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Different expression of MMPs/TIMP-1 in human atherosclerotic lesions. Relation to plaque features and vascular bed

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Abstract

Background: Proteolytic imbalance might determine arterial remodeling and plaque destabilization in atherosclerotic vessels. The aim of this study was to examine differences in the patterns of metalloproteinases (MMPs) and MMP inhibitor (TIMP-1) expression in advanced human atheromas, both in relation to the plaque features and the vascular bed involved. *Methods and results:* Immunohistochemistry for MMP-1, -3, -9 and TIMP-1 as well as the collagen content were measured in vascular sections from patients undergoing peripheral revascularization (carotid $n = 11$, femoral $n = 23$) and aorto-coronary bypass surgery (mammary arteries $n = 20$, as controls). Increased expression of all MMPs was detected in atherosclerotic as compared with control sections ($P < 0.01$). Aneurismal plaques showed a significant increase of MMP-1 and-3 and a reduction in total collagen ($P < 0.05$) in relation to occlusive lesions. Calcification areas in atherosclerotic plaques were consistently associated with increased TIMP-1 expression ($P < 0.01$). Finally, MMP-9 expression was higher in occlusive lesions from carotid than femoral arteries ($P < 0.01$). *Conclusions:* Aneurysm lesions expressed higher MMP-1 and-3 expression than occlusive plaques, and MMP-9 was mainly detected in carotid as compared with femoral arteries. TIMP-1 was associated with arterial calcification. These differences in the MMPs/TIMP-1 expression might determine the evolution of advanced atherosclerotic plaques and contribute to its vulnerability.

Keywords: Metalloproteinases; TIMP-1; Atherosclerosis; Calcification; Collagen; Aneurysm

1. Introduction

Atherosclerosis involves multiple processes including endothelial dysfunction, inflammation, vascular proliferation and extracellular matrix (ECM) degradation. Advanced atherosclerotic disease is characterized by rupture, hemorrhage and thrombus formation [1,2]. Converging lines of evidence point to a dynamic regulation of collagen synthesis and breakdown in the atherosclerotic plaque. The net result, dissolution of collagenous matrix in the fibrous cap due to overexpression of active metalloproteinases (MMPs), renders this structure weak, friable and susceptible to rupture when exposed to hemodynamic stress [3]. Moreover, during atherosclerosis, the proteolytic balance shifts towards increased proteolysis causing degradation of ECM contributing to aneurysm formation [4]. MMPs are zinc-dependent endopeptidases that degrade many molecules of the ECM. They can be divided into four major subclasses, generally based on substrate specificity; collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), and membrane-type (MT-MMPs). Immunohistochemistry studies have demonstrated that MMPs are expressed by cells present within atheromas, but not in normal arteries [5,6]. Excessive or inappropriate expression of MMPs is known to contribute to the pathogenesis of cardiovascular diseases (atherosclerosis plaque rupture and aneurysm formation). There is evidence of increased expression of some MMPs, particularly gelatinases and/or stromelysin-1, in abdominal aortic aneurysms [7–10]. MMP activity is controlled by specific inhibitors (TIMPs); four members of the tissue inhibitor family have been identified: TIMP-1, -2, -3, and -4 [11,12]. Their overexpression reduces neointimal development in experimental models of vascular injury

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and may provide protection against plaque destabilization [13,14].

Little is known as to the relative contribution of each MMP and TIMP in determining the evolution of atherosclerotic lesions. We hypothesized that the susceptibility for an atherosclerotic vessel to develop occlusive or aneurismal lesions could be determined by differences in MMPs expression. It is also unknown whether MMPs and/or TIMP-1 are expressed differently in atherosclerotic lesions depending on the affected segment of the arterial tree. Therefore, we examined the expression of MMP-1, -3, -9 and TIMP-1 in advanced human atheroma both in relation to the plaque features and the vascular bed involved.

2. Materials and methods

2.1. Vascular specimens

Vascular tissue samples were obtained from 36 male patients (67.3 ± 1.6 years) undergoing peripheral revascularization procedures (carotid endarterectomy = 11, femoro-popliteal revascularization = 25). Surgical intervention criteria were internal carotid stenosis > 75% and critical limb ischemia due to femoro-popliteal obstruction or femoral aneurysm (diameter > 20 mm). The following cardiovascular risk factors were recorded preoperatively: arterial hypertension (systolic blood pressure > 139 mm Hg and/or diastolic blood pressure > 89 mm Hg and/or use of antihypertensive drugs), dyslipidemia (total cholesterol ≥220 mg/dl and/or HDL cholesterol ≤40 mg/dl and/or LDL cholesterol ≥140 mg/dl and/or use of cholesterol lowering drugs), obesity (body mass index ≥30 kg/m^2), smoking (≥one cigarette a day), and diabetes (fasting glucose ≥126 mg/dl and/or use of oral antidiabetics).

