Diagn Interv Radiol 2011; 17:290–296

INTERVENTIONAL RADIOLOGY

ORIGINAL ARTICLE

TGFβ/activin signaling pathway activation in intimal hyperplasia and atherosclerosis

Rahmi Öklü, Robin Hesketh, Stephan Wicky, James Metcalfe

PURPOSE

Intimal hyperplasia and atherosclerosis are the Achilles' heels of vascular interventions. Many cytokines and growth factors have been shown to mediate these pathological processes. There are conflicting data concerning the expression of transforming growth factor-β1 (TGFβ1) antigen in human intimal hyperplasia and atherosclerotic lesions and conflicting views about whether TGFβ1 is pro- or anti-atherogenic. The presence of TGFβ1 is not sufficient to infer activation of its signaling pathway because TGFβ1 may be present in inactive complexes.

MATERIALS AND METHODS

A sensitive immuno-fluorescence assay (cyanine-3 tyramide signal amplification system) was used on human coronary artery and aorta sections with early or advanced stage lesions to detect TGFβ1, activin, Smad2-P, a marker of the activated TGFβ1/activin pathway and components of latent TGFβ complexes.

RESULTS

All antigens were readily detected in the media and neointima of early stage lesions. The levels were either reduced or undetectable in the media of advanced lesions but were increased in the neointima in areas of high cell density. In marked contrast to activin, TGFβ1 and LAP1 expression levels were closely correlated with Smad2-P throughout the artery wall.

CONCLUSION

Discrepancies in previous data for TGFβ1 expression are probably due to assay sensitivity. TGFβ1, but not activin, expression is consistently correlated with Smad pathway activation in the artery wall. The pattern of Smad2 activation supports a model in which TGFβ/activin signaling is anti-atherogenic in the media of normal artery walls but is equally compatible with an anti-atherogenic or pro-atherogenic response to TGFβ/activin in the neointima of lesions.

Key words: • *atherosclerosis* • *coronary artery disease* • *vascular intima* • *immunohistochemistry*

From the Department of Vascular Imaging and Interventions (R.Ö. ✉ *roklu@partners.org*, S.W.), Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts, USA; and the Department of Biochemistry (R.H., J.M.), University of Cambridge, Cambridge, United Kingdom.

Received 30 August 2010; revision requested 20 September 2010; revision received 22 September 2010; accepted 23 September 2010.

Published online 15 October 2010
DOI 10.4261/1305-3825.DIR.3844-10.1

Cardiovascular disease is the major cause of morbidity and mortality in the developed world (1). The major cause of death is associated with atherosclerosis of the coronary, cerebral and renal arteries. Principal risk factors, such as cigarette smoking, hypertension, obesity, and elevated plasma cholesterol levels, may promote the development of vascular disease (2, 3). Although lifestyle changes and the use of drugs to lower plasma cholesterol levels have had a substantial impact on mortality, atherosclerosis remains the major cause of death (4, 5).

Atherogenesis is a complex phenomenon involving interplay between inflammatory system components, lipids and the cells of the arterial wall (2, 6). To provide clues to the interactions involved in atherogenesis, changes in gene expression patterns in the arterial cells during the course of lesion development must be defined. A better understanding of the molecular mechanisms of atherosclerosis will help identify new targets for drug design and may enable early diagnosis for therapeutic intervention.

The early stages of atherosclerosis are thought to represent a protective response to injury or dysfunction of the endothelium that lines the arterial wall (2, 6, 7). Some of the possible causes of such injury and dysfunction are oxidized and/or elevated LDL (low-density lipoprotein), hypertension, mechanical stress, free radicals (e.g., those produced by cigarette smoking), elevated plasma homocysteine, and infectious microorganisms, such as the herpes virus and *Chlamydia pneumoniae* (1, 2, 7–10). As a consequence of injury, the impaired endothelium initiates an inflammatory reaction. The expression of adhesion molecules and the secretion of cytokines and growth factors are locally increased in the region of injury (6).

Monocytes and T-lymphocytes infiltrate the injured regions of the vessel wall (9). The secretion of growth factors and cytokines from these cells initiates the repair process (2). If repair is not achieved, these cells remain in the sub-endothelial layer, and their numbers increase (9). In response to the secretions released from cells involved in the inflammatory response, the cells of the arterial wall may proliferate, migrate, differentiate, secrete more growth factors, demonstrate derangements of lipid metabolism, and synthesize connective tissue proteins, such as collagen and fibronectin (11). These processes contribute to the formation of the neointima, which is derived from the intima and formed in response to endothelial injury or dysfunction. For example, platelet-derived growth factor (PDGF), which is produced by cells of the arterial wall (e.g., endothelial cells and vascular smooth muscle cells [VSMCs]), is one of the most potent chemotactic agents for VSMCs and a potent mitogen for VSMCs (12, 13). Thus, PDGF secretions may promote the migration and proliferation of VSMCs in the human arterial wall and contribute to the formation of the neointima.

Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) secreted by activated monocytes *in vitro* and *in vivo* lead to a significant increase in the expression of vascular cell adhesion molecule-1 (VCAM-1 [14]), intercellular adhesion molecule-1 (ICAM-1 [15]), and E-selectin in endothelial cells (16). An increase in the expression of adhesion molecules in turn leads to the recruitment of more inflammatory cells to the injured region (11). VSMCs may become activated and switch to the dedifferentiated phenotype, which is characterized by increased levels of rough endoplasmic reticulum (17). Dedifferentiated VSMCs are thought to be important in lesion progression because they proliferate and deposit connective tissue proteins into the extracellular matrix (ECM [6]).

Platelets are another source of growth factors that may further promote lesion progression (18). Platelet adhesion to the injured or dysfunctional endothelium leads to the activation and subsequent release reaction of α-granules, which contain large concentrations of various growth factors and cytokines (e.g., PDGF [2, 19]). Although the role of platelets in lesion development is still unclear, it is possible that degranulation and the release of growth factors may influence the cells of the intima. Platelet degranulation may also lead to the migration and proliferation of VSMCs and monocytes in the vascular intima.

If the response to injury or dysfunction of the endothelium is incomplete, the response continues indefinitely. Early stage lesions eventually progress into more life-threatening advanced lesions in which necrosis and accumulation of a lipid core, lesion rupture, and thrombo-embolism are common (2, 17). Advanced lesions may produce symptoms of angina pectoris or myocardial infarctions, but early stage lesions are almost invariably clinically silent (17, 18). However, acute rupture of lesions at various stages may lead to near or total artery occlusion (17).

Vascular stent placement represents one scenario in which the response to injury can presumably lead to restenosis and the sequelae associated with vessel narrowing. Transforming growth factor-β (TGFβ) has been shown to be one of the critical players in mediating intimal hyperplasia and its progression. TGFβ inhibits the proliferation and migration of vascular smooth muscle cells and maintains their fully differentiated phenotype *in vitro* (20). These data, as well as those from *in vivo* studies (21, 22), suggest that control of TGFβ activity is potentially important for homeostasis in the normal arterial vessel wall and that abnormalities in TGFβ signaling may be important in atherogenesis (20).

All three mammalian isoforms of TGFβ are secreted by cells *in vitro* in inactive forms (23). The active TGFβ homodimer (25 kDa), which is noncovalently bound to latency-associated peptides (LAP), constitutes the small latent complex (SLC), and the covalent attachment of the latent TGFβ-binding protein (LTBP) to LAP forms the large latent complex (LLC [23]).

There are conflicting data for detecting both the TGFβ1 antigen and the TGFβ1 signal-transducing receptors (TGFBRI/TGFBRII hetero-oligomers) in normal arteries and atherosclerotic plaques (24–26). Using chromogenic immunoperoxidase, Bobik and colleagues (24) reported significant levels of TGFβ1 antigen in the neointima of aortic lesions but little or no TGFβ1 antigen in the media of normal or atherosclerotic aorta. The distribution of both TGFBRI and TGFBRII antigens was highly heterogeneous in the media and neointima of early and advanced stage lesions. It was concluded that only the TGFβ1 antigen that was co-localized with the expression of both receptors was likely to be functional, and this was only observed in the neointima of Stage II and III lesions (fatty streaks). From these data, it was suggested that TGFβ1 is likely to be pro-atherogenic. In contrast, McCaffrey and colleagues (26) reported that the pattern of TGFβ1 antigen expression was similar to that of TGFBRI and TGFBRII in the fibrous cap of advanced lesions (Stage V). In marked contrast to either study, Ihling and colleagues (25) reported that in 11/11 atherosclerotic human carotid arteries and 5/5 normal coronary arteries both TGFBRI and TGFBRII antigens were highly expressed in all regions of the arterial wall.

These studies did not directly address the question of whether the TGFβ is active because there is no antibody that distinguishes active TGFβ homodimers from TGFβ in SLCs (27). However, the activation of the Smad signaling pathway by TGFβ or activin leads to phosphorylation of Smad2 to Smad2-P (23), and Smad2-P levels provide an assay for the activation of the TGFβ/activin signaling pathway (28).

In this study, the causes of discrepancies in the detection of TGFβ1 antigen in previous studies were analyzed by the use of multiple antibodies and detection systems, including Cy-3-TSA, which has been reported to be much more sensitive than chromogenic immunoperoxidase detection (29). Immuno-Cy-3-TSA assays for TGFβ1, LAP1, activin-A and Smad2-P were used to determine whether the TGFβ/activin signaling pathway was activated and whether the expression of any of the antigens examined was correlated with Smad2-P. In addition, these data were correlated with our previously reported expression patterns of LTBP1 (30), which were acquired for the same tissue specimens.

