experiments. Culture media were changed to 5% FBS in DMEM for EC (**A, B**) and 0 or 2% FBS in DMEM for pericytes (**C, D**). Plates were placed immediately under either hypoxic (5% O₂, 5% CO₂) or normoxic (21% O₂, 5% CO₂) conditions as indicated. After 4 days incubation, cells were pulsed for 4 hr with ³H-thymidine at 2 μCi/ml (**A, C**) or counted (**B, D**). Bars represent the mean ± SD.

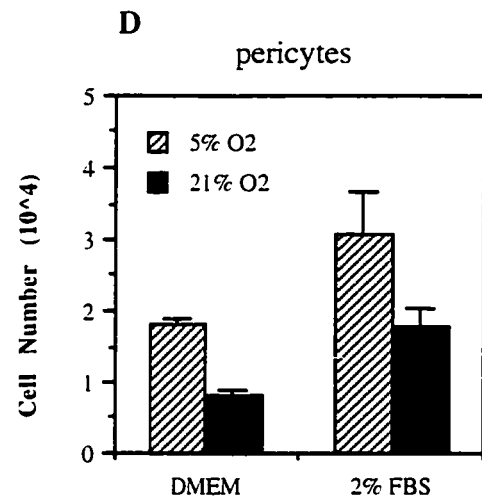
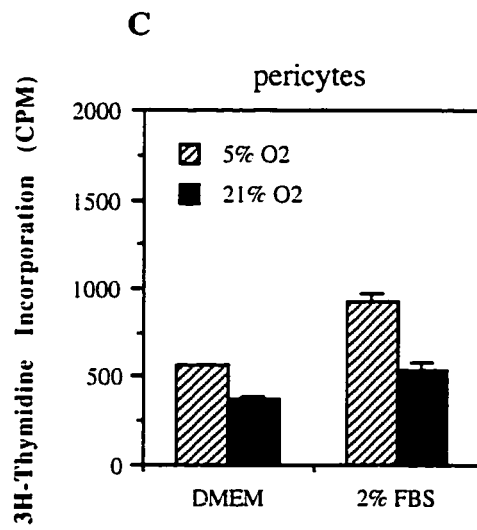
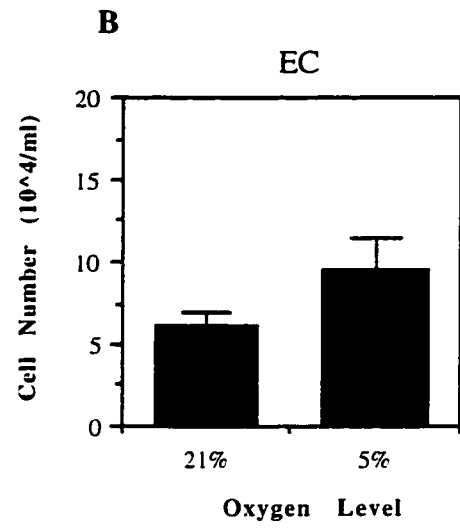
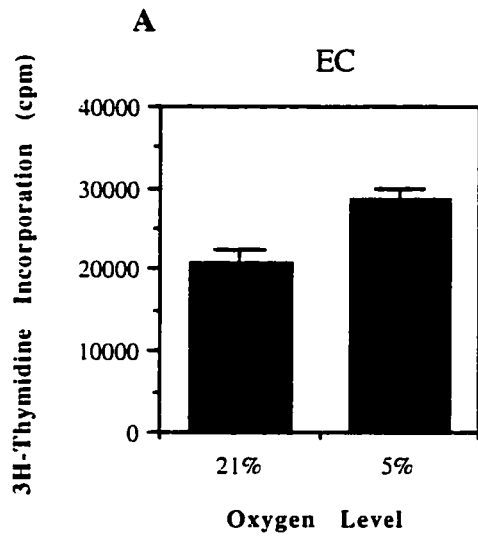


Figure 3.3. BrdU labeling of retinal EC cultured in hypoxic or normoxic incubators.

Retinal EC were seeded at a density of 10,000 cells/3 cm² on 35-mm dishes and grown for 24 hr before treatment. The cells were exposed to (A) normoxia atmosphere (21% O₂) or (B) hypoxic atmosphere (5% O₂) for 3 days in 5% FBS/DMEM. Cells were labeled with BrdU (10 μM) for 2 hr to measure DNA synthesis.

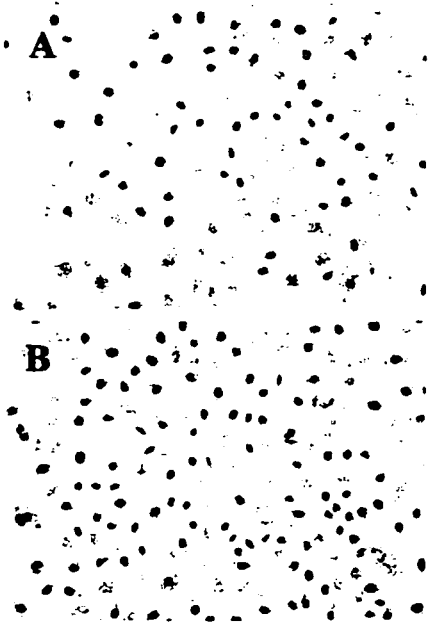


Figure 3.4. Mitogenic effects of VEGF and/or bFGF on DNA synthesis in retinal EC cultured under hypoxia and normoxia.

Retinal EC were plated as described in Methods. After 20 hr incubation with 10% FBS/DMEM, cells were treated with (A) VEGF (0, 2, 5, 10, 20, 30, 50 ng/ml), (B) bFGF (0, 2, 5, 10, 20 ng/ml), or (C) bFGF + VEGF (2+5, 5+10 ng/ml, respectively) in the presence of 5% FBS/DMEM, under either hypoxic (5% O₂, 5% CO₂) or normoxic (21% O₂, 5% CO₂) conditions. After 2 days' incubation, cells were pulsed for 4 hr with ³H-thymidine at 2 μCi/ml. Bars represent the mean ± SD.

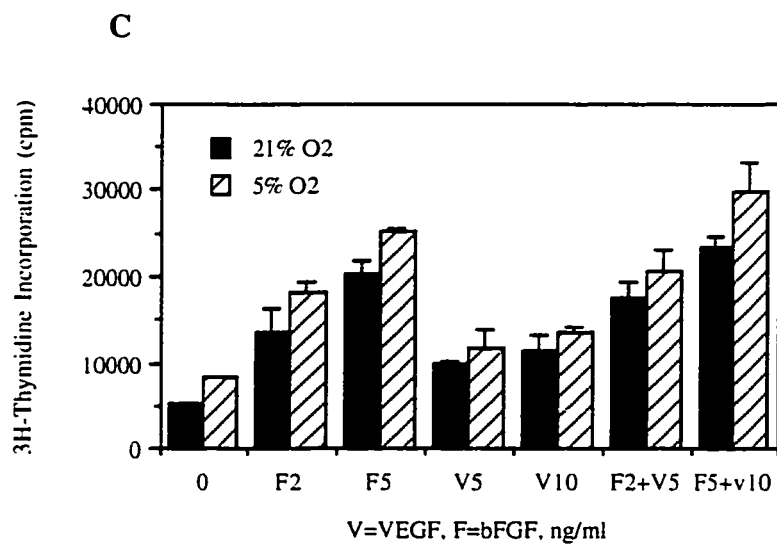
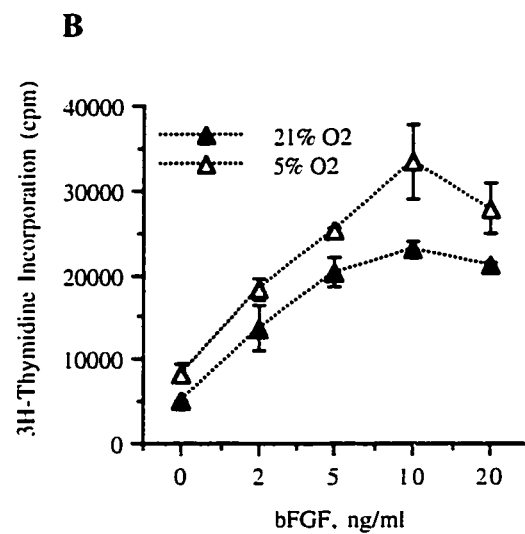
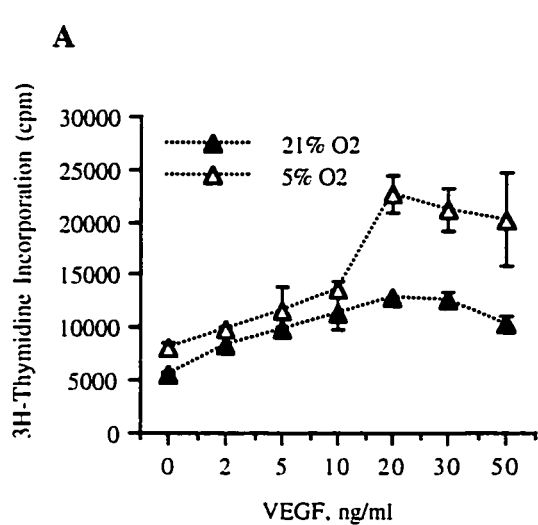
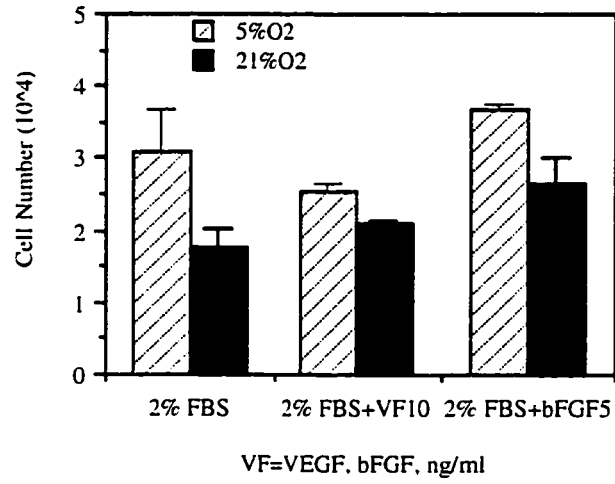


Figure 3.5. Effects of VEGF or bFGF on pericyte proliferation under hypoxia or normoxia in vitro.

Pericytes were plated as described in Methods. After 20 hr, cells were treated with VEGF (10 ng/ml) or bFGF (5 ng/ml) in the presence of 2% FBS/DMEM. Immediately, plates were placed under either hypoxic (5% O₂, 5% CO₂) or normoxic (21% O₂, 5% CO₂) conditions, respectively. After 4 days' incubation, cells were (A) counted, or (B) pulsed for 4 hr with ³H-thymidine at 2 μCi/ml. Bars represent the mean ± SD.

A



B

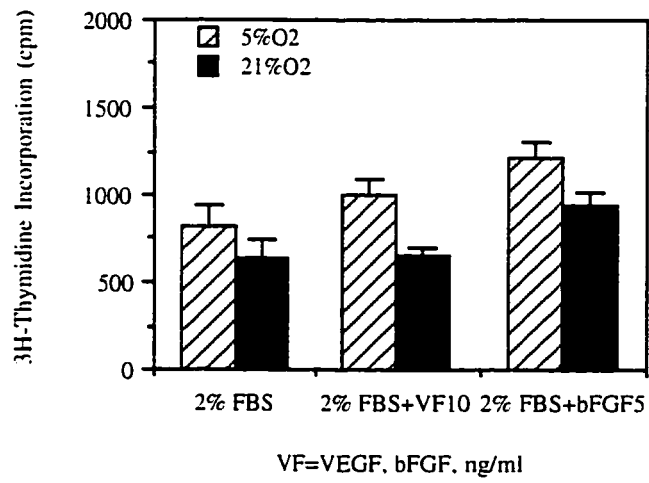


Figure 3.6. Effect of mitogens on retinal capillary EC grown on collagen gels (phase-contrast microscopy).

(A) Retinal EC were plated on the top of a collagen I gel for 2 hrs. A few cells started spreading on the gel. (B) Control EC form a monolayer in basic growth medium. The cells were closely apposed to each other and exhibited some degree of alignment in response to growth medium. (C) Shown is a phase contrast view under higher magnification of cell invasion within a collagen gel; the plane of focus is beneath the surface monolayer (m). The capillary sprout (s) is indicated by the arrow. (D) bFGF (10 ng/ml)-treated EC after 48 hr. Numerous EC change orientation from the confluent monolayer and invade beneath the monolayer. (E) VEGF (20 ng/ml)-treated EC after 48 hr. (F) VEGF (20 ng/ml)- and bFGF (10 ng/ml)-treated EC after 48 hr.

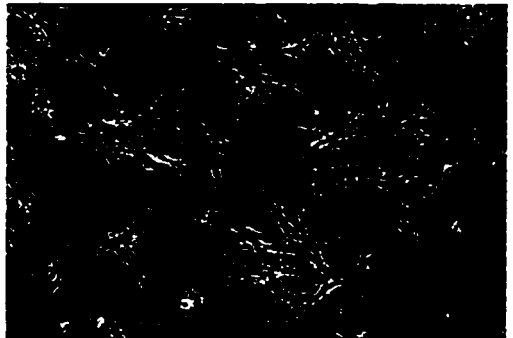
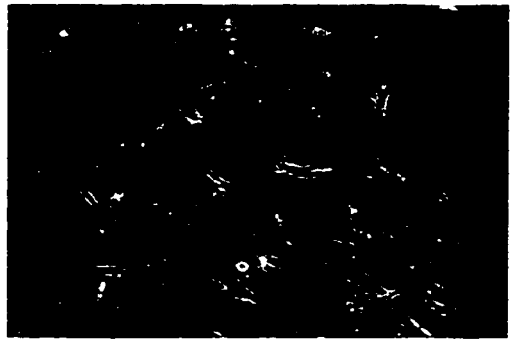
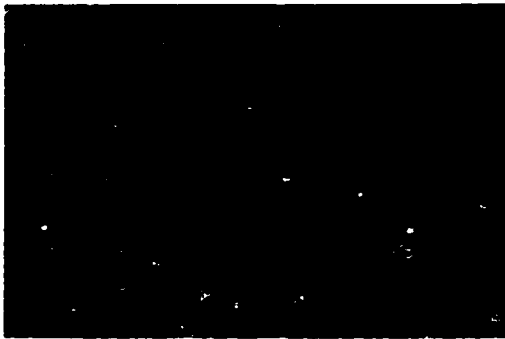
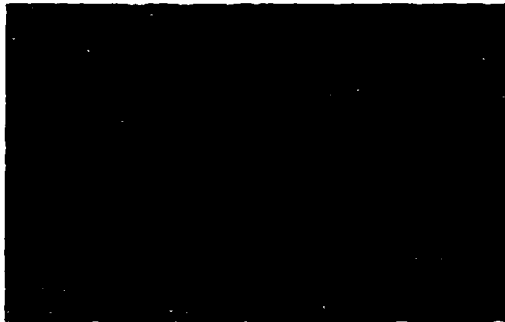
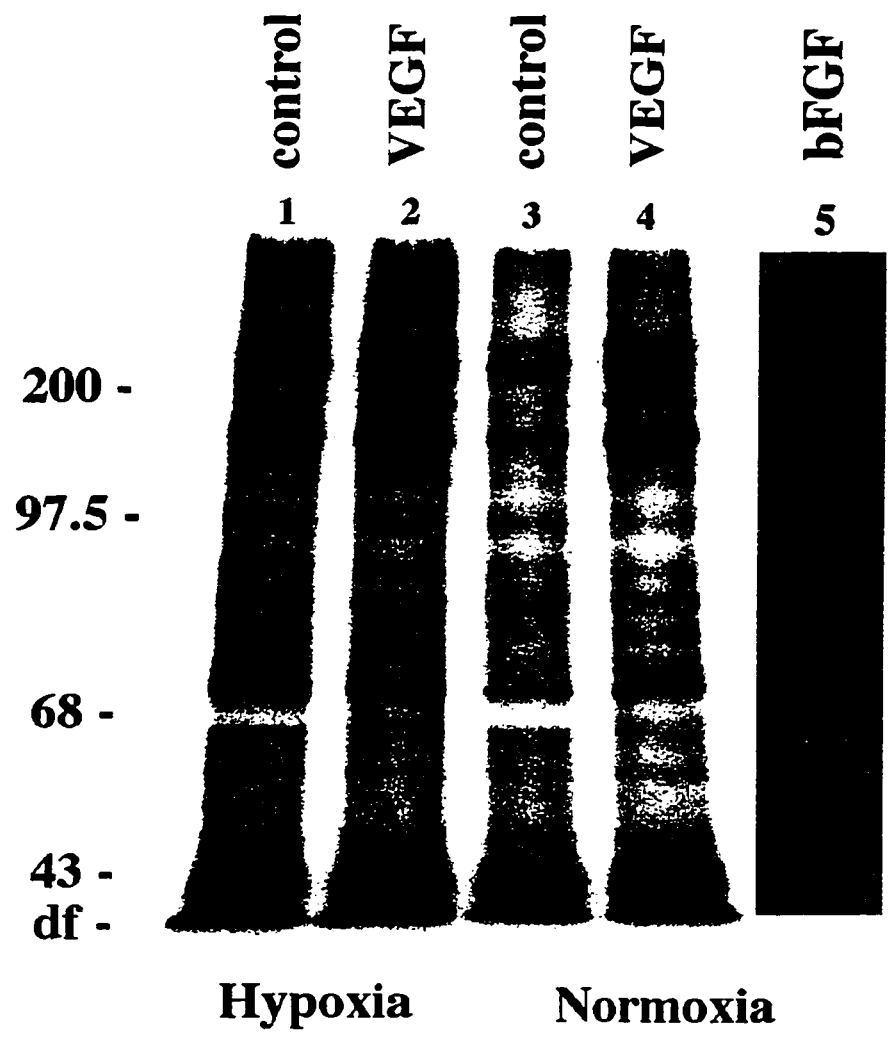


Figure 3.7. Comparison of proteins secreted by retinal EC before and after exposure to VEGF, bFGF, or hypoxia.

