**Beta-Type Transforming Growth Factor Specifies Organizational Behavior in Vascular Smooth Muscle Cell Cultures**

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**Abstract.** In culture, vascular smooth muscle cells (SMC) grow in a “hill-and-valley” (multilayered) pattern of organization. We have studied the growth, behavioral organization, and biosynthetic phenotype of rat aortic SMC exposed to purified platelet-derived growth regulatory molecules. We show that multilayered growth is not a constitutive feature of cultured SMC, and that beta-type transforming growth factor (TGF-β) is the primary determinant of multilayered growth and the hill-and-valley pattern of organization diagnostic for SMC in culture. TGF-β inhibited, in a dose-dependent manner, the serum- or platelet-derived growth factor-mediated proliferation of these cells in two-dimensional culture, but only when cells were plated at subconfluent densities. The ability of TGF-β to inhibit SMC growth was inversely correlated to plating cell density. When SMC were plated at monolayer density (5 x 10⁴ cells/cm²) to allow maximal cell-to-cell contact, TGF-β potentiated cell growth. This differential response of SMC to TGF-β may contribute to the hill-and-valley pattern of organization. Unlike its effect on other cell types, TGF-β did not enhance the synthesis of fibronectin or its incorporation into the extracellular matrix. However, the synthesis of a number of other secreted proteins was altered by TGF-β treatment. SMC treated with TGF-β for 4 or 8 h secreted markedly enhanced amounts of an Mr 38,000-D protein doublet whose synthesis is known to be increased by heparin (another inhibitor of SMC growth), suggesting metabolic similarities between heparin- and TGF-β-mediated SMC growth inhibition. The data suggest that TGF-β may play an important and complex regulatory role in SMC proliferation and organization during development and after vascular injury.

**Materials and Methods**

**Cell Culture**

Rat aortic SMC were grown from explants as described (14), and were subcultured in Waymouth’s medium containing 10% FBS, 100 U/ml penicillin,
and 0.1 mg/ml streptomycin. Cells were maintained in an humidified 37°C, 5% CO₂ atmosphere and were utilized in the third to sixth passage. Serum-free medium (12) was prepared as a 1:1 mixture of DMEM and F12 media supplemented with 0.1 mg/ml BSA and 5 μg/ml transferrin. Cultures were photographed on a Fluovert inverted microscope (E. Leitz, Wetzlar, Federal Republic of Germany) using a ×4 or ×10 phase-contrast objective. For low-magnification photography, cells were stained with Coomassie Blue to aid visualization.

**Growth Factors**

PDGF was purchased from Bethesda Research Laboratories (Gaithersburg, MD). The PDGF was purified from fresh porcine platelets and was >90% pure by silver-stained SDS-PAGE. The PDGF preparations contained <0.2% TGF-β. Porcine platelet TGF-β was purchased from R&D Systems, Inc. (Minneapolis, MN), and was shown to be >96% pure by silver-stained SDS-PAGE, amino acid analysis, and NH₂-terminus sequencing. The half-maximal activity of colony formation in soft agar (28) was determined to be 1 ng/ml.

**Cell Growth Assays**

Cell growth assays were performed in triplicate in 16-mm wells (12-well plates). Except where noted, cells were plated at 5 × 10⁴ cells/cm² in 5% FBS and allowed to attach and spread for 24 h before addition of test medium. At the time of harvest, medium was removed, cells were washed twice in PBS, and 1.0 ml of 0.4% trypsin/EDTA was added to each well. Cell numbers were determined electronically on a model ZM Coulter counter (Coulter Electronics, Inc., Hialeah, FL). All tissue culture chemicals were obtained from Gibco (Grand Island, NY). Where data are expressed as percent inhibition of growth, values were derived according to this formula: % inhibition = [1 - (net growth of treated cells)/(net growth of control cells)] × 100, where the net growth of cells represents the difference between the plating density and the final cell density.

