Differences in Matrix Metalloproteinase-1 and Matrix Metalloproteinase-12 Transcript Levels Among Carotid Atherosclerotic Plaques With Different Histopathological Characteristics

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Background and Purpose—Previous studies have shown that atherosclerotic lesions express a number of matrix metalloproteinases (MMPs). Here we investigated whether transcript levels of MMP-1, -3, -7, -9, and -12 in carotid atherosclerotic plaques were correlated with histological features and clinical manifestations.

Methods—Atherosclerotic plaques (n=50) removed from patients undergoing carotid endarterectomy were classified histologically using a system proposed by Virmani et al, and MMP-1, -3, -7, -9, and -12 transcript levels in these tissues were quantified by real-time reverse-transcriptase polymerase chain reaction.

Results—Compared to plaques with a thick fibrous cap, those with a thin cap had a 7.8-fold higher MMP-1 transcript level (P=0.006), MMP-3, -7, and -12 were 1.5-fold, 1.8-fold, and 2.1-fold, respectively, higher in thin cap plaques, but the differences did not reach statistical significance. MMP-12 transcript levels were significantly increased in ruptured plaques compared with lesions without cap disruption (P=0.001). MMP-9 transcript levels were similar among the different types of lesion. MMP-1 and -12 transcript levels were significantly higher in plaques from patients with amaurosis fugax, than in those from asymptomatic patients (P=0.029 and P=0.008 for MMP-1 and MMP-12, respectively), than in those from patients with stroke (P=0.027 and P=0.001, respectively), and than in those from patients with transient ischemic attack (P=0.046 and P=0.008, respectively).

Conclusions—These data support a role of MMP-1 and -12 in determining atherosclerotic plaque stability. (Stroke. 2004;35:1310-1315.)

Key Words: carotid arteries ■ atherosclerosis ■ metalloproteinases ■ gene expression

Atherosclerosis is a chronic, progressive process that may span several decades.1 In a histological classification system proposed by the American Heart Association (AHA), atherosclerotic lesions are divided into 6 types representing different stages of the disease.2 A modification of this system has been proposed by Virmani et al3 and endorsed by Stary,4 differentiating advanced lesions into those with a thick fibrous cap and those with a thin one. Atherosclerotic plaques with a thin fibrous cap are considered to be prone to rupture, which can result in an acute ischemic event, whereas plaques with a thick cap are more stable and have a lower complication rate.5

The formation and progression of atherosclerotic lesions involves degradation of vascular matrix proteins and remodeling of the vascular wall.5 Macrophages in atherosclerotic lesions produce a number of matrix metalloproteinases (MMPs).6–10 These enzymes are capable of degrading various matrix proteins11 and may play an important role in atherosclerotic lesion development and progression. Overexpression of these enzymes in advanced lesions may contribute to thinning of the plaque cap and the development of ischemic events resulting from plaque rupture.12

In this study, we measured the transcript levels of MMP-1, -3, -7, -9, and -12 in carotid atherosclerotic plaques to investigate whether they were expressed at different levels in different lesion types and whether their expression levels correlated with clinical manifestations.
Materials and Methods

Carotid Atherosclerotic Specimens
Atherosclerotic plaques (n = 50) removed from patients undergoing carotid endarterectomy at Southampton General Hospital were subjected to histopathological examinations and quantifications for MMP-1, -3, -7, -9, and -12 transcripts were performed as described. Immediately after a plaque was removed from the patient, it was divided into multiple transverse sections. The plaque section in the internal carotid artery closest to the bifurcation was immediately snap-frozen in liquid nitrogen and then stored at -80°C before extraction of RNA for MMP transcript measurement. The plaque section in the common carotid artery closest to the bifurcation was fixed in formaldehyde and then embedded in paraffin wax for subsequent histological examination. The study was approved by the local research ethics committee and all subjects gave written consent.