Cultured retinal EC were incubated as described in Methods. Cells were labeled with ^3H -proline for 24 hr in an hypoxic or normoxic incubator. Proteins were analyzed by SDS-PAGE on 4% / 7% acrylamide slab gels in the presence of 50 mM DTT. ^3H -proline labeled proteins were visualized by autoradiography. Loading is equivalent to 2×10^5 cells per lane. Hypoxic condition (lane 1, and 2); normoxic condition (lane 3, 4, and 5). Molecular weight standards are indicated by the arrowheads on the left: 200 kDa, 97.5 kDa, 68 kDa, and 43 kDa. df= dye front.



**CHAPTER 4: TGF- β 1 INDUCES APOPTOTIC CELL DEATH IN
CULTURED RETINAL ENDOTHELIAL CELLS BUT NOT
PERICYTES:ASSOCIATION WITH DECREASED EXPRESSION OF
P21^{WAF1/CIP1}**

4.1. Summary of Chapter 4

Transforming growth factor- β 1 (TGF- β 1) regulates a variety of cellular functions. In several types of cells, for example, it acts as a growth inhibitor and an inducer of apoptotic cell death. Although one of the important modulators in retinal vascular development and retinal neovascularization, the effects of TGF- β 1 on retinal microvascular cells are not fully defined. We have found that proliferation of both bovine retinal EC and pericytes was inhibited by TGF- β 1 in a concentration-dependent manner. However, only retinal EC lost viability after exposure to increasing concentrations of TGF- β 1 (up to 10 μ g/ml) in the presence of 2% fetal bovine serum. Dying EC exhibited the morphological and biochemical characteristics of apoptosis. Fragmented nuclei and chromatin condensation were apparent after staining with the fluorochrome Hoechst 33258 and the reagent ApopTag; moreover, gel electrophoresis of DNA from TGF- β 1-treated EC revealed degradation of chromatin into the discrete fragments typically associated with apoptosis. The addition of anti-TGF- β 1 neutralizing antibody abolished the apoptotic cell death induced by TGF- β 1. Since not all the EC in a given culture died after exposure to TGF- β 1, we separated the apoptosis-sensitive cells from those resistant to TGF- β 1-mediated apoptosis and determined the expression of several proteins associated with this apoptotic pathway. Apoptosis of EC mediated by TGF- β 1 was associated with a decreased level of the cyclin-dependent kinase inhibitor p21^{waf1/cip1}, relative to that observed in the apoptosis-resistant cells. In contrast, the translation product of the tumor suppressor gene p53 was increased in the TGF- β 1-treated, apoptotic cells. I thus propose that p21^{waf1/cip1} and p53 function in distinct pathways that are protective or permissive, respectively, for the apoptotic signals mediated by TGF- β 1.

4.2. Introduction to Chapter 4

Retinal angiogenesis plays a pivotal role in the development of retinal vasculature and pathological retinal neovascularization. The process of retinal angiogenesis is regulated through both stimulatory and inhibitory signals that include VEGF, bFGF, and TGF- β 1 (D'Amore, 1994). VEGF and bFGF are considered to be promoters of angiogenesis that regulate endothelial cell survival, proliferation, migration, and extracellular proteolytic activities (Alon et al., 1995; Montesano et al., 1986; Stone and Maslim, 1997). In contrast, the role of TGF- β 1 in angiogenesis is more complicated and has thus been controversial: TGF- β 1 has been described as angiogenic or anti-angiogenic, *in vivo* and *in vitro* (Phillips et al., 1993; Pierce et al., 1995; Madri et al., 1988). A current hypothesis is that the positive or negative regulation of angiogenesis by TGF- β 1 depends on the context of the experimental model or the stage of the angiogenic process under study (Pepper, 1997). TGF- β 1 is nevertheless a critical factor in vascular development, since TGF- β 1-deficient mice have defective vasculogenesis that results, in part, in abnormal capillary structure (Dickson et al., 1995).

The effects of TGF- β 1 on retinal angiogenesis are not entirely clear. Retinal microvasculature is composed of EC and pericytes. Interactions between these cells affect retinal neovascularization, a sequel common to diabetic retinopathy, retinopathy of prematurity, and other ocular retinopathies. TGF- β 1 is synthesized by most cells in a latent form that requires activation for interaction with cell-surface receptors. The mechanisms of activation of latent TGF- β 1 *in vivo* are not well-understood; however, studies *in vitro* have shown that interaction between EC and pericytes or smooth muscle cells leads to the activation of secreted TGF- β 1 (Antonelli-Orlidge et al., 1989). In mammals, the retinal microvasculature contains the highest number of pericytes (Shepro and Morel, 1993; Kohner et al., 1991). It is therefore possible that TGF- β 1, secreted locally by retinal microvascular cells, could affect the function of retinal capillary EC and pericytes. This cytokine might maintain vascular quiescence under normal conditions, since it inhibits EC proliferation and migration (Roberts and Sporn, 1989). Moreover, studies *in vivo* have demonstrated that TGF- β is localized in the retinal ganglion cell layer and pigment epithelium (Anderson et al., 1995; Lutty et al., 1993); further, elevated levels of TGF- β were detected in vitreous aspirates from patients with proliferative diabetic retinopathy (Gaudric et al., 1990; Esser et al., 1997). These and other studies (Tanihara et al., 1997; Orlidge and D'Amore, 1987; Sato and Rifkin, 1989) provide convincing evidence that

TGF- β 1 regulates certain functions of retinal EC and pericytes and is likely to mediate selected aspects of retinal neovascularization.

TGF- β 1 is a 25-kDa homodimeric polypeptide that exerts pleiotropic effects on cellular proliferation, cellular differentiation, and extracellular matrix production (Kingsley, 1994). The actions of TGF- β are clearly multifocal and depend on the target cell type, its associated substrate, and the presence of other growth factors. TGF- β 1 inhibits cell proliferation, including that of EC and pericytes, in the G1 phase of the cell cycle. Recent interest has been focused on the role of TGF- β 1 in cell death. For example, TGF- β 1 induced apoptosis in cultured human umbilical vein EC (Tsukada et al., 1995), bovine glomerular capillary EC (Choi and Ballermann, 1995), bovine adrenal microvascular EC (Mandriota and Pepper, 1997), hepatocytes (Fabregat et al., 1996), uterine epithelial cells (Rotello et al., 1991), and several other types of cultured cells (Yamamoto et al., 1996; Ohta et al., 1997; Iio et al., 1995; Landstrom et al., 1996; Lomo et al., 1995; Mathieu et al., 1995; Selvakumaran et al., 1994). However, the mechanisms of TGF- β 1-induced apoptosis among the different cell types appear to be dissimilar, although data on this point are limited.

Since TGF- β 1 is considered to be an integral cytokine in the retinal vasculature and endothelial apoptosis might play a significant role in retinal neovascularization, we have asked whether TGF- β 1 induces apoptotic death in cells cultured from the retinal microvasculature. We present evidence that TGF- β 1 inhibits the proliferation of both retinal EC and pericytes. However, TGF- β 1 induced specifically apoptotic cell death in retinal EC, but not in pericytes, in a concentration-dependent manner. Furthermore, apoptosis of retinal EC was associated with decreased expression of the cyclin-dependent kinase inhibitor p21^{waf1/cip1}, whereas the product of the tumor suppressor gene p53 was augmented in apoptotic EC, relative to levels in nonapoptotic cells. p21^{waf1/cip1} and p53 might thus act on distinct pathways that mediate the inhibition or stimulation, respectively, of apoptosis by TGF- β 1.

4.3. Materials and Methods

Cell Culture Conditions for Study of Apoptosis

Retinal microvascular EC were grown and maintained in DMEM containing 10% FBS, in the absence of retinal extract and heparin, in a humid atmosphere of 5% CO₂ at 37°C for 1-2 days prior to the experiments. Cells were detached by a rinse with

$\text{Ca}^{+2}/\text{Mg}^{+2}$ -free PBS (Sigma) followed by exposure to 0.05% trypsin-0.02% EDTA (Gibco). Detached cells were resuspended in DMEM containing 10% FBS and antibiotics, and were plated on fibronectin-coated dishes or wells for 24-48 hr (all cells were attached and spread within this interval.) Subsequently, the culture medium was changed to 2% FBS/DMEM containing various concentrations of TGF- β 1 (R&D Systems, Minneapolis, MN), or in 2% FBS/DMEM alone, for 24-48 hr. Pericytes plated on plastic wells were treated identically.

DNA Synthesis Assay

Cells were plated in 24-well plates at a density of 2×10^4 EC or 1×10^4 pericytes/well in 10% FBS in DMEM and were grown for 48 hr. The culture medium was changed to 2% FBS in DMEM, and TGF- β 1 was added subsequently. Control cultures were grown in DMEM containing 2% FBS only. Cultures were incubated for 48 hr and were exposed to 2 $\mu\text{Ci/ml}$ [methyl- ^3H]-thymidine (81.3 Ci/mmol, 1 mCi/ml; New England Nuclear, Boston, MA) for the last 4 hr. They were washed three times with PBS, incubated in ice-cold 10% trichloroacetic acid for 1 hr, washed twice with anhydrous ethanol at -20°C , and air-dried. 300 μl of NaOH (0.2N) was added to each well for 30 minutes at 68°C . The cpm incorporated into DNA were quantified by liquid scintillation counting in 3 ml Ecolume (ICN, Irvine, CA).

Cell Viability Assays

Determination of cell viability was done by a trypan blue-exclusion assay. Cells were plated and treated as described above. Attached cells were treated with trypsin, resuspended, and incubated with trypan blue dye for 2-5 min. Live cells were counted by hemacytometer.

Blocking experiments were performed on retinal EC plated on fibronectin (FN)-coated 48-well plates in DMEM containing 10% FBS for 48 hr. The medium was changed to DMEM with either 2% or 5% FBS. TGF- β 1 (2 ng/ml) or TGF- β 1 (2 ng/ml) + anti-TGF- β neutralizing antibody (R&D Systems, 7 $\mu\text{g/ml}$) was added simultaneously to different wells. Control wells contained DMEM with 2 or 5% FBS only. After a 24 hr incubation, a cell viability assay was performed as described above.

Double-Staining with ApopTag and Hoechst 33258 Dye

EC in 2% FBS/DMEM were incubated on FN-coated, 35-mm dishes for 24 hr, in the presence or absence of TGF- β 1 (4 ng/ml). Cells were fixed with 2% paraformaldehyde in PBS and were rendered permeable with 0.5% Nonidet P-40 (NP-40) for 5 min. The cells were next incubated with digoxigenin-dUTP terminal dioxynucleotide transferase and were subsequently labeled with fluorescein-conjugated antibody to digoxigenin, according to the manufacture's protocol (ApopTag plus, In Situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD). Morphological changes in the nuclear chromatin of cells undergoing apoptosis were also detected by a counterstain with Hoechst 33258 flurochrome (4 μ g/ml in distilled water, Molecular Probes Inc., Eugene, OR) for 10 min at room temperature. The slides were mounted with 80% glycerol and were photographed under a Nikon fluorescence microscope. Detached cells in TGF- β 1-treated dishes were collected, pelleted, fixed in 2% paraformaldehyde for 20 min, and washed with PBS. The cells were resuspended in PBS at 10^6 cells/100ul, 20 ul of which was pipetted onto one of several slides. The cells were stained with ApopTag and Hoechst 33258 as described above and were photographed.

Gel Analysis of DNA Fragmentation

Cultured retinal EC were collected with a cell scraper, centrifuged (500 x g, 5 min), and washed with cold PBS. The cell pellet was resuspended in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.2% Triton X-100, pH 7.5). After a 30 min incubation on ice, the lysate was centrifuged at 13,000 g for 20 min at 4°C. The supernate was collected and was incubated with RNAase A (50 μ g/ml) for 30 min at 37°C. DNA was purified by phenol-chloroform extraction and by precipitation at -70°C for 24 hr after addition of 2 volumes of ice-cold ethanol and 1/10 volume of 3M sodium acetate (pH 5.3). The DNA was subsequently washed with cold 70% ethanol, air-dried, and dissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.5). The sample was resolved by electrophoresis in a 1.5% agarose gel. DNA was visualized by staining with ethidium bromide (0.5 μ g/ml), and the gel was photographed under UV light with a Polaroid camera.

Western Blot Analyses

Cells were lysed in a NP-40 buffer (0.5% NP-40, 10% glycerol, 1mM DTT, 2.5 mM ethylene glycol bis (β -aminoethyl ether)-tetraacetate (EGTA), 5 mM EDTA, 150 mM NaCl, 50 mM Hepes, (pH 7.4), 1 mM NaF, and 0.5 mM Na vanadate) containing a

complete protease inhibitor cocktail (Boehringer Mannheim Biochemical, Indianapolis, IN). Protein concentrations were determined by a Bradford assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. Cell lysates (20 μ g protein) were solubilized in SDS-PAGE sample buffer and were heated for 5 min at 100°C with 50 mM DTT. Proteins were resolved by SDS-PAGE on 4 /15% polyacrylamide minigels and were electrotransferred onto nitrocellulose membranes (0.45- μ m pore size). The blots were stained with 0.1% amido black (in 10% acetic acid and 20% methanol) to verify equal loading and transfer efficiency. Blots were blocked with 5 % nonfat dry milk in PBS containing 0.05% Tween 20 overnight at 4°C and were probed with 1 μ g/ml mouse anti-human p53 monoclonal antibody (Pharmingen, San Diego, CA) and 1 μ g/ml mouse anti-human WAF1(ab-1) monoclonal antibody (Oncogene Research Product, Cambridge, MA) for 2 hr at room temperature. After several washes in PBS with 0.05% Tween 20, signals were detected by horseradish peroxidase-conjugated secondary goat anti-mouse antibody at a dilution of 1:2000 (Bio-Rad Laboratories, Richmond, CA) for 45 min at room temperature. After extensive washes, electrochemical luminescence was developed (Amersham). Blots were exposed to RP X-Omat film (Eastman Kodak, Rochester, NY). The blot was stripped with stripping buffer (100 mM β -mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), and 2% w/v SDS), and anti-enolase IgG (a gift from Dr. Lindsay Miles, Scripps Institute and Research Foundation, La Jolla, CA) was used to reprobe the blot as an internal control.