Vascular mammary artery segments ($n = 20$) from patients undergoing aorto-coronary bypass surgery (mean age 62.5 ± 1.9 years) were used as controls.

The study protocol was approved by the Research Ethics Committee of the University Clinic of Navarra, and written informed consent was obtained for all participants.

2.2. Analysis of atherosclerotic lesions

Vascular segments were removed carefully at surgery to preserve the plaque structure. The samples were cut into pieces transversely and immediately fixed in 4% paraformaldehyde for 4 h, before paraffin embedding. The histological sections were stained with hematoxilin–eosin and van Gieson and examined with a microscope (Nikon Optiphot-2, Japan).

All peripheral samples corresponded to advanced atherosclerotic plaques (types V–VIII), based on the AHA histological classification [15]. They were divided into occlusive or aneurismal, attending to surgical and histological criteria as proposed by the Ad Hoc Committee on Reporting Standars [16]. Occlusive arterial disease was defined as stenosis obstruction of the arterial vessel due to atherosclerotic plaque enlargement resulting in impairment of blood flow. Arterial aneurysm was defined as a permanent and localized dilatation with at least 50% increase of the normal vessel diameter.

2.3. Immunohistochemistry for MMPs and TIMP-1

Serial sections were analyzed by immunohistochemistry as previously described [17], using specific monoclonal antibodies anti-MMP-1 (2 μg/ml, Oncogene, USA), anti-MMP-3 (3.3 μg/ml, Oncogene), anti-MMP-9 (1.3 μg/ml, Oncogene) which recognize both active and latent forms and a polyclonal antibody anti-TIMP-1 (20 μg/ml, Biogenesis, UK). All sections were incubated with secondary biotinylated antic-Fc (Dako, Denmark), developed with avidin–biotin-peroxidase complex (ABC, Dako) and 3-3′-diaminobenzidine hydrochloride (Sigma, USA) and counterstained with Harris' hematoxylin.

Monoclonal anti CD-68 (0.2 μg/ml, Dako), and anti-α-actin (0.3 μg/ml, Dako), were used for detection of macrophages and smooth muscle cells (SMC), respectively.

A visual grading scale was used for assessing the extension of MMPs and TIMP-1 immunostaining as previously reported [17]. Three observers characterized sections independently in a blinded fashion, by comparison against a control section using the grading scale: 0 = no positive cells; 1 ≤ 25% of the plaque area with positive cells; 2 ≤ 50%; and 3 ≥ 50%.

2.4. Staining of collagen by picrosirius red

Sirius red staining was used to identify interstitial collagen on serial 3 μm sections as described previously [18]. Vascular sections stained for collagen were analyzed using a polarized filter, which characterized type I (red/yellow) and type III (green) collagen fibrils [19]. Analysis of sirius red collagen staining was performed with a personal computer-based quantitative OPTIMAS 5.2 color image analysis. The percentage of the plaque area stained for collagen types I and III was recorded.

2.5. Statistical analysis

Values are expressed as mean ± S.E.M. Differences in the collagen content and proteolytic balance between aneurysm and occlusive lesions were performed by Mann–Whitney-*U*-test and regression analysis. Kendall's tau–b correlation was used to assess the association between percentage of plaque area stained for collagen with values for MMPs and TIMP-1 semiquantitive scores. Differences in the proteolytic balance between femoral and carotid arteries were studied by Fisher exact test and *t*-student or Mann–Whitney-*U*-test, as appropriate. Statistical signifi-

Table 1
Cardiovascular risk factors in patients undergoing revascularization procedures in relation to atherosclerotic plaque features

	Control arteries ($n=20$)	Advanced atherosclerotic lesions		
		Total ($n=36$)	Occlusive ($n=23$)	Aneurysm ($n=13$)
Age (year)	62.5 ± 1.9	67.3 ± 1.6	67.4 ± 2.0	66.7 ± 2.8
Hypertension (%)	60.9	58.6	57.1	62.5
Dyslipidemia (%)	52.2	71.4	71.4	71.4
Diabetes mellitus (%)	18.2	30.8	40	32.5
Obesity (%)	26.1	25.4	21.1	30.5
Smoking (%)	60.9	75.9	77.3	71.5

cance was indicated by a value of $P<0.05$. All calculations were performed with SPSS 9.0.

