Potentiation by Hypercholesterolemia of the Induction of Aortic Intramural Synthesis of Plasminogen Activator Inhibitor Type 1 by Endothelial Injury

Hirofumi Sawa, Burton E. Sobel, Satoshi Fujii

Accumulation of plasminogen activator inhibitor type 1 (PAI-1) in the arterial wall may accelerate atherogenesis by inhibiting fibrinolysis, diminishing proteolysis of extracellular matrix proteins, or modifying migration of vascular smooth muscle cells. Increased intramural expression of the PAI-1 gene is induced by thrombosis. To determine whether it occurs also in response to a sustained mechanical insult to endothelium, hypercholesterolemia, or both, rabbits were subjected to sustained aortic injury induced by implantation of indwelling polyethylene tubing, to hyperlipidemia induced by cholesterol and peanut oil feeding over a period of 8 weeks, or both. Sustained vascular injury alone did not increase plasma PAI-1. However, hypercholesterolemia with or without mechanically induced vascular injury increased plasma PAI-1 twofold. The expression of PAI-1 mRNA in aorta (Northern blots) was significantly increased when vascular injury was combined with hyperlipidemia. In situ hybridization showed that the increase with mechanical injury alone occurred in endothelial cells covering the neointima (positive for factor VIII and thrombomodulin), in abnormally differentiated vascular smooth muscle cells (positive for embryonic myosin heavy chain), and in macrophages (positive for the RAM-11 anti-macrophage antibody). Qualitatively similar but much more marked increases in PAI-1 gene expression were seen when arterial injury was accompanied by hypercholesterolemia. Neither vitronectin, known to stabilize PAI-1, nor vitronectin mRNA increased in liver. However, immunocytochemistry and Western blots demonstrated marked aortic accumulation of vitronectin protein with hyperlipidemia, particularly in subendothelial fibrotic regions, accompanied by increased neointimal vitronectin mRNA as shown by in situ hybridization. These results suggest that increased synthesis and stabilization of vascular PAI-1 may potentiate accumulation of extracellular matrix, thereby accelerating atherosclerosis. (Circ Res. 1993;73:671-680.)

KEY WORDS • vascular injury • PAI-1 gene expression • vitronectin • atherosclerosis

Intimal thickening in arteriosclerotic vessels is a manifestation of migration and proliferation of smooth muscle cells from the media and intimal infiltration of monocyte/macrophages, some of which are transformed into foam cells.1 Both processes may be associated with increased procoagulant activity of the luminal surface.2 Despite some obvious differences, vasculopathy in cholesterol-fed rabbits mimics some aspects of atherosclerosis in human subjects. The intimal thickening seen1 resembles that in normocholesterolemic rabbits subjected to intravascular mechanical injury.3,4 Because of our interest in factors predisposing to the development of restenosis after angioplasty and the potentially deleterious effects of hypercholesterolemia on its severity, we performed the present study in rabbits subjected to both hypercholesterolemia and mechanically induced vascular injury.

Plasminogen activator inhibitor type 1 (PAI-1) attenuates fibrinolysis in plasma. In tissue, PAI-1 can modify cell migration and tissue repair.5,6 The present study was performed to determine whether hypercholesterolemia, sustained mechanically induced intravascular injury, or both elicited changes in vascular PAI-1 that may affect the balance between thrombosis and thrombolysis and accelerate vasculopathy. The results obtained show that intramural PAI-1 expression is increased by either mechanically induced intravascular injury or hypercholesterolemia and that the increases are much more marked when the two insults occur concomitantly.

Materials and Methods

Goat anti-rabbit thrombomodulin antibody was kindly provided by Dr Naomi L. Esmon (Oklahoma Medical Research Foundation); anti-macrophage antibody, RAM-11, by Dr Allen M. Gown (University of Washington); anti-myosin heavy chain antibodies, SM1 and SMemb, by Dr R. Nagai (University of Tokyo, Japan)7-11; anti-rabbit vitronectin antibody, EMR1a/212D, and rabbit vitronectin cDNA by Dr T. Takano (Teikyo University, Japan); and PAI-1 cDNA by Dr

Received October 20, 1992; accepted June 21, 1993.

From the Cardiovascular Division, Washington University School of Medicine, St Louis, Mo.

