PLATELET-DERIVED GROWTH FACTOR-BB STIMULATES FIBRONECTIN GENE EXPRESSION IN FIBROBLASTS ISOLATED FROM RAT THORACIC AORTA

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ZHANG

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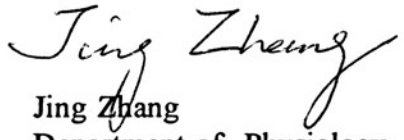
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ABSTRACT

Title of Dissertation: Platelet-derived growth factor-BB stimulates fibronectin gene expression in cultured fibroblasts isolated from rat thoracic aorta

Jing Zhang, Doctor of philosophy, 1994

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Platelet-Derived Growth Factor (PDGF) and fibronectin are implicated in cell proliferation and matrix expansion. It is possible that PDGF stimulates cell proliferation via induction of fibronectin synthesis from fibroblasts. Fibroblasts isolated from rat thoracic aorta were used in these studies because they produce a large amount of fibronectin. Therefore, the role of PDGF-BB in fibronectin gene expression in cultured fibroblasts was investigated. Northern blot analysis demonstrated that PDGF-BB induces fibronectin mRNA in a time-and dose-dependent manner. Elevated fibronectin mRNA levels were detected at 4 hours and peaked at 6 hours (120% increase, $P<0.001$) after PDGF-BB (20 ng/ml) treatment. The effects of actinomycin D and cycloheximide on fibronectin mRNA synthesis in the presence of PDGF-BB were examined. Actinomycin D blocked the fibronectin mRNA increase induced by PDGF-BB. Cycloheximide produced a greater increase in fibronectin mRNA levels. These results suggest transcriptional and translational control by PDGF-BB. Slot blot and western blot analysis demonstrated a 21% increase ($P<0.05$) of fibronectin levels in the intracellular

compartment 8 hours after PDGF-BB (20 ng/ml) treatment and a 20% increase ($P<0.01$) in the cell culture medium 12 hours after PDGF-BB (20 ng/ml) addition. PDGF-BB produced a dose-dependent increase of intracellular fibronectin levels and fibronectin secretion into the cell culture medium. Insulin-like growth factor-I (IGF-I) also regulates cell proliferation. Since PDGF and IGF-I stimulate cell proliferation individually, the interaction between PDGF-BB and IGF-I was examined. PDGF-BB acted synergistically with IGF-I on the stimulation of fibronectin mRNA (276% increase, $P<0.001$) and fibronectin levels (115% increase, $P<0.001$) after exposure of cell cultures to both growth factors (20 ng/ml each) simultaneously. These studies suggest that PDGF-BB may have a regulatory effect on fibronectin gene expression. The interaction between growth factors and matrix proteins may play a role in smooth muscle cell proliferation and extracellular matrix expansion.

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by

Jing Zhang

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the degree of Doctor of Philosophy

June 1994

DEDICATION

to

Dr. Matie Shou, my beloved husband

and

Baoyuan Qi and Junlan Zhang, my dear dad and mom

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SPECIFIC AIMS

Hypertension and atherosclerosis are characterized by smooth muscle cell proliferation and extracellular matrix expansion. The molecular mechanisms causing these pathologic changes are not clear. This research will investigate the role of platelet-derived growth factor (PDGF) in stimulating fibronectin gene expression in rat thoracic aortic fibroblasts. The interaction between growth factors and extracellular matrix proteins may be responsible for the smooth muscle cell proliferation and extracellular matrix expansion. Specific aims of this research are to examine

(1) whether PDGF-BB affects fibronectin mRNA levels in primary cultured fibroblasts isolated from rat thoracic aortae;

(2) whether PDGF affects fibronectin levels;

(3) the interaction of PDGF-BB and IGF-I on stimulating fibronectin gene expression.

This research will examine how PDGF-BB regulates fibronectin which could mediate cell proliferation and matrix expansion in the cardiovascular system. Further, it will help to reveal the molecular mechanisms of hypertension and atherosclerosis.

SIGNIFICANCE

Hypertension and atherosclerosis, the two major forms of vascular disease in the United States and Western Europe, share a number of features. The frequency of both diseases increases with age. The risk of atherosclerosis is greatly increased in individuals with hypertension. Both diseases are characterized by smooth muscle cell proliferation and extracellular matrix expansion. In atherosclerosis, the central cellular feature is proliferation of smooth muscle cells in the arterial intima of larger arteries. As these smooth muscle lesions enlarge, lipid accumulates, thrombosis occurs, the lumen is narrowed, and patients die of infarction. In hypertension, smooth muscle cell proliferation or hypertrophy and extracellular matrix deposition in small arteries results in increased wall mass and a narrowed lumen. The small vessel change is thought by some to be the reason of the increased peripheral resistance that causes high blood pressure. This common role of accumulation of smooth muscle cells and matrix proteins suggests that control of smooth muscle cell proliferation and extracellular matrix expansion may be critical to both diseases.

Smooth Muscle Cell Proliferation in Atherosclerosis and Hypertension

Virchow (1856) recognized the presence of cell proliferation in atherosclerosis over a century ago. His view of the cellular events as a reaction to the accumulation of toxic materials in the vessel wall was largely neglected while research was directed at the equally important issue of lipid accumulation in lesions. This began to change in the

latter part of the 1960s and early 1970s. Experimental evidence in animals, and observations in humans, supported the idea that the initial step in lesion formation, prior to an increase in intimal lipids, was the formation of focal masses of proliferated smooth muscle cells in the intima (Ross *et al.* 1976, Parker *et al.* 1966, Haust *et al.* 1960). These observations began to lead to a possible understanding of the mechanism.

The major cellular participants in atherosclerosis are monocyte/macrophages, vascular smooth muscle cells, T lymphocytes, platelets and endothelial cells. According to "the response to injury" hypothesis, endothelial cell dysfunction is perhaps one of the earliest events in atherosclerosis (Ross 1986b). Leukocyte adhesion to the damaged endothelium and subsequent infiltration into the arterial wall is followed by their activation and elaboration of various cytokines and growth factors. Among the factors released by these activated leukocytes are substances, such as PDGF (Ross *et al.* 1990), that have the potential to initiate the migration of medial smooth muscle cells into the neo-intima and their subsequent proliferation. During later phases of plaque formation, endothelial dysfunction may progress to endothelial cell death and detachment. Exposure of the underlying extracellular matrix leads to platelet microthrombus formation and degranulation with the consequential release of platelet associated growth factors and chemotactic agents.

The history of our understanding of the role of smooth muscle cell proliferation in hypertension is less clear. Arteriolar hypertrophy in hypertension was described as early as 1868 by Johnson, and the concept that these thickened vessel walls might increase peripheral resistance was offered by Ewald in 1877. These morphological observations meant little until physiologists began to understand the central role of smooth

muscle contractility in controlling resistance to flow through small arteries. Because only a small proportion of cases of hypertension is readily explained on the basis of classical renal, adrenal, or sympathetic neural mechanisms (Folkow 1982), abnormal function of the arterial smooth muscle cell must play a central role. The most obvious is that hypertension results from a general change in smooth muscle contractility or responsiveness.

Hypertension is a major risk factor for strokes, myocardial infarction, and peripheral vascular disease (Kannel and Sorlie 1975). Hypertension can accelerate atherosclerosis in experimental animal models as well as in human (Chobanian 1983, McGill *et al.* 1985). Hypertension causes a large number of functional and morphological alterations in the vessel wall, including hypertrophy and increased turnover of endothelial cells (Chobanian 1983, Haudenshild *et al.* 1980), hypertrophy, polyploidy, and intimal migration of medial smooth muscle cells (Chobanian 1983, Lichtenstein *et al.* 1986), adhesion and subendothelial migration of blood mononuclear cells (Haudenshild *et al.* 1980), and extracellular matrix accumulation (Chobanian 1983). The molecular mechanisms whereby increased intravascular pressure affects the vessel wall *in vivo* are unknown. However, studies using cultured cells have indicated that a number of different polypeptide growth factors can influence vascular cell function and proliferation. Several growth factors have been shown to be made *in vitro* by endothelial cells (Ross *et al.* 1986a, Sitaras *et al.* 1987, Gospodarowicz *et al.* 1987), smooth muscle cells (Gospodarowicz *et al.* 1987, Majesky *et al.* 1988, Winkles *et al.* 1987), and blood mononuclear cells (Ross *et al.* 1986a, Sporn *et al.* 1987, 1988). Multiple effects of different growth factors on these cell types led to suggestions that complex autocrine and

paracrine control mechanisms exist *in vivo* in vascular tissue (Ross *et al.* 1986b, Sporn *et al.* 1988).

Growth Factors and Cell Proliferation

Growth factors are a group of polypeptides which appear in both tissue and blood. The role of polypeptide growth factors in stimulating the proliferation of cells and in maintaining their viability has been increasingly appreciated. Growth factors also play roles in differentiation, development, chemotaxis and activation of inflammatory cells, tissue repair and disease. Growth factors differ from hormones in that they usually act through paracrine and autocrine mechanisms. However, growth factors have been identified in plasma and may act as hormones as well. Growth factors are synthesized and secreted by both normal and transformed cells. Abnormal secretion of growth factors by normal cells probably results in disease characterized by a proliferative cellular response or by fibrosis.

Platelet-derived Growth Factor

Platelet-derived growth factor (PDGF) was first identified as a factor in platelets which allowed the growth of fibroblasts *in vitro* (Kohler *et al.* 1974). Further characterization of this factor demonstrated that it is a potent mitogen for all cells of mesenchymal origin, including smooth muscle cells and glial cells. Subsequent purification and chromatographic analysis demonstrated the presence of two closely related proteins, termed PDGF-I and PDGF-II (Deuel *et al.* 1981). These proteins were found to be of similar size and amino-acid composition and to possess similar mitogenic

properties, but differed only in the extent of glycosylation. PDGF-I is about 30 kD and contains about 7% carbohydrate. PDGF-II is about 28 kD and contains about 4% carbohydrate.

The structure and isoforms. PDGF is a dimeric polypeptide consisting of two disulfide linked chains, termed A and B (Johnsson *et al.* 1984). The A and B chains of PDGF are 56% identical to each other throughout the mature PDGF molecule (Betsholtz *et al.* 1986). The B-chain of PDGF is encoded by the *c-sis* protooncogene which is the normal cellular homologue of the transforming oncogene of the simian sarcoma virus (Waterfield *et al.* 1983, Doolittle *et al.* 1983, Johnsson *et al.* 1984). The human A chain is encoded on chromosome 7 (Betsholtz *et al.* 1986), while the human B chain is encoded on chromosome 22 (Swan *et al.* 1982), and these two genes are often expressed independently of each other (Alitalo *et al.* 1987, Papayannopoulou *et al.* 1987). Independent regulation of expression of the A and the B chains allows the production of at least three different PDGF-related molecules, the A-A and the B-B homodimers, and also the A-B heterodimer. Other modifications, including differential proteolysis and glycosylation can potentially give rise to an entire spectrum of PDGF-related proteins. Human platelet PDGF is thought to be an A-B heterodimer (Stroobant *et al.* 1984). The presence of multiple forms of closely related molecules raises the possibility that the individual PDGF-related proteins are tailored to specific physiological roles.

Biological activity. PDGF is an extraordinarily potent mitogen for most mesenchymally derived connective-tissue-forming cells. After exposure to fibroblasts,

smooth muscle, or glial cells, PDGF induces increased thymidine incorporation within 12 to 16 hours (maximal by 24 hours) and leads to cell doubling between 30 and 36 hours (Ross *et al.* 1987). Functionally, PDGF also appears to play a significant role in a number of other biological processes, including vasoconstriction, chemotaxis, stimulating collagen and collagenase synthesis, and calcium mobilization (Berk *et al.* 1986, Hart *et al.* 1988, Chan *et al.* 1987, Berridge *et al.* 1984, Rose *et al.* 1986a).

The PDGF receptors. The PDGF receptor was first identified as a 180- to 190-kD membrane glycoprotein by the covalent cross-linking of ^{125}I-labeled PDGF (AB heterodimer form) to intact cells (Glenn *et al.* 1982) or to membrane preparations (Williams *et al.* 1984). The receptor can be found on vascular smooth muscle cells, fibroblasts, and glial cells, but is not present on endothelial cells or on most hematopoietic cells. The PDGF receptor belongs to a subfamily of tyrosine kinase receptors that includes the insulin receptor, epidermal growth factor receptor, colony-stimulating factor-I receptor, and insulin-like growth factor receptor. The structure of the PDGF receptor is very close to that of the CSF-I receptor (Rossel *et al.* 1987) and *c-kit* protooncogene (Qiu *et al.* 1988) (Fig. 1). The most striking structural features are the organization of the extracellular region into five immunoglobulin-like domains, D_1 to D_5. The other distinctive characteristic of this class of receptors is that there is a large region that interrupts the coding sequence of the tyrosine kinase domain (Yarden *et al.* 1986). This large region is called the "kinase insert" (KI) region of the receptor. The KI region of the PDGF receptor, CSF-I receptor, and *c-kit* protein are different in specific sequences and length, but are found at precisely the same location within their respective

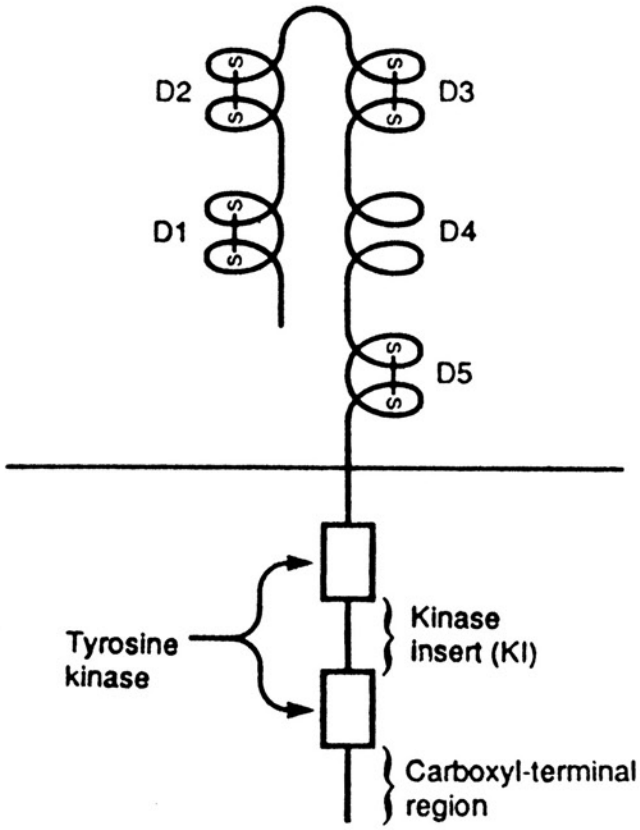


Figure 1. The structure of PDGF receptor (Williams 1989)

kinase domains. It is possible that the KI sequences represent a structural "excursion" from the sequences that actually form the active site of the kinase. The cytoplasmic region contains sequences homologous to other tyrosine kinases. The other features of the PDGF receptor are the presence of a single membrane-spanning segment and a "juxtamembrane" region that connects the first kinase domain with the transmembrane domain (Yarden *et al.* 1986). Little is known about the juxtamembrane region except that its length of approximately 47 amino acids is highly conserved among the receptor tyrosine kinases. The carboxyl-terminal domain of the PDGF receptor is distinctive in sequence but has no easily predictable structure, and its function is also unknown.

It has been demonstrated that there are two distinct PDGF receptors, α-PDGF receptor (α-PDGFR) and ß-PDGF receptor (ß-PDGFR). They are coded by different

genes, and usually expressed at the same time (Matsui *et al.* 1989). The PDGF isoforms bind with different affinities to two distinct cell surface receptors (Fig. 2). The PDGF α-

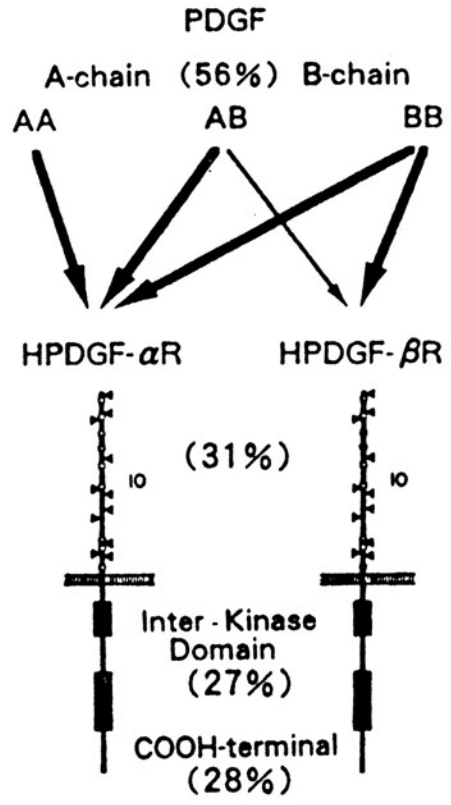


Figure 2. The PDGF isoforms bind with two receptors (Matsui 1991)

receptor binds all three isoforms with high affinity, while the PDGF ß-receptor binds only PDGF-BB with high affinity and PDGF-AB with lower affinity (Claesson-Welsh *et al.* 1988, 1989, Östman *et al.* 1989). The amino acid identity between the two receptors varies from 30% in the extracellular part to 87% in the most N-terminal part of the tyrosine kinase domain (Nister *et a*l. 1991). Binding of PDGF to the extracellular part of either receptor type leads to dimerization of receptor molecules, followed by activation of the receptor tyrosine kinase (Heldin *et al.* 1989, Bishayee *et al.* 1989, Kanakaraj *et al.*

1991, Seifert *et al.* 1989). The functional difference between the α-and ß-PDGF receptors is important to define their distinct physiological roles (Hammacher *et al.* 1989). The ratio of the two receptors might vary from one cell type to another or according to the proliferation and differentiation stages. Moreover, the two receptors might trigger a partially distinct down-stream pathway to transduce the ligand signal. Additional regulatory flexibility may be provided by the different ability of three PDGF isoforms to interact with the two receptors. This regulatory flexibility may be important to reveal the wide spectrum specificity of tissues and cells whose function is differentially controlled by PDGFs.

After binding of PDGF to its receptor, a number of immediate changes occur within the cell (Williams 1989). These include activation of tyrosine kinase, hydrolysis of phosphatidylinositol (PI) (Habenicht *et al.*1986), alterations of cellular pH (L'Allmain *et al.*1984), increase in cytosolic calcium levels (Ives *et al.* 1987), a dramatic change in the cytoskeleton (Bockus *et al.* 1984), increased expression of a group of genes (Kelly *et al.* 1983, Greenberg *et al.* 1985, Sukhatme *et al.* 1987, Almendral *et al.* 1988, Cochran *et al.* 1983), elevation of cellular cyclic adenosine monophosphate (cAMP) (Rozengurt *et al.* 1983), and internalization and degradation (downregulation) of the receptor. Although all these changes occur, none can be related directly to the mitogenic effects of PDGF (Williams 1989).

Several PDGF receptor substrates have recently been identified. These include phospholipase C-γ (PLCγ) (Morrison *et al.* 1990), GTPase-activating protein (GAP) (Molloy *et al.* 1989, Kaplan *et al.* 1990), and the 85 kDa subunit of the phosphatidylinositol 3-kinase (Carpenter *et al.* 1990). Each has been shown to undergo

rapid tyrosine phosphorylation and/or physical association with PDGF receptors in response to PDGF triggering (Morrison *et al.* 1990, Molloy *et al.* 1989, Carpenter *et al.* 1990, Kaplan *et al.* 1990, Kazlauskas *et al.* 1990). PLCγ hydrolyzes phosphatidylinositol 4,5-biphosphate into two second messengers, 1,2-diacrylglycerol and inositol 1,4,5-triphosphate. The former activates protein kinase C, and the latter promotes the release of Ca^{2+} from intracellular stores (Berridge 1987). GAP enhances hydrolysis of *ras*-GTP to *ras*-GDP which normally inactivates *ras* function (McCormick 1989, Trahey 1987). Phosphatidylinositol 3-kinase phosphorylates the inositol ring of phosphatidylinositol at the D3 position, but there is yet no clue as to the biological functions of such metabolites (Auger *et al.* 1989, Whitman *et al.* 1988).

