

Expression of Fibrinolytic Genes in Atherosclerotic Abdominal Aortic Aneurysm Wall

A Possible Mechanism for Aneurysm Expansion

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Abstract

Expansion of atherosclerotic abdominal aortic aneurysm (AAA) has been attributed to remodeling of the extracellular matrix by active proteolysis. We used *in situ* hybridization to analyze the expression of fibrinolytic genes in aneurysm wall from eight AAA patients. All specimens exhibited specific areas of inflammatory infiltrates with macrophage-like cells expressing urokinase-type plasminogen activator (u-PA) and tissue-type PA (t-PA) mRNA. Type 1 PA inhibitor (PAI-1) mRNA was expressed at the base of the necrotic atheroma of all specimens and also within some of the inflammatory infiltrates where it frequently colocalized in regions containing u-PA and t-PA mRNA expressing cells. However, in these areas, the cellular distribution of the transcripts for t-PA and u-PA extended far beyond the areas of PAI-1 expression. These observations suggest a local ongoing proteolytic process, one which is only partially counteracted by the more restricted expression of PAI-1 mRNA. An abundance of capillaries was also obvious in all inflammatory infiltrates and may reflect local angiogenesis in response to active pericellular fibrinolysis. The increased fibrinolytic capacity in AAA wall may promote angiogenesis and contribute to local proteolytic degradation of the aortic wall leading to physical weakening and active expansion of the aneurysm. (*J. Clin. Invest.* 1995. 96:639–645.) **Key words:** abdominal aortic aneurysm • angiogenesis • plasminogen activator • plasminogen activator inhibitor • *in situ* hybridization

Introduction

Atherosclerosis is considered to be a chronic inflammatory process with characteristic macrophage and lymphocyte infiltrates

in the vessel wall (for review see references 1 and 2). These infiltrates have been demonstrated in aortic wall of atherosclerotic aneurysm as well as in the wall of nondilated aortas affected by occlusive disease and are considered secondary to cell-mediated immune responses (3). Macrophages serve as antigen presenting cells to T lymphocytes and also synthesize growth factors and cytokines, which in turn, may be expected to regulate gene expression in the vessel wall (4).

The genesis of atherosclerotic aortic aneurysm is associated with remodeling of extracellular matrix (ECM),¹ including breakdown of structural components of the vascular wall (5). This change may reflect increased collagenolytic and elastolytic activity in atherosclerotic aneurysm wall (6, 7). For example, type IV collagenase mRNA was detected in cells surrounding vasa vasorum within the plaque (8), and macrophage elastase may induce aortic aneurysm in rats (9). Furthermore, enlargement of elastase-induced aortic aneurysm was shown to correlate with the presence of inflammatory infiltrates within the aortic wall (10). These observations suggest that the enzymes required for ECM degradation are synthesized and subsequently activated *in situ* within the aneurysm wall.

Although the fibrinolytic system may also play a role in the process of ECM remodeling, little is known about its expression in the vessel wall. In this system, two plasminogen activators (PAs), tissue-type (t-PA) and urokinase-type (u-PA), generate plasmin from the inactive proenzyme plasminogen. Plasmin is a trypsin-like proteolytic enzyme capable of directly degrading some components of the ECM and may initiate the degradation of others by activating metalloproteinases (11, 12). The activity of both PAs is modulated by PA inhibitor type 1 (PAI-1), a serine protease inhibitor, which circulates in complex with vitronectin and is stabilized through this interaction (13, 14). Vitronectin also has been detected in the atherosclerotic vessel wall (15, 16), raising the possibility that PAI-1 antigen may be bound to vitronectin in the diseased vessel wall at sites distal from its sites of synthesis. Early studies demonstrated fibrinolytic activity in atherosclerotic human aorta (17) and recent Northern blotting analyses have demonstrated increased t-PA and u-PA gene expression in aortic aneurysm wall compared with nondilated atherosclerotic aortic wall and normal

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1. *Abbreviations used in this paper:* AAA, abdominal aortic aneurysm; ECM, extracellular matrix; PA, plasminogen activator; PAI-1, type 1 plasminogen activator inhibitor; t-PA, tissue-type PA; u-PA, urokinase-type PA.

aorta (18). In addition, t-PA (19) and u-PA (18) immunoreactivities were shown to reside within the aneurysm wall, indicating that the transcripts of both genes are efficiently translated. Recently, plasmin immunoreactivity was demonstrated in abdominal aortic aneurysm (AAA) extracts as well (20). These data suggest that the fibrinolytic balance in the aneurysmatic vessel wall is disturbed, an abnormality that may lead to increased plasmin generation, activation of metalloproteinases, and structural deterioration of the vascular wall.

