Unstable Carotid Plaques Exhibit Raised Matrix Metalloproteinase-8 Activity

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Background—The fibrous cap of atherosclerotic plaques is composed predominantly of type I and III collagen. Unstable carotid plaques are characterized by rupture of their cap, leading to thromboembolism and stroke. The proteolytic mechanisms causing plaque disruption are undefined, but the collagenolytic matrix metalloproteinase (MMP) -1, -8, and -13 may be implicated. The aim of this study was to quantify the concentrations of these collagenases in carotid plaques and to determine their relationship to markers of plaque instability.

Methods and Results—Atherosclerotic plaques were collected from 159 patients undergoing carotid endarterectomy. The presence and timing of carotid territory symptoms were ascertained. Preoperative embolization was recorded by transcranial Doppler. Each plaque was assessed for histological features of instability. Plaque MMP concentrations were quantified with ELISA. Significantly higher concentrations of active MMP-8 were observed in the plaques of symptomatic patients (20.5 versus 11.4 ng/g; \( P < 0.0002 \)), in plaques of emboli-positive patients (22.7 versus 13.5 ng/g; \( P = 0.0037 \)), and in those plaques showing histological evidence of rupture (20.8 versus 14.7 ng/g; \( P = 0.0036 \)). No differences were seen in the levels of MMP-1 and MMP-13. Immunohistochemistry, in situ hybridization, and colocalization studies confirmed the presence of MMP-8 protein and mRNA within the plaque, which colocalized with macrophages.

Conclusions—These data suggest that the active form of MMP-8 may be partly responsible for degradation of the collagen cap of atherosclerotic plaques. This enzyme represents an attractive target for drug therapy aimed at stabilizing vulnerable plaques. (Circulation. 2004;110:337-343.)

Key Words: atherosclerosis ■ carotid arteries ■ collagen ■ metalloproteinases ■ plaque

Rupture of the cap of atherosclerotic plaques is considered to be a crucial step in the development of myocardial infarction and stroke.\(^1,2\) Cap disruption exposes the underlying thrombogenic plaque core to the bloodstream, leading to thrombosis with subsequent embolism. It has been suggested that plaque rupture is caused by a localized imbalance in proteolytic activity,\(^3\) and of particular interest is the family of enzymes known as the matrix metalloproteinases (MMPs).\(^4\)

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The cap of the plaque is composed predominantly of type I and III collagen.\(^5\) MMP-1, MMP-8, and MMP-13 are collagenases that preferentially degrade these fibrillar collagens. Degradation occurs by cleavage of the collagen molecule at the distinct Gln(775)–Ile(776) position, which generates 2 fragments that are further degraded by nonspecific proteases, including MMP-9. Previous reports have shown significantly raised MMP-9 concentrations in unstable carotid plaques.\(^6\) However, intact collagen type I and III molecules are not substrates for this enzyme, so MMP-9 cannot be solely responsible for plaque rupture.\(^7,8\) The enzymes responsible for plaque disruption must be defined to facilitate the development of pharmacotherapy aimed at stabilizing vulnerable atherosclerotic plaques.

The collagenases MMP-1 and MMP-13 have been implicated in plaque rupture,\(^9,10\) but concentrations of these enzymes within the plaque are low, and evidence of a direct association with plaque instability is lacking. Until recently, only neutrophils were considered capable of expressing MMP-8 (“neutrophil collagenase”), but it is now known that endothelial cells, smooth muscle cells, and macrophages are also able to perform this function.\(^11\) The discovery that MMP-8 is expressed by cells commonly found in atheroma (as opposed to neutrophils, which are not) led to the hypothesis that this enzyme may be involved in plaque rupture. The aim of this study was to quantify the concentrations of the collagenolytic MMPs (MMP-1, MMP-8, and MMP-13) in stable and unstable carotid plaques in an attempt to define the
pharmacological profile required for inhibitors with potential to cause plaque stabilization.

Methods

Study Design
The local research ethics committee approved the study, and all patients gave written informed consent. The study group comprised 159 patients undergoing carotid endarterectomy, all having >70% carotid stenosis.

Patient information was collected preoperatively and included age, sex, medication, and history of smoking, ischemic heart disease, hypertension, or diabetes. Carotid stenosis severity was measured by duplex ultrasound.

