Original Article

Expression of Matrix Metalloproteinases and Endogenous Inhibitors in Abdominal Aortic Aneurysm and Aortoiliac Occlusive Disease (Syndrome Leriche)

(abdominal aortic aneurysm / aortoiliac occlusive disease / matrix metalloproteinases / syndrome Leriche)

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Abstract. Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play a complex role in the pathogenesis of atherosclerosis. We compared (1) the histopathological findings in patients with abdominal aortic aneurysms (AAA) and aortoiliac occlusive disease (AOD); (2) the expression of MMP-2/MMP-9 and TIMP-1/TIMP-2 in aortic layers, inflammatory cells and smooth muscle cells (SMCs), aiming to identify the common underlying pathogenic mechanisms of the disease development. Samples were obtained from 30 patients with AAA and 30 with AOD. Aortic histology and immunohistochemistry were performed to evaluate inflammatory changes and MMP and TIMP expression. Thrombosis and ulceration were more frequent in AOD than in AAA. The MMP-9 expression was elevated in all aortic layers of AAA patients and in media/adventitia of AOD patients, mainly followed by lower expression of its inhibitor TIMP-1. Higher MMP-9 expression was also found in SMCs and macrophages of both AAA and AOD specimens, while higher TIMP-1/TIMP-2 were predominantly observed in the lymphocytes and macrophages of the aneurysm. These results showed that both conditions exhibited increased MMP-9 expression; however, the MMP expression pattern differed to some degree between the aneurysms and occlusive disease. The variations in molecular mechanisms underlying dilatative/stenosing disease warrant further investigation.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are implicated in a variety of physiological processes such as embryonic development, morphogenesis, apoptosis and tissue remodelling. Based on the substrate specificity, MMPs are divided into subclasses such as gelatinases, elastases, and collagenases. They are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs). The imbalance between MMPs and their TIMPs has been shown to contribute to different pathologies such as cancer, rheumatoid arthritis and aneurysm formation (Aziz and Kuivaniemi, 2007). MMP-2 and MMP-9 degrade denatured fibrillar collagen (gelatin), elastin, and native IV, V, and VII collagen along with other extracellular matrix (ECM) components. MMP-2 is constitutively expressed in SMCs and fibroblasts of the aortic media, and MMP-9 may be produced by inflammatory cells such as neutrophils and macrophages (Ruddy et al., 2008). The importance of MMP-9 in aneurysm development was further supported in a murine model, where aneurysm induction was inhibited in MMP-9 knockout mice (Longo et al., 2002).

Aneurysm is defined as a permanent localized dilation of an artery whose diameter is at least 50 % greater

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Abbreviations: AAA – abdominal aortic aneurysms, AOD – aortoiliac occlusive disease, ECM – extracellular matrix, HE – haematoxylin-eosin, HP – histopathological, MMPs – matrix metalloproteinases, SMCs – smooth muscle cells, TIMPs – tissue inhibitors of metalloproteinases.

than the expected normal size. The vessel dilatation and weakening is caused by lysis of ECM, primarily of elastin and collagen, in the aortic media and adventitia (Keeling et al., 2005). The majority of aortic aneurysms are localized in the abdominal aorta (Abraha et al., 2016). Aneurysms lead to death due to complications of aneurysm expansion and rupture (Keisler and Carter, 2015).

Aortoiliac occlusive disease (AOD), also referred to as Leriche syndrome, is the triad of claudication, impotence and absent femoral pulses due to thrombotic occlusion of the abdominal aorta just above the site of its bifurcation (Leriche and Morel, 1943). The complications in AOD include clot formation, development of acute ischaemia, and in severe disease it may result in distal limb amputation in cases of systemic disease (Wooten et al., 2014).

