

Cell Surface Thrombospondin Is Functionally Essential for Vascular Smooth Muscle Cell Proliferation

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Abstract. Thrombospondin (TS) is an extracellular glycoprotein whose synthesis and secretion by vascular smooth muscle cells (SMC) is regulated by platelet-derived growth factor. We have used a panel of five monoclonal antibodies against TS to determine an essential role for thrombospondin in the proliferation of cultured rat aortic SMC. All five monoclonal antibodies inhibited SMC growth in 3-d and extended cell number assays; the growth inhibition was specific for anti-TS IgG. The effects of one antibody (D4.6) were examined in detail and were found to be reversible and dose dependent. Cells treated with D4.6 at 50 μ g/ml (which resulted in a >60% reduction in cell number at day 8) were morphologically identical to control cells. D4.6-treated SMC were analyzed by flow cytometry and were found to be arrested in the G₁ phase of the cell cycle. To determine a possible cellular site of action of TS in cell growth, SMC were

examined by immunofluorescence using a polyclonal antibody against TS. TS was observed diffusely bound to the cell surface of serum- or platelet-derived growth factor-treated cells. The binding of TS to SMC was abolished in the presence of heparin, which prevents the binding of TS to cell surfaces and inhibits the growth of SMC. Monoclonal antibody D4.6, like heparin, largely abolished cell surface staining of TS but had no detectable effect on the cellular distribution of fibronectin. These results were corroborated by metabolic labeling experiments. We conclude that cell surface-associated TS is functionally essential for the proliferation of vascular SMC, and that this requirement is temporally located in the G₁ phase of the cell cycle. Agents that perturb the interaction of TS with the SMC surface, such as heparin, may inhibit SMC proliferation in this manner.

THROMBOSPONDIN (TS)¹ is a platelet- and cell-derived homotrimeric glycoprotein of *M*_r 450,000 (see Silverstein et al., 1986; Lawler 1986; Majack and Bornstein, 1987 for recent reviews). The complete amino acid sequence of thrombospondin has recently been deduced from sequencing cDNAs from endothelial (Lawler and Hynes, 1986) and fibroblast (Dixit et al., 1986b) libraries. TS is released from platelet alpha-granules upon activation and plays an essential role in the secretion-dependent phase of platelet aggregation (Leung, 1984). In addition, TS is secreted by a variety of cells in culture and may be a transient component of the extracellular matrix (see review by Majack and Bornstein, 1987). TS binds a variety of ligands including heparin (Lawler and Slayter, 1981; Dixit et al., 1984b), collagen (Lahav et al., 1982; Mumby et al., 1984), fibronectin (Lahav et al., 1982), fibrinogen (Dixit et al., 1984a), plasminogen (Silverstein et al., 1984, 1986), and plasminogen activator (Silverstein et al., 1985, 1986). The formation of a ternary complex among TS, plasminogen, and plasminogen activator may be of particular importance extravascularly for the facilitation

of plasmin generation in nonfibrin-containing environments such as the extracellular matrix (see review by Silverstein et al., 1986).

A variety of evidence suggests that TS may play an important role in the growth response of mesenchymal cells. In vascular smooth muscle cell (SMC) cultures, TS synthesis is regulated by platelet-derived growth factor (PDGF) (Majack et al., 1985). This regulation is effected at the mRNA level (Majack et al., 1987). The rapid induction of the TS message, together with its superinducibility in the combined presence of PDGF and cycloheximide, suggests that TS may be a member of the "competence" gene family that mediates the mitogenic response of cells to PDGF (Kelly et al., 1983; Cochran et al., 1983, 1984). Once secreted, TS binds to the SMC surface and extracellular matrix (Majack et al., 1985). TS plays a poorly understood role in the facilitation of the SMC response to epidermal growth factor (Majack et al., 1986). Finally, heparin-like molecules, which are potent inhibitors of SMC growth (Clowes and Karnovsky, 1977; Castellet et al., 1981), inhibit the binding of TS to the SMC surface (Majack et al., 1985) and prevent the TS/epidermal growth factor-mediated mitogenesis (Majack et al., 1986).

In this report we present antibody inhibition data support-

1. *Abbreviations used in this paper:* PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TS, thrombospondin.

ing the hypothesis that cell surface TS is functionally required for the proliferation of vascular SMC.

Materials and Methods

Cell Culture

Rat aortic SMC were grown from explants from the vessels of male Sprague-Dawley rats as described previously (Majack and Clowes, 1984). Cells were subcultured in Waymouth's medium supplemented with 5% FBS and were used in the third through sixth passage. All culture materials were purchased from Gibco (Grand Island, NY). Cultures were photographed with a Leitz Fluovert inverted microscope using phase-contrast optics.

