INSULIN-LIKE GROWTH FACTOR-I STIMULATES FIBRONECTIN GENE EXPRESSION IN RAT VASCULAR SMOOTH MUSCLE CELLS AND GLOMERULAR MESANGIAL CELLS

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ABSTRACT

Title of Dissertation: Insulin-Like Growth Factor-I Stimulates Fibronectin Gene Expression in Rat Vascular Smooth Muscle Cells and Glomerular Mesangial Cells

Terry A. Tamaroglio, Doctor of Philosophy, 1993

Dissertation directed by: Chu-Shek Lo, Ph.D.
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Elevated fibronectin levels have been observed in the matrix of diabetic blood vessels and renal glomeruli. Growth factors have been implicated as possible regulators of matrix production. Therefore, the role of Insulin-Like Growth Factor-I (IGF-I) on fibronectin gene expression and synthesis in rat thoracic aortic vascular smooth muscle cells (SMC) and glomerular mesangial cells (MC) was investigated. Northern blot analysis demonstrated a time and dose-dependent increase of fibronectin mRNA levels. Significant levels ($P<0.05$) were observed 4 hours (SMC) and 8 hours (MC) after the addition of an optimum dose of 10 ng/ml IGF-I. When incubated in the presence of actinomycin D and IGF-I, the fibronectin response was blocked. However, cycloheximide and IGF-I increased fibronectin mRNA levels even more than the response induced by the growth factor alone. These results suggest transcriptional and translational control by IGF-I. Western and slot blot analysis demonstrated a 25% ($P< 0.05$) and 29% ($P<0.01$) increase in fibronectin secreted into the SMC culture media 4 and 8 hours after IGF-I addition, respectively. IGF-

I had no effect on cellular SMC fibronectin levels. However, a 22% ($P<0.01$), 32% ($P<0.001$), and 19% ($P<0.01$) increase in cellular MC fibronectin levels were observed after 4, 8, and 12 hours of IGF-I treatment, respectively. A 46% increase ($P<0.01$) of fibronectin secreted into the culture media 8 hours after IGF-I addition was also observed. The fibronectin mRNA and protein synthesis by both cell types were specific for IGF-I and <u>not</u> secondary to increased total RNA or total protein synthesis at the times reported. The effect of insulin on the IGF-I-mediated mesangial cell fibronectin gene expression was also explored. Fibronectin mRNA levels were higher in mesangial cells treated with IGF-I and insulin than those treated with IGF-I alone. Insulin alone was unable to stimulate fibronectin mRNA synthesis. These results introduce IGF-I as an important regulator of SMC and MC fibronectin synthesis. Altered IGF-I regulation of fibronectin may be an early event in the development of microvascular and macrovascular diseases.

INSULIN-LIKE GROWTH FACTOR-I STIMULATES FIBRONECTIN GENE EXPRESSION IN RAT VASCULAR SMOOTH MUSCLE CELLS AND GLOMERULAR MESANGIAL CELLS

by

Terry A. Tamaroglio

Dissertation submitted to the Faculty of the Department of Physiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1993

DEDICATION

I dedicate this dissertation to my advisor, Dr. Chu-Shek Lo.

He was not only my teacher, but a father and a friend.

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I thank Dr. Clough, Dr. Terris, Dr. Murano, and Dr. Cote for their time and assistance as a member of my graduate committee.

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I thank my sister Tammy for her assistance in the lab, her understanding in the "ways of science", and her constant confidence in my efforts even during the most difficult times.

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Lastly, I would like to thank "my partner in crime", Jorge, for his friendship during these past 5 years. I thank him for the wonderful memories in the midst of many hardships which we endured.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

A. Specific Goal

The goal of this dissertation was to investigate the role of Insulin-like growth factor-I (IGF-I) on fibronectin gene expression in rat thoracic aortic smooth muscle cells and glomerular mesangial cells. It is well established that diabetics have a greater risk of acquiring vascular disease. The factors linking diabetes and cardiovascular complications are not clearly defined. These chronic complications are categorized into microvascular diseases and macrovascular diseases. Lesions of both categories are characterized by extracellular matrix expansion and enhanced cellular proliferation. It has been established that levels of the extracellular matrix protein, fibronectin, are increased in the matrix of diseased blood vessels and renal glomeruli. Growth factors have recently been implicated as possible regulators of matrix production. The vascular smooth muscle cells and mesangial cells both respond to IGF-I as well as synthesize the growth factor itself. IGF-I is an important growth factor in regulating normal cellular functions of these two types of cells in an endocrine, paracrine and/or autocrine fashion. Cellular actions regulated by IGF-I could be altered in the diabetic state. Therefore, I planned to examine if IGF-I stimulates fibronectin synthesis.

B. BACKGROUND AND SIGNIFICANCE

1. Complications of Diabetes Mellitus

Diabetes mellitus exhibits symptoms of polyuria, glycosuria, and acidosis. Although the discovery of insulin in 1921 by Banting and Best prolongs and improves the quality of life associated with this disease, about 10 million people are still afflicted with its symptoms and/or consequent complications (Marble et al., 1985). Of these 10 million people, they fall into one of two broad categories of diabetes: Insulin-Dependent (IDDM or type I) and Non-Insulin Dependent (NIDDM or type II). These two types of diabetics have a greater than normal chance of acquiring vascular disease (Ruderman and Haudenschild, 1984). Diabetes can be associated with varying degrees of cardiovascular complications ranging from macrovascular diseases which affect medium or large sized arteries to microvascular diseases which affect capillaries of various organs such as the kidney and the eyes. Some of these conditions are further complicated with hypertension. The association between diabetes and vascular disease has been established (Tzagournis and Skillman, 1989; Eliahou, 1988). However, it is still not clear why diabetes is a risk factor for vascular complications.

a. Macrovascular Disease

Macrovascular disease is the collective name encompassing

various degrees of accelerated atherosclerosis associated with diabetes mellitus. The resulting lesions found in diabetes are the same as those in non-diabetics diagnosed with atherosclerosis. The earliest manifestation of atherosclerosis is the fatty streak, an accumulation of lipid containing cells known as foam cells (Smith et al., 1968). The fatty streak can then progress to the fibrocellular plaque characterized by an infiltration of monocytes, macrophages, and highly proliferative smooth muscle cells. These plaques also exhibit an increased production and deposition of matrix proteins (Pearson et al., 1977). In the final stages of the disease, the accumulation of lipid and matrix narrows the vascular lumen resulting in the clinical consequences of compromised cardiovascular function, i.e., heart attacks and strokes.

The hyperlipidemic state commonly associated with diabetes has been suggested as a possible cause of atherosclerosis (Goldberg, 1981). Altered insulin levels may link hyperlipidemia to diabetes (Tzagournis, 1986). Elevated insulin levels enhance lipoprotein synthesis through a stimulatory effect on the enzyme lipoprotein lipase. Low insulin levels present in type I diabetes may also be related to hyperlipidemia because of a decreased clearance of lipids as well as an increased release of free fatty acids. Trapping of circulating lipids in the vascular intima could be caused by glycosylation. Hyperglycemia has been established to cause

protein glycosylation found in various angiopathies related to diabetes. Hemoglobin, matrix proteins, and lipid carrier protein are all susceptible to glycosylation. These glycosylated carrier proteins may somehow facilitate binding and accumulation of lipids in the vascular intima (Steinbrecher and Witzum, 1984).

b. Smooth Muscle Cells in Macrovascular Disease

The role of vascular smooth muscle cells in the increased extracellular matrix of these lesions has been studied extensively (Ross and Glomset, 1973). Smooth muscle cells can now be isolated and grown In Vitro (Ross, 1971; Chamley-Campbell et al., 1979). They are spindle-shaped cells of mesenchymal origin that, when cultured in medium supplemented with 5-10% serum, proliferate in overlapping layers. This very characteristic pattern of growth has been termed the "hills and valleys" pattern. Their cytoplasm contains typical organelles, i.e., ribosomes, mitochondria, endoplasmic reticulum and cytoskeletal elements such as actin, myosin, and tropomyosin. The distribution of organelles and cytoskeletal elements depends on the cultured phenotype. Studies have demonstrated that these phenotypes change from a "contractile state" to a "synthetic state" (Manderson et al., 1989; Stadler et al., 1989). The synthetic state correlates with a decreased amount of myofilaments and an increase in organelles. Smooth muscle cells can be distinguished from possible contaminating fibroblasts through

immunohistochemistry and electron microscopy techniques. Fluorescein staining of smooth muscle cell-specific α-actin positively identifies the smooth muscle cell from the fibroblast. Abundant myosin filaments assume a longitudinal and parallel orientation whereas fibroblasts contain smaller amounts of scattered bundles. (Chamley-Campbell et al., 1979). Electron microscopy of smooth muscle cells reveals dense bodies and basal laminae containing laminin, collagen IV, and proteoglycans. In addition, interstitial matrix consisting of fibronectin, collagens I and III, thrombospondin, and elastin accumulates between individual cells (Carey, 1991).

The smooth muscle cells of fibromuscular plaques in diabetic blood vessels show an altered state of proliferation and matrix production. In Vitro studies have attempted to explain this change in proliferative activity. Clowes et al. (1989) proposed a model based upon studies involving the smooth muscle cell response to arterial injury. Based upon metabolic and/or mechanical perturbations that alter or physically damage the endothelial cell barrier, the smooth muscle cell response has been divided into 4 stages: an initial proliferation, migration from the media to intima, intimal proliferation, and deposition of matrix. The possible role of regulatory factors such as hormones, growth factors, and hyperglycemia in macrovascular disease is being explored.

c. Microvascular Disease

Microvascular disease involves increased matrix

deposition into the basement membranes of capillaries. A chronic microvascular complication prevalent in insulin-dependent diabetics is diabetic nephropathy, sometimes called diabetic glomerulosclerosis. This complication affects 20-40% of Type I diabetics (Pickup and Williams, 1992). Microscopic observations of the diabetic kidney show thickened basement membranes of the glomerular capillaries and mesangium expansion manifested as increased cellular proliferation and matrix deposition (Ditscherlein, 1988; Steffes et al., 1989). These changes consequently alter the filtration barrier leading to proteinuria, a clinical sign of deteriorating renal function. A progressively declining glomerular filtration rate in these diabetic patients ultimately leads to total renal failure. Histological studies reveal the presence of collagen IV, VI, laminin, and fibronectin in the glomeruli (Striker et al., 1984; Funabiki et al., 1990). They also show that mesangial expansion involves diffuse or focal deposition of collagen IV, VI, laminin, and fibronectin in different types of glomerulonephritis. The diabetic kidney can also develop diffuse or focal depositions of matrix, the latter being commonly known as the Kimmelstiel-Wilson nodule. One prevalent hypothesis for increased glomerular matrix deposition is a defective glucose metabolism. Since glucose uptake in the kidney is not insulin dependent, an increased glucose uptake into the cell may metabolically trigger the increase in matrix production. This suggestion is supported

by the observation that incubating fibroblasts in a high glucose medium results in an increase in collagen production (Villee and Powers, 1977). More recent work has supported the diabetic effect on matrix production at the molecular level. Poulsom et al. (1988) found increased laminin mRNA levels in diabetic rat kidneys while Roy et al. (1990) observed elevated fibronectin in diabetic rats and endothelial cells incubated in high glucose medium. Diabetic nephropathy has also been associated with hyalinized afferent and efferent arterioles (Kincaid-Smith et al., 1985) containing abundant amounts of interstitial collagens I and III. Hyalinization narrows arteriolar lumens thereby affecting peripheral resistance. This lesion suggests a possible link between hypertension and type I diabetics with nephropathy (Christlieb et al., 1981; Feldt-Rasmussen et al., 1990).

d. Mesangial Cells in Microvascular Disease

The mesangium consists of two types of cells depending upon their location. The intrinsic mesangial cell is found between the intraglomerular capillary loops while the extraglomerular mesangial cell is located in the juxtaglomerular apparatus. Since the development of techniques to culture mesangial cells (Foidart et al., 1981; Kreisberg and Karnovsky, 1983), research has grown rapidly to characterize its structure and function (Striker and Striker, 1985; Schlondorff, 1987; Kreisberg, 1988). The intrinsic mesangial cell is a mononucleated, stellate-shaped cell

possessing many cytoplasmic processes. When grown in culture medium supplemented with 10-20% serum, they form monolayer sheets or multilayer formations depending on cell density. Like vascular smooth muscle cells, mesangial cells contain actomyosin fibrils (Pease, 1968). These are positively identified through immunofluorescent staining of actin by NBD-phallacidin (Kreisberg et al., 1985). Other cytoskeletal proteins which distinguish these cells from glomerular epithelial or endothelial cells are vimentin and desmin. However, mesangial cells do not positively stain for cytokeratin and factor VIII, proteins present in epithelial cells and endothelial cells, respectively (Mene et al., 1989). Mesangial cells were considered to be structural supports for glomerular capillary loops, but they are now known to possess many other functions. Mesangial cell cytoskeletal structures are responsible for contractile responses to vasoactive substances thereby modulating the glomerular filtration rate (Bernik, 1969). Mesangial cells can contract in response to a number of vasoactive substances such as angiotensin II, arginine-vasopressin, histamine, and platelet activating factor as well as generate some vasoactive substances such as prostaglandins (Ausiello et al., 1980; Foidart et al., 1980; Ardaillou et al., 1985).

The mesangial cell secretes a matrix which consists primarily of types I, III, IV, and V collagens, laminin, glycosaminoglycans, and fibronectin (Ishimura et al., 1989).

When these cultured cells reach confluency, areas where they form multilayers and synthesize matrix appear (Sterzel et al., 1986). These areas have been termed "hillocks". The association between mesangial matrix expansion and diabetes has been known for years, but its cause is still under much speculation. In 1974, Hägg observed that 15 month old diabetic rats had expanded mesangial regions when compared to controls. Osterby (1974) studied the mesangium of diabetic patients of 3.5 to 5 years and saw a positive correlation between the duration of diabetes and mesangial matrix expansion. A recent study demonstrates that mesangial cells, In Vitro, produce more matrix proteins in the presence of high glucose (Ayo et al., 1989). These studies propose that increased synthesis of matrix proteins in later stages of the disease is probably secondary to metabolic abnormalities. Therefore, insulin has been the target of many nephropathy related studies.

Previous studies have shown that insulin may prevent diabetic glomerulosclerosis. Insulin supplementation appears to decrease the rate of matrix expansion (Osterby, 1974; Rasch, 1979). Another study demonstrated that increased collagen IV levels present in glomeruli from diabetic rats could be controlled by insulin treatment (Cohen and Khalifa, 1977). Abrass et al. (1988) confirmed this possible relationship by comparing different matrix compositions among normal, diabetic, and insulin-treated rats. Controlling

normal glucose and insulin levels by either islet or whole-pancreas transplantation has also been shown to decelerate or even prevent matrix expansion (Mauer et al., 1974, Orloff et al., 1986). These studies suggest that insulin deficiency may be involved in mesangial expansion. Is its effect on matrix production direct or secondary to glycemic control? Is insulin deficiency the only contributing factor? Insulin resistant models of diabetes suggest the possibility that other factors may be involved in the diabetic kidney.

In both microvascular and macrovascular disease, two features generally exist: cellular proliferation and matrix expansion. Experiments involving matrix proteins and cultured cells have established new roles for extracellular matrices (Carey, 1991). There is evidence suggesting that extracellular matrix proteins can modulate cellular functions including adhesion, differentiation, and proliferation. Any knowledge on these new roles may lead to an understanding of cell-matrix interactions that may be disrupted in these vascular lesions associated with diabetes.

2. The Structure and Function of Extracellular Matrix

Extracellular matrices are networks of different proteins deposited around cells that synthesize them. They consist of different types of collagens, elastin, proteoglycans, (eg., heparan sulfate), and glycoproteins such as laminin and fibronectin. Because of the well known tensile strength of

collagens and elastin, extracellular matrices have been clearly established as an important structural component of tissues. Proteoglycans are those gel-like substances which enable diffusion of nutrients, metabolites, and hormones. Laminin and fibronectin attach these proteins to the cell so that cellular adhesion to other structures may be possible. Basement membranes are well characterized forms of extracellular matrices (Sage, 1982). These are thin layers of matrix proteins that separate epithelial cells from endothelium. They are present beneath the endothelium of respiratory and digestive tissues, vascular structures, renal glomeruli, cornea, and the dermal-epidermal junction.

Immunofluorescence techniques not only identified the protein components of basement membranes but also revealed that cells can have their own distinct matrix. Vascular smooth muscle cells are surrounded by their own basement membrane containing collagen, laminin, and heparan sulfate. An interstitial matrix of fibronectin, thrombospondin, collagens, elastin and dermatan sulfate separates these cells from each other and endothelial cells. Both cell types contribute to the formation of this interstitial matrix. Mesangial matrix contains collagens, large amounts of fibronectin, and small amounts of laminin and thrombospondin providing support for the glomerular capillaries. In contrast, the glomerular basement membrane (GBM) is a separate and distinct matrix that possesses three distinct layers:

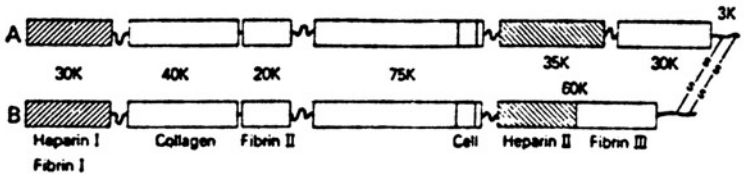
lamina rara interna, lamina densa, and lamina rara externa. These layers contain collagen IV and large amounts of thrombospondin and laminin. The GBM forms a filtration barrier between the endothelium and the epithelial podocytes. This specialized matrix wraps around the glomerular capillaries and the mesangial cell (Sakai and Kriz, 1987). The mesangial cell contacts the GBM through mesangial processes at areas called mesangial angles. These are areas where the GBM deviates from its pericapillary route to cover the mesangium. The perimesangial portion of the GBM illustrates its intimate relationship with the mesangial cell and its matrix. Sakai and Kriz demonstrated that the GBM and mesangium together construct a structural unit for contraction necessary for modulating glomerular filtration. Very little matrix is found between the mesangial cell and endothelial cell. However fibronectin, an extracellular matrix protein, has been detected at this interface (Courtroy et al., 1980). Fibronectin may be the bridge that facilitates important communication between the two cells. The glomerulus is a good example of two distinct extracellular matrices coexisting to provide structural and functional unity among the endothelial, epithelial, and mesangial cell.

Fibronectin synthesis is augmented by the smooth muscle cell in macrovascular disease and the mesangial cell in microvascular disease. Because it is an extracellular matrix glycoprotein that may play a major role in cell behavior, cell

adhesion, and matrix organization (Ruoslahti, 1988; Yamada, 1981), fibronectin is a potentially important regulatory factor in these two pathologies.

a. Structure and Types of Fibronectin

Fibronectin is a glycoprotein found in the plasma and cell. Plasma fibronectin and cellular fibronectin are nearly identical in amino acid and carbohydrate composition (Yamada et.al., 1977; Vuento et al., 1977; Vaheri and Mosher, 1978). However, they differ in molecular weight and solubility. Plasma fibronectin is made by the liver and circulates at a concentration of 0.3 mg/ml. The cellular form is relatively insoluble except at an alkaline pH. While they both can exist as dimers of disulfide-linked subunits, cellular fibronectin can be either monomers or polymers (Mosher, 1980). Cultured cells produce the cellular fibronectin found at cell surfaces or in culture media. As the following picture describes, cellular fibronectin is a dimer consisting of 2 subunits linked by carboxy-terminal disulfide bonds having a molecular weight of 500,000 daltons.