**SDS-PAGE**

SMC were grown in 5% FBS in 35-mm diam tissue culture wells and were treated with 10 ng/ml TGF-β as indicated. Metabolic labelings were performed by culturing cells for 2 h in serum-free medium lacking methionine in the presence of 50 μCi/ml [³⁵S]methionine (New England Nuclear, Boston MA). Radiolabeled culture medium proteins were harvested into protease inhibitors at 4°C to yield a final concentration of 25 mM EDTA, 1 mM N-ethylmaleimide, 45 μg/ml pepstatin, and 0.9 mM phenylmethylsulfonyl fluoride. Proteins were precipitated in 10% trichloroacetic acid, dissolved in SDS-PAGE sample buffer containing 50 mM dithiothreitol, and resolved on 6% or 10% polyacrylamide gels. Cell layer proteins were washed twice with PBS, lysed in 95°C sample buffer containing 50 mM dithiothreitol, and resolved on 6% or 10% polyacrylamide gels. Gels were fixed, dried, and exposed to film at -70°C. The position of migration of fibronectin was determined by immunoprecipitation as described (15). Molecular mass estimates were derived from comparisons with the positions of migration of known molecular mass standards.

**Figure 1.** Kinetics of SMC growth in 5% FBS and in response to PDGF. SMC were plated in 5% FBS at 5 × 10⁴ cells/cm² in triplicate 16-mm wells and allowed to attach for 24 h. On day 0 (24 h after plating), cells were exposed to either (squares) fresh 5% FBS or (circles) 0.5% FBS supplemented with 10 ng/ml PDGF. Cells were fed fresh serum or PDGF on day 4 and cells were counted electronically at 2-d intervals. Note that SMC proliferating in response to PDGF achieve a lower saturation density than do cells exposed to 5% FBS.

**Figure 2.** Morphologic appearance of SMC, at saturation density, after culture in PDGF or 5% FBS. SMC were cultured as described in the legend to Fig. 1. On day 8, cultures were fixed in buffered 3% paraformaldehyde, stained with Coomassie Blue, and photographed. (a) SMC cultured in 0.5% FBS supplemented with 10 ng/ml PDGF. (b) SMC cultured in 5% FBS. Note that cultures exposed to PDGF reach monolayer saturation density and never exhibit multilayered growth or a "hill-and-valley" pattern of organization. Cells grown in 5% FBS, in contrast, are multilayered and present a hill-and-valley appearance typical of cultured SMC. Bar, 200 μm.
Results

Growth Kinetics and Morphologic Organization of SMC Cultured in Serum or PDGF

SMC proliferate in culture in response to FBS or in response to appropriate purified growth factors in the presence of "maintenance" levels of serum or plasma. Cells become quiescent if maintained in serum-free medium or in 0.5% FBS for periods of 48-72 h (15, 16). However, in the additional presence of 10 ng/ml PDGF (sufficient to elicit a substantial mitogenic response [16]), proliferation continues albeit at a slower rate than for SMC maintained in FBS (Fig. 1). SMC grown in low serum and PDGF reached saturation densities of 1-2 × 10^6 cells/cm^2, whereas cells grown in FBS reached threefold greater densities (5 × 10^6 cells/cm^2). The morphologic appearance, at confluency, of cultures grown under these conditions is presented in Fig. 2. As shown, cells cultured in FBS grew to overconfluence and organized into the hill-and-valley pattern characteristic of vascular SMC in culture. In contrast, SMC maintained in 0.5% FBS and PDGF reached only monolayer density and never exhibited multilayered growth. We conclude that multilayered growth is not a constitutive feature of vascular SMC in vitro, and that a factor in serum, other than PDGF, is the primary determinant of this growth pattern. We have investigated the possibility that this determinant is TGF-β.

Effects of TGF-β on SMC Growth and Organization in Culture

As shown previously (2), TGF-β is a potent inhibitor of bovine aortic SMC growth in vitro, when given in concert with 5% FBS or in platelet-poor plasma supplemented with epidermal growth factor (EGF). We have used a 4-d cell number assay (described in Materials and Methods) to assess the effects of increasing amounts of TGF-β on the proliferation of SMC prepared from the rat aorta. TGF-β was found to be a potent inhibitor of SMC proliferating in response to 5% FBS (not shown) or 0.5% FBS supplemented with 10 ng/ml PDGF (Fig. 3), with a half-maximal effect observed at concentrations <1 ng/ml. A maximal effect (>70% inhibition) was seen at concentrations ≥10 ng/ml (Fig. 3). TGF-β did not stimulate SMC proliferation when added alone (at 10 ng/ml) to cells maintained in 0.5% FBS.