The Role of PDGF in Smooth Muscle Cell Proliferation

PDGF is a mitogen and chemoattractant (Grotendorst *et al.* 1981, Albini *et al.* 1988) for vascular smooth muscle cells *in vitro*. Thus, it may be involved in at least two important aspects of atherogenesis--the proliferation of smooth muscle cells and their migration into the intima of the vessel wall (Schwartz *et al.* 1986, Ross 1986b). PDGF would be released into the vessel wall following platelet adherence and degranulation *in vivo*. Also, endothelial cells (Barrett *et al.* 1984, Gay and Winkles 1990), smooth muscle cells (Majesky *et al.* 1988, Sjolund *et al.* 1988, Hosang and Rouge 1989), and activated blood monocytes (Martinet *et al.* 1986) in culture produce PDGF. RNA gel blot (Barrett and Benditt 1988) and *in situ* (Wilcox *et al.* 1988) hybridization studies have demonstrated that PDGF mRNA is expressed by vascular cells in normal human vessels and in atherosclerotic plaques. In addition, Ross *et al.* (1990) detected PDGF B-chain

protein in macrophages during all stages of atherogenesis. Therefore, one may hypothesize that PDGF could play a significant role in the hyperplastic response characteristic of atherosclerosis and hypertension.

Smooth muscle cells isolated from the intima adjacent to regions of endothelial cell damage displayed 10 times the PDGF concentration of those taken from the media of uninjured arteries (Walker *et al.* 1986). There is also evidence that the rapid proliferation of smooth muscle cells *in vitro* was associated with endogenous PDGF-like protein synthesis, and smooth muscle cell phenotypic modulation (Nilsson *et al.* 1985).

In vitro studies of human smooth muscle cells have shown that cells derived from the atheromatous plaques can significantly stimulate cultured smooth muscle cell growth. Some of this stimulatory effect can be attributed to PDGF because the addition of antibodies to PDGF reduced, but did not eliminate, the increased growth rate (Libby *et al.* 1988a). Other substances such as interleukin-1, fibroblast growth factor or insulin-like growth factor may have contributed to the mitogenic potential of the plaque extract (Libby *et al.* 1988b, Winkles *et al.* 1987, Clemmons *et al.* 1985b). These and other investigations found that cells derived from atheromatous plaques synthesized detectable quantities of PDGF mRNA (Libby *et al.* 1988a, Barrett *et al.* 1987, 1988).

Examination (using *in situ* hybridization) of human tissue removed during surgery has revealed the cells that have the potential to produce PDGF *in vivo* (Wilcox *et al.* 1990). Almost all cell types of the intima derived from the atheromatous plaques gave a positive response to either mRNA for PDGF-A, PDGF-B or both. Localization of PDGF receptors within the intima coincide with those cells that synthesize PDGF (wilcox *et al.* 1990).

PDGF receptors are expressed at increased levels in atherosclerotic plaques (Rubin *et al.* 1988). This expression was specifically on the smooth muscle cell within the intima and was not found in the media, either adjacent to or distant from the lesion. The increased PDGF receptor expression was associated mainly with areas of increased density of active T cells and macrophages. Rubin *et al.* hypothesized that increased expression of PDGF receptors is a result of the presence of these cells (Rubin *et al.* 1988).

Experiments demonstrated angiotensin II and α-adrenergic stimulation can stimulate PDGF A-chain gene expression in cell cultures and experimental animals (Naftilan *et al.* 1989, Majesky *et al.* 1990). Receptor research also demonstrated that deoxycorticosterone acetate (DOCA)/salt hypertension induced a threefold increase in aortic steady-state PDGF ß-receptor mRNA levels (Sarzani *et al.* 1991). Aortic PDGF ß-receptor expression also was higher in spontaneously hypertensive rats (SHRs) (Sarzani *et al.* 1991). All evidence suggests that PDGF is involved in the paracrine/autocrine regulation of smooth muscle cell proliferation of atherosclerosis and hypertension.

The Role of PDGF in Fibroblast Proliferation

PDGF is a potential mediator of fibroblast proliferation (Dinarello 1988, Shimokado *et al.* 1985). Experiments have demonstrated that PDGF can cause rabbit papillary fibroblast (Knecht *et al.* 1991), human synovial fibroblast (Butler *et al.* 1989), and human skin fibroblast proliferation (Raines *et al.* 1989; Bonner *et al.* 1990). In human skin fibroblasts, the first increase in [^{3}H]thymidine incorporation in response to PDGF-AA, PDGF-BB and PDGF-AB was seen at 16 hours and was maximal at 24 hours.

Maximal [^{3}H]thymidine incorporation, detected at 24 to 26 hours in response to PDGF-BB, was approximately 6 times that seen in response to PDGF-AA. The human dermal fibroblasts have both types of PDGF receptors (Smits *et al.* 1992), and there are more PDGF-BB binding sites than PDGF-AA binding sites (Raines *et al.* 1989).

PDGF is related to many proliferative disorders of fibroblastic origin. Smits *et al.* (1992) using immunohistochemical and *in situ* hybridization techniques found that high expression of PDGF β-receptor mRNA and protein was found in malignant tumors, and also in some benign lesions, such as dermatofibroma. In contrast, high expression of PDGF α-receptor mRNA was only found in fully malignant lesions, such as malignant fibrous histiocytoma. These data indicate that an autocrine growth stimulation via the PDGF β-receptor could occur in an early phase of tumorigenesis, and may be a necessary but insufficient event for the progression into fully malignant human connective tissue lesions.

Insulin-like Growth Factors

Insulin-like growth factors were discovered in 1957 by Salmon and Daughaday when it was observed that rat serum contained a growth hormone-dependent factor(s) capable of inducing the incorporation of ^{35}S into cartilage (sulfation factor activity, SFA) (Salmon *et al.* 1957). Investigators in 1963 described factors isolated from human serum that had insulin-like effects on muscle and adipose tissue and were not suppressed by the addition of insulin antiserum (nonsuppressible insulin-like activity, NSILA) (Froesch, *et al.* 1963). In the 1960s, studies by Temin and others indicated that cell proliferation in some cell lines was dependent on the presence of specific factors in serum, whereas in

other lines the cells produced their own growth-promoting substances. One such activity was termed "multiplication-stimulating activity (MSA). When it later became apparent that all three of these activities represented a similar group of substances with a much wider biological activity, the workers in the field agreed to introduce the term somatomedin in 1972 (Daughaday *et al.* 1972). It was not until 1978 that any member of this family was chemically characterized and its primary structure determined. Rinderknecht *et al.* successfully purified NSILA from a Cohn fraction of human plasma, resolving this activity to be due to two biologically active peptides that they termed insulin-like growth factor-I (IGF-I) and IGF-II (Rinderknecht *et al.* 1976, 1978a, b).

The structure of IGF-I. IGF-I is a single chain peptide, 70 amino acids in length, with 3 intra-chain disulfide bridges (Rinderknecht *et al.* 1978a, b). As with pro-insulin, regions of this growth factor peptide may be delineated into four domains, A, B, C and D; the A and B domains are very similar to the corresponding domains of pro-insulin, with which they share 43% homology. IGF-I has a similar tertiary structure to pro-insulin (Blundell *et al.* 1983). The receptor binding site of IGF-I is near the end of its B domain and the amino terminal end of the B domain is involved in binding IGF binding proteins (IGF-BPs) (Sheikh *et al.* 1987), while the A domain appears to be involved in the mitogenic response (Cascieri *et al.* 1988).

The IGF binding proteins. IGFs circulate in blood bound to one of a number of binding proteins. IGF-BP3 (IGF-BP53), which accounts for approximately 80% of the total binding capacity, is present as a glycosylated 150 kDa binding complex in human,

rat and porcine serum (Baxter 1988, Zapf *et al.* 1988, Gopinath *et al.* 1989). The 150 kDa complex appears to represent a stable reservoir for the IGFs and its plasma concentration is dependent on levels of circulating growth hormone (Zapf *et al.* 1979, 1980). IGF-BP1 (IGF-BP28), the other major circulating IGF-BP, is found as a 25.3 kDa species in plasma, and represents an unglycosylated, growth hormone-independent component. Other IGF binding species are found in serum, with molecular weights ranging from 18 to 160 kDa. These are thought to represent post-translational modifications of IGF-BP3 (Hossenlopp *et al.* 1986, Hardouin *et al.* 1987) and IGF-BP1 (Koistinen *et al.* 1986, Baxter *et al.* 1987, Lee *et al.* 1988).

The IGF-I receptor. The IGF-I receptor is structurally and functionally very similar to the insulin receptor (Ullrich *et al.* 1986) (Fig. 3). The functional receptor is a 300-350 kDa dimmer, each half consisting of one α (130 kDa) and ß subunit (95 kDa) which are disulfide linked. The α subunits form the major part of the extracellular domain, and are involved in ligand binding. The ß subunits contain the transmembrane region and an intracellular tyrosine kinase catalytic domain, which shows 84% homology with the insulin receptor. Ligand binding is followed by autophosphorylation of tyrosine residues on the ß subunit. Although IGF-I, IGF-II and insulin all bind to the IGF-I receptor, IGF-II binds with lower affinity than IGF-I and insulin with lower affinity than IGF-II (Steele-Perkins *et al.* 1988). The mitogenic effect of insulin was mediated by the IGF-I receptor: an anti-IGF-I receptor monoclonal antibody reduced the insulin and IGF-I induced mitogenic effect, whereas an anti-insulin receptor antibody was without significant effect (Banskota *et al.* 1989a, b).

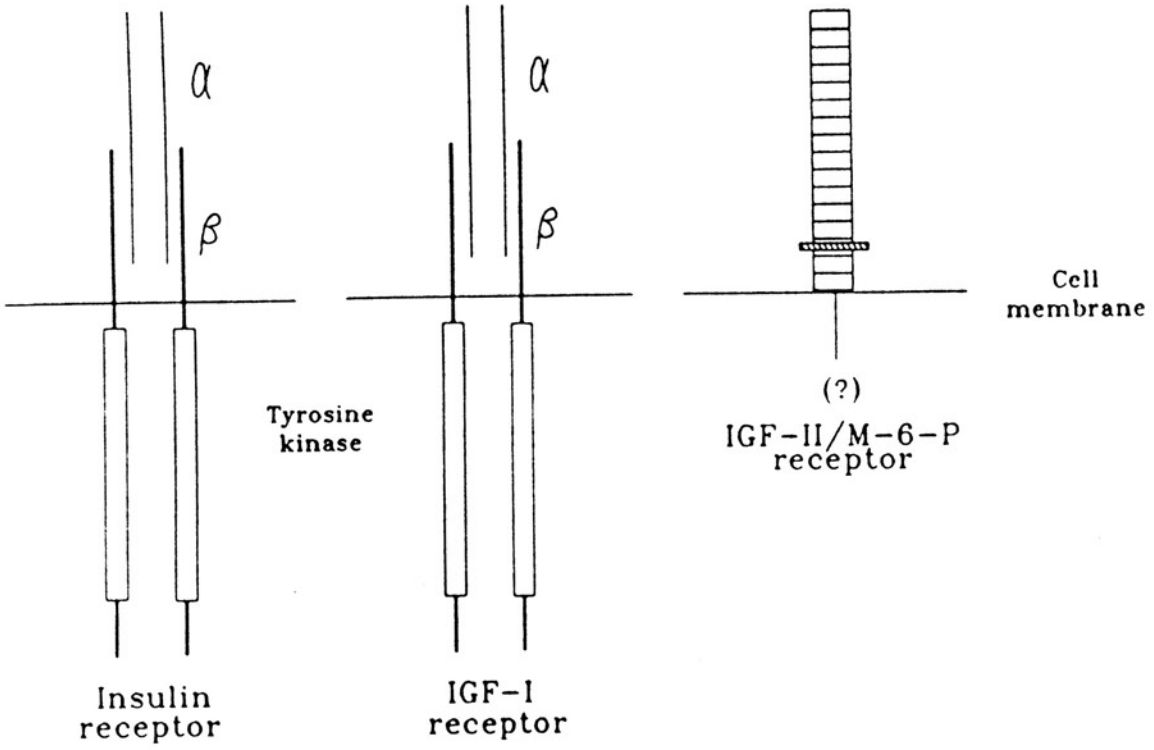


Figure 3. The structure of IGF-I receptor (Ferns *et al.* 1991a)

Biosynthesis and biological effects. The liver is the primary site of IGF-I synthesis in humans and rats (Schwander *et al.* 1983, Scott *et al.* 1985, Schimpff *et al.* 1980). IGF-I has also been produced in culture by fibroblasts from both humans and rats (Atkinson, *et al.* 1980; Adams *et al.* 1983, Clemmons *et al.* 1981a). In fact, the rate of IGF-I production by adult fibroblasts (50 ng/10^6cells/48hrs) is similar in magnitude to that for hepatocytes. However, that these cells represent a major source of IGF-I *in vivo* is speculative (Scott, *et al.* 1985). A variety of other organs including kidney, lung, pancreas, testes, neural tissue and heart may contribute to the circulating levels of IGF-I (D'Ercole *et al.* 1984, Sara et al 1982).

The two main biological actions of the IGFs may be summarized as an insulin-

like metabolic action and a growth-promoting action. Both IGF-I and IGF-II stimulate DNA synthesis and cell proliferation. IGFs are involved in embryonic development (D'Ercole *et al.* 1980), angiogenesis (Hansson *et al.* 1989), tumorigenesis (Gray *et al.* 1987, Tricoli *et al.* 1986, Höppener *et al.* 1988), neural function (Tannenbaum *et al.* 1983, Mulholland *et al.* 1988), and wound healing (Hansson *et al.* 1987, Rappolee *et al.* 1988).

The Role of IGF-I in Hypertension and Atherosclerosis

Although there is no definitive proof that the IGFs play a major role in atherosclerosis and hypertension, there is mounting circumstantial evidence that they do so. Most of the cell types implicated in these processes are capable of expressing IGF-I, IGF-I receptor, IGF-BPs, or a combination of these proteins. Serum and platelets contain a high proportion of mitogenic activity (>50%) that can not be attributed to PDGF (Ferns *et al.* 1991b). It follows, therefore, that platelets contain other potent mitogens that may contribute to intimal cell proliferation. Moreover, arterial injury is accompanied by a rapid and long-lasting induction of IGF-I mRNA expression (Cercek *et al.* 1990), suggesting an autocrine or paracrine role for IGF-I in the vascular response to injury.

Vascular endothelial cells have been shown to have receptors for IGF-I, using a modified Langendorff perfused heart system, and *in vitro* (Bar *et al.* 1984, 1988). Hansson *et al.* (1989) showed that immunoreactive IGF-I was expressed by endothelial cells following injury. Receptors for IGFs have been demonstrated on vascular smooth muscle cells *in vivo* and *in vitro* (Bornfeldt *et al.* 1988, Cascieri *et al.* 1986, Jialal *et al.* 1985). *In vitro* IGF-I itself is not a good mitogen for smooth muscle cells. But IGF-I acted additively or synergistically with other growth factors, such as PDGF, fibroblast

growth factor (FGF) and epidermal growth factor (EGF), on induction of mitogenic response and receptor expression (Clemmons *et al.* 1984, 1985b, Banskota *et al.* 1989a, Pfeifle *et al.* 1984, 1987). The gene for the human IGF-I receptor is expressed by fibroblasts (Rosenfeld 1982). Both fibroblasts and smooth muscle cells can synthesize IGF-I endogenously (Clemmons *et al.* 1981b, 1985a, b, Weidman *et al.* 1979).

The recent cloning of the genes for both IGFs has permitted investigation of tissue IGF expression by northern blotting and *in situ* hybridization. A possible autocrine role for IGF-I in the rat aorta has been demonstrated. IGF-I mRNA was detected in the aortic media by *in situ* hybridization and northern analysis (Sarzani *et al.* 1989). Cercek *et al.* (1990) found that balloon catheter de-endothelialization of the rat aorta results in a rapid and sustained elevation in IGF-I mRNA. The peak response was observed at 7 days, though expression remains above basal levels beyond 14 days.

Although PDGF is one of the major platelet associated mitogens, other potent mitogens are also present. Karey *et al.* (1989) have purified and characterized human platelet derived IGF-I. Thus platelet adherence and degranulation at sites of arterial de-endothelializations would be expected to result in the release of high local concentrations of IGFs, which may act on cells with the vessel wall, or affect the function of the platelets themselves.

Extracellular Matrix and Cell Proliferation

Most cells in multicellular organisms are in contact with an intricate meshwork of interacting, extracellular macromolecules that constitute the extracellular matrix. These versatile protein and polysaccharide molecules are secreted locally and assemble into an

organized meshwork in the extracellular space of most tissues. Until recently, the vertebrate extracellular matrix was thought to serve mainly as a relatively inert scaffolding that stabilized the physical structure of tissues. But now it is clear that the matrix plays a far more active and complex role in regulating the behavior of the cells that contact it—influencing their development, migration, proliferation, shape, and metabolic functions. The macromolecules that constitute the extracellular matrix are secreted by local cells, especially fibroblasts, which are widely distributed in the matrix. Two of the main classes of extracellular macromolecules that make up the matrix are the collagens and the polysaccharide glycosaminoglycans.

The noncollagen glycoproteins of the extracellular matrix have been relatively neglected until recently. A good deal is now known about fibronectin. Fibronectin first attracted attention when it was discovered to be present in greatly reduced amounts on the surface of fibroblasts derived from tumors compared to normal fibroblasts (Vaheri *et al.* 1975, 1976, Olden *et al.* 1977).

Fibronectin

Fibronectin was first isolated from human plasma by Morrison *et al.* in 1948 and termed " cold-insoluble globulin". There are two type fibronectins—cell surface (cellular) fibronectin, and plasma fibronectin. Cell surface fibronectin is a major constituent of the cell surface of many cultured cells and has a subunit molecular weight of between 200,000 and 250,000 (Hynes 1976, Yamada *et al.* 1976, Mosesson 1977, Grinnell 1978). Plasma fibronectin is a dimeric glycoprotein with subunit polypeptides of 200,000 to 220,000 that circulates in vertebrate blood (Morrison *et al.* 1948, Mosesson *et al.* 1970,

1975, Mosher 1975, Iwanaga *et al.* 1978).

The structure of fibronectin. The fibronectin molecule is a dimmer: it consists of two similar subunits which are joined at one end by disulfide bonds (Fig. 4). The

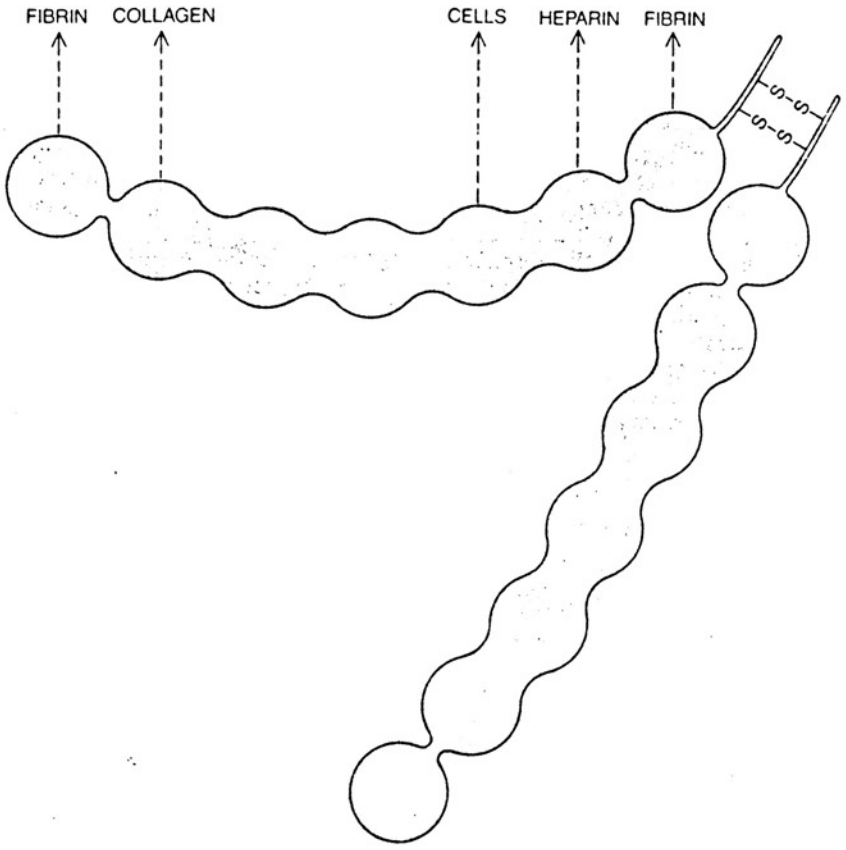


Figure 4. The structure of fibronectin (Hynes 1986)

protein chain of each subunit forms an elongated structure 60 to 70 nanometers long and two to three nanometers thick; that structure in turn is subdivided into a series of smaller domains, within each of which the protein chain is tightly folded. Fibronectin is produced mainly by fibroblasts (Ruoslahti *et al.* 1974). Endothelial cells (Stenmam *et al.* 1978) and smooth muscle cells (Stenmam *et al.* 1977) also can synthesize fibronectin. Fibronectin has been implicated functionally in the regulation of several cellular precesses,

including adhesion, differentiation, motility and transformation (Mosher 1989, Hynes 1990).

