Prospective, Randomized, Double-Blind Trial Investigating the Effect of Doxycycline on Matrix Metalloproteinase Expression Within Atherosclerotic Carotid Plaques

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Background and Purpose—Elevated levels of matrix metalloproteinases (MMPs), particularly MMP-1 and MMP-9, have been implicated in plaque rupture. It has been suggested that inhibition of MMPs may stabilize vulnerable atherosclerotic plaques and improve clinical outcome. The aim of the study was to investigate the ability of doxycycline, a nonspecific MMP inhibitor, to reduce MMP concentration in carotid atheroma.

Methods—The study design was a prospective, double-blind randomized trial. One hundred patients requiring carotid endarterectomy were randomized to receive 200 mg/d doxycycline or placebo for 2 to 8 weeks before surgery. During endarterectomy, carotid plaques were retrieved. The concentrations of MMPs and doxycycline were determined in the atherosclerotic tissue by enzyme-linked immunosorbent assay and high-performance liquid chromatography, respectively. Clinical events were recorded, as was the rate of preoperative embolization (transcranial Doppler).

Results—Analysis of endarterectomized specimens demonstrated a mean doxycycline concentration of 6.0 μg/g wet weight in treated patients. Administration of doxycycline significantly reduced the concentration of MMP-1 in carotid plaques from a mean of 14.8 to 10.3 ng/100g wet weight ($P=0.038$). This difference was due to decreased MMP-1 transcript ($P<0.001$). There was no difference in any other MMP (MMP-2, -3, or -9) or tissue inhibitor of matrix metalloproteinases–1 or –2.

Conclusions—Doxycycline penetrated atherosclerotic plaques with acceptable tissue levels. This resulted in a reduction in MMP-1 concentration because of decreased expression. (Stroke. 2002;33:2858-2865.)

Key Words: atherosclerosis ■ drug therapy ■ metalloproteinases

Acute disruption of an atherosclerotic plaque is a prelude to the onset of clinical ischemic events, including stroke and myocardial infarction. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that act as the physiological regulators of the extracellular matrix. A review of the available evidence suggests that these enzymes play a pivotal role in the evolution and development of atherosclerotic plaques.\(^1\) In recent years, the role of MMPs in acute plaque disruption has received increasing attention because proteolytic disruption of the fibrous cap overlying an atheromatous plaque may be a final common pathway in many clinical events.

Previous studies have identified the presence of several MMPs within atherosclerotic plaques, of which MMP-1 and MMP-9 appear to be the most important. Galis and colleagues\(^2\) described a localized increase in the expression of MMP-1, MMP-3, and MMP-9 in the vulnerable shoulder and cap of the plaque, in tandem with an increase in overall proteolytic activity. Several investigators have now described increased expression of MMP-1 in vulnerable regions of atherosclerotic lesions\(^1,4\) and suggested that this enzyme may be partly responsible for plaque rupture.

More recent work has clearly demonstrated an increase in the level and expression of MMPs in unstable carotid plaques.\(^5,7\) Experimental and clinical evidence has demonstrated that vulnerable atherosclerotic plaques may be characterized by an imbalance between MMPs and their inhibitors and that artificially enhancing antiproteolytic mechanisms may stabilize these plaques.\(^8,9\)

Pharmacotherapy to prevent plaque disruption is an attractive option. Hypothetically, inhibition of MMPs within the atherosclerotic plaque may prevent disruption of the shoulders and fibrous cap of the plaque and thus inhibit plaque rupture. Doxycycline is a member of the tetracycline antibiotic family that, apart from its antibacterial action, is also a potent nonselective MMP inhibitor. Several investigations have demonstrated that doxycycline may inhibit MMP expression and activity in a number of vascular disorders.\(^10,12\) The aim of the present study was to determine the effect of doxycycline on MMP concentration within carotid plaques.
Methods

Protocol
The study was designed as a double-blind, randomized, controlled trial to evaluate the effects of doxycycline on MMP production within carotid plaques of patients undergoing carotid endarterectomy. The Leicestershire Research Ethics Committee approved the study.

