Role of the Latent TGF-β Binding Protein in the Activation of Latent TGF-β by Co-Cultures of Endothelial and Smooth Muscle Cells

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Abstract. Transforming growth factor beta (TGF-β) is released from cells in a latent form consisting of the mature growth factor associated with an aminoterminal propeptide and latent TGF-β binding protein (LTBP). The endogenous activation of latent TGF-β has been described in co-cultures of endothelial and smooth muscle cells. However, the mechanism of this activation remains unknown. Antibodies to native platelet LTBP and to a peptide fragment of LTBP inhibit in a dose-dependent manner the activation of latent TGF-β normally observed when endothelial cells are cocultured with smooth muscle cells. Inhibition of latent TGF-β activation was also observed when cells were co-cultured in the presence of an excess of free LTBP. These data represent the first demonstration of a function for the LTBP in the extracellular regulation of TGF-β activity and indicate that LTBP participates in the activation of latent TGF-β, perhaps by concentrating the latent growth factor on the cell surface where activation occurs.

TRANSFORMING growth factor-β (TGF-β)[1] is a member of a family of molecules, including Mullerian inhibiting substance, inhibins, activins, and bone morphogenic proteins that are potent regulators of cell growth and development (Roberts and Sporn, 1990; Lyons and Moses, 1990; Massague, 1990). TGF-β has been isolated from a variety of tissues and has a broad spectrum of effects on many cell types. TGF-β stimulates the growth of cells of mesenchymal origin (Shipley et al., 1985) and inhibits the growth of epithelial (Tucker et al., 1984), endothelial (Frater-Schroder et al., 1986), and lymphoid (Kerhl et al., 1986*a*, *b*) cells. It induces matrix biosynthesis by stimulating the expression of matrix components, such as proteoglycans (Chen et al., 1987; Bassols and Massague, 1988), collagen and fibronectin (Ignotz et al., 1987; Raghow et al., 1987), and protease inhibitors, including plasminogen activator inhibitor type 1 (PAI-1) (Laiho et al., 1986; Pepper et al., 1990) and tissue inhibitor of metalloprotease (Edwards et al., 1987) as well as decreasing the expression of matrix degrading proteases such as collagenase (Edwards et al., 1987). TGF-β is chemotactic for fibroblasts (Postlewaite et al., 1987), macrophages (Wahl et al., 1987), and smooth muscle cells (Koyama et al., 1990), and inhibits the migration of endothelial cells (Heimark et al., 1986; Muller et al., 1987; Sato and Rifkin, 1989). However, although it is clear that TGF-β is an important molecule in regulating many aspects of cellular physiology, it is not clear how the activity of this growth factor is controlled.

One level of control that appears to be important is the activation of TGF-β from its latent form. TGF-β is secreted from cells predominantly, if not exclusively, in a latent form that is unable to bind the TGF-β receptor and is inactive (Pircher et al., 1986; Lawrence et al., 1985). The latent TGF-β molecule is synthesized as a 390-amino acid proprotein that is processed at an arg-arg cleavage site between residues 278 and 279. Mature TGF-β1 is a 25-kD homodimer of 112-amino acid polypeptides derived from the carboxy terminus of the proprotein (Derynck et al., 1985). The latency associated peptide (LAP) is a 75-kD homodimer of two 249-amino acid polypeptides representing the NH_2-terminal remnant of the proprotein. These two proteins, mature TGF-β and the LAP, remain associated through noncovalent interactions. The dissociation of the LAP from mature TGF-β renders TGF-β biologically active. Yet, how this activation is mediated in vivo is unknown.

A high molecular weight form (~210,000) of latent TGF-β has been identified in platelets (Miyazono et al., 1988; Wakefield et al., 1988). This form of latent TGF-β was shown to contain a protein of ~160 kD that is joined to the LAP through a disulfide bond (Miyazono et al., 1988). The latent TGF-β binding protein (LTBP) has recently been cloned and sequenced (Kanzaki et al., 1990; Tsuji et al., 1990). The protein contains several copies of motifs found in other proteins. There are 16 EGF-like domains that, in other proteins, have been reported to be involved in pro-

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; BS^3, bis(sulfosuccinimidyl) suberate; BSM, bovine smooth muscle; LAP, latency associated peptide; LTBP, latent TGF-β binding; PA, plasminogen activator; TGF-β, transforming growth factor-β.

tein–protein interactions (Apella et al., 1988). At least two of these EGF-like domains contain hydroxyasparagine post-translational modifications (Kanzaki et al., 1990). In addition, the sequence contains an RGD and a laminin B2-like sequence. Both these sequences may mediate protein–protein interactions with cell surface molecules such as integrins (Ruoslahti and Pierschbacher, 1987; Sasaki and Yamada, 1987). However, the function of LTBP is not understood, although it is known that the addition of LTBP does not confer latency on mature recombinant TGF-β1 (Kanzaki et al., 1990) and that LTBP may be required for the proper assembly and secretion of latent TGF-β (Miyazono et al., 1991).

Latent TGF-β in medium conditioned by cultured cells can be activated by transient treatment with either acid, base, heat, or chaotrophic agents (Lawrence et al., 1985). Latent TGF-β can also be activated either by proteolysis of the latent complex (Lyons et al., 1988, 1990) or by alteration of carbohydrate structures within the LAP (Miyazono and Heldin, 1990). In addition to treatments with exogenous agents, latent TGF-β has been shown to be activated when bovine aortic endothelial (BAE) cells are co-cultured with bovine pericytes or bovine smooth muscle (BSM) cells (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989; Sato et al., 1990). The mechanism of this co-culture activation is not well understood, but appears to involve plasmin because inhibitors of plasmin prevent the activation of latent TGF-β in co-cultures (Sato and Rifkin, 1989; Sato et al., 1990). The reaction also appears to be surface mediated (Sato and Rifkin, 1990; Dennis and Rifkin, 1991).

