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Detailed localization of type-1 plasminogen activator inhibitor mRNA expression and antigen in atherosclerotic plaque on human coronary artery

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Summary *Objective*: To accurately localize type-1 plasminogen activator inhibitor (PAI-1) both in atherosclerotic and normal human coronary arteries and to determine which cells are responsible for its production.
Design: Immunohistochemistry and in situ hybridization over paraffin embedded samples obtained from patients subjected to heart transplant in 'La Fe' University Hospital, Valencia, Spain.
Results: PAI-1 mRNA is clearly detectable in atherosclerotic coronary artery, both in endothelial cells and in smooth muscle cells (SMCs) in the connective tissue of the thickened intima, while in the core of the plaque macrophages are the main PAI-1 producer cells. This pattern is coincident with PAI-1 antigen localization. In arteries appearing to be normal, PAI-1 antigen and mRNA were only slightly detectable or not at all.
Conclusion: PAI-1 expression and antigen are increased in endothelial cells from atherosclerotic arteries. PAI-1 mRNA and antigen are also present in the SMCs from thickened arteries and in macrophages in the core of the plaque. These results suggest that PAI-1 may be involved in the development, evolution and final outcome of the atherosclerotic plaque.

INTRODUCTION

Type-1 plasminogen activator inhibitor (PAI-1) is the physiological inhibitor of both urokinase (uPA) and tissue type (tPA) plasminogen activator, and appears to be one of the primary regulators of the fibrinolytic system in vivo.[1,2] PAI-1 biosynthesis is induced by several cytokines and growth factors in a number of cell lines,[3,4] and the overexpression of this inhibitor may compromise normal fibrin clearance mechanisms and thus promote pathological fibrin deposition when the clotting cascade is activated.[5]

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Thrombosis and atherosclerosis are two major factors in the pathogenesis of ischemic cardiopathy. The initial observations of Chakrabarti,[6] who reported that coronary ischemic processes involve a fibrinolytic hypofunction, were confirmed by other authors,[7,8] and attributed to an increase in PAI activity,[9–11] due basically to a rise in PAI-1.[12]

A relationship between impaired fibrinolytic activity and acute myocardial infarction has been described.[13] Moreover, it has been reported that there is a decrease in the fibrinolytic activity of coronary patients, primarily due to an increase in circulating levels of PAI-1.[14–20] In fact, PAI-1 has been found to be an independent risk factor for reinfarction in young patients[18,21] in whom PAI-1 activity correlated with the reinfarction risk in the 3 years following the first infarction.[22]

In addition to its directly thrombogenic role, PAI-1 can also contribute to vascular ischemic processes by favoring the development of atherosclerotic disease. In prospective

studies on young infarct patients, increased PAI-1 levels were found to be significantly associated with the number of stenosed vessels[23] or vessel damage.[24]

The mechanism by which PAI-1 could contribute to the development of the atherosclerotic lesions remains unclear, but increased PAI-1 synthesis in atherosclerotic arteries has been detected,[25,26] and the levels of PAI-1 mRNA seem to be related to the severity of atherosclerosis.[25] In addition to this increased PAI-1 synthesis detected in atherosclerotic arteries, there may be certain circumstances which are sometimes found in coronary patients, like elevated levels of triglycerides or glucose, that can favor this synthesis.

It has recently been reported[27] that monocytes produce and secrete relatively large amounts of PAI-2, while macrophages and foam cells contain all their PAI-2 as an intracellular protein. It has been suggested that this intracellular PAI-2 could protect macrophages against cell death, especially in the atherosclerotic plaque where lipoproteins can be cytotoxic to cells.

The presence of fibrinolytic activators and inhibitors in the atherosclerotic plaque can also be related to the cellular migration process associated with the plaque development,[28] and with the plaque disruption prior to acute myocardial infarction.[26] Thus, accurate localization of fibrinolytic compounds and cells responsible for their production is needed if we are to understand the fibrinolytic system's role in the atherosclerotic process.

Previous studies have detected PAI-1 mRNA in macrophages and smooth muscle cells (SMCs) in human atherosclerotic coronary artery[29] and other arteries,[26,30,31] but the information about the localization of the mRNA expression and about the antigen localization is contradictory. For example, some of these studies[31] localized PAI-1 mRNA in media and adventitia cells (while others found it in strayed cells, SMCs, foam cells or macrophages). This contradictory information about the exact localization of PAI-1 is probably due to the difficulty of obtaining good specimens and controls. For example, some researchers[28] use arteries with a thickened intima, which means a previous pathological stage for atherosclerotic plaque as controls.