The structure of the fibronectin polypeptides varies somewhat depending on the cellular source of the protein. These variations are due to alternative splicings of the fibronectin mRNA (Ruoslahti 1988). The biological significance of the alternative splicings is not known. But they may affect the functions of fibronectin. The site in fibronectin that promotes cell attachment is in the middle portion of the fibronectin polypeptide. This segment contains the sequence Arg-Gly-Asp (RGD), the recognition of which, in surface-bound fibronectin, results in the attachment of the cells to that surface. Main Heparin-binding site, another important binding site for cell attachment, is located near the COOH-terminus of the fibronectin polypeptide (Ruoslahti 1988).

Cells interact with fibronectin at the RGD cell attachment site and at the heparin-binding sites. The specificity of the interaction seems to come from the RGD site, while the binding at the heparin-binding site plays an augmenting role. The RGD site is recognized by the RGD-directed receptors (Ruoslahti et al, 1986, 1987) that belong to the integrin superfamily of proteins (Hynes 1987), while cell-surface proteoglycans bind to the heparin-binding sites (Rapraeger *et al.* 1987).

Fibronectin receptors. The mammalian fibronectin receptors and other RGD-directed receptors are belonging to the integrin receptor family. Integrin receptors are typically heterodimers of two subunits α and β (Ruoslahti *et al.* 1987, Pytela *et al.* 1985, 1986) (Fig. 5). The α subunits consist of two polypeptides disulfide-linked to one another. The β subunit is a single polypeptide with a molecular weight of about 140,000

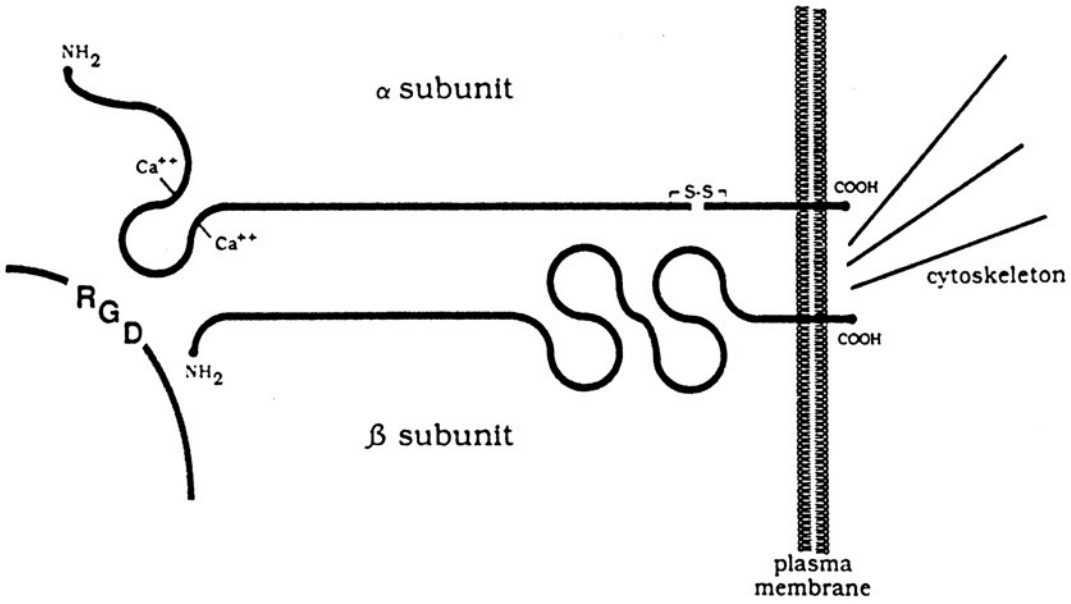


Figure 5. The structure of integrin receptor (Ruoslahti 1988)

(Ruoslahti 1988). Both subunits are transmembrane proteins. In the α subunit, the smaller polypeptide anchors the subunit to the membrane. The larger α subunitpolypeptide contains several short sequences homologous to known Ca^{2+}-binding sites in other proteins. In the β subunit, a segment with a high disulfide content is shown as bends in the polypeptide. Both subunits are thought to participate in the binding of the ,Arg-GLy-Asp cell attachment site of fibronectin.

The Role of Fibronectin in Cell Proliferation

Vascular complications of several diseases are associated with changes in the extracellular matrix and accompanying intracellular changes in vascular cells (Thyberg *et al.* 1990). Since interactions between protein of the extracellular matrix and cell receptors, called integrins, are known to influence cell structure, it is plausible that

changes in the expression of components of the extracellular matrix could have a causative role in the development of the resulting vascular lesions.

Histochemical studies have documented the presence of fibronectin in aortic tissue, and changes in fibronectin content have been reported in different disease states, such as atherosclerosis, hypertension and diabetes (Jensen *et al.* 1983, Rasmussen *et al.* 1989, Orelhov *et al.* 1984, Smith *et al.* 1986, Phan-Thanh *et al.* 1987, Stenman *et al.* 1980). A study by Glukhova *et al.* (1989) showed that different alternately spliced forms of fibronectin are selectively localized in the intima and media, and following either balloon injury to the rat aorta or in human aortic atherosclerotic lesions, a selective accumulation of an alternatively spliced form of fibronectin was found within the intimal lesion. A study has shown that steady-state mRNA levels for rat aortic fibronectin increased several-fold in deoxycorticosterone acetate (DOC)/salt-treated and angiotensin II-infused rats and in the spontaneously hypertensive rat (SHR) (Takasaki 1990). There was a three to sixfold increase in fibronectin biosynthesis by aortic rings taken from rats treated with deoxycorticosterone/salt (Saouaf *et al.* 1991). Interactions between fibronectin and cultured vascular smooth muscle cells induced a phenotypic change from a contractile to synthetic state that is a prerequisite for the cellular proliferation (Chamley-campbell *et al.* 1979, Thyberg *et al.* 1983). Smooth muscle cells from atherosclerotic lesions of human arteries, as well as those from experimental intimal thickening in animal arteries, express the synthetic or fibroblast-like phenotype (Dilley *et al.* 1987, Nilsson *et al.* 1986, Ross 1986b, Schwartz *et al.* 1986). *In vivo*, a similar change in the differentiated properties of the smooth muscle cells appears to be one of the initial events in the development of an atherosclerotic lesion (Ross *et al.* 1986b, Schwartz 1986).

Vascular lesions are a major complication of diabetes. The vascular lesions also are characterized by smooth muscle cell proliferation. Increased biosynthesis and processing of fibronectin in fibroblasts from diabetic mice (Phan-Thanh *et al.* 1987) also suggest that the smooth muscle cell changes that occur during hypertension and atherosclerosis may be due to fibronectin-smooth muscle cell interactions. These findings support the hypothesis that fibronectin is involved in the vascular smooth muscle autocrine and/or paracrine regulation in hypertension and atherosclerosis.

PDGF May Affect Cell Proliferation by Fibronectin

As previously indicated, both PDGF and fibronectin are involved in proliferation of vascular smooth muscle cells. Are there any relationships between PDGF and fibronectin? Because the cell response elicited by PDGF is very similar to the effects of fibronectin on cell proliferation, it is reasonable to consider the possibility that PDGF may affect cell proliferation by induction of fibronectin. A few reports have suggested that PDGF was able to stimulate fibronectin gene expression (Blatti *et al.* 1988, Allen-Hoffmann *et al.* 1990). Human dermal fibroblasts transfected with a human *c-sis* cDNA (coding for the platelet-derived growth factor B-chain) increase fibronectin levels and gene expression (10-fold) relative to control. It was demonstrated that one of the biological functions of PDGF B-chain isoforms is to modulate fibronectin synthesis (Allen-Hoffmann *et al.* 1990). The present studies were undertaken to determine the role of PDGF-BB, one of the PDGF isoforms, in the stimulation of fibronectin gene expression in vascular fibroblasts isolated from rat thoracic aorta.

MATERIALS AND METHODS

Materials

Human recombinant PDGF-BB was purchased from UBI (Lake Placid, NY). The mitogenic stimulation is determined by ^{3}H-thymidine incorporation using human foreskin fibroblasts. The dose for half maximal stimulation is 0.9 ng/ml. Human recombinant IGF-I was from UBI. Biological activity of IGF-I was determined via a radio-receptor assay using human placental cell membranes. The dose for half maximal displacement is 1.6 ng/ml. Collagenase, elastase and Bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum and newborn calf serum were from Whittaker Bioproducts (Walkersville, MD). Cell culture medium-199 was purchased from Gibco/BRL (Grand Island, NY). Polyclonal anti-rat fibronectin antibody and monoclonal anti-rat α-smooth muscle actin antibody, Goat anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC) and goat anti-mouse antibody conjugated with FITC were from Sigma. Polyclonal anti-rat Factor VIII antibody was from Calbiochem (La Jolla, CA). Normal goat serum was from Cappel Laboratories (Cochranville, PA). *E. Coli* containing the fibronectin gene and the 18s gene was obtained from Dr. R. O. Hynes (MIT). Restriction endonuclease ECORI was purchased from Gibco/BRL. Agarose and polyacrylamide were from Sigma. Low melting point agarose gel was from FMC BioProducts (Rockland, ME). Elutip-d columns were purchased from Scleicher & Schuell (Kneene, NH). DECA primer DNA labelling kits

were purchased from Ambion Inc. (Austin, TX). [α-^{32}P] dCTP 3000 ci/nM was from NEW/DUPONT (Boston, MA). Anti-rat IgG antibody conjugated with alkaline phosphatase, Nitroblock and phosphatase substrate CSPD were purchased from Tropix (Bedford, MA). PVDF membranes were from Millipore (Bedford, MA).

Fibroblast Isolation and Culture

Wistar-Kyoto (WKY) rats (male, 160-180 g body weight) were purchased from Taconic Farms (Germantown, NY). WKY rats were sacrificed by guillotine and aortae were rapidly dissected and placed in a beaker of ice-cold Dulbecco's Phosphate-Buffered Saline (PBS) (PH 7.4). After washing out blood and cleaning pericentitial tissue, aortae were opened longitudinally. The intima were scraped out with a scalpel blade. The remaining tissue was subjected to an enzyme solution containing collagenase and elastase (Collagenase 2 mg/ml, Elastase 2 mg/ml, Bovine Serum Albumin 2 mg/ml), digesting the tissue for about 30 min at 37°C. The adventitial layer was then carefully peeled away from the media under a microscope. The adventitia were pooled, minced and placed in a fresh enzyme solution for about 2 hours to produce single-cell suspensions for plating. The suspensions were centrifuged and the pellets were seeded into a Petri dish with cell culture medium-199 (M-199) supplemented with 20% fetal bovine serum (FBS). The cells were incubated at 37°C in an atmosphere of 5% CO_2-95% air. The medium was replaced twice a week. After several generations the subculture medium was switched to M-199 supplemented with 10% newborn calf serum (NCS). All manipulations were performed in the cell culture hood. The fibroblasts between passages 10 to 20 were used for experiments.

Characterization of Fibroblasts

Even when the intima and media of rat thoracic aorta have been removed, it is still possible that smooth muscle cells or endothelial cells contaminate the cell cultures. Immunohistochemical techniques were used to identify the fibroblast. For example, α-Smooth muscle actin is specific to smooth muscle cells and Factor VIII is present only in endothelial cells. Therefore, immunofluorescent staining of α-smooth muscle actin and factor VIII helped distinguish fibroblasts from smooth muscle cells and endothelial cells. Since we know that the fibroblast is the major cell producing fibronectin, fibronectin produced by fibroblasts isolated from rat thoracic aortae was confirmed by immunofluorescent stain.

The cells were grown on glass coverslips for 3 to 4 days and then fixed in 3.7% formaldehyde for 10 min at 4°C. Before staining, the coverslips were treated with acetone for 5 min at -20°C to increase the permeability of the cell membrane. To avoid a dirty background, non-specific binding sites were blocked by incubating the glass coverslips with normal goat serum at a 1:20 dilution in Hank's Buffered Salt Solution (HBSS) overnight at 4°C. The first antibodies (polyclonal anti-rat fibronectin antibody, polyclonal anti-rat Factor VIII antibody, mouse monoclonal anti-rat α-smooth muscle actin antibody) were added to the coverslips, respectively, and incubated with the cells for 3 or 4 hours at 4°C at a dilution of 1:20 in HBSS. Excess antibodies were washed out from the coverslips with HBSS. The second antibody (goat anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC) or goat anti-mouse IgG antibody conjugated with FITC was added and incubated with the coverslips at 4°C for another 3

to 4 hours. The coverslips were then rinsed with HBSS and viewed under fluorescence microscopy.

Isolation of Plasmid DNA Containing Fibronectin cDNA and 18s DNA

This procedure is based on the alkaline lysis method (Sambrook *et al.* 1989) utilizing the detergent sodium dodecyl sulfate (SDS) and NaOH to lyse *E. coli* and release nucleic acids and plasmid DNA. Potassium acetate was added to neutralize the solution which allows plasmid DNA to reanneal while precipitating most denatured genomic DNA and proteins. Plasmid DNA was further purified from any unprecipitated genomic DNA by a cesium chloride (CsCl) gradient containing ethidium bromide. Nicked plasmid or genomic DNA, being linear, binds more ethidium bromide than plasmid DNA resulting in a lower density. This allows plasmid to travel further down the CsCl gradient and be visualized via ultraviolet (UV) light as a separated fluorescent band. Then, the band can be extracted.

E. Coli containing the fibronectin gene or 18s gene were grown on a shaker overnight at 37°C in 25 ml Luria-Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract, 1% NaCl and 0.35 N NaOH plus 50 μg/ml ampicillin. The 25 ml of bacteria were amplified by growing in 500 ml LB medium supplemented with 50μg/ml ampicillin and incubated overnight shaking at 37°C. Then, the bacteria were spun down at 4°C and the pellets 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. Proteins in the mixture were precipitated by the addition of 3 M potassium acetate. After a subsequent centrifugation, the supernatant containing DNA

was filtered through a Whatman No.1 filter and precipitated overnight at -20°C in ethanol. The precipitated DNA was spun down and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 11 gm CsCl. Ethidium bromide (10 mg/ml) was added to each sample allowing the DNA to be visualized under UV light. The samples were aliquoted into 5 ml Beckman quick seal tubes and centrifuged for at least 16 hours at 64,000 rpm at 20°C. After centrifugation, ultraviolet light disclosed two thin bands in the tubes. The lower one was plasmid DNA. The plasmid DNA was collected with a syringe connected to an 18 gauge needle. Ethidium bromide was washed out from plasmids 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 and the samples allowed to stand overnight at -20°C to precipitate DNA. The samples were spun down and the pellets washed with 70% ethanol. Plasmid DNA was kept in 0.05 volumes of 3 M ammonium acetate and 2.5 volumes of ethanol at -20°C. Before using, the samples were spun and resuspended in TE buffer. The plasmid DNA was quantitated by reading at A_{260} by a spectrophotometer.

Isolation of Fibronectin cDNA Probe

Fibronectin cDNA was isolated via restriction endonuclease cutting of a plasmid containing the fibronectin gene. Plasmid DNA was incubated with restriction endonuclease ECORI in a reaction buffer containing 50 mM Tris/HCl-pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl for 1 hour at 37°C. The reaction was terminated by the addition of loading buffer (20% Ficoll 400, 0.1 M Na-EDTA, 1% SDS, and 0.25% xylene cyanol). The fibronectin cDNA fragments were separated from the remaining plasmid fragments

by agarose 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 about 2 hours. After electrophoresis, the DNA gel was checked on an ultraviolet transilluminator. The fibronectin cDNA band was identified by comparing the bands obtained from plasmid DNA with the bands obtained from DNA standard running in the same gel. The fibronectin cDNA band was excised from the gel under ultraviolet illumination. Further extraction and purification of fibronectin cDNA from the agarose gel were performed through the use of the Elutip-d column containing a DNA binding resin. The low melting point gel was melted in low salt solution (0.2 M NaCl, 20 mM Tris/HCl-pH 7.5, 1 mM EDTA) at 65°C. Then, the gel mixture was applied to the column and passed through the column slowly. The bound cDNA was eluted from the column by slowly washing the column with high salt solution (1 M NaCl, 20 mM Tris/HCl-pH 7.5, 1 mM EDTA). The cDNA was precipitated in two volumes of TE buffer and six volumes of 100% ethanol overnight at -20°C. Before using, the cDNA was resuspended in TE buffer and quantitated by reading at A_{260}.

Fibronectin mRNA Isolation and Analysis

Total RNA was obtained by the use of phenol extraction (Sambrook *et al.*, 1989). This method utilizes SDS lysing of the cells thereby releasing nucleic acids and proteins while inhibiting RNA degrading enzymes. Phenol at acid pH was used to separate nucleic acid-associated proteins from RNA.

The cell cultures were rinsed with PBS two times to get rid of cell culture medium. The cells were scraped out and lysed with SDS (10 mM EDTA-pH 8.0, 0.5%

SDS, 0.1M Na-Acetate). After sonication, the lysate RNA was extracted by the addition of acidic phenol (pH 3.7-4.5) and subsequent centrifugation at 4°C for 15 to 20 min. The top aqueous layers containing RNA were carefully collected and precipitated overnight at -20°C in 0.07 M Tris-pH 8.0, 0.87 M NaCl, and 100% ethanol. The samples were spun and RNA pellets washed with 70% ethanol one time. To avoid RNA degradation, it is better to keep the RNA sample in 0.05 volumes of 3 M ammonium acetate and 2.5 volumes of ethanol at -20°C. Before using, the samples were spun and resuspended in diethyl pyrocarbonate (DEPC) treated water and quantitated at A_{260}.

mRNA Agarose Gel Electrophoresis

Fifteen µg total RNA were added to a mixture containing 1X MOPS, 50% formamide and denatured with incubation at 65°C for 15 minutes. After mixing with 0.2 volumes of formaldehyde, 2 µl loading buffer (1 mM EDTA-pH 8.0, 50% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue) and 2 µl 1 µg/ml ethidium bromide, the sample was applied to a well in a slab gel containing 1.2% agarose in 1X MOPS and 6% formaldehyde. The gel was submerged in 1X MOPS and run at 80 volts until the leading dye front had run about 50% of the gel length.

Once the electrophoresis was completed, the RNA bands were photographed on an ultraviolet transilluminator. DEPC treated water was used to wash out formaldehyde from the gel. Capillary transfer in 20X SSPE (17.4% NaCl, 2.76% NaH_2PO_4, 1.24% EDTA, pH 7.4) was used to transfer the RNA to a nylon membrane. The gel was placed on top of a strip of filter paper which had been soaked in 20X SSPE. This paper was draped over a plastic plate and allowed to hang into a container of 20X SSPE. A sheet

of nylon, cut to the dimensions of the gel and soaked in DEPC treated water followed by 20X SSPE was placed on the gel. A two inch stack of brown paper towels, all cut to the dimensions of the gel, was placed on top of the nylon. A second plastic plate was placed on top to insure contact between the layers of paper. After overnight transfer, the nylon was baked for 2 hours at 80°C in an oven or exposed to ultraviolet irradiation on a transilluminator for 4 min to fix the RNA to nylon. This step permitted it to be incubated in prehybridization buffer without loss of the RNA.

