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J Clin Invest. 1994;93(5):2048-2055. https://doi.org/10.1172/JCI117199.

Research Article

Transforming growth factor-beta 1 (TGF-beta 1) has been implicated in mediating smooth muscle cell (SMC) growth after vascular injury. Studies examining TGF-beta-induced growth of cultured SMC have identified only modest mitogenic effects which are largely dependent on autocrine production of platelet-derived growth factor-AA (PDGF-AA). Recent studies have suggested, however, that TGF-beta also may have delayed growth effects independent of PDGF-AA. The aims of the present studies were to examine the effects of TGF-beta on chronic growth responses of cultured SMC. Results demonstrated that TGF-beta elicited a delayed growth response (24 fold increase in 3H-TdR incorp. from 48-72 h) and enhanced SMC production of PDGF-AA (eightfold increase at 24 h). Neutralizing antibodies to PDGF-AA, however, inhibited only 10-40% of delayed TGF-beta-induced growth. Co-treatment with TGF-beta transiently delayed epidermal growth factor (EGF)-, basic fibroblast growth factor (bFGF)-, or PDGF-BB-induced entry into S phase but enhanced the delayed growth responses to these growth factors by 16.0-, 5.8-, or 4.2-fold, respectively. Neutralizing antibodies to PDGF-AA had no effect on these synergistic responses and exogenous PDGF-AA did not increase growth responses to EGF, bFGF, or PDGF-BB. In summary, TGF-beta induces marked delayed growth responses, alone and in combination with EGF, bFGF or PDGF-BB, that are largely independent of PDGF-AA.

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TGF-β Promotes Proliferation of Cultured SMC via Both PDGF-AA-dependent and PDGF-AA-independent Mechanisms

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Abstract

Transforming growth factor-β1 (TGF-β1) has been implicated in mediating smooth muscle cell (SMC) growth after vascular injury. Studies examining TGF-β-induced growth of cultured SMC have identified only modest mitogenic effects which are largely dependent on autocrine production of platelet-derived growth factor-AA (PDGF-AA). Recent studies have suggested, however, that TGF-β also may have delayed growth effects independent of PDGF-AA. The aims of the present studies were to examine the effects of TGF-β on chronic growth responses of cultured SMC. Results demonstrated that TGF-β elicited a delayed growth response (24 fold increase in ^{3}H-TdR incorp. from 48–72 h) and enhanced SMC production of PDGF-AA (eightfold increase at 24 h). Neutralizing antibodies to PDGF-AA, however, inhibited only 10–40% of delayed TGF-β–induced growth. Co-treatment with TGF-β transiently delayed epidermal growth factor (EGF)–, basic fibroblast growth factor (bFGF)–, or PDGF-BB–induced entry into S phase but enhanced the delayed growth responses to these growth factors by 16.0-, 5.8-, or 4.2-fold, respectively. Neutralizing antibodies to PDGF-AA had no effect on these synergistic responses and exogenous PDGF-AA did not increase growth responses to EGF, bFGF, or PDGF-BB. In summary, TGF-β induces marked delayed growth responses, alone and in combination with EGF, bFGF or PDGF-BB, that are largely independent of PDGF-AA. (*J. Clin. Invest.* 1994. 93:2048–2055.) Key words: smooth muscle cells • transforming growth factor-β • epidermal growth factor • basic fibroblast growth factor • platelet-derived growth factor

Introduction

Smooth muscle cell (SMC)[1] proliferation plays a major role in the vascular reparative response after mechanical injury. There is evidence that a variety of factors regulate this SMC growth including angiotensin-II (1, 2), basic fibroblast growth factor (bFGF) (3), platelet-derived growth factor (PDGF) (4, 5), and transforming growth factor-β1 (TGF-β1) (6). TGF-β1 is a 25-kD growth factor released during acute phases of injury by degranulating platelets (7) and secreted during chronic phases of lesion development by SMCs (6) and activated macrophages (8). Majesky et al. (6) demonstrated that TGF-β1 mRNA levels were elevated within 6 h of balloon embolectomy catheter-induced injury of rat carotid artery, and remained elevated for at least 2 wk. Immunohistochemical studies of vessels 2 wk after injury revealed that a large majority of intimal cells expressed TGF-β1 protein in an intracellular pattern consistent with active synthesis. Furthermore, infusion of recombinant TGF-β1 into rats for 48 h, 2 wk after carotid artery injury, increased [^{3}H]thymidine labeling indices of intimal cells by fourfold. These data strongly suggest that TGF-β is present, both acutely and chronically, and may influence growth of SMC at sites of vascular injury.

Our understanding of the role of TGF-β in myointimal lesion formation after injury has been confounded by in vitro observations that TGF-β can both inhibit as well as stimulate growth of cultured SMC. Several studies (7, 9, 10), including one from this laboratory (11), have shown that TGF-β is a potent inhibitor of growth factor- or serum-induced proliferation. In contrast, other investigators have found that TGF-β stimulated SMC proliferation. Majack et al. (12) showed that TGF-β stimulated growth of postconfluent rat SMC and inhibited growth of subconfluent cells. Battegay et al. (13) found that TGF-β stimulated growth of cultured human SMC in a bimodal, concentration dependent manner. Both Majack et al. and Battegay et al. found that TGF-β stimulation of growth was associated with increased production of PDGF-AA, mimicked by treatment with exogenous PDGF-AA and partially inhibited by neutralizing antibodies to PDGF-AA. The effects of TGF-β on SMC growth were modest in both of these studies; maximal [^{3}H]thymidine incorporation induced by TGF-β was 200% of control in the studies of Majack et al. and 400% of control in the studies of Battegay et al. Based on the results of these studies, TGF-β is currently thought to have modest effects on SMC proliferation which are mediated primarily through autocrine production of PDGF-AA.

