Virchows Arch (2000) 437:521–527
DOI 10.1007/s004280000271

ORIGINAL ARTICLE

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Distribution pattern of matrix metalloproteinases 1, 2, 3, and 9, tissue inhibitors of matrix metalloproteinases 1 and 2, and α2-macroglobulin in cases of generalized AA- and AL amyloidosis

Received: 26 May 2000 / Accepted: 26 May 2000 / Published online: 31 August 2000

Abstract Matrix metalloproteinases (MMPs) degrade basement membranes and connective tissue and play an essential role in the homeostasis of the extracellular matrix which is disrupted by the deposition of amyloid. This immunohistochemical study investigated the distribution pattern of matrix metalloproteinases (MMP-1, -2, -3, and -9) and their inhibitors [α2-macroglobulin (α2-M), tissue inhibitors of MMPs (TIMP)-1, and TIMP-2] in human AA- and AL amyloid deposits. Specimens of liver, kidney, and spleen from 22 autopsy cases were investigated. Nine patients had suffered from generalized AA amyloidosis, eight from generalized AL amyloidosis, and five from rheumatoid arthritis or tuberculosis with no histological evidence of amyloid. In all amyloidotic and non-amyloidotic patients, each protease and protease inhibitor was detected in almost every organ investigated. In the amyloidotic cases, there was no indication that a specific protease or protease inhibitor was absent or expressed, but a difference was observed in their spatial distribution patterns. The most noticeable difference was found in immunostaining of amyloid. Only MMP-1, -2, and -3, and α2-M were present in AA amyloid deposits, and only TIMP-1 and TIMP-2 were found in deposits of AL amyloid. This is the first study to show that MMP-1, -2, and -3 are present in AA amyloid deposits. They may be involved in tissue remodeling or in proteolysis of the precursor and fibril proteins.

Keywords Amyloid · Protease · Protease inhibitors · Matrix metalloproteinases

Introduction

Physiological processes involving remodeling of the extracellular matrix, such as wound healing, embryogenesis, angiogenesis, and the female reproductive cycle, require the activity of matrix metalloproteinases (MMPs). This group of proteases degrades basement membranes and connective tissue and plays an essential role in the homeostasis of the extracellular matrix. An imbalance in the expression and/or activity of MMPs can have important consequences in the development of diseases such as cancer and Alzheimer's disease [4, 5, 20, 32]. The latter is a cerebral amyloid disease. Amyloidoses are characterized by local, organ-limited, or generalized proteinaceous deposits of autologous origin showing fibrillar protein deposits with specific tinctorial and structural properties [6, 7, 10]. Approximately 45% of all generalized amyloidoses are AA amyloidosis, and the acute phase protein serum amyloid A (SAA) is the precursor of the AA fibril protein deposited in this disease. In the West, AA amyloidosis is commonly associated with chronic rheumatic and idiopathic inflammatory diseases [9, 14, 23, 28]. Of patients with rheumatoid arthritis (RA), 5.8% eventually develop AA amyloidosis, and their lifespan is shortened by 7.7 years [18]. Patients suffering from RA exhibit significantly increased serum levels of MMP-1, -2, -3 and -9 compared with healthy controls, and the serum level of MMP-3 correlates with the C-reactive protein level and the erythrocyte sedimentation rate [11, 12, 13]. MMP-3 is not an independent marker of joint disease in RA, but is correlated with systemic inflammation [26]. Inflammatory cytokines are potent inducers of some MMPs, including MMP-1, -3, and -9, and they may increase transcription by as much as 100-fold [16, 19, 33].

