EXTENDED REPORT

Gelatinase expression and proteolytic activity in giant-cell arteritis

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Objectives: Gelatinases (MMP2 and MMP9) are expressed in giant-cell arteritis (GCA) and are thought to play a role in vessel disruption. However, their activation status and enzymatic activity have not been evaluated. Our aim was to investigate the distribution and proteolytic activity of gelatinases in GCA lesions at different stages.

Methods: Expression of MMP2, MMP9, MMP2-activator MMP14 and their natural inhibitors TIMP1 and TIMP2 was determined by real-time PCR and immunohistochemistry in temporal artery sections from 46 patients and 12 controls. MMP activation status and enzymatic activity were assessed by gelatin and film in situ zymography.

Results: Vascular smooth muscle cells from normal specimens constitutively expressed pro-MMP2 and its inhibitor TIMP2 with no resulting proteolytic activity. In GCA MMP2, MMP9 and MMP14 were strongly expressed in their active form by infiltrating leucocytes. Inflamed arteries also expressed TIMP1 and TIMP2. However, the MMP9/TIMP1 and MMP2/TIMP2 ratios were higher in patients compared with controls, indicating an increased proteolytic balance in GCA which was confirmed by in situ zymography. Maximal gelatinase expression and activity occurred at the granulomatous areas surrounding the internal elastic lamina (IEL). Myointimal cells also expressed MMPs and exhibited proteolytic activity, suggesting a role for gelatinases in vascular remodelling and repair.

Conclusions: GCA lesions show intense expression of gelatinases. Activators and inhibitors are regulated to yield enhanced gelatinase activation and proteolytic activity. Distribution of expression and proteolytic activity suggests that gelatinases have a major role not only in the progression of inflammatory infiltrates and vessel destruction but also in vessel repair.

Giant-cell arteritis (GCA) is a granulomatous vasculitis involving large and medium-sized arteries.[1] Histopathological patterns observed in involved vessels suggest that leucocytes invade the vessel wall through the adventitial vasa vasorum and surrounding small vessels.[2 3] This interpretation is supported by immunopathological studies showing that adhesion molecules necessary for leucocyte recruitment are mainly expressed by vasa vasorum.[4] Inflammatory infiltrates subsequently extend towards the adventitia and the medial layer where they undergo granulomatous differentiation.[1 2] At this stage, inflammatory cells can be additionally recruited through inflammation-induced neovessels.[4 5]

To invade the vessel wall, infiltrating leucocytes need to break the basement membrane of the vasa vasorum, and to migrate through the interstitial matrix. As inflammatory cells proceed across the artery wall, the internal elastic lamina (IEL) is disrupted, allowing the progression of leucocytes, as well as myointimal cells towards the intima.[2 6] Among the proteolytic systems participating in this process, gelatinases (MMP2 and MMP9) may have an important role, given their elastinolytic activity and their unique ability to degrade basement membranes.[7–9] Rupture of elastic fibres may lead to deleterious consequences such as the development of aortic aneurysms, an increasingly recognised complication of GCA.[10 11] The relevance of gelatinases in vascular destruction has been demonstrated, indeed, in animal models of aortic aneurysms.[12]

As with other proteolytic systems, MMP activity is tightly regulated at several levels. Gelatinase production is transcriptionally regulated, but post-transcriptional control of enzymatic activity is even more crucial. Gelatinases are secreted as inactive zymogens and need to be activated by proteolytic cleavage.[7 8 13] MMP2 is activated at the cell surface through a unique multistep pathway requiring MMP14 and tissue inhibitor of metalloproteinase 2 (TIMP2).[13 14] Active MMP2 is, in turn, one of the most efficient activators of MMP9.[7 8 13] Gelatinase activity is subsequently modulated by interaction with their natural inhibitors, TIMPs, by forming noncovalent 1:1 stoichiometric complexes. TIMP2 preferentially inhibits MMP14 and MMP2, whereas TIMP1 is a potent inhibitor of MMP9.[8 13]

Gelatinases are known to be expressed in GCA.[15–19] However, molecules modulating gelatinase activity such as MMP14 or TIMPs have not been evaluated or have been detected in only a few cases. In order to gain a better understanding of the physiopathological role of gelatinases in GCA, the aims of our study were to investigate the expression and distribution of gelatinases, TIMPs and MMP2-activator MMP14 at the mRNA and protein level, and to determine gelatinase activation status and resulting proteolytic activity in GCA lesions.

