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Collagen Degradation in the Abdominal Aneurysm

A Conspiracy of Matrix Metalloproteinase and Cysteine Collagenases

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Growth and rupture of abdominal aortic aneurysms (AAAs) result from increased collagen turnover. Collagen turnover critically depends on specific collagenases that cleave the triple helical region of fibrillar collagen. As yet, the collagenases responsible for collagen degradation in AAAs have not been identified. Increased type I collagen degradation products confirmed collagen turnover in AAAs (median values: <1, 43, and 108 ng/mg protein in control, growing, and ruptured AAAs, respectively). mRNA and protein analysis identified neutrophil collagenase [matrix metalloproteinase (MMP)-8] and cysteine collagenases cathepsin K, L, and S as the principle collagenases in growing and ruptured AAAs. Except for modestly increased MMP-14 mRNA levels, collagenase expression was similar in growing and ruptured AAAs (anterior-lateral wall). Evaluation of posttranslational regulation of protease activity showed a threefold increase in MMP-8, a fivefold increase in cathepsins K and L, and a 30-fold increase in cathepsin S activation in growing and ruptured AAAs. The presence of the osteoclastic proton pump indicated optimal conditions for extracellular cysteine protease activity. Protease inhibitor mRNA expression was similar in AAAs and controls, but AAA protein levels of cystatin C, the principle cysteine protease inhibitor, were profoundly reduced (>80%). We found indications that this secondary deficiency relates to cystatin C degradation by (neutrophil-derived) proteases. ***(Am J Pathol 2007, 170:809–817; DOI: 10.2353/ajpath.2007.060522)***

Abdominal aortic aneurysm (AAA) is a common pathology and a major cause of death because of rupture.[1,2] The hallmark pathology of AAA is a persistent proteolytic imbalance that results in excess matrix destruction and progressive weakening of the arterial wall. A number of matrix metalloproteinases (MMPs) (in particular the gelatinases MMP-2 and -9)[1,3] have been implicated as primary proteolytic culprits in the disease, but it is dubious whether these proteases are directly responsible for the weakening and ultimate failure of the aortic wall. Biomechanical studies invariably show that the mechanical stability of the arterial wall essentially relies on fibrillar collagens in media and adventitia.[4–6] These structural collagens are highly resistant toward proteolytic degradation, and the only mammalian proteases that have been shown to cleave the native triple helical region of fibrillar collagen are the classic collagenases of the MMP family[7] [ie, MMP-1, -8, and -13 and the membrane type-1 MMP (MT-1 MMP or MMP-14[8])], as well as selected members of the cysteine protease family (ie, cathepsin K,[9,10] L,[11] and possibly S[12]).

Several reports indicate expression of these collagenases in AAA on an individual basis, but comparative data regarding expression of the collagenases, and their possible relationship to rupture of the aneurysm,[13,14] are not available. Moreover, available studies[15] do not address the critical and complex posttranslational regulation of protease activity that involves controlled secretion of an inactive proenzyme, activation of the proenzyme, and rapid inhibition of protease activity by specific and nonspecific inhibitors.

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To characterize collagenases involved in AAA growth and to test whether rupture is associated with increased collagenase expression, we used an integrated approach. We first confirmed excess fibrillar collagen turnover in AAA and ruptured AAA wall samples through quantification of collagen degradation products and next established mRNA expression profiles of the MMP and cysteine collagenases by semiquantitative real-time polymerase chain reaction (RT-PCR). Because this approach does not provide information on the post-transcriptional regulation of protease activity, we quantified tissue expression of specific inhibitors of proteases activity and applied specific protease activity assays and Western blot analyses to address the post-translational regulation of protease activity.[16,17] Data from this study characterize members of the cysteine protease family, cathepsin K, L, and S, along with neutrophil collagenase (MMP-8), as the primary collagenases in AAA and ruptured AAA.

Materials and Methods

Patients

Tissue from the anterior-lateral aneurysm wall was obtained during elective surgery for asymptomatic AAA (>5.5 cm or larger, growing AAA) or during emergency surgery (ruptured AAA). Aortic patches removed along with the renal artery during kidney explantation from brain-dead, heart-beating, adult organ donors were used as controls. Samples were immediately halved. One half was snap-frozen in CO_2-cooled isopentane or liquid N_2 and stored at −80°C for later analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensen's solution, 120 hours), and paraffin-embedded for histological analysis. Sample collection and handling was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center, Leiden, The Netherlands.

RNA Isolation and Real-Time Competitive LightCycler PCR

RNA isolation and semi quantitative mRNA analysis using real-time competitive LightCycler PCR (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) were performed following protocols detailed by Lindeman and colleagues.[17] All mRNA data were normalized on basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Tissue Homogenization

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in lysis buffer [10 mmol/L Tris, pH 7.0, 0.1 mmol/L $CaCl_2$, 0.1 mol/L NaCl, and 0.25% (v/v) Triton X-100]. This protocol releases both soluble as well as membrane-bound proteases. Samples were subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C, snap-frozen, and stored at −80°C until use. Homogenates were normalized on the basis of their protein content (Pierce, Rockford, IL).

Collagen Degradation Assay

Collagen turn-over was assessed by the CTX assay for type I collagen degradation (Serum Cross laps; Nordic Biosciences, Milsbeek, The Netherlands). This assay is based on the detection of a neo-epitope that is released on cleavage of the GVG/L peptide bond in the C-terminal telopeptide of α2(I)-chain of mature type I collagen.

