Metalloproteinase-2 and -9 in Giant Cell Arteritis
Involvement in Vascular Remodeling

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Background—Both matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) have been postulated to play roles in the pathophysiology of giant cell arteritis (GCA) because of their ability to degrade elastin. Understanding the specific mediators of arterial damage in GCA could lead to new therapeutic targets in this disease.

Methods and Results—Temporal artery biopsy specimens were obtained from 147 consecutive patients suspected of GCA. Clinical and histopathological data were collected according to protocol. Using immunohistochemistry, we compared the expression of MMP-2 and MMP-9 in the temporal artery biopsies of both GCA cases (n = 50) and controls (n = 97). MMP-9 was found more frequently in positive than in negative temporal artery biopsies (adjusted odds ratio [OR], 3.20; \( P = 0.01 \)). In contrast, the frequency of MMP-2 was not significantly different between positive and negative biopsies (adjusted OR, 2.18; \( P = 0.22 \)). Both MMP-2 and MMP-9 were found in macrophages and giant cells near the internal elastic lamina and in smooth muscle cells and myofibroblasts of the media and intima. MMP-9 was also found in the vasa vasorum. MMP-9 but not MMP-2 was associated with internal elastic lamina degeneration, intimal hyperplasia, and luminal narrowing, even after adjustment for possible confounding variables.

Conclusions—MMP-9 appears more likely than MMP-2 to be involved in the pathophysiology of GCA. MMP-9 not only participates in the degradation of elastic tissue but also is associated with intimal hyperplasia, subsequent luminal narrowing, and neangiogenesis. The expression of MMP by smooth muscle cells implicates these cells as potential secretory cells in GCA. (Circulation. 2005;112:264-269.)

Key Words: temporal arteritis | immunohistochemistry | metalloproteinases | muscle, smooth | vasculitis
issues conducted to date have included only small numbers of temporal artery biopsy (TAB). More precise knowledge of the molecular mechanisms underlying degeneration of the IEL and intimal hyperplasia could help us discover new therapeutic targets designed to prevent the vascular destruction and end-organ complications of GCA.

In the present study, we investigated the relationship between the immunohistochemical expression of MMP-2 and MMP-9 and the histopathological features of GCA in temporal arteries.

Methods

Clinical and Epidemiological Data

Between January 1997 and March 2002, 147 consecutive TABs were performed in our hospital because of clinical suspicion of GCA. All patients who underwent TAB at our center (a public, tertiary care hospital) were admitted through the emergency room after either self-referral or referral by their primary care provider. By protocol, all patients with suspected GCA are admitted. If the level of clinical suspicion for GCA is sufficiently high, treatment with glucocorticoids is begun immediately (before TAB), consistent with the standard of care for this disease. Clinical information is collected according to a defined protocol: age, gender, American College of Rheumatology (ACR) classification criteria, symptom duration before biopsy, and days of glucocorticoid treatment before the biopsy. These data were collected by investigators blinded to the results of the MMP studies.

Histochemistry and Immunohistochemistry

Tissues were fixed in 10% formaldehyde. After paraffin embedding, between six and eight 4-μm sections were used for histochemical or immunohistochemical studies. Hematoxylin and eosin staining was used for histological diagnosis. Verhoeff–van Gieson staining for elastic fibers was performed to visualize the IEL, and Masson’s trichrome staining was used to differentiate between collagen and muscular tissue.