Studies show that abdominal aortic aneurysms (AAA) and AOD are related to underlying atherosclerotic disease (Golledge and Norman, 2010; Clair and Beach, 2015). However, it is still unclear whether atherosclerosis precedes the AAA. As indicated in a recent study by Peshkova et al. (2016), several theories exist regarding the AAA aetiology. One is that atherosclerosis-driven changes in the aortic wall underlie AAA pathology, and the inflammatory mechanisms promoting atherosclerosis contribute to AAA. The second possibility is that different risk factors promote AAA and atherosclerosis separately, and they thus develop independently without greatly influencing each other. The third possibility is that the same risk factors promote both AAA and atherosclerosis via similar mechanisms, although additional factors are required for AAA induction. Thus, it is still unclear whether atherosclerosis can cause AAA development, and whether inflammatory infiltrates and mediators represent a common denominator for atherosclerosis and AAA development (Peshkova et al., 2016). Based on the premise that atherosclerosis stimulates AAA development, all patients with AAA are still considered to have significant atherosclerosis and should be considered for indicated therapy, as advised by AHA guidelines, in which AAA is considered an atherosclerotic equivalent (Golledge and Norman, 2010; Gerhard-Herman et al., 2016).

In atherosclerosis, MMPs play a role in neointima formation and SMC migration after vascular injury. Atherosclerotic human vessels display increased levels of the members of the gelatinase subclass, MMP-2 and MMP-9, as compared with healthy ones and destruction of elastic media was shown to depend on the increased activity of elastolitic matrix metalloproteinases (MMPs) in the aortic wall of AAA and AOD (Azevedo et al., 2014).

Thus, the objective of this study was to compare the histopathological (HP) findings in patients with AAA and AOD: immunohistochemical expression of MMP-2/MMP-9 and their respective inhibitors, TIMP-2/TIMP-1, in all aortic layers, inflammatory cells (neutrophils and macrophages) and SMCs, aiming to identify the common underlying pathogenic mechanisms and to get further insight into the biological processes of the disease development.

Material and Methods

Patients

The study included 60 patients who underwent surgery for AAA (30 cases) and AOD (30 cases) at the Department of Surgery, University Clinical Centre of the Republic of Srpska. The study was approved by the Ethics Committee of the Clinical Centre and the protocol was consistent with the World Medical Association Declaration of Helsinki. Patients with AAA and AOD operated as urgent surgical cases and those with previous surgery as well as patients with aneurysms at other localization were excluded from study. Clinical data were assessed by reviewing each patient's medical history (Table 1). AAA and AOD were more frequent in males than females. Hypertension was present in almost all patients with AAA and AOD. The size of AAA was measured by angiography and it ranged from 3.5 to 7.5 cm (median 5.47 cm).

Histological specimens

Infrarenal aortic wall specimens were taken during the surgery. The samples were immediately fixed in formaldehyde in the operating theatre, processed for histology, and sections were cut and stained by the routine haematoxylin-eosin (HE) method and analysed by light microscopy to examine the following parameters: cholesterol crystals, calcification, inflammatory infiltrate (0 – negative, 1 – slight, 2 – moderate, and 3 – severe), extracellular lipids, thrombosis and ulceration (0 – no, 1 – yes).

Immunohistochemistry

Immunohistochemical analysis of MMP-2, MMP-9, TIMP-1, and TIMP-2 was performed in 5 μm sections prepared from paraffin-embedded human AAA walls and abdominal aorta with AOD. Specimens were processed as follows: for retrieval of antigens, a high-temperature technique was used (histological slides were placed in 10 mmol/l citrate buffer, pH 6.0, and heated at

Table 1. Clinical data of the patients with AAA and AOD

Group	Number of patients	Age (years) Min/Max/Med	Sex (F/M)	Chronical course Asymptomatic/ Symptomatic	Diabetes Yes/No	Hypertension Yes/No
AAA	30	46/80/67.77	4/26	15/15	4/26	25/5
AOD	30	49/82/65.80	10/20	0/30	4/26	24/6

96 °C for 20 min in a PT module). Primary monoclonal antibodies for MMP-2 (mouse monoclonal antihuman MMP-2 antibody, A-Gel VC2), TIMP-1 (mouse monoclonal antihuman TIMP-1 antibody, 102D1), TIMP-2 (mouse monoclonal antihuman TIMP-2 antibody, 3A4), and rabbit polyclonal antihuman MMP-9 antibody for MMP-9 (all by Lab Vision-USA, Fermont, CA) were used. For MMP-9, TIMP-1 and TIMP-2, pre-diluted (ready-to-use) antibodies were used, while anti-MMP-2 antibody was used at 1 : 100 dilution. All slides were incubated in the primary antibody overnight at 4 °C, followed by incubation with secondary antibodies (UltraVision LP Detection System: HRP polymer/DAB Plus Chromogen; LabVision-USA) for 25 min at room temperature. The binding reaction was detected using 3,3'- diaminobenzidine.