Antibodies

Polyclonal and monoclonal antibodies against purified human platelet TS were prepared and characterized as previously described (Galvin et al., 1985; Dixit et al., 1985a, b, 1986a). Monoclonal antibody A2.5 recognizes an epitope on the heparin-binding domain of the TS molecule; A6.1 and A4.1 recognize the trypsin-resistant 70-kD core; C6.7 recognizes the platelet-binding domain; and D4.6 recognizes the 50-kD fragment containing the fibrinogen-binding domain. Antibodies A6.1 and D4.6 preferentially recognize TS prepared in the presence of EDTA and are thus conformation dependent. The monoclonal antibodies used in the experiments reported here were isolated on a protein A-sepharose affinity column (Bio-Rad Laboratories, Richmond, CA) and were stored as stock concentrations of 1–2 µg/ml. Antibodies were filter sterilized before use. The polyclonal antibody recognized all chymotryptic fragments of TS on Western blots. Antibody MIF11, against the T antigen of SV-40, was a generous gift of Drs. L. R. Gooding and Ann Berger. Dr. John MacDonald kindly provided the monoclonal antibodies (M-53 and N-294) against human fibronectin.

Growth Assays

All assays were performed in Waymouth's medium supplemented with 5% FBS with or without the indicated concentration of antibody. SMC were plated at densities of 5×10^3 cells/cm² in 16-mm-diam wells. Cells were counted electronically with a coulter counter (model ZM; Coulter Electronics, Luton, England) 24 h later (= day-0 control) and at the indicated time points. Antibodies were added to fresh culture medium 24 h after plating. Cultures were not refed for the duration of the experiment. All experiments were performed in triplicate wells and were done a minimum of three times. The percent inhibition of growth was derived by the following formula:

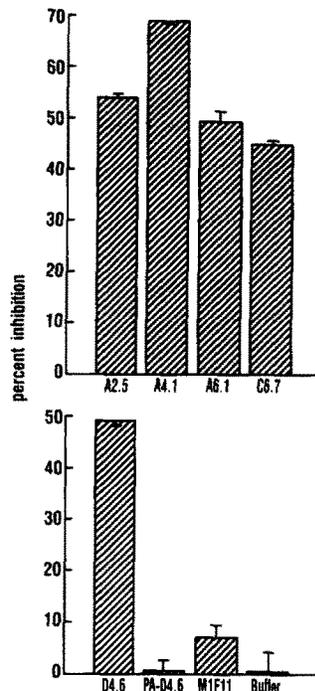
$$\% \text{ inhibition} = \left(1 - \frac{\text{net growth of treated cells}}{\text{net growth of control cells}} \right) \times 100.$$

Values for the net growth of cells were derived by subtracting the cell number after plating (at day 0) from the final experimental cell number (at day 3).

Flow Microfluorimetry

Flow cytometric analysis was performed on control cells on day 3 and on D4.6-treated cells on day 5 (see Results). Cells were harvested by mild trypsinization and prepared for analysis as described by Adams et al. (1981). Samples ($1-2 \times 10^6$ cells) were gently pelleted and resuspended in 3 ml cold Puck's saline without Mg²⁺ and glucose. Cells were then fixed by three rapid additions of ice-cold absolute ethanol, with vigorous pipetting between additions. Fixed samples were stored at 4°C, then were pelleted and resuspended at $1-2 \times 10^6$ cells/ml in a 0.9% NaCl, 15 mM MgCl₂ staining solution containing 50 µg mithramycin (Dome Laboratories, West Haven, CT) per ml. Mithramycin-stained cells were analyzed for DNA content using a FACS-II (Becton-Dickinson & Co., Sunnyvale, CA) and a nuclear data acquisition computer (model ND660; Nuclear Data, Inc., Schaumburg, IL). DNA histograms were analyzed mathematically for cell cycle phase distribution on an IBM 3031 computer using a program based on the multiple Gaussian model first proposed by Fried (1977). This method has been described in detail by Gray and Dean (1980) and was implemented here using the nonlinear estimation program NONLIN (Metzler et al., 1974).

