Connective Tissue Proteinases and Inhibitors in Abdominal Aortic Aneurysms

Involvement of the Vasa Vasorum in the Pathogenesis of Aortic Aneurysms

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Recent studies have shown that increases in proteolytic activity are associated with abdominal aortic aneurysms (AAAs). We have studied samples of the dilated aortic wall, taken during corrective surgery for AAAs, in terms of the number, type, and tissue location of connective tissue proteinases and their inhibitors. Five distinct caseinolytic serine proteinases and six gelatinolytic metalloproteinases were resolved by molecular weight by use of sodium dodecyl sulfate–substrate gel electrophoresis. Isoforms of the $M_r$, 92,000 neutrophil gelatinase were identified by immunoprecipitation of biosynthetically labeled organ culture media. About 50% of the total radiolabeled protein secreted by AAA organ cultures was identified as the $M_r$ 30,000 glycoprotein, tissue inhibitor of metalloproteinase (TIMP), by immunoprecipitation. Both TIMP and gelatinase were localized to the vasa vasorum by immunoperoxidase staining. However, interstitial collagenase could not be detected by any method. These results suggest the involvement of the vasa vasorum in the maintenance and possibly the genesis of AAAs.

(Arteriosclerosis and Thrombosis 1991;11:1667-1677)

The development of irreversible dilations of the infrarenal aorta has been attributed to hemodynamic disturbances as well as to structural and metabolic changes within the vessel wall.1,2 Chemical and histological analyses have demonstrated significant decreases in the two major structural proteins, collagen and elastin, in dilated vascular tissue from patients undergoing corrective surgery for abdominal aortic aneurysm (AAA) disease.3,4 Experimentally, it has been shown that circumferential arterial wall stress is borne primarily by elastin and that collagen maintains the integrity of the arterial wall, as enzymatic hydrolysis of collagen results in vessel rupture, whereas degradation of elastin induces dilation only.5 A retrospective study of patients whose previously asymptomatic aneurysms ruptured after recent laparotomy suggested that connective tissue degradation in the weakened aneurysm wall could be induced by major surgical operation.4 Such studies demonstrate that an imbalance occurs in the metabolism of specific connective tissue components, which favors net degradation over synthesis only in certain regions of the vasculature and only at specific times.

Patients with AAAs have been shown to have deficiencies in connective tissue crosslinking6 as well as increased levels of both blood and tissue proteinase activities.7-9 More recently, two reports have suggested that the ratios of elastase-like activity to $\alpha$-1-antiproteinase activity is increased in aortic tissue taken from patients with ruptured AAAs compared with similar tissues taken from patients with atherosclerotic occlusive disease.10,11 However, these studies failed to identify the enzyme species responsible for the proteolytic activity, the origin, or the location of these activities within the vessel wall.

Increased activity of proteinases may result from increased de novo synthesis, enhanced conversion of zymogen precursors, and/or increased ratios of activated proteinases to tissue inhibitor concentrations in the vicinity of the actual lesion.12
The purpose of the present study was threefold: 1) to identify and characterize connective tissue proteinases and their inhibitors in both normal aortic tissue and dilated aortas from patients undergoing surgery for AAA; 2) to determine whether these enzyme and inhibitor species are synthesized and secreted by the cells of the diseased aortic wall or whether they are produced at remote sites and carried to the dilated region by the general circulation; and 3) to examine the approximate location of enzymes and inhibitors within the aortic wall through the use of immunohistochemical techniques.

The results of these studies show that specific connective tissue proteinases and inhibitors are synthesized and secreted by aortic tissues. Localization of these species to the vasa vasorum strongly suggests that a neovascular response may contribute to the pathogenesis of AAA disease.

**Methods**

**Reagents**

Chemicals were purchased from the following sources: Sulfur-35-labeled-methionine (specific activity, 1,400 Ci/nmol) from Amersham Corp., Arlington Heights, Ill.; carbon-14-labeled protein standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) from New England Nuclear, Boston, Mass.; formalin-fixed *Staphylococcus aureus* cell walls (Zysorbin) from Zymed Laboratories, Inc., South San Francisco, Calif.; gelatin, casein, elastin, Brij-35, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, aminophenylmercuric acetate (APMA), and N-tosyl-L-phenylalanine chloromethyl ketone–trypsin from Sigma Chemical Co., St. Louis, Mo.; and preextracted electrophoresis protein standards and gelatin-Sepharose from Bethesda Research Laboratories (BRL), Gaithersburg, Md.

Monospecific sheep antiserum to purified rabbit collagenase (CL), previously shown to crossreact with human CL, and monospecific sheep antiserum to purified human amniotic fluid tissue inhibitor of metalloproteinase (TIMP), characterized as described previously, were generous gifts of Gillian Murphy, Strangeways Research Laboratories, Cambridge, UK. Monospecific sheep antiserum to purified human neutrophil gelatinase (GL) was prepared as described previously.

