Clinical and Biochemical Results of the Metalloproteinase Inhibition with Subantimicrobial Doses of Doxycycline to Prevent Acute Coronary Syndromes (MIDAS) Pilot Trial


Background—Vulnerable plaque demonstrates intense inflammation in which macrophages secrete matrix metalloproteinases (MMPs) that degrade the fibrous cap, ultimately leading to rupture, in situ thrombosis, and an associated clinical event. Thus, inhibition of MMP activity or more general suppression of vascular inflammation are attractive targets for interventions intended to reduce plaque rupture. We hypothesized that subantimicrobial doses of doxycycline (SDD) (20 mg twice daily) would benefit patients with coronary artery disease by reducing inflammation and MMP activity and thus possibly prevent coronary plaque rupture events.

Methods and Results—We conducted a prospective, randomized, double-blind, placebo-controlled pilot study of 6 months of SDD or placebo treatment to reduce inflammation and prevent plaque rupture events. A total of 50 patients were enrolled, of whom 24 were randomized to placebo and 26 to SDD. At 6 months, there was no difference in the composite endpoint of sudden death, fatal myocardial infarction (MI), non-fatal MI, or troponin-positive unstable angina in SDD compared with placebo-treated patients (8.4% versus 0%, \(P = 0.491\)). Biochemical markers of inflammation were assessed in plasma at study entry and after 6 months of therapy in 30 patients. In SDD-treated patients, high-sensitivity C-reactive protein (CRP) was reduced by 46% from 4.8±0.6 µg/mL to 2.6±0.4 µg/mL (\(P = 0.007\)), whereas CRP was not significantly reduced in placebo patients. Interleukin (IL)-6 decreased from 22.1±3.7 pg/mL at baseline to 14.7±1.8 pg/mL at 6 months in SDD-treated patients (\(P = 0.025\)) but did not decrease significantly in placebo-treated patients. On zymography, pro-MMP-9 activity was reduced 50% by SDD therapy (\(P = 0.011\)), whereas it was unchanged by placebo treatment.

Conclusion—SDD appears to exert potentially beneficial effects on inflammation that could promote plaque stability. These findings should be investigated in a larger study. (Arterioscler Thromb Vasc Biol. 2004;24:733-738.)

Key Words: inflammation ■ plaque rupture ■ metalloproteinase ■ cytokines ■ antibiotics

Disruption of atherosclerotic plaque in the coronary arteries is the final common pathophysiology of most cases of sudden ischemic cardiac death, acute myocardial infarction (MI), and unstable angina.1–3 Sites of plaque rupture are characterized macroscopically by a rent or erosion in the shoulder of the fibrous cap overlying the atheroma with superimposed thrombus. At the microscopic level, plaque vulnerable to rupture demonstrates intense inflammation with infiltration by macrophages, lymphocytes, and mast cells.1–3 In an inflammatory environment, macrophages secrete matrix metalloproteinases (MMPs) that progressively degrade the collagenous components of the fibrous cap. Ultimately, when the structural integrity of the fibrous cap is overwhelmed by the mechanical stresses imposed on it, the fibrous cap fractures, inciting in situ thrombosis and an associated clinical event.1–3 Thus, inhibition of MMP activity or more general suppression of vascular inflammation are attractive targets for interventions intended to reduce plaque rupture.

The antibiotic doxycycline at subantimicrobial doses inhibits MMP directly and has more generalized anti-inflammatory properties by virtue of its ability to inhibit several cytokine and protease mediators of inflammation.4 We hypothesized that subantimicrobial doses of doxycycline (SDD) as a consequence of these properties would benefit patients with coronary artery disease by reducing inflammation and MMP activity and thus possibly prevent coronary plaque rupture events.

Methods

This study was a randomized, prospective, placebo-controlled, double-blind, single-center pilot study of patients admitted with symptomatic coronary artery disease to the Jack D. Weiler Hospital of the Albert Einstein College of Medicine/Montefiore Medical Center (D.L.B.), New York, NY; Beth Israel Medical Center (D.L.B.), New York, NY; and the Department of Oral Biology and Pathology (H.-M.L., L.M.G.), School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, NY.

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in 3 months after randomization, and each patient or their physician was contacted by phone or mail to ascertain whether an endpoint event occurred at completion of the study. Acute MI was defined as chest pain lasting at least 20 minutes and creatine kinase greater than or equal to twice the upper limit of normal, with CK-MB elevated above the 99th percentile of the normal range. Unstable angina was defined as chest pain lasting at least 5 minutes associated with elevated troponin levels. Analysis was by the intention-to-treat principle.