Patients were recruited from a “single visit” carotid clinic, where patients with carotid disease underwent a vascular consultation and subsequent duplex evaluation. All patients who were considered to require an elective carotid endarterectomy were eligible for the study. Patients with hypersensitivity to tetracycline or systemic lupus erythematosus or who required urgent surgery were excluded from the study. Patients were selected for carotid endarterectomy on the basis of a duplex scan that demonstrated a carotid stenosis >70% (peak systolic velocity >230 cm/s). Indications for carotid endarterectomy in the 100 randomized patients were stroke in 34, transient ischemic attacks in 27, amaurosis fugax in 26, and central retinal artery occlusion in 3; 7 patients were asymptomatic.

Randomization was performed after written informed consent was obtained. Patients were randomized to doxycycline 100 mg twice daily or placebo. After randomization, patients were given a provisional date for surgery 2 to 8 weeks from their initial consultation. Some patients required further medical evaluation or specialized investigations that were organized at this stage. Patients remained on the same medication until surgery. The day before surgery, patients were admitted, and a plasma sample was collected for determination of doxycycline concentration. A carotid duplex scan was repeated at this time, and patients were monitored for preoperative embolization (transcranial Doppler). Carotid endarterectomy was performed in a standardized manner, and the carotid plaque was retained for estimation of MMP concentration, doxycycline penetration, histological analyses, and MMP expression.

The primary outcome measure for the study was the concentration of MMPs and tissue inhibitor of MMPs (TIMPs) in the endarterectomy carotid plaque (with particular reference to MMP-1 and -9). Overall, 19 patients were withdrawn from the trial because they did not undergo carotid endarterectomy within the specified time period and thus plaque MMP concentrations could not be determined.

Secondary outcomes were preoperative cerebral embolization, histological plaque analysis, and doxycycline concentration in serum and plaque. Clinical events were also recorded and reported on an intention-to-treat basis (preoperative transient ischemic attack, stroke, and death) but were not considered an end point of the trial.

The sample size for the study was calculated to detect a difference in MMP-1 concentration between the groups with an effect size of 0.75. At a significance level of 0.05 and a power of 0.9, 70 patients would be needed to refute the null hypothesis. An anticipated dropout rate of 30% necessitated that 100 patients be recruited.

Assignment and Masking
The unit of randomization was the patient. Patients were assigned to placebo or doxycycline 100 mg twice daily by a randomly generated numerical sequence. Placebo and doxycycline were packaged in identical capsules and supplied in receptacles containing a 6-week supply. These were renewed with identical drug if necessary. The investigators were blinded as to treatment allocation until all samples had been analyzed.

Sample Collection
A blood sample was obtained from patients the day before surgery, and plasma was collected for determination of serum doxycycline concentration. Plasma samples were stored at −80°C until use. Carotid endarterectomy was performed with a standard technique and with minimal manipulation of the specimen. After endarterectomy, the carotid plaque was divided longitudinally through the point of maximum stenosis. An attempt was made to divide the plaque equally with reference to areas of ulceration, thrombosis, or hemorrhage. One half was snap-frozen for later MMP extraction. The other half was divided longitudinally again, with the specimens prepared for histological analysis and doxycycline quantification.

Doxycycline Concentration
Carotid plaques were snap-frozen in liquid nitrogen and stored at −80°C. Doxycycline concentrations in plasma samples and carotid plaques were quantified with a high-performance liquid chromatography-based methodology for this study.15

MMP Extraction and Quantification
MMP and TIMP levels were quantified by gelatin zymography and enzyme-linked immunosorbent assay (ELISA) as previously described.3 Briefly, plaques were stored at −70°C until MMPs were extracted. During the extraction process, samples were dialyzed to remove molecules with a molecular weight <12 000 Da (doxycycline, 512 Da). The active and latent forms of MMP-2 and MMP-9 were analyzed with substrate gel zymography. The total concentrations of MMP-1, -2, -3, and -9, TIMP-1, and TIMP-2 were quantified with the Biotrack assay system (Amersham, UK).5

MMP-1 Expression
Reverse-transcription polymerase chain reaction (RT-PCR) was performed to confirm and compare the expression of MMP-1 within the plaque. In selected samples, total RNA was extracted from the tissue with Trizol reagent (Life Technologies), and RT-PCR was performed using sequences detailed below, according to established methodology.6 MMP-1 expression was analyzed in only 9 plaques because most plaques had insufficient tissue to allow for MMP and doxycycline analysis, histological examination, and mRNA extraction. Molecular analysis was possible only in very bulky plaques. Samples were therefore selected for expression studies on the basis of weight and were not consecutive.