Histopathological Examinations
The atherosclerotic plaques were classified histologically using a system proposed by Virmani et al.3 and endorsed by Star.4 In brief, pathological intimal thickening was defined as a lesion containing fibrous cap atheromas were lesions with a well-formed necrotic core and an underlying fibrous cap thicker than 200 μm. Thin fibrous cap atheromas were lesions with a thick (<200 μm) fibrous cap and an underlying necrotic core. Ruptured plaques were fibroatheromas with cap disruption with an underlying necrotic core.3

Quantification of MMP-1, -3, -7, -9, and -12 Transcripts
Total cellular RNA was extracted using an RNAgents total RNA isolation system (Promega). The mRNA fraction of the total cellular RNA was converted to cDNA by reverse transcription with an oligo-dT15 primer, moloney murine leukemia virus reverse-transcriptase (Promega) and RNasin ribonuclease inhibitor (Promega). Real-time polymerase chain reaction (PCR) was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR primers and probes (Table 1) were designed using the Primer Express program (Applied Biosystems). Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed as described. MMP gene was subtracted from the Ct value of a reference transcript to standardize for the amounts of RNA template and efficiencies of reverse transcription. The resulting change in Ct values was then converted to a linear form using 2^-ΔΔCt and used in subsequent statistical analysis.

Statistical Analyses
Correlation analysis was performed to calculate Pearson correlation coefficients between the MMP transcript levels. One-way ANOVA analyses were performed to examine differences in MMP transcript levels among different types of atherosclerotic lesion and among patient groups with different manifestations. If a 1-way ANOVA analysis showed that there was a difference among the group means, post hoc pair-wise multiple comparisons (least significant difference) were performed to determine which means differ. The statistical analyses were performed using the SPSS software (version 11.5, SPSS Inc), with the exception of the linear trend test presented in Table 3, which was performed using the StatsDirect program (StatsDirect Ltd). P < 0.05 was considered significant.

Results
Demographic and clinical features of the subjects are summarized in Table 2. Of the 50 atherosclerotic plaques studied, there were 5 early lesions (pathological intimal thickening), 33 fibrous cap atheromas, 9 thin fibrous cap atheromas, and 3 ruptured plaques.3

MMP Transcript Levels in Different Types of Atherosclerotic Lesions
MMP-1, -3, -7, -9, and -12 transcript levels in the atherosclerotic plaques were measured by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) using an ABI Prism 7700 Sequence Detection System. A representative output from the system is shown in Figure 1. The mean MMP-1, -3, -7, -9, and -12 transcript levels in pathological intimal thickening lesions, plaques with a thick fibrous cap,
plaques with a thin fibrous cap, and ruptured plaques, classified according to the Virmani system\textsuperscript{3,4} are shown in Figure 2. MMP-1 was 7.8-fold higher in those with a thin fibrous cap than in those with a thick fibrous cap ($P=0.006$). MMP-3, -7, and -12 were 1.5-fold, 1.8-fold, and 2.1-fold, higher in thin cap plaques, respectively, but the differences did not reach statistical significance. MMP-12 was significantly higher in ruptured plaques than in pathological thickening lesions ($P=0.001$), thick cap plaques ($P<0.001$), and thin cap plaques ($P=0.007$). MMP-9 transcript levels were similar in all 4 types of lesion.

There were positive correlations between the levels of MMP-1, -7, and -12 transcripts, with a correlation coefficient ($r$) of 0.650 ($P=0.001$) between MMP-1 and -7, 0.653 ($P<0.001$) between MMP-1 and -12, and 0.785 ($P<0.001$) between MMP-7 and -12. There was also a nominal correlation between MMP-7 and -9 ($r=0.419$, $P=0.046$). In contrast, MMP-3 levels were not correlated with the levels of other MMPs.

**MMP Transcript Levels in Atherosclerotic Plaques and Clinical Manifestations**

Forty percent of early lesions (pathological intimal thickening) and 15.2% of plaques with a thick fibrous cap were asymptomatic, whereas all plaques with a thin fibrous cap and all ruptured plaques were symptomatic (Table 3). Amaurosis fugax was seen in 9.1% of patients with thick cap plaque, 22.2% of patients with thin cap plaque, 33.3% of patients with ruptured plaque, but in none of the patients with only pathological thickening (Table 3).

