

Role of PDGF-A Expression in the Control of Vascular Smooth Muscle Cell Growth by Transforming Growth Factor- β

Richard A. Majack,* Mark W. Majesky,† and Linda V. Goodman*

*The Upjohn Company, Kalamazoo, Michigan 49001; †Department of Pathology, University of Washington, Seattle, Washington 98195

Abstract. Transforming growth factor- β (TGF- β) is a multifunctional regulatory peptide that can inhibit or promote the proliferation of cultured vascular smooth muscle cells (SMCs), depending on cell density (Majack, R. A. 1987. *J. Cell Biol.* 105:465–471). In this study, we have examined the mechanisms underlying the growth-promoting effects of TGF- β in confluent SMC cultures. In mitogenesis assays using confluent cells, TGF- β was found to potentiate the stimulatory effects of serum, PDGF, and basic fibroblast growth factor (bFGF), and was shown to act individually as a mitogen for SMC. In gene and protein expression experiments, TGF- β was found to regulate the expression of PDGF-A and thrombospondin, two potential mediators of SMC proliferative events. The induction of thrombospondin protein and mRNA was density-

dependent, delayed relative to its induction by PDGF and, based on cycloheximide experiments, appeared to depend on the de novo synthesis of an intermediary protein (probably PDGF-A). The relationship between PDGF-A expression and TGF- β -mediated mitogenesis was investigated, and it was determined that a PDGF-like activity (probably PDGF-A) was the biological mediator of the growth-stimulatory effects of TGF- β on confluent SMC. The effects of purified homodimers of PDGF-A on SMC replication were investigated, and it was determined that PDGF-AA was mitogenic for cultured SMC, particularly when used in combination with other growth factors such as bFGF and PDGF-BB. The data suggest several molecular mechanisms that may account for the ability of TGF- β to promote the growth of confluent SMC in culture.

THE replication of vascular smooth muscle cells (SMC)¹ is tightly controlled in the adult animal and normally occurs only during tissue repair and angiogenesis. Excessive or uncontrolled SMC replication can be an important contributing factor to a number of vascular disease states including arteriosclerosis, hypertension, and the reocclusion of vascular grafts and endarterectomies (7, 34, 38, 47). Much is now known concerning the factors that contribute to SMC growth control, based on studies of SMC replication in vitro. PDGF has been extensively implicated as an important positive regulator of SMC replication (34, 35). At least three forms of PDGF are known to exist (for review, see reference 14): homodimers of the A-chain of PDGF, homodimers of the B-chain of PDGF (products of the *c-sis* oncogene), and A-B heterodimers. The three dimeric forms of PDGF interact selectively with at least two forms of PDGF receptors (12, 13, 14). The specific contributions of the

different forms of PDGF in the control of SMC growth have not been completely elucidated.

Other growth and accessory factors are probably equally important in SMC growth control. Those relevant to this study include basic fibroblast growth factor (bFGF) (for review, see reference 16), thrombospondin (TS) (21–24), and transforming growth factor- β (TGF- β)² (3, 10, 20). TGF- β is of particular interest in the biology of vascular SMC in that it is very abundant in platelets (2, 3), is secreted by vascular endothelial and SMC (1), and can either promote or inhibit the replication of SMC in vitro, depending on culture conditions. When SMC are maintained in two-dimensional culture, TGF- β can be a potent growth inhibitor (3, 20), but only when the cells are sparse (20).

When the cells are confluent (20) or in soft agar (3), TGF- β potentiates SMC growth. A combination of these growth-inhibitory and growth-promoting effects is believed responsible for the establishment and maintenance of the “hill-and-valley” morphology common to SMC in culture (20). In a separate study (10), we have shown that the differential effects of TGF- β on SMC replication may result from an in-

Address correspondence to Dr. R. A. Majack, Synergen, Inc., 1885 33rd Street, Boulder, CO 80301.

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CS, calf serum; FN, fibronectin; SMC, smooth muscle cell; TGF- β , transforming growth factor- β ; TS, thrombospondin.

2. Unless otherwise specified, “TGF- β ” refers to TGF- β ₁ homodimers.

teraction of TGF- β with distinct receptor phenotypes expressed by SMC as a function of cell density. In this study, we describe the mechanisms whereby TGF- β promotes the growth of vascular SMC in confluent culture. We show that TGF- β can facilitate the growth of vascular SMC via the induced expression of PDGF, similar to previously reported data for fibroblasts and AKR-2B cells (18, 43). In addition, we describe some of the growth-promoting effects of PDGF-AA.

Materials and Methods

Cell Culture

Rat aortic SMC were derived from explants as previously described (21). Cells were maintained in culture in 5% calf serum (CS) in Waymouth's medium in the absence of antibiotics, and were used for experiments in the third through seventh passages. Growth-arrested cells were obtained by maintaining cultures in 0.5% CS for 72 h as indicated. All tissue culture reagents were from Gibco Laboratories (Grand Island, NY).

Growth Factors

TGF- β from porcine platelets was purchased from R and D Systems (Minneapolis, MN), as was porcine platelet PDGF (PDGF-BB) and human platelet PDGF (primarily PDGF-AB) (see references 6 and 11). Recombinant human PDGF-AA was purchased from Collaborative Research, Inc. (Waltham, MA). The PDGF used for radioreceptor assays was prepared from outdated human platelets as described (32). Human recombinant basic FGF was prepared by Synergen, Inc. (Boulder, CO).

RNA Preparation

Total RNA from cultured cells was prepared using the SDS-proteinase K method (36). The quantity and purity of the samples were determined by spectrophotometric analysis by reading absorbances at 260 and 280 nm. Cells to be used for RNA preparations were grown in 150-mm diameter dishes and were washed 2–3 \times with cold (4°C) PBS before lysis. Typically, the yield of total RNA for sparse cells (plated at 1×10^3 cells/cm²) was 50 μ g RNA/dish. For confluent cells (plated at 5×10^4 cells/cm²), yields averaged 180 μ g total RNA/dish.

