Thrombospondin Causes Activation of Latent Transforming Growth Factor-β Secreted by Endothelial Cells by a Novel Mechanism

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Abstract. Thrombospondin (TSP) forms specific complexes with transforming growth factor-β (TGF-β) in the α granule releasate of platelets and these TSP-TGF-β complexes inhibit the growth of bovine aortic endothelial cells (BAE). In these studies, we report that TSP stripped of associated TGF-β (sTSP) retained growth inhibitory activity which was partially reversed by a neutralizing antibody specific for TGF-β. Since BAE cells secrete latent TGF-β, we determined whether sTSP activates the latent TGF-β secreted by BAE cells. Cells were cultured with or without sTSP and then the conditioned medium was tested for the ability to support TGF-β–dependent normal rat kidney (NRK) colony formation in soft agar. Medium conditioned with sTSP showed a dose- and time-dependent ability to stimulate BAE-secreted TGF-β activity, reaching maximal activation by 1–2 h with 0.4 μg/ml (0.9 nM) sTSP. The sTSP-mediated stimulation of TGF-β activity is not dependent on serum factors and is not a general property of extracellular matrix molecules. The sTSP-mediated stimulation of TGF-β activity was blocked by a mAb specific for sTSP and by neutralizing antibodies to TGF-β. Activation of BAE cell secreted latent TGF-β by sTSP can occur in the absence of cells and apparently does not require interactions with cell surface molecules, since in conditioned medium removed from cells and then incubated with sTSP, activation occurs with kinetics and at levels similar to what is seen when sTSP is incubated in the presence of cells. Serine proteases such as plasmin are not involved in sTSP-mediated activation of TGF-β. Factors that regulate the conversion of latent to active TGF-β are keys to controlling TGF-β activity. These data suggest that TSP is a potent physiologic regulator of TGF-β activation.

TGF-β is a member of a family of growth, differentiation, and morphogenesis autocrine and paracrine factors (3, 26). TGF-β can affect diverse cellular functions in virtually all cell types. Depending on the cell types and its extracellular environment, these effects can be either positive or negative. TGF-β inhibits the proliferation of endothelial cells in vitro (31), but stimulates angiogenesis in vivo (39). TGF-β has also been shown to enhance or inhibit the proliferation of fibroblasts depending on the nature of the substrate and the mitogens present (3). Myoblast differentiation can also be induced or blocked by TGF-β depending on the availability of mitogens (25, 45).

TGF-β1 is a disulfide-linked homodimer that is synthesized as part of a latent precursor molecule (26). The latent precursor molecule is 390 amino acids in length and consists of an NH_2-terminal 278–amino acid latency-associated peptide (LAP)[1] and the COOH-terminal 112 amino acids which constitute the active domain (15–17). The proregion of TGF-β is unique in that it remains non-covalently attached to the active region after intracellular proteolytic processing and secretion (17). Association of the LAP with the mature peptide region confers latency: the LAP-associated growth factor is unable to interact with its cellular receptors. The LAP contains three N-linked glycosylation sites, two of which have mannose-6-phosphate residues (8, 27, 38). These carbohydrate structures may be important for latency since endoglycosidase F treatment leads to activation of TGF-β (27). The disulfide-bonded dimeric structure of LAP is critical for latency, since site-directed mutagenesis of critical cysteine residues (cys 223, 225) in the LAP abolishes activity (9). The active domain contains nine conserved cysteine residues that participate in inter- and intrachain disulfide bonding (28).

TGF-β is secreted by most cell types as a latent complex (28, 37). Since TGF-β synthesis and TGF-β receptor expression are not highly regulated, primary regulation of TGF-β activity occurs by controlling conversion of the latent TGF-β complex to the active molecule. Physiochemical activation can occur by extremes of pH, heat, chaotropic agents, and deglycosylation (6, 27, 28, 37). Activation in vivo is more complex and not well understood. There is evidence from cell culture models that activation may occur through bind-

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; bFGF, basic FGF; LAP, latency associated peptide; NRK, normal rat kidney; sTSP, TSP stripped of associated TGF-β; TSP, thrombospondin.

ing of the latent molecule to mannose-6-phosphate receptors (12, 21), by plasmin-mediated proteolytic processing (4, 23, 40, 41), and/or by processing in acidic cellular microenvironments (20). In some systems, activation of latent TGF-β by plasmin is relatively inefficient (41). In addition, there are reports of TGF-β activation occurring independently of these mechanisms (19). These results suggest that additional mechanisms of latent TGF-β activation may exist.

We have shown previously that thrombospondin (TSP), a disulfide-linked trimer (450,000 daltons) present in connective tissues and in platelet α granules, is associated with TGF-β as an active complex in the releasate of stimulated platelets (5, 14, 30, 34). TSP is synthesized and secreted by cells in vitro, where it is then able to bind various cellular receptors and/or become incorporated into the extracellular matrix (reviewed in 5, 14, 30). TSP is present transiently in wound environments and its synthesis is rapidly induced by growth factors, including TGF-β. TSP, like TGF-β, has varied effects on cell growth and differentiation. TSP stimulates the proliferation of fibroblasts (36) and smooth muscle cells (24), and in contrast, it inhibits the proliferation of endothelial cells (2, 42). TSP may also serve as both an attachment protein and an anti-adhesive molecule as shown by its ability to cause disassembly of focal adhesions in endothelial cells (32).

In the present studies, we show that TSP activates latent TGF-β secreted by bovine aortic endothelial (BAE) cells in culture. TSP-mediated activation of TGF-β occurs independently of plasmin activity and does not require interactions with cell surface molecules. Based on these data, we propose that TSP activates latent TGF-β through a novel mechanism. Furthermore, TSP-TGF-β interactions may act as a physiologic means of regulating TGF-β activity.

Materials and Methods

Thrombospondin Purification

TSP was purified as previously described (34). Briefly, 8–10 U of fresh human platelets were purchased from the Birmingham American Red Cross and washed with Hepes wash buffer (10% ACD, 0.05 M Hepes, 0.15 M NaCl, and 5 mM Dextrose, pH 7.6). The platelets were thrombin-stimulated and the platelet releasate was applied to a heparin–Sepharose CL-6B (Pharmacia, Piscataway, NJ) affinity column pre-equilibrated with TBS-C (0.01 M Tris-HCl, 0.15 M NaCl, 0.1 mM CaCl), pH 7.4. The bound TSP was eluted with 0.55 M NaCl/TBS with 1 mM CaCl and applied to a A0.5 M gel filtration column (Bio-Rad Laboratories, Richmond, CA) pre-equilibrated with TBS-C, pH 11, to remove associated TGF-β. Purity was assessed by SDS-PAGE using Coomassie blue or silver staining. No contaminating TGF-β activity was found associated with sTSP in normal rat kidney (NRK) soft agar colony formation assays.

