Transforming Growth Factor- β Complexes With Thrombospondin

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Thrombospondin (TSP) was demonstrated to inhibit the growth of bovine aortic endothelial cells, an activity that was not neutralized by antibodies to TSP or by other agents that block TSP-cell interactions but that partially was reversed by a neutralizing antibody to transforming growth factor- β (TGF- β). Similar to TGF- β , TSP supported the growth of NRK-49F colonies in soft agar in a dose-dependent manner, which required epidermal growth factor and was neutralized by anti-TGF- β antibody. Chromatography of a TSP preparation did not separate the TGF- β -like NRK colony-forming activity from high molecular weight protein. However, when chromatography was performed at pH 11, this activity was dissociated from TSP. These results suggest that at least some growth modulating activities of TSP are due to TGF- β associated with TSP by strong non-covalent forces. Most of the active TGF- β released from platelets after degranulation was associated with TSP, as demonstrated by anti-TSP immunoaffinity and gel permeation chromatography. ^{125}I -TGF- β binds to purified TSP in an interaction that is specific in the sense that bound TGF- β could be displaced by TGF-depleted TSP but not significantly by native TSP, heparin, decorin, α_2 -macroglobulin, fibronectin, or albumin. Hence, TGF- β can bind to TSP, and the complex forms under physiological conditions. Furthermore, TSP-associated TGF- β is biologically active, and the binding of TGF- β to TSP may protect TGF- β from extracellular inactivators.

INTRODUCTION

Thrombospondin (TSP)¹ is a trimeric glycoprotein (M_r = 450,000) that is secreted from the alpha granules of stimulated platelets, synthesized by cultured cells, and is a transient component of extracellular matrices during wound repair, embryogenesis, and in neoplastic tissues (reviewed in Frazier, 1987; Mosher, 1990). Although TSP can serve as a substrate for the attachment of certain cell types, it appears to act primarily as a modulator of cell adhesion by causing a loss of focal adhesions (Murphy-Ullrich and Höök, 1989), a phenomenon that ap-

pears to be required for the progression of mitosis and cell migration. In addition, TSP has been reported to regulate the proliferation of different cell types. TSP was found to stimulate the proliferation of fibroblasts (Phan *et al.*, 1989) and to promote the growth of aortic smooth muscle cells in synergy with epidermal growth factor (Majack *et al.*, 1986). In contrast, TSP was reported to inhibit the proliferation of a mouse lung capillary endothelial cell line (Taraboletti *et al.*, 1990) and of bovine adrenal-cortical endothelial cells (Bagavandoss and Wilks, 1990). This spectrum of regulatory effects on the in vitro proliferation of different cell types is reminiscent of the growth stimulatory/inhibitory effects of transforming growth factor- β (TGF- β). This cytokine belongs to a family of relatively small polypeptides that act as potent modulators of cell growth and differentiation (reviewed in Barnard *et al.*, 1990; Massagué, 1990). Like TSP, TGF- β is stored in the alpha-granules of platelets and can be released on degranulation of these cells. In platelets, TGF- β occurs primarily

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¹ Abbreviations used: BAE, bovine aortic endothelial; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; IgG_{2a}, monoclonal anti-TSP IgG; TGF- β , transforming growth factor- β ; Tris, tris(hydroxymethyl)aminomethane; TSP, thrombospondin.

as an inactive precursor that is associated with a high molecular weight binding protein (Miyazono *et al.*, 1988). However, platelet releasates also contain an active form of TGF- β (Slivka and Loskutoff, 1991). Activation of the pro-form of TGF- β may represent a regulatory step controlling TGF- β activity. In addition, the availability of active TGF- β can be controlled through interactions with extracellular inactivators, such as α_2 -macroglobulin (O'Connor-McCourt and Wakefield, 1987) and the proteoglycan, decorin (Yamaguchi *et al.*, 1990).

We report that a major fraction of the active form of TGF- β released from platelets is associated with TSP. Furthermore, TSP purified from platelets contains associated TGF- β , which is responsible for at least part of the observed growth modulatory activities of the TSP preparations. TGF- β binds with a high affinity to TSP—an interaction that may represent an important mechanism in the balance of TGF- β activity.

