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研究——

Activation of Latent TGF-β at the Vascular Wall : Roles of Endothelial Cells and Smooth Muscle Cells

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Introduction

Cytokines and growth factors play important roles in the physiology and pathophysiology of the vascular system. The vascular wall is primarily composed of endothelial cells (ECs) and mural pericytes or smooth muscle cells (SMCs). Pericytes are cells that surround ECs in capillary vessels or post-capillary venules, while SMCs are present in large vessels. ECs and mural cells are able to produce certain cytokines or growth factors, and these factors may affect the functions of vascular cells in autocrine or paracrine manners.

Transforming growth factor-β (TGF-β), a family of 25-kD homodimeric peptide, is one of these cytokines secreted from both ECs and mural cells, and has potent activities on cell growth, motility, and differentiation (1-3). TGF-β is localized at the vascular wall and may express profound effects on the vasculature (4, 5). In vitro, TGF-β1 inhibits the migration and proliferation of ECs (6), while TGF-β1 stimulates or inhibits the proliferation of SMCs depending on the cell density or its concentration (7, 8). TGF-β1 dependent stimulation of SMC proliferation is mediated by the induction of autocrine PDGF synthesis (8). In vivo, exogenously administered TGF-β1 enhances intimal thickening of the arterial wall after balloon-catheter injury (4). Neutralizing anti TGF-β antibody inhibited intimal thickening after balloon-catheter injury (9). Direct transfer of the actively mutated TGF-β1 gene into arteries induces fibrocellular hyperplasia in the intima (10). These observations suggest that TGF-β plays a role in the progression of atherosclerosis.

Latency of TGF-β

A characteristic feature of TGF-β is that TGF-β is normally secreted in a biologically inactive latent form (11, 12). There are three components of the LTGF-β complex: the mature TGF-β, the TGF-β latency-associated peptides (LAP), and the LTGF-β binding protein (LTBP) (13, 14). LAP is a 75-kD homodimer representing the NH2-terminal remnant of the proTGF-β. LAP determines the latency of the complex by binding to the mature TGF-β through noncovalent interactions. LTBP, a protein of 125～160-kD derived from a distinct gene (15, 16), is attached to LAP by a disulfide bond, and is required for the assembly and secretion of LTGF-β from cells (17). LTGF-β composed of the mature TGF-β, LAP, and LTBP is denoted as the large LTGF-β complex, whereas the complex composed of the mature TGF-β and LAP is denoted as the small

LTGF-β complex. Both ECs and mural cells secrete large LTGF-β (18). Thus, the post-secretional activation of LTGF-β is a limiting step for expression of its activity (Fig. 1). LTGF-β is activated in a test tube by proteolysis of the latent complex with plasmin and cathepsin D (19), or by alteration of carbohydrate structures within the LAP (20). Plasmin cleaves the carboxyl-terminal portion of LAP and releases mature TGF-β from the LTGF-β complex (21).

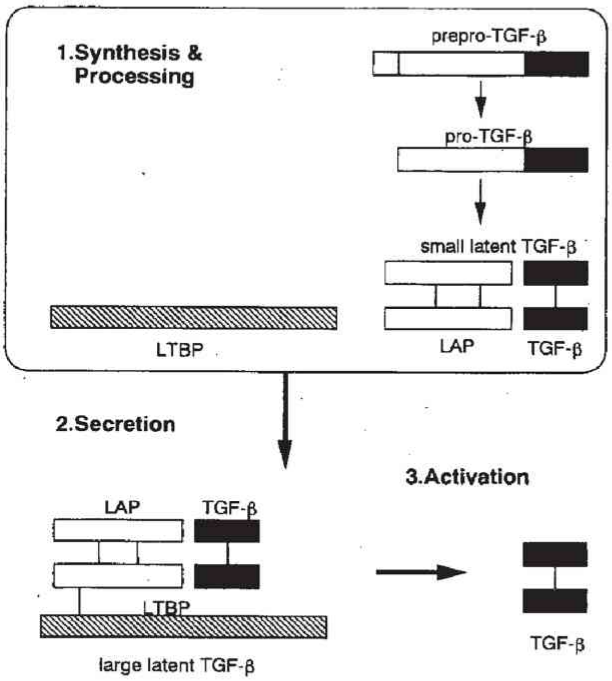

Figure 1 Synthesis, secretion, and activation of TGF-β. TGF-β is synthesized and secreted in a latent form. Therefore, post-secretional activation is required for the expression of its activity.