Northern Analysis

For Northern analysis, RNA samples were denatured by glyoxylation and separated by electrophoresis through a 1.3% agarose gel formed in 10 mM phosphate buffer (pH 6.8). The RNA was transferred electrophoretically to Genescreen Plus hybridization membranes (New England Nuclear, Boston, MA) overnight at 4°C at 20 V in tris acetate buffer containing EDTA, then was cross-linked to the filters by exposure to UV for 3 min. The filters were prehybridized for 10–30 min in 0.5 M sodium phosphate (pH 7.2) containing 1 mM EDTA, 1% BSA, and 7% SDS, then were hybridized overnight at 65°C in the same buffer containing the DNA probe. Filters were washed in two changes of 0.1 \times SSC/1% SDS at 65°C, and then were exposed to Kodak XAR-5 film with Dupont Cronex intensifying screens at –70°C. To verify that equal amounts of RNA were loaded in each lane, relative levels of 28 and 18S ribosomal RNA were compared and the blots were probed with control cDNAs.

SDS-PAGE

SMC protein biosynthesis was analyzed by SDS-PAGE, as previously described (21). Briefly, SMC were metabolically labeled for 1 or 2 h with [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) in medium lacking methionine. Radiolabeled proteins from SMC culture supernatants were precipitated in 10% TCA, dissolved in Laemmli sample buffer containing DTT, and resolved on 10% polyacrylamide gels containing SDS. Proteins were visualized by autoradiography at –70°C using Kodak XAR-5 film. Densitometric analysis of protein bands was performed on underexposed films (to insure linear scanning conditions) using a Haake-Buchler scanning densitometer (Joyce-Loebl, Malden, CA). Constitutively expressed internal reference proteins were used as standards.

DNA Probes

cDNA probes were oligo-labeled with [³²P]dCTP using random primers and DNA polymerase (Pharmacia Fine Chemicals, Uppsala, Sweden). Typically, 200–300 ng of purified insert DNA was labeled to a specific activity of 10^8 – 10^9 cpm/ μ g. cDNA probes used included a 1.5-kb Bam HI fragment of a cDNA encoding human endothelial cell plasminogen activator inhibitor, a 600-bp Pst I fragment of a cDNA encoding mouse type I procollagen, a 900-bp Sph I/Bam HI fragment of a cDNA encoding human fibronectin (FN), a 700-bp Pst I fragment of a cDNA encoding mouse β -actin, a 1.2 kb Pst I fragment of a genomic clone encoding the *v-sis* oncogene (PDGF B-chain), a 1.3-kb Eco RI fragment of a cDNA encoding the A chain of human PDGF, and a 1.2 kb Eco RI/Sac I fragment of a cDNA encoding human TS.

Mitogenesis Assays

The mitogenic activity of TGF- β and other growth factors was assessed by determining the uptake of [³H]thymidine in control (growth-arrested) and stimulated cells. Because initial experiments showed a clear difference between the kinetics of S-phase entry in sparse and confluent cells, sparse cells were pulse-labeled for 1 h at 16 h poststimulation (during the peak of S-phase) while confluent cells were pulse-labeled for 1 h at 24 h. Briefly, the cells were labeled with 1 μ Ci/ml [³H]thymidine in serum-free medium for 1 h, washed, and precipitated with 10% TCA. Cells were solubilized in 0.3 N NaOH and the extent of uptake of [³H]thymidine was determined by scintillation counting. All experiments were done in triplicate, and data were normalized to a control value of 100% and presented as mean \pm SEM. To corroborate the thymidine incorporation data, for certain experiments nuclear labeling indices were determined by autoradiography after exposure of the cells to [³H]thymidine over a 32-h period as previously described (22).

PDGF Radioreceptor Assay

Levels of PDGF-like molecules in media conditioned by SMC were estimated by radioreceptor assay as previously described (5). Cultures of human diploid fibroblasts were incubated with increasing amounts of SMC-conditioned medium for 3 h at 4°C, rinsed with cold PBS, and then incubated with ¹²⁵I-labeled PDGF (5 ng/ml) for 1 h at 4°C. The cultures were rinsed three times with cold binding rinse, and bound ¹²⁵I-labeled PDGF was solubilized with 2% Triton X-100. Purified human platelet PDGF, consisting primarily of AB heterodimers (11), was used as the standard. This assay detects PDGF-AA homodimers as well as PDGF-AB heterodimers.

Results

TGF- β Regulates Connective Tissue mRNAs in a Density Independent Fashion

In a previous study (20), we showed that TGF- β exerted a bidirectional effect on SMC growth, depending on cell density. To test the hypothesis that TGF- β may exert these differential effects via a density dependent regulation of connective tissue gene expression (a common cellular response to TGF- β) (15, 30, 33), we determined mRNA levels for several connective tissue genes under conditions favoring TGF- β -mediated growth inhibition (sparse SMC) and growth promotion (confluent SMC). RNA was prepared from sparse proliferating SMC, sparse SMC growth inhibited by TGF- β (10 ng/ml; 72 h), confluent SMC, and confluent SMC exposed to 10 ng/ml TGF- β for 72 h. The RNA was then subjected to Northern analysis as described in Materials and Methods. Steady-state mRNA levels for PAI-1 were identical in control and TGF- β -treated cells, but were consistently higher in sparse cells. Message levels for β -actin were decreased by TGF- β under both culture conditions, while transcripts for fibronectin and type I collagen were induced by TGF- β independent of cell density (data not shown). These

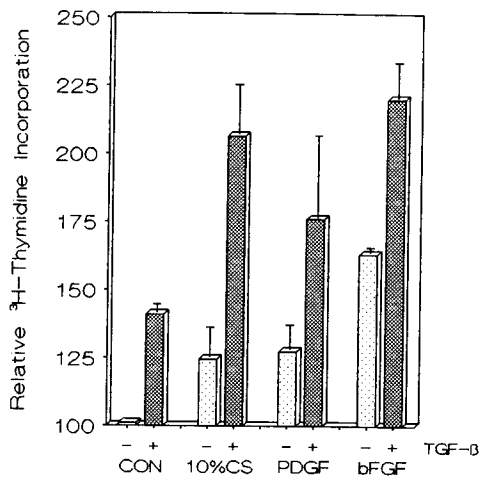


Figure 1. TGF- β potentiates mitogenicity in confluent SMC cultures. Confluent SMC cultures were growth-arrested by serum deprivation (0.5% for 72 h), exposed to the indicated factors for 24 h in the presence (+) or absence (-) of TGF- β , pulse-labeled for 1 h with 1 μ Ci/ml [³H]thymidine, and then precipitated with 10% TCA. The amount of [³H]thymidine incorporated into DNA was determined by scintillation counting, and all data were normalized to a value of 100% for the control (CON) cultures. TGF- β added alone (at 10 ng/ml) elicited a mitogenic response in confluent cultures and, when given in combination with other factors, potentiated the mitogenic responses to 10% CS, porcine platelet PDGF (10 ng/ml), and bFGF (10 ng/ml). In sparse cell cultures, TGF- β inhibited the mitogenic responses to each of these factors at the same concentrations.

results likely reflect a direct effect of TGF- β on the expression of these genes, and suggest that the growth-inhibitory effects of TGF- β in SMC are not causally related to connective tissue production, as recently suggested for another cell type (29).