MATERIALS AND METHODS

Materials

The following items were purchased: Dulbecco's modified Eagle's medium (DMEM; Cell-Gro, Mediatech, Herndon, VA); fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT); 500 μ g/ml trypsin-2.2 mM EDTA (GIBCO, Grand Island, NY); and bovine serum albumin (BSA), fucoidan, thrombin, nonimmune IgG, and heparin (Sigma Chemical, St. Louis, MO). Human platelet TGF- β 1 was obtained from either R and D Systems (Minneapolis, MN) or from Oncomembrane (Seattle, WA). Recombinant human TGF- β used in some experiments was generously provided by Dr. Daniel Twardzik (Oncogen, Seattle, WA). 125 I-platelet TGF- β was obtained from Biomedical Technologies, (Stoughton, MA), or 125 I-recombinant TGF- β was obtained from Dupont New England Nuclear (Boston, MA). Epidermal growth factor was a generous gift of Dr. B. Lynn Allen-Hoffmann (Department of Pathology, University of Wisconsin). Neutralizing antibodies to TGF- β were obtained from R and D Systems or Oncomembrane. Monoclonal antibodies to specific domains of TSP were gifts from Dr. Vishva Dixit (University of Michigan) and Dr. Deane Mosher (University of Wisconsin). A rabbit antibody to TSP was also provided by Dr. Mosher. Rabbit anti-human tenascin was obtained from Telios Pharmaceuticals (San Diego, CA). Human fibronectin was a gift of Dr. Deane Mosher.

TSP Purification

TSP was purified from fresh thrombin-stimulated human platelets purchased from the Birmingham American Red Cross by using heparin-Sepharose CL-6B (Pharmacia, Piscataway, NJ) affinity chromatography, followed by gel permeation chromatography on a column of A0.5m resin (Bio-Rad, Richmond, CA) (Murphy-Ullrich and Mosher, 1985). For preparations of "stripped" TSP (TGF- β free), the gel permeation step was performed at pH 11 in 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.15 M NaCl. Stripped TSP was then dialyzed against several changes of 0.01 M Tris-HCl, pH 7.35, 0.15 M NaCl, (TBS) with 0.1 mM CaCl. Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie blue or silver staining.

Cells

Bovine aortic endothelial (BAE) cells were provided by Dr. Robert Auerbach (Department of Zoology, University of Wisconsin) and had been characterized in Dr. Auerbach's laboratory. Cells were routinely

cultured in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine, and 20% FBS as previously described (Murphy-Ullrich and Höök, 1989). Clone 49F of NRK cells (American Type Culture Collection, Rockville, MD, CRL 1570) was provided by Dr. B. Lynn Allen-Hoffmann. Stocks were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine, and 10% heat-inactivated calf serum (Hyclone Laboratories) as described (Allen-Hoffmann *et al.*, 1988). All cells were negative when tested for mycoplasma.

NRK Colony Formation in Soft Agar

TGF- β activity was assayed by determining colony formation by NRK cells in soft agar according to a previously described method (Allen-Hoffmann *et al.*, 1988), except that assays were performed in 24-well tissue culture plates. The cells were incubated at 37°C, 5% CO₂ for 7 d, and the number of colonies > 62 μ m (>8–10 cells) in diameter was counted. Experiments were performed either in duplicate or triplicate as indicated in the figure legends.

BAE Cell Proliferation Assays

BAE cells were plated at 1000 cells/well in 1 ml of DMEM with 10% FBS in 24-well tissue culture plates. The cells were grown overnight and rinsed twice with serum-free DMEM. Subsequently, 1 ml of test substance in DMEM supplemented with 2.5% FBS was added to each well in triplicate (day 0). On days 2 and 4, cells received fresh aliquots of test sample in volumes <200 μ l without removing the original media. Cells were then incubated for 48 h. Culture media were removed and attached cells were harvested with 0.5 ml trypsin-EDTA. The number of harvested cells was determined with a model ZM Coulter Counter (Coulter Electronics, Hialeah, FL).