Materials and methods

Section preparation

This study was approved by the Huntingdon Local Research Ethics Committee. Informed consent for the use of coronary and aortic tissue was obtained from 21 patients undergoing cardiac transplantation. Additional normal aorta samples were obtained from two donor hearts. Coronary artery samples (n=22; 17 patients) and aortic samples (n=6, 6 patients; n=2, 2 donors) were mounted in embedding medium and snap-frozen in liquid nitrogen. Tissue sections (8.5 μm) were thaw-mounted onto slides and stored at -85 °C. Lesions in coronary artery and aorta sections were classified according to the criteria of Stary and colleagues (9, 17) using Oil-Red-O, hematoxylin and eosin. Early stage lesions are defined throughout as Stages I-III, and advanced lesions are defined as Stages IV-VI.

Immunohistochemistry

Immunohistochemistry was performed using Cy-3-TSA (NEN Life Sciences, Waltham, Massachusetts, USA). Primary detection antibodies were TGFβ1, polyclonal affinity purified AF-101-NA (0.5 ng/mL; R&D Systems, Minneapolis, Minnesota, USA) or monoclonal MAB240 (0.62 μg/mL to 25 μg/mL; R&D Systems); LAP1, monoclonal MAB246 (1.25 μg/mL;

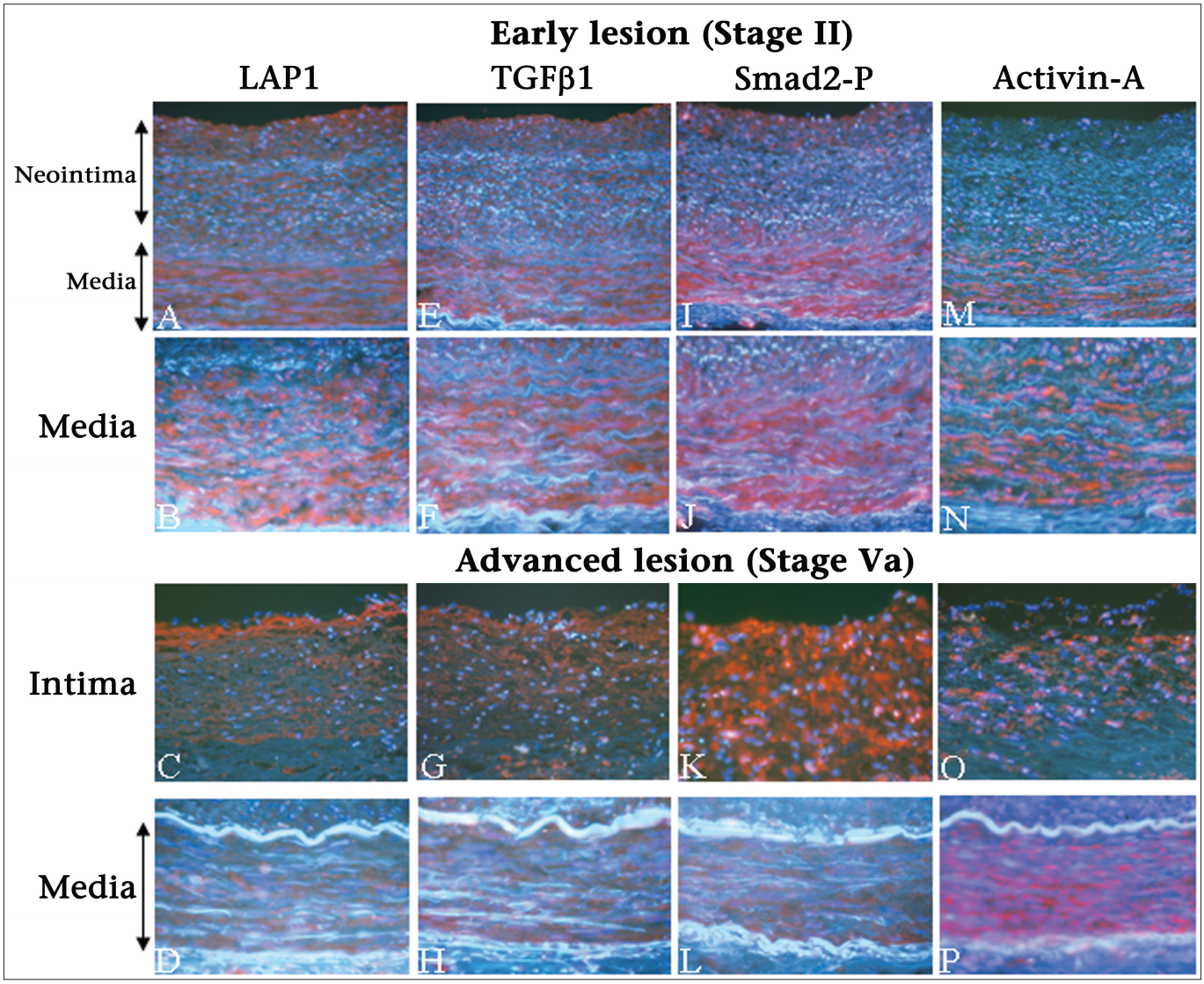

Figure 1. a–p. Expression patterns of LAP1 **(a–d)**, TGFβ1 **(e–h)**, Smad2-P **(i–l)** and activin **(m–p)** in serial sections. Antigens were visualized with Cy-3-TSA *(red)* and nuclei with DAPI *(blue)*. Expression patterns are shown for a representative early stage lesion (Stage II) and an advanced lesion (Stage Va). Original magnification, 200x **(a, e, i, m** and **c, g, k, o)**; 120x **(b, f, j, n)**; and 400x **(d, h, l, p)**.

R&D Systems); LTBP1, monoclonal MAB388 (5 µg/mL; R&D Systems) or polyclonal PAB39 (1:800; gift of Prof. K. Miyazono); activin, polyclonal AF338 (5 µg/mL; R&D Systems) specific for activin-A and monoclonal MCA950S (1:200; Serotec, Kidlington, UK) specific for all activins and inhibin; Smad2-P, polyclonal antibody 06-829 (1:400; Upstate Biotechnology, Waltham, Massachusetts, USA); von Willebrand Factor (vWF), polyclonal antibody A0082 (1:500; Dako, Carpinteria, California, USA). All secondary antibodies were biotinylated (1:800; Biotin-SP; Jackson ImmunoResearch, West Grove, Pennsylvania, USA). After treatment with streptavidin-conjugated horseradish peroxidase (1:500; NEN Life Sciences), sections were incubated with Cy-3-TSA