Regulació de la producció de gelatinases (MMP2 i MMP9) pels limfòcits.

Implicació en malalties inflamatòries i síndromes limfoproliferatives

Tesi presentada per

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per a optar al grau de Doctora en Bioquímica

per la Universitat de Barcelona

Tesi dirigida per la Dra. Ma. Cinta Cid Xutglà

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Universitat de Barcelona, IDIBAPS

Barcelona, Març 2006

Expressió i Activitat de Gelatinases en les Lesions Inflamatòries de l'Arteritis de Cèl·lules Gegants

SEGON ESTUDI:

Anàlisi de l'expressió de metal·loproteïnases i de la seva activitat proteolítica en una malaltia inflamatòria crònica: l'arteritis de cèl·lules gegants. Estudi de mecanismes inductors potencials i correlació topogràfica de l'expressió d'integrines leucocitàries amb les MMPs.

Aquest treball ha donat lloc al següent article: Gelatinase Expression and Activity in Giant-Cell Arteritis Lesions. Gelatinolytic activity of MMP2 and MMP9 is associated with local co-expression of leukocyte integrins and MMP2 activator MMP14. Segarra M, García-Martínez A, Sánchez M, Hernández-Rodríguez J, Lozano E, Grau JM, Cid MC. (Abstract presentat al congrés American College of Rheumatology Annual Scientific Meeting 2005 i manuscrit enviat a publicar).

GELATINASE EXPRESSION AND ACTIVITY IN GIANT-CELL ARTERITIS LESIONS.

Gelatinolytic activity of MMP2 and MMP9 is associated with local co-expression of leukocyte integrins and MMP2 activator MMP14.

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Short title: Relationship between gelatinase (MMP2 and MMP9) and integrin expression in GCA.

Key words: matrix metalloproteinases, integrins, inflammation, vasculitis

Results partially presented at the Annual Scientific Meeting of the American College of Rheumatology, San Diego, CA, November 2005.

Supported by Ministerio de Ciencia y Tecnología y Fondo Europeo de Desarrollo Regional (FEDER) (SAF 02/03307 and SAF 05/06250), Fondo de Investigación Sanitaria (FIS 00/0689), and Generalitat de Catalunya (SGR 05/0300), Ana García-Martínez was supported by a research award from Hospital Clínic and from Fundació Pedro Pons.

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ABSTRACT

Objectives: To investigate the expression and activity of matrix metalloproteinases MMP2 and MMP9 in giant-cell arteritis (GCA).

Methods: Immunohistochemical detection of MMP2, MMP9 and MMP2-activator MMP14 was performed in temporal artery sections from 46 patients with GCA and 12 controls. Topographic relationship with integrin αvβ3, α4, and α5 expression was assessed in serial sections. MMP and pro-inflammatory cytokine IL-1β, TNFα and IL-6 mRNA was measured by real-time PCR. MMP enzymatic activity was assessed by in situ zymography.

Results: Vascular smooth muscle cells from normal temporal arteries constitutively expressed MMP2. In GCA specimens MMP2, MMP9 and MMP14 were mainly expressed by infiltrating leukocytes. No correlation was found between MMP and IL-1β or TNFα expression. A negative correlation was observed between MMP9 and IL-6 expression ($r = -0.732$, $p = 0.001$). Maximal MMP expression and enzymatic activity occurred at the granulomatous areas were topographic relationship with integrin expression was observed. In arteries with fully developed lesions, no correlation was observed between MMP expression and IEL disruption or intimal hyperplasia. MMP expression scores were significantly lower in patients who had received corticosteroid treatment.

Conclusions: MMP expression and activity predominated in the granulomatous areas where concomitant expression of leukocyte integrins was observed, indicating the relevance of contact-dependent mechanisms in their regulation. The lack of correlation between MMP expression and IEL degradation suggest that regulation of MMP activity may be more relevant than regulation of MMP expression and suggest that other elastinolytic enzymes may also participate in vessel wall disruption.

INTRODUCTION

Giant cell arteritis (GCA) is a vasculitis of the elderly characterized by granulomatous inflammation of large and medium-sized arteries (1, 2). Histopathologic evaluation of the various degrees of inflammation appearing in temporal artery biopsies, suggests that infiltrating lymphocytes invade the vessel wall through the adventitial *vasa vasorum* and surrounding small vessels (3, 4). This interpretation is supported by immunopathologic studies showing that endothelial adhesion molecules necessary for leukocyte recruitment are mainly expressed by adventitial *vasa vasorum* (5). Furthermore, leukocytes arround them, strongly express endothelial cell adhesion molecule receptors, namely integrins of the $\alpha 2$ subfamily and VLA4, as it has been demonstrated for leukocytes migrating to specific compartments in other diseases and in experimental models (6, 7). In addition, leukocytes surrounding *vasa vasorum* express MRP8 and MRP14, S100-derived proteins expressed by phagocytes recently recruited into tissues (8). Inflammatory infiltrates subsequently extend towards the adventitia and the medial layer where they undergo granulomatous differentiation (4). At this stage, inflammatory cells can be additionally recruited through inflammation-induced neovessels which typically appear in GCA inflammatory lesions (1, 9).

To infiltrate the vessel wall, infiltrating leukocytes need to break a natural barrier, the basement membrane of the *vasa vasorum*, and to migrate through the interstitial matrix. As the inflammatory process evolves, the internal elastic lamina (IEL) is also disrupted, allowing the progression of leukocytes, as well as myointimal cells towards the intima (10). By altering the structural integrity of the vessel wall, rupture of elastic fibers may lead to late deleterious consequences such as the development of aneurysms. Among the proteolytic systems participating in this process,

gelatinases (MMP2 and MMP9) may have an important role given their ability to degrade basement membrane components and elastin (11-13). Like other proteolytic systems, MMP activity is tightly regulated at several levels. Gelatinase production by inflammatory cells is transcriptionally regulated but post-transcriptional regulation is at least as relevant. Gelatinase expression is induced in lymphocytes and macrophages by cytokines such as IL-1 and TNFα, and other inflammatory mediators (14-19), some of which are known to be produced in GCA (20). However, the most powerful inducer not only of gelatinase expression but also of gelatinase release and activation is contact-dependent and it is mediated by integrin engagement (14, 18). Gelatinases, like most MMPs are secreted as inactive zymogens and need to be activated by proteolytic cleavage. Integrin engagement not only induces gelatinase synthesis and release but also expression of MMP14 which is the more efficient physiologic activator of MMP2 (11, 12). Gelatinase activity is subsequently modulated by interactions with their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

Gelatinase and TIMP1 and 2 expression in GCA lesions has been previously documented but in very small series of patients (21-24) or in patients who have received corticosteroid treatment for variable periods of time (25). Given that gelatinase biologic functions do not solely depend on their expression, the aims of our study were to investigate the expression of gelatinases and MMP14 in temporal artery lesions from patients with GCA in order to assess their topographic relationship with soluble inducers and, particularly, with leukocyte integrins, and to determine the resulting proteolytic activity by in situ gelatin zymography.