PATIENTS AND METHODS

Patients

We studied 46 patients with biopsy-proven GCA. Thirty-three patients had received no treatment before the temporal artery excision, whereas the remaining 13 had received 1 mg/kg/day of prednisone for 9±2.5 days (mean ± SEM). Unless otherwise indicated, only treatment-naive patients were considered in quantitative measurements. Twelve normal temporal arteries from patients in whom GCA was initially considered but

Abbreviations: GCA, giant-cell arteritis; IEL, internal elastic lamina; VSMC, vascular smooth muscle cells

subsequently excluded served as controls. In all of them, symptoms were related to other conditions, and in none of them was the clinical suspicion strong enough to prescribe treatment in spite of a negative biopsy. The study was approved by the Ethics Committee of our institution, and all patients signed informed consent.

Specimens were embedded in OCT, snap-frozen in isopenthane prechilled in liquid nitrogen and stored at −80°C. Additional fragments from 6 patients and 2 controls were directly frozen in liquid nitrogen and stored at −80°C in order to perform gelatin zymography and western-blot analysis.

Histopathological evaluation

Temporal artery biopsies were classified according to the extension of inflammatory infiltrates. Sixteen specimens had inflammatory infiltrates limited to the vasa vasorum and adventitial layer. The remaining 30 had fully developed lesions with inflammatory infiltrates extending through the entire artery wall. The topographic distribution of MMPs was separately evaluated in both groups. Additional aspects evaluated were IEL integrity and extent of intimal hyperplasia. Elastic lamina was stained with 1% Shikata's orcein (Scharlau Chemie S.A., Barcelona, Spain) in 70% ethanol. IEL disruption was scored as follows: 1, IEL preserved in >80% of the circumference; 2, IEL preserved in 50–80%; 3, IEL preserved in 30–50%; and 4, IEL remaining in <30%. Intimal hyperplasia was scored from 0 to 4 as described.[5]

Immunostaining

Serial 4–6-μm temporal artery sections from the 46 patients and 12 controls were incubated with the following primary antibodies: monoclonal mouse antihuman MMP9 (clone GE-213) (Chemicon International, Inc., Temecula, CA) at 1/1000 dilution, polyclonal rabbit antihuman MMP2 (Chemicon) at 1/500 dilution, polyclonal rabbit antihuman MMP14 (Chemicon) at 1/250 dilution, monoclonal mouse antihuman TIMP1 (clone Ab-2) (Calbiochem, Cambridge, MA) at 1/40 dilution and monoclonal mouse antihuman TIMP2 (clone Ab-1) (Calbiochem) at 1/40 dilution. Immunoglobulins obtained from the same species were used as negative controls. Immunodetection was carried out with an HRP-labelled polymer conjugated to secondary antibodies (EnVision kit from Dako, Carpinteria, CA), as reported.[20 21]

Quantification of the immunostaining at the granulomatous area was performed in the 30 specimens with fully developed lesions according to a semiquantitative 0–4 score, as described.[20 21]

mRNA quantification

Total RNA was obtained from 150 serial sections (5 μm thick) per biopsy using TRIzol (Invitrogen, Carlsbad, CA). RNA could be obtained from 35 patients (27 untreated and 8 treated) and from the 12 controls. One microgram of total RNA was reverse-transcribed to cDNA using the Archive kit (Applied Biosystems, Foster City, CA). Samples were stored at −20°C until use.

MMP2, MMP9, MMP14, TIMP1 and TIMP2 expression was measured by real-time quantitative PCR using specific Assay-on-Demand Taqman Gene expression probes from Applied Biosystems.[20 21]

Film in situ zymography (FIZ)

Topographic distribution of gelatinase activity was assessed by FIZ (Fuji Photo Film Co., Ltd, Tokyo, Japan). Five-micrometre-thick cryostat sections from the same biopsy samples used for immunostochemistry were applied to 7-μm polyester membranes cross-linked with gelatin or with gelatin containing the gelatinase inhibitor (1,10)-phenanthroline as a control for specificity. Films were incubated for 20 h in a moist chamber at 37°C and stained with 0.5% Amido Black 10B (Sigma) in 70% methanol, 10% acetic acid for 10 min and distained by washing in 70% methanol, 10% acetic acid. Gelatinase activity was visualised as destained areas on a dark-blue background.

Gelatin zymography

Frozen temporal artery samples (0.5 cm long) from 6 patients and 2 controls, were homogenised in 1 ml of TRIzol. Given the substantial amount of tissue required, this study could not be extended to more specimens. One hundred micrograms of total protein per sample, extracted according to the manufacturer's instructions, was subjected to gelatin zymography as described.[22 23]

Western-blot

Twenty micrograms of protein obtained from the homogenised arteries was subjected to SDS-PAGE and transferred onto nitrocellulose. Membranes were incubated overnight at 4°C with a polyclonal rabbit antihuman MMP14 (Chemicon) at 1:1000 dilution. Immunodetection was performed as published.[23]

Statistics

The Mann–Whitney U test was used to compare quantitative variables and Spearman test for correlations, and the Fisher exact test was used for contingency tables.