reagent for 7 min. TSA-Cy3 staining patterns were compared with those from a chromogenic immunoperoxidase (12-min reaction with Nova-Red substrate kit; Vector, Burlingame, California, USA) reaction, as described previously (24). To detect nuclei, sections were counterstained with 300 nM DAPI (Molecular Probes) for 10 min and mounted with Prolong-Antifade (Molecular Probes, Invitrogen, Carlsbad, California, USA). For negative controls, an appropriate control IgG (AB-101-C and MAB002, R&D Systems; R5256, Sigma, Sigma-Aldrich, St. Louis, Missouri, USA) was substituted for the primary antibody. All negative control samples showed no detectable Cy-3 or Nova-Red above background.

Results

TGFβ1 and LAP1 antigens

Immunofluorescence images of coronary artery sections are compared in Figure 1 for an early stage lesion with a highly cellular neointima (Stage II) and an advanced lesion containing a lipid core (Stage Va). These images are representative of all samples of early and advanced lesions at the same stages. The presence of an intact endothelial layer in all samples was confirmed by immunostaining for vWF.

TGFβ1 antigen was readily detected in all coronary artery sections using polyclonal antibody AF-101-NA (Fig.

1e-h), but there was no detectable signal when using the monoclonal antibody MAB240 in any of the sections examined, although the antibody is an effective TGFβ1 capture agent in ELISAs.

In the early stage lesions, the expression of both TGFβ1 and LAP1 was much higher in the media than in the endothelial layer and the underlying neointima (Fig. 1a, b, e and f). TGFβ1 and LAP1 antigens were present both in the cytoplasm of the smooth muscle cells and in the extracellular matrix. Similar distributions of TGFβ1 and LAP1 antigen were also detected in the media of all of the advanced lesions (Fig. 1d and h), but the expression levels of both antigens were reduced as compared to the media of the early stage lesions.

The expression of both TGFβ1 and LAP1 antigens was much higher in the cellular regions (e.g., the fibrous cap) of the advanced lesions than in the neointima of the early stage lesions (compare Fig. 1c, g with a, e). TGFβ1 and LAP1 antigens in these regions were mainly extracellular, and minimal antigen was detected in the cytoplasm. Regions of low cell density in the neointima showed reduced levels of expression for both antigens.