PATIENTS AND METHODS

Patients

The study group consisted of 46 patients with biopsy-proven GCA. Thirty-three patients had received no treatment before the temporal artery excision whereas the remaining 13 had received treatment with 1 mg/Kg/day of prednisone for 9 ± 2.5 (mean ± SEM). Unless otherwise indicated only untreated patients were considered in quantitative measurements. Twelve normal temporal arteries from patients in whom GCA was initially suspected but subsequently ruled out served as controls.

All specimens were embedded in OCT, snap-frozen in isopenthane pre-chilled in liquid nitrogen, and stored at -80ºC until processing.

Histopathologic evaluation

Haematoxylin-eosin stained temporal artery sections from GCA patients were classified according to the extension of the inflammatory infiltrates. Sixteen GCA specimens had inflammatory infiltrates limited to adventitial *vasa vasorum* and adventitial layer. The remaining 30 had fully-developed lesions with inflammatory infiltrates extending through the entire artery wall. The topographic distribution and scoring of MMPs and integrins were separately evaluated in both groups. Additional aspects evaluated were the IEL integrity and the extent of intimal hyperplasia. Elastic lamina was stained with 1% Shikata's orcein (Scharlau Chemie S.A., Barcelona, Spain) in 70% ethanol. Disruption of IEL 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.(9)

Immunostaining

Serial 4-6 μm cryostat temporal artery sections obtained from the above individuals were air-dried and fixed with cold acetone. Sections were incubated with the primary antibodies depicted in Table 1. Immunoglobulins obtained from the same species as the primary antibodies were used as negative controls at the same concentrations. Immunodetection was carried out with an HRP-labeled polymer conjugated to secondary antibodies (EnVision kit from Dako, Carpinteria, CA).

Quantification of the immunostaining was performed according to a semiquantitative 0-4 score, as described (26, 27). Scoring was performed by 3 investigators (MS, AGM, and MCC) blinded to the patients' clinical information. There was agreement in 90% of the measurements. For the remaining 10% a consensus was achieved after re-evaluation.

mRNA quantification

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

MMP2, MMP9, MMP14, TNFα, IL-1β and IL-6 expression was measured by real-time PCR using specific Assay-on-Demand Taqman Gene expression probes from Applied Biosystems. PCR reaction was performed with 2μl of cDNA together with Taqman PCR Universal Master Mix (Applied Biosystems) and the corresponding primers and probe. Each sample was tested twice. PCR reaction conditions were those recommended by the manufacturer. PCR reaction was monitorized by measuring the

fluorescence signal after each cycle with ABI Prism 7900 sequence detection system (Applied Biosystems). C_T values (cycle number where fluorescence overpassed a fixed threshold) were obtained for each target probe and normalized with the corresponding C_T values for the internal control (GUSb). mRNA quantity was expressed as relative units.

Film in situ zymography (FIZ)

Topographic distribution of gelatinase activity was assessed in temporal artery sections by FIZ (Fuji Photo Film Co., Ltd Tokyo, Japan). Five μm thick cryostat sections from the same biopsy samples used for immunostochemistry were applied to 7μm 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 hours in a moist chamber at 37ºC. Subsequently, membranes were air dried and stained with 0.5% Amido Black 10B (Sigma) in 70% methanol, 10% acetic acid for 10 minutes and distained by washing in 70% methanol, 10% acetic acid. Gelatinase activity was visualized as distained areas on a dark-blue background.

Statistics

Mann-Whitney U test was used to compare quantitative variables and Spearman test for correlations. 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 could be detected in normal temporal arteries by immunohistochemistry. However, MMP2 was expressed by vascular smooth muscle cells (VSMC) in the medial layer, as it has been reported by others (21-23, 25) (figure 1). MMP9 and MMP14 mRNA could be detected at very low concentrations in temporal arteries from controls but were significantly more abundant in samples from patients with GCA (figure 2). No differences were found in MMP2 mRNA between patients and controls, further supporting constitutive MMP2 expression in non-inflamed temporal arteries.

In GCA lesions, MMP2 was not only expressed by VSMC at the media but also by infiltrating leukocytes and, in some cases, by myointimal cells at the intimal layer. MMP9 and MMP14 expression was restricted to inflammatory cells, except MMP2 and MMP9 that could also be observed in some endothelial cells.

As shown in figure 1, MMP expression varied according to the extent of inflammatory involvement. In specimens with *vasa vasorum* and adventitial inflammation only, MMP9 and MMP14 were moderately expressed by adventitial inflammatory cells. MMP2 was also expressed by inflammatory cells and also by preserved VSMC at the media, similarly to normal arteries. In GCA arteries with fully-developed inflammatory lesions, MMPs were intensively expressed by activated macrophages in the granulomatous areas at the media and at the intima/media junction, and the intensity of immunostaining was stronger. In these arteries, where VSMC were partially destroyed, MMP2 expression also predominated at the granulomatous areas, following a different pattern from that observed in normal arteries or in arteries with

only adventitial inflammation, where MMP2 expression was restricted to VSMC (figure 1).

Immunostaining scores for all 3 MMPs 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 induced by cell interactions with ECM proteins (18), MMP2 and MMP14 mRNA weakly correlated ($r=0.39$, $p=0.048$). Surprisingly, a negative correlation was observed between MMP2 and MMP9 mRNA concentration ($r=-0.45$, $p=0.019$). No significant correlation was observed between MMP9 and MMP14 mRNA concentrations.

Relationship between pro-inflammatory cytokine and MMP expression in GCA

We next investigated the potential relationship between MMP expression and pro-inflammatory cytokines TNFα, IL-6 and IL-1β, that have been shown to be associated with MMP production in several systems (28-31). As previously described (27), pro-inflammatory cytokine mRNA was higher in patients than controls (figure 2) and immunohistochemical detection predominated in the granulomatous areas (data not shown). No significant correlation was found between IL-1β or TNFα and MMP mRNA expression. Surprisingly, a negative correlation was observed between IL-6 and MMP9 mRNA concentration. No correlation was found between Immunohistochemical scores for MMPs and pro-inflammatory cytokines in patients with fully-developed lesions (table 2).

Relationship between MMP and integrin expression

We next explored the topographic relationship between integrins and MMP expression in GCA lesions. As shown in figure 3, αvβ3 and α5 integrins were constitutively expressed by endothelial cells and by VSMC in normal arteries. No expression of the leukocyte α4 was observed in normal controls. In GCA samples, all 3 integrins were strongly expressed by infiltrating leukocytes and predominated at the granulomatous area in fully-developed lesions. α5 was also expressed by myointimal cells.

Leukocyte integrins were strongly expressed by leukocytes in invading fronts progressing from the adventitia to the media (figure 4). Strong co-expression of MMPs was observed in these areas, indicating that MMPs may have an important pro-inflammatory role by allowing the progression of inflammatory infiltrates through the artery wall (figure 4).

Gelatinolytic activity of MMP2 and MMP9 in GCA

Given that MMP expression does not necessarily parallel MMP function, we assessed MMP2 and MMP9 enzymatic activity by FIZ.