Re-endothelialization after the injury of endothelial monolayer

In the case of the injury of endothelial monolayer, remaining endothelial cells migrate, proliferate, and repair the denuded area. Certain growth factors including fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) have potent effects on ECs and may accelerate this process. Among them, bFGF is produced by a variety of cell types including ECs (22). bFGF produced by ECs stimulates plasminoge activator (PA) synthesis, migration, proliferation of ECs in an autocrine or paracrine manner (23-25). When a confluent monolayer of bovine aortic ECs was wounded with a razer blade in vitro, the remaining ECs migrated into a denuded area. Anti-bFGF antibody blocked this migration (23). Moreover, ECs at the wound edge expressed urokinase-type PA (u-PA) activity, and anti-bFGF antibody blocked the expression of uPA activity, indicating that these phenomena were dependent on endogenous bFGF. Biro et al. showed the increased synthesis of bFGF in migrating ECs (26). The induction of u-PA activity in migrating ECs is derived from increased expressions of both u-PA and u-PA receptors, and these expressions are dependent on endogenous bFGF (25, 27).

During the migration of ECs, the degradation of extracellular matrices is indispensable. Proteinases, which are involved in the degradation of extracellular matrices, are PA-plasmin and matrix metalloproteinases (MMPs) like collagenase and stromelysin (28). ECs synthesize two types of PA : u-PA and tissue-type PA (t-PA) and their specific inhibitor, plasminogen activator inhibitor 1 (PAI-1). Moreover, ECs synthesize a latent form of MMPs and their specific inhibitor ; tissue inhibitor of metalloproteinase 1 (TIMP-1). PA converts ubiquitous plasminogen to plasmin, and plasmin converts latent MMP to an active form. Therefore, this cascade of proteinases is required for the expression of their activities. Furthermore, the proteinase activities are determined by the balance between proteinases and their specific inhibitors (Fig. 2). However, it is not simply a matter of amounts of proteinases and their inhibitors. There are cell-surface receptors for u-

PA and t-PA, and cell-surface binding sites for plasminogen and plasmin (Fig. 3). The u-PA receptor is a glycosyl-phosphatidylinositol anchored membrane protein (29), and u-PA binds to the receptor with its N-terminal non-catalytic domain (30). PAI-1 promptly binds PAs and quenches PA activity in the fluid phase. However, once bound to its receptor, u-PA becomes relatively resistant to the inhibition by PAI-1 and expresses its proteinase activities at pericellular milieu, converting cell-surface-bound plasminogen to plasmin. Cell-surface-bound plasmin degrades laminin, fibronectin, and proteoglycan; components of extracellular matrices. Plasmin also activates latent MMPs and activated MMPs degrade collagen, a major component of extracellular matrices. In this way, cell-surface PA-plasmin plays a fundamental role in the degradation of vascular basement membrane and interstitial matrices.

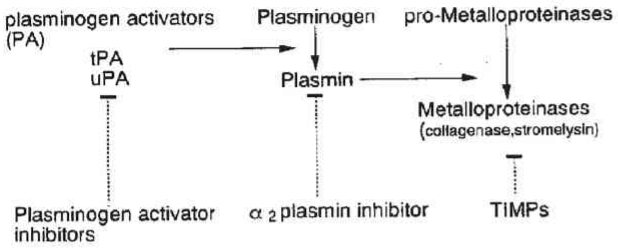

Figure 2 Proteinases for matrix degradation and their specific inhibitors. ECs produce 2 types of PA, tPA and uPA, and their specific inhibitor PAI-1. ECs also produce a latent form of matrix metalloproteinases and their specific inhibitor TIMP-1. PAs convert ubiquitous zymogen, plasminogen to plasmin. Plasmin activates latent metalloproteinases.