The expression patterns of TGFβ1 and LAP1 antigens were very similar within each stage of the early and advanced lesions in coronary artery sections. However, in a previous study of the aorta using chromogenic immunoperoxidase detection, no TGFβ1 antigen was detected in the media of early or advanced stage lesions (24). We examined aortic sections from two donors and four recipients (all normal artery or early stage lesions) using the same antibodies and Cy-3-TSA detection system used for the coronary artery sections. Similar patterns of TGFβ1 (and of LAP1, Smad2-P, activin-A and LTBP1 antigens) were detected in the media of the normal aorta sections as in the early stage lesions of the coronary artery sections (Figs. 1 and 2). In contrast, when TRITC-labeled secondary antibody was used in place of the Cy-3-TSA, TGFβ1 was only weakly detected above background autofluorescence (not shown); with Nova-Red chromogenic immunoperoxidase, TGFβ1 was barely detectable, if at all (compare Fig. 1a, c with b, d). Thus, the detection of TGFβ1 in the media is critically dependent on both the antibody and the sensitivity of the detection system used.

Smad2-P antigen

The TGFβ1 antibodies in ELISAs do not distinguish between active TGFβ1 homodimers and TGFβ1 in SLCs (27); hence, antigen detection does not identify active TGFβ1. Smad2-P expression was therefore examined to determine whether the TGFβ1 detected in sections might be active. For the early stage coronary artery lesions, Smad2-P antigen expression was much higher in the media than in the neointima (Fig. 1i and j), and a similar pattern was observed in the early stage lesions in aorta sections. However, in the media of the advanced lesions, Smad2-P was reduced as compared to the levels detected in the media of the early stage lesions (compare Fig. 1j and l), whereas Smad2-P expression was increased in the neointima of the advanced lesions in regions of high cell density (compare Fig. 1i and k). The pattern of Smad2-P expression was therefore strongly correlated with TGFβ1 and LAP1 in the media and neointima of both early and advanced stage lesions. Smad2-P was detected in both the cytoplasm and nucleus at all sites.

Activin

The above data are consistent with regulation of the Smad2 pathway by TGFβ1. However, activins also regulate the same pathway, and the presence of activin antigen has been reported in the vessel walls of human aortas (31). We therefore examined the expression of activins in the coronary artery sections to compare with the patterns of Smad2-P expression. Antibodies to the A subunit (binding all activins and inhibin) and antibodies to the active βA dimer (binding activin-A only) showed very similar patterns of expression, consistent with previous data showing that activin-A is the major activin antigen detected in the human aorta (31).

In the early stage lesions, activin expression was much higher in the media than in the neointima, where the antigen was barely detectable (Fig. 1m and n). However, in the media and the neointima of the advanced lesions, activin expression was greater than the corresponding levels detected in the media and neointima of the early stage lesions (compare Fig. 1m, n with o, p). In the advanced lesions, activin in the neointima was mostly detected in regions of high cell density (e.g., fibrous caps). All of the activin detected was both cytoplasmic and extracellular.

The relative levels of activin and Smad2-P expression were inversely related in the media of the advanced lesions as compared with the media of the early stage lesions, and there was no consistent correlation between activin and Smad2-P expression, in marked contrast to TGFβ1.

LTBP1 antigen

We previously showed (30) that the LTBP1 antigen is easily detectable in the media of early stage lesions but is not detected in the media of advanced lesions using either the monoclonal or polyclonal antibody. Very high levels of LTBP1 were consistently detected in the endothelial layer, overlying lower levels of uniform expression in the neointima of early stage lesions. In the neointima of advanced lesions, LTBP1 antigen levels were very heterogeneous and were mainly restricted to areas of high cell density (e.g., the fibrous cap), with intense staining at the endothelial surface.

TGFβ1 and LAP1 protein expression in both the early and advanced stage lesions was closely correlated with Smad2-P levels, whereas the expression of LTBP1 was distinct. In particular, there was no correlation between TGFβ1 and LTBP1 at the luminal surface of the early or advanced stage lesions, whereas LTBP1 was very strongly expressed. Conversely, LTBP1 was not detectable in the media of the advanced lesions, whereas low levels of TGFβ1 were observed.

Discussion

TGFβ1 antigen expression in the coronary artery and aorta

The consistency of the data for antigen detection obtained for different lesions of the same stage in this and previous studies (24–26) suggests that sample heterogeneity is unlikely to be a major cause of the discrepancies in antigen detection. However, we have shown that the ability to detect TGFβ1 antigen in the media of early lesions, for example, depends critically on the sensitivity of the detection system. TGFβ1 antigen was readily detectable in the media using the Cy3-TSA detection system (see Fig. 2a); however, it was much harder to detect above