As shown in figure 5, in spite of constitutive MMP2 expression by VSMC, normal temporal arteries did not exhibit gelatinolytic activity. In arteries with inflammatory infiltrates limited to the adventitia, a weak gelatinolytic activity could be observed in the inflamed regions. In fully-developed lesions, a strong gelatinolytic activity was observed. Gelatinolytic activity predominated at the granulomatous areas at the media and at the intima/media junction where co-expression of leukocyte integrins and MMP2-activator MMP14 was observed in serial sections (figure 5). The

specificity of the signal was supported by the lack of enzymatic activity observed in the presence of (1,10)-phenanthroline (data not shown).

Potential role of gelatinases in vascular destruction, remodelling and regeneration in GCA

IEL was significantly more disrupted in temporal arteries with fully-developed lesions than in those with adventitial inflammation only (odds ratio 31, 95% CI 3.02-329.1, p=0.0011), supporting a relevant elastinolytic activity of macrophages at the granulomatous area and the potential participation of MMP2 and MMP9 in this process, given that the maximum gelatinolytic activity was also localized in this area. IEL rupture positively correlated with the extent of intimal hyperplasia (r=0.5, p=0.036), suggesting that myointimal cells may take advantage of the breakdown of this natural barrier to migrate to the intima.

However, in samples with fully-developed lesions, no significant correlation was observed between MMP immunohistochemical expression 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). Taken together these findings suggest that MMPs probably have a role in disrupting IEL but variations in the intensity of expression in fully-developed lesions do not have a significant impact on IEL degradation. Accordingly, no significant correlation was found between MMP expression scores and intimal hyperplasia scores.

Effect of corticosteroid treatment on MMP expression in GCA

Samples from patients who had received corticosteroid treatment had indeed significantly lower immunohistochemical scores for MMP2, MMP9, and MMP14 than samples excised from untreated patients (figure 6). However, no significant differences

in mRNA levels could be found between untreated patients and those who had received treatment. This is possibly due to the fact that patients from whom RNA could be extracted had been treated for 6 ± 1 days whereas, as overall, patients from whom immunostained sections were evaluated had received treatment for longer periods of time (9 ± 2.5 days).

DISCUSSION

Infiltrating leukocytes were the main source of MMPs in GCA and the distribution and intensity of their expression varied according to the extent of inflammatory infiltrates. In fully-developed lesions, MMP expression highly predominated at the granulomatous areas at the media and at the intima/media junction.

Although there was a significant positive correlation between expression of MMP2, MMP9, and MMP14 at the more stable protein level, we were surprised to observe a significant inverse correlation between MMP2 and MMP9 transcripts. There are several possible explanations for this finding. MMP9 is mainly expressed by infiltrating leukocytes, and its active transcription may somehow reflect inflammatory activity. Smooth muscle cells are highly damaged in severely inflamed arteries. Intense MMP9 expression may reflect more intense inflammatory activity and higher VSMC destruction, reducing their constitutive MMP2 expression. On the other hand, it has been recently shown that MMP2-null mice develop more severe lesions in various models of chronic inflammatory diseases (32, 33). Esparza *et al*. recently showed that lymphocytes obtained from MMP2-null mice produce more MMP9 and a down-regulatory effect of MMP2 on MMP9 expression driven by MMP2 interaction with membrane bound MMP14 may transmit inhibitory signals for MMP9 expression (32). A decrease in MMP2 production might then further up-regulate MMP9.

Gelatinase expression was not always associated with enzymatic activity. In fact, normal specimens and samples disclosing inflammatory infiltrates restricted to the adventitia did not show gelatinolytic activity in the media in spite of significant MMP2 expression by VSMC suggesting that, in normal arteries, MMP2 is produced as a pro-enzyme and additional stimuli are required for its activation. In GCA lesions, areas with

strong gelatinolytic activity combined the expression of the three MMPs, suggesting a functional relationship between them. In fact, MMP14 is the best known physiological activator of MMP2 which, in turn, may also activate MMP9 (11, 12).

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 (34). This location suggests, indeed, a role for gelatinases in the destruction of IEL, typically found in GCA. 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 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.

When we investigated the expression of potential inducers of gelatinase expression we did not find any significant correlation between MMP expression and local expression of pro-inflammatory cytokines IL-1β or TNFα, indicating that other factors contribute to MMP induction in GCA. Surprisingly, IL-6 mRNA negatively correlated with MMP9 expression. IL-6, highly expressed in temporal arteries from patients with GCA, has some anti-inflammatory functions (35). It down-regulates excessive IL-1 and TNFα production and might also contribute to down-regulate MMP9 expression in GCA. Alternatively other factors may exert opposite effects on both molecules.

Maximal MMP2, MMP9 and MMP14 expression occurred in inflamed areas with strong integrin expression by infiltrating leukocytes. Topographic relationship between integrins, MMPs and gelatinolytic activity together with the lack of association between pro-inflammatory cytokine and MMP expression suggest that contact-dependent mechanisms are major regulators of MMP expression and activation in GCA.

Integrins and MMPs were co-expressed by leukocytes invading the vessel wall from the adventitial layer. We have recently shown that integrin induced gelatinase release is regulated by pathways controlling lymphocyte migration (18). In addition, it has been recently shown that MMP14, a transmembrane and potentially signalling molecule, is required for monocyte migration and endothelial transmigration (36). Taken together, these findings suggest that, in addition to their potential contribution to IEL degradation and vessel destruction, gelatinases may have important pro-inflammatory roles in GCA, by allowing the progression of inflammatory cells through the artery wall.

The development of intimal hyperplasia is a significant source of morbidity in patients with vasculitis. However, it provides a mechanism reinforcing the vessel wall when IEL is destroyed. Intimal hyperplasia correlated, indeed, with the extent of IEL disruption, as it has been observed by others (37). IEL degradation may promote intimal hyperplasia by facilitating myointimal cell migration towards the intima. While intact IEL favours a quiescent/contractile VSMC phenotype, elastin fragments promote the differentiation of VSMC into migratory and secretory myointimal cells (38). However, no significant correlation was found between the extent of intimal hyperplasia and the intensity of MMP expression in arteries with fully-developed lesions. In this regard, MMP may have a dual function in vascular remodelling: by

disrupting IEL they may promote myointimal cell migration but, at the same time, increased MMP expression and activity may prevent excessive matrix deposition and lumen occlusion.

In appearance, our results differ from those published by Rodríguez-Pla *et al.* who found correlation between MMP9 expression and IEL disruption and intimal hyperplasia. However in that study no histological categorization of the biopsies according to the extent of inflammation was performed and it is possible that the entire series included specimens with predominant adventitial inflammation, which, according to our results, have more preserved IEL and less MMP expression scores. In the absence of histopathologic categorization, higher MMP9 expression may also reflect more extended inflammatory infiltrates which are usually associated with more prominent IEL destruction and intimal hyperplasia (9).