Complementary DNA Labeling

A random primer DNA labelling kit was used to incorporate [α-^{32}P] dCTP 3000 Ci/nM into the cDNA. This method uses random hexanucleotides as primers which bind to the denatured cDNA (25 ng). The large subunit of DNA polymerase I (Klenow fragment) polymerizes a mixture of unlabeled dATP, dGTP and dTTP along with the labelled dCTP, using the purified cDNA as the template. The reaction was run for 2 hours at 37°C and terminated by removal of the unincorporated nucleotides from the labelled cDNA with a passage over the Elutip-d column. The dCT^{32}P-labelled cDNA fragments were then be incubated with the northern blot.

Northern Blotting

The hybridization was carried out using a published procedure (Church and Gilbert, 1984). Prehybridization took at least 2 hours at 65°C in a hybridization buffer containing 1% BSA, 7% SDS, 0.5 M phosphate-pH 7.0 and 0.001 M EDTA. 100 µg/ml sonicated and denatured salmon sperm DNA was added at the time of hybridization.

Hybridization was performed for 16-24 h at 65°C using cDNA probes labeled with ^{32}P by the random hexamer priming procedure. After hybridization, membranes were washed for 10 min 1 to 3 times at 65°C with washing buffer containing 1% SDS, 0.04 M phosphate, 0.001 M EDTA. A γ-ray monitor was used to monitor the radioactivity of the membranes. After getting an optimal signal, blots were exposed to X-ray films between two intensifying screens for 5 to 10 days at -80°C. Developed films were scanned by laser densitometry and analyzed using software NIH image 1.5.4. The term "relative intensity" used in this dissertation denotes the relative density of fibronectin mRNA bands in northern blots.

SDS-PAGE Gel Electrophoresis

One-dimensional polyacrylamide gel electrophoresis under denaturing conditions was used to separate proteins based on the basis of molecular size. After solubilizing all the proteins by boiling in the presence of SDS and urea, an aliquot of the protein solution was applied to a gel lane, and the individual proteins or their subunits were separated electrophoretically.

SDS-polyacrylamide gel usually consists of a separating and a stacking gel. Before making a gel, a gel cast was assembled using two clean glass plates (one with comb) and a rubber spacer. A 7% separating gel was made by mixing 9.3 ml 30% acrylamide/0.8% biacrylamide, 10 ml 4X Tris.Cl/SDS-pH 8.8, and 20.7 ml distal water. The mixture was degassed 10 to 15 min and 0.4 ml 10% ammonium persulfate and 0.03 ml TEMED were added. A syringe connected to a long needle was used to transfer the separating gel solution to the gel cast along an edge of the spacer to a height of about 11

cm. The gel was polymerized within 30 min at ambient temperature. A 4% stacking gel was made by mixing 1.95 ml 30% acrylamide/0.8% biacrylamide, 3.75 ml 4X Tris/SDS-pH 6.8, and 9.15 ml distilled water. After degassing and the addition of ammonium persulfate and TEMED, the gel mixture was carefully poured into the cast, until the height of the solution in the cast was about 3 cm. After 30 to 45 min polymerization, the plate with comb and rubber spacer were carefully removed without tearing the gel.

The gel was placed on the electrophoresis unit and two buffer chambers were filled with 2 liters of SDS/electrophoresis buffer (1.5% Tris-base, 7.2% glycine and 0.5% SDS). Two stacks of filter papers that had been soaked in SDS/electrophoresis buffer connected the gel and electrodes via touching one side to the top or bottom of the gel and draping the other side into the buffer chambers.

The protein to be analyzed was diluted 1:1.5 (vol/vol) with sample buffer (1% SDS, 8 M urea, 1% Dithiothreitol, 0.001 M H_3PO_4). After boiling 5 min at 100°C to denature the protein, and adding bromophenol blue, the samples were applied to the gel and run at 40 mA for about 3-4 hours at 12-15°C.

When electrophoresis had been completed, the samples were transferred from the gel to PVDF membrane. A piece of PVDF membrane, cut to the same dimensions as the gel and wetted with methanol followed by distilled water, was placed on top of the gel. Two stacks of filter papers, about 20 layers each, cut to the same dimensions as the gel and soaked in transfer buffer (25 mM Tris-base, 192 mM glycine, 0.0375% SDS), sandwiched the gel. Then, the filter paper-gel sandwich was transferred onto the anode plate of the transfer unit. The cathode plate was placed on top of the sandwich. Transfer usually takes about 2-3 hours at 0.8 mAmps/cm^2. After transforation, the gel was stained

with coomassie blue to insure completed transfer. The membrane was kept in washing buffer (0.002% Tween-20, 2 mM Tris-HCl, 0.09% NaCl, 0.002% NaAzide, pH 7.4), until ready for western blotting.

Slot Blotting

To get an optimal protein concentration applied to the slot blot, different dilutions of protein samples with 2% deoxycholate were prepared. The samples were applied to a Filtration Manifold System (Gibco/BRL). The proteins were bound to the PVDF membrane via a vacuum connected to the manifold. The membrane containing sample proteins can be kept in washing buffer for three days, until ready for the chemiluminescent western blotting.

Non-radioactive Chemiluminescent Western Blotting

After the protein samples were transferred to PVDF membranes by slot blotting or SDS-polyacrylamide gel transfer, the membranes were gently washed in blocking/antibody incubation buffer (5% dry, non-fat milk; 0.1% Tween-20; 2 mM Tris-HCl; 0.09% NaCl; 0.002% NaAzide; pH 7.4) for one hour. The first antibody, anti-rat fibronectin antibody from rabbit antiserum, at a concentration of 1:25000 diluted in blocking/antibody incubation buffer, was added to the membranes and incubated for another 1 hour. The first antibody was washed out by several washings in washing buffer. The second antibody, anti-rat IgG antibody conjugated with alkaline phosphatase, was added at a 1:35000 dilution in blocking/antibody incubation buffer and incubated with the membrane for 15 minutes. Membranes were washed in washing buffer to get a clean

background. To enhance the chemiluminescent signal on PVDF membranes, a 5 minute incubation in Nitroblock was carried out. Finally, the membranes were subjected to substrate CSPD solution at a final concentration of 0.0024 mM. After five minute reaction, the wet membranes were wrapped in plastic wrap allowing the reaction to proceed for another 15 minutes. The membranes were then exposed to Kodak XAR-OMAT X-ray films for 1 to 3 minutes. Developed films were scanned by laser scanner and analyzed using software NIH image 1.5.4. The fibronectin levels were determined via comparing the density measurement obtained from fibronectin standard and the density measurement obtained from samples.

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons test were used to determine statistical significance. Differences were considered significant at $P<0.05$. Software Instat version 2.01 were used for statistical analysis.

RESULTS

Cell Characterization

Morphology. Hematoxylin-Eosin stain was used to examine the cell morphology. Fibroblasts isolated from rat thoracic aorta showed predominant bi- and tripolar morphologies. The cell border was smooth and there were small processes at each pole (Fig. 6). At confluence, the fibroblasts aligned in parallel arrays giving the cell cultures a swirling appearance.

Immunofluorescent stain. α-smooth muscle actin stain: anti-rat α-smooth muscle actin antibody is a monoclonal antibody specific to smooth muscle cells. Immunofluorescent staining of fibroblasts isolated from rat thoracic aorta with monoclonal anti-α-smooth muscle actin antibody was performed to estimate the purity of the fibroblast cell cultures. The staining was negative in the fibroblasts (top, Fig. 7). Smooth muscle cells isolated from rat thoracic aorta were used as a positive control. The bright white color in the picture denoted that the smooth muscle cells contain α-smooth muscle actin (bottom, Fig. 7). This evidence demonstrated that the fibroblast cell cultures were not contaminated by smooth muscle cells.

Factor VIII stain: Factor VIII is a factor which is only present in endothelial cells. To find out whether there was endothelial cell contamination, the cell cultures were reacted with rat polyclonal anti-Factor VIII antibody (Fig. 8). The negative results in fibroblasts isolated from thoracic aorta suggested that there was no endothelial cell contamination in these cultures.

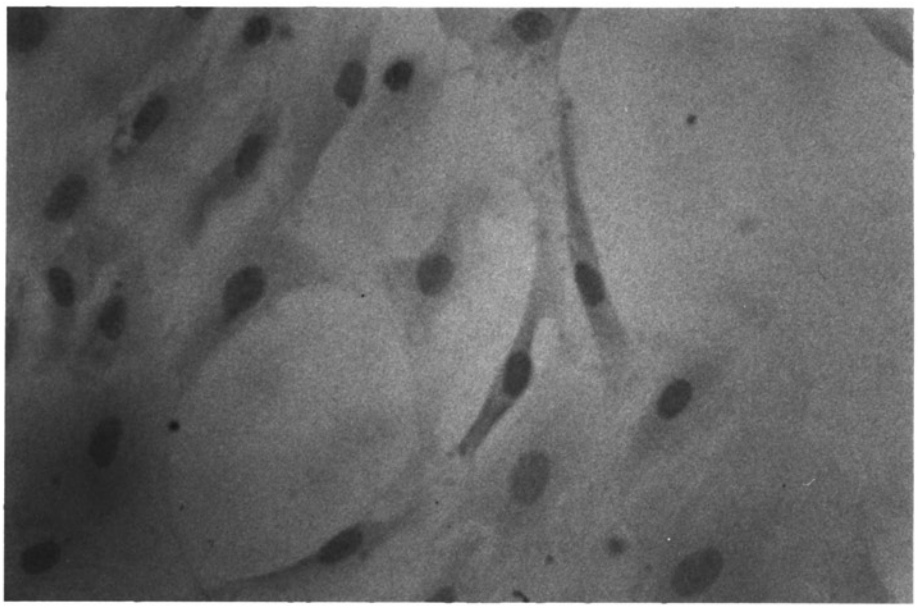
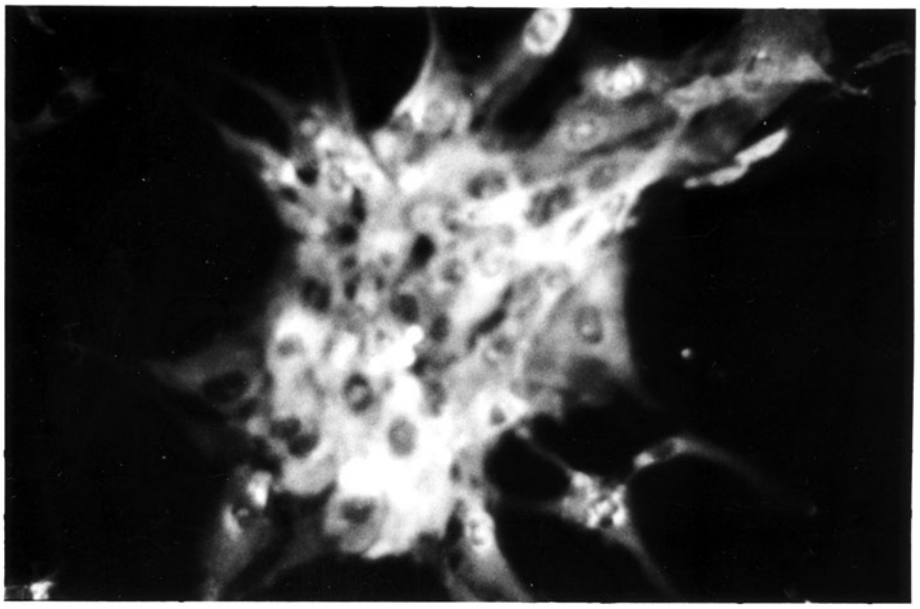


Figure 6. Hematoxylin-Eosin stain of cultured fibroblasts isolated from WKY rat thoracic aorta.

Figure 7. Immunofluorescent stain of cultured fibroblasts (top) and smooth muscle cells (bottom) isolated from WKY rat thoracic aorta with monoclonal anti-rat α-smooth muscle actin antibody. The bright white color in the picture is α-smooth muscle actin.





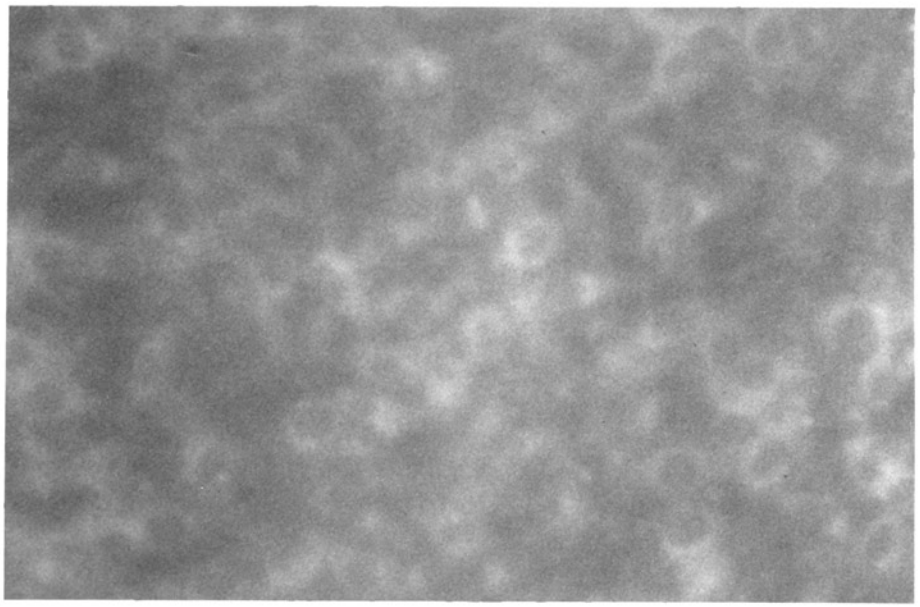


Figure 8. Immunofluorescent stain of cultured fibroblasts isolated from rat thoracic aorta with polyclonal anti-rat Factor VIII antibody.

The presence of fibronectin in the cell cultures was confirmed by immunofluorescent staining with rat polyclonal anti-fibronectin antibody. A large amount of fibronectin production (bright white color in the picture), was detected in these cultures (Fig. 9).

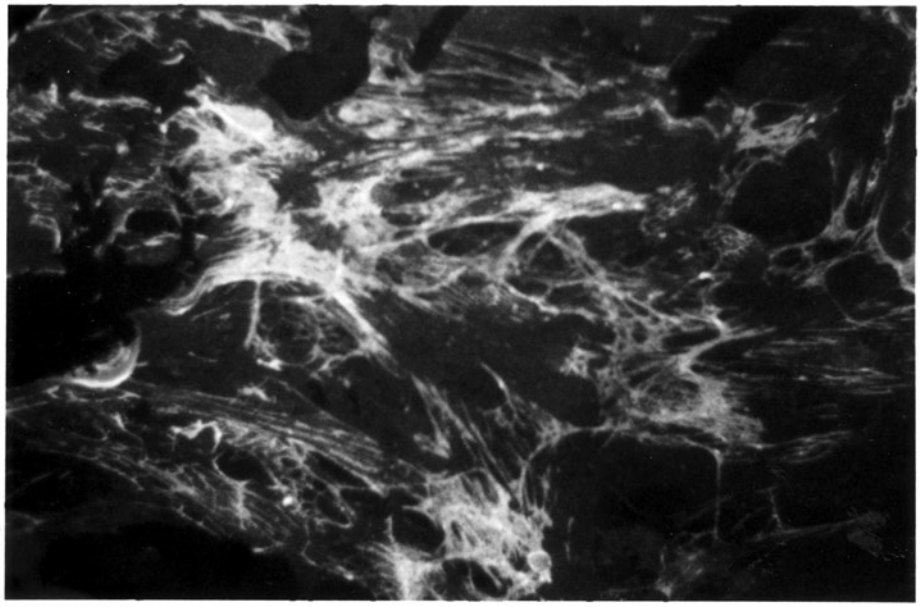
Fibronectin mRNA Response to PDGF-BB

Fibronectin mRNA time-response experiments. PDGF-BB produced rapid and transient stimulation of fibronectin gene expression in vascular fibroblasts (Fig. 10, 11). Cell cultures were treated with PDGF-BB with a final concentration of 20 ng/ml, fibronectin mRNA levels were assessed at 2, 4, 6 and 8 hours after PDGF-BB addition. In the northern blot analysis, an increase in fibronectin mRNA levels after PDGF treatment was first detected at 4 hours and appeared maximal (1.2-fold above normal values) at 6 hours (Fig. 10, 11). These increases were transient, and returned to basal levels by 14 hours. No further changes in fibronectin mRNA levels were found at 24 hours (data not shown).

In northern blotting analysis, 18s ribosomal RNA was used as an internal control. Uniform 18s ribosome RNA bands denote (1) uniform application of the RNA samples to the gel; (2) uniform transfer of the RNA samples from the gel to the nylon; (3) the increase of fibronectin mRNA levels was a specific response to growth factors and not due to a generalized effect on RNA synthesis (Fig. 10, 13, 16, 19, 22).

Total RNA levels at different time points after PDGF-BB addition showed no difference when compared to untreated control ($P > 0.05$ vs. control) (Fig. 12).

Figure 9. Immunofluorescent stain of cultured fibroblasts isolated from rat thoracic aorta with (top) and without (bottom) polyclonal anti-fibronectin antibody. The bright white color in the picture is fibronectin.





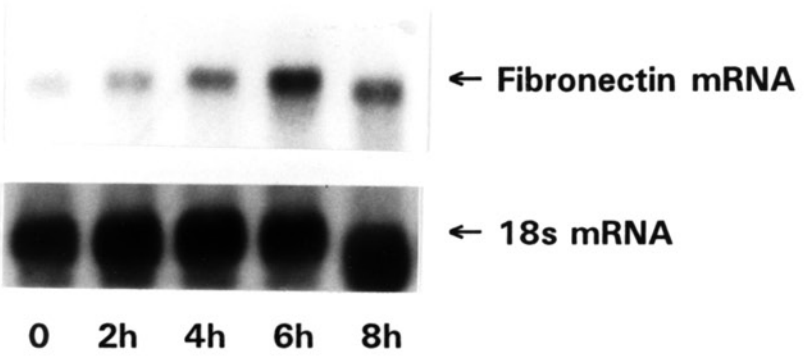


Figure 10. Northern blot of fibronectin mRNA levels in response to PDGF-BB in time-response experiments. Total RNA (15 μg/lane), extracted from 20 ng/ml PDGF-BB treated fibroblasts at different time points, was probed with cDNA for fibronectin (top). Same blot was stripped and reprobed with cDNA for 18s mRNA (bottom).