(Yamada, 1981)

Interspersed between the globular domains are unfolded regions of polypeptides which enable fibronectin to stretch for the purpose of adhesion. Its 2500 amino acid sequence contains

three types of repeats (Petersen et al., 1983). Type I amino acid repeat is located at the amino and carboxy ends. Type II repeat is also found at the amino terminal while type III comprises the middle of the sequence. Different splicings of these repeats by various cells create different fibronectin molecules. These slightly different proteins still retain similar functions. The different types of amino acid repeats generate unique domains which confer fibronectin's binding activity and multiple biological activities. Fibronectin mediates collagen binding to cells through a 30-40 kD globular domain (Ruoslahti et al., 1979a; Hahn and Yamada, 1979a). This collagen binding domain can be isolated through affinity chromatography on solubilized gelatin, also known as denatured collagen I (Balian et al., 1979) . Although fibronectin binds strongly to gelatin, it has lesser affinities for other native collagens. It only binds to native collagen I at 37°C (Engvall et al., 1981) and unfolded collagen IV (Engvall et al., 1982). This phenomenon suggests that some degree of denaturation is required, yet fibronectin binds to native collagen fibrils In Vivo. Therefore, the physiological significance of this binding still remains unclear.

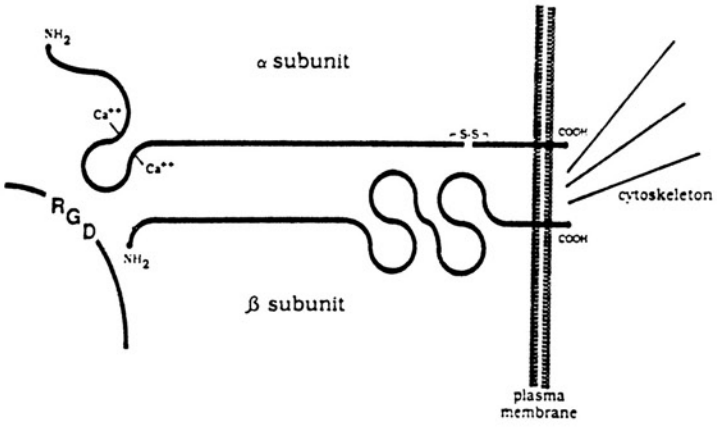
Another major domain near the amino terminal end contains a fibrin binding site and heparin binding site (Petersen et al., 1983). The fibrin binding site is important for fibronectin incorporation into blood clots. Since fibrin binds weakly to cells, fibronectin facilitates cellular

attachment to blood clots that is necessary for wound healing. The heparin binding site is important for strengthening collagen-fibronectin binding (Johansson and Hook, 1980). There is also an additional heparin binding site found near the carboxy terminal that appears to have stronger binding activity (Skorstengaard, et al., 1986). Other glycosaminoglycans such as dermatan sulfate and others of the hyaluronic acid binding type can attach and enhance fibronectin-collagen binding (Ruoslahti and Engvall, 1980; Schmidt et al., 1987). Although a structural domain has not been identified, glycosaminoglycan interaction with fibronectin appears to involve a specific, unique site. The implications of heparin/glycosaminoglycan binding sites suggest fibronectin may be an important matrix organizer.

The middle section of the fibronectin molecule contains a cell attachment site (Hahn and Yamada, 1979b; Ruoslahti and Hayman, 1979b). Different fragments of this site reveal an RGD (Arginine-Glycine-Aspartic Acid) sequence that appears to be necessary for cell binding (Pierschbacher and Ruoslahti, 1984). This same study demonstrated a loss of cell binding by synthetic peptides with modified RGD sequences. Soluble synthetic peptides containing the RGD sequence also prevent cell attachment to the culture plates (Hayman et al., 1985). The RGD sequence located in the cell binding domain appears to be the necessary element which enables fibronectin to mediate cell to cell or cell to matrix adhesion.

b. Fibronectin Receptors

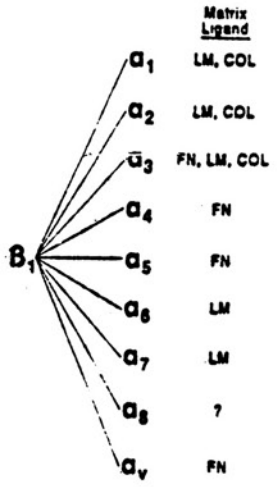
Fibronectin interacts with cells and extracellular matrix via binding to a RGD-directed receptor belonging to the integrin family of matrix receptors (Hynes, 1987). Integrin receptors are transmembrane heterodimers consisting of an α and ß polypeptide subunit. The structure of the fibronectin receptor consisting of these two subunits is shown in the diagram.



(Ruoslahti, 1988)

Multiple α and ß chains have been identified (Akiyama et al., 1990). While similarities exist amoung the various α chains and among the various ß chains, these two chains are genetically and structurally distinct from each other. The α subunit is composed of two disulfide-linked polypeptide chains and determines substrate specificity. The ß subunit is a single chain possessing an area of high disulfide bond density which is responsible for the "bends" found in this structure. This subunit may be more important for interaction

with cytoskeletal components. Integrin receptor groups are identified by the type of ß chain which is associated with the various α subunits. The $ß_1$ integrins, the largest group, can combine with at least nine different α chains (Adler, 1992). As the following picture describes, combinations of different α subunits with the $ß_1$ subunit can bind the same matrix protein ligand.



LM, laminin; COL, collagen;, and FN, fibronectin

(Adler, 1992)

The fibronectin receptor and other matrix protein receptors (eg., laminin and collagen) belong to this group of integrin receptors. These receptors are found on almost all cell types and are important in cell/matrix adhesion. Another receptor group is the $ß_2$ integrins. These receptors are found on leukocytes and mediate adhesions with endothelium and complement byproducts. The $ß_3$ integrins are important for cell/cell and cell/matrix interactions of platelets,

endothelial cells, and leukocytes (Albeda and Buck, 1990).

Ligand binding to the fibronectin receptor requires RGD recognition by both subunits and the presence of divalent cations (Ruoslahti, 1988). The α subunits contain sequences homologous to Ca^{2+} binding sites further supporting the requirement of Ca^{2+} for receptor binding. Other possible roles for these Ca^{2+} binding sites are still unknown. Both mesangial cells and smooth muscle cells possess fibronectin receptors. The integrin receptors, $\alpha_3ß_1$ and $\alpha_5ß_1$, on mesangial cells bind fibronectin (Cosio et al., 1990). Smooth muscle cells also have RGD-directed $\alpha_5ß_1$ fibronectin receptors which establish a link to actin filaments present in the cytoplasm (Thyberg et al., 1990). Smooth muscle cell adhesion occurs when seeded on a plate coated with RGD-containing peptides (Thyberg et al., 1990). Mesangial cell adhesion to the plastic substrate was inhibited when incubated with medium containing soluble RGD peptides (Simonson et al., 1989). These RGD peptides also affected cellular morphology and phenotype suggesting fibronectin's role in cellular differentiation.

c. Synthesis and Location of Fibronectin

Fibronectin is synthesized as a prepropeptide which enters the rough endoplasmic reticulum via a signal peptide. The signal peptide is then cleaved from the precursor, and the resulting propeptide enters the golgi apparatus where final processing to the mature fibronectin molecule begins.

Posttranslational modifications including removal of the signal peptide, phosphorylation, sulfation, and glycosylation occur in the rough endoplasmic reticulum and golgi apparatus. Mature fibronectin is secreted into the culture medium or binds to the cell surface. The amount of glycosylation varies according to the fibronectin producing source. It appears that glycosylation protects fibronectin from degradation. However, new evidence suggests that certain carbohydrate moieties may affect its binding capacities (Zhu et al., 1984). More data on these functions will help answer the physiological importance for differing degrees of glycosylation by different cells.

Fibronectin can be found in extracellular matrices of many mesenchymal-derived cells and some basement membranes through immunohistochemistry. Fibronectin is present primarily in the tunica intima of blood vessels (Stenman and Vaheri, 1978). Both endothelium and vascular smooth muscles contribute to its production and incorporation (Takasaki et al., 1990). Another prominent location is the renal glomerulus (Courtroy et al., 1980). Large amounts are found in the mesangium with smaller amounts in the lamina rarae of the glomerular basement membrane. Fibronectin synthesis can be affected by various factors such as cellular phenotype and cellular density. For example, fibronectin synthesis by NILH hamster cells changes according to the "prior history of the cells" (Senger et al., 1983). This study demonstrated that

subconfluent cells synthesize smaller amounts of fibronectin when compared to those reaching confluency. In addition, the culture age and duration of cellular quiescence appeared to determine fibronectin production. The longer the confluent NILH hamster cells remained quiescent, the greater the production of fibronectin. However, if the aged cells were restimulated to grow, fibronectin synthesis was decreased. Mesangial cells at varying densities exhibit different immunofluorescent patterns of fibronectin distribution (Simonson et al., 1989). Others have investigated this regulation at the transcriptional level. Smooth muscle cells have different amounts of steady state fibronectin mRNA levels during pre- and postconfluent stages (Liau and Chan, 1989). More fibronectin mRNA levels are detected at postconfluent stages. Over a period of time, these mRNA levels gradually increase while approaching a "density-arrested" state. However, collagen III mRNA levels do not change until the density-arrested state is achieved. This shows that gene expression of some matrix proteins may be more dependent on cell density than is that of others.

Diseased states such as diabetes affect fibronectin synthesis. Fibroblasts from genetically diabetic KK mice have increased [^{35}S]methionine incorporation into fibronectin (Phan-Thanh et al., 1987). Renal biopsies from diabetic kidneys and other diseases demonstrate increased immunofluorescent detection of fibronectin, especially in the

mesangium (Weiss et al., 1979). The increased fibronectin synthesis may be an early indicator of diabetic nephropathy, but how diabetes alters fibronectin synthesis is still unknown.

Changes in cell phenotype also result in altered fibronectin biosynthesis. For example, intimal thickening found in atherosclerosis is associated with increased fibronectin deposition (Stenman et al., 1980) and the presence of highly proliferative "synthetic" smooth muscle cells. Upon arterial injury by denudation, the highly proliferative smooth muscle cells have fewer myofilaments than the contractile cell would usually possess, suggesting a synthetic phenotype (Manderson et al., 1989). Atherosclerotic lesions also possess a fibronectin RNA variant, extra domain A sequence (ED-A), not detected in normal tunica media of arteries (Glukhova et al., 1989). This suggests that certain RNA transcripts of the fibronectin gene are phenotype dependent and thereby play an important role in the development of atherosclerosis.

d. Fibronectin Effect on Cellular Proliferation and Differentiation

While cellular phenotype can regulate fibronectin synthesis, the opposite case is also true. Fibronectin potentially regulates phenotypic changes by cells. It induces phenotype modulation of smooth muscle cells from the contractile state to the synthetic state (Hedin and Thyberg, 1987). This modulation is slower when cells are plated on

laminin and collagen IV (Hedin et al., 1988). This effect suggests a way by which cells can alter their metabolism and proliferation by sensing the matrix they produce. A small body of literature suggests stimulation of proliferation by fibronectin (Herman and Castellot, 1987; Humphries and Ayad, 1984). These studies found an induction of cellular growth on fibronectin-coated surfaces. They also discovered that other matrix proteins or varying combinations stimulated different responses by the smooth muscle cells. For example, endothelial derived matrix elicits a slower growth rate than the rate stimulated by fibronectin. Matrix secreted by smooth muscle cells from spontaneously hypertensive rats has greater stimulatory growth potential than smooth muscle cell matrix from normotensive (WKY) rats (Scott-Burden et al., 1989). The mesangial cell also responds to extracellular matrix (Simonson et al., 1989). Fibronectin and collagen stimulate growth much more than laminin and collagen I. These studies propose that some matrix proteins are more critical for cellular growth. Factors affecting the production and synthesis of these growth stimulating matrix proteins will greatly influence the proliferative capacity of the cell. Knowledge of matrix regulation may lead to some answers regarding altered cellular states of vascular diseases.

e. Growth Factor Regulation of Matrix Production

This relatively new area of research probably arose from discoveries that some matrix proteins can potentially regulate

cellular growth. A small amount of data is available implying that some matrix proteins are under control by growth factors. PDGF appears to stimulate transcription of thrombospondin in smooth muscle cells (Majack et al., 1987). Smooth muscle cells synthesize fibronectin and collagen when exposed to Transforming Growth Factor-ß (Ignotz and Massague, 1986). IGF-I and glucose together have been shown to regulate proteoglycan synthesis in mesangial cells (Moran et al., 1991) which may explain the abnormal proteoglycan metabolism implicated in diabetes mellitus. New observations such as these will potentially target which matrix proteins are growth factor responsive. This information may unveil new possible mechanisms by which growth factors mediate cellular growth through matrix production and organization. Combining this information with knowledge of cell-matrix interactions, a more complete picture of how the interaction between extracellular matrix and soluble factors affect cell behavior will be available.

3. Growth Factors in Diabetes Mellitus

Growth factors are polypeptides found in blood and tissue. They promote and modulate growth, cellular differentiation, metabolism, and tissue repair. These peptides bind to receptors on target cells and trigger intracellular transduction pathways resulting in a response. However, unlike hormones they are also produced and released

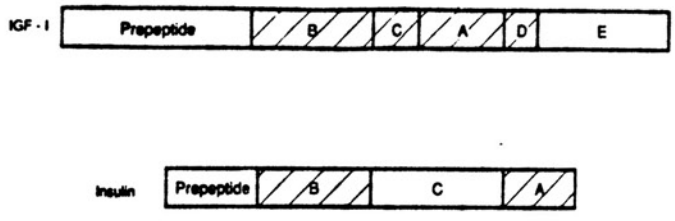
locally by multiple sources. Cells involved in diabetes-associated vascular lesions such as smooth muscle cells, endothelial cells, fibroblasts, and mesangial cells are targets as well as producers of various growth factors. Therefore, autocrine or paracrine mechanisms of macrovascular and microvascular disease possibly exist conferring potentially relevant roles of growth factors in diabetes mellitus (Koschinsky et al., 1987). One particular growth factor important for normal smooth muscle cell (Clemmons and Van Wyk, 1985) and mesangial cell (Conti et al., 1988b) function is Insulin-Like Growth Factor I (IGF-I).

a. Insulin-like Growth Factor I

Insulin-like growth factor I (IGF-I) was identified in 1957 by Salmon and Daughaday as a "sulfation factor activity" that mediated growth hormone's action on sulfate incorporation into developing cartilage. When its insulin-like activity was discovered, it was renamed Somatomedin-C (Froesch et al., 1963). When Dulak and Temin (1973) identified a peptide "exhibiting a multiplication-stimulating activity" (MSA) distinct from somatomedin-C, the names of IGF-I (somatomedin-C) and IGF-II (MSA) were officially given to this new class of polypeptides (Daughaday et al., 1987a). Along with its metabolic properties, IGF-I possesses mitogenic properties In Vitro. Since its discovery in 1957, the structural and functional properties of IGF-I have been characterized. (Zapf and Froesch, 1986a; Roberts and LeRoith, 1992).

b. Structural and Functional Characteristics of IGF-I

IGF-I has a structure very similar to insulin. It is a single polypeptide chain 70 amino acids long consisting of four different domains designated A, B, C, and D. Like insulin, the A and B regions are joined by a C region. However, unlike insulin, the C region of IGF-I is not cleaved during posttranslational processing but retained in its mature form. The B region is believed to be important for receptor binding while the A region provides for its mitogenic properties (Sara and Hall, 1990). Although these two regions have approximately 40% homology with insulin, IGF-I alone possesses a D region at the carboxy terminal. The function of this domain is still unknown. A diagram which demonstrates these similarites and differences between insulin and IGF-I is shown below.



Domains of IGF-I and Insulin Precursors

(Roberts and LeRoith, 1992)

IGF-I is synthesized by multiple organs such as the liver, lungs, heart, kidney, and testes. IGF-I synthesis follows the typical constitutive secretory pathway of peptide

hormones. After transcription and translation into a pre-propeptide, the IGF-I precursor enters the endoplasmic reticulum via a signal peptide. The signal peptide is cleaved off, and the resulting pro-IGF-I enters the Golgi apparatus where final processing to the mature form commences. The plasma concentration in humans is about a hundred fold higher than the concentration of insulin. However, specific binding proteins for IGF-I prevent large amounts of the circulating free form that may result in a potentially harmful physiological response. These soluble proteins are important for modulating IGF-I functions in either a stimulatory or inhibitory fashion (Clemmons, 1991). They can also be found in extracellular fluids such as lymph, milk, and cerebral spinal fluid. The most abundant binding protein in plasma to which the majority of IGF-I and IGF-II are bound is IGFBP-3. While having the highest affinity for IGF-I, it binds with the growth factor plus an acid labile unit to form a 150,000 MW complex. While IGFBP-3 is usually saturated with both IGFs, other binding proteins, IGFBP-1 and IGFBP-2, are largely found unsaturated in the blood. These two proteins, while important for fetal development, may also be important for IGF-I transport out of the blood but this remains to be clearly established. These were the first three binding proteins to be discovered and analyzed. During the past two years, IGFBP-4, 5, and 6 have been discovered (Cohick and Clemmons, 1993). Binding protein research is now focusing on differential

synthesis in various cell types, how they are regulated and their effect on IGF-I effects.

c. In Vivo Actions

Early In Vivo studies established a classical hormonal axis between growth hormone and IGF-I. GH stimulates IGF-I production in various organs such as the liver, kidney, heart, lung, and pancreas. Increased IGF-I levels then exert negative feedback on GH secretion. The GH/IGF-I hormonal axis is further strengthened by clinical presentations of acromegaly and Laron dwarfism. Acromegaly patients afflicted with high levels of GH have elevated levels of IGF-I (Clemmons et al., 1980). The opposite is true for Laron dwarfs who have correlating low levels of GH and IGF-I (Daughaday et al., 1987b). When exogenous GH is administered to hypophysectomized rats, IGF-I secretion is restored. Purified IGF-I stimulates growth of these GH-deficient rats thus supporting IGF-I as a mediator of GH (Schoenle et al., 1985). Much research is available regarding the GH/IGF-I axis in the kidney (Hammerman, 1989). Infusion of GH into normal as well as hypophysectomized rats stimulates a rise in glomerular filtration rate and renal plasma flow. However, upon a short term infusion of GH, glomerular filtration rate and renal plasma flow increase as circulating levels of IGF-I increase while GH remains at control levels. This suggests that renal function changes are indirect actions of GH through IGF-I production.