Immunohistochemical staining was performed by the streptavidin-biotin peroxidase method with 3,3′-diaminobenzidine (DAB) as a chromogen using the automated Dako EnVision TechMate System. The paraffin-embedded sections of the TAB specimens were treated in xylene and dipped in a gradient of ethanol (once in 99% ethanol, once in 95% ethanol, and once in water). The sections were incubated in EDTA (150°C, 45 minutes). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were then incubated with a monoclonal or a polyclonal antibody specific for each antigen. Human CD68+ macrophages were identified with the use of monoclonal antibody (KP1, 1:200; Dako). MMP-2 was detected by a mouse monoclonal antibody to human MMP-2 (CA-4001, 15:1000; Vitro-Neomarkers). MMP-9 was localized by a rabbit polyclonal antibody directed toward the medial region of human CD31 mouse monoclonal antibody to human MMP-9 (CA-4001, 15:1000; Vitro-Neomarkers). Anti–human CD31 and anti–human smooth muscle actin monoclonal mouse antibody was used to detect SMCs (1A4, 1:100; Dako). Incubation times were 20 minutes for anti–human smooth muscle actin, 30 minutes for anti-Cd68, 45 minutes for MMP-9, and 60 minutes for MMP-2 and anti–human CD31. The sections were then incubated with ENVISION Techmate kit (Dako cytometry) for 30 minutes at room temperature, followed by 3,3′-DAB tetrahydrochloride (Dakocytomation) for 10 minutes (K 4007 HRP, mouse [DAB+] for CD68+ macrophages, MMP-2, anti–human CD31, and anti–human smooth muscle actin; and K 4011 HRP, rabbit [DAB+] for MMP-9). The sections were then counterstained with Mayer’s hematoxylin. Antigen retrieval with pressure cooking at 2 atm with citrate buffer, pH 6, for 5 minutes was used for anti-Cd68 antibodies and for 5 minutes for anti–human CD31 and anti–human smooth muscle actin, followed by 5 minutes at room temperature.

Positive and negative controls were included in each colorimetric assay. Positive controls recommended by the manufacturer were used, which consisted on placenta for MMPs, skin biopsies of cutaneous polyarteritis nodosa for CD68+ macrophages, endothelial cells for anti–human CD31, and smooth muscular biopsy for anti–human smooth muscle actin. As negative controls, samples of the same specimens without the primary antibody were used.

Histopathological Data

An experienced pathologist (P.H.-R.) divided the biopsies into 2 groups according to the histopathological classification criteria of the ACR:2 positive for GCA (cases) and negative (controls). The absence of both lymphoplasmacytic and multinucleated giant cell infiltrates in the sample led to the categorization of a sample as negative. The following pathological findings were recorded on every biopsy: the presence of multinucleated giant cells, IEL degeneration, intimal hyperplasia, luminal narrowing, calcifications, macrophages, and expression and localization of MMP-2 and MMP-9. The degrees of IEL degeneration were scored as follows: 0 = intact, 1+ = focal rupture, 2+ = ruptures that were up to half the vessel circumference in length, to 3+ = lesions that were more than half the vessel circumference in length.14 The changes in intimal thickness were scored with a semiquantitative scale: 0 = absence, 1+ = mild (25% occlusion of the lumen when the intima was readily discernible), 2+ = moderate (25% to 50% occlusion), to 3+ = severe (>50% occlusion).13 The intensity of MMP staining was scored from 0 (no staining) to 1+ (mild or moderate staining) or 2+ (most intense), as defined in previous studies.6,9

Statistical Analysis

Pearson’s χ² test was used to study the association between 2 categorical variables when the expected value in at least 80% of the table cells was >5. Fisher’s exact test was applied when those conditions were not met and in case of symmetric tables. The Shapiro-Wilk test was used to assess the normality of the quantitative variables. To study the association between a quantitative and a categorical variable with 2 categories, a Student’s t test was applied when the quantitative variable followed a normal distribution. The Mann-Whitney U test was used when the distribution was not normal.

To analyze the correlation between MMP expression, IEL degeneration, and the presence of intimal hyperplasia, the Spearman test was applied. In evaluating the association between TAB result and MMP expression, odds ratios (ORs) and 95% CIs were calculated. A fixed logistic regression model was applied to study variables that could have affected this relationship. The independent variables included in the model were age (dichotomized as <70 and ≥70 years), gender, days of symptom duration before the TAB, and days of treatment with glucocorticoids before the biopsy. Using these variables, we derived models for both MMP-2 and MMP-9. Adjusted ORs and 95% CIs were calculated for all independent variables.

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Hosmer and Lemeshow’s test was used to evaluate goodness of fit. All analysis were performed with the SPSS program for Windows, version 8.0. Values of P<0.05 were considered significant.

Results

Clinical Data

Clinical records were available for review for 46 of the 50 patients (92%) with positive TAB and 79 of the 97 (84%) with negative TAB. All 46 patients with positive TABs whose clinical records were available for review met the ACR classification criteria for GCA.13 The principal clinical features of patients with positive and negative TABs, reported previously,16 are summarized in Table 1.

