

# The Mechanism for the Activation of Latent TGF- $\beta$ during Co-culture of Endothelial Cells and Smooth Muscle Cells: Cell-type Specific Targeting of Latent TGF- $\beta$ to Smooth Muscle Cells

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**Abstract.** Transforming growth factor- $\beta$  (TGF- $\beta$ ) is secreted in a latent form and activated during co-culture of endothelial cells and smooth muscle cells. Plasmin located on the surface of endothelial cells is required for the activation of latent TGF- $\beta$  (LTGF- $\beta$ ) during co-culture, and the targeting of LTGF- $\beta$  to the cellular surface is requisite for its activation. In the present study, the cellular targeting of LTGF- $\beta$  was examined. We detected the specific binding of <sup>125</sup>I-large LTGF- $\beta$ 1 isolated from human platelets to smooth muscle cells but not to endothelial cells. A mAb against the latency-associated peptide (LAP) of large LTGF- $\beta$ 1 complex, which blocked the binding of <sup>125</sup>I-large LTGF- $\beta$ 1 to smooth muscle cells, inhibited the

activation of LTGF- $\beta$  during co-culture. The binding of <sup>125</sup>I-large LTGF- $\beta$ 1 could not be competed either by mannose-6-phosphate (300  $\mu$ M) or by the synthetic peptide Arg-Gly-Asp-Ser (300  $\mu$ g/ml). These results indicate that the targeting of LTGF- $\beta$  to smooth muscle cells is required for the activation of LTGF- $\beta$  during co-culture of endothelial cells and smooth muscle cells. The targeting of LTGF- $\beta$  to smooth muscle cells is mediated by LAP, and the domain of LAP responsible for the targeting to smooth muscle cells may not be related to mannose-6-phosphate or an Arg-Gly-Asp sequence, both of which have been previously proposed as candidates for the cellular binding domains within LAP.

**T**RANSFORMING growth factor- $\beta$  (TGF- $\beta$ )<sup>1</sup>, a member of a family of 25-kD homodimeric polypeptides, is produced and secreted by a variety of cell types including endothelial cells and smooth muscle cells and has potent activities on cell growth, motility, and differentiation (for review see Lyons and Moses, 1990; Massague, 1990; Robert and Sporn, 1992). However, TGF- $\beta$  is usually secreted as a biologically inactive latent form (Pircher et al., 1984; Wakefield et al., 1987). There are three components of the latent TGF- $\beta$  (LTGF- $\beta$ ) complex: (a) the mature TGF- $\beta$ ; (b) the TGF- $\beta$  latency-associated peptide (LAP); and (c) the latent TGF- $\beta$  binding protein (LTBP) (Miyazono et al., 1988; Wakefield et al., 1988; Tsuji et al., 1990). LAP is a 75-kD homodimer representing the NH<sub>2</sub>-terminal remnant of the

proTGF- $\beta$ . LTBP is a protein of 125–160 kD that is attached to LAP by a disulfide bond, and is required for the assembly and secretion of LTGF- $\beta$  (Miyazono et al., 1991). LTGF- $\beta$  composed of the mature TGF- $\beta$ , LAP, and LTBP is denoted the large LTGF- $\beta$  complex, whereas the complex composed of the mature TGF- $\beta$  and LAP is denoted the small LTGF- $\beta$  complex. It has been reported that both endothelial cells and smooth muscle cells secrete large LTGF- $\beta$  (Flaumenhaft et al., 1993). Since TGF- $\beta$  is secreted in a latent form, the dissociation of mature TGF- $\beta$  from the latent complex is essential for expression of its biological activities postsecretion. Although LTGF- $\beta$  can be activated by transient acid, base, heat, or chaotropic agents like urea in a test tube, the mechanism of the activation of LTGF- $\beta$  in vivo remains unexplained. LTGF- $\beta$  is activated by proteolysis of the latent complex with plasmin and cathepsin D (Lyons et al., 1988), or by alteration of carbohydrate structures within the LAP (Miyazono and Heldin, 1989). Plasmin cleaves LAP and releases mature TGF- $\beta$  from LTGF- $\beta$  (Lyons et al., 1991). In tissue culture conditions, LTGF- $\beta$  is activated by co-culture of bovine endothelial cells and bovine pericytes/smooth muscle cells. The TGF- $\beta$  formed inhibits the migration and proliferation of bovine endothelial cells (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). The activation

