 Activation of Matrix-Degrading Metalloproteinases by Mast Cell Proteases in Atherosclerotic Plaques

Jason L. Johnson, Christopher L. Jackson, Gianni D. Angelini, Sarah J. George

Abstract—Recent studies suggest that mast cell–derived neutral proteases can activate matrix-degrading metalloproteinases (MMPs). We have investigated the role of the mast cell proteases tryptase and chymase in the activation of MMPs in human carotid endarterectomy specimens (atherosclerotic, n=32) and postmortem carotid arteries (control, n=17). In vitro degranulation of mast cells in atherosclerotic carotid arteries by compound 48/80 caused a significant increase in MMP activity. Addition of the nonselective tryptase inhibitor antipain, the specific trypsinlike protease inhibitor 4-amidinophenylmethanesulfonyl fluoride, and the chymase inhibitor chymostatin reduced this increase in MMP activity by 30±6%, 23±6%, and 9±2%, respectively. Immunocytochemistry identified significantly higher numbers of tryptase-containing cells (mast cells) and cells expressing MMP-1 and MMP-3 in the “shoulder” regions of atherosclerotic artery lesions compared with the tunica media of control arteries. Dual immunocytochemistry showed collocaltion of MMP-1 and MMP-3 with mast cells in the shoulder regions. Degranulation was observed in 78±5% (mean±SEM) of mast cells in this area, whereas nonactivated mast cells were observed in all other areas. In situ zymography revealed caseinolytic and gelatinolytic activity in these areas. In conclusion, in vitro mast cell degranulation, which releases mast cell proteases, in carotid arteries increases MMP activity. Furthermore, elevated MMP-1 and MMP-3 expression is collocated with increased numbers of degranulated mast cells and with greater MMP activity in the shoulder regions of atherosclerotic plaques. Activation of MMPs by mast cell–derived proteases may be an important mechanism in atherosclerotic plaque destabilization. (Arterioscler Thromb Vasc Biol. 1998;18:1707-1715.)

Key Words: atherosclerosis ■ mast cells ■ plaque rupture ■ matrix metalloproteinase ■ tryptase

Atherosclerosis is a complex disease characterized by smooth muscle cell proliferation and migration, cholesterol deposition, infiltration of mononuclear cells, and extracellular matrix degradation.1,2 Myocardial infarction and stroke are commonly caused by the rupture of atherosclerotic plaques, a process that often occurs at the “shoulder” regions of the plaque.3–5 Smooth muscle cells, macrophage-derived foam cells, T lymphocytes, and endothelial cells are present in the shoulder regions, and all have been shown to synthesized matrix-degrading metalloproteinases (MMPs).6–11 Recent studies have shown increased expression of interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and gelatinase B (MMP-9) in the shoulder regions of atherosclerotic plaques.12–14 The MMP family is capable of degrading all of the components of the extracellular matrix in blood vessels, and therefore, these enzymes are normally tightly regulated. In addition to regulation by their endogenous inhibitors, the tissue inhibitors of metalloproteinases and by transcriptional regulation, the activity of MMPs is also regulated by the necessity for proteolytic activation of secreted proenzyme forms. Proteolytic activation of pro-MMPs has been achieved in vitro by using trypsin, chymotrypsin, plasma kallikrein, plasmin, thermolysin,15 chymase, and tryptase.16 Furthermore, MMPs can activate each other; eg, activated MMP-3 can activate MMP-1.17 Mast cells play pivotal roles in many biological responses, including hypersensitivity reactions, inflammation, tissue remodeling, and angiogenesis, and they are involved in the pathogenesis of chronic degenerative diseases such as asthma and rheumatoid arthritis.18 In addition, the observation of elevated numbers of mast cells in the shoulder regions of atherosclerotic plaques compared with normal arteries has led to the suggestion that mast cells may be involved in the development of atherosclerotic lesions.19–22 Mast cells produce a range of soluble mediators, including histamine, heparin, leukotrienes, cytokines, growth factors, and neutral proteases. All mast cells in blood vessels have been shown to express the neutral protease tryptase, and ~40% of them also contain another neutral protease, chymase.20,23 These enzymes serve as specific markers for mast cells.24 Chymase can directly degrade components of the basement membrane, including collagen types IV and V, laminin, fibronectin, and elastin.25 Tryptase can degrade pericellular matrix proteins such as fibronectin.26 These proteases also activate pro-
TABLE 1. Effect of Mast Cell Degranulation by Compound 48/80 on Protease Activity

