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

Yasufumi Sato,* Fumio Okada,§ Mayumi Abe,* Tadashi Seguchi,† Michihiko Kuwano,§ Seiji Sato,§ Akiko Furuya,§ Nobuo Hanai,§ and Tatsuya Tamaoki§

*Department of Internal Medicine 1 and †Biochemistry, Oita Medical University, 1-1 Idaigaoka, Hasama-machi, Oita-gun, Oita 879-55, and §Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., LTD., 3-6-6 Asahi-machi, Machida City, Tokyo 194, Japan

Abstract. Transforming growth factor-β (TGF-β) 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-β (LTGF-β) during co-culture, and the targeting of LTGF-β to the cellular surface is requisite for its activation. In the present study, the cellular targeting of LTGF-β was examined. We detected the specific binding of 125I-large LTGF-β1 isolated from human platelets to smooth muscle cells but not to endothelial cells. A mAb against the latency-associated peptide (LAP) of large LTGF-β1 complex, which blocked the binding of 125I-large LTGF-β1 to smooth muscle cells, inhibited the activation of LTGF-β during co-culture. The binding of 125I-large LTGF-β1 could not be competed either by mannose-6-phosphate (300 μM) or by the synthetic peptide Arg-Gly-Asp-Ser (300 μg/ml). These results indicate that the targeting of LTGF-β to smooth muscle cells is required for the activation of LTGF-β during co-culture of endothelial cells and smooth muscle cells. The targeting of LTGF-β 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.

Transforming growth factor-β (TGF-β), 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-β 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-β (LTGF-β) complex: (a) the mature TGF-β; (b) the TGF-β latency-associated peptide (LAP); and (c) the latent TGF-β binding protein (LTBP) (Miyazono et al., 1988; Wakefield et al., 1988; Tsuji et al., 1990). LAP is a 75-kD homodimer representing the NH2-terminal remnant of the proTGF-β. 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-β (Miyazono et al., 1991). LTGF-β composed of the mature TGF-β, LAP, and LTBP is denoted the large LTGF-β complex, whereas the complex composed of the mature TGF-β and LAP is denoted the small LTGF-β complex. It has been reported that both endothelial cells and smooth muscle cells secrete large LTGF-β (Flaumenhaft et al., 1993). Since TGF-β is secreted in a latent form, the dissociation of mature TGF-β from the latent complex is essential for expression of its biological activities postsecretion. Although LTGF-β can be activated by transient acid, base, heat, or chaotrophic agents like urea in a test tube, the mechanism of the activation of LTGF-β in vivo remains unexplained. LTGF-β 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-β from LTGF-β (Lyons et al., 1991). In tissue culture conditions, LTGF-β is activated by coculture of bovine endothelial cells and bovine pericytes/smooth muscle cells. The TGF-β formed inhibits the migration and proliferation of bovine endothelial cells (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). The activation...
of LTGF-β 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-β to the cell surface appears to be required for the activation of LTGF-β during co-culture of two cell types (Dennis and Rifkin, 1991; Flaumenhaft et al., 1993). However, the characterization of the cellular targeting of LTGF-β has not been previously done.

In the present study, we isolated large LTGF-β1 (LLTGFL-β1) from human platelets, and examined the binding of 125I-LLTGFL-β1 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-β were examined. We found that 125I-LLTGFL-β1 bound only to smooth muscle cells but not to endothelial cells, and that at least LAP of the LTGF-fl complex was responsible for the cellular targeting to smooth muscle cells. By eliminating cellular targeting of LTGF-β with a mAb against LAP, the activation of LTGF-β during coculture 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-β1

Isolation of LLTGFL-β1 from human platelets was performed by the method described previously (Okada et al., 1989). Isolated LLTGFL-β1 was labeled with 125I by chloramine-T method (Oono et al., 1982). The specific activity of 125I-LLTGFL-β1 was 6.93 × 105 cpm/ng. 125I-LLTGFL-β1 was analyzed by SDS-PAGE as follows. 125I-LLTGFL-β1 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). 125I-LLTGFL-β1 or cross-linked 125I-LLTGFL-β1 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-β1 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 125I-LLTGFL-β1 in DME containing 0.1% BSA at 4°C for 3 h. 100-fold concentration of unlabeled LLTGFL-β1 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 μM of mannose-6-phosphate (Sigma Immunochemicals, St. Louis, MO), or 300 μg/ml of Arg-Gly-Asp-Ser (Sigma Immunochemicals) were added during the incubation period.

Preparation of mAbs against LAP or LTBP

Isolated LTGF-β1 was treated with 6 M urea to dissociate mature TGF-β 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 diazylated against PBS. Adult SD rats or BALB/c mice were immunized with intraperitoneal injection of LAP-LTBP complex-KLH (Hemocianin Keyhole Limpet) conjugate mixed with aluminum gel and Borodetta 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-UI (P3-UI), using polyethylene glycol No. 1000. The fused cells were suspended in the hypoxanthine/aminopterine/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 μl of antigen solution (10 μg 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 μl 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-azinobis-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 chromatography 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 coculture migration experiments, 1.5 × 104 PASM cells were plated after the wounding, and incubated for 24 h in DME containing 0.1% BSA. mAbs, mature TGF-β1 (R & D Systems, Minneapolis, MN), polyclonal rabbit anti-TGF-β antibody (100 μg/ml protein A-purified IgG) (R & D Systems), or each of mAbs (10 μg/ml IgG) was added during the migration periods. Migrated PAE cells were determined by counting the migrated cells in four different fields (×100).