Specific Immunocapture MMP and Cathepsin Activity Assays

MMP-1, -8, -9, -13, and -14 (MT1-MMP) activity assays (Amersham Biosciences, Buckinghamshire, UK) were performed according to the suppliers' recommendations. In short, target proteases were captured by an immobilized specific antibody on microtiter plates, and the proteolytic activation of a modified proenzyme by the captured protease was used to quantify the protease activity.[17] MMP activity was quantified using recombinant MMP as standard. These assays have been shown to allow sensitive and specific assessment of active MMP as well as pro-MMP (on activation of latent MMP by a mercuric salt (*p*-aminophenylmercuric acetate) in *in vitro* systems. Conversely, assessment of active MMP in more complex samples such as tissue homogenates is generally hampered by rapid inactivation of active proteases because of the high levels of endogenous protease inhibitors that are present during initial sample preparation. Indeed, preliminary studies failed to indicate active MMPs in both aneurysmal and normal aortic wall homogenates; hence, only latent MMP activity (ie, on *p*-aminophenylmercuric acetate activation of the captured proenzyme) was assessed.

Cathepsin K activity was measured by a novel activity assay, based on the same principle as the MMP assays.[17] We developed a similar assay for assessment of cathepsin S activity; however, because of dissociation of the cathepsin S-cystatin C complex in the incubation steps required in the test, this assay measures both active cathepsin S as well as cathepsin S that was previously bound to cystatin C (cystatin C-complexed cathepsin S). Costar Stripwell plates were coated (2 hours, 37°C) with 1 μg/ml cathepsin S-specific monoclonal antibody (TNO-1503). This antibody does not cross-react with cathepsin K (<0.5%), L (0%), or V (<0.1%) and does not interfere with the enzyme activity. Purified cathepsin S (Calbiochem, Merck Biosciences, Darmstadt, Germany) or sample in binding buffer (20 mmol/L HEPES, 1 mmol/L ethylenediaminetetraacetic acid, and 0.1% Triton X-100, pH 6.5) were incubated for 16 hours at 4°C. Plates were subsequently washed four times with capture buffer (20 mmol/L HEPES, pH 6.5), and captured active cathepsin S was quantified through activation of a modified prourokinase variant (UKcatS) in detection buffer (20 mmol/L HEPES, 1 mmol/L ethylenediaminetetraacetic acid, and

Table 1. Patient Characteristics

	AAA (n = 17)	Ruptured AAA (n = 15)	P value
Age (years, mean ± SD)	72.4 ± 6.2	72.5 ± 9.9	P = ns
Male/female	14/3	13/2	P = ns
AAA diameter (cm, mean ± SD)	6.9 ± 1.3	7.7 ± 1.4	P = ns
Location AAA			
Infrarenal	11	11	P = ns
Juxtarenal	3	4	
Suprarenal	3	0	
Smoking			
Never	7	8	P = ns
Stopped	7	5	
Current	3	2	

ns, not significant.

0.1% Triton X-100, pH 8.5). Cathepsin S activation of the proenzyme was quantified using a chromogenic peptide substrate (S-2444). Cathepsin S activity was calculated from a standard curve using recombinant enzyme, and expressed in ng/ml. Thresholds for cathepsin K and S activity assays were 0.001 and 0.05 ng/ml, respectively.

Western Blot Analysis

Western blot analysis was used to quantify protease-inhibitor complexes. Preliminary analysis showed that the primary antibodies used in the analysis allowed analysis of both pro- and active forms of the respective proteases and that the standard denaturing conditions required for Western blot analysis resulted in full dissociation of MMP-8-TIMP-1 and cathepsin-cystatin C complexes, thus indicating that the analysis allows assessment of MMP-8-TIMP and cathepsin K-, L-, and S-cystatin C complexes.

Western blot analyses for these proteases as well as for cystatin C and TIMP-1 were performed as described in Kleemann and colleagues,[18] using the following antibodies: anti-human cathepsin K (IM55L; Calbiochem, Breda, The Netherlands), anti-cathepsin L (AF952; R&D Systems, Abingdon, UK), anti-cathepsin S (sc-6505; Santa Cruz Biotechnology, Heerhugowaard, The Netherlands), anti MMP-8 (MAB3316; Chemicon, Chemicon Europe, Ltd., Chandlers Ford, UK), anti-cystatin C (sc-16989; Santa Cruz Biotechnology), and anti-TIMP-1 (Ab8229; Chemicon). All samples were normalized on the basis of total actin [anti-actin (sc-1615; Santa Cruz Biotechnology)] levels. All secondary antibodies were obtained from Santa Cruz Biotechnology. Immunoblots were visualized and quantified using Super Signal West dura extended duration substrate (Perbio Science, Etten-Leur, The Netherlands), LabWorks 4.6 software and the luminescent image workstation (UVP, Cambridge, UK).

Immunohistochemistry

Immunohistochemistry was performed using 4-μm deparaffinized, ethanol-dehydrated tissue sections. Sections were incubated overnight with a polyclonal antibody against MMP-8 (Medix Biochemica, Milsbeek, The Netherlands) or by a polyclonal antibody against the 100-kd transmembrane subunit of human osteoclast v-H^+ATPase (a generous gift from Dr. M.A. Harrison, School of Biochemistry and Molecular Biology, University of Leeds, Leeds, UK).[16] Conjugated biotinylated anti-goat or rabbit anti-IgG were used as secondary antibodies. Sections were stained with Nova Red (Vector Laboratories, Burlingame, CA) and counterstained with Mayer hematoxylin. Controls were performed by omitting the primary antibody.