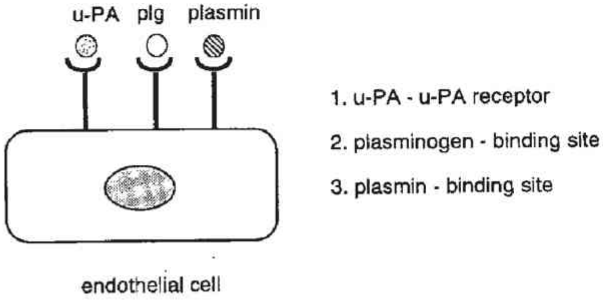

Figure 3 Cell associated PA, plasminogen, and plasmin. ECs express receptors for u-PA, and binding sites for both plasminogen and plasmin. This cell-associated PA-plasmin system is involved in pericellular proteolysis.

Activation of Latent TGF-β by ECs and Mural Pericytes/SMCs Interaction

(1)Role of ECs

Almost all cell types including ECs and mural cells produce and secrete TGF-β, and most cells have receptors for TGF-β. As TGF-β expresses profound effects on most cells even in a very low concentration, the expression of its effects must be tightly regulated. In this context, the latency of TGF-β is one of the most important characteristics. Although the mechanism for activation of LTGF-β in vivo is poorly understood, LTGF-β was found to be activated quite efficiently by the interaction of ECs and mural cells in tissue culture conditions (31). When a confluent monolayer of bovine aortic ECs was wounded, and bovine retinal pericytes or bovine aortic SMCs were co-cultured immediately after the wounding, mural cells inhibited EC migration. This inhibition was abrogated by anti-TGF-β antibody. While ECs and mural cells secreted LTGF-β in homotypic culture conditions, active TGF-β was found to be generated during co-culture of ECs and mural cells with contact between the two cell types. Antonelli-Orlidge et al. independently reported that the inhibition of EC proliferation by co-culture with pericytes/SMCs was mediated by activated TGF-β (32). Plasmin cleaves LAP and

releases mature TGF-β from the LTGF-β complex (21). The expression of PA activity by migrating ECs suggests that the activation of LTGF-β during co-culture is dependent on the PA-plasmin activity of ECs. This idea was supported by the following observations. Simultaneous addition of serine protease inhibitors (aprotinine, e-aminocaproic acid, or a rather specific inhibitor for plasmin ; a2-plasmin inhibitor) almost completely neutralized the inhibitory effect of mural cells on EC migration (31). Moreover, removal of plasminogen from the tissue culture conditions or the addition of lipoprotein (a), which competes with plasminogen, blocked the activation of LTGF-β during co-culture (33). Therefore, the activation of LTGF-β by the interaction of ECs and mural cells appears to be mediated by PA-plasmin activity expressed on the surface of ECs (Fig. 4).

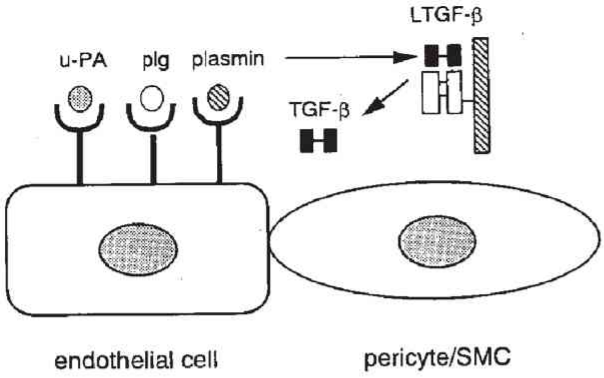

Figure 4 Activation of latent TGF-β during co-culture of ECs and mural cells. Role of ECs. Migrating ECs express PA-plasmin on their cell surface. Plasmin on the surface of ECs activates LTGF-β during co-culture.

