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Role of Latent TGF-β1 Binding Protein in Vascular Remodeling

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Transforming growth factor-β (TGF-β) is secreted as a latent, high molecular weight complex, which is composed of TGF-β, a latency associated peptide (LAP) and a latent TGF-β binding protein (LTBP). In this study, we report on the role of LTBP in vascular remodeling. 0.01-5 ng/ml of LTBP stimulated the migration activities of cultured rat arterial smooth muscle cells (SMC) about 4-7 fold compared with control in vitro. The maximal activity of SMC migration by LTBP was 75% of that by 10 ng/ml of PDGF-BB. A checker board analysis showed that the migration by LTBP was chemotactic, not chemokinetic. By cross-linking experiment, LTBP associated with 80-120 kd cell surface protein of SMC, suggesting that a part of LTBP can bind with SMC. Furthermore, LTBP was more strongly expressed in the intimal layer than in the medial layer of BCI artery. These results suggest that LTBP plays an importantrole in the initial stage of arterial intimal thickening through the acceleration of SMC migration from the medial to intimal layer and is one of the essential factors influencing vascular remodeling.

The most prominent features of the atherogenic process are the migration and proliferation of arterial wall cells and the deposition of lipid and extracellular matrix in the arterial wall (1). These changes in arteries lead to what is known as vascular remodeling. It has been reported that many growth factors and cytokines play important roles in vascular remodeling (2, 3).

Transforming growth factor-β (TGF-β), one of the essential factors in vascular remodeling, has been found to control extracellular matrix protein synthesis and degradation by arterial smooth muscle cells (SMC) and to regulate SMC migration and proliferation bifunctionally depending on species, cell phenotype, growth conditions and interactions with other growth factors in vitro (4, 5). Moreover, TGF-βs are secreted from producer cells as latent, high molecular weight complexes (large latent complexes) (6). The large latent complex of TGF-β1 is composed of mature TGF-β1, the N-terminal remnant of TGF-β1 precursor dimer (latency associated peptide, LAP) and a latent TGF-β1 binding protein (LTBP) (7, 8). It has been reported that TGF-β1 affects tumorigenesis, fibrosis and immune and inflammatory systems in studies of transgenic mice and of the targeted expression of TGF-β1 (9-11), and stimulates the intimal thickening of arteries by the enhancement of extracellular matrix synthesis in vivo (12, 13). LAP has an essential function in the latency of TGF-β complex by non-covalent association with mature TGF-β (14).

LTBP is a 125-210 kd glycoprotein that is disulphide-bonded to LAP in the latent complex of TGF-β (15, 16). LTBP occurs in various sizes probably due to proteolytic processing or altenative splicing. 60-70% of the LTBP structure is composed of two different types of cysteine-rich repeat sequences, one being 16-18 epidermal growth factor (EGF)-like repeats and the other 3-4 copies of LTBP specific eight cysteine repeats. LTBP is a member of a superfamily of proteins that contains EGF-like repeats and eight cysteine repeats, and it includes LTBP-1, -2, -3 and -4 and fibrillin-1 and -2 (17-22). In an in vitro system, LTBP has no direct role in TGF-β latency, but it has been shown to have an essential role in the assembly and secretion of TGF-β complex, and in the association of latent TGF-β complex with extracellular matrix and the subsequent activation of TGF-β (23, 24). It has been reported that LTBP is detected in the arterial wall under pathological condition, as for example, in rheumatoid arthritis (25), but there are no reports about the LTBP function in the arterial wall or in atherosclerosis.

In the present study, we report on the function of LTBP in arterial wall cells in vitro, and on its distribution in an artery with intimal thickening in vivo. The significance of LTBP in vascular remodeling is discussed.

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MATERIALS AND METHODS

Chemicals. Sources of materials were as follows: fetal bovine serum (FBS, Lot No. 37K0325), GIBCO Laboratories (Grand Island, NY, USA); platelet-derived growth factor-BB (PDGF-BB), R&D System Inc. (Minneapolis, MN, USA); ^{125}I, New England Nuclear (Boston, MA, USA); Protein G-Sepharose, Pharmacia LKB (Uppsala, Sweden); Bis (sulfosuccinimidyl) suberate, Pierce (Rockford, IL, USA). LTBP was purified from Escherichia coli transfected with its cDNA (LTBP-1, short form), and its purity was determined by SDS-PAGE, silver staining and immunoblotting. The molecular weight of this recombinant LTBP is about 180 kd.