**Patient Information and Sample Collection**

Nine pathological samples were taken from approximately the same dilated region of the aorta during corrective surgery for aneurysmal disease. Two normal aortic samples approximately 2 cm from the common iliac arteries were obtained from accident victims who participated in the Organ Donor Program at the Kidney Transplant Service, University of California San Francisco Medical Center. Tissue specimens were placed in phosphate-buffered saline (PBS) at 0°C immediately after excision and kept on ice for less than 2 hours.

**Preparation of Samples for Biochemical Analysis**

**Extraction of frozen tissue.** Samples were either frozen at −80°C or drained and pulverized under liquid N2 with a mortar and pestle. Pulverized samples were then stored at −20°C until further processing. Unless specifically stated, all tissue extractions and processing were performed at 0–4°C. For initial experiments in which tissue extracts were first analyzed by SDS-substrate gel electrophoresis, approximately 0.5 g tissue was extracted by four to five strokes of a dounce homogenizer in the presence of 0.5 ml 5% SDS, 2% sucrose, 125 mM tris(hydroxymethyl)aminomethane (Tris) HCl (pH 6.8), and 0.05% bromophenol blue (2× substrate gel buffer). The homogenates were then centrifuged, and supernatants were applied directly to SDS-substrate gels (see below). For all other experiments, 0.5 g frozen tissue was homogenized in a mortar and pestle with 1 ml 30 mM Tris HCl (pH 7.6) and 10 mM CaCl2. After brief centrifugation, pellets were resuspended in 1.0 ml Tris or PBS buffer and analyzed for CL activity.

 Supernatants were collected and divided into separate aliquots for 1) determination of protein concentration, 2) analysis of enzyme and enzyme inhibitor activities in soluble assays, 3) immunoprecipitation with antisera against CL, GL, and TIMP followed by SDS–PAGE, or 4) visualization of proteolytic activities by electrophoresis on SDS-substrate gels.

**Organ culture.** Media conditioned by tissue specimens in vitro were obtained by organ culture. Tissue specimens stored in PBS on ice were minced in Dulbecco's modified Eagle's medium (DMEM) (supplemented with 10% fetal bovine serum [FBS]) in a sterile hood, blotted dry on gauze, and placed on sterile 0.45-μm Millipore filters. Filters were then placed on wire screens and set in Costar organ culture dishes to which 1.0 ml medium was added. Duplicate cultures were incubated at 37°C for 72 hours in either DMEM with 10% FBS or methionine-free DMEM with 10% FBS containing 50 μCi [35S]methionine. After incubation, organ culture media were removed, centrifuged, and frozen at −20°C until ready for processing.

Unlabeled media were thawed and analyzed for enzyme and enzyme inhibitor activities in soluble assays and by SDS-substrate gel electrophoresis as described below. Biosynthetically labeled secreted proteins present in culture media were either concentrated by precipitation by addition of 0.5% SDS and 0.02 M quinine sulfate followed by washing with 80% acetone and incubation with gelatin-linked Sepharose, or immunoprecipitated as described below.

**Biochemical Analysis**

**Determination of protein concentration.** The concentration of total protein in samples of tissue homogenates was determined by the method of Lowry, as modified by Peterson, with crystalline bovine serum albumin as the standard.
Soluble enzyme assays. Aliquots of supernatant from tissue homogenates and unlabeled organ culture media were either assayed directly or incubated first with trypsin at a final concentration of 10 µg/ml for 30 minutes at ambient temperature followed by addition of soybean trypsin inhibitor at 50 µg/ml. Samples were also activated by addition of APMA at a final concentration of 2 mM followed by incubation at 37°C for 3-4 hours.

Collagenase Activity. Vertebrate CL activity was determined before and after activation by the [14C]collagen fibril assay.18

Caseinase Activity. Nonspecific caseinolytic activity was determined before and after activation by the [14C]caseinase assay.18

Gelatinase Activity. Nonspecific gelatinolytic activity was determined before and after activation by the [14C]gelatinase assay.15

Collagenase inhibitory activity. Before determination of CL inhibitory activity, all samples were diluted with Tris/calcium buffer to yield a protein concentration of 1 mg/ml. Inhibition of vertebrate CL was determined by incubating samples with 0.1-1.0 unit CL, obtained from tissue plasminogen activator-treated rabbit synovial fibroblasts for 2-3 hours and then assaying for residual CL activity. One unit of CL inhibitory activity is defined as that amount of inhibitor giving 50% inhibition of 2 units CL.19

Sodium dodecyl sulfate—substrate gel analysis. SDS-substrate gels (Zymograms) were prepared as previously described.20 Type I gelatin, casein, or insoluble elastin was added to the standard Laemmli acrylamide polymerization mixture at a final concentration of 1 mg/ml. Aqueous or detergent extracts or unconcentrated organ culture media were mixed 3:1 with substrate sample buffer, and 5–10 µl was loaded immediately without boiling into wells of a 4% acrylamide Laemmli stacking gel apparatus (Idea Scientific, Corvallis, Ore.). Gels were run at 15–20 mA/gel at 4°C. After electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking for 30 minutes at ambient temperature, followed by rinsing and incubation at 37°C overnight in substrate buffer (50 mM Tris HCl [pH 8], 5 mM CaCl₂, and 0.2% NaCl). After incubation the gels were stained for 15–30 minutes in 0.5% Coomassie blue R-250 in a mixture of acetic acid/isopropanol/water (1:3:6), destained in water, photographed, and dried for permanent record.