<table>
<thead>
<tr>
<th>Protease</th>
<th>Control 48/80</th>
<th>Atherosclerotic 48/80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptase</td>
<td>2.4 ± 0.5</td>
<td>4.9 ± 1.4</td>
</tr>
<tr>
<td>Chymase</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>MMP</td>
<td>6.3 ± 0.8</td>
<td>5.7 ± 0.3</td>
</tr>
</tbody>
</table>

Chymase and trypsin activity is expressed as mmol/L nitroaniline per hour per gram (control, n = 5; atherosclerotic, n = 5), and MMP activity is expressed as mmol/L activated MMP-2 per minute per gram (control, n = 3; atherosclerotic, n = 4). Results are stated as mean ± SEM.

*Indicates significant difference from unactivated control.

MMPs: trypsin activates pro–MMP-3 and chymase activates both pro–MMP-1 and pro–MMP-3.16,27 Therefore, release of trypsin and chymase from mast cells could initiate an amplification cascade whereby pro–MMP-1 and pro–MMP-3 are activated, which in turn activate other MMPs, including MMP-1, MMP-2, and MMP-9, leading to matrix degradation, plaque instability, and possibly, plaque rupture.

Although active MMPs have been shown to be present in the shoulder regions of atherosclerotic plaques,12,13 the way in which they become activated is unknown. In these studies, we have examined the effect of mast cell degranulation on MMP activation in the human atherosclerotic internal carotid artery.

Methods

Reagents
All materials were purchased from Sigma except for those stated below. Unconjugated mouse monoclonal anti-human mast cell tryptase antibody (AA1), normal goat serum, biotinylated goat anti-mouse and goat anti-rabbit IgG, and horseradish peroxidase–labeled swine anti-rabbit antibodies were purchased from Dako. Avidin/biotin blocking kit, β-galactosidase–conjugated avidin D, and nuclear fast red were purchased from Vector Laboratories. Mouse monoclonal anti-human chymase antibody was purchased from Biogenes. Resorufin-labeled universal protease substrate and ZnCl2, and 0.05% Brij 35, pH 7.5) for up to 4 hours at 37°C. Increase in fluorescence was monitored every 30 minutes by using an excitation wavelength of 328 nm and an emission wavelength of 393 nm. Recombinant human MMP-2 was activated with 1 mmol/L p-aminophenylmercuric acetate (APMA) and used to establish standard curves of the rate of increase in fluorescence per minute (n = 6).

The rate of increase in fluorescence per minute in the samples was expressed as nanomoles per liter of activated MMP-2 per gram wet weight. MMP activity was inhibited by the addition of standard MMP inhibitors (20 mmol/L EDTA, 100 μmol/L Ro 31-9790). The role of proteases in MMP activation was examined by the addition of protease inhibitors: 100 μmol/L EDTA, 100 μmol/L Ro 31-9790, 1 μmol/L aprotinin, 40 μmol/L APMSF, 1 μmol/L aprotinin, 10 μmol/L E-64, or 1 μmol/L APMSF.

TABLE 2. Effect of Protease Inhibitors on MMP Activity After Compound 48/80 Mast Cell Degranulation in Atherosclerotic Arteries

<table>
<thead>
<tr>
<th>Protease Inhibitor</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (n = 4)</td>
<td>62 ± 11*</td>
</tr>
<tr>
<td>Ro 31-9790 (n = 4)</td>
<td>100 ± 0*</td>
</tr>
<tr>
<td>Antipain (n = 6)</td>
<td>30 ± 6*</td>
</tr>
<tr>
<td>APMSF (n = 4)</td>
<td>23 ± 6*</td>
</tr>
<tr>
<td>Chymostatin (n = 4)</td>
<td>9 ± 2*</td>
</tr>
<tr>
<td>Aprotinin (n = 4)</td>
<td>24 ± 20</td>
</tr>
<tr>
<td>E-64 (n = 4)</td>
<td>23 ± 17</td>
</tr>
<tr>
<td>Pepstatin A (n = 4)</td>
<td>13 ± 7</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM percent inhibition of MMP activity in the presence of the protease inhibitors compared with noninhibited controls.