The results were semi-quantitatively evaluated by observing the presence of yellowish-brown particles within the cytoplasm. For the assessment of stained cells (lymphocytes, macrophages and SMCs), 100 randomly chosen cells were observed for staining. The results of immunohistochemical staining were scored as 0 (< 10 % of the cells), 1 (11 %–25 % of the cells), 2 (26 %– 50% of the cells) and 3, when > 50 % of the cells were stained. For analyses, the cases with scores 2 and 3 were considered to have elevated MMP and TIMP expression in the observed cells. The expression of MMPs and their tissue inhibitors was graded separately for the intima, media and adventitia, as well as for the inflammatory cells (lymphocytes and macrophages) and SMCs.

Statistics

Statistical analyses were performed with SPSS 23.0 for Windows (SPSS Inc., Chicago, IL). To compare the frequency score distribution for HP findings, Fisher's exact test for 2 × 2 contingency tables or χ^2 test for 4 × 4 tables were used. To compare the MMP/TIMP expression scores in aortic layers, the rank-based nonparametric Kruskal-Wallis H test (H is tested as a χ^2 variable), appropriate for non-normal distributed data sets, followed by Dunn's multiple comparison test was used. The differences in the expression score frequencies between SMCs and each inflammatory cell type separately were tested by the χ^2 test. The P values < 0.05 were considered significant.

Results

Histopathology

The semi-quantitative grading in AAA and AOD specimens (Table 2) showed that thrombosis and ulceration were more frequent in AOD than in AAA (P = 0.007 and P = 0.03, respectively), while accumulation of lipids in foam cells was not statistically different (P = 0.612) (Fisher's exact test). There was no significant difference in accumulated cholesterol crystals (P = 0.170), calcification (P = 0.189), or degree of inflammatory infiltrate (P = 0.635) (χ^2 test) between AAA and AOD specimens.

MMP and TIMP expression in AAA

Table 3 and Fig. 1 present data on the scores of MMP and TIMP expression in the aortic layers, inflammatory cells and SMCs in the specimens from AAA patients. A significant difference in the expression scores of the examined parameters was found in each aortic layer (media, H = 17.03, P = 0.0007; intima, H = 19.61, P = 0.0002; adventitia, H = 25.54, P < 0.0001, Kruskal-Wallis test).

Dunn's post hoc test revealed a significant difference (P < 0.05) in the expression scores between MMP-2 and MMP-9 in the media, intima and adventitia. An elevated MMP-9 level was observed in 46.67 % (media), 63.33 % (intima) and 50 % (adventitia) of patients. There was also a significant difference in MMP-9 vs. TIMP-1 expression in the media and adventitia (P < 0.05). Negative and low TIMP-1 expression was observed in 80 % (media) and 93.33 % (adventitia) of patients (Table 3A).

Analysis of the expression scores between SMCs and inflammatory cells (χ^2 test) revealed a significant difference in the MMP-9 (P = 0.008), TIMP-1 (P = 0.0145) and TIMP-2 (P < 0.0001) level between SMCs/lymphocytes; and in the TIMP-1 (P < 0.0001) and TIMP-2 (P = 0.0003) level between SMCs/macrophages.

Elevated MMP-9 expression was noticed in 60 % of SMCs (18 patients) and in 66.67 % of macrophages (20 patients), while 30 % of lymphocytes had high MMP-9 expression. Enhanced expression of TIMPs was observed in 50 % and 86.6 % (TIMP-1), and in 73.3 % and 60 % (TIMP-2) of lymphocytes and macrophages, respectively, Table 3B.