### Clinical Study Endpoints

The primary clinical endpoint was the composite of sudden death, fatal or nonfatal MI, or troponin-positive unstable angina during the 6 months after randomization. Patients were interviewed or examined 6 months after randomization, and each patient or their physician was contacted by phone or mail to ascertain whether an endpoint event occurred at completion of the study. Acute MI was defined as chest pain lasting at least 20 minutes and creatine kinase greater than or equal to twice the upper limit of normal, with CK-MB elevated above the 99th percentile of the normal range. Unstable angina was defined as chest pain lasting at least 5 minutes associated with elevated troponin levels. Analysis was by the intention-to-treat principle.

### Biochemical Analyses

The primary biochemical endpoint was CRP level after 6 months of SDD or placebo therapy. Non-fasting blood samples were drawn from 30 patients at study entry and study termination. Plasma was obtained by centrifugation and stored at −70°C until analysis. CRP levels in plasma were measured by high-sensitivity ELISA for C-reactive protein (CRP) (ICN Pharmaceuticals, Diagnostic Division, Costa Mesa, Calif). Plasma samples were diluted 100-fold with sample diluent containing phosphate-buffered bovine serum albumin solution before use. Ten μL of diluted specimen and undiluted CRP standards were pipetted into appropriate wells and 100 μL of CRP enzyme conjugate reagent was then added to the reaction mixture. All subsequent steps were performed as described in the commercial assay kit, and yellow color was read at 450 nm with a microtiter well reader. This assay has a sensitivity of 0.4 ng/mL and the mean recovery was 100.4%.

Levels of IL-1β, IL-6, IL-10, and TNF-α were measured by commercially available ELISA kits (Biosource International, Camarillo, Calif). The concentrations were expressed as pg/mL. Plasma concentrations of MMP-9 were measured by an ELISA (Oncogene Research Products, Boston, Mass). This assay detects free and tissue inhibitor of metalloproteinase (TIMP)-1–bound MMP-9 with a sensitivity of 0.1 ng/mL.

Gelatin zymography was performed using a modification of a technique described previously. In brief, 2.5 μL of plasma samples were loaded on to precast 10% SDS-PAGE gels containing 0.1% (1 mg/mL) denatured type I collagen (gelatin) as substrate (Invitrogen Life Technologies, Carlsbad, Calif). After electrophoresis (125 V) for 2 hours at 4°C, the slab gels were incubated for 30 minutes with 2.5% Triton X-100 at 22°C, then incubated with developing buffer (50 mmol/L Tris/HCl with 0.2 mol/L NaCl, 10 mmol/L CaCl₂, pH 7.6) overnight at 37°C. The gels were stained with Coomassie Blue R-250, destained with 20% methanol/10% acetic acid, and the molecular weights of the gelatinolytic zones were compared with standards of pro-MMP-2 (72kDa) and pro-MMP-9 (92kDa) (Oncogene Research Products). Identical controls were used in all gels. The gelatin zymograms were then scanned using a Kodak Scientific Imaging System (Eastman Kodak, Rochester, NY) to determine the relative activity of MMP-2 and MMP-9. The data were presented as densitometric units.

### Sample Size and Power Calculations

The sample size necessary to detect a 20% reduction in a mean CRP of 4.0 mg/L in the placebo group with an alpha of 0.05 and a beta of 0.8 was 10 patients per arm. A cohort of 50 patients was determined to have a power of 0.20 to detect a 50% reduction in the composite endpoint assuming a 6-month event rate of 32% in the control group.

### Statistical Analysis

Differences between the 2 treatment groups were assessed by the Student t test for continuous variables and χ² or Fisher exact test, as appropriate, for categorical variables. ANOVA was used for within-group comparisons. Statistical calculations were performed with SPSS software.
TABLE 2. Angiographic and Procedural Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo (n=26)</th>
<th>Doxycycline (n=26)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction (%)</td>
<td>63±10</td>
<td>64±8</td>
<td>0.785</td>
</tr>
<tr>
<td>1-vessel CAD (%)</td>
<td>49</td>
<td>30</td>
<td>0.273</td>
</tr>
<tr>
<td>2-vessel CAD (%)</td>
<td>33</td>
<td>58</td>
<td>0.084</td>
</tr>
<tr>
<td>3-vessel CAD (%)</td>
<td>11</td>
<td>12</td>
<td>0.917</td>
</tr>
<tr>
<td>PCI (%)</td>
<td>79</td>
<td>77</td>
<td>0.848</td>
</tr>
<tr>
<td>GP IIb/IIIa inhibitor (%)</td>
<td>29</td>
<td>31</td>
<td>0.002</td>
</tr>
<tr>
<td>Post-PCI MI (%)</td>
<td>4.2</td>
<td>0</td>
<td>0.293</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; PCI, percutaneous coronary intervention; GP, glycoprotein; MI, myocardial infarction.