In spite of the potential importance of these observations, little is known about the mechanism(s) responsible for the increase in PAs, and the identity of the cells producing these enzymes remains to be determined. In this report, we used *in situ* hybridization to detect the expression of the major physiologic components of the fibrinolytic system in the normal vessel wall and in the wall of the atherosclerotic AAA. These studies not only identified specific areas of t-PA and u-PA synthesis which may result in local PA predominance, but also colocalized these sites with regions of intense inflammatory infiltration and angiogenesis. These results suggest a linkage between inflammation, enhanced fibrinolysis, atherosclerotic plaque neovascularization, and potential breakdown of the vessel wall.

Methods

Study population, tissue selection, and preparation

Specimens of vascular tissue were obtained with informed consent from eight consecutive patients undergoing elective resection of AAA. All patients presented with sizable aneurysm exceeding 6 cm in diameter. None of the patients suffered from rupture of AAA at the time of surgery. Segments of aneurysm wall were analyzed macroscopically and in each case were observed to be involved with severe atherosclerosis. Normal appearing aortic wall samples were collected from three young brain-dead organ donors (ages 22, 24, and 29 yr). None of these samples exhibited clinical or pathological evidence of atherosclerosis. Specimens were immediately fixed in 10% formalin at room temperature for 24 h, embedded in paraffin, and cut into 3–5 μm sections.

In situ hybridization

In situ hybridization was performed as described previously (21) using ^{35}S -labeled riboprobes. Both sense and antisense probes were routinely labeled to specific activities between 0.5 and 2×10^8 cpm/ μg RNA. In all cases, the exposure time for t-PA, u-PA, and PAI-1 was 6 wk. The total surface area of each slide was scanned for positive cells by using both regular light microscopy and epifluorescence at magnifications ranging from 40- to 800-fold. Although there was some variation in the signal intensity between different areas of the same specimen, as well as among the eight vessels studied, at least 50% of the inflammatory infiltrates exhibited a positive signal. The following cRNA probes were used.

PAI-1. A 1.3-kb human PAI-1 cDNA fragment (22) was subcloned into pGEM-3 (Promega, Madison, WI). An antisense probe was synthesized with SP6 RNA polymerase after linearization of the template with *Stu*I, whereas a sense probe was synthesized with T7 RNA polymerase after linearization with *Hind*III.

t-PA. A 360-bp human t-PA cDNA fragment (23) (kindly provided by Dr. T. Ny, University of Umea, Sweden) was subcloned into pGEM-1 (Promega), linearized with *Eco*RI, and transcribed with SP6 RNA polymerase for antisense probe, or linearized with *Hind*III and transcribed with T7 RNA polymerase for a sense probe.

u-PA. A 1.2-kb human u-PA cDNA *Pst*I/*Bam*HI fragment (57329; American Type Culture Collection, Rockville, MD) was subcloned into pSP73 (Promega), linearized with *Hind*III, and transcribed with SP6 RNA polymerase for an antisense probe, or linearized with *Eco*RI, and transcribed with T7 RNA polymerase for a sense probe.

Immunohistochemistry

Immunohistochemistry was performed essentially as described (21, 24). Briefly, endogenous peroxidase was quenched by treating formaldehyde-fixed, paraffin-embedded tissue sections (3–5 μm) with 1% H_2O_2 . The slides were then treated with pronase (Calbiochem-Novabiochem, La Jolla, CA) to expose masked epitopes and with 5% normal horse serum to block nonspecific protein binding sites. The slides were incubated with antibodies to H endothelial antigen (clone BNH9; Amac, Inc., Westbrook, ME) at a dilution of 1:30 for 60 min. The slides were rinsed, incubated with biotinylated horse anti-mouse IgG, followed by avidin-peroxidase complex, and then developed with DAB chromogen. Finally, the slides were counterstained with Gills hematoxylin (Richard Allen Medical, Richland, MI). Parallel slides were analyzed with antibodies to vWF. Lymphocytes were recognized by their small size, smooth, round and basophilic nucleus, and minimal cytoplasm. Macrophages were identified by their large size and cytoplasmic volume, and by the presence of less basophilic nuclei.

Unfortunately, the formaldehyde-fixed and paraffin-embedded tissues were not especially useful for detecting either residual enzymatic activities (e.g., PAs, plasmin, metalloproteinases) or u-PA and t-PA antigens. In separate studies, we showed that treatment of fresh human vascular tissue (iliacal arteries and veins) with 10% formaldehyde destroyed the u-PA and t-PA antigens recognized by antibodies 3639 for u-PA and ESP-4 for t-PA (kindly provided by R. Hart, American Diagnostica Inc., Greenwich, CT).

Results

All atherosclerotic specimens presented inflammatory infiltrates within the thickened intima, media, and adventitia (Fig. 1 A). The infiltrating cells were morphologically identified as lymphocytes and macrophages as described in Methods. The aortic wall at these sites of inflammation was abundant in vascular capillaries as revealed by staining for H endothelial antigen (Fig. 1 B), an endothelial cell marker, and some of the capillaries were filled with red blood cells. In contrast, regions of noninflamed tissue from atherosclerotic specimens were relatively devoid of capillaries (Fig. 1 C, compare left side of photo with right side). Quantitation of the immunohistochemical results for H endothelial antigen revealed that 16–22 capillaries were present in each microscopic field of inflammatory infiltrate compared with 0–2 capillaries in noninfiltrated areas ($\times 400$; 15 fields each analyzed). Except for the adventitial layer where numerous arterioles were visualized (not shown), normal aortas lacked both areas of inflammatory infiltrates and areas of increased vascularity (Fig. 1 D). Similar results were obtained using tissue sections stained for vWF, another endothelial cell marker protein (not shown).