The aim of the present study is to extend and complete previous information about PAI-1 expression and localization in order to determine its role in the development and outcome of coronary artery disease.

MATERIALS AND METHODS

Samples

We examined 30 atherosclerotic and 10 normal appearing segments of coronary arteries from 16 patients subjected to heart transplant in 'La Fe' University Hospital, Valencia, Spain. All these patients were in advanced stages of coronary artery disease.

The coronary arteries were separated from the recipient's heart, washed and fixed immediately after the extraction. For immunohistochemistry and in situ hybridization, the sections were fixed in 10% paraformaldehyde overnight at 4°C and embedded in paraffin blocks. Microscopic examination revealed some zones where the arteries appeared normal and others where they had a pathological appearance. The pathological segments included different stages of atherosclerotic lesions, from the initial lesion (type I) with mild thickening of the intima to type V (fibroatheroma).[32] Segments with complicated lesions (type VI) were excluded.

Immunohistochemistry

The immunohistochemistry study was performed as described earlier.[33] Fixed, paraffin-embedded tissue blocks were cut into 3.5 μm sections using a microtome (Microm, Walldorf, Germany), placed onto pretreated slides (Fisher Scientific, Pittsburgh, PA, USA) and air dried. The sections were deparaffinized in xylene, cleared in 95% ethanol, incubated in 3% hydrogen peroxide in methanol for 10 min and rehydrated through graded ethanol. After washing with Tris-buffered saline (TBS), the sections were treated with an increasing concentration of Triton X-100 (0.1% to 1%) in TBS for permeabilization. To unmask PAI immunoreactivity, the sections were incubated at 37°C for 5 min with 0.23% (wt/vol) pepsin (2830 U/mg, Worthington Biochemica Corporation, Freehold, NJ, USA) in 0.01 N HCl. To prevent non-specific binding, tissue sections were subsequently incubated at 22°C for 1 h with 1% normal goat serum in TBS. The sections were then incubated with either 10 μg/ml of affinity purified rabbit anti-human PAI-1 (American Diagnostica Inc, Greenwich, CT, USA) or 10 μg/ml of preimmune (normal) rabbit IgG. After washing with 1% Triton-TBS (3 × 3 min), biotinylated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories inc, West Grove, PA, USA), diluted 1:100 with TBS containing 0.05% Tween 20, was added and allowed to react for 15 min at room temperature. The tissue sections were washed again with 1% Triton-TBS (3 × 3 min), incubated with streptavidin-peroxidase conjugate prepared according to the manufacturer's instructions for 10 min at room temperature, washed with 1% Triton-TBS (3 × 3 min), and then treated with a freshly prepared aminoethylcarbazole (AEC) (Zymed, San Francisco, CA, USA) chromogen containing 0.02% hydrogen peroxide at room temperature for 15 min. Finally, the sections were counterstained with Mayer's hematoxylin for 3 min at room temperature, rinsed well with tap water and mounted in GVA-mount (Zymed).

The following antibodies were used to determine the identity of cells expressing PAI-1 mRNA or protein: anti-human von Willebrand factor as a marker of endothelial cell; anti-smooth muscle-α actin to identify SMCs; and anti-human macrophage, CD-68 (KP1) to identify macrophages (Dako, Copenhagen, Denmark).

Probes

For in situ hybridization, the probe used was prepared employing a human 1.3-kb PAI-1 cDNA fragment,[34,35] corresponding to the PAI-1 coding region, and subcloned into the pGEM-3Z vector (Promega Biotec, Madison, WI, USA). The vector was linearized with the restriction enzyme EcoRI and used to make an antisense riboprobe labeled with ^{35}S-labeled UTP (specific activity, 1200 Ci/mmol; Amersham International plc, Buckinghamshire, UK) by in vitro transcription using SP6 RNA polymerase (Promega). The DNA template was removed by digestion using RQ1 DNAse for 15 min at 37°C and the riboprobe was purified by phenol extraction and ethanol precipitation. The vector was also used to make a sense control probe following linearization with the restriction enzyme HindIII and in vitro transcription using T7 RNA polymerase.