RESULTS

MMP expression in temporal artery biopsies from patients with GCA and controls

No MMP9 or MMP14 expression was detected by immunohistochemistry in normal arteries. In contrast, MMP2 was expressed by vascular smooth muscle cells (VSMC) in the medial layer, as previously observed[15–17 19] (fig 1). MMP9 and MMP14 mRNA were detected at low concentrations in normal biopsies but were significantly more abundant in samples from patients with GCA (fig 2). No differences were found in MMP2 mRNA between patients and controls, further supporting constitutive MMP2 expression in non-inflamed arteries.

MMP9 and MMP14 were detected by immunohistochemistry in all specimens with GCA lesions and were mainly expressed by inflammatory cells. Although, at the mRNA level, no differences were found in MMP2 expression between patients and controls, immunostaining revealed important differences in MMP2 distribution. In normal arteries, MMP2 was mainly expressed by VSMC, whereas in GCA specimens MMP2 was expressed not only by remaining VSMC but also, and more intensively, by infiltrating leucocytes (fig 1). The pattern of MMP expression varied according to the extent of inflammatory involvement (fig 1). In specimens with inflammatory infiltrates restricted to vasa vasorum and adventitial layer, MMPs were expressed by adventitial inflammatory cells, and MMP2 was also expressed by preserved VSMC at the media, similarly to normal arteries. In GCA arteries with fully developed lesions, VSMC were destroyed, and MMPs were intensively expressed by the granulomatous infiltrates at the media and intima/media junction. MMP2, MMP9 and MMP14 were also expressed by myointimal cells at the hyperplasic intima (fig 1).

Immunostaining scores for all 3 MMPs were significantly correlated (MMP2 vs MMP9, $r = 0.80$, $p<0.0001$; MMP2 vs MMP14, $r = 0.78$, $p<0.0001$; MMP9 vs MMP14, $r = 0.78$, $p<0.0001$). In accordance with previous studies showing coordinated expression of MMP2 and MMP14,[22 23] a significant correlation between MMP2 and MMP14 mRNA was observed ($r = 0.39$, $p = 0.048$). Intriguingly, there was a negative

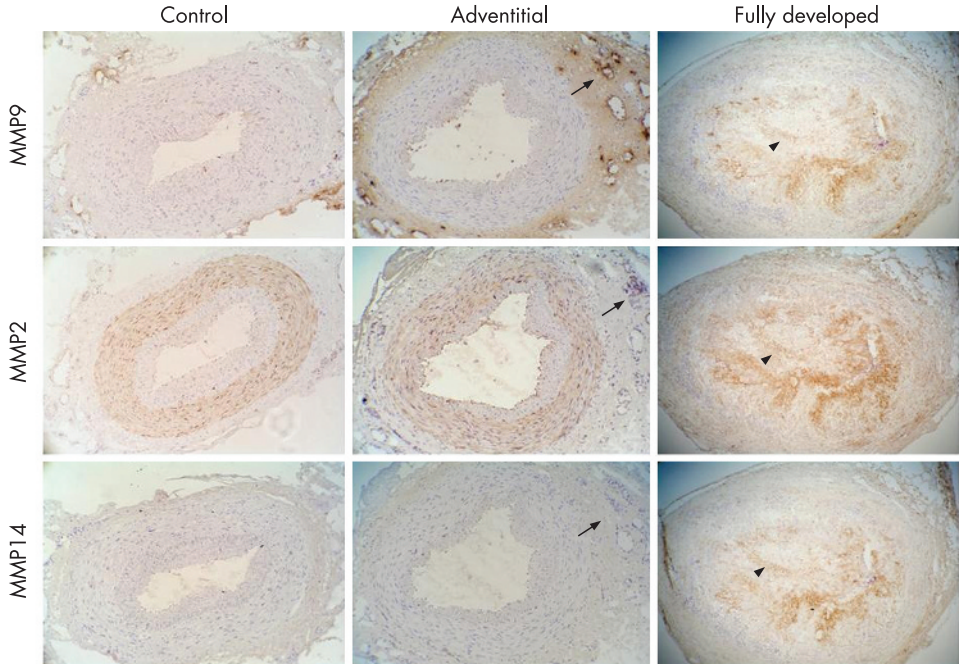

Figure 1 Expression of MMP2, MMP9 and MMP14 in serial sections of temporal arteries from controls and from patients with GCA, according to the extent of inflammatory infiltrates. In specimens with inflammatory infiltrates involving vasa vasorum and adventitia only, MMP expression was similar to controls, with some MMP2 and MMP9 expression by inflammatory cells (arrows). In fully developed lesions, expression predominates at the granulomatous areas. MMP expression by myointimal cells surrounding the lumen can also be observed (arrowheads).

correlation between MMP2 and MMP9 mRNA concentration ($r = -0.45$, $p = 0.019$).