Results

Of 230 patients screened, 50 were randomized a mean of 5 days after admission (Figure 1). Twenty-six patients received SDD and 24 patients received placebo. One patient in the placebo group discontinued treatment because of the development of a skin rash. Baseline clinical characteristics of patients randomized to SDD or placebo did not differ significantly (Table 1). Hypertension, diabetes, and current smoking did not differ significantly between groups. The total, low-density lipoprotein, and high-density lipoprotein cholesterol values did not differ significantly between groups. Statin therapy was similar in both groups. Most patients in both groups underwent percutaneous coronary intervention. The 6-month outcomes are presented in Table 3. Clinical endpoints were uncommon and the composite endpoint did not differ significantly between groups.

Plasma samples from 30 subjects who consented to return for follow-up 6 months after randomization were analyzed for high-sensitivity C-reactive protein (CRP), IL-1β, TNF-α, IL-6, IL-10, MMP-2, and MMP-9. As shown in Table 4, at baseline there was no significant difference in the mean high-sensitivity CRP concentration between the placebo and SDD groups (5.2±0.8 µg/mL versus 4.8±0.6 µg/mL, P=NS). After 6 months, when compared with the pretreatment values, there was no significant change in the high-sensitivity CRP levels of the placebo group (5.2±0.8 µg/mL versus 4.9±0.7 µg/mL, P=NS), whereas the SDD-treated patients demonstrated a 46% reduction in high-sensitivity CRP levels (4.8±0.6 µg/mL versus 2.6±0.4 µg/mL, P=0.007). Moreover, after 6 months, high-sensitivity CRP was reduced by 47% in the SDD group compared with the placebo group (P=0.008).

The cytokines IL-1β and TNF-α were not detected in the plasma of any patient. The plasma levels of IL-10 did not change significantly in patients randomized to SDD or placebo after 6 months. In contrast, when compared with the pretreatment values, IL-6 levels were reduced 32% in the SDD patients from 23 pg/mL to 14 pg/mL (P<0.025), whereas IL-6 levels in placebo-treated patients did not change significantly. However, IL-6 levels did not differ significantly between the SDD and placebo groups after 6 months of treatment (P=0.615).

Plasma levels of MMP-9 protein, as assessed by ELISA, were not significantly reduced in SDD- or placebo-treated patients compared with their respective baseline values (Table 4). Moreover, the MMP-9 protein levels were not significantly different between the 2 groups at the 6-month time periods (P=0.732). However, zymographic assessment of MMP-2 and MMP-9 did reveal some patterns of change. Based on comparison with the bands produced by commercial standards for MMP-9 and MMP-2, the bands visualized in plasma samples of both SDD and placebo groups represented pro-forms of MMP-2 (72 kDa) and MMP-9 (92 kDa). Smaller molecular-weight-activated forms were not detected on zymography. Scanning densitometry of the gelatin zymograms revealed a 38% reduction in pro-MMP-2 activity compared with baseline values (P=0.248). Pro-MMP-2 activity in the placebo group did not change between study
entry and 6-month follow-up (P=0.983). Pro-MMP-2 activity was reduced by 55% after 6 months of treatment with SDD compared with placebo (P=0.284).

Densitometric analysis of the zymograms for the pro-MMP-9 forms of gelatinase did reveal statistically significant effects of SDD treatment. Examination of the 92 kDa band alone or analysis of this band in combination with higher molecular weight forms of MMP-9 revealed that, compared with baseline values, SDD treatment reduced the activity of this gelatinase by 43% (P=0.028) and 50% (P=0.011), respectively. The gelatinolytic activity of the 92 kDa and higher-molecular-weight forms of MMP-9 were reduced by 54% in the SDD-treated patients compared with those treated with placebo (P=0.004). Gelatinolytic activity of the 92 kDa band alone was reduced 30% by SDD treatment compared with placebo (P=0.169). Among placebo-treated patients, there was no change from baseline to 6-month values in activity of either form of MMP-9.

Representative zymograms showing the plasma gelatinase activity (MMP-2 and MMP-9) in SDD- and placebo-treated patients are presented in Figure 2. Six months of SDD treatment resulted in consistent reduction in the zones of lysis compared with baseline indicative of inhibition of in vitro MMP activity. However, no consistent pattern was observed in placebo-treated patients.