Bristol Healthcare National Health Service Trust Ethics Committee (reference E3111).

Tryptase, Chymase, and MMP Activity Assays
Carotid endarterectomy specimens and postmortem carotid arteries were chopped into 1-mm squares and added to 0.15 mol/L Tris-HCl, pH 7.6. To half of the samples 5 μg/mL compound 48/80, a specific mast cell degranulation agent,26 was added, and they were incubated for 15 minutes at 37°C to induce mast cell degranulation. All samples were microfuged at 13 000 rpm for 3 minutes and the supernatant decanted. The supernatant was assayed in the trypsin, chymase, and MMP assays.

Tryptase and chymase activities of the supernatant were assayed by using the chromogenic substrates N-benzoyl-D,L-arginine-p-nitroanilide and N-succinyl-L-phenylalanine-p-nitroanilide, respectively.26 In brief, 100 μL of supernatant was added to 0.9 mmol/L N-benzoyl-D,L-arginine-p-nitroanilide in trypsin buffer or 0.2 mmol/L N-succinyl-L-phenylalanine-p-nitroanilide in chymase buffer. The rate of change in absorbance was measured over 10 minutes at 410 nm for trypsin activity and overnight at 405 nm for chymase activity. With the use of a nitroaniline standard, curves of the absorbance at 405 and 410 nm were calculated, and the trypsin and chymase activities detected in the samples were expressed as millimoles per liter of nitroaniline per hour per gram wet weight. To test specificity, trypsin activity was inhibited with 100 μg/mL antipain (a nonselective trypsin inhibitor) and 40 μg/mL APMSF (a specific trypsinlike protease inhibitor), and chymase activity was inhibited with 100 μg/mL chymostatin (a chymase inhibitor). The percent inhibition of enzyme activity for each protease inhibitor was calculated.

The MMP activity of the supernatant was measured in a fluorometric assay with the use of a fluorescein substrate that is susceptible to degradation by all MMPs.29 Supernatant (300 μL) was incubated with 1 mmol/L 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Leu-(2,4-dinitrophenylamino)-Ala-Ala-Arg-NH2 in MMP assay buffer (100 mmol/L NaCl, 100 mmol/L Tris, 10 mmol/L CaCl2, 20 μmol/L ZnCl2, and 0.05% Brij 35, pH 7.5) for up to 4 hours at 37°C. Increase in fluorescence was monitored every 30 minutes by using an excitation wavelength of 328 nm and an emission wavelength of 393 nm. Recombinant human MMP-2 was activated with 1 mmol/L p-aminophenylmercuric acetate (APMA) and used to establish standard curves of the rate of increase in fluorescence per minute (n = 6).

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pepstatin. The rate of MMP activity in the presence of these inhibitors was calculated per milligram wet weight and was expressed as a percentage of the compound 48/80–degranulated control.

**Immunocytochemistry**

Immunocytochemistry using monoclonal anti-human mast cell tryptase antibodies was used to identify the location of the mast cells and determine the number of degranulated mast cells. In brief, serial 3-μm-thick paraffin sections were dewaxed and rehydrated, and endogenous peroxidase activity was inhibited with 3% H₂O₂. Sections were permeabilized by incubation in 0.1% calf thymus trypsin and 0.1% CaCl₂ for 10 minutes at 37°C. Sections were blocked with nonimmune goat serum and then incubated with monoclonal anti-human mast cell tryptase antibodies (diluted 1:50), followed by biotinylated goat anti-mouse IgG (diluted 1:200), and then horseradish peroxidase–labeled ExtrAvidin (diluted 1:400). Color was developed by incubation in 0.05% 3,3′-diaminobenzidine, and sections