Table 2. Histopathological analysis in AAA and AOD

	Cholesterol	Calcification	Inflammatory infiltrate	Lipids	Thrombosis	Ulceration
Groups/Scores	0 1 2 3	0 1 2 3	0 1 2 3	0 1	0 1	0 1
AAA (N)	4 12 2 12	1 8 12 11	1 18 9 2	29 1	29 1	29 1
AOD (N)	7 13 5 5	1 11 4 14	2 21 5 2	27 3	13 17	11 19
χ^2 P value	0.170	0.189	0. 635	0.612	0.007	0.030

Haematoxylin-eosin staining score: cholesterol crystals, calcification, inflammatory infiltrate (0 – negative, 1 – slight, 2 – moderate, 3 – severe); extracellular lipids, thrombosis, ulceration (0 – no, 1 – yes); (N) – number of patients

Table 3. Immunohistochemical staining scores of MMPs and TIMPs in AAA patients

A)

	MMP-2				MMP-9				TIMP-1				TIMP-2				P value (Kruskal-Wallis)
Groups/Scores	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	
media (N)	14	15	1	0	4	12	8	6*	14	10	3	3#	10	15	3	2	0.0007
intima (N)	4	12	12	2	3	8	4	15*	1	1	11	17	1	10	11	8	0.0002
adventitia (N)	18	10	2	0	5	10	6	9*	16	12	2	0#	14	14	2	0	< 0.0001

Dunns' test ($P < 0.05$): * MMP-2 vs. MMP-9; # MMP-9 vs. TIMP-1

B)

SMCs (N)	10	14	5	1	2	10	5	13	10	14	6	0	9	16	5	0	
lymphocytes (N)	10	14	6	0	4	16	10	0	9	6	8	7	1	7	15	7	
χ^2 P value	0.7700				0.0008				0.0145				< 0.0001				
macrophages (N)	7	10	11	2	1	9	7	13	0	4	13	13	0	12	11	7	
χ^2 P value	0.2860				0.8687				< 0.0001				0.0003				

χ^2 P value (χ^2 test): SMCs vs. lymphocytes; SMCs vs. macrophages
Expression scores: 0 (< 10% of the cells), 1 (11 %–25 % of the cells), 2 (26 %–50 % of the cells), 3 (> 50% of the cells); (N) – number of patients

