Increased Matrix Metalloproteinase-9 Activity in Unstable Carotid Plaques

A Potential Role in Acute Plaque Disruption

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Background and Purpose—Acute disruption of atherosclerotic plaques precedes the onset of clinical syndromes, and studies have implicated a role for matrix metalloproteinases (MMPs) in this process. The aim of this study was to establish the character, level, and expression of MMPs in carotid plaques and to correlate this with clinical status, cerebral embolization, and histology.

Methods—Plaques were obtained from 75 consecutive patients undergoing carotid endarterectomy and divided into 4 groups according to symptomatology (group 1, asymptomatic; group 2, symptomatic >6 months before surgery; group 3, symptomatic within 1 to 6 months; group 4, symptomatic within 1 month). All patients underwent preoperative and intraoperative transcranial Doppler monitoring. Plaques were subjected to histological examination and quantification of MMPs by zymography and ELISA.

Results—The level of MMP-9 was significantly higher in group 4 (median 125.7 ng/mL for group 4, median <32 ng/mL for all other groups; \( P=0.003 \)), with no difference in the levels of MMPs 1, 2, or 3. Furthermore, the MMP-9 concentration was significantly higher in plaques undergoing spontaneous embolization \( (P=0.019) \) and those with histological evidence of plaque instability \( (P<0.03) \). In situ hybridization demonstrated increased MMP-9 expression in highly symptomatic plaques in areas of intense inflammatory infiltrate.

Conclusions—The concentration, production, and expression of MMP-9 is significantly higher in unstable carotid plaques. If this proves to be a causal relationship, MMP-9 may be a strong candidate for pharmacotherapy aimed at stabilizing plaques and preventing stroke. \( (Stroke. 2000;31:40-47.) \)

Key Words: atherosclerosis ■ metalloproteinases ■ carotid arteries ■ cardiovascular diseases

Acute disruption of atherosclerotic plaques precedes the onset of clinical syndromes, and studies have implicated a role for matrix metalloproteinases (MMPs) in this process. The aim of this study was to establish the character, level, and expression of MMPs in carotid plaques and to correlate this with clinical status, cerebral embolization, and histology.

Subjects and Methods

Patients
Seventy-five consecutive patients admitted for carotid endarterectomy into a single vascular surgical unit were entered into this study between September 1996 and April 1997. Local ethical committee approval was obtained for the procurement of specimens, and all patients gave full informed consent for the study. A clinical history was obtained from each patient, with particular care taken to establish the number and duration of ischemic events, together with a record of the time between the last symptom and the operation. All patients underwent a thorough neurological examination. Each was then assigned preoperatively to 1 of 4 symptom groups on the basis of their symptom-free duration, with group 1 (n=20) consisting of all asymptomatic patients (either with severe bilateral disease or as part of the Asymptomatic Carotid Surgery Trial); group 2 (n=16), those with symptoms >6 months preoperatively; group 3 (n=18), those with symptoms <6 months but >1 month preoperatively; and group 4 (n=21), all those with symptoms only in the month before surgery. Focal cerebral ischemic events were defined as transient ischemic attack, amaurosis fugax, central retinal artery occlusion, or cerebrovascular accident. Those patients with vague symptoms such as dizziness and headaches but an absence of definite focal cerebral events were assigned to group 1. All patients were undergoing long-term aspirin therapy (75 or 150 mg/d), and there was no difference between the groups in the use of other medications, including lipid-lowering agents, or in serum lipid levels.

Preoperative Imaging and Emboli Detected
All patients underwent a preoperative Duplex ultrasound assessment of the carotid plaque for quantification of the degree of stenosis. All had >70% stenosis, with no difference between the groups in the percentage stenosis (Kruskal-Wallis test, \( P>0.05 \)). Patients were monitored with transcranial Doppler (TCD) preoperatively for 30
minutes, as well as intraoperatively during the dissection phase of the operation. This aimed to identify those with ongoing particulate microembolization, which is highly indicative of plaque instability.\textsuperscript{1} Continuous TCD monitoring of the ipsilateral middle cerebral artery was performed with a SciMed PC Dop 842 TCD. Signals were recorded on digital audiotape for offline analysis and interpretation of embolic signals as described previously.\textsuperscript{2} For the purpose of the present study, emboli were only recorded if they occurred in the preoperative period or the dissection phase of the operation, and after the internal carotid artery was clamped, recording was discontinued.