Figure 1. Inhibition of SMC growth by monoclonal antibodies against TS. Rat aortic SMC were plated at 5×10^3 cells/cm², allowed to attach and spread for 24 h, then allowed to proliferate in 5% FBS in the absence or presence of monoclonal antibodies (at 50 µg/ml) against human platelet TS. Cell numbers were determined electronically at day 0 (24 h after plating) and at day 3. The percent inhibition of growth was determined as described in Materials and Methods. The inhibitory activity of D4.6 (bottom) was abolished by preabsorption of the antibody to TS-Sepharose (PA-D4.6). MIF11, a control antibody directed against the T antigen of SV-40, had no inhibitory effect. Similarly, cells treated with antibody vehicle alone (Buffer) or with monoclonal antibodies against fibronectin (see text) were not growth inhibited.



Immunofluorescence

SMC were plated at sparse or confluent densities on glass coverslips and allowed to attach and spread for 24 h. Cultures were left untreated or were exposed to 100 µg/ml heparin or 50 µg/ml D4.6 for 48 h. Cells were washed extensively in PBS, then were fixed for 10 min at 4°C in 2.5% formaldehyde. Under these conditions, cell membranes were left intact and only extracellular staining was observed. After several PBS washes, nonspecific binding sites were blocked with serum homologous to the species of origin of the secondary (labeled) antibody. Cells were exposed to a 1:10 dilution of primary antibody (polyclonal rabbit anti-human TS or polyclonal goat anti-rat fibronectin) for 60 min at room temperature. The fibronectin antiserum was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and immunoprecipitated a single radiolabeled band from SMC culture medium. FITC-labeled secondary antibodies were used at 1:500 dilutions. Cells were photographed on a Zeiss Photomicroscope II using fluorescence or phase-contrast imaging.

SDS-PAGE

SMC were plated at sparse density and allowed to attach and spread for 24 h. Metabolic labeling was performed by culturing cells with 40–60 µCi/ml [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) in Waymouth's medium lacking methionine and supplemented with 0.1 mg/ml BSA and 100 µg/ml heparin or 50 µg/ml D4.6 as indicated. Metabolically labeled cell layers and culture medium were harvested into protease inhibitors as previously described (Majack et al., 1985). Radiolabeled proteins were precipitated in 10% trichloroacetic acid, dissolved in Laemmli sample buffer, neutralized as required with 0.5M NaOH, and resolved on 6% polyacrylamide gels in the presence of 50 mM dithiothreitol. Gels were fixed, dried, and exposed to x-ray film.

Results

We have used a well-characterized panel of monoclonal antibodies against human platelet TS (Galvin et al., 1985; Dixit