We found significantly lower immunostaining scores in samples from patients who had received corticosteroid treatment. MMP9 and MMP14 promoters have NF-κB binding sites and, accordingly, their expression is decreased by corticosteroids (39). Although MMP2 promoter is quite different, the decrease in MMP2 expression achieved with corticosteroids can be explained by multiple mechanisms. Corticosteroids decrease lymphocyte and macrophage activation, and, consequently, reduce integrin avidity and MMP induction and activation driven by cell contact (40).

In summary, gelatinases and MMP2 activator MMP14 are strongly expressed in GCA inflammatory lesions, particularly at the granulomatous areas. The biologic functions of MMPs in GCA appear to be complex. According to our results, they may have pro-inflammatory effects by allowing the progression of infiltrating leukocytes and may also contribute to IEL disruption and vascular remodelling although additional enzymes are probably involved in this process.

Table 1. Antibodies used in the study.

Antigen description	Type, origin	Clone	Source	Working dilution
VLA4, α chain (CD49d, α4)	monoclonal, mouse	HP2/1	Immunotech, Marseille, France	1/200
VLA5, α chain (CD49e, α5)	monoclonal, mouse	SAM1	Immunotech, Marseille, France	1/200
αvβ3 (CD51/CD61)	monoclonal, mouse	LM609	Chemicon Inc., Temecula, CA	1/100
MMP2	polyclonal, rabbit		Chemicon Inc., Temecula, CA	1/500
MMP9	monoclonal, mouse	GE-213	Chemicon Inc., Temecula, CA	1/1000
MMP14	polyclonal, rabbit		Chemicon Inc., Temecula, CA	1/250
TNFα	polyclonal, rabbit		Genzyme, Minneapolis, MN	1/500
IL-1β	monoclonal, mouse	B1	R&D Systems, Minneapolis, MN	1/50
IL-6	monoclonal, mouse	6708.111	R&D Systems, Minneapolis, MN	1/50

Table 2. Correlation between MMP and cytokine expression in temporal arteries from patients with GCA.

	mRNA quantification			*Immunohistochemistry*		
	MMP2	**MMP9**	**MMP14**	**MMP2**	**MMP9**	**MMP14**
TNFα	r= -0.046 p= 0.855	r= 0.265 p= 0.287	r= -0.077 p= 0.76	r= 0.112 p= 0.702	r= 0.27 p= 0.35	r= 0.337 p= 0.192
IL-6	p= 0.224 r= 0.372	r= -0.732* p= 0.001	r= -0.181 p= 0.473	r= 0.045 p= 0.884	r= 0.207 p= 0.497	r= 0.192 p= 0.530
IL-1β	r= -0.001 p= 0.997	r= -0.205 p= 0.414	r= -0.186 p= 0.416	r= 0.025 p= 0.928	r= 0.189 p= 0.484	r= 0.392 p= 0.133

FIGURE 1

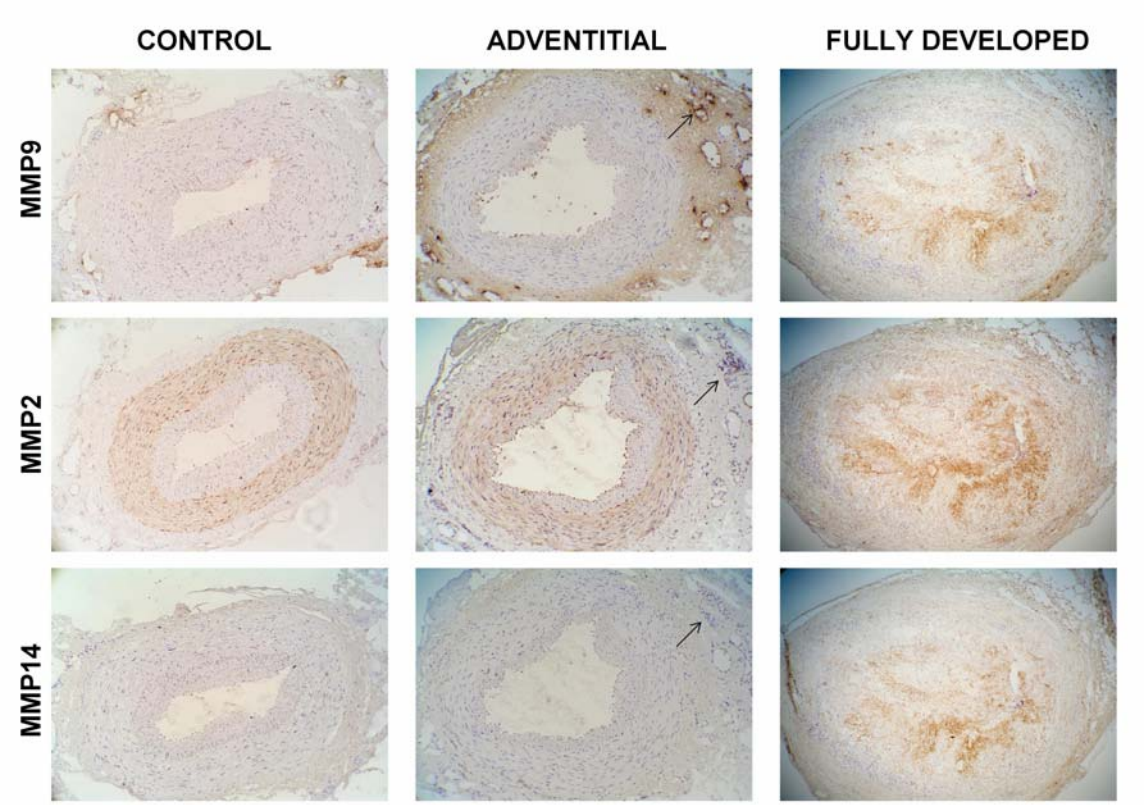

Figure 1: Expression of MMP2, MMP9, and MMP14 in serial sections from temporal arteries from controls and from patients with GCA, according to the extent of inflammatory infiltrates. In specimens with inflammatory infiltrates involving *vasa vasorum* and adventitia only (arrows), MMP expression was similar to controls, with some MMP2 and MMP9 expression in inflammatory cells.