In situ Hybridization

In situ hybridizations were carried out essentially as described.[33,35] Briefly, prior to hybridization the paraffin sections (3–5 μm) were pretreated sequentially with xylene, 2 × SSC, paraformaldehyde and 1 μg/ml proteinase K (Boehringer Mannheim Biochemica, Mannheim, Germany). The slides were then prehybridized for 1–2 h at 42°C in prehybridization buffer (50% (wt/vol) formamide/0.3M NaCl/20 mM Tris-HCl, pH 8.0/5 mM EDTA/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/10% (wt/vol) dextran sulphate/ 10 mM dithiothreitol). The hybridizations were performed by adding 600 000 cpm of ^{35}S-labeled riboprobe in 20 μl of prehybridization buffer containing 2.5 mg/ml of t-RNA and incubated at 55°C overnight. The sections were washed with 2 × SSC, treated with RNase A (Boehringer Mannheim Biochemica), washed with 2 × SSC and finally washed at 60°C for 2 h in 0.1 × SSC. All the SSC solutions contained 10 mM 2-mercaptoethanol and 1 mM EDTA. The sections were washed in 0.5 × SSC without 2-mercaptoethanol, dehydrated in a graded alcohol series containing 0.3 M ammonium acetate, dried, coated with NTB2 emulsion (Eastman Kodak Co, New Haven, CT, USA) and exposed in the dark at 4°C for 2–10 weeks. Slides were developed, fixed, washed and counterstained with Gill's hematoxylin and eosin (Sigma Chemica Co, St Louis, MO, USA).

RESULTS

Histological examination of the coronary artery segments shows the normal appearing zone with the typical intima (I) separated from the media (M) by the internal elastic lamina (arrows) (Fig. 1, panels A and C). Coronary artery segments with atherosclerotic lesions exhibit the global thickening of intima (I) and, at one side, the atherosclerotic plaque (type III)[32] (Fig. 1, panels B and D, arrowheads).

Representative segments of normal and atherosclerotic areas were processed for in situ hybridization and immunohistochemical analysis. In endothelial cells, PAI-1 mRNA expression is clearly detectable in the altered coronary artery (Fig. 2, panel D), while no signal is present in the normal zones (Fig. 2, panel C). Furthermore, the PAI-1 antigen clearly marks the cellular morphology of the endothelial cells in the atherosclerotic area (Fig. 2, panel B), while only a slight signal is present on the endothelial surface of the control arteries (Fig. 2, panel A, arrow).

The PAI-1 protein and mRNA expression was increased at different stages of atherosclerotic lesions in comparison with the normal area, and this expression apparently depended on the number of cells with a PAI-1 signal present in the area studied.

The pattern of PAI-1 antigen distribution and PAI-1 mRNA expression on the endothelial surface and plaque core from a representative atherosclerotic artery is shown in Figure 3. In the thickened intima, PAI-1 expression is clearly detectable by in situ hybridization using an antisense probe, mainly in the endothelial cell but also in the SMCs in the intima (panel C, arrowheads). Sections were analyzed using a sense probe as control, and no specific signal was detected after 10 weeks of exposure (panel D). In addition, these cells show a prominent specific signal for PAI-1 antigen (panel A). No signal was detected when a non-immune antibody was use as a control (panel B).

Detailed analysis of a representative atherosclerotic plaque is shown in Figure 3 in panels E to H. The PAI-1 antigen is clearly present (panel E) in macrophages (arrows) inside the atherosclerotic plaque. This area is a magnification of Figure 1, panel B. There was no staining in this area when a non-immune antibody was used as a control to assess the specificity of this signal (panel F). The same pattern was observed in the in situ hybridization studies when an antisense probe was used to detect PAI-1 mRNA (panel G). In these atherosclerotic plaques, some (but not all) macrophages have strong PAI-1 mRNA expression. Again, the use of a cRNA sense probe for PAI-1 as control gives no signal after 10 weeks of exposure (panel H). No signal was obtained for either PAI-1 antigen or PAI-1 mRNA in media or adventicia cells.