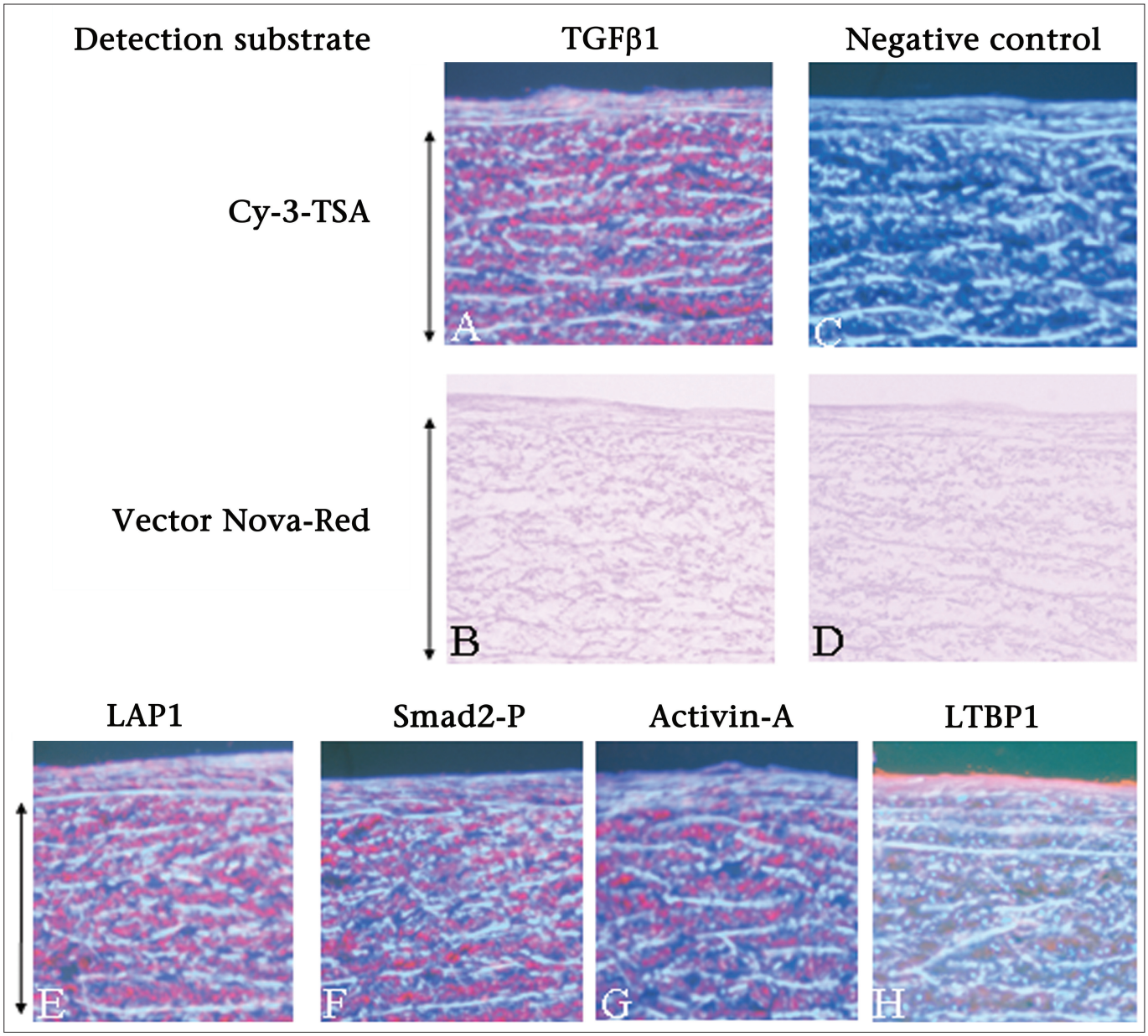

Figure 2. a–h. Expression patterns of LAP1 **(e)**, TGFβ1 **(a, b)**, Smad2-P **(f)**, activin **(g)** and LTBP1 **(h)** antigens in serial sections of a normal aorta (donor). Antigens and nuclei were visualized in **a, c, e–h** with Cy-3-TSA *(red)* and nuclei with DAPI *(blue)*. TGFβ1 antigen was visualized by Vector-NovaRed in **b**. Corresponding controls omitting primary antibody are shown in **c** and **d**. ↔ denotes media layer of artery. Original magnification, 200x.

background autofluorescence using TRITC-labeled secondary antibodies, and it was at the limit of detection when using the chromogen immunoperoxidase detection technique used in previous studies (24–26). Furthermore, wide variations in the ability of different antibodies to detect TGFβ1 antigen in the artery wall are apparent from the inability of a monoclonal antibody to detect TGFβ1 at sites in adjacent sections where the polyclonal antibody was effective. The consistency of data between studies is therefore likely to depend on the use of appropriate antibodies and detection systems for TGFβ and related antigens.