Immunoprecipitation and sodium dodecyl sulfate—polyacrylamide gel electrophoresis. Unactivated tissue homogenate supernatants and organ culture media were immunoprecipitated with monospecific antiserum and analyzed by SDS-substrate gel electrophoresis or SDS-PAGE. Immunoaffinity chromatography.

Immunoprecipitation. Two hundred microliters of tissue sample or culture media was first preincubated with Zysorbin and centrifuged to remove protein A binding material, according to previously published procedures.18 The resulting supernatants were incubated for 20 minutes at 37°C with monospecific and nonimmune antisera, followed by incubation with Zysorbin at 0°C for 20 minutes. After centrifugation the pellets were washed two times with 10 mM Tris HCl (pH 8.2), 150 mM NaCl, 1 mM methionine, 0.5% Nonidet-P40, and 1 mg/ml ovalbumin followed by a final wash in the same buffer without ovalbumin. Biosynthetically labeled proteins were eluted from protein A–immunoglobulin G complexes by resuspension of the pellets in Laemmli21 sample buffer, followed by heating to 100°C for 3 minutes. Unlabeled proteins from both tissue extracts and culture media were eluted by resuspension of the pellets in substrate gel sample buffer (10% SDS, 4% sucrose, 0.25 M Tris HCl [pH 6.8], and 1% bromophenol blue), followed by incubation at 37°C for 5 minutes. Resuspended pellets were then centrifuged, and supernatants were analyzed by either SDS-PAGE or SDS-substrate gel electrophoresis.

Biosynthetically labeled culture media were also incubated for 5 minutes at ambient temperature with gelatin-linked Sepharose (BRL). After a brief centrifugation, the resin was washed twice with substrate gel buffer, and bound material was eluted by heating to 100°C in the presence of Laemmli sample buffer. Eluted proteins were then analyzed by SDS-PAGE, followed by autoradiography.

Sodium dodecyl sulfate—polyacrylamide gel electrophoresis. For analysis of biosynthetically labeled proteins, samples were electrophoresed on 10% polyacrylamide gels (acrylamide/bis, 29:1) according to the method of Laemmli,21 followed by autoradiography.

Immunohistochemical Analysis

Tissue specimens stored in PBS on ice were cut into approximately 1-cm² pieces and fixed overnight in 2.5% paraformaldehyde/PBS, pH 7.5. After fixation, samples were rinsed twice in cold PBS, placed in OCT for 30 minutes, and frozen at −70°C. Tissues were cryosectioned at 7 µm onto gelatin-coated slides. Sections were then stained with sheep anti-rabbit CL, rabbit anti-human GL, and sheep anti-human TIMP antibodies, as described above, which were used at dilutions of 1:100, 1:1,000, and 1:10, respectively, for 45 minutes at room temperature. Sections were then stained with a biotinylated goat anti-sheep or goat anti-rabbit second antibody (Vector Labs, Burlingame, Calif.), then treated with 0.3% H₂O₂ for 30 minutes to eliminate endogenous peroxidase activity. Sections were then stained with VectorStain ABC reagent (Vector Labs) for 30 minutes and then for 5 minutes with the substrate 3,3′-diaminobenzidine according to the manufacturer’s directions. Slides were photographed with a Zeiss photomicroscope at a magnification of ×200.

Results

Aqueous Extraction of Proteinases and Inhibitors

Tissue samples were first homogenized in aqueous buffered media, and the activities were quantified by
Table 1. Proteinase and Inhibitor Activities in Aortic Tissue Extracts

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Collagenase</th>
<th>Caseinase</th>
<th>Gelatinase</th>
<th>TIMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activities (μg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td>No.</td>
<td>Mean±SD</td>
<td>No.</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unactivated</td>
<td>7.0±5.6</td>
<td>2</td>
<td>54±5.6</td>
<td>1</td>
</tr>
<tr>
<td>Activated</td>
<td>49±5.0</td>
<td>2</td>
<td>156±...</td>
<td>1</td>
</tr>
<tr>
<td>Diseased</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unactivated</td>
<td>58±48</td>
<td>2</td>
<td>92±28</td>
<td>3</td>
</tr>
<tr>
<td>Activated</td>
<td>67±32</td>
<td>4</td>
<td>775±634</td>
<td>3</td>
</tr>
</tbody>
</table>

Proteolytic activities were determined by assaying duplicate samples of aqueous tissue extracts made from individual pathological or normal specimens. Values indicated are units of activity per gram of protein in extract. Means are values of activities from different individuals. SD is standard deviation of the mean. No. is number of tissue samples from different individuals. Normal and diseased samples were either assayed before (unactivated) or after (activated) incubation for 30 minutes at 37°C with 2 mM aminophenylmercuric acetate. ND, not determined; TIMP, tissue inhibitor of metalloproteinase.

use of native interstitial collagens, casein, and gelatin as substrates. The supernatants from these extracts had total protein concentrations ranging from 1 to 9 mg/ml, with an average of about 4 mg/ml (data not shown). The data in Table 1 were normalized to 1 mg/ml for comparison purposes.