Results of recent studies have suggested that TGF-β may also have delayed effects on SMC growth that are not mediated solely by PDGF-AA production. For example, we found that angiotensin-II (Ang-II) enhanced growth responses of SMC derived from spontaneously hypertensive rats (SHR) to epidermal growth factor (EGF), bFGF, and PDGF-BB and that these effects were delayed and dependent on autocrine production of TGF-β (14). The effect of Ang-II on growth responses to PDGF-BB are unlikely to be mediated via autocrine production of PDGF-AA since PDGF α receptors, the only known effectors of PDGF-AA actions, should have been fully activated by the saturating concentrations of PDGF-BB used in these experiments. Using cultured rabbit SMC, Janat and Liau

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Received for publication 23 September 1993.

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CM, conditioned media; MLE, mink lung epithelial; SFM, serum-free medium; SHR, spontaneously hypertensive rats; SMC, smooth muscle cell.

J. Clin. Invest.
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0021-9738/94/05/2048/08 $2.00
Volume 93, May 1994, 2048–2055

(15) showed that TGF-β alone had no effect on [^{3}H]thymidine incorporation. However, similar to our observations, cotreatment with TGF-β and PDGF-BB resulted in a growth effect which was 10-fold greater than that elicited by PDGF-BB alone. This effect was delayed, with maximal effects observed 30 h after treatment with TGF-β + PDGF-BB as opposed to 16 h after treatment with PDGF-BB. Taken together, results of these studies suggest that TGF-β–induced increases in PDGF-AA production cannot explain TGF-β–induced growth under all conditions, and that TGF-β has effects on SMC growth that are distinct from those previously described.

The aims of the present studies were: (*a*) to examine the effects of exogenous TGF-β, alone as well as in combination with EGF, bFGF, or PDGF-BB, on acute and chronic growth responses of cultured SMC, and (*b*) to examine the contribution of PDGF-AA in this process by determining whether exogenous PDGF-AA, alone or in combination with EGF, bFGF, or PDGF-BB, stimulated growth of SHR-derived SMC, and whether neutralizing antibodies to PDGF-AA inhibited TGF-β–induced growth effects.

Methods

Smooth muscle cell culture. SMCs were isolated from aorta of spontaneously hypertensive or Sprague-Dawley rats by enzymatic digestion as previously described (16). The cells were cultured in medium containing a 1:1 formulation of Dulbecco's modified Eagle's Medium (DME; GIBCO BRL, Gaithersburg, MD) and Ham's F12 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), L-glutamine (0.68 mM; Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were harvested for passaging at subconfluence with a trypsin-EDTA (0.05% trypsin, 0.02% EDTA, GIBCO BRL) solution. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. SMC identity and the purity of cultures was verified by immunocytochemical analysis using antibodies specific for smooth muscle α-actin (17).

SMC between passages 6 and 19 were plated at 3×10^3 cells/cm^2 in serum containing media. They were grown to confluence and then growth arrested in a defined serum free medium (SFM) containing a 1:1 formulation of DME and Ham's F12 supplemented with transferrin (5 μg/ml, Sigma Chemical Co.), insulin (5×10^{-7} M, Sigma Chemical Co.), ascorbate (0.2 mM, Sigma Chemical Co.), selenium (38 nM, Sigma Chemical Co.), glutamine, and penicillin/streptomycin. This SFM has been shown to maintain SMC in a quiescent, non-catabolic state and to promote expression of SMC-specific contractile proteins (18).

Fibroblast cell culture. 3T3 Swiss albino mouse fibroblasts were obtained from American Type Culture Collection (Rockville, MD) and maintained in DME supplemented with L-glutamine, penicillin/streptomycin, and 10% FBS. Passaging was at subconfluence using similar methods to those described above. The cells were plated at 3×10^3 cells/cm^2 and grown to subconfluence in DME supplemented with L-glutamine, penicillin/streptomycin and 10% FBS. The cells were then growth arrested for 8–24 h in DME supplemented with L-glutamine, penicillin/streptomycin and 1% FBS. Conditioned media experiments were performed as described in figure legends.

Radioreceptor assay of PDGF concentration. This assay is essentially the same as that described by DiCorleto and Bowen-Pope (19). Briefly, subconfluent human foreskin fibroblasts were plated in 1% human plasma-derived serum. Between 2 and 10 d later, the cells were placed on an ice tray and washed with 0.5 ml of ice-cold binding medium (Dulbecco-Vogt medium without bicarbonate containing 25 mM Hepes buffer, pH 7.2, and bovine serum albumin [BSA]). The medium was removed and aliquots of conditioned medium from TGF-β or vehicle-treated SMC were added in binding medium. The cultures were incubated at 4°C with gentle mixing for 2 h. The test substance was removed and the cells were rinsed with binding medium. ^{125}I-PDGF (0.2 ng) was then added and the incubation was continued for two more hours. The media was removed and the cells washed three times with 1 ml cold phosphate-buffered saline containing BSA. Bound radioactivity was determined by solubilizing the cells with 1% Triton X-100 in water containing BSA. Nonspecific binding, measured in the presence of at least a 100-fold excess of unlabeled crude growth factor, was < 5% of specific binding. Standard curves were derived using wells containing various amounts of unlabeled PDGF.