The expression of fibrinolytic genes in normal and diseased vascular tissue was analyzed by *in situ* hybridization. Although a strong signal for u-PA mRNA was present in macrophage-like cells in subintimal inflammatory infiltrates in all samples, it was especially prominent in six out of eight specimens (Fig. 2, A–C). In contrast, the noninfiltrated segments were relatively devoid of u-PA signal (Fig. 2 B, compare area of inflammatory cells in upper right with areas devoid of inflammatory cells in lower left), and u-PA mRNA was not detectable in normal aorta (Fig. 2 D). The u-PA transcript was also present in isolated inflammatory cells scattered in the thickened intima and thinned media (not shown). Arteriolar luminal and capillary endothelial cells within the infiltrate (Fig. 2 B), as well as lymphocytes identified by their condensed, basophilic nucleus and minimal cytoplasm, appear to be devoid of u-PA mRNA signal (Fig. 2 C).

Interestingly, t-PA mRNA also was readily detectable in all

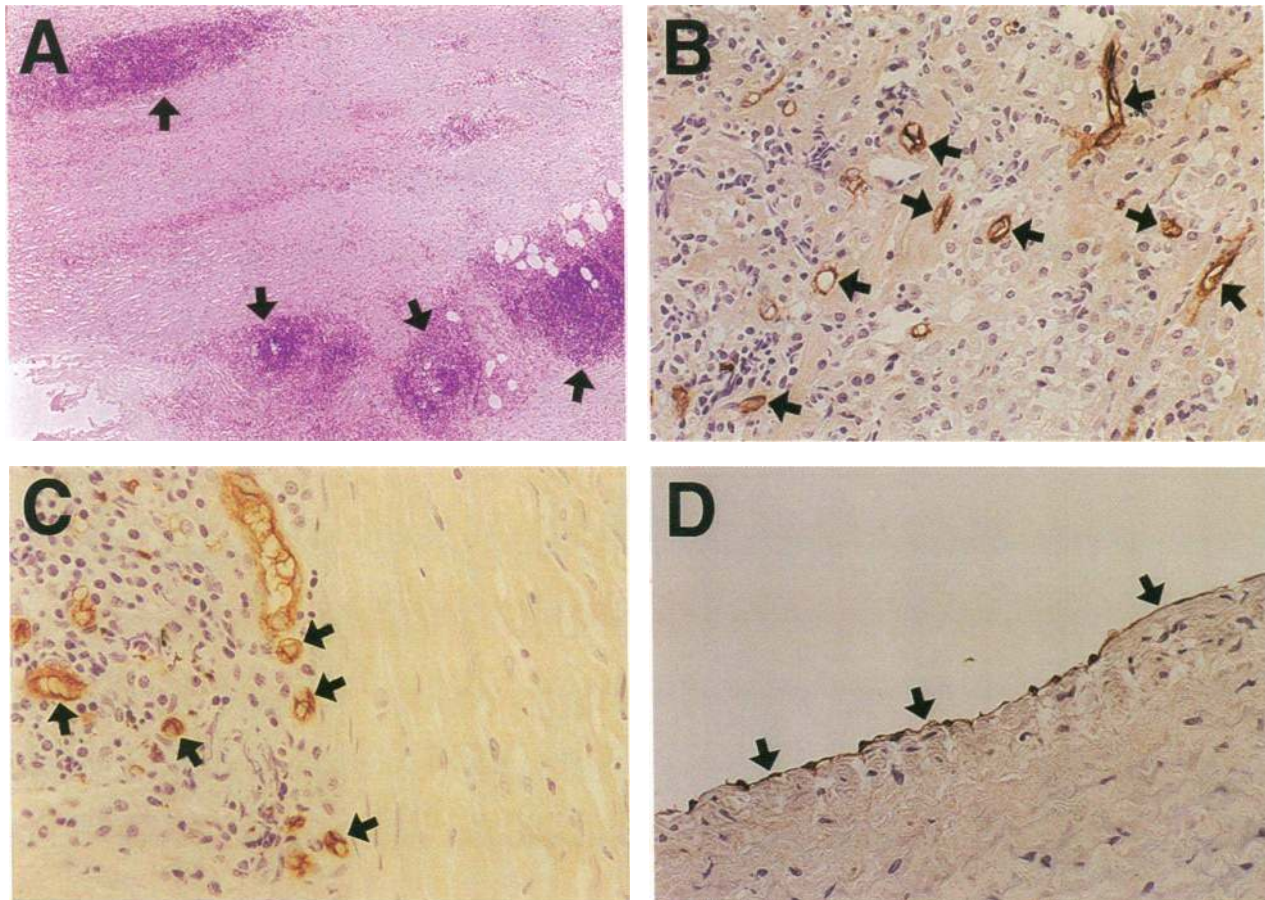


Figure 1. Neovascularization in inflammatory infiltrates of the AAA wall. Atherosclerotic vessel wall specimens from AAA and apparently normal aortas were analyzed by hematoxylin and eosin staining and immunohistochemistry for endothelial H antigen. (A) Atherosclerotic aneurysm wall of the abdominal aorta shown at relatively low power, $\times 100$. Multiple foci of inflammatory infiltrates are visualized within the vessel wall (arrows). Hematoxylin and eosin staining. (B) Atherosclerotic aneurysm wall. An inflammatory infiltrate composed of macrophages and lymphocytes and containing multiple capillaries (arrows). Staining for H antigen, $\times 400$. (C) Atherosclerotic aneurysm wall. Transition between inflammatory infiltrate and noninfiltrated vessel wall. Note that tissue beyond the inflammatory infiltrate is devoid of small vessels (right) (H antigen), $\times 400$. (D) Normal aorta. Intimal endothelium (arrows) stained for endothelial H antigen. Note absence of capillaries within the wall, $\times 400$.