Symptomatology
A detailed clinical history was obtained from each patient with particular reference to carotid territory ischemic events. The timing of the most recent symptom was used for dividing patients into groups for comparison (see below). Patients with no symptoms in the preceding 6 months were classified as asymptomatic.

Cerebral Embolization
Transcranial Doppler (TCD) insonation of the middle cerebral artery was performed for 30 minutes preoperatively and during the dissection phase of the operation to detect spontaneous cerebral embolization, a reliable marker of carotid plaque instability. Any emboli occurring after carotid artery clamping were considered to be unrelated to plaque instability. The numbers of emboli were recorded.

Groups
In the absence of a consensus definition for plaque instability, we divided patients into the following groups so that multiple comparisons could be performed: group 1, symptoms <6 months preoperatively versus asymptomatic; group 2, emboli positive versus emboli negative; group 3, histological feature positive versus histological feature negative; group 4, symptoms <2 weeks preoperatively versus symptoms >2 weeks preoperatively (or asymptomatic); and group 5, symptoms <2 weeks preoperatively and/or emboli positive versus symptoms >2 weeks preoperatively (or asymptomatic) and emboli negative (or emboli unknown). The results for groups 4 and 5 are available only in the online Data Supplement.

Collection of Specimens
The carotid plaque was divided longitudinally through the point of maximum stenosis. Half was snap-frozen in liquid nitrogen and stored at −80°C, and half was placed in 4% paraformaldehyde solution for 24 hours.

MMP Quantification
Carotid plaques were defrosted before MMP and tissue inhibitor of metalloproteinase (TIMP) extraction using a standardized method. ELISA was used to quantify MMP-1, MMP-8 (total and active), MMP-13, TIMP-1, and TIMP-2 concentrations (Amersham). The MMP-8 activity ELISA used the pro form of a detection enzyme that is transformed by captured active MMP-8 into an active detection enzyme through a single proteolytic event. This activated detection enzyme can then be measured with a specific chromogenic peptide substrate. Total MMP-8 levels can be measured on the same assay enzyme can then be measured with a specific chromogenic peptide substrate. Total MMP-8 levels can be measured on the same assay enzyme can then be measured with a specific chromogenic peptide substrate.

Histological Analysis
Plaques were paraffin embedded, sectioned, and stained with hematoxylin and eosin and elastic van Gieson. An experienced histopathologist (J.L.J.) blinded to sample identity and symptoms graded sections for the presence of 6 histological features previously shown to be associated with plaque instability.

Immunohistochemistry and Colocalization Studies
MMP-8 immunohistochemistry was performed in a selected group of samples using a monoclonal antibody (R&D Systems) to confirm the presence of MMP-8 protein in the plaque. Colocalization studies for smooth muscle cells, leukocytes, macrophages, and neutrophils were performed using antibodies to smooth muscle actin (Sigma) and cluster of determinants (CD) 45, CD68 (Dako), and CD15 (Becton Dickinson) antigens, respectively.

In Situ Hybridization
A nonisotopic in situ hybridization technique was performed in a selected group of samples with a digoxigenin-labeled oligonucleotide probe cocktail based on published sequences: 5'-TCGACAGTCTCCGACTCCATTTTCCTCGAT-3', 5'-CGGAAC-GACAGAAGGTTGATACAGAAGTC-3', 5'-TTGTGAAGAGAACATTTCGTTAAGAC-3', and 5'-CTTGATCTTAAA-CCAATTCATTCCCT-3'.

Paraffin-embedded sections were deparaffinized and incubated with proteinase K at 10 μg/mL for 1 hour at 37°C. Slides were washed in water, dehydrated in graded ethanol, and dried for 2 minutes. They were then incubated (10 minutes at 65°C followed by 2 hours at 37°C) with a digoxigenin-labeled MMP-8 oligonucleotide cocktail (or its sense equivalent as a control) in hybridization buffer (30% formamide, 0.6 mol/L NaCl, 10% dextran sulfate, 50 mmol/L Tris, pH 7.5, 0.1% sodium pyrophosphate, 0.2% Ficoll, and 5 mmol/L EDTA).

Stringency washes were carried out twice with 2× SSC/30% formamide at 37°C for 10 minutes. Slides were then incubated in filtered blocking solution for 10 minutes, followed by alkaline-phosphatase–conjugated ovine anti-digoxigenin for 1 hour. NBT/BCIP chromogen solution was applied, and slides were incubated in the dark. Slides were checked microscopically until maximum signal occurred with minimum background and finally were washed and mounted.