[^{3}H]thymidine incorporation. Relative rates of DNA synthesis were assessed by determination of [^{3}H]thymidine incorporation into trichloroacetic acid (TCA) precipitable material. Cells were pulsed with [^{3}H]-thymidine (1 μCi/ml, 6.7 Ci/mmol; New England Nuclear, Boston, MA) and then washed with a calcium- and magnesium-free phosphate buffered saline (PBS) (NaCl 137 mM, Na_2HPO_4 8.1 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, pH 7.4). This was followed by 10-min washes with 10% TCA, first at 4°C and then at room temperature. Cells were then dissolved in 1 N NaOH and placed in Ready-Safe scintillation fluid (Beckman Instruments, Inc., Palo Alto, CA). Counting was done with a Wallac LKB scintillation counter.

Growth curves. Cultures were washed with PBS, harvested with trypsin-EDTA, diluted with 0.9% NaCl (Columbia Diagnostics Inc., Springfield, VA) and counted using an Electrozone Celloscope (Particle Data, Inc., Elmhurst, IL) with orifice size of 95 μm and sample volume of 500 μl.

Reagents. TGF-β derived from human platelets was obtained from R&D Systems (Minneapolis, MN). Recombinant human EGF, bFGF, PDGF-BB, and PDGF-AA were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). PDGF-AA neutralizing antibodies (pfa4) were a kind gift of Dr. Michael Pech (F. Hoffmann-La Roche, Basel, Switzerland). Control antibodies for PDGF-AA neutralizing antibody experiments were α-actin monoclonal antibodies (Sigma Chemical Co.) PDGF-AA polyclonal neutralizing antibody was purchased from Genzyme Corp.

Statistical analysis. Results are presented as mean ± standard deviation unless otherwise stated. One way analysis of variance followed by the Newman-Keuls' multiple range test was used to analyze data. A *P* value of less than or equal to 0.05 was considered statistically significant. Triplicate wells were analyzed for each experiment and each experiment was performed independently a minimum of three times. Data shown are from representative experiments.

Results

TGF-β induced a delayed increase in [^{3}H]thymidine incorporation in cultured aortic SMC. Since TGF-β levels are increased in vascular lesions for at least 2 wk after injury (6), the initial aim of these studies was to determine the effects of exogenous TGF-β on mitogenesis of SHR-derived SMC, maintained in a defined serum-free medium, at acute, as well as chronic, time points. Previous studies (14) demonstrated that TGF-β was not mitogenic for SHR-derived SMC within the initial 24 h after treatment. In contrast, results of the present studies demonstrated that TGF-β elicited a concentration dependent increase in [^{3}H]thymidine incorporation at both 24–48 h (2–4-fold) and 48–72 h (Fig. 1). SMC growth was observed at concentrations of 10 pM and greater with an ED_{50} for this effect of 20–30 pM. Maximal effects were observed at concentrations of 40 pM or greater. The magnitude of TGF-β–induced increases in [^{3}H]-thymidine incorporation (48–72 h after treatment) increased with passage number. The response averaged threefold in cells at passages 5–9 versus 49-fold in cells at passages 15–19 times.

TGF-β–stimulated production of PDGF-AA by SHR-derived SMC. To determine if delayed mitogenic effects of TGF-β (100 pM) in SHR-derived SMC were mediated by PDGF-AA

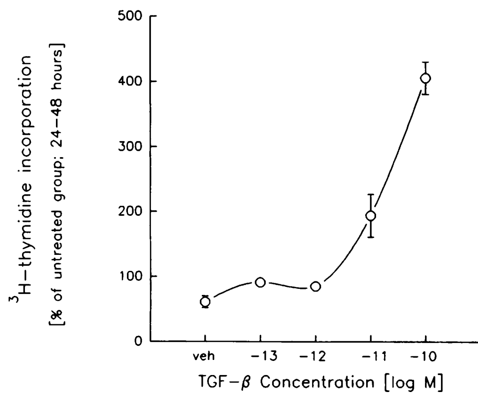

Figure 1. Concentration dependence of growth responses to TGF-β1. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (various concentrations) or vehicle added. The SMC were pulsed with [^{3}H]-thymidine (2 μCi/ml) 48 h after treatment and [^{3}H]thymidine incorporation was assayed 24 h later as described in Methods. The control group was treated similarly but without the addition of TGF-β1 or vehicle.

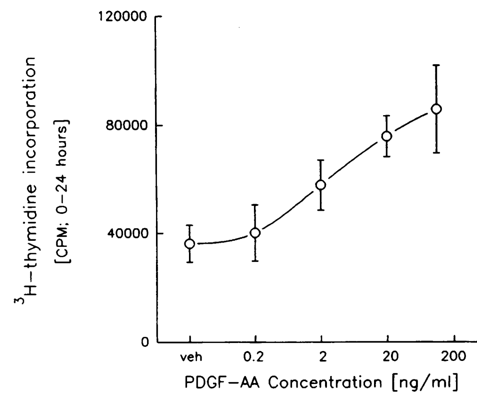

Figure 3. Concentration dependence of growth responses to PDGF-AA. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and PDGF-AA (various concentrations) or vehicle added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) at time of treatment and [^{3}H]thymidine incorporation was assayed 24 h later. The control group was treated similarly but without the addition of PDGF-AA or vehicle.

as previously reported in SMC derived from Sprague-Dawley rats (12) or humans (13), PDGF-AA levels were measured in conditioned media from TGF-β–treated and vehicle-treated SMC using a radioreceptor competition assay. Results demonstrated an eightfold increase in PDGF concentration in conditioned media (CM) 24 h after TGF-β as compared with vehicle treatment (Fig. 2). PDGF-AA levels remained elevated for at least 48 h (Fig. 2). Despite increased levels of PDGF-AA in CM from TGF-β–treated SMC (TGF CM), there was no increase in mitogenic activity for SMC in TGF CM as compared with CM from vehicle treated SMC (Cnt CM) (data not shown). Human recombinant PDGF-AA also stimulated only modest increases in [^{3}H]thymidine incorporation and required concentrations far in excess of that in TGF CM (Fig. 3). Results indicate that although TGF-β stimulated production of PDGF-AA, these cells responded weakly to either endogenous or exogenous PDGF-AA. In contrast to effects on SHR-derived SMC, PDGF-AA is a potent mitogen for 3T3 fibroblasts which express abundant PDGF-α receptors (20). TGF CM had increased mitogenic activity for 3T3 cells compared to Cnt CM that was partially inhibited by a monoclonal antibody (designated pfa4) that has previously been shown to neutralize rat PDGF-AA (21) (Fig. 4).