The low number of normal aorta samples in our experiments precluded the possibility of accurately comparing differences between normal and diseased tissue proteolytic activities. However, the data in Table 1 indicate that the collagenolytic activities present in either normal or diseased aortic tissues were nearly 10-fold lower than caseinolytic and gelatinolytic activities in these same samples. In addition, the data show that proteinase zymogens may be present in these tissues because in every case, activation of aqueous extracts with APMA or trypsin resulted in an increase in activity.

Because proteinase species may not be adequately released from endogenous substrates by aqueous extraction and because previous workers had reported that CL-like activities were found exclusively in particulate suspensions of diseased vascular tissue, the resuspended tissue pellets of aqueous homogenates were assayed for CL activity. A maximum of 33 units/g was found in diseased aortas, consistent with the relatively low level of activity found in the cleared supernatants (data not shown).

Table 1 also indicates that the activity levels of tissue inhibitors (TIMP) present in these samples were several orders of magnitude less than proteinase activities. This finding suggests that either the actual concentration of TIMP was very low or the majority of inhibitor was bound to active proteinases and therefore not demonstrable by these procedures.

In summary, based on activities of aqueous tissue extracts, it appears that CL was not the major proteolytic species in these samples and that unbound tissue proteinase inhibitors were found at low levels.

Detergent Extraction of Proteinases

The anionic detergent SDS was used to extract both soluble and membrane- and/or substrate-bound enzyme species to confirm the results obtained with the use of aqueous buffer extraction and in vitro assays. It is known that proteinase activities measured by incubation of crude culture media or tissue homogenates with native substrates under in vitro assay conditions do not accurately reflect the concentrations of either active or proenzyme species due to the presence of variable concentrations of tissue inhibitors. We used the technique of SDS-substrate gel electrophoresis (zymography) to separate enzyme–inhibitor complexes while simultaneously characterizing proteinases according to their molecular weights.

Figure 1 shows the enzymatic profile of several different tissue homogenates by use of this technique. The samples shown in Figure 1 are representative of the nine pathological and two normal aortic tissues that we obtained. At least five distinct zones of...
substrate clearing were evident with the use of gelatin as a substrate. The molecular weights ($M_r$) of the major activities were estimated to be 250,000, 150,000, 92,000, 66,000, and 60,000. When casein was the substrate in the gel, broad zones of degradation were evident at approximate $M_r$ s of 150,000, 70,000–80,000, 40,000, and 35,000–38,000. Caseinolytic activity was also observed to migrate at the dye front and therefore, the molecular weight(s) of this activity were below $M_r$ 25,000.

Previous studies with the SDS-substrate gel system have demonstrated that enzyme concentrations are roughly proportional to the size and extent of substrate clearing and that gelatin is particularly sensitive to very small quantities of enzyme. Figure 1 shows that of the four aortic samples from diseased patients, all have large amounts of gelatinolytic activity in the region of $M_r$ 92,000. Normal sample B, however, contains relatively low levels of activity in this region. It is also evident that the two sharp zones of lysis at $M_r$ 60,000–66,000 are present in equal amounts in all but diseased sample C. The lower of these two bands in normal sample B is relatively weak.

For comparison, culture media taken from human dermal fibroblasts (Figure 1, lane A), which are known to secrete interstitial procollagenase at $M_r$ 55,000–60,000 and type IV CL at $M_r$ 66,000 show one major band at $M_r$ 66,000. A minor zone of lysis representing procollagenase can also be seen in sample A at $M_r$ 55,000; however, none of the aortic samples contained traces of this band.

In contrast to the results on gelatin gels, all diseased samples had approximately the same levels of caseinolytic zones at $M_r$ 70,000–80,000 (Figure 1). In addition, it is evident that the normal sample has much lower activity in this region. At least two samples (Figure 1, lanes C and F) had caseinolytic zones at $M_r$ 40,000–43,000.

To show which proteinase species were giving rise to the activities quantified by in vitro assay of aqueous tissue extracts (Table 1), these same extracts were electrophoresed on SDS-substrate gels. The profiles of activities observed were the same as those seen when detergent-extracted samples were analyzed by this technique (data not shown). Therefore, aqueous extraction of vascular tissue releases the same complement of proteinases in approximately the same ratios relative to SDS extraction.