specimens with an especially strong hybridization signal being apparent in six out of eight atherosclerotic aortic wall samples. Again, the signal was localized to infiltrating cells morphologically resembling macrophages (Fig. 3, A–C). Cells resembling lymphocytes demonstrated no positive t-PA signal (Fig. 3 C). Although t-PA mRNA expression was evident in areas of u-PA expression, t-PA positive cells also were found beyond areas of u-PA expression within the infiltrate, in areas of medial destruction (not shown). In the normal aorta, a weak t-PA signal was detected but only in luminal endothelial cells (Fig. 3 D).

As reported previously (25, 26), the PAI-1 gene was expressed in subintimal macrophage-like or mesenchymal-like cells at the base of the necrotic atheroma in all specimens (Fig. 4 A). In addition, a few cells in the necrotic core (not shown), and in macrophage-like or mesenchymal-like cells within and at the periphery of the inflammatory infiltrates, also stained for PAI-1 (Fig. 4 B) (detected in six out of eight specimens). Within some of the inflammatory infiltrates, the PAI-1 transcript was detected in cells in close proximity to u-PA and t-PA mRNA expressing cells (not shown). However, it should be noted that u-PA and t-PA expression within cells of the inflammatory infiltrates extended beyond the areas of colocalization with PAI-1 mRNA positive cells. In contrast, the base of

the necrotic core lacked u-PA or t-PA mRNA, whereas the PAI-1 transcript was easily detectable. Some of the cells expressing PAI-1 mRNA in areas containing the inflammatory infiltrates were arranged in a ring-like manner, suggestive of capillary endothelial cells (Fig. 4 C). In addition, scattered subintimal macrophage- and mesenchymal-like cells (assumed to be smooth muscle cells) expressed the PAI-1 transcript (not shown). A positive PAI-1 signal was also present in luminal endothelial cells of adventitial vessels (not shown). In the normal aorta, luminal endothelial and a few subintimal cells expressed PAI-1 mRNA (Fig. 4 D). Control hybridizations using sense u-PA, PAI-1, and t-PA riboprobes were uniformly negative (not shown).

Discussion

In this study, we have demonstrated an abundance of vascular capillaries specifically confined to areas of inflammatory infiltrates within the aneurysm wall (Fig. 1). This vascular proliferation was minimal in areas devoid of inflammation within the same specimens and was absent in the normal aortic wall. These findings corroborate previous demonstrations of neovascularization in atherosclerotic plaques from nondilated vessels (27, 28).