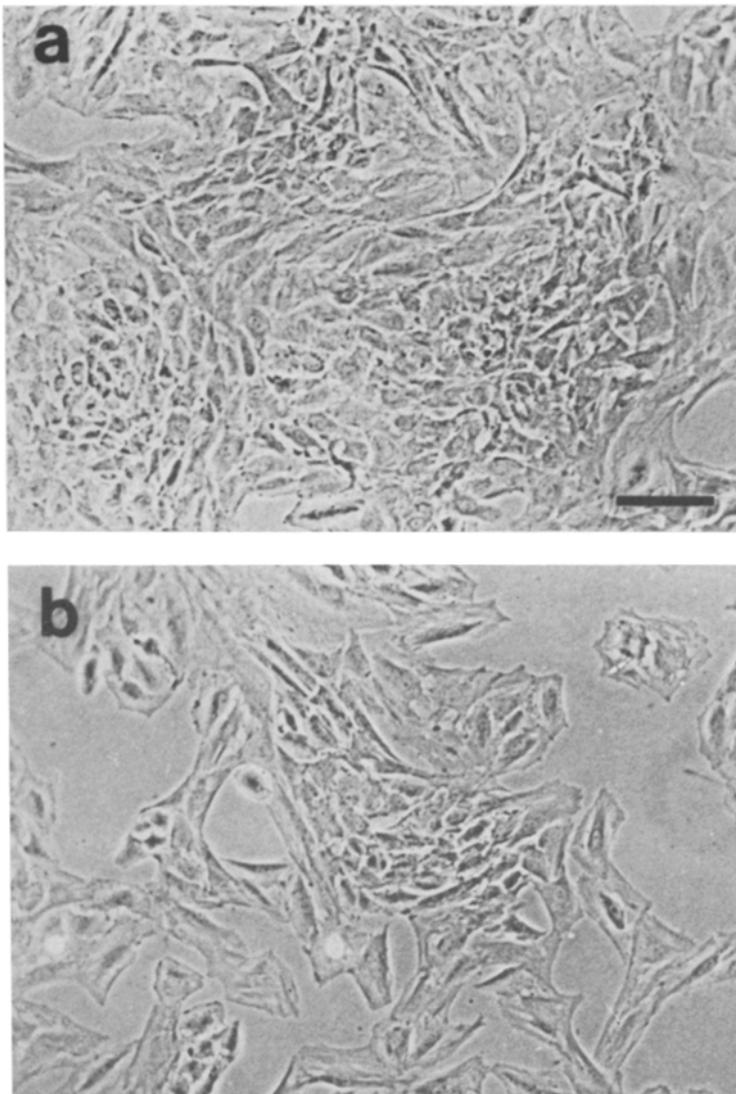


Figure 2. Morphological appearance of control (*a*) and D4.6-treated SMC (*b*). SMC were plated at 5×10^3 cells/cm², allowed to attach and spread for 24 h, then were grown for 6 d in 5% FBS with or without 50 μ g/ml D4.6. Antibody-treated cells (*b*) showed a reduced cell number but exhibited no evidence of cell detachment, cell rounding, or cytotoxicity. Bar, 100 μ m.

et al., 1985*a, b*, 1986*a*) to assess the role of TS in SMC growth. SMC were plated at a density of 5×10^3 cells/cm² and allowed to proliferate for 3 d (day 0 of the experiment is 24 h after plating) in medium supplemented with 5% FBS. Preliminary assays established that, under these conditions, SMC exhibited a >10-fold increase in cell number without surpassing monolayer density (i.e., the cultures were not multilayered). Monoclonal antibodies were tested initially at 50 μ g/ml. Fig. 1 presents a compilation of data from a series of experiments (*n* always >3) expressed as percent inhibition of growth (see Materials and Methods). Five monoclonal antibodies (A2.5, A4.1, A6.1, C6.7, and D4.6) were tested for growth-inhibitory activity in 3-d assays; inhibitory activity ranged from 45 to almost 70%. The four antibodies shown in the Fig. 1 (*top*) inhibited growth but also, in separate assays, inhibited SMC adhesion and/or spreading on TS substrates (Varani, J., personal communication). Only D4.6 had no significant effect on cellular adhesion or spreading (see also Varani et al., 1986; Roberts et al., 1987). We there-

fore chose to study the growth-regulatory effects of D4.6 in greater detail.

As presented in Fig. 1 (*bottom*) a number of controls convinced us that the effects of these antibodies were TS specific. Preabsorption of D4.6 on a TS-sepharose affinity column removed the inhibitory activity. Similarly, addition of an irrelevant IgG antibody (M1F11, against the Big T antigen of SV-40) or the vehicle buffer alone did not inhibit SMC growth. In addition, two monoclonal antibodies against plasma fibronectin (known to interact with fibronectin in extracellular matrices) had little (N-294) or no (M-53) growth-inhibitory activity (not shown). SMC treated with D4.6 showed no morphological alterations and remained fully spread and attached (Fig. 2). While differences in cell density were obvious, no noticeable alterations in behavioral organization (i.e., cell grouping or multilayering) were observed.

The effects of D4.6 were concentration dependent over a range of 1–50 μ g/ml (Fig. 3). Addition of 100 μ g/ml antibody resulted in a slightly greater inhibitory effect. However, at

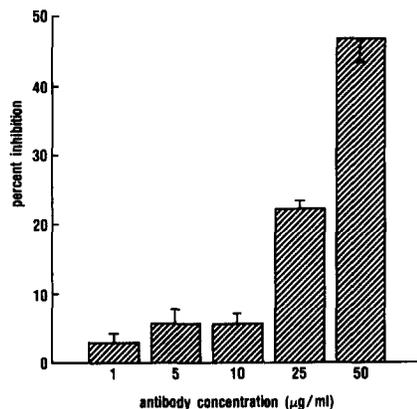


Figure 3. Concentration-dependent inhibition of SMC growth by D4.6. Cell number assays were performed as described in the legend to Fig. 1 and the percent inhibition of growth was determined as described in Materials and Methods. SMC were treated with increasing amounts of D4.6 (1–50 µg/ml) and showed a dose-dependent inhibition of growth over the 3-d assay period.

this concentration, SMC showed some rounding and detachment. We therefore used a concentration of 50 µg/ml for subsequent experiments.

Fig. 4 illustrates the growth-inhibitory effects of 50 µg/ml D4.6 as a function of time. An inhibitory effect was observed at 1 d after addition of antibody and increased with time (to nearly 70% inhibition at day 8). Note that all previous experiments were 3 d in duration when the inhibition is somewhat less.