MMP-1 and -12 transcript levels were significantly higher in the plaques from patients with amaurosis fugax than in those from asymptomatic patients ($P=0.029$ and $P=0.008$ for

<table>
<thead>
<tr>
<th>TABLE 2. Characteristics of Subjects and Atherosclerotic Plaques</th>
</tr>
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<tbody>
<tr>
<td>Mean±SD (continuous variables) or Proportion (categorical variables)</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
</tr>
<tr>
<td>Body mass index</td>
</tr>
<tr>
<td>Smoking history (yes/no)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
</tr>
<tr>
<td>Diabetes (type 1/type 2/no)</td>
</tr>
<tr>
<td>Hyperlipidemia (yes/no)</td>
</tr>
<tr>
<td>History of claudication or critical limb ischemia (yes/no)</td>
</tr>
<tr>
<td>Clinical presentation</td>
</tr>
<tr>
<td>Asymptomatic</td>
</tr>
<tr>
<td>Stroke</td>
</tr>
<tr>
<td>Transient ischemic attack</td>
</tr>
<tr>
<td>Amaurosis fugax</td>
</tr>
<tr>
<td>Plaque type</td>
</tr>
<tr>
<td>Pathological intimal thickening</td>
</tr>
<tr>
<td>Fibrous cap atheroma</td>
</tr>
<tr>
<td>Thin fibrous cap atheroma</td>
</tr>
<tr>
<td>Ruptured plaque</td>
</tr>
</tbody>
</table>

Figure 1. Representative real-time RT-PCR results. The graph shows fluorescence signals accumulated at different PCR cycles. Each curve represents 1 PCR.
MMP-1 and MMP-12, respectively; Figure 3), those from patients with stroke (P \( \leq \) 0.027 and P \( \leq \) 0.001, respectively; Figure 3), and those from patients with transient ischemic attack (P \( \leq \) 0.046 and P \( \leq \) 0.008, respectively; Figure 3). MMP-1 transcript levels were higher also in plaques from patients with stroke and plaques from patients with transient ischemic attack than in plaques from asymptomatic patients (Figure 3), but the differences did not reach statistical significance. There was no significant association between clinical manifestations and MMP-3, -7, and -9 transcript levels (Figure 3).

**Discussion**

The aims of this study were to compare the levels of gene expression of a number of key MMPs between different types of atherosclerotic lesion and to relate these to clinical manifestations. Using the quantitative real-time RT-PCR method, we found that MMP-1 transcript levels were nearly 8-fold higher in thin cap plaques than in thick cap plaques, which supports the finding of Sukhova et al\(^{12}\) who showed that thin cap plaques had higher MMP-1 and -13 protein levels than thick cap plaques. We also observed 1.5- to 2-fold higher MMP-3, -7, and -12 transcript levels in thin cap plaques compared with thick cap plaques, although these differences did not reach statistical significance. In addition, the expression of MMP-12 was found to be significantly higher in ruptured atherosclerotic plaques than in lesions without cap disruption. In contrast, the expression levels of MMP-9, which have been suggested to be related to plaque instability, were found to be similar among the different types of lesion. Taken together, these observations indicate a link of MMP-1 and possibly MMP-12, but not MMP-9, with plaque instability. These observations are in agreement with results from a recent study using the gene knockout technique, which showed that the frequency of plaque rupture is reduced in MMP-12 knockout mice but surprisingly is increased in MMP-9 knockout mice, suggesting that members of the MMP family have diverse effects on plaque stability.\(^{16}\)

**Table 3. Clinical Manifestations Versus Types of Atherosclerotic Plaque**

<table>
<thead>
<tr>
<th></th>
<th>Asymptomatic</th>
<th>Stroke</th>
<th>TIA</th>
<th>AMF</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathological intimal thickening</td>
<td>2 (40.0%)</td>
<td>2 (40.0%)</td>
<td>1 (20.0%)</td>
<td>0 (0.0%)</td>
<td>5 (100.0%)</td>
</tr>
<tr>
<td>Thick cap plaque</td>
<td>5 (15.2%)</td>
<td>10 (30.2%)</td>
<td>15 (45.5%)</td>
<td>3 (9.1%)</td>
<td>33 (100.0%)</td>
</tr>
<tr>
<td>Thin cap plaque</td>
<td>0 (0%)</td>
<td>2 (22.2%)</td>
<td>5 (55.6%)</td>
<td>2 (22.2%)</td>
<td>9 (100.0%)</td>
</tr>
<tr>
<td>Ruptured plaque</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (66.7%)</td>
<td>1 (33.3%)</td>
<td>3 (100.0%)</td>
</tr>
</tbody>
</table>

\( X^2_{(df=1)} \) for linear trend (M) = 8.19, P = 0.0042.