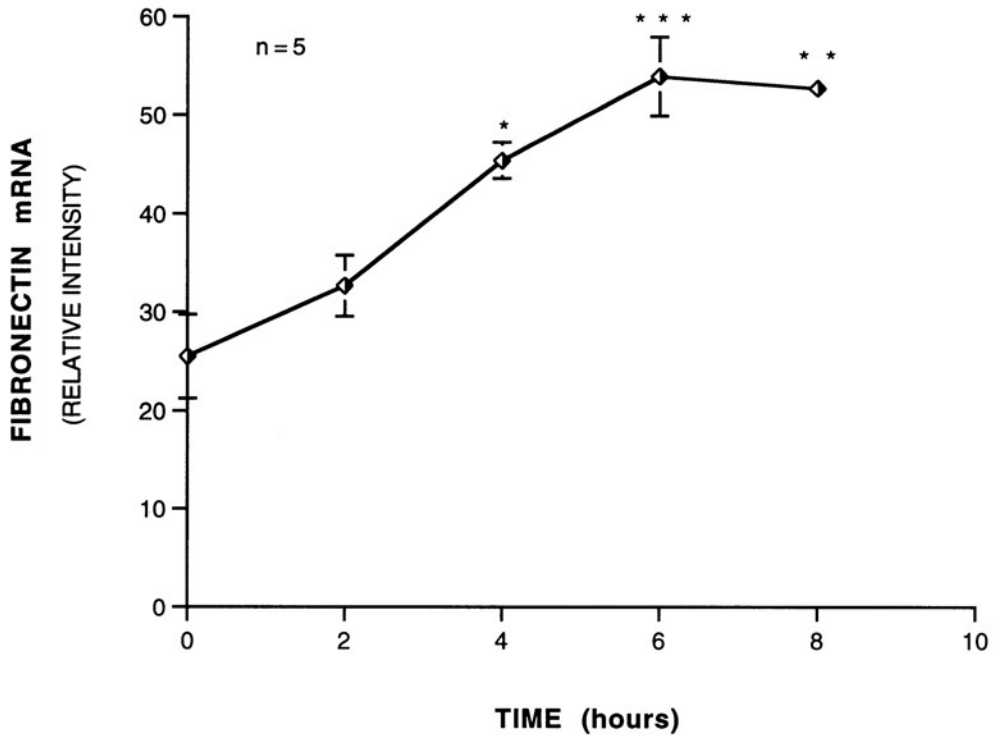


Figure 11. Fibronectin mRNA levels in response to PDGF-BB in time-response experiments. Fibroblasts isolated from rat thoracic aorta were treated with 20 ng/ml PDGF-BB. Fibronectin mRNA levels obtained from 15 μg total RNA was analyzed at 2, 4, 6 and 8 hours after PDGF-BB addition. n = 5. * $P < 0.05$ vs. untreated control. ** $P < 0.01$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

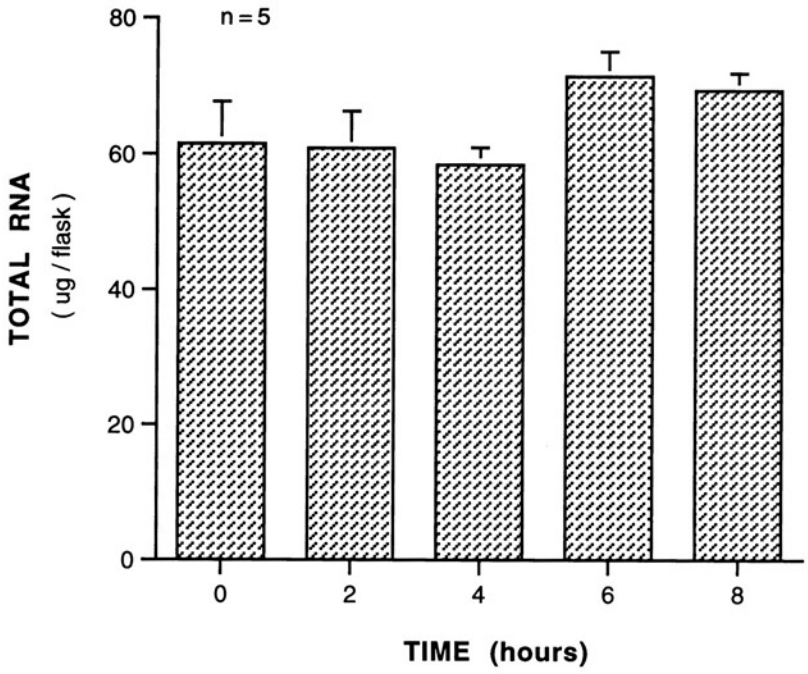


Figure 12. Total RNA levels in response to PDGF-BB in time-response experiments. The fibroblasts were treated with 20 ng/ml PDGF-BB. Total RNA was extracted at 2, 4, 6 and 8 hours after PDGF-BB addition. n = 5. $P > 0.05$ vs. untreated control.

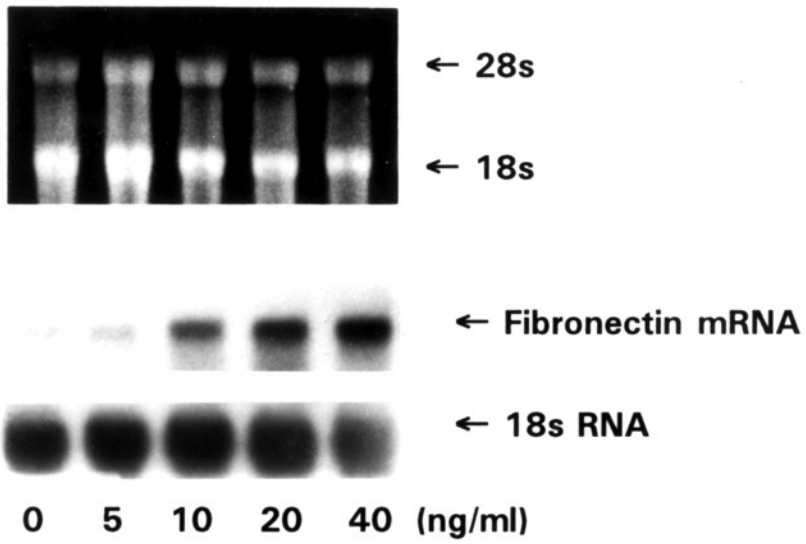


Figure 13. Northern blot of fibronectin mRNA levels in response to PDGF-BB in dose-response experiments. Total RNA (15 μg/lane), from fibroblasts treated with PDGF-BB at a concentration of 5, 10, 20 and 40 ng/ml for 6 hours, was probed with cDNA for fibronectin mRNA (middle). Same blot was stripped and reprobed with cDNA for 18s mRNA (bottom). Top panel is ethidium bromide stained total RNA samples used for northern blotting. FN, fibronectin.

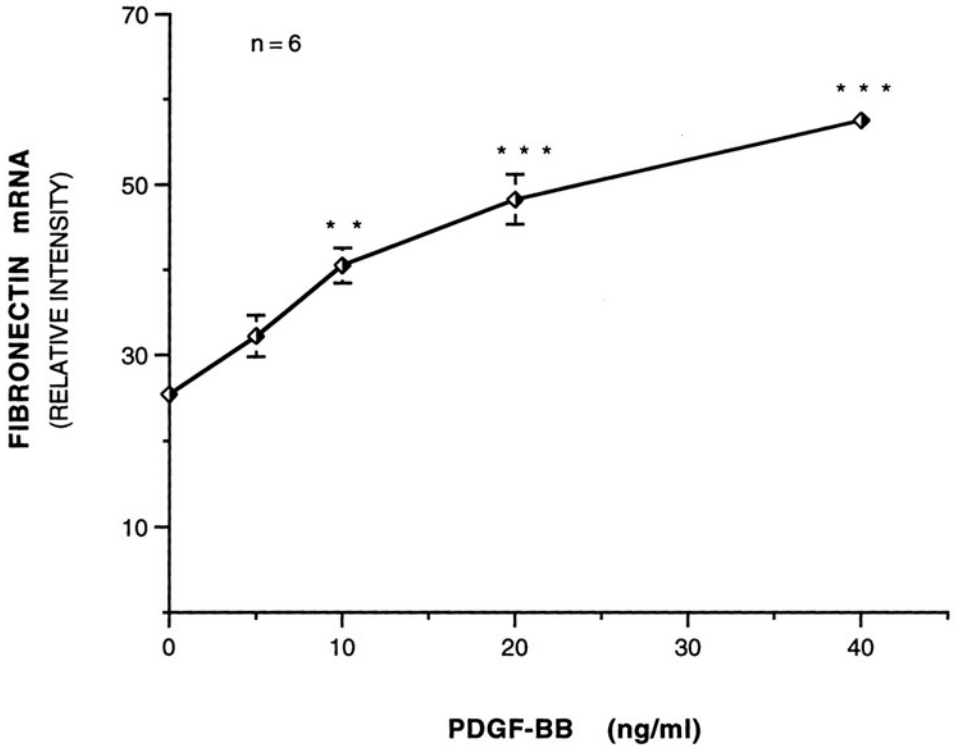


Figure 14. Fibronectin mRNA levels in response to PDGF-BB in dose-response experiments. Fibroblasts isolated from rat thoracic aorta were treated with 5, 10, 20 and 40 ng/ml PDGF-BB. Fibronectin mRNA levels were analyzed at 6 hours after PDGF-BB addition by northern blotting. n = 6. ** $P < 0.01$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

Fibronectin mRNA dose-response. PDGF-BB stimulated fibronectin mRNA in vascular fibroblasts in a dose-dependent manner (Fig. 13, 14). Fibroblasts were treated with PDGF-BB at a final concentration of 5, 10, 20 and 40 ng/ml respectively. Fibronectin mRNA levels were assessed 6 hours after PDGF-BB addition. Significant increases in fibronectin mRNA levels were found after exposure of cell cultures to 10 ng/ml PDGF-BB. Further increases in PDGF-BB concentrations produced gradual increases in fibronectin mRNA levels. After exposure of cell cultures to 40 ng/ml PDGF-BB, fibronectin mRNA levels were 1.3-fold above normal values (Fig. 14).

Assurance that equal amounts of total cellular RNA were loaded per lane was made by ethidium bromide staining of the major ribosomal RNA bands of the samples used for northern blot analysis (Fig. 13). Ethidium bromide at a concentration of 1 mg/ml was added to the total RNA samples before the samples were applied to the agarose gel. Ethidium bromide staining also helped determine whether there was RNA degradation in the sample.

The total mRNA levels at different PDGF-BB dosages showed no significant variation ($P > 0.05$ vs. untreated control) (Fig. 15).

Fibronectin mRNA in Response to IGF-I

IGF-I also produced a fast stimulation of fibronectin mRNA in vascular fibroblasts (Fig. 16, 17). In the time-response, fibroblasts were treated with 20 ng/ml IGF-I, and fibronectin mRNA levels were assessed at 2, 4, 6 and 8 hours after IGF-I addition. Significant increases in fibronectin mRNA levels were detected at 6 hour after PDGF-BB addition. The fibronectin mRNA levels at 8 hours is approximately 2.5-

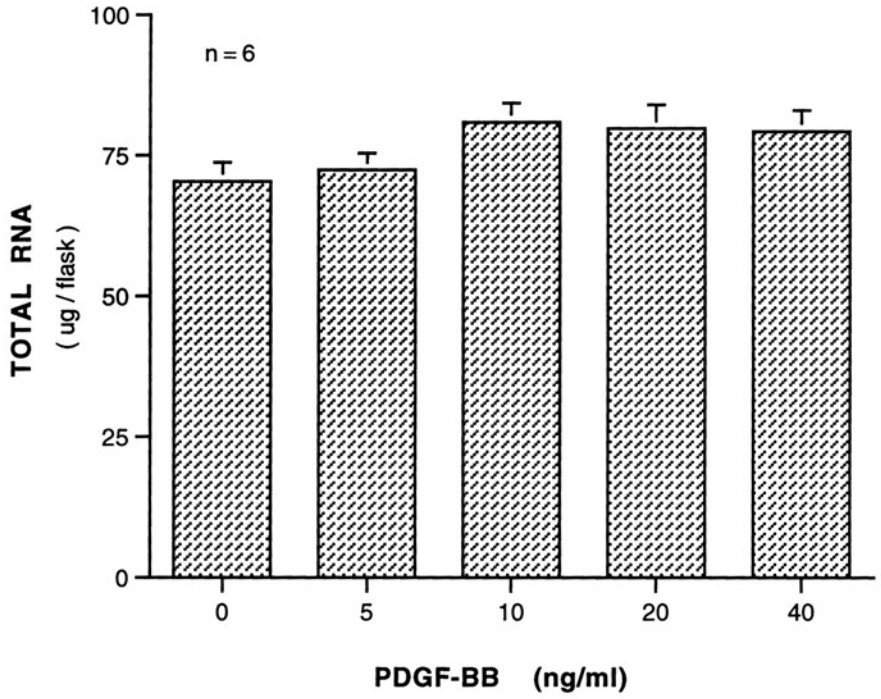


Figure 15. Total RNA levels in response to PDGF-BB in dose-response experiments. Fibroblasts isolated from rat thoracic aorta were treated with 5, 10, 20 and 40 ng/ml PDGF-BB respectively. Total RNA was extracted 6 hours after PDGF-BB treatment. n = 6. $P > 0.05$ vs. control.

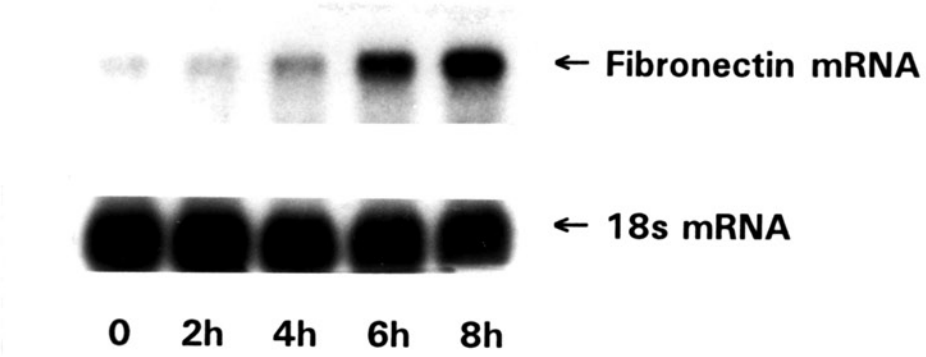


Figure 16. Northern blot of fibronectin mRNA levels in response to IGF-I in time-response experiments. Total RNA (15 µg/lane), from fibroblasts treated with IGF-I (20 ng/ml) for 2, 4, 6 and 8 hours respectively, was probed with cDNA for fibronectin mRNA (top). Same blot was stripped and reprobed with cDNA for 18s mRNA (bottom).

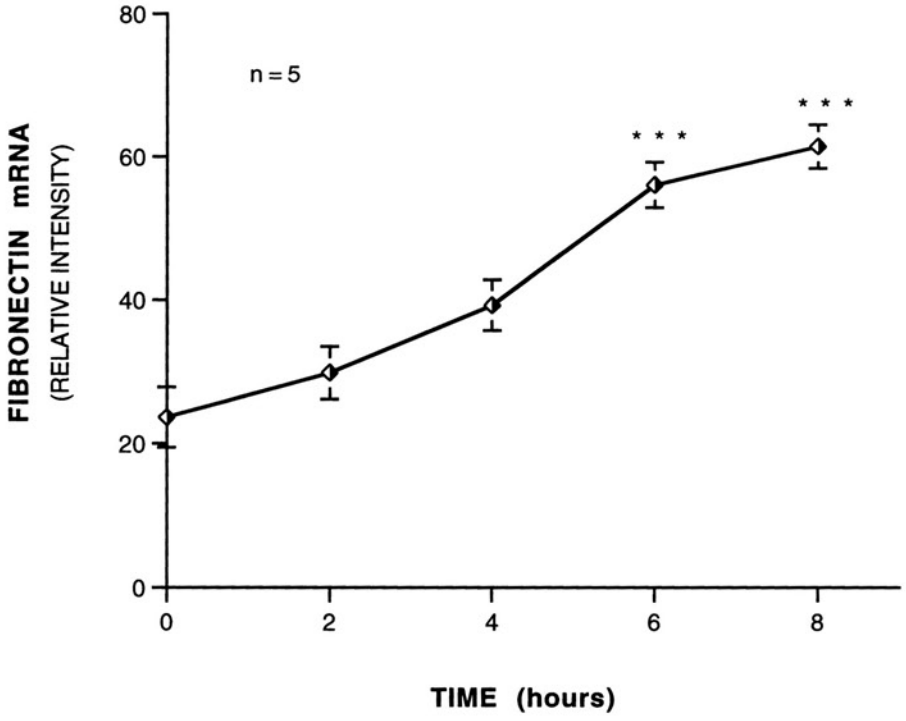


Figure 17. Fibronectin mRNA levels in response to IGF-I in time-response experiments. Fibroblasts isolated from rat thoracic aorta were treated with 20 ng/ml IGF-I. Total RNA was extracted at 2, 4, 6 and 8 hours after IGF-I addition. n = 5. *** $P < 0.001$ vs. untreated control.

fold above the normal values (Fig. 17).

No difference was found in total RNA levels at different time points after IGF-I addition ($P > 0.05$ vs. untreated control) (Fig. 18).

Interaction Between PDGF-BB and IGF-I on Stimulating Fibronectin mRNA Levels

The above results demonstrate that either PDGF-BB or IGF-I was able to stimulate fibronectin mRNA expression individually in vascular fibroblasts. To further determine whether PDGF-BB could act additively or synergistically with IGF-I, the interaction between PDGF-BB and IGF-I was examined by exposure of cell cultures for 6 hours to PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) simultaneously. The results show a synergistic increase (276%) in fibronectin mRNA levels in cells exposed to both PDGF-BB and IGF-I (Fig. 19, 20). There was an 85% increase of fibronectin mRNA levels in fibroblasts treated with PDGF-BB (20 ng/ml) and a 93% increase in cells treated with IGF-I (20 ng/ml).

No difference was found in total RNA levels between growth factor treated and untreated samples ($P > 0.05$) (Fig. 21).

The Effect of Cycloheximide and Actinomycin D on Fibronectin mRNA Synthesis

To elucidate the possible mechanisms underlying the fibronectin mRNA response to PDGF-BB, PDGF-BB (20 ng/ml) was added to cell cultures in the presence of cycloheximide (a final concentration of 36 mM/ml), a protein synthesis inhibitor, and actinomycin D (a final concentration of 40 mM/ml), a transcription inhibitor. Cycloheximide resulted in a greater increase in fibronectin mRNA levels (Fig. 22).

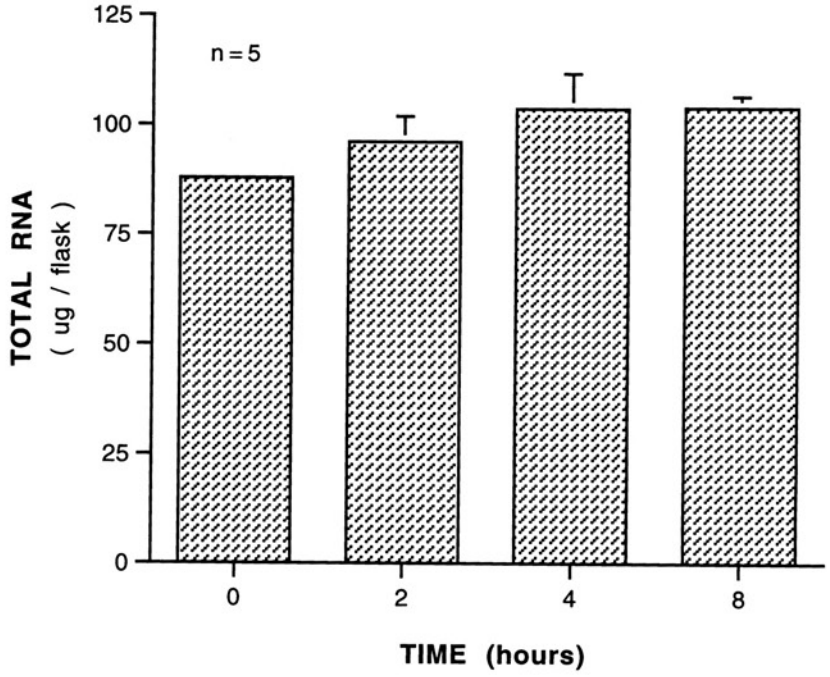


Figure 18. Total RNA levels in response to IGF-I in time-response experiments. The fibroblasts isolated from rat thoracic aorta were treated with 20 ng/ml IGF-I. Total RNA was extracted at 2, 4, 6 and 8 hours after IGF-I addition. n = 5. $P > 0.05$ vs. untreated control.

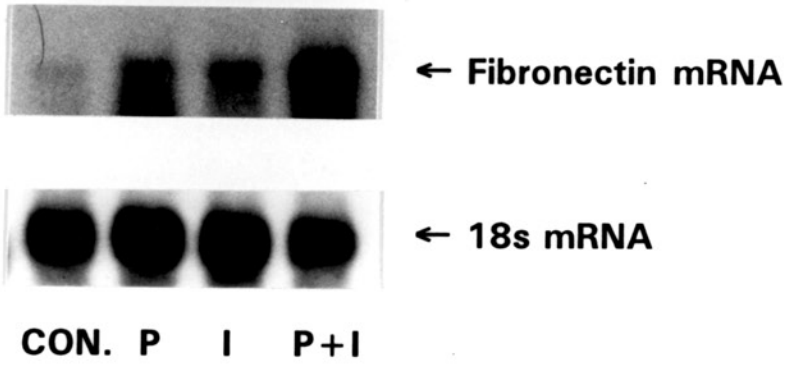


Figure 19. Northern blot of fibronectin mRNA levels in response to PDGF-BB and IGF-I in fibroblasts isolated from rat thoracic aorta. Total RNA (15 μg/lane), from fibroblasts treated for 6 hours with either PDGF-BB (20 ng/ml) (P) or IGF-I (20 ng/ml) (I), or both together (20 ng/ml each) (P+I), was probed with cDNA for fibronectin mRNA (top). Same blot was stripped and reprobed with cDNA for 18s mRNA (bottom). CON., control.