Discussion
The most significant findings of this prospective, randomized, double-blind, placebo-controlled pilot study were that SDD reduced the levels of high-sensitivity CRP, IL-6, and pro-MMP-9 activity in patients with active coronary artery disease. These findings, if verified in a larger study, may have profound implications for the prevention of acute coronary events. The current study, however, was underpowered to detect any differences in clinical outcomes related to SDD therapy.

Plaque Rupture
Rupture of coronary artery atherosclerotic plaque is the major cause of sudden death, MI, and unstable angina.1-3 Thus, these acute coronary syndromes are a leading cause of morbidity and mortality in the industrialized world and prevention of plaque rupture would have significant public health implications. Plaque rupture occurs in a milieu of unstable plaque structure, inflammation, and matrix degradation. The unstable atheroma is one characterized by a thin fibrous cap overlying a large lipid pool. Immunohistochemical studies of human atherosclerotic plaque have shown that macrophages, T lymphocytes, and, to a lesser extent, mast cells are the most prominent cells. Subpopulations of these inflammatory cells express activation markers, indicating their ability to produce cytokines and other inflammatory mediators.1-3

Table 4. Inflammatory Mediators, Cytokines, and Matrix Metalloproteinases in the Study Population Before and After Treatment

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=13)</th>
<th></th>
<th></th>
<th></th>
<th>Doxycycline (n=17)</th>
<th></th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>6 Months</td>
<td></td>
<td></td>
<td>Baseline</td>
<td>6 Months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>5.2±0.8</td>
<td>4.9±0.7</td>
<td>0.789</td>
<td>0.007</td>
<td>4.8±0.6</td>
<td>2.6±0.4</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>22.8±3.5</td>
<td>17.4±3.7</td>
<td>0.209</td>
<td>0.025</td>
<td>22.1±3.7</td>
<td>14.7±1.8</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>0.9±0.4</td>
<td>0.5±0.5</td>
<td>0.156</td>
<td>0.313</td>
<td>1.3±0.6</td>
<td>0.5±0.3</td>
<td>0.313</td>
<td></td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>TNF-α (pg/mL)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-9 protein (ng/mL)</td>
<td>20.4±2.8</td>
<td>13.6±1.5</td>
<td>0.086</td>
<td>0.235</td>
<td>17.9±2.7</td>
<td>12.8±1.4</td>
<td>0.235</td>
<td></td>
</tr>
<tr>
<td>MMP-9 92 kDa+ higher MW (units)</td>
<td>595±82</td>
<td>597±61</td>
<td>0.982</td>
<td>0.011</td>
<td>550±103</td>
<td>276±79</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>MMP-9 92 kDa only (units)</td>
<td>343±45</td>
<td>338±50</td>
<td>0.945</td>
<td>0.028</td>
<td>417±76</td>
<td>238±56</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>MMP-2 activity (units)</td>
<td>137±32</td>
<td>134±35</td>
<td>0.983</td>
<td>0.248</td>
<td>96±21</td>
<td>60±21</td>
<td>0.248</td>
<td></td>
</tr>
</tbody>
</table>

hsCRP indicates high-sensitivity C-reactive protein; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; ND, not detected; MW, molecular weight.

Figure 2. Representative gelatin zymograms of patients treated with placebo (top row) or subantimicrobial doses of doxycycline (bottom row). Bands at 92 kDa represent pro-matrix metalloproteinase (MMP)-9 and at 72 kDa represent pro-MMP-2. Each patient sample is numbered and presented on adjacent lanes. The first lane (B) is before treatment and the second lane is after 6 months of treatment.
The foci of inflammation surrounding the vulnerable plaque appear to occur in the context of systemic inflammation. Elevations in markers of systemic inflammation such as CRP are able to identify patients at risk for plaque rupture as well as patients with a poor prognosis after plaque rupture. Recent evidence suggests that CRP is not only a marker of inflammation but also is a contributor to atheroma development, progression, and instability. Recent data have demonstrated the ability of CRP to induce LDL uptake, leukocyte activation and adhesion, chemokine production and tissue factor, and plasminogen activator inhibitor-1 expression while inhibiting vascular nitric oxide production. Thus, therapeutic reduction in CRP may have salutary effects on vascular health. The CRP levels of patients in this study were significantly greater than those reported in healthy populations, reflecting their recent hospital admission with acute coronary ischemia. Of interest, the 47% reduction of CRP was greater than that seen in previous studies of statins or thiazolidinediones. The mechanism of reduction of high-sensitivity CRP by SDD in this study is unclear. However, IL-6, which induces CRP production and release by the liver, was also reduced by SDD. The SDD-related reduction in high-sensitivity CRP may then result from the upstream inhibition of IL-6, a direct inhibitory effect on high-sensitivity CRP synthesis, or both.