Cells

BAE cells were isolated from aortas obtained at a local abattoir, and were characterized by Dil-AcLDL and staining for Factor VIII antigen. Cells were cultured in DME (Cell-Gro, Mediatech, Herndon, VA) supplemented with 4.5 g/liter glucose, 2 mM glutamine, and 20% FBS (Hyclone Laboratories, Logan, UT) as previously described (32). Clone 49F of NRK cells (American Type Culture Collection, Rockville, MD) were provided by Dr. Lynn-Allen Hoffmann, UW-Madison. Stocks were cultured in DME supplemented with 4.5 g/liter glucose, 2 mM glutamine, and 10% calf serum (CS) (Hyclone Laboratories, Logan, UT) as described (1). Calf serum was tested and chosen for low levels of active TGF-β. Mink lung epithelial cells (CCL 64) (MvlLu) were cultured in MEM (Cell-Gro, Mediatech) supplemented with 4.5 g/liter glucose, 2 mM glutamine, and 10% FBS. CHO cells were provided by Dr. Jeff Esko (University of Alabama at Birmingham). Stocks were cultured in F-12 (Cell-Gro, Mediatech) supplemented with 10% FBS. All cells tested negative for mycoplasma contamination.

NRK Colony Formation in Soft Agar

TGF-β activity was assayed by determining colony formation by NRK cells in soft agar assays as described (1) except assays were performed in 24 well tissue culture plates. Briefly, 5% Noble agar (Difco, Detroit, MI) was diluted 10-fold in 10% calf serum/DME and 0.5 ml of this 0.5% agar dilution was added per well to a 24-well tissue culture plate as a base layer, and allowed to harden. 0.2 ml sample containing 5 ng EGF was combined with 0.6 ml 0.5% agar and 0.2 ml (2×10^3) of a NRK cell suspension in 10% calf serum/DME. 0.5 ml of this 0.3% agar sample solution was added to the cooled base layer and the plates were incubated for 7 d at 37°C, 5% CO_2. The number of colonies greater than 62 μm (>8–10 cells) in diameter were counted. Experiments were performed in triplicate.

BAE Cell Proliferation Assays

BAE cells were plated at 5,000 cells per well in 1 ml of DME with 20% FBS in 24-well tissue culture plates and incubated overnight at 37°C, 5% CO_2. The cells were rinsed once in serum-free DME. Test samples in 0.5 ml 2.5% FBS DME was added to each well in triplicate (day 0). On day 2, cells received fresh aliquots of test sample in 0.5 ml without removing the original media (to give a final volume of 1 ml). Cells were grown for another 2 d, then culture media was removed and cells were trypsinized with 0.5 ml trypsin-EDTA (GIBCO BRL, Gaithersburg, MD) and harvested. The number of harvested cells was determined using a model ZM Coulter Counter (Coulter Electronics, Hialeah, FL).

Preparation of BAE Conditioned Media

BAEs were plated at a density of 100,000 cells in a 25 cm^2 flask in 20% or 0.2% FBS/DME and incubated overnight at 37°C, 5% CO_2. This density was determined by comparing the ability of sTSP to activate latent TGF-β in sparse, sub-confluent, and confluent BAE cultures. This cell density showed the greatest difference in levels of active TGF-β between control and TSP-treated media. Flasks were rinsed once with 2 ml serum-free DME and then test samples were added in 2.5 ml of DME with either 0.2 or 20% FBS as specified in the figure legends. The flasks were incubated for additional times at 37°C, 5% CO_2. Conditioned media was collected, centrifuged at 1,200 rpm for 5 min to remove cellular debris, and stored at 4°C in polypropylene tubes for no more than 3 d before testing in NRK soft agar assay to determine TGF-β activity.

Activation of Purified Recombinant Latent TGF-β by sTSP

13 nM sTSP was incubated with 2 nM purified recombinant latent TGF-β in a final volume of 0.5 ml in PBS for 2 h at 37 or 4°C in siliconized microfuge tubes. Samples were then tested in NRK colony forming soft agar assays for TGF-β activity.

Additional Materials

The following items were purchased: ε-aminocaproic acid (EACA), Aprotinin, pepstatin A, and α2 anti-plasmin (Sigma Chemical Co., St. Louis, MO), E-64, carboxypeptidase, leupeptin (Calbiochem Corp., San Diego, CA), cystatin (Boehringer-Mannheim Corp., Indianapolis, IN). Recombinant TGF-β (rTGF-β) and purified recombinant latent TGF-β (LTGF-β) were generous gifts of Dr. Daniel Twardzik (Bristol-Myers Squibb, Seattle, WA). Tenascin was a gift of Dr. Harold Erickson, Duke University. Laminin was obtained from Dr. Magnus Höök (Institute of Biotechnology, Houston, TX).

Antibodies

Mouse anti-TSP 133 was raised against stripped TSP and developed using the mAb Core facility at UAB. This antibody is a IgG_{2b} which recognizes the 50-kD chymotryptic fragment of stripped TSP by Western blotting. Mab TSP-B7 ascites was raised against human platelet releasate and is specific for TSP (11) (Sigma Chemical Co.). Mouse monoclonal and rabbit polyclonal anti-TSP antibodies, raised against native TSP, were generous gifts of Dr. Deane Mosher, University of Wisconsin (Madison, Wisconsin) and

mouse anti-TSP antibodies to specific regions of native TSP were generous gifts of Dr. Vishva Dixit (University of Michigan).

A chicken anti-TGF-β antibody was purchased from Oncomembrane (Seattle, WA) and a mouse monoclonal anti-TGF-β antibody was purchased from Genzyme Corp. (Cambridge, MA). Anti-vitronectin monoclonal and polyclonal antibodies were purchased from Telios (San Diego, CA). A polyclonal anti-platelet factor 4 antibody was purchased from Atlantic Antibodies (Scarborough, ME). Mouse monoclonal anti-basic FGF (bFGF) was obtained from Upstate Biotech. Inc. (Lake Placid, NY).