Activation status of MMPs in GCA

In order to assess the activation status of gelatinases in GCA lesions, temporal artery protein extracts were subjected to gelatin zymography (fig 3A). Interestingly, in control arteries, both MMP9 and MMP2 pro-enzymes could be detected by this sensitive method, even though immunohistochemistry was only able to detect MMP2. No activated forms of gelatinases were detected in normal samples. In contrast, in fully inflamed arteries, activated forms of both gelatinases were present. Interestingly, in the artery with inflammatory infiltrates restricted to the adventitia with a preserved media, the activated form of MMP9 was apparent, but MMP2 was mostly present in its latent form, as in control arteries (fig 3A).

MMP14 detection by western blot in temporal artery biopsy extracts disclosed a 45-kDa form in addition to the 57-kDa active species (fig 3B). The 45-kDa form is inactive and results from proteolytic removal of the catalytic domain of MMP14 by active MMP2 and by active MMP14 itself.[24 25] This constitutes a counter-regulatory mechanism limiting MMP14 activity. The generation of the 45-kDa form requires, thus, full activation of the MMP14/MMP2 system, and its detection in lesions further indicates that the MMP2/MMP14 system is functionally operative. As shown in fig 3B, active MMP14 and particularly its 45-kDa form were more abundant in GCA patients than in controls.

TIMP counterbalance

Like MMP2, its inhibitor TIMP2 was constitutively expressed by VSMC in normal biopsies, at both the mRNA and protein level (fig 4 and on-line figure). As mentioned, TIMP2 is necessary for MMP2 activation by MMP14.[13 14] Constitutive TIMP2 expression guarantees that MMP2 can be activated when required but, at the same time, maintains constitutive MMP2 functionally inactive in quiescent arteries.

In GCA samples, TIMP1 mRNA was significantly upregulated, whereas TIMP2 mRNA significantly decreased compared with controls (fig 4). Immunohistochemical analysis showed that, in GCA biopsies, TIMP1 was expressed by inflammatory cells, whereas TIMP2 was expressed by both VSMC and infiltrating leucocytes (on-line figure). Distribution of gelatinases was, thus, coincident with that of their respective inhibitors and may constitute a counter-regulatory mechanism restricting the destructive potential of gelatinases, However, the MMP9/TIMP1 and MMP2/TIMP2 mRNA ratios were significantly higher in GCA specimens compared with controls (fig 4). In agreement with this observation, the intensity and extent of immunostaining were lower for TIMPs than for MMPs. MMP9 median score was 3 (range 1–4), whereas the TIMP1 median score was 2 (range 1–3); $p = 0.015$. The MMP2 median score was 3 (range 1–4), whereas the TIMP2 median score was 2 (1–3); $p = 0.03$. (on-line figure). These findings indicate that, in inflammed arteries, MMP/TIMP balance favours proteolytic activity.

Gelatinolytic activity of MMP2 and MMP9 in GCA

As shown in fig 5, in accordance with the inactive status of MMP2 expressed by VSMC, normal temporal arteries did not exhibit proteolytic activity. In arteries with inflammatory infiltrates limited to the adventitia, a weak gelatinolytic activity could be observed in areas with inflammatory infiltrates. No gelatinolytic activity could be observed in the media, as in control specimens. In fully developed lesions, a strong gelatinolytic activity was observed in inflamed zones, particularly in granulomatous areas at the media and at the intima/media junction. Myointimal cells also exhibited proteolytic activity (fig 5). Gelatinase activity, thus, required co-expression of MMP-9, MMP2 activator MMP14 and activated MMP2, which in turn may activate MMP9. According to the elevated MMP/TIMP ratio observed, colocalisation of MMPs with their respective inhibitors did not prevent gelatinolytic activity (on-line figure). Unfortunately, this system could not discern how much MMP9 or MMP2, respectively, contributed to the resulting proteolytic activity.