I^{125} -TGF- β Binding Assays

Binding of 125 I-TGF- β to immobilized TSP was determined using a solid-phase binding assay (Mooradian *et al.*, 1989). Briefly, 96-well Immulon 2 (Becton-Dickinson, Lincoln Park, NJ) plates were coated with either 2.5 pmol TSP or BSA in 50 μ l at 37°C for 16 h. Additional protein binding sites were blocked by incubation with 0.1% BSA in binding buffer (10 mM phosphate buffer, pH 7.4, 0.1 M NaCl, 0.1 mM CaCl) for 90 min at 37°C, and the wells were then washed once with washing buffer (binding buffer with 0.05% Triton-X 100). 125 I-TGF- β was incubated in binding buffer with the wells for 2 h at 37°C. Wells were washed twice, and bound radioactivity was counted in the isolated wells.

RESULTS

Preparations of TSP Inhibit the Growth of BAE Cells

In initial experiments, we tried to reproduce the reported effects of TSP as an inhibitor of endothelial cell growth. BAE cells were exposed to increasing concentrations of isolated TSP in media supplemented with 2.5% FBS for a period of up to 6 d, and the number of cells in the cultures was determined. In cultures treated with TSP, the proliferation of BAE cells was inhibited greatly, and BAE cell number substantially was reduced throughout the course of the assay relative to cultures grown in media with 2.5% FBS alone. There was no significant cell death observed, and the effects were reversible. The inhibition of BAE cell growth was dose-dependent (Figure 1). After 4 d in culture, an essentially total block of cell growth was observed with 30 μ g/ml TSP, and 50% inhibition of growth was achieved with 1–3 μ g/ml TSP.

Figure 1. Cell growth of BAE cells in response to TSP. BAE cells seeded at 1000 cells/well were grown overnight in 10% FBS, washed with DMEM, and then treated with varying concentrations of TSP in 2.5% FBS on days 0 and 2. Cell number was determined on day 4. Results are expressed as means of triplicate determinations $\pm$ SD.

In most subsequent experiments, 3 μ g/ml TSP was routinely used; this concentration of TSP inhibited cell growth to $43 \pm 12\%$ (mean $\pm$ SD) ($n = 15$) relative to cells grown in 2.5% FBS.

The Cell Growth-Regulating Activities of TSP Are Inhibited by Anti-TGF- β But Not by Anti-TSP

The growth-regulating activities of TSP resemble that of TGF- β (reviewed in Heimark *et al.*, 1986; Müller *et al.*, 1987; Takehara *et al.*, 1987; Barnard *et al.*, 1990; Massague, 1990), a protein that, like TSP, is stored in the alpha granules of platelets. A neutralizing antibody to TGF- β reversed the inhibitory effects of the TSP preparation on BAE cell growth in a concentration-dependent manner. The inhibitory activity of 3 μ g/ml TSP could be reduced by a maximum of 60% by 20 μ g/ml of anti-TGF- β neutralizing antibody (Figure 2). Higher concentrations of antibody caused no additional reversal of growth inhibition. Antibody alone had no effect on cell growth. Addition of antibodies to TSP did not affect the proliferation modulating activity of the TSP preparations (Figure 2). Antibodies tested included a rabbit polyclonal and several mouse monoclonal antibodies (Jaffe *et al.*, 1983; Majack *et al.*, 1988), one of which (A2.5) previously has been shown to block TSP-dependent disassembly of focal adhesion plaques (Murphy-Ullrich and Höök, 1989). Furthermore, heparin and fucoidan, which interfere with the binding of TSP to BAE cells (Murphy-Ullrich and Mosher, 1987), did not reduce the ability of TSP preparations to inhibit BAE proliferation. This suggested that the growth-inhibitory activity of TSP was at least partially due to a TGF- β -like substance present in these preparations. In other assays,

the presence of immunoreactive TGF- β could also be detected in an enzyme-linked immunosorbent assay in which the TSP preparation was adsorbed to microtiter wells and probed with anti-TGF- β antibody. From these assays, it was estimated that the TSP preparation contained the equivalent of 0.2 ng TGF- β /µg TSP.