FIGURE 2

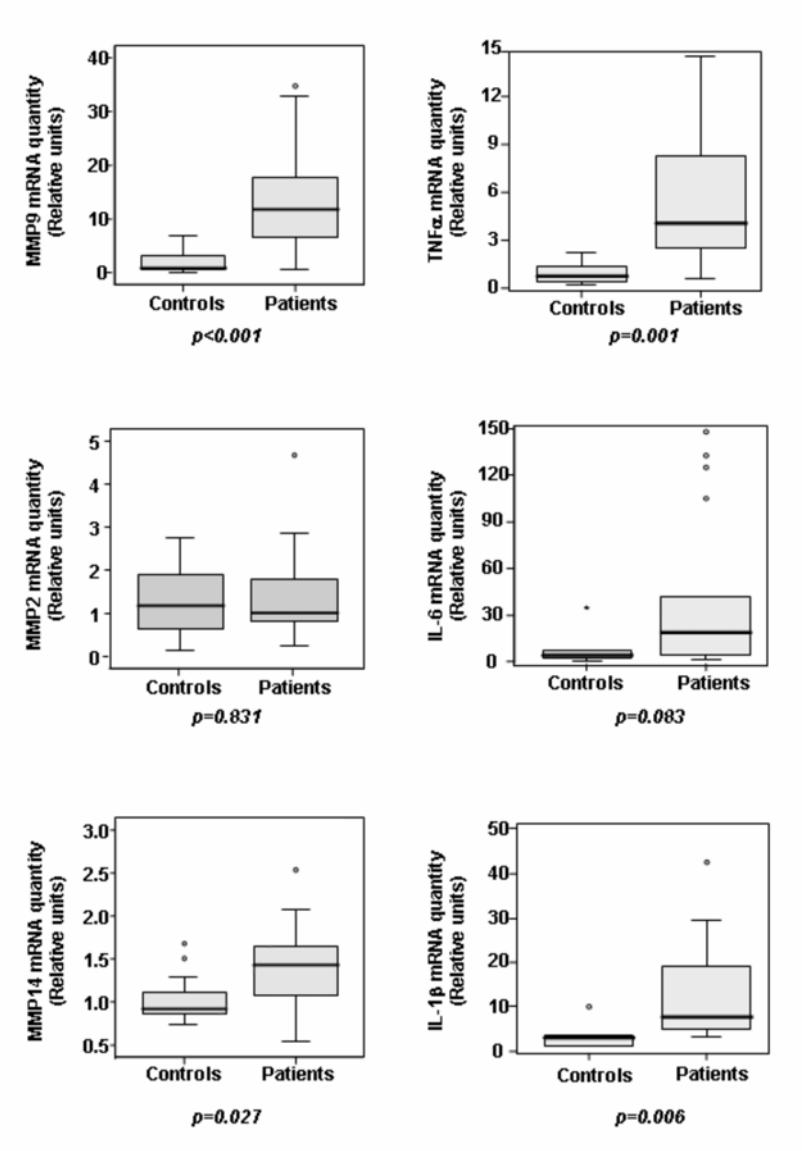

Figure 2: MMP (MMP9, MMP2 and MMP14) and proinflammatory cytokine (TNFα, IL-6 and IL-1β) mRNA levels in temporal arteries from untreated GCA patients and controls.

FIGURE 3

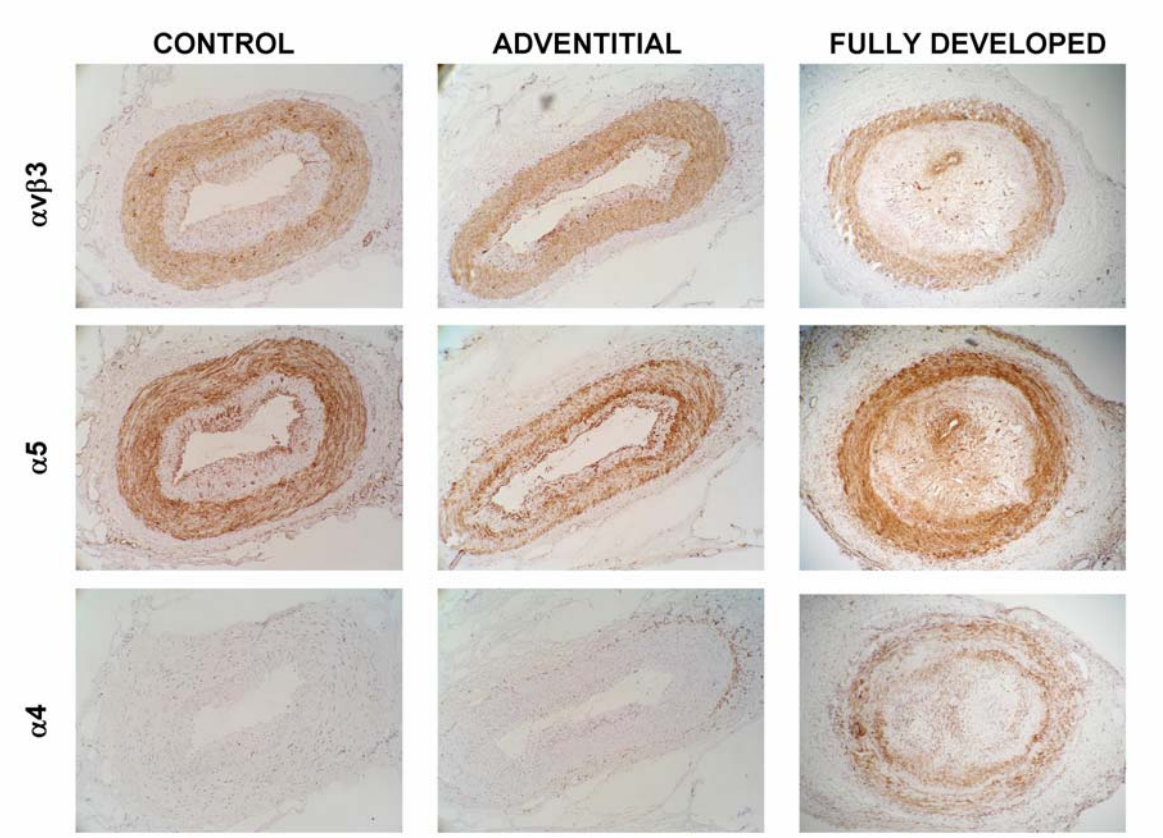

Figure 3: Integrin expression in temporal artery biopsies from controls and from patients with GCA, according to the extent of inflammatory involvement.