SAA itself stimulates production and secretion of MMP-2 and MMP-3 in synovial fibroblasts [16], while MMP-1 and MMP-3 are able to degrade rabbit acute phase SAA [17]. Since circulating SAA is the source of the AA fibril protein deposited in AA amyloidosis, it is tempting to speculate that SAA may exert a local effect on MMP secretion and concentration before or during amyloid formation and that MMPs are involved in the pathogenesis of AA amyloidosis. MMPs may participate in amyloidogenesis by either processing SAA or by remodeling the interstitial matrix. The deposition of amy-

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Table 1 Age and gender distribution, basic diseases, and cause of death among the cases studied

	AA amyloidosis	AL amyloidosis	Controls
No. of cases	9	8	5
Age (years, range)	56 (35–70)	63 (50–73)	65 (54–75)
Gender (male:female)	6:3	3:5	2:3
Underlying disease (*n*)	Rheumatoid arthritis (4), Rosai-Dorfman disease (1), unknown (4)	Plasmacytoma (3), immunocytoma (1), primary amyloidosis (4)	Rheumatoid arthritis (4), tuberculosis (1)
Causes of death (*n*)	Cardiac failure (5), pulmonary thrombembolism (2), renal failure (1), acute appendicitis (1)	Cardiac failure (4), myocardial infarction (2), septic shock (1), gastrointestinal bleeding (1)	Ischemic colitis (1), septic shock (1), myocardial infarction (1), dissecting aneurysm (1), tuberculosis (1)

loid disrupts the integrity of the vascular and interstitial matrix. Laminin, collagen IV, fibronectin, and highly sulfated glycosaminoglycans are co-deposited with amyloid, and the translation of laminin-, entactin-, and collagen IV-genes is changed during amyloidogenesis [1, 30]. Many of these basement membrane proteins and components are putative substrates of MMPs [21].

Evidence of spatial and temporal relationships between MMPs and amyloid formation is an essential prerequisite for discussion and further analysis of their pathophysiological significance. As yet, only a few studies have investigated the significance of MMPs in AA amyloid deposits. In two previous studies, immunostaining was used to investigate the spatial relationship of MMPs with amyloid, but only human neutrophil elastase was found within or near amyloid [27, 31]. In view of the potential significance of MMPs during amyloidogenesis, we performed an immunohistochemical study to investigate the spatial relationship of MMPs (MMP-1, -2, -3, and -9) and their inhibitors [α2-macroglobulin (α2-M), tissue inhibitors of MMPs (TIMP)-1, and TIMP-2] with AA amyloid deposits. Specimens from patients with AL amyloidosis, which is not commonly associated with an acute phase response, and from patients with a chronic inflammatory disease without amyloidosis, served as controls.

Materials and methods

Twenty-two patients were selected from a consecutive series of 3277 autopsies performed from 1991 to 1999. Selection was based on autopsy reports which stated either the presence of a generalized amyloidosis or a basic disease known to cause AA amyloidosis. Deparaffinized serial sections were used throughout the study. The presence of amyloid was demonstrated by the appearance of green birefringence from alkaline alcoholic Congo red staining under polarized light [22]. Of the 22 patients, 17 had suffered from generalized amyloidosis. Specimens from the liver, kidney, and spleen were available in 17, 15, and 13 cases, respectively. Variable amounts of amyloid were present in each organ as vascular and interstitial deposits. Amyloid was classified immunohistochemically as previously described using antibodies directed against AA amyloid, amyloid P-component, transthyretin, β2-microglobulin, and λ- and κ-light chain (all Dako, Denmark) [24]. Amyloid deposits from nine patients showed immunostaining with antibodies directed against AA amyloid, and these were classified as generalized AA amyloidosis. Specimens from four patients showed no immunostaining of the amyloid deposits with antibodies directed against AA amyloid, transthyretin, and β2-microglobulin. However, these cases were immunoreactive with antibodies directed against λ- or κ-light chain, and they were classified as AL amyloidosis. The amyloid deposits of four patients stained for amyloid P-component only. Two of these patients had suffered from a plasmacytoma, while the other two had no disease commonly associated with either AA- or AL amyloidosis. These cases were classified as myeloma-associated or primary AL amyloidosis, respectively.