Activation of the Smad signaling pathway

Smad2-P is generated in response to TGFβ and activin signaling (23). Our data show that, in marked contrast to activin, the expression patterns of TGFβ1 and LAP1 antigens were closely correlated with each other and with Smad2-P antigen expression in the early and advanced stage lesions. The data suggest that TGFβ1, rather than activin, is the major activator of the pathway in the media of advanced lesions, but it does not allow the contributions of the two ligands to the production of Smad2-P to be distinguished elsewhere.

The close correlation between TGFβ1 and LAP1 antigen expression is consistent with the ability of the TGFβ1 antibodies to detect the active TGFβ1 homodimer and TGFβ1 in SLCs *in vitro* with similar sensitivity. The correlation of both antigens with Smad2-P is most

simply accounted for by assuming that TGFβ1 activity is determined mainly by the amount of SLC produced, rather than by variations in the SLC proteolytic activation rate.

As we showed previously (30), there was a marked difference in the expression pattern of LTBP1 antigen as compared with LAP1 or TGFβ1, particularly at the luminal surface of both the early and advanced stage lesions; consequently, no consistent correlation between LTBP1 expression and Smad2-P was found. We also showed previously that the *in situ* hybridization patterns for LTBP1 mRNA do not account for the patterns of protein expression (30). Furthermore, the LTBP1 protein at the luminal surface of lesions does not appear to be a component of LLCs because the LAP1 and LTBP1 expression patterns are very different, although the LAP1 antibody recognizes LAP1 in both LLCs and SLCs (27). The data do not allow for a clear distinction between models in which LTBP1 is produced independently of SLC components at the luminal surface of the neointima and those in which LTBP1 from SLCs is slowly degraded and therefore accumulates at some sites in the neointima, relative to LAP1 and TGFβ1 antigens. Inefficient conversion of LLCs to SLCs and TGFβ in the neointima may result from insufficient proteolytic activity in the neointima as compared with the media. However, this difference may also be due, in whole or in part, to the switch to LTBP1Δ41 isoform expression, if this isoform is relatively resistant to proteolysis (32). The deposition of LTBP1 from the circulation may also contribute to the high levels detected at the luminal surface.

Implications for atherogenesis

It has been suggested that the increase in the TGFβ1 antigen, together with the presence of the TGFβ1 signaling receptors detected in the neointima of advanced lesions, indicates that TGFβ is pro-atherogenic (24–26). However, it seems unlikely that the role of TGFβ in atherogenesis can be deduced simply from the presence of TGFβ antigen and its receptors. Furthermore, our finding that TGFβ1 antigen expression is closely correlated with Smad2 signaling pathway activation in the media and neointima of both early and late stage lesions does not fully resolve this issue. For example, it is possible that the expression of TGFβ1 in the media of early stage lesions and the reduced levels in advanced lesions reflect a decreased anti-atherogenic effect of TGFβ1 in the media as lesions develop. In this model, the increases in TGFβ1 antigen and the activation of Smad signaling in the neointima of advanced lesions may represent a compensatory protective mechanism against lesion development (e.g., by strengthening the fibrous cap, where there is strong Smad signaling) that is overridden by atherogenic stimuli as lesions develop. Alternatively, the increased TGFβ1/activin expressed in the neointima of lesions may represent a contributory mechanism to lesion development, as suggested previously (24).

Two observations suggest that the former view is more likely. It seems very unlikely that the TGFβ1/activin and Smad2 signaling clearly detected in the media of normal coronary artery and aorta contribute to atherogenesis in the normal vessel wall. Similarly, TGFβ1 antigen expression has been observed at high levels in mouse and rat arteries, which are not normally susceptible to atherogenesis (21, 33). It is more plausible that there are mechanisms to protect the normal artery wall against atherogenic stimuli, analogous to the anti-thrombotic mechanisms described for the arterial endothelium. Furthermore, we have shown that the aortas of mice heterozygous for the *TGFβ1* allele are susceptible to lipid lesion formation when fed a high-fat diet, whereas their wild-type controls are resistant (22). The reduced level of TGFβ1 antigen in the aortic wall of heterozygous mice is therefore correlated with increased susceptibility to lipid lesion formation. The correlation between antigen and Smad2-P patterns in human arteries cannot definitively resolve whether TGFβ is pro- or anti-atherogenic or indeed whether TGFβ has any effect in modulating lesion development, but the evidence points more plausibly to a protective effect.

Acknowledgments

This work was supported by a program grant from the British Heart Foundation (BHF) to JM. RÖ is a Koç scholar.

Conflict of interest disclosure

There are no conflicts of interest to disclose from any author.