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1. *Abbreviations used in this paper:* TGF- $\beta$ , transforming growth factor- $\beta$ ; LAP, latency-associated peptide; LTBP, latent TGF- $\beta$  binding protein; LTGF- $\beta$ , latent TGF- $\beta$ ; LLTGF- $\beta$ , large latent TGF- $\beta$ ; PA, plasminogen activator; PAE, porcine aortic endothelial; PASM, porcine aortic smooth muscle.

of LTGF- $\beta$  during co-culture is mediated by plasminogen activator (PA)-plasmin activity (Sato and Rifkin, 1989). PA-plasmin is localized on the cellular surface (for review see Mignatti and Rifkin, 1993). Targeting of LTGF- $\beta$  to the cell surface appears to be required for the activation of LTGF- $\beta$  during co-culture of two cell types (Dennis and Rifkin, 1991; Flaumenhaft et al., 1993). However, the characterization of the cellular targeting of LTGF- $\beta$  has not been previously done.

In the present study, we isolated large LTGF- $\beta$ 1 (LLTGF- $\beta$ 1) from human platelets, and examined the binding of  $^{125}$ I-LLTGF- $\beta$ 1 to endothelial cells or smooth muscle cells. Moreover, mAbs against LAP and LTBP were developed, and the roles of LAP and LTBP on the activation and targeting of LTGF- $\beta$  were examined. We found that  $^{125}$ I-LLTGF- $\beta$ 1 was bound only to smooth muscle cells but not to endothelial cells, and that at least LAP of the LTGF- $\beta$  complex was responsible for the cellular targeting to smooth muscle cells. By eliminating cellular targeting of LTGF- $\beta$  with a mAb against LAP, the activation of LTGF- $\beta$  during co-culture was blocked.

## Materials and Methods

### Cells

Porcine aortic endothelial (PAE) cells were isolated by scraping an endothelial layer from the porcine aorta with a blade. The cells were plated on a gelatin-coated plastic dish, and cultivated in DME containing 5% FCS. Porcine aortic smooth muscle (PASM) cells were isolated from the same tissue using the explant technique, and were grown in DME containing 5% FCS.

### Isolation and Iodination of Human Platelet Large LTGF- $\beta$ 1

Isolation of LLTGF- $\beta$ 1 from human platelets was performed by the method described previously (Okada et al., 1989). Isolated LLTGF- $\beta$ 1 was labeled with  $^{125}$ I by chloramine-T method (Ono et al., 1982). The specific activity of  $^{125}$ I-LLTGF- $\beta$ 1 was  $6.93 \times 10^5$  cpm/ng.  $^{125}$ I-LLTGF- $\beta$ 1 was analyzed by SDS-PAGE as follows.  $^{125}$ I-LLTGF- $\beta$ 1 was cross-linked with 2 mM bis (sulfosuccinimidyl) Suberate (Pierce Chemical Co., Rockford, IL) for 20 min at 4°C as described (Flaumenhaft et al., 1993).  $^{125}$ I-LLTGF- $\beta$ 1 or cross-linked  $^{125}$ I-LLTGF- $\beta$ 1 was run on a 7.5% SDS gel under reducing or nonreducing conditions and iodinated proteins were visualized by autoradiography.

### Binding Assay of Large LTGF- $\beta$ 1 to PAE Cells or PASM Cells

PAE cells and PASM cells were cultured separately in 35-mm plastic dishes. After the cells reached confluence, the binding experiments were performed. The cells were preincubated in serum-free DME containing 0.1% BSA for 5 h at 37°C. Next, the cells were washed with PBS and incubated with  $^{125}$ I-LLTGF- $\beta$ 1 in DME containing 0.1% BSA at 4°C for 3 h. 100-fold concentration of unlabeled LLTGF- $\beta$ 1 were added to determine a non-specific binding. After the incubation, the cells were washed with ice-cold PBS three times, and the cell-associated radioactivities extracted by 0.5% Triton X-100 were determined. In several experiments, either mAbs as described below, 300  $\mu$ M of mannose-6-phosphate (Sigma Immunochemicals, St. Louis, MO), or 300  $\mu$ g/ml of Arg-Gly-Asp-Ser (Sigma Immunochemicals) were added during the incubation period.