In five patients who had suffered from either rheumatoid arthritis or tuberculosis, no information had been recorded on amyloid deposition. Specimens of liver were available from four of these patients, and kidneys and spleen were available from five patients. Congo red-stained paraffin sections of all 14 specimens did not show any amyloid deposits. Therefore, these cases were regarded as suitable controls. Age, gender distribution, basic diseases, and cause of death are summarized in Table 1.

Immunodetection of endoproteases and protease inhibitors was performed with monoclonal antibodies directed against MMP-1 (dilution 1:100), MMP-2 (dilution 1:30), MMP-3 (dilution 1:50), MMP-9 (dilution 1:50), TIMP-1 (dilution 1:25; all Oncogene, Cambridge, UK), and TIMP-2 (dilution 1:20; Quartett, Berlin, Germany). Further immunostaining was performed using a polyclonal antibody directed against α2-M (dilution 1:500; Biotrend, Cologne, Germany). Macrophages and histiocytic cells were identified using an antibody directed against CD68 (monoclonal; dilution 1:40; Dako, Denmark). Prior to immunostaining, the specimens were pre-treated with 10 mM ethylene diamine tetraacetic acid (EDTA; 2×10 min, 450 W microwave oven; TIMP-2, CD68). Immunoreaction was visualized with the avidin biotin complex method applying a Vectastain ABC alkaline phosphatase kit (distributed by Camon, Wiesbaden, Germany) or UltraTech HRP Streptavidin-Biotin Universal Detection System (Immunotech, France). AEC, fast red, neufuchsin, and 3,3-diaminobenzidine-tetrahydrochloride served as chromogens. The specimens were counterstained with hematoxylin.

Fig. 1 Tubulointerstitial deposits of AA amyloid in the kidney of a patient who had suffered from rheumatoid arthritis: immunodetection of α2-macroglobulin spatially related to amyloid deposits. Congo red staining in polarized light (**A**) and immunostaining with anti-α2-macroglobulin and hematoxylin counterstain (**B**). Magnifications ×50 (**A**) and ×100 (**B**) ▶

Fig. 2 Vascular and tubulointerstitial deposits of AA amyloid in the kidney (**A–D**) of a patient who had suffered from rheumatoid arthritis. Matrix metalloproteinase (MMP)-2 was found intracellularly in close proximity to the deposits (**E**) and within the amyloid deposits (**F**). **A**, **B** Congo red staining in polarized light; **C**, **D** immunostaining with anti-AA amyloid; **E**, **F** immunostaining with anti-MMP-2. Magnifications ×40 (**A**), ×50 (**B**), ×100 (**C, E**), and ×150 (**D, F**)