FIGURE 4

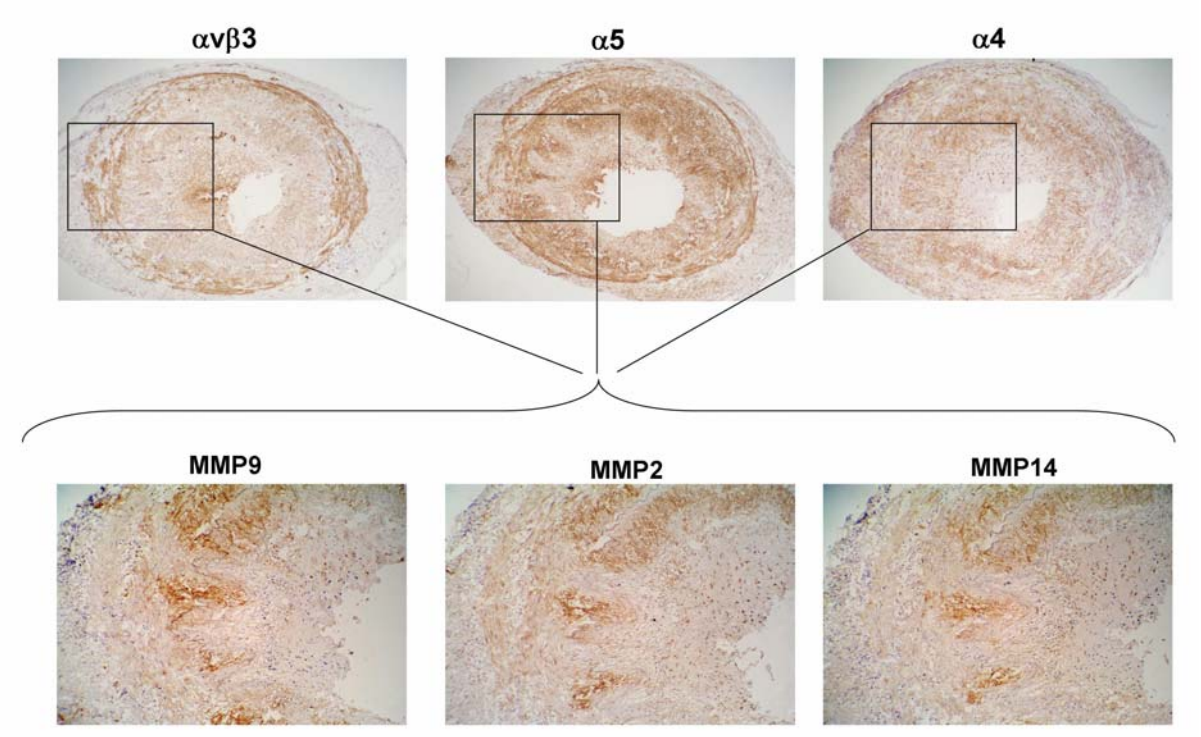

Figure 4: Co-expression of strong integrin and MMP expression in invading fronts of inflammatory cells. Upper pannels are serial temporal artery sections immunostained for integrins. Lower pannels correspond to higher magnifications of the insets immunostained for MMPs.

FIGURE 5

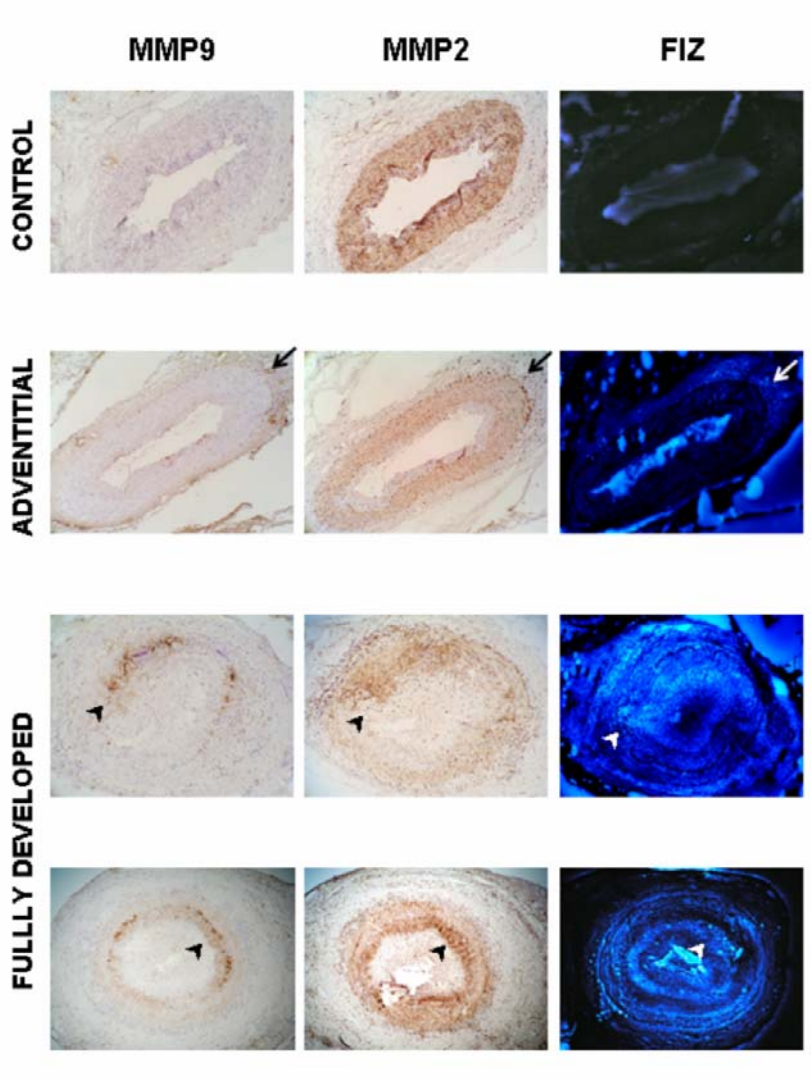

Figure 5: Gelatinolytic activity of MMP2 and MMP9 according to the extension of inflammatory infiltrates. Serial sections of temporal arteries from normal controls and from patients with GCA with different extent of inflammatory involvement were immunostained for MMPs and subjected to film in situ gelatin zymography (FIZ). 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, particularly in the granulomatous areas (arrowheads). Adventitial inflammatory infiltrates also disclose slight gelatinolytic activity (arrows).

FIGURE 6

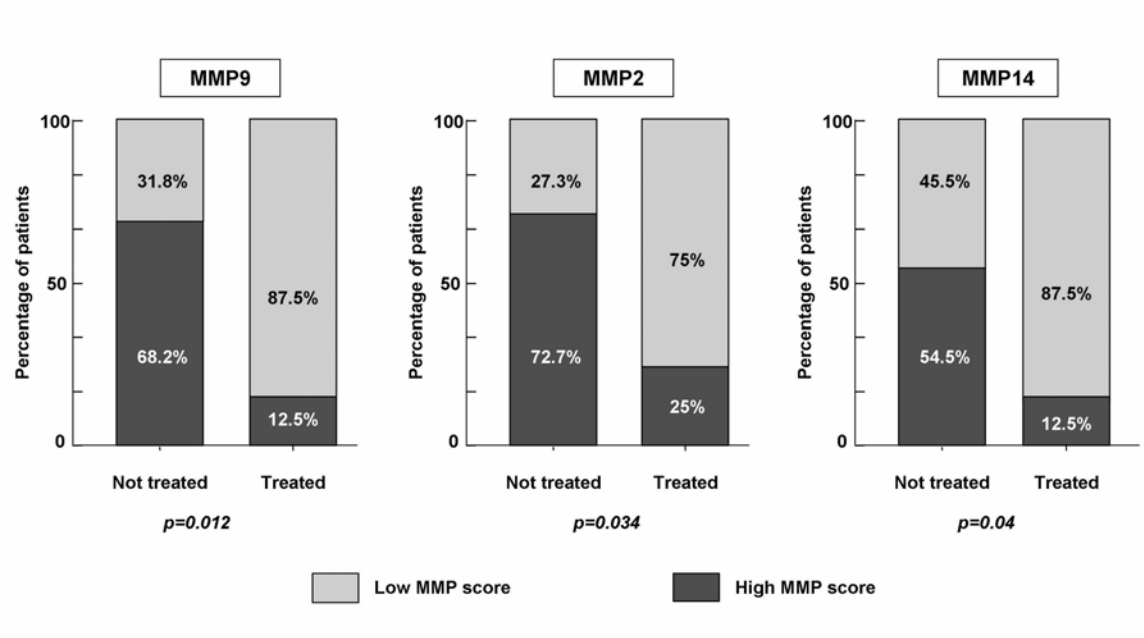

Figure 6: Percentage of patients with high vs low MMP expression scores according to corticosteroid treatment.