Effect of corticosteroids on MMP expression

Samples from patients who had received corticosteroids had significantly lower immunohistochemical scores for MMPs than samples from untreated patients (fig 6). However, no significant differences in MMP2 and MMP14 mRNA levels

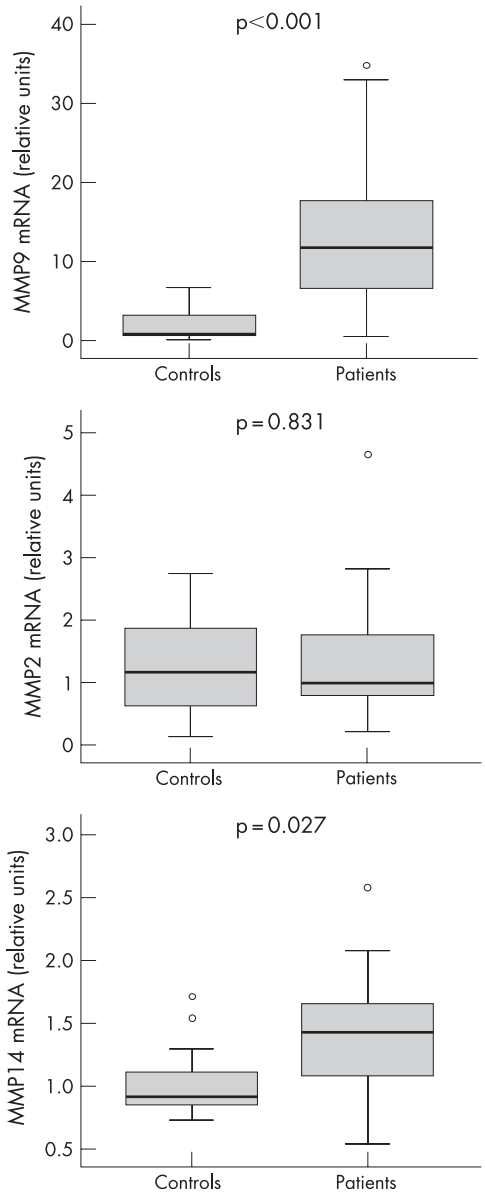

Figure 2 MMP9, MMP2 and MMP14 mRNA concentrations in temporal arteries from 27 untreated GCA patients and 12 controls.

could be found between untreated patients and those who had received treatment (MMP2: 1.02 (0.23–4.67) vs 1.43 (0.58–6.93), p = 0.43; MMP14: 1.44 (0.55–2.54) vs 1.32 (0.83–2.48), p = 0.81). MMP9 mRNA clearly tended to decrease, but the difference did not reach statistical significance (11.74 (0.59 – 34.78) vs 5.08 (2.29–23.55), p = 0.11).

Potential role of gelatinases both in vascular destruction and vascular remodelling

High (3–4) IEL disruption scores were significantly more frequent in temporal arteries with fully developed lesions than in those with adventitial inflammation only (OR 31, 95% CI 3.02–329.1, p = 0.0011), supporting a relevant elastinolytic activity of macrophages at the granulomatous area. The participation of MMP2 and MMP9 in this process is supported by the localisation of maximal gelatinolytic activity in the granulomatous area. IEL rupture positively correlated with the extent of intimal hyperplasia (r = 0.5, p = 0.036), suggesting that, through MMP expression, myointimal cells may contribute

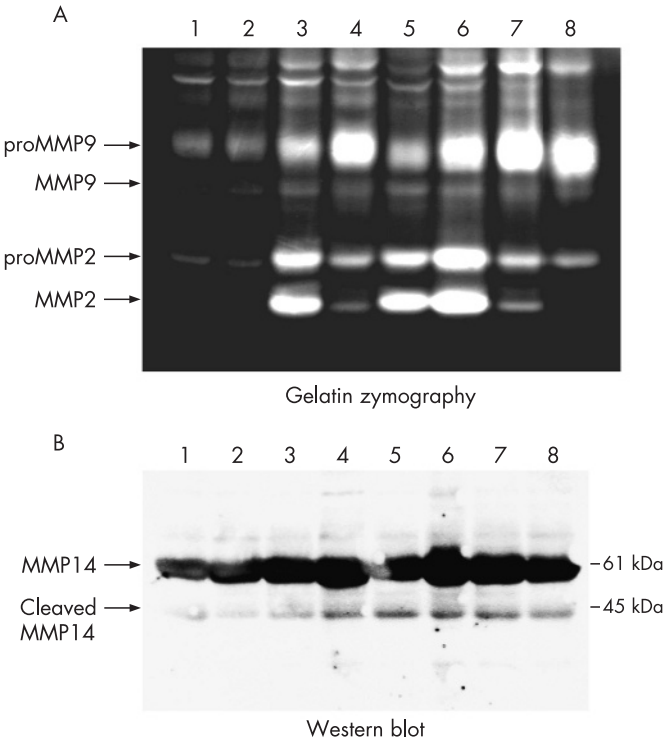

Figure 3 Activation status of MMPs. (A) Gelatin zymography of temporal artery extracts disclosing gelatinolytic bands corresponding to zymogens and activated forms of MMP2 and MMP9. Lanes 1–2 are controls, lanes 3–7 samples with fully developed lesions, and lane 8 corresponds to a specimen with adventitial inflammation only. (B) Detection of MMP14 in the same samples by western blot.

and, at the same time, take advantage of the breakdown of IEL to migrate towards the intima.