Correspondence to Satoshi Fujii, MD, Cardiovascular Division, Washington University School of Medicine, 660 South Euclid Ave, Box 8086, St Louis, MO 63110.
T.C. Wun (Monsanto Corporation, St Louis, Mo).12 Purified human PAI-1 protein and goat anti-human PAI-1 antibody were purchased from American Diagnostica (Greenwich, Conn); anti-α-actin antibody, HHF-35, from Enzo Diagnostics (Syosset, NY); and anti-factor VIII–related antigen antibody from Atlantic Antibodies (Scarborough, Me). Hydrogen peroxide, hematoxylin, trypsin, poly-L-lysine, diethanolamine, RNAse, and diethyl pyrocarbonate were obtained from Sigma Chemical Co (St Louis, Mo); paraformaldehyde, methanol, calcium chloride, xylene, formamide, Permunt, Wheaton staining jars, cover slips, and micro slide glasses from Fisher Scientific (Pittsburgh, Pa); proteinase K, Asp 718, and transfer RNA from Boehringer Mannheim (Indianapolis, Ind); Eco RI and Sal I from United States Biochemical (Cleveland, Ohio); PBluescript SK+ vector and RNA transcription kits from Stratagene (La Jolla, Calif); RNAid from Bio 101 (La Jolla, Calif); Elite ABC kits and 3,3'-diaminobenzidine (DAB) substrate kits from Vector (Burlingame, Calif); DPX from BDH Laboratory (Poole, England); nitrocellulose membrane and Elutip from Schleicher & Schuell (Keene, NH); and emulsion NTB-2, fixer, and developer D-19 from Kodak/International Biotechnologies (New Haven, Conn).

Procedures in Animals

All procedures in animals conformed to the “Position of the American Heart Association on Research Animal Use” (November 11, 1984) and were approved by the Animal Studies Committee at Washington University. New Zealand White rabbits weighing 3.2±0.2 kg (range, 2.7 to 3.8 kg, n=31) were fed either a 2% cholesterol/6% peanut oil diet (Purina chow) or a standard laboratory diet for 8 weeks. In some control and some cholesterol-fed animals, persistent aortic injury was induced by aseptic insertion of indwelling polyethylene tubing 4 weeks after initiation of either dietary regimen.4 For this purpose, the rabbits were anesthetized with 20 mg/kg ketamine IM and 8 mg/kg xylazine IM. A sterile 17- to 20-cm length of polyethylene tubing (Intramedic PE-60, Clay Adams, Parsippany, NJ; diameter, 1.22 mm) was inserted into the aorta via the right femoral artery and advanced to the descending thoracic aorta. The exterior end was fixed to muscle. Sham operation was performed in two animals, with cannulation only advanced to 1 cm from the entry of the right femoral artery. Four weeks after cannulation, the aortas were excised and characterized.

The four groups of animals studied included those with no mechanically induced injury and a normal diet (group 1, n=8); those with mechanically induced vascular injury and a normal diet (group 2, n=8); those with no mechanically induced vascular injury and a high-cholesterol diet (group 3, n=7); and those with mechanically induced vascular injury and a high-cholesterol diet (group 4, n=8).

Blood samples were obtained at time 0 and 4 and 8 weeks after initiation of the study for assay of PAI-1 activity in plasma harvested by centrifugation at 2000g at 4°C for 15 minutes. Aliquots were frozen immediately in liquid nitrogen for storage at −70°C until assay. Plasma PAI-1 activity was assayed spectrophotometrically.14 One arbitrary unit (AU) of plasma PAI-1 activity was defined as the amount of activity that inhibited 1 IU of tissue-type plasminogen activator (t-PA) completely over a period of 10 minutes. Serial dilutions of rabbit plasma were used to construct standard curves. Plasma cholesterol was assayed enzymatically (Sigma Chemical Co, kit #352-20).

The harvested aortas were characterized histologically and immunohistochemically. Aortic PAI-1 gene expression was quantified by Northern blotting and localized by in situ hybridization.

Immunohistochemistry

Serial frozen sections adjacent to those used for in situ hybridization were analyzed immunohistochemically for PAI-1 antigen and immunocytochemically for cell type. HHF-35 antibodies were used for identification of smooth muscle cells, anti-SM1 for cells with adult myosin heavy chains, anti-SMemb for cells with embryonic myosin heavy chains, RAM-11 for macrophages, and anti-rabbit thrombomodulin and anti-factor VIII–related antigen for endothelial cells.15 Cross-reactivity of antibody against human PAI-1 with rabbit PAI-1 was verified as described previously.16