### Preparation of mAbs against LAP or LTBP

Isolated LLTGF- $\beta$ 1 was treated with 6 M urea to dissociate mature TGF- $\beta$  from LAP-LTBP complex. LAP-LTBP complex was further isolated by gel filtration chromatography using a Superose 6 column (Pharmacia-LKB Biotechnology, Uppsala, Sweden) in the presence of 6 M urea. Isolated LAP-LTBP complex was dialyzed against PBS. Adult SD rats or BALB/c

mice were immunized by intraperitoneal injection of LAP-LTBP complex-KLH (Hemocianin Keyhole Limpet) conjugate mixed with aluminium gel and Bordetella pertussis, followed by six boosters of antigen at intervals of 1 wk. The animals which showed a high reactivity to the antigen were sacrificed 3 d after the last boost. The spleen cells from the animals were fused with murine myeloma cells, P3-X63.Ag8-U1 (P3-U1), using polyethylene glycol No. 1000. The fused cells were suspended in the hypoxanthine/aminopterin/thymidine medium and distributed into 96-well culture plates. The cells were cultured for 14 d and their supernatants were analyzed by ELISA. ELISA was performed as follows. 50  $\mu$ l of antigen solution (10  $\mu$ g protein/ml in PBS) was placed in each well of 96-well E.I.A. micro titration plate (Flow Laboratories, Mclean, VA), and left for overnight at 4°C. After blocking with 1% BSA in PBS, 50  $\mu$ l of diluted mAbs were added to each well, and were left for 2 h at room temperature. After washing the plate, peroxidase-labeled rabbit anti-rat or anti-mouse Igs (Dakopatts, Glostrup, Denmark) were allowed to react for 2 h at room temperature. 2,2-azino bis 3-ethyl benzothiazoline-6-sulfonic acid diammonium was used as a substrate of the enzyme reaction and the samples were measured at 415 nm. The hybridoma cells which produced antibodies against LAP or LTBP were subsequently cloned.

### Purification and Characterization of mAbs

The mAbs were purified by an affinity chromatographical technique using a protein G-Sepharose column (Pharmacia-LKB Biotechnology) from ascites obtained from nude mice inoculated with hybridomas. The subclass of mAbs was determined by ELISA using a mAb typing kit (Zymed Labs., Inc., S. San Francisco, CA). Reactivities of mAbs against LAP or LTBP were determined by Western blotting technique. The LAP-LTBP complex was applied to SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane. After being blocked with 1% BSA in PBS, proteins on the membrane were exposed to react with mAbs for 2 h at room temperature. After being washed, the membrane was exposed to peroxidase-labeled rabbit anti-rat or anti-mouse Igs (Dakopatts) for overnight at 4°C. Immune complexes on the membrane were visualized by an immunostaining kit HRP (Konica, Tokyo, Japan).

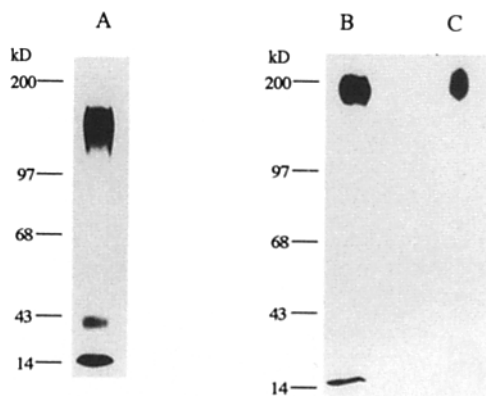
### Migration of PAE Cells and Co-culture Migration of PAE Cells in the Presence of PASM Cells

Each of these experiments was performed as previously described (Sato and Rifkin, 1988, 1989). Briefly, PAE cells were grown in a 35-mm plastic dish. When the cells became confluent, the monolayer was wounded with a razor blade and incubated for 24 h in DME containing 0.1% BSA. For the co-culture migration experiments,  $1.5 \times 10^4$  PASM cells were plated after the wounding, and incubated for 24 h in DME containing 0.1% BSA. mAbs, mature TGF- $\beta$ 1 (R & D Systems, Minneapolis, MN), polyclonal rabbit anti-TGF- $\beta$  antibody (100  $\mu$ g/ml protein A-purified IgG) (R & D Systems), or each of mAbs (10  $\mu$ g/ml IgG) was added during the migration periods. Migrated PAE cells were determined by counting the migrated cells in four different fields ( $\times 100$ ).

## Results

Large LTGF- $\beta$ 1 isolated from human platelets was iodinated and characterized by SDS-PAGE as described in Materials and Methods. Under reducing conditions, mature TGF- $\beta$ 1, LAP, and LTBP were found to be iodinated (Fig. 1, lane A). Under nonreducing conditions, two bands of LAP-LTBP complex and mature TGF- $\beta$ 1 were observed (Fig. 1, lane B). When cross-linked  $^{125}$ I-LLTGF- $\beta$ 1 was applied to SDS-PAGE under non-reducing conditions, a single band of 200 kD, the large LTGF- $\beta$ 1 complex, was observed (Fig. 1, lane C).