To determine whether LTBP is involved in the activation of latent TGF-β, we analyzed the effect of anti-LTBP antibodies, as well as competition with free LTBP, on the generation of TGF-β in co-cultures of BAE and BSM cells. The results indicate that the participation of the LTBP is required for the activation of latent TGF-β in this system.

Materials and Methods

Antibodies

Anti-LTBP IgG was purified from a rabbit antiserum to native human platelet LTBP (Ab 39; Kanzaki et al., 1990) using protein A–Sepharose (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ). IgG purified from a rabbit antiserum to a peptide corresponding to amino acids 1111–1122 of the sequence of LTGF (Kanzaki et al,. 1990) was also used. LTBP was purified from platelets as previously described (Miyazono et al., 1988). Fab fragments were generated by papain digestion of anti-LTBP antibody and isolated from the eluate following chromatography on a protein A–Sepharose column (Harlow and Lane, 1988).

Cross-linking and Western Blot Analysis of Conditioned Media

To prepare conditioned media, BAE and BSM cells were plated separately at a density of 15×10^6 cells per T150 flask in αMEM containing 10% calf serum. Cells were incubated for 2 h to allow them to attach to the bottom of the flask and rinsed with serum-free αMEM. They were then incubated with 10 ml of serum-free medium for 12 h. This medium was discarded and replaced with 10 ml per flask of fresh αMEM containing aprotinin (1 μg/ml), pepstatin (0.5 μg/ml), and leupeptin (0.5 μg/ml) for 24 h. Conditioned media were collected and clarified by centrifugation at 2,500 *g* for 15 min in tubes siliconized with Sigmacoat. Clarified conditioned media were cross-linked with 2 mM bis(sulfosuccinimidyl) suberate (BS^3) (Pierce Chemical Co., Rockford, IL) for 20 min at 4°C in coated tubes. These media were concentrated 50- to 180-fold in Centriprep-30 and Centricon-10 concentrator units (Amicon Division, Beverly, MA) at 4°C. Both the ultrafiltration units and membranes were pretreated with 0.1% BSA in PBS. Samples were stored at −20°C.

To perform Western blots, samples were diluted 1:4 (v/v) in sample buffer (10% SDS, 42% glycerol, 180 mM Tris, pH 6.3), boiled for 5 min, and analyzed by SDS-PAGE using 5–16% gradient slabgels. Proteins were transferred to nitrocellulose paper (0.45-μm pore size; Schleicher and Schuell, Keene, NH) for 12 h at 80 V in ice and subjected to immunoblotting using 8 μg/ml rabbit anti-LTBP IgG or 8 μg/ml rabbit anti-porcine TGF-β1 IgG. Bound antibodies were visualized using ^{125}I-protein A followed by autoradiography.

Immunoblotting of Platelet LTBP

50 ng of purified LTBP was run overnight at 8 mA on a 5–16% gradient gel. The proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) via semidry transfer (1 3/4 h) using the continuous buffer system (39 mM glycine, 48 mM tris, 0.0375% SDS, 20% methanol) on the Multiphor II Nova Blot System (Pharmacia LKB Biotechnology, Piscataway, NJ).

The membrane was blocked in blotting buffer (PBS 5% nonfat dry milk, 150 mM NaCl, 0.1% Tween-20, 0.02% azide) for 30 min and incubated for 1 h with an antibody prepared against purified platelet LTBP (Ab39) diluted 1:3,000 in blotting buffer. All dilutions and washes were performed in blotting buffer except where indicated. After removal of the primary antibody, the blot was washed 4 × 15 min, and goat α-rabbit IgG-alkaline phosphatase (Promega Corp., Madison, WI) diluted 1:7,500 was added. After 1 h, the blot was washed 8 × 15 min and 3 × 5 min in PBS-0.1% Tween-20. The blot was developed for 2 h in BCIP/NBT phosphatase substrate and stopped by washing with PBS.

Preparation of Conditioned Media

To prepare homotypic conditioned medium, confluent cultures of the cells were trypsinized and seeded separately in either 35-mm plastic dishes or 24-well Linbro plates (Flow Lab., Inc., McLean, VA) in 10% calf serum at a cell density of 8×10^4 BAE and 4×10^4 BSM cells per cm^2. After 1.5 h of incubation at 37°C, this medium was replaced with 0.1 ml/cm^2 serum-free αMEM containing 0.1% BSA. After 5 h, the medium was collected and centrifuged to remove debris. Conditioned medium from homotypic cultures mixed 1:1 (BAE/BSM cell) was used as a control for medium conditioned by heterotypic cultures.

Heterotypic conditioned medium was prepared by trypsinizing confluent cultures of BAE and BSM cells and seeding the two cell types together in either 35-mm or 24-well Linbro plates at a cell density of 4×10^4 BAE and 2×10^4 BSM cells per cm^2. After 1.5 h of incubation at 37°C, this medium was replaced with 0.1 ml/cm^2 serum-free αMEM containing 0.1% BSA. After 5 h, the medium was collected and centrifuged.

To acid treat conditioned medium, medium was acidified to pH 2.0 with HCl and neutralized after 30 min at room temperature with NaOH. To activate latent TGF-β by proteases, conditioned medium was incubated for 2 h with plasmin at 37°C. After this incubation, subsequent proteolysis was inhibited by the addition of aprotinin (10 μg/ml).