**Figure 1.** Location of mast cells in atherosclerotic and control arteries: a, fibrous cap; b, shoulder region; and c, adventitia of an atherosclerotic plaque stained for mast cell tryptase. d, Intima; e, media; and f, adventitia of a control carotid artery stained for mast cell tryptase. Positive cells stained brown (which appear black and are marked by arrows), whereas cytoplasm of negative cells remained unstained. Nuclei are stained blue (which appear grey). Scale bar in A represents 25 μm and applies to all panels.
were counterstained with hematoxylin before mounting. Tryptase-positive cells (mast cells) were stained brown with blue nuclei, whereas negative cells had blue nuclei only. A negative control for which the anti-trypase antibody was substituted with nonimmune mouse IgG was always included. Mast cells in the fibrous cap, shoulder, and adventitial regions of atherosclerotic arteries and in intimal, medial, and adventitial regions of control arteries were counted in three 0.25-mm² fields and expressed as a percentage of the total number of nucleated cells. Mast cells with tryptase-positive granules observed as “halos” in their immediate pericellular vicinity were classified as degranulating. Immunocytochemistry for tryptase and chymase protein was also carried out on 5-μm-thick frozen sections in a similar manner to that described above. The number of tryptase-and-chymase–containing mast cells and the number of tryptase-only–containing mast cells were determined.

Immunocytochemistry for MMP-1 and MMP-3 was carried out in a similar manner to that described for mast cells. The primary and secondary antibodies were substituted with rabbit anti-human MMP-1 or MMP-3 antisera (diluted 1:500 and 1:2000, respectively) and biotinylated goat anti-rabbit IgG (diluted 1:400). The color substrate was 3-amin-9-ethylcarbazole. Negative controls, for which the rabbit antisera were preabsorbed with 100 ng of the appropriate peptide, were always included. To demonstrate the specificity of the rabbit anti–MMP-1 and –MMP-3 antisera, Western blots using purified recombinant human MMP-1, MMP-2, MMP-3, and MMP-9 were performed. The numbers of MMP-positive cells in the same regions as examined for mast cells were scored on a 4-point scale, where 0 = no positive cells; 1 = <10% positive cells; 2 = 10% to 50% positive cells; and 3 = >50% positive cells.30 The reproducibility of assessments was tested for interobserver variation by using an unweighted k test (κ = 0.75).

Dual immunocytochemical labeling of mast cells and MMPs was carried out by using an indirect 2-stage method. Sections were dewaxed, rehydrated, and permeabilized, and endogenous peroxidase activity was inhibited as described above. Mast cells were detected with monoclonal anti-human mast cell tryptase antibodies as described above, with 3,3′-diaminobenzidine as the chromogenic substrate, yielding a brown product. MMP-1 and MMP-3 were detected as described above with the addition of 2 drops of avidin blocking solution per milliliter of goat serum and 2 drops of biotin blocking solution per milliliter of primary antibody. Peroxidase-labeled ExtrAvidin was substituted with β-galactosidase avidin D, and 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranosidase was used as the chromogen, yielding a green-blue product. Sections were counterstained with nuclear fast red.

**Figure 2.** Identification of chymase-and-tryptase–containing and tryptase-only–containing mast cells. Representative immunocytochemistry of serial frozen sections of the shoulder region of an atherosclerotic plaque for mast cell chymase (A) and mast cell tryptase (B). Black color indicates positive protein staining, whereas nuclei stained blue (which appear grey). Arrows in A and B indicate the 6 mast cells containing both chymase and tryptase, whereas B contains 6 tryptase-only–containing mast cells. Scale bar in A represents 50 μm and applies to both panels.