However, in samples with fully developed lesions, no significant correlation was observed between MMP immunohistochemical scores and IEL disruption scores (MMP9 r = 0.22, p = 0.38; MMP2 r = 0.34, p = 0.17; MMP14 r = 0.22, p = 0.38) or intimal hyperplasia scores.

DISCUSSION

Gelatinase expression has been previously reported in GCA,[15–19] but this is the first attempt to investigate gelatinase functional regulation and activity. Indeed, gelatinase expression was not always associated with enzymatic activity. Normal specimens and samples with infiltrates restricted to the adventitia did not disclose gelatinolytic activity in the media in spite of significant MMP2 expression by VSMC. These findings indicate that constitutively expressed MMP2 is not functionally active and that additional stimuli (ie, inflammation or injury) are required for its activation. As shown by gelatine zymography, MMP2 was produced, indeed, as a pro-enzyme in normal arteries whereas, in GCA lesions, MMP2 was activated, likely as a consequence of concomitant induction of MMP14. Moreover, MMP2 inhibitor TIMP2, intensively expressed by VSMC in normal specimens, decreased in GCA, favouring MMP-2 proteolytic balance in inflamed arteries.

MMP9 was strongly upregulated and activated in GCA. MMP9 inhibitor TIMP1, which is induced by inflammatory mediators,[26] was also upregulated in GCA. However, MMP9/TIMP1 ratio favoured proteolysis in lesions. Maximal gelatinolytic activity occurred in areas where coexpression of the three MMPs investigated was observed in inflammatory or in myointimal cells. These findings suggest that gelatinolytic activity in GCA requires coordinated regulation of the whole

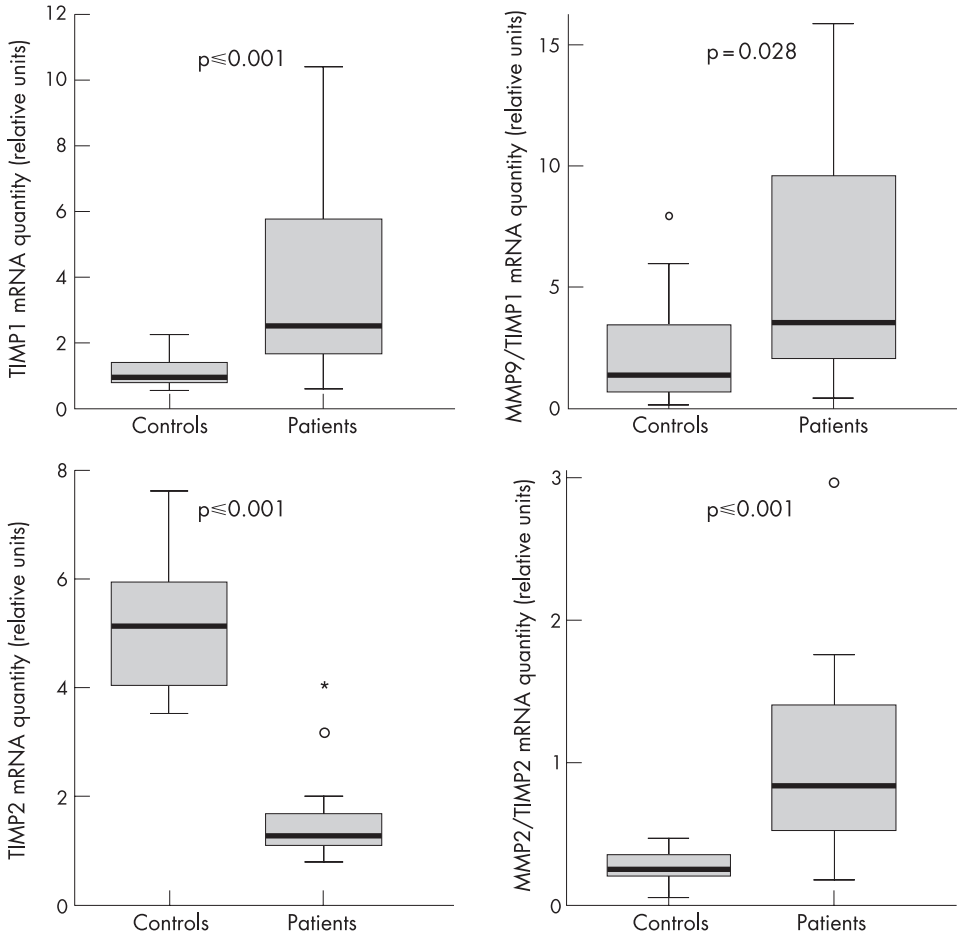

Figure 4 TIMP1 and TIMP2 mRNA concentrations in temporal arteries from 27 untreated GCA patients and 12 controls. MMP9/TIMP1 and MMP2/TIMP2 ratios in the same specimens.

system: increased expression of MMP9 and MMP14, activation of MMP2 by MMP14, subsequent activation of MMP9 by MMP2, and absolute or relative downregulation of TIMPs.