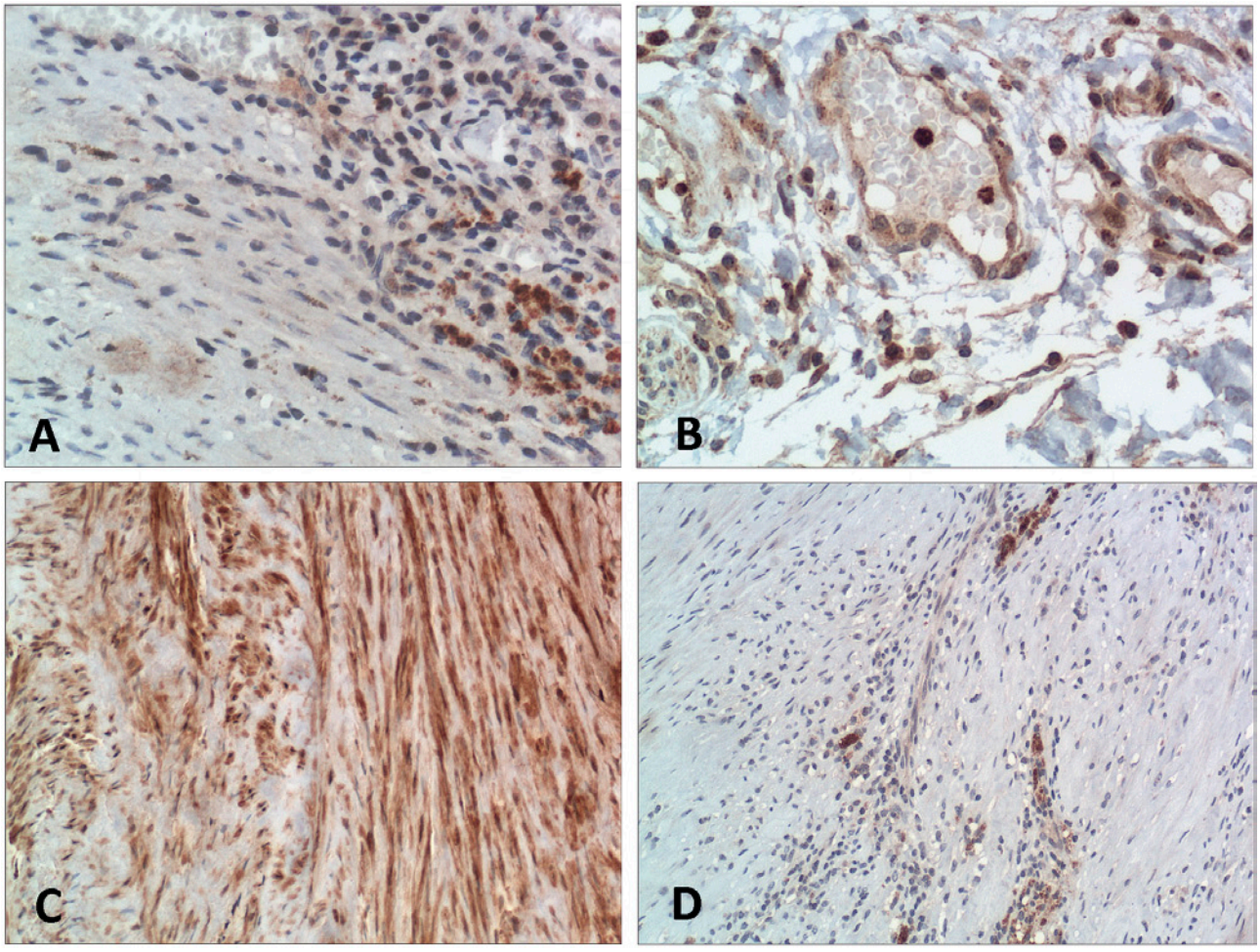

Fig. 1. Expression of MMPs and TIMPs in AAA samples. **A**) immunostaining of MMP-2 expression in inflammatory cells and SMCs (original magnification ×20), **B**) immunostaining of MMP-9 expression in inflammatory cells (original magnification ×20), **C**) immunostaining of MMP-9 expression in SMCs (original magnification ×10), **D**) immunostaining of TIMP-1 expression in inflammatory cells and SMCs (original magnification ×10)

Table 4. Immunohistochemical staining scores of MMPs and TIMPs in AOD patients

A)

	MMP-2				MMP-9				TIMP-1				TIMP-2				P value (Kruskal-Wallis)
Groups/scores	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	
media (N)	16	9	4	1	5	10	4	11*	6	18	5	1#	11	16	3	0	0.0004
intima (N)	10	8	12	0	8	6	10	6	2	6	14	8	2	12	12	4	0.0077
adventitia (N)	21	8	1	0	10	10	4	6*	24	5	0	1	18	12	0	0	0.0002

Dunns' test ($p < 0.05$): * MMP-2 vs. MMP-9; # MMP-9 vs. TIMP-1

B)

SMCs (N)	15	10	4	1	4	11	2	13	7	20	3	0	12	18	0	0	
lymphocytes (N)	17	10	3	0	8	10	7	5	11	14	4	1	6	18	5	1	
χ^2 P-value	0.7368				0.0520				0.3779				0.0460				
macrophages (N)	10	11	9	0	6	8	7	9	2	11	11	6	3	11	13	3	
χ^2 P-value	0.3971				0.2234				0.0012				0.0001				

χ^2 P-value (χ^2 test): SMCs vs. lymphocytes; SMCs vs. macrophages
Expression scores: 0 (< 10% of the cells), 1 (11 %–25 % of the cells), 2 (26 %–50 % of the cells), 3 (> 50 % of the cells); (N) – number of patients