Primers were designed according to GeneBank data. The 3-antisense primer was complementary to bases 1496 through 1518 in the 3-untranslated region (5-GGACTCACACCATGTGTTTTCC-3), and the 5-sense primer was defined by bases 1114 through 1135 of the hemopexinlike domain (5-GGGCTGTTCAGG-GACAGAATGT-3) of MMP-1. Amplification of cDNA was performed with Taq DNA polymerase with 40 cycles of denaturation, annealing, and extension at 94°C, 59°C, and 72°C, respectively, for 1 minute. Negative controls included samples without the primers and polymerase. MMP-1 expression was compared with β-actin by densitometric analysis of ethidium bromide–stained gels.

Histological Analysis
Immediately after endarterectomy, samples were placed in fresh 4% paraformaldehyde. After decalcification, samples were paraffin embedded and sectioned at 4-μm intervals. Sections were stained with hematoxylin and eosin, elastic van Gieson, and monoclonal antibodies for MMP-1 and -9 (R&D Systems).14 An experienced histopathologist (L.J.) who was blinded to patient identity and randomization then evaluated sections. Plaques were characterized with reference to the presence or absence of cap foam cells, cap thinning, intraplaque hemorrhage, plaque necrosis, ulceration, and calcification.

Preoperative Embolization
Patients were monitored with transcranial Doppler insonation of their ipsilateral middle cerebral artery for 30 minutes the day before surgery. The number of emboli was recorded and characterized as a marker of plaque instability.5,6

Statistical Analysis
Distribution of the data sets was analyzed with the Kolmogorov-Smirnov test. Because data were normally distributed, results were expressed as mean values with SD. Differences in continuous variables were analyzed with the unpaired t test. Discrete variables were compared with Fisher’s exact test.
Results

Participant Flow and Follow-Up

Patient demographics at randomization are illustrated in Table 1. Overall, 100 patients were randomized. The trial profile is summarized in Figure 1. There were 19 patients who were withdrawn from the trial before carotid endarterectomy. Most were patients who required specialists’ opinions or investigation of comorbid medical conditions. These patients had their surgical procedure deferred for 6 months after randomization and were therefore withdrawn from the primary study. Two patients had a discrepancy between their duplex stenosis performed at randomization and the day before surgery. Repeat examinations determined that the degree of carotid stenosis was <70% and operative intervention was considered inappropriate (Table 2).

Interestingly, 3 patients in the placebo group developed carotid occlusion between randomization and carotid surgery compared with 1 patient in the doxycycline-treated group.

Doxycycline Concentration

No patients in the placebo group demonstrated evidence of doxycycline within plasma or carotid plaque. Patients receiving doxycycline had a mean±SD plasma doxycycline concentration of 3.5±1.3 μg/mL 3 hours after ingestion.

In the cohort of patients receiving active drug, the mean±SD concentration of doxycycline within the excised endarterectomy tissue was 6.0±2.6 μg/g wet weight.

MMP Concentration

The concentrations of MMP-1, -2, -3, and -9 and TIMP-1 and -2 were quantified in the carotid plaques with ELISA. The results are illustrated in Table 3. The main finding of the study was a significant reduction in MMP-1 within the cohort treated with doxycycline. Interestingly, the ratio of TIMP-1 to MMP-1 was increased in the treated group, which suggested a change in enzyme balance toward inhibition of collagenolysis. This difference, however, failed to reach statistical significance. Gelatin zymography demonstrated no signifi-