Antibody. Rabbit polyclonal antibody (Ab 39) against the free form of LTBP purified from human platelets was a gift from Dr. Kohei Miyazono (Department of Biochemistry, The Cancer Institute, Tokyo, Japan). Ab 39 cross-reacts with rat, the specificity of which has been reported (26, 27).

Cell culture. Smooth muscle cells (SMC) were explanted from the thoracic aorta of the male Wistar rat essentially by the method of Fischer-Dzoga et al (28). Primary culture and subculture were carried out in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were subcultured at a split of 1:2 and used at the 4th-6th passages for the following experiments.

Migration assay of SMC. Migration of SMC was assayed by a modification of Boyden's chamber method using a polycarbonate filter (Neuro Probe Inc., MD, USA) with pores of 5.0 μm diameter (29). Cultured SMC were trypsinized and suspended at a concentration of 5.0×10^5 cells/ml of DMEM supplemented with 1% FBS. Then the SMC suspension was placed in the upper chamber and DMEM with various concentrations of LTBP or other migration factors in the lower chamber. The chamber was incubated at 37 °C in 5% CO_2 and 95% air for 4h. SMC on the upper side of the filter were scraped off and the filter was removed. Then SMC that had migrated to the lower side of the filter were fixed in methanol, stained with hematoxylin and eosin, and counted by light microscopy ($\times$100) for quantitation of SMC migration. Migration activity was expressed as the mean number of migrated cells seen in five different fields (F).

Cross-linking and immunoprecipitation of LTBP with rat SMC. Iodination of LTBP was carried out by the mild chloramine T method as described by Frolik et al. (30) Cross-linking and immunoprecipitation experiments were performed as previously described (31). In brief, confluent SMC in 6-well plates were incubatedwith serum free DMEM for 24 h, and then cells were washed with binding buffer (PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 1 mg/ml BSA) and incubated on ice in the same buffer with 100 pM ^{125}I-LTBP in the presence or absence of excess unlabeled LTBP for 3 h. Cells were washed, and cross-linking was done in the binding buffer without BSA together with 0.25 mM Bis for 30 min on ice. Supernatants from solubilized cells (cell lysates) containing the same amounts of protein were immunoprecipitated with Ab 39 and protein G-Sepharose, and the immune complexes were subjected to analysis by SDS gel electrophoresis using 5% polyacrylamide gel, followed by autoradiography.

Balloon catheter injury (BCI). Male Wistar rats weighing 400 g were used for this experiment. BCI of the left common carotid artery was carried out using a 2 F Fogarty balloon catheter as described by Igarashi et al (32). This was done under sterile conditions under anesthesia with sodium pentobarbital (30 mg/kg, i. p.), and met the ethical standards regarding experimental animals. Two or four weeks after BCI, the left common carotid arteries were removed and used for immunohistochemistry.

Immunohistochemical study of LTBP in arteries. The arteries were snap-frozen and stored at $-80°$ C. They were sectioned serially at 6μm thickness and fixed in acetone.

For the identification of LTBP, we used 100 $\times$ diluted anti-LTBP antibody (Ab 39). The staining of sections was followed by the method of ABC peroxidase immunohistochemistry described by Waltenberger et al (26).

Statistical methods. The significance of differences was evaluated by Student's t test and Dunnet's test.

TABLE 1

Effect of LTBP on Migration of Rat SMC

	Factors (ng/ml)	Migration activity (cells/F)	Relative migration activity (fold)
	0	8.07 ± 3.68	1
	0.001	18.5 ± 11.2	2.3
	0.01	*34.4 ± 19.2	4.3
	0.1	*42.2 ± 21.0	5.2
LTBP	0.5	*48.4 ± 14.9	6.0
	1	*56.0 ± 13.9	6.9
	2	*48.6 ± 12.6	6.0
	5	*37.4 ± 23.0	4.6
	10	15.3 ± 5.76	1.9
PDGF-BB	10	*73.8 ± 23.0	9.1

Note. Migration activities by various concentrations of LTBP were assayed in rat medial SMC by modified Boyden chamber method, and were expressed as the mean number of migrated cells seen in five different fields (F) and the value relative to control. *: $p<0.01$, ($n=10$).