(2) Role of Mural Pericytes/SMCs

Role of mural cells in the activation of LTGF-β during co-culture is need to be characterized. Activation of LTGF-β by plasmin during co-culture of ECs and mural cells and localization of PA-plasmin on the surface of E suggest that LTGF-β must be targeted to the cell surface for its activation. LAP contains two potential cellular binding domains: mannose-6-phosphate and an Arg-Gly-Asp sequence (34, 35). It is shown that LTGF-β is able to bind to the cell surface via cation-independent mannose-6-phosphate /insulin-like growth factor Ⅱ (IGF-Ⅱ) receptor (35). Dennis and Rifkin reported that an excess amount of mannose-6-phosphate or antibody to the IGF-II receptor prevented the activation of LTGF-β during co-culture of ECs and SMCs (36). Therefore, they speculated that the LTGF-β complex binds to the cell surface via IGF-II receptor, and that this binding is required for its activation during co-culture. Another molecule that may be involved in the targeting of LTGF-β is LTBP. LTBP contains 16 EGF-like domain repeats and three copies of a motif with eight cysteine residues (15, 16). The EGF-like domain repeats have been proposed to be involved in the protein-protein interaction. Flaumenhaft et al. showed that the antibody to LTBP or an excess amount of free LTBP inhibited the activation of LTGF-β during co-culture of ECs and SMCs (18). Thus, they speculated that LTBP directs LTGF-β to the activation site on the cell surface with these EGF-like domain repeats. However, these two observations did not directly show the cellular targeting of LTGF-β.

The targeting of LTGF-β by the binding of ^{125}I-large LTGF-β 1 to ECs or SMCs was examined. It was found that ^{125}I-large LTGF-β 1 bound specifically to SMCs, but not to ECs. Thus, the targeting of LTGF-β appears to be cell-type specific to mural SMCs. Scatchard analysis revealed that there were high affinity and low affinity binding sites on a SMC: a high affinity site with a dissociation constant of about 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. To under-

stand the precise roles of LAP and/or LTBP on the targeting and activation of LTGF-β during co-culture of ECs and pericytes/SMCs, 10 monoclonal antibodies (mAbs) that recognized either LAP or LTBP were developed. Each mAb was added to the co-culture wound migration assay, and one of the mAbs against LAP (KM704) was found to neutralize the inhibitory effect of SMCs on the migration of ECs. No mAbs against LTBP were capable of blocking the activation of LTGF-β during co-culture. Furthermore KM704, which prevented the activation of LTGF-β during co-culture, blocked the binding of ^{125}I-large LTGF-β1 to SMCs. This demonstrates for the first time that LTGF-β is selectively targeted to SMCs but not to ECs, and that elimination of targeting of LTGF-β to SMCs appears to block the activation of LTGF-β during co-culture of ECs and SMCs. This implies a unique mechanism for the activation of LTGF-β during co-culture (Fig. 5). ECs express PA-plasmin activity on their cellular surface. However, as LTGF-β is not targeted to ECs, the activation of LTGF-β is not efficient in homotypic culture conditions. By contrast, LTGF-β is targeted to pericytes/SMCs, but pericytes/SMCs do not express enough PA activity to activate LTGF-β in homotypic culture conditions. During co-culture of ECs and pericytes/SMCs with cell contact, PA-plasmin localized on the surface of ECs efficiently activated LTGF-β targeted to pericytes/SMCs. It is possible that a cell which posesses both PA activity and the binding sites for LTGF-β could activate LTGF-β in homotypic culture conditions. As described, LAP contains two potential cellular binding domains: mannose-6-phosphate and an Arg-Gly-Asp sequence. Therefore mannose-6-phosphate or an Arg-Gly-Asp sequence in LAP might be responsible for the targeting of LTGF-β to SMCs. The binding of ^{125}I-large LTGF-β to SMCs showed no competitive inhibition by either mannose-6-phosphate or the synthetic peptide Arg-Gly-Asp-Ser. These findings suggest that neither the mannose-6-phosphate nor an Arg-Gly-Asp sequence in LAP is involved in the targeting of LTGF-β to SMCs, and that KM704 might recognize an entirely different domain of LAP responsible for the targeting of LTGF-β to SMCs (37).