TABLE 1. Patient Characterization at Randomization

<table>
<thead>
<tr>
<th></th>
<th>Doxycycline (n=50)</th>
<th>Placebo (n=50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62.2±9</td>
<td>67.7±9.6</td>
<td>0.40</td>
</tr>
<tr>
<td>Carotid stenosis, %</td>
<td>80.6±7.7</td>
<td>79.2±9.7</td>
<td>0.44</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>28 (56)</td>
<td>21 (42)</td>
<td>0.23</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>16 (32)</td>
<td>15 (30)</td>
<td>1.0</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>15 (30)</td>
<td>16 (32)</td>
<td>0.83</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>4 (8)</td>
<td>5 (10)</td>
<td>0.74</td>
</tr>
<tr>
<td>Ischemic heart disease, n (%)</td>
<td>15 (30)</td>
<td>18 (36)</td>
<td>0.53</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>41 (82)</td>
<td>36 (72)</td>
<td>0.36</td>
</tr>
<tr>
<td>β-Blocker, n (%)</td>
<td>7 (14)</td>
<td>8 (16)</td>
<td>1.0</td>
</tr>
<tr>
<td>ACE inhibitor, n (%)</td>
<td>4 (8)</td>
<td>4 (8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Calcium channel blocker, n (%)</td>
<td>8 (16)</td>
<td>7 (14)</td>
<td>1.0</td>
</tr>
<tr>
<td>Diuretic, n (%)</td>
<td>15 (30)</td>
<td>14 (28)</td>
<td>1.0</td>
</tr>
<tr>
<td>Statin therapy, n (%)</td>
<td>15 (30)</td>
<td>16 (32)</td>
<td>1.0</td>
</tr>
<tr>
<td>Time to surgery, d</td>
<td>67.3 (37.3)</td>
<td>68.3 (27.4)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

ACE indicates angiotensin-converting enzyme. Continuous variables are presented as mean±SD, and comparison was done with an unpaired t test. Discrete variables were analyzed with Fisher’s exact test.

TABLE 2. Characteristics of Patients Who Were Withdrawn From the Trial

<table>
<thead>
<tr>
<th></th>
<th>Doxycycline (n=6, n)</th>
<th>Placebo (n=13, n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical comorbidity requiring specialist investigation or opinion that resulted in a delay in surgery &gt;6 mo</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Death (MI)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Stroke</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Carotid occlusion</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Noncompliance with medication</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Repeat duplex &lt;70% stenosis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Refused surgery</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction.

Figure 1. Trial flow diagram. Detailed reasons for patient withdrawal are given in Table 2.
cant differences between the groups with respect to MMP-2 or -9 in pro or active form (data not shown).

In the patients randomized to doxycycline, there was no relationship between the duration of therapy and MMP-1 levels (Pearson’s correlation coefficient, −0.003; P=0.98) (Figure 2).

**MMP-1 Expression**

MMP-1 expression was analyzed in a subset of 9 plaques (Figure 3). Expression was measured with semiquantitative RT-PCR and was compared with β-actin. RT-PCR demonstrated a significant reduction in the ratios of MMP-1 to β-actin in the plaques derived from patients receiving doxycycline compared with control subjects (P=0.0002; 95% CI, 0.26 to 0.13).

**MMP-1 Immunocytochemistry**

Immunocytochemistry of untreated plaques with monoclonal antibodies to MMP-1 demonstrated intense staining in areas of macrophage infiltration in the shoulders of the carotid plaques. MMP-1 was predominantly identified in the cytoplasm of macrophages. Plaques treated with doxycycline demonstrated a similar pattern of staining as control tissues, with MMP-1 demonstrated predominantly in the shoulder regions. The intensity of staining was reduced compared with control tissue. No MMP-1 was identified in smooth muscle cells (Figure 4).

**Histological Analysis**

The histological characterization of the carotid plaques is illustrated in Table 4. There were no significant differences in plaque morphology between the 2 groups.

**Preoperative Embolization**

In this study, 1 patient receiving doxycycline (2.2%) and 4 patients on placebo (10.8%) had preoperative evidence of cerebral embolization (P=0.17, Fisher’s exact test).

**Discussion**

The present study has demonstrated that the nonspecific metalloproteinase inhibitor doxycycline was concentrated in atherosclerotic tissue and caused significant inhibition of MMP-1 in carotid plaques. The tissue concentrations of the other potentially important MMPs or their physiological inhibitors were not affected. Doxycycline has been advocated as a possible therapeutic agent in several vascular disorders.11,15 Doxycycline has direct actions on MMPs through