Early works by J. Zapf and E.R. Froesch established that IGF-I has direct metabolic actions similar to those of insulin. A bolus injection of IGF-I given to hypophysectomized and normal rats stimulated glucose uptake and glycogen incorporation (Zapf et al, 1986b). However, these insulin-like IGF-I responses were not as effective as those stimulated by insulin itself. A long-term subcutaneous infusion of IGF-I induces additional effects including an increase in body weight, thymidine incorporation, and tibial epiphyseal width. Zapf suggested that multiple IGF-I responses appear upon different types of administration. An acute bolus injection of IGF-I saturates binding proteins resulting in more free protein to potentially cross-react to insulin receptors and thereby trigger glucose metabolism. Long term infusion allows time for binding proteins to associate with IGF-I. Therefore, IGF-I levels in the circulation do not saturate their receptors and thus trigger predominantly mitogenic actions.

d. In Vitro Actions

IGF-I stimulates tritiated thymidine incorporation in smooth muscle cells (Pfeifle et al., 1987a) and mesangial cells (Conti et al., 1988a). Platelet-Derived Growth Factor (PDGF) potentiates this mitogenic effect. The dual control by IGF-I and PDGF was first shown in BALB/c3T3 cells demonstrating the specific roles for each growth factor in the regulation of cellular growth (Stiles et al., 1979). This

study described PDGF as a competence factor enabling cells to replicate DNA while IGF-I behaved as a progression factor facilitating them to enter into the S phase of the cell cycle. Both smooth muscle cells and mesangial cells demonstrated an increase in thymidine incorporation in response to IGF-I and PDGF (Clemmons, 1985; Doi et al., 1989). Potentiation of this effect occurred when both mitogens were added to the cell cultures. PDGF also stimulates production and secretion of IGF-I (Clemmons and Van Wyk, 1985) suggesting a possible autocrine mechanism of IGF-I as well as paracrine regulation by PDGF. An alteration of the dual regulation by IGF-I and PDGF may be a causative factor in increasing smooth muscle cell proliferation of diabetic vascular lesions. Mesangial cells also synthesize and release IGF-I (Conti et al., 1988b). However, factors affecting its synthesis and release are not known. IGF-I may regulate proliferation of mesangial cells and other glomerular cells in an autocrine and paracrine fashion. Perturbations of this regulation could result in the abnormalities found in nephropathy.

Insulin is another mitogen that affects smooth muscle cell proliferation. (Stout et al., 1975; Stout, 1991; Pfeifle and Ditschuneit, 1981). A stronger proliferative effect by insulin occurs in combination with serum. These observations suggest that other growth factors in the serum enhance insulin's mitogenic effect. Because IGF-I has similar structure and function, its effect was compared to that of

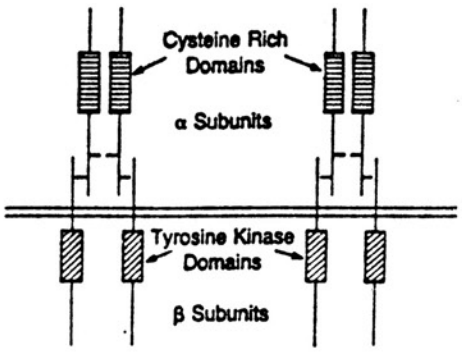
insulin. The effect of IGF-I on thymidine incorporation by the smooth muscle cells was more potent than that of insulin, but their effects were additive at near-physiological concentrations (King et al., 1985). Not only does insulin act synergistically with IGF-I for cellular proliferation of smooth muscle cells, but may also regulate serum IGF-I levels. Insulin-deficient animals have lower levels of serum IGF-I (Maes et al, 1983). Maes et al. also noticed that IGF-I can be increased upon insulin treatment. However, insulin may not be an absolute requirement for IGF-I function. For example, Scheiwiller et al. (1986) found that IGF-I restored growth in diabetic rats. Therefore, insulin may regulate circulating IGF-I levels but not necessarily its action. However, they share common actions in various target cells and may have important modulatory interactions with each other that are not yet discovered (Pfeifle et al., 1987b). Evidence for diabetic tissue levels of IGF-I still remains to be clearly established. Ikeda et al. (1988) observed decreased amounts of immunodetectable IGF-I in the diabetic liver and kidney, but Werner et al. (1990) detected no change in IGF-I mRNA levels in the kidney. Data regarding insulin in the mesangial cell is also available. Pharmacological doses of insulin stimulate thymidine uptake while much lower concentrations of IGF-I are sufficient for the same effect (Conti et al., 1988a; Arnqvist et al., 1988). Unlike the smooth muscle cell, insulin and IGF-I in the mesangial cell do not have an

additive effect. These findings may relate to diabetics undergoing insulin treatment. These patients may have higher than normal plasma insulin concentrations which could directly or indirectly through IGF-I stimulate proliferation associated with vascular lesions.

e. IGF-I Receptor

IGF-I binds to an insulin-like receptor (Rechler and Nissley, 1986) which consists of two extracellular α subunits each bound to a transmembrane ß subunit via disulfide bonds. The α subunits function as ligand binding domains while signal transduction cascades are initiated by the ß subunit. The Ligand binding to the IGF-I receptor induces a conformational change activating the tyrosine kinase of the ß subunit. This kinase activation results in a receptor autophosphorylation followed by a phosphorylation/dephosphorylation of proteins which act as second messengers for the cellular response. The following picture demonstrates the great structural similarity between the IGF-I and the insulin receptor.

Insulin



IGF-I

(Roberts and LeRoith, 1992)

Although the IGF-I receptor parallels the insulin receptor in structure, it is encoded by genes that are distinct from its similar counterpart. Binding of a monoclonal antibody to the IGF-I receptor confirms its unique structural identity (LeBon et al., 1986). Current IGF-I research is investigating the signal transduction pathways of the IGF-I receptor to determine any similarites or differences from the insulin receptor.

Structural similarities allow for cross-reaction between the binding of IGF-I and insulin to their receptors. IGF-I interacts with the insulin receptor but with a 100 fold lower affinity than for its own receptor. Similarly, insulin binds to the IGF-I receptor with a much lower affinity. Competitive inhibition studies established the presence of IGF-I receptors in smooth muscle cells (Pfeifle et al., 1987a; King et al., 1985) and mesangial cells (Conti et al., 1988a; Arnqvist et al.,1988). These studies also demonstrated that increasing concentrations of insulin inhibit radiolabeled IGF-I binding to its receptors thus confirming cross-reactivity. Therefore, both peptides have the potential to promote the same biological effects. For example, IGF-I stimulates glucose metabolism via insulin receptors in adipocytes while insulin stimulates DNA synthesis in human fibroblasts through IGF-I receptors. Since insulin and IGF-I share cross-reactivity, dual receptor regulation may exist. Insulin's effect on IGF-I receptor regulation is being explored. For

example, the presence of insulin inhibits IGF-I induced stimulation of tyrosine aminotransferase in rat hepatoma cells (Heaton et al., 1984). On the other hand, insulin enhances IGF-I binding in smooth muscle cells (King et al., 1985).

IGF-I binding in smooth muscle cells may also be regulated by other growth factors. Although PDGF does not bind to IGF-I receptors in competitive inhibition assays, it does enhance IGF-I binding (Pfeifle et al., 1987a). The same study also revealed that IGF-I stimulates PDGF binding to the PDGF receptor. Thus, IGF-I can even have many regulatory interactions with other peptides that are not structurally similar. The ability of IGF-I to have multiple interactions may be the reason for different pathological manifestations of diabetes mellitus.

C. Specific Aims

Recent studies have been conducted to determine if IGF-I and its receptor play a relevant role in diabetic nephropathy and atherosclerotic lesions associated with macrovascular disease. An increased number of IGF-I receptors has been found in kidneys of streptozotocin-induced diabetic rats (Werner et al., 1990). A study using mesangial cells isolated from diabetic mice (db/db) exhibited more IGF-I receptors than in its normal counterparts (Oemar et al., 1991). Transgenic mice expressing growth hormone demonstrated high levels of IGF-I and renal lesions similar to those found

in diabetes mellitus (Doi et al., 1988). IGF-I appears to be a factor in atherosclerosis since an induction of IGF-I mRNA is observed in the aorta after balloon injury (Cercek et al., 1990) and abdominal coarctation (Fath et al., 1993). IGF-I gene expression has been detected in smooth muscle cells and may be a potent growth factor for developing or regenerating blood vessels (Delafontaine et al., 1991a).

Since gene expression of IGF-I and its receptor is possibly altered by the diabetic state, IGF-I may be an important stimulus for the increased fibronectin synthesis found in these lesions. Insulin and IGF-I have common metabolic and mitogenic actions as well as potential regulatory actions on each other. Since increased insulin receptors have also been observed in diabetic mesangial cells (Oemar et al., 1991), the modulation of the IGF-I induction of fibronectin gene expression in mesangial cells by insulin is also a possibility. In summary, the three primary aims of this dissertation are:

1. Characterize the primary tissue cultures of rat thoracic aortic smooth muscle cells and glomerular mesangial cells.
2. Determine the effect of IGF-I on protein and mRNA levels of fibronectin in smooth muscle cell and mesangial cell cultures.
3. Determine the effect of insulin on the IGF-I induction of fibronectin mRNA in mesangial cell cultures.

II. METHODS

A. Smooth Muscle Cell Culture

Cells were obtained from thoracic aortae of 250-300 gm Wistar-Kyoto (WKY) male rats (Charles River Breeding Lab, Boston, MA) according to the protocol by Owens et al. (1986). The intima was removed, and the media containing smooth muscle cells was dissected from the adventitia. Smooth muscle cells were dissociated with collagenase (262U/ml) and elastase (7U/ml). The isolated cells were grown in Medium 199 (Gibco/BRL, Gaithersburg, MD) supplemented with 5% newborn calf serum and 5% Nuserum (Collaborative Biomedical, Bedford, MA). Cells between passages 15 and 25 were used for experiments.

B. Mesangial Cell Culture

Mesangial cells were cultured from glomeruli from 100-150 gm male, Sprague-Dawley rats (Charles River Breeding Lab, Boston, MA). Glomeruli were isolated through sequential sieving of the renal cortex (Foidart et al., 1981). Rat kidneys were removed and the medulla dissected from the cortex. The cortex was homogenized in phosphate-buffered saline (PBS) and filtered through three sieves of pore sizes, 297μ, 149μ, and 74μ. Glomeruli trapped on the 74μ sieve were collected into PBS. They were washed three times by gravity sedimentation to remove contaminating tubules. Glomeruli were

then spun at 500 rpm for about 1 minute and placed into 25 cm^2 culture flasks containing RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) and 2.5 μg/ml fungizone (Gibco/BRL). After 12 days, mesangial cells were identified and grown in 75 cm^2 flasks. Cells between passages 15-20 were used for experiments.

C. Immunofluorescence Utilizing Fibronectin, Factor VIII, and Cytokeratin Antibodies

Cells were grown on glass coverslips for 3-4 days and then fixed in 3.7% formaldehyde for 10 minutes at room temperature. Before adding antibodies, the coverslips were placed in acetone at -20°C for 5 minutes. The coverslips were then removed and air dried. Non-specific sites were blocked by incubation with a 1:20 dilution of goat serum in Hank's Buffered Salt Solution (HBSS) overnight at 4°C. The coverslips were then incubated with the primary polyclonal anti-rat antibody from the rabbit (Sigma, St. Louis, MO; Calbiochem, La Jolla, CA) at a dilution of 1:20 in HBSS for 1.5 hours at 4°C. Excess antibody was washed from the coverslips with HBSS before adding 1:20 anti-rabbit FITC (fluorescein isothiocyanate)-IgG from the goat for 1 hour at 4°C. The coverslips were then rinsed with HBSS and viewed via fluorescence microscopy.

D. α-Smooth Muscle Cell Actin Identification

Smooth muscle cells on glass coverslips were fixed in 3.7% formaldehyde and then placed in acetone at -20°C for 5 minutes. Non-specific binding sites were blocked by incubating the coverslips for 10 minutes in 1% normal goat serum at room temperature. The slides were then incubated for 1 hour at room temperature with a mouse monoclonal anti-α-smooth muscle actin (Sigma). The slides were washed with PBS 4 times and incubated with a goat anti-mouse FITC-IgG antibody for 20 minutes at room temperature. Slides were washed 4 times with PBS and then viewed via fluorescent microscopy.

E. Stress Fiber Identification

Cells were grown on glass coverslips and then fixed in 3.7% formaldehyde for 10 minutes at room temperature. After 2 washes in HBSS, acetone was applied to the coverslip and placed in -20°C for 5 minutes. The coverslip was air dried before adding 2 units of Bodipy-phallicidin (Molecular Probes Inc., Eugene OR) for 1.5 hours at room temperature. Excess toxin was then washed from the coverslip with HBSS. The cells were then viewed via fluorescence microscopy.

F. Isolation of Total RNA from Cell Cultures

Total RNA was obtained by via a phenol extraction method (Sambrook et al., 1989). This method utilized sodium dodecyl sulfate (SDS) and EDTA to lyse cells while releasing nucleic acids and proteins and inhibiting RNA degrading enzymes at the

same time. Phenol at an acid pH was then used to separate nucleic acid-associated proteins from RNA.

Culture medium was removed from the cultures and flasks rinsed with PBS. The cells were lysed in a solution containing 10 mM EDTA, pH 8.0 and 0.5% SDS. The flasks were rinsed with a solution containing 0.1 M sodium-acetate, 10 mM EDTA and the solution pooled with the lysates in appropriate eppendorfs. RNA was extracted by the addition of phenol and subsequent centrifugation (15,000 rpm) at 4°C. The top, aqueous layers containing RNA were precipitated in 0.07 M Tris, pH 8.0, 0.87 M NaCl, and 100% ethanol. Before use, RNA pellets were resuspended in diethyl pyrocarbonate (DEPC) treated water and quantitated at A_{260}/A_{280}.

G. Isolation of Plasmid DNA Containing Fibronectin cDNA

This procedure was based on the alkaline lysis method (Sambrook et al., 1989) utilizing the detergent, SDS, and NaOH to lyse E. coli so as to release its nucleic acids and the plasmid DNA. Potassium acetate was added to neutralize the solution thereby allowing plasmid DNA to reanneal while precipitating most denatured genomic DNA and proteins. Plasmid DNA is further purified from any unprecipitated genomic DNA by a cesium chloride (CsCl) gradient containing ethidium bromide. Nicked plasmid or genomic DNA, being linear, bound more ethidium bromide than unnicked plasmid DNA resulting in a higher density. This allowed for the unnicked

plasmid to travel farther down the CsCl gradient and be visualized via ultraviolet light as a separate fluorescent band to be extracted.

E. coli containing the fibronectin gene were grown overnight at 37°C with constant agitation in 25 ml LB medium plus 50 μg/ml ampicillin. The bacteria was amplified by incubation in 500 ml LB medium plus 50 μg/ml ampicillin with constant agitation at 37°C. The bacterial suspension was spun at 4°C and the resulting pellet resuspended in a solution containing 50 mM glucose, 25 mM Tris, and 10 mM EDTA. The resuspended bacteria were lysed by gentle shaking in a 0.2 M NaOH/1% SDS solution. Protein in the mixture was then precipitated by the addition of 3 M potassium acetate. After a subsequent centrifugation, the supernatants containing DNA were filtered through a Whatman No.1 filter and precipitated overnight at -20°C in ethanol. The precipitated DNA was spun and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 11 gm CsCl. Then, ethidium bromide (10 mg/ml) was added to each sample. The samples were aliquoted into 5 ml Beckman quick seal tubes and centrifuged for at least 16 hours at 65,000 rpm at 20°C.

The plasmid band in the gradient was visualized by UV lighting and collected with an 18 gauge needle inserted into the tube. Ethidium bromide was extracted from the plasmid DNA samples by mixing an equal volume of TE buffer saturated with anhydrous isopropanol. Two volumes of TE buffer and 6 volumes

of 100% ethanol were added to the DNA samples and allowed to precipitate overnight. After centrifugation at 12,000 rpm, the pellets were washed in 70% ethanol, redissolved in buffer, and transferred to eppendorfs for another precipitation with 6 M ammonium acetate and 100% ethanol overnight at -20°C. Before use, the samples were spun and pellets resuspended in TE buffer ready to be quantitated at A_{260}/A_{280}.

H. Isolation of the Fibronectin cDNA Probe

Fibronectin cDNA (500 base pairs) was isolated from the plasmid (received from Dr. R.O. Hynes, MIT) by use of restriction endonucleases. Plasmid DNA was incubated for 1 hour at 37°C in a reaction buffer (50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl) containing the restriction endonuclease, ECORI (Gibco/BRL). The reaction mixture was terminated by the addition of a loading buffer (20% Ficoll 400, 0.1 M EDTA, 1% SDS, and 0.25% xylene cyanol). The cut fibronectin cDNA fragment was then separated from the remaining plasmid fragment by gel electrophoresis. The reaction mixture was applied to a 1% low melting point agarose gel containing 0.5 μg/ml ethidium bromide and run at 60 volts for 2 hours. The fluorescent cDNA band was identified and excised from the gel under ultraviolet illumination. It was then extracted and purified from the agarose gel through the use of the Elutip-d column containing a DNA binding resin (Scleicher & Schuell, Keene, NH). This procedure involved

melting the agarose in a low salt solution (0.2 M NaCl, 20 mM Tris/HCl, pH 7.5, 1 mM EDTA) and applying it to the column. The bound DNA was then eluted from the column by application of a high salt solution (1 M NaCl, 20 mM Tris/HCl, pH 7.5, 1 mM EDTA). DNA was precipitated in ethanol and resuspended in TE buffer ready to be quantitated.

I. Radiolabeling of Fibronectin cDNA via Random Primer Extension

This method by Feinberg and Vogelstein (1983) utilized hexanucleotide primers that bind to single stranded DNA. These primers allowed the Klenow fragment, DNA polymerase I without exonuclease activity, to synthesize a new DNA strand incorporating the radioactively labeled cytidine triphosphate (CTP). The radioactively labeled DNA strands were then released from their complementary strand by heat denaturation and used to bind with fibronectin mRNA.

The random primer extension labeling kit by Dupont/NEN Research Products (Bedford, MA) was used. About 25-30 ng of fibronectin cDNA was denatured in a boiling water bath for 5 minutes and then quickly chilled. A mixture of dGTP, dATP, and dTTP, ^{32}P-labeled dCTP and hexanucleotide primers in a reaction buffer (250 mM Tris-HCl, 25 mM $MgCl_2$, 2 mg/ml bovine serum albumin, and 10 mM 2-mercaptoethanol) was added to the fibronectin cDNA. After the final addition of the Klenow fragment, the reaction proceeded for approximately 3 hours at

room temperature. Radiolabeled cDNA was then separated from unincorporated nucleotides by use of the Elutip-d Column. Just prior to Northern blot hybridization, fibronectin cDNA was denatured by boiling and quickly chilled so radiolabeled single strands were available to bind to the target mRNA.

J. Northern Blot Analysis

This method developed by Thomas (1980) enabled levels of mRNA to be determined. Total RNA was fractionated by gel electrophoresis and transferred onto a nylon membrane. The mRNA of interest was targeted by incubating the membrane with a radioactively labeled complementary DNA. Binding of cDNA to mRNA is visualized through X-ray exposure and development.