Wound Assays for BAE Cell Migration

Wound assays were performed as previously described (Sato and Rifkin, 1988). Briefly, confluent monolayers of BAE cells in 35-mm dishes were wounded with a razor blade. After wounding the cells were washed with PBS and further incubated in αMEM containing 0.1% BSA for 20 h. The cells were fixed with absolute methanol after the incubation and stained with Giemsa. Cells that had migrated from the edge of the wound were counted in successive (seven) 125-μm increments at 100× using a light microscope with an ocular grid. The cell number represents the mean from at least four different fields.

^{3}H-Thymidine Incorporation Assay

The inhibition of ^{3}H-thymidine incorporation by CCl 64 mink lung cells as an assay for TGF-β has been previously described (Tucker et al., 1984). Briefly, the CCl 64 cells were trypsinized, centrifuged, and resuspended in DME containing 0.2% FCS. 100 μl of medium containing 8×10^4 cells was transferred to a 96-well plate and incubated for 2 h at 37°C. The medium was removed from the wells, replaced with the indicated additions, and allowed to incubate overnight. The additions were replaced with 100 μl of ^{3}H-thymidine (1 μCi/ml) in DME containing 0.2% FCS and incubated for 2 h at 37°C. After this incubation, the cells were fixed with 100

μl of a 3:1 (vol/vol) solution of methanol:acetic acid for 10 min, washed twice with 80% methanol, incubated with 100 μl of a 0.5% trypsin solution for 30 min, and solubilized with an additional 100 μl of a 20% solution of SDS. The amount of radioactivity that remained associated with the cells was quantitated with a Beckman LS3801 beta scintillation counter (Beckman Instruments, Fullerton, CA).

Plasminogen Activator Assay

Confluent cultures of BAE cells grown in 96-well plates were preincubated in serum-free αMEM for 10 h, and then incubated overnight with the indicated additions. After this incubation, the cells were washed twice with PBS and extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1. 10 μl of cell extract was assayed for plasminogen activator (PA) activity using a ^{125}I-fibrin plate assay (Gross et al., 1982).

Results

Detection of LTBP in Conditioned Media

To explore the possibility that LTBP is involved in the activation of latent TGF-β in co-cultures of BAE and BSM cells, we first determined whether these cells secreted LTBP and, if so, whether this protein occurs in association with a high mol wt latent TGF-β complex. Medium conditioned by equal numbers of either BAE or BSM cells were cross-linked, concentrated and analyzed by SDS-PAGE. Western blot analysis of cross-linked conditioned medium using anti-TGF-β antibody (Fig. 1 *A*) shows that both BAE and BSM secrete TGF-β as a high mol wt latent TGF-β complex evident as a band migrating with an apparent mol wt of 180,000–205,000. The lack of significant reactivity corresponding to lower mol wt forms of TGF-β suggests that the vast majority of TGF-β secreted by BAE and BSM occurs in a high mol wt complex. When a companion gel was analyzed by immunoblotting using anti-LTBP antibody, bands were observed with the same apparent mol wt as those detected with the anti-TGF-β antibody (Fig. 1 *B*). Incubation of a sample of the cross-linked conditioned medium after SDS-PAGE with nonimmune serum yielded no positive bands (Fig. 1 *C*). Thus, both BAE and BSM cells appear to release TGF-β as a high mol wt complex containing LTBP. The relatively narrow distribution of molecular weight of the latent TGF-β complex when compared to that observed by Miyazono et al. (1991) probably reflects shorter incubation times and the absence of serum during our incubation. The absence of any lower molecular weight bands in Fig. 1 *B* suggests that no free LTBP is secreted into the medium by either cell type.

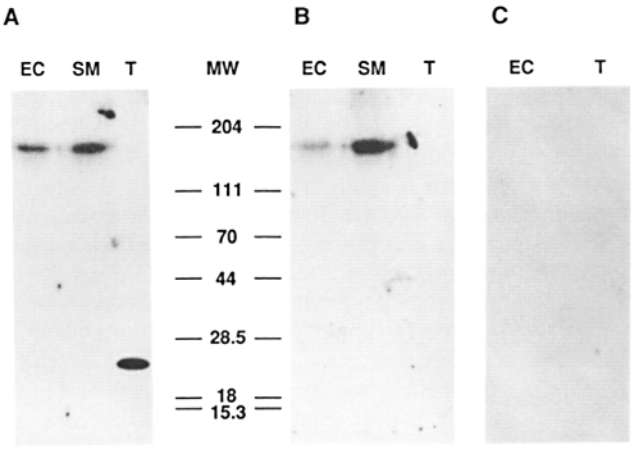

Figure 1. Western blot of conditioned media from BAE and BSM cells probed with anti-TGF-β and anti-LTBP IgG. Medium conditioned by BAE or BSM was chemically crosslinked using BS^3, concentrated, and analyzed by SDS-PAGE under nonreducing conditions. Proteins were transferred to nitrocellulose paper and probed with either (*A*) anti-TGF-β, (*B*) anti-LTBP IgG, or (*C*) nonimmune IgG. Bound antibodies were visualized using ^{125}I-Protein A followed by autoradiography. Lanes *EC* contain conditioned medium from BAE cells, lanes *SM* contain conditioned medium from BSM cells, and lanes *T* contain 50 ng of rTGF-β1.