Although, at the protein level, there was a positive correlation between MMP2, MMP9 and MMP14 expression scores in granulomatous areas, a negative correlation between MMP2 and MMP9 transcripts was observed. Since MMP9 is mainly expressed by infiltrating leucocytes, increased MMP9 transcripts may indicate intense inflammatory activity, resulting in destruction of the medial layer and decreasing the contribution

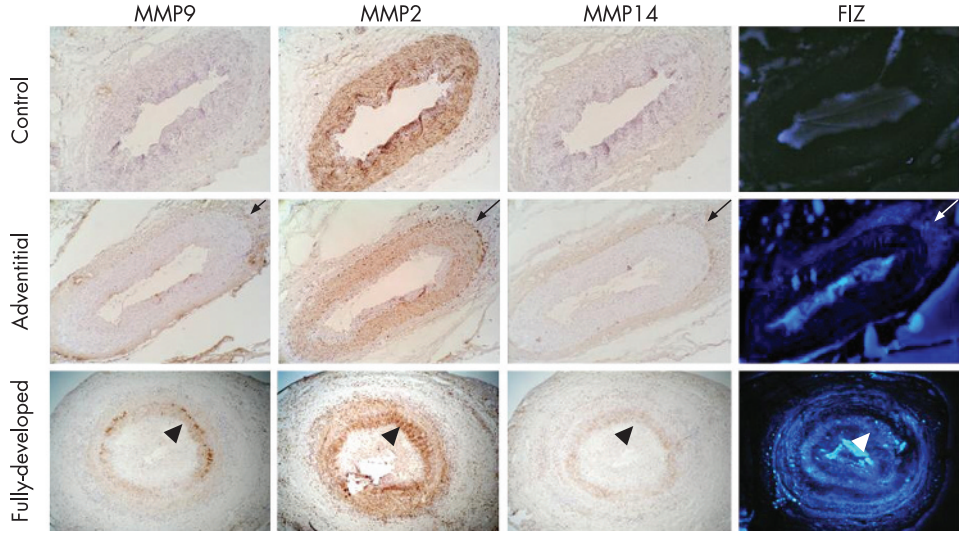

Figure 5 Gelatinolytic activity of MMP2 and MMP9 according to the extension of inflammatory infiltrates. In spite of strong MMP2 expression in the media of normal temporal arteries and of specimens with adventitial inflammation only, gelatinolytic activity appears only in the areas with inflammatory infiltrates (arrows). Activity is maximal in fully developed lesions, particularly at the granulomatous areas (arrowheads). Gelatinolytic activity can also be observed in the hyperplastic intima of fully developed lesions.