Total RNA (10 μg from mesangial cells, 15 μg from smooth muscle cells) was denatured in a MOPS buffer (5 mM sodium acetate, 1 mM EDTA, 20 mM 3-[N-Morpholino]propanesulfonic acid, pH 7.0) containing 50% formamide and 6% formaldehyde at 65°C for 15 minutes. Loading buffer (1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) and ethidium bromide (1 mg/ml) were added for visualization of the RNA after electrophoresis in a 1.2% formaldehyde agarose gel. Afterwards, the RNA was transferred to a neutral-charged nylon membrane (Stratagene, La Jolla, CA) by capillary transfer for at least 18 hours. The blot was then baked at 80°C for 2 hours and incubated in 0.1X SSPE, 0.5% SDS at 65°C for 1 hour. The blot was prehybridized in a buffer containing 5X SSPE, 1X

Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 250 μg/ml sonicated salmon sperm DNA, 50% formamide, and 8% dextran-sulfate for at least 3 hours at 42°C. Hybridization was carried out for approximately 20 hours at 42°C in the prehybridization buffer containing approximately 25-30 ng of fibronectin cDNA labeled with ^{32}P-CTP (3000 Ci/mmole). The blot was washed one time in 1X SSPE/ 1% SDS at 60°C for 15 minutes and rinsed in 0.1X SSPE/0.5% SDS at room temperature. The membrane was then air-dried, wrapped in plastic wrap, and exposed to Kodak XAR-OMAT X-ray film (Rochester, NY) with intensifying screens at -70°C for 5-7 days.

K. Protein Extraction

After various periods of IGF-I incubation, medium was collected, and fibronectin extracted from the cultured cells according to the protocol by Senger et al. (1983). Total protein from the smooth muscle cells was obtained by scraping the cells into a solution containing 2% deoxycholate (DOC), 0.02 M Tris, pH 8.0 solution plus 2 mM N-Ethyl Maleimide (NEM) and 2 mM Phenylmethlysulfonyl fluoride (PMSF). These DOC protein extracts were quantitated by the method of Lowry et al. (1951). Fibronectin in the medium and cell extracts were analyzed via western and slot blot analysis.

L. Western Blot Analysis

Western blot analysis (Towbin et al., 1979; Burnett,

1981) is used for protein identification. Like southern and northern blotting, proteins are electrophoretically separated in a SDS-polyacrylamide gel and transferred to nitrocellulose or nylon membranes. The proteins of interest are identified through use of specific antibodies. These antibodies are then detected through the binding of secondary antibodies traditionally labeled with ^{125}I or enzymes such as horseradish peroxidase or alkaline phosphatase. Visualization of the protein is performed through autoradiography or color respectively. Recently, chemiluminescent techniques that allow autoradiographic detection without the use of radioactive isotopes have been developed. These techniques utilize special substrates which are broken down by horseradish peroxidase or alkaline phosphatase releasing light that can be detected on X-ray film.

Protein samples were denatured in sample buffer containing 8 M urea, 50 mg/ml dithiothreitol, and 0.01 M H_3PO_4, pH 6.8 overnight at room temperature (Corcoran and Proudman, 1991). 1μl of a 0.1% bromophenol blue solution was added to the samples before application to the 3.0/7.0% SDS-polyacrylamide gel. Protein samples in the gel were electrophoresed at 40 mAmps for approximately 4-5 hours and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Inc., Bedford, MA) between filter papers soaked in transfer buffer (25 mM Tris-base, 192 mM glycine, pH 8.3) at a current of 0.8mA/cm^2 for 2 hours.

Chemiluminescence detection of proteins on the PVDF membrane was based on the protocol by Tropix, Inc. (Bedford, MA). After transfer, the membrane was incubated in blocking buffer (5% non-fat milk, 0.1% Tween-20, 0.02% sodium azide in tris-buffered saline (TBS), pH 7.4) for 1 hour at room temperature with constant agitation. The membrane was then incubated in blocking buffer containing a 1:25,000 dilution of anti-rat fibronectin antibody at room temperature for 1.0 hour. Unbound antibody was washed from the membrane two times for 10 minutes in a wash buffer (0.02% Tween-20, 0.02% sodium azide in TBS, pH 7.4) and one time in blocking buffer for 5 minutes. Incubation with an alkaline phosphatase conjugated anti-rabbit IgG antibody at a dilution of 1:35,000 in blocking buffer proceeded for 15 minutes at room temperature. The membrane was then washed four times in the wash buffer for ten minutes each in order to eliminate any non-specific binding of antibody. In order to detect proteins by chemiluminescence, two 5 minute washes in a assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide, pH 10) were necessary. To enhance the chemiluminescent signal on PVDF membranes, a 5 minute wash in Nitroblock (Tropix, Inc.) was performed. The membrane was rinsed two times in the assay buffer for 5 minutes and incubated in the assay buffer containing the substrate, AMPPD (Tropix, Inc.) for 5 minutes. The membrane was then wrapped in plastic wrap and exposed to Kodak XAR-OMAT film for 5 minutes. The film was then

developed.

M. Slot Blot Analysis

Protein samples were diluted in transfer buffer and applied to a Filtration Manifold System (Gibco/BRL). Proteins were bound to the membrane via a vacuum connected to the manifold. The membrane was then placed in wash buffer until ready for the chemiluminescent detection described above.

N. Autoradiograph Quantitation

Northern blots and slot blots were quantitated through densitometric analysis using a Xerox Datacopy GS Plus image scanner in conjunction with the MacIntosh programs Adobe Photoshop, version 2.01 and NIH Image, version 1.49. Values were statistically compared using an unpaired Student's t-test (Instat, version 2.0).

O. Chemicals

All chemicals were of analytical reagent grade and molecular biology grade. Human, recombinant IGF-I was obtained from Boeringher-Mannheim (Indianapolis, IN).

For a flow chart of these methods, see Appendix 1.

III. RESULTS

A. Characterization of the Primary Tissue Cultures

1. **Smooth Muscle Cells**

Smooth Muscle Cells from rat thoracic aortae were grown as described by Owens et al. (1986). Hematoxylin and Eosin staining of smooth muscle cells near confluency demonstrated the "hills and valleys" pattern of growth typical of smooth muscle cells (figure 1). Positive immunofluorescent staining using the monoclonal antibody to the α-smooth muscle actin confirmed the identity of the cells as well as a lack of contamination by either endothelial cells or fibroblasts (figure 2). Production of fibronectin by smooth muscle cells In Vitro was verified through immunofluorescent staining of fibronectin using a polyclonal anti-rat fibronectin antibody (figure 3).

2. **Mesangial Cells**

Mesangial cells were grown from glomeruli (figure 4A) as described by Foidart et al. (1981). After 7 days, rat glomeruli attached to the culture flasks from which cellular outgrowths appeared (figure 4B). By 12 days, mesangial cells were identified (figure 4C). The cultured cells were mononucleated and stellate-shaped typical of mesangial cell morphology. Stress fibers, which are bundles of actin and myosin filaments, could be shown via a fluorescein-conjugated

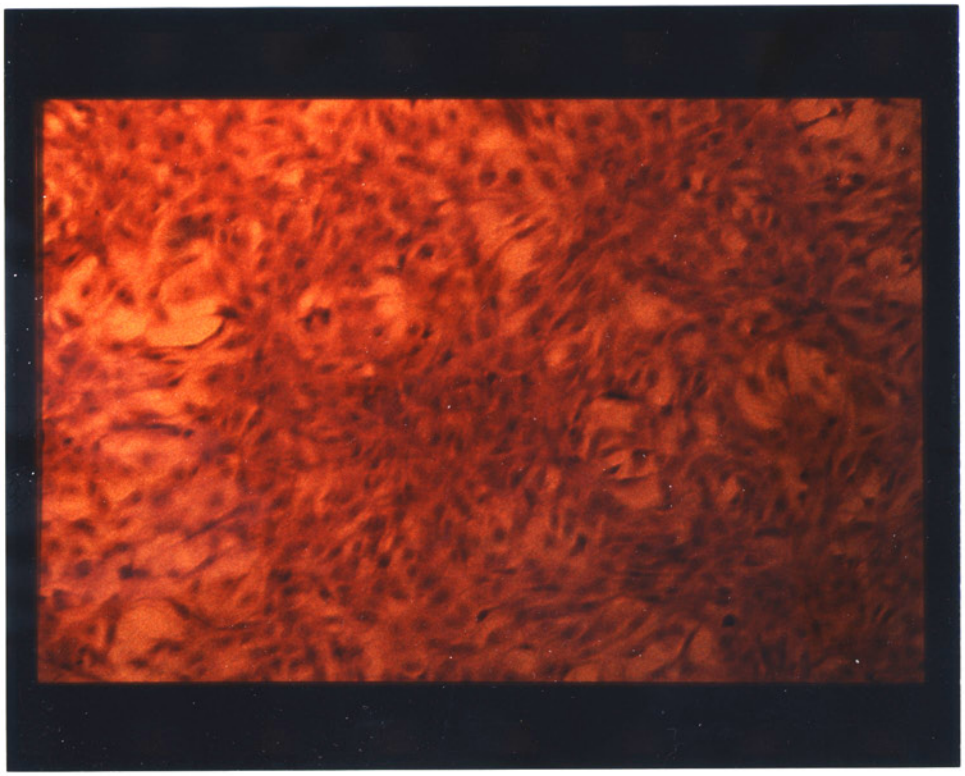


Figure 1. Hematoxylin and Eosin Stain of Cultured Smooth Muscle Cells. Confluent smooth muscle cells were stained to demonstrate the characteristic "hills and valleys" pattern of growth.

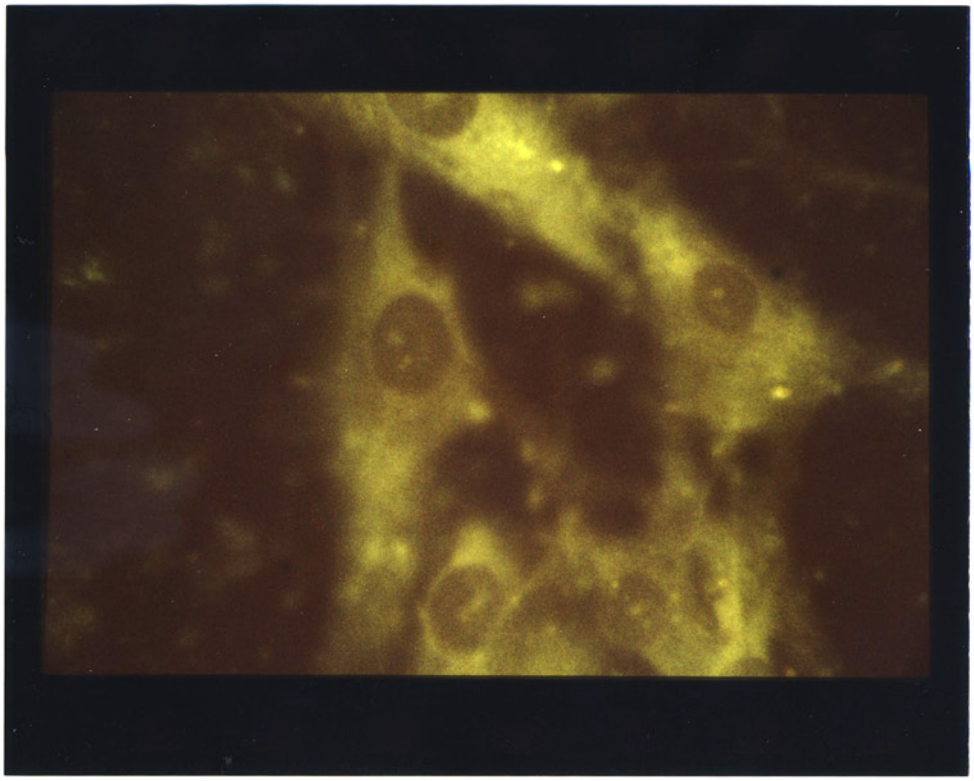
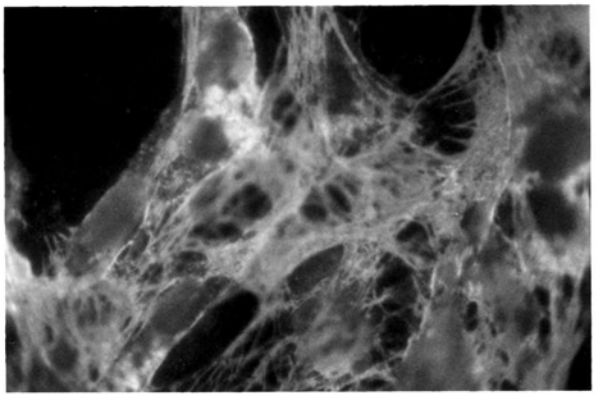
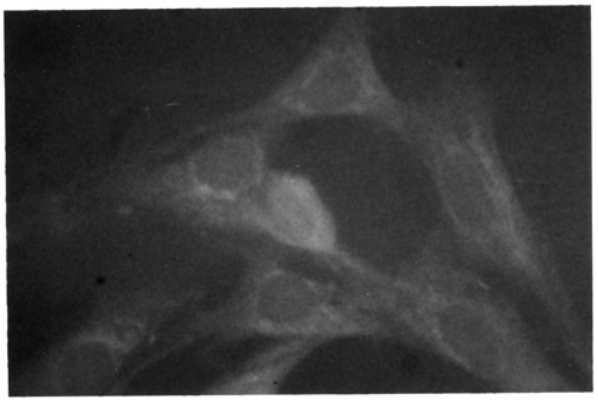


Figure 2. Identification of the α-Smooth Muscle Cell Actin. The specific α-smooth muscle cell actin filaments were demonstrated through the binding of a **monoclonal** antibody to the α-actin. The green fluorescence confirmed the presence of the specific α-actin filaments and therefore the identity of the cells.

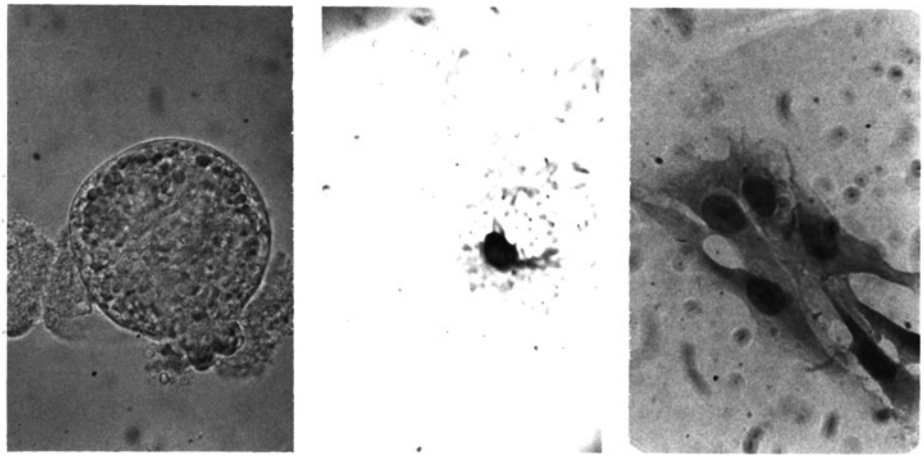


(A)



(B)

Figure 3. Production of Fibronectin by Cultured Smooth Muscle Cells. A) Fibronectin production by smooth muscle cells In Vitro was confirmed by immunofluorescence using a polyclonal anti-rat fibronectin antibody. B) A control stain in the absence of the fibronectin antibody exhibited no intense fluorescence as shown in (A).



(A) (B) (C)

Figure 4. Tissue Culture of Rat Mesangial Cells. A) A micrograph of a rat glomerulus used to obtain mesangial cells. B) Cellular outgrowths from an attached glomerulus after 7 days of culture. C) Mononucleated, stellate-shaped mesangial cells after 12 days of culture.

phallotoxin specific for actin filaments. Mesangial cells had abundant actin filaments running longitudinally throughout its cytoplasm (figure 5). Cultured mesangial cells also produced fibronectin In Vitro (figure 6). Contamination by glomerular epithelial cells and endothelial cells was also determined through immunofluorescence. Mesangial cell cultures did not fluoresce when incubated with antibodies to the cytokeratin antigen, specific for epithelial cells (figure 7), and antibodies to the Factor VIII antigen, specific for endothelial cells (figure 8). Therefore, the mesangial cell cultures were not contaminated by either epithelial cells or endothelial cells.