The cellular targeting of LTGF- $\beta$  was determined by the binding of  $^{125}$ I-LLTGF- $\beta$ 1 to PAE cells or PASM cells. It was found that  $^{125}$ I-LLTGF- $\beta$ 1 bound specifically to PASM cells, but did not bind to PAE cells (Fig. 2 A). Thus, the targeting of LTGF- $\beta$  appears to be cell-type specific to PASM cells. A Scatchard analysis revealed that there were high- and low-affinity binding sites on a PASM cell (Fig. 2 B): a high-affinity site with a dissociation constant of  $\sim 140$  pM and



**Figure 1.** Autoradiography of  $^{125}\text{I}$ -LLTGF- $\beta$ 1 isolated from human platelets. (Lane A)  $^{125}\text{I}$ -LLTGF- $\beta$ 1 run on a 7.5% SDS gel under reducing conditions. (Lane B)  $^{125}\text{I}$ -LLTGF- $\beta$ 1 run on a 7.5% SDS gel under nonreducing conditions. (Lane C) Cross-linked  $^{125}\text{I}$ -LLTGF- $\beta$ 1 run on a 7.5% SDS gel under nonreducing conditions. Iodinated proteins were visualized by autoradiography.

1,264 sites per cell and a low-affinity site with a dissociation constant of about 5.1 nM and 13,850 sites per cell.

It has been suggested that LAP and/or LTBP play a role in the activation of latent TGF- $\beta$  during co-culture (Dennis and Rifkin, 1991; Flaumenhaft et al., 1993). To clarify possible roles of LAP and/or LTBP on the activation of LTGF- $\beta$ , we developed 10 hybridoma lines which produced mAbs that recognized either LAP or LTBP isolated from human platelets (Table I). We examined the effects of mAbs on the activation of LTGF- $\beta$  during co-culture of endothelial cells and smooth muscle cells. The migration of PAE cells was determined by the wound migration assay, as described in Materials and Methods. When PAE cells were co-cultivated with PASM cells after wounding, PASM cells inhibited the migration of PAE cells by  $\sim 50\%$  of control after a 24-h incubation (Fig. 3). 10  $\mu\text{g}/\text{ml}$  of each mAb was added to co-cultures, and we observed that KM704, which recognizes

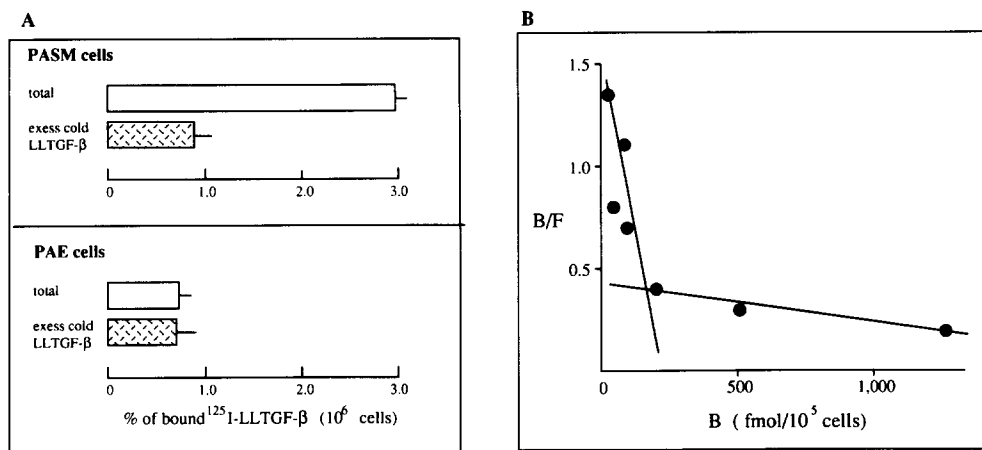
**Table I.** Anti-human Platelet LAP and LTBP mAbs

mAbs	Recognition site	Species	Subclass
KM698	LAP	Rat	IgG2a
KM699	LTBP	Rat	IgG2a
KM700	LTBP	Rat	IgG1
KM701	LTBP	Rat	IgG1
KM702	LAP	Rat	IgG2a
KM703	LTBP	Rat	IgG2a
KM704	LAP	Rat	IgG2a
KM705	LTBP	Rat	IgG1
KM706	LTBP	Rat	IgG1
KM707	LAP	Mouse	IgG1

mAbs against human platelet LAP and LTBP were developed and characterized as described in Materials and Methods.