References

1. Braunwald E. Shattuck lecture. Cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med 1997; 337:1360–1369.
2. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999; 340:115-126.
3. Eckel RH, Krauss RM. American Heart Association call to action: obesity as a major risk factor for coronary heart disease. AHA Nutrition Committee. Circulation 1998; 97:2099–2100.
4. Treasure CB, Klein JL, Weintraub WS, et al. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. N Engl J Med 1995; 332:481–487.
5. Shepherd J, Cobbe SM, Ford I, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med 1995; 333:1301–1307.
6. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362:801–809.
7. Anderson TJ. Assessment and treatment of endothelial dysfunction in humans. J Am Coll Cardiol 1999; 34:631–638.
8. Libby P, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. Circulation 1997; 96:4095–4103.
9. Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1994; 89:2462–2478.
10. Steinberg D. Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 1997; 95:1062–1071.
11. Raines EW, Ross R. Multiple growth factors are associated with lesions of atherosclerosis: specificity or redundancy? Bioessays 1996; 18:271–282.
12. Bornfeldt KE, Raines EW, Nakano T, Graves LM, Krebs EG, Ross R. Insulin-like growth factor-I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. J Clin Invest 1994; 93:1266–1274.
13. Heldin CH. Structural and functional studies on platelet-derived growth factor. EMBO J 1992; 11:4251–4259.
14. Cybulsky MI, Gimbrone MA, Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991; 251:788–791.
15. Printseva O, Peclo MM, Gown AM. Various cell types in human atherosclerotic lesions express ICAM-1. Further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3. Am J Pathol 1992; 140:889–896.
16. Shimizu Y, Newman W, Gopal TV, et al. Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. J Cell Biol 1991; 113:1203–1212.

17. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995; 92:1355–1374.
18. Yeghiazarians Y, Braunstein JB, Askari A, Stone PH. Unstable angina pectoris. N Engl J Med 2000; 342:101–114.
19. Gawaz M, Neumann FJ, Dickfeld T, et al. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 1998; 98:1164–1171.
20. Grainger DJ, Metcalfe JC. A pivotal role for TGF-beta in atherogenesis? Biol Rev Camb Philos Soc 1995; 70:571–596.
21. Grainger DJ, Metcalfe JC, Grace AA, Mosedale DE. Transforming growth factor-beta dynamically regulates vascular smooth muscle differentiation in vivo. J Cell Sci 1998; 111 (Pt 19):2977–2988.
22. Grainger DJ, Mosedale DE, Metcalfe JC, Bottinger EP. Dietary fat and reduced levels of TGFbeta1 act synergistically to promote activation of the vascular endothelium and formation of lipid lesions. J Cell Sci 2000; 113 (Pt 13):2355–2361.
23. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998; 67:753–791.
24. Bobik A, Agrotis A, Kanellakis P, et al. Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. Circulation 1999; 99:2883–2891.
25. Ihling C, Technau K, Gross V, Schulte-Monting J, Zeiher AM, Schaefer HE. Concordant upregulation of type II-TGF-beta-receptor, the cyclin-dependent kinases inhibitor P27Kip1 and cyclin E in human atherosclerotic tissue: implications for lesion cellularity. Atherosclerosis 1999; 144:7–14.
26. McCaffrey TA, Du B, Fu C, et al. The expression of TGF-beta receptors in human atherosclerosis: evidence for acquired resistance to apoptosis due to receptor imbalance. J Mol Cell Cardiol 1999; 31:1627–1642.
27. Grainger DJ, Mosedale DE, Metcalfe JC. TGF-beta in blood: a complex problem. Cytokine Growth Factor Rev 2000; 11:133–145.
28. Brodin G, ten Dijke P, Funa K, Heldin CH, Landstrom M. Increased smad expression and activation are associated with apoptosis in normal and malignant prostate after castration. Cancer Res 1999; 59:2731–2738.
29. Werner M, Von Wasielewski R, Komminoth P. Antigen retrieval, signal amplification and intensification in immunohistochemistry. Histochem Cell Biol 1996; 105:253–260.
30. Oklu R, Hesketh R, Wicky S. Localization of latent transforming growth factor-β binding protein-1 (LTBP1) in human coronary atherosclerotic plaques. Paper presented at 2010 Arterioscler Thromb Vasc Biol Conference; Abstract P376; April 8, 2010; San Francisco, USA.
31. Engelse MA, Neele JM, van Achterberg TA et al. Human activin-A is expressed in the atherosclerotic lesion and promotes the contractile phenotype of smooth muscle cells. Circ Res 1999; 85:931–939.
32. Oklu R, Hesketh R, Wicky S. Expression of latent transforming growth factor-β binding protein-1 (LTBP1) isoforms coronary atherosclerosis. Paper presented at 2010 Arterioscler Thromb Vasc Biol Conference; Abstract P171; April 8, 2010; San Francisco, USA.
33. Grainger DJ, Kemp PR, Liu AC, Lawn RM, Metcalfe JC. Activation of transforming growth factor-beta is inhibited in transgenic apolipoprotein(a) mice. Nature 1994; 370:460–462.