Flow microfluorimetry was used to generate DNA histograms of control and D4.6-inhibited cells, to determine the cell cycle compartment affected by D4.6. Control cells were examined at day 3, while D4.6-treated cells were examined

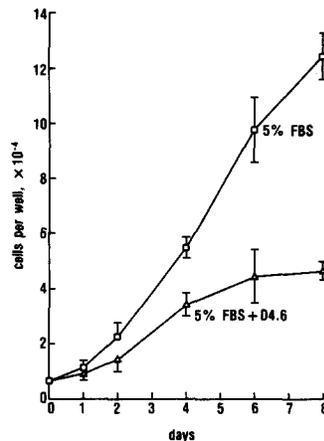


Figure 4. Time course of SMC growth inhibition by D4.6. SMC were plated at 5×10^3 cells/cm², allowed to attach and spread for 24 h, then were grown in 5% FBS for 8 d in the absence (□) or presence (Δ) of 50 µg/ml D4.6. Cell numbers were determined electronically at the indicated time points. D4.6-treated SMC showed a markedly reduced growth rate and final cell density and were essentially quiescent by 6 d of treatment.

at day 5. As shown in Fig. 4, under these conditions cell densities are identical, control cells are in exponential growth, and D4.6-treated cells are nearly quiescent. D4.6-treated SMC showed a 36% decrease in S phase and G₂/M phase cells and a concomitant increase in G₁ cells (not shown), suggesting that growth arrest occurs in the G₁ phase of the cell cycle.

We next used immunofluorescence techniques to determine the distribution of TS in SMC cultures. SMC at several densities were examined after formaldehyde fixation and staining with a polyclonal antibody against TS. Because this fixation protocol does not disrupt the integrity of the cell membrane, the staining observed reflects only extracellular (cell surface) TS. As shown in Fig. 5 *a*, immunostaining of SMC near monolayer confluency revealed a uniform distribution of TS over the cell surface. As previously reported for methanol-fixed, permeabilized SMC (Majack et al., 1985), the staining was dependent upon exposure of the cells to se-

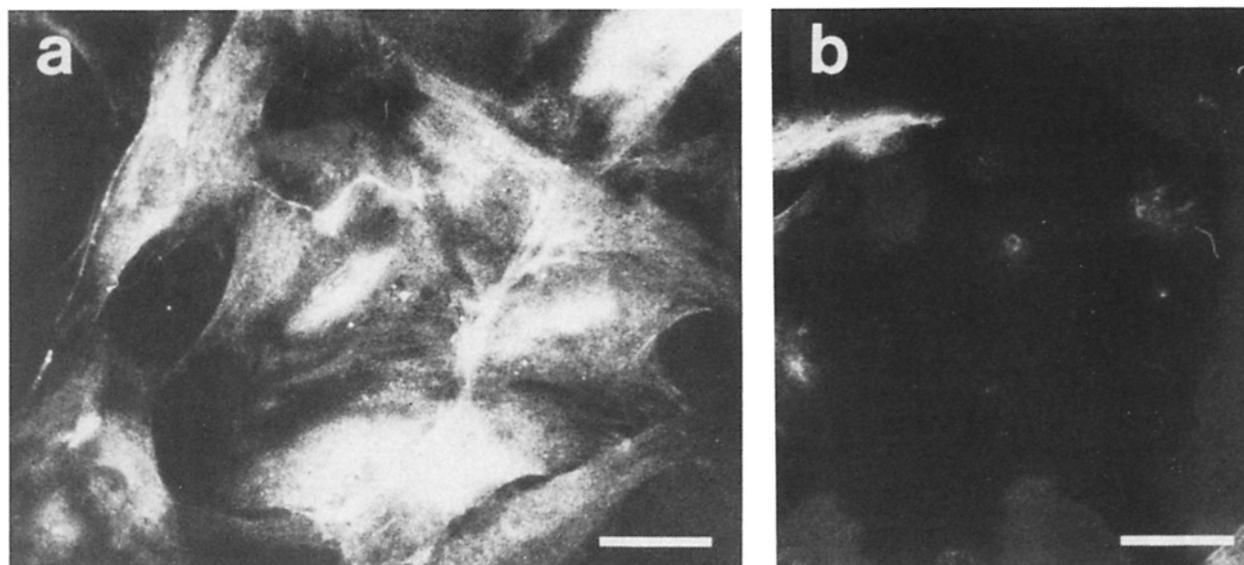


Figure 5. Immunofluorescence localization of TS in SMC cultures. The distribution of TS on SMC surfaces was examined using immunofluorescence techniques as described in Materials and Methods. (*a*) Uniform distribution of TS on the SMC surface under control culture conditions. (*b*) Lack of immunostaining for TS on SMC (at the same cell density as in *a*) treated with 100 µg/ml heparin for 48 h before fixation. Cells maintained in 0.5% FBS or stained with secondary antibody only also exhibited no cell surface staining. Bars, 50 µm.