TSP Preparations Support NRK Growth in Soft Agar

Perhaps the most specific assay for TGF- β is its ability to support colony formation by NRK cells in soft agar. Therefore, a number of TSP preparations were tested for their effects on the growth of NRK cells in soft agar. TSP preparations supported NRK cell colony formation in a dose-dependent manner (Figure 3A), which was maximal at ~ 10 μ g/ml of protein. The level of colony formation observed with 10 μ g added TSP corresponded to the level of colony formation obtained with 3 ng TGF- β ; however, the response level varied with different TSP preparations. This could reflect variability in amounts of TGF- β present in various TSP preparations. Colony formation stimulated by TSP preparations was dependent on the presence of epidermal growth factor, as is colony formation supported by TGF- β . NRK cell

Figure 2. Growth inhibition of BAE cells by TSP is partially reversed by anti-TGF- β antibody but not by anti-TSP antibody. BAE cells were seeded on 24-well plates as described in Figure 1 and grown for 4 d in the presence of 2.5% FBS, FBS + 3 μ g TSP, FBS + 3 μ g TSP + 20 μ g rabbit anti-TGF- β (R and D Systems), FBS + 3 μ g TSP + a 1:50 dilution of a rabbit anti-TSP antibody, or with FBS + antibodies. Fresh TSP and antibodies were added on day 2, and cell number was determined on day 4. Results are expressed as means of triplicate determinations $\pm$ SD.

colony formation promoted by TSP preparations could be blocked by a rabbit-neutralizing antibody against TGF- β (Figure 3B). During internal controls, nonimmune rabbit IgG had no effect on TGF- β activity, and an irrelevant antibody, rabbit anti-tenascin IgG, had no effect on activity in soft agar assays using TSP.

The TGF- β -Like Activity can be Dissociated From TSP

Isolated TSP was subjected to gel permeation chromatography under physiological conditions on an A0.5-M column, and the fractions collected were analyzed for protein content and TGF- β activity as measured by the ability to support NRK cell colony formation in soft agar. Colony formation activity largely coelutes with the leading portion of the peak of absorbance at 280 nm, corresponding to the TSP peak (Figure 4A). These data show that a TGF- β -like activity copurifies with TSP and suggest that TSP and TGF- β may be associated in a complex. In attempts to dissociate the putative TSP-TGF- β complex, the TSP preparation was treated at pH 11 and the gel permeation column was eluted at the same pH. When collected fractions were analyzed for TSP and for TGF- β activity, the colony formation activity was eluted at a position expected for uncomplexed TGF- β , well separated from the TSP-containing fractions (Figure 4B). The stripped TSP preparations retained full biological activity in the focal adhesion assay (Figure 3 and Murphy-Ullrich and Höök, 1989). These data suggest that TSP and TGF- β can form a complex associated by forces that are disrupted at pH 11.

Active TGF- β is Released From Platelets in Association With TSP

The soluble release products (releasate) of thrombin-stimulated platelets were fractionated on a gel permeation column (Bio-Gel A0.5M) eluted in 0.01 M Tris-HCl (pH 7.35), 0.15 M NaCl with 0.1 mM CaCl to determine the macromolecular form of the released TGF- β activity. Fractions were analyzed with a monoclonal antibody to TSP and a rabbit polyclonal antibody to TGF- β in Western blots and by soft agar assays to determine the distribution of TGF- β activity and of immunoreactive TGF- β and TSP. The results of the soft agar assays showed that the biologically active TGF- β was primarily present in the fractions near the v_0 , which contained high molecular weight components such as the immunoreactive TSP (Figure 5A). Activity present in these fractions was neutralized by anti-TGF- β antibody. The primary molecular form of the TGF- β in these fractions was $\sim 25,000$ Da, as determined by Western blots run under nonreducing conditions. There was a small amount of TGF- β immunoreactivity and biological activity found in later fractions. There was a TGF- β immunoreactive component in Western blots with an apparent molecular mass of ~ 60 Da (elution volume 120–

Figure 3. TSP supports NRK-49F cell colony formation in soft agar and this activity is neutralized by anti-TGF- β antibody. (A) NRK-49F cells were incubated with increasing concentrations of TSP and examined for the ability to support colony formation in soft agar in the presence of calf serum and epidermal growth factor. On day 7, colonies $> 62 \mu\text{m}$ in diameter were enumerated by phase microscopy. Results are expressed as the means of triplicate determinations $\pm$ SD. (B) NRK cells were incubated with 3 ng TGF- β , 10 μg TSP, proteins and 10 μg of a rabbit anti-TGF- β neutralizing antibody or with antibody alone. Colonies were counted on day 7 as above.