Histopathological Studies

Of the 147 TABs, 50 (34%) were positive for GCA and 97 (66%) were negative. Giant cells were observed in 36 of the
MMP Localization

In the positive TABs, both MMP-9 and MMP-2 were detected in giant cells and macrophages within the media and intima, particularly those that congregate along the IEL, and in myofibroblasts and SMCs of the media and intima. Among the negative TABs, the MMPs were expressed in myofibroblasts and SMCs of the media and the intima (Figure 1). In addition, expression of MMP-9 but not MMP-2 was observed in the SMC layer of the vasa vasorum in 12 TABs (10 positive, 2 negative) in all layers of the arterial wall (Figure 2). Neither MMP-9 nor MMP-2 was detected in the adventitia.

Discussion

In our study, the largest investigation to date of the expression of MMP in TABs, MMP-9 but not MMP-2 was associated with the histopathological diagnosis of GCA. Our results indicate that MMP-9 expression is associated specifically with IEL degeneration, intimal hyperplasia, and luminal narrowing. This is further supported by the statistically significant correlation found. Both MMP-2 and MMP-9 were detected in inflammatory cells, as well as in SMCs and myofibroblasts. Only MMP-9, however, was found in the SMC layer of the vasa vasorum in some TABs.

Weyand et al6 reported the presence of MMP-2 in macrophages located near the IEL and in giant cells in 5 temporal arteritis cases they studied and suggested a role for this MMP in the pathogenesis of GCA. Other investigators, however, have reported that MMP-2 is ubiquitously expressed by several cell types within the arterial wall, including SMCs, fibroblasts in temporal arteries both with and without vasculitis,8,9 and macrophages in GCA arteries. Sorbi et al7 found that the serum concentration of MMP-9 and its gelatinase activity were significantly higher in patients with GCA who had received no treatment than in a control group. These findings support a role for MMP-9 in the pathophysiology of GCA, yet the number of patients in that study was small (12 cases, 12 controls).

In GCA, activated T cells and macrophages play important roles in disease pathophysiology, forming granulomatous reactions in the arterial wall. T-cell activation occurs in the adventitia, where the vasa vasorum provide a port of entry for inflammatory cells. On stimulation, T cells secrete interferon-gamma (IFN-γ), a cytokine that regulates effector functions of macrophages throughout the arterial wall. Recruited macrophages differentiate into distinct subsets of effector cells that are injurious to tissues, producing MMP and reactive oxygen intermediates. Macrophages and multinucleated giant

### Table 1. Clinical Variables in Patients With Positive and Negative TABs

<table>
<thead>
<tr>
<th>Feature</th>
<th>Positive TABs</th>
<th>Negative TABs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (SD), y</td>
<td>76 (7.2)</td>
<td>73 (11)</td>
<td>0.06</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>38 (76)</td>
<td>60 (61.9)</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean duration of symptoms</td>
<td>2.3 (1.9)</td>
<td>4.5 (7.3)</td>
<td>0.31</td>
</tr>
<tr>
<td>Median duration of treatment</td>
<td>8 (7)</td>
<td>7.5 (13)</td>
<td>0.72</td>
</tr>
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</table>

### Table 2. Association Between MMP-9 Expression and Histopathological Findings on All TABs

<table>
<thead>
<tr>
<th>Features</th>
<th>TABs Expressing MMP-9,* %</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giant cells†</td>
<td>47.2</td>
<td>21.4</td>
<td>3.28</td>
<td>0.78–13.77</td>
</tr>
<tr>
<td>Intimal hyperplasia‡</td>
<td>28.1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IEL degeneration‡</td>
<td>33</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal narrowing</td>
<td>42.1</td>
<td>20.2</td>
<td>2.88</td>
<td>1.30–6.38</td>
</tr>
<tr>
<td>Calcifications</td>
<td>32.2</td>
<td>21.6</td>
<td>1.73</td>
<td>0.82–3.64</td>
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</tbody>
</table>

*Proportion of biopsies expressing MMP in which a given feature is present/absent. †Only calculated considering the biopsies positive for GCA. ‡OR and 95% CI not calculable.
cells also provide growth and angiogenic factors that support the response of the artery to injury. The maladaptive reaction of the artery is believed to lead to the formation of lumenoclusive intimal hyperplasia.18

