Localization of Latent Transforming Growth Factor-\(\beta\) Binding Protein-1 in Human Coronary Atherosclerotic Plaques

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**Background:** Transforming growth factor-\(\beta\) (TGF-\(\beta\)) and its receptors have been detected by immunohistochemistry in the normal vessel wall and in atherosclerotic lesions of human coronary arteries. However, TGF-\(\beta\) is normally secreted as an inactive complex associated with a latent TGF-\(\beta\)-binding protein (LTBP). Therefore, detection of TGF-\(\beta\) antigen only in the arterial wall does not imply the activated form of the growth factor.

**Methods and Results:** In situ hybridization and immunohistochemistry demonstrated LTBP1 mRNA and protein expression throughout the media and intima of early coronary artery lesions, with the highest levels of protein at the luminal surface. In advanced lesions, LTBP1 mRNA and protein were detected mainly in regions of high cell density, such as the fibrous cap.

**Conclusions:** Assays of the TGF-\(\beta\) signalling pathway will be required to determine the activity associated with TGF-\(\beta\) antigen in the vessel wall. (Circ J 2011; 75: 196–200)

**Key Words:** Atherosclerosis; Coronary heart disease; Genes; Immunohistochemistry; Molecular biology

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is a multifunctional cytokine that regulates a variety of cellular processes, including proliferation, differentiation and migration in a wide range of cell types. For example, TGF-\(\beta\) inhibits the in vitro proliferation and migration of vascular smooth muscle cells, and maintains their differentiated phenotype in vitro and in vivo. Control of TGF-\(\beta\) activity is therefore potentially important for homeostasis of the arterial wall and abnormalities in TGF-\(\beta\) signaling may be critical in the pathogenesis of vascular disease. Both TGF-\(\beta\) type I and type II receptors (TGFBR1 and TGFBR2) have been detected in normal human coronary and carotid arteries, the aorta and in atherosclerotic plaques. However, atherosclerotic plaques have been examined for an inactivating mutation in the TGFBR2 which inhibits TGF-\(\beta\) signaling through heterodimer formation of TGFBR1 and TGFBR2 complexes. The mutation was detected in 1 study in a high proportion of plaques, but not in the other. Consequently the issue of whether TGF-\(\beta\) antigen in the vessel wall is biologically active and whether it affects lesion development remains unresolved.

TGF-\(\beta\) is generally secreted in its inactive form as a large latent complex (LLC) consisting of the active form of the cytokine non-covalently associated with latency-associated peptides (LAPs), I of which is covalently attached to the latent TGF-\(\beta\)-binding protein (LTBP), of which there are 4 types, LTBP1–4. Association of the 25-kDa active TGF-\(\beta\) homodimer with the LAPs is sufficient to render the complex biologically inactive in vitro and in vivo, whereas LTBP1 has been implicated in the normal assembly and secretion of TGF-\(\beta\). LTBP1 may also target the latent complex to the cell surface and extracellular matrix, and therefore regulate access to activating enzymes that release the active 25-kDa homodimer. The addition of excess free LTBP1, or a peptide fragment of LTBP1, inhibits activation of TGF-\(\beta\) in the LLC. However, the presence of LTBP1 in the human arterial wall and in atherosclerotic lesions has not been reported. We have therefore examined the expression of LTBP1 mRNA by in situ hybridization (ISH) and of LTBP1 protein by immunohistochemistry in human coronary atherosclerotic plaques. Other isoforms, LTBP2–4, in atherosclerosis were not investigated, primarily because their relationship with TGF-\(\beta\) assembly, secretion and activation have not been as extensively demonstrated.

**Methods**

**Study Population and Sample Preparation**

The study population consisted of 11 patients referred for cardiac transplantation between June 1997 and February...
The study was approved by the Huntingdon Local Research Ethics Committee and informed consent for use of tissue for experimentation was given by each patient.

Human coronary arteries (n=16) were dissected immediately following cardiectomy, mounted in embedding medium (Cryo-M-Bed; Bright Instrument Company Ltd) and snap-frozen in liquid nitrogen. Tissue sections (8.5 μm) were prepared using a cryostat, thaw-mounted onto Superfrost Plus slides (BDH Laboratory Supplies), and stored at –85°C. Samples from 2 groups classified according to the established criteria of Stary et al22,23 using haematoxylin and eosin and Oil-Red-O staining (early lesion, stage II and III, no atheroma-tous plaques, n=6; advanced lesion, stages V and VI, n=10) were examined.