Statistical Analysis
Analyses of the 5 groups detailed above were carried out. Discrete variables were presented as actual numbers (and percentages) and compared by use of Fisher’s exact test. The continuous variable of age was normally distributed and thus presented as a mean (and total range) and compared by use of the unpaired t test. The other continuous variables did not show normal distributions. They were therefore presented as median values (and interquartile ranges) and compared by use of the Mann-Whitney U test. Multiple analyses were performed, so significance was assumed at P<0.01.

Results

Symptoms
Of 159 patients, 90 experienced symptoms in the 6 months before surgery (transient ischemic attack, 36; amaurosis fugax, 21; stroke with recovery, 13; stroke with residual deficit, 9; central retinal artery occlusion, 1; combination of above, 10), with 19 of these having symptoms within the preoperative 2 weeks. There were no significant differences between the symptom groups in terms of age, risk factors, carotid stenosis, or medication (Table 1 and Online Table 4).

Cerebral Embolization
Twenty-six percent of patients had emboli detected in the preoperative or dissection phase. Embolization status was undefined in 11 patients (4 were symptomatic; 0 within the preoperative 2 weeks) because of the absence of TCD windows.
Spontaneous embolization was significantly more likely in symptomatic compared with asymptomatic patients (41% versus 11%; \(P<0.0001\); Table 1). Patients experiencing symptoms in the 2 preoperative weeks were particularly likely to be emboli positive compared with the remainder of the group (79% versus 21%; \(P<0.0001\); Online Table 4).

There were no significant differences in age, risk factors, carotid stenosis, or medication between emboli groups (Table 2).

**Histology**

Table 1 shows the distribution of plaque histological features related to symptom groups. The incidence of plaque rupture and intraplaque hemorrhage tended to be higher among symptomatic patients (\(P=0.0361\) and 0.0416, respectively). No differences were seen for the other histological features.

**MMP and TIMP Concentrations**

Tables 1 and 2 show the plaque concentrations of collagenases and TIMPs related to plaque instability (as defined by symptomatology and embolization). There were significantly higher plaque concentrations of both active MMP-8 (20.5 versus 11.4 ng/g; \(P=0.0002\)) and total MMP-8 (275 versus 191 ng/g; \(P<0.0001\)) in symptomatic patients (Table 1).

Significantly higher concentrations of active MMP-8 were also seen in unstable compared with stable plaques according...
to the following definitions: emboli positive (22.7 versus 13.5 ng/g; \( P = 0.0037 \); Table 2), symptoms <2 weeks preoperatively (40.5 versus 14.7 ng/g; \( P = 0.0023 \); Online Table 4), and symptoms <2 weeks preoperatively and/or emboli positive (23.6 versus 13.0 ng/g; \( P = 0.0001 \); Online Table 5). No significant differences were seen in the levels of total MMP-8 in groups 2, 3, 4, or 5 and in the levels of MMP-1 and MMP-13 in any of the groups.

There was a significantly higher concentration of TIMP-1 in the plaques of symptomatic patients (Table 1), although no such difference existed in the other groups.

There were significantly higher levels of active MMP-8 in those plaques with histological evidence of rupture (20.8 versus 14.7 ng/g; \( P = 0.0036 \); Table 3). No significant difference was demonstrated for MMP-1, MMP-8 (total), or MMP-13 (data not shown).

Immunohistochemistry and Colocalization Studies
Immunohistochemistry identified MMP-8 protein in carotid plaques that colocalized to CD45-positive cells, also shown to be strongly CD68 positive, confirming their identity as macrophages. There was no evidence of MMP-8 localizing to SMA-positive smooth muscle cells or CD15-positive neutrophils (Figure 1).

Focal, weak staining for MMP-8 was detected in clusters of cells within asymptomatic plaques. These cells were strongly CD68 positive, weakly positive for CD45, and negative for CD15 and SMA, consistent with macrophages (Figure 2).

**In Situ Hybridization**
In situ hybridization performed in a selection of symptomatic plaques localized MMP-8 mRNA to the same site as the macrophage infiltration, supporting its origin from this cell population (Figure 1).