138 ml), which did have some activity in the soft agar assays. Later fractions, which eluted between 162 and 180 ml, had TGF- β immunoreactivity migrating just above the TGF- β standard at ~ 30 kDa. Fractions eluting between 90 and 114 ml, which had no activity in soft agar assays, blotted a peak of ~ 100 kDa, which could represent the inactive proform. There was no TSP or

Figure 4. TGF- β activity in soft agar assays coelutes with TSP and can be separated from TSP by chromatography at pH 11. (A) Two milligrams of TSP were applied to an A0.5-M gel filtration column ($v_0 = 40$ ml, $v_t = 125$ ml) in TBS with 0.1 mM CaCl at pH 7.4. Absorbance at 280 nm (—□—) was used to monitor elution of TSP. Certain fractions (0.4 ml) were assayed in soft agar assays for activity (—▲—). Colonies formed $>62 \mu\text{m}$ were counted on day 7. (B) Purified TSP (1 mg) was applied to the same column equilibrated in 0.01 M Tris base, pH 11. NaCl (0.15 M) and fractions were monitored for absorbance at 280 nm (—□—) and assayed for ability to support NRK growth in soft agar (—▲—). Under these conditions, soft agar growth-promoting activity could be dissociated from TSP.

25-kDa form of TGF- β detected in any of these later fractions.

In other experiments, releasate was applied to a column of monoclonal anti-TSP IgG (IgG_{2a}) coupled to CNBr-activated Sepharose (Jaffe *et al.*, 1983). TGF- β activity measured by the soft agar assays was determined for both the bound and unbound fractions. Most of the TGF- β activity (60% of the recovered activity) bound to the immobilized antibody and was eluted from the column with TBS (pH 11) but not with 0.2 M NaCl (Figure 5B). There was no binding of TGF- β to non-immune IgG_{2a} coupled to Sepharose. These results suggest that in the material released from platelets after thrombin stimulation, a significant portion of the biologically active TGF- β occurs in complex with TSP.

Figure 5. TSP is complexed with TGF- β in the platelet alpha granule release products. (A) Releasate (5 ml) from 8 units of fresh thrombin-stimulated platelets was applied to an A0.5-M gel filtration column in TBS with 0.1 mM CaCl ($v_0 = 70$ ml, $v_t = 220$ ml). Fractions (3.0 ml) were collected and analyzed by monitoring absorbance at 280 nm (—●—), SDS-PAGE, Western blotting under nonreducing conditions with a monoclonal anti-TSP and a rabbit anti-TGF- β antibody, and by soft agar assays (▨). Soft agar activity was found primarily in fractions eluting at 72–96 ml, which correspond to immunoreactivity for TSP and for TGF- β that stained a 25-kDa band (insert: lane A, column fraction with soft agar activity; lane B, TGF- β). There was some colony formation in fractions eluting at 132–186 ml that correspond to immunoreactivity for TGF- β with apparent molecular mass of ~ 60 kDa eluting between 120 and 138 ml and ~ 30 kDa eluting between 162 and 180 ml. A 100-kDa band eluted between 102 and 114 ml, which was inactive in soft agar assays, was recognized by anti-TGF- β . Absorbance at 280 nm eluting at the v_t was due to p-nitrophenyl p'guanidinobenzoate in the releasate. (B) Two milliliters of releasate were applied to a monoclonal anti-TSP antibody-Sepharose column. Pass-through fractions were recycled through the column and unbound proteins were washed with TBS with 0.1 mM CaCl and then with 0.2 M NaCl. Bound proteins were eluted with 0.01 M Tris, 0.15 M NaCl, at pH 11. All fractions, including fractions not shown, were analyzed as described above. TGF- β activity as determined by Western blots and activity in soft agar assays was eluted with the TSP during the pH 11 treatment (38–43 ml) (▨). There was residual TGF- β and TSP activity in the pass through fractions; however, no TGF- β or TSP was detected in the wash fractions.