RESULTS

Effect of LTBP on the Migration of Rat SMC

First, we examined the effects of LTBP on the migration and proliferation of rat SMC in vitro.

Migration activity of rat SMC by addition of 0.001-10 ng/ml of LTBP increased 1.9-6.9 fold compared with that of control SMC with no addition of LTBP; the maximal activity was observed at 1 ng/ml of LTBP (Table 1). The migration activity at 1 ng/ml of LTBP was 75% of the maximal activity by PDGF-BB at 10 ng/ml. However, 0.001- 10 ng/ml of LTBP had no effects on the proliferation of rat SMC (data not shown).

Checker Board Analysis of Rat SMC Migration by LTBP

To clarify whether SMC migration by LTBP is unidirectional, we examined it by checker board analysis.

When only the lower chamber contained 100 pg/ml of LTBP, the migration activity was 5.0 fold compared with that without LTBP in either the upper or lower chamber (Fig. 1). But when only the upper chamber contained 100 pg/ml of LTBP or both chambers contained 100 pg/ml of LTBP, the migration activity was 1.5 fold or 1.8 fold, respectively. These results show that most of the SMC migration by LTBP was due to chemotaxis, not to chemokinesis.

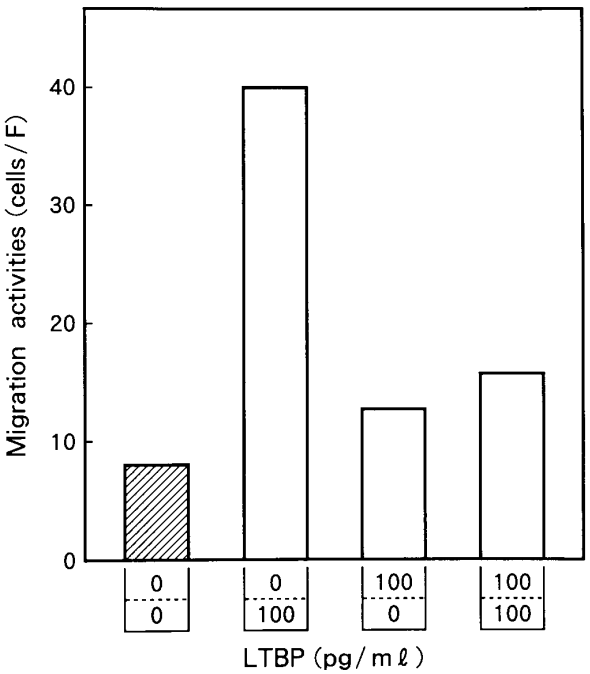

FIG. 1. Checker board analysis of rat SMC migration by LTBP. 100 pg/ml of LTBP was added only to the lower chamber (a chemotaxis model), and added to the upper chamber or both chambers (a chemokinesis model). The experiments were repeated six times using different lots of SMC, and typical data are shown.

Binding of LTBP with Rat SMC

To determine the mechanism of expression of SMC migration by LTBP, binding of LTBP with SMC was examined by the method of cross-linking and immunoprecipitation.

A band of 260-300 kd was observed in the cell lysate from SMC cross-linked with LTBP (Fig. 2, lane 2). But this band was not detected in control with an excess of unlabeled LTBP (Fig. 2, lane 1), and also not in control immunoprecipitated with non-immune serum (Fig. 2, lane 3), suggesting that this band shows the binding of SMC with LTBP. The molecular weight of a band of SMC cross-linked with LTBP is 260-300 kd and that of LTBP with this binding assay is about 180 kd, indicating that LTBP associates with 80-120 kd cell surface protein of SMC.

Immunohistochemical Study of LTBP in Rat BCI Artery

To clarify the in vivo effect of LTBP on an artery, the distribution of LTBP was studied in arteries by the method of immunohistochemistry.