**Procurement of Tissue Specimens**

Carotid plaques were obtained immediately after endarterectomy. All operations were performed with standard surgical techniques and with minimal manipulation of the specimen. The endarterectomy was extended in a caudal direction to include a sample of nondiseased common carotid artery proximal to the plaque but in continuity with the plaque, to act as a negative paired control. This did not involve a significant change to the standard operative technique. The plaque and control tissue were divided longitudinally through the most apparent lesion or the area of tightest stenosis. These were then processed for quantification of the major subtypes of MMP and tissue inhibitor of MMP (TIMP), as well as for histological analysis.

**Plasma Samples**

Blood was taken from each patient 24 hours before surgery for the measurement of plasma MMP levels by ELISA.

**MMP Quantification**

MMP and TIMP levels were quantified by zymography, which, while semiquantitative, differentiates between active and latent enzyme forms, as well as by quantitative ELISA. The tissue was snap-frozen in liquid nitrogen and stored at \(-70^\circ\text{C}\) until extraction by the method of Vine and Powell as previously described.\textsuperscript{3} Briefly, tissue was weighed, then homogenized in buffer containing phenylmethylsulfonyl fluoride (0.1 mM/L; Sigma). After centrifugation, the supernatant was dialysed for 18 hours at 4°C. The protein concentration was standardized for each sample to 0.9 mg/mL with PBS, which was found in preliminary experiments to be within the linear range for densitometric quantification (data not shown).

Proteins with gelatinolytic activity were identified by use of substrate gels prepared by incorporation of gelatin (1 mg/mL; Sigma) into a 10% SDS-polyacrylamide gel, as previously described.\textsuperscript{4} The molecular weight of each band was estimated by comparison with the positions of known molecular-weight standards (Bio-Rad). The relative density of each lytic band was determined from negative photographic images of gels with a Pharmacia LKB Imagemaster scanning densitometer. The product of the optical density and area of the band was compared directly with a standardized positive control (media conditioned by HT-1080 human fibrosarcoma cells) to obtain a ratio comparable between gels, as previously described.\textsuperscript{3} The identities of the lytic zones on the gelatin zymograms were confirmed as MMP-2 and MMP-9 (72 and 92 kDa, respectively) by immunoblotting. Further quantification was performed by ELISA techniques for MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-1/MMP-1 complex with Biotrak assay systems (Amersham), which have been validated for use with human tissue homogenates.\textsuperscript{5} The TIMP-1/MMP-1 complex assay recognizes activated MMP-1 that has subsequently been complexed with TIMP-1. It does not recognize free MMP-1 or TIMP-1.

**Histology**

Immediately after removal, the section of plaque for histological analysis was placed in fresh 4% paraformaldehyde solution. After overnight decalcification, the samples were paraffin embedded and sectioned at 4-μm intervals. These were stained with hematoxylin and eosin, elastic Van Giesen, and monoclonal antibodies for MMP-9 (R&D Systems). Sections were then evaluated by an experienced histopathologist (L.J.) who was blinded to the clinical findings and identity of each patient. Four sections from each plaque were examined for the presence of plaque rupture, plaque cap thinning, intraplaque hemorrhage, intraplaque fibrosis, core necrosis, and cap foam cells and graded for the degree of staining for MMP-9. Plaques were also classified as necrotic, fibrous, or calcific on the basis of the predominant component of the plaque, as previously described by Carr et al.\textsuperscript{3}

**mRNA Detection**

Reverse-transcription polymerase chain reaction (RT-PCR) was performed to confirm the expression of MMP-9. Total RNA was extracted from intact carotid tissue by use of TriZol reagent (Life Technologies) according to the manufacturer’s protocol. Reverse transcription was performed with AMV-RT enzyme and oligo-dT\(_{18}\) primers (Promega) as directed in the enzyme literature. Amplification of specific sequences was performed by standard PCR methodology, and primers were designed according to sequences obtained from the GenBank database (sense 5'-AAGGATCCGACTATGACACCGAGCCGTCGTGAGCTCGTGA-3' and antisense 5'-AAGAATTCTGGCGCCCGTAGGCTCGTGA-3'). All reaction products were analyzed on a 1% agarose gel, stained with ethidium bromide, and photographed under 254-nm ultraviolet illumination.