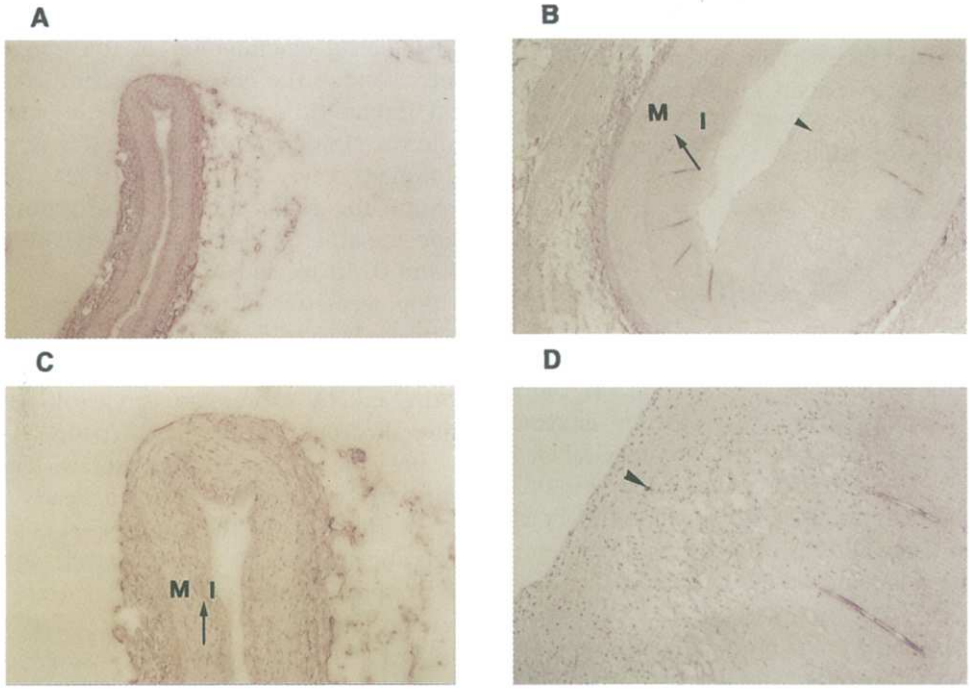

Fig. 1 Morphological differences between atherosclerotic area (panels B, D) and a normal appearing zone (panels A, C) of representative human coronary arteries. Magnification: X50 (panels A, B); X125 (panels C, D). Arrows indicate the internal elastic lamina and arrowheads signal the atherosclerotic plaque. (I) intima. (M) media.

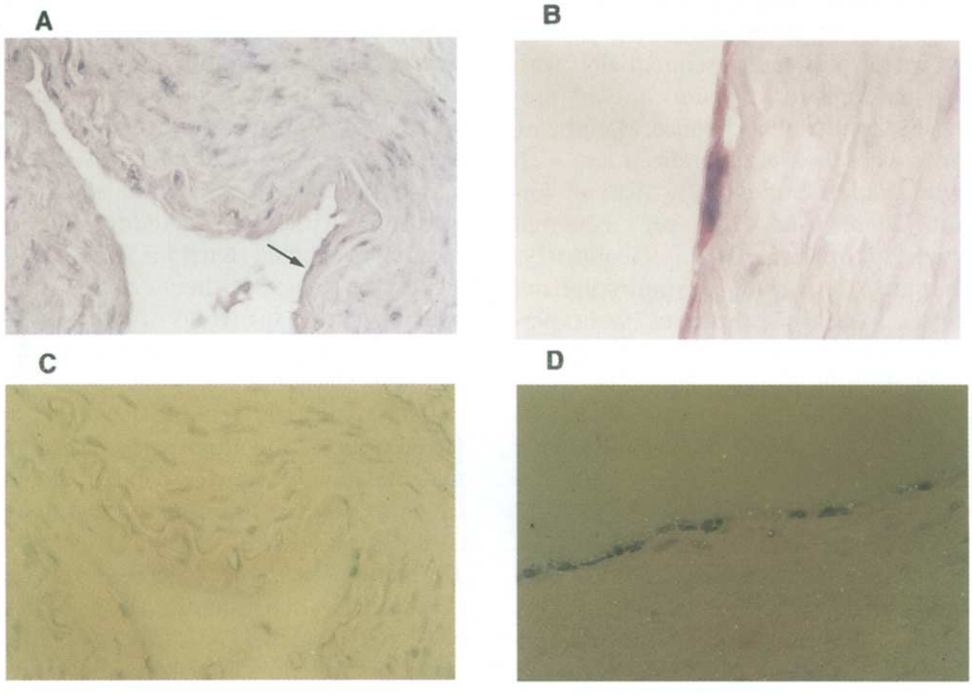

Fig. 2 Immunohistochemistry (panels A, B) and in situ hybridization analysis (panels C, D) of PAI-1 antigen and mRNA expression respectively, on endothelial surface of normal (panels A, C) or atherosclerotic zones of coronary arteries (panels B, D). Magnification: X500 (panels A, C, D); X1250 (panel B). Arrow signals a weak PAI-1 antigen signal on the endothelial surface.