**Table 3. Concentrations in the Carotid Plaques of Patients Receiving Doxycycline 200 mg/d or Placebo**

<table>
<thead>
<tr>
<th>MMP</th>
<th>Doxycycline, ng protein/100 g wet weight tissue</th>
<th>Placebo, ng protein/100 g wet weight tissue</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>10.33±7.02</td>
<td>14.76±10.9</td>
<td>0.038</td>
<td>0.24–8.62</td>
</tr>
<tr>
<td>MMP-2</td>
<td>585.7±263.9</td>
<td>555.6±243.4</td>
<td>0.61</td>
<td>−146–85.8</td>
</tr>
<tr>
<td>MMP-3</td>
<td>31.0±12.6</td>
<td>33.5±17.2</td>
<td>0.47</td>
<td>−4.38–9.32</td>
</tr>
<tr>
<td>MMP-9</td>
<td>309±198.5</td>
<td>396±311.9</td>
<td>0.15</td>
<td>−33.5–208</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>15 560±6316</td>
<td>14 710±6169</td>
<td>0.56</td>
<td>−3774–2062</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>4904±216</td>
<td>5337±172.5</td>
<td>0.36</td>
<td>−50.3–137.2</td>
</tr>
<tr>
<td>TIMP-1/MMP-1</td>
<td>1938±1328</td>
<td>1410±1035</td>
<td>0.068</td>
<td>−4–1001</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Comparative analysis used the unpaired t test.

**Figure 2.** MMP-1 concentration in patients treated with doxycycline as a function of duration of therapy. Best-fit linear regression line with 95% CIs is demonstrated. There was no relationship between concentration and time (Pearson’s correlation coefficient, −0.14, P=0.41).

**Figure 3.** Ethidium bromide gel comparing MMP-1 expression in patients receiving either doxycycline or placebo. β-Actin controls were used to standardize samples. Patients receiving doxycycline demonstrated a significant reduction in MMP-1 expression in their carotid plaques (P<0.001).
reversible inhibition of the enzyme and reduction of gene transcription.\textsuperscript{16} In addition to these effects, doxycycline reduces the levels of interleukin (IL-1\textsubscript{a}, IL-1\textsubscript{b}, IL-6)\textsuperscript{17} and nitric oxide synthase,\textsuperscript{18} which may act to reduce proteolytic activity, as well as having effects on arterial diameter and vessel wall fragmentation.\textsuperscript{19}

To be effective in reducing MMP activity in vascular lesions, doxycycline must be efficiently delivered to the tissues. Previous studies have demonstrated that tetracyclines localize to atherosclerotic plaques. Franklin et al\textsuperscript{20} revealed that tetracycline rapidly penetrated the aortic wall and achieved a median concentration of 2.9 \mu g/g tissue wet weight, which was sufficient for biological activity. In the present study, 200 mg/d of orally administered doxycycline resulted in a mean concentration of 6.1 \mu g doxycycline/g wet weight of carotid plaque and a serum level of 3.5 \mu g/mL. Recent observations from Prall et al\textsuperscript{21} have suggested that doxycycline concentrations >2 \mu g/mL are likely to be effective in reducing MMP concentrations in clinical studies.

This reduction in MMP-1 observed in the present study has confirmed previous reports that have demonstrated inhibition of MMP-1 by relatively low concentrations of doxycycline in other tissues.\textsuperscript{22} The inhibition observed in human carotid plaques may have significant clinical consequences. Recent experimental studies have suggested that MMP-1 may play a significant role in atherogenesis and plaque rupture. Chen et al\textsuperscript{23} demonstrated that losartan reduced lipid deposition within a rabbit model and that this effect may be linked to a reduction in MMP-1 expression. Similarly, other groups have observed that some HMG CoA reductase inhibitors alter smooth muscle accumulation and collagen deposition in a hyperlipidemic model and have postulated that MMP-1 may play a pivotal role in this process.\textsuperscript{24,25}

The function that MMP-1 plays in human plaque rupture has not been completely defined, although evidence suggests a pivotal role. Elevated levels of MMP-1 have been demonstrated in unstable atheromatous carotid plaques,\textsuperscript{4} and the enzyme has been localized to activated white cells.\textsuperscript{26} Interestingly, oxidized low-density lipoprotein is known to stimulate MMP-1 expression in human endothelial cells.\textsuperscript{27} As well as its direct proteolytic capacity, MMP-1 plays an integral role in the cascade of MMP production and activation that occur in the tissues. MMP-1 has the ability to cleave type I, II, and III collagen and plays a central role in the activation of MMP-2 and -9.\textsuperscript{28} The pleiotropic effects of MMP-1 make this enzyme an attractive target for pharmacotherapy to reduce proteolysis in unstable carotid plaques.