In Vitro *Cystatin C Degradation*

Putative cystatin C degradation by MMP and serine proteases was evaluated *in vitro*. To that end, human cystatin C (5 ng/ml; Biovendor, Brno, Czech Republic) was incubated (24 hours, 37°C) with preactivated MMP-9 (0.3 ng/ml; Invitek, Leusden, The Netherlands), preactivated MMP-8 (0.3 ng/ml; Chemicon) or the serine protease neutrophil elastase (0.1 ng/ml; Calbiochem) in a 50 mmol/L Tris, pH 7.5, buffer containing 1.5 mmol/L NaCl, 1 μmol/L Zn^+, 0.5 mmol/L Ca^{2+}, 0.01% Brij, and 2 E/ml heparin, and remaining cystatin C was quantified by Western blotting (see above).

Statistical Analysis

mRNA expression (Ct values) was compared by Student's *t*-test or Wilcoxon-Mann-Whitney *U*-test. Results of activity assays and Western blots were analyzed by the Wilcoxon-Mann-Whitney *U*-test to compare the different groups. Putative correlations between cystatin C protein concentrations and cysteine protease mRNA and protein expression were analyzed by Pearsons' test. P values <0.05 were considered significant.

Results

Patients

Baseline clinical characteristics of the patients are provided in Table 1. Two of the AAA patients and two of the ruptured AAA patients had aneurysms elsewhere and one of the AAA patients had a family history of AAA. Because of national regulations, clinical data, other than

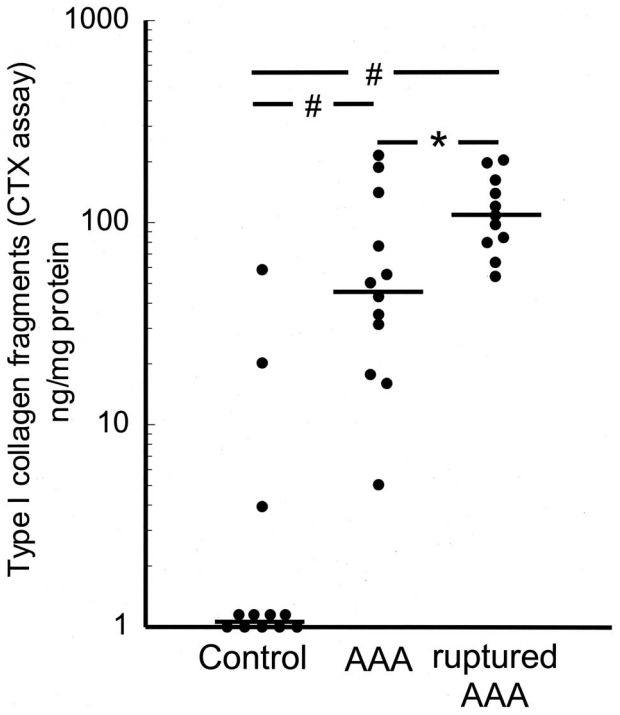

Figure 1. Collagen degradation (CTX assay for collagen type I degradation) in aortic wall samples of control aorta, growing AAAs, and ruptured aneurysms (ruptured AAAs). $^{*}P < 0.02$; $^{\#}P < 0.001$.

sex and age, was not available for the controls; however, all donor organs were considered eligible for transplantation. The median age of the donors was 48 years (range, 27 to 76 years) and 45% were male.

Increased Collagen Turnover in AAA and Ruptured AAA

Sharply increased type I collagen carboxyterminal telopeptide fragments [CTX enzyme-linked immunosorbent assay (ELISA)] in AAA (AAA versus controls, $P < 0.001$; Figure 1) and a further increase in ruptured AAA (ruptured AAA versus AAA, $P < 0.02$) confirmed increased fibrillar collagen degradation in aneurysmal disease.

mRNA Expression Profiles of MMP and Cathepsin Collagenases and Their Inhibitors

Normalized mRNA expression of MMP collagenases (MMP-1, -8, -13, -14), the gelatinase MMP-9 (an MMP gelatinase that is frequently associated with AAA), cysteine collagenases, and the endogenous inhibitors of protease activity is shown in Figure 2, A and B, and the normalized (GAPDH = 0) number of amplification cycles (Ct values) is provided in Table 2. Our findings confirm prominent expression of MMP-9 in AAA as well as in ruptured AAA ($P < 0.01$; Figure 2A, Table 2); however, with the sole exception of a modest increase in MMP-14 expression in ruptured AAA ($P < 0.05$), mRNA expression of MMP collagenases was similar and low in all three study groups (Figure 2A, Table 2). Expression of the