B. IGF-I Effect on Fibronectin mRNA Levels

1. Smooth Muscle Cells

Northern blot analysis demonstrated that fibronectin mRNA levels in the smooth muscle cells respond to IGF-I in a time and dose dependent fashion. A set of sixteen $25cm^2$ tissue culture flasks was seeded with 30×10^4 cells and allowed to grow to confluency for approximately 6 days. After 48 hours of quiescence in serum-free M199 medium, IGF-I at a final concentration of 10 ng/ml IGF-I (1.3×10^{-9} M) was added to the cultures. After 1, 4, 8, and 12 hours of IGF-I incubation, total RNA was extracted and fibronectin mRNA analyzed. Fibronectin mRNA gradually increased to significant levels 4 hours after IGF-I addition and began declining

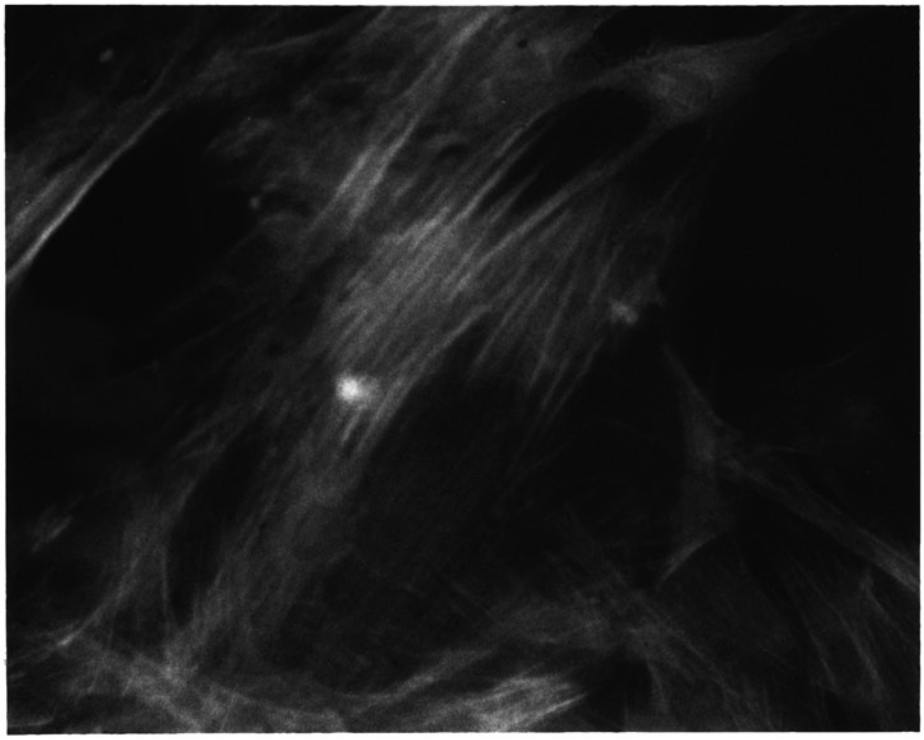
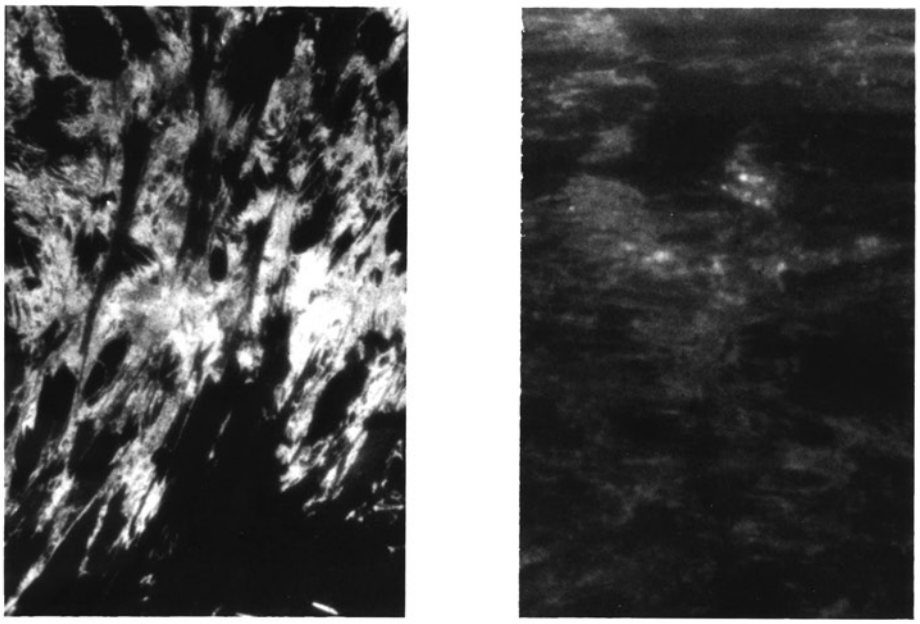
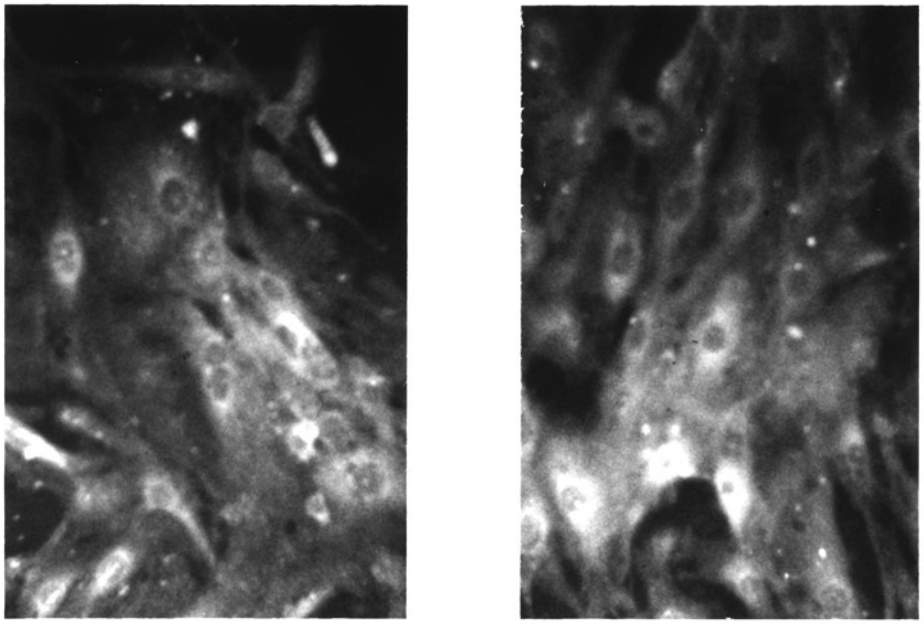


Figure 5. Stress fibers of Mesangial Cells. The presence of stress fibers typical of mesangial cells was demonstrated through the binding of a fluorescein-conjugated phallotoxin specific for the actin filament. The fluorescence displayed above demonstrates the existence of abundant actin filaments longitudinally running throughout the cytoplasm of the cells.



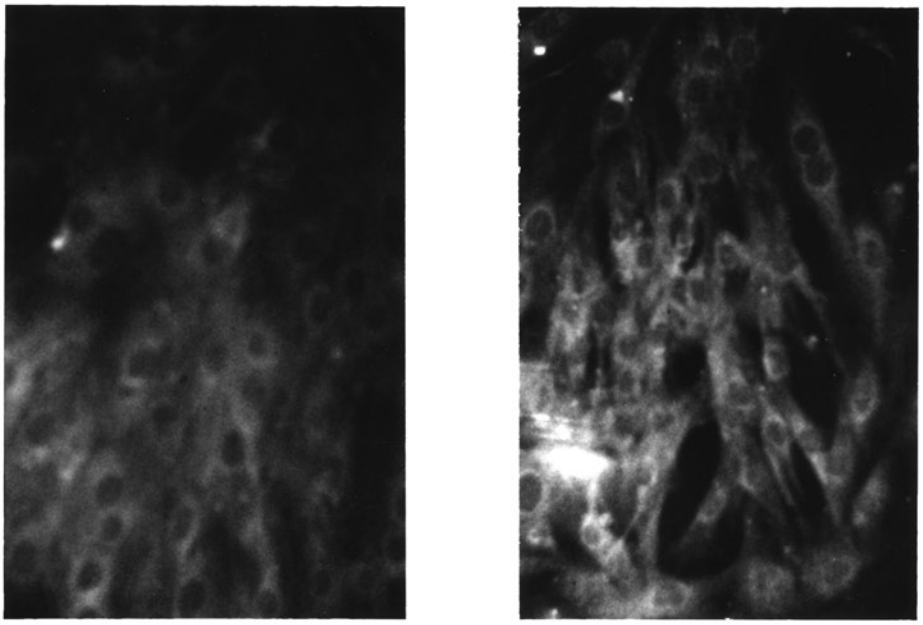
(A) **(B)**

Figure 6. Fibronectin Production by Cultured Mesangial Cells. A) Fibronectin production by mesangial cells In Vitro was confirmed by immunofluorescence using a polyclonal anti-rat fibronectin antibody. B) A control stain in the absence of the fibronectin antibody lacked the intense fluorescence as shown in (A).



(A) **(B)**

Figure 7. Determination of Mesangial Cell Culture Contamination by Epithelial Cells. A) Mesangial cells were incubated with anti-cytokeratin antibodies specific for the epithelial cells. The intensity of fluorescence was similar to control conditions containing no antibody as shown in (B). Therefore, no epithelial cells were present in the cultures.



(A) **(B)**

Figure 8. Determination of Mesangial Cell Culture Contamination by Endothelial Cells. A) Mesangial cells were incubated with anti-Factor VIII antibodies specific for the endothelial cells. The intensity of fluorescence was similar to control conditions containing no antibody as shown in (B). Therefore, no endothelial cells were present in the cultures.

thereafter (figure 9A). Densitometric quantitation of the fibronectin mRNA bands confirmed the increase of fibronectin mRNA with time (figure 9B). At these same time points, IGF-I did not induce any significant increase in total RNA synthesis (figure 9C). Under the same conditions as described above, the smooth muscle cells were incubated with various final concentrations of 0, 2.5, 5.0, 10, and 20 ng/ml IGF-I, respectively for 4 hours. Fibronectin mRNA increased in a dose-dependent fashion and reached significant levels at 10-20 ng/ml (figure 10A). Densitometric quantitation of the fibronectin mRNA bands confirmed the dose-dependent increase (figure 10B). The physiological concentration of 10 ng/ml (1.3×10^{-9} M) IGF-I was chosen in order to mimic the In Vivo conditions as much as possible. This concentration has also been determined as the amount which binds to 50% of the available IGF-I receptors (King et al., 1985). The 18S ribosomal RNA levels also showed no change in response to IGF-I and confirmed equal loading of RNA samples onto the gel (figure 9A and 10A). In addition, ethidium bromide stained RNA gels visualized under ultraviolet illumination also displayed equal loading of RNA samples (figure 11).

To determine the potential sites at which IGF-I regulates fibronectin mRNA synthesis, RNA and protein synthesis inhibitors were added in conjunction with IGF-I. Actinomycin D, which inhibits RNA synthesis, or cycloheximide, which inhibits protein synthesis, was added to the smooth muscle

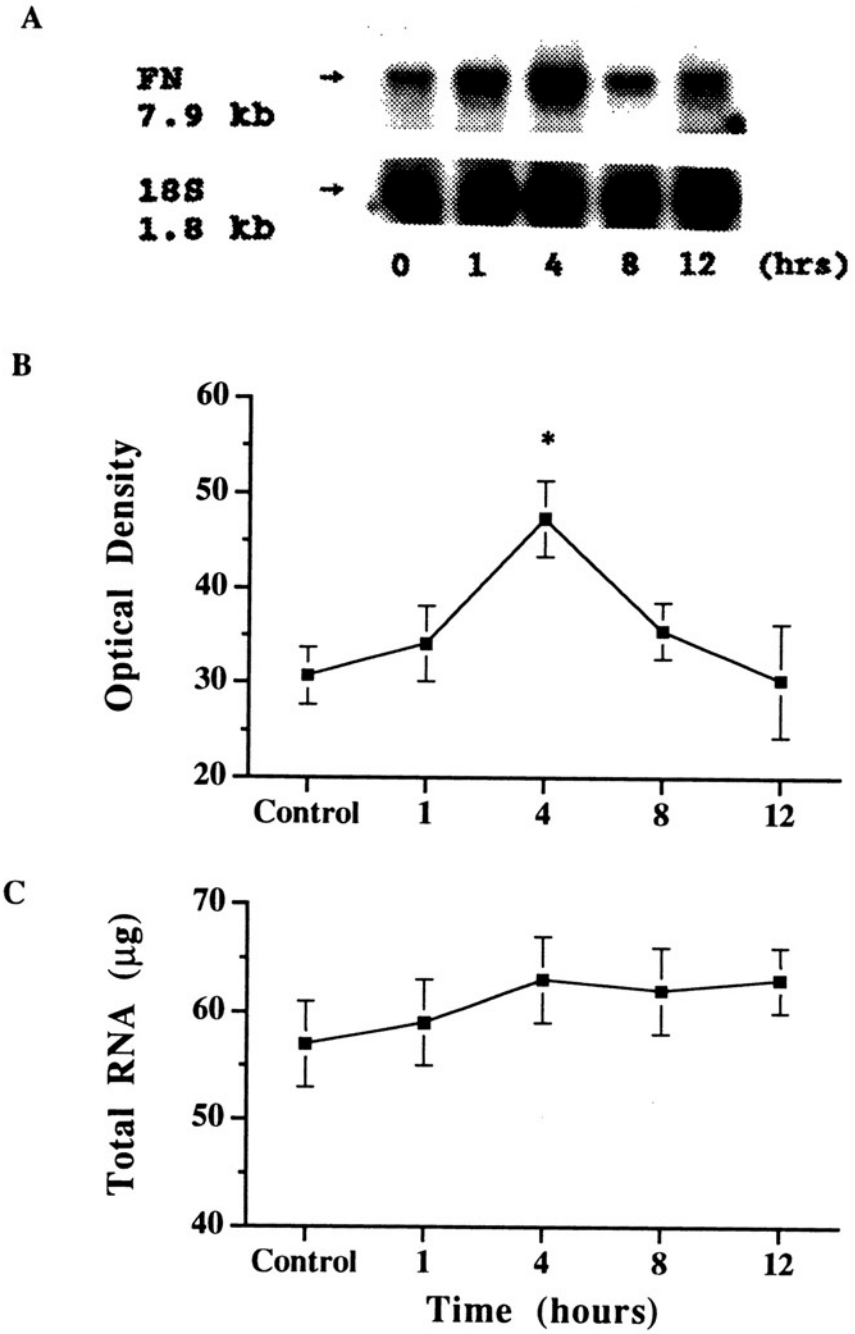
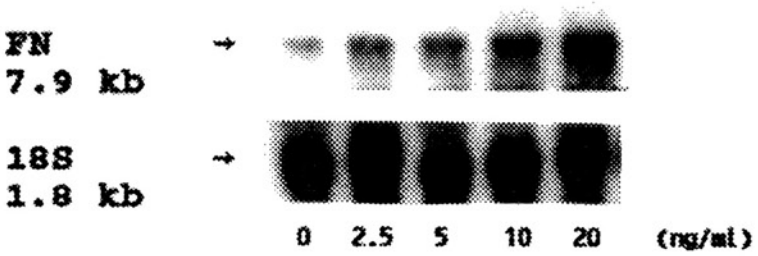


Figure 9. Time Response of Smooth Muscle Cell Fibronectin mRNA Levels to IGF-I. A) Northern blot of fibronectin mRNA (FN) after 1, 4, 8, and 12 hours of IGF-I treatment. Also shown are the 18S rRNA levels as the internal control. B) Northern blots were quantitated and fibronectin mRNA levels expressed as relative optical density units. Each point represents the mean ± SEM (n=8). *P<0.05 vs. control. C) Total RNA from the cultures were quantitated. Each point represents the mean ± SEM (n=8).

A



B

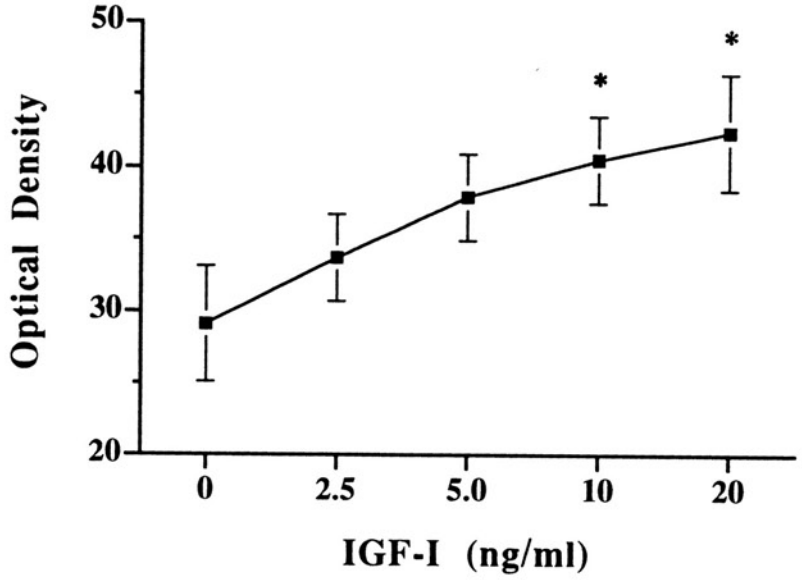


Figure 10. Dose Response of Smooth Muscle Cell Fibronectin mRNA Levels to IGF-I. A) Northern blot of fibronectin mRNA (FN) in response to various concentrations of IGF-I. Also shown are the 18S rRNA levels as the internal control. B) Northern blots were quantitated and fibronectin mRNA levels expressed as relative optical density units. Each point represents the mean ± SEM (n=8). *P<0.05 vs. control.

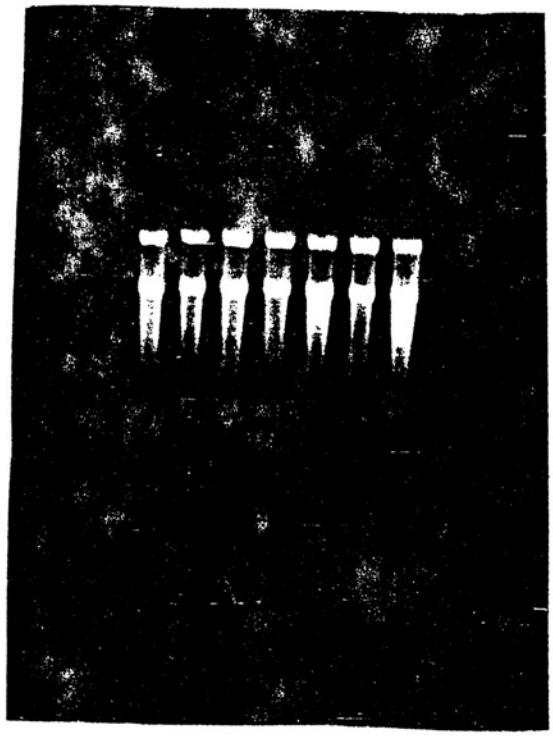


Figure 11. Ultraviolet (UV) Visualization of Total RNA Agarose Gels. Upon UV illumination, the fluorescence emitted from the ethidium bromide-bound total RNA was observed. Uniform fluorescent intensity of the individual RNA samples as shown above confirmed equal loading of the samples onto the agarose gel.

cells at final concentrations of 40 μM and 36 μM, respectively, 1 hour before the addition of 10 ng/ml IGF-I. Four hours after a co-incubation of IGF-I plus the inhibitor, Northern blot analysis demonstrated an inhibition of the IGF-I-induced increase of fibronectin mRNA by actinomycin D. However, an even greater induction of fibronectin mRNA was seen in the presence of cycloheximide and IGF-I (figure 12).

2. **Mesangial Cells**

Northern blot analysis demonstrated that fibronectin mRNA levels in the mesangial cells also respond to IGF-I in a time and dose dependent fashion. A set of sixteen flasks was seeded with 35 X 10^4 cells and allowed to grow to confluency for approximately 6 days. After 48 hours of quiescence in RPMI medium supplemented with 2% FBS, a final concentration of 10 ng/ml IGF-I (1.3 x 10^{-9} M) was added to the cultures and total RNA extracted at 1, 4, 8, and 12 hours, respectively. Fibronectin mRNA gradually increased to significant levels 8 hours after IGF-I addition and began declining thereafter (figure 13A). Densitometric quantitation of the fibronectin mRNA bands confirmed the increase of fibronectin mRNA with time (figure 13B). At these same time points, total RNA showed no significant change in response to IGF-I stimulation (figure 13C). Under these same conditions, the mesangial cells were incubated with various final concentrations of 0, 2.5, 5.0, 10, and 20 ng/ml IGF-I, respectively for 8 hours.

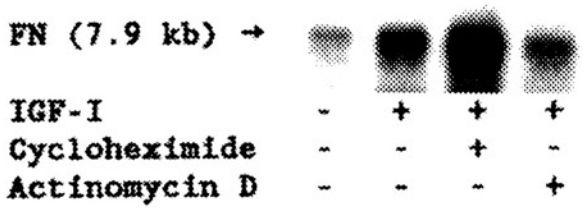


Figure 12. Effects of Actinomycin D and Cycloheximide on the IGF-I-Induction of Smooth Muscle Cell Fibronectin mRNA Levels. A northern blot of smooth muscle cell fibronectin mRNA levels (FN) in response to a 4 hour treatment with actinomycin D (40 μM, final concentration) or cycloheximide (36 μM, final concentration) plus 10 ng/ml IGF-I.