3. Results

3.1. Immunohistochemistry for MMPs/TIMP-1 in control and atherosclerotic human arteries

MMPs expression was measured in 36 arteries from patients undergoing revascularization procedures and presenting one or more cardiovascular risk factors. Samples were divided into two groups: carotid endarterectomy ($n=11$) and femoral revascularization ($n=25$). As control, we used mammary arteries ($n=20$) without macroscopic atherosclerotic lesions from patients undergoing aorto-coronary bypass surgery. Both groups were of similar ages and had no differences for cardiovascular risk factors (Table 1). Overall, human advanced atherosclerotic lesions showed overexpression of all MMPs and TIMP-1, which appeared most abundant in lipid- and macrophage-rich atheromatous compared with fibrous (SMC-rich) plaques, and localized mostly in regions prone to rupture and in areas surrounding the lipid core (Fig. 1). Semiquantitative image analysis showed that staining for MMP–1, -3, -9 and TIMP-1 antigen in atheromatous plaques significantly ($P<0.01$) exceeded those in

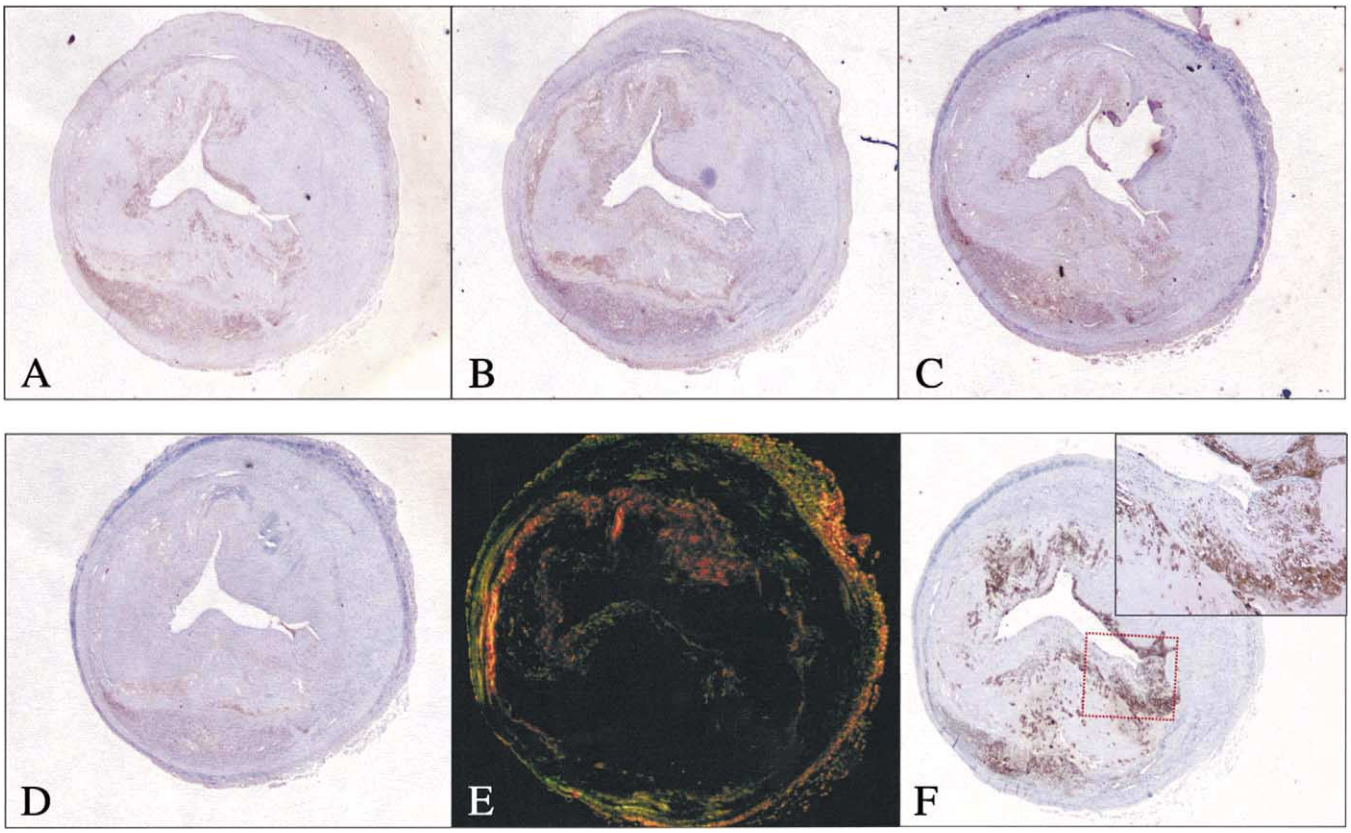

Fig. 1. Immunohistochemical and sirius red staining photographed under polarized light, of human advanced atherosclerotic lesion. Extense positive signal for MMP-1 (panel A), MMP-3 (panel B) and MMP-9 (panel C) expression is shown, in contrast to a weaker TIMP-1 expression (panel D). Total collagen was markedly reduced in areas stained for MMPs (Panel E). MMPs were mainly expressed in areas showing intense positivity for macrophage (CD-68) staining (Panel F). Magnified detail of panel F shows a local accumulation of macrophages that could predispose to plaque rupture.