In Situ Zymography

Caseinolytic and gelatinolytic activity was located in atherosclerotic (n = 12) and control (n = 8) carotid arteries by in situ zymography as described previously.31 In brief, for caseinolytic in situ zymography, 8-μm-thick frozen sections were applied to slides coated with 1 mg/mL Resorufin-labeled universal protease substrate in incubation medium (50 mmol/L Tris, 50 mmol/L NaCl, 10 mmol/L CaCl₂, and 0.05% [wt/vol] Brij 35, pH 7.6) supplemented with 1% (wt/vol) agarose. Sections were covered in incubation medium and incubated.
Frozen segments of atherosclerotic (n=14) and control (n=13) carotid arteries were crushed under LN₂, and proteins were extracted as described previously. In brief, the frozen samples (~100 mg) were crushed under LN₂ with a mortar and pestle and added to 400 μL of extraction buffer (2 mmol/L NaCl, 100 μmol/L Tris, 1 μg/mL aprotinin, and 1 μmol/L PMSF). Total MMP-1 and MMP-3 (the pro-form, active form, and tissue inhibitor of metalloproteinase–bound MMP) levels were quantified with sandwich-based ELISA systems according to the manufacturer’s instructions. Tissue extracts from atherosclerotic (n=14 for MMP-1, n=10 for MMP-3) and control (n=13 for MMP-1, n=8 for MMP-3) carotid arteries were diluted to fall within the linear range of the assays. Samples were assayed in duplicate. Owing to the varying histological composition, size, and wet weight of the samples, the protein concentration of all tissue extract samples was determined by using a bicinchoninic acid protein assay with BSA as the standard. The concentrations of MMP-1 and MMP-3 were expressed per microgram of total protein.

**Statistical Analysis**

Values are expressed as mean±SEM. Mean values in the MMP, tryptase, and chymase activity assays were compared with controls by using the 1-sample Student’s t test. Mean values of MMP-1– and MMP-3–positive cell scores of atherosclerotic and control arteries were compared with the Mann-Whitney U test for nonparametric data. Mean values for MMP-1 and MMP-3 protein levels and the numbers of mast cells in atherosclerotic and control arteries were compared by using Student’s t test for unpaired data. Differences were considered statistically significant when P≤0.05.

**Results**

**Effect of Mast Cell Degranulation on Tryptase, Chymase, and MMP Activity**

Incubation with compound 48/80 caused tryptase activity to increase significantly (4-fold, P=0.04) in atherosclerotic carotid arteries (n=6); however, tryptase activity was not affected in control carotid arteries (n=3) (Table 1). The tryptase inhibitors antipain and APMSF significantly reduced tryptase activity in atherosclerotic carotid arteries by 92±14% and 81±18%, respectively (P<0.01). Mast cell degranulation with compound 48/80 did not cause a detectable increase in chymase activity in atherosclerotic (n=5) or control (n=3) carotid arteries compared with the nonactivated controls (Table 1).

MMP activity was significantly increased in atherosclerotic carotid arteries (n=14) after mast cell degranulation with compound 48/80 (1.5±0.2-fold, P=0.018) (Table 1). This increase was blocked by standard MMP inhibitors (Table 2). Addition of antipain, APMSF, and chymostatin significantly inhibited MMP activity by 30±6% (P=0.001), 23±6% (P=0.034), and 9±2% (P=0.01) (Table 2), respectively, demonstrating the involvement of tryptase and chymase in the increased MMP activity. Addition of aprotinin, E-64, or pepstatin A did not significantly inhibit the increase in MMP activity caused by mast cell degranulation (Table 2).

**TABLE 3. Quantification of MMP-1– and MMP-3–Positive Cells in Control and Atherosclerotic Arteries**

<table>
<thead>
<tr>
<th></th>
<th>Atherosclerotic (n=18)</th>
<th>Control (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibrous Cap</td>
<td>Shoulder Region</td>
</tr>
<tr>
<td>MMP-1</td>
<td>1.9±0.2*</td>
<td>2.9±0.1*</td>
</tr>
<tr>
<td>MMP-3</td>
<td>0.6±0.2*</td>
<td>1.5±0.3*</td>
</tr>
</tbody>
</table>

*Number of MMP–positive cells in the fibrous cap, shoulder regions, and adventitia of atherosclerotic arteries and in the intima, media, and adventitia of control arteries was subjectively scored on a scale of 0 to 3: 0, no positive cells; 1, <10% positive cells; 2, between 10% and 50% positive cells; 3, >50% positive cells. Results are expressed as mean±SEM.

*Indicates significant difference from control (P≤0.05, Mann-Whitney U test).
Higher concentrations of antipain, APMSF, and chymostatin did not increase the level of inhibition of tryptase, chymase, or MMP activity (data not shown).