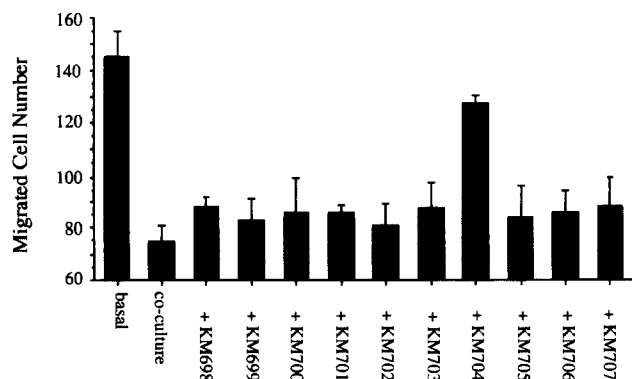
LAP, neutralized the inhibitory effect of PASM cells on the migration of PAE cells (Fig. 3). The neutralizing effect of KM704 was concentration dependent, and 10  $\mu\text{g}/\text{ml}$  of KM704 almost completely neutralized the inhibitory effect of PASM cells on the migration of PAE cells (Fig. 4). The effect of KM704 was further characterized as follows. KM704 (10  $\mu\text{g}/\text{ml}$ ) did not affect the basal migration of PAE cells (Fig. 5). Exogenously added mature TGF- $\beta$ 1 (1 ng/ml) inhibited the migration of PAE cells, and KM704 did not affect the inhibitory effect of exogenous mature TGF- $\beta$ 1 (Fig. 5). Co-cultivation of PASM cells inhibited the migration of PAE cells, and the inhibition by PASM cells was abrogated by both neutralizing polyclonal anti-TGF- $\beta$  antibody (100  $\mu\text{g}/\text{ml}$ ) and KM704 (10  $\mu\text{g}/\text{ml}$ ) (Fig. 5). These findings confirm that KM704, a mAb against LAP, blocks the activation of LTGF- $\beta$  during co-culture of PAE cells and PASM cells. We did not observe any mAbs against LTBP capable of blocking the activation of LTGF- $\beta$  during co-culture.

The targeting of LLTGF- $\beta$  to the cell surface is believed to be required for the activation of TGF- $\beta$  during co-culture and that targeting is mediated through LAP and/or LTBP (Dennis and Rifkin, 1991; Flaumenhaft et al., 1993). There-



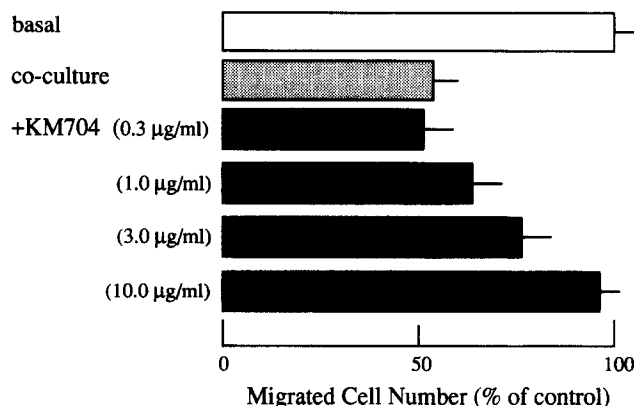
**Figure 2.** The binding of  $^{125}\text{I}$ -LLTGF- $\beta$ 1 to porcine cells. (A) PAE cells or PASM cells were preincubated at 37°C for 5 h in serum-free DME containing 0.1% BSA. The cells were washed with PBS and incubated with  $^{125}\text{I}$ -LLTGF- $\beta$ 1 (0.2 ng) at 4°C for 3 h in DME containing 0.1% BSA. 100-fold concentration of unlabeled LLTGF- $\beta$ 1 was added to determine nonspecific binding. After the incubation, the cells were washed with ice-cold PBS three times and the cell associated radioactivities were determined. Bound  $^{125}\text{I}$ -

LLTGF- $\beta$ 1 to the cells as a percent of added  $^{125}\text{I}$ -LLTGF- $\beta$ 1 was expressed. Values are means of triplicated samples. (B) Scatchard Analysis of the binding of  $^{125}\text{I}$ -LLTGF- $\beta$ 1 to PASM cells. PASM cells were incubated with varying concentrations of  $^{125}\text{I}$ -LLTGF- $\beta$ 1 at 4°C for 3 h in DME containing 0.1% BSA. After the incubation, the cells were washed with ice-cold PBS three times and the cell-associated radioactivities were determined. The cell-associated radioactivities were analyzed by the Scatchard procedure.