REFERENCES

1. Cid MC, Font C, Coll-Vinent B, Grau JM. Large vessel vasculitides. Curr Opin Rheumatol 1998;10(1):18-28.

2. Salvarani C, Cantini F, Boiardi L, Hunder GG. Polymyalgia rheumatica and giant-cell arteritis. N Engl J Med 2002;347(4):261-71.

3. Esteban MJ, Font C, Hernandez-Rodriguez J, Valls-Sole J, Sanmarti R, Cardellach F, et al. Small-vessel vasculitis surrounding a spared temporal artery: clinical and pathological findings in a series of twenty-eight patients. Arthritis Rheum 2001;44(6):1387-95.

4. Hernandez-Rodriguez J, Villar I, Garcia-Martinez A, Font C, Esteban MJ, Sanmarti R, et al. Histopathologic findings in temporal artery biopsies from 171 patients with giant-cell arteritis (GCA): Relationship with prospectively recorded clinical manifestations. Arthritis Rheum 2005;52(9):S222-S 521 Suppl. S.

5. Cid MC, Cebrian M, Font C, Coll-Vinent B, Hernandez-Rodriguez J, Esparza J, et al. Cell adhesion molecules in the development of inflammatory infiltrates in giant cell arteritis: inflammation-induced angiogenesis as the preferential site of leukocyte-endothelial cell interactions. Arthritis Rheum 2000;43(1):184-94.

6. Laffon A, Garcia-Vicuna R, Humbria A, Postigo AA, Corbi AL, de Landazuri MO, et al. Upregulated expression and function of VLA-4 fibronectin receptors on human activated T cells in rheumatoid arthritis. J Clin Invest 1991;88(2):546-52.

7. Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature 1992;356(6364):63-6.

8. Foell D, Hernandez-Rodriguez J, Sanchez M, Vogl T, Cid MC, Roth J. Early recruitment of phagocytes contributes to the vascular inflammation of giant cell arteritis. J Pathol 2004;204(3):311-6.

9. Cid MC, Hernandez-Rodriguez J, Esteban MJ, Cebrian M, Gho YS, Font C, et al. Tissue and serum angiogenic activity is associated with low prevalence of ischemic complications in patients with giant-cell arteritis. Circulation 2002;106(13):1664-71.

10. Kimmelstiel P, Gilmour MT, Hodges HH. Degeneration of elastic fibers in granulomatous giant cell arteritis (temporal arteritis). AMA Arch Pathol 1952;54(2):157-68.

11. Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta 2005;1755(1):37-69.

12. Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2002;2(9):657-72.

13. Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG. Human 92- and 72-kilodalton type IV collagenases are elastases. J Biol Chem 1991;266(12):7870-5.

14. Esparza J, Vilardell C, Calvo J, Juan M, Vives J, Urbano-Marquez A, et al. Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines. A process repressed through RAS/MAP kinase signaling pathways. Blood 1999;94(8):2754-66.

15. Leppert D, Hauser SL, Kishiyama JL, An S, Zeng L, Goetzl EJ. Stimulation of matrix metalloproteinase-dependent migration of T cells by eicosanoids. The Faseb Journal: Official Publication of the Federation of American Societies For Experimental Biology 1995;9(14):1473-81.

16. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. T cell gelatinases mediate basement membrane transmigration in vitro. Journal of Immunology (Baltimore, Md.: 1950) 1995;154(9):4379-89.

17. Romanic AM, Graesser D, Baron JL, Visintin I, Janeway CA, Madri JA. T cell adhesion to endothelial cells and extracellular matrix is modulated upon transendothelial cell migration. Laboratory Investigation; a Journal of Technical Methods and Pathology 1997;76(1):11-23.

18. Segarra M, Vilardell C, Matsumoto K, Esparza J, Lozano E, Serra-Pages C, et al. Dual function of focal adhesion kinase in regulating integrin-induced MMP-2 and MMP-9 release by human T lymphoid cells. FASEB J 2005; 1875-1877.

19. Xia M, Leppert D, Hauser SL, Sreedharan SP, Nelson PJ, Krensky AM, et al. Stimulus specificity of matrix metalloproteinase dependence of human T cell migration through a model basement membrane. J Immunol 1996;156(1):160-7.

20. Weyand CM, Goronzy JJ. Medium- and large-vessel vasculitis. N Engl J Med 2003;349(2):160-9.

21. Nikkari ST, Hoyhtya M, Isola J, Nikkari T. Macrophages contain 92-kd gelatinase (MMP-9) at the site of degenerated internal elastic lamina in temporal arteritis. Am J Pathol 1996;149(5):1427-33.

22. Sorbi D, French DL, Nuovo GJ, Kew RR, Arbeit LA, Gruber BL. Elevated levels of 92-kd type IV collagenase (matrix metalloproteinase 9) in giant cell arteritis. Arthritis Rheum 1996;39(10):1747-53.

23. Tomita T, Imakawa K. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in giant cell arteritis: an immunocytochemical study. Pathology 1998;30(1):40-50.

24. Wagner AD, Goronzy JJ, Weyand CM. Functional profile of tissue-infiltrating and circulating CD68+ cells in giant cell arteritis. Evidence for two components of the disease. J Clin Invest 1994;94(3):1134-40.

25. Rodriguez-Pla A, Bosch-Gil JA, Rossello-Urgell J, Huguet-Redecilla P, Stone JH, Vilardell-Tarres M. Metalloproteinase-2 and -9 in giant cell arteritis: involvement in vascular remodeling. Circulation 2005;112(2):264-9.

26. Hernandez-Rodriguez J, Segarra M, Vilardell C, Sanchez M, Garcia-Martinez A, Esteban MJ, et al. Elevated production of interleukin-6 is associated with a lower incidence of disease-related ischemic events in patients with giant-cell arteritis: angiogenic activity of interleukin-6 as a potential protective mechanism. Circulation 2003;107(19):2428-34.

27. Hernandez-Rodriguez J, Segarra M, Vilardell C, Sanchez M, Garcia-Martinez A, Esteban MJ, et al. Tissue production of pro-inflammatory cytokines (IL-1beta, TNFalpha and IL-6) correlates with the intensity of the systemic inflammatory response and with corticosteroid requirements in giant-cell arteritis. Rheumatology (Oxford) 2004;43(3):294-301.

28. Chu SC, Yang SF, Lue KH, Hsieh YS, Wu CL, Lu KH. Regulation of gelatinases expression by cytokines, endotoxin, and pharmacological agents in the human osteoarthritic knee. Connect Tissue Res 2004;45(3):142-50.

29. Hunder GG, Bloch DA, Michel BA, Stevens MB, Arend WP, Calabrese LH, et al. The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis. Arthritis Rheum 1990;33(8):1122-8.

30. Xie Z, Singh M, Singh K. Differential regulation of matrix metalloproteinase-2 and -9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1beta. J Biol Chem 2004;279(38):39513-9.