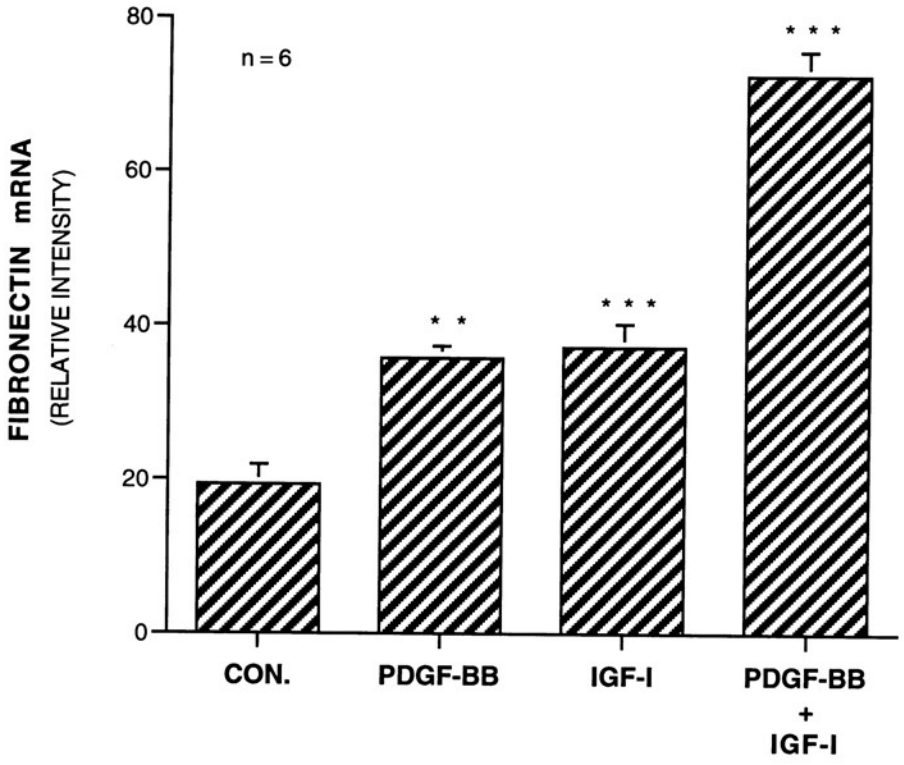


Figure 20. Fibronectin mRNA levels in response to PDGF-BB and IGF-I. Fibroblasts were treated with PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) respectively, or treated with PDGF-BB and IGF-I (20 ng/ml each) simultaneously. Fibronectin mRNA levels were estimated by northern blot analysis at 6 hours after growth factor addition. n = 6. ** $P < 0.01$ vs. untreated control. *** $P < 0.001$ vs. untreated control. CON., control.

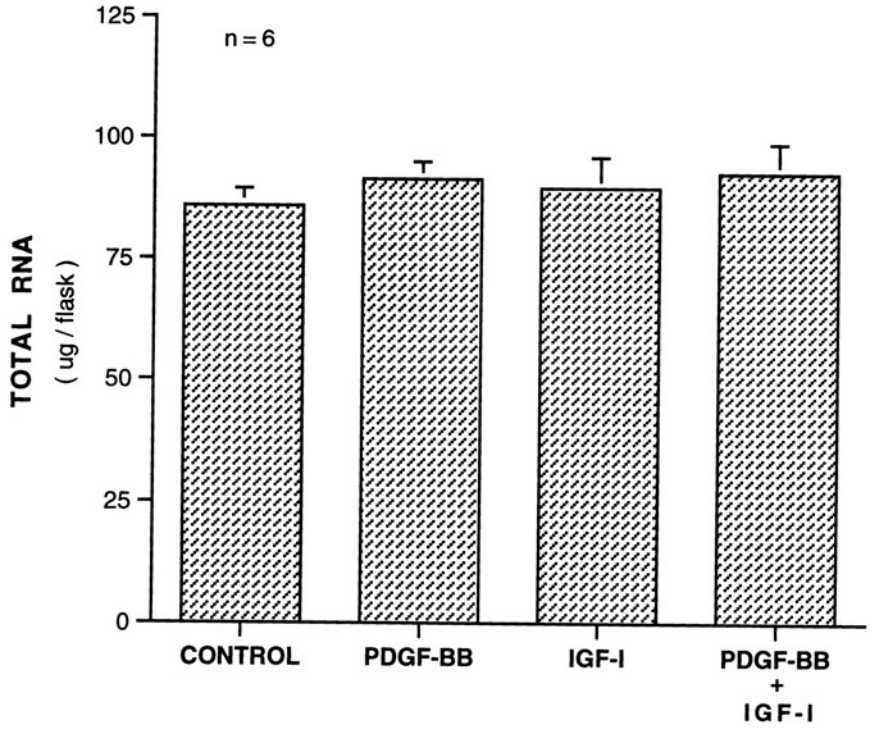


Figure 21. Total RNA levels in response to PDGF-BB and IGF-I. Vascular fibroblasts were exposed to PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) respectively, or exposed to PDGF-BB and IGF-I simultaneously. Total RNA was extracted at 6 hour after growth factor addition. n = 6. $P > 0.05$ vs. untreated control.

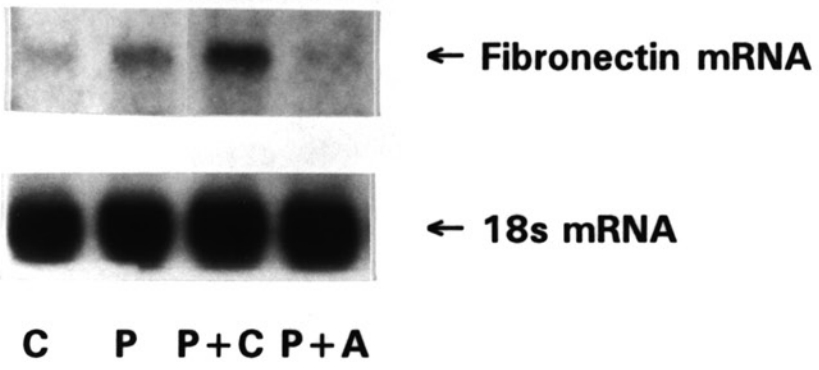


Figure 22. Northern blot of the effects of cycloheximide and actinomycin D on fibronectin mRNA expression. Total RNA (15 μg/lane), from fibroblasts treated with PDGF-BB (20 ng/ml) only (P), PDGF-BB plus cycloheximide (36 mM/ml) (P+C), and PDGF-BB plus actinomycin D (40 mM/ml) (P+A), was probed with cDNA for fibronectin mRNA (top). Same blot was stripped and reprobed with cDNA for 18s mRNA (bottom). C, untreated control.

Actinomycin D blocked the increase of fibronectin mRNA levels induced by PDGF-BB (Fig. 22).

Fibronectin Standard Serial Dilution

A serial fibronectin standard (rat serum fibronectin) dilution, from 80 ng/well to 2.5 ng/ml, was made to obtain optimal fibronectin concentration and anti-fibronectin antibody concentration applied to slot blotting (top, Fig. 23). A concentration-intensity curve was drawn according to the relative intensity of fibronectin standard obtained from slot blotting (bottom, Fig. 23). The relative intensity of fibronectin increased rapidly at concentrations of 2.5 to 20 ng/well. The curve started reaching a plateau at a concentration of 20 to 40 ng/well. Fibronectin standard, 10 ng/well, was applied to every blot.

SDS-PAGE Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis was used to separate fibronectin molecules from other proteins. After western blotting analysis, only one fibronectin band was detected in the blot (Fig. 24) suggesting that (1) the fibronectin antibody was specific to fibronectin. No non-specific binding was detected; (2) slot blotting can be used to examine the fibronectin levels.

PDGF-BB stimulated fibronectin levels can also be demonstrated in the western blot. A significant increase in fibronectin levels after PDGF-BB (20 ng/ml) treatment was detected in both cell culture medium and cell samples (Fig. 24).

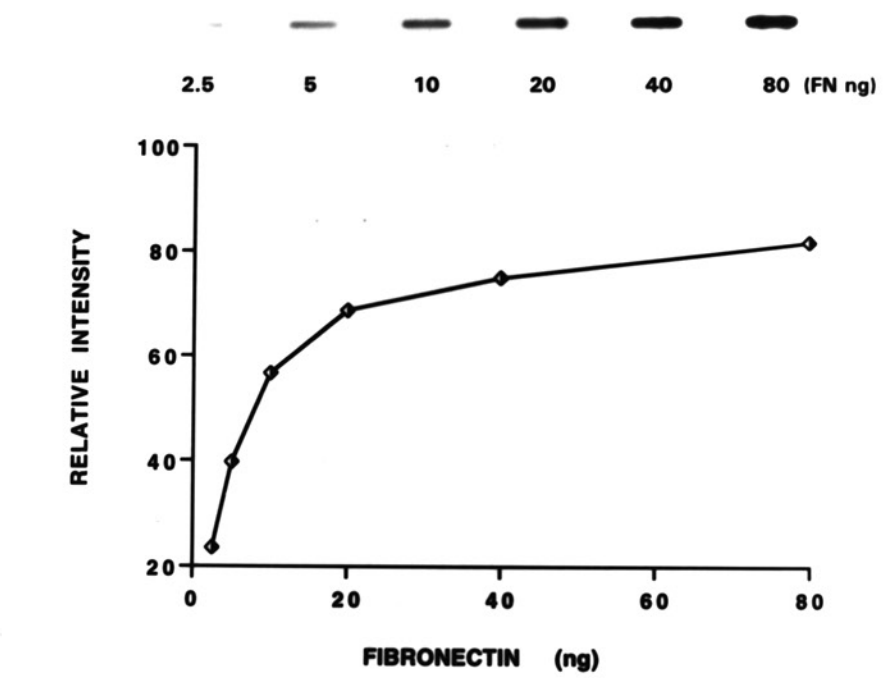


Figure 23. Slot blot of serial diluted rat serum fibronectin (top). Bottom panel is the concentration-intensity curve of rat serum fibronectin drawn according to the relative intensity of fibronectin obtained from slot blotting. FN, fibronectin.

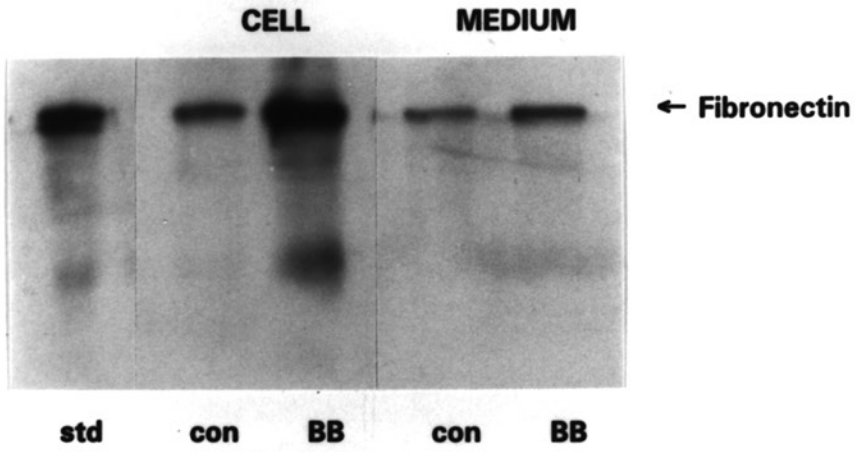


Figure 24. Western blot of fibronectin produced by fibroblasts isolated from rat thoracic aorta. Total protein extracted from fibroblasts was separated by SDS-PAGE gel electrophoresis and was probed by anti-fibronectin antibody. std, fibronectin standard; con, control; BB, PDGF-BB (20 ng/ml) treated.

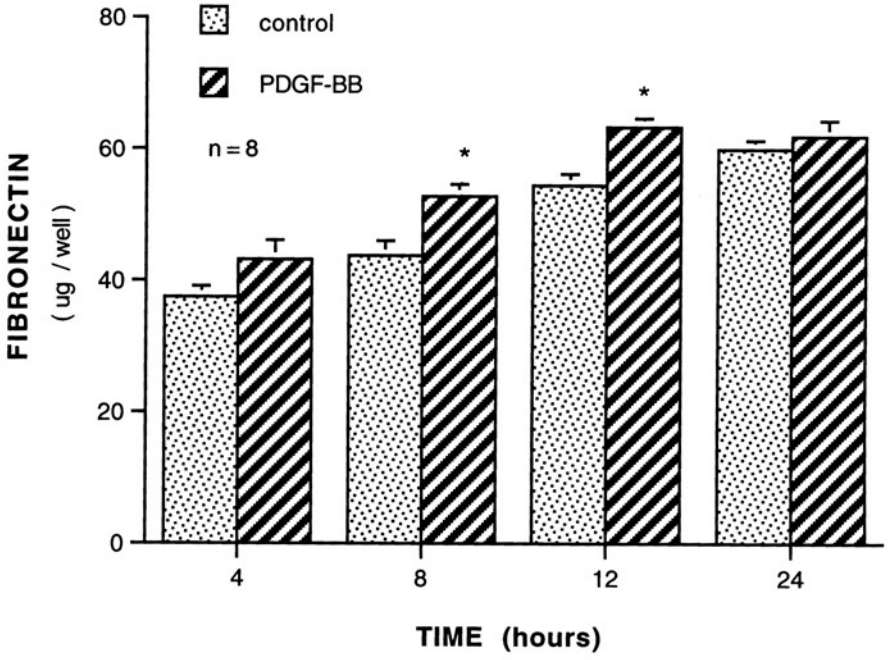


Figure 25. Intracellular fibronectin levels in response to PDGF-BB in time-response experiments. Fibroblasts were treated with 20 ng/ml PDGF-BB for 4, 8, 12 and 24 hours respectively. Fibronectin levels were assessed by slot blotting and western blotting. n = 8. * $P < 0.05$ vs. untreated control.

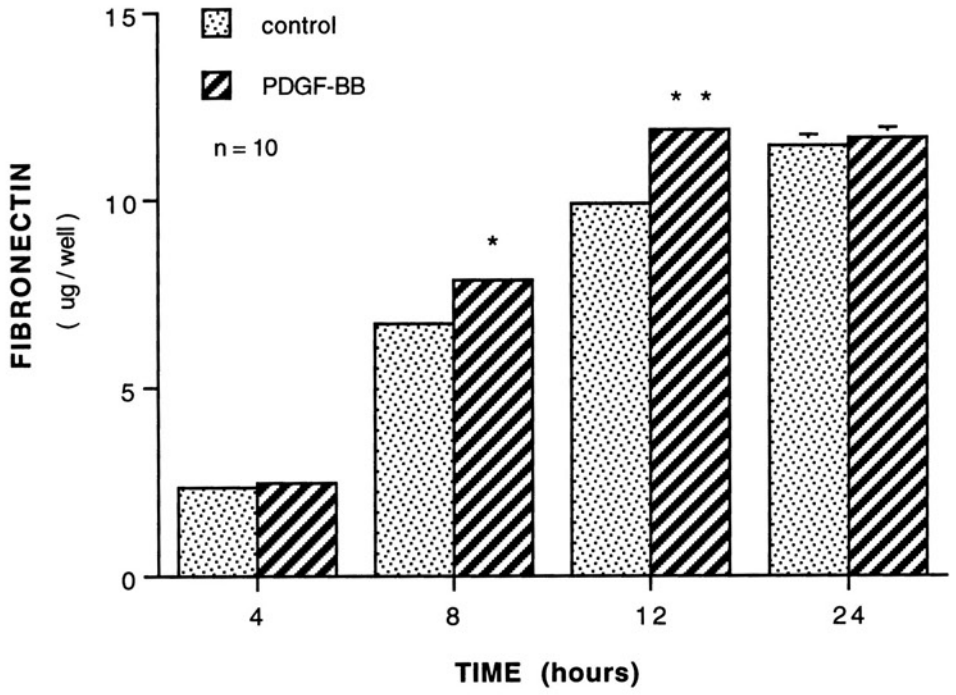


Figure 26. Medium fibronectin levels in response to PDGF-BB in time-response experiments. Fibroblasts were treated with PDGF-BB (20 ng/ml) for 4, 8, 12 and 24 hours respectively. Fibronectin levels were assessed by slot blotting and western blotting. n = 10. * $P < 0.05$ vs. untreated control. ** $P < 0.01$ vs. untreated control.

Fibronectin Response to PDGF-BB

Fibronectin time-response experiments. PDGF-BB stimulated fibronectin levels in both the intracellular compartment and the cell culture medium (Fig. 25, 26). Cell cultures were treated with PDGF-BB at a final concentration of 20 ng/ml and fibronectin levels in the cell and in the cell culture medium were evaluated at 4, 8, 12 and 24 hours after PDGF-BB addition by slot blotting and western blotting. Significant increase of cellular fibronectin levels (21% higher than that of untreated control) was detected at 8 hours and lasted to 12 hours after PDGF-BB treatment (Fig. 25). In the cell culture medium, a maximal increase of fibronectin levels (20% higher than that of untreated control) occurred at 12 hours after PDGF-BB addition (Fig. 26). The fibronectin response to PDGF-BB also was fast and transient. No difference in fibronectin levels between treated and untreated samples was detected at 24 hours after PDGF-BB treatment.

The total protein levels in the PDGF-BB treated group increased at 24 hours after PDGF-BB addition. There was no significant increase in total protein levels between treated and untreated groups at any time point (Fig. 27).

Fibronectin dose-response experiments. Dose-dependent increases in fibronectin levels in responding to PDGF-BB were demonstrated in both the intracellular compartment and the cell culture medium (Fig. 28, 29). Cell cultures were treated with 5, 10, 20 and 40 ng/ml PDGF-BB. Fibronectin levels in the cell and cell culture medium were assessed at 12 hours after PDGF-BB addition. Significant increases in fibronectin levels were detected after exposed cells to 10 ng/ml PDGF-BB in the cell (Fig. 28) and to 20 ng/ml PDGF-BB in cell culture medium (Fig. 29). After 20 ng/ml PDGF-BB

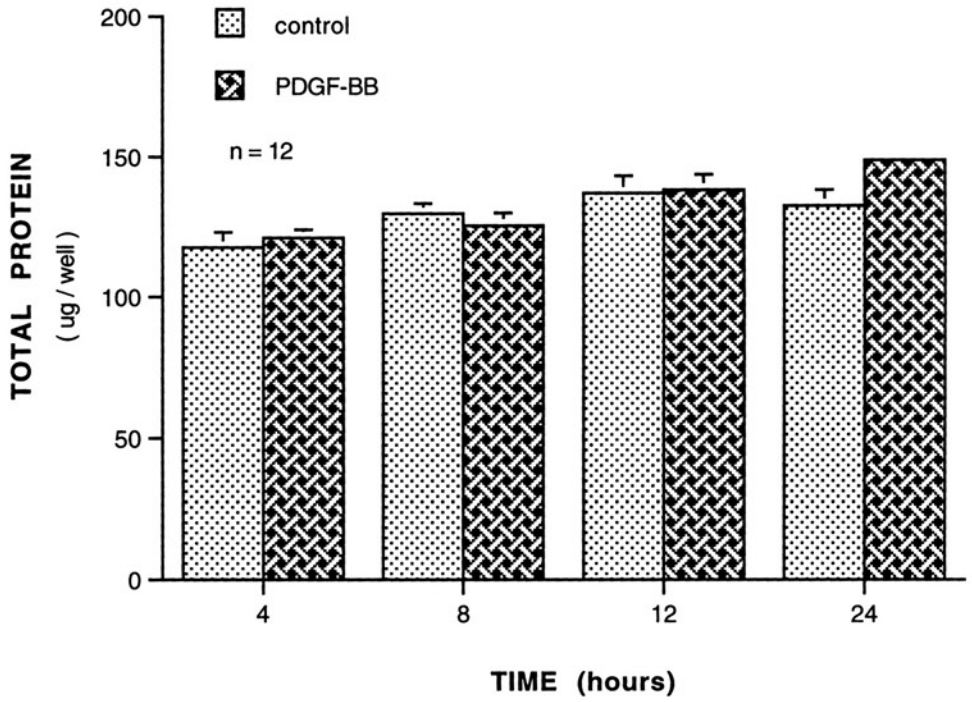


Figure 27. Total protein levels in response to PDGF-BB in time-response experiments. Fibroblasts were treated with PDGF-BB (20 ng/ml) for 4, 8, 12 and 24 hours respectively. Total protein was measured by Lowry Assay. n = 12.