**Mast Cell Location**

The distribution of mast cells in atherosclerotic and control carotid artery specimens was examined by immunocytochemistry (Figure 1: a, b, and c, atherosclerotic; d, e, and f, control). A significantly higher proportion of mast cells was identified in the shoulder regions (4.9±1.3%) and fibrous caps (2.5±1%) of atherosclerotic plaques than in the media (0±0%, \(P=0.031\)) and intima (0.9±0.2%, \(P=0.048\)) of control arteries. In contrast, the proportion of mast cells in the adventitia was significantly lower in atherosclerotic arteries (2.6±1.3%) than in control arteries (5.3±0.3%, \(P=0.019\)). Therefore, in the whole atherosclerotic artery, there are 2-fold more mast cells than in the controls. In the majority of cases, the mast cells present in the shoulders of atherosclerotic plaques were located in regions of neovascularization. Degranulation was observed in 78±5% of mast cells in the shoulder and fibrous cap regions of atherosclerotic plaques, whereas in control arteries, few mast cells were activated in any area. No staining was detected in the negative control sections (data not shown). In serial frozen sections (n=4), only 37±7% of tryptase-containing mast cells also contained chymase (Figure 2), confirming previous findings. 20,23

**Location of MMP-1 and MMP-3 Proteins**

Western blots demonstrated that the rabbit anti-human MMP-1 and MMP-3 antisera were specific for the respective proteins and that the antisera identified both the active and the pro- forms of the MMP (Figure 3). Staining of serial sections with anti–MMP-1 and anti–MMP-3 antisera showed a strikingly similar pattern to that seen for mast cells in atherosclerotic plaques (data not shown). The small amount of positive staining that was detected in the control arteries was restricted to the adventitia. High levels of MMP-1– and MMP-3–positive cells were observed in the fibrous cap and shoulder regions of atherosclerotic arteries (n=18) compared with control vessels (n=15) (Table 3). No signal was detected when the antisera were preabsorbed with the appropriate peptides.

**Collocation of Mast Cells and MMP-1 and MMP-3 Proteins**

Double labeling of atherosclerotic plaques showed that the mast cells in the fibrous cap, especially in the shoulder region, were located in zones containing cytoplasmic and extracellular MMP-1 and MMP-3 proteins (Figure 4A and 4B, respectively). The orange color detected after double labeling for tryptase and MMPs develops as an artifact of combining the 2 color substrates and is not specific (Figure 5). No staining was detected in the negative controls (data not shown).
Immunocytochemistry using serial sections demonstrated that MMP-1 and MMP-3 expression was not detected in mast cells (data not shown).

**Location of MMP Activity**

Our immunocytochemical studies do not discriminate between the latent and active forms of MMPs. Therefore, serial frozen sections of freshly excised vessel segments were analyzed by in situ zymography (Figure 4C). Areas of substrate lysis, which indicate caseinolytic activity, were greatest in all plaques examined (n=6) in the shoulder regions, where increased expression of MMP-1, MMP-3, and mast cells was also detected (Figure 4A and 4B). Addition of the MMP inhibitors Ro 31-9790 (Figure 4D) or EDTA (data not shown) to the incubation buffer abolished the caseinolytic activity. The addition of the mast cell protease inhibitors antipain and chymostatin did not affect the observed caseinolytic activity (Figure 6). The control arteries (n=3) and the uninvolved areas of the atherosclerotic specimens did not exhibit any enzymatic activity. Gelatinolytic activity was observed in the shoulder regions of all plaques (data not shown).

**Quantification of MMP-1 and MMP-3 Proteins**

ELISA assays showed significantly higher levels of total MMP-1 (2.7±0.5 ng/mg of total protein) and MMP-3 (3.8±1.1 ng/mg of total protein) in atherosclerotic plaques than in control arteries (MMP-1, 1.6±0.3 ng/mg of total protein, P=0.046; MMP-3, 0.9±0.2 ng/mg of total protein, P=0.050).

**Discussion**

Matrix degradation within atherosclerotic plaques is thought to cause weakening and to lead to erosion and rupture.3-5 It has been suggested that mast cells contribute to this process by releasing neutral proteases such as tryptase and chymase.19,21-23,33 These enzymes, which are released from mast cells by degranulation, may act indirectly by activation of...
MMPs or directly by digestion of plaque matrix proteins. Although previous studies have found increased numbers of mast cells, MMP-1, MMP-3, and MMP-9-immunopositive cells; and net MMP activity in the shoulder regions of atherosclerotic plaques, no direct collocation or involvement of mast cells in MMP activation has been reported.