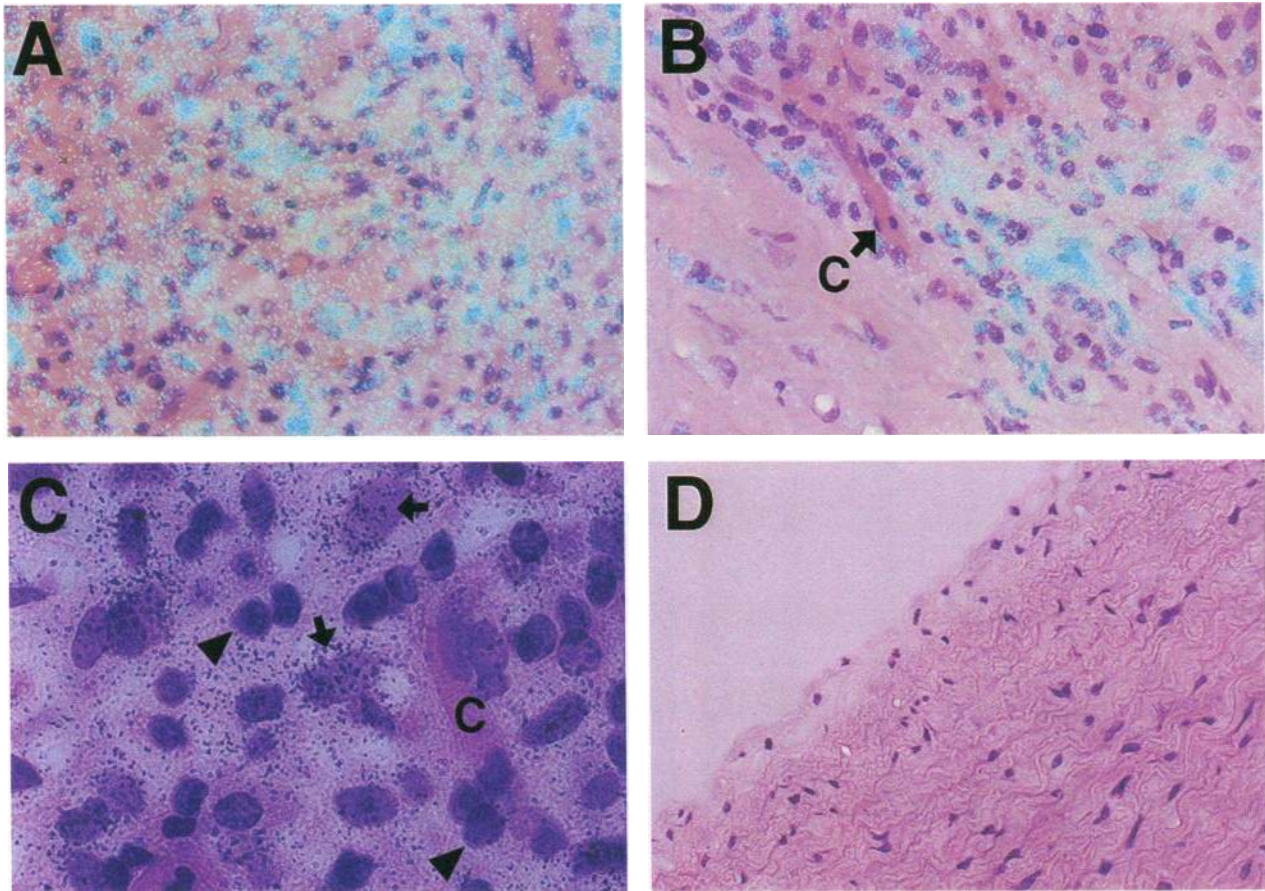


Figure 2. Expression of u-PA mRNA in AAA wall. Atherosclerotic aneurysm wall and normal aortas were analyzed for u-PA gene expression by in situ hybridization (see Methods). (A) Atherosclerotic aneurysm wall. Cells within the infiltrate express u-PA transcript, $\times 400$, epiluminescence. (B) Atherosclerotic aneurysm wall. Transition between inflamed (upper right) and noninflamed (lower left) area. The u-PA transcript is primarily restricted to cells within the infiltrate, $\times 400$, epiluminescence. Note capillary (C, arrow) within the infiltrate. (C) Atherosclerotic aneurysm wall. Large cells resembling macrophages (arrows) within the inflammatory infiltrate are expressing u-PA mRNA (black dots). Note the negative lymphocytes (arrowheads) and capillary (C), $\times 1,000$, bright-field. (D) Normal aorta. The u-PA transcript was undetectable throughout the vessel wall, $\times 400$, epiluminescence.

The degree of plaque neovascularization was correlated previously with the severity of atherosclerosis (29), and inflammatory infiltrates are most abundant in aortic aneurysm wall (3). Since the infrarenal abdominal aorta lacks vasa vasorum (30), the presence of multiple capillaries is obviously related to the disease process. The development of newly formed vasa vasorum in conjunction with peripheral atherosclerosis is well documented (28, 31), and the potential grave clinical consequences of these changes have been appreciated. For example, neovascular capillaries are fragile and may promote intimal hemorrhage and subsequent rupture of the atheroma (32–34). This scenario is also supported by studies correlating macrophage density in human atherosclerotic aorta with the tendency for plaque rupture (35).

The fibrinolytic system has been implicated in the process of angiogenesis (36), and in this report we additionally demonstrate elevated expression of u-PA (Fig. 2), t-PA (Fig. 3), and PAI-1 (Fig. 4) mRNAs in cells also present within the inflammatory infiltrates. The cells expressing these fibrinolytic genes were in close proximity to the numerous capillaries present in the infiltrates, suggesting an active process of pericellular proteolysis which could promote neovascularization. This concept is supported by in vitro studies, which demonstrate activa-

tion of the cellular fibrinolytic system by angiogenic factors (37, 38) and by substances released from atherosclerotic carotid plaques (39) and tumor fragments (40). Moreover, studies of the murine reproductive system (41) revealed the expression of u-PA mRNA in proliferating capillary sprouts, and PAI-1 mRNA expressing cells were detected in close proximity to the cells expressing u-PA. These latter observations support our findings of partial colocalization of PAs and PAI-1 mRNA within the boundaries of the inflammatory infiltrates and suggest an active interplay between these two opposing activities during angiogenesis. Although the actual signals that mediate the increased expression of fibrinolytic genes in the atherosclerotic vessel await elucidation, both $\text{TNF}\alpha$ (42, 43) and basic fibroblast growth factor (44) have been detected in the atherosclerotic vessel wall and both stimulate PA and PAI-1 gene expression by vascular endothelial and smooth muscle cells.