Immunohistochemical assays were performed conventionally as previously described.16 Briefly, sections were incubated in methanol containing 0.25% hydrogen peroxide for 15 minutes to quench endogenous peroxidase. After washes with phosphate-buffered saline (PBS), the sections were incubated for 10 minutes at 37°C with diluted (1.5%) serum (horse serum for assay of HHF-35 and RAM-11 and goat serum for assay of PAI-1 antigen, factor VIII–related antigen, and thrombomodulin). The sections were then incubated with primary antibody at 37°C for 30 minutes, with biotinylated secondary antibody at 37°C for 30 minutes, and with avidin/biotin peroxidase complexes for 30 minutes. Peroxidase was visualized with DAB and counterstained with hematoxylin. For immunostaining of PAI-1 antigen, factor VIII–related antigen, and thrombomodulin, sections were pretreated with trypsin (0.1% wt/vol trypsin, 0.1% wt/vol CaCl2, 0.05 mol/L Tris-HCl, pH 7.6) at 37°C for 10 minutes.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

For immunoblots with ERM1a/212D anti-rabbit vitronectin antibody, 4-cm segments of the descending thoracic aortas were removed, washed with PBS, and homogenized in 10 mmol/L sodium phosphate buffer (pH 7.0) containing 2% sodium dodecyl sulfate (SDS) and 2 mmol/L phenylmethylsulfonyl fluoride (3 mL). Samples were heated at 90°C for 2 minutes and centrifuged at 12 000g for 5 minutes. The supernatant fractions were stored at −70°C until assay.12 Total protein was quantified with the use of Bio-Rad (Richmond, Calif) protein assay reagents (#500-0111).

Crude protein extracts (50 μg) were applied to 10% polyacrylamide gels under reducing conditions. Protein was transferred to nitrocellulose membranes in 25 mmol/L Tris-HCl, 192 mmol/L glycine, and 10% methanol with a Trans-Blot apparatus (Bio-Rad) at a constant voltage of 100 V for 1 hour. Nitrocellulose membranes were blocked by incubation with 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 2% bovine serum albumin (BSA) at 4°C overnight with agitation followed by 3 rinses of 20 minutes each with PBS.
containing 0.5% Tween 20. Membranes were then incubated with primary antibody (EMR1a/212D, 1:1000) at 37°C for 1 hour with agitation and washed in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.2% SDS, 0.5% BSA, and 0.5% Triton X-100 for 40 minutes and in 50 mmol/L Tris, 150 mmol/L NaCl, and 2% BSA for 10 minutes. After rinsing, they were incubated with 125I goat anti-mouse IgG (1.3x10^6 cpm/mL) at 37°C for 1 hour with agitation, rinsed with the same buffer, and air dried. For autoradiography, membranes were incubated with Kodak X-OMAT AR film at −70°C and developed. Autoradiographic signals were quantified by scanning densitometry (LKB Ultrascan XL laser densitometer, Pharmacia-LKB, Piscataway, NJ).

**Preparation of RNA and Northern Blots**

Tissues were removed rapidly by dissection, minced, rinsed twice in PBS at 4°C, frozen quickly in liquid nitrogen, and stored at −70°C. Total RNA was isolated, quantified spectrophotometrically (at 260 nm), and subjected to electrophoresis on 1.5% agarose/formaldehyde gels. Northern blotting was performed after capillary transfer of the RNA to nylon membranes (Biodyne, Pall BioSupport Co, Glen Cove, NY). Integrity of the RNA isolated, consistency of loading, and transfer of RNA were verified by ethidium bromide staining of ribosomal RNA.

The nylon membranes were baked at 80°C for 1 hour in a vacuum oven. Prehybridization was performed in a solution of 50% deionized formamide, 10x Denhardt’s solution, 0.05 mol/L Tris-HCl, 1.0 mol/L NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 100 µg/mL calf thymus DNA for 6 hours at 42°C. cDNA probe was labeled with [32P]dCTP by the random primer method (with DNA labeling kits purchased from the Boehringer Mannheim Corp.). Hybridizations with PAI-1 (500 000 cpm/mL), glyceraldehyde-6-phosphate dehydrogenase (GAPDH, 150 000 cpm/mL), and vitronectin (300 000 cpm/mL) labeled probes were performed at 42°C for 24 hours with the same solutions as those used for prehybridization. Cross-reactivity of human PAI-1 and GAPDH probes with rabbit probes were verified previously. Membranes were washed in 2x sodium chloride/sodium citrate (SSC) (1x SSC=0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7.0) at room temperature for 5 minutes and washed with 2x SSC and 1% SDS at 60°C for 6 minutes. Radioactivity in specific bands was assayed with an Ambis radioisotopic scanner (Automated Microbiology Systems, San Diego, Calif). Autoradiography was performed at −70°C with Kodak X-OMAT AR film.