**TABLE 2. Comparison of Emboli Groups in Terms of Patient Characteristics, Plaque Histological Features, and Plaque MMP and TIMP Concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Emboli Positive (n=42)</th>
<th>Emboli Negative (n=106)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age (range), y</td>
<td>68.4 (54–80)</td>
<td>70.0 (50–86)</td>
<td>0.2356</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>30 (71)</td>
<td>75 (71)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>35 (83)</td>
<td>88 (83)</td>
<td>1.0000</td>
</tr>
<tr>
<td>IHD, n (%)</td>
<td>18 (43)</td>
<td>46 (43)</td>
<td>1.0000</td>
</tr>
<tr>
<td>HTN, n (%)</td>
<td>23 (55)</td>
<td>73 (69)</td>
<td>0.1275</td>
</tr>
<tr>
<td>DM, n (%)</td>
<td>7 (17)</td>
<td>20 (19)</td>
<td>0.8181</td>
</tr>
<tr>
<td>Carotid stenosis (IQR), %</td>
<td>80 (80–90)</td>
<td>80 (70–90)</td>
<td>0.0721</td>
</tr>
<tr>
<td>Statin use, n (%)</td>
<td>9 (21)</td>
<td>44 (42)</td>
<td>0.0234</td>
</tr>
<tr>
<td>Aspirin use, n (%)</td>
<td>36 (86)</td>
<td>90 (85)</td>
<td>1.0000</td>
</tr>
<tr>
<td><strong>Histology, n</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque rupture</td>
<td>8 (19)</td>
<td>19 (18)</td>
<td>1.0000</td>
</tr>
<tr>
<td>IP</td>
<td>13 (31)</td>
<td>23 (22)</td>
<td>0.2885</td>
</tr>
<tr>
<td>Cap thinning</td>
<td>24 (57)</td>
<td>68 (64)</td>
<td>0.4562</td>
</tr>
<tr>
<td>Intraplaque fibrin</td>
<td>18 (43)</td>
<td>50 (47)</td>
<td>0.7156</td>
</tr>
<tr>
<td>Plaque necrosis</td>
<td>21 (50)</td>
<td>53 (50)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Cap foam cells</td>
<td>21 (50)</td>
<td>51 (48)</td>
<td>0.8571</td>
</tr>
<tr>
<td><strong>Plaque concentrations, median, ng/g (IQR)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>47.8 (17.4–101.4)</td>
<td>29.1 (12.1–101.6)</td>
<td>0.2491</td>
</tr>
<tr>
<td>MMP-8 (active)</td>
<td>22.7 (9.8–53.2)</td>
<td>13.5 (6.2–25.4)</td>
<td>0.0037*</td>
</tr>
<tr>
<td>MMP-8 (total)</td>
<td>271 (196–330)</td>
<td>238 (140–307)</td>
<td>0.0780</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.77 (0.46–1.16)</td>
<td>0.57 (0.28–0.84)</td>
<td>0.0787</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>7747 (4316–13 540)</td>
<td>7716 (5205–11 470)</td>
<td>0.7465</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>874 (378–1405)</td>
<td>803 (499–1295)</td>
<td>0.6987</td>
</tr>
</tbody>
</table>

*Significant.

**TABLE 3. Comparison of Plaque Active MMP-8 Concentration in Presence or Absence of 6 Histological Features Associated With Plaque Instability**

<table>
<thead>
<tr>
<th>Histological Feature</th>
<th>Present</th>
<th>Absent</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque rupture</td>
<td>20.8 (13.2–49.0)</td>
<td>14.7 (6.3–26.4)</td>
<td>0.0036*</td>
</tr>
<tr>
<td>IP</td>
<td>21.4 (10.9–53.5)</td>
<td>13.4 (6.7–25.7)</td>
<td>0.0173</td>
</tr>
<tr>
<td>Cap thinning</td>
<td>13.0 (7.2–25.4)</td>
<td>20.8 (6.6–32.6)</td>
<td>0.1972</td>
</tr>
<tr>
<td>Intraplaque fibrin</td>
<td>15.6 (6.2–33.3)</td>
<td>16.9 (7.2–26.4)</td>
<td>0.8479</td>
</tr>
<tr>
<td>Plaque necrosis</td>
<td>17.3 (8.4–30.1)</td>
<td>14.7 (6.7–27.1)</td>
<td>0.2719</td>
</tr>
<tr>
<td>Cap foam cells</td>
<td>16.6 (6.0–24.3)</td>
<td>16.4 (7.2–32.6)</td>
<td>0.3269</td>
</tr>
</tbody>
</table>

*PH indicates intraplaque hemorrhage.