4.4 Results

Effect of TGF- β 1 on Cell Proliferation and Viability

After approximately 20 hr of culture in the presence of TGF- β 1, we observed a significant number of detached retinal EC. 3 H-Thymidine incorporation assays indicated that TGF- β 1 inhibited DNA synthesis of EC in a concentration-dependent manner (Fig. 4. 1A). We examined cell viability by trypan blue assay and found that the number of live cells was decreased significantly as a function of increasing concentration of TGF- β 1 (Fig. 4.1B), in agreement with the data shown in Fig. 4. 1A. Decreases in both DNA synthesis and cell numbers were likely due to 1) temporary arrest of the cell cycle, or 2) induction of cell death (possibly apoptosis). A TGF- β 1-blocking experiment was performed first to ensure that the observed effects on EC were indeed due to TGF- β 1. Retinal EC were

grown for 24 hr on fibronectin-coated wells in DMEM with 10 % FBS for 24 hr; subsequently, the concentration of FBS was reduced to 2%, and TGF- β 1 and TGF- β 1 plus anti-TGF- β 1 neutralizing antibody were added to different wells. As shown in Figure 4. 2, the anti-TGF β 1 antibody abrogated cell death, as cell numbers in the presence of the antibody were nearly identical to those of control cells, and their morphology was similar to that of control cells (data not shown). The experiment was also repeated in 5% FBS, with identical results (not shown).

In contrast, no detached cells were observed in pericyte cultures after addition of TGF- β 1 (up to 10 ng/ml for 2 days). Incorporation of ^3H -thymidine by pericytes was decreased in a concentration-dependent manner after a 48 hr incubation with TGF- β 1 (Fig. 4.3A). However, simultaneous experiments showed no significant change in cell number as a function of increasing concentration of TGF- β 1 (Fig. 4. 3B). The data indicate that DNA synthesis in pericytes was inhibited by TGF- β 1, without appreciable changes in cell number over a period of 48 hr. These results contrast sharply with those observed for EC. However, after prolonged periods of culture in the presence of TGF- β 1(1 wk), decreases in pericyte cell number were seen (not shown).

TGF- β 1-Induced Cell Death Occurs by Apoptosis

After retinal endothelial monolayers were incubated with TGF- β 1 for 20 hrs, many cells exhibited shrinkage, nuclear and cytoplasmic condensation, membrane blebbing, and loss of cell-cell contact. Subsequently, the cells began to break apart into small apoptotic bodies, became detached, and floated in culture dishes (Fig. 4. 4). To observe the condensed and fragmented nuclei and to identify potentially apoptotic cells, we performed double labeling with Hoechst 33258 dye and ApopTag reagent (Fig. 4. 5). The images on the left side of Figure 4. 5 show Hoechst staining on control cells (A), TGF- β 1-treated cells (B), and detached cells after TGF- β 1 treatment (C). ApopTag labeling of the same fields is shown in a-c (Fig.4. 5). Arrows in (B) indicate the brightly-staining, fragmented nuclei that were also labeled with the ApopTag reagent (b). These profiles were not seen in control cells (A, a). Anti-TGF- β 1 neutralizing antibody blocked the cell fragmentation induced by TGF- β 1 (not shown). Detached, floating cells collected from the cultures that had been incubated with TGF- β 1 are shown in C. All the detached cells exhibited fragmented and condensed nuclei (C) and were stained with ApopTag (c). Few intact nuclei were observed in the collected, detached cells. Analysis of DNA from TGF- β 1-treated EC

revealed fragmentation of chromatin into nucleosomal ladders (Fig. 4. 6). These data were indicative of apoptosis, or programmed cell death, in retinal EC.

Interestingly, not all of the retinal EC were sensitive to TGF- β 1-induced apoptosis. We removed the detached, apoptotic cells and the apoptotic cells which were rounded but still attached to the substrate. The remaining, attached EC were incubated with Hoechst 33258 dye and ApopTag reagent. Almost all the remaining cells showed intact nuclei and were not labeled by ApopTag. This population thus appeared to be resistant to TGF- β 1-mediated apoptosis (Fig. 4. 4 C). Moreover, these cells exhibited a change in morphology after exposure to TGF- β 1 in the presence of 2% FBS. Despite a more elongate shape, relative to untreated retinal EC, they lacked smooth muscle- α -actin and exhibited uptake of DiI-ac-LDL (data not shown).

Although some retinal EC with apoptotic morphology were found in control cultures (likely a consequence of the decrease in FBS from 10% to 2%), the number was insignificant relative to that seen in cultures treated with TGF- β 1.

Expression of p53 and p21^{waf1/cip1} Proteins in EC and Pericytes

To ask whether the apoptosis induced by TGF- β 1 was associated with changes in the expression of p53 and/or p21^{waf1/cip1}, we made cell extracts from either control or TGF- β 1-treated EC. Detached cells (TGF- β 1 apoptosis-sensitive cells), as well as attached cells (TGF- β 1 apoptosis-resistant cells), from TGF- β 1-treated cultures were analyzed separately. Monoclonal antibodies against p53 and p21^{waf1/cip1} were used simultaneously on the same nitrocellulose blot. The apoptotic EC exhibited increased expression of p53 protein relative to that of attached cells and/or untreated control cells (160% over control) (Fig. 4. 7A). Attached cells contained similar levels of p53 in comparison to the control cells. Interestingly, p21^{waf1/cip1} was decreased significantly in apoptotic detached cells, relative to control cells and/or attached cells (5.5% of control). However, the attached cells expressed essentially the same amount of p21^{waf1/cip1} as control cells. Staining with amido black (not shown) and immunoblotting with anti-enolase IgG (Fig. 4. 7A) confirmed nearly equal loading of cell-extract protein (20 μ g/lane).

The same experiments were performed with pericytes (Figure 4. 7B). p53 and p21^{waf1/cip1} proteins remained relatively unchanged between the control (untreated) and TGF- β 1 (2 and 7 ng/ml, respectively)-treated samples. p21^{waf1/cip1} was barely

detectable, and p53 was relatively low, in comparison to that of EC. Probing with anti-enolase IgG showed nearly equal loading of all the samples (20 µg/lane).

4.5. Discussion

Retinal microvascular EC are unique among vascular endothelia (Thieme et al., 1995; Schor and Schor, 1986), as they are subject to regulation by a) the various cytokines, ECM components, and matricellular proteins produced by retinal cells (including neurons) (Tanihara et al., 1997; Yan et al., 1998); b) by interactions with pericytes, astrocytes, and Muller cells (Jiang et al., 1995; Puro, 1995); and c) by local changes in oxygen (Chan-Ling et al., 1995). Although the propagation of pure cultures of retinal microvascular EC in vitro has been difficult, these cells, isolated successfully from several species, have been studied with respect to their biochemical properties, interaction with cells such as pericytes and astrocytes, and responses to exogenous factors and matrix proteins, all of which contribute to the process of retinal neovascularization.

In this report, we demonstrate that TGF-β1 induces apoptotic cell death in cultured retinal EC, but not in retinal pericytes. Apoptosis is obvious in retinal EC after 20 hr of incubation with exogenous TGF-β1 as a function of concentration. Since it is known that serum deprivation induces endothelial apoptosis (Araki S et al., 1990a; 1990b), we attempted to minimize this effect by performing our experiments in 2% FBS, in which both retinal EC and pericytes are viable. The extent of cell death in 2% vs. 10% FBS was insignificant, relative to that observed in the TGF-β1-treated cells. We also found that the degree of apoptosis induced by TGF-β1 was slightly lower in 5% vs. 2% FBS. This result indicates that the apoptosis we described is not due to serum deprivation, although serum might compensate partially for some of the apoptotic effects induced by TGF-β1.

Our data also show that TGF-β1 inhibited ³H-thymidine incorporation in both retinal EC and pericytes. The mechanism(s) of TGF-β-induced antiproliferation is (are) not fully understood, but for many cell types it is clear that TGF-β1 causes growth arrest in the G1 phase of the cell cycle (Massague, 1990; Sporn and Roberts, 1992). This arrest is due in part to the regulation of G1 cyclins/cyclin-dependent kinases by induction of their inhibitors p21^{waf1/cip1}, p27^{Kip1}, p16, and p15^{INK4B}, and by the reduction of c-myc (Koff et al., 1993; Ewen et al., 1993; Geng et al., 1993). Another target gene for the growth-inhibitory activity of TGF-β1 is p53 (Suzuki et al., 1992; Raynal et al., 1994; Landesman et al., 1997). We investigated whether p53 and p21^{waf1/cip1} were involved in

the regulation of retinal endothelial apoptosis for the following reasons: 1) both proteins are involved in growth inhibition; 2) p53 mediates some, but not all, forms of apoptosis (Clarke et al., 1994, Strasser et al., 1994); 3) some forms of p53-induced apoptosis are associated with increased expression of p21^{waf1/cip1} (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), and 4) p21^{waf1/cip1} can block apoptosis during cell differentiation (Wang and Walsh, 1996; Shi et al., 1994).

Retinal EC were not uniformly sensitive to TGF- β 1-mediated apoptotic cell death. In the presence of 2 ng/ml TGF- β 1 in 2% FBS/DMEM, approximately 30% of the cells were apoptotic. The TGF- β 1-sensitive cells (detached) exhibited significantly reduced levels of p21^{waf1/cip1}; in contrast, elevated levels of p21^{waf1/cip1} were found in the TGF- β 1-resistant cells (attached) in comparison to detached cells (Fig. 4. 7A). Induction of p21^{waf1/cip1} thus appears to be correlated with a resistance to TGF- β 1-mediated apoptosis in retinal EC. It is also possible that, in the presence of TGF- β 1, p21^{waf1/cip1} protects EC that are growth-arrested from cell death. In fact, this hypothesis appears to be valid for myocyte differentiation in low serum (Wang and Walsh, 1996), and similar observations have been reported in other cell types in vitro (Shi et al., 1994; Poluha et al., 1996). The change in morphology that we observed in the apoptosis-resistant EC cultured in TGF- β 1 was in fact reminiscent of studies by Arciniegas et al. (1992), who found that TGF- β 1 promoted the differentiation of EC into smooth muscle-like cells in vitro.

The p53 protein is a sequence-specific DNA-binding protein that acts as a transcription factor, and the gene encoding p21^{waf1/cip1} contains a p53-responsive element. Exposure of cells to ionizing radiation or to DNA-damaging agents results in the accumulation of high levels of p53 protein, and in the subsequent stimulation of p21^{waf1/cip1} and cell cycle arrest, presumably for repair of the damaged DNA or for apoptosis. In contrast, TGF- β 1 can effect rapid transcriptional induction of p21^{waf1/cip1} through a p53-independent pathway (Datto et al., 1995). Nevertheless, p53 does appear to mediate growth inhibition induced by TGF- β 1 (Raynal et al., 1994; Reiss et al., 1993; Ewen et al., 1995; Mogi et al., 1994), e.g., TGF- β 1 controls the intracellular localization as well as the phosphorylation pattern and the stability of p53 protein (Landesman et al., 1997; Suzuki et al., 1992). Both p53 and p21^{waf1/cip1} thus participate in one or more pathways that transduce signals via TGF- β 1 receptors. It has been shown that proliferating cells which subsequently became apoptotic exhibited an induction of p53 followed by an increase in p21^{waf1/cip1} (Stromblad et al., 1996; Symonds et al., 1994; Gansauge et al.,

1997; Boudreau et al., 1996). The induction of apoptosis might result from concomitant but conflicting signals, such as stimulation of DNA synthesis and growth arrest (Stromblad et al., 1996). In our experimental system, however, the retinal cells are, for the most part, growth-inhibited, and the accumulation of p53 was not associated with activation of p21^{waf1/cip1} in cells undergoing TGF- β 1-mediated apoptosis. p53 might therefore regulate apoptosis in these cells through a stimulation of bax and a diminution of the bcl-2 pathway, since p53 is a transcriptional repressor of bcl-2 and an activator of bax (Miyashita et al., 1994a; 1994b). Alternatively, p53 might affect regulators other than p21^{waf1/cip1}, bcl-2, or bax. As there are no anti-bcl-2 antibodies that crossreact with the bovine protein, we were unable to determine the bcl-2 : bax ratio in retinal cells exhibiting elevated levels of p53.

Other functions of TGF- β 1 might also contribute to the apoptotic response that we observed. For example, interactions between cells and extracellular matrix proteins are critical for cell survival (Frisch and Francis, 1994; Meredith et al., 1993; Boudreau et al., 1995), and TGF- β 1 regulates deposition and degradation of matrix proteins and integrin expression on EC (Massague, 1990; Lyons and Moses, 1990). Therefore, detachment of retinal EC might be due in part to changes in extracellular matrix mediated by TGF- β 1. The distribution of TGF- β 1 receptors on retinal capillary EC should also be considered. Expression of TGF- β receptors (type I, type II, and betaglycan) is constitutive in most cell types (Massague, 1992), and types I and II have been identified as signaling receptors for TGF- β 1. A recent report indicated that the type II receptor mediated the anti-proliferative effect, whereas the type I receptor was responsible for the matrix-biosynthetic response of EC to TGF- β 1 (Sankar et al., 1996). Determination of both the levels and types of TGF- β 1 receptors on retinal EC will provide valuable insight into the apoptotic effect that we have described here.

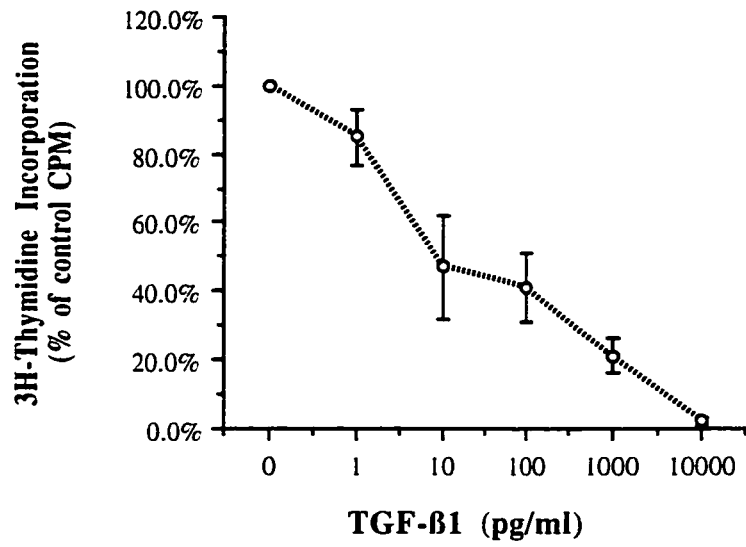
One of the unique features of retinal capillaries is the unusually high ratio (1:1) of pericytes to EC (Kohner et al., 1991). Both their proximity and interactive capabilities predict functional, intercellular regulation. When we investigated the proliferative response of pericytes to TGF- β 1, we found that proliferation was inhibited by TGF- β 1, but that pericytes were resistant to TGF- β 1-mediated apoptosis. This result might be due in part to the lack of response of the p53 and p21^{waf1/cip1} genes to TGF- β 1 in pericytes. Furthermore, p53 and p21^{waf1/cip1} do not appear to be critical for the inhibition of pericyte cell growth by TGF- β 1.

In this study we have shown that retinal microvascular EC undergo a programmed cell death in response to TGF- β 1. The mechanisms underlying this phenomenon likely involve multiple pathways, one of which is associated with decreased levels of the cyclin-dependent kinase inhibitor p21^{waf1/cip1}. The induction of retinal endothelial apoptosis by TGF- β 1 could lead to the regression of retinal neovascular capillaries and could therefore form the basis for potential therapeutic strategies for neovascular retinopathy.

Figure 4.1. Effect of TGF- β 1 on retinal EC: DNA synthesis and cell proliferation.

Retinal EC (2×10^4) were incubated in 10% FBS/DMEM for 48 hr. Subsequently, media were changed to 2% FBS in DMEM containing TGF- β 1 (0, 1, 10, 100, 1000, or 10,000 pg/ml) for 48 hr. Cells were pulsed during the last 4 hr with ^3H -thymidine at 2 $\mu\text{Ci/ml}$ (**A**), or were counted as trypan blue-negative cells by hemacytometer (**B**). Data are mean \pm SD; values were normalized to control counts or to cpm incorporated by controls cultured in 2% FBS/DMEM without TGF- β 1.