¹²⁵I-TGF- β Specifically Binds To TSP

To further characterize the interaction between TSP and TGF- β , the binding of ¹²⁵I-TGF- β to immobilized TSP was examined. Binding of ¹²⁵I-TGF- β was time-dependent and reached a maximum after 2 h of incubation. Excess unlabeled TGF- β inhibited binding of 0.5 ng ¹²⁵I-TGF- β to wells coated with 0.7 ng TSP (ID_{50} = 125 ng). To examine the specificity of ¹²⁵I-TGF- β binding to immobilized TSP, a number of unlabeled proteins were tested in attempts to displace labeled TGF- β during a 2-h incubation at 37°C. In these assays, stripped TSP displaced 50% of total ¹²⁵I-TSP bound to immobilized native TSP, whereas native TSP, heparin, α_2 -macroglobulin, fibronectin, or BSA did not displace significant amounts (<20%) of labeled TGF- β (Figure 6). In experiments not shown, decorin released an average of 20% of bound labeled TGF- β . The inability of native TSP to displace bound TGF- β may be due to prior occupancy of TGF- β binding sites on native TSP.

The binding of soluble TSP to ¹²⁵I-TGF- β was also examined by gel permeation chromatography in Tris-buffered saline. When ¹²⁵I-TGF- β was eluted in the absence of TSP, the radiolabel was eluted at a K_{av} of 0.76 (Figure 7A). The elution position of a major portion of the labeled material was shifted to a K_{av} of 0.08 after the ¹²⁵I-TGF- β was incubated with stripped TSP before application to the gel permeation column (Figure 7B).

Figure 6. Stripped TSP can displace ¹²⁵I-TGF- β from immobilized TSP. Ninety-six-well plates were coated with 0.9 μ g (2 pmol) native TSP (nTSP)/well and binding assays were performed with 0.125 ng (0.005 pmol) ¹²⁵I-TGF- β as described in MATERIALS AND METHODS, except that the assay was conducted for 3 h. Wells were then washed, and 67 pmol of sample were then incubated with the wells for 2 h at 37°C, washed, and remaining bound radioactivity determined. Stripped TSP (sTSP), but not native TSP (nTSP), effectively displaced bound radioactivity. Nonspecific binding was determined in the presence of 100-fold excess unlabeled recombinant TGF- β . Results are expressed as the means of triplicate determinations, except for the displacement with BSA, which is the average of duplicate determinations.

Figure 7. TSP and TGF- β complex with each other in solution. (A) ¹²⁵I-TGF- β (11 ng, $\sim 1.1 \times 10^6$ cpm) was applied to A0.5-M column (v_o = 30 ml, v_i = 105 ml) in TBS with 0.01 mM CaCl₂. Elution of ¹²⁵I-TGF- β was monitored using a gamma counter. (B) One milliliter of 52 μ g/ml stripped TSP was incubated with 11 ng ¹²⁵I-TGF- β for 2 h at 37°C and then applied to column. The elution position of the TGF- β incubated with the TSP was shifted toward the void volume. (C) Incubation of 11 ng ¹²⁵I-TGF- β with 1 mg BSA did not result in a shift in elution volume when applied to the column.

Using the same column, we found that TSP alone typically eluted at a similar position. The observed shift in elution position indicates a specific binding of TGF- β to soluble TSP and is probably not due to nonspecific protein-protein interactions, because preincubations of ¹²⁵I-TGF- β with 0.1% BSA (Figure 7C). Similarly we

found that fibronectin, or fibrinogen did not result in a significant shift in elution position.