31. Yoo HG, Shin BA, Park JS, Lee KH, Chay KO, Yang SY, et al. IL-1beta induces MMP-9 via reactive oxygen species and NF-kappaB in murine macrophage RAW 264.7 cells. Biochem Biophys Res Commun 2002;298(2):251-6.

32. Esparza J, Kruse M, Lee J, Michaud M, Madri JA. MMP-2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity. Faseb J 2004;18(14):1682-91.

33. Itoh T, Matsuda H, Tanioka M, Kuwabara K, Itohara S, Suzuki R. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. J Immunol 2002;169(5):2643-7.

34. Rittner HL, Kaiser M, Brack A, Szweda LI, Goronzy JJ, Weyand CM. Tissue-destructive macrophages in giant cell arteritis. Circ Res 1999;84(9):1050-8.

35. Hegde S, Pahne J, Smola-Hess S. Novel immunosuppressive properties of interleukin-6 in dendritic cells: inhibition of NF-kappaB binding activity and CCR7 expression. Faseb J 2004;18(12):1439-41.

36. Matias-Roman S, Galvez BG, Genis L, Yanez-Mo M, de la Rosa G, Sanchez-Mateos P, et al. Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. Blood 2005;105(10):3956-64.

37. Kaiser M, Younge B, Bjornsson J, Goronzy JJ, Weyand CM. Formation of new vasa vasorum in vasculitis. Production of angiogenic cytokines by multinucleated giant cells. Am J Pathol 1999;155(3):765-74.

38. Karnik SK, Brooke BS, Bayes-Genis A, Sorensen L, Wythe JD, Schwartz RS, et al. A critical role for elastin signaling in vascular morphogenesis and disease. Development 2003;130(2):411-23.

39. Aljada A, Ghanim H, Mohanty D, Triparthy D, Dandona P. Hydrocortisone suppress intranuclear activation protein -1 (AP-1) binding activity in mononuclear cells and plasma matrix metalloproteinases 2 and 9 (MMP2 and MMP9). J Endocrinol 2001;86:5988-9.

40. Buttgereit F, Straub RH, Wehling M, Burmester GR. Glucocorticoids in the treatment of rheumatic diseases: an update on the mechanisms of action. Arthritis Rheum 2004;50(11):3408-17.

RESUM DELS RESULTATS

1. L'expressió dels gens de MMP9 i MMP14 és significativament superior en les artèries afectades d'arteritis de cèl·lules gegants respecte artèries normals procedents de pacients control. L'expressió del transcrit de MMP2 no presenta diferències entre controls i pacients.

2. En les artèries normals es detecta immunohistoquímicament l'expressió constitutiva de les integrines $\alpha 5$ i $\alpha v\beta 3$ i la gelatinasa MMP2 en la capa muscular llisa i en l'endoteli principal.

3. Les artèries inflamades expressen MMP9, MMP2 i MMP14 fonamentalment en la zona granulomatosa amb la intersecció amb la làmina elàstica. La gradació de l'expressió immunohistoquímica està significativament correlacionada entre totes tres MMPs.

4. L'expressió de MMPs, tant a nivell immunohistològic com gènic, no es correlaciona amb l'expressió de les citocines proinflamatòries TNFα, IL6 i IL1β presents en les artèries de pacients amb arteritis de cèl·lules gegants.

5. Les artèries afectades per l'arteritis de cèl·lules gegants també expressen la integrina limfocitària $\alpha 4$ definint l'infiltrat inflamatori. L'expressió de les integrines $\alpha 5$ i $\alpha v\beta 3$ és molt intensa i es magnifica en la zona granulomatosa. A més, $\alpha 5$ està fortament expressada en tota l'extensió de l'infiltrat inflamatori, en canvi $\alpha v\beta 3$ gairebé no s'expressa en l'infiltrat de l'adventícia.

6. Les MMPs es co-expressen amb les integrines leucocitàries, dibuixant la progressió de l'avenç de l'infiltrat.

7. Les artèries normals, malgrat expressar MMP2 constitutivament, no tenen activitat gelatinolítica. En canvi, les artèries patològiques tenen una activitat proteolítica

màxima focalitzada en la zona granulomatosa, on es dóna la major co-localització de MMPs i integrines.

8. Tot i que l'expressió de gelatinases no es correlaciona amb els graus de destrucció de l'elàstica, observem una major destrucció de la làmina elàstica en les artèries que tenen lesions granulomatoses ben desenvolupades en la zona d'unió íntima/mitja respecte aquelles que presenten només afectació adventicial. A més, els graus de ruptura de la làmina elàstica es correlacionen positivament amb els graus d'hiperplàsia de la capa íntima.

9. L'ús terapèutic de glucocorticoids disminueix significativament l'expressió de MMP9, MMP2 i MMP14 a nivell immunohistològic atès que en el grup de pacients que havien estat tractats prèviament al moment de la biòpsia presenten nivells d'expressió més febles que els pacients no tractats.

CONCLUSIONS

L'arteritis de cèl·lules gegants és una malaltia inflamatòria crònica que afecta a vasos grans i mitjans, com és el cas de l'artèria temporal. L'expressió de MMPs en les lesions d'arteritis de cèl·lules gegants era prèviament coneguda a partir d'estudis immunohistoquímics de sèries petites. Aquest és el primer treball que inclou una sèrie gran de pacients verges de tractament on s'investiga, a més de l'expressió de MMPs per immunohistoquímica, la quantitat de mRNA, els mecanismes d'inducció i l'estat d'activació de les gelatinases. Dels resultats obtinguts podríem concloure:

1. Les artèries afectades amb arteritis de cèl·lules gegants tenen una expressió important de MMPs (MMP9, MMP2 i MMP14) que es correspon amb la localització de l'activitat gelatinolítica. En canvi, en les artèries normals, tot i expressar MMP2 de forma constitutiva, no s'observa gelatinolisi. Aquest fet

suggereix que en els controls s'expressa el zimogen de MMP2 i en canvi els efectes derivats del procés inflamatori, com la co-localització amb MMP14 i integrines leucocitàries, provoquen l'activació de MMP2.

2. L'expressió de gelatinases en l'artèria està coordinada amb la co-expressió d'integrines. Novament aquesta observació suporta el fet que aquest mecanisme exerceix una potent inducció en la producció i activació de MMPs en les cèl·lules inflamatòries, superior a les citocines pro-inflamatòries estudiades.

3. La intensitat de l'expressió de MMP2 i MMP9 no sembla ser la única responsable de la destrucció de la làmina elàstica interna, ja que no existeix correlació entre el grau de destrucció i l'expressió de gelatinases. Probablement la regulació de la seva activitat elastinolítica i potser la contribució d'altres enzims també juguen un paper important.

4. El tractament amb glucocorticoids podria evitar la destrucció proteolítica de l'artèria ja que redueix molt significativament l'expressió de MMPs. D'altra banda aquesta acció podria contribuir a l'evolució de les lesions cap a un estadi cicatricial fibrosant degut a la disminució de la degradació de la matriu extracel·lular.