Delayed mitogenic effect stimulated by TGF-β was partially mediated by autocrine production of PDGF-AA. Treatment with pfa4 antibody inhibited only 10–40% of TGF-β–induced mitogenesis measured 48–72 h after treatment (Fig. 5). Similar results were obtained with a polyclonal antibody preparation

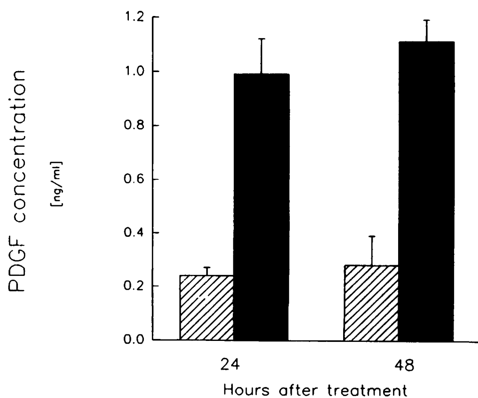

Figure 2. PDGF concentration in conditioned media from TGF-β1 treated SMC. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle added. 24 or 48 h later, BSA (1 mg/ml) was added and CM removed and frozen. PDGF levels were measured using radioreceptor competition assays as described in Methods. (▨ Control; ■ TGF-β1 treated).

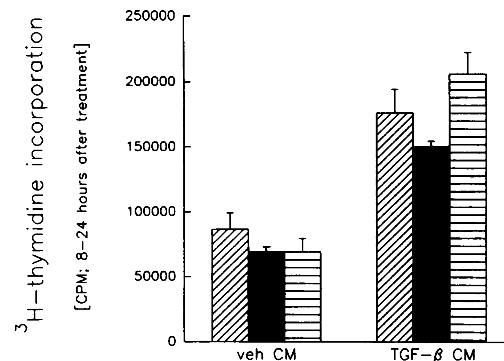

Figure 4. Effect of PDGF-AA neutralizing antibody on mitogenic activity in conditioned media from TGF-β1–treated SMC. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle added. 48 h later, CM was harvested by adding BSA (1 mg/ml) and removing CM from SMC. CM was added to 3T3 cells which had been grown to confluence and growth arrested for 24 h. PDGF-AA neutralizing antibody (pfa4; 10 μg/ml) or control antibody (Cnt Ab) were added. SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) 8 h after addition of CM and [^{3}H]thymidine incorporation assayed 16 hour later. (*veh CM,* conditioned media from vehicle treated SMC; *TGF-β CM,* conditioned media from TGF-β1 treated SMC; *Ab,* antibody). (▨) conditioned media; (■) CM + PDGF – AA antibody; (⊟) CM + control antibody.

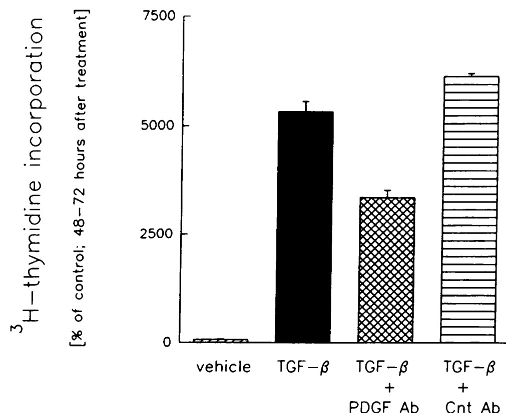

Figure 5. Effect of PDGF-AA neutralizing antibody on TGF-β–induced SMC mitogenesis. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle plus or minus PDGF-AA neutralizing antibody (pfa4; 10 μg/ml) or control antibody were added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) 48 h after treatment and [^{3}H]thymidine incorporation assayed 24 h later.

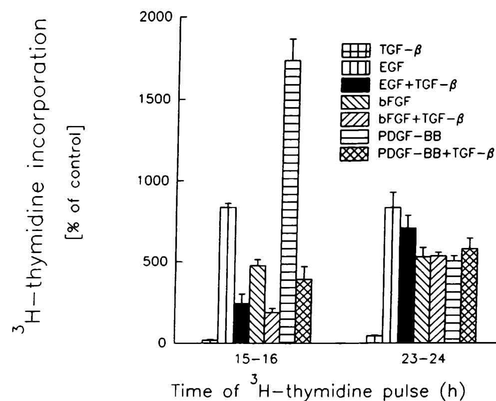

Figure 6. Effects of TGF-β1 on acute growth responses of SMC to EGF, bFGF, or PDGF-BB. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle plus or minus EGF (1.7 nM), bFGF (1.25 nM) or PDGF-BB (0.7 nM) added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) at either 15 or 23 h after treatment and [^{3}H]thymidine incorporation assayed 1 hour later. The control group was treated similarly but without the addition of growth factors.