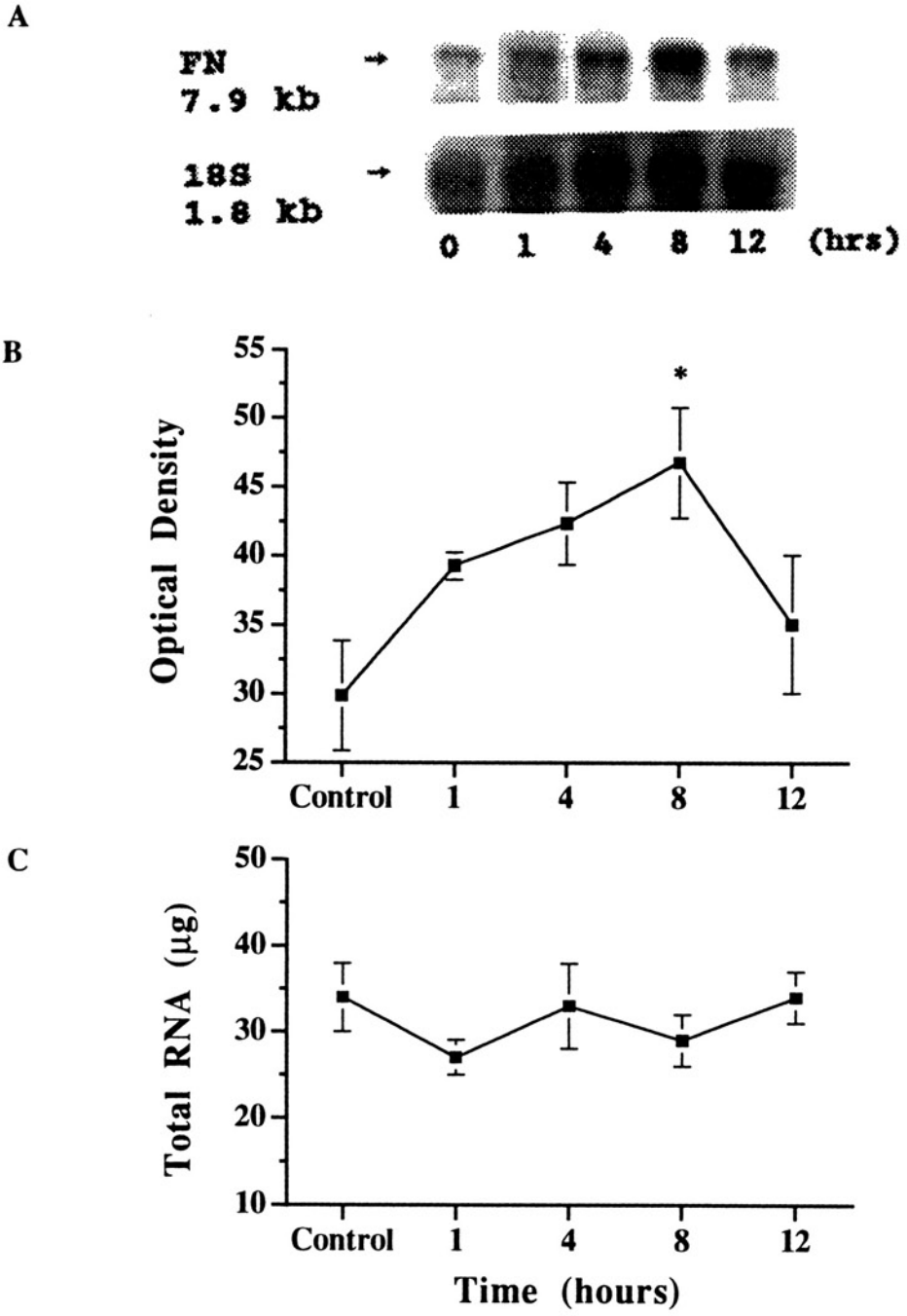


Figure 13. Time Response of Mesangial Cell Fibronectin mRNA Levels to IGF-I. A) Northern blot of fibronectin mRNA (FN) after 1, 4, 8, and 12 hours of IGF-I treatment. Also shown are the 18S rRNA levels as the internal control. B) Northern blots were quantitated and fibronectin mRNA levels expressed as relative optical density units. Each point represents the mean ± SEM (n=5). *P<0.05 vs. control. C) Total RNA from the cultures were quantitated. Each point represents the mean ± SEM (n=5).

Fibronectin mRNA increased in a dose-dependent fashion and reached significant levels at 10 ng/ml (figure 14A). Densitometric quantitation confirmed this result (figure 14B). The physiological concentration of 10 ng/ml IGF-I was chosen for all subsequent experiments. This concentration has also been determined as the amount which binds to 50% of the available IGF-I receptors (Arnqvist et al., 1988; Conti et al., 1988). The 18S ribosomal RNA levels which served as a control for equal sample loading showed no change in response to IGF-I. Ethidium stained RNA gels also confirmed equal loading of RNA samples.

Potential sites at which IGF-I regulates fibronectin mRNA synthesis were also investigated in mesangial cells. Actinomycin D or cycloheximide was added to mesangial cell cultures at final concentrations of 40 μM and 36 μM, respectively, 1 hour before an additional 8 hour co-incubation with 10 ng/ml IGF-I. Northern blot analysis demonstrated a response identical to that by the smooth muscle cells. An inhibition of the IGF-I-induced increase of fibronectin mRNA was observed in the presence of actinomycin D. However, an even greater induction of fibronectin mRNA was seen in the presence of cycloheximide and IGF-I (figure 15).

C. IGF-I Effect on Fibronectin Protein Levels

1. **Smooth Muscle Cells**

In order to determine if the IGF-I-mediated fibronectin

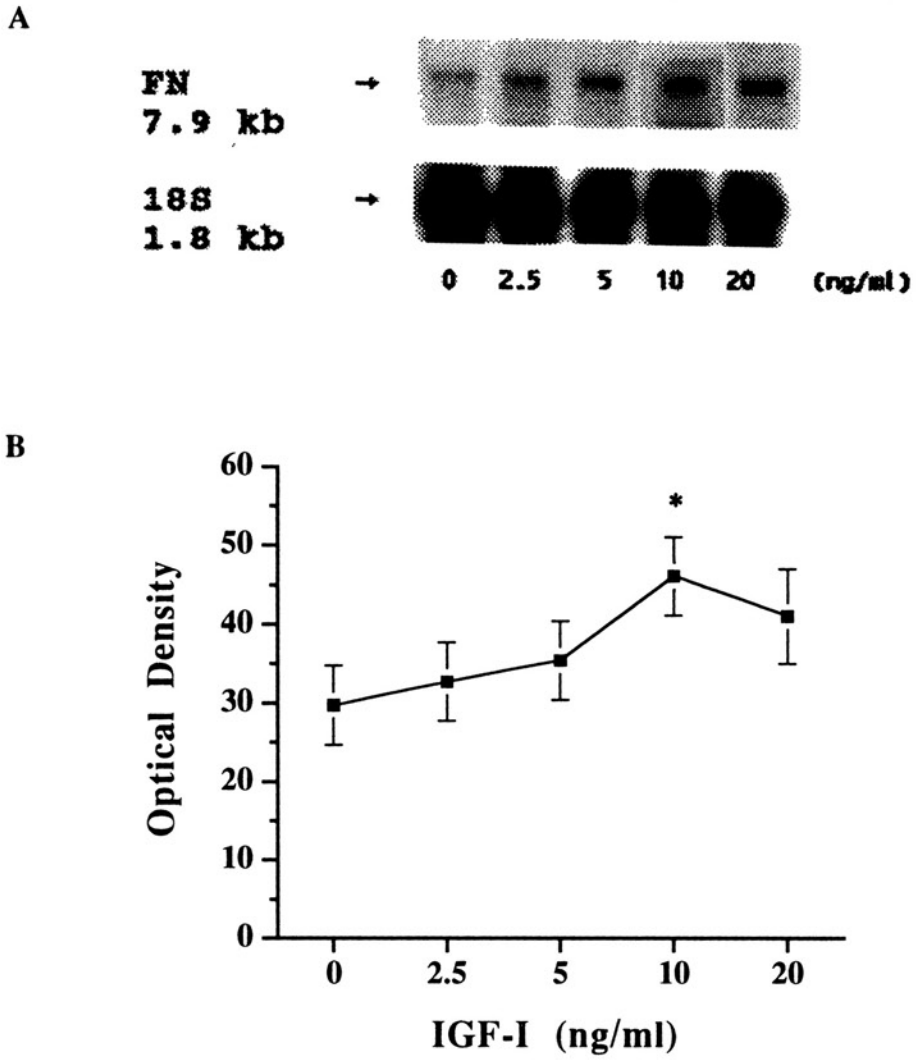


Figure 14. Dose Response of Mesangial Cell Fibronectin mRNA Levels to IGF-I. A) Northern blot of fibronectin mRNA (FN) in response to various concentrations of IGF-I. Also shown are the 18S rRNA levels as the internal control. B) Northern blots were quantitated and fibronectin mRNA levels expressed as relative optical density units. Each point represents the mean ± SEM (n=6). *P=0.05 vs. control.

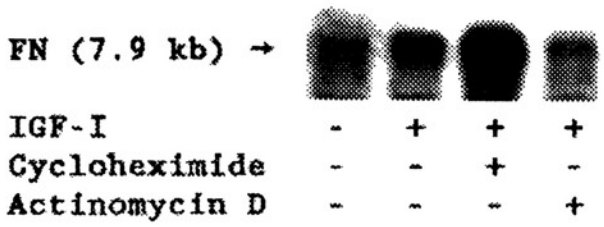


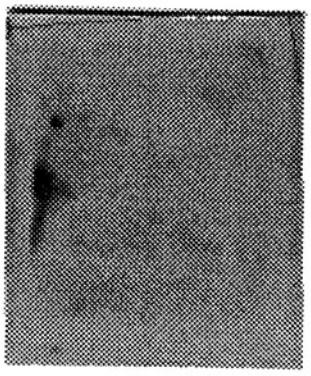
Figure 15. Effects of Actinomycin D and Cycloheximide on the IGF-I-Induction of Mesangial Cell Fibronectin mRNA Levels. A northern blot of mesangial cell fibronectin mRNA levels (FN) in response to a 8 hour treatment with actinomycin D (40 μM, final concentration) or cycloheximide (36 μM, final concentration) plus 10 ng/ml IGF-I.

mRNA induction results in an increased synthesis and/or secretion of fibronectin, levels of the matrix protein were quantitated in cellular protein extracts and culture media of smooth muscle cells. Cells were cultured in a 24 well tissue culture dish (1.6 cm^2/well) at a density of 3 X 10^4. After a 48 hour period of quiescence in serum free M199, a final concentration of 10 ng/ml IGF-I was added to the cultures. Culture medium was then collected and total cellular protein extracted as described in the Methods section. Total protein in cellular extracts and culture medium were separated by SDS-polyacrylamide gel electrophoresis and fibronectin was detected by chemiluminescent western blotting analysis. A single protein band, which co-migrated with a pure rat fibronectin standard, was observed in the cellular extracts and the culture media. The western blot also demonstrated an increase in fibronectin secreted into the media of smooth muscle cell cultures treated with IGF-I. Fibronectin levels showed no change in the IGF-I-treated cell extracts (figure 16A). The western blot was also incubated in the absence of the fibronectin antibody to confirm that the protein band was indeed fibronectin and not due to non-specific binding by the alkaline phosphatase antibody. When the fibronectin antibody was absent, a protein band was not evident verifying the specificity of the fibronectin antibody (figure 16B).

Since fibronectin existed as a single band in the western blot analysis, slot blot analysis was used to investigate a

FN SM+ SM- SC+ SC-

(A)



(B)

Figure 16. Western blot of Smooth Muscle Cell Fibronectin Levels. A) Fibronectin detected in cellular protein extracts (SC) and culture media (SM) from control (-) and IGF-I-treated (+) smooth muscle cell cultures. A pure rat fibronectin standard (FN) is also shown. B) A western blot incubated without the anti-rat fibronectin antibody.

time profile of the IGF-I-mediated fibronectin synthesis and also confirm the effect observed in the western blot shown in figure 16A. After 2, 4, 8, and 12 hours of IGF-I treatment, cell extracts and culture media were collected. Using a chemiluminescent slot blot procedure, optimal antibody concentrations were first determined by probing various amounts of a pure rat fibronectin standard. The fibronectin and alkaline phosphatase conjugated antibody concentrations used in these experiments were able to detect between 0 and 20 ng of fibronectin in a linear fashion (r^2=0.93; figure 17). Samples were diluted so as to be confident that fibronectin differences were within the linear range of detection. Examples of fibronectin detected in the serial dilutions of the cell extracts and culture media along with the linear range of fibronectin standards are shown in figure 18. These dilutions were used to quantitate fibronectin levels in the cultures. In addition, 12 ng of the pure rat fibronectin standard was applied to each slot blot for normalizing data to the standard curve shown in figure 17. A significant increase of fibronectin secreted into the culture media was observed 4 and 8 hours after IGF-I (10 ng/ml) addition (Table 1). However, no change in cellular fibronectin levels from IGF-I-treated cultures was observed. In order to confirm that the IGF-I-induced fibronectin secretion into the culture medium was not reflective of an increase in total protein synthesis, total protein from these same cultures were

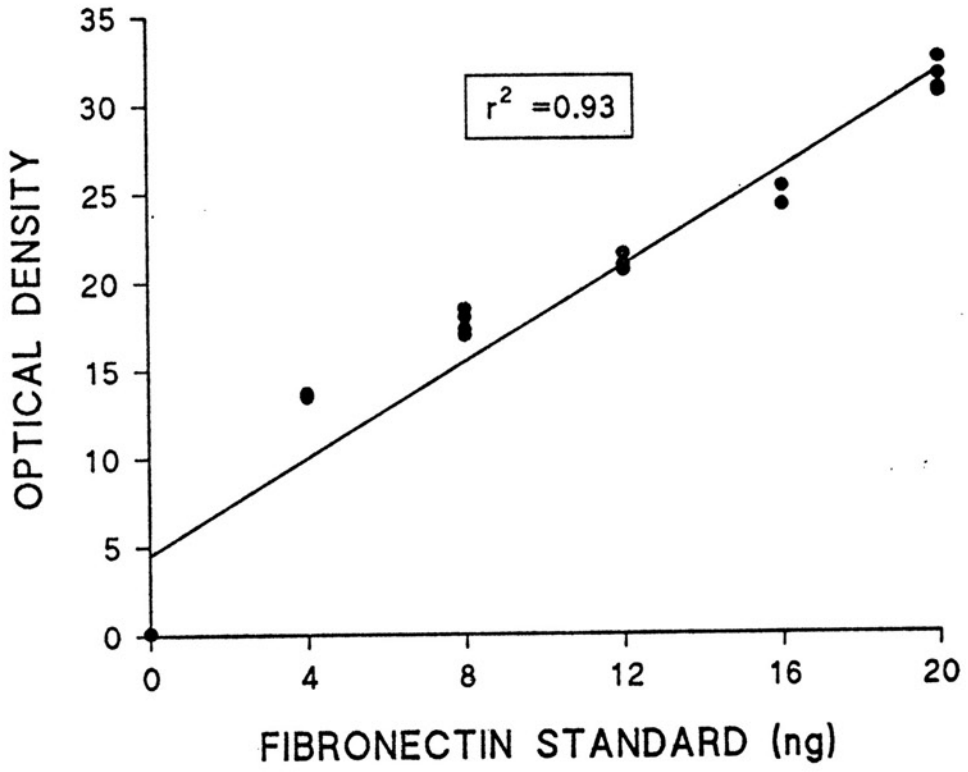


Figure 17. Determination of the Linear Range of Fibronectin Detection for Slot Blot Analysis. A slot blot of various amounts of pure rat fibronectin. Fibronectin levels were quantitated and expressed as relative optical density units which were then analyzed using linear regression.

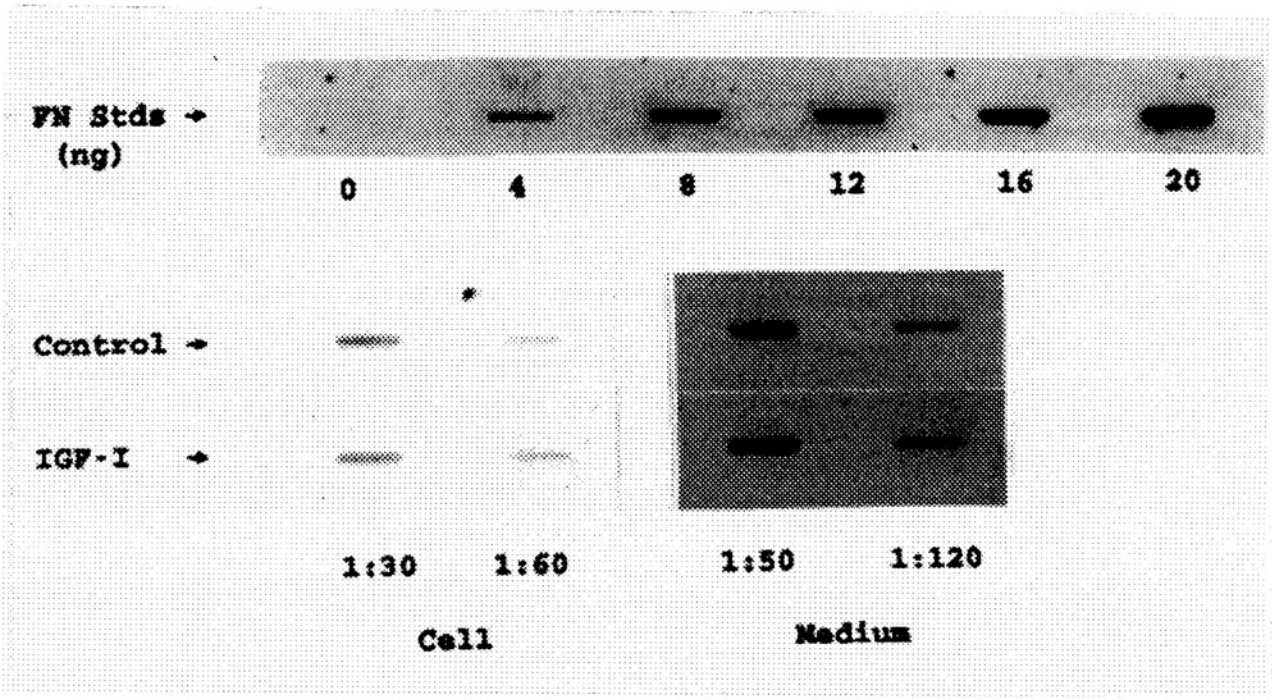


Figure 18. Slot Blot Analysis of Fibronectin in Smooth Muscle Cell Cultures. Top) Linear range of fibronectin detection (FN Stds). Bottom) Examples of fibronectin detected in serial dilutions of the cell extracts and culture media, both from control and IGF-I-treated cultures. These dilutions were used to quantitate fibronectin differences in the linear range of detection.