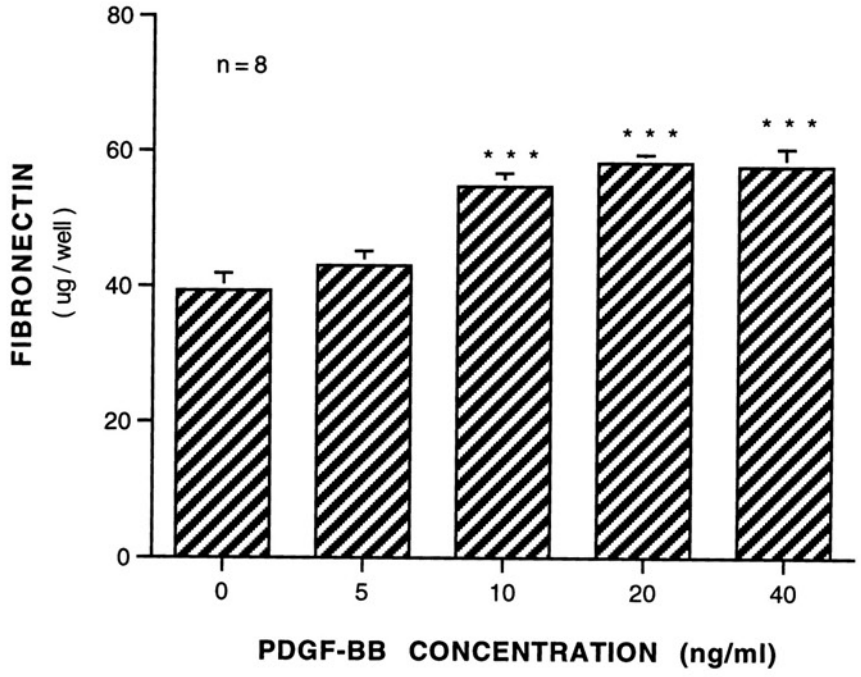


Figure 28. Intracellular fibronectin levels in response to PDGF-BB in dose-response experiments. Fibroblasts were incubated with PDGF-BB at concentrations of 5, 10, 20 and 40 ng/ml for 12 hours. Fibronectin levels were assessed by slot blotting and western blotting. n = 8. *** $P < 0.001$ vs. untreated control.

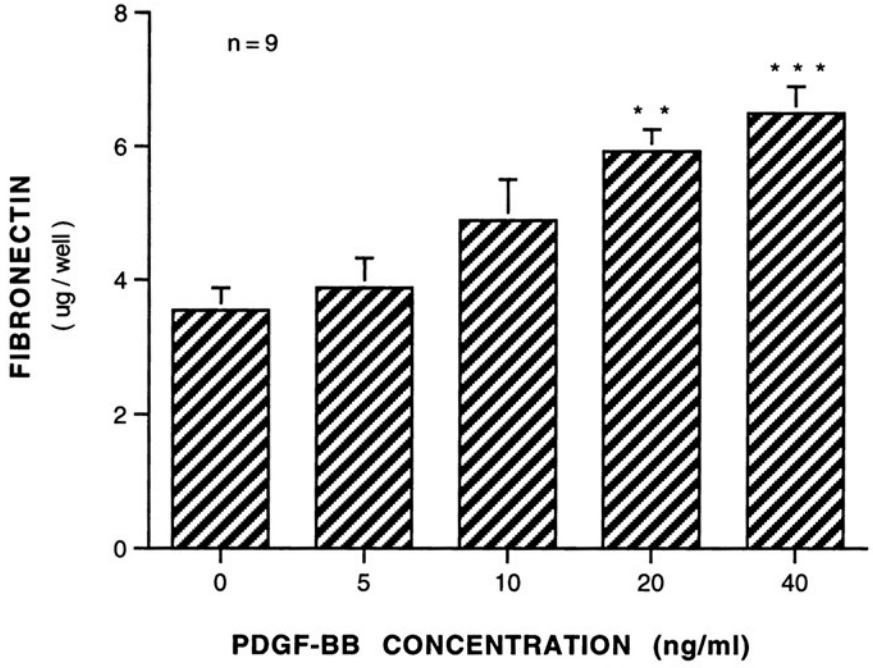


Figure 29. Medium fibronectin levels in response to PDGF-BB in dose-response experiments. Fibroblasts were incubated with PDGF-BB at concentrations of 5, 10, 20 and 40 ng/ml for 12 hours. Fibronectin levels were assessed by slot blotting and western blotting. n = 9. ** $P < 0.01$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

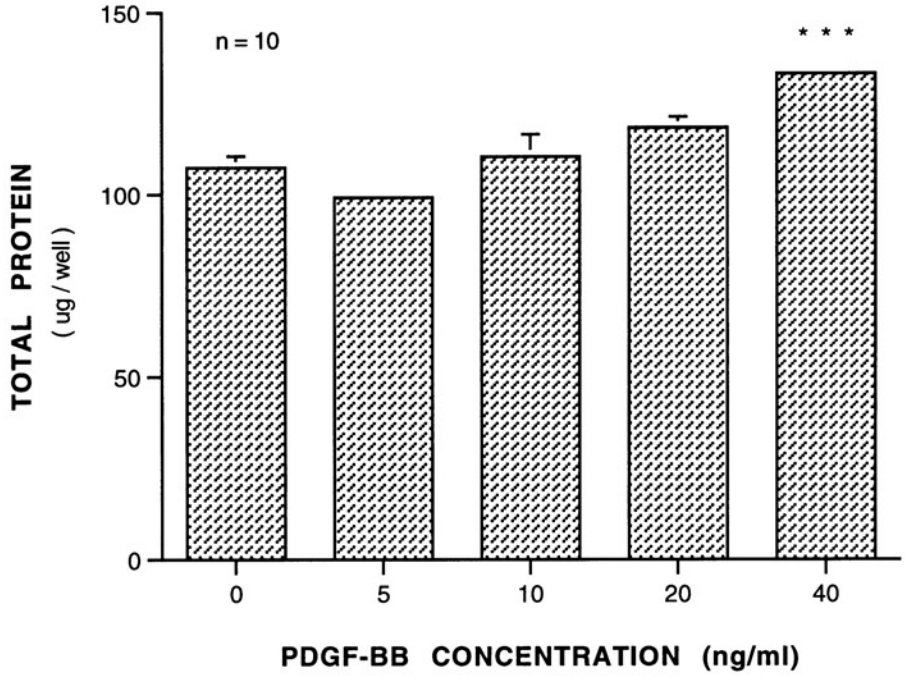


Figure 30. Total protein levels in response to PDGF-BB in dose-response experiments. Fibroblasts were incubated with IGF-I at concentrations of 5, 10, 20 and 40 ng/ml for 12 hours. Total protein was measured by Lowry Assay. n = 10. *** $P < 0.001$ vs. untreated control.

addition, intracellular and extracellular fibronectin levels increased 48% and 67%, respectively.

Total protein levels obtained from cells exposed to 40 ng/ml PDGF-BB were higher than that of untreated control group (Fig. 30).

Fibronectin Response to IGF-I

Fibronectin time-response experiments. Cell cultures were incubated with 20 ng/ml (final concentration) IGF-I for 4, 8, 12 and 24 hours respectively. Fibronectin levels in the cells and culture medium were evaluated separately. IGF-I induced both intracellular fibronectin levels and fibronectin secretion into the cell culture medium. Increases in fibronectin levels were only detected at 8 hours after IGF-I addition in the fibroblasts (Fig. 31) and 12 hours in the cell culture medium (Fig. 32). IGF-I produced a 25% and 28% increase in fibronectin levels in the cells and cell culture medium, respectively.

At 12 and 24 hours after IGF-I addition, total protein levels in fibroblasts increased in both treated and untreated groups (Fig. 33). However, no significant increase was found between the treated and untreated group at the same time points.

Fibronectin dose-response experiments. Increased fibronectin levels response to IGF-I was dose dependent (Fig. 34, 35). Fibroblasts were incubated with 5, 10, 20 and 40 ng/ml IGF-I (final concentration), respectively, for 12 hours. Fibronectin levels were assessed by slot blotting. IGF-I at a concentration of 10 ng/ml induced a significant increase in both intracellular and extracellular fibronectin. Increasing IGF-I concentration

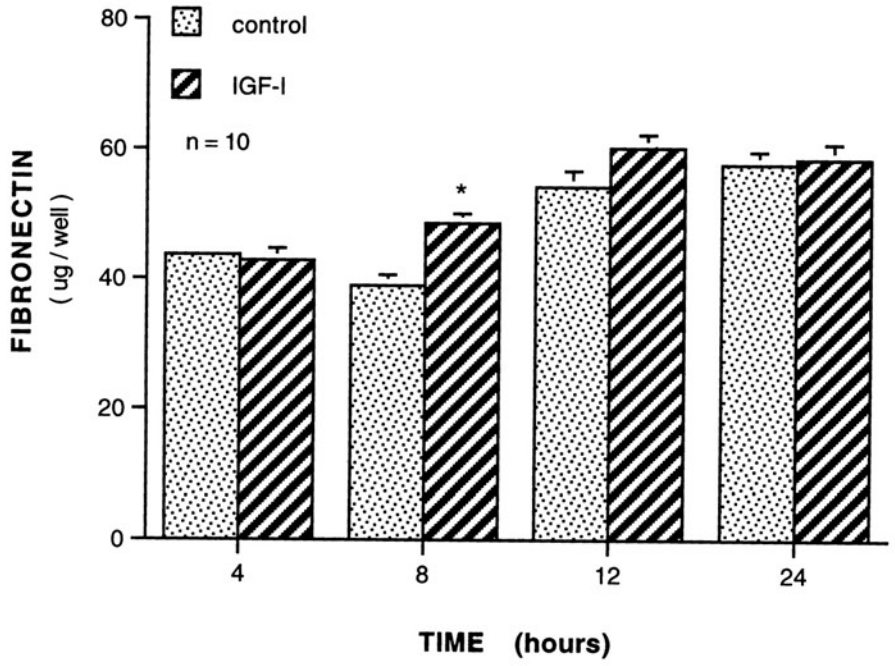


Figure 31. Intracellular fibronectin levels in response to IGF-I in time-response experiments. Fibroblasts were exposed to IGF-I (20 ng/ml) for 4, 8, 12 and 24 hours respectively. Fibronectin levels were assessed by slot blotting and western blotting. n = 10. * $P < 0.05$ vs. untreated control.

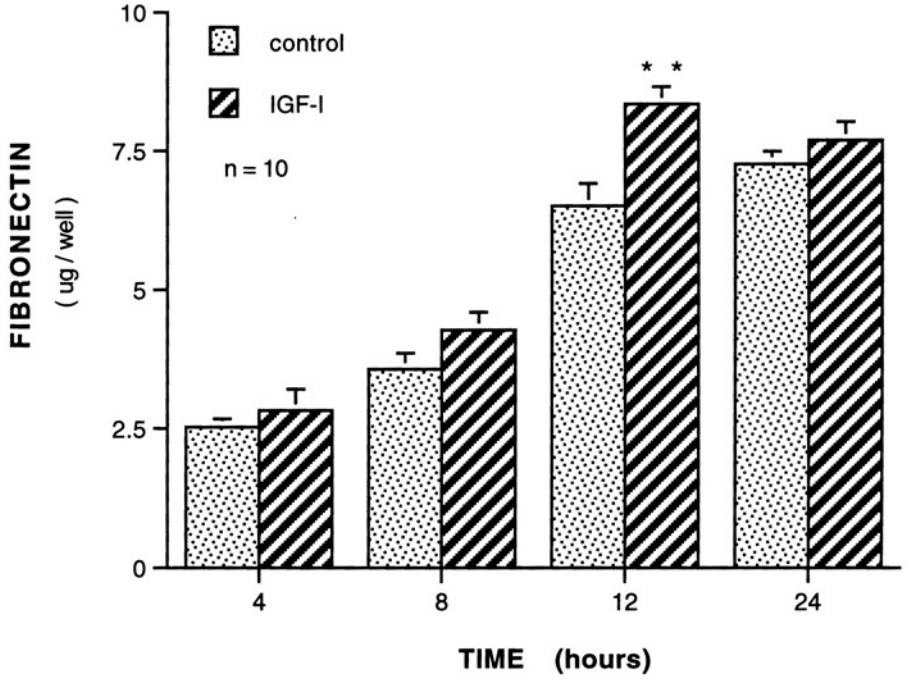


Figure 32. Medium fibronectin levels in response to IGF-I in time-response experiments. Fibroblasts were exposed to IGF-I (20 ng/ml) for 4, 8, 12 and 24 hours respectively. Fibronectin levels were evaluated by slot blotting and western blotting. n = 10. ** $P < 0.01$ vs. untreated control.

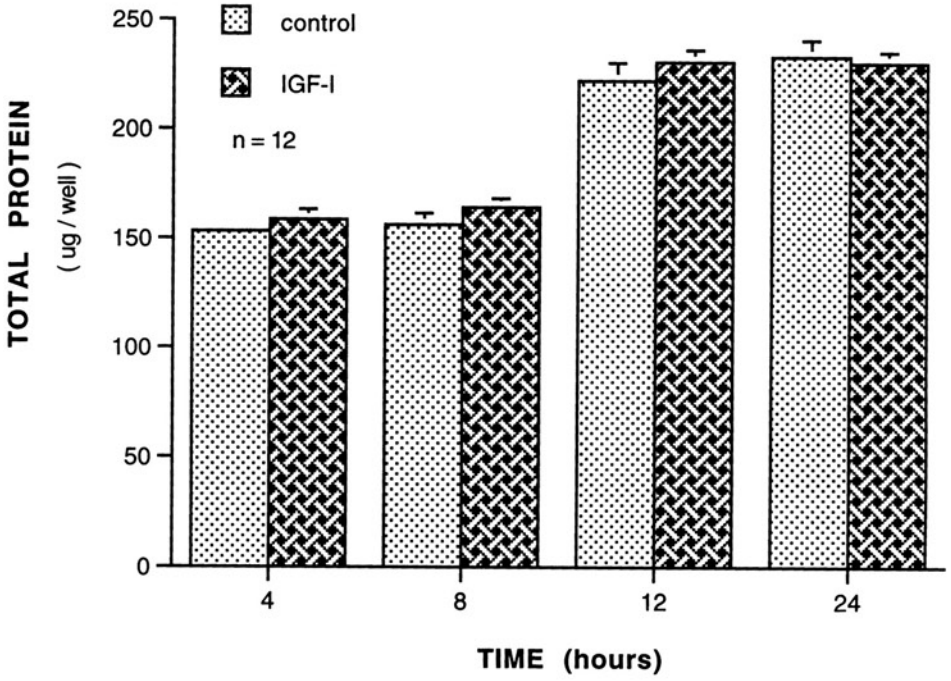


Figure 33. Total protein levels in response to IGF-I in time-response experiments. Fibroblasts were exposed to IGF-I (20 ng/ml) for 4, 8, 12 and 24 hours respectively. Total protein was measured by Lowry Assay. n = 12.

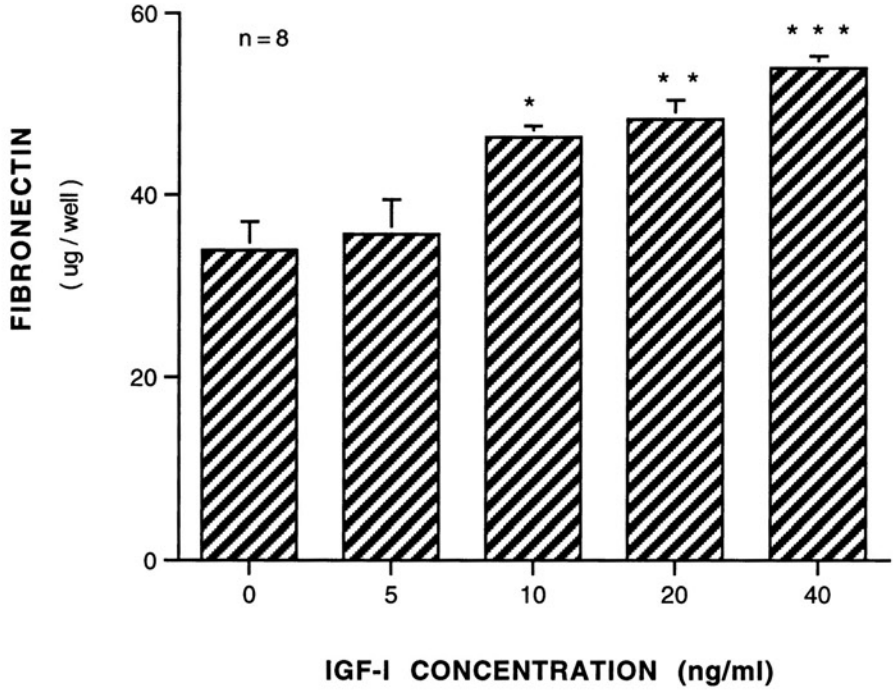


Figure 34. Intracellular fibronectin levels in response to IGF-I in dose-response experiments. Fibroblasts were treated with IGF-I at a final concentration of 5, 10, 20 and 40 ng/ml for 12 hours. Fibronectin levels were assessed by slot blotting and western blotting. n = 8. * $P < 0.05$ vs. untreated control. ** $P < 0.01$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

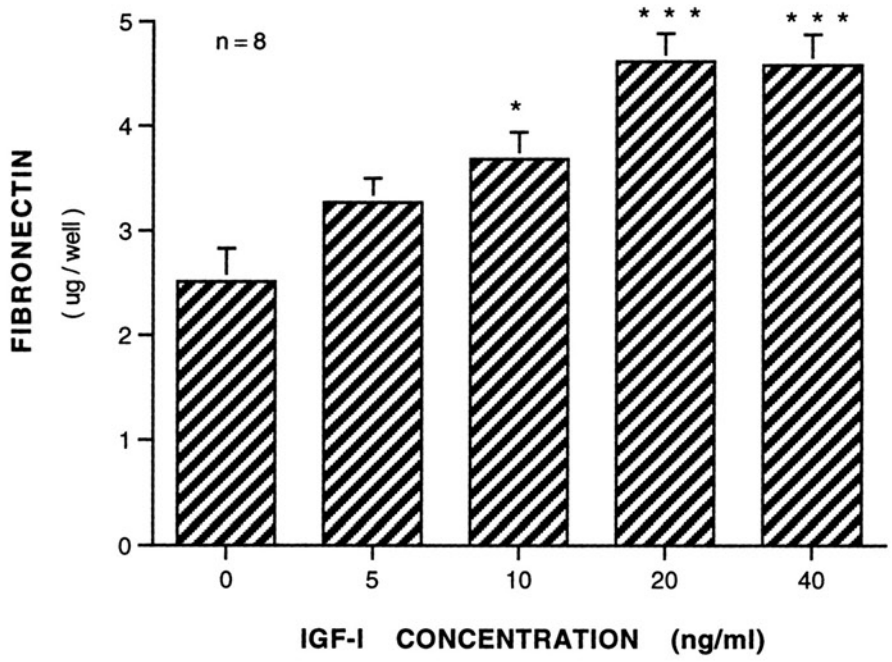


Figure 35. Medium fibronectin levels in response to IGF-I in dose-response experiments. Fibroblasts were treated with IGF-I at a final concentration of 5, 10, 20 and 40 ng/ml for 12 hours. Fibronectin levels were assessed by slot blotting and western blotting. n = 8. * $P < 0.05$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

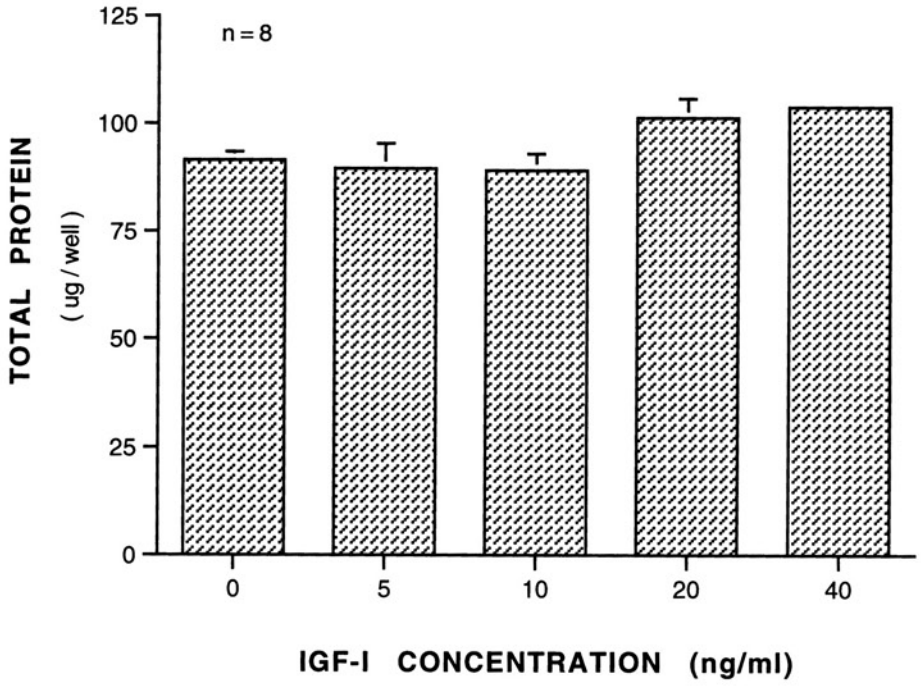


Figure 36. Total protein levels in response to IGF-I in dose-response experiments. Fibroblasts were treated with IGF-I at a final concentration of 5, 10, 20 and 40 ng/ml for 12 hours. Total protein was measured by Lowry Assay. n = 8.

to 40 ng/ml, produced a 58% increase in fibronectin levels in the cells (Fig. 34) and a 80% increase in the cell culture medium compared with the untreated control (Fig. 35).