First, we stained rat normal carotid artery, but the staining of LTBP was very weak in the arterial wall (data not shown). Next, arteries two weeks after BCI were studied as the model of intimal thickening. Neither medial nor intimal layers from rat BCI arteries were stained with non-immune serum (Fig. 3A), but intimal layers were stained strongly with Ab 39, whereas the staining of medial layers was very weak (Fig. 3B). The staining of LTBP was observed in extracellular space. We also had the same results in arteries four weeks after BCI (data not shown).

DISCUSSION

The present results showed that LTBP stimulated the chemotaxis of rat SMC at concentrations of 0.01-5 ng/ml in vitro, and that in the BCI artery LTBP appeared mainly in the intimal layer. The purity of LTBP was estimated by SDS-PAGE, silver staining and immunoblotting using Ab 39 (data not shown), indicating that LTBP function in this study was only due to LTBP.

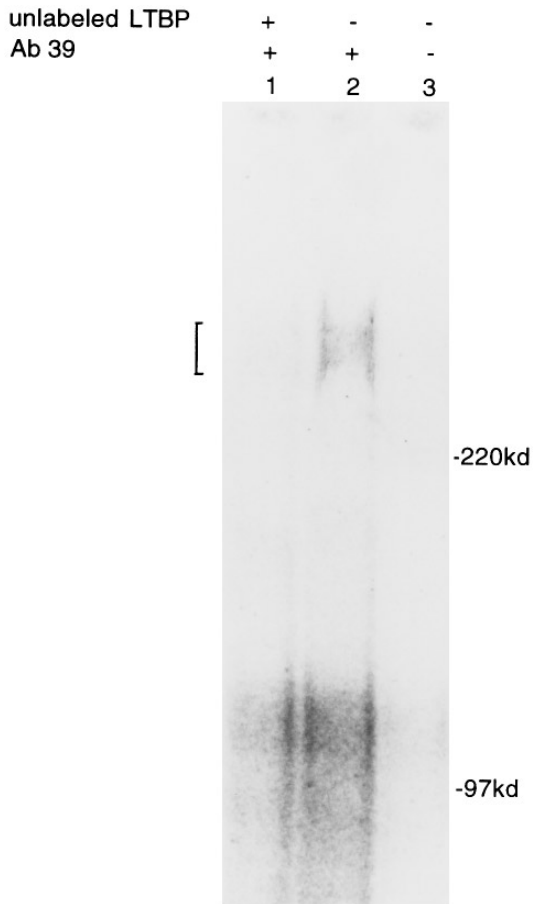

FIG. 2. Cross-linking and immunoprecipitation of ^{125}I-LTBP with rat SMC. Rat SMC was cross-linked with ^{125}I-LTBP only (lanes 2,3) or ^{125}I-LTBP and unlabeled LTBP (lane 1). Cell lysates from SMC were immunoprecipitated with Ab 39 (lanes 1,2) or non-immune serum (lane 3). [shows specific binding complex of LTBP with a binding protein of SMC detected in lane 2, and its molecular weight was about 260-300 kd.