The morphologic appearance of control and TGF-β-inhibited cells was determined, as part of our dose-response assays (Fig. 3), on day 4. As shown in Fig. 4a, control cells (proliferating in response to PDGF alone) grew to a monolayer and exhibited a "cobblestone" pattern of organization. In contrast, TGF-β-treated SMC organized into multilayered foci concomitant with a markedly reduced final cell density.

\[ \text{Figure 3. Effects of TGF-β on the proliferation of SMC in vitro.} \]

SMC were plated in 5% FBS at 5 × 10^3 cells/cm^2 in triplicate 16-mm wells and allowed to attach for 24 h. On day 0 (24 h after plating), the cultures received 0.5% FBS supplemented with 10 ng/ml PDGF alone or in concert with increasing amounts of TGF-β. Cells were counted electronically on (squares) day 0 or (circles) day 4. Note that TGF-β-treated SMC show marked growth inhibition.

\[ \text{Figure 4. Morphologic appearance of control and TGF-β-treated SMC.} \]

SMC were cultured as described in the legend to Fig. 1. On day 4, cultures were fixed in buffered 3% paraformaldehyde, stained with Coomassie Blue, and photographed. (a) SMC cultured in 0.5% FBS supplemented with 10 ng/ml PDGF. (b) SMC cultured in 0.5% FBS supplemented with 10 ng/ml PDGF and 10 ng/ml TGF-β. Note that TGF-β-treated SMC, despite marked growth inhibition, organized into multilayered aggregates ("hills") similar to those formed by SMC proliferating in response to 5% FBS. Bar, 200 μm.
(Fig. 4 b). In the dose-response assays, inhibition of SMC growth by TGF-β correlated with the ability of the cultures to form multilayers.

Organizational Behavior of SMC in Response to PDGF and TGF-β

To further investigate the effects of TGF-β on multilayered growth of SMC, we studied the organizational behavior of SMC maintained in a defined media (12), to eliminate possible combinatorial effects of TGF-β with factors present in 0.5% FBS. After a 48-h incubation in defined medium, growth factors were added singly or in combination, and organizational morphologies were examined at subconfluent or confluent densities. We have previously shown that the defined medium used for these experiments is sufficient to support the mitogenic response of SMC to PDGF (16).

As shown in Fig. 5, SMC maintained in defined medium supplemented with PDGF and/or TGF-β exhibited different organizational behaviors. After 4–8 d of serum-free culture, SMC appeared randomly dispersed on the culture surface; cells were moderately spread and ovoid rather than spindle-shaped (not shown). In contrast, cells treated with TGF-β alone were not randomly distributed over the culture dish but instead were organized into “fascicles” of cells, often with the outermost cells aligned circumferentially around the perimeter of the fascicle, enclosing an innermost group of randomly oriented cells (Fig. 5 a). Typically, this type of organizational behavior was observed within 4 d after addition of TGF-β. Cells maintained in TGF-β did not proliferate and did not reach confluent densities. SMC treated with PDGF alone appeared, at sparse density, as randomly spread, individual cells with a more spindle-shaped morphology (not shown). At confluence, PDGF-treated cells exhibited a monolayered, “cobblestone” pattern (Fig. 5 b), identical to that illustrated above (Fig. 2 a) for SMC proliferating in PDGF.

![Figure 5](image)
and 0.5% FBS. When SMC were treated with PDGF and TGF-β in concert, cells appeared more elongate and aggregated into multilayered foci, even at low density (Fig. 5, c and d). In confluent areas of the cultures, cells organized into distinct multilayered nodules and exhibited the hill-and-valley organization diagnostic for SMC in culture (Fig. 5 e).

We therefore conclude, on the basis of these data, that TGF-β specifies the multilayered pattern of growth of cultured aortic SMC.