raised in goats which has been shown to neutralize rat PDGF-AA (22) (data not shown). To ensure that sufficient neutralizing antibody was present throughout the experiment, the pfa4 antibody was used at concentrations 33 fold greater than the IC_{50} which blocked binding of PDGF-AA (2 ng/ml) to NIH 3T3 cells (22a). Additionally, control studies revealed that: (*a*) daily addition of neutralizing antibody had similar effects on TGF-β–induced mitogenesis as did one time addition (data not shown), and (*b*) sufficient antibody was present in CM at 48 h to neutralize 1 ng/ml of rat PDGF-AA (obtained from SMC CM) or 5 ng/ml of human recombinant PDGF-AA (based on assays of mitogenic activity for 3T3 cells). Taken together, these studies demonstrate that TGF-β enhanced SHR-derived SMC production of PDGF-AA but that this effect was only partially responsible for delayed growth effects stimulated by TGF-β.

TGF-β delayed EGF-, bFGF-, or PDGF-BB–induced entry into S phase in confluent, growth-arrested SMC. Saltis et al. (23) have previously shown that low concentrations of TGF-β (40 pM) modestly enhanced growth responses of SHR-derived SMC to EGF, bFGF, PDGF-AB, or PDGF-BB within the initial 48 h of treatment. The aim of the present studies was to determine the effects of higher concentrations of TGF-β (100 pM) on growth responses to EGF, bFGF, or PDGF-BB, at both acute and delayed time points. Results demonstrated that TGF-β (100 pM) inhibited EGF-, bFGF-, or PDGF-BB–induced [^{3}H]thymidine incorporation by 71, 61, and 77%, respectively when measured between 15 and 16 h after treatment (Fig. 6). This effect was transient, however, in that TGF-β had no effect on EGF-, bFGF-, or PDGF-BB–induced [^{3}H]-thymidine incorporation measured at 23–24 h (Fig. 6) or 0–24 h (Fig. 7). Taken together, these data show that TGF-β (100 pM) delayed EGF-, bFGF-, or PDGF-BB–induced entry into S phase but did not have an overall inhibitory effect on growth responses of SMC during the initial 24 h after treatment.

TGF-β induced a delayed, synergistic increase in growth responses of SMC to EGF, bFGF, or PDGF-BB. Subsequent studies examined the effects of TGF-β on growth responses to EGF, bFGF, or PDGF-BB at later time points. Results demonstrated that co-treatment with TGF-β (100 pM) markedly increased EGF-, bFGF-, or PDGF-BB-induced growth responses measured from 48 to 72 h after treatment (Fig. 7). For example, TGF-β increased the growth responses to EGF, bFGF, or PDGF-BB by 16.0-, 5.8-, or 4.2-fold, respectively, as compared with treatment with EGF, bFGF, or PDGF-BB, alone. TGF-β–induced increases in [^{3}H]thymidine incorporation were accompanied by corresponding increases in cell number (Fig. 8). Treatment with TGF-β, EGF, bFGF, or PDGF-BB alone resulted in 28, 50, 36, or 89% increases in cell number, respectively, 7 d after a single treatment. Co-treatment with TGF-β and EGF, bFGF, or PDGF-BB increased cell number by 212, 117, or 188%, respectively, compared with controls. Consistent

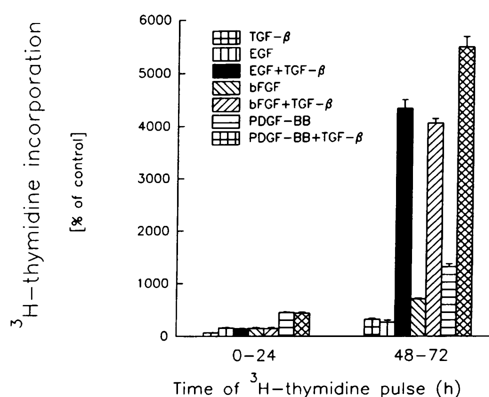

Figure 7. Delayed effects of TGF-β1 on SMC growth responses to EGF, bFGF or PDGF-BB. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle plus or minus EGF (1.7 nM), bFGF (1.25 nM) or PDGF-BB (0.7 nM) added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) at either 0 or 48 h after treatment and [^{3}H]thymidine incorporation assayed 24 h later. The control group was treated similarly but without the addition of growth factors.

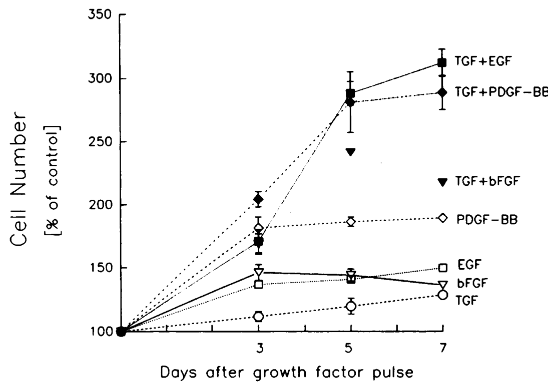

Figure 8. Delayed effects of TGF-β1 on EGF-, bFGF-, or PDGF-BB–induced SMC proliferation. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle plus or minus EGF (1.7 nM), bFGF (1.25 nM) or PDGF-BB (0.7 nM) added. The SMC were trypsinized and cell counts done at 3, 5, or 7 d. The control group was treated similarly but without the addition of growth factors.

with [^{3}H]thymidine incorporation data, the largest increase in cell number occurred between three and five days after treatment.