Elastin was also used as the substrate during polymerization of the substrate gels (see "Methods"). Consistent with previous investigations with the SDS-substrate gel system, the extent of substrate clearing observed on elastin gels was not sufficient to allow accurate determination of individual proteinases; however, the zones of elastin degradation produced by proteinases from vascular tissue appeared to correlate well with the zones observed on casein gels (data not shown).

Figure 2. Zymograms showing characterization of proenzyme and active proteinase species from aortic aneurysms. Aliquots (10 μl) of aqueous tissue extracts made from three representative diseased aortas were either left untreated (u) or were activated (a) with 10 mM aminophenylmercuric acetate for 50 minutes at 37°C, then electrophoresed on gelatin (lanes A–C) and casein (lanes D–F) gels. Asterisks to the right of each gel indicate distinct zones of proteolytic activity. Molar weight markers (M.W.; $\times 10^3$) are shown to the left of each gel.

Characterization of Proenzyme and Active Enzymes

APMA-activated and untreated samples were electrophoresed on SDS-substrate gels to further identify and characterize the proteinases associated with vascular tissue. For clarity, the major zones of substrate degradation have been marked with asterisks to the left of each gel as shown in Figure 2. When gelatin was used as the substrate, at least five major zones of clearing could be identified in unactivated samples: $M_r$ 250,000, 120,000–150,000, 92,000, 75,000–85,000, and 66,000. In addition, a broad zone of gelatinolytic activity could be seen in lane B at $M_r$ 43,000–45,000 (Figure 2). Lanes A, B, and C on gelatin gels are equivalent to lanes D, E, and F on casein gels and represent separate extracts from three different patients.

It was evident that several of these zones consisted of multiple bands, and therefore it was of interest to see which were associated with precursors and which were generated by conversion of these precursors (proenzymes,zymogens, or "latent" enzymes) into active proteinases. The latter group of proteinases were the easiest to identify based on their appearance when samples were activated; zones at $M_r$ 75,000–80,000 and at 60,000 were included in this group (Figure 2). Precursors were more difficult to identify based on their disappearance on activation; the first three higher-molecular-weight zones may be included in this group.

There were five major lysis zones observed when vascular tissues were electrophoresed on casein gels: $M_r$ 200,000, 85,000, 75,000–80,000, 40,000–45,000, and 35,000–40,000 (Figure 2). Several of these zones were quite diffuse, and others were clearly resolved into two or three bands (Figure 2, lane E). The only
conversion product that could be positively identified on casein gels was the zone at $M_r$, 75,000–80,000 (Figure 2, lane D).

**Identification of Metalloproteinases and Serine Proteinases**

All enzyme activities giving rise to zones of lysis on gelatin gels were identified as metalloproteinases by incubating portions of the same gel with proteinase inhibitors (Figure 3). Both 1,10-phenanthroline and EDTA completely blocked the gelatinolytic activities extracted from vascular tissues, whereas the serine proteinase inhibitor PMSF had no effect (Figure 3, lane D). As a control, the serine proteinase, pancreatic elastase, was inhibited by PMSF by use of this technique (data not shown).

In contrast to the results obtained on gelatin gels, little if any of the caseinolytic zones were affected by divalent metal ions chelators. Zones at $M_r$, 75,000–80,000 and 55,000–70,000 appeared to be the most sensitive to PMSF; however, total inhibition of all zones was not possible. The results presented in Figure 3 are representative of five separate experiments with this technique (data not shown).

When rabbit brain capillary endothelial cell proteinases were electrophoresed along with those extracted from human aortas, complete inhibition of the rabbit enzymes was obtained on both gelatin and casein with 1,10-phenanthroline (data not shown), and this was consistent with the fact that the enzymes secreted by these cells were metalloproteinases.

**Secretory Products of Aortic Aneurysm Organ Cultures**

Although the vascular tissues obtained from patients after corrective surgery for aneurysmal disease were rinsed to remove blood and adherent serum proteins before extraction and analysis, it was possible that contamination occurred, and this would necessarily confuse the results of both the in vitro and the SDS-substrate gel analysis of proteinases. To positively identify the proteinases and their inhibitors that were synthesized by aneurysmal vascular tissue. The large amount of bovine serum albumin present in the culture media prevented the electrophoretic resolution of proteins between $M_r$, 50,000–93,000; however, the general distribution of biosynthetically labeled products was evident (Figure 4, lanes A and B). The secreted proteins shown in Figure 4 were synthesized by tissues excised from two different regions of the aorta; one sample was taken from the dilated region of the aorta and another from an undilated region, caudal to the aneurysm itself (see figure legend). A preliminary examination of the two profiles indicated...
small differences in the number and intensity of incorporated label.

Antisera against purified CL did not precipitate any biosynthetically labeled proteins from these aortic tissues in the region \( M_r 45,000-57,000 \) (Figure 4, lanes C and D), which is where human procollagenase and active CL migrate on SDS-polyacrylamide gels.\(^{22}\) As a control, we precipitated CL from other pathological organ culture media to ensure that the antisera were effective (data not shown). Similarly, precipitation of the media with preimmune serum showed negligible nonspecific proteins (data not shown).