A



B

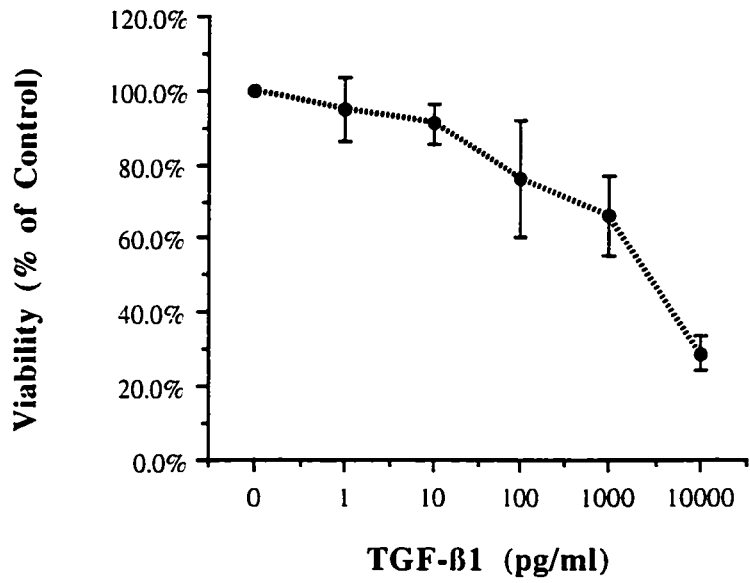


Figure 4.2. Anti-TGF- β 1 neutralizing antibody blocks the inhibitory effect on EC proliferation induced by TGF- β 1.

Retinal EC were plated in 48-well culture plates coated with fibronectin. After adherence, cells received 2% FBS/DMEM (control); TGF- β 1 (2 ng/ml) in 2% FBS/DMEM; or TGF- β 1 (2 ng/ml) + anti-TGF- β 1 neutralizing antibody (7 μ g/ml) in 2% FBS/DMEM. After 24 hr, live cells were counted by hemacytometer. Data are mean \pm SD.

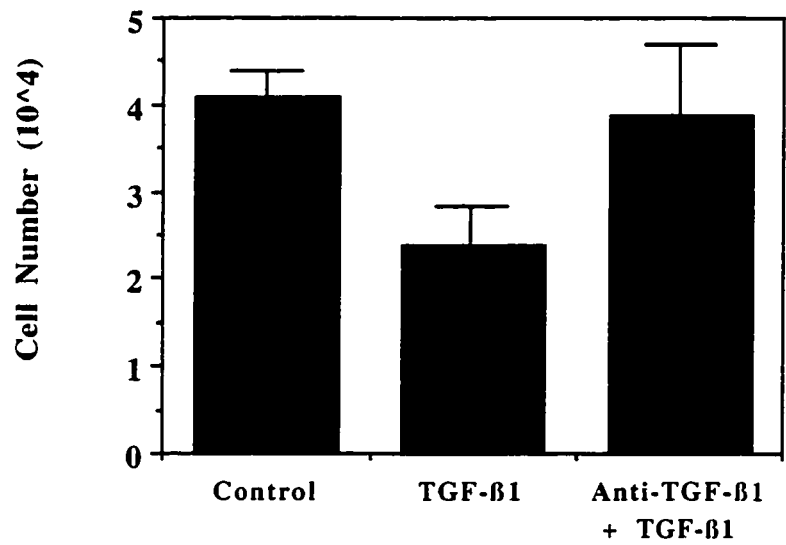


Figure 4.3. Effect of TGF- β 1 on DNA synthesis and cell proliferation in retinal pericytes.

Retinal pericytes (1×10^4) were incubated in 10% FBS/DMEM for 24 hr. Subsequently, media were changed to 2% FBS in DMEM containing TGF- β 1 (0, 1, 10, 100, 1000, or 10,000 pg/ml). After an incubation of 48 hr, cells were pulsed for the last 4 hr with ^3H -thymidine at 2 $\mu\text{Ci/ml}$ (A) or were counted by hemacytometer (B). Data are mean \pm SD.

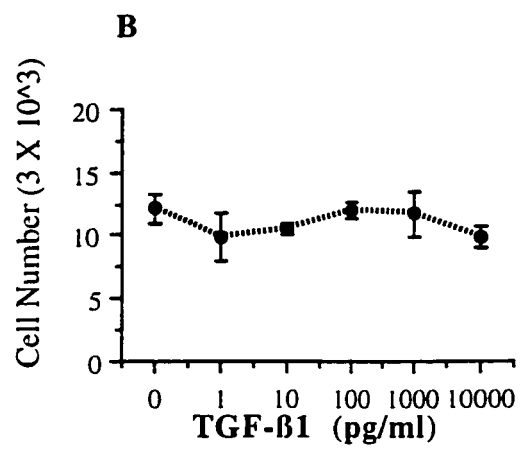
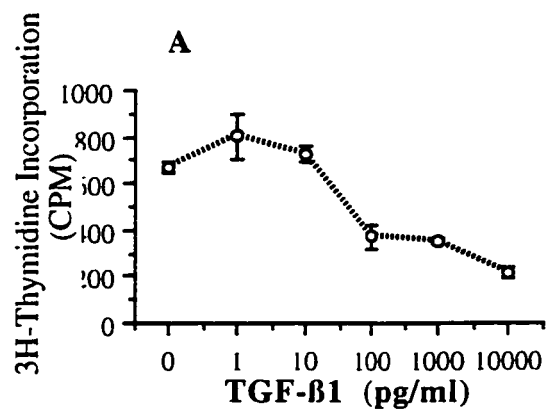


Figure 4.4. Changes in morphology of retinal EC after treatment with TGF- β 1. (A) EC in 2% FBS/DMEM for 24 hr. (B) Cells treated for 24 hr with 4 ng/ml TGF- β 1 in 2% FBS/DMEM. (C) TGF- β 1 non-apoptotic EC: cells were treated for 24 hr with 4 ng/ml TGF- β 1 in 2% FBS/DMEM. Detached cells, and cells that were rounded but partially attached, were removed by several washes. Arrows indicate elongated cells that have lost their typical cobblestone morphology.

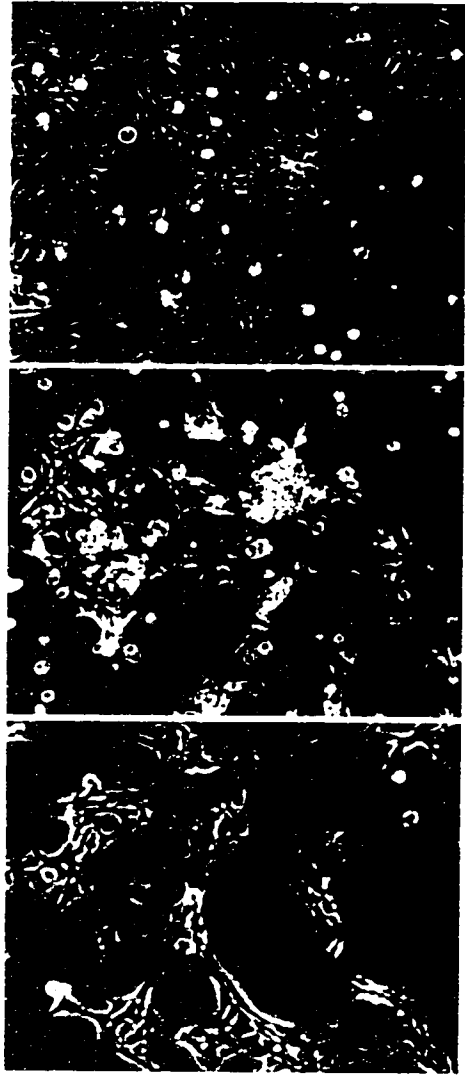


Figure 4.5. TGF- β 1 induces DNA fragmentation in retinal EC.

(**A**) Control EC in 2% FBS/DMEM for 24 hr. (**B**) EC treated with TGF- β 1(4 ng/ml) in 2% FBS/DMEM for 24 hr. (**C**) Detached cells (**B**) were collected, pelleted, and spread on slides. DNA was stained with Hoechst 33258 fluorochrome (**A,B** and **C**). The fluorescence micrographs show condensed and fragmented apoptotic cell nuclei (arrows in **B** and **C**). (**a, b and c**) ApopTag staining of the same fields shown in **A, B**, and **C**. Arrows at **b** and **c** indicate apoptotic cells that correspond to the fragmented DNA detected by the Hoechst 33258 dye in **B** and **C**.

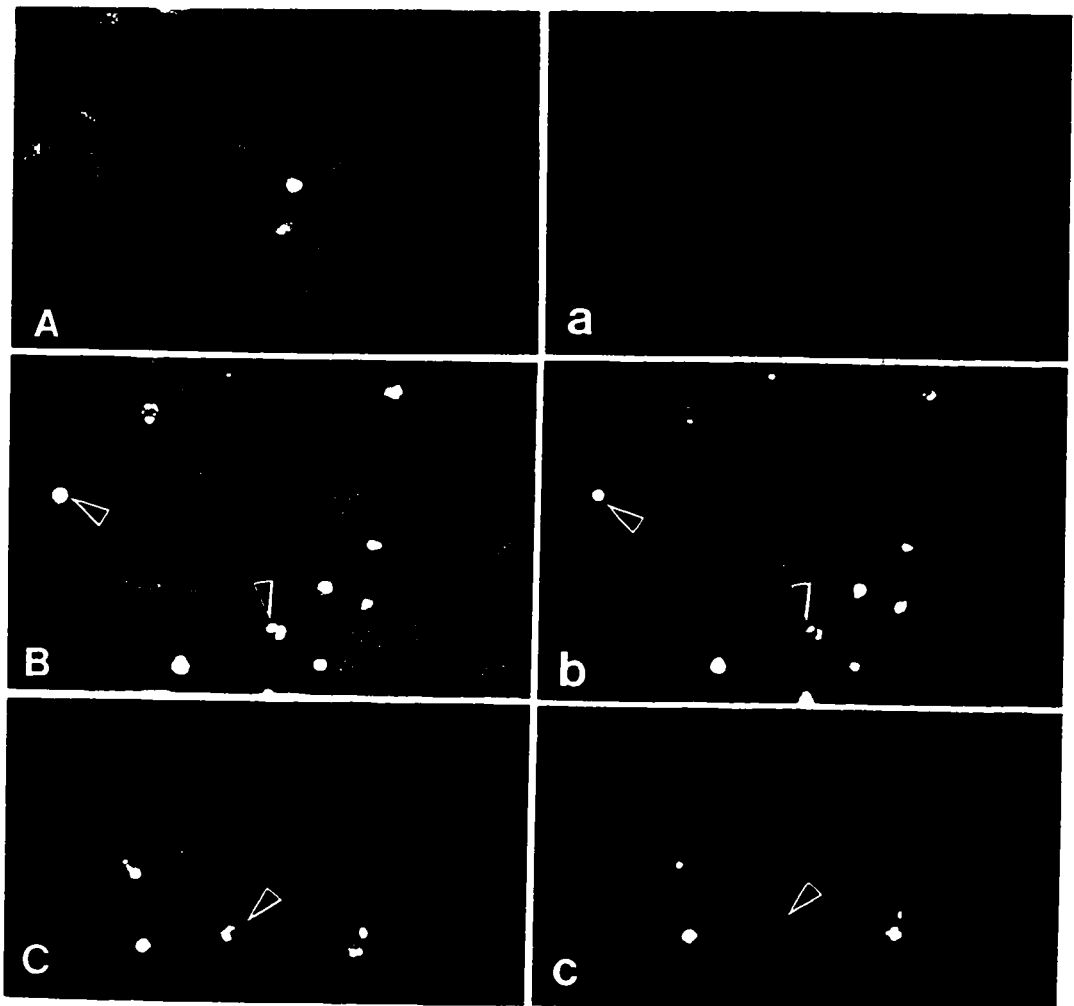


Figure 4.6. Electrophoresis of DNA from control and TGF- β 1-treated retinal EC. Retinal EC were incubated in 4 ng/ml of TGF- β 1 for 24 hr or in 2% FBS/DMEM alone (Control) for 24 hr. DNA was extracted from treated cells and was resolved by electrophoresis on a 1.5% agarose gel. DNA bands were visualized by staining with ethidium bromide. Lane 1: DNA standards (bp). Lane 2: control cells, DNA extract. Lane 3: TGF- β 1-treated cells, DNA extract.

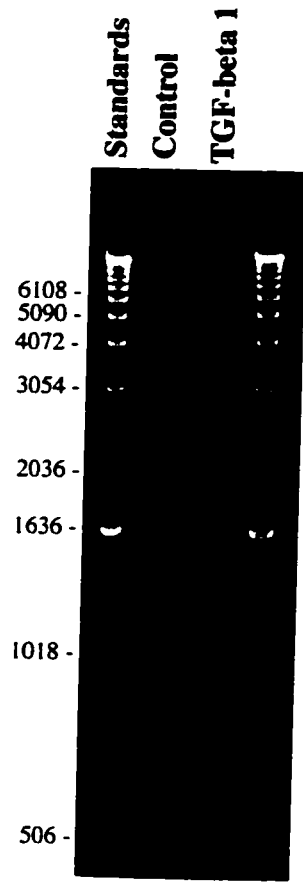
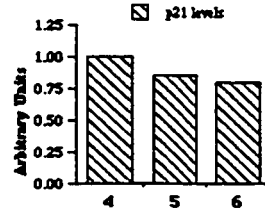
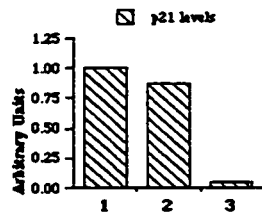
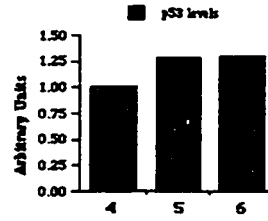
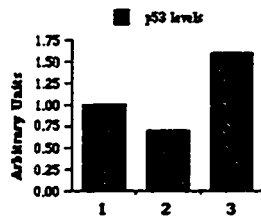
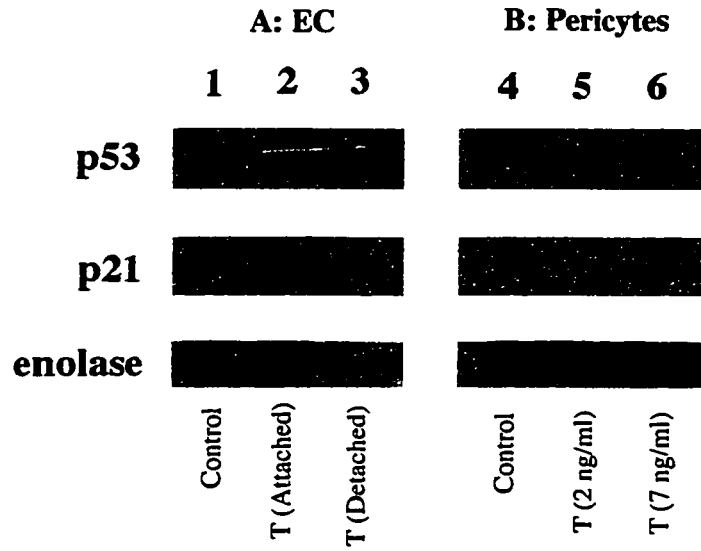


Figure 4.7. Expression of p21^{waf1/cip1} and p53 by retinal EC and pericytes in response to TGF- β 1.

20 μ g of protein per lane, extracted from EC or pericytes 24 or 48 hr after addition of TGF- β 1, was resolved by SDS-PAGE. Immunoblotting was performed subsequently with anti-WAF1(p21) and anti-p53 antibodies, followed by appropriate secondary antibodies. The blot was stripped and reprobed with an anti-enolase antibody as an internal control. **(A)**, EC: Lane 1, Control (2% FBS/DMEM); Lane 2, TGF- β 1 (2 ng/ml, attached cells); Lane 3, TGF- β 1 (2 ng/ml, detached cells). **(B)**, Pericytes: Lane 4, Control (2% FBS/DMEM, 48 hr); Lane 5, TGF- β 1 (2 ng/ml, 48 hr); Lane 6, TGF- β 1 (7 ng/ml, 48 hr). T, TGF- β 1. Histogram below **A** and **B** shows results of scanning densitometry of p53 and p21^{waf1/cip1}. The data shown are representative of three experiments. Data were normalized to the internal loading control (enolase) and were plotted relative to p53 and p21^{waf1/cip1} levels in control (untreated) samples.



CHAPTER 5: SPARC IS EXPRESSED IN GANGLION CELLS AND ASTROCYTES IN NORMAL BOVINE RETINA

5.1. Summary of Chapter 5

It is reported that SPARC plays an important role in angiogenesis. However, the role of SPARC in retinal angiogenesis has not been investigated. The purpose of this chapter is to describe the localization of SPARC in bovine retina in vivo.