Results

TSP Stripped of TGF-β Activity Inhibits the Growth of BAE Cells

Previous results from our lab and others have shown that TSP inhibits BAE cell growth (2, 42). We observed that active TGF-β associated with the TSP was partially responsible for growth inhibition of BAE cells (34). To examine whether TSP stripped of associated TGF-β (sTSP) activity inhibited BAE growth, we repeated cell proliferation assays using sTSP. BAE cells were exposed to increasing concentrations of either native TSP (TGF-β activity associated with TSP), or sTSP (no associated TGF-β activity) in media supplemented with 2.5% FBS for a period of 4 d, at which time cell number was determined. Native TSP and sTSP significantly inhibited the proliferation of BAE cells as compared with 2.5% FBS alone (Fig. 1). Furthermore, the dose-response curves were nearly identical for native and stripped TSPs. No significant cell death was observed. The inhibition of BAE proliferation by TSP was concentration dependent with 1 μg/ml sTSP inhibiting 75% of growth. Cells grown in the presence of sTSP assumed a more elongated, fibroblastic shape and had prominent nucleoli (Fig. 2 *a*) as compared with the polygonal cells in the 2.5% FBS media control (Fig. 2 *e*). Similarly, TGF-β treated cells were elongated with numerous processes and prominent nucleoli (Fig. 2 *c*).

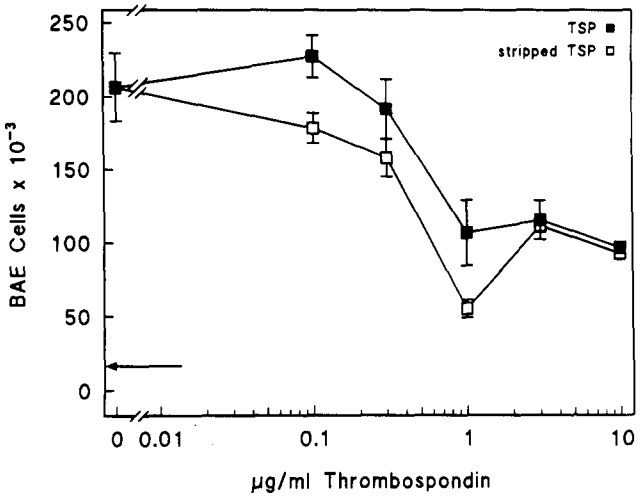

Figure 1. Inhibition of BAE cell proliferation by TSP. BAE cells were seeded at 5,000 cells per well in 24-well plates and incubated overnight in DME supplemented with 20% FBS. They were then washed with DME, and treated with varying concentrations of TSP (native or stripped) in DME containing 2.5% FBS on days 0 and 2. Final cell number was determined on day 4. Cell number on day 0 was 15,640 ± 2925 cells/well (*arrow*). Results are expressed as means of triplicate determinations ±SD.

A neutralizing antibody to TGF-β reversed the growth inhibitory effects of sTSP by 42% (Fig. 3). Addition of the neutralizing antibody to TGF-β to wells containing sTSP also caused a partial reversion to a smaller, more polygonal cell, characteristic of normal BAE cells (Fig. 2 *b*). Similar results were obtained with both mouse and chick anti-TGF-β antibodies. In contrast, a polyclonal antibody (gift of Dr. Deane Mosher, University of Wisconsin, Madison, Wisconsin) and various mAbs specific for native TSP were not able to neutralize sTSP-mediated growth inhibition (data not shown). These results are similar to what was observed in growth inhibition experiments using native TSP (34). Antibodies alone did not affect cell growth. These data suggest that growth inhibition of BAE cells by TSP stripped of associated TGF-β activity is at least partially due to a TGF-β-dependent component.

sTSP Activates TGF-β in BAE Conditioned Media

Since sTSP-mediated BAE growth inhibition is partially TGF-β dependent, it is possible that sTSP incubation with BAE cells is causing activation of endogenous latent TGF-β. TGF-β is secreted from endothelial cells as an inactive molecule (18) and it is not entirely clear how endothelial cell latent TGF-β is activated. To test the hypothesis that sTSP activates latent TGF-β secreted by endothelial cells, sTSP was added to BAE cells in DME with 0.2% FBS for 0–48 h. Aliquots of the conditioned media were tested in NRK colony forming soft agar assays for the presence of TGF-β activity. sTSP at 0.4 μg/ml (0.9 nM) was able to increase colony forming activity in the conditioned media by 2–3-fold as compared with conditioned medium alone (Fig. 4). Increases in TGF-β activity were observed as early as 15 min after addition of sTSP to cells and persisted above control levels for at least 48 h. Similar levels of activation were observed when cells were conditioned in media where serum levels were raised from 0.2 to 20%, suggesting that sTSP-mediated stimulation of TGF-β activity is independent of serum factors.

Stimulation of TGF-β Activity Is Dependent on sTSP Concentration

To assess whether the stimulation of TGF-β activity in BAE conditioned media was dependent on the concentration of sTSP present, varying doses of sTSP ranging from 10 ng to 10 μg were added to BAE cells in 2.5 ml of media. Concentrations of sTSP between 40 and 400 ng/ml (100–1,000 ng added) were effective at stimulating NRK colony formation in soft agar (Fig. 5). The maximal response was repeatedly observed with 1 μg sTSP/25 cm^2 flask (0.4 μg/ml, or 0.9 nM). When compared with recombinant TGF-β (rTGF-β), the level of maximal NRK colony formation induced by sTSP correlated to ~0.1 ng/ml of TGF-β activity.

To rule out that the increase in TGF-β activity in the NRK soft agar assays was due to sTSP acting at the level of the NRK cells, we tested whether sTSP affected mature TGF-β activity and whether anti-sTSP antibody 133, which inhibits sTSP-stimulation of TGF-β activity in the conditioned medium, affected TGF-β activity in the NRK assay. As shown in Table I, there was no modulation of TGF-β activity by either sTSP or anti-TSP antibodies, nor did sTSP by itself