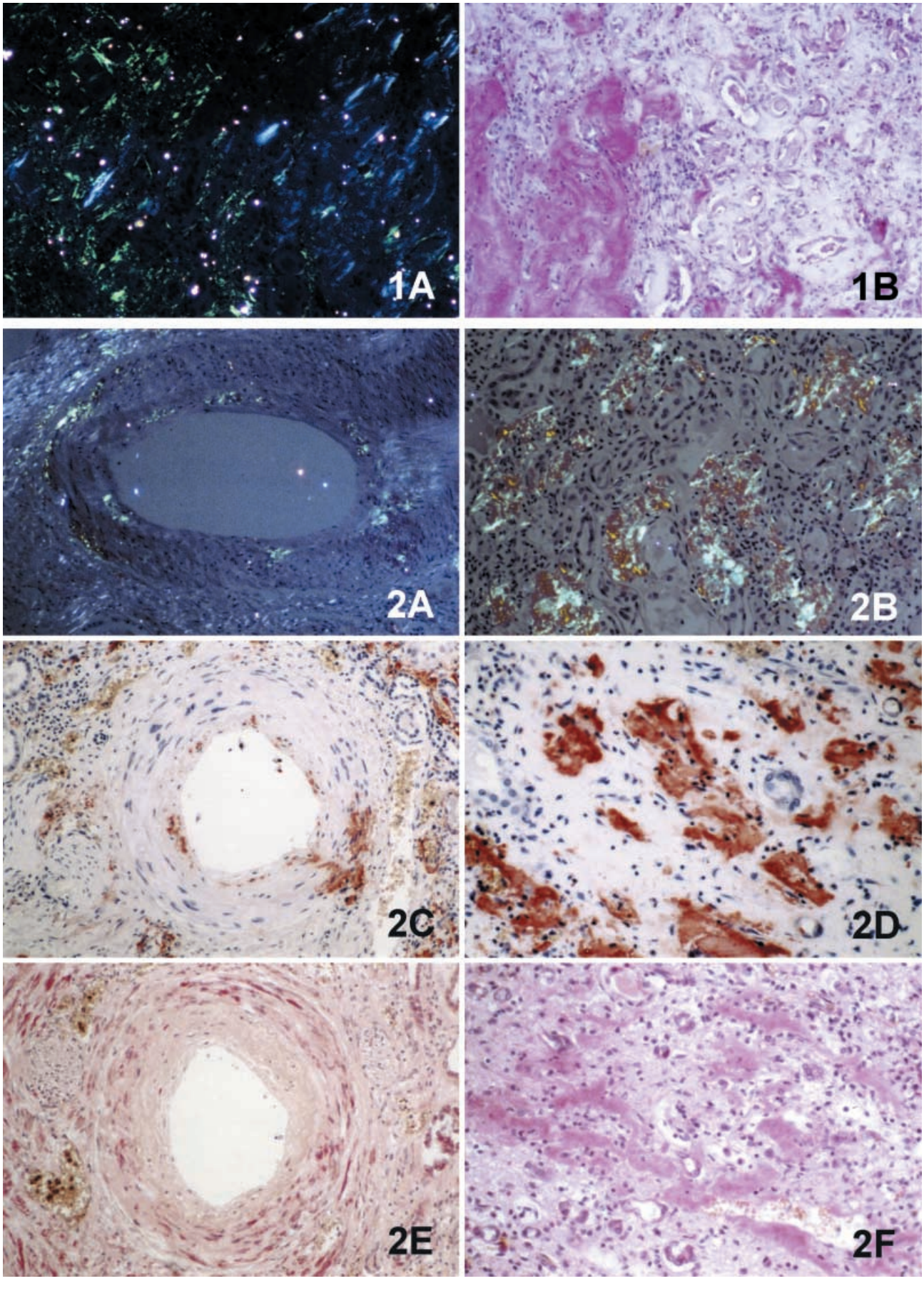
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Table 2 Distribution pattern of proteases and protease inhibitors spatially related to AA- and AL amyloid deposits. *n/m* number of positive cases/number of organs investigated; *MMP* matrix metalloproteinase; *TIMP* tissue inhibitors of matrix metalloproteinases