**In Situ Hybridization**

In situ hybridizations were performed as described by Simmons et al19 with tissue sections prepared from rabbits anesthetized with ketamine and xylazine and perfusion fixation in vivo via the left ventricle with simultaneous removal of blood from the right atrium. The perfusates were physiological saline followed with 4% paraformaldehyde in 0.02 mol/L sodium phosphate buffer, pH 7.3 at 0 to 4°C. Tissue was harvested and incubated in the same fixative for 4 hours at 4°C, washed with PBS twice, and stored at −70°C. Sections (6 to 10 µm) were cut in a cryostat and placed on 0.01% poly-L-lysine-coated slides. Sections from animals in each of the experimental groups were mounted juxtaposed to those from controls for comparison. Slides were postfixed, dehydrated in graded alcohol, dried in a vacuum desiccator for 1 hour, and stored at −70°C until processed for hybridization by treatment with proteinase K (1 µg/mL proteinase K in 0.1 mol/L Tris HCl, pH 8.0, and 50 mmol/L EDTA) and 0.25% acetic anhydride in 0.1 mol/L triethanolamine, rinsing with 2x SSC and dehybridation.

To construct riboprobes, PBluescript SK+ plasmid containing a 0.9-kb-length human PAI-1 cDNA insert was linearized by restriction digestion with Eco RI or Sal I (for antisense and sense, respectively) and transcribed with T7 or T3 RNA polymerase in the presence of 35S-uridine triphosphate (UTP). For the rabbit vitronectin probe, a 0.9-kb fragment of rabbit vitronectin cDNA was cut from pUC540E and subcloned in a PBluescript SK+ plasmid. The template DNA was linearized with ASP718 or Eco RI (to yield antisense and sense probes, respectively) and transcribed with T3 or T7 RNA polymerase in the presence of 35S-UTP. After transcription, DNase (0.67 U/µL) was added to digest any remaining template. Transcripts were isolated from unincorporated nucleotides with RNaid.

After quantification of radioactivity by liquid scintillation spectrometry, an RNA probe (2.5x10^4 cpm) was mixed with 2.5 mg transfer RNA and 0.05 mol/L dithiothreitol. The final volume was adjusted to 1.0 mL with a 0.1% solution of diethyl pyrocarbonate, heated for 5 minutes at 65°C, added to 4 mL of hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1x Denhardt’s solution, 0.3 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), and maintained at 65°C for 4 hours with agitation, rinsed with the same buffer, and air dried. For autoradiography, membranes were incubated with Kodak X-OMAT AR film at −70°C and developed. Autoradiographic signals were quantified by scanning densitometry (LKB Ultrascan XL laser densitometer, Pharmacia-LKB, Piscataway, NJ).

**Plasma Plasminogen Activator Inhibitor Type 1 Activity in Rabbits From Each Group**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Diet</th>
<th>Mechanical Injury</th>
<th>0 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>Normal</td>
<td>−</td>
<td>7.3±4.5</td>
<td>6.1±3.0</td>
<td>5.7±4.1</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Normal</td>
<td>+</td>
<td>6.7±4.3</td>
<td>5.9±2.4</td>
<td>4.1±3.4</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>1% C/6% PNO</td>
<td>−</td>
<td>7.0±3.0</td>
<td>9.7±3.4</td>
<td>16±7.6***</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>1% C/6% PNO</td>
<td>+</td>
<td>6.7±2.6</td>
<td>6.2±4.3</td>
<td>16±10+**$</td>
</tr>
</tbody>
</table>

PAI-1 indicates plasminogen activator inhibitor type 1; N, number of animals per group; C, cholesterol; and PNO, peanut oil.

*P<.05 compared with values at 0 weeks.
†P<.05 compared with values at 4 weeks.
‡P<.05 compared with values in group 1.
§P<.05 compared with values in group 2.
−20°C. After addition of 75 μL of hybridization solution, slides were covered with coverslips, sealed with DPX to prevent drying, and incubated at 60°C for 20 hours.