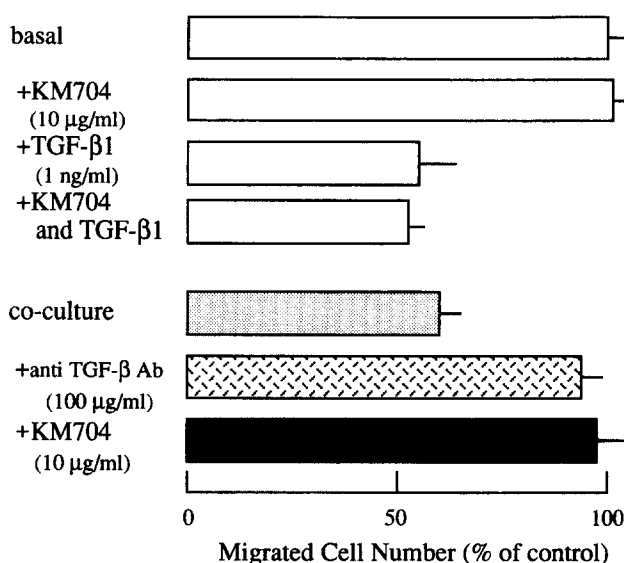


**Figure 3.** The effects of mAbs on the inhibition of PAE cell migration by PASM cells. A confluent monolayer of PAE cells was wounded with a razor blade and incubated in DME containing 0.1% BSA for 24 h. For the co-culture migration assay,  $1.5 \times 10^4$  of PASM cells were plated after the wounding of PAE cells and incubated for 24 h. Each of mAbs (10  $\mu\text{g/ml}$ ) was added to the co-culture migration assay. The number of PAE cells that had migrated was determined as described in Materials and Methods. Values are means and SDs of eight random fields in duplicated samples.

fore, the effect of mAbs on the targeting of large LTGF- $\beta$  to PASM cells was examined. We found that KM704 (10  $\mu\text{g/ml}$ ) blocked the binding of  $^{125}\text{I}$ -LLTGF- $\beta$ 1 to PASM cells, whereas other mAbs did not (Table II). Thus, LAP was found to be involved in the targeting of LTGF- $\beta$  to PASM cells, and the elimination of the targeting of LTGF- $\beta$  to PASM cells by KM704 blocked the activation of latent TGF- $\beta$  during co-culture. LAP contains two potential cellular binding domains: mannose-6-phosphate and an Arg-Gly-Asp sequence. Therefore, we examined whether mannose-6-phosphate or an Arg-Gly-Asp sequence in LAP might be responsible for the targeting of LLTGF- $\beta$  to PASM cells. The binding of  $^{125}\text{I}$ -LLTGF- $\beta$  to PASM cells showed no competitive inhibition by either mannose-6-phosphate (300  $\mu\text{M}$ ) or the synthetic peptide Arg-Gly-Asp-Ser (300  $\mu\text{g/ml}$ ). In the same experiment, the binding of  $^{125}\text{I}$ -LLTGF- $\beta$  to PASM cells was again



**Figure 4.** Dose-dependent neutralization of the co-culture inhibition by KM704. Wound migration assays were performed as described in Fig. 3. KM704 (0.3, 1, 3, 10  $\mu\text{g/ml}$ ) was added to the co-culture migration assays. The number of PAE cells that had migrated was determined. Values are means and SDs of eight random fields in duplicated samples.



**Figure 5.** The effect of KM704 on the activation of LTGF- $\beta$  during co-culture of PAE cells and PASM cells. Effects of KM704 and/or exogenous TGF- $\beta$ 1 on the basal migration of PAE cells were examined. KM704 (10  $\mu\text{g/ml}$ ), exogenous mature TGF- $\beta$ 1 (1  $\text{ng/ml}$ ), or KM704 (10  $\mu\text{g/ml}$ ), plus exogenous mature TGF- $\beta$ 1 (1  $\text{ng/ml}$ ) were added to the wound migration assays and incubated for 24 h. Effects of polyclonal neutralizing anti-TGF- $\beta$  antibody and KM704 on the co-culture migration were examined. Polyclonal neutralizing anti-TGF- $\beta$  IgG (100  $\mu\text{g/ml}$ ) or KM704 (10  $\mu\text{g/ml}$ ) were added to the co-culture migration assay and incubated for 24 h. The number of PAE cells that had migrated was determined. Values are means and SDs of eight random fields in duplicated samples.

blocked by KM704 (10  $\mu\text{g/ml}$ ) (Fig. 6). These results suggest that neither the mannose-6-phosphate nor an Arg-Gly-Asp sequence in LAP is involved in the binding of  $^{125}\text{I}$ -LLTGF- $\beta$  to PASM cells, and that KM704 might recognize an entirely different domain of LAP responsible for the targeting of LTGF- $\beta$  to PASM cells.