The abnormally high level of PA gene expression (i.e., t-PA [Fig. 3], u-PA [Fig. 2], or both mRNAs) within the inflammatory infiltrates of the aneurysm wall of all eight specimens is particularly significant since elevated t-PA or u-PA may result in increased local plasmin generation. In these studies, six samples exhibited a strong signal for both u-PA and t-PA, while the other two samples demonstrated intense expression

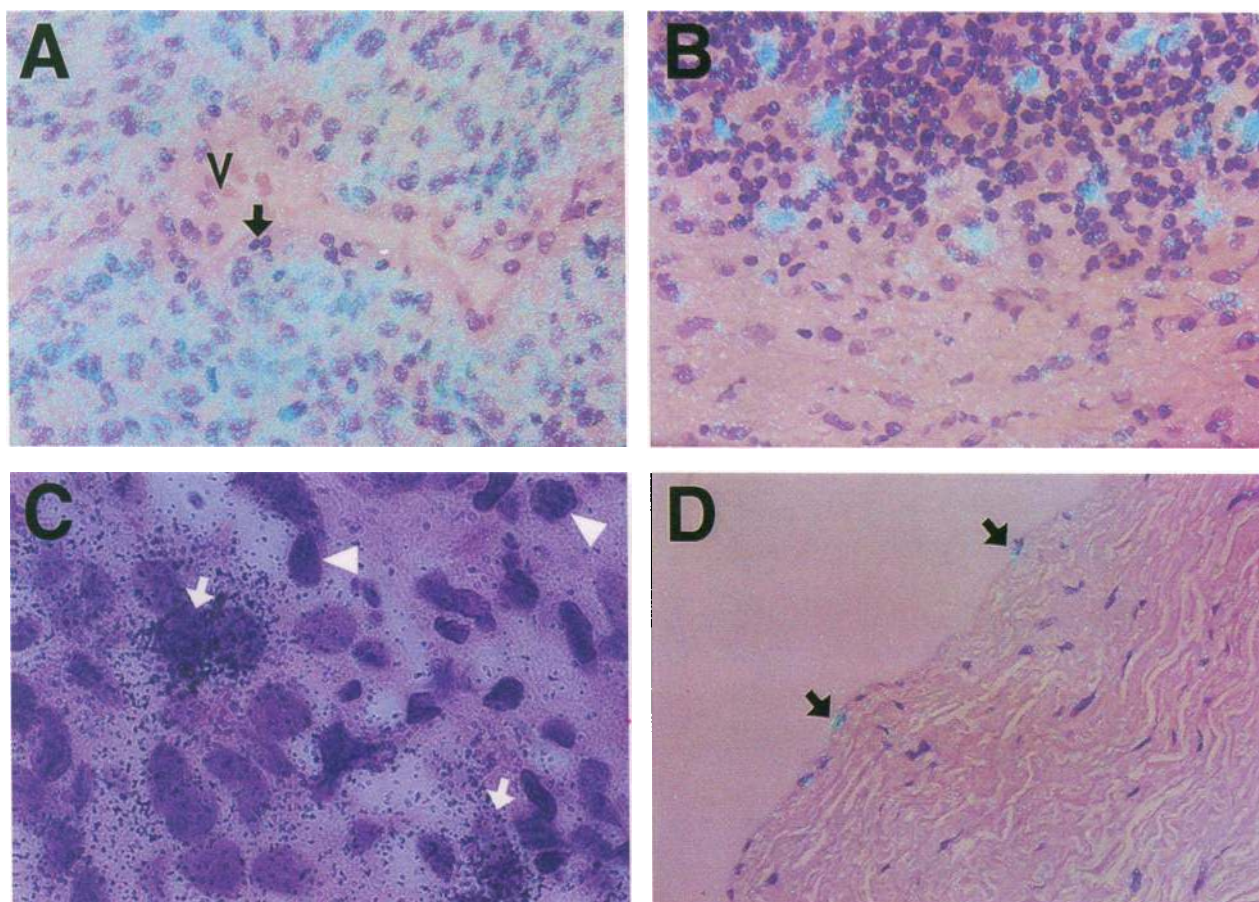


Figure 3. Detection of t-PA mRNA in AAA wall. Vascular specimens were analyzed for t-PA gene expression by in situ hybridization as in Fig. 2. (A) Atherosclerotic aneurysm wall. Cells in the inflammatory infiltrate express t-PA mRNA. Note negative endothelial (arrow) and vessel (V), $\times 400$, epiluminescence. (B) Atherosclerotic aneurysm wall. Transition from infiltrated (upper section) to noninfiltrated (lower section) vessel wall. Note that the tissue beyond the infiltrate is relatively devoid of t-PA signal, $\times 400$, epiluminescence. (C) Atherosclerotic aneurysm wall. Large cells resembling macrophages (arrows) within the inflammatory infiltrate are expressing t-PA mRNA (black dots). Note the negative lymphocytes (arrowheads), $\times 1,000$, bright-field. (D) Normal aorta. The t-PA transcript was only detected in luminal endothelial cells (arrows), $\times 400$, epiluminescence.

of either u-PA or t-PA. In the latter two cases, the level of expression of the second PA was clearly above background, but not as intense as in the other six samples. This variation may be related to different stages of the disease process. In this regard, there also was a wide range of variation in PA mRNA expression by cells within the intimal and medial inflammatory infiltrates within the same specimen. However, at least 50% of the infiltrates revealed significant expression of fibrinolytic genes. These variations may be attributed to differences in cellular composition of the inflammatory infiltrates.