TGF- β Facilitates Mitogenesis in Confluent, but Not Sparse, SMC Cultures

Sparse and confluent SMC cultures were growth arrested by serum deprivation (0.5% CS for 72 h), and then exposed to serum or purified growth factors for 16 h (sparse cells) or 24 h (confluent cells) before a 1-h pulse labeling with [³H]thymidine (1 μ Ci/ml). As shown in Fig. 1, TGF- β elicited a mitogenic response in quiescent confluent SMC cultures and potentiated the mitogenicity of serum, porcine platelet PDGF (consisting primarily of PDGF-BB), and human basic FGF. The relatively weak stimulation of mitogenesis observed was consistent among experiments and results from the fact that over-confluent, density-arrested cultures were used in these studies. Addition of TGF- β alone to sparse SMC did not elicit a mitogenic effect, and was always inhibitory when added in combination with bovine serum, PDGF from human or porcine platelets, or bFGF (data not shown).

To verify the ability of TGF- β to act to elicit SMC mitogenesis, nuclear labeling indices were determined on sparse and confluent SMC labeled with [³H]thymidine during incubation with or without TGF- β for 32 h. Samples were then processed for autoradiography. Growth-arrested SMC incubated without TGF- β showed relatively high labeling index values of $31.2 \pm 1.6\%$ (possibly due to density-dependent

activation of various autocrine/paracrine activities). Nonetheless, confluent SMC incubated in the presence of TGF- β exhibited a marked increase in the percent of labeled nuclei to $71.9 \pm 3.8\%$ (Fig. 2). In contrast, TGF- β had no stimulatory or inhibitory effect on the basal labeling index (<10%) of sparse, growth-arrested cultures of SMC. TGF- β , or an autocrine factor induced by TGF- β , therefore elicits a mitogenic signal in confluent, but not sparse, SMC cultures.

Thrombospondin Protein and mRNA Are Expressed during TGF- β -mediated Mitogenesis

Our previous studies (21) have shown that SMC express increased amounts of two secreted proteins (sp55 and TS) after mitogenic stimulation by serum or PDGF. Since TS secretion is believed to be essential for SMC replication (23) and may, in fact, exert growth-promoting effects of its own (22, 39, 40), we sought to determine if TS were induced during TGF- β -mediated mitogenesis. In addition, a comparison of the kinetics of TS induction during TGF- β -mediated mitogenesis with that occurring during PDGF-mediated mitogenesis should contribute to our understanding of the mechanisms underlying TGF- β -mediated SMC replication. Growth-arrested SMC cultures were treated with TGF- β (10 ng/ml) for 0–24 h, and then pulsed for 2 h with [³⁵S]methionine. Radiolabeled secreted proteins were analyzed by SDS-PAGE, autoradiography, and quantitative scanning densitometry. Addition of TGF- β to quiescent confluent SMC resulted, within 4 h, in the induction of synthesis and secretion of TS and sp55. Similar experiments were performed using human platelet PDGF, and the data were quantitated using scanning densitometry. As shown in Fig. 3, the TGF- β -mediated induction of TS was delayed 2 h as compared to the PDGF-mediated induction. Furthermore, the effect was longer in duration and of lesser magnitude in the TGF- β -treated cultures. Similar results were observed for sp55 (data not shown). Neither sp55 nor TS protein levels were induced by TGF- β in sparse SMC, at least within the time frame examined.

We next examined the ability of TGF- β to induce TS mRNA levels in quiescent confluent SMC, using Northern analysis of RNA prepared from cells treated for 1–5 h with 10 ng/ml TGF- β . As shown in Fig. 4, TGF- β caused a gradual rise (first apparent at 2–3 h) in TS message levels, reaching threefold induction by 5 h. These data should be interpreted in view of our previous studies (24) that demonstrated a significant (fourfold) induction of TS mRNA in SMC within 30 min of treatment with PDGF. The data demonstrate that, similar to TS protein levels (Fig. 2), the induction of TS mRNA by TGF- β also occurs with a delayed time course relative to its induction by PDGF. TS message levels remained high at 24-, 48-, and 72-h time points in confluent cells only (see Fig. 5). Consistent with our protein data, TS mRNA was not induced by TGF- β under sparse culture conditions at any of the time points examined (0–6-, 24-, 48-, and 72-h) (Fig. 5).

The stimulation of TS mRNA accumulation by TGF- β appeared to be indirect in nature (Fig. 6). Confluent, quiescent SMC were treated with actinomycin D or cycloheximide before a 4-h treatment with TGF- β , cytoplasmic dot blots were prepared, and the blots were hybridized with labeled TS cDNA. In the presence of actinomycin D (an inhibitor of DNA-dependent RNA transcription), the inductive effect of