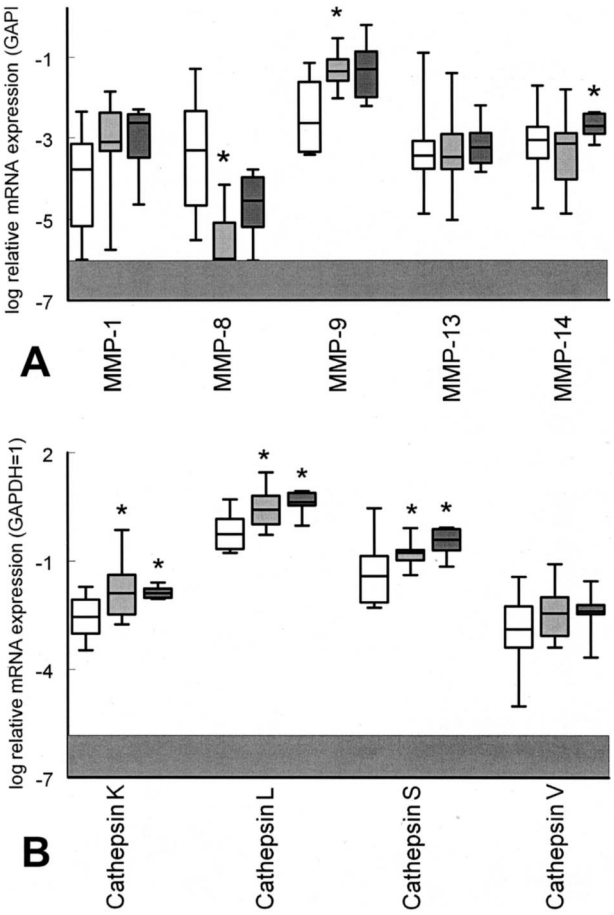

Figure 2. A: Relative mRNA expression (GAPDH = 1) of MMP collagenases and the gelatinase MMP-9 in the infrarenal aorta of controls, growing AAAs, and ruptured aneurysms. $^{*}P < 0.05$ versus controls. **B:** Relative mRNA expression (GAPDH = 1) of the cysteine collagenases in the infrarenal aorta of controls, growing AAAs, and ruptured AAAs. $^{*}P < 0.05$ versus controls.

cysteine collagenases cathepsin K, L, and S, on the other hand, was equally increased in both AAA and ruptured AAA ($P < 0.04$; Figure 2B, Table 2). Cathepsin V mRNA expression was similar and low in all groups. mRNA expression for the tissue inhibitors of MMP (TIMPs) as well as cystatin C, the cognate inhibitor of cysteine protease activity, is shown in Table 2. Expression of TIMP-1 and -3, the most prominently expressed TIMPs in the aortic wall, was similar and high in all study groups, whereas a moderate increase in TIMP-2 expression was observed in ruptured AAA ($P < 0.05$). Cystatin C expression was similar and high in all three study groups.

Protease Activity Assays and Western Blot Analysis

Posttranslational regulation of protease activity was evaluated by specific protein activity assays and Western blot analysis. Preliminary studies did not indicate direct MMP activity in the tissue homogenates (ie, activities below the detection limit of the respective assays (1.4, 5.0, 2.7, 8.1, and 0.2 ng/ml for MMP-1, -8, -9, -13, and -14, respectively); hence only latent (pro) forms (ie, on activation of

Table 2. Normalized (GAPDH = 0) ΔCt Values for MMP and Cathepsin Collagenases, MMP-9, and Their Inhibitors in Control Aorta, AAA, and Ruptured AAA

	Control (n = 11)	AAA (n = 17)	Ruptured AAA (n = 15)
MMP-1	14.2 [7.8 to 20.0]	9.7 [5.3 to 13.6]	9.4 [6.6 to 20]
MMP-8	11.9 [4.3 to 20]	15.9 [13.4 to 20.0]*	16.5 [12.6 to 20.0]*
MMP-9	7.7 (3.4)	4.0 (1.4)*	4.9 (1.8)*
MMP-13	11.5 (4.4)	10.1 (2.3)	9.9 (2.2)
MMP-14	10.1 (2.8)	10.7 (2.7)	9.4 (1.0)*
Cathepsin K	7.9 (2.3)	5.8 (2.3)*	6.4 (1.3)*
Cathepsin L	0.4 (1.9)	−1.6 (2.2)*	−2.3 (0.9)*
Cathepsin S	4.0 [−1.5 to 7.6]	2.2 [0.3 to 4.0]*	1.4 [−1.3 to 3.9]*
Cathepsin V	9.3 (3.5)	8.0 (2.4)	8.4 (2.1)
TIMP-1	−1.6 (1.6)	−2.7 (2.0)	−2.2 (0.9)
TIMP-2	5.7 (1.0)	4.2 (1.3)	4.9 (0.6)*
TIMP-3	1.5 (1.1)	1.6 (2.4)	2.0 (1.1)
Cystatin C	−3.1 (1.2)	−3.1 (1.1)	−3.1 (1.3)

Mean (SD) is in parentheses and median (range) is in brackets. Ct values represent the number of amplification cycles required before reaching a predefined threshold in the real-time PCR. All values were normalized on basis of duplex measurement of GAPDH expression. Normalized Ct values (ΔCt values) inversely relate to the mRNA expression (ie, a negative ΔCt value indicates expression exceeding GAPDH expression, whereas high Ct values indicate low mRNA expression). A ΔCt value of 20 reflects the detection limit of the assay (40 cycles).