Table 2
Expression of individual MMPs and TIMP-1 in relation to atherosclerotic plaque features

	Control arteries ($n=20$)					Advanced atherosclerotic lesions ($n=36$)				
Score	0	1	2	3	Median	0	1	2	3	Median
MMP-1	9	5	6	0	1	2	9	13	12	2**
MMP-3	8	12	0	0	1	4	13	15	4	2**
MMP-9	20	0	0	0	0	14	9	7	6	1**
TIMP-1	20	0	0	0	0	11	10	7	8	1**

The median values for the semiquantitative immunohistochemical score are shown. ** $P<0.01$ as compared with control arteries.

control arteries, which showed a weak immunoreactivity for MMP-1 and MMP-3, whereas no positive signal for MMP-9 and TIMP-1 was detected (Table 2).

A great variability in the collagen content was observed in atherosclerotic plaques, with the stained area in advanced plaques being 64.7 ± 3.8% of the total area (range 10.6–97.3%). As expected, the total collagen content negatively correlated with MMP-1 expression ($r=-0.3$, $P<0.05$). In general, the percentage of red fibers corresponding to type I collagen exceeded the content of green fibers corresponding to type III collagen (40.0 ± 4.0 vs. 25.0 ± 3.3%, $P<0.01$). The percentage of collagen type III negatively correlated with MMP-1, -3, and -9 ($r=-0.2$, $r=-0.3$ and $r=-0.5$, respectively, $P<0.02$), whereas no correlation was observed for type I collagen.

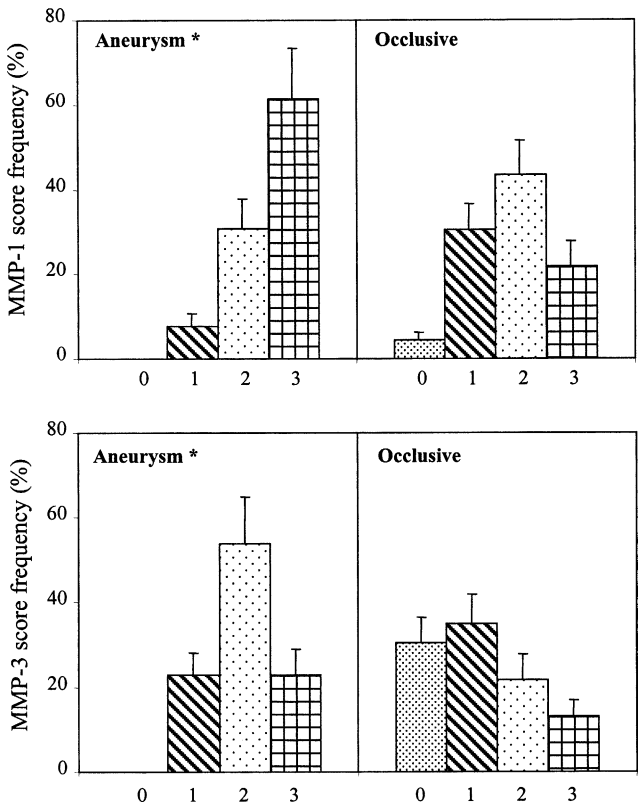

Fig. 2. Percentage of scored values for MMP-1 and MMP-3 in aneurysm ($n=13$) and occlusive ($n=23$) lesions. *, $P<0.05$ as compared with occlusive lesions.

3.2. Expression of MMPs/TIMP-1 in aneurysm compared with occlusive atherosclerotic disease

Human atherosclerotic plaques were separated by conventional morphological characteristics into occlusive ($n=23$, 63.9%) and aneurismal ($n=13$, 36.1%). A significant increase of MMP-1 and MMP-3 could be demonstrated in arterial aneurysms in relation to occlusive lesions ($P<0.05$); however, no differences in the expression of MMP-9 and TIMP-1 were observed (Fig. 2). A significant reduction in total collagen content was also found in aneurysm as compared with occlusive plaques (53.2 ± 6.8 vs. 70.1 ± 4.2%, $P<0.05$). The observed reduction was mainly due to a decrease in type I fibers (31.3 ± 5.9. vs. 44.1 ± 5.1%), in contrast with the practically unaltered content in type III collagen (22.0 ± 4.0 vs. 26.4 ± 4.5%) (Fig. 3).