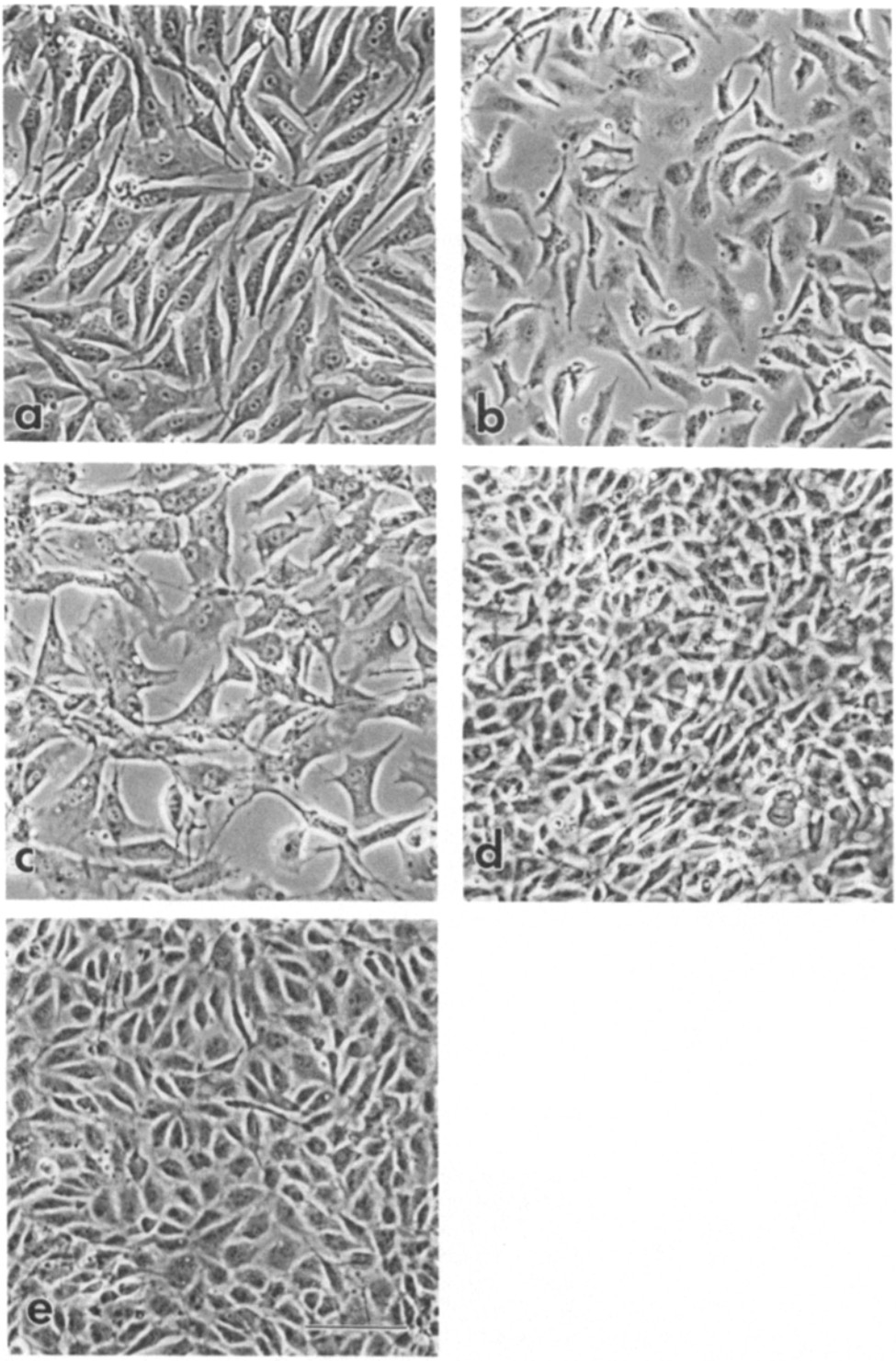

Figure 2. The altered shape of BAE cells grown in sTSP is partially reversed by anti-TGF-β antibody. Cells were seeded at 5,000 cells per well in 24-well plates, grown overnight in DME with 20% FBS, washed, and refed on days 0 and 2 with DME with 2.5% FBS and (*a*) 3 μg/ml sTSP; (*b*) 3 μg/ml sTSP + 2.5 μg/ml mouse anti-TGF-β; (*c*) 1 ng/ml recombinant TGF-β; (*d*) 1 ng/ml recombinant TGF-β + 2.5 μg/ml mouse anti-TGF-β; or (*e*) no additions. Phase micrographs of these cells were photographed on day 4. Bar, 100 μM.

stimulate colony formation. sTSP also does not activate the latent TGF-β present in the 0.2% FBS (Table I).

Stimulation of TGF-β Activity in BAE Conditioned Media Is Specific for sTSP

Other extracellular matrix proteins were tested for their ability to activate endothelial cell secreted latent TGF-l/β. Equimolar amounts of tenascin, fibronectin, BSA, or laminin did not stimulate TGF-β activation (Fig. 6). Basic FGF, in contrast to a previous report (13), did not stimulate increased TGF-β activity in our system (Fig. 6). These results show that stimulation of TGF-β activity in BAE conditioned medium is not a general property of extracellular matrix molecules, including TGF-β–binding molecules such as fibronectin, and therefore is a specific property of sTSP.

Antibodies to sTSP Inhibit Stimulation of TGF-β Activity by sTSP

To eliminate the possibility that the observed increase in TGF-β activity was due to potential components associated

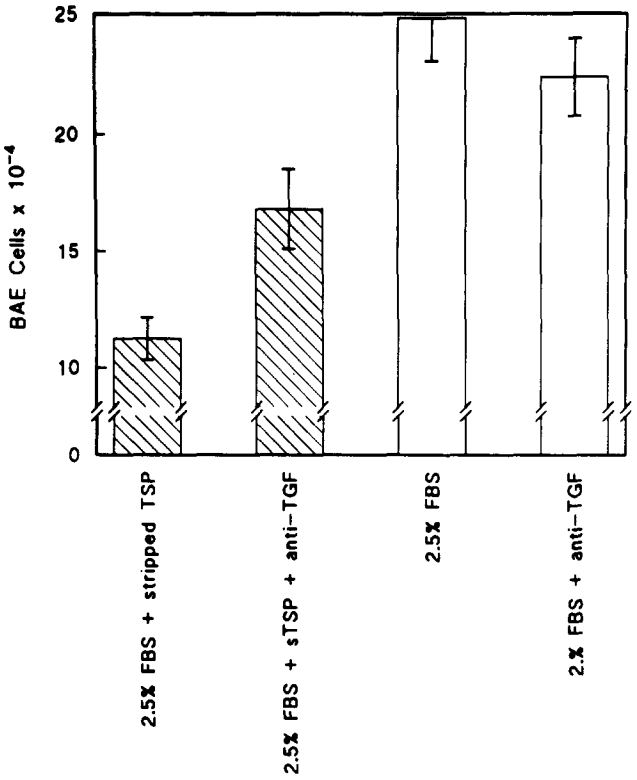

Figure 3. Inhibition of BAE proliferation by sTSP is sensitive to anti-TGF-β neutralizing antibody. BAE cells were seeded at 5,000 cells per well and incubated overnight in DME with 20% FBS, washed once with DME, and then grown in DME with 2.5% FBS supplemented on days 0 and 2 with either 3 μg/ml sTSP, 3 μg/ml sTSP + 1 μg/ml chicken anti-TGF-β, or 1 μg/ml chicken anti-TGF-β. Cell number on day 0 was 13,840 ± 600 cells per well. Cells were harvested and cell number per well was determined on day 4. Results are expressed as means of triplicate determinations ±SD.