Proteases/ protease inhibitor	Organ	AA amyloidosis (25 specimens)				AL amyloidosis (20 specimens)			
		Adjacent to amyloid deposits		Within amyloid deposits amyloid		Adjacent to deposits		Within amyloid	
		n/m	(%)	n/m	(%)	n/m	(%)	n/m	(%)
MMP-1	Total	12/25	(48)	2/25	(8)	5/20	(25)	0/20	
	Kidney	3/7	(43)	1/7	(14)	2/8	(25)	0/8	
	Liver	3/9	(33)	1/9	(11)	3/8	(38)	0/8	
	Spleen	6/9	(66)	0/9		1/4	(25)	0/4	
MMP-2	Total	20/25	(80)	4/25	(16)	13/20	(65)	0/20	
	Kidney	6/7	(86)	3/7	(43)	6/8	(75)	0/8	
	Liver	7/9	(78)	0/9		4/8	(50)	0/8	
	Spleen	7/9	(78)	1/9	(11)	3/4	(75)	0/4	
MMP-3	Total	20/25	(80)	3/25	(12)	12/20	(60)	0/20	
	Kidney	6/7	(86)	3/7	(43)	5/8	(63)	0/8	
	Liver	7/9	(77)	0/9		5/8	(63)	0/8	
	Spleen	7/9	(77)	0/9		2/4	(50)	0/4	
MMP-9	Total	2/25	(8)	0/25		1/20	(5)	0/20	
	Kidney	1/7	(14)	0/7		0/8		0/8	
	Liver	0/9		0/9		1/8	(13)	0/8	
	Spleen	1/9	(11)	0/9		0/4		0/4	
TIMP-1	Total	15/25	(60)	0/25		10/20	(50)	1/20	(5)
	Kidney	5/7	(71)	0/7		4/8	(50)	1/8	(13)
	Liver	4/9	(44)	0/9		4/8	(50)	0/8	
	Spleen	6/9	(67)	0/9		2/4	(50)	0/4	
TIMP-2	Total	4/25	(16)	0/25		7/20	(35)	4/20	(20)
	Kidney	3/7	(43)	0/7		3/8	(38)	2/8	(25)
	Liver	0/9		0/9		3/8	(38)	1/8	(13)
	Spleen	1/9	(11)	0/9		1/4	(25)	1/4	(25)
α2-macroglobulin	Total	23/25	(92)	5/25	(20)	19/20	(95)	0/20	
	Kidney	7/7	(100)	3/7	(43)	7/8	(88)	0/8	
	Liver	7/9	(78)	1/9	(11)	8/8	(100)	0/8	
	Spleen	9/9	(100)	1/9	(11)	4/4	(100)	0/4	

The specificity of immunostaining was controlled using positive controls recommended by the manufacturers and by omitting the primary antibodies.

Results

Detection of proteases and protease inhibitors

In all amyloidotic (22 cases) and non-amyloidotic patients (5 cases), each protease and protease inhibitor was detected in almost every organ investigated. The intensity of immunostaining varied within each case and between different cases. In general, the proteases and protease inhibitors were found in endothelial cells of arteries, veins, capillaries, or sinusoids (all protease inhibitors and proteases except MMP-9), hepatocytes (all protease inhibitors and proteases except MMP-9), bile duct epithelium (all protease inhibitors and proteases except MMP-9), renal tubular epithelium (all proteases and protease inhibitors), smooth muscle cells of vessel walls (all proteases and protease inhibitors), erythrocytes (MMP-1, -2, -3, TIMP-1, α2-M), polymorphonuclear cells (MMP-1, -2, -3, -9, TIMP-1, and TIMP-2, α2-M), histiocytes (MMP-1, -2, -3), and multinucleated histiocytic giant cells (MMP-1, -2, -9, TIMP-1). In three organ specimens from different patients, specific proteases were not detected. MMP-9 was not expressed in two kidney specimens and one liver specimen (one case with AA amyloidosis and two cases with AL amyloidosis). However, MMP-9 was present in other organ specimens from the same patients. The cellular expression pattern of proteases and protease inhibitors showed no obvious difference between amyloidotic and non-amyloidotic cases.

In addition, in cases with generalized amyloidosis, proteases and protease inhibitors were spatially related to amyloid. The proteases and protease inhibitors were found either within the amyloid deposits (Fig. 1 and Fig. 2) or adjacent to the deposits where they were extracellular (MMP-2 and MMP-3, TIMP-1, and α2-M) or intracellular (MMP-1, -2, -3, -9, TIMP-1, TIMP-2, and α2-M). Intracellular immunostaining was confined to cells which were either attached to or enclosed by amyloid deposits (Fig. 2).