Results

Large LTGF-β1 isolated from human platelets was iodinated and characterized by SDS-PAGE as described in Materials and Methods. Under reducing conditions, mature TGF-β1, LAP, and LTBP were found to be iodinated (Fig. 1, lane A). Under nonreducing conditions, two bands of LAP-LTBP complex and mature TGF-β1 were observed (Fig. 1, lane B). When cross-linked 125I-LLTGFL-β1 was applied to SDS-PAGE under non-reducing conditions, a single band of 200 kD, the large LTGF-β1 complex, was observed (Fig. 1, lane C).

The cellular targeting of LTGF-β was determined by the binding of 125I-LLTGFL-β1 to PAE cells or PASM cells. It was found that 125I-LLTGFL-β1 bound specifically to PASM cells, but did not bind to PAE cells (Fig. 2 A). Thus, the targeting of LTGF-β 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 ~140 pM and
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-β 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-β, 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-β 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 ~50% of control after a 24-h incubation (Fig. 3). 10 μg/ml of each mAb was added to co-cultures, and we observed that KM704, which recognizes 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 μg/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 μg/ml) did not affect the basal migration of PAE cells (Fig. 5). Exogenously added mature TGF-β1 (1 ng/ml) inhibited the migration of PAE cells, and KM704 did not affect the inhibitory effect of exogenous mature TGF-β1 (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-β1 antibody (100 μg/ml) and KM704 (10 μg/ml) (Fig. 5). These findings confirm that KM704, a mAb against LAP, blocks the activation of LTGF-β during co-culture of PAE cells and PASM cells. We did not observe any mAbs against LTBP capable of blocking the activation of LTGF-β during co-culture.

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

### 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.
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 × 10^4 of PASM cells were plated after the wounding of PAE cells and incubated for 24 h. Each of mAbs (10 μg/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.

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 × 10^4 of PASM cells were plated after the wounding of PAE cells and incubated for 24 h. Each of mAbs (10 μg/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-β to PASM cells was examined. We found that KM704 (10 μg/ml) blocked the binding of 125I-LLTGF-β1 to PASM cells, whereas other mAbs did not (Table II). Thus, LAP was found to be involved in the targeting of LTGF-β to PASM cells, and the elimination of the targeting of LTGF-β to PASM cells by KM704 blocked the activation of latent TGF-β 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-β to PASM cells. The binding of 125I-LLTGF-β to PASM cells showed no competitive inhibition by either mannose-6-phosphate (300 μM) or the synthetic peptide Arg-Gly-Asp-Ser (300 μg/ml). In the same experiment, the binding of 125I-LLTGF-β to PASM cells was again blocked by KM704 (10 μg/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 125I-LLTGF-β to PASM cells, and that KM704 might recognize an entirely different domain of LAP responsible for the targeting of LTGF-β to PASM cells.

Discussion

The activation of LTGF-β during co-culture was previously

Table II. The Effects of mAbs on the Specific Binding of 125I-LLTGF-β1 to PASM cells

| mAbs    | The specific binding of 125I-LLTGF-β1 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 125I-LLTGF-β1 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. Each of mAbs (10 μg/ml) was added simultaneously with 125I-LLTGF-β1. After the incubation, the specific binding was determined. Bound 125I-LLTGF-β1 to the cells as a percent of added 125I-LLTGF-β1 was expressed.
as LTGF-B is not targeted to endothelial cells, the activation of PA-plasmin activity on their cellular surface. However, latent LTGF-B is selectively targeted to smooth muscle cells, but not to endothelial cells, and that elimination of targeting of LTGF-B 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 targeting of LTGF-B to smooth muscle cells that involves the binding of LTGF-B to target cells.

Evidence supports the concept that the cellular targeting of LTGF-B is required for the activation during co-culture (Dennis and Rifkin, 1991; Flamenhaft 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-B is selectively targeted to smooth muscle cells but not to endothelial cells, and that elimination of targeting of LTGF-B 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-B 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-B is not targeted to endothelial cells, the activation of LTGF-B is not efficient in homotypic culture conditions, and is observed only when PA activity is highly enhanced by exogenous bFGF (Flamenhaft et al., 1992). On the contrary, LTGF-B is targeted to smooth muscle cells, but smooth muscle cells do not express enough PA activity to activate LTGF-B 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-B targeted to smooth muscle cells. The other candidate for the binding domain in LAP, a new 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 LTGF-B to smooth muscle cells. Thus, the responsible domain in LAP for the targeting of LTGF-B to smooth muscle cells remains uncertain until the recognition site of KM704 in LAP is identified. LTBP, another molecule of large LTGF-B complex, is also suggested to be involved in the targeting and activation of LTGF-B during co-culture, although we did not observe any mAbs against LTBP which affect the targeting and activation of large LTGF-B during co-culture, we cannot exclude the possibility that LTBP participates in the targeting of large LTGF-B. 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-B to the cell surface. Further study is required.
to clarify the binding site of large LTGF-β on the surface of smooth muscle cells and the possible relationship between LAP and LTBP in the mechanism of targeting of large LTGF-β.

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