SPARC/osteonectin is a matricellular, counter-adhesive glycoprotein that disrupts cell-matrix interactions, interacts with growth factors and components of extracellular matrix, and modulates the cell cycle, but it appears to subserve only minor structural roles. SPARC is expressed in a variety of tissues during embryogenesis and remodeling, and is thought to regulate vascular morphogenesis and cellular differentiation. Although generally limited in normal adult tissues, SPARC is expressed at significant levels in the adult central nervous system. Using a monoclonal antibody against bovine bone osteonectin, we have determined the localization of SPARC in newborn (3 day-old) and adult (4-8 year-old) normal bovine retinas. SPARC was present in the soma of ganglion cells, and strong reactivity was found in ganglion cell axons. Muller cells displayed no immunoreactivity, but SPARC was present in retinal astrocytes that were identified by the astrocyte marker glial fibrillary acidic protein (GFAP). Newborn calf retina showed a staining pattern similar to that of adult retina but exhibited significantly reduced levels of SPARC. Minimal levels of SPARC protein were also detected in some capillaries of the inner retina of both newborn and adult animals, whereas large vessels were negative. The presence of SPARC in the retina was confirmed by Western blotting of retinal extracts. These data indicate that SPARC originating from both neurons and glia of the inner retina could be a potentially important modulator of pathological retinal neovascularization. The increased expression of SPARC in adult relative to newborn retinal tissue also suggests that SPARC has an ongoing role in the maintenance of retinal functions.

5.2. Introduction to Chapter 5

SPARC, also termed osteonectin, is a highly conserved extracellular glycoprotein. Widespread expression of SPARC in tissues undergoing developmental or injury-related remodeling and morphogenesis has been reported (Sage and Bornstein, 1991; Sage et al., 1986; 1989; Lane and Sage, 1994). Unlike adhesive extracellular proteins such as fibronectin, laminin, vitronectin or fibrillar collagens, SPARC belongs to a group of matricellular proteins, defined as secreted components that do not serve structural roles but interact with growth factors, specific components of the ECM, and various cell-surface receptors (Bornstein, 1995). SPARC exerts a counter-adhesive activity which modulates cell shape through interference with molecules that support cell adhesion. This counter-adhesive function allows cell movement or shape changes required for development or remodeling. Microinjection of anti-SPARC antibodies into the blastocoel of *Xenopus* embryos resulted in deformed eyes and embryonic axes (Purcell et al., 1993), and in the nematode *Caenorhabditis elegans*, altered morphology and viability have also been shown to be coincident with the inappropriate expression of SPARC (Schwarzbauer and Spencer, 1993). These functional studies in embryos and the widespread expression of SPARC during development (Holland et al., 1987; Sage et al., 1989; Mundlos et al., 1992) indicate that SPARC plays an important (but ill-defined) role in the development of many tissues including the eye. In contrast, the expression of SPARC in adult tissues is generally more limited (Lane and Sage, 1994), e.g., SPARC has been identified principally in tumors (Schulz et al., 1988; Porter et al., 1995) and in tissues undergoing remodeling (Sage et al., 1989; Reed et al., 1993; Raines et al., 1992). Since SPARC mRNA and protein appear to be expressed at significant levels in the normal adult brain (Mendis et al., 1994a; 1995), SPARC might have an ongoing role in the adult central nervous system.

Two SPARC-related family genes have recently been identified. SC1 (synaptic cleft protein 1) was isolated from a rat brain library (Johnston et al., 1990) and QR-1 (quail retina protein 1) from embryonic quail retina (Guermah et al., 1991). SC1 is highly enriched in neural tissue and is localized within neurons in the central nervous system in both developing and adult animals (McKinnon et al., 1996; Mendis et al., 1996a; 1996b; Soderling et al., 1997). QR-1 is a retina-specific gene and encodes an ECM protein which plays an important role during retinal differentiation (Casado et al., 1995). To our knowledge, there is no detailed report of SPARC expression in retina. We have therefore investigated the immunocytochemical localization of SPARC in the normal bovine retina which is a widely accepted model for in vitro studies of microvascular functions.

5.3. Materials and Methods

Animals and Preparation of Tissue

Bovine eyes from four normal adult cows (4-8 year-old) and two calves (3-day-old) were obtained from a local slaughterhouse and were transported to the laboratory on ice within 2 hr after death. The anterior segment, lens, and vitreous were removed, and the posterior eyecup was fixed by immersion in methyl Carnoy solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) overnight at 4°C. Small pieces of retinas were dehydrated in ethanol solutions (70, 80, 95, and 100%) and were embedded in paraffin.

Retinal tissue for immunoblotting was dissected from fresh adult eyes, rinsed with sterile PBS containing a complete protease inhibitor cocktail (Boehringer Mannheim Biochemica), and homogenized in lysis buffer (0.5% NP-40, 10% glycerol, 1mM DTT, 2.5 mM EGTA, 5 mM EDTA, 150 mM NaCl, 50 mM Hepes (pH 7.4), 1 mM NaF, and 0.5 mM Na vanadate) with complete protease inhibitor cocktail (same as above) with a Teflon-glass homogenizer. After incubating for 20 min on ice, the homogenized sample was centrifuged at 13,000 g for 10 min at 4°C. The protein concentration of the supernate was assayed with the Bradford reagent (Pierce), and the supernate was aliquoted and stored at -70°C.

Immunohistochemistry

Serial 5 micrometer-thick paraffin sections were cut in transverse or tangential planes, deparaffinized, and rehydrated through a series of ethanol solutions before washing in PBS. Nonspecific binding sites were blocked by incubation in 10% normal goat serum in PBS for 2 hr at room temperature. Sections were immunostained with monoclonal mouse anti-bovine osteonectin antibody (mouse IgG1, 1:250 dilution of a 3.7 mg/ml stock solution; Haematologic Technologies Inc., Essex Jct., VT) for 2 hr at room temperature in a humid chamber with agitation. Adjacent serial sections or the same section was incubated with polyclonal rabbit anti-human GFAP (1:1000 dilution of a 8.7 mg/ml stock solution; BioMakor, Rehovot, Israel). Negative controls included replacement of primary antibodies by normal mouse IgG1 or PBS. Sections were washed 3 times for 10 min each in PBS, and were incubated in fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or Texas red-conjugated goat anti-mouse IgG (1:200) for 1 hr at room temperature. After 2 washes with PBS, cell nuclei were counterstained for 30 min with Hoechst 33258 fluorochrome (0.1 µg/ml in distilled water, Flow Laboratories, Inc. McLean, VA). After a brief rinse with PBS, sections were mounted in 50% glycerol and were photographed with a Nikon

fluorescence microscope with wavelength-specific filters. Osteonectin antibody binding was also visualized using a mouse IgG-avidin-biotinylated-peroxidase complex (Vector Laboratory) developed with diaminobenzidine and hydrogen peroxide.

Whole Mount Immunohistochemistry

A portion of the adult bovine retina extending from disc to periphery was removed from the eyecup without contamination of pigment epithelium or other tissues. The retina was washed with PBS and placed in 30% sucrose to freeze on dry ice. Subsequently, tissue was thawed at room temperature and rinsed with PBS several times after removal of sucrose. Subsequently, the tissue was fixed for 1 hr in methanol at room temperature, washed in PBS, and incubated with 5% normal goat serum with 0.2% Triton X-100 overnight at 4°C. Primary antibodies (mouse anti-osteonectin and rabbit anti-GFAP, at the same concentration as above) were incubated with the tissue for 2 days at 4°C. After a thorough washing (24 hr), secondary antibodies (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or Texas red-conjugated goat anti-mouse IgG) were incubated with the tissue for 5 hr on a shaker at room temperature. After an overnight wash in PBS, a glass slide was inserted below the retina tissue, and a brush was used to move and spread the retina flat upon the slide (nerve fiber layer face up). Excess solution was drained off and the retina was coverslipped with glycerin.

Western Blot

Retinal tissue was assayed for SPARC by SDS-PAGE followed by immunoblotting. The frozen aliquots were thawed, solubilized in SDS-PAGE sample buffer (Laemmli 1970), and heated for 5 min at 100°C with 50 mM DTT. Proteins (40 and 80 µg/lane) were analyzed by SDS-PAGE on 4% /10% polyacrylamide minigels. Proteins were fractionated and electrotransferred to nitrocellulose membranes (0.45-µm pore size). The blots were stained with 0.1% amido black (in 10% acetic acid and 20% methanol), and were photographed. Subsequently, the blot was blocked with 1% nonfat milk in PBS containing 0.05% Tween 20 overnight at 4°C, incubated with mouse anti-bovine osteonectin (1:1000) for 2 hr, followed by rabbit anti-mouse IgG. The bound protein-immunoglobulin complexes were detected with ¹²⁵I-labeled protein A at 0.5 µCi/ml (New England Nuclear) and were visualized by autoradiography.

5.4. Results

In adult bovine retina, staining with anti-SPARC IgG was restricted to the ganglion cell layer (GCL), nerve fiber layer (NFL), and to some of the retinal capillaries (Fig. 5. 1B,C). Control retinas showed no staining in the corresponding areas (Fig. 5. 1A). Although SPARC is believed to be closely related to vascular functions (Sage and Vernon 1994), there were only low levels of immunoreactivity in some of the capillaries in the inner capillary beds within the NFL and GCL and the two capillary beds along the inner and outer borders of the inner nuclear layer (Fig. 5. 1C and inset). Medium and large blood vessels in the inner retina did not show any immunoreactivity with the monoclonal antibody. In contrast, many cell bodies in the GCL and all axon bundles in the NFL were stained for SPARC (Fig.5. 1B, C).

Hoechst 33258 (the DNA-binding fluorochrome bis trihydrochloride) binds to nuclear chromatin of all retinal nuclei. Figure 5. 2A is a double exposure of a section double-labeled with Hoechst 33258 and anti-SPARC IgG. All the nuclei of the cells of the outer nuclear layer, inner nuclear layer, and GCL were labeled by Hoechst 33258. The bovine GCL contains large ganglion cell bodies (arrowheads) and neurons which could be either smaller ganglion cells or displaced amacrine cells (small arrow). Astrocyte cell bodies (large arrow) lie within the axon bundles in the NFL. In all animals, SPARC protein was detected in large ganglion cell somas (Fig. 5. 2A, arrowheads; 5. 2B). The cytoplasm and large primary dendrites were heavily labeled by the antibody, but the nucleus was devoid of reactivity (Fig. 5. 2B). Figure 2B shows a single large ganglion cell with labeled cytoplasm and primary dendrite. Without a specific amacrine cell marker, it is difficult to distinguish displaced amacrines from small ganglion cells. Regardless of type, none of the smaller neurons was labeled by the anti-SPARC antibody. High levels of SPARC protein also were present in most of the ganglion cell axons within the NFL throughout the retina (Figs. 5. 1, 2, 3, and 4A). In Fig.5. 2B, two small axon bundles are cut in cross section and show large and small labeled axons.

Muller cells run perpendicularly through all retinal layers with end feet forming the inner border of the retina. These cells showed no immunoreactivity with the anti-SPARC antibody (Fig 5. 2, curved arrows), but strong reactivity with anti-GFAP antibody (data not shown). The other large cellular population in the retina which exhibited SPARC immunoreactivity was the astrocyte glia. Astrocyte cell bodies are confined to the GCL and NFL of the bovine retina. Their long processes surround ganglion cell axons and extend within and along the NFL axon bundles. Fig. 5. 3A shows a cross section of a NFL axon bundle which was double-labeled for Hoechst 33258 and SPARC; Fig.3B shows the same

bundle labeled for GFAP. All of the cell bodies except the small vessels in Fig.3A are astrocytes because they contain GFAP (Fig.3B). These astrocytes are labeled for SPARC (arrowheads). A single astrocyte cell body shown at higher power contains SPARC (Fig.3C, arrowhead) and GFAP (Fig.3D, arrowhead). In comparison to the strong staining reaction of ganglion axons with the anti-SPARC antibody, the labeling of astrocyte cell soma and processes was relatively weak. Because the thin astrocyte processes wrap around the axons which contain high levels of SPARC, it is difficult to identify SPARC in the astrocyte processes in sections.

Wholemout immunohistochemistry was done to provide a three-dimensional view of the NFL axon bundles. Double labeling with the focal plane at the level of the NFL showed that the axons were heavily stained by anti-SPARC IgG (Fig. 5. 4A), and the astrocyte processes associated with the same axon bundles were labeled with the anti-GFAP antibody (Fig. 5. 4B). Data from the wholemount confirmed further the observation in the retinal sections for axonal labeling with anti-SPARC IgG (Fig. 5. 1). By comparing the double labeling for SPARC and GFAP in the wholemounts and by careful focusing through the axon bundles, we confirmed the staining pattern seen in sections, namely, that the astrocyte cytoplasm and its long process were stained with anti-SPARC IgG, but at a significantly reduced level compared to that of the ganglion neurons.

The 3-day-old calf retinas showed a neuronal and glial staining pattern similar to that of the adult bovine retina, but the apparent level of SPARC protein in the calf retina was significantly lower. Figure 5. 5 illustrates the difference in intensity of staining between bovine (Fig. 5. 5A) and calf retina (Fig. 5. 5B). The ganglion cell bodies (not seen in Fig. 5. 5B) were only lightly labeled compared to axon staining in calf retina. Compared to adult retina, only some of the axons were heavily labeled in the calf NFL and these appeared to be large axons. The large vessels in both adult and calf revealed no SPARC immunoreactivity (Fig. 5. 5, arrows).

The presence of SPARC protein in the retina was confirmed by SDS-PAGE of adult retinal extracts (Fig. 5. 6, lanes 1 and 2) and by subsequent immunoblotting with the anti-SPARC antibody (Fig. 5. 6, lanes 3 and 4). The molecular weight of SPARC on SDS-PAGE is 43 kDa (Sage et al. 1984). The anti-SPARC monoclonal antibody reacts with sequence which is not common to other SPARC-like proteins, e.g., SC1 and QR1. Because it recognizes only one band at 43kDa, coincident with the molecular weight of SPARC, this blot excludes the possibility that the antibody also recognizes other SPARC-

like proteins such as SC1 or QR1, which have greater apparent molecular weights by SDS-PAGE analysis.

5.5. Discussion

Our data demonstrate for the first time that SPARC protein is present in bovine retina and is confined to the ganglion cell soma and axon, astrocytes, and some capillaries. Although the function of SPARC in the retina and elsewhere in the central nervous system remains obscure, our identification of the cell types that serve as the most likely source of the protein in the retina represents a first step in the characterization of SPARC as a regulatory protein in this tissue.

Immunohistochemical labeling patterns are known to vary under different conditions of fixation and tissue processing, and in different species. We have found that methyl Carnoy solution is the best fixative for preservation of the antigenicity of SPARC. The monoclonal antibody used in our study was produced against bovine bone osteonectin and reacts specifically with SPARC protein of bovine tissues. Our immunoblotting assay confirmed that the antibody recognizes specifically the 43 kDa band which is coincident with the molecular weight of SPARC on SDS-PAGE.

SPARC is a well-characterized matricellular glycoprotein that modulates cell-matrix interactions. Because SPARC can disrupt cellular contacts with ECM and therefore alter cell shape, its major function has been referred to as counter-adhesion, which leads to cell rounding. It also has been shown to modulate the expression and interaction of a variety of matrix components and growth factors (Lane and Sage, 1994). The limited expression of SPARC in adult tissues, particularly in quiescent tissues and cells, and the increased expression in tissues undergoing repair or remodeling, is consistent with these functions. However, in the central nervous system, there appears to be a constitutive, relatively robust expression of SPARC. In fact, studies have reported low levels of SPARC protein and mRNA in the embryonic nervous system (Bassuk et al., 1993; Nomura et al., 1989), and significant levels of SPARC in the normal adult brain in regions enriched in synapses (Mendis et al., 1995). In the adult brain, some counter-adhesive glycoproteins such as SPARC and SC1 are associated with the ECM and are thought to regulate adhesive events at synaptic areas and prevent further neurite outgrowth (Mendis et al., 1994b). We have demonstrated that SPARC is expressed predominantly in ganglion cell soma and especially axons in the bovine retina. This distribution suggests an ongoing association with axon transport, axon growth and cell shape maintenance. In the retinal plexiform layers where

synapses are concentrated, SPARC was not detected; an active role for SPARC related to retinal synapses is thus excluded.