The present study failed to detect any differences in the concentrations of other MMPs. Previous studies have demonstrated a reduction in MMP-9 levels in vascular tissue. Curci et al investigatd MMP-9 production and expression in patients with abdominal aortic aneurysms.\textsuperscript{29} A significant reduction of MMP-9 was demonstrated in the doxycycline treated patients by immunoblotting and RT-PCR. Previous studies from our group demonstrated that highly symptomatic carotid plaques were characterized by elevated MMP-9 levels.\textsuperscript{5} Unpublished observations on cultured human monocytes suggested that serum levels of doxycycline <2 \mu g/mL doxycycline reduced MMP-9 secretion by \textasciitilde 20\%. In the present investigation, the MMP-9 concentration in carotid plaques from the doxycycline-treated group was reduced by a similar amount but failed to reach statistical significance.

One criticism of the present study was that MMP protein concentration, rather than MMP activity, was used as the primary end point. Ideally, it would have been preferable to quantify the extent of MMP-dependent elastolytic and collagenolytic action through the use of activity assays. However, these assays have been technically difficult and unreliable in the past\textsuperscript{29} and have not been successfully implemented in our laboratory. Despite these limitations, doxycycline reduced the concentration of MMP-1, and RT-PCR suggested that this action was mediated by reduced gene transcription.

In the long term, inhibition of MMP-1 in high-risk carotid or coronary plaques might be expected to reduce

\begin{table}[h]
\centering
\caption{Morphological Characteristics of Carotid Plaques} \label{table:4}
\begin{tabular}{|l|c|c|c|}
\hline
 & Doxycycline & Placebo & \textit{P} \\
\hline
Cap foam cells, n & 1.65 \pm 1.02 & 1.36 \pm 0.83 & 0.17 \\
\hline
Cap thinning, n (%) & 32 (72) & 23 (62) & 0.21 \\
\hline
Plaque hemorrhage, n (%) & 19 (43) & 18 (49) & 0.82 \\
\hline
Plaque necrosis, n (%) & 25 (57) & 21 (57) & 1.0 \\
\hline
Plaque rupture, n (%) & 11 (25) & 7 (19) & 0.59 \\
\hline
Calcification, n (%) & 30 (68) & 22 (60) & 0.32 \\
\hline
\end{tabular}
\begin{flushleft}
Discrete variables were analyzed with Fisher’s exact test.
\end{flushleft}
\end{table}
the incidence of plaque rupture or progression. The present study was powered to investigate MMP concentrations in atherosclerotic tissue, so a reduction in clinical events was not expected. However, several clinical findings warrant comment, although they were not considered end points of the clinical trial. Overall, 4 patients in the trial developed a carotid occlusion between randomization and surgery: 3 in the placebo group and 1 in the doxycycline cohort. This was an unexpectedly high number (the usual rate in our center is <1.5%) but did represent a tendency toward a lower occlusion rate in patients treated with doxycycline. Clearly, a larger cohort would be required to investigate the clinical effects of MMP inhibition on clinical outcome, but these initial data show some promise.

There are a number of limitations in the trial. The primary concern was the number of patients who were withdrawn before carotid endarterectomy. Some had a clinical event that served as a definitive end point, but most were withdrawn because they required specialist medical treatment before endarterectomy. In retrospect, it may have been more appropriate not to randomize these patients until their medical comorbidity had been evaluated and treated. Patients also received doxycycline for variable times before surgery, but there was no relationship between MMP levels and duration of treatment.

The trial did not address the role of the antimicrobial effect of doxycycline on carotid atheroma. Numerous studies have demonstrated an association between carotid atheroma and Chlamydia pneumoniae. It has been suggested that eradication of chlamydia may improve clinical events in patients with coronary atherosclerosis, although definitive results are awaited. Doxycycline has activity against chlamydia in atherosclerotic plaques, and experimental evidence has suggested that anticlimamydial therapy may reduce plaque volume.

Despite these limitations, the present study suggests that doxycycline administration to patients with unstable atherosclerotic plaques was associated with inhibition of MMP-1. Further studies are required to determine the role of MMP inhibition in modulating adverse clinical events in patients with widespread atheroma.

Acknowledgments

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References


MMP Inhibition and the Development of Cerebrovascular Atherosclerosis: The Road Ahead

It has been 40 years since the first member of the matrix metalloproteinase (MMP) family of specialized enzymes was described. Structural, molecular, and biochemical approaches have subsequently contributed to piecing together the puzzle of how MMPs work and how they contribute to the pathophysiology of various disease processes. Because degradation of the extracellular matrix scaffold enables reshaping of tissue, participation of MMPs has become the object of intense recent scientific interest in relation to pathogenic events involved in vascular remodeling.