A well-established, nonsotopic RNA in situ hybridization technique was performed with digoxigenin-labeled oligonucleotide probes based on published sequences. The oligonucleotide sequences for probe synthesis were as follows: ACTGGCAGGTTTCTCCAT-CAGCATTTGCCGT, TCCGGCAGTATGGAATGTACTAAGCC-CCAGC, GTTGACGGCATCTGCCACCCGGACCTCAAAGGGC, GCTCCCTGCTCCTCAGAGAACTGCCAGTFA, and GCG-GCTCTCTCAAAGACCGAGTCCAGTGC. Sections were deparaffinized, rinsed in 2 × SSC, and incubated with 100 μL of proteinase K (2 μL/mL) for 60 minutes at 37°C. After they were washed, the slide samples were prehybridized with 50 μL of prehybridization solution and incubated for 1 hour at 37°C. Digoxigenin-11-dUTP-labeled probes were added to each prehybridized slide in 50 μL of fresh prehybridization solution and left at 37°C overnight. The slides were then washed in 2 × SSC/30% formamide twice, after which they were incubated in filtered blocking solution for 10 minutes. Tissue sections were then incubated in antidigoxigenin alkaline phosphatase, washed twice in TBS, then incubated in substrate buffer for 5 minutes. Subsequently, each slide was incubated in the dark in 200 μL of substrate containing 8 μL/mL nitro blue tetrazolium, 8 μL/mL BCIP, and 1 μL/mL levamisole. Slides were checked microscopically until maximum signal occurred before background developed, then they were washed and mounted in aqueous mountant.

**Statistical Analysis**

All results are expressed as median values and interquartile ranges. Risk factors and individual histological features were analyzed by the \(\chi^2\) test, and densitometry and ELISA results were compared by the Kruskal-Wallis ANOVA test. Differences in MMP levels between histological features and emboli detection were analyzed by the nonpaired, nonparametric Mann-Whitney U test. Significance was assumed with a \(P\) value <0.05.

**Results**

**Patient Demographics**

There was no significant difference between the major demographic features or cardiovascular risk factors between these 4 groups (\(\chi^2\) test, \(P>0.05\)) (Table 1).

**Histological Examination**

The microscopic appearance of the plaques was qualified in all cases, and the results are shown in Table 2. Only 2 microscopic features were found to be significantly more common in the most recently symptomatic plaques, namely, plaque rupture and intraplaque hemorrhage.
Substrate Gel Zymography

Gelatin zymography and subsequent immunoblotting revealed the presence of MMP-2 and MMP-9 in both active and inactive forms (Figure 1). The amount of active and inactive MMP-9, as quantified by densitometric analysis, was significantly higher in the most recently symptomatic group than in the other 3 groups of patients (Figures 1 and 2) \((P < 0.001\) for both, Kruskal-Wallis). There were no significant differences between the levels of active or latent MMP-2.

Enzyme-Linked Immunoabsorbent Assay

The median value for the absolute concentration of MMP-9 as determined by ELISA was 4 times higher in those plaques from symptom group 4 than from the other 3 groups (Figure 3), and this was highly significant on statistical analysis \((P = 0.001\) for both, Kruskal-Wallis). There were no significant differences between the levels of active or latent MMP-2.

Cerebral Embolization

TCD monitoring detected spontaneous particulate embolization in 21 patients (Table 2). There was a significant increase in the rate of embolization in the most recently symptomatic group compared with the other 3 groups \((P < 0.01\). There were no strokes.

The level of MMP-9 was significantly higher in those plaques from patients in whom spontaneous embolization was detected \((median 31.5 \text{ ng/mL for those without embolization versus 72 \text{ ng/mL for those with; } P = 0.017\). There was no significant difference in the levels of the other MMP subtypes or in the levels of TIMPs 1 or 2.

Histological Examination and MMP-9 Levels

There was a significantly higher concentration of MMP-9 in those plaques with histological evidence of instability, in particular intraplaque hemorrhage, plaque necrosis, and plaque rupture (Figure 4). There was no association between the other MMP subtypes or TIMPs and any of the histological features studied.

Immunocytochemistry, RT-PCR, and In Situ Hybridization

Immunostaining for MMP-9 confirmed the presence of the enzyme within the plaque, revealing intense staining around the plaque core, especially in the plaque shoulder and cap (Figure 5a). This corresponded to areas of intense inflammatory infiltration, predominantly macrophages.

In situ hybridization for MMP-9 mRNA revealed a similar pattern of staining in the cap and shoulder of the plaque.
(Figure 5b), confirming MMP-9 production in the plaque. Furthermore, RT-PCR demonstrated the presence of mRNA for MMP-9 in carotid homogenates (Figure 6).