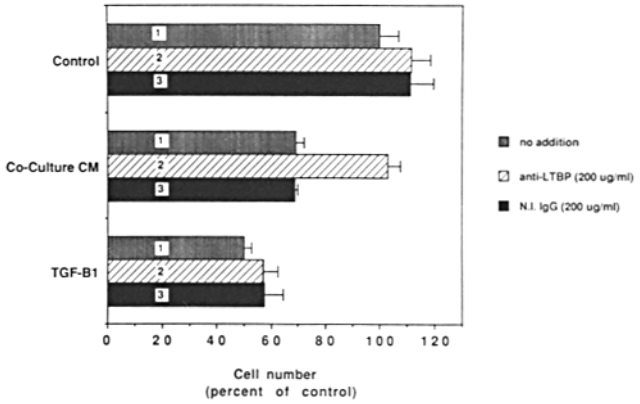

Figure 2. Effect of antibodies to LTBP on generation of an inhibitor of BAE movement in heterotypic cultures. Confluent cultures of BAE cells were wounded as described in Experimental Procedures. Serum-free media (*Control*), conditioned media from co-cultures of BAE and BSM cells (*Co-culture CM*), or serum-free media containing recombinant TGF-β1 (300 pg/ml) (*TGF-β1*) were placed on the cells. Nonimmune IgG (200 μg/ml) or anti-LTBP IgG (200 μg/ml) were added either directly to the medium (*control* and *TGF-β1*) and placed on the wounded BAE monolayer or incubated with the co-cultures (*Co-culture CM*) for 20 h before being placed on the wounded monolayer. The cells were allowed to migrate overnight, and the monolayers were fixed and stained, and the number of cells that had crossed the original wound margin were counted. Data are expressed as a percent of migration observed in a control untreated wound. The average number of cells that had migrated from the origin in the control sample was 117.

Anti-LTBP Antibody Inhibits Generation of TGF-β in Heterotypic Cultures

To test whether LTBP plays a role in the activation of latent TGF-β, the generation of TGF-β by co-cultures of BAE and BSM cells in the presence of antibody to native LTBP was monitored. The TGF-β generated in heterotypic co-cultures can be quantitated by the ability of the co-culture conditioned medium to block the migration of BAE cells from the edge of a wounded monolayer (Sato and Rifkin, 1989; Sato et al., 1990). Medium conditioned by co-cultures of BAE and BSM cells contains active TGF-β (Fig. 2, *Co-Culture CM*, row *1*) as previously demonstrated by receptor competition assays (Sato et al., 1990) and with a TGF-β neutralizing antibody (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). Medium conditioned by these cell types in the presence of anti-LTBP IgG, however, showed no evidence of TGF-β activity (Fig. 2, *Co-Culture CM*, row *2*). Medium conditioned in the presence of nonimmune antibody did contain TGF-β activity (Fig. 2, *Co-Culture CM*, row *3*). Anti-

Table I. Assay of Active TGF-β in Conditioned Media from Co-Cultured BAE and BSM Cells in the Presence of Various Additions

Sample	TGF-β(pg/ml)
Conditioned medium	
Alone	17 ± 1.2
+LTBP antibody (200 μg/ml)	<2
+TGF-β antibody (50 μg/ml)	<2
+LTBP Fab (50 μg/ml)	<2
+N.I. antibody (200 μg/ml)	21 ± 1.5
Acid-treated conditioned medium	
Alone	1,100 ± 90
+LTBP antibody (200 μg/ml)	1,000 ± 172

Co-cultures of BAE and BSM cells (as described in Materials and Methods) were incubated in αMEM or with the indicated additions for 6 h. The conditioned media from the first group of these cultures were transferred directly onto confluent monolayers of BAE cells. The conditioned media from the second group of these cultures were acidified to pH 2.0 for 30 min and neutralized before transfer onto confluent monolayers of BAE cells. Cells were incubated for 12 h and then extracted in 0.5% Triton X-100 and assayed for PA activity. The amount of TGF-β present in the co-culture conditioned medium was calculated by reference to a standard curve using recombinant TGF-β1.

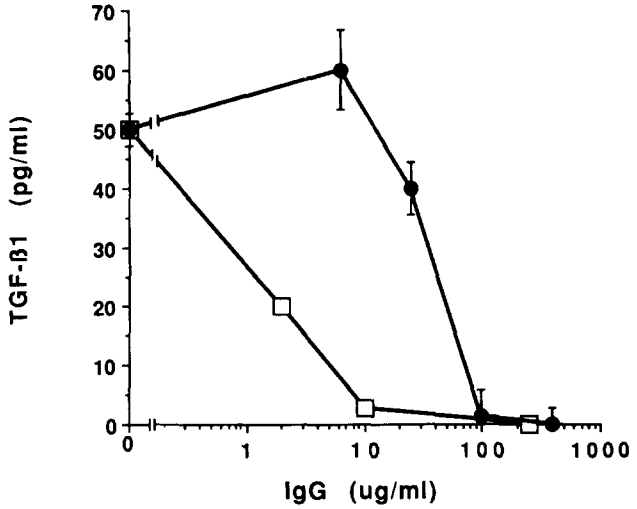

Figure 3. Dose-dependent inhibition of TGF-β generation in heterotypic cultures by LTBP antibody. Co-cultures of BAE and BSM cells were incubated in αMEM containing the indicated concentrations of anti-LTBP IgG for 6 h. The conditioned media from these cultures were then transferred onto confluent monolayers of BAE cells for 12 h. After incubation, the cells were extracted in 0.5% Triton X-100 and assayed for PA activity. The degree of inhibition of PA activity was converted to pg/ml of TGF-β by reference to a standard curve obtained using recombinant TGF-β1 added to BAE cells. □, anti-LTBP IgG; ●, anti-LTBP peptide IgG.

LTBP IgG did not affect the movement of BAE cells into a denuded area normally observed within 20 h after wounding (Fig. 2, *Control*), nor did anti-LTBP IgG effect the inhibitory activity of recombinant TGF-β1 on the migration of BAE cells (Fig. 2, *TGF-β*, row *2*).