We incubated carotid endarterectomy specimens with the mast cell degranulator compound 48/80, which caused a 2-fold increase in the activity of mast cell trypsin. The levels of chymase detected in the carotid arteries was much lower than that of trypsin, and no increase in chymase activity was observed after compound 48/80 activation. The addition to plaque supernatants of the tryptase inhibitors antipain and APMSF or the chymase inhibitor chymostatin abolished the activity of the enzymes. Mast cell degranulation by compound 48/80 also caused an increase in MMP activity, which was abolished by the addition of standard MMP inhibitors. This shows that mast cell degranulation leads to an increase in MMP activity. The addition of trypsin and chymase inhibitors reduced the increase in MMP activity by 30% and 10%, respectively, suggesting that chymase plays a lesser though significant role in MMP activation than trypsin. The requirement for the extended incubation to detect chymase activity and the detection of fewer chymase-and-tryptase-containing mast cells than tryptase-only-containing mast cells support the hypothesis that there are rather low concentrations of this protease in atherosclerotic plaques.

Because incubation with trypsin and chymase only reduced MMP activity by ≈40%, ≈60% of the increase in MMP activity is not caused by mast cell proteases. The addition of serine, cysteine, and aspartic protease inhibitors did not affect the MMP activity, suggesting that these classes of protease are not involved in MMP activation and that this remaining 60% may be due to MMP autoactivation. The increase in MMP activity detected after mast cell degranulation with compound 48/80 is not the result of the release of preformed MMPs from mast cells, since previous studies have shown that MMP activity is not detectable in isolated mast cells.

Significantly higher numbers (2-fold) of mast cells were detected in atherosclerotic arteries compared with control arteries, which is correlated with the finding of 2-fold higher levels of trypsin in the atherosclerotic arteries compared with controls. Mast cells account for 5% and 2.5% of the nucleated cells in the shoulder region and fibrous cap, respectively, whereas in control arteries mast cells account for only 1% of the intimal cells. The majority of the mast cells (78±5%) in the rupture-prone regions of the atherosclerotic plaques were degranulated, suggesting that their neutral proteases were available to interact with other mediators in these regions. This finding is in accord with previous studies that have shown that the number of activated mast cells at sites of coronary artery thrombotic atheromatous erosion or rupture is considerably increased.

Current knowledge suggests that mast cell degranulation is stimulated in vivo by several mechanisms. The best-understood stimulus is the binding of an allergen to mast cell–bound IgE. In addition, “histamine releasing factor,” which is secreted by immunocompetent cells such as T lymphocytes and macrophages present in the shoulder regions of atherosclerotic plaques, can also stimulate mast cell degranulation. Furthermore, matrix degradation during atherosclerotic plaque development may result in the generation of breakdown products, including collagen-derived peptides, which have also been shown to induce mast cell degranulation. More recently, it has been suggested that oxidized LDL present in the atherosclerotic plaques may induce mast cell degranulation in atherosclerosis either directly or through recruitment of leukocytes. In addition, trypsin has been shown to activate mast cells, highlighting a possible amplification cascade.

Our results show that the release of neutral proteases from mast cells in atherosclerotic plaques causes MMP activation, which may degrade the extracellular matrix and destabilize the plaque. However, there was no significant increase in MMP activity in control carotid arteries. This may be because lower numbers of mast cells are present in the control arteries. This conclusion is supported by the finding that trypsin activity is unaffected by mast cell degranulation in the control arteries. Furthermore, it may be because lower levels of MMPs are expressed in the control arteries than in the atherosclerotic plaques, and therefore there is less MMP available for activation.

In conclusion, our results indicate that mast cells accumulate and degranulate in the shoulder regions and fibrous caps of carotid artery atherosclerotic plaques, where expression of total MMP-1 and MMP-3 is elevated and net MMP activity is located. The in vitro assays demonstrate directly that trypsin and chymase released from mast cells activate MMPs. Together these results suggest that mast cells may play an important role in MMP activation in atherosclerotic plaques.

Acknowledgments
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References

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