Table 1. Effect of IGF-I on Smooth Muscle Cell Fibronectin Levels

	Medium		Cell	
Time	Control	IGF-I	Control	IGF-I
2 hours	70.7 ± 9	81.9 ± 10	25.5 ± 2	29.5 ± 4
4 hours	99.4 ± 8	124.4 ± 5*	26.8 ± 5	26.8 ± 4
8 hours	131.9 ± 4	169.9 ± 7**	23.5 ± 3	28.5 ± 4
12 hours	153.3 ± 9	170.7 ± 17	24.4 ± 4	29.9 ± 2

After the addition of 10 ng/ml IGF-I to smooth muscle cell cultures for 2, 4, 8, and 12 hours, culture medium was collected and protein extracted as described in the Methods section. Fibronectin (ng/μg protein) was then quantitated through slot blot analysis. Each value represents the mean ± SEM (n=5-6). *P<0.05 vs. control; **P<0.01 vs. control.

quantitated. IGF-I did not stimulate an increase in total protein synthesis during a period of 12 hours (Table 2).

2. **Mesangial Cells**

Cells were cultured in a 24 well tissue culture dish (1.6 cm^2/well) at a density of 3 X 10^4. After a 48 hour period of quiescence in RPMI supplemented with 2% FBS, a final concentration of 10 ng/ml IGF-I was added to the cultures. The culture medium was then collected and total cellular protein extracted. Total protein in the cell extracts and culture media were separated by SDS-polyacrylamide gel electrophoresis and fibronectin was detected by chemiluminescent western blotting analysis. A single protein band, which co-migrated with a pure rat fibronectin standard, was observed in the cellular extracts and the culture media (figure 19). In addition, the western blot demonstrated an increase in cellular fibronectin levels as well as fibronectin secreted into the culture media form cultures treated with IGF-I.

Since fibronectin from mesangial cell cultures also existed as a single band in the western blot analysis, slot blot analysis was used to quantitate a time profile of fibronectin levels in response to IGF-I and confirm the effect observed in the western blot shown in figure 19. After 2, 4, 8, and 12 hours of 10 ng/ml IGF-I treatment, cell extracts and culture media were collected. These samples were also diluted

Table 2. Total Smooth Muscle Cell Protein Levels After IGF-I Treatment

Time	Control	IGF-I
2 hours	92.5 ± 2	87.5 ± 3
4 hours	71.0 ± 4	73.0 ± 1
8 hours	80.0 ± 2	77.0 ± 1
12 hours	79.0 ± 4	82.0 ± 3

Total cellular protein (μg) in smooth muscle cell cultures were quantitated and compared to control values. Each value represents the mean ± SEM (n=5-6). At all time points, there was no statistically significant difference between control and treated samples.

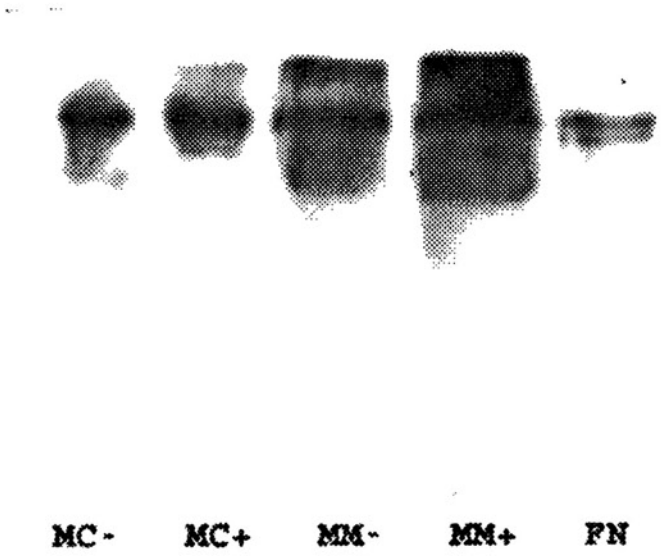


Figure 19. Western blot of Mesangial Cell Fibronectin Levels. Fibronectin detected in cellular protein extracts (MC) and culture media (MM) from control (-) and IGF-I-treated (+) mesangial cell cultures. A pure rat fibronectin standard (FN) is also shown.

so as to be confident that the fibronectin differences were within the same linear range of detection previously described. An example of serial dilutions of the cell extracts and culture media along with the linear range of fibronectin standards are shown in figure 20. These dilutions were used to quantitate fibronectin in the mesangial cell cultures. A 12 ng fibronectin standard was also used to normalize data to the standard curve (Figure 17). To determine whether there were any significant amounts of fibronectin present in the 2% FBS culture medium, an equal volume of RPMI medium plus 2% FBS was applied to the PVDF membrane and analyzed. Fibronectin present in the 2% FBS displayed a negligible signal when compared to the experimental samples (Figure 21). Therefore, fibronectin detected in the mesangial cell culture medium and cellular protein extracts originated from the cells rather than the 2% serum. A significant increase of fibronectin in the cell extracts was observed at 4, 8, and 12 hours after IGF-I (10 ng/ml) addition (Table 3). IGF-I also stimulated a significant increase in fibronectin secreted into the culture media 8 hours after IGF-I addition. During the same time course of 12 hours, IGF-I did not stimulate any significant increase in total protein synthesis by the cultured mesangial cells (Table 4).

D. Regulatory Effect of Insulin on the Mesangial Cell Fibronectin mRNA Response to IGF-I

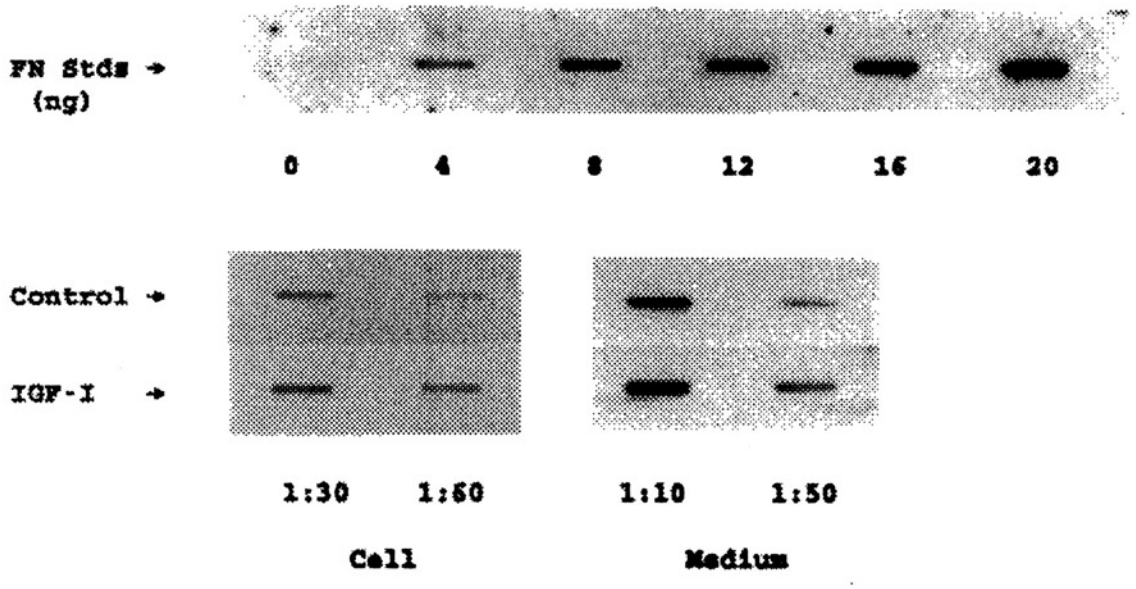


Figure 20. Slot Blot Analysis of Fibronectin in Mesangial Cell Cultures. Top) Linear range of fibronectin detection (FN Stds). Bottom) Examples of fibronectin detected in serial dilutions of the cell extracts and culture media, both from control and IGF-I-treated cultures. These dilutions were used to quantitate fibronectin differences in the linear range of detection.

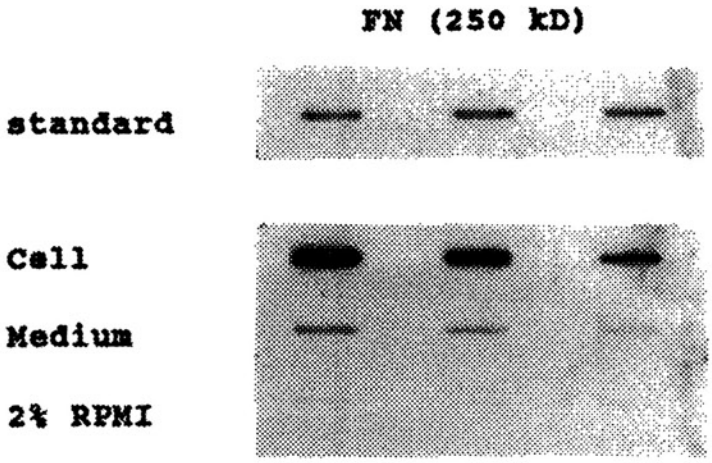


Figure 21. Comparison of Fibronectin Present in Mesangial Cell Cultures and 2% FBS-supplemented RPMI Medium. A slot blot of fibronectin levels in an equal volume of the mesangial cell protein extract (cell), mesangial cell culture medium, and RPMI medium plus 2% FBS diluted at the following ratios (from left to right): Cell - 1:35, 1:70, 1:140; Medium and 2% RPMI - 1:50, 1:100, 1:200. A triplicate application of 12 ng of pure rat fibronectin (standard) is also shown.

Table 3. Effect of IGF-I on Mesangial Cell Fibronectin Levels

	Medium		Cell	
Time	Control	IGF-I	Control	IGF-I
2 hours	39.4 ± 4	42.3 ± 7	62.8 ± 7	71.3 ± 7
4 hours	40.0 ± 2	42.6 ± 2	69.5 ± 3	85.1 ± 4*
8 hours	42.2 ± 3	61.5 ± 5*	69.0 ± 2	91.0 ± 3**
12 hours	40.2 ± 2	40.0 ± 1	70.4 ± 3	83.6 ± 5*

After the addition of 10 ng/ml IGF-I to mesangial cell cultures for 2, 4, 8, and 12 hours, culture medium was collected and protein extracted as described in the Methods section. Fibronectin (ng/μg protein) was then quantitated through slot blot analysis. Each value represents the mean ± SEM; 2 and 4 hours: n=7-8, 8 and 12 hours: n=10-12. *$P \leq 0.01$ vs. control; **$P < 0.001$ vs. control.

Table 4. Total Mesangial Cell Protein Levels After IGF-I Treatment

Time	Control	IGF-I
2 hours	60.0 ± 5	61.3 ± 5
4 hours	56.0 ± 8	59.3 ± 8
8 hours	62.5 ± 5	63.8 ± 6
12 hours	62.5 ± 4	66.8 ± 3

Total Protein (μg) in the cell extracts of mesangial cell cultures were quantitated and compared to control values. Each value represents the mean ± SEM (n=8). At all time points there was no statistically significant difference between control and treated samples.

The possible regulatory effect of insulin on the IGF-I-mediated fibronectin gene expression in mesangial cell cultures was also explored. The fibronectin mRNA response to insulin alone as well as IGF-I plus insulin was compared to that response elicited by IGF-I alone. After 48 hours of quiescence, a final concentration of 10 ng/ml IGF-I and/or a final physiological concentration of 5 ng/ml (8×10^{-10} M) insulin was added to the mesangial cells. After 8 hours, fibronectin mRNA levels were analyzed by Northern blot analysis. Fibronectin mRNA levels were higher in mesangial cells treated with IGF-I and insulin than those treated with IGF-I alone. However, insulin alone was not able to stimulate fibronectin mRNA synthesis (Figure 22).

Figure 22. Modulation of the IGF-I-Induced Mesangial Cell Fibronectin mRNA Synthesis by Insulin. A northern blot of mesangial cell fibronectin mRNA in response to IGF-I, insulin, and IGF-I plus insulin.

IV. DISCUSSION

These results propose that IGF-I is a potential regulator of fibronectin synthesis in rat aortic smooth muscle cells and glomerular mesangial cells. Northern blot experiments demonstrated that fibronectin mRNA synthesis responds to IGF-I in a time and dose-dependent fashion. Total RNA in the cell cultures did not change upon IGF-I stimulation during a period of 12 hours. Therefore, the induction of fibronectin mRNA levels by IGF-I is probably an effect that is not due to a general increase in total RNA. RNA and protein synthesis inhibitors were used to determine the sites of action of the IGF-I-induced fibronectin gene expression. The smooth muscle cells and mesangial cells responded in an identical fashion. The IGF-I-induced fibronectin mRNA levels were inhibited by actinomycin D. This result suggests that IGF-I affects fibronectin mRNA synthesis at the transcriptional level. More molecular studies, i.e. nuclear run-on assays, would be necessary to determine whether IGF-I regulates pre- or post-transcriptional mechanisms. When smooth muscle cells and mesangial cells were exposed to cycloheximide and IGF-I, fibronectin mRNA levels were enhanced even more than by IGF-I alone. Differential effects of cycloheximide on cellular responses have been reported. In response to the PDGF plus cycloheximide, Majack et al. (1987) observed a superinduction of thrombospondin mRNA while Eriksson et al. (1991) found an

attenuation of PDGF α and ß receptor expression. There are still no definitive explanations for these effects. Increased fibronectin mRNA levels could be the result of increased mRNA stability induced by IGF-I and/or cycloheximide. IGF-I could also regulate the synthesis of a "protein" which acts as a negative feedback signal for fibronectin mRNA synthesis. By blocking protein synthesis, the negative feedback signal is inhibited and greater fibronectin mRNA levels are observed. More molecular studies are needed to accurately interpret these findings.

Fibronectin is a glycoprotein which exists as a soluble form in the plasma and an insoluble form in the extracellular matrices. Cultured cells produce fibronectin which can be secreted into the culture media or bound to the cell surfaces. Fibronectin was quantitated in cellular protein extracts and culture media to determine whether the elevated fibronectin mRNA levels after IGF-I stimulation result in a larger production of the protein by the smooth muscle cells and mesangial cells. Fibronectin produced by the smooth muscle cells was present in the cellular compartment as well as in the medium compartment. Fibronectin levels, which co-migrated with the purified rat fibronectin, were higher in the culture media while cellular fibronectin remained unchanged after IGF-I treatment. Quantitation of fibronectin production through slot blot analysis showed a significant increase of fibronectin secreted into the culture media 4 and 8 hours

after IGF-I addition. During this same time period, IGF-I did not stimulate total protein synthesis thereby suggesting that the fibronectin response to IGF-I is not indicative of a generalized increase in total protein. Another issue to address is the parallel increase of fibronectin secreted into the media of smooth muscle cell cultures not treated with IGF-I. Senger et al. (1983) suggested that fibronectin synthesis in NILH8 hamster embryo cells is dependent on culture density and culture age. In the same way, fibronectin synthesis in smooth muscle cell cultures may also be dependent on these two factors. The experiments used confluent control cultures maintained quiescent for 48 hours plus an additional experimental period of 12 hours. Therefore, the "basal" fibronectin synthesis observed may be exhibiting regulation by the confluent state as well as the total time in culture. IGF-I somehow increases this basal secretion significantly at 4 and 8 hours.

Fibronectin produced by the mesangial cells was also present in the cellular compartment and the medium compartment. Western blots revealed that fibronectin in the cell extracts and the culture media co-migrated with a purified rat fibronectin standard as a single protein band. Levels of this matrix protein in both compartments were also increased after 8 hours of incubation with IGF-I. Slot blot analysis was also used to obtain a time profile of the IGF-I effect on fibronectin production by the mesangial cells.

A significant increase of cellular fibronectin levels was observed at 4, 8, and 12 hours after IGF-I addition. IGF-I also stimulated an increase in fibronectin secreted into the culture medium after 8 hours of IGF-I treatment. During these periods, total protein synthesis did not change in response to IGF-I. Thus, the fibronectin response by the mesangial cells is not indicative of increased total protein synthesis by IGF-I. In summary, fibronectin mRNA levels and fibronectin protein levels of both the smooth muscle cells and the mesangial cells respond to IGF-I. These observations suggest that increased fibronectin levels may be secondary to increased mRNA levels. Since the results indicated the possible control of fibronectin mRNA by IGF-I at the transcriptional and translational level, it remains to be established whether transcription and/or translational processing of fibronectin mRNA levels is critical for the increased synthesis and secretion.

The time and dose response experiments are consistent with previous studies that demonstrated increased fibronectin deposition by the smooth muscle cells (Stenman et al., 1980; Glukhova et al., 1989). These studies also indicate that a potential regulator could be altered in vascular disease. However, the primary sources of IGF-I for smooth muscle cell function need to be establish so as to target possible sites of pathological alteration. IGF-I circulating in the plasma may stimulate fibronectin deposition. According to the injury

hypothesis of atherosclerosis (Ross, 1986; Clowes et al., 1989), smooth muscle cell proliferation begins after endothelial injury when these cells become exposed to blood-borne substances not normally present with an intact endothelium. Upon some mechanical or metabolic injury, increased fibronectin deposition by smooth muscle cells may occur as a result of their greater exposure to plasma IGF-I not normally present with an intact or healthy endothelial barrier. However, IGF-I stimulation through autocrine and paracrine mechanisms must also be considered. There are a number of studies suggesting that platelet-derived growth factor (PDGF) regulates IGF-I gene expression and secretion in smooth muscle cell cultures (Clemmons and Van Wyk, 1985; Delafontaine et al., 1991b). PDGF secreted from the platelets at the site of injury may induce IGF-I secretion from the smooth muscle cell. Consequently, IGF-I triggers fibronectin gene expression and production.

In summary, these smooth muscle cell studies propose that IGF-I-induced fibronectin synthesis may be an early event in the formation of diabetes-associated atherosclerotic lesions. An altered stimulus induced by the diabetic state, whether it be a mechanical or metabolic injury, could enhance IGF-I to stimulate the increased fibronectin present in these macrovascular complications. Because of its ability to bind other matrix proteins (Ruoslahti and Engvall, 1980), fibronectin has been identified as a potential matrix

organizer. Therefore, fibronectin synthesis may be the early catalyst for additional deposition of extracellular matrix proteins. Although In Vitro data cannot be directly correlated with In Vivo states, the IGF-I induction of fibronectin poses an interesting interpretation to explain the migration and phenotypic modulation of the smooth muscle cells in the tunica intima of the atherosclerotic lesions. If fibronectin is not incorporated into the matrix, the secreted protein may act as a "chemoattractant-like" molecule to other smooth muscle cells in the tunica media. There are reports that describe cellular migration mediated by fibronectin (Ruoslahti, 1988). Once bound to the cells, fibronectin could facilitate their migration into the tunica intima where atherosclerotic lesions progress. The initial stimulation of fibronectin secretion may also be the activator for phenotypic modulation of the contractile smooth muscle cells to the synthetic (or proliferative) phenotype which possess the capability to produce more fibronectin. Thus, the matrix accumulation contributes to the progressive occlusion of the blood vessel lumen leading to compromised cardiac function.