**Differential Effects of TGF-β on SMC Proliferation**

To explain the apparent paradox between the ability of TGF-β to inhibit the growth of SMC cultures while simultaneously promoting the formation of multilayered nodules, we hypothesized that TGF-β may exert differential, density-dependent effects on SMC proliferation. To test this possibility, SMC were plated at 1 × 10^4, 5 × 10^3, 1 × 10^4, or 5 × 10^4 cells/cm^2 in either 16-mm wells (for growth studies) or in 35-mm wells (for morphologic studies). Cells were grown in 5% FBS in the absence or presence of 10 ng/ml TGF-β. As presented in Fig. 6, TGF-β was a potent inhibitor of the growth of sparsely plated SMC. However, as plating density increased, the efficacy of TGF-β as a growth inhibitor was decreased. The proliferation of cells plated at monolayer density (5 × 10^4 cells/cm^2) was augmented by TGF-β. The morphologic appearance of SMC cultures after TGF-β treatment under these conditions is also presented in Fig. 6. These data, which imply a selective ability of TGF-β to stimulate the growth of cells already in aggregated conditions (in close contact with their neighbors), suggest a plausible mechanism by which TGF-β may promote the hill-and-valley pattern of organization.

**Effects of TGF-β on the SMC Biosynthetic Phenotype**

Previous studies that used other cell types (11, 23) have suggested that TGF-β may exert certain of its growth-regulatory effects via enhancement of fibronectin synthesis. Fibronectin has also been implicated in the formation of "nodules" in cultures of vascular SMC (3). These observations led us to investigate a possible role for fibronectin in the TGF-β-mediated effects described above. First, we used metabolic labeling techniques to determine comparative levels of fibronectin synthesis in control and TGF-β-treated SMC. Control cells and cells treated with 10 ng/ml TGF-β for 0–46 h were metabolically labeled for 2 h with [35S]methionine in the continued presence of TGF-β. Radiolabeled secreted and cell layer proteins were resolved by SDS-PAGE and visualized by autoradiography. No significant increase was noted in fibronectin synthesis (identified by specific immunoprecipitation) at any time point studied (Fig. 7). We also employed immunofluorescence techniques to detect possible differences in the distribution of fibronectin in control vs. TGF-β-treated SMC. Control cells and cells cultured in 0.5% FBS supplemented with 10 ng/ml TGF-β, 10 ng/ml PDGF, or both factors for 72 h, then were fixed and stained with a goat antibody to rat fibronectin as previously described (14). No differences were observed in the distribution of fibronectin in the SMC matrix or on the cell surface under these three conditions. We predict that the TGF-β-mediated morphologic changes described above are due to determinants other than fibronectin.

Fig. 7 presents an SDS-PAGE analysis of the SMC biosynthetic phenotype after treatment of cells with TGF-β for 0, 2, 4, 8, or 24 h. No differences in protein synthesis were detected in cell layer samples; these data are not presented. Several differences in the relative production of secreted proteins were noted. We observed selective increases in proteins with M, 90,000, 38,000, and 22,000 D and a decrease in proteins with M, 92,000 and 55,000 D. These proteins are of particular interest in view of the regulation of their production in other culture conditions. For example, PDGF treat-
Figure 7. Effects of TGF-β on the SMC biosynthetic phenotype. SMC were plated in 5% FBS and allowed to reach subconfluent densities in 35-mm wells. Fresh medium supplemented with 5% FBS and 10 ng/ml TGF-β was added to experimental wells at appropriate times. Cultures were subsequently metabolically labeled by incubation for 2 h in [35S]methionine-labeling medium with or without fresh TGF-β. Labeled secreted proteins were harvested and resolved as described in Materials and Methods. The indicated times represent the total length of time cells were exposed to TGF-β, including the 2-h labeling period. Molecular masses of specific regulated proteins (>10^3 D) are shown to the right. Note that TGF-β did not stimulate synthesis of fibronectin (FN). SMC secreted increased amounts of an M_92,000-D protein and decreased amounts of proteins with M_92,000 and 55,000 D as a function of time of exposure to TGF-β. SMC treated with TGF-β for 4 or 8 h secreted increased amounts of an M_38,000-D protein doublet whose synthesis is also regulated by heparin (13). Synthesis of an M_22,000-D protein was enhanced at 8 h but was unchanged at other time points examined.