TGF-β induced increases in growth responses to EGF, bFGF, or PDGF-BB were concentration dependent with effects dependent on TGF-β concentrations greater than 10 pM (Fig. 9). Daily addition of TGF-β (100 pM) did not alter growth responses to TGF-β or TGF-β + EGF, as opposed to one time addition, suggesting that delayed growth responses to TGF-β were not dependent on changing TGF-β concentrations (data not shown).

TGF-β–induced increases in growth responses to EGF, bFGF, or PDGF-BB were not mediated by autocrine production of PDGF-AA. To determine whether autocrine production of PDGF-AA was required for the synergistic growth responses

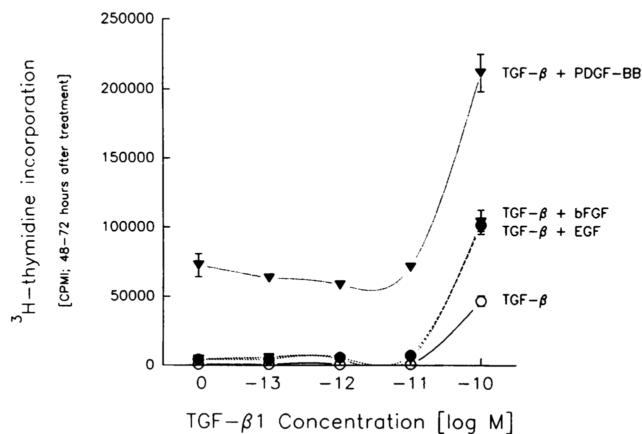

Figure 9. Concentration dependence of the effects of TGF-β1 on growth responses to EGF, bFGF, or PDGF-BB. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (various concentrations) or vehicle plus or minus EGF (1.7 nM), bFGF (1.25 nM0 or PDGF-BB 0.7 nM) added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) 48 h after treatment and [^{3}H]thymidine incorporation was assayed 24 h later as described in Methods. The control group was treated similarly but without the addition of growth factors.

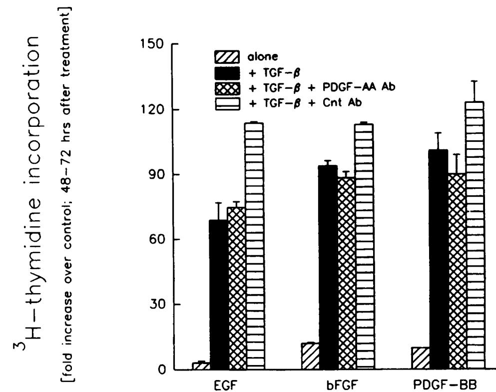

Figure 10. Effects of neutralizing antibody to PDGF-AA on TGF-β1 synergism with EGF, bFGF or PDGF-BB. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle plus or minus EGF (1.7 nM), bFGF (1.25 nM), or PDGF-BB (0.7 nM) added. PDGF-AA neutralizing antibody (pfa4; 10 μg/ml) or control antibody were added to the indicated groups. The SMC were pulsed with [^{3}H]-thymidine (2 μCi/ml) 48 h after treatment and [^{3}H]thymidine incorporation assayed 24 h later. The control group was treated similarly but without the addition of growth factors.

seen with TGF-β + EGF, TGF-β + bFGF, or TGF-β + PDGF-BB, the effects of PDGF-AA neutralizing antibodies on these growth responses were examined. Results demonstrated that neutralizing antibodies to PDGF-AA did not inhibit synergistic growth responses to TGF-β + EGF, TGF-β + bFGF, or TGF-β + PDGF-BB (Fig. 10). Conditioned media from TGF-β treated SMC did not increase growth responses to EGF (data not shown) and treatment with exogenous PDGF-AA did not enhance growth responses to EGF, bFGF or PDGF-BB (Fig. 11).

TGF-β–enhanced growth responses to EGF in SMC primary lines which are unresponsive to exogenous PDGF-AA. We have identified a number of SMC primary lines derived from Sprague-Dawley rats that do not express PDGF α receptors and in which PDGF-AA elicits no measurable growth response (22). These cells were used to further investigate whether TGF-

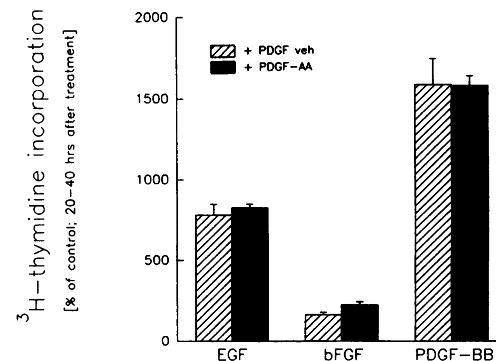

Figure 11. Effects of PDGF-AA on growth responses of SMC to EGF, bFGF or PDGF-BB. SMC derived from SHR were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and PDGF-AA (0.7 nM) or vehicle plus or minus EGF (1.7 nM), bFGF (1.25 nM), or PDGF-BB (0.7 nM) added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) 20 h after treatment and [^{3}H]-thymidine incorporation was assayed 20 h later. The control group was treated similarly but without the addition of growth factors.