In fibronectin dose-response experiments, no significant variation of total protein levels of samples was found after treating cell cultures with different IGF-I concentrations (Fig. 36).

Interaction Between PDGF-BB and IGF-I in Stimulating Fibronectin Levels

That PDGF-BB acted synergistically with IGF-I in stimulating fibronectin levels in both the intracellular compartment and cell culture medium (Fig. 37, 38). Cell cultures exposed to PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) simultaneously for 12 hours produced a 115% increase in intracellular fibronectin levels, while a 30% and 33% increase in fibronectin levels were detected after cells were treated with PDGF-BB (20 ng/ml) or IGF-I (20 ng/ml), respectively (Fig. 37). In the cell culture medium, similar results were obtained. There was a 116% increase in medium fibronectin levels after cell cultures were treated with both PDGF-BB and IGF-I (20 ng/ml each) simultaneously. Cell cultures treated with PDGF-BB (20 ng/ml) or IGF-I (20 ng/ml) respectively, increased fibronectin levels by 26% and 29% (Fig. 38).

No difference in total protein levels between both growth factor treated and untreated groups was detected ($P > 0.05$) (Fig. 39).

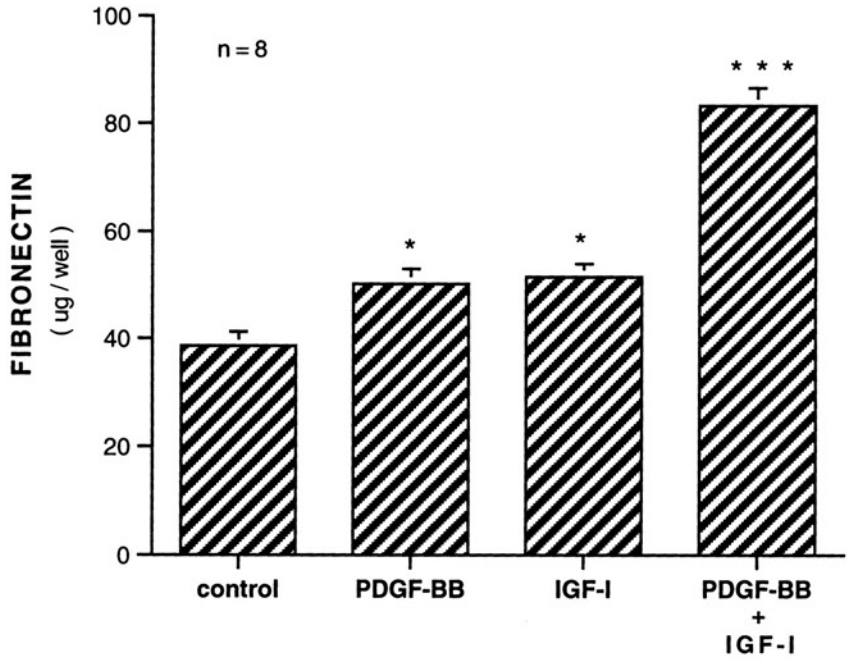


Figure 37. Intracellular fibronectin levels in response to PDGF-BB and IGF-I. Fibroblasts were exposed to PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) respectively, or exposed to PDGF-BB and IGF-I (20 ng/ml each) simultaneously. Fibronectin levels were assessed by slot blotting and western blotting at 12 hours after growth factor addition. n = 8. * $P < 0.05$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

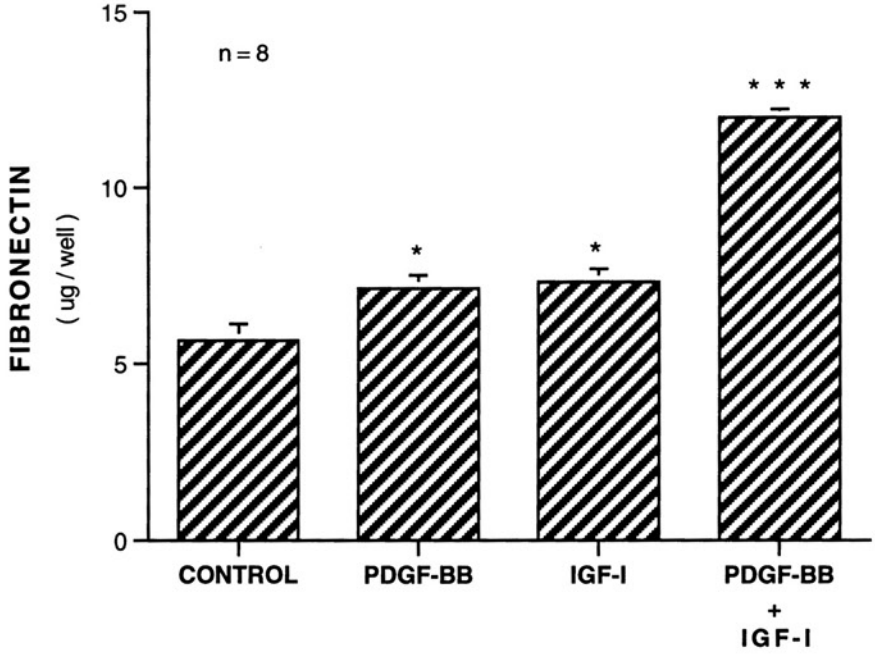


Figure 38. Medium fibronectin levels in response to PDGF-BB and IGF-I. Fibroblasts were exposed to PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) respectively, or exposed to PDGF-BB and IGF-I (20 ng/ml each) simultaneously. Fibronectin levels were assessed by slot blotting and western blotting at 12 hours after growth factor addition. n = 8. * $P < 0.05$ vs. untreated control. *** $P < 0.001$ vs. untreated control.

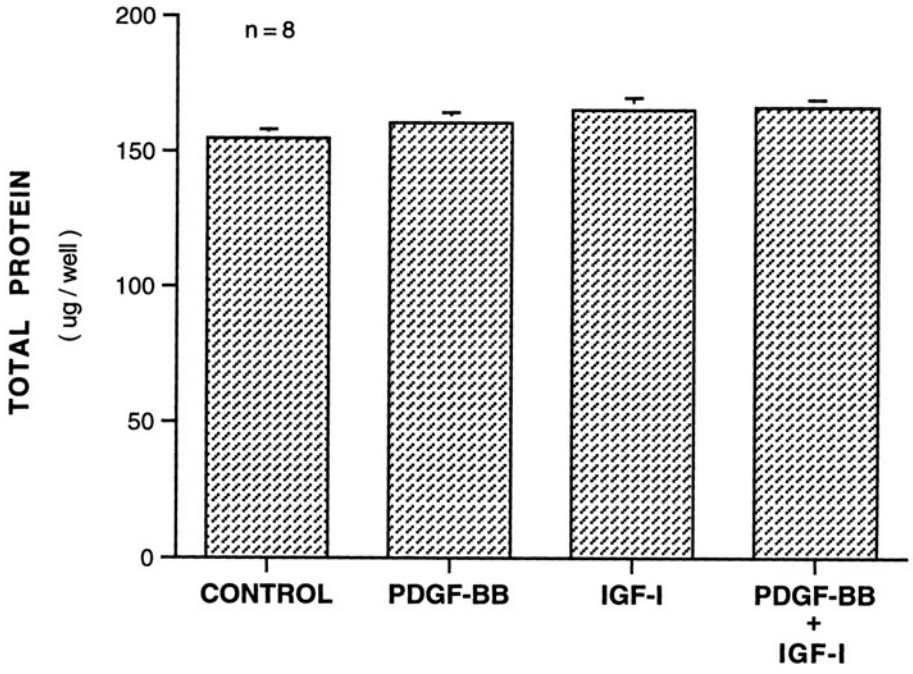


Figure 39. Total protein levels in response to PDGF-BB and IGF-I. Fibroblasts were exposed to PDGF-BB (20 ng/ml) and IGF-I (20 ng/ml) respectively, or exposed to PDGF-BB and IGF-I (20 ng/ml) simultaneously. Total protein levels were measured by Lowry Assay. n = 10.

DISCUSSION

In recent years there have been many reports indicating that changes in the composition of the extracellular matrix have profound effects on cell proliferation and differentiation. Physiological and pathological processes involving cell growth and differentiation, such as wound healing, atherosclerosis and hypertension are also associated with complex patterns of extracellular matrix accumulation (Grinnell 1984). The remarkable parallelism that exists between the types of cellular responses elicited by PDGF and the effects of the extracellular matrix on cell proliferation led to the possibility that a key event in the action of PDGF could be the induction of changes in composition and/or abundance of extracellular matrices. Growth factors regulate fibronectin gene expression have been reported in recent years. Studies by Blatti *et al.* (1988) and Allen-Hoffmann *et al.* (1990) suggested that PDGF stimulates fibronectin gene expression in AKR-2B cells and human fibroblasts. Transforming growth factor-ß (TGF-ß) has been shown to regulate the expression of fibronectin and other components of the extracellular matrix in fibroblasts and aortic endothelial cells (Ignotz and Massague 1986, Madri *et al.* 1988, 1989, Roberts *et al.* 1988, Penttinen *et al.* 1988). In addition to PDGF and TGF-ß, substances shown to influence fibronectin expression in cultured cells include glucocorticoid (Dean *et al.* 1988), cAMP (Dean *et al.* 1988), interleukin-6 (Hagiwara *et al.* 1990), epidermal growth factor (Blatti *et al.* 1988), glucose (Roy *et al.* 1990), heparin (Liau *et al.* 1989), and tumor necrosis factor (Mauviel *et al.* 1988).

The present studies demonstrates that PDGF-BB, one of the isoforms of PDGF-related molecules enhances fibronectin gene expression in cultured rat thoracic aortic fibroblasts. Fibroblasts isolated from rat thoracic aorta were used in these studies because they synthesize a large amount of fibronectin which in turn may stimulate vascular smooth muscle cell proliferation. Since PDGF-BB is the most potent growth factor among the three isoforms (Heldin *et al.* 1988) and PDGF-BB binding sites are most abundant in human fibroblasts (Hart *et al.* 1988), the role of PDGF-BB in fibronectin gene expression in vascular fibroblasts were investigated.

Fibronectin mRNA Response to PDGF-BB

Northern blot analysis demonstrated that PDGF-BB induces a fast and transient fibronectin mRNA increase. Elevated fibronectin mRNA levels in response to PDGF-BB were time and dose-dependent (Fig. 25, 26, 28, 29). The induction of fibronectin mRNA levels by PDGF-BB is specific because 18s RNA levels did not vary significantly after PDGF-BB addition (Fig. 12, 15). In addition, total RNA levels did not change in the time and dose response experiments after PDGF-BB treatment (Fig. 12, 15) suggesting that the increase of fibronectin mRNA levels was a specific response to PDGF-BB, not a generalized effect on total RNA levels (Fig. 12, 15).

To explore the interaction between PDGF-BB and IGF-I on fibronectin gene expression, the effects of IGF-I on fibronectin mRNA levels were investigated. IGF-I produced a 2.5-fold increase of fibronectin mRNA levels at a concentration of 20 ng/ml (Fig. 16, 17). There was no change in total RNA levels at different time points suggesting that the fibronectin mRNA response was specific to IGF-I (Fig. 18).

To elucidate the possible mechanisms underlying the fibronectin mRNA response to PDGF-BB, the effects of actinomycin D and cycloheximide on PDGF-BB induced fibronectin mRNA levels were examined. Inhibition of the PDGF-BB induced fibronectin mRNA levels by actinomycin D (Fig. 22) suggests that PDGF-BB regulates fibronectin mRNA synthesis at the transcriptional level. When fibroblasts were exposed to both cycloheximide and PDGF-BB, fibronectin mRNA levels were enhanced even more than by IGF-I alone (Fig. 22). It is possible that increased fibronectin mRNA levels could be the result of increased mRNA stability, presumably through the inhibition of the synthesis of ribonucleases which degrade the mRNA. It is also possible that cycloheximide could block the synthesis or activity of a short lived negative regulator of fibronectin gene expression. A superinduction of thrombospondin mRNA and an attenuation of PDGF α and ß receptor mRNA by cycloheximide were reported (Majack *et al.* 1987, Eriksson *et al.* 1991).

Effect of PDGF-BB on Fibronectin Levels

Fibronectin biosynthesis is influenced by many factors, but fibronectin mRNA is the major determinant (Hynes 1990). If PDGF and IGF-I enhance the fibronectin mRNA levels, the change in fibronectin mRNA levels should correspond to a change in fibronectin levels. The effects of PDGF-BB and IGF-I on fibronectin levels were examined. PDGF-BB and IGF-I enhance fibronectin levels in the cell samples and in the culture media, respectively. The effects of PDGF-BB and IGF-I on fibronectin levels were also dose-dependent. The peak response of fibronectin mRNA induced by PDGF-BB and IGF-I occured at 6-8 hours after PDGF-BB addition (Fig. 10, 11), and the

maximal fibronectin response to PDGF-BB appeared at 8 hours in the cell extracts and 12 hours in the cell culture medium after PDGF-BB treatment (Fig. 25, 26). The increase of intracellular fibronectin in response to PDGF-BB was about 2 hours later than the increase of fibronectin mRNA levels suggesting that fibronectin synthesis is a fast response. The percent increase in fibronectin mRNA levels (120% increase response to PDGF-BB and 259% increase response to IGF-I, Fig. 11, 17) is much higher than that in fibronectin levels (21% increase response to PDGF-BB in the cell extracts and 20% increase in the cell culture medium, 25% increase response to IGF-I in the cell extracts and 28% increase in the cell culture medium, Fig. 25, 26, 31, 32). Even fibronectin mRNA is one of the major factors which affect fibronectin levels, post-transcriptional modifications at the level of protein synthesis, covalent modification, matrix assembly, or degradation can also influence fibronectin levels. It is possible that while PDGF-BB stimulates fibronectin mRNA levels, it also accelerates other processes which decrease fibronectin levels, such as increased fibronectin degradation, decreased post-transcriptional modification or translation. Increased incorporation of fibronectin into the matrix by TGFß in chick fibroblasts was reported (Ignotz and Massagué 1986).

It is interesting to note that fibronectin levels in the cell extracts and cell culture medium increase with time in fibroblast cultures grown in serum-free medium (Fig. 25, 26, 31, 32) in the absence of PDGF-BB and IGF-I, respectively. The above observations suggest that other factors in the medium, except serum and exogenous PDGF-BB and IGF-I may also influence fibronectin levels. Since fibroblasts are known to produce IGF-I and PDGF-like molecules (Clemmons *et al.* 1981a, 1983, 1985b, Paulsson *et al.* 1987, Raines *et al.* 1989), it is possible that the increase of fibronectin levels in the serum-free

cultures is induced by these endogenous growth factors. Some low molecular nutrients contained in cell culture medium 199, such as amino acids and vitamins, may also influence fibronectin levels by either stimulating synthesis or inhibiting degradation.

Total protein levels in response to PDGF-BB did not varied significantly in the time response experiments. PDGF-BB increase total protein levels in fibroblasts at high concentration (40 ng/ml) (Fig. 30), but the fibronectin increase responded to PDGF-BB was detected at much lower concentration (10 ng/ml in cell extracts and 20 ng/ml in cell culture medium, Fig. 28, 29). No significant increase in total protein levels between the IGF-I treated and the untreated groups was detected in both time and dose-experiments. These observations suggest that the increase of fibronectin levels in response to IGF-I and PDGF-BB (at concentration 20 ng/ml or less) was a specific response to IGF-I and PDGF-BB, not a generalized effect on total protein levels. Increased total protein levels induced by PDGF-BB at high concentration (40 ng/ml or more) may caused by enhanced protein synthesis in the cells or cell proliferation.

Interaction Between PDGF-BB and IGF-I

Since PDGF and IGF-I induce fibronectin mRNA and fibronectin levels in aortic fibroblast cultures respectively, it is important to examine whether these two growth factors act synergistically in enhancing fibronectin mRNA and fibronectin levels. The present studies demonstrate that there was a significant percent increase in fibronectin mRNA (276%, Fig. 19, 20) in the fibroblasts treated with both PDGF-BB and IGF-I than either PDGF-BB (85%) or IGF-I (93%) alone. Similarly, there was a significant percent increase in cellular fibronectin levels (115%, Fig. 37) in the fibroblasts treated with both

PDGF-BB and IGF-I than either PDGF-BB (30%, Fig.37) or IGF-I (33%, Fig. 37) alone. The above observation suggest that PDGF-BB and IGF-I act synergistically in fibronectin gene expression. It is compatible with reports that PDGF acted additively or synergistically with IGF-I. For example, PDGF and IGF-I have been shown to produce a synergistic or additive effect on control of cell growth (Stiles *et al.* 1979, Banskota *et al.* 1989a), induction of DNA synthesis (Pledger *et al.* 1977, 1978) and expression of the protooncogene c-myc, a growth related gene (Banskota *et al.* 1989b).

The mechanisms underlying the interaction of PDGF-BB and IGF-I are not clear. Pfeifle *et al.* (1984) demonstrated that 3 hours transient exposure of smooth muscle cells to PDGF increases IGF-I receptor expression and enhances smooth muscle cell sensitivity to IGF-I. Later, they found that PDGF increases IGF-I binding to the receptor in a dose dependent manner and that PDGF synergises with IGF-I in a smooth muscle cell mitogenesis assay (Pfeifle *et al.* 1987). Bornfeldt *et al.* (1990) have shown that PDGF regulates the expression of the IGF-I as well as that of the IGF-I receptor in cultured vascular smooth muscle cells.

The present studies demonstrated that PDGF-BB stimulated fibronectin mRNA and fibronectin levels in cultured fibroblasts isolated from rat thoracic aorta. The functional significance of altered fibronectin expression in vascular fibroblasts is difficult to assess at present. Fibronectin has been implicated as one of the early response genes that may be rapidly expressed when cells are exposed to certain growth factors that initiate cell cycle changes (Blatti *et al.* 1988). However, despite the multiplicity of functions that have been attributed to fibronectin in both physiological and pathological

processes, it is not possible at present to establish with certainty what role fibronectin has in vascular tissue. The consistent and long-lived increase in aortic fibronectin mRNA levels in aging or hypertensive rats suggest a possible role for fibronectin in mediating the morphological and functional changes that accompany those conditions, but additional studies concerning the localization of fibronectin in aorta and the relative contribution of specific isoforms of fibronectin will be necessary to determine if such a role exists. These studies also suggest that interaction between growth factors and extracellular matrix may play a role in the regulation of cell proliferation and extracellular matrix expansion which occur in atherosclerosis and hypertension.

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