Interestingly, in both aneurysm and occlusive lesions, overexpression of TIMP-1 was consistently associated with calcification areas in the atherosclerotic plaque (Fig. 4). The observed increase in TIMP-1 expression correlated significantly with the presence of type I collagen ($r=0.6$, $P<0.01$) under polarized light, thereby increasing the typeI/typeIII collagen ratio when compared with noncalcified lesions (5.9 ± 1.7 vs. 1.5 ± 0.3, $P<0.02$).

3.3. Expression of MMPs/TIMP-1 in relation to the vascular bed

Atherosclerotic plaques were classified according to vascular bed and morphological characteristics. As expected, femoral aneurysms ($n=13$) showed higher MMP-1 ($P<0.01$) and MMP-3 ($P<0.01$) expression than femoral occlusive lesions ($n=12$). Said analysis could not be performed on carotid arteries, since they were all occlusive.

Comparisons between occlusive lesions in relation to the vascular bed involved, either carotid ($n=11$) or femoral ($n=12$), were performed. Significant changes were observed for the MMP-9 expression in different sections of the arterial tree ($P<0.01$). Occlusive carotid lesions had significantly higher MMP-9 expression than similar lesions in the femoral arteries (Fig. 5). However, no differences in the total collagen content nor in other MMPs and TIMP-1 expression could be demonstrated between similar lesions in relation to the vascular bed.