Because SPARC contains a follistatin domain capable of binding growth factors (Hemmati-Brivanlou and Melton, 1994), modulation of growth factors and matrix components represent two possible roles for SPARC in retinal tissue. During retinal development or in the ischemic retina, astrocytes, Muller cells, and ganglion cells have been shown to synthesize vascular endothelial growth factor (VEGF) in response to hypoxia (Shima et al., 1996; Stone et al., 1995). The Muller cells and ganglion cells also express transforming growth factor (TGF)- β (Anderson et al., 1995). It is possible that the SPARC produced in the GCL/NFL could interact with these growth factors and regulate retinal angiogenesis. This hypothesis is supported by studies in vitro showing that SPARC regulates endothelial cell proliferation in part through an interaction with VEGF (C. Kupprion and H. Sage, unpublished). Retinal neovascularization is regulated by various molecular cues such as growth factors, extracellular matrix components, and environmental factors such as hypoxia. Nothing is known concerning the regulation of SPARC itself in the retina, and its potential interaction with other molecules in the retina. However, the localization of SPARC and VEGF in the retina, and the close relation to the superficial layer of retinal vessels, provide strong evidence that SPARC could directly or indirectly (e.g., through VEGF or other factors) regulate retinal angiogenesis. In addition, some of the capillaries (EC) were labeled by anti-SPARC IgG, although large vessels in the inner retina were unreactive. Since not all the capillaries were stained with the anti-SPARC antibody, it is likely that the amounts of SPARC in some of the vessels were too minimal to be detected by immunocytochemistry. In addition, the attenuated endothelial cell cytoplasm is often difficult to visualize by immunocytochemical methods (Lane and Sage, 1994).

Our identification of SPARC in the adult and newborn bovine retina, together with the demonstration of SPARC family proteins SC1 and QR1 in the central nervous system (Casado et al., 1996; Mendis et al., 1996b), suggest that SPARC and SPARC-like proteins are important not only in the development of the retina, but also in the maintenance of its normal functions in the mature retina. More detailed study will be required to elucidate the precise functions of these proteins in both the developing and mature retina.

Figure 5.1. Immunohistochemical localization of SPARC protein in the adult bovine retina.

(A) Control section of bovine retina exposed to mouse IgG₁ instead of primary anti-SPARC IgG. (B, C) Longitudinal sections of bovine retina exposed to anti-SPARC IgG. Immunohistochemistry was performed with an avidin-biotin-peroxidase method (A-C). Axon bundles in the nerve fiber layer and associated ganglion cell bodies (arrowheads) were specifically labeled by the antibody. Some of the capillaries in the inner retina were also reactive (arrows). Inset shows a higher magnification of capillaries labeled by anti-SPARC IgG. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; NFL, nerve fiber layer. Bar = 10 μ m.

ONL

INL

GCL

NFL

A

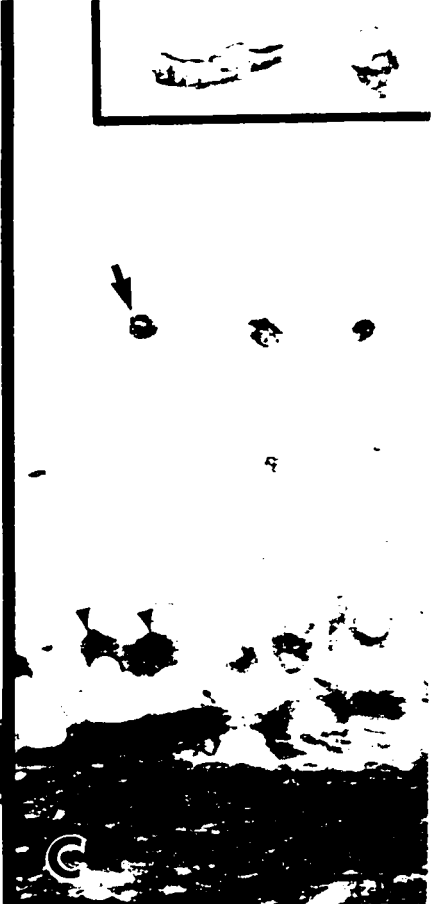
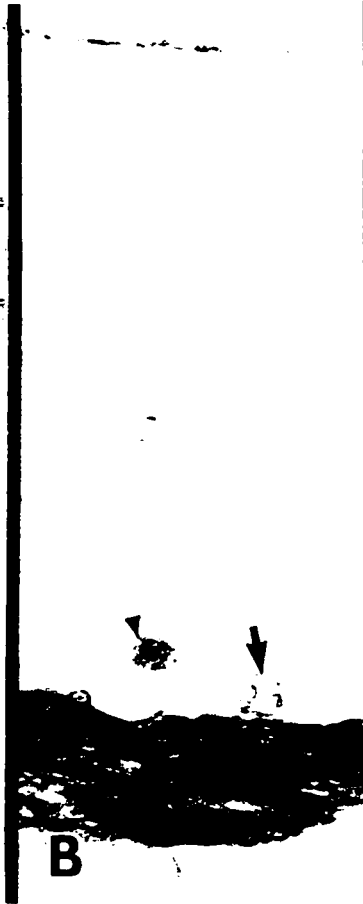


Figure 5.2. Epifluorescence micrographs of bovine retina illustrating the immunolabeling patterns revealed with anti-SPARC IgG.

(A) Double exposure of Hoechst 33258 and anti-SPARC IgG followed by goat anti-mouse IgG conjugated with Texas Red. All nuclei of retinal cells were labeled by Hoechst 33258. SPARC is present in ganglion cytoplasm (arrowheads) and axons. Astrocyte nuclei (black arrow), displaced amacrine cells (white arrow), and Muller cells (curved white arrow) are indicated. (B) A dendrite and cytoplasm of a single ganglion cell are labeled by anti-SPARC IgG. Two axon bundles are shown. Curved arrow denotes Muller cells. GC, ganglion cell, D, dentrite. Bar=10 μ m.

Figure 5.3. Co-localization of SPARC and GFAP in bovine retinal astrocytes.

Double antibody staining for (A,C) anti-SPARC IgG and (B,D) anti-GFAP IgG was performed. Cytoplasm containing SPARC (red, arrowheads) coincides with GFAP (green, arrowheads) surrounding the axon bundles in the nerve fiber layer of the calf retina (A,B) and adult retina (C,D). Bar=10 μ m.

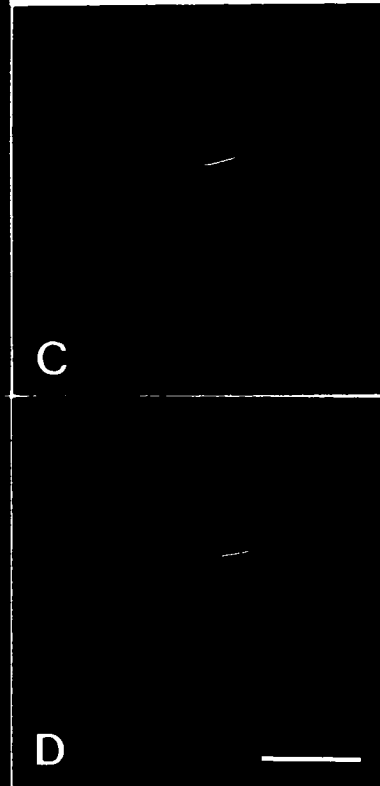
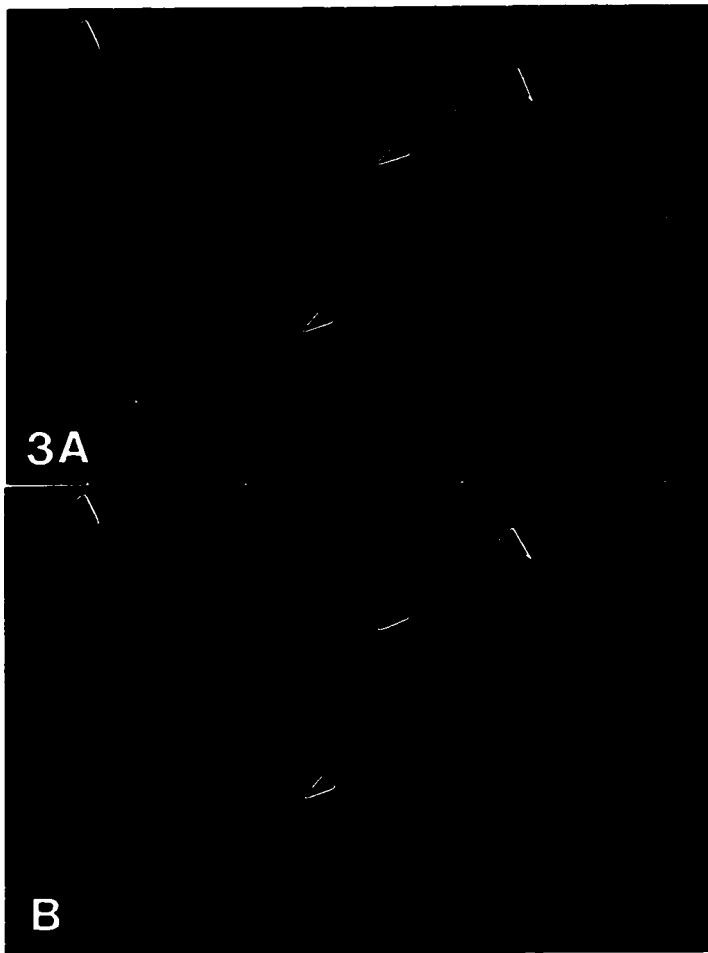
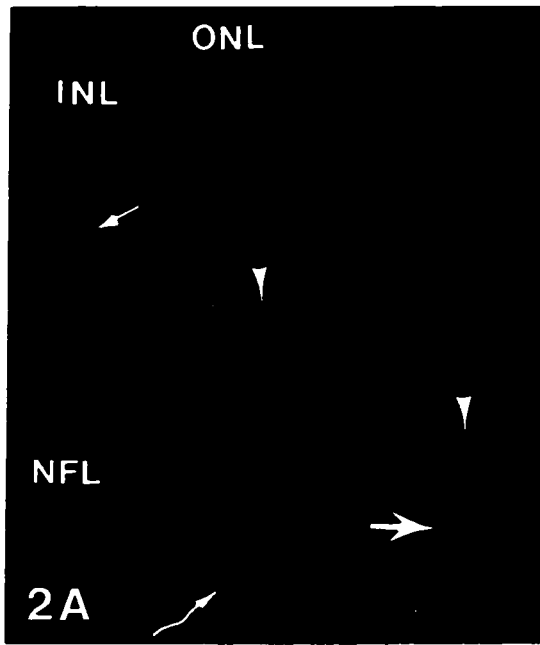


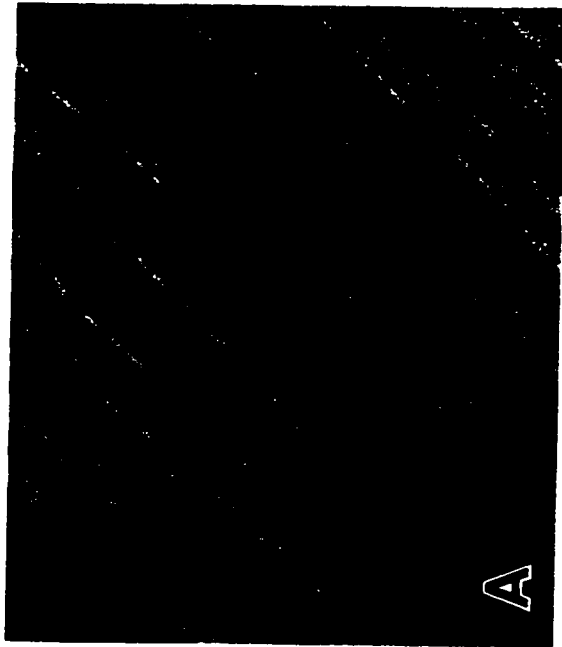
Figure 5.4. Wholemount of adult bovine retina double-stained with (A) anti-SPARC IgG, and (B) anti-GFAP IgG.

The nerve fiber layer was the superficial layer. Astrocyte processes are shown (arrows) along with axons which were labeled by anti-SPARC IgG (arrows).

Bar=10 μm .



B



A

Figure 5.5. Expression of SPARC protein in adult and calf retina. Tangential sections of nerve fiber layer: **(A)** adult bovine, **(B)** 3-day old calf. Large nerve bundles accompany a large vessel (arrows). Sections exposed to anti-SPARC IgG followed by Texas red-conjugated goat anti-mouse IgG. Bar=10 μ m.

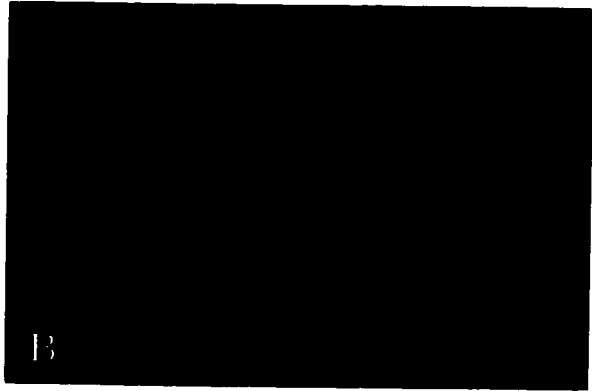
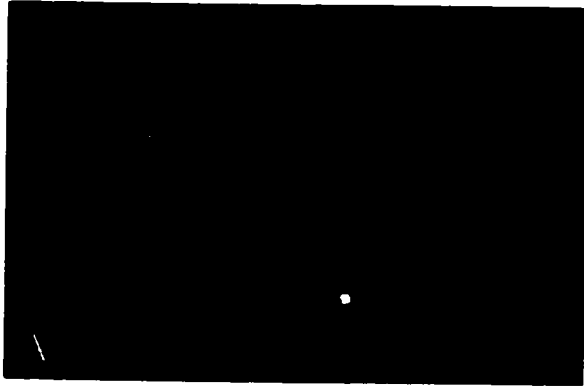
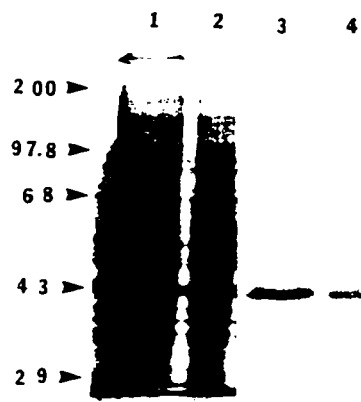


Figure 5.6. Immunoblotting analysis of SPARC protein in the bovine retina. Protein isolated from bovine retina were separated on polyacrylamide gels (4/10 %) in the presence of DTT and were transferred electrophoretically to a nitrocellulose membrane. Lanes 1 and 2 represent aliquots of 80 μg and 40 μg , respectively, that were transferred to the blot and stained with amido black. Lanes 3 and 4 represent an autoradiogram of lanes 1 and 2 after exposure to anti-SPARC IgG, followed by [^{125}I] protein A, respectively. Molecular weight standards ($\text{kD} \times 10^{-3}$) are indicated on the left.



CHAPTER 6: POTENTIAL ROLE OF SPARC IN RETINAL ANGIOGENESIS AND FUTURE STUDIES

The localization of SPARC in bovine retina has been detailed in Chapter 5. Its high level of expression in adult retina is an exception, since SPARC expression in adult tissues is generally limited and has been associated with epithelium exhibiting high rates of turnover and/or tumors (Lane and Sage, 1994; Sage et al., 1989a,b). Why is so much SPARC expressed in adult retina? Is SPARC involved in regulating retinal angiogenesis or is SPARC a major inhibitor which maintains the normal quiescence of retinal EC? In this chapter, I report on studies to elucidate the functions of SPARC in retinal angiogenesis.