Whereas the inhibition of BAE migration is a sensitive assay for TGF-β, it requires relatively large volumes of medium and reagents. The inhibition of PA activity of confluent BAE cells by TGF-β can be performed in 96-well plates and provides a convenient assay for quantitating TGF-β generation by co-cultured cells. PA activity in BAE cells is decreased by mature rTGF-β1 in a dose-dependent manner with an ED_{50} of 15–20 pg/ml (Flaumenhaft and Rifkin, 1992). The amount of TGF-β generated by heterotypic cocultures can be determined by quantitating the amount of inhibition of PA activity by heterotypic conditioned medium and comparing this to the inhibition of PA activity by standard rTGF-β1. Table I shows the amount of TGF-β as measured by the PA assay generated in the medium of co-cultures of BAE and BSM cells incubated in the presence of the indicated additions. The inhibitory effect of anti-TGF-β IgG indicates again that the activity in the co-culture conditioned medium is TGF-β and that the PA assay under these conditions is specific for TGF-β1. The observation that anti-LTBP IgG decreases the amount of TGF-β activity in heterotypic culture conditioned medium, whereas nonimmune IgG has no effect, confirms the data obtained with the migration assay (Fig. 2). A Fab fragment derived from the anti-LTBP IgG also inhibited the generation of TGF-β in co-cultures, demonstrating that the antibody does not act by forming large aggregates with the LTBP and precipitating the latent TGF-β from solution (Table I). The fact that acid-treated conditioned medium from cells co-cultured in the presence of anti-LTBP IgG contains at least as much TGF-β as control co-culture conditioned medium demonstrates that the anti-LTBP IgG does not inhibit the generation of TGF-β by preventing the secretion of latent TGF-β. Inhibition of activation of latent TGF-β by anti-LTBP IgG occurs in a dose-dependent manner with an ED_{50} of 1–2 μg/ml (Fig. 3). Antibody raised against a synthetic peptide of LTBP also inhibited activation in a dose-dependent manner with an ED_{50} of approximately 50 μg/ml (Fig. 3). The difference in potencies between the two antibodies is consistent with the observation that the peptide antibody has a lower affinity for native LTBP than the antibody to the purified protein (data not shown).

Anti-LTBP IgG Does Not Interfere with Plasmin Cleavage of Latent TGF-β or rTGF-β1 Activity

One mechanism by which anti-LTBP IgG might inhibit the activation of latent TGF-β in co-culture is to prevent plasmin from cleaving the LAP of the latent TGF-β complex (Lyons et al., 1990; Sato et al., 1990). To test this possibility, conditioned medium from homotypic cultures of BSM cells was treated with purified plasmin in the absence or presence of anti-LTBP IgG. A ^{3}H-thymidine incorporation assay was used to monitor the generation of active TGF-β because the presence of plasmin inhibitors used to stop the reaction would interfere with the PA assay. Incubation of conditioned medium with increasing amounts of plasmin under control conditions resulted in a decrease in ^{3}H-thymidine incorporation (Fig. 4). This effect was blocked by the inclusion of a TGF-β neutralizing antibody proving that the decrease in DNA synthesis resulted from TGF-β generated via plasmin. The addition of the LTBP antibody to the conditioned medium before the addition of plasmin did not block the plasmin-mediated generation of TGF-β. In fact, a slight stimulation was noted. Nonimmune IgG had a small effect. Thus, inhibition of latent TGF-β activation by anti-LTBP IgG is not the result of inhibition of plasmin cleavage.

Anti-LTBP IgG might act directly on the mature TGF-β generated in heterotypic conditions. However, rTGF-β1 inhibits PA activity in BAE cells in a dose-dependent manner

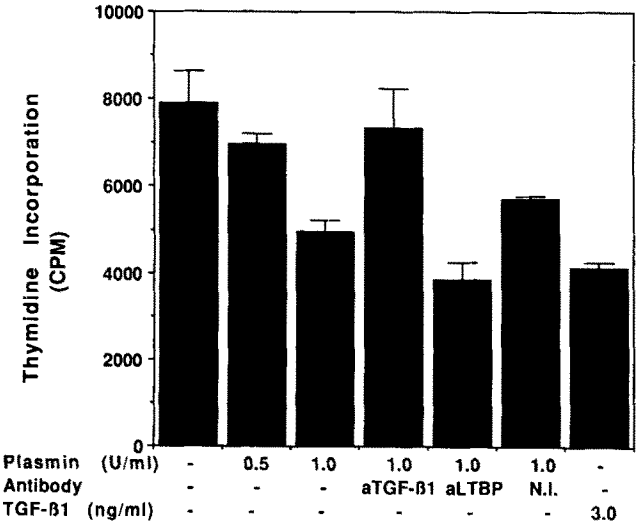

Figure 4. Effect of anti-LTBP antibody on the generation of active TGF-β1 from BSM cell conditioned media by purified plasmin. BSM cells were incubated for 20 h in αMEM, and the conditioned media were collected. The samples were incubated with plasmin (1.0 U/ml unless otherwise noted) either alone or in combination with antibody to LTBP (200 μg/ml), nonimmune IgG (200 μg/ml), or TGF-β1 IgG (200 μg/ml) for 2 h at 37°C, and then aprotinin (10 μg/ml) for 15 min. The samples were transferred onto confluent monolayers of CCl 64 cells, incubated for 12 h, and assayed for ^{3}H-thymidine incorporation as described in Materials and Methods.