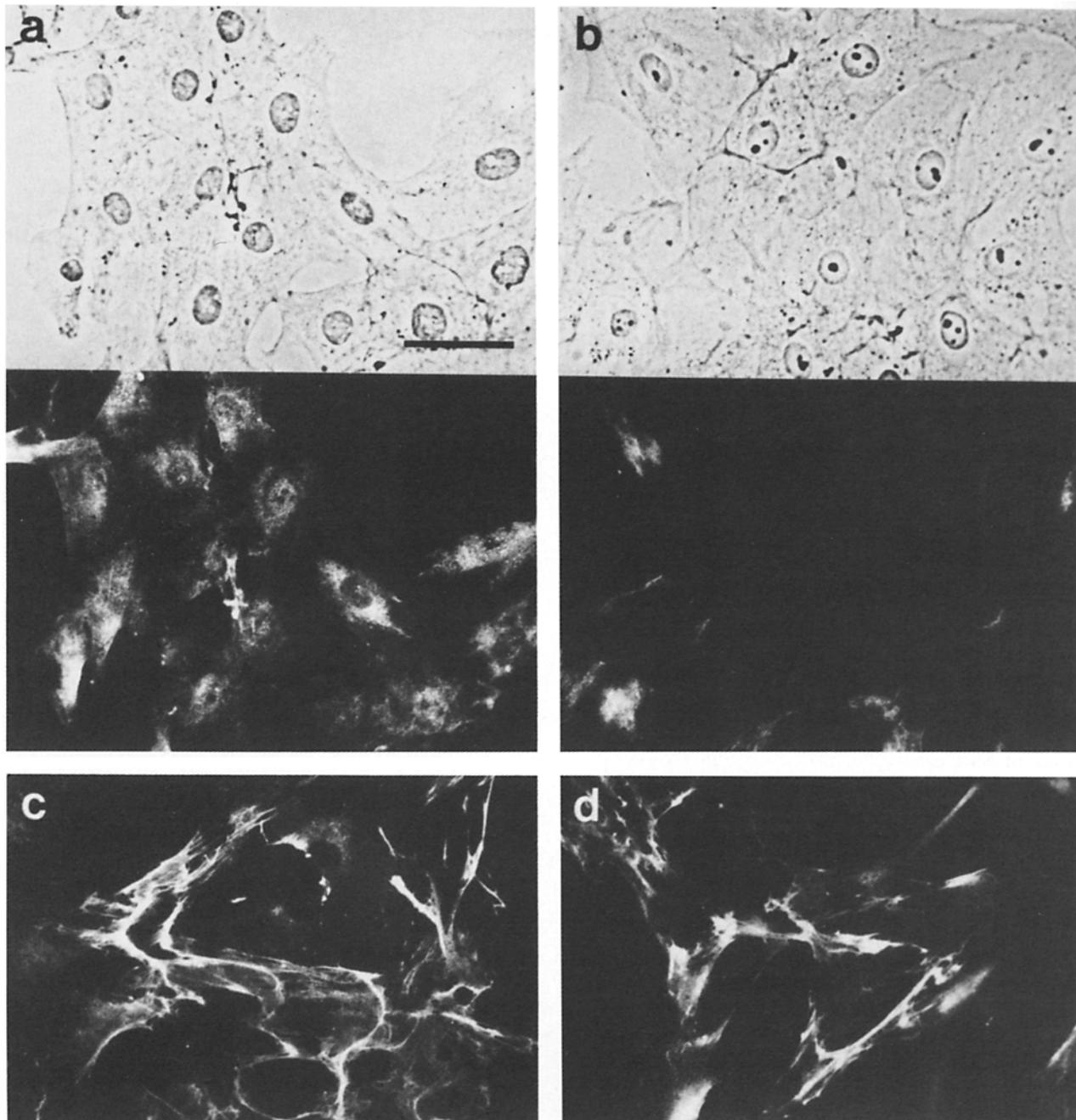


Figure 6. Immunofluorescence distribution of TS and fibronectin in control and D4.6-treated SMC. The distribution of TS and fibronectin on the cell surfaces of SMC cultured in the absence or presence of D4.6 was examined using immunofluorescence techniques as described in Materials and Methods. (a) Paired phase-contrast and immunofluorescence photographs of control SMC showing extensive surface staining for TS. (b) Paired phase-contrast and immunofluorescence photographs of SMC treated for 48 h with 50 $\mu\text{g}/\text{ml}$ D4.6, showing a marked reduction in the amount of surface-associated TS. (c) Distribution of fibronectin on the surface of control SMC. (d) Distribution of fibronectin on the surface of D4.6-treated SMC. Note that D4.6 reduces the levels of stainable TS on the SMC surface without affecting the cell surface distribution of fibronectin. Bar, 50 μm .

rum or PDGF and could be markedly reduced by preaddition of heparin to the culture medium (Fig. 5 b). No immunofluorescence was observed if the primary antibody was omitted from the staining protocol or if the primary antibody was preabsorbed to purified TS.