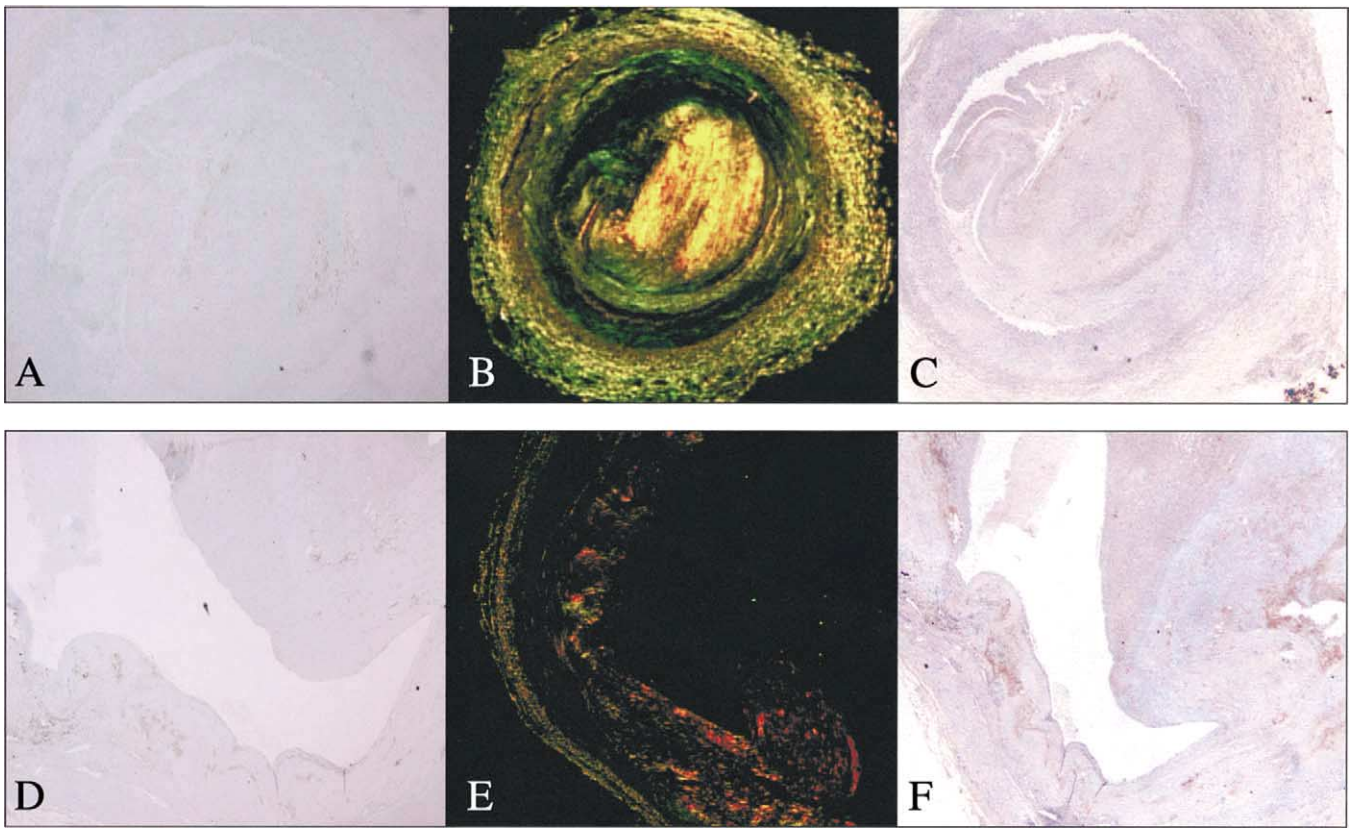

Fig. 3. Immunohistochemical and sirius red staining photographed under polarized light, of occlusive (panels A–C) and aneurysm (panels D–F) lesions. Weak positive signal for MMP-1 (panel A) and MMP-3 (panel C) expression is shown in occlusive lesion, in contrast to extense MMP-1 (panel D) and MMP-3 (panel F) expression in aneurysms. Total collagen was markedly reduced in aneurysm (Panel E) as compared with occlusive lesion (panel B).

4. Discussion

The MMPs family of enzymes contributes to both normal and pathological tissue remodeling. Inappropriate remodeling underlies the pathogenesis of advanced atherosclerotic plaques, the substrate for acute cardiovascular syndromes, including acute myocardial infarction, stroke and peripheral arterial disease [20]. Our data demonstrate the differences in the expression of one or more MMPs might influence the type of lesion, therefore, determining the final evolution of the advanced atherosclerotic plaque. We also show the regional differences in the vascular bed involved might account for a different proteolytic pattern in the atherosclerotic lesion.

As compared with control arteries, advanced vascular lesions showed overexpression of MMP-1, -3, -9 and TIMP-1 throughout the atherosclerotic plaque, predominantly within the shoulder region, colocalizing with the presence of macrophage infiltrating cells, a characteristic feature of unstable atherosclerotic plaques [5,21]. Collagen is the major component of the ECM of the vessel wall and comprises up to 60% of total protein. The collagen fibers being responsible for tensile strength and elastic resiliance are mainly composed of types I and III collagen. We found that overexpression of MMPs in advanced lesions was associated with a reduced proportion of type III collagen, most likely weakening the plaque structure [22]. These data are consistent with a growing body of evidence showing that MMPs expression is restricted to specific plaque regions, influencing plaque development, composition and stability [5,21,23].

However, higher MMPs/TIMP-1 expression was observed in aneurysm as compared with occlusive vascular sections, MMP-1 and-3 being significantly increased in aneurysm atherosclerotic lesions. This proteolytic pattern could be responsible for the expansive remodeling characteristic of aneurysm lesions, rather than for determining an occlusive atherosclerotic plaque. The observed differences could not be attributed to a particular cardiovascular risk factor because the presence of dyslipidemia, arterial hypertension, diabetes and smoking, were similar between both groups. There are conflicting reports on the expression of MMPs in relation to aneurysms [7–10], but in general, the majority of the studies have reported the involvement of at least MMP-2 and MMP-9 in abdominal aortic aneurysms [24]. A novel finding of our study was an increased expression of MMP-1 and-3, but not MMP-9 in femoral aneurysms, suggesting that regional differences in the proteolytic mechanisms may be operating in different arterial beds. Whether MMP-1 and-3 are causally related to the presence of aneurysm in