The IGF-I induction of fibronectin gene expression and synthesis also corroborates the potential importance of IGF-I in microvascular diseases of diabetes mellitus. These time and dose response experiments introduce a potential early event in the development of diabetic nephropathy. Alteration of the IGF-I-mediated fibronectin gene expression by the

diabetic state could be a mechanism to explain the increased deposition of fibronectin into the mesangial matrix of the diabetic kidney. The precise change in IGF-I function and availability under diabetic conditions is still under much deliberation. Two possible sources of IGF-I for fibronectin synthesis are the plasma and the extracellular fluid which contains locally synthesized IGF-I from a particular organ. Low serum levels of IGF-I are associated with insulin-deficient diabetic animals (Maes et al., 1983). Therefore, the observed increase in IGF-I receptors in diabetic rat kidneys (Werner et al., 1990) as well as cultured diabetic mesangial cells (Oemar et al., 1991) may be secondary to the primary defect of depressed circulating IGF-I levels. Decreased circulating IGF-I levels would cause an upregulation of IGF-I receptors by mesangial cells resulting in an increased sensitivity to IGF-I. Consequently, an enhanced response of fibronectin synthesis by the cells ensues. On the other hand, transgenic mice expressing growth hormone exibited elevated plasma levels of IGF-I and renal lesions similar to diabetic nephropathy (Doi et al., 1988). In this case, high levels of plasma IGF-I were associated with nephropathy-like lesions. However, the transgenic mice study proposes that growth hormone may also be involved, and thus circulating IGF-I cannot be implicated as the sole cause. Because IGF-I is produced by various cells of the kidney including mesangial cells, local synthesis of IGF-I must also be considered as a

potential source of IGF-I for stimulating fibronectin synthesis. Local IGF-I levels in diabetic rats have been investigated (Ikeda et al., 1988; Werner et al., 1990). These studies observed a decrease in immunoreactive IGF-I levels in the diabetic kidney, but the cause for the decline still remains to be defined. Werner et al. (1990) reported no change in renal IGF-I mRNA levels, thereby suggesting that decreased tissue levels of IGF-I is secondary to lower circulating IGF-I levels. Although the mechanism for decreased tissue IGF-I levels is under much deliberation, depressed local production of IGF-I should also be considered as a possible stimulus for the reported increase in IGF-I receptors. Still, additional data is necessary to fully determine whether diabetic alterations in endocrine, paracrine, or autocrine mechanisms of IGF-I are responsible for the pathological effects found in the kidney.

Hyperglycemia is an important factor in diabetes mellitus, and its effect on matrix production has been explored. High glucose in the culture medium caused an increase in collagen synthesis by cultured fibroblasts (Villee and Powers, 1977) and fibronectin synthesis by cultured endothelial cells (Roy et al., 1990). Two other studies also investigated high glucose on matrix production by cultured mesangial cells. An increase in fibronectin (Ayo et al., 1990) and collagen IV (Danne et al., 1993) were observed when cultured mesangial cells were incubated in a high glucose

medium. Although glucose appears to be an important factor in matrix protein synthesis, these studies propose IGF-I as another regulator of extracellular matrix production. Coincidently, Moran et al. (1991) investigated the effect of IGF-I and glucose on protein and proteoglycan synthesis in cultured mesangial cells. They reported a modulation of IGF-I-mediated protein and proteoglycan synthesis by high glucose conditions. IGF-I and glucose may interact together to regulate various cellular functions. It would be interesting to examine how different concentrations of glucose modulate the IGF-I-induction of fibronectin synthesis. Diabetes may alter IGF-I regulation of fibronectin production through glucose perturbations.

Insulin is a peptide that shares similar structure to IGF-I. Both insulin and IGF-I mediate common metabolic and mitogenic effects via their own, but similar receptors (Froesch and Zapf, 1985). They also appear to have some dual regulatory action on each other. For example, a recent study by Zenobi et al. (1992) demonstrated suppressed insulin secretion upon IGF-I infusion into fasting humans. Just as IGF-I appears to regulate insulin synthesis, insulin may also regulate IGF-I synthesis. For example, low circulating IGF-I levels associated with insulin deficiency in diabetes have been observed (Maes et al., 1983). Upon insulin treatment, IGF-I levels returned to near normal levels. Although insulin may regulate IGF-I synthesis, it may or may not be necessary

for the full expression of IGF-I effects. Growth of insulin deficient diabetic rats was still restored when treated with IGF-I (Scheiweiller et al., 1986). However, another study demonstrated that insulin inhibited the IGF-I-induced stimulation of tyrosine aminotransferase in rat hepatoma cells (Heaton et al., 1984). All these studies implicate a special regulatory interaction between IGF-I and insulin. Therefore, potential modulation of the IGF-I-mediated mesangial cell fibronectin gene expression by insulin was explored. We observed a further increase in fibronectin mRNA levels in mesangial cells treated with IGF-I and insulin when compared to those treated with IGF-I alone. However, insulin alone did not stimulate fibronection mRNA. It is well established that mesangial cells possess small amounts of insulin receptors. Supraphysiological concentrations of insulin are necessary to cross-react with IGF-I receptors and stimulate cellular proliferation (Arnqvist et al., 1988; Conti et al., 1988a). In the present studies, a physiological concentration of insulin (8×10^{-10} M) was added to the cultures. Therefore, cross-reactivity with IGF-I receptors by insulin is unlikely for the enhanced fibronectin response. Studies on the signal transduction pathways of IGF-I and insulin via their respective receptors in cultured mesangial cells would help to elucidate how insulin modulates IGF-I-induced fibronectin gene expression. A common intracellular signal generated by the insulin and the IGF-I receptor is a possible reason for

the enhancement of fibronectin mRNA levels. Larger amounts of insulin receptors have been observed in cultured diabetic mesangial cells (Oemar et al., 1991). Therefore, alteration of the IGF-I-induced fibronectin gene expression may be secondary to upregulated insulin receptors. Greater insulin sensitivity in the mesangial cells would enhance IGF-I action on fibronectin mRNA synthesis by generating a stronger intracellular signal upon receptor activation. A critical number of insulin receptors might also be required for insulin to independently stimulate fibronectin gene expression resulting in an additive effect. This speculation is analogous to IGF-I action on cellular proliferation. It has been proposed that IGF-I alone does not stimulate proliferation. However, if cells increase the number of their IGF-I receptors via some stimulus, i.e., PDGF, IGF-I can then independently stimulate growth (Baserga, 1992). This study introduces new questions for potential roles of insulin in the diabetic kidney.

Fibronectin was found in the cellular protein extracts as well as the culture medium of the smooth muscle cells and mesangial cells. Although both cells responded to IGF-I by an increase in fibronectin synthesis, the predominant IGF-I effect was detected in different cellular compartments. In order to speculate on the results of these studies, it must be noted that the cellular protein extracts contained intracellular fibronectin as well as fibronectin bound to the

cell surface. It was interesting to observe that fibronectin in the cell extracts of the smooth muscle cells did not change in response to IGF-I. A possible explanation is an accelerated post-translational processing (i.e., phosphorylation, sulfation, glycosylation) and/or secretion of fibronectin. Therefore, a greater number of completely processed fibronectin is secreted from the cell. In the absence of IGF-I, less fibronectin is completely processed for secretion and therefore would still be found in the cytoplasm of the cells. Because these cell extracts also include fibronectin bound to the cell surface, a matrix fraction must be considered. These results appear to indicate the possibility that fibronectin secreted by the cultured smooth muscle cells is not readily incorporated into its extracellular matrix since cellular levels did not change. An increased fibronectin production in response to IGF-I was therefore detected in the culture media. On the other hand, the IGF-I induction of fibronectin synthesis occurred predominantly in the cellular compartment of the mesangial cell cultures. However, these results cannot be conclusively interpreted as an augmented intracellular accumulation or an accelerated translational processing and secretion. Mesangial cell processing of fibronectin may be slower than in the smooth muscle cells. In any case, the slot blot results of the mesangial cells may be explained in two ways. If IGF-I accelerates translation of mRNA into fibronectin precursors,

more fibronectin would accumulate in the cytoplasm of the cell. On the other hand, IGF-I could also increase post-translational processing and secretion of fibronectin which is subsequently incorporated into the matrix. In either case, an increase of fibronectin levels would be found in the cell extracts.

Processing of pre-existing fibronectin precursors into active forms by the smooth muscle cells and mesangial cells must also be considered. IGF-I could stimulate post-translational processing of fibronectin precursors through conformational changes which present more antigenic sites to which the antibody binds. Therefore, the increased fibronectin levels may be a result of an IGF-I effect on a specific intracellular pool of fibronectin precursors. Studies which separate different compartments of fibronectin would be needed to definitively confirm these speculations.

The slot blot results also reveal that fibronectin in the mesangial cell cultures is a protein most likely to be found associated with the cell surface rather than a secreted protein present in the culture media as in the case of the smooth muscle cells. Comparison of mesangial cell fibronectin levels in cellular extracts and culture media agree with a study by Simonson et al. (1989) which also compared fibronectin in both compartments. They observed larger amounts of fibronectin associated with the cell than fibronectin secreted into the culture media. Using

immunofluorescence techniques, fibronectin was also detected in the mesangial cell matrix. Therefore, elevated cellular fibronectin levels could reflect an increased incorporation into the matrix. Meanwhile, the smooth muscle cells demonstrated the IGF-I effect as a significant increase of fibronectin secreted into the culture media. These differences of fibronectin deposition agree with anatomical observations. Mesangial cells exhibit a prominent matrix of its own between the glomerular capillaries. However, smooth muscle cells have a relatively small area of matrix around them. The only prominent matrix structure in the artery is the basment membrane. This matrix structure is maintained not only by smooth muscle cells but also by endothelial cells. Therefore, fibronectin secreted by smooth muscle cells may be important for the basement membrane rather than incorporation into extracellular matrix.

Comparison of fibronectin production by the smooth muscle cells and mesangial cells also prompted speculation regarding the availability of fibronectin receptors to bind its secreted ligand. Mesangial cells possess $\alpha_3ß_1$ and $\alpha_5ß_1$ fibronectin receptors (Cosio et al., 1990), while smooth muscle cells possess only $\alpha_5ß_1$ receptors (Thyberg et al., 1990). It is possible that mesangial cells have more fibronectin receptors available for binding the increased amount of fibronectin, and thus the IGF-I effect is detected in the cellular compartment. Smooth muscle cells, however, may have fewer receptors to bind

the increased fibronectin which is inevitably secreted into the culture medium. In this case, the IGF-I effect is detected primarily in the medium compartment. Studies investigating whether IGF-I regulates the fibronectin receptor would help to discover different regulatory mechanisms between these two cells.

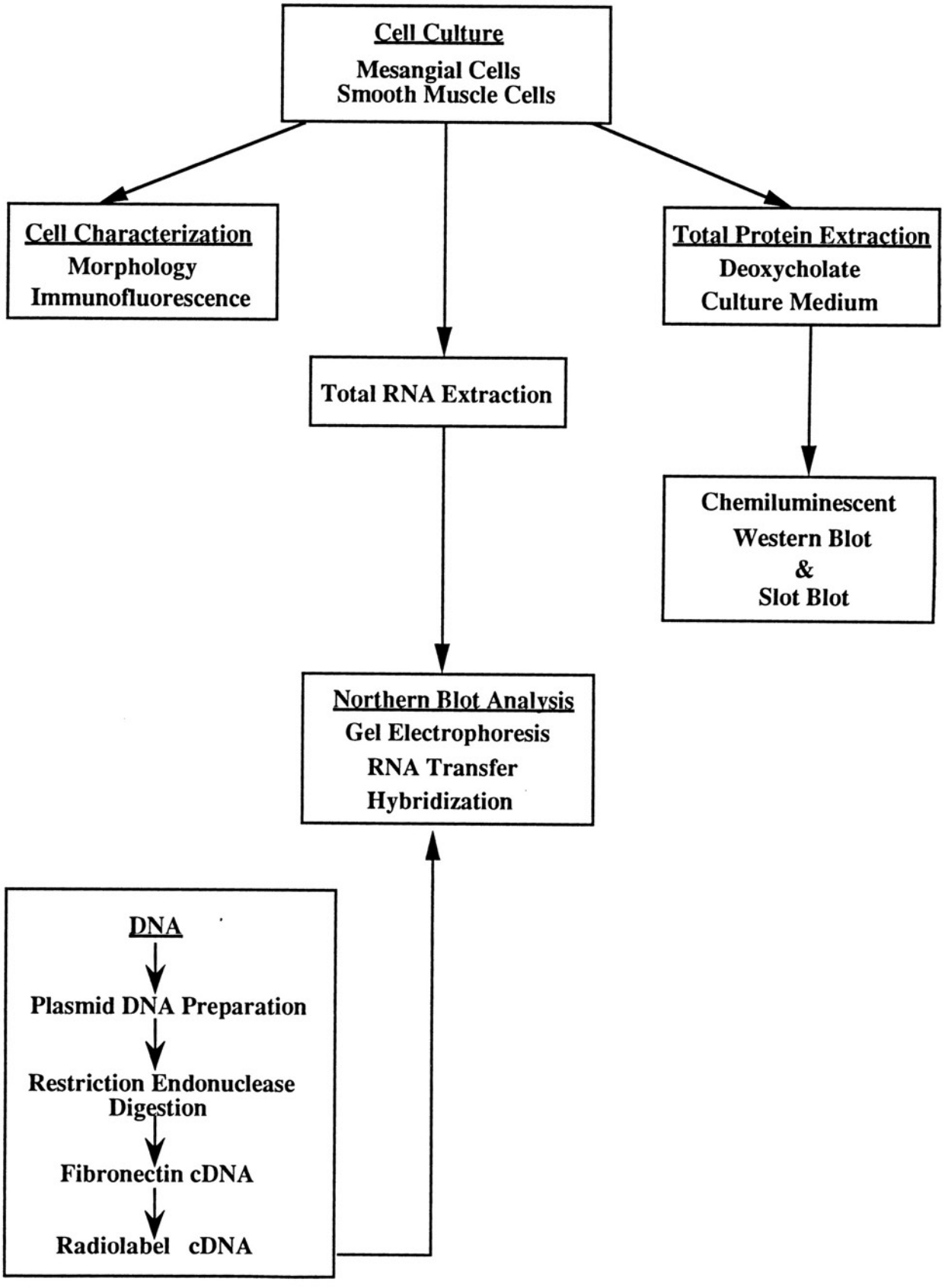
This dissertation also poses an interesting interpretation to explain the association between the extracellular matrix expansion and enhanced cellular proliferation typical of these macrovascular and microvascular lesions. The capability of IGF-I to stimulate fibronectin synthesis introduces a mechanism by which a growth factor indirectly regulates cellular growth through the production of a matrix protein. Appendix 2 demonstrates how fibronectin could be a mediator of cellular growth induced by IGF-I. If fibronectin can be truly established as a direct mediator of cellular proliferation, increased levels of this matrix protein, resulting from an altered IGF-I signal, may be the link to the increased cell growth found in these diabetic-associated vascular lesions.

V. CONCLUSION

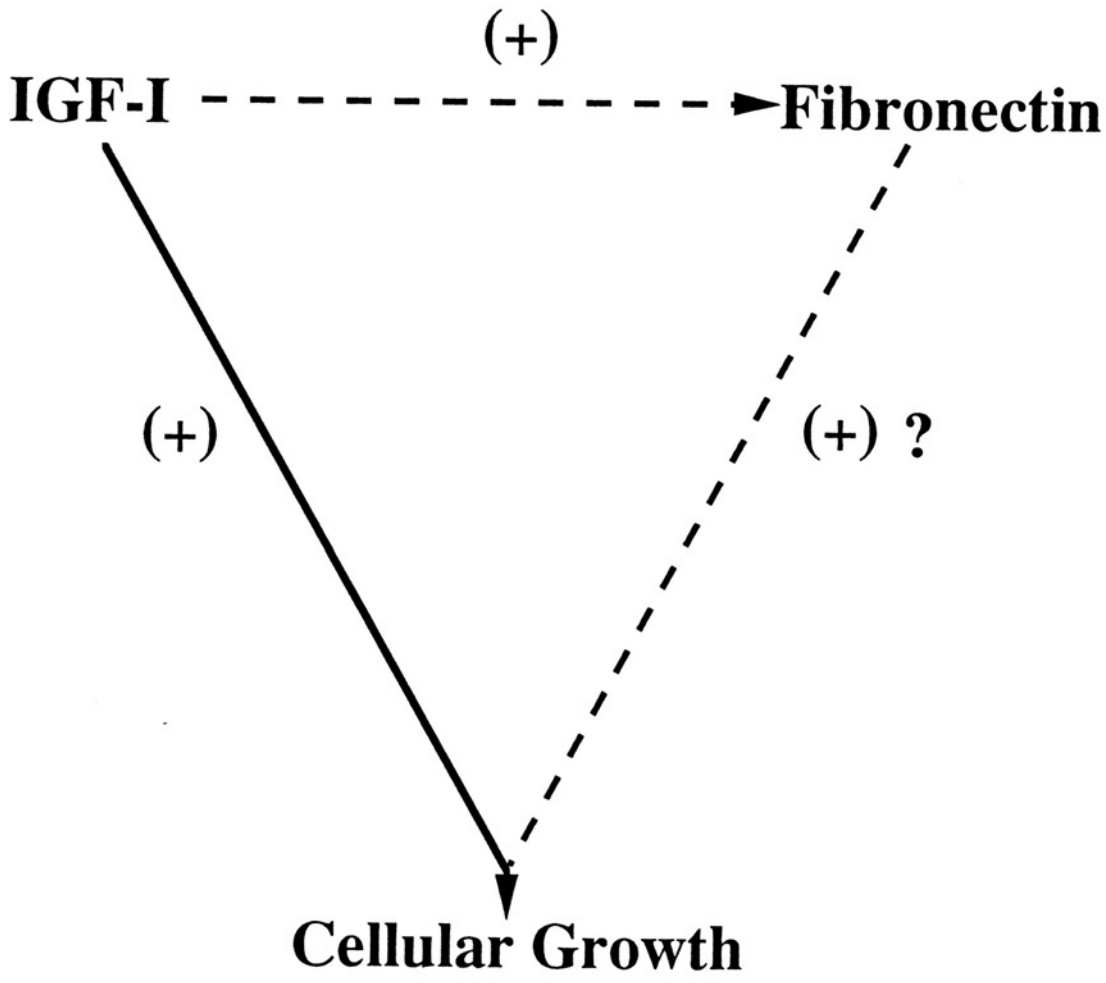
Fibronectin gene expression in response to IGF-I may be an early event in the development of diabetes-associated vascular complications. Perturbations of circulating IGF-I concentrations associated with diabetes may be an important factor in the development of macrovascular and microvascular lesions. Studies investigating how diabetes alters this process are needed to identify early targets for therapeutic intervention. While insulin therapy is necessary for regulating blood glucose levels, this dissertation also proposes another important consideration for achieving proper insulin balance in diabetics. In closing, information on matrix regulation could provide new targets for understanding the events involved in the progression of diabetes-associated vascular complications.

VI. APPENDICES

Appendix 1. Methods



Appendix 2.



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