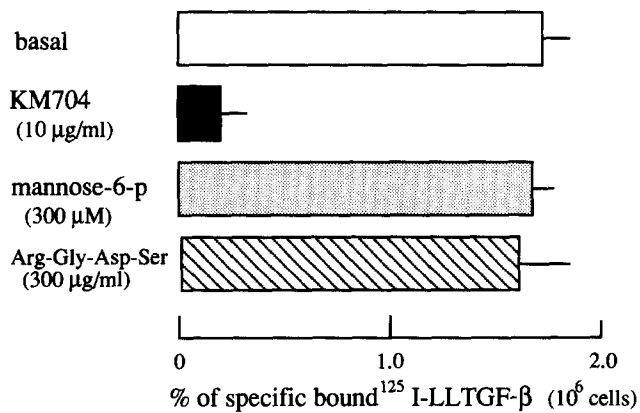
## Discussion

The activation of LTGF- $\beta$  during co-culture was previously

**Table II.** The Effects of mAbs on the Specific Binding of  $^{125}\text{I}$ -LLTGF- $\beta$ 1 to PASM cells

mAbs	The specific binding of $^{125}\text{I}$ -LLTGF- $\beta$ 1 to PASM cells (percent of control)
KM698	92
KM699	107
KM700	101
KM701	113
KM702	94
KM703	93
KM704	24
KM705	105
KM706	101
KM707	103

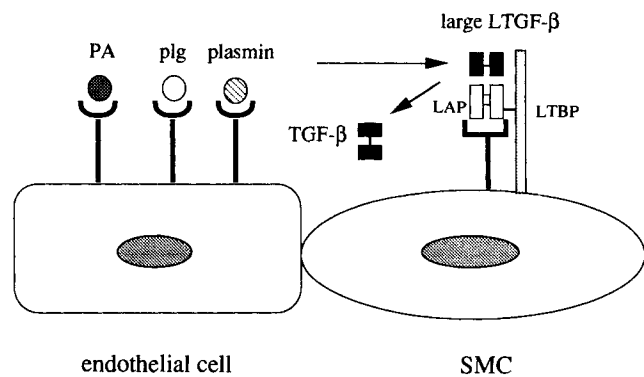
PASM cells were incubated with  $^{125}\text{I}$ -LLTGF- $\beta$ 1 for 3 h in DME containing 0.1% BSA as described in Fig. 2. 100-fold concentration of unlabeled LLTGF- $\beta$ 1 was added to determine nonspecific binding. Each of mAbs (10  $\mu\text{g/ml}$ ) was added simultaneously with  $^{125}\text{I}$ -LLTGF- $\beta$ 1. After the incubation, the specific binding was determined. Bound  $^{125}\text{I}$ -LLTGF- $\beta$ 1 to the cells as a percent of added  $^{125}\text{I}$ -LLTGF- $\beta$ 1 was expressed.



**Figure 6.** The effects of mannose-6-phosphate or the synthetic peptide Arg-Gly-Asp-Ser on the specific binding of <sup>125</sup>I-LLTGF-β1 to PASM cells. PASM cells were incubated with <sup>125</sup>I-LLTGF-β1 (0.2 ng) for 3 h in DME containing 0.1% BSA as described in Fig. 2. 100-fold concentration of unlabeled LLTGF-β1 was added to determine nonspecific binding. Mannose-6-phosphate (300 μM), Arg-Gly-Asp-Ser (300 μg/ml), or KM704 (10 μg/ml) was added simultaneously with <sup>125</sup>I-LLTGF-β1. After the incubation, the specific binding of <sup>125</sup>I-LLTGF-β1 to PASM cells were determined. Bound <sup>125</sup>I-LLTGF-β1 to the cells as a percent of added <sup>125</sup>I-LLTGF-β1 was expressed.

demonstrated by the combination of bovine aortic or capillary endothelial cells and bovine aortic smooth muscle cells or bovine retinal pericytes (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989; Sato et al., 1990). Here, we observed that LTGF-β was activated during co-culture by using a porcine cell system as well. This activation was also observed using a human system (data not shown), and so the activation of LTGF-β during co-culture of endothelial cells and smooth muscle cells appears to be general and not restricted to the bovine system.