After hybridization reactions were complete, slides were washed with 4×SSC for 1 hour, treated with RNase (2% RNase A in 0.5 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) for 30 minutes at 37°C, washed with 2×SSC, 1×SSC, and 0.5×SSC for 10 minutes each, and incubated in 0.1×SSC for 30 minutes at 75°C. They were then dehydrated, dried in a vacuum desiccator, dipped in emulsion (Kodak NTB-2 diluted 1:1 with water), placed in light-proof containers, stored at 4°C for 2 weeks, and developed with Kodak developer D-19 and fixer and then counterstained with hematoxylin and eosin.
Fig 1. Histological sections showing localization of plasminogen activator inhibitor type 1 (PAI-1) mRNA in aorta. A, The distribution of PAI-1 mRNA in a mechanically injured aorta from a hypercholesterolemic rabbit. This section was hybridized with a 35S-labeled cRNA probe for PAI-1 mRNA. B, Dark-field photomicrograph of the same section shown in A. PAI-1 mRNA is evident as white grains. C, Higher magnification of the distribution of PAI-1 mRNA in A. Exuberant intimal thickening is evident. Neoendothelial cells covering the intima express PAI-1 mRNA (arrows; arrowheads point to internal elastic lamina). D, Localization of PAI-1 mRNA in the aorta from a hypercholesterolemic rabbit. The extent of intimal thickening is less than that seen in mechanically injured aortas from hypercholesterolemic rabbits. Positive hybridization signals are seen in neoendothelial cells (arrows; arrowheads point to internal elastic lamina). E, The distribution of PAI-1 mRNA in a section from a mechanically injured aorta from a normocholesterolemic rabbit. Only minimal accumulation of foam cells is seen in an only slightly thickened intima. Neoendothelial cells express PAI-1 mRNA (arrows; arrowheads point to internal elastic lamina). F, A section from an aorta from a control animal (no mechanical injury and a standard diet) hybridized with an antisense cRNA probe for PAI-1 mRNA. Only sparse signals are evident. The intima is not thickened. G, A section from a mechanically injured aorta from a hypercholesterolemic rabbit hybridized with a negative control, sense cRNA probe for PAI-1 mRNA. No positive signal is seen, consistent with the specificity of the anti-sense probe used for the positive hybridizations. H, Localization of PAI-1 antigen in the neoendothelial cells covering the thickened intima of a mechanically injured aorta from a hypercholesterolemic rabbit. Strong dark brown positive immunostaining is evident along the luminal surface of the neointima. Magnification for A and B, ×50; for C through H, ×2000.

At least four separate sections from each aorta were used for in situ hybridization and immunohistochemical analysis with a Nikon microscope (Nikon, Tokyo, Japan), performed by two independent observers.

Statistics

Data are mean±SD. Differences were assessed by analysis of variance followed by the Fisher's least significant difference post hoc tests for comparisons within multiple groups. Statistical significance was defined as P<.05.

Results

Plasma Cholesterol

Cholesterol values in animals fed a normal diet remained within the normal range for rabbits regardless of the presence or absence of mechanical injury to the aorta (26±13 and 27±8 mg/dL, with and without injury, respectively, after 8 weeks). In contrast, cholesterol feeding increased plasma cholesterol to 3056±397 and 2998±466 mg/dL in animals with or without mechanically induced aortic injury.

Plasma PAI-1

At the time of initiation of either a normal or a high-cholesterol diet, plasma PAI-1 activity was comparable in all groups (Table). After 4 weeks, mean plasma PAI-1 activity did not increase significantly despite cholesterol feeding. However, after 8 weeks it did increase (P<.05). Mechanically induced vascular injury appeared to exert no influence on plasma PAI-1. Plasma PAI-1 activity in the two animals that had undergone sham operation was not altered 4 weeks after surgery.
FIG 3. Northern blot analysis of plasminogen activator inhibitor type 1 (PAI-1) mRNA and Western blot analysis of vitronectin. A, Northern blot analysis of aortic PAI-1 mRNA. Total RNA (20 μg) was isolated from aortic wall (n=15) and assayed for PAI-1 mRNA. mRNA was quantified by radioisotopic scanning with calibration of PAI-1 mRNA radioactivity with respect to that in GAPDH mRNA as described in the text. Error bars are standard deviations. *P<.05 compared with results from control animals. B, Autoradiograms from experiments in four representative animals are shown. Ethidium bromide staining of the same gels demonstrated equivalent amounts of ribosomal RNA (28S). Lanes 1 through 4 show results from a representative experiment in each of the four groups of animals (groups 1 through 4, respectively). C, Western blot detection of vitronectin in aortas and liver. Antibody to vitronectin delineated two bands, one of 75 kD and a less prominent band of 65 kD. Migration of molecular weight markers, run in parallel, is shown to the right. The expression of vitronectin was most significant in the aortas from hypercholesterolemic rabbits (lane 4), whereas aortas from control rabbits exhibited the least expression (lane 3). Liver vitronectin (lane 2) was not increased in hypercholesterolemic compared with control rabbits (lane 1).
**Fig 4.** Sections showing distribution of vitronectin in liver and aorta. A, The distribution of vitronectin mRNA in liver from an animal fed a standard diet that was not subjected to mechanically induced aortic injury. The section was hybridized with a 35S-labeled anti-sense cRNA probe specific for vitronectin mRNA. Strongly positive hybridization signals are evident in the parenchymal liver cells. B, An adjacent section hybridized with a sense probe (negative control) for vitronectin mRNA. No positive signal is seen, consistent with the specificity of the anti-sense probe used for the positive hybridizations that were observed. C, The distribution of vitronectin mRNA in regions of intimal thickening in a mechanically injured aorta from a hypercholesterolemic rabbit. A positive hybridization reaction is evident in the foam cells (arrows). The extent of expression of vitronectin mRNA is less than that of PAI-1 mRNA. D, An adjacent section hybridized with a sense cRNA probe for vitronectin (negative control). No positive signal is seen, consistent with the specificity of the anti-sense probe used for the positive hybridizations that were observed. E, A section of a mechanically injured aorta from a hypercholesterolemic rabbit stained immunocytochemically for vitronectin antigen. A dark brown positive immunostaining reaction to vitronectin is evident in the neointima and in the media. Arrowheads delineate the internal elastic lamina. Magnification for A and B, ×1600, and for C through E, ×2000.