In spite of the dramatic increase in t-PA and u-PA mRNAs, we were unable to detect plasmin activity in our samples because of the fixation process used. However, plasmin probably was generated since plasmin activity and antigen (20) were detected previously by other investigators in extracts of unfixed aneurysmal wall. We also were unable to consistently detect t-PA or u-PA antigens in our samples using immunohistochemical approaches (our unpublished observations). Again, we believe that this failure was due to the destruction of the epitopes recognized by our antibodies. This conclusion is based on the observation that 10% formalin fixation destroyed the immunoreactivity of t-PA and u-PA detected in other vascular samples (our unpublished observation). It is unlikely that the t-PA and

u-PA mRNAs were not translated since elevated levels of t-PA and u-PA antigens were detected in extracts or frozen sections of aneurysmal wall (18). In any case, increased expression of the PA system may induce secondary increases in local metalloproteinase activity (45), which may collectively weaken the vessel wall. This concept is consistent with the detection of stromelysin in extracts of AAA (46), and of type IV collagenase mRNA in cells surrounding vasa vasorum in the aortic aneurysm wall (8). Thus, the highly localized activation of the fibrinolytic system may facilitate regional angiogenesis and may also promote a more diffuse proteolytic state, adversely affecting the integrity of aneurysm wall.

The main cellular components of the inflammatory infiltrates within the atherosclerotic wall are macrophages and T lymphocytes (1, 3). We demonstrated a strong signal for u-PA, t-PA, and PAI-1 mRNA in macrophage-like cells in the inflammatory infiltrates, consistent with the documented synthetic potential of macrophages (47). Previous studies of PAs in normal human vessels revealed the presence of t-PA within the luminal endothelium, whereas u-PA was not detected (48). In vitro studies demonstrate that actively proliferating endothelial cells elaborate u-PA during angiogenesis and that u-PA expression by these cells in vitro ceases after the initial angiogenic response