MMPs are hypothesized to play a pivotal role in the pathogenesis of several central nervous system disorders (reviewed in Reference 2). Indeed, increased levels of expression of MMP-9 (gelatinase B) and MMP-2 (gelatinase A) have been observed in Alzheimer’s disease, stroke, multiple sclerosis, and amyotrophic lateral sclerosis. Aberrant activity of MMPs was also observed during human brain focal ischemia, cardioembolic stroke, and atherogenesis of intracranial arteries. There is now convincing evidence of increased MMP activity during acute plaque disruption. The hypothesis that MMPs contribute to weakening of atherosclerotic plaques is especially attractive for the potential development of therapeutic interventions aimed at preventing plaque disruption and its clinical sequelae. Accordingly, intervention experimental studies demonstrated that the MMP inhibitor KB-R7785 has a protective efficacy against focal cerebral ischemia in mice. Moreover, MMP inhibition (with the inhibitor batimastat, BB-94) attenuates mechanisms involved in experimental thrombolyis-induced hemorrhage. Furthermore, an elegant study demonstrates that there were no differences in systemic hemodynamic parameters and gross cerebrovascular anatomy between wild-type mice and mutant mice with a targeted knockout of the MMP-9 gene. After induction of focal ischemia, similar reductions in cerebral blood flow were obtained. In the MMP-9 knockout mice, ischemic lesion volumes were significantly reduced compared with wild-type littermates in male and female mice. In normal wild-type mice, the MMP inhibitor batimastat also significantly reduced ischemic lesion size. However, batimastat had no detectable protective effect when administered to MMP-9 knockout mice subjected to cerebral ischemia. These data further corroborate the concept that MMP-9 plays a deleterious role in the development of brain injury after brain ischemia. In another study, wild-type and MMP-2 knockout mice were subjected to permanent and transient occlusions of the middle cerebral artery. MMP-9 levels were increased in all brains after ischemia. MMP-2 levels did not show a significant increase in wild-type mice and were not detectable in knockout mice. Equivalent ischemic reductions in perfusion in wild-type and knockout mice were observed. These data indicate that MMP-2 does not contribute to acute tissue damage in this model of focal cerebral ischemia. Therefore, further studies are needed to investigate specific pathophysiological mechanisms involving MMP (and its isoforms) in the development of brain ischemic damage. In this light, the use of the broad MMP inhibitor KB-R7785 or batimastat (or newly developed drugs) should be developed preclinically, promoting the design of more focused studies in humans.

On the other hand, doxycycline has well-described anticollagenase properties and is used in the treatment of α1-antitrypsin deficiency panangiitis. Moreover, this drug was proposed to suppress the growth of abdominal aortic aneurysms and was used in the treatment of recalcitrant recurrent corneal erosions, which is associated with increased MMP activity. These studies provided the “soil” for a new study appearing in this issue of Stroke. This study, done in patients elected for carotid endarterectomy, demonstrates that 200 mg/d doxycycline penetrated atherosclerotic carotid plaques with acceptable tissue levels. This intervention study also induced a reduction in MMP-1 tissue expression in carotid specimens. MMP-2 and MMP-9 activities were not...
affected by the drug, and there was no clinical difference in patient outcome.\textsuperscript{14} No causal relationship can be postulated in a correlation study, but future studies should evaluate MMP activities rather than MMP protein levels, and a large cohort of patients is needed for evaluating differences in outcome. Because arterial MMP levels increase with age,\textsuperscript{5} patients should be also age stratified, and doxycycline must be administered following reproducible protocols in terms of doses and days of treatment before to investigate clinical endpoints. Indeed, the natural history of atherosclerosis is different in intracranial arteries compared with extracranial arteries.\textsuperscript{5,15,16} Until adult age, increased MMP activities are associated with increased endogenous resistance to oxidative stress in intracranial but not extracranial arteries.\textsuperscript{5} This balance is lost in elderly patients.\textsuperscript{9} Thus, the beneficial effects of doxycycline seen in this issue of \textit{Stroke}\textsuperscript{14} can decrease with increasing age.

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Prospective, Randomized, Double-Blind Trial Investigating the Effect of Doxycycline on Matrix Metalloproteinase Expression Within Atherosclerotic Carotid Plaques
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