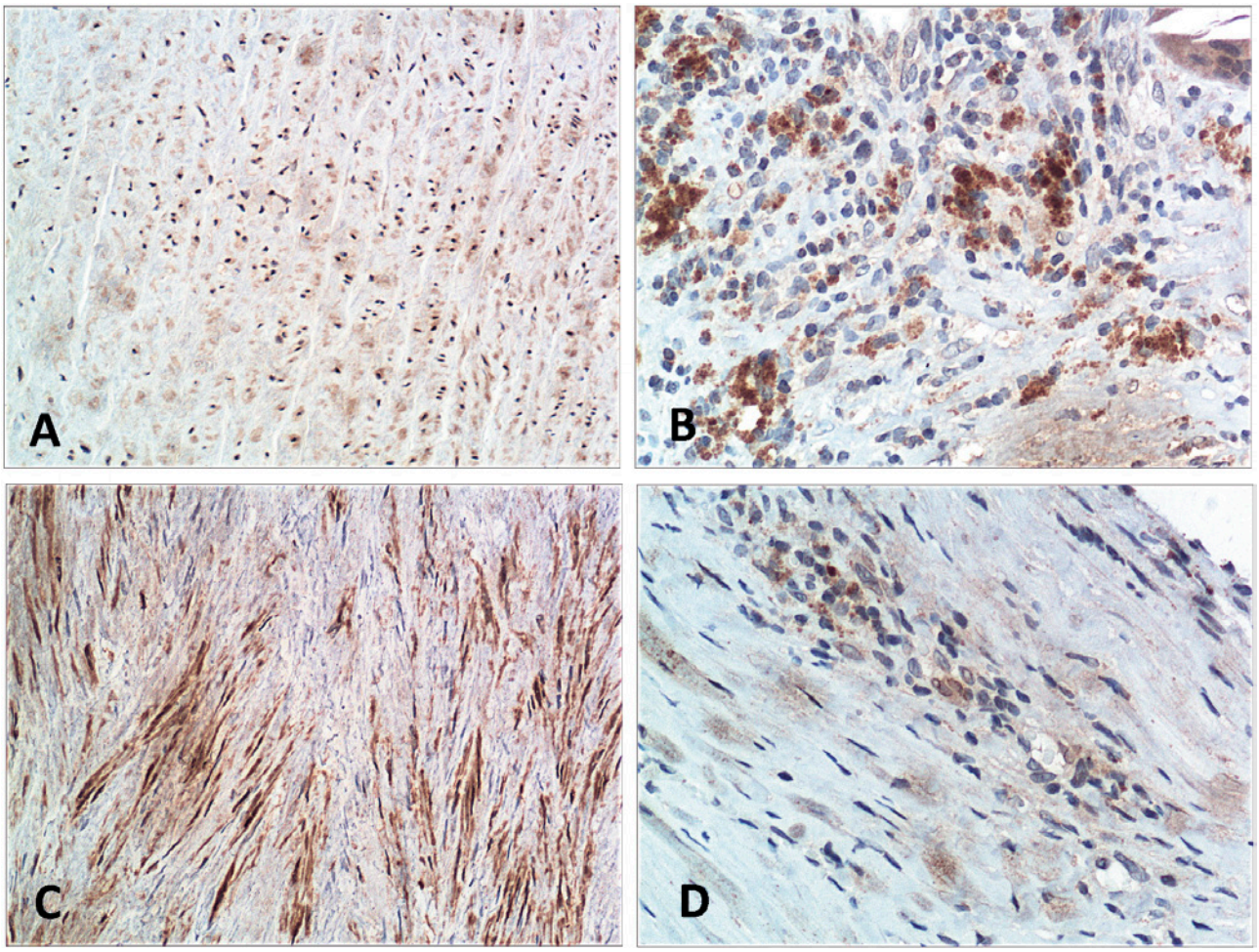

Fig. 2. Expression of MMPs and TIMPs in AOD samples. **A**) immunostaining of MMP-2 expression in inflammatory cells (original magnification ×10), **B**) immunostaining of MMP-9 expression in inflammatory cells (original magnification ×10), **C**) immunostaining of MMP-9 expression in SMCs (original magnification ×10), **D**) immunostaining of TIMP-1 expression in inflammatory cells and SMCs (original magnification ×20)