Control and D4.6-arrested cells (day 5) were stained with a polyclonal antibody against TS or with a polyclonal anti-

body against fibronectin. The TS antibody used recognizes all chymotryptic fragments of TS on Western blots and should, therefore, detect TS immunologically even in the presence of bound D4.6. As presented in Fig. 6, D4.6-treated SMC possessed significantly less stainable TS on their cell surfaces than did control cells. Thus D4.6 appears to mimic heparin in its ability to reduce cell surface TS (see Fig. 5 b).

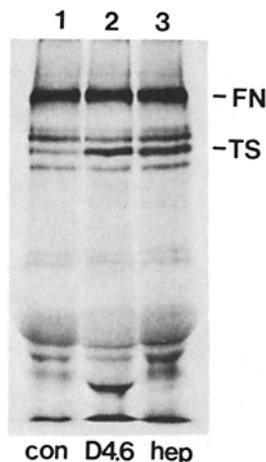


Figure 7. Effects of D4.6 and heparin on levels of TS in SMC culture supernatant. Sparsely plated, proliferative SMC were metabolically labeled for 2 h with [³⁵S]methionine in the absence (lane 1, *con*) or presence of 50 µg/ml D4.6 (lane 2, *D4.6*) or 100 µg/ml heparin (lane 3, *hep*). Radiolabeled proteins from the culture supernatant were resolved by SDS-PAGE and visualized by autoradiography. The positions of migration of fibronectin (*FN*) and thrombospondin (*TS*) are indicated. Treatment of SMC with D4.6 or heparin increases relative levels of TS in the culture medium; both of these agents decrease the amount of immunodetectable TS on the SMC surface (see Fig. 5 and 6) and, concomitantly, inhibit SMC proliferation.

Duplicate wells were stained to visualize the distribution of fibronectin (Figure 6, *c* and *d*). There were no detectable differences in the relative amounts or distribution of cell surface fibronectin in control vs. D4.6-inhibited cells, suggesting that D4.6 does not inhibit proliferation through a generalized disruption of the extracellular matrix. Instead, the inhibitory effect appears to correlate with a reduced presence of TS on the SMC surface.

An alternate possible explanation for the reduction in surface TS staining after D4.6 treatment is that D4.6 may bind to surface-associated TS *in situ* and sterically inhibit the binding of the polyclonal antibody used for immunostaining. We have addressed this issue by metabolically labeling SMC with [³⁵S]methionine for 2 h in the absence or presence of D4.6 or heparin. Our previous work (Majack et al., 1985) documented the ability of heparin to block the incorporation of newly synthesized TS into the SMC matrix; a concurrent increase in the amount of labeled TS in the culture supernatant was a characteristic of this effect. As shown in Fig. 7, treatment of SMC with D4.6 or heparin resulted in increased levels of TS in the culture medium. Subsequent treatment of the D4.6-treated culture supernatant with protein A-Sepharose revealed that a majority of the secreted TS was associated with antibody. Cell layer-associated TS could not be visualized, in these experiments, by SDS-PAGE and autoradiography. These labeling data, together with the immunostaining data presented in Fig. 6, are fully consistent with the hypothesis that D4.6, like heparin, prevents the interaction of newly synthesized TS with the SMC surface.

Discussion

Extracellular or cell surface proteins appear to play important roles in the control of cellular proliferation. Membrane-associated growth-inhibitory proteins may function in density-dependent growth arrest (Lieberman and Glaser, 1981). Proteases secreted in response to growth factors (e.g., Gal and Gottesman, 1986) may play important roles in degradation of the extracellular matrix, a process believed essential

for cell proliferation. Other cell surface molecules, such as the receptorlike protooncogenes *fms* and *neu* (Manger et al., 1984; Drebin et al., 1985; Sherr et al., 1985; Stern et al., 1986) or the 83-kD protein described by Weiland et al. (1986), may play unknown but growth-facilitative roles. Components of the extracellular matrix, which may influence cell shape, adhesion, or spreading, also appear to exert growth-regulatory functions (Gospodarowicz and Ill, 1980). In this report we provide further evidence that TS, a PDGF-regulated cell surface protein, may play an important functional role in the proliferation of vascular SMC.