MMP-9, one of the few enzymes that can degrade elastin,5 has previously been detected in regions of IEL interruption in temporal arteries with GCA,7–9 as we have seen. These observations are consistent with the involvement of MMP-9 in the degradation of the elastic tissue in GCA. Although not described in GCA previously, increased MMP expression and activity have been found to be associated with development of neointimal arterial lesions and SMC migration after arterial balloon injury in experimental models.19 In contrast, MMP inhibition decreases SMC migration in vitro and in vivo.20 After appropriate signals, SMCs in the media are believed to undergo a phenotypic change, reverting from contractile to secretory cells that migrate into the intima, where their proliferation ultimately leads to intimal hyperplasia.21 This, in turn, leads to luminal narrowing, a common feature of GCA.22 Experimental studies have demonstrated that SMC migration is stimulated by platelet-derived growth factor (PDGF) and fibroblast growth factor, which produce this stimulant effect in vitro at least partially through the activation of MMP-2 and MMP-9.23 Because a key role has been assigned to PDGF for intimal hyperplasia and luminal stenosis in GCA,24 we postulate that part of the stimulatory effect on SMC caused by PDGF is mediated by MMP-9.

The concept that the SMCs of the media are indeed the cell of origin of the proliferating fibroblastlike cells in the hyperplastic intima remains open to debate.25 An alternative hypothesis is that the adventitia is the site of origin of these migrating myofibroblasts.26,27 In our study, however, the absence of staining for MMP in the adventitia supports the SMCs of the media as the cells responsible for intimal hyperplasia in GCA.

Vascular SMCs are also considered to play a central role in atherosclerosis.28 Whereas SMCs produce collagen, which provides the structural support for the vessel wall, activated SMCs and macrophages secrete MMPs that degrade collagen and elastin.29 Although SMCs were once considered to play a passive role in GCA pathophysiology, their ability to secrete PDGF is now recognized.24 Our findings support the concept that SMCs and myofibroblasts (cells that resemble SMCs) located in the media and intima of the temporal arteries also play an active role in GCA secreting MMPs. These cells could be a primary source of MMPs, thereby assuming a direct role in the vascular remodeling in GCA.

The expression of several MMPs has been described in the endothelium of the vasa vasorum in aortic atherosclerotic lesions,30 abdominal aortic aneurysms,31 and stenosis of an implanted vascular prosthesis.32 Detection of MMP within the SMC layer of the vasa vasorum, however, has not been

### TABLE 4. Logistic Regression Models for MMP-9 and MMP-2 and TAB Result

<table>
<thead>
<tr>
<th>Variables</th>
<th>β</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1: MMP-9</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>...</td>
<td>1</td>
<td>...</td>
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<tr>
<td>≥70</td>
<td>−0.022</td>
<td>0.98</td>
<td>0.38–2.50</td>
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<td></td>
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<tr>
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<td>...</td>
<td>1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Male</td>
<td>−0.670</td>
<td>0.51</td>
<td>0.21–1.24</td>
<td>0.14</td>
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<tr>
<td>Days before the biopsy</td>
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<td>0.99–1.00</td>
<td>0.47</td>
</tr>
<tr>
<td>Duration of treatment</td>
<td>−0.019</td>
<td>0.98</td>
<td>0.95–1.01</td>
<td>0.23</td>
</tr>
<tr>
<td>Model 2: MMP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt;70</td>
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<td>1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>≥70</td>
<td>0.108</td>
<td>1.11</td>
<td>0.44–2.81</td>
<td>0.82</td>
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<tr>
<td>Gender</td>
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</tr>
<tr>
<td>Female</td>
<td>...</td>
<td>1</td>
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</tr>
<tr>
<td>Male</td>
<td>−0.665</td>
<td>0.51</td>
<td>0.22–1.23</td>
<td>0.13</td>
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<td>Days before the biopsy</td>
<td>−0.001</td>
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<td>0.99–1.00</td>
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<tr>
<td>Duration of treatment</td>
<td>−0.020</td>
<td>0.98</td>
<td>0.95–1.01</td>
<td>0.23</td>
</tr>
</tbody>
</table>