MMP and TIMP expression in AOD

Table 4 and Fig. 2 present data on the scores of MMP and TIMP expression in the aortic layers, inflammatory cells and SMCs in the specimens from AOD patients. As in AAA patients, a significant difference in the expression scores was found in each aortic layer (media, $H = 18.25$, $P = 0.0004$; intima, $H = 11.91$, $P = 0.0077$; adventitia, $H = 19.92$, $P = 0.0002$, Kruskal-Wallis test).

Dunn's post hoc test revealed a difference in the expression scores between MMP-2 and MMP-9 in the media and adventitia ($P < 0.05$). Higher MMP-9 expression scores were found in 50 % (15 patients) and 53.33 % (16 patients) in the media and adventitia, respectively. There was also a difference in MMP-9 vs. TIMP-1 expression ($P < 0.05$) in the media, in which negative and low TIMP-1 expression was found in 80 % of patients (Table 4A).

Analysis of the expression scores between SMCs and inflammatory cells (χ^2 test) showed a borderline significant difference in MMP-9 ($P = 0.052$) and TIMP-2 ($P = 0.046$) between SMCs/lymphocytes; and in TIMP-1 ($P = 0.0012$) and TIMP-2 ($P = 0.0001$) between SMCs/macrophages.

Elevated MMP-9 expression was noticed in 50 % of SMCs (15 patients) and in 53.33 % of macrophages (16 patients), while 40 % of lymphocytes had high MMP-9 expression. Enhanced expression of TIMP-1 and TIMP-2 was observed in macrophages of 56.66 % and 53.33 % patients, respectively. Only 20 % of patients had elevated TIMP-2 expression in lymphocytes, Table 4B.

Discussion

Arterial remodelling is now being recognized as an important determinant in vascular pathology. Arterial restructuring requires breakdown of the extracellular matrix, which is supported by non-specific up-regulation of the MMPs (Pasterkamp et al., 2000). Of the MMPs, MMP-2 and MMP-9 are considered to be central to the vascular remodelling processes (Chase and Newby, 2003). In this study, we compared the expression of MMP-2, MMP-9 and their inhibitors TIMP-2 and TIMP-1, respectively, in the aortic wall, SMCs and inflammatory cells of patients with dilatative and occlusive pathology. All specimens taken from AAA and AOD patients had various advanced atherosclerotic lesions, and the findings point to certain similarities in MMP and TIMP expression in both pathologic conditions.

In the analysis of MMP/TIMP expression in aortic layers, we noticed higher MMP-9 expression in the whole aortic wall of AAA patients, while in AOD subjects, the MMP-9 expression was elevated in the media and adventitia. Previously, similar quantities of MMP-9 were reported in both aneurysmal and occlusive aortic tissue (Crowther et al., 2000). The elevation of MMP-9 expression was mainly followed by lower TIMP-1 expression scores. Different MMP and TIMP expression in the aortic wall layers observed in this study is in accordance with previous data (Petersen et al., 2000; Liapis and Paraskevas, 2003).

However, we noticed lower MMP-2 expression in the aortic layers in both diagnoses when compared to MMP-9. It is known that MMP-2 is synthesized by the SMCs of the intima and media wall as well as by adventitial fibroblasts (Newby, 2005). MMP-2 derived from mesenchymal lineage cells may play a prominent role in the initiation of ECM changes that cause aneurysmal dilatation. Evidence suggests that MMP-2 was the dominant elastolytic enzyme in the wall of small early aneurysms, with MMP-9 becoming most prominent as the inflammatory infiltrate increased in density. It was demonstrated that AAAs smaller than 5.5 cm had greater MMP-2 activity than larger aneurysms, indicating that early AAA growth is directed by MMP-2 (Freestone et al., 1995; Saratzis and Bown, 2014). Our results of higher MMP-9 and lower MMP-2 expression may also be in accordance with these findings, since our specimens consisted of AAA that ranged from 3.5 to 7.5 cm.