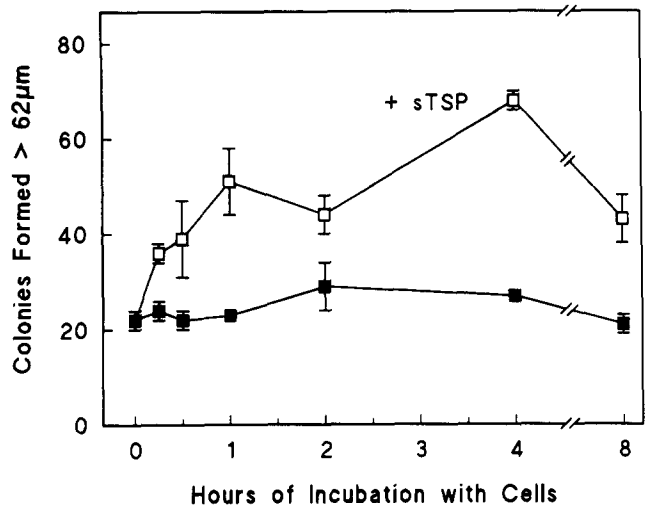

Figure 4. Kinetics of sTSP activation of TGF-β in 0.2% FBS BAE conditioned media (CM). BAE cells seeded at 1 × 10^5 cells/25 cm^2 flask were grown overnight in DME supplemented with 0.2% FBS and 2 mM glutamine. 0.4 μg/ml sTSP (0.9 nM) was added to the flasks and incubated for 15 min to 8 h at 37°C, 5% CO_2. Aliquots (0.2 ml/sample) of conditioned medium with (□) or without (■) added sTSP were tested in the NRK colony forming soft agar assay. Results are expressed as means of triplicate determinations ±SD.

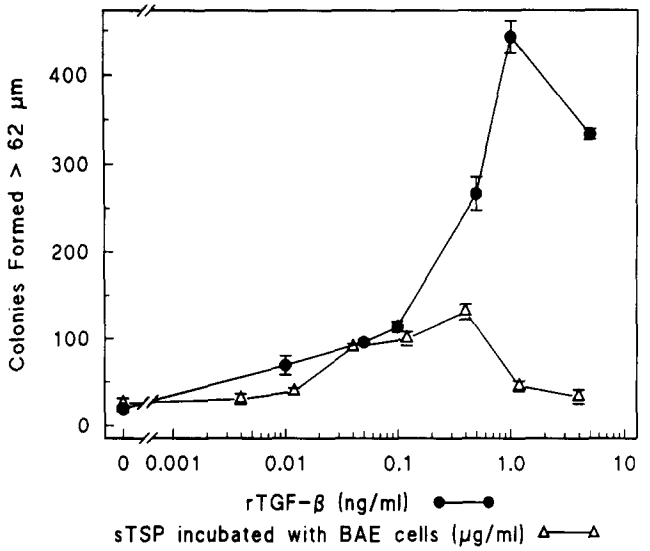

Figure 5. Activation of TGF-β in BAE conditioned media by sTSP is dose dependent. BAE cells were seeded at 1 × 10^5 cells/25 cm^2 flask in DME with 20% FBS and incubated overnight. Flasks were rinsed once with DME, and then increasing amounts of sTSP in 2.5 ml of DME with 0.2% FBS were incubated an additional 48 h. Aliquots of these conditioned media were tested in the NRK soft agar colony forming assays to determine TGF-β activity (△). Different concentrations of rTGF-β were also tested in the same set of NRK soft agar assays (●). Results are expressed as means of triplicate determinations ±SD.

with sTSP, we attempted to block stimulation with antibodies to TSP. mAb 133 ascites, which recognizes an epitope in the 50-kD chymotryptic fragment of sTSP, completely inhibited the stimulation of TGF-β activity by sTSP (Fig. 7). mAb TSP-B7 ascites, which is specific for the 70-kD core of plate-

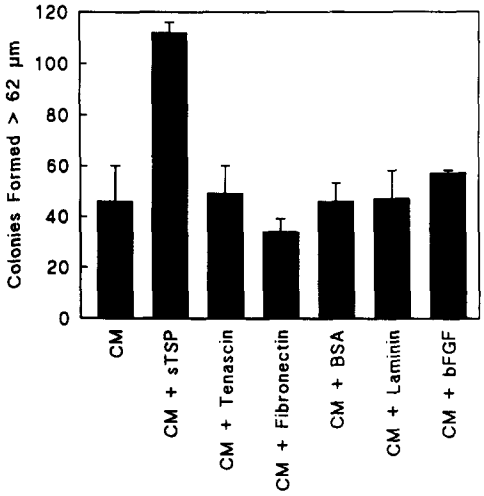

Figure 6. Stimulation of active TGF-β in BAE conditioned media (CM) is specific for sTSP. BAE cells seeded at 1 × 10^5 cells/25 cm^2 flask were grown overnight in DME supplemented with 20% FBS and then washed with DME. Then 0.4 μg/ml sTSP or equimolar amounts (0.9 nM) of various proteins were then added to 2.5 ml of 20% FBS/DME and incubated with the cells for 48 h at 37°C, 5% CO_2. bFGF was used at 1 ng/2.5 ml. Aliquots of conditioned medium (0.2 ml/sample) were tested in NRK colony forming soft agar assays to determine TGF-β activity. Similar results were obtained when cells were conditioned in DME with 0.2% FBS. Results are expressed as the means of triplicate determinations ±SD.