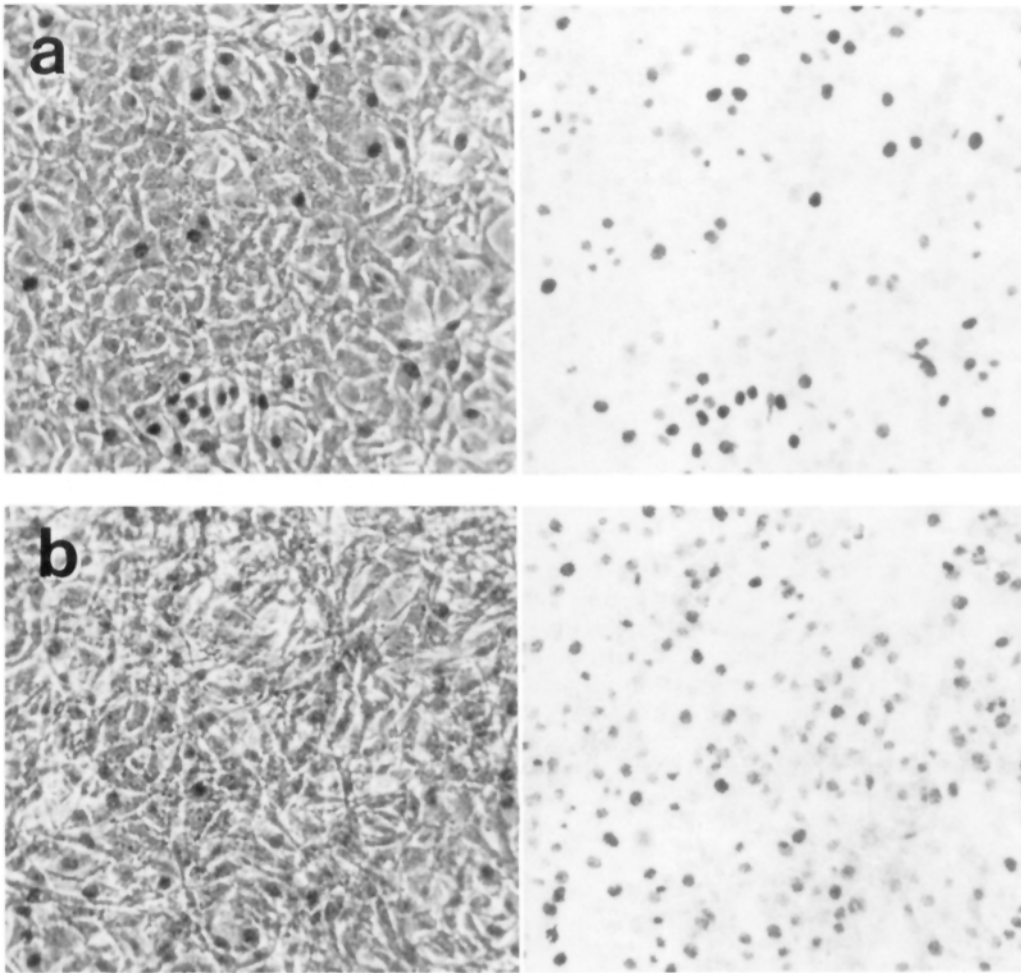


Figure 2. Addition of TGF- β to confluent quiescent SMC results in an increase in the nuclear labeling index. Confluent SMC cultures were growth-arrested by serum deprivation, then were labeled with [3 H]thymidine in the absence (a) or presence (b) of 10 ng/ml TGF- β for 32 h. Cultures were then fixed, coated with emulsion and processed for autoradiography. The percent labeling index for control cells (a) was $31.2 \pm 1.6\%$, compared with $71.9 \pm 3.8\%$ for cells treated with TGF- β (b). Paired photomicrographs of SMC cultures were taken with phase-contrast (left) and bright field optics (right).

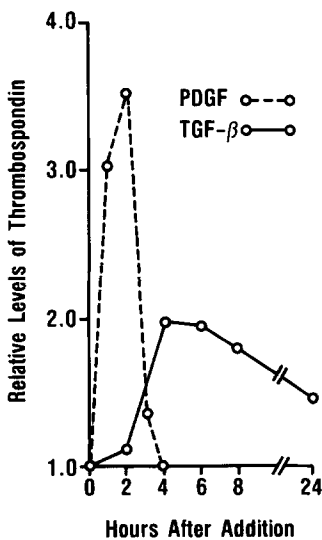


Figure 3. Induced secretion of thrombospondin by TGF- β occurs with delayed kinetics relative to its induction by PDGF. SMC at confluent densities were treated with 10 ng/ml human platelet PDGF or TGF- β for the indicated times and secreted proteins were analyzed as described in Materials and Methods. Autoradiographs were scanned and the relative optical density of TS was determined and normalized to constitutively expressed proteins. Secretion of TS occurs with delayed kinetics in response to TGF- β . In addition, the effect was generally of longer duration and of lesser intensity as compared to that mediated by PDGF.

TGF- β on TS message levels was completely abolished. As previously shown (24), cycloheximide alone exerted a slight inductive effect on TS mRNA (presumably due to stabilization of the TS message). Addition of TGF- β together with cycloheximide did not increase TS mRNA levels above those observed for cycloheximide alone. These data are in contrast to the synergistic effects of PDGF and cycloheximide, that combine to “superinduce” the TS message in similar experiments reported previously (24) and repeated here (Fig. 6). The data suggest that the stimulatory effect of TGF- β on TS message levels requires de novo protein synthesis and is therefore indirect in nature.

TGF- β -treated SMC Express Induced Amounts of PDGF-A mRNA and Secrete Increased Amounts of PDGF-like Protein

Because the induction of TS protein and mRNA by TGF- β was delayed and indirect, and because the pattern of secreted proteins induced during TGF- β -mediated mitogenesis was identical to that induced by PDGF, we examined the possibil-

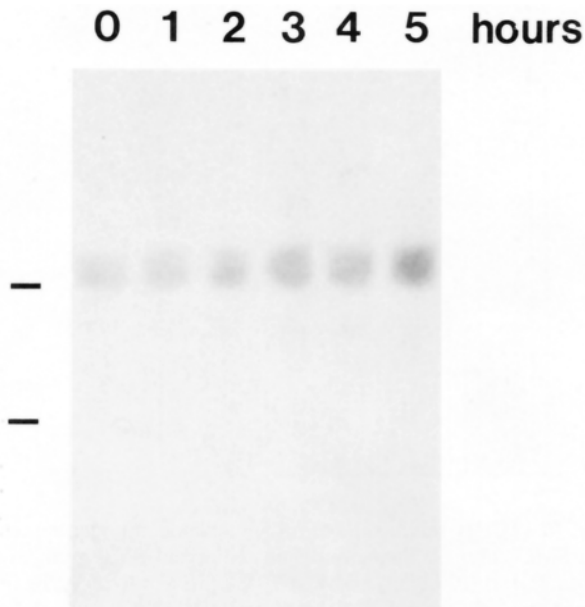


Figure 4. TS mRNA levels are elevated within 3 h after addition of TGF- β to quiescent confluent SMC. SMC at confluent densities were growth-arrested by serum deprivation, then were treated with 10 ng/ml TGF- β for the indicated times. 10 μ g of total RNA per lane was subjected to Northern analysis for thrombospondin mRNA levels. The induction of TS mRNA levels was gradual and was detectable only after 2–3 h of addition of TGF- β to the cultures.