Regarding the TIMP expression, McMillan et al. (1995) found that in AAA and AOD specimens, TIMP-1 and TIMP-2 were localized in the adventitia. In our study, we observed hihger expression of both TIMPs in the intima. The different MMP and TIMP expression in the aortic wall layers observed in this study is in accordance with previous data that indicated involvement of MMPs/TIMPs in AAA and AOD in aneurysmal and plaque instability (Petersen et al., 2000; Liapis and Paraskevas, 2003). However, the clear ratio has not been fully elucidated and data are still conflicting. It is known that extensive remodelling of the aortic wall in AAA and AOD involves synthesis and degradation of structural matrix proteins, in which the biomechanical properties of the vessel largely depend on the collagen proportion. Such compensatory repair process has been shown in human AAA and AOD and in animal models (Defawe et al., 2003).

Multiple cell types, including SMCs and inflammatory cells, populate the wall of an AAA and AOD. Each of these cell types is capable of producing proteases. In previous studies, inflammatory cells have been implicated as the major source of MMPs in the aneurysm development (Freestone et al., 1995; LeMaire et al., 2005). Phenotypic changes in SMCs and transition to the synthetic type from the contractile one has been associated with MMP expression and activity, since SMCs produce MMP-1, -2, -3, -7, -9, and -14 (Galis and Khatri, 2002; Beamish et al., 2010). According to our results, MMP-9 appears to be the predominant metalloproteinase expressed in AAA and in syndrome Leriche, since we recorded elevated expression of MMP-9 in SMCs and macrophages.

Consistent with these results is the fact that during the formation of the aneurysm, extensive infiltration of inflammatory cells (macrophages and lymphocytes) is observed in all aortic layers (Reeps et al., 2014). Mesen-

chymal cells do not express MMP-9 under normal conditions, but SMC production can be induced by a proinflammatory milieu, as occurs in AAA tissue (Gurjar et al., 2001). Conversely, macrophages produce relatively large amounts of MMP-9. The findings obtained on an animal model are consistent with histologic studies showing macrophages to be the primary source of MMP-9 in human AAA tissue (Longo et al., 2002). It was also reported that occlusive atherosclerotic lesions had higher MMP-9 expression (Orbe et al., 2003).

Many lines of evidence have suggested that high levels of MMP-2 and MMP-9 may contribute to the aneurysm formation (Ailawadi et al., 2009) and progression of AAA toward rupture (Petersen et al., 2000). The increased release of MMP-9 from inflammatory cells in the abdominal aorta causes continued growth of AAA (Theruvath et al., 2012) and failure of vascular grafts (Johnson et al., 2001), where MMP-2 and MMP-9 overexpression was noticed at neointimal lesions after exposure to arterial pressure (Chung et al., 2005).

In our study, elevated staining of TIMP-1 and TIMP-2 was predominantly observed in lymphocytes and macrophages of AAA patients. Originally, TIMPs were thought to function exclusively as endogenous inhibitors of MMP activity. However, recent reports have shown that TIMPs have a much broader spectrum of targets than originally believed. Mounting evidence points towards a function of TIMPs in a variety of biological processes such as apoptosis, cell survival, growth, migration, differentiation, angiogenesis, inflammation and ECM remodelling, and may play a central role in the process of cardiovascular remodelling (Vanhoutte and Heymans, 2010).

TIMP-1 levels were found to be unchanged or elevated in human atherosclerotic plaques, whereas TIMP-2 was also abundant (Newby, 2008). 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 (Orbe et al., 2003). Lesauskaite et al. (2006) demonstrated that in the wall of AAA, TIMP-1 was expressed more by inflammatory cells than by SMCs, whereas expression of TIMP-2 did not differ significantly between SMCs and inflammatory cells.

Despite similarities underlying the development of AAA and AOD, including increased MMP and TIMP expression, it is still unclear why some patients develop aneurysm and some occlusive arterial disease. It is possible that differences in the expression pattern of MMPs and TIMPs may determine whether atherosclerotic aorta will develop dilatative or stenosing disease.

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