**Changes in the Aortas**

The appearance of the aortic lesions was consistent in each group, and the morphology of cells in the aortas examined was indicative of the following and was seen in all of the aortas examined.

Mechanically injured aortas from hypercholesterolemic rabbits exhibited abundant white to yellow atheromatous plaques that protruded into the lumen. Some large plaques contained grumous fluid. As shown histologically, the atheromatous plaques were composed of neoendothelium, fibrous caps, necrotic centers, and thickened media. The intima in control aortas consisted of a flat monolayer of endothelial cells without thickening or smooth muscle or foam cell infiltration. Aortas from animals subjected to sham operation did not exhibit any histological changes. In mechanically injured aortas from hypercholesterolemic rabbits, marked intimal thickening was evident, consistently exceeding that in vessels subjected to mechanical injury without concomitant hypercholesterolemia or in vessels from ani-
mals with hypercholesterolemia alone (Fig 1). The internal elastic lamina was generally intact. Atheromatous plaques were covered completely by neointima that stained positively with anti-factor VIII–related antigen and anti-thrombomodulin (results not shown). Fibrous caps contained smooth muscle cells, macrophages, foam cells, lymphocytes, and connective tissue. In the necrotic centers, cell debris, lipid, and foam cells were present.

In plaques in mechanically injured aortas from hypercholesterolemic rabbits, foam cells were abundant, generally centrally, and were surrounded by fibrous tissue in the thickened intima and media. They exhibited positive immunoreactions with SMemb antibody (anti-embryonic myosin heavy chain) (results not shown) and RAM 11 anti-macrophage monoclonal antibody, but not with SM1 (anti-adult myosin heavy chain) (Fig 2). Thus, the cells had characteristics of macrophage/monocytes and embryonic smooth muscle cells. In contrast, plaques in mechanically injured vessels from normocholesterolemic rabbits exhibited intimal thickening attributable predominantly to deposition of smooth muscle cells with only a few scattered foam cells present. The media contained only rare, small foci of foam cells. Plaques from hypercholesterolemic animals without mechanically induced vascular injury comprised primarily intimal foam cells with only a few smooth muscle cells and occasional medial foci of foamy macrophages.

**Cellular Locus of Increased Aortic PAI-1 mRNA**

Aortas from rabbits in each of the four groups (n=3 for each) were characterized by in situ hybridization. The identities of specific cell types were determined by immunocytochemistry. In aortas from control and sham-operated animals, PAI-1 mRNA was not detectable (Fig 1). In contrast, mechanically injured aortas from hypercholesterolemic rabbits exhibited strong positive hybridization signals in neointimal cells (Fig 1) overlying the plaques, in foam cells within the plaques, and in some smooth muscle cells beneath the internal elastic lamina (Fig 2). The cells were found to be endothelial cells, as judged from the positive immunoreaction with antibodies to factor VIII–related antigen and to thrombomodulin (results not shown); to be foam cells positive to RAM-11 and SMemb (results not shown); and as smooth muscle cells positive to HHF-35 and SM1 (Fig 2).

PAI-1 mRNA was expressed, but much less intensely, in injured aortas harvested from normocholesterolemic as opposed to hypercholesterolemic rabbits. Thus, in mechanically injured aortas from normocholesterolemic rabbits, PAI-1 mRNA was detected in neointimal cells (Fig 1), in foam cells in the thickened intima, and in smooth muscle and foam cells in the media. However, the numbers of cells exhibiting positive hybridization signals were much lower than those in mechanically injured aortas from hypercholesterolemic rabbits.