ity that TGF- β regulates the production of PDGF. Sparse and confluent SMC were growth arrested early in G₁ by maintaining the cultures for 72 h in low (0.5%) serum. Total RNA was isolated from SMC treated with 10 ng/ml TGF- β for

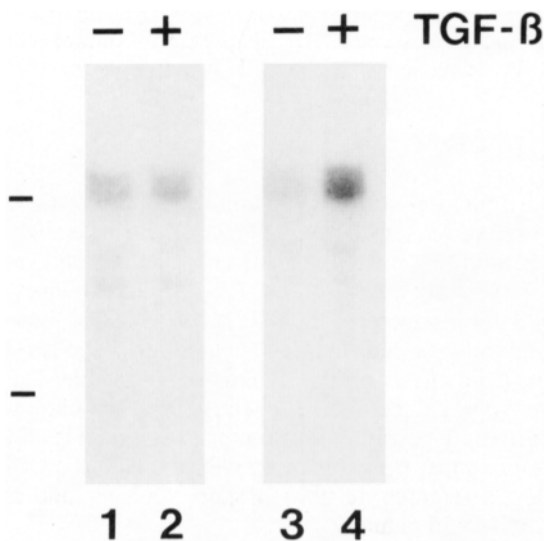


Figure 5. TS mRNA levels are selectively increased in TGF- β -treated confluent cells. SMC at sparse (lanes 1 and 2) and confluent (lanes 3 and 4) densities were left untreated (lanes 1 and 3) or were exposed to 10 ng/ml TGF- β for 72 h (lanes 2 and 4). 10 μ g of total RNA was subjected to Northern analysis for TS message. Messenger mRNA levels for TS are markedly increased in confluent cells stimulated with TGF- β while message levels in sparse inhibited cells are unaffected.

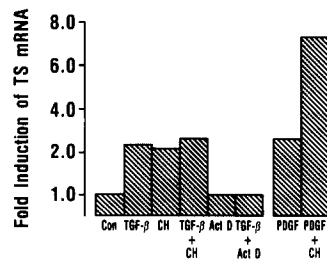


Figure 6. The increase in thrombospondin mRNA levels in response to TGF- β appears to be indirect. SMC at confluent densities were growth-arrested by serum deprivation, then were treated for 4 h with TGF- β with or without cycloheximide (CH) or actinomycin D (Act D). Control

cells (Con) received no additions. Cytoplasmic dot blots were prepared and hybridized with a cDNA specific for TS. The resultant autoradiographs were quantitated by scanning densitometry. Both TGF- β and CH, when added alone, caused increases in steady-state TS mRNA levels; TGF- β did not further enhance the response to CH when cells were treated with TGF- β and CH together. For comparison, the inductive effects of a 2-h PDGF treatment with and without prior CH treatment (which “superinduces” the TS message) (24) are also shown.

0–24 h and Northern blots were prepared. The blots were probed with cDNAs specific for the A and B chains of PDGF. Message for PDGF B-chain (*c-sis*) could not be detected in SMC under quiescent (serum-starved) or TGF- β -treated conditions (data not shown). The same Northern blots revealed strong hybridization signals when probed for A-chain message (Fig. 7). Three distinct mRNA species in SMC are frequently detected by A chain probes, corresponding to message sizes of 2.9-, 2.3-, and 1.7-kb. TGF- β treatment of quiescent SMC resulted in the rapid accumulation (first apparent at 1 h) of the 2.3-kb species of A-chain mRNA (Fig. 1). Identical results were obtained when RNA was examined from sparse or confluent SMC.

Radioreceptor assays were used to estimate the amount of PDGF-like protein in control and TGF- β -treated SMC-conditioned media. Cells at sparse or confluent densities were growth arrested in 0.5% CS for 72 h, then were treated with TGF- β for 24 h. The culture supernatants were removed and replaced with serum-free media with or without TGF- β ; cells were allowed to condition this medium for an additional 24 h. PDGF-like activity in culture supernatants of untreated SMC, as well as in serum-free media incubated in the absence of cells, was below reliable limits of detection for the assay. In contrast, both sparse and confluent SMC secreted readily detectable amounts of PDGF-like activity (0.15–0.41 ng/ml) after TGF- β treatment. We therefore conclude that TGF- β stimulates PDGF gene expression and secretion by

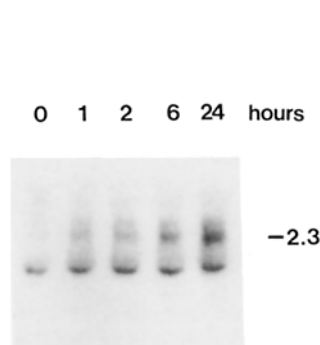


Figure 7. TGF- β regulates steady-state mRNA levels for the A-chain of PDGF. Quiescent confluent SMC were treated with 10 ng/ml TGF- β for the indicated times. 10 μ g of total RNA per lane was subjected to Northern analysis for PDGF A-chain mRNA. A rapid and sustained increase in A-chain transcripts (2.3-kb species) occurs in response to TGF- β treatment.