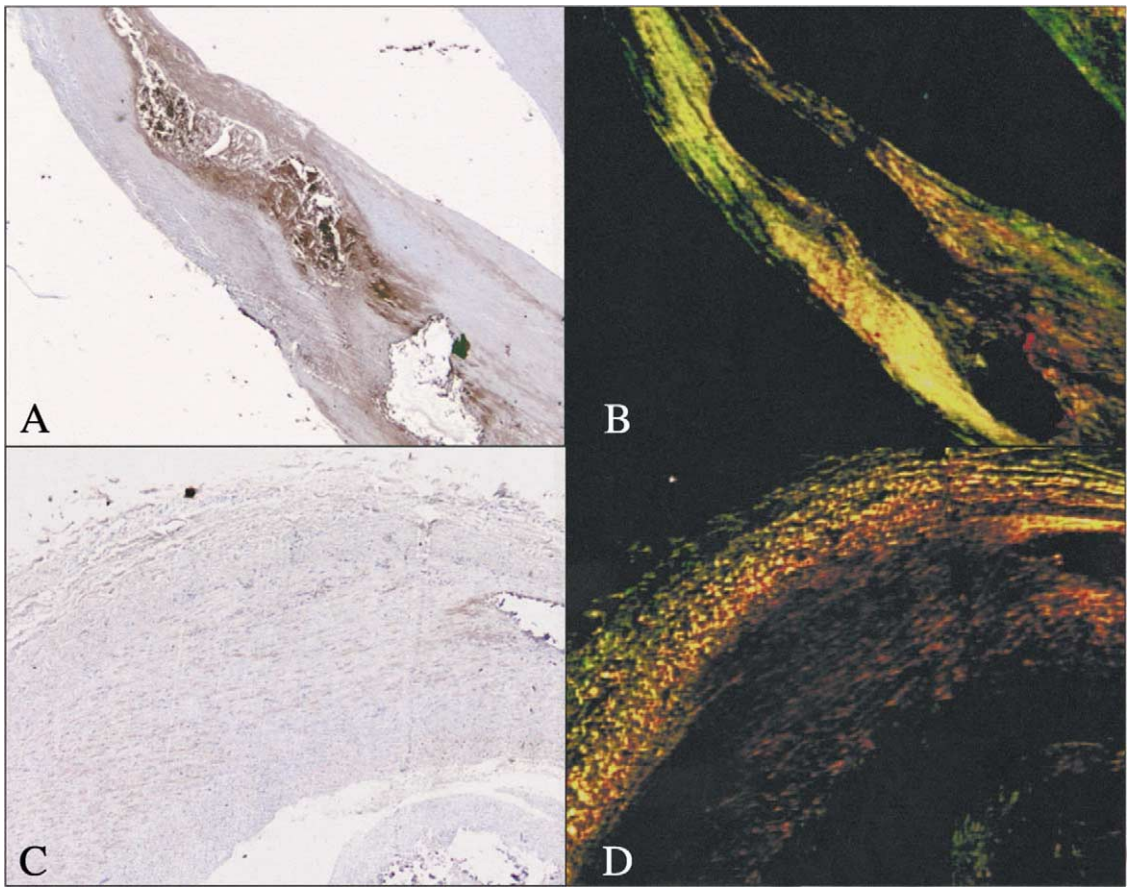

Fig. 4. Immunostaining for TIMP-1 (panels A, C) and collagen (panels B, D) in calcified lesions from carotid (panels A, B) and femoral (panels C, D) arteries. Extensive positive signal for TIMP-1 was associated with calcification areas, both in carotid (panel A) and femoral (panel C) arteries, associated with type I collagen (panels B, D).

the femoral territory is, at present, unknown. MMP-1 has been related to cardiac hypertrophy, dilation and systolic dysfunction in the hearts of transgenic mice [25]. On the other hand, MMP-3 would contribute to plaque destabilization by degrading ECM components, and could also promote aneurysm formation by degrading the elastic lamina [9]. These effects may be directly mediated by MMP-3 or by activation of other proMMPs [7].

Interestingly, for the first time, we show that TIMP-1 expression was mainly increased in fibrocalcific lesions, suggesting a possible role of TIMP-1 in arterial calcification. The degree of vascular calcification has been shown to correlate with plaque burden. However, it is uncertain whether calcification is a marker for plaque instability [26]. Recent studies showing that pharmacological inhibition of MMPs attenuates ventricular dilation, increases collagen content, and stabilizes atherosclerotic plaque, lead us to speculate that TIMP-1, a natural inhibitor of MMPs, could well provide protection against plaque instability [13,27,28]. The observed correlation between TIMP-1 and increased type I collagen would also support a role for this inhibitor in the maintenance of vascular integrity.

As compared with femoral arteries, carotid occlusive atherosclerotic lesions showed higher MMP-9 expression. There are no studies directly assessing the expression of different MMPs and TIMPs in separated regions of the arterial tree. Blood vessels have long been known to have many organ-specific properties [29], which might partially explain the regional differences in atherosclerotic lesions. The diversity of cell functions may be important in physiology and pathophysiology, allowing different responses within or between vascular beds [30]. As to whether high MMP-9 (gelatinase B) expression could determine a different evolution of the atherosclerotic lesion in the carotid territory in relation to other peripheral atherosclerotic-prone sites is unknown at present. Despite this different proteolytic pattern regarding the vascular beds analyzed, the total plaque collagen content was not changed significantly. It can be argued that the main substrates for MMP-9, gelatin and collagen types IV and V, are not directly detected by sirius red staining. Moreover, most of carotid arteries (ten out of 11) showed calcification areas and, therefore, also overexpression of TIMP-1. Therefore, since overall proteolytic activity depends on the relative concentration of the active enzymes and their inhibitors [12], we could speculate