We have shown that a panel of monoclonal antibodies, which recognize distinct epitopes along the TS molecule, can inhibit the proliferation of SMC in culture. These data are an extension of earlier studies that indirectly suggested some growth-regulatory function for TS. Other data that imply such a role for TS include (*a*) the rapid induction of the TS mRNA and protein after stimulation of SMC with PDGF (Majack et al., 1985, 1987), (*b*) similarities in the effects of metabolic inhibitors on mRNA levels for TS (Majack et al., 1987) and other PDGF-induced growth-regulatory proteins such as *c-myc* (Kelly et al., 1983) and *c-fos* (Cochran et al., 1984), (*c*) the enhanced response of SMC to epidermal growth factor after exposure of the cells to purified TS (Majack et al., 1986), and (*d*) the apparent correlation between the inhibition of SMC growth by heparin (Clowes and Karnovsky, 1977; Castellot et al., 1981) and the ability of heparin to block the binding of TS to cell membranes (McKeown-Longo et al., 1984; Majack et al., 1985; Roberts et al., 1985). In this report we show that D4.6 reduces the association of TS with the SMC surface and concomitantly inhibits cellular proliferation. We propose that cell surface-associated TS is functionally essential for the proliferation of vascular SMC.

Monoclonal antibodies to five different epitopes along the TS molecule were roughly equivalent in their abilities to inhibit SMC growth (Fig. 1). This surprising observation does not allow us to conclude which domain(s) of the TS molecule is important for SMC growth. The observed data may result from the ability of TS to interact with cell surfaces via multiple sites, as hypothesized by Roberts et al. (1987). In their studies, antibodies A2.5, A6.1, and C6.7 all inhibited melanoma cell attachment and spreading. Similar data have been reported by Varani et al. (1986) for squamous carcinoma cells. The interaction of fibronectin with cell surfaces may also occur via multiple sites (Akiyama et al., 1985). Alternately, the data may reflect an ability of the antibodies to induce conformational changes in TS that affect the functional characteristics of the molecule, as shown previously for antibody A2.5 (Galvin et al., 1985). Little is known about the conformational state of TS *in vivo*. Our data suggest that the conformation of TS in cell culture conditions may be similar to that induced by EDTA, since the conformation-dependent antibodies A6.1 and D4.6 (Dixit et al. 1986a) are effective in preventing SMC proliferation. This contention is supported by the attachment data cited above (in which A6.1 was shown to be an effective inhibitor of cell attachment) and by immunolocalization studies using D4.6 as a primary antibody (personal observations).

What is the function of cell-associated TS and how might this function be involved in cell growth control? Silverstein and co-workers (1984, 1985, 1986) have shown that TS inter-

acts with plasminogen and plasminogen activator in a ternary complex that facilitates the formation of plasmin in nonfibrin-containing environments. Furthermore, the plasmin generated remains bound to TS and is protected from inactivation by plasmin inhibitors (Silverstein et al., 1986). TS may therefore play an important role in the extracellular matrix via its ability to facilitate the matrix degradation and remodeling that accompanies the proliferation of mesenchymal cells. This concept is consistent with other reports that have described a very short (<90 min) half-life of extracellular TS (McKeown-Longo et al., 1984), and with our unpublished data that suggest that surface-bound TS may be capped, presumably after use, and accumulated at the trailing edge of the cells. Further experimentation is clearly needed to clarify the functional role and metabolic fate of TS on the cell surface.

Finally, we feel that the data presented in this paper provide further evidence that heparin-like molecules may inhibit SMC growth through a mechanism involving TS (Majack et al., 1985, 1986). The molecular mechanism by which heparin inhibits SMC growth (Clowes and Karnovsky, 1977; Castellot et al., 1981) and migration (Majack and Clowes, 1984) is not known but is likely to be external given the small amount of input heparin internalized during binding assays (Castellot et al., 1985). After growth stimulation, SMC in culture produce a TS-rich extracellular matrix (Majack et al., 1985). In the presence of heparin, the incorporation of TS into the SMC surface and matrix is markedly inhibited (Majack et al., 1985). We now show that D4.6, like heparin, blocks the interaction of TS with SMC and, concomitantly, cell proliferation. The data suggest important growth-regulatory functions for TS interactively associated with cell surfaces.

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