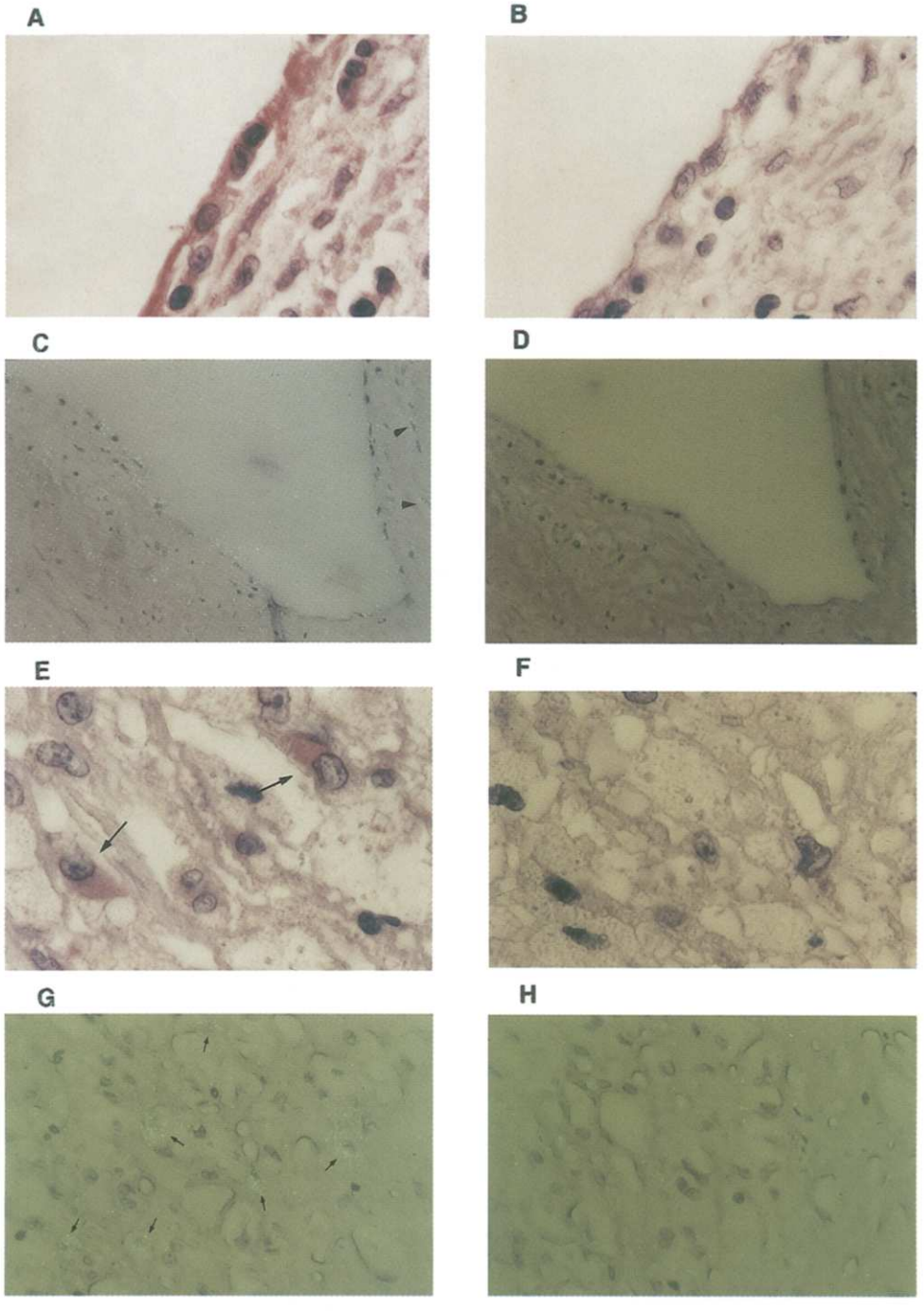

Fig. 3 Immunohistochemistry (panels A, B, E, F) and in situ hybridization analysis (panels C, D, G, H) of PAI-1 antigen and mRNA expression respectively, on endothelial surface (panels A, B, C, D) or atherosclerotic plaque core (panels E, F, G, H) in atherosclerotic areas of human coronary arteries. Arrows in panel G indicate macrophages producing PAI-1 mRNA evidenced as green grains. Arrowheads in panel C show SMCs producing PAI-1 mRNA in the thickened intima. Panels D and H show the result of in situ hybridization when a sense probe for PAI-1 was used as a control. In panel E, arrows signal macrophages positive for PAI-1 antigen stained in red. Panels B and F show the results of immunohistochemistry when a non-immune IgG was used as a control. Magnification: X250 (panels C, D); X500 (panels G, H); X1250 (panels A, B, E, F).