In aortas from hypercholesterolemic rabbits in which PAI-1 mRNA was expressed in plaques associated with or without mechanical injury and in plaques from normocholesterolemic rabbits in which it was expressed only in association with mechanical injury, PAI-1 antigen colocalized with PAI-1 mRNA along the luminal surface of the aorta (Fig 1), in vascular smooth muscle cells, and in macrophages.

**Expression of PAI-1 and Vitronectin**

Northern blot analyses of aortic RNA showed that only the 3.2-kb form of PAI-1 mRNA was expressed (Fig 3), consistent with our previous observations in tissues from rabbits. Compared with the amounts of PAI-1 mRNA in aortas from controls, PAI-1 mRNA was increased in the vessels from cholesterol-fed animals without mechanically induced injury and in aortas that had been subjected to a mechanical insult in either the presence or absence of hypercholesterolemia (Fig 3). For purposes of comparison, liver RNA was assayed as well. In contrast to the case for aortas, the concentrations of PAI-1 mRNA in liver did not differ in animals in the different groups (data not shown).

Vitronectin mRNA was not detectable in aortas from any of the four groups of rabbits. It was abundant in liver, consistent with previous observations by others in other species. However, the levels of vitronectin mRNA in liver did not vary significantly between groups (data not shown).

In contrast to results of assays of vitronectin mRNA, assays of vitronectin protein in extracts of aortas from rabbits in each of the four groups were positive (Fig 3). Rabbit vitronectin was detected in both 75-kD and 65-kD bands. Only scanty amounts were detectable in extracts from aortas from control rabbits. More than fivefold greater amounts were present in extracts from hypercholesterolemic rabbits devoid of mechanically induced intravascular injury. Comparable increases were seen in extracts of aortas from hypercholesterolemic rabbits in which mechanical insults had been induced. In contrast and compared with results in controls, vitronectin was not increased in extracts of liver from any of the groups.

**Vitronectin mRNA and Protein**

Vitronectin mRNA in liver was expressed to a similar extent in each of the four groups of animals studied and was localized in parenchymal cells, as was vitronectin protein (Fig 4). Thus, hypercholesterolemia did not increase the gene expression of hepatic vitronectin. However, in contrast to normocholesterolemic animals, the hypercholesterolemic rabbits with mechanically induced aortic injury exhibited vitronectin mRNA in foam cells (Fig 4). In these animals, immunoreactive vitronectin protein was evident as well, in vascular smooth muscle cells in the neointima and media of the aortas (Fig 4). Strong immunoreactivity was also present in the basal lamina of the vascular wall. Comparable increases were seen in aortas from hypercholesterolemic rabbits in which mechanical insults had not been implemented.

**Discussion**

This study was performed to determine whether the local, intramural, genetic expression of PAI-1 is increased by aortic endothelial injury, and if so, whether the increase is more intense when hypercholesterolemia is present. Mechanically induced aortic injury elicited vascular lesions characterized primarily by accumulation of neointimal smooth muscle cells, probably reflecting both local proliferation and migration of vascular
smooth muscle cells from the media. Hypercholesterolemia per se (without mechanical injury) led to qualitatively different lesions composed of foam cells largely confined to the intima. The combination of mechanical injury plus hypercholesterolemia led to complex lesions with both accumulation of smooth muscle cells in the intima and infiltration of macrophages into the intima. Furthermore, expression of PAI-1 mRNA was intense in cells of both types as well as in the neointimal endothelium.

Thus, local, intramural vascular injury elicits increased genetic expression of PAI-1 in several cellular components in the induced lesions. Concomitant hypercholesterolemia potentiates the increase. These observations are consistent with the hypothesis that accumulation of mitogenic constituents of thrombi and extracellular matrix in turn exacerbating cellular proliferation and migration in complex plaques results, in part, from intramural inhibition of proteinolysis secondary to increased, local genetic expression of PAI-1.

The induction of PAI-1 gene expression as opposed to the changes in plasma PAI-1 seen with hypercholesterolemia appeared to be associated specifically with vascular lesions. Thus, PAI-1 activity in plasma increased only modestly and comparably in hypercholesterolemic rabbits with or without mechanical insults consistent with the generally normal values of plasma fibrinolytic activity seen in most patients with generalized atherosclerosis.21 However, the results of the Northern blot analyses of aorta and in situ hybridization studies in the present study demonstrate that the severity of the vascular lesions induced parallels the increase in PAI-1 gene expression in the neointima. The most marked increases in PAI-1 mRNA within the aortic wall occurred in hypercholesterolemic animals in which mechanically induced aortic injury had been induced as well. It is possible that the increase in plasma PAI-1 activity in hypercholesterolemic rabbits with or without mechanical insults may be derived from other sources, such as liver parenchymal and liver endothelial cells.