**Immunohistochemistry**

LTBP1 protein in human coronary artery sections was detected using a monoclonal antibody (mAb) 388 (5 μg/ml; R&D Systems) and a rabbit polyclonal antibody (pAb) 39 (1:50; gift of Dr Kohei Miyazono), followed by biotin-conjugated anti-mouse IgG (1:100; Sigma) or biotin-conjugated anti-rabbit IgG (1:20; Sigma), respectively. After incubation with streptavidin-conjugated horseradish peroxidase (HRP, 1:500; Sigma), LTBP1 immunoreactivity was visualized using a Cyanine-3 Tyramide Signal Amplification kit (TSA; NEN Life Sciences) according to the manufacturer’s instructions. Tyramide staining patterns were compared with conventional fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG and FITC-labelled anti-rabbit IgG secondary antibodies (1:50; Sigma). The reactivity of the antibody mAb 388 with LTBP1 was confirmed by western blotting under reducing conditions using purified platelets as the source of LTBP1, which yielded a single band of 160 kDa (not shown).

Endothelium was visualized using a rabbit polyclonal antibody against von Willebrand factor (1:500, Dako; HRP-conjugated anti-rabbit IgG (1:500; Sigma) and the Cyanine-3 TSA kit). The presence of platelet specific microparticles was investigated using a goat polyclonal antibody against CD42 (1:100, Santa Cruz Biotechnology; HRP-conjugated anti-goat IgG (1:500; Sigma) and the Cyanine-3 TSA kit). Smooth muscle cell (SMC) differentiation status was analyzed using a FITC-labelled SM-α-actin monoclonal antibody (1:500, Sigma).

All incubations were carried out at 37°C for 60 min with the primary antibody, 30 min with the secondary antibody and 15 min with streptavidin–HRP. Sections were counterstained for 10 min with 300 nmol/L 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and mounted with Prolong-Antifade (Molecular Probes). For controls, the primary antibody was omitted from the staining protocol and substituted with equal amounts of non-immune IgG (Sigma).

**ISH**

Isolation of a LTBP1 fragment (681 bp) common to all reported LTBP1 spliced variants was obtained using reverse transcriptase-polymerase chain reaction (RT-PCR) and total RNA isolated from adult human aorta.24 The forward primer (5′-CCATGGTGCCACGTGCTAAACTTATC-3′) and
Comparison of LTBP1 antigen detection methods in coronary artery sections of a stage I lesion. (A) LTBP1 antigen (red) was detected using the monoclonal antibody 388 and the Cyanine-3-TSA system. (B) LTBP1 antigen was detected using the monoclonal antibody 388 and a FITC-labeled anti-mouse IgG (green). (C) LTBP1 antigen was detected using the polyclonal antibody 39 and a FITC-labeled anti-rabbit IgG (green). Arrows indicate the internal elastic membrane of the arterial wall. Original magnification ×200. FITC, fluorescein isothiocyanate; LTBP, latent TGFβ-binding protein; TGF, transforming growth factor.

Figure 2.

Results and Discussion

Differentiation Status of SMCs in the Coronary Artery Sections

Sections stained with haematoxylin and eosin typical of an early lesion with a highly cellular neointima (stage II) and of 2 more advanced lesions (stage Va and stage Vc) are shown in Figures 1A, F and K, respectively. Sections from the same lesions stained by immunofluorescence for the SMC differentiation marker, SM-α-actin, are shown in Figures 1B, G and L. The neointima of early lesions showed less SM-α-actin staining than the media and very little staining in the layer of cells towards the luminal surface. In the stage Vc lesions the amount of SM-α-actin was also markedly reduced at the neointimal surface, but was similar in the cellular interior of the neointima and the media (Figure 1G). The neointima of stage Va lesions showed only heterogeneous expression of SM-α-actin near the surface, with very little detectable antigen in the lipid core (Figure 1I). The most prominent reduction in SM-α-actin staining occurred mainly in areas of high cell density in the fibrous connective tissue layer near the luminal surface (Figure 1G) and in areas of very low cell density in the lipid core of advanced lesions (Figure 1L). These observations are consistent with previous studies on SMC differentiation in such lesions.