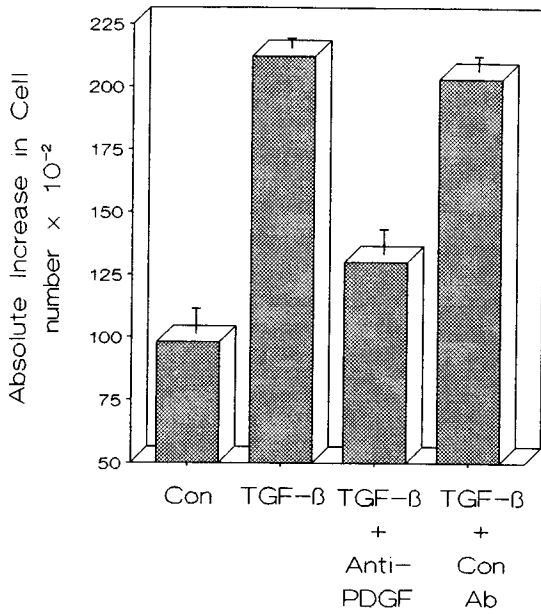


Figure 8. Antibodies against PDGF inhibit the ability of TGF-β to promote the growth of cultured SMC. Confluent quiescent SMC were exposed to TGF-β in the presence or absence of 20 mg/ml anti-human platelet PDGF or control IgG. Cell numbers were determined at 0 and 48 h, and the absolute increase in cell number (over $T = 0$ control values) was calculated. Antiserum against PDGF resulted in a significant reduction in the increase in cell number elicited by TGF-β.

SMC cultured under sparse and confluent conditions. Since only the A-chain mRNA can be detected (by Northern analysis), we presume the secreted PDGF to consist primarily of PDGF A-A homodimers. It is possible, however, that PDGF-B

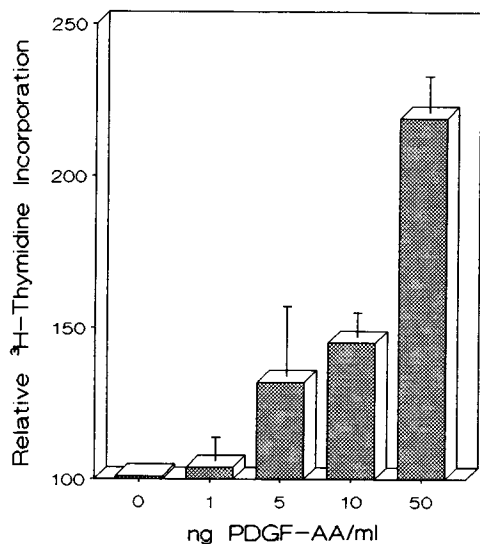


Figure 9. PDGF-AA is mitogenic for cultured vascular SMC. Confluent quiescent SMC were exposed to the indicated concentrations of PDGF-AA for 24 h and pulse-labeled with [³H]thymidine for 1 h to determine relative rates of DNA synthesis. PDGF-AA was able to elicit a significant mitogenic response at concentrations >5 ng/ml; maximum DNA synthesis was induced at 50 ng/ml.

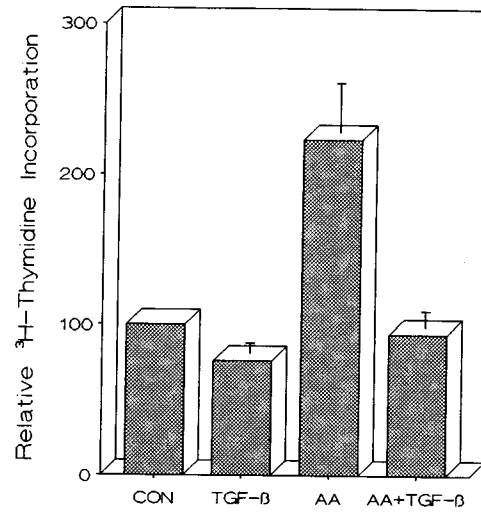


Figure 10. TGF-β inhibits PDGF-AA-mediated mitogenesis in sparse SMC cultures. Sparsely plated, quiescent SMC were exposed to TGF-β (10 ng/ml) and PDGF-AA (50 ng/ml) alone or in combination. Cells were pulse-labeled with [³H]thymidine at 16 h. PDGF-AA, but not TGF-β was mitogenic for sparse SMC. TGF-β inhibited the mitogenesis induced by PDGF-AA.

may be expressed at very low levels, and that small amounts of PDGF-AB and/or -BB may be present.

Induced PDGF Expression Accounts for the Mitogenic Activity of TGF-β

To determine the possible role of PDGF-like proteins in mediating the growth-promoting effects of TGF-β, we tested anti-PDGF IgG for their ability to neutralize the increase in

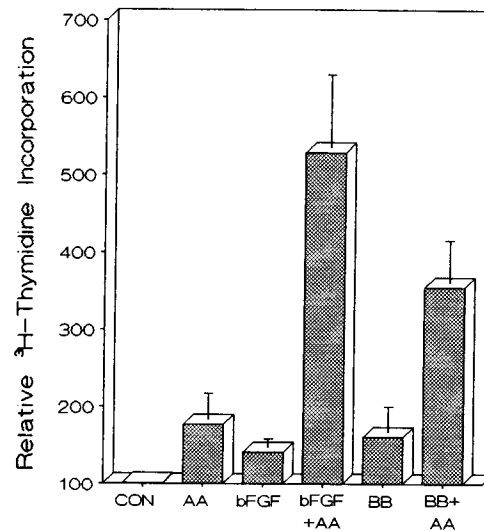


Figure 11. PDGF-AA acts cooperatively with bFGF and PDGF-BB to stimulate SMC mitogenesis. Confluent quiescent SMC were exposed to the indicated growth factors for 24 h, and then were pulse-labeled with [³H]thymidine. While PDGF-AA (50 ng/ml), bFGF (10 ng/ml), and PDGF-BB (50 ng/ml) each gave relatively weak mitogenic responses when added alone, when PDGF-AA was added in combination with bFGF or PDGF-BB, the mitogenic responses were markedly enhanced.

cell number observed in TGF- β -treated confluent cultures. The antibodies used (a generous gift of E. Raines and R. Ross, University of Washington) were prepared against human platelet PDGF (which is predominantly PDGF-AB) but show some neutralizing ability against PDGF-AA (31). As shown in Fig. 8, very high amounts of this antisera (20 mg/ml) significantly reduced the increase in cell number induced by TGF- β over a 48-h period. Control antisera (also at 20 mg/ml) did not block the TGF- β mediated increase in cell number. On the basis of these data, we concluded that the induced expression of a PDGF-like activity was primarily responsible for the growth-promoting effects of TGF- β .