*$P < 0.05$ versus control.

captured latent MMPs) of the respective MMP collagenases were assessed. MMP-9 activities were included as positive control. Activation of captured latent MMP proteases revealed prominent MMP-8 [350 (161 to 622) ng eq/mg protein versus 275 (56 to 1361) ng eq/mg protein, median, (range); P = ns] and MMP-9 [84 (15 to 334) ng eq/mg protein versus 118 (68 to 1484) ng eq/mg protein in AAA and ruptured AAA, respectively, P = ns] activities, as well as marginal MMP-13 [37 (21 to 49) ng eq/mg and 34 (21 to 44) ng eq/mg protein, P = ns] proenzyme expression in AAA and ruptured AAA, respectively. Activities for MMP-1 remained below the detection threshold of the assay.

Immunohistochemical analysis of MMP-8 expression (Figure 3) showed that MMP-8 expression was primarily confined to infiltrating neutrophils, thus accounting for the apparent discrepancy between minimal MMP-8 mRNA expression and abundant pro-MMP-8 activities.

Unlike MMPs, cysteine protease are not readily activated by small molecular compounds; hence the cathepsin K activity assay only allows analysis of active cathepsin K. Analogous to assays for active MMP, the cathepsin

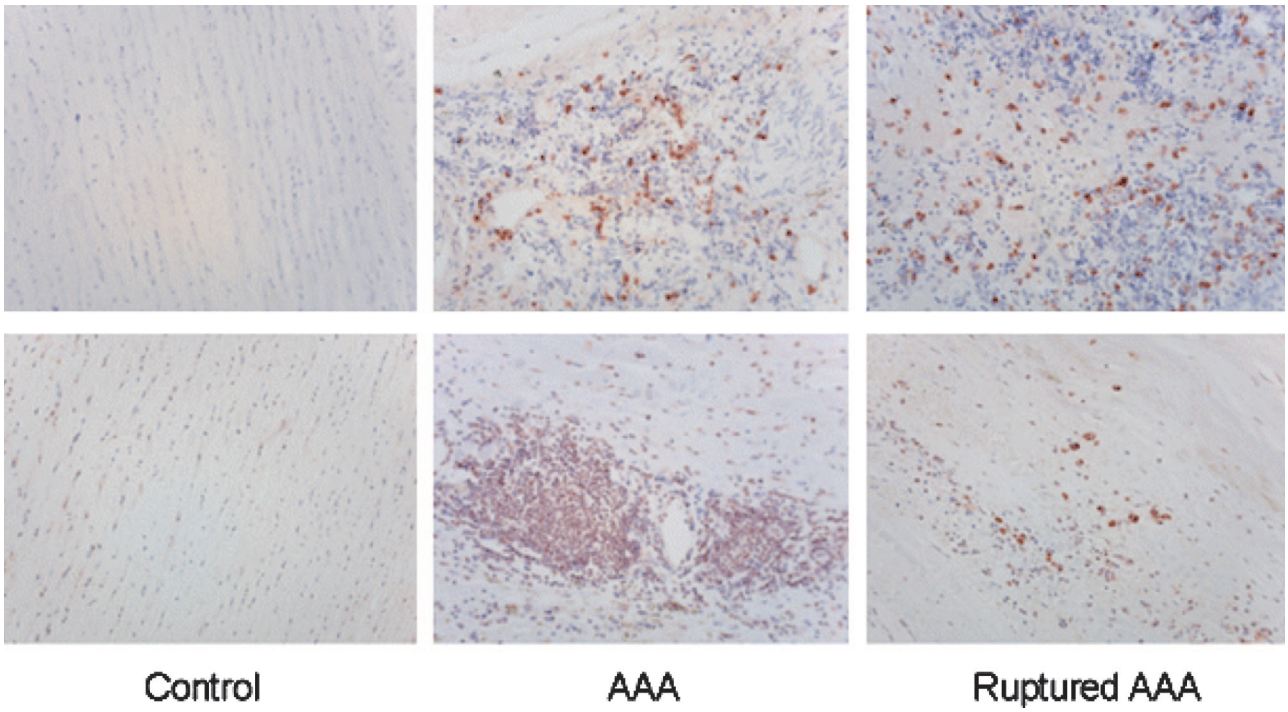

Figure 3. Immunohistochemical staining of MMP-8 (top) and the osteoclastic proton pump v-H^+-ATPase (bottom) in normal control aorta, AAA, and ruptured AAA. MMP-8 is expressed in infiltrating neutrophils, whereas v-H^+-ATPase is primarily expressed in monocytes/macrophages and to a lesser extent in smooth muscle cells.