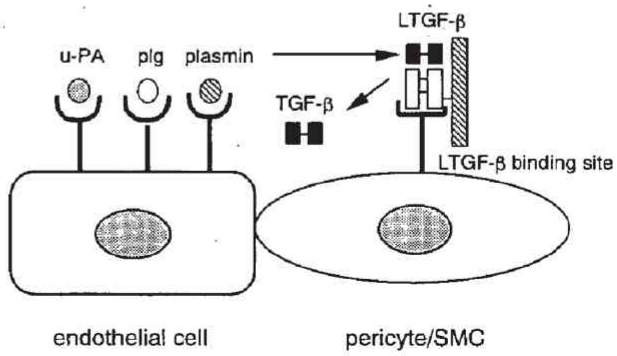

Figure 5　Activation of latent TGF-β during co-culture of ECs and mural cells. Role of mural cells. Latent TGF-β is specifically targeted to mural cells. During co-culture of ECs and mural cells, plasmin localized on the surface of ECs activates latent TGF-β targeted on mural cells.

(3) Regulation of the Activation of Latent TGF-β by the Induction of PAI-1

Once active TGF-β is generated during co-culture of ECs and mural cells, active TGF-β inhibits the migration and proliferation of ECs (31, 32). Moreover, there is more than a 20 fold increase of PAI-1 in protein level, and the PA activity of ECs is inhibited during co-culture (38). Less than 10% of LTGF-β was activated during co-culture. Since PA-plasmin is responsible for the activation of LTGF-β and TGF-β induces PAI-1 during co-culture, TGF-β may regulate its own generation by interfering with PA-plasmin activity via the induction of PAI-1. Conditioned medium (CM) was collected every 12 hours and the TGF-β activity

in CM was determined. Active TGF-β was found only in the first 12 hr of co-culture CM, and no active TGF-β was found during the subsequent hours 12 to 36. However, when CM was collected in the presence of antibody to PAI-1, the generation of active TGF-β during co-culture proceeded at a constant rate for at least 36 hr. When antibody to PAI-1 was removed at the 24 hr point, the generation of active TGF-β was lost during hours 24 to 36. Therefore, the activation of LTGF-β by the interaction of ECs and mural cells appears to be self-regulated and determined by the levels of PA and PAI-1 (Fig. 6).

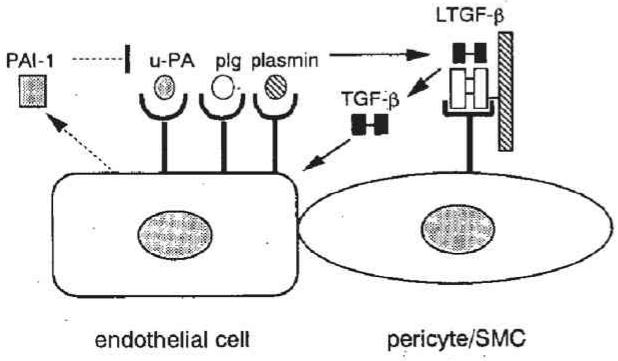

Figure 6 Regulation of the activation of latent TGF-β. Once LTGF-β is activated, active TGF-β induces PAI-1. PAI-1 inhibits PA activity of ECs, and prevents the further activation of LTGF-β.

Conclusion

While ECs and pericytes are physiologically in contact at capillaries or post-capillary venules, ECs and SMCs are normally separated by internal elastic lamina in large vessels. However, there are cell contacts between ECs and intimal SMCs at the site of the atherosclerotic lesion. When ECs are injured, the remaining ECs induce PA activity and migrate into the denuded area. At the atherosclerotic lesion, LTGF-β targeted to SMCs is efficiently activated by PA-plasmin on the surface of migrating ECs. Activated TGF-β inhibits the repair of endothelial layer and induces PAI-1 which may cause thrombosis.

Acknowledgment

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