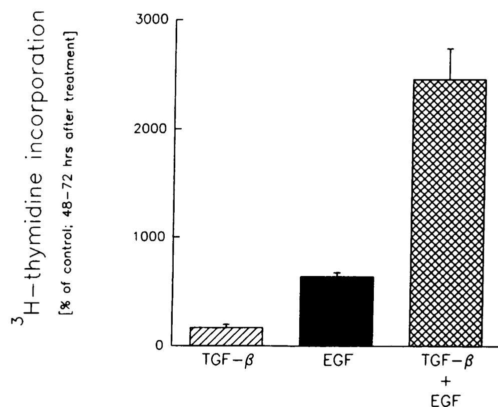

Figure 12. Delayed effects of TGF-β1 on SMC growth responses to EGF in a SMC line which was unresponsive to PDGF-AA. SMC derived from Sprague-Dawley rats were grown to confluence and growth arrested in SFM. 4 d later the SFM was changed and TGF-β1 (100 pM) or vehicle plus or minus EGF (1.7 nM) added. The SMC were pulsed with [^{3}H]thymidine (2 μCi/ml) 48 h after treatment and [^{3}H]-thymidine incorporation assayed 24 h later. The control group was treated similarly but without the addition of growth factors.

β enhanced growth was dependent on PDGF-AA. Results demonstrated that exogenous PDGF-AA had no effect on [^{3}H]-thymidine incorporation from 0 to 72 h after treatment in these cells (data not shown). In contrast, TGF-β or EGF stimulated two- or sevenfold increases in [^{3}H]thymidine incorporation respectively, between 48 and 72 h and co-treatment with TGF-β and EGF stimulated a 24-fold increase in [^{3}H]thymidine incorporation (Fig. 12) as well as a significant increase in cell number (data not shown).

Discussion

TGF-β is a multifunctional growth factor which is present, both acutely and chronically, at sites of vascular injury. Effects of endogenous TGF-β on SMC in injured vessels are unknown, although infusion of TGF-β 2 wk after vascular injury has been shown to promote SMC mitogenesis (6). Studies to date have shown that TGF-β stimulates modest increases in [^{3}H]-thymidine incorporation of cultured SMC which is largely dependent on autocrine production of PDGF-AA (12, 13). Results of the present studies extend these earlier findings by demonstrating that higher concentrations of TGF-β also have pronounced, delayed effects on SMC growth. TGF-β (100 pM)-induced an average 24- (range 3–49) fold increase in [^{3}H]thymidine incorporation when measured 48–72 h after treatment. This effect on SMC growth is much larger than observed in prior studies (12, 13). Additionally, TGF-β stimulated a marked delayed growth response to EGF, bFGF, or PDGF-BB in SHR-derived SMC and to EGF in Sprague-Dawley-derived SMC. These data are consistent with earlier studies demonstrating that TGF-β enhanced growth responses to EGF in bovine SMC (7), to bFGF or PDGF in rat SMC (12) and to PDGF-BB in rabbit SMC (15). Similarly, in SHR-derived SMC, Saltis et al. (23) showed that, while TGF-β (40 pM) alone did not stimulate an increase in cell number, treatment with TGF-β increased the proliferative response to EGF or bFGF by ~ 160% and the proliferative response to PDGF-AB or PDGF-BB by ~ 60%. The concentrations of TGF-β used in those studies was less, and the onset of action earlier, than in the present studies. Taken together, these studies suggest that TGF-β effects on growth of cultured rat SMC are concentration dependent with a relatively modest effect seen within 48 h of TGF-β treatment and a larger effect seen within 72 h of treatment at TGF-β concentrations of 100 pM or greater.

Results of the present studies demonstrate that TGF-β promotes SMC growth via both PDGF-AA–dependent and PDGF-AA–independent mechanisms. Consistent with earlier studies in rat (12) or human SMC (13), we found that TGF-β increased production of PDGF-AA in SHR-derived SMC, that exogenous PDGF-AA was mitogenic for these cells and that neutralizing antibodies to PDGF-AA partially inhibited TGF-β–induced growth. Novel findings of the present studies were that TGF-β had delayed growth promoting activity, both alone and in combination with EGF, bFGF, or PDGF-BB and that autocrine production of PDGF-AA played a minor role in mediating these effects. Evidence supporting the latter conclusion includes: (*a*) neutralizing antibodies (either monoclonal or polyclonal) to PDGF-AA inhibited, at most, only 40% of TGF-β–induced growth and had no effect on TGF-β enhanced growth responses to EGF, bFGF, or PDGF-BB measured between 48 and 72 h; (*b*) exogenous PDGF-AA stimulated only a modest increase in [^{3}H]thymidine incorporation and did not enhance growth responses to EGF, bFGF or PDGF-BB; (*c*) conditioned media from TGF-β–treated SMC did not have increased mitogenic activity for cultured SMC, alone or in combination with EGF, bFGF, or PDGF-BB, when compared with conditioned media from vehicle treated SMC; and (*d*) TGF-β enhanced growth responses to EGF in a primary SMC culture line which did not respond mitogenically to PDGF-AA.

There are several potential mechanisms by which TGF-β may stimulate SMC growth independent of PDGF-AA production. One possibility is that TGF-β may stimulate SMC production of secondary factors, in addition to PDGF-AA, which have growth promoting activity. Results demonstrated that there was increased mitogenic activity for 3T3, but not SMC, in TGF CM compared with Cnt CM which was only partially inhibited by neutralizing antibodies to PDGF-AA. This suggests that if secondary factors are produced which mediate TGF-β effects on SMC, they were not transferrable in conditioned media. A second possibility is that TGF-β may increase SMC growth factor receptor expression. Janat and Liau (15) showed that TGF-β increased PDGF β-receptor mRNA in rabbit SMC, concurrent with TGF-β enhancement of growth responses to PDGF-BB. However, to explain the current results by this mechanism would require TGF-β treatment to simultaneously increase expression of EGF, bFGF, and PDGF receptors. Additionally, co-treatment with TGF-β (100 pM) has delayed, synergistic effects on α-thrombin– or Ang-II–induced growth (23a) implying that TGF-β would also have to increase expression of α-thrombin and Ang-II receptors. A third possibility is that TGF-β enhancement of protooncogene expression may explain TGF-β actions. Recent studies (15) have shown that co-treatment with TGF-β and EGF has a synergistic effect on *c-myc* expression. These effects have, however, been observed within 8 h of treatment and there is no evidence to suggest that a similar effect occurs at delayed time points.