A band at \( M_r 92,000 \) was readily observable in the precipitates from both organ cultures with anti-GL, with more proteinase present in the culture from undilated aortas (Figure 4, lanes E and F). Previous work with metalloproteinases found in the culture media taken from a variety of cells and tissues grown in vitro have shown that proteolytic activities were often regulated by proteinase inhibitors that were synthesized by the same cultures.\(^{12,14,20,24}\) The major proteinase inhibitor found in these studies was an \( M_r 28,500 \) glycoprotein called TIMP, which forms very high-affinity complexes \((K_i \leq 10^{-16})\) with active metalloproteinases. Precipitation of the media from dilated and undilated regions of aneurysmal aortas with the antisera against purified TIMP shows a large amount of protein centered at approximately \( M_r 30,000 \) (Figure 4, lanes G and H). Examination of the profile of total secreted proteins in these media show that TIMP accounts for approximately 50% of all radiolabeled proteins.

When labeled media from the organ cultures were incubated with gelatin-linked Sepharose, the gelatin-binding proteins eluted from the resin showed major bands that comigrated with bands precipitated with anti-GL IgG (Figure 4, lanes I and J). Other major gelatin-binding proteins had \( M_r 68,000, 50,000, 38,000-40,000, \) and 30,000. The undilated tissue sample appeared to secrete more gelatin-binding protein at \( M_r 92,000 \) than did the dilated sample.

To show that the proteinase activities observed on SDS-substrate gels were similar to those found in the

Figure 5. Photomicrographs showing localization of proteinase and inhibitor in normal aortas (upper panel) and aortic aneurysms (lower panel) by immunoperoxidase staining. Sections of paraformaldehyde-fixed vascular tissue from normal aortas and aortic aneurysms were incubated with anti-gelatinase immunoglobulin G (IgG) (GL), anti-collagenase IgG (CL), anti–tissue inhibitor of metalloproteinase IgG (TIMP), or preimmune serum (Pre-Imm Serum), followed by washing and incubation with biotinylated rabbit anti-sheep IgG and finally by avidin-linked horseradish peroxidase as described in “Methods.” Stained sections were then viewed by light microscopy, and photomicrographs were divided into lumina–media and media–adventitia to facilitate viewing. Magnification, ×320.
culture media from aneurysmal aortas, both aqueous extracts and organ culture media were compared on SDS-substrate gels. Most of the gelatinolytic zones present in extracted tissue were also present in organ culture media, with the notable exception of the zone at $M_r 75,000-80,000$ (data not shown). Because this zone was identified as a possible conversion product after activation of some higher-molecular-weight precursor (see Figure 2, lanes A and C), the data indicate that organ cultures secrete proteinasezymogens that are not activated under the conditions of these experiments. Caseinolytic zones produced by organ culture media were also the same as those observed in tissue extracts, but organ cultures degraded far less substrate.

Finally, to demonstrate which of the proenzymes and their activated forms corresponded to the GL identified in the media from organ cultures, both aqueous extracts and unlabeled organ culture media were immunoprecipitated with the same anti-GL antiserum used in the experiments shown in Figure 4, and the precipitated proteins were eluted and then electrophoresed on gelatin gels. The anti-GL antiserum removed proteinases from both aqueous extracts and organ culture media that produced lysis zones on gelatin gels at $M_r 92,000$; however, an additional zone at $M_r 75,000-85,000$ was present in the immunoprecipitate from aqueous extracts (data not shown).

**Immunohistochemical Analysis**

Normal and diseased aortas were stained with the anti-CL, anti-GL, and anti-TIMP antibodies, as described in the “Methods” section. Typical staining patterns of the normal aorta were easily elicited for GL and TIMP (Figure 5A). GL was localized predominantly in the adventitia, whereas TIMP was found both in the media and adventitia, particularly in the vasa vasorum. CL could not be detected by this method.

Diseased aortas showed strong staining of all three regions of the vessel wall with the anti-GL antibody (Figure 5B). The vasa vasorum was strongly delineated by this antibody. TIMP was localized in the luminal region of the wall, adjacent to the atheromatous plaque, and in the adventitia.

**Discussion**

**Detergent Versus Aqueous Buffer Extraction**

The complement of proteinases present in both normal and aneurysmal aortic tissue vary in number, molecular weight, and substrate specificity. It was found that aqueous extraction of homogenized vascular tissue yielded the same number, size, and type of proteinases as did extraction with the denaturing detergent SDS. This allowed extensive characterization of these enzymes, as well as their quantification by conventional substrate incubation assays. **Quantification and Characterization of Proteolytic Activities**

Connective tissue proteinases in biologic fluids and tissues may be present in one of several different compartments and functional pools depending on their state of activation, substrate binding, and interaction with inhibitors. Measurement of enzymatic activity in tissue homogenates represents perhaps the least accurate method of determining enzyme concentration because of the complexity of these regulatory mechanisms.