TIA indicates transient ischaemic attack; AMF, amaurosis fugax.
Among the MMPs examined in this study, only MMP-9 was found to be expressed at considerable levels in early lesions, ie, pathological intimal thickening lesions. It has been shown that smooth muscle cells in atherosclerotic lesions express MMP-9.9 Because the main constituent of pathological intimal thickening lesions is smooth muscle cells, the observation that MMP-9 but not the other MMPs studied is expressed at significant levels in these early lesions is consistent with findings from other studies that MMP-9 plays a major role in vascular smooth muscle cell migration and proliferation in atherogenesis.17,18 Each vascular smooth muscle cell is surrounded by a basement membrane. Migration of smooth muscle cells entails breakdown of this extracellular barrier, and this is dependent on the secretion of MMP-9, which can degrade the major constituent protein, ie, type IV collagen, in the basement membrane.17

The results of this study also suggest an association between increased MMP-1 and -12 expression and the development of amaurosis fugax in patients with carotid atherosclerosis. Although MMP-1 transcript levels were also higher in plaques from patients with stroke and in those from patients with transient ischemic attack than they were in plaques from asymptomatic patients, the differences did not reach statistical significance. The finding that there is an association of increased MMP-1 and -12 expression with the development of amaurosis fugax but that there is no significant association between the expression of these MMPs and the development of stroke and transient ischemic attack suggest that these different complications of carotid atherosclerosis might result from different mechanisms. Previous studies have suggested that the development of amaurosis fugax is a result of embolism caused by small emboli from ruptured carotid plaques, whereas the development of transient ischemic attack and stroke has more complicated mechanisms, relating not only to carotid plaque rupture but also to the degrees of intracranial stenosis and impaired collateral cerebral circulation.19,20 This could explain the observation of this study that amaurosis fugax, but not stroke or transient ischemic attack, was significantly associated with increased MMP-1 and MMP-12 expression and unstable carotid atherosclerotic plaques.

The focus of this study was on gene expression levels of a number of key MMPs in different types of atherosclerotic lesion and their relationships with clinical manifestations. A relevant question as to what types of cell in the atherosclerotic lesion express these MMPs has been addressed by other studies. It has been shown that MMP-1, -3, -7, -9, and -12 are expressed primarily by macrophages in the shoulder region of the atherosclerotic plaque and the border between the lipid core and overlying fibrous areas.6,7,10 In addition, previous studies have also shown that MMP mRNA levels correspond to the levels of MMP proteins and proteolytic activities in atherosclerotic lesions.7,12

It is likely that plaque rupture involves a battery of different proteases, and therefore further studies are required to examine a larger set of candidate genes for plaque instability. Strong candidates would include the other 2 collagenases, ie, MMP-8 and MMP-13, both of which have been shown to be expressed in atherosclerotic lesions, and the latter has been found to be expressed at higher levels in lipid-rich plaques.12,21 In addition to MMPs, serine and cysteine proteases might also have an impact on atherosclerotic plaque stability. Recent studies have revealed that members of the serine proteinase family, such as neutrophil elastase21 and plasminogen activators,22 and members of the
cysteine proteinase family, such as cathepsins B, K, L, and S, are expressed in atherosclerotic plaques and might also have an impact on plaque stability.

Atherosclerosis is a systemic disease and patients often have multiple lesions in different arteries. In the present study, only 1 carotid plaque per patient was available for examination. It is possible that additional atherosclerotic plaques were present in the vasculature in some of these patients, and they might have different MMP expression profiles and histopathological features from the plaques examined in this study. However, the carotid plaques examined in this study were the plaques that prompted the carotid endarterectomy operations and seemed likely responsible for the clinical symptoms in these patients.

In summary, the results of this study support a role of MMP-1, and possibly also MMP-12, in determining atherosclerotic plaque stability and the development of amaurosis fugax. It is conceivable that inhibiting these MMPs could stabilize atherosclerotic plaques and improve the clinical outcome, and it is encouraging that a prospective, randomized, double-blind trial has shown that doxycycline can reduce MMP-1 expression in carotid atherosclerotic plaques.

Acknowledgments

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References

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