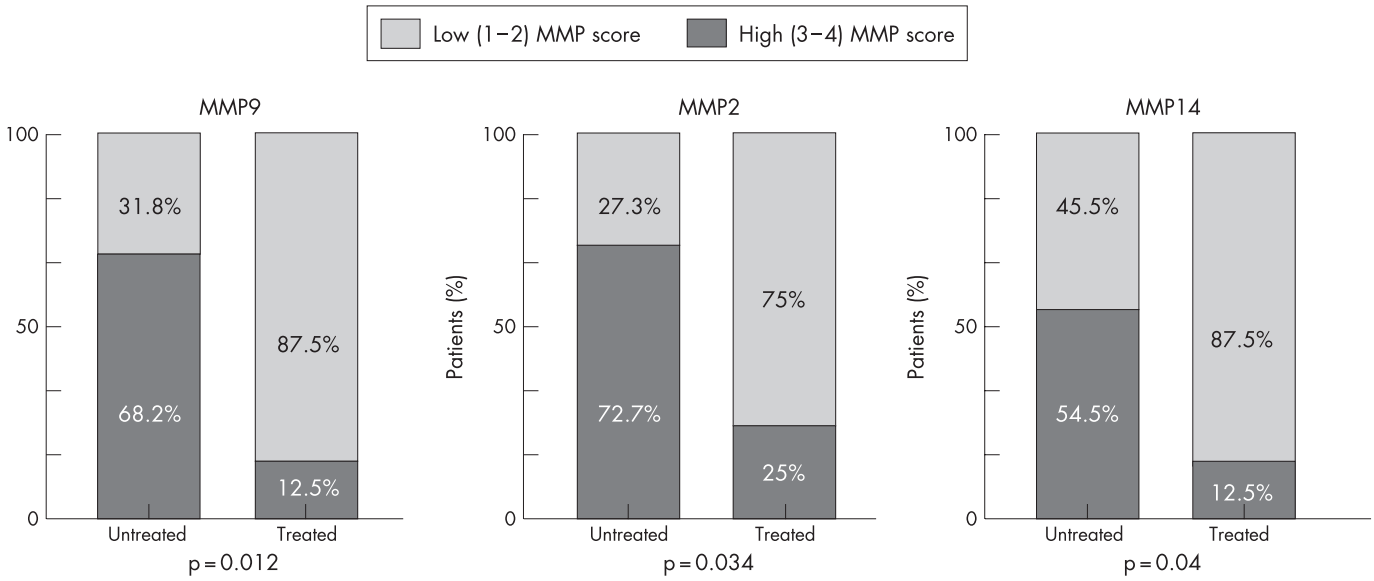

Figure 6 Effect of corticosteroid treatment on MMP expression in the granulomatous areas. Graphs show the percentage of specimens disclosing high (3–4) versus low (0–2) immunohistochemical scores, evaluated in the 30 samples with fully developed granulomatous lesions (22 from treatment-naive patients and 8 from patients treated with corticosteroids).

of VSMC to MMP2 expression. Moreover MMP2-deficient mice have increased production of MMP9, and a negative regulation of MMP2 on MMP9 expression has been demonstrated.[27 28]

Production of active MMPs by infiltrating leucocytes may be crucial in the development of vascular inflammation by allowing leucocyte progression across the vessel wall.[29 30] Moreover, gelatinases modulate bioavalability and cleavage of many cytokines, chemokines and growth factors, resulting in additional regulatory functions in inflammatory diseases. The recent observation that MMP2-null mice develop more severe lesions in various models of chronic inflammatory diseases[27 28] indicates that the roles of MMPs in inflammation are far more complex than anticipated.

Gelatinases may contribute to vessel-wall disruption in GCA, which may convey severe complications such as the development of aneurysms. Maximal gelatinolytic activity occurred in the granulomatous areas where activated macrophages have been demonstrated to have additional destructive activities, such as production of reactive oxygen species and nitric oxide.[31] This location suggests, indeed, a role for gelatinases in the destruction of IEL. In fact, specimens with adventitial inflammation only, which had no gelatinolytic activity at the media, had significantly more preserved IELs than arteries with fully developed lesions, which showed significant enzymatic activity at the intima/media junction. However, in specimens with fully developed lesions, no correlation was found between the intensity of gelatinase expression and the extent of IEL disruption. This observation indicates that regulation of MMP enzymatic activity may have a stronger functional impact than regulation of MMP expression. Moreover, elastin-degrading enzymes produced in the granulomatous area may include enzymes other than MMP2 or MMP9.

In specimens with fully developed inflammatory lesions, myointimal cells also expressed MMPs. The development of intimal hyperplasia is a significant source of morbidity in patients with vasculitis.[1 5] However, it provides a mechanism reinforcing the vessel wall when IEL is destroyed. Intimal hyperplasia correlated, indeed, with the extent of IEL disruption, as observed by others.[32] IEL degradation may promote intimal hyperplasia by facilitating myointimal cell activation and migration towards the intima.[1 2 33] However, in arteries with fully developed lesions, no significant correlation was found between the extent of intimal hyperplasia and the intensity of MMP expression. MMP may have a dual function in vascular remodelling: by disrupting IEL may promote and allow myointimal cell migration but, at the same time, increased MMP expression and activity may prevent excessive matrix deposition and lumen occlusion.

Immunostaining scores at the granulomatous area were significantly lower in samples from patients who had received corticosteroids. Interestingly, although MMP9 mRNA tended to decrease, no significant differences were found in MMP mRNA concentrations. Corticosteroids exert post-transcriptional and post-translational regulatory activities, which may regulate protein and mRNA levels differently,[34–36] and the treatment period may not have been long enough to downregulate the whole system efficiently. Moreover, although MMP expression in granulomatous areas may decrease upon corticosteroid treatment, MMP, and particularly MMP2, mRNA may be upregulated in regenerating VSMC during the process of vascular repair.

In summary, increased MMP expression and activity are prominent in GCA. The pattern of MMP expression and activity supports complex roles not only in vessel-wall inflammation and disruption, but also in vascular remodelling and repair. A better understanding of the specific roles of various MMPs at different disease stages is necessary before MMPs can be considered therapeutic targets to limit vessel inflammation and destruction in GCA.

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