**Discussion**
The atherosclerotic plaque is a dynamic structure that undergoes continuous remodeling of the extracellular matrix on which its structural integrity depends. Acute changes within the plaque, such as intraplaque hemorrhage, cap rupture, and cap ulceration, are a prelude to the onset of clinical ischemic events. Recent work suggests that each phase of the atherosclerotic process may be mediated by a series of enzymes called MMPs, the main physiological regulators of the extracellular matrix. Secreted in a latent proenzyme form by a range of cell types, including inflammatory cells, fibroblasts, and smooth muscle cells, they require activation by limited proteolysis, a process that is tightly controlled by specific TIMPs. There is evidence linking MMPs to disease states in which tissue degradation plays a key role, including aneurysmal disease. Their role in pathological states has led to the development of specific inhibitors, some of which are currently involved in clinical trials.

Although proteolytic enzymes have been identified in plaques, no previous study has accurately quantified the levels of the major subtypes within human plaques. In this study, we hypothesized that a localized increase in the level of MMPs may be associated with plaque instability and the onset of clinical events. The aim of the study was to establish the level of the major MMP/TIMP subtypes within carotid plaques and correlate them with clinical and histological features of plaque instability.

The present study has clearly shown that there is a localized increase in the concentration of MMP-9 in the most unstable carotid plaques based on recent focal cerebral ischemic events and has demonstrated expression of MMP-9 within this tissue. Plaques with other features highly indicative of instability, namely, histological evidence of plaque rupture and intraplaque hemorrhage and the detection of spontaneous cerebral particulate embolization, also demonstrated a significant increase in the level of MMP-9 compared with plaques from less symptomatic patients. There were, however, no significant differences in the level of TIMP-1, its major physiological inhibitor. Our data demonstrated no differences in the levels of MMP-1, MMP-2 (both active and
latent), and MMP-3. These results suggest that MMP-9 may play a key role in acute plaque disruption leading to the onset of symptoms. Recent work in patients with acute coronary syndromes supports this theory, showing a sharp but transient increase in plasma levels of MMP-9.12 We found no difference in plasma MMP levels, although this may well be related to the broader cohort of patients within each group.

Previous studies have identified the presence of several MMPs within atherosclerotic plaques. Henney et al13 demonstrated the presence of mRNA for MMP-3 in coronary plaques by in situ hybridization, whereas the expression of MMP-1 in carotid plaques was described by Nikkari.14 Galis and colleagues15 described localized increases in MMP-9 surrounding the lipid core of plaques, particularly in the shoulder and cap of the plaque, and demonstrated the presence of both latent and active forms of the enzyme by zymography. However, in all of these studies, the enzyme levels were not quantified, although the latter study reported an increase in overall proteolytic activity in the vulnerable regions of the plaque by in situ zymography. Additionally, in previous studies, the procurement of specimens was not standardized, and the MMP activity could not be related to patient symptomatology. Brown et al16 attempted to associate MMP-9 with plaque instability, documenting intracellular localization of the enzyme in all patients with unstable angina, indicating active synthesis, compared with only 30% of those with stable angina.

Further work is required to establish the precise cause of this localized increase in the level of MMP-9. Inflammation seems to play a key role in destabilization, although alternative factors such as genetic variation, infectious agents, and others require clarification.

Previous studies have shown that the site of plaque rupture is characterized by an intense inflammatory infiltration,17 which consists predominantly of macrophages, foam cells, and T lymphocytes, and it has been hypothesized that this inflammatory infiltration plays a key role in the destabilization of the plaque.18 It appears that this infiltrate within the plaque undergoes a period of activation at the time of acute coronary syndromes, and the associated release of proteolytic enzymes may lead to destabilization of the plaque.19,20 However, the events leading up to this activation remain unclear. Macrophages are certainly potent producers of MMP-9.21 Furthermore, increased expression of MMP-1 and MMP-3 has been reported within carotid plaques in association with macrophages13,14,22,23 and mast cell infiltration,24 whereas active synthesis of MMP-2 has been demonstrated within aortic plaques.25

A genetic variation in the MMP-3 promoter has been associated with the progression of coronary atherosclero-