Matrix Metalloproteinases

Matrix metalloproteinases are among the inflammatory mediators produced by activated plaque macrophages, lymphocytes, and smooth muscle cells. The MMPs are members of a family of related enzymes that are secreted into the extracellular space primarily by macrophages. Each of the enzymes requires zinc and calcium activity for enzymatic activity. Individually, these enzymes degrade one or more extracellular membrane proteins and collectively they are capable of degrading all of the major extracellular matrix components. The activity of MMP is controlled at several levels. First, MMPs are under transcriptional regulation. Their synthesis may be induced by cytokines. Second, after synthesis and secretion into the extracellular space, the enzymes must be activated, generally by proteolytic cleavage of the proenzyme. After MMPs are activated, they may be inhibited by a family of endogenous inhibitors known as tissue inhibitors of metalloproteinases. The expression of several MMPs is increased in human atheroma, and increased levels of MMP-9 have been correlated with plaque rupture. Thus, the reduction in pro-MMP-9 activity by SDD may have important implications for promoting plaque stability.

Doxycycline

Tetracycline treatment has been associated with a reduced risk of subsequent MI. Although the mechanism of this reduction in risk is unknown, the tetracycline family of antibiotics, including SDD, is capable of entering the arterial wall, where it may inhibit cytokine and protease mediators of several inflammatory cascades that may contribute to plaque instability. Tetracycline has been shown to block endotoxin-induced secretion of tumor necrosis factor and IL-1 in cultured monocytes, downregulate the expression and activity of MMP-2 and MMP-9, and inhibit IL-6 expression. Finally, this family of antibiotics is capable of inhibiting the activity of secreted MMPs by virtue of their ability to bind the calcium and zinc required for maintenance of proper conformation of the MMPs. The ability of doxycycline to limit the expansion of abdominal aortic aneurysms also appears to be related to inhibition of MMPs.

An alternative explanation for an effect of doxycycline on coronary events may lie in its ability to treat Chlamydia pneumoniae. There appears to be an association between C pneumoniae and coronary disease that, if causal, would suggest a role of anti-chlamydial antibiotics in prevention of coronary events. To avoid the potential confounding of our results by treatment of C pneumoniae, a subantimicrobial dose of doxycycline was administered. However, such a beneficial effect seems unlikely on the basis of several recent large randomized trials indicating no effect of anti-chlamydial antibiotics other than tetracyclines on reduction in coronary events.

Clinical Implications

Atherosclerosis is increasingly recognized as an inflammatory disease with many features in common with inflammatory diseases of other organs. Furthermore, the degree of inflammation as measured by high-sensitivity CRP correlates with prognosis among patients with established coronary disease as well as healthy individuals. It is unknown whether elevated levels of high-sensitivity CRP simply identify individuals with an increased risk of events or actually causes these events by virtue of its pro-atherogenic properties. Nevertheless, the prospect of a therapeutic agent with the ability to reduce CRP, IL-6, and MMP-9 has significant clinical implications. Non-pharmacological interventions such as weight loss and exercise have been associated with a reduction in inflammation. Pharmacotherapy with aspirin, statins, and thiazolidinediones also appear to reduce inflammation. However, these interventions have pleiotropic benefits that may be difficult to separate from their anti-inflammatory actions. Doxycycline in subantimicrobial doses, without other potentially beneficial properties, might be an appropriate candidate to test the inflammation hypothesis.

Limitations

Several important limitations should be borne in mind when interpreting the results of this preliminary pilot study. First, the sample size was small and not all patients were included in the biochemical analysis. However, there were no significant differences between patients who did and did not participate in the biochemical analysis (data not shown). Second, we did not obtain doxycycline levels and thus cannot document compliance with the SDD regimen or correlate levels to alterations in inflammatory markers. Third, the zymographic analysis only detected an SDD-related inhibition of in vitro MMP-9 activity. It remains to be demonstrated in future studies if SDD treatment inhibits in vivo MMP-9 activity. Finally, the subjects of this study were generally patients who had a recent acute coronary syndrome with
residual elevation of high-sensitivity CRP at study entry. The same results might not have been obtained in a more stable group of patients with coronary disease.

Conclusions

In summary, a 6-month course of SDD appears to result in the same results might not have been obtained in a more stable group of patients with coronary disease.

References

Clinical and Biochemical Results of the Metalloproteinase Inhibition with Subantimicrobial Doses of Doxycycline to Prevent Acute Coronary Syndromes (MIDAS) Pilot Trial

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