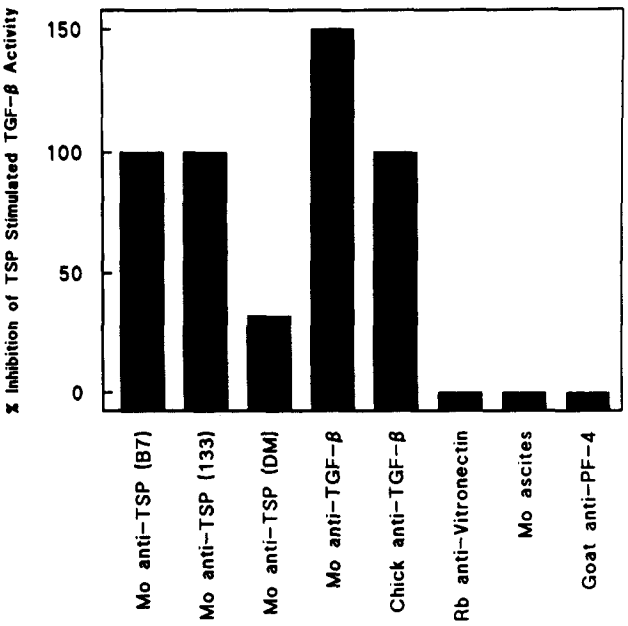

Figure 7. Percent inhibition of sTSP-stimulated TGF-β activity by specific antibodies to TGF-β and sTSP. BAE cells were seeded at 1×10^5 cells/25 cm^2 flask in DME with 20% FBS and incubated overnight and then rinsed with serum-free DME. 1 μg sTSP, 1 μg sTSP + 1:100 mAb TSP-B7 ascites, 1 μg sTSP + 1:100 mAb 133 ascites, 1 μg sTSP + 10 μg mouse anti-TSP (DM, gift of Deane Mosher), 1 μg sTSP + 2.5 μg mouse anti-TGF-β (Genzyme), 1 μg sTSP + 10 μg chick anti-TGF-β (Oncomembrane), 1 μg sTSP + rabbit anti-vitronectin (Telios), 1 μg sTSP + 1:100 control ascites, and 1 μg sTSP + 10 μg goat anti-platelet factor-4 (PF-4), were added in 2.5 ml DME with 0.2% FBS/flask and incubated 48 h at 37°C, 5% CO_2. Antibodies alone were also used as controls and had no effect. Aliquots of conditioned medium (0.2 ml/sample) were tested in NRK colony forming soft agar assays to determine TGF-β activity. Variations among triplicates was <10%.

let TSP (11) also blocked this effect of sTSP. However, another mAb which recognizes an epitope in the 70-kD fragment of native TSP (gift of Dr. Deane Mosher) only inhibited sTSP activation of latent TGF-β by 32%. Antibodies alone had no effect on these assays and did not interfere with rTGF-βs ability to form colonies in soft agar (Table I).

Table I. sTSP Does Not Affect the Activity of Mature rTGF-β or Stimulate TGF-β Activity in FBS

	Number of colonies formed
EGF	39 ± 1
EGF + rTGF-β	240 ± 3
EGF + rTGF-β sTSP	241 ± 2
EGF + sTSP	49 ± 12
EGF + sTSP	40 ± 4
EGF + 0.2% FBS	41 ± 5
EGF + 0.2% FBS + sTSP	41 ± 4

The NRK colony forming assay was performed as described in Materials and Methods. All samples contained 5 ng/ml EGF in 10% calf serum/DME, except the samples in which EGF was in 0.2% FBS as indicated. sTSP (1 μg/ml) was preincubated with 1 ng/ml rTGF-β for 2 h at 37°C, and compared for relative activity versus 1 ng/ml rTGF-β or 1 μg/ml sTSP. Anti-sTSP antibody mAb 133 (10 μg/ml) was pre-incubated with rTGF-β for 2 h, 37°C before addition to NRK cells in soft agar. sTSP (3 μg/ml) was also incubated with 0.2% FBS for 2 h, 37°C and tested for relative activity versus sTSP (3 μg/ml) or 0.2% FBS. All samples were tested in the NRK soft agar assay for 7 d at 37°C, 5% CO_2. Results are expressed as the means of triplicate determinations ±SD.

Colony formation was also TGF-β dependent, since a polyclonal chicken anti-TGF-β antibody and a monoclonal mouse anti-TGF-β neutralizing antibody completely inhibited colony formation (Fig. 7). These results show that the factor activated by sTSP in BAE conditioned medium is TGF-β.

In contrast, antibodies to vitronectin (both monoclonal and polyclonal), platelet factor-4, bFGF, and control ascites, did not inhibit the stimulation by sTSP (Fig. 7 and data not shown). These data show that increases in TGF-β activity observed in the NRK soft agar assays are not due to the presence of commonly associated matrix and platelet proteins, but are dependent on sTSP and TGF-β.

sTSP Stimulation of TGF-β Activity in BAE Conditioned Medium Occurs Independently of Binding to the Cell Surface

A proposed mechanism of latent TGF-β activation in vivo is through binding to and internalization of latent TGF-β by mannose-6-phosphate receptors and subsequent processing in acidifying vesicles or processing by plasmin at the cell surface (12, 20, 21). Experiments were performed to determine whether sTSP requires interactions with cell surface molecules to activate latent TGF-β. After incubating BAE cells in DME with 0.2% FBS overnight, the media was removed from the culture flasks and incubated in polypropylene tubes in the presence or absence of sTSP (0.4 μg/ml) for the indicated times. This was done in direct comparison with sTSP incubated in the presence of cells. Aliquots of the conditioned medium were then tested in NRK colony forming soft agar assays for sTSP-mediated activation of TGF-β. These data show that sTSP is able to activate TGF-β in the absence of cells to a similar extent and with similar kinetics as shown in Fig. 4 in which sTSP was incubated in the presence of cells (Fig. 4). Cell-conditioned media incubated with sTSP in the absence of cells demonstrated increased TGF-β activity as early as 15 min after addition of sTSP. Maximal levels were reached by 2 h and persisted above baseline for at least 48 h (Fig. 8). Thus, in contrast to previously reported mechanisms of activation, TSP-mediated activation of latent TGF-β does not require interactions with cell surface molecules.

sTSP-mediated Stimulation of TGF-β Activity Is Insensitive to Serine Protease Inhibitors

Previous studies have shown that plasmin can activate latent TGF-β in vitro (22, 23). In cocultures of endothelial and smooth muscle cells, plasmin levels have been shown to be upregulated, thus activating latent TGF-β (40, 41). The common motif is the involvement of a serine protease in the activation of latent TGF-β. Therefore, we tested the effects of different serine protease inhibitors on the activation of TGF-β by sTSP in BAE-conditioned medium. TGF-β activation by sTSP was tested against a panel of serine protease inhibitors (Table II). sTSP (0.4 μg/ml) was incubated with ε-aminocaproic acid (EACA, 0.3 mM), aprotinin (6 mM), and α2-antiplasmin (0.6 μM). The concentrations of these inhibitors were chosen based on previous studies (40) and dose-response assays. These inhibitors were unable to in-

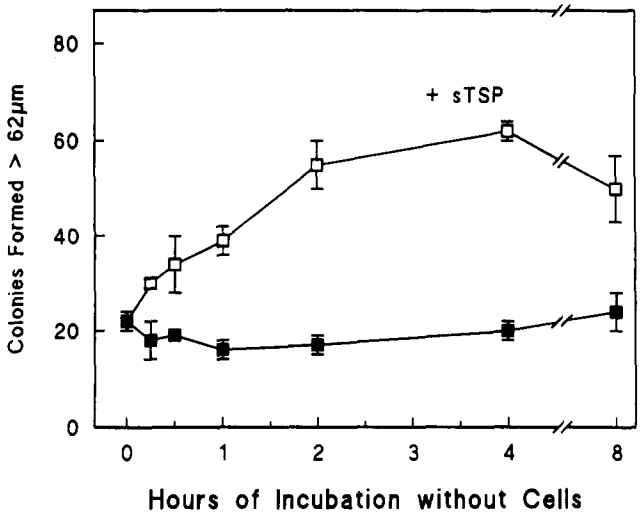

Figure 8. sTSP stimulation of TGF-β activity in BAE conditioned medium occurs independently of binding to the cell surface. BAE cells were seeded at 1 × 10^5 cells/25 cm^2 flask and incubated overnight in DME supplemented with 0.2% FBS. The cell-conditioned medium was removed, centrifuged to remove debris, and then incubated in sterile polypropylene tubes in the presence or absence of 0.4 μg/ml sTSP for 15 min to 8 h. Aliquots of media with (□) or without (■) sTSP were tested in the NRK soft agar assay to determine TGF-β activity. All results are expressed as the means of triplicate determinations ±SD.