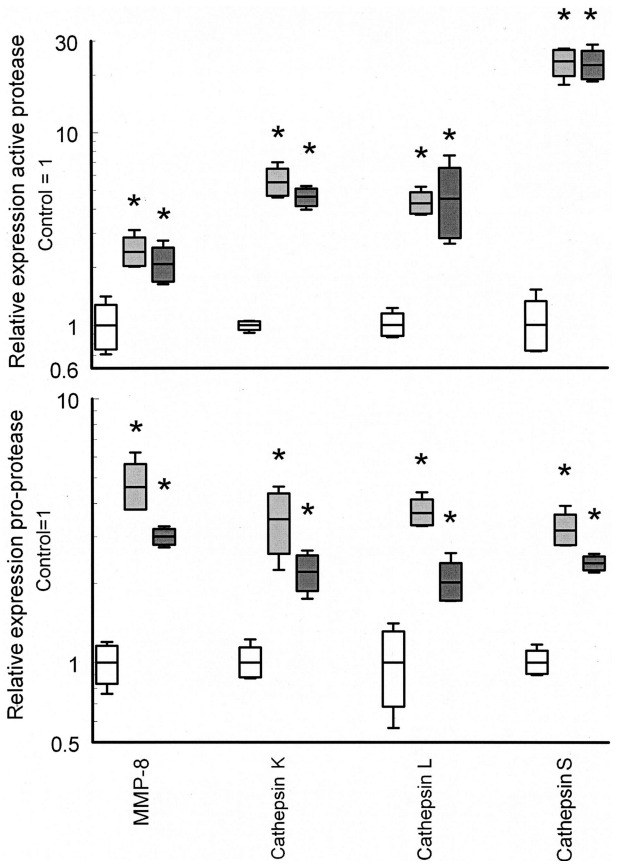

Figure 4. Increased expression of the activated forms of the collagenases MMP-8, cathepsin K, L (24- and 28-kd bands[1]), and S in growing AAAs (**light gray boxes**) and ruptured aneurysms (**dark gray boxes**) compared with control aorta (**white boxes**). $^{*}P < 0.05$ versus controls.

K activity assay did not reveal net cathepsin K activity in AAA and ruptured AAA (detection threshold 0.001 ng/ml). We used a novel assay for the quantification of cathepsin S activity; however, do to the dissociation of cathepsin S-cystatin C complex in the assay, this assay measures both active cathepsin S as well as cystatin C-complexed cathepsin S. We found significant activities for cathepsin S in both AAA and ruptured AAA [37.3 (16.3 to 78.4) ng eq/mg protein and 49 (11.6 to 93.4) ng eq/mg protein (median ranges) in AAA and ruptured AAA, respectively; P = ns].

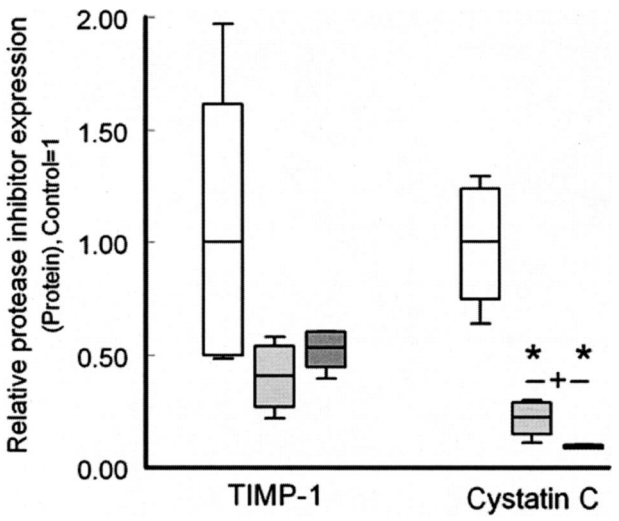

Figure 5. Relative protein expression of the principal inhibitor of MMP activity (TIMP-1) and of cystatin C, the principal inhibitor of cysteine protease activity in control infrarenal aorta (**white boxes**), growing AAAs (**light gray boxes**), and ruptured AAAs (**dark gray boxes**). $^{*}P < 0.05$ versus controls; $^{+}P < 0.05$ AAA versus ruptured AAAs.

Although detectable cathepsin S activities and absent MMP and cathepsin K activity may identify cathepsin S as the primary collagenase in AAA and ruptured AAA, these results mostly likely reflect the rapid inactivation of active proteases by excess endogenous protease inhibitors during the preparation of the tissue homogenates. It was reasoned that formation of protease-inhibitor complexes critically depends on preceding protease activation and that quantification of these complexes thus provides an indirect means of establishing preceding protease activation. We performed Western blot analysis for pro and active forms of MMP-8, cathepsin K, L, and S that indeed showed strongly increased activation of these proteases in growing AAAs and ruptured AAAs (Figure 4). No differences were found between growing AAAs and ruptured AAAs.

Extracellular cathepsin K and L activity and stability critically depends on formation of an acidic pericellular microenvironment. In osteoclasts such an environment is created by the osteoclastic *trans*-membranous proton pump (v-H^{+}-ATPase). Expression of this proton pump in cysteine protease-expressing mononuclear cells and smooth muscle cells in AAA and ruptured AAA (Figure 3) shows that conditions required for extracellular cathepsin K and L activities are present in aneurysmal disease.

TIMP-1 and Cystatin C Protein Expression

Protein expression of TIMP-1, the principal TIMP in the arterial wall, as well as expression of cystatin C was evaluated by Western blot analysis. Data in Figure 5 suggest a trend toward lower TIMP-1 protein expression in AAA and ruptured AAA; however, this difference did not reach significance ($P < 0.1$). Cystatin C protein levels were significantly reduced in growing AAA ($P < 0.05$) and further ruptured AAA ($P < 0.05$ versus growing AAA). Decreased cystatin C protein levels along with abundant cystatin C mRNA expression in AAA suggests that the decreased cystatin C levels in AAA are secondary and may relate to increased cystatin C consumption as result of increased cysteine protease activity or alternatively may reflect increased cystatin C catabolism. We found no indication for an association between reduced cystatin C protein levels and cysteine collagenase mRNA or protein expression (data not shown). However, the observed inverse relationship between active MMP-8 and cystatin C protein levels ($r = -0.78$, $P < 0.05$) in growing AAA suggests that cystatin C deficiency is secondary and may result from proteolytic degradation by MMP-8 or other neutrophil-derived proteases. Indeed, *in vitro* experiments showed that cystatin C is degraded by various neutrophil-derived proteases such as MMP-8 and the serine protease neutrophil elastase, and to a lesser extend by MMP-9 (Figure 6).