Discussion

In culture, vascular SMC exhibit a characteristic "hill-and-valley" pattern of organization. This growth pattern has been extensively described in SMC cultures derived from a variety of species (5, 9, 30) and is considered a diagnostic feature of these cells in culture (5). In this report we show that the ability to organize into multilayers is not a constitutive feature of SMC in vitro. Rather, multilayered growth appears to be specified by the response of these cells to TGF-β, a recently described regulatory peptide that controls the growth and differentiation of a variety of cell types (10, 18, 19, 27). TGF-β is found in platelets (1, 2, 6, 22, 23) in concentrations roughly equivalent to that of PDGF (1). The effects of TGF-β on arterial SMC function may therefore be of considerable importance in vascular biology given the probable involvement of platelet products in atherogenesis, wound healing, and other vessel wall pathologies (25). Our data suggest that TGF-β may regulate a number of SMC functions including proliferation (2), behavioral organization, and biosynthetic phenotype.

The formation and maintenance of contact-inhibited monolayer cultures has, until recently, been considered a feature exclusive (among vascular cells) to cultured endothelium. Although clonal variability in organizational behavior of cultured SMC has been described (17), Walker et al. (29) have recently provided one example of a population of SMC that exhibits monolayered growth. In their studies, phenotypically distinct SMC populations were cultured from the intima and media of carotid arteries after injury. Intimal-derived SMC secreted increased amounts of PDGF-like molecules, possessed fewer PDGF receptors, and exhibited a "cobblestone" monolayer growth pattern similar to that of aortic media-derived SMC proliferating in response to PDGF alone (Figs. 2 and 4). Similar results have been described by Seifert et al. (26) for neonatal SMC. The data imply that an additional characteristic of neonatal or neonatal SMC proliferating in vivo may be a lack of responsiveness to TGF-β, a possibility which is currently being investigated. Multilayered growth therefore does not appear to be a constitutive feature of cultured arterial SMC. Rather, the organizational behavior of SMC in culture may vary according to the type of growth-regulatory molecules presented to the cells. The tendency to exhibit multilayered growth undoubtedly reflects an ability of TGF-β to differentially regulate SMC growth as well as an increased ability of SMC to interact, in a nonpolarized fashion, with all surfaces of its neighboring cells. This ability may be an important requisite for the formation of three-dimensional structures at appropriate time points during development or tissue repair.

In other cell systems (11, 23), TGF-β appears to exert its growth-regulatory effects via stimulation of synthesis of fibronectin, a ubiquitous cell surface and extracellular matrix protein. SMC treated with TGF-β for up to 48 h showed no alteration in fibronectin synthesis or cell surface distribution of immunoreactive protein. Although our data cannot rule out subtle molecular alterations in fibronectin structure, we predict that the effects of TGF-β on SMC growth and behavioral organization are mediated by a determinant other than fibronectin.

Other effects of TGF-β on the SMC biosynthetic phenotype were observed in our studies. Of particular interest is the TGF-β-mediated increase in synthesis of a secreted M_38,000-D protein. This protein appears identical to an M_38,000-D protein whose synthesis and secretion is markedly enhanced by treatment of rat SMC with heparin (13), a potent inhibitor of SMC migration (14), and proliferation (4, 8). The characteristics of regulation of this protein by heparin have been fully described (13). In contrast to the very rapid induction of secretion of this protein by heparin, enhanced production of the M_38,000-D protein was not ob-
served until 4 h of treatment with TGF-β. Nevertheless, the data suggest a common metabolic relationship between the mechanisms of heparin- and TGF-β-mediated growth inhibition. Finally, the Mr 38,000-D protein may relate to a protein of similar size secreted by swine aortic SMC after formation of “nodules” (multilayered aggregates) in culture (20, 21). Our data predict potentially important relationships among TGF-β, SMC growth inhibition in general, the formation of multilayered aggregates (“nodules”) of SMC, and expression of an Mr 38,000-D secreted protein.

The author gratefully acknowledges the excellent secretarial and technical assistance provided by Jeanne L. Obreiter and Christine K. Castle.

Received for publication 19 December 1986, and in revised form 13 March 1987.

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