β indicates regression coefficient.
described previously. Development of vasa vasorum involves the release of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor, but much of the process is poorly defined.\(^2\) On the other hand, VEGF is known to upregulate MMP-9 expression in T lymphocytes\(^3\) and vascular SMCs.\(^2\) The upregulation of MMP in vascular SMCs induced by VEGF is concomitant with accelerated migration of SMCs, suggesting a role for VEGF in angiogenesis stimulating MMP production by vascular SMCs.\(^2\) Recently, it was demonstrated that minocycline can inhibit VEGF-induced human aortic SMC migration and that this effect is mediated, at least in part, through the inhibition of MMP-9 mRNA transcription.\(^2\) We postulate that in GCA, VEGF induces angiogenesis through the induction of MMP-9 expression in the SMCs of newly formed microvessels. MMP-9 then assists in the migration of these vessels through the vascular extracellular matrix.

Our study has certain potential limitations. First, the fact that MMP-9 was not found in all positive TABs must be reconciled with other pieces of evidence suggesting an important role for this enzyme. MMP-9 is analogous in this sense to giant cells, which also are not found in all TABs from patients with GCA. The remodeling of the vascular wall in GCA is a dynamic process; histological studies permit only a “static” picture of the inflammatory cascade at the moment of TAB. Because MMP is subject to enzymatic degradation\(^3\) and because the timing may affect the histopathological findings, the absence of MMP-9 immunostaining in some arteries with histological alterations otherwise consistent with GCA does not allow us to exclude a contribution of MMP-9 even in those cases, albeit detectable MMP-9 may not have been present at the time of the TAB. The presence of both MMP-2 and MMP-9 in some negative temporal arteries that otherwise present age-related changes may suggest that they might also play a role in these age-related findings. Second, the expression of MMP documented by immunohistochemistry does not provide any information about its enzymatic activity because zymogens lack activity and MMP inhibitors may block activated MMP.\(^3\) Finally, we have to take into consideration that other extracellular protease systems are likely to be involved in the vascular remodeling associated with GCA. However, the definition of these other systems is beyond the scope of this project. On the other hand, one of the strengths of our studies is the large number of TABs studied. The size of our study may have permitted observations overlooked in previous investigations limited by a relatively small number of TABs.

In summary, our data indicate that MMP-9 expression is associated with the histological diagnosis of GCA. The SMCs of the media and intima are the cells most likely to be the origin of the hyperplastic neointima. MMP-9 may also

Figure 1. Serial sections and different stainings from same temporal artery with GCA. A, Hematoxylin and eosin staining in which inflammatory infiltrate with multinucleated giant cells is observed in all layers of arterial wall. Marked intimal hyperplasia completely occludes lumen vessel (×40). B, Verhoeff–van Gieson staining for elastic fibers showing nearly complete degradation of IEL (×40). C, Masson's trichrome staining highlighting adventitial and intimal collagen in green and medial SMCs in red (×40). D, Anti-CD68+ immunohistochemical staining for macrophages, positive in media and near IEL (×40). E, Mildly positive (1+) immunohistochemical staining for MMP-2 near IEL (×40). F, Detail of E showing mild staining for MMP-2 in cytoplasm of macrophages and giant cells (×400). G, Marked (2+) immunohistochemical staining for MMP-9 in media and intima near IEL (×40). H, Detail of G showing staining for MMP-9 in cytoplasm of macrophages, in myofibroblasts, and in SMCs. Intensity of staining for MMP-9 is greater than that for MMP-2 (×400).

Figure 2. Different staining of same temporal artery with GCA. A, Immunohistochemical staining for actin in SMCs and myofibroblasts (×100). B, Anti-CD31+ immunohistochemical staining for endothelial cells (EC) (×100). C, Immunohistochemical staining for MMP-9 showing evidence of MMP-9 in SMCs but not in endothelial cells of vasa vasorum (×400).
contribute to the neoangiogenesis observed in GCA because this enzyme is observed in the SMCs of the vasa vasorum. Strategies designed to inhibit the effects of MMP-9, perhaps in concert with the inhibition of other mediators, would be appealing approaches to the therapy of this disease.

Acknowledgments
This study was funded by the Vall d’Hebron Hospital Foundation. We thank Anna Solsoma and María José Trujillo for their expert technical assistance.

References
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Circulation. 2005;112:264-269; originally published online July 5, 2005;
doi: 10.1161/CIRCULATIONAHA.104.520114
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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