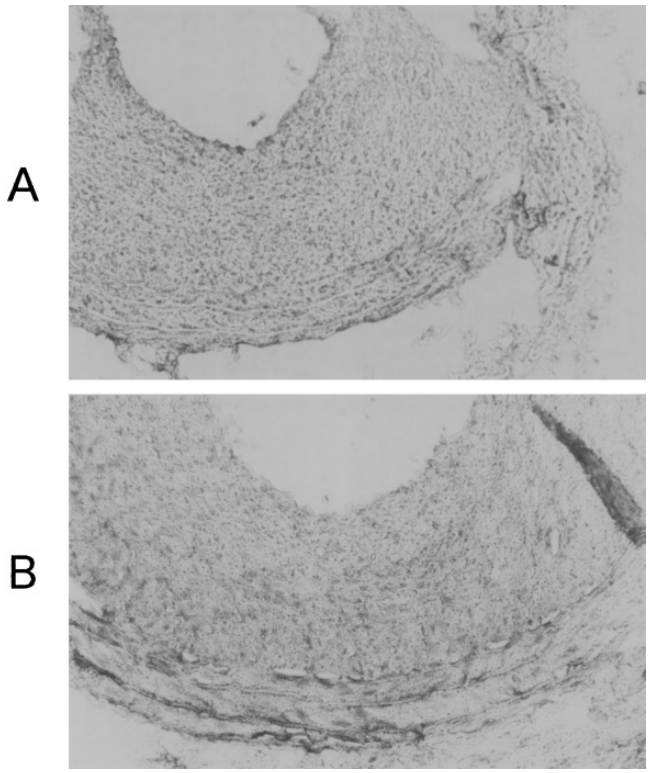

FIG. 3. Immunohistochemistry of LTBP in rat BCI artery. Rat arteries two weeks after BCI were stained with non-immune serum (A) or Ab 39 (B).

Taipale et al (33) reported that LTBP-1 associates covalently with cellular fibronectin, one of the important factors in the extracellular matrix, within 2 h after the secretion from cells. Nunes et al (34) reported that the covalent association between LTBP-1 and extracellular matrix is transglutaminase dependent. From these reports, it seems certain that LTBP added to cultured SMC binds with extracellular matrix. However, our cross-linking experiment showed that LTBP associated with about 80-120 kd cell surface protein of SMC, different from fibronectin whose molecular weight is 220 kd in reduced condition, suggesting that a part of LTBP can bind with the surface of SMC. Indeed, Cam et al (35) reported that LTBP was located at the epithelial-mesenchymal junction by immunostaining. Moreover, it was reported that 5-100 μg/ml of fibronectin stimulated SMC migration in an in vitro system (36), but the LTBP concentration for the stimulation of SMC chemotaxis was very low (0.01-5 ng/ml) compared with fibronectin, suggesting that there is little possibility that LTBP stimulates SMC chemotaxis as a function of fibronectin by its association with fibronectin. We cannot, however, rule out the possibility that LTBP might function through the association with non-fibronectin extracellular matrix.

LTBP-1 consists of 17 EGF-like repeats and 4 copies of eight cysteine repeats. There are several reports concerning the portions where LTBP binds with other proteins. Saharinen et al (37) and Gleizes et al (38) reported that eight cysteine repeats associate with LAP by binding studies of LAP and deleted mutant LTBP-1. Taipale et al (33) reported that the N-terminal region of LTBP-1 binds covalently with extracellular matrix. We previously reported that human LTBP-1 contains a RGD sequence (15). The reacted region of LTBP-1 with SMC remains to be elucidated.

LTBP has an important role in the assembly and secretion of latent TGF-β complex, and in the association of latent TGF-β complex with extracellular matrix and possibly the subsequent activation of latent TGF-β in an in vitro system (23, 24). Dallas et al (39) reported that LTBP appears to play a role in bone formation in vitro, and also that its localization in vivo is suggestive of a role in bone development and formation. However, very little is known about the regulation and function of LTBP in arteries and atherosclerosis. BCI is one of several models of arterial intimal thickening accompanied by increases of SMC and extracellular matrix (40). In BCI arteries, LTBP was expressed in the intimal layer more strongly than in the medial layer, indicating that the concentration of LTBP is higher in the intima than media. In the in vitro system, the chemotaxis of SMC was stimulated by LTBP. Considering these results, it is possible to suppose that LTBP is crucially involved in the initial stage of intimal thickening through the stimulation of the migration of SMC from the medial to the intimal layer of an artery, and that LTBP is one of the essential factors affecting vascular remodeling.

It has been reported that LTBP is secreted not only in a latent complex but also in free form without LAP and TGF-β in a culture system (23). It would also be of interest to find out whether LTBP occurs in free form in an artery, as we have known for some time that LTBP in the in vitro system has an individual function. By immuno- histochemical study, TGF-β1 stainings of the intimal and medial layers of a BCI artery were quite similar (data not shown), indicating that LTBP and TGF-β1 are regulated differently in the intimal layer. These findings suggest that the free form of LTBP exists in the intimal layer of the BCI artery and contributes to intimal thickening.

In summary, LTBP arises mainly in the intimal layer of arteries and possibly is significantly involved in vascular remodeling.

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