The activation of LTGF-β during co-culture requires the contact of two cell types, and is mediated by PA-plasmin activity (Sato and Rifkin, 1989; Kojima et al., 1991). Since endothelial cells have the ability to produce PA and secrete LTGF-β, it is not clear why the activation of LTGF-β requires secondary cells. It is reported that endothelial cells treated with exogenous bFGF highly enhance PA activity and activate LTGF-β in homotypic culture conditions (Flaumenhaft et al., 1992). However, this does not explain the mechanism of activation during co-culture of endothelial cells and smooth muscle cells. Evidence supports the concept that the cellular targeting of LTGF-β is required for the activation during co-culture (Dennis and Rifkin, 1991; Flaumenhaft et al., 1993); but it remains obscure why activation requires two different cell types, as well as contact between these two cell types. Our present study is the first we know of to show that LTGF-β is selectively targeted to smooth muscle cells but not to endothelial cells, and that elimination of targeting of LTGF-β to smooth muscle cells appears to block the activation during co-culture of endothelial cells and smooth muscle cells. This implies a unique mechanism for cellular activation of LTGF-β during co-culture of endothelial cells and smooth muscle cells (Fig. 7). Endothelial cells express the PA-plasmin activity on their cellular surface. However, as LTGF-β is not targeted to endothelial cells, the activation



**Figure 7.** Proposed model of latent TGF-β activation during co-culture of endothelial cells and smooth muscle cells. Endothelial cells express PA-plasmin on their cellular surface. However, latent TGF-β is not targeted to endothelial cells, but to smooth muscle cells. When endothelial cells and smooth muscle cells contact each other, PA-plasmin localized on endothelial cell surface activates latent TGF-β targeted to smooth muscle cells.

of LTGF-β is not efficient in homotypic culture conditions, and is observed only when PA activity is highly enhanced by exogenous bFGF (Flaumenhaft et al., 1992). On the contrary, LTGF-β is targeted to smooth muscle cells, but smooth muscle cells do not express enough PA activity to activate LTGF-β in homotypic culture conditions. And during co-culture of endothelial cells and smooth muscle cells with cell contact, PA-plasmin localized on the surface of endothelial cells efficiently activates LTGF-β targeted to smooth muscle cells. It is also possible that a cell that possesses both PA activity and the binding sites for LTGF-β could activate LTGF-β in homotypic culture conditions.

A mAb against LAP, KM704, blocked the binding of <sup>125</sup>I-LLTGF-β1 to PASM cells. Therefore, LAP appears to play an important role in the targeting of LTGF-β. LAP is reported to bind to the mannose-6-phosphate/insulin-like growth factor II receptor via mannose-6-phosphates attached to LAP (Kovacina et al., 1989). However, the binding of <sup>125</sup>I-LLTGF-β1 to PASM cells could not be competed by large amounts of mannose-6-phosphate. The other candidate for the binding domain in LAP, an Arg-Gly-Asp sequence (Derynck et al., 1985), is not supported by the demonstrated failure of large amounts of synthetic peptides containing an Arg-Gly-Asp sequence to competitively inhibit the binding of <sup>125</sup>I-LLTGF-β1 to PASM cells. Thus, the responsible domain in LAP for the targeting of LTGF-β to PASM cells remains uncertain until the recognition site of KM704 in LAP is identified. LTBP, another molecule of large LTGF-β complex, is also suggested to be involved in the targeting and activation of LTGF-β during co-culture (Flaumenhaft et al., 1993). Although we did not observe any mAbs against LTBP which affect the targeting and activation of large LTGF-β during co-culture, we cannot exclude the possibility that LTBP participates in the targeting of large LTGF-β. LTBP contains 16 EGF-like domain repeats and three copies of a motif with eight cysteine residues (Kanzaki et al., 1990; Tsuji et al., 1990), and the EGF-like domain repeats are proposed to be involved in protein-protein interaction. It is possible that LTBP acts together with LAP in the targeting of large LTGF-β to the cell surface. Further study is required

to clarify the binding site of large LTGF- $\beta$  on the surface of smooth muscle cells and the possible relationship between LAP and LTBP in the mechanism of targeting of large LTGF- $\beta$ .

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