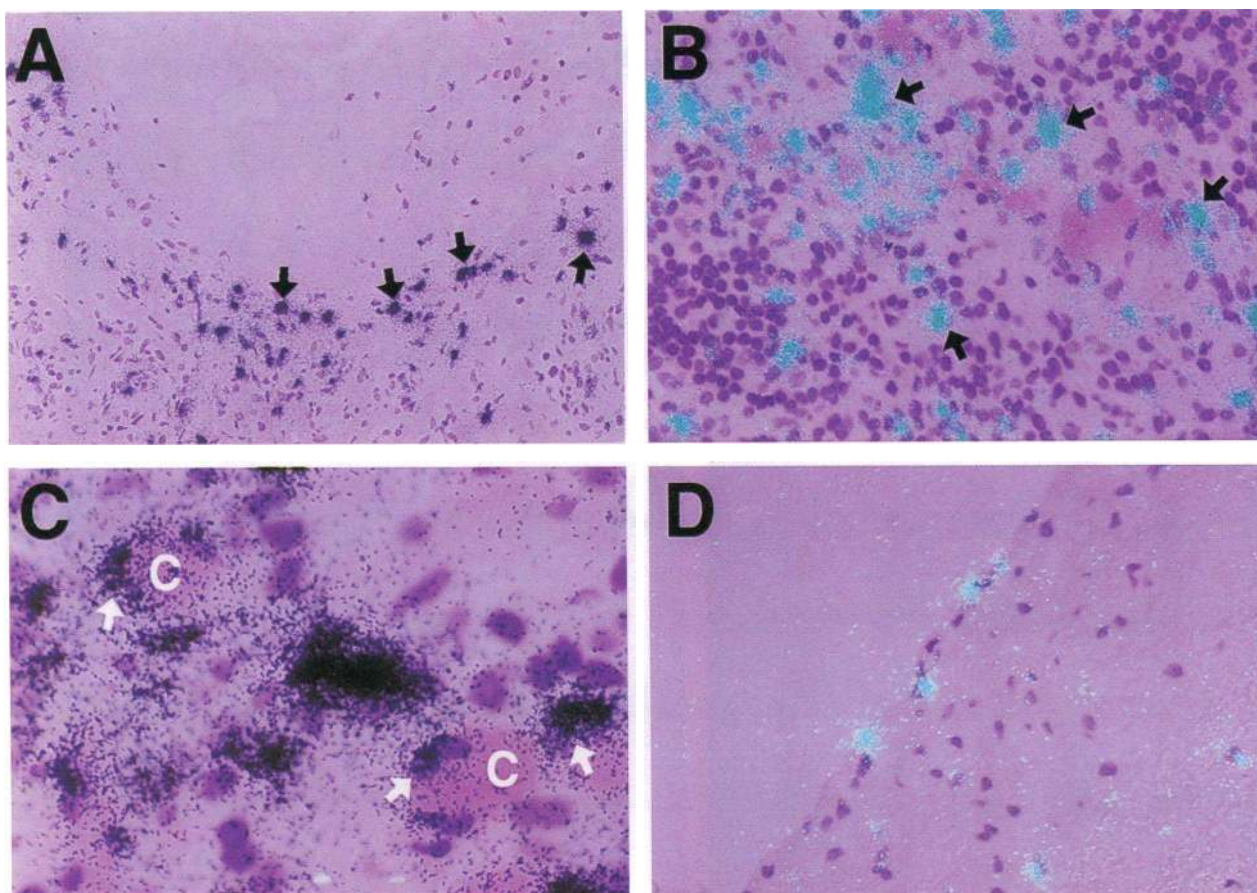


Figure 4. PAI-1 gene expression in the AAA wall. Tissue samples were analyzed for the expression of PAI-1 mRNA by in situ hybridization (see Methods). (A) Atherosclerotic aneurysm wall. The PAI-1 transcript is expressed in cells (arrows) aligned at the base of the necrotic atheroma core, $\times 200$, bright-field. (B) Atherosclerotic aneurysm wall. Macrophage-like cells expressing PAI-1 mRNA (arrows) within the inflammatory infiltrate, $\times 400$, epiluminescence. (C) Atherosclerotic aneurysm wall. PAI-1 mRNA expression in circumferentially arranged cells (arrows) which depict cross-section of ring-like structures, assumed to be small caliber capillaries (C) within an inflammatory infiltrate, $\times 1,000$, bright-field. (D) Normal aorta. PAI-1 mRNA signal is detected in luminal endothelium and in a few subintimal cells, $\times 400$, epiluminescence.

(41). Our failure to detect u-PA mRNA in endothelial cells in vivo (Fig. 2) is not inconsistent with this observation since the likelihood of identifying the replicating cells in any blood vessel is extremely small in cross-sectional analysis. Thus, most of the endothelial cells detected in vivo are part of established blood vessels and thus should be chronologically beyond the period of active angiogenesis.

We (25) and others (26) previously reported an abundance of PAI-1 mRNA positive cells mainly around the base of the atherosclerotic plaque in both nondilated and aneurysmal aortic wall of patients undergoing reconstructive vascular surgery. In these instances, PAI-1 mRNA was frequently detected in areas devoid of inflammatory cells. The level of PAI-1 mRNA and the degree of aortic wall atherosclerosis were positively correlated, suggesting that local decreases in fibrinolytic capacity may accelerate atherogenesis by facilitating thrombosis and fibrin deposition (25, 26). Comparison of the sites of PAI-1 biosynthesis with sites of PAI-1 deposition is important since this inhibitor interacts with vitronectin (13) and vitronectin is present in the diseased vessel wall (15, 16). This consideration raises the possibility that PAI-1 may accumulate in the diseased vessel wall at sites distinct from those of biosynthesis. This possibility is under investigation. It should be noted that the base of the plaque was practically devoid of PA-expressing cells, consistent

with the idea that these sites are in fact prothrombotic. In contrast, the dramatic upregulation of fibrinolysis at specific sites of inflammatory infiltrates in AAA wall may result in a highly localized process of plasmin generation and proteolysis, facilitating both neovascularization and local degradation of ECM, and promoting aortic aneurysm expansion.

In summary, the vascular wall of the aortic atherosclerotic aneurysm seems to host an uneven distribution and imbalanced expression of the various components of the fibrinolytic system, causing major pathophysiological sequelae. Areas of increased thrombogenicity, like the base of the plaque, coexist and border discrete areas of inflammatory infiltrates that were demonstrated to harbor proteolytic predominance. It is reasonable to hypothesize that sites of increased proteolytic activity may contribute to localized neovascularization and promote the rapid breakdown of ECM components which result in mural weakening, inevitable expansion, and eventual rupture of untreated aortic aneurysm.

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