PDGF-AA Is Weakly Mitogenic for Sparse and Confluent SMC in Culture

While PDGF-AB and PDGF-BB are known mitogens for cultured vascular SMC (32, 35), several recent abstracts offered contradictory statements concerning the mitogenic effects of PDGF-AA on cultured SMC (Blank, R. S., M. H. Corjay, and G. K. Owens. 1989. *FASEB [Fed. Am. Soc. Exp. Biol.] J.* 3:A611 [abstr.]; Gibbons, G. H., R. E. Pratt, and V. J. Dzano. 1989. *J. Cell Biochem. Suppl.* 13E:191 [abstr.]). Because our data strongly suggest a role for PDGF-AA in the control of SMC replication by TGF- β , we sought to determine the effects of AA homodimers on the replication of the rat aortic SMC used in this study. As presented in Fig. 9, recombinant human PDGF-AA caused a dose-dependent increase in [3 H]thymidine uptake by confluent SMC, with a maximal effect observed at 50 ng/ml. As presented below, AA homodimers were also weakly mitogenic for sparse SMC. In additional experiments not shown, PDGF-AA (at 50 ng/ml) was found to stimulate the expression of thrombospondin and sp55, similar to the effects of PDGF-AB (human platelet PDGF) (21). These are expected results given that PDGF-AA and -AB are believed to interact with a common receptor (6, 12-14).

The Growth Inhibitory Effects of TGF- β Override the Mitogenic Effects of PDGF-AA in Sparse SMC Cultures

As noted above, PDGF-A was induced by TGF- β in both sparse and confluent cultures, while TGF- β -mediated mitogenesis was observed only in confluent cultures. Conceivably, this differential effect could result from an ability of PDGF-AA to override the growth inhibitory effects of TGF- β , coupled with a lack of responsiveness of sparse SMC to PDGF-AA. To test this possibility, we treated quiescent sparse SMC with TGF- β (10 ng/ml) and AA homodimers (50 ng/ml) alone and in combination. As shown in Fig. 10, PDGF-AA was clearly mitogenic for sparse cells, and this mitogenicity was completely inhibited by the simultaneous addition of TGF- β . These data are fully consistent with the demonstrated ability of TGF- β to inhibit serum-, bFGF-, PDGF-BB-, and PDGF-AB-mediated mitogenesis in sparse SMC cultures. The data suggest that sparse SMC respond to TGF- β with a dominant growth inhibitory signal that is completely dissociable from its effects on growth factor production.

PDGF-AA Acts Cooperatively with Other Growth Factors during SMC Mitogenesis

If PDGF-A expression is responsible for the potentiation of

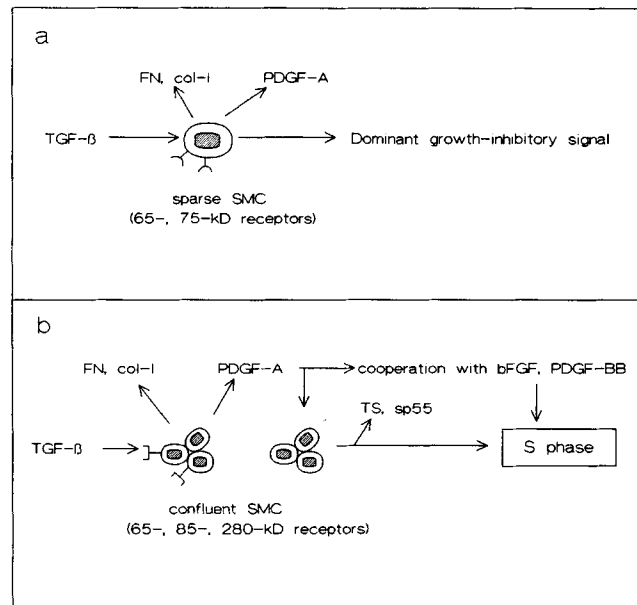


Figure 12. Hypothetical roles for TGF- β , TGF- β receptors, and PDGF-AA in the control of SMC growth in vitro. Sparse and confluent cultured SMC express distinct TGF- β receptor phenotypes (10). Exposure of sparse SMC to TGF- β (a) results in the induced expression of FN, type I collagen (*col-I*), and PDGF-A. Cell growth in response to a variety of stimuli is inhibited, suggesting a dominant growth inhibitory block somewhere in the G₁ phase of the cell cycle. When confluent SMC are exposed to TGF- β (b), cell growth in response to a variety of stimuli is enhanced or unaffected rather than inhibited, suggesting that confluent SMC are refractory to the growth inhibitory effects of TGF- β . The TGF- β -mediated enhancement of proliferation results from the mitogenic activity of the induced PDGF (which we presume to be primarily AA homodimers), as well as from the increased expression of TS and the ability of PDGF-AA to act cooperatively with other mitogens such as bFGF and PDGF-BB.

SMC mitogenesis that occurs when TGF- β is added in combination with serum or other growth factors (see Fig. 1), then purified PDGF-AA should act similarly to potentiate mitogenesis. To test this hypothesis, we performed mitogenesis assays on quiescent confluent SMC using recombinant PDGF-AA, porcine platelet PDGF (PDGF-BB), and bFGF. As presented in Fig. 11, PDGF-AA (50 ng/ml) acted in concert with bFGF (10 ng/ml) and PDGF-BB (50 ng/ml) to stimulate mitogenesis under these conditions. Similar results were obtained in experiments using sparse cells.

Discussion

Multifunctional regulatory peptides such as PDGF (for review, see references 14, 35) and TGF- β (for review, see references 28, 44, 45) are believed to play important roles in vascular biology during development, in homeostatic tissue maintenance, and in tissue repair after injury. TGF- β has been shown to regulate the behavioral organization of vascular SMC in culture (20) and may inhibit (3, 20) or promote (3, 20) SMC replication, depending on culture conditions. In a previous study, we have provided evidence that the differential effects of TGF- β on SMC growth may result from an interaction of TGF- β with distinct, selectively expressed,

cell-surface receptors (10). In this study, we have continued our examination into the effects of TGF- β on SMC replication and gene expression in vitro with an emphasis on elucidating the mechanisms underlying the growth promoting effects of TGF- β on confluent SMC.