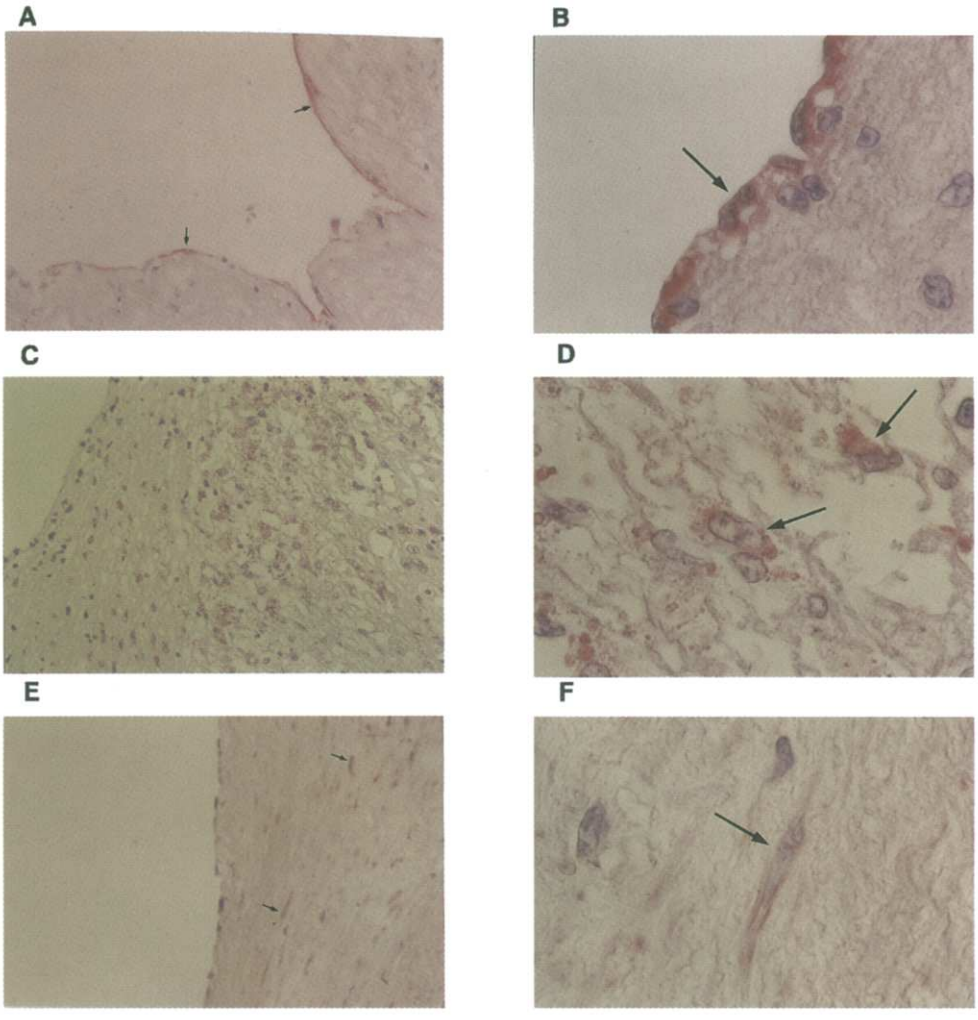

Fig. 4 Histogenetic marker expression in atherosclerotic coronary arteries. Sections were stained with antibodies to identify ECs (anti-human von Willebrand factor) (panels A, B), macrophages (CD68) (panels C, D) and SMCs (anti-human smooth muscle actin, panels E, D). Arrows signal the positive cells for each histogenetic marker. Magnification: X250 (panels A, C, E); X1250 (panels B, D, F).