*Significant.
MMP-8 mRNA was not detected in a selection of asymptomatic plaques, although the same plaques did show weak staining for MMP-8 protein in macrophages (Figure 2). This apparent discrepancy probably relates to the level of MMP-8 synthesis falling below the sensitivity threshold of in situ hybridization.

**Discussion**

Sudden alterations in atherosclerotic plaque structure such as cap rupture and intraplaque hemorrhage frequently precede ischemic cardiovascular events. Studies have shown that there is often an intense inflammatory infiltrate around the rupture site that is probably responsible for destabilization of the plaque. There is now considerable evidence that excessive proteolysis leads to plaque rupture, with MMPs being the major group of enzymes involved in this process. The gelatinase MMP-9 has been highlighted as one of the most important enzymes in this regard. Brown et al initially showed increased MMP-9 synthesis in atherectomy specimens removed from patients with unstable as opposed to stable angina, and more recently, increased levels of MMP-9 have been demonstrated in unstable carotid plaques.

However, type I and III collagen molecules, which account for the load-bearing strength of the plaque cap, are not substrates for the MMP-9 enzyme. There have been reports that high concentrations of other gelatinases (eg, MMP-2) can degrade type I collagen in an in vitro environment devoid of TIMPs. Nonetheless, it is likely that in vivo only the interstitial collagenases (MMP-1, MMP-8, and MMP-13) are capable of degrading these fibrillar collagens and therefore must play a role in the pathogenesis of cap rupture.

MMP-8 (neutrophil collagenase) has only very recently been identified in atherosclerotic tissue, a finding that suggested that this enzyme may play a role in the pathogenesis of carotid disease. This study by Herman et al suggested the possibility that there may be an excess of MMP-8 in histologically characterized vulnerable plaques compared with stable ones and showed that MMP-8 tended to colocalize with cleaved collagen.

The suggestion of a role for MMP-8 in plaque rupture is further implicated by the present study of 159 patients. This study is the first to show increased concentrations of the active form of a collagenase, MMP-8, in clinically defined unstable carotid plaques. Active and total concentrations of MMP-8 were higher in plaques from patients with carotid territory symptoms in the 6 months before surgery, whereas active MMP-8 was raised in patients with symptoms in the preoperative fortnight and in those with cerebral embolization. This suggests that the active form of the enzyme is most important in modulating plaque rupture.

In our study, MMP-8 colocalized with macrophages demonstrated to be synthesizing MMP-8 mRNA. These findings confirm the pivotal role of macrophage infiltration in plaque destabilization. Recently, evidence has revealed an increase in macrophage-rich areas in unstable coronary and carotid plaques and has suggested increased macrophage activation.
in the plaques of those patients suffering from acute coronary syndromes. It remains unknown as to whether raised MMP levels cause rupture of the atherosclerotic plaque or are a result of it. Attempts to develop animal models of plaque rupture have been largely unsatisfactory. Proof of a causative role will remain a point of debate until a randomized controlled trial of an MMP inhibitor compared with placebo is completed. Such inhibitors have been used in cancer studies, but there are significant concerns over the high incidence of musculoskeletal side effects. In a recent study of MMI270 (a direct inhibitor of MMP-2, MMP-8, and MMP-9), 39 patients of a total of 92 suffered significant arthralgia and/or myalgia, in addition to 18 patients who developed a widespread maculopapular rash.

Doxycycline is a nonspecific MMP inhibitor. Numerous studies have assessed its potential to influence the development of vascular disease. Most work in this area has focused on the possibility that doxycycline could prevent abdominal aortic aneurysm expansion by blocking the proteolytic activity of MMPs. One randomized controlled trial relating to the effects of an MMP inhibitor on carotid......
plaques has been published.29 This showed that patients taking oral doxycycline for a period of 2 to 8 weeks while awaiting carotid endarterectomy had lower concentrations of MMP-1 in their retrieved plaques than patients taking placebo. No difference was seen in the levels of MMP-2, -3, or -9 or of TIMP-1 or -2. Although trends toward lower rates of carotid occlusion and preoperative embolization were seen in the treated group, they did not reach significance.

We believe that the results of the present study represent a significant step forward in our understanding of the biological mechanisms likely to be involved in plaque rupture. Combined, MMP-8 and MMP-9 can degrade all the major structural components of the plaque extracellular matrix; therefore, any pharmacotherapy aimed at plaque stabilization should target both of these enzymes.

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References

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