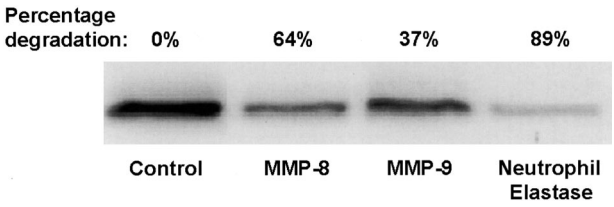

Figure 6. Cystatin C degradation by neutrophil proteases MMP-8, MMP-9, and neutrophil elastase *in vitro*. Cystatin C and respective proteases were incubated for 24 hours in a 100:1 mol/mol ratio.

Discussion

Biomechanical[4–6] and clinical studies[19] demonstrate that the mechanical strength of the vascular wall relies on the structural collagen network in the media and adventitia. Increased collagen turnover that is not adequately matched by collagen deposition is held responsible for the growth and ultimate rupture of AAA.[1] However, the proteases responsible for the increased collagen turnover have not been identified. Load-bearing collagens within the arterial wall are predominantly type I/III fibrillar collagens that are highly resistant toward proteolysis. Degradation of these collagens critically depends on the action of specific collagenases that are able to destabilize the triple helix of native fibrillar collagen.[20] Destabilized collagen helices can than be further degraded by less specific proteases such as the gelatinases MMP-2 and -9, and the stromelysins MMP-3 and -10. Hence, degradation of collagen matrix in arterial wall primarily dependent on the initial action of specific collagenases (ie, the classic MMP collagenases as well as selected members of the cysteine protease family).

Several reports indicate expression of MMP as well as cysteine collagenases in AAA on an individual basis,[21–26] but these studies[15] are not quantitative and do not address the important posttranslational regulation of protease activity, which involves controlled activation of the inactive proenzyme and subsequent inactivation by specific and nonspecific endogenous inhibitors.[16,27] Moreover, the possible involvement of increased collagenase activity[13,14] as the underlying cause of rupture has not been addressed in detail.

To confirm increased collagen turnover in AAA and to identify candidate collagenases involved in AAA growth and possible rupture, we used an integrated approach that involved evaluation of collagen degradation, expression of all MMP and cysteine collagen-degrading enzymes, and the posttranslational regulation of protease activity. Putative increases in aortic wall collagen turnover[1] were evaluated by the Serum Crosslaps ELISA. This ELISA specifically detects C-telopeptide fragments that are released on proteolytic cleavage of native type I fibrillar collagen. Sharp increases in C-telopeptide fragments in AAA wall samples, and an even further increase in wall samples of ruptured AAA, confirms increased fibrillar collagen degradation in AAA and corroborates earlier observations of increased collagen degradation in ruptured AAA.[13,14]

To identify collagenases responsible for the excess collagen degradation, we first explored mRNA expression of the classic collagenases (namely the MMP collagenases, MMP-1, -8, -13, and -14) by semiquantitative real-time PCR. We included expression of MMP-9, a gelatinase that is prominently expressed in AAA, as the positive control. Findings from the mRNA analysis confirmed prominent expression of MMP-9 in AAA[3] and ruptured AAA. With the exception of a modest increase in MMP-14 expression in the ruptured AAA, analysis did not indicate increased MMP collagenase mRNA expression in growing AAA or ruptured AAA. We used specific immunocapture-protease activity assays to validate the MMP mRNA data. These activity assays have been shown to allow quantification of active proteases[16] and, after *in vitro* activation of the latent MMPs, assessment of the pool of pro-MMP.[16] Direct assessment of MMP collagenases (ie, active enzymes) did not reveal detectable protease activity in the tissue homogenates (all activities were below the detection threshold of the assay). Although this finding may indicate that all collagenases present are in their inactive, latent form, it most likely reflects a technical limitation when assessing protease activity in complex biological samples such as tissue homogenates. Under such conditions, high levels of endogenous inhibitor will rapidly inactivate any active protease present, thus resulting in the absence of detectable protease activity.

Findings for the latent (pro) MMPs (ie, on *in vitro* activation of the latent proteases) primarily paralleled findings from mRNA analysis and indicated significant expression of proMMP-9 but only minimal expression of the collagenases pro-MMP-1 and -13. Indicating that the absolute expression of MMP-1 and -13 in AAA[23–25] is low, suggesting that their contribution to collagen degradation in growing and ruptured AAA is limited. Prominent pro-MMP-8 activities sharply contrast with minimal MMP-8 mRNA expression, and our activity data actually put MMP-8 on par with MMP-9 as the most prominently expressed MMP in AAA. Neutrophil MMP-8 is a stored secondary granule protein that is transiently expressed during the late myeloid maturation pathway of neutrophils.[28,29] Immunohistochemical analysis confirmed MMP-8 abundance in growing and ruptured AAA and showed that MMP-8 is predominantly expressed in infiltrating neutrophils, thus accounting for the apparent discrepancy between MMP-8 mRNA and protein expression.