Serial sections adjacent to those used for PAI-1 detection were processed for immunohistochemical identification of endothelial cells, macrophages and SMCs (Fig. 4). Figure 4 shows the positive staining for von Willebrand factor in endothelial cells (panels A and B). The signal is colocalized with the PAI-1 expression (Fig. 3, panel A). Moreover, the cell-rich areas in which the PAI-1 signal is observed in Figure 3 (panels E and G) also stained intensively for CD68-positive macrophages (Fig. 4, panels C and D). Panels E and F show the positive staining for smooth muscle α actin.

DISCUSSION

The vascular expression of PAI-1 has been extensively studied both in vitro and in vivo.[34,35] Some cellular lines, like those derived from endothelial cell, SMC and fibroblasts, are known to be prone to increase the PAI-1 synthesis levels in culture by cytokine induction.[1,4] On the other hand, atherogenesis and plaque disruption are complex processes which involve many cellular types and inflammatory events,[32,36] while PAI-1 appears to be a key protein, both in the control of cell migration and for its role in fibrin degradation.[5] For this reason, several reports describing PAI-1 synthesis and antigen localization in human atherosclerotic arteries have been published,[25,26,28–31,37,38] although only one of them[29] deals with coronary arteries and it only studies two samples. Moreover, in these reports there is some contradictory information about the cell types involved in PAI-1 expression, PAI-1 antigen localization and, finally, the differences observed between normal and pathological arteries. These contradictions could be a consequence of the difficulty involved in obtaining a good series of specimens. On the other hand, the heterogenous materials compacted inside the necrotic core in some

plaques are prone to develop high background levels, especially in immunohistochemical analysis or in situ hybridization when an enzymatic development step is used.

We studied human coronary arteries from 16 patients subjected to heart transplant, using pathological and normal appearing zones from the same patients separately. Although biochemical changes could also be responsible for an altered PAI-1 expression in artery zones that appear normal, the difference between the PAI-1 expression of the normal and pathological samples in our study rules out this possibility.

Our results show that endothelial synthesis of PAI-1 mRNA increases in human atherosclerotic coronary arteries. These results contrast with previous reports that PAI-1 mRNA was not detected in endothelial cells from atherosclerotic coronary arteries.[29]

PAI-1 is synthesized not only by endothelial cells but also by SMCs in arteries with fibrointimal proliferation, while in the core of the atherosclerotic plaque PAI-1 is produced mainly by macrophages. Detailed analysis of the atherosclerotic plaque revealed a prominent PAI-1 antigen signal in macrophages (Fig. 3, panel E). These results are confirmed by in situ hybridization (Fig. 3, panel G). In our study, SMCs were not, in general, responsible for PAI-1 synthesis in the core of atherosclerotic plaque, although a few of them seem to produce a detectable signal (data not show). In contrast with previous reports,[31] no PAI-1 mRNA signal was detected in medial and adventicia layers below the plaque.

The role of macrophages in atherosclerosis has been clearly demonstrated,[39] and the fibrinolytic system appears to have to do with the entry of monocyte/macrophage into the evolving plaque and the re-cycling of macrophages from the plaque during regression.[40]

It is well known that PAI-1 expression is highly inducible by TNFα and TGFβ,[3,4,34,35] and there are also reports on cellular growth from human vascular lesions being stimulated by TGFβ1.[41] Interestingly, the pattern of PAI-1 expression described here in atherosclerotic lesions coincides with that described for βig-h3,[42] a TGFβ-inducible gene. This raises the possibility that PAI-1 expression in these cells can be up-regulated by TGFβ.

The locally increased production of PAI-1 mRNA detected in the atherosclerotic plaque can contribute to intramural lesions in advanced aged plaques, and may be determinant in the triggering of the thrombotic events subsequent to plaque disruption in collaboration with procoagulant factors like tissue factor.[43] However, PAI-1 overexpression can also play an important role in the developing of the atherosclerotic plaque[28] by promoting cellular migration and infiltration, or in the process of plaque disruption by controlling the proteolytic activities in the extracellular matrix.[26]

In conclusion, there is a strong PAI-1 mRNA expression associated with endothelial cells and SMCs in connective tissue of thickened intima. In the core of atherosclerotic lesions in human coronary arteries, macrophages are the primary cells producing PAI-1 mRNA. This PAI-1 expression can be related to the development, evolution and final outcome of the atherosclerotic plaque. Further research on the agents and mechanisms controlling the expression of PAI-1 in the coronary atherosclerotic lesions could provide new therapeutic approaches to control the evolution of coronary artery disease.

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