Modular Structure of SPARC and Its Functional Domains

SPARC displays a high degree of interspecies sequence conservation (Villarreal et al, 1989) which suggests the importance for conserving function. The protein was isolated as a 43 kd monomer from proliferating cells (Sage et al., 1984; 1981; Otsuka et al., 1994), from bone tissues (named osteonectin) (Termine et al., 1981), and from a basement membrane tumor (named BM-40) (Mann et al., 1987). According to predicted secondary structure, SPARC has three modules and four distinct functional domains (Fig. 6. 1). To understand the domain functions of SPARC, peptides representing unique sequence motifs have been synthesized. Locations of the synthetic peptides are indicated in Fig. 6. 2. Murine SPARC cDNA consists of a 17 amino acid signal sequence followed by a secreted protein of 283-287 amino acids (Fig. 6.2; Engel et al., 1987). Domain I (aa 3-51) is highly acidic and binds calcium with low affinity (Engel et al., 1987). Peptide 1.1 (aa 3-21), within domain I, inhibits cell spreading, blocks chemotaxis induced by bFGF, enhances synthesis of PAI-1, and decreases fibronectin and TSP-1. Domain II (aa 52-132) contains 10 cysteines (Cys) and has a sequence similar to a repeated domain in follistatin, an inhibitor of TGF- β -like cytokines (Lane and Sage, 1994). Peptide 2.1 (aa 55-74) is an inhibitor of cell proliferation; peptide 2.3 (aa 114-130) contains the sequence KGHK and has been shown to stimulate cell proliferation and angiogenesis. Domain III (132-227) contains α -helical segments, binds to collagen, and has an endogenous protease-sensitive site (Lane and Sage, 1994). Peptide 3.4 has been designed as a control peptide, since it has

no effects on cell spreading or proliferation. Domain IV (228-285) binds Ca^{++} with high affinity because of an EF hand-like loop. Peptide 4.2, derived from this region, inhibits cell spreading and proliferation. A disulfide bond between Cys-255 and Cys-271 stabilizes the conformation of this domain (Mann et al., 1987). Mutant peptide 4.2AKA, in which the two disulfide-bonded Cys (position 255 and 271) were changed to Ala residues, and residue Asp-258 was replaced to Lys substitution, therefore it fails to perform the functions exhibited by 4.2 wt (Sage et al., 1994).

Hypothesis I

I hypothesize that SPARC, which has antiadhesive properties that can cause cell rounding, will induce retinal endothelial apoptosis in a dose-dependent manner in vitro.

SPARC, along with TSP and tenascin, belongs to the matricellular protein group, presently defined as a nonhomologous group of extracellular, regulatory macromolecules that mediate cell-matrix interactions but do not contribute significantly to ECM structure (Bornstein, 1995). The principal function of SPARC is counteradhesion; it can selectively disrupt cellular contacts with matrix and therefore cause cell shape change. Exogenous SPARC inhibits cell spreading and induces cellular rounding (Lane and Sage 1990, Everitt and Sage, 1992). Furthermore, NH_2 -terminal peptide 1.1 and COOH terminal EF-hand peptide 4.2 (Fig.6. 2) have been identified as regions of SPARC that inhibit cell spreading. Moreover, SPARC disrupts focal adhesions, alters the distribution of actin, and enhances the permeability of EC monolayers via cell rounding (Murphy-Ullrich et al., 1995; Goldblum et al., 1994). All these reported studies clearly indicate that SPARC can change cell shape.

Most cells require attachment to an ECM and subsequent spreading for survival, growth, and function. This dependence of cell growth and survival on substrate attachment is called anchorage dependence; the apoptosis or programmed cell death resulting from lack of anchorage has been named "anoikis". It has been well established that spread cells survive and grow better than cells with a more rounded shape. The work by Chen et al. further indicated that ECM controls cell shape, which in turn governs whether individual cells grow or die (Chen et al., 1997). The matricellular protein TSP-1 and its peptides have been reported to induce endothelial apoptosis (Guo et al., 1997).

Experiments For Testing Hypothesis I

A. SPARC Is Secreted By Cultured Retinal EC

In Chapter 5, the expression of SPARC in retina *in vivo* has been demonstrated. *In vitro*, cultured retinal EC synthesized and secreted a large amount of SPARC into the culture medium (Fig. 2.7, and 3.7). Figure 6.3 shows immunocytochemical staining of cultured retinal EC with anti-SPARC IgG, in which a granular, perinuclear staining of the Golgi apparatus was seen (Fig. 6. 3A). Furthermore, the presence of SPARC in cultured retinal EC and pericytes was confirmed by SDS-PAGE and subsequent immunoblotting with the anti-SPARC antibody (Fig. 6. 3B). These *in vivo* and *in vitro* data indicated that expression of SPARC is related closely to retinal capillary cells. It is thus hypothesized that SPARC is involved in regulating the functions of these retinal capillary cells.

B. Morphology of Retinal EC After Exogenous SPARC Treatment

Retinal EC were plated in 35-mm dishes in 10% FBS/DMEM for 24 hr, medium was subsequently changed to 2% or 5% FBS /DMEM with addition of SPARC. SPARC was purified from murine parietal yolk sac (PYS)-2 cell culture medium (Sage et al., 1989; Funk and Sage, 1991; provided by S. Funk), or baculovirus rSPARC was used in the study (provided by A. Bradshaw). The concentration of protein solubilized in PBS was determined by measurement of absorbance at 280 nm using an extinction coefficient of (calculated from the amino acid composition of murine SPARC). Concentrations used on retinal EC were 0, 5, 10, 15, and 20 $\mu\text{g/ml}$.

Measurement of endotoxin (LPS): Levels of bacterial LPS in PYS-SPARC, rSPARC, and SPARC peptides preparations were measured with the *Limulus Amebocyte* Lysate (Associate of Cape Cod, Inc., Falmouth, MA), according to the manufacture's instructions.

Staining of Hoechst 33258 Dye: Method is the same as described in Chapter 4.

I measured the levels of contaminating bacterial LPS in different preparations of SPARC and its peptides. Preparations of SPARC contained LPS at 0.3-0.6 ng/ μg of PYS-SPARC; 0.1-0.2 ng/ μg of rSPARC; and 0.4 or less pg/ μg of SPARC peptides. Considering the relatively high contamination of LPS in the SPARC preparations, and the sensitivity of bovine EC to LPS in comparison to human EC (Funk and Sage, 1993), the experiments always included a LPS control. Figure 6.4A-C shows the morphology of retinal EC after a 4 hr exposure to rSPARC (B) or LPS (C) equivalent to the level contained in the SPARC preparation (B). Either PYS-SPARC (data not shown) or rSPARC caused cell rounding significantly at 4 hr. Nuclear staining with Hoechst 33258 (D-F) or Apoptag reagent (data not shown) indicates that the rounded cells underwent apoptotic cell death

under these experimental conditions. The fragmented nuclei were obvious in the SPARC-treated cells (E), but not in the control cells (D). However, the LPS control cells (F) also exhibited the same phenotype as shown in the SPARC-treated cells, indicating that SPARC-induced apoptosis might be caused by the contaminating LPS in the SPARC. I therefore designed an experiment to inactivate the activity of SPARC but not LPS. rSPARC was heated at 70° C for 3 hr and boiled for 10 min. Because LPS can not be inactivated by heating at autoclave temperature (Q. Yan, E.H. Sage, unpublished data), the heating treatment will only deplete SPARC activity (Goldblum et al., 1994). Figure 6. 4G-I shows the morphology of retinal EC after incubation with rSPARC (G), boiled rSPARC (H), and LPS control (I). The degree of cell rounding in the three treatments appeared very similar at 20 hr. These data suggested that the level of LPS contamination in SPARC preparations is sufficient to cause apoptosis. Another possibility, however, is that SPARC was degraded by heating and the degraded SPARC fragments had the ability to induce apoptosis. To test this possibility, I tested all available SPARC peptides. As described above, all the peptides tested contained very low levels of LPS. None of them (peptides 1.1, 2.1, 3.4, and 4.2; concentration range 0.2 mM-1 mM) caused cell rounding as observed in SPARC-treated cells (data not shown). Staining with Hoechst 33258 dye did not detect fragmented nuclei in the SPARC peptide-treated cells (data not shown). From these experiments, I would conclude that SPARC or its peptides cannot induce retinal endothelial apoptotic cell death in vitro under these reported experimental conditions.

Hypothesis II

SPARC is an inhibitor of retinal neovascularization. Under normal physiological conditions, SPARC could be one of the endogenous inhibitors which maintain the quiescence of retinal EC.

It is now well-recognized that the normal retinal vascular state reflects a complex balance of endogenous positive and negative modulators of angiogenesis. Loss of this balance leads to neovascularization in many pathological conditions. The inhibitors in retinal angiogenesis have not been clearly identified. Given my study in Chapter 5 (Yan et al., 1998), and previous studies that 1) SPARC mRNA and protein were upregulated in proliferating EC (Iruela-Arispe et al., 1995; 1991a,b), 2) SPARC caused growth-arrest of EC in mid-G1 (Funk and Sage, 1991, 1993), and 3) SPARC inhibited endothelial proliferation induced by VEGF via an interaction with VEGF (Kupprion et al., 1998,

submitted), it is possible that SPARC might be an appropriate candidate for a retinal angiogenic inhibitor.

Experiments for Testing Hypothesis II

Endothelial proliferation is considered to be the most important component in the process of retinal angiogenesis. To test if SPARC or its peptides can inhibit retinal endothelial proliferation, ^3H -thymidine incorporation was used in a proliferation assay. Methods are the same as described previously. Briefly, cells were plated in 24-well plates with 10% FBS/DMEM for 24 hr to let cells attach and spread. Subsequently, rSPARC, or peptides at various concentrations were added to the cells (for detail, see Figure legends). In some experiments, retinal extract (see Chapter 2) was used as a mitogen to stimulate cell proliferation, and SPARC or peptides were added to observe whether they could neutralize the mitogenic effect induced by retinal extract. Reagents were incubated with cells for 20 hr, and were pulsed for 3-4 hr.

Peptide 2.1 is a peptide of 19 amino acid residues that has been shown to inhibit DNA synthesis by EC and fibroblasts (Funk and Sage, 1991, 1993), and had no apparent effect on endothelial morphology. Peptide 4.2 has been reported to be a potent inhibitor of DNA synthesis by EC (Sage et al., 1995), and also modulates cell shape (Lane and Sage, 1990). These two peptides were tested for biological activity in an assay of ^3H -thymidine incorporation into retinal EC DNA. Figure 6. 5 shows that these peptides inhibited DNA synthesis in a dose-dependent manner. Peptide 3.4 had no effect on cell proliferation (Fig. 6. 5A) and has been used as a control peptide. In addition, a mutant peptide 4.2 (4.2 AKA) (Figure. 6.2), was unable to inhibit ^3H -thymidine incorporation into retinal endothelial DNA. LPS (the concentration found in the peptide solutions) showed no effects on ^3H -thymidine incorporation or cell shape (data not shown).

Retinal EC are surrounded by various factors secreted by local retinal tissue in vivo. To test if SPARC or its peptides can neutralize or inhibit the mitogenic effects induced by factors released from retinal tissue, retinal extract was added to cells to stimulate DNA synthesis. Figure 6.6 shows clearly that either rSPARC or peptide 4.2 significantly inhibited cell proliferation induced by retinal extract.

Further Studies

Experiments in vitro indicated that SPARC and its peptides are potent inhibitors of retinal endothelial replication, since they effectively inhibited proliferation induced by

retinal extract. Therefore, SPARC could be a potential candidate for a retinal angiogenic inhibitor. To test this hypothesis, more studies are needed and are described below.

I. In Vitro 3-Dimensional Angiogenesis Model

For studies of angiogenesis, the 2-dimensional dish culture system may not reflect or mimic the situation in vivo, although it is an acceptable assay of cell proliferation. Animal models, although available, have attendant drawbacks of not being able to define precisely the molecular components of the angiogenic process. Montesano and Orci developed a novel experimental system in vitro (Montesano et al., 1983), in which the interactions between EC and the surrounding ECM are re-established, and angiogenesis can be recapitulated and analyzed under well-defined conditions. I have used this 3-dimensional assay in the studies described in Chapter 3. This angiogenesis model in vitro is not only able to assay cell proliferation, but also cell migration, ECM degradation, and tube formation. Therefore, I propose to test SPARC and its peptides in this model in vitro.

II. Wild Type (WT) vs. SPARC-Knock Out (KO) Mice

SPARC-KO mice are available in our laboratory. It has been found that SPARC KO mice exhibit aberrant dermal wound repair. The development of retinal vasculature in these KO mice has not been examined. If SPARC plays a major role in retinal angiogenesis, I should expect an abnormality in retinal vessels if SC1 or another molecule does not compensate SPARC's functions in retina. It may be necessary to generate a double-KO of SC1 and SPARC since both of them have similar and potentially overlapping functions (ref). The redundancy could mask the function of one or both proteins, and single KO mice may therefore appear relatively normal.

It is possible that SPARC KO mice may develop a normal retinal vasculature. This certainly can not rule out the importance of this molecule in retinal angiogenesis, as discussed above. Under pathological circumstances, the role of this protein might become obvious. Therefore, to generate a murine model of proliferative retinopathy in WT and SPARC-KO mice seems important and potentially exciting.

III. How SPARC Regulates Pericyte Functions

As an integral part of the microvascular wall, pericytes are believed to participate in the synthesis of the vascular basement membrane, regulation of endothelial functions, and formation of new blood vessels. Since pericytes are more numerous in vessels undergoing

angiogenesis than in resting vessels (Schlingemann et al., 1990), pericytes indeed seem to be involved in the process of angiogenesis, although their specific role is unclear.

It was interesting to find that pericytes synthesize SPARC *in vitro* (Fig. 6.3B). How SPARC regulates pericyte' function has never been investigated. Because of the importance of TGF- β in the regulation of retinal capillary cells (Chapter 4) and the considerable role of pericytes in the secretion of activated TGF- β 1, it is hypothesized that SPARC can regulate TGF- β 1 expression in retinal pericytes. The basis for the hypothesis is related to previous reports (Wrana et al., 1988) and the preliminary data from our group (A Francki, personal communication), showing that SPARC upregulates TGF- β 1 expression in murine mesangial cells and fibroblasts. Kidney glomerular mesangial cell has been considered as vascular pericyte in some cases due to the similarities with pericytes (Marra et al., 1996).

In the preceding chapters, the isolation and characterization of retinal microvascular EC and pericytes have been reported, and primate retinal EC were first time cultured successfully *in vitro*. The effects of VEGF, bFGF, and TGF- β 1 on retinal capillary cells have been investigated. bFGF was a more potent mitogen than VEGF on retinal EC under normoxic condition; but under hypoxic condition, VEGF was a more potent mitogen than bFGF. VEGF and bFGF acted synergistically upon retinal EC in collagen gel assay. TGF- β 1 inhibited the proliferation of both retinal EC and pericytes in a concentration-dependent manner. TGF- β 1 induced specifically apoptotic cell death in retinal EC but not pericytes. The retinal EC apoptosis was associated with a decreased level of p21^{waf1/cip1}, whereas p53 was augmented. The distribution pattern of SPARC in retina was described. Although the examination of SPARC's functional roles in retinal angiogenesis was not fully completed in this project, it is hoped the observations presented here will provide the ideas for designing future study. The study in this project will contribute to a better understanding of retinal angiogenesis.

Figure 6.1. Modular structure of SPARC and related proteins.

The three modules are based on sequence data from SPARC, QR1, SC1/hevin, testican, and follistatin-related protein (E.H Sage, 1997). They exhibit significant identity in the extracellular Ca^{++} -binding module, and somewhat less identity in the follistatin-like module. The specificity of each protein is thought to be determined by the N-terminal domain, which appears to be the least conserved within this family.

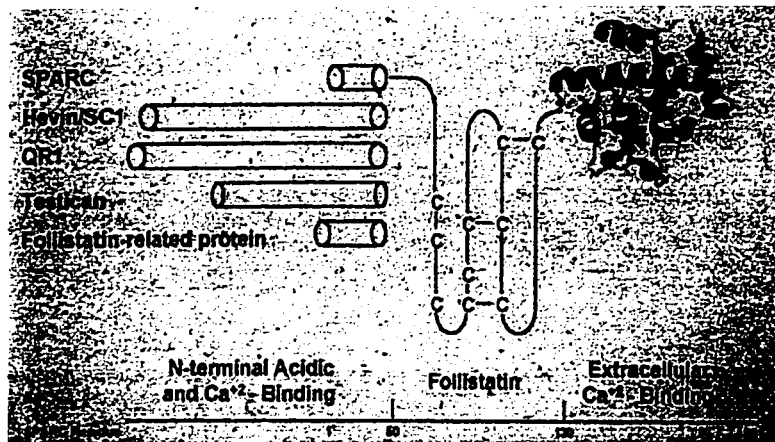


Figure 6.2. Four domains and synthetic peptides of murine SPARC. SPARC peptides that have been used in this work are underlined. Brackets separate four predicted domains. Mutant 4.2 (4.2 AKA) sequence is shown.

domain I

MRAWIFFLLCLAGRALAAP]QOQTEVAEEIVEEE

1.1

TVVEETGVPVGANPVQVEMGEFEDGAEETVEE

domain II

VVAD][NPCQNHCKHGKVCELDESNTPMCVC

2.1

QDPTSCPAPIQEFKVCSDNKNKTFDSSCHFFAT

domain III

KCTLEGTKKGKHLHDYIGPC][KYIAPCLDSEL

2.3

TEFPLMRDWLKNVLTLYERDEGNLLTEKQ

KLRVKKIHENEKRLEAGDHPVELLARDFEKNY

3.4

domain IV

NMYIFPVHWQFGQLDQHPIDGY][LSHTE LAPLR

4.2

APLIPMEHCTTRFFETCDLDNDKYIALEEWAGC

A K

A

(4.2 AKA)

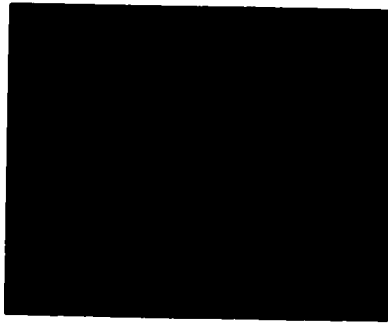
FGIKEQDINKDLVI

Figure 6.3. Retinal EC express SPARC in vitro.