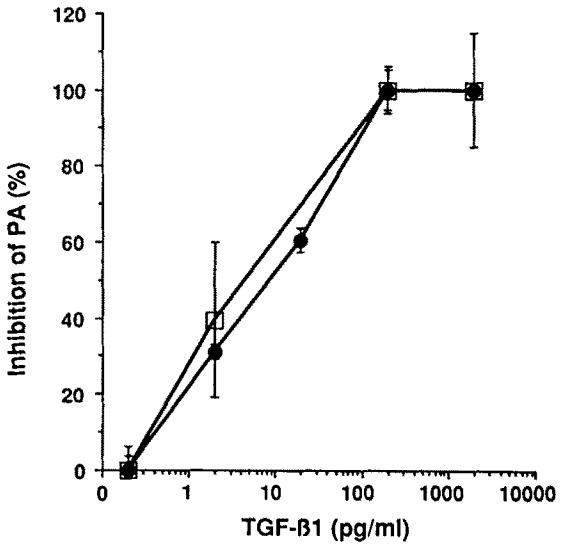

Figure 5. Effect of anti-LTBP antibody on inhibition of PA activity by recombinant TGF-β1. Confluent cultures of BAE cells were incubated in αMEM containing the indicated concentrations of TGF-β1 with or without anti-LTBP IgG (200 μg/ml). After incubation the cells were extracted in 0.5% Triton X-100 and assayed for PA activity. □, TGF-β1 alone; ●, TGF-β1 plus anti-LTBP IgG.

equally in the absence or presence of antibody to LTBP (Fig. 5). Furthermore, although anti-TGF-β IgG demonstrates an inhibitory effect if added to heterotypic cultures either during or after the medium is conditioned, anti-LTBP IgG only inhibits TGF-β activity if added during the conditioning of the co-culture medium (data not shown). Thus, the anti-LTBP IgG blocks the generation of TGF-β in heterotypic culture but does not directly inhibit the activity of TGF-β.

Purified LTBP Inhibits Generation of Latent TGF-β in Heterotypic Cultures

Because the LTBP contains structural motifs that are involved in protein–protein interactions in other proteins, anti-LTBP IgG may inhibit latent TGF-β activation by preventing the high mol wt latent TGF-β complex from associating with a binding site, perhaps on the cell surface. To address this possibility, an experiment was conducted in which the ability of added free LTBP to inhibit the generation of TGF-β by co-cultures was determined. The rationale for conducting this experiment is that if a binding site for LTBP did exist on the cell surface, then free excess LTBP might compete with the LTBP in the high molecular weight latent TGF-β complex for the putative LTBP binding site and inhibit the activation of the latent TGF-β. Thus, the effect of excess free LTBP on latent TGF-β activation was determined by quantitating the generation of TGF-β in co-cultures incubated with free excess LTBP.

The amount of LTBP used in initial experiments was at least 60-fold greater on a molar ratio than the estimated amount of high molecular weight latent TGF-β (1 ng/ml) in heterotypic conditioned medium (Table I). The addition of purified free LTBP completely inhibited the generation of TGF-β in co-cultures of BAE and BSM cells (Fig. 6 *A*), whereas the addition of free LTBP denatured by boiling for 15 min did not. The purified LTBP contained one major band as visualized by staining after SDS-PAGE (Fig. 6 *B*). Fig. 7 demonstrates that the inhibition of the generation of TGF-β by LTBP occurred in a dose-dependent manner with an ED_{50} of ~2 ng/ml of LTBP. Acid treatment of media conditioned by heterotypic cultures demonstrated the presence of ~8 ng/ml of latent TGF-β whether the media were formed in the absence or presence of excess LTBP. Therefore, excess LTBP did not act by inhibiting the secretion of latent TGF-β. Furthermore, excess LTBP did not act by inhibiting the secretion of latent TGF-β. Furthermore, LTBP did not inhibit the activity of mature recombinant TGF-β1 (Fig. 8). This experiment suggests that the LTBP does not directly inhibit TGF-β, but inhibits the generation of mature TGF-β from the latent complex.

Discussion

Activation of Latent TGF-β

TGF-β is a potent modulator of cell growth and differentiation for many cell types (Lyons and Moses, 1990; Roberts and Sporn, 1990; Massague et al., 1990). It is present in serum and in cell culture conditioned medium at concentrations well above the levels necessary for the mature form to elicit in vitro cellular responses. However, the molecule exists primarily as a high mol wt latent complex (Miyazono et al., 1988; Wakefield et al., 1988) from which it must be released in order to bind to its cell surface receptor. The mechanism by which active TGF-β is released from the latent complex is not well understood, but it is assumed that this process is important in the regulation of TGF-β activity in vivo.

Physiological activation of latent TGF-β has been proposed to occur in homotypic cultures of human erythroleukemia cell lines (Piao et al., 1990), granulosa cell lines

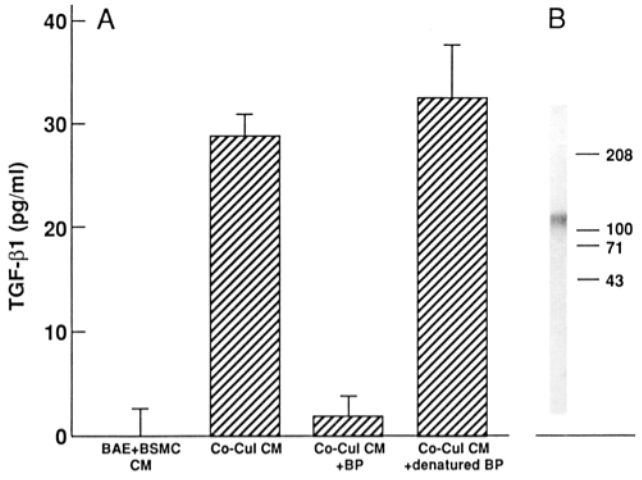

Figure 6. Inhibition of TGF-β generation in heterotypic culture by purified LTBP. (*A*) Co-cultures of BAE and BSM cells were incubated in αMEM containing no addition, purified LTBP, or heat-denatured purified LTBP for 5 h. The conditioned media from these cultures and from control BAE and BSM cells were cultured separately, mixed, and then transferred onto confluent monolayers of BAE cells and incubated for 12 h. After incubation, the cells were extracted in 0.5% Triton X-100 and assayed for PA activity. The amount of TGF-β present in the co-culture medium was calculated by reference to a standard curve using recombinant TGF-β1. (*B*) Immunoblot of purified LTBP. 50 ng of LTBP purified from human platelets was analyzed on a 5–16% SDS-polyacrylamide gel, transferred to Immobilon-P membrane, and analyzed with antibody Ab39 prepared against purified LTBP.