A major finding of this report is that CL activity could not be found in significant amounts in either normal aortas or aortic aneurysms at this stage of the disease. This lack of activity was found not to be due to masking of active CL by tissue inhibitors such as TIMP, in contrast to our earlier studies of cells grown in culture. The average CL activities found in vascular tissues are negligible relative to the activities found in other pathological tissues, such as rheumatoid joints, diseased gingiva, or malignant tumors. This is particularly noteworthy because previous workers have implicated CL as one of the major proteinases responsible for aneurysm formation and rupture. Our findings suggest that at the stage of aneurysm development where corrective surgery is indicated, CL production appears either to have already ceased or not yet begun at the level of de novo enzyme synthesis. Whether CL secretion and activation heralds the imminence of aortic rupture remains to be shown.

The low levels of "collagenase" activities found in these samples (see Table 1) may be the result of several different enzymes. The fact that previous workers have found increased levels of CL activities to be present within the aortic aneurysm wall suggests that the method used for assaying interstitial CL inadvertently measured other proteolytic activity(s). For instance, it is possible that very low concentrations of CL provide the initial cleavage of collagen fibrils and that these become substrates for GL and possibly caseinase as well. Alternatively, the background level of denatured collagen present in the assay may also yield these results, supporting the idea that there are no CLs present in these samples.

With substrate incubation assays, caseinase and GL were found at high levels, the average activities of which were several times higher than those of CL. This observation is consistent with the analysis of aortic tissue by SDS-substrate gel electrophoresis and indicates that the major substrates of the proteinases present in aortic tissue may be a modified product of the native types I and III collagen fiber, a collagen type other than interstitial collagen, or other connective tissue components. In this respect, it is of interest that the crosslinking defects documented in patients with AAA may provide excellent substrates for the GL found at high levels in these tissues.

Caseinolytic activity is a nonspecific marker for all known proteinases. Most serine and metalloprotein-
ases are efficient caseinases; for instance, pancreaticase and neutrophil elastase, as well as macrophage elastase, rapidly degrade casein.

Casein degradation is used to monitor the purification of various proteoglycan and type IV collagen-degrading neutral proteinases found in tissues undergoing repair and in the media of connective tissue cells grown in culture. Two specific metalloproteinases, stromelysin and type IV CL, have been extensively characterized at the molecular level. Both of these enzymes are efficient caseinases, with molecular sizes in the range of those observed on substrate gels of aortic tissue. The results of our inhibition studies shown in Figure 3 suggest that the residual zones of lysis left on casein gels after incubation with PMSF may be metalloproteinases; however, positive identification awaits further investigation.

The molecular sizes of the major caseinolytic enzymes are in the range of plasmin/plasminogen and both urokinase and tissue plasminogen activator and their respective conversion products. These enzymes are known to be involved in extracellular matrix degradation and may contribute significantly to tissue destruction in AAA.

The high levels of both active and trypsin-activatable caseinase found in aneurysmal aortas suggest that matrix degradation is an ongoing process characteristic of these tissues (Table 1 and Figures 1-3). Elastase-like activities have been found at increased levels in aortic aneurysms compared with those from aortic tissue from patients with atherosclerotic occlusive disease. It is possible that the caseinolytic activity measured in the present study is equivalent to the elastase-like activity measured by previous workers, and therefore the data presented are consistent with the idea that bothzymogen and activated proteinases capable of degrading elastin are elevated in aortic aneurysms relative to the normal aorta.

GLs are frequently found in situations where interstitial CLs have been identified and have been shown to act synergistically with interstitial CL to effect type I and III collagen degradation. There are two well-characterized gelatin-binding metalloproteinases found in human tissues. One, which is found in the neutrophil secretory granule and which is referred to as "neutrophil gelatinase," has high specificity for denatured interstitial collagens and native type V collagen. This enzyme is found in at least three molecular weight forms: M, 225,000, 130,000, and 92,000. The latter two forms are found on reduction of sulphydryl groups and/or on serine proteinase exposure. These isoforms of neutrophil GL have also been found in several other cell types and tissues, including fibroblasts, capillary endothelial cells, macrophages, and bone cultures. The fact that neutrophil GL is found at high levels in AAA suggests an ongoing tissue repair process.

The other gelatin-binding proteinase, called type IV CL, has recently been characterized at the molecular level. This enzyme is one member of the family of metalloproteinases that shares sequence homology with interstitial CL and stromelysin. Expression of these enzymes is correlated with tumorogenic transformation, and type IV CL has been implicated in the tumor invasion process as the key activity required for migration across the basement membrane.