hibit sTSP-mediated activation of TGF-β and had no effect on rTGF-β activity in soft agar assay. Due to evidence that TSP can interact with these serine proteases (7), sTSP was also tested for associated plasmin and thrombin activity using enzyme assays measuring generation of chromogens from specific substrates (Boehringer-Mannheim Corp.). No associated plasmin or thrombin activity was detected in sTSP and there was no generation of plasmin activity in sTSP-conditioned medium as compared with control conditioned medium (data not shown).

These data show that in contrast to activation of endothelial cell-derived latent TGF-β by bFGF or in coculture systems, latent TGF-β activation by sTSP does not involve serine proteases such as plasmin.

Table II. sTSP-mediated Stimulation of TGF-β Activity Is Insensitive to Serine Protease Inhibitors

Protease inhibitor	Concentration	Percent inhibition of sTSP-stimulated Tgf-β activity (conditioned media)	Percent inhibition of rTGFβ activity
sTSP +			
EACA	0.3 mM	0	0
Aprotinin	6 mM	0	0
α_2-antiplasmin	0.6 μM	0	0

BAE cells were seeded at 1 × 10^5 cells/cm^2 flask in DME with 20% FBS and incubated overnight. Flasks were washed with DME, and 1 μg sTSP + protease inhibitor was added to each flask in DME with either 2.5 ml 0.2% or 20% FBS and then incubated with cells an additional 48 h. Aliquots of conditioned medium were tested in NRK colony forming soft agar assays to determine TGF-β activity. Recombinant TGF-β (5 ng/ml) was also incubated with the inhibitors and assayed for colony forming activity. Inhibitors alone were also added to the conditioned medium and had no effects on the assay (data not shown). Results are representative of several different experiments each performed in triplicate. Variations among triplicates were <10%.

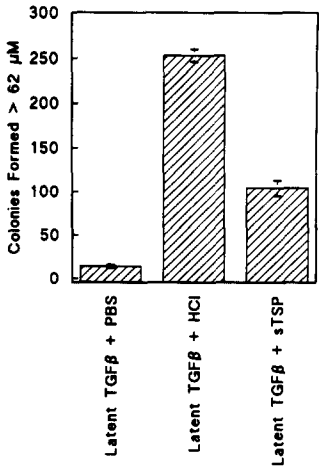

Figure 9. sTSP activates purified recombinant latent TGF-β. 13 nM sTSP was incubated with 2 nM purified recombinant latent TGF-β for 2 h at 4°C in siliconized microfuge tubes. 4 mM HCl was used at an equal volume as sTSP as a positive control. The final volume was brought to 0.5 ml with PBS. Samples were then tested in NRK colony forming soft agar assay for TGF-β activity. Results are expressed as the means of triplicate determinations ±SD.

sTSP Can Activate Purified Recombinant Latent TGF-β

To determine if sTSP was activating latent TGF-β without the involvement of cell-secreted factors, sTSP was incubated with purified recombinant latent TGF-β for 2 h and then assayed for TGF-β activity. sTSP was able to activate recombinant latent TGF-β at both 37 and 4°C (Fig. 9 and Schultz-Cherry, S., S. Ribeiro, J. E. Ranchalis, O. R. Twardzik, J. E. Murphy-Ullrich, manuscript in preparation). sTSP at 13 nM could activate approximately half of the acid-activatable latent TGF-β. These results show that sTSP is able to activate latent TGF-β directly, without the involvement of cell-secreted factors such as proteases.

Discussion

In these studies, we describe a novel mechanism for activation of latent TGF-β secreted by endothelial cells that is dependent upon the platelet and extracellular matrix protein, TSP. sTSP inhibited BAE cell growth and caused morphological changes that were at least partially TGF-β dependent. This led us to propose that these TGF-β–dependent responses were due to activation of endogenous BAE latent TGF-β by added sTSP. Indeed, these studies show that incubation of sTSP with BAE cells or BAE conditioned medium generates TGF-β activity as measured by EGF-dependent NRK colony formation in soft agar. Activation is related to the concentration of sTSP added to the cells and occurs fairly rapidly, within 15–120 min, and persists for at least 48 h. The increases in TGF-β activity occurring with sTSP treatment are most likely due to conversion of latent to active TGF-β and are not due to increased synthesis of TGF-β, since activation of latent TGF-β by sTSP can occur in BAE conditioned media in the absence of cells.

It is interesting that levels of TGF-β activity peak at 2–4 h and do not increase with time. It is possible that after the initial activation of latent TGF-β by sTSP, which occurs within 15 min, the available binding sites for TGF-β on sTSP become saturated. We have initial evidence suggesting the activation of latent TGF-β and the binding of active TGF-β are due to the same region of TSP (Schultz-Cherry, S., J. Lawler, J. E. Murphy-Ullrich, manuscript in preparation).

Once the latent TGF-β is activated by TSP, this region could bind to the newly activated TGF-β, potentially preventing further interactions. This may explain why the levels of active TGF-β remain steady or are even slightly decreased at incubation times greater than 4 h. sTSP may also play a role in prolonging the half-life of active TGF-β.

Although several matrix proteins have been shown to bind active TGF-β (29, 34, 35, 44), activation of latent TGF-β is not a general property of extracellular matrix molecules or of TGF-β binding proteins, since equimolar concentrations of tenascin, fibronectin, BSA, or laminin were unable to activate latent TGF-β secreted by endothelial cells. Serum cofactors do not appear to be important in sTSP-mediated activation since activation occurs to a similar extent under high and low serum conditions.