(A) Retinal EC were grown on 35-mm dish for 24 hr. Immunocytochemical staining with anti-SPARC IgG, followed by a secondary antibody conjugated with Texas red was performed.

(B) Proteins (20 μ g) extracted from cultured retinal EC or pericytes were resolved by SDS-PAGE. Immunoblotting was performed subsequently with anti-SPARC IgG, followed by horseradish peroxidase-conjugated secondary goat anti-mouse antibody.

A



B

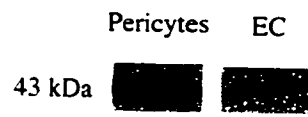


Figure 6.4. SPARC contaminated with LPS causes retinal endothelial apoptosis in culture.

(A) Retinal EC in 2% FBS/DMEM for 4 hr. (B) Cells treated with 10 $\mu\text{g/ml}$ rSPARC in 2% FBS/DMEM for 4 hr. (C) Cells treated with 2.6 ng/ml LPS (equivalent amount of LPS to that in 10 $\mu\text{g/ml}$ SPARC) in 2% FBS/DMEM for 4 hr. Hoechst 33258 fluorochrome was used to label DNA in retinal EC treated with (D) 2% FBS/DMEM; (E) 10 $\mu\text{g/ml}$ rSPARC in 2% FBS/DMEM; and (F) 2.6 ng/ml LPS in 2% FBS/DMEM. Retinal EC cultured in 2% FBS/DMEM were treated with (G) rSPARC for 20 hr; (H) boiled rSPARC for 20 hr; and (I) LPS control for 20 hr.

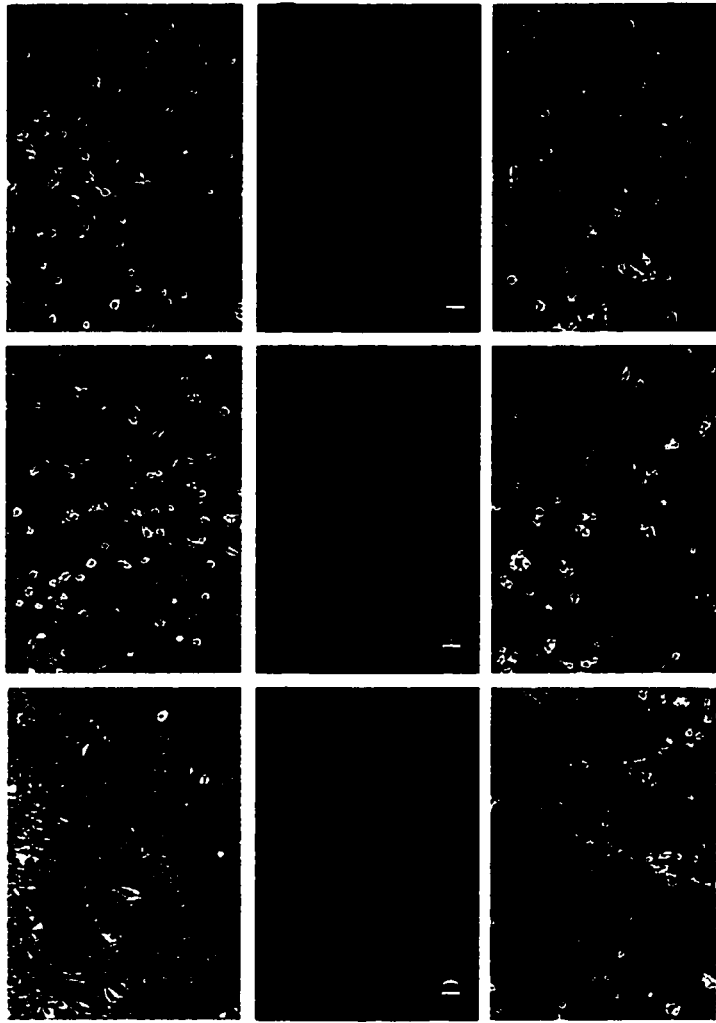
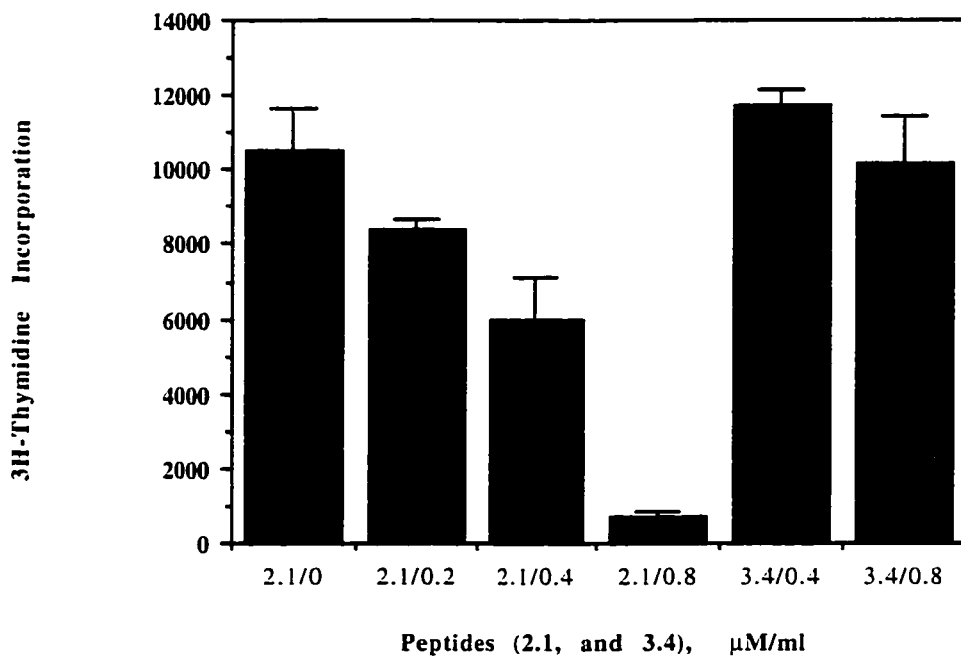


Figure 6.5. SPARC peptides 2.1 and 4.2 inhibit DNA synthesis in cultured retinal EC.

Bovine retinal EC were incubated in 10% FBS/DMEM for 24 hr. Subsequently, media were changed to 10% FBS/DMEM containing (A) peptides 2.1 (0, 0.2, 0.4, 0.8 $\mu\text{M}/\text{ml}$) and peptide 3.4 (0.4, 0.8 $\mu\text{M}/\text{ml}$); or (B) peptide 4.2 (0, 0.2, 0.4, 0.8 $\mu\text{M}/\text{ml}$), and 4.2AKA (0.4, 0.8 $\mu\text{M}/\text{ml}$) for 24 hr. Cells were pulsed during the last 3-4 hr with ^3H -thymidine at 2 $\mu\text{Ci}/\text{ml}$. Data are mean \pm S.D..

A



B

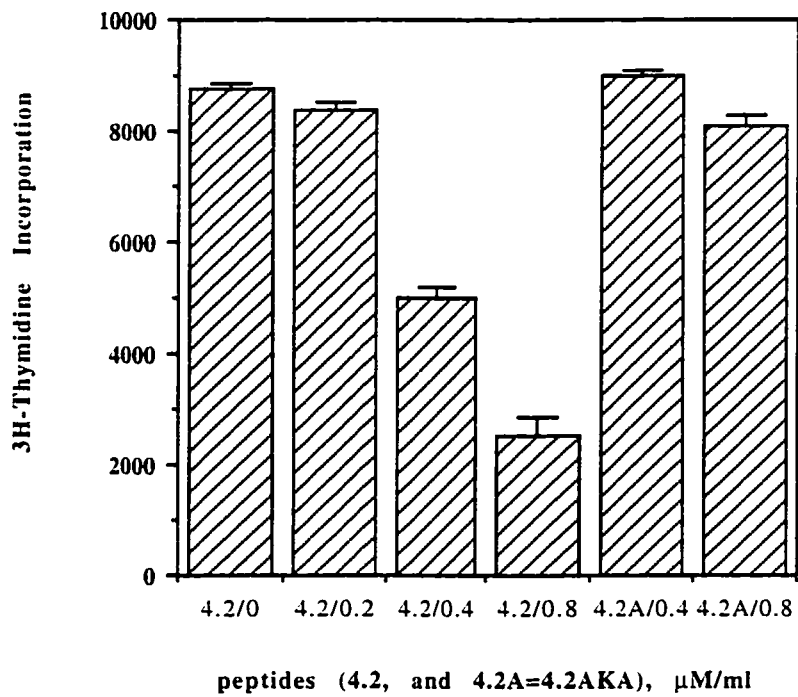
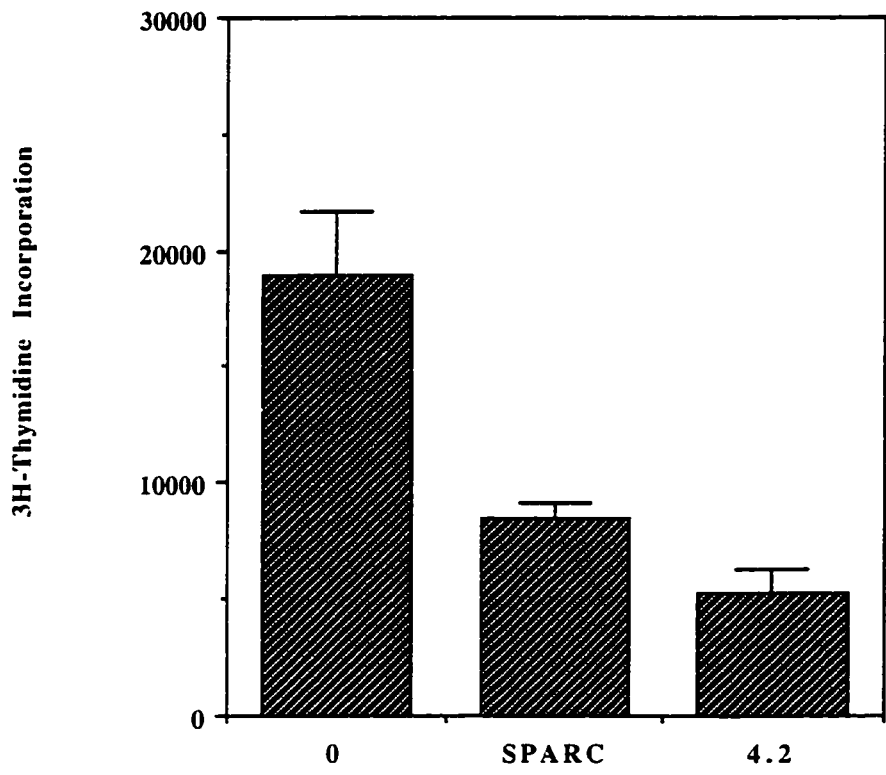


Figure 6.6. SPARC and peptide 4.2 neutralize the proliferative effect of retinal extract on retinal EC in vitro.

Retinal EC were incubated in 10% FBS/DMEM for 24 hr. Subsequently, media were changed to 15 μ l/ml retinal extract in 10% FBS/DMEM containing (1) no additions (control); (2) rSPARC (10 μ g/ml); or (3) peptide 4.2 (1 μ M) for 24 hr. Cells were pulsed during the last 3-4 hr with 3 H-thymidine at 2 μ Ci/ml. Data are mean \pm S.D..



END NOTES

Chapter 2 was published in *Invest. Ophthalmol Vis. Sci.* 1996; 37:2185-2194.

Chapter 4 was published in *J Cell. Biochem.* 1998; 70:1-14.

Chapter 5 was published in *J Histochem. Cytochem.* 1998; 46:3-10.

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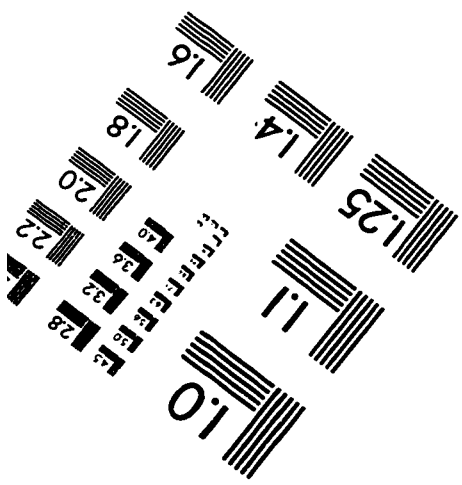
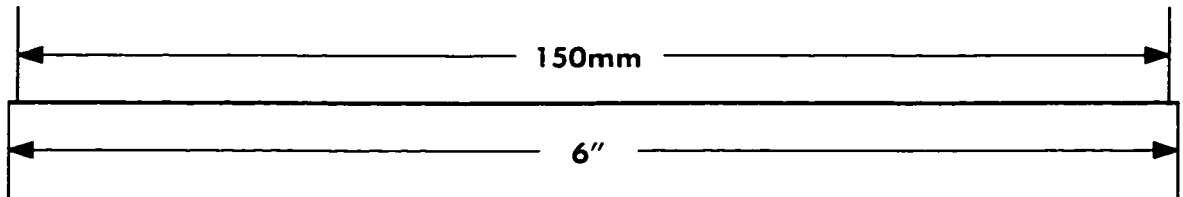
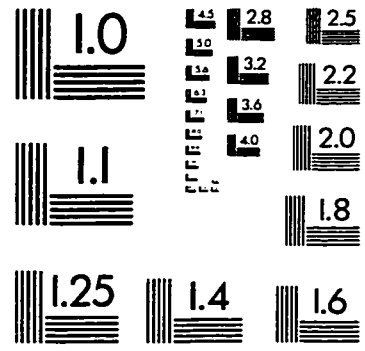
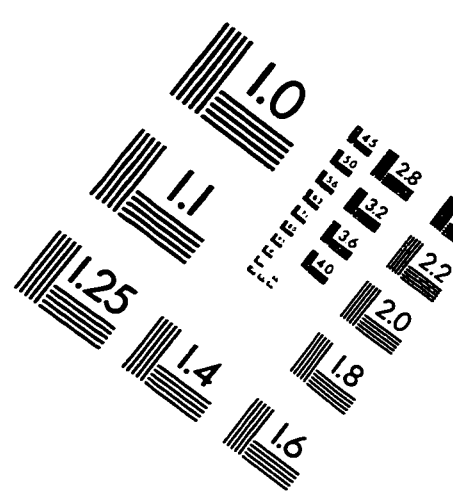
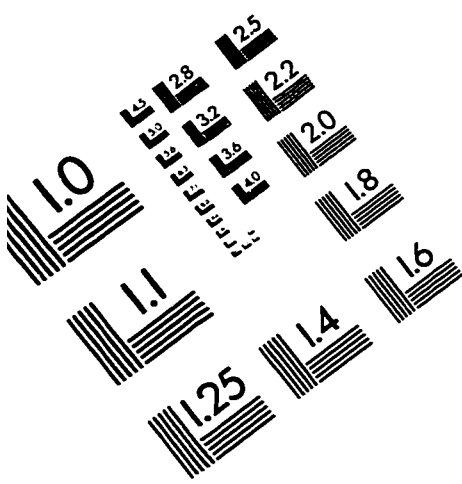
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PUBLISHED ABSTRACTS

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IMAGE EVALUATION TEST TARGET (QA-3)



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