Although a human PAI-1 probe was used for measurement of PAI-1 gene expression, sequence similarity of the PAI-1 gene among species reported made Northern blot and in situ hybridization possible.15,18 In fact, Northern blot performed with a rabbit PAI-1 probe cloned from rabbit liver cDNA using the polymerase chain reaction gave identical results (unpublished observation).

Several factors may mediate the local increase in PAI-1 genetic expression that we observed. Platelet-derived growth factor (PDGF), a chemotactic and mitogenic stimulus of vascular smooth muscle cells,1 may be involved. The mRNAs of PDGF A and B chains and of the PDGF-β receptor are present in cells within plaques,22 and PDGF has been shown to be capable of stimulating PAI-1 gene expression in vascular smooth muscle cells in culture.23 Thus, PDGF may increase vascular wall PAI-1 gene expression in an autocrine or paracrine fashion. In addition, basic fibroblast growth factor, transforming growth factor-β, and tumor necrosis factor-α, all of which can induce PAI-1 synthesis in endothelial cells in culture,24-26 may participate.

Because the endothelium from aortas from normal rabbits exhibited virtually no PAI-1 mRNA, it seems likely that the increase in endothelial cell PAI-1 gene expression we observed depended on mediators known to alter endothelial cell function such as interleukin-1, tumor necrosis factor-α,26,27 or transforming growth factor-β, shown by us and others to increase PAI-1 gene expression when released from platelets.25,26

PAI-1 is secreted by endothelial cells in a polar fashion.28 Basally secreted PAI-1 and PAI-1 elaborated by vascular smooth muscle can be deposited in the extracellular matrix.29,30 This process may render the vessel wall refractory to remodeling and repair otherwise mediated by proteases, facilitating cell migration.

Vitronectin, known to stabilize PAI-1,3 accumulates in the aortic wall after mechanically induced injury, especially in the hypercholesterolemic rabbits. In these animals, the concentration of vitronectin relative to that of total proteins in aorta was higher than that in livers. Accumulation of vitronectin in atherosclerotic lesions without alteration in plasma vitronectin levels has been observed also in Watanabe heritable hyperlipidemic rabbits.12 The accumulation may contribute to augmentation of local activity of PAI-1 in the walls of diseased vessels, possibly attenuating the activity of cell surface plasmin and the activity of plasminogen activator receptor/ligand complexes.31

Mural thrombi were not observed in this study, possibly because the vascular insult was relatively mild. Thus, the regions had intact endothelial lining and a modest quantity of intimal smooth muscle cell layers. Locally increased expression of PAI-1 may contribute to atherogenesis indirectly as well as directly by predisposing to local thrombosis adjacent to or within the vessel wall. The histopathology of atheromatous lesions supports the view that recurrent thrombotic events may exacerbate formation of complex plaques.32 Increased PAI-1 gene expression in atherosclerotic lesions has been observed also in human aortas,33 supporting the importance of this molecule in human pathobiology of atherosclerosis. Thus, increased PAI-1 in thrombi15 and in neointimal lesions may accelerate development of complex plaques. Accordingly, identification of mediators of the locally increased PAI-1 gene expression and development of methods designed to attenuate the increase may ultimately improve the prevention and treatment of atherosclerotic vascular disease.

Acknowledgments

This work was supported in part by National Institutes of Health grant HL-17646, SCOR in Coronary and Vascular Diseases, and by a grant-in-aid from the American Heart Association, Missouri Affiliate (S.F.). We thank Dr Jeffrey E. Saffitz for help with the immunocytochemical and in situ hybridization procedures, Dr Naomi L. Esmon for supplying anti-rabbit thrombomodulin antibody, Dr Ryozo Nagai for supplying anti-rabbit myosin heavy chain antibodies, Dr Tatiana Takano for supplying anti-rabbit vitronectin antibody and cDNA, Pilar Herrero for statistical support, Denise Nachowiak, John Botz, Jeffrey Labuda, and Pamela Lundius for technical assistance, and Barbara Donnelly and Kelly Hall for secretarial services.

References


Potentiation by hypercholesterolemia of the induction of aortic intramural synthesis of plasminogen activator inhibitor type 1 by endothelial injury.

H Sawa, B E Sobel and S Fujii

Circ Res. 1993;73:671-680
doi: 10.1161/01.RES.73.4.671

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/73/4/671

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/