Expression of LTBP1 mRNA

ISH showed that LTBP1 mRNA was expressed at similar, uniform levels throughout the neointima and media of early lesions (Figure 1C). In the advanced lesions the neointimal expression of LTBP1 mRNA was very heterogeneous (Figures 1H, M) compared with that seen in the neointima of early lesions (Figure 1C) with extensive areas of low LTBP1 mRNA expression. However, in areas of high cell density in the neointima of advanced lesions (Figure 1H), and particularly in regions of dense connective tissue at fibrous caps (Figure 1M), high levels of LTBP1 mRNA expression were detected. Within these regions expression was restricted to a minor proportion (15–20%) of the cells (Figures 1H, M). The levels of LTBP1 mRNA in the media were similar in all samples within each group of lesions (compare media in Figures 1C and H).

Expression of LTBP1 Protein

In this study the experiments on LTBP1 protein expression were performed with both mAb 388 and pAb 39. The patterns of LTBP1 antigen expression using both antibodies detected by the tyramide signal amplification system and FITC-labelled anti-IgG were very similar (Figure 2) and all data shown are for the mAb.

LTBP1 protein in early lesions consistently showed very high levels of expression in the endothelial layer overlying lower levels of uniform expression in the neointima and further reduced levels in the media (Figure 1D). It is therefore clear that the LTBP1 protein levels near the luminal surface are substantially higher, relative to LTBP1 mRNA expression, than those observed in the media (compare Figures 1C and D). These results imply that factors other than the amount of mRNA determine LTBP1 protein levels. For example, it is possible that dedifferentiated SMCs in the neointima drive translation of LTBP1 mRNA into protein more efficiently than in the media. Alternatively, LTBP1 protein stability may be substantially higher in the neointima (eg, if the LLC is not degraded proteolytically to release TGFβ). The high levels of LTBP1 protein near the luminal surface might also be explained by accumulation of LLC from the circulation, although there are no reports of LTBP1 protein in platelet-poor plasma. Deposition in the lesion of cells from the circulation that express very high levels of the protein might also account for the high levels of LTBP1 protein near the luminal surface. Platelets are a major source of LLC in the circulation and we therefore used immunofluorescent

Figure 1.
staining with anti-CD42 antibody to assay for the presence of platelet microparticles carrying glycoprotein IIb/IIIa. No specific staining was detected in early or late lesions, indicating that platelet deposition and granule release are unlikely to account for the observed accumulation of LTBP1.

LTBP1 protein expression was very heterogeneous in the neointima of advanced lesions compared with early lesions, with areas of low expression interspersed with regions of high expression in areas of high cell density close to the luminal surface (Figure 1I). In marked contrast to the early lesions, the high levels of LTBP1 protein in regions of high cell density in the advanced lesions correlated with expression of high levels of LTBP1 mRNA in the same regions (compare Figures 1M and N). However, it was not possible to be certain that the cells that produced high levels of mRNA were also responsible for the high levels of protein.

In the media there was little difference in mRNA expression between early and advanced lesions (Figures 1C, H). In contrast, LTBP1 protein levels were much reduced in the media of advanced lesions compared with the media of early lesions, where extensive expression of the LTBP1 protein was readily detectable (Figures 1E, J, O).

Conclusions
This study indicates that there are marked differences in the expression pattern of LTBP1 mRNA and protein in early lesions compared with advanced lesions. LTBP1 mRNA expression was uniformly detected in the early lesions, but was heterogeneous in the neointima of the advanced lesions. LTBP1 protein expression was also heterogeneous, most markedly in the media of the vessel wall where some protein was detected in early lesions but none detected in the advanced lesions, even though similar mRNA levels were observed in all samples. Heterogeneity in LTBP1 expression may reflect changes in TGFβ secretion or may represent free LTBP1 independent of TGFβ. This study does not distinguish these possibilities or the consequences for TGFβ signaling. Presence of LTBP1 in the vessel wall and its differential expression in atherosclerosis implies an added complexity to the role TGFβ may play in the arterial wall. Alternative interpretations have been made of the likely role of TGFβ in the development of atherosclerotic plaques, based mainly on the levels and distribution of TGFβ/antigen and receptors detected. However, it is well established that LTBP1 is important in the secretion of TGFβ from cells as complexes in which TGFβ has no biological activity. Furthermore, no antibodies have been described that distinguish the mature 25-kDa TGFβ homodimer from the small latent complexes of large latent complexes. Thus, no studies in which TGFβ antigen has been localized in human atherosclerotic plaques are available any role of TGFβ activity in human atherosclerotic plaque development remains unresolved.

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References