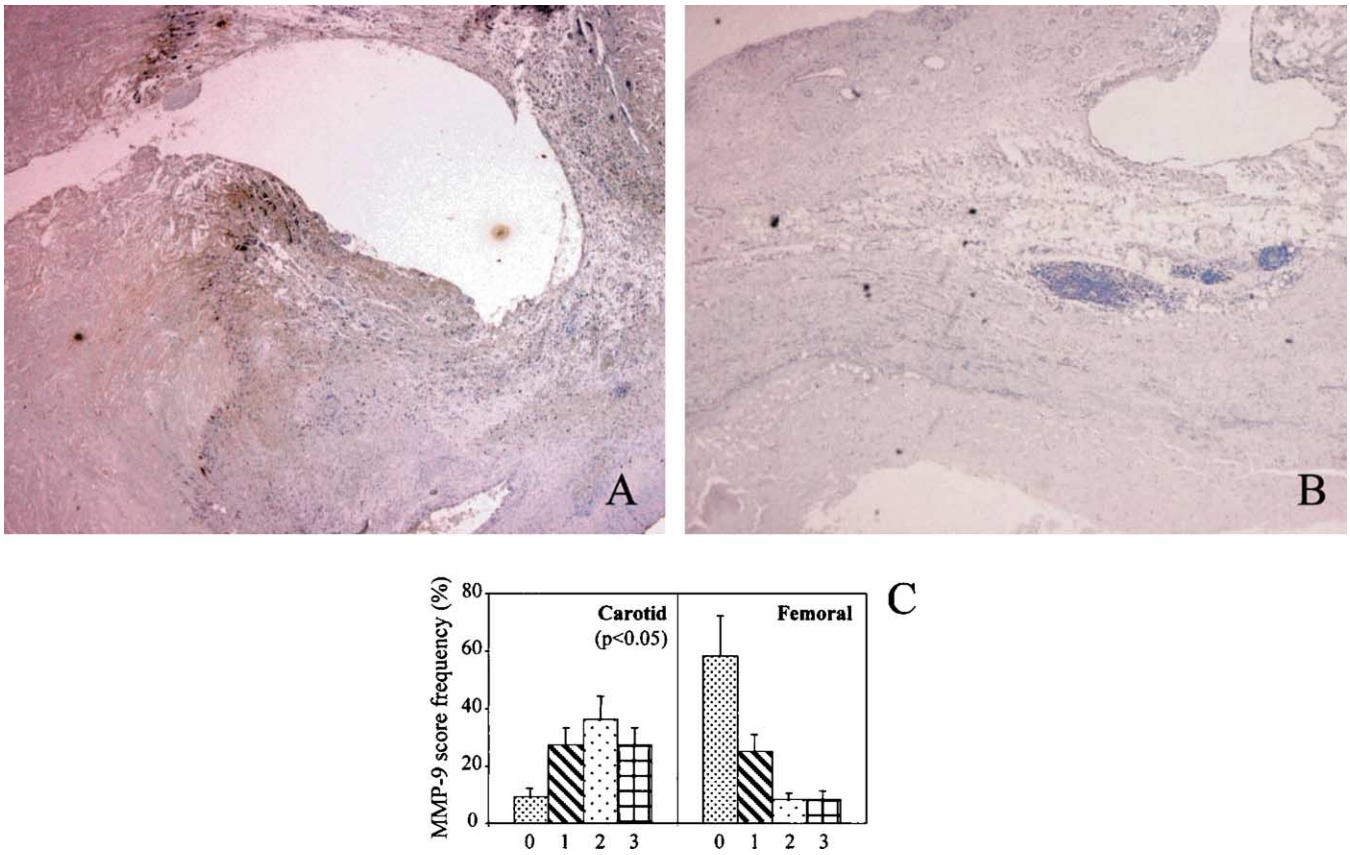

Fig. 5. Immunostaining for MMP-9 in carotid (panel A) and femoral (panel B) arteries with occlusive lesions. Panel C shows the percentage for the MMP-9 semiquantitative score comparing carotid and femoral arteries. *, $P<0.05$ as compared with femoral occlusive lesions.

that the upregulation of TIMP-1 in carotid plaques would counterbalance the process of collagen digestion.

Our study has some limitations. Other MMPs are also playing a role in arterial advanced atherosclerotic plaques. In immunohistochemical staining, the presence of MMPs assessed by immunohistochemistry does not imply increased catalytic capacity [31]. In addition, matrix catabolism in the cardiovascular system involves enzymes other than the MMP family, like catepsins and cystatins. Finally, a causal relationship between matrix degrading action and plaque rupture has yet to be demonstrated.

In conclusion, we show that a different vascular proteolytic pattern is present in occlusive as compared with aneurysm atherosclerotic disease. We also showed differences in the vascular proteolytic pattern in relation to the vascular bed involved. Further studies are warranted to assess whether the observed differences might determine the evolution of advanced human atherosclerotic plaques and contribute to the vulnerability of atherosclerotic lesions.

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