DISCUSSION

In prior studies, preparations of TSP have been shown to stimulate the in vitro proliferation of smooth muscle cells (Majack *et al.*, 1986) and fibroblasts (Phan *et al.*, 1989) and to inhibit the growth of endothelial cells (Bagavandoss and Wilks, 1990; Taraboletti *et al.*, 1990). In the present communication, we have reproduced the growth-inhibitory effects in endothelial cells and show that this activity is at least partially due to TGF- β associated with TSP. Furthermore, we and others (Phan *et al.*, 1989) find that TSP preparations stimulate fibroblast growth; however, TSP purified at pH 11 to remove associated TGF- β has no stimulatory effect on fibroblast proliferation. These data suggest that some of the reported biological activities of TSP preparations may be attributed to TGF- β activity. The results also show that TSP acts as a carrier for the active form of TGF- β and that TSP may modulate growth factor activity, possibly by protecting TGF- β from inactivation.

Both TSP and TGF- β are stored in platelet alpha granules. TGF- β released from platelets is present primarily in a 110-kDa latent form, which is associated with a high molecular weight (M_r 125–160,000) binding protein, distinct from TSP (Miyazono *et al.*, 1988). The binding protein interacts with the amino-terminal propeptide of the latent TGF- β molecule (Miyazono *et al.*, 1988). The 25-kDa active form of TGF- β is thought to be generated by proteolytic cleavage of the amino-terminal propeptide by enzymes, such as plasmin (Lyons *et al.*, 1990; Sato and Rifkin, 1989). Some of the TGF- β released from platelets is in the active form (Slivka and Loskutoff, 1991). In the present study, we demonstrate that most of the active TGF- β released from platelets is associated with TSP. We could not detect the latent proform of TGF- β by Western blot analysis in fractions containing TSP. In addition, exposure of TSP to alkaline conditions known to activate latent TGF- β caused no additional increase in NRK cell colony formation as compared with untreated TSP, suggesting that there is no latent TGF- β associated with TSP. These data do not rule out the possibility that the latent form is able to bind to TSP. It is possible that the latent TGF- β bound to TSP is rapidly and efficiently activated, thereby preventing detection of the larger form of TGF- β . The binding of TSP to TGF- β does not appear to be mediated by potentially contaminating proteoglycans because TSP digested with heparitinase or chondroitinase ABC does not show significantly reduced binding of TGF- β and heparin prebound to TSP slightly reduced, not enhanced, TGF- β binding. Taken together, these data suggest that active TGF- β is associated with TSP under physiological conditions and that the binding of TGF- β may regulate its activity. Similarly, active TGF-

β_2 has been shown to copurify with alpha-fetoprotein in amniotic fluid (Altman *et al.*, 1990).

TGF- β bound to TSP appears to be recognized by its receptor(s) because it has biological activity. This is in contrast to TGF- β bound to alpha₂-macroglobulin or decorin, which results in inactivation of the cytokine (O'Connor-McCourt and Wakefield, 1987; Yamaguchi *et al.*, 1990). Because TGF- β bound to TSP is not displaced effectively by decorin or alpha₂-macroglobulin at an enormous molar excess, it is possible that growth factor bound to TSP is protected from extracellular inactivators. If this hypothesis is correct, TSP could potentiate and prolong the biological effects of active TGF- β . For example, TSP stripped of associated exogenous TGF- β still inhibits the growth of BAE cells by a mechanism that is neutralized by anti-TGF- β antibody but not by anti-TSP antibodies (Murphy-Ullrich and Schultz-Cherry, unpublished data). Although the mechanism responsible for this observation is not yet clear, it is possible that this effect is the consequence of TSP complexing with and protecting an endogenous TGF- β -like factor. Alternatively, TSP may cause an increase in expression of active TGF- β and/or its receptors. Furthermore, it was demonstrated recently that an angiogenesis inhibitor whose expression is controlled by a tumor-suppressor gene is related to an alternatively spliced form of TSP (Good *et al.*, 1990). TSP also has been shown to be antiangiogenic in vitro (Iruela-Arispe *et al.*, 1991). Although the role of TGF- β in in vivo angiogenesis is complex and not fully understood (Yang and Moses, 1990), it is possible that TSP-TGF- β interactions may influence certain stages of the angiogenic process. These hypothetical mechanisms presently are being investigated.

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