Alternative explanations for the synergistic growth responses observed with TGF-β and EGF, bFGF, or PDGF-BB are that TGF-β exerts generalized intracellular or extracellular effects which enhance SMC growth responses to multiple

growth factors. TGF-β has been shown to increase SMC production of extracellular matrix and this may account for the delayed growth promoting activity through alterations in cell–matrix interactions. In particular, TGF-β has been shown to increase expression of fibronectin, collagen, and elastic fiber proteins. Ignotz and Massague (24) demonstrated that TGF-β–induced anchorage-independent growth and enhanced expression of fibronectin in cultured chick embryo fibroblasts. Furthermore, inhibitors of fibronectin binding blocked, and exogenous fibronectin mimicked, TGF-β induction of anchorage-independent growth. In SMC, Majack et al. (12) and Janat and Liau (15) demonstrated that TGF-β enhanced SMC expression of thrombospondin. Majack et al. (12) also showed that co-treatment with thrombospondin and EGF resulted in a synergistic proliferative response. TGF-β has been shown to enhance SMC expression of α_5 and β_3 classes of integrin (25, 26), but these effects have not been directly linked to SMC growth. TGF-β has also been shown to regulate levels of extracellular matrix degradation by reducing the synthesis of enzymes involved in matrix proteolysis and increasing levels of proteolytic inhibitors (e.g., plasminogen activator inhibitor-1) (27).

Results of the present studies add to the growing evidence that TGF-β stimulates SMC growth in vitro (12, 13), as well as in vivo (6). There is, however, extensive evidence that TGF-β can also inhibit growth of cultured SMC (7, 9, 10). The mechanisms responsible for the bifunctional growth effects of TGF-β on SMC are not known. However, results of recent studies suggest that the multiplicity of TGF-β functions may be due to differential expression of TGF-β receptors (28, 29, 30). Chen et al. (30) demonstrated that overexpression of a truncated type II receptor mutant acted in a dominant negative fashion in mink lung epithelial (MLE) cells resulting in complete loss of type II receptor binding and TGF-β–induced growth inhibition. In contrast, there was no change in type I binding or in TGF-β–induced increases in fibronectin, plasminogen activator inhibitor 1 or jun B mRNA expression. Massague and coworkers (29), using mutant MLE cells, have shown that type I receptors are necessary for TGF-β–induced growth inhibition. Taken together, these studies suggest that both type I and type II receptors are required to mediate the growth inhibitory effect of TGF-β in MLE cells, whereas type I receptors alone can mediate other effects of TGF-β including matrix production. In SMC, Goodman and Majack (31) demonstrated that confluent, growth arrested SMC expressed lower levels of 65 kD (now referred to as type I receptors), and higher levels of 280 kD (type III receptors) binding proteins relative to proliferating, subconfluent SMC. Levels of 85-kD (type II receptors) binding protein expression were similar in both groups. TGF-β inhibited growth of rat SMC at subconfluent densities but potentiated growth of SMC at confluent densities suggesting that these effects were mediated by differential TGF-β–binding protein expression. Taken together, these studies suggest that TGF-β receptor expression could play a role in determining growth responses of cultured cells to TGF-β and that this will be an important area of research for further understanding of TGF-β effects on SMC growth.

In the present studies, TGF-β delayed EGF-, bFGF-, or PDGF-BB–induced entry of SHR-derived SMC into S phase. Co-treatment with TGF-β markedly inhibited EGF-, bFGF-, or PDGF-BB–induced [^{3}H]thymidine incorporation when measured 15–16 h after treatment. This effect was transient, however, and TGF-β had no effect on EGF-, bFGF-, or PDGF-BB–induced growth over the initial 24 h of treatment. Reversible growth inhibitory effects of TGF-β have been seen in SMC, epithelial, endothelial and fibroblast cultures. These effects have been shown to be due to TGF-β interference with the ability of cells to traverse a stage in late G_1 phase. Using bovine or human SMC, Reddy and Haure (32) found that TGF-β inhibition of serum stimulated growth resulted from reversible arrest at a point in the cell cycle located 1–2 h from S phase. A more prolonged growth inhibitory activity of TGF-β has also been observed in serum stimulated SMC (11). Potential insight into cellular events associated with TGF-β–induced growth arrest was provided by recent studies by Koff et al. (33) They showed that TGF-β treated MLE cells failed to stably assemble cyclin E-Cdk2 complexes or accumulate cyclin E-associated kinase activity.

In summary, these studies provide further data regarding the effects of TGF-β on SMC growth. TGF-β (100 pM) elicited marked, delayed growth responses, both alone and in combination with EGF, bFGF or PDGF-BB. TGF-β enhanced SHR-derived SMC production of PDGF-AA which partially mediated TGF-β–induced growth. However, TGF-β markedly enhanced growth responses to EGF, bFGF, or PDGF-BB through mechanisms that were largely independent of PDGF-AA. Further studies will be required to identify the mechanisms whereby prolonged treatment with TGF-β enhances SMC growth resposiveness in cultured SMC and to determine whether similar effects occur in vivo.

Acknowledgements

This work was supported in part by Public Health Service grants RO1-HL-38854 and PO1-HL-19242 from the National Institutes of Health and by National Research Service Award Training Grant T32 HL07355.

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