### TABLE 3. MMP/TIMP Concentrations Determined by ELISA

<table>
<thead>
<tr>
<th>MMP/TIMP</th>
<th>Symptom Group</th>
<th>1 (n=20)</th>
<th>2 (n=16)</th>
<th>3 (n=18)</th>
<th>4 (n=21)</th>
<th>Control (n=75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td></td>
<td>4.66 (1.91–9.03)</td>
<td>2.92 (2.11–5.56)</td>
<td>3.78 (2.55–6.58)</td>
<td>5.99 (2.92–10.03)</td>
<td>12.62 (5.42–21.20)</td>
</tr>
<tr>
<td>MMP-2</td>
<td></td>
<td>187.37 (126.39–507.63)</td>
<td>169.37 (112.68–459.88)</td>
<td>183.56 (98.60–275.03)</td>
<td>166.72 (142.14–255.21)</td>
<td>202.69 (159.53–258.68)</td>
</tr>
<tr>
<td>MMP-3</td>
<td></td>
<td>4.34 (3.07–7.41)</td>
<td>3.74 (2.58–5.43)</td>
<td>5.26 (4.84–6.70)</td>
<td>5.69 (3.43–7.10)</td>
<td>8.36 (5.53–11.12)</td>
</tr>
<tr>
<td>MMP-9</td>
<td></td>
<td>31.5 (24.5–75.4)</td>
<td>30.5 (17.0–51.5)</td>
<td>30.6 (14.9–113.7)</td>
<td>125.7 (62.4–165.8)</td>
<td>8.58 (4.82–20.93)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td></td>
<td>542.42 (418.49–674.67)</td>
<td>600.58 (440.02–871.01)</td>
<td>551.67 (335.94–879.33)</td>
<td>819.73 (595.29–1384.72)</td>
<td>335.39 (257.40–512.55)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td></td>
<td>63.94 (20.85–152.19)</td>
<td>47.69 (25.12–98.47)</td>
<td>44.46 (21.24–87.98)</td>
<td>41.52 (30.62–79.01)</td>
<td>108.86 (72.96–150.07)</td>
</tr>
<tr>
<td>MMP-1/TIMP-1</td>
<td>complex</td>
<td>3.65 (3.20–4.26)</td>
<td>3.60 (2.27–4.80)</td>
<td>3.35 (2.43–4.49)</td>
<td>4.17 (2.74–6.08)</td>
<td>3.23 (2.65–4.17)</td>
</tr>
</tbody>
</table>

Values are medians (interquartile ranges), in ng/mL.
sis, and it is possible that such genetic variation affects other members of the MMP family. There may also be a role for other factors in the cascade of MMP regulation, such as the plasminogen system, oxidized LDL, and Chlamydia pneumoniae, each of which is undergoing further investigation.

A potential criticism of this study is the grouping of patients by symptom-free duration. This was justified by the significant increase in cerebral emboli detected in group 4. A number of studies have highlighted the importance of spontaneous cerebral embolization in determining the most unstable plaques, and in the present study, >50% of the recently symptomatic patients had evidence of such emboli. Also, the incidences of plaque rupture and hemorrhage were greater in plaques from patients in group 4. However, both features were identified in patients from each of the symptom groups. Previous studies have identified coronary plaque disruption at postmortem examination in patients who died of noncardiac causes, and thus it seems probable that such acute changes can occur in the carotid vessels without causing symptoms. Conversely, histological features of instability were not detected in some patients in the most recently symptomatic group. One limitation of the present study was that by necessity, only a small proportion of each plaque was examined microscopically, and it may well be that features were missed in some patients. This may particularly apply to the identification of cap foam cells, which have been shown in previous studies to be more common in symptomatic plaques and which may be missed when a small number of individual sections is examined.

Figure 5. Immunostaining for MMP-9 (a) and in situ hybridization (b) revealing intense staining for MMP-9 and MMP-9 mRNA, respectively, around the shoulder of a plaque from group 4 (shown by the arrow).
Again, limited plaque tissue restricted the study in terms of MMP expression. Although we have shown production of the enzyme in the plaque, this requires further quantification by ELISA RT-PCR and Northern blotting.

In summary, although many of the factors that predispose to the early development of the atherosclerotic lesion have been identified, there remains uncertainty as to the reasons why, after years of indolent growth, a plaque should suddenly undergo the acute changes that predispose to the onset of symptoms. This study has identified significantly higher levels of active and latent MMP-9 in the most unstable carotid plaques, as determined by patient symptomatology, spontaneous particulate cerebral embolization, and histological features of instability. Such a localized increase has the potential to cause the acute plaque disruption that precedes the onset of symptoms in both the coronary and cerebral circulations. MMP-9 represents an attractive target for pharmacotherapy to prevent plaque destabilization, and a variety of pharmaceutical agents have been shown to inhibit MMP activity. We recognize that a causal relationship could only be concluded from a formal randomized, controlled trial of an MMP inhibitor in patients with significant stenoses.

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References


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