The activity assays did not indicate net MMP-8 activity. Failure to detect any appreciable MMP-8 activity most likely relates to inactivation of active proteases by excess endogenous protease inhibitor during preparation of tissue homogenates. This notion is supported by our observation of increased MMP-8 inhibitor complexes (Western blot analysis) in growing and ruptured AAA. Formation of these complexes critically depends on protease activation, and assessment of protease-inhibitor complexes thus provides a means of establishing preceding protease activation. We validated Western blot analysis as a means of quantifying protease inhibitor complexes and found abundant expression of the active 28-kd MMP-8 form in growing and ruptured AAA, thus showing that

MMP-8 activation had occurred in AAA and ruptured AAA.

Although the MMP-collagenases are referred to as the classic collagenases, it is now apparent that selected members of the cysteine family of proteases are involved in remodeling of the collagen matrix as well.[30] Extracellular activities of cathepsin K and L have been recognized as critical factors in bone turnover[31] and endothelial stem cell trafficking,[32] and evidence from animal studies identifies cathepsin K and S as critical factors in remodeling of the atherosclerotic plaque.[33] Sharply increased C-telopeptide fragments (CrossLaps ELISA) in aortic wall samples of AAA and an even further increase in ruptured AAA show that the cysteine proteases are also involved in collagen degradation in growing and ruptured AAA.[34] We analyzed mRNA expression of the cysteine proteases cathepsin K, L, S, and V[35] as well as expression of cystatin C, the cognate endogenous inhibitor of extracellular cysteine protease activity. In contrast to the data for the MMP-collagenases, this analysis indicated clear increases in mRNA expression of cathepsin K, L, and S in both AAA as well as ruptured AAA. Again, no apparent differences were found between growing and ruptured AAA. Activation[36,37] and stability of cysteine proteases cathepsin K and L critically relies on an acidic pericellular environment.[38–40] In osteoclasts such a microenvironment is created by a transmembraneous proton pump v-H^+-ATPase.[41] We performed immunohistochemical staining for this osteoclastic v-H^+-ATPase[17] and found abundant expression of this proton pump in infiltrating mononuclear cells and to a lesser extend in the vascular smooth muscle cells in growing and ruptured AAA, indicating that the optimal conditions required for pericellular cysteine protease activity may indeed exist in aneurysmal disease.

We used novel specific activity assays based on the same principle as the MMP activity assays to evaluate cathepsin K[17] and S activities in AAA and ruptured AAA. Akin to the MMP activity assays, the cathepsin K activity assay did not indicate net cathepsin K activity, but we did observe significant cathepsin S activity in growing AAAs and ruptured AAAs in the cathepsin S activity assay. Abundance of activated cathepsin K by Western blot analysis shows that cathepsin K activation occurs in AAA and indicates that failure to detect active cathepsin K in the activity assay presumably reflects inactivation of active cathepsin K by the endogenous inhibitors during preparation of the tissue homogenates. Abundant cathepsin S activities in the novel cathepsin S assay may identify cathepsin S as the principal collagenase in AAA and ruptured AAA; however, we found indications that observed cathepsin S activities relate to dissociation of the cathepsin S-cystatin C complex during the washing steps required in the cathepsin S activity assay, an effect that is not seen in MMP and cathepsin K activity assays.

Reported deficiencies in cystatin C, the principle inhibitor of extracellular cysteine protease activity[42,43] in AAA may amplify the role of the cysteine proteases. It was postulated that these deficiencies occur at the transcriptional level and relate to transforming growth factor-β deficiency.[42] However, our data point to a different mechanism. Reduced protein levels, albeit similar cystatin C mRNA expression, along with the inverse relationship between tissue MMP-8 and cystatin C levels and our *in vitro* data showing that cystatin C is degraded by various neutrophil-derived proteases such as neutrophil elastase and MMP-8, suggest that cystatin C deficiency in AAA is secondary and may relate to cystatin C degradation by neutrophil-derived proteases. Such a mechanism is not known for cystatin C, but a similar gain of function mechanism[44] has previously been described for the serpin α1-proteinase inhibitor.[45] Eliason and colleagues[46] recently showed that neutrophil depletion inhibits aneurysm formation in the elastase model of aneurysm formation but also observed that, although neutrophils are critical for the process of aneurysm formation in this model, their contribution is independent of MMP-8 (as well as of MMP-2 and -9). Neutrophil-mediated cystatin C degradation may well explain the putative prominent role of neutrophils in the process of aneurysm formation.

In conclusion, our results confirm excess collagen degradation in AAA and ruptured AAA and identify MMP-8 and the cysteine proteases cathepsin K, L, and S that are expressed along with the osteoclastic proton pump v-H^+-ATPase as the principle collagenolytic culprits in AAA. Our findings confirm and extend findings from Wilson and colleagues[47] but do not indicate increased MMP or cysteine collagenase expression in the anterior aneurysmal wall as the cause of rupture,[47,48] yet we cannot exclude that local increases in cysteine collagenase activities at the site of rupture contribute to rupture of the aneurysm. Reduced cystatin C protein expression along with increased collagen degradation products in the anterior aneurysmal wall of ruptured aneurysms points to an alternative mechanism and suggests that protease inhibitor deficiency rather than increased protease expression may contribute to AAA rupture. Pharmaceutical inhibition of cysteine protease activity[49] and/or manipulation of neutrophil activation[50] may provide a pharmaceutical means of stabilizing AAA.[51]

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