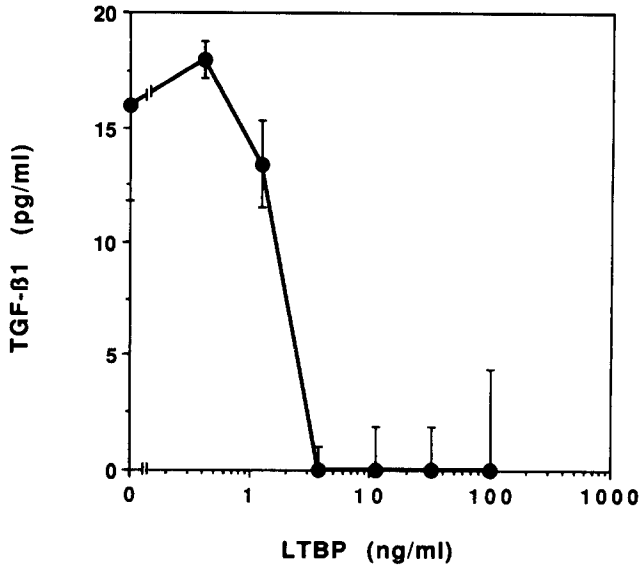

Figure 7. Dose-dependent inhibition of TGF-β generation in heterotypic culture by purified LTBP. Co-cultures of BAE and BSM cells were incubated in αMEM containing increasing amounts of purified LTBP. The conditioned media from these cultures were transferred onto confluent monolayers of BAE cells and incubated for 12 h. After incubation, the cells were extracted in 0.5% Triton X-100 and assayed for PA activity. The amount of TGF-β present in the co-culture medium was calculated by reference to a standard curve using recombinant TGF-β1.

plated on fibronectin (Kim and Schomberg, 1989), osteoclasts activated by vitamin A (Oreffo et al., 1989), macrophages exposed to γ-interferon (Twardzik et al., 1990), keratinocytes treated with retinoic acid (Glick et al., 1989), and mammary carcinoma cells and fibroblasts treated with antiestrogens (Knabbe et al., 1987; Colletta et al., 1990). Endogenous activation of latent TGF-β also occurs in co-cultures of BAE and BSM cells (or pericytes) (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). Activation of latent TGF-β by heterotypic cultures of BAE and BSM cells requires cell-to-cell contact (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989), binding of latent TGF-β to mannose-6-phosphate receptors (Dennis and Rifkin, 1991), PA and plasmin activity (Sato and Rifkin, 1989; Sato et al., 1990), and is complete within 6 h of co-culturing (S. Kojima, unpublished observation). The concentration of active TGF-β attained in the conditioned medium of co-cultured cells appears to be 15–50 pg/ml. Though this quantity represents only 2–5% of the total latent TGF-β present in co-culture conditioned medium, it is near or above the ED_{50} of recombinant mature TGF-β in several assays of TGF-β such as inhibition of ^3H-incorporation into CCl 64 mink lung cells (Tucker et al., 1984), inhibition of PA activity in BAE cells (Flaumenhaft and Rifkin, 1992), and inhibition of BAE migration (Sato et al., 1990). Thus, the molecular mechanism of latent TGF-β activation remains largely unknown even though in vitro systems have been developed in which biologically significant amounts of TGF-β are produced.

The activation of latent TGF-β may resemble the surface catalyzed activation of coagulation factors in which the binding of zymogens and cofactors to specific cell surface molecules increases the efficiency of activation by orders of magnitude. The activation of prothrombin may be a useful paradigm for latent TGF-β activation (Furie and Furie, 1988). Prothrombin circulates at relatively high levels compared to those necessary to initiate coagulation, the inactive

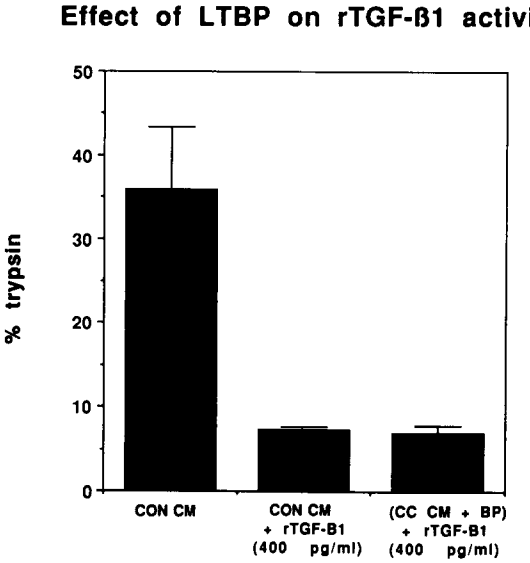

Figure 8. Effect of LTBP on the ability of recombinant TGF-β1 to induce a decrease in fibrinolytic activity in BAE cells. Confluent cultures of BAE cells were incubated in αMEM containing no addition, rTGF-β1 (400 pg/ml), or rTGF-β1 (400 pg/ml) plus LTBP (500 ng/ml). After an overnight incubation, the cells were extracted in 0.5% Triton X-100 and assayed for PA activity.

amino terminal region contains specific domains that target prothrombin to surface activation complexes of factor X_a and V_a, and prothrombin activation is, to a degree, self-regulating. Most of these features are repeated in other proteolytic activation reactions and represent an effective way to control extracellular activities.