In Vitro Responses of Sparse SMC to TGF- β

Fig. 12 presents a comprehensive summary of our data concerning the hypothesized roles of TGF- β , TGF- β receptors, PDGF-A, and cell density in the control of SMC replication in vitro. Vascular SMC maintained at sparse density in culture express two predominant TGF- β binding proteins on their cell surfaces, with receptor-ligand complexes of $M_r = 65,000$ and $75,000$ (10). Because the ability of TGF- β to act as a growth inhibitor for SMC correlates with the expression of the 75-kD "receptor," we have hypothesized that this receptor-subunit mediates a growth inhibitory response in these cells (10). Sparse SMC express increased amounts of fibronectin and type I collagen mRNA in response to TGF- β (as do confluent cells). TGF- β -treated sparse SMC express markedly increased amounts of the PDGF-A message and secrete increased amounts of PDGF-A homodimers, which, as we have shown in this study (Figs. 9-11), are clearly mitogenic for rat aortic SMC. It seems obvious that TGF- β elicits a dominant growth inhibitory response in sparse SMC that is unrelated to its effects on connective tissue or PDGF-A expression. TGF- β can inhibit the growth of sparse SMC replicating in 5% CS (20), and can inhibit the mitogenic response of sparse SMC to EGF (3), PDGF-AB, PDGF-BB, PDGF-AA, bFGF, and CS (this study). The mechanisms underlying the growth inhibitory effect of TGF- β on sparse SMC are presently unknown and are the focus of ongoing investigation.

In Vitro Responses of Confluent SMC to TGF- β

Exposure of confluent vascular SMC to TGF- β results in the accumulation of FN, type I collagen, and PDGF-A mRNA transcripts with kinetics similar to that observed in sparse SMC. As shown in our previous work (20) and reiterated in this study (Fig. 1), confluent SMC cultures appear refractory to the growth inhibitory effects of TGF- β . Without such a TGF- β -mediated block to cellular proliferation, the cells can respond to autocrinely produced PDGF alone or in concert with other growth factors (Fig. 11), resulting in growth promotion. It should be emphasized here that, although we have concentrated on the effects of induced PDGF-A in TGF- β -mediated growth stimulation, our data do not rule out the involvement of other growth factors in this process.

TS Expression during TGF- β -mediated Mitogenesis

TS, an $M_r = 450,000$ homotrimeric secreted glycoprotein, is expressed during TGF- β -mediated mitogenesis (Figs. 3-5). Thrombospondin was previously shown to be regulated by PDGF in vascular SMC (21), and the regulation was shown to occur at the mRNA level (17, 24). TS is believed to be essential for SMC proliferation (23), and may play a role in the response of SMC to other growth factors (22, 40). Pettinen et al. (30) have demonstrated, in 3T3 fibroblasts, the induction of TS message by TGF- β ; however, it was not determined if this was a direct effect or one requiring the synthesis of a protein intermediate (e.g., PDGF). Our data, which show that the TGF- β -induced expression of TS is density-depen-

dent, of slow onset, and cycloheximide-sensitive, strongly suggest that the effect is indirect, mediated via the induced secretion of a PDGF-like activity (probably PDGF-AA). Regardless of the underlying mechanism, the expression of TS during TGF- β -mediated mitogenesis suggests an additional way in which TGF- β can positively influence SMC growth in confluent cultures (22, 39).

Role of PDGF-A in SMC Growth Control

TGF- β is known to regulate the expression of PDGF genes in several systems, including vascular endothelial cells (8, 46), leukemia cells (27), AKR-2B cells (18), and human fibroblasts (43); autocrine PDGF-mediated growth stimulation has been proposed for the latter two examples. Vascular SMC are known to express PDGF-A under certain culture conditions (19, 25, 41, 42), but the functional consequences of A-chain expression have not been elucidated. Several conflicting preliminary reports exist that address the issue of the mitogenicity of PDGF-A on cultured SMC. In this study, we have demonstrated that AA homodimers are weakly mitogenic for cultured rat aortic SMC (Figs. 9-11). In addition, we provide evidence that PDGF-AA can act synergistically with other growth factors (e.g., bFGF and PDGF-BB) to stimulate SMC mitogenesis. The available evidence therefore suggests that the induced expression of PDGF-A can facilitate SMC replication in two ways: PDGF-AA can act individually as a mitogen, and can cooperate with other growth factors to facilitate their mitogenic activity. As demonstrated by the data presented in Fig. 10, the TGF- β -mediated induction of PDGF-A in sparse SMC would not be expected to influence DNA synthesis since, in sparse cells, TGF- β inhibits mitogenesis in response to PDGF-AA and all other growth stimuli tested to date.

The demonstration that TGF- β can facilitate the replication of confluent SMC may explain the heretofore puzzling observations that TGF- β mRNA is induced in the vascular wall during tissue repair following balloon catheterization (Majesky, M. W., M. A. Reidy, D. R. Twardzik, and S. M. Schwartz. 1989. *FASEB [Fed. Am. Soc. Exp. Biol.] J.* 3:A298 [abstr.]), and during vascular wall remodeling associated with the development of hypertension (37). Other recent studies have shown that infusion of TGF- β after balloon catheterization injury of the rat carotid artery results in a significant stimulation of SMC replication in the neointima (M. Reidy, personal communication). Thus, SMC in vivo may respond, as could be predicted, as "confluent" SMC do in vitro. We propose that TGF- β may be an important regulatory molecule involved in many aspects of vascular growth, tissue repair, and development. Our data emphasize the multifunctionality of TGF- β in vascular biology, and show that some of this multifunctionality may arise from the induced production of other autocrine regulatory factors. The data also demonstrate that the pleiotropic effects of TGF- β can be functionally dissociated from one another, and strongly suggest that the in vivo responses of SMC to TGF- β may vary considerably depending on the biological situation.

The authors gratefully acknowledge Carla Worland for secretarial assistance and for her excellent contributions to the preparation of this manuscript; Drs. C. Betsholtz, C.-H. Heldin, V. Dixit, G. Vogeli, V. Groppi, L. Ericson, K. Marotti, and R. Hynes for providing cDNA probes; Dr. D. Bowen-Pope and colleagues for performing PDGF radioreceptor assays

on SMC-conditioned media; and E. Raines and Dr. R. Ross for their generous gift of antibodies against PDGF. We would also like to thank Dr. M. Reidy for communicating data on the in vivo effects of TGF- β on SMC replication before their publication.

This work was supported in part by National Institutes of Health grants HL-03174 and HL-07312.

Received for publication 12 January 1990 and in revised form 17 March 1990.

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