Type IV CL is secreted as a zymogen of M, 72,000 and is activated to an M, 66,000 form by organonemical trypsin. It is likely that the bands observed on gelatin gels (Figures 1-3) from aortic aneurysms at these molecular weights represent type IV CL. Although this possibility remains speculative pending positive identification with specific antisera, a highly suggestive finding is the elution profile of the gelatin-linked resin shown in Figure 4. It is clear that a gelatin-binding protein of approximately M, 66,000 is synthesized and secreted by aneurysm tissue (Figure 4, lanes I and J).

**Implications of Proteinase and Inhibitor Location in Aortic Tissue**

Theories of the pathogenesis of AAA disease remain controversial. Histopathological studies of the disease show that focal inclusions of a basophilic-staining material accumulate between degraded elastic and fibromuscular layers of the media. These lesions, referred to as "cystic medial necrosis," suggest the presence of ongoing tissue repair and/or inflammatory processes. Active proteinases such as CLs, GLs, and elastases are usually found at high levels in such areas. We found that neutrophil GL and TIMP were concentrated in focal patches in the intima-media; however, interstitial CL could not be identified by any method. The significance of these findings and their relation to cystic medial necrosis await further investigation.

Previous studies of metalloproteinases secreted from stimulated capillary endothelial cells grown in vitro have shown that enzymatic activities are regulated very effectively by TIMP, which is a major secretory product of these cells. We found that TIMP was synthesized and secreted in large quantities by aortic aneurysm tissue, representing approximately 50% of the total protein secreted by organ cultures. However, as Table 1 indicates, the amount of TIMP activity present in aortic tissue was several orders of magnitude below the level of GL measured in the same tissue. From the high synthetic rate of TIMP in these tissues, it is possible that this inhibitor is rapidly escaping from its localization in the vessel wall. Alternatively, TIMP may be bound to active metalloproteinases in tissue extracts, making it unavailable for detection by soluble inhibition assays.

As demonstrated by the recent purification of TIMP from bovine aortic smooth muscle cells, this low-molecular-weight inhibitor is most likely synthesized in large amounts by the vascular media. Consistent with our immunolocalization data, TIMP may be released into the general circulation from the vasa vasorum, where it also appears to be synthesized.

Observations by previous workers that trauma such as laparotomy can induce aneurysm rupture postop-
eratively stimulated much interest in the possible involvement of systemic enzymes, activators, and inhibitors that may rapidly modulate aortic wall degradation. Several recent reports have shown that alterations in both systemic and tissue elastase-like activity are present in patients with AAA. By analogy to the pathogenic mechanisms observed in cigarette smokers who develop emphysema of the lung, the ratios of elastase-like activity to α-1-protease inhibitor activity was significantly higher in aortic tissue from patients with AAA compared with those with atherosclerotic occlusive disease. Although such studies implicate aortic elastin metabolism as a major factor in the pathogenesis of AAA, they neglect the possibility that other connective tissue proteinase and inhibitor systems may contribute significantly to this process. Our results show that the activities of the “classical elastases,” that is, those of the neutrophil and the pancreas, are negligible in aortic tissue from AAA patients. However, recent reports of elastase secretion by atherectomy tissue suggest that local synthesis of elastase may contribute to the pathogenesis of AAA (J. Cohen, personal communication).

Forty years ago, aortic aneurysms were thought to be caused by ischemic lesions of the medial wall as a result of either atherosclerotic or thrombotic occlusion of the vasa vasorum or of the vessels feeding the vasa. Other workers have suggested that infrarenal AAAs develop because this portion of the human aorta lacks adequate nutritional support owing to a scarcity of medial vasa vasorum. Regardless of the cause, once medial wall weakness has established that there is a genetic predisposition to connective tissue abnormalities may develop AAAs in the presence of atherosclerotic occlusive disease.

The relation between AAA and atherogenesis has been debated for many years. It is now well established that there is a genetic predisposition to arterial aneurysm formation, which may be related to a systemic connective tissue disorder. Although the risk factors for these two diseases are different, there are two pathological features associated with atherosclerotic disease that may contribute to aneurysm formation: 1) Lipid accumulation and deposition may alter the synthesis and structural integrity of native connective tissue proteins, predisposing to matrix degradation and vessel deformation/dilation. 2) The autocrine and paracrine factors secreted by invading monocytes and stimulated medial cells may diffuse into an area where cells comprising the inner limit of the vasa vasorum are located, inducing neovascularization and its associated increase in proteolytic activity.

Alternatively, initial deformation and dilation resulting from hemodynamic forces may cause ischemia and stimulate vasa proliferation. In light of the well-documented neovascular response in atherosclerosis, patients with a genetic predisposition to connective tissue abnormalities may develop AAAs with a systemic connective tissue disorder. 42,52

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KEY WORDS • neutrophil gelatinase • tissue inhibitor of metalloproteinase • vasa vasorum • neovascularization • aortic aneurysms • pathogenesis
Connective tissue proteinases and inhibitors in abdominal aortic aneurysms. Involvement of the vasa vasorum in the pathogenesis of aortic aneurysms.
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doi: 10.1161/01.ATV.11.6.1667

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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