Although soluble latent TGF-β from conditioned medium can be activated by purified plasmin (Lyons et al., 1988), the concentrations of plasmin required (1 U/ml) are nonphysiologic. Thus, it is likely that plasmin cleavage of latent TGF-β occurs on the cell surface where both latent TGF-β and plasmin can be concentrated and the activity of plasmin enhanced. The concentration of latent TGF-β on the cell surface may occur through its association with the cation-independent mannose-6-phosphate receptor (Kovacina et al., 1989; Purchio et al., 1988). Mannose-6-phosphate and anti-mannose-6-phosphate receptor antibody appear to inhibit activation by blocking the binding of latent TGF-β to the cell surface (Dennis and Rifkin, 1991). However, the mannose-6-phosphate receptor is present at a high concentration in many cell types and would not be expected to confer specificity to the activation reaction. Furthermore, latent TGF-β bound to mannose-6-phosphate receptor might be rapidly internalized. Thus, a potential role of the LTBP is to target the latent TGF-β to a molecular assembly responsible for the catalytic activation of latent TGF-β. Additionally, the LTBP may decrease the rate of internalization of latent TGF-β upon binding the mannose-6-phosphate receptor. We are currently testing this hypothesis.

It is also worthwhile to consider the significance of the absolute amount of TGF-β formed in these cocultures and its possible relevance to in vivo activities. In the co-culture system only 2–5% of the total latent TGF-β is found in the activated state. Although it might appear that a system that generates such a small amount of TGF-β is of no consequence, several considerations suggest that this level of activation may have biological significance. First, LTGF-β1 activation in the co-culture system is self-regulating (Sato et al., 1990); the TGF-β that is formed inhibits subsequent activation through increased expression of plasminogen activator inhibitor 1 (PAI-1) by both BAEs and SMCs. Thus, once PAI-1 expression is stimulated by the TGF-β that is initially produced, the cells continue to secrete latent TGF-β but do not convert it to TGF-β. Therefore, at later times, the ratios of active to latent TGF-β1 are skewed by the continuous production of latent TGF-β. At early time points in the co-culture system 20% of the total TGF-β formed may be active (S. Kojima, unpublished observation). Second, the concentration of TGF-β attained (20–40 pg/ml) is at the ED_{50} for many of the biological effects of TGF-β1. Third, the interactions of the LTBP and the mannose-6-phosphate residues of LTGF-β1 with cell surface moieties will increase the local concentration of latent TGF-β, and the surface activation will enhance the pericellular concentration of the active growth factor. The interaction of TGF-β with other matrix and cell surface molecules might also produce stimulatory concentrations. Finally, if the in vivo response of cells to TGF-β mimics the in vitro responses, the activation of a high percentage of the latent TGF-β would generate TGF-β1 concentrations 10–100-fold above the ED_{50} of the cytokine for its receptor condition. This would create a condition in which modulation of TGF-β effects would be difficult as large amounts of active ligand would have to be removed to attain the nonstimulated state.

Role of the LTBP in the Activation of Latent TGF-β

LTBP has recently been shown to have a role in the proper assembly and secretion of TGF-β (Miyazono et al., 1991). Tsuji et al. (1990) have speculated that LTBP may function in the activation of latent TGF-β. Our data indicate a role for the LTBP in the activation of latent TGF-β in heterotypic cultures. Western blot analysis demonstrates that the vast majority of latent TGF-β secreted by both BAE and BSM cells contains the LTBP. Although it is possible that some free latent TGF-β occurs without LTBP, the level of such a species must be relatively small to remain undetected in our assay. We have observed inhibition of latent TGF-β activation by an antibody to native platelet LTBP, an antibody to a synthetic peptide corresponding to a 12-amino acid sequence within the native platelet LTBP, and a Fab fragment of the native platelet LTBP antibody. These antibodies probably block activation by preventing a specific domain in LTBP from interacting with a surface component involved in the formation of a surface complex required for the activation of latent TGF-β. Inhibition of activation by addition of excess LTBP most likely occurs through competition of exogenous LTBP with the LTBP occurring in the high mol wt complex. The unlikely possibility that a contaminant in the LTBP preparation is inhibiting activation has, however, not been excluded. Together these data form strong support for the hypothesis that the LTBP plays an essential role in the activation of latent TGF-β in heterotypic cultures.

Analysis of the primary structure of human LTBP revealed the presence of a number of motifs involved in protein–protein interactions (Kanzaki et al., 1990). Of particular interest with regard to this possibility are the multiple EGF-like repeats, at least two of which contain a β-hydroxylated asparagine, an RGD sequence, and a laminin B2-like sequence. These sequences are involved in cell surface protein–protein interactions in a variety of molecules (Appella et al., 1988; Ruoslahti and Pierschbacher, 1987; Sasaki and Yamada, 1987). In addition, the LTBP contains three copies of a motif with eight cysteine residues. The organization of the eight cysteine-containing domains and EGF-like repeats has recently also been described in the matrix protein fibrillin (Maslen et al., 1991). Our studies predict that the LTBP should interact with a cell surface or matrix macromolecule. Although we have some indirect indications of a cell surface or localization of the LTBP, attempts to demonstrate directly binding of LTBP to the cell surface in a saturable manner have thus far been unsuccessful due to the fact that we have been unable to radiolabel the LTBP to a high specific activity. Although it is presently impossible to determine which sequences confer this putative targeting activity on LTBP, analysis of rat LTBP revealed neither an RGD sequence nor a laminin B2-like sequence suggesting that these may not be critical to the function of LTBP (Tsuji et al., 1990). Further studies on the role of LTBP in the activation of latent TGF-β should determine with what structures the LTBP interacts and what parts of the LTBP are involved in this interaction.

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