In these studies, sTSP is apparently activating latent TGF-β secreted by BAE cells and not the latent TGF-β present in the FBS used in the medium, since TGF-β activity levels are not increased in FBS incubated with added sTSP as compared with control FBS. There is evidence that there are active TGF-β–TSP complexes normally present at low levels in these fluids, since when aliquots of FBS or BAE conditioned medium are applied to a monoclonal anti-TSP–CNBr Sepharose affinity column, active TGF-β elutes with the TSP fractions (Ribeiro S., S. Schultz-Cherry, and J. E. Murphy-Ullrich, manuscript in preparation). It is possible that the TSP present in the serum and the endogenous TSP secreted by the endothelial cells are already complexed with TGF-β and therefore lack available binding sites for interactions with latent TGF-β synthesized during the course of the experiments.

It is not likely the sTSP-mediated activation of latent TGF-β is the result of sTSP acting as an adhesive protein or by causing increased internalization of TGF-β by the NRK cells, since sTSP incubated with rTGF-β causes no increase in colony number as compared with rTGF-β alone and anti-sTSP antibody which neutralizes TSP-mediated activation of latent TGF-β in conditioned medium had no effect on mature TGF-β activity in the NRK assays.

The B7 and 133 mAbs effectively inhibit TGF-β activation by sTSP. These antibodies both recognize epitopes in the 50–70 kD chymotryptic core of TSP (11 and Schultz-Cherry, S., S. Ribeiro, J. Ranchilso, O. R. Twardzik, and J. E. Murphy-Ullrich, unpublished data). This is consistent with recent data from our lab that show that the 50-kD fragment of sTSP binds TGF-β and furthermore, that this 50-kD fragment can activate TGF-β in the conditioned media assays (Schultz-Cherry, S., and J. E. Murphy-Ullrich, manuscript in preparation).

The observation that most antibodies raised against native platelet TSP were unable to inhibit sTSP-mediated activation of TGF-β whereas a monoclonal raised against sTSP was able to completely block activation, suggests that bound TGF-β blocks the epitope recognized by mAb 133 in sTSP or alternately, that there are conformational differences between native and stripped TSPs. There may be definite conformational requirements that determine TSP–TGF-β interactions. These could involve disulfide-bond influenced tertiary structure, since heat denaturation and reduction and alkylation of sTSP abolish sTSP activity in the conditioned medium assays (data not shown). These possibilities remain to be clarified.

It has been shown previously in coculture systems (12, 21) that latent TGF-β requires interactions at the cell surface for activation. In contrast, sTSP does not require interactions with the cell surface to activate TGF-β. Activation of latent TGF-β in BAE-conditioned medium removed from cells occurs with kinetics and to levels similar to what is observed when sTSP is incubated in the presence of cells. Furthermore, sTSP is able to activate latent TGF-β secreted by cells (CHO mutants pgsA 745) which lack TSP receptors (10, 33). In addition, data show that sTSP binds and activates purified recombinant latent TGF-β (Fig. 9 and Schultz-Cherry, S., S. Ribeiro, J. Ranchilso, O. R. Twardzik, and J. E. Murphy-Ullrich, manuscript in preparation). These data show that activation most likely occurs through direct TSP–TGF-β interactions.

The activation of latent TGF-β by plasmin in endothelial cell systems is well documented (22, 23, 40, 41). In cocultures of endothelial cells and pericytes, activation of latent TGF-β was related to increased production of plasmin and was blocked by plasmin/serine protease inhibitors (40, 41). In contrast, activation of TGF-β by sTSP in BAE conditioned media was not sensitive to serine protease or specific plasmin inhibitors. Furthermore, we were unable to detect any plasmin activity associated with sTSP or any increases in plasmin activity in sTSP-treated BAE conditioned medium as compared to control conditioned medium. When inhibitors of other classes of protease (cysteine, aspartyl) were tested, we found either no effect or that the cysteine protease inhibitors, cystatin and E-64, inhibited TSP-mediated stimulation of TGF-β. However, cystatin was determined to be cytotoxic to the endothelial cells and E-64 was shown to interfere with the binding of TGF-β to sTSP. Furthermore, E-64 had no inhibitory effect on sTSP-mediated stimulation of BAE conditioned medium removed from the cells. In addition, calpastatin, a specific inhibitor of the cysteine protease, calpain, did not affect sTSP stimulation of TGF-β activity in BAE conditioned medium. We also observed that sTSP was able to activate purified recombinant TGF-β with similar kinetics when tested at both 4 and 37°C (Fig. 9 and Schultz-Cherry, S., S. Ribeiro, J. Ranchilso, O. R. Twardzik, and J. E. Murphy-Ullrich, manuscript in preparation). Together these data strongly suggest that sTSP activation of latent TGF-β occurs independently of proteolytic activity.

sTSP-mediated activation of TGF-β is not restricted to endothelial cells since incubation of sTSP with MvlLu and CHO cells (wild-type and glycosaminoglycan-deficient mutants) results in increased TGF-β activity (data not shown). Thus activation of latent TGF-β by sTSP may be a general mechanism for paracrine control of TGF-β activity by cells that synthesize TGF-β in the latent form. It will be important to examine the processing of the TGF-β once it is activated by sTSP. It is not known whether the pro-region of the latent molecule remains associated with the sTSP.

Activation of TGF-β by sTSP is efficient, since levels of colony formation in sTSP-treated conditioned medium were 80% of the total alkaline-activatable TGF-β present in control BAE conditioned medium (data not shown). The levels of TGF-β activity generated by sTSP-treatment of BAE cells are physiologically significant (~250 pg TGF-β activity per ~8×10^5 cells over 48 h). These two observations strongly suggest that TSP interactions with latent TGF-β may play a substantive role in activation of TGF-β in vivo.

These results show that TSP is a potent modulator of

TGF-β activity, acting through novel mechanisms. In addition to activating TGF-β, TSP may serve as a carrier molecule to prolong the otherwise extremely short half-life of active TGF-β in body fluids (43). TSP may also serve to locally deposit active TGF-β at appropriate sites in tissues. Since TSP is released in large quantities from stimulated platelets and its synthesis is rapidly induced by growth factors, it is reasonable to suggest that TSP may enhance the ability of TGF-β to stimulate wound healing. Alternately, TSP-TGF-β interactions may serve to facilitate the excessive fibrosis stimulated by TGF-β in some pathological states. These possibilities remain to be explored.

The authors gratefully acknowledge the technical assistance of Mr. Manuel A. Pallero in the characterization of the mAb 133 and to Ms. Frances Allen and the UAB Hybridoma Core Facility for producing the monoclonals (NIH grant 5P60 AR20614). We would also like to thank Dr. Daniel Twardzik and Ms. Jane Ranchalis at Bristol Myers Squibb, Seattle, WA, for recombinant mature and latent TGF-β.

These studies were supported by grant HL44575 to J. E. Murphy-Ullrich and by a predoctoral stipend from the Department of Pathology to S. Schultz-Cherry.

Received for publication 9 February 1993 and in revised form 15 May 1993.

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