The prevalence of proteases and protease inhibitors spatially related to amyloid varied between AA- and AL amyloidoses (Table 2).

MMP-1, -2, and -3 displayed a spatial relationship which was more common with AA amyloid (48%, 80%,

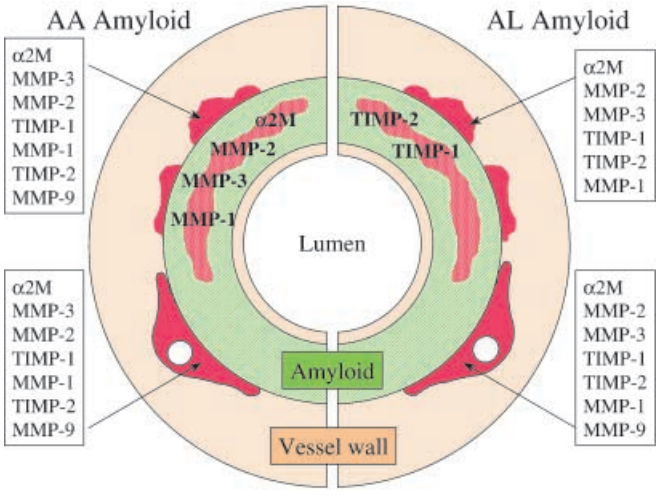

Fig. 3 Diagrammatic summary of the distribution pattern of proteases and protease inhibitors among AA- and AL amyloidoses. Vertical listing according to frequency of immunohistochemical detection. *Red coloration* indicates immunostaining of amyloid, intracellular immunostaining of cells within or immediately attached to amyloid deposits, and interstitial immunostaining in the immediate vicinity of amyloid deposits

and 80%, respectively) than with AL amyloid (25%, 65%, and 60%, respectively). While deposits of AA amyloid immunostained for MMP-1, -2, and -3, AL amyloid did not show immunostaining for any MMP. In AL amyloidoses, immunostaining was confined primarily to cells attached to the deposits. Among the MMPs, MMP-9 was least commonly spatially related to amyloid with frequencies of 8% (AA amyloid) and 5% (AL amyloid).

TIMP-1 and α2-M were commonly found in a spatial relationship with amyloid, with no obvious difference between AA- and AL amyloidosis. Both protease inhibitors were either attached intracellularly to amyloid or found interstitially within the deposits. However, the amount of immunostaining for α2-M (Fig. 1) far exceeded that for TIMP-1. TIMP-2 was the least common protease inhibitor found attached to or within amyloid; it was detected within some AL amyloid deposits in four specimens. Table 2 summarizes the distribution pattern of the proteases and protease inhibitors spatially related to AA- and AL amyloid deposits.

Intracellular immunostaining in close proximity to amyloid

Intracellular immunostaining for proteases and protease inhibitors within or in close proximity to amyloid deposits was found in endothelial cells (MMP-1, -2, -3, TIMP-1, α2-M), hepatocytes (MMP-2 and MMP-3, TIMP-1 and TIMP-2, α2-M), $CD68^+$-histiocytes (MMP-1), renal tubular epithelium (TIMP-1, α2-M), $CD68^-$-smooth muscle cells of vessel walls (all proteases and protease inhibitors), and very occasionally in polymorphonuclear cells (MMP-9, TIMP-2).

$CD68^+$-histiocytes were present adjacent to amyloid deposits in all cases of AA amyloidosis and in four (50%) cases of AL amyloidosis. Occasionally, a diffuse, cell-free immunostaining of amyloid deposits for CD68 was noted. Figure 3 is a diagrammatic summary of the distribution pattern of proteases and protease inhibitors among AA- and AL amyloidoses.

Discussion

The immunohistochemical studies reported here present morphological evidence of the expression of different MMPs and their inhibitors in patients with generalized amyloidosis. Each of the proteases and protease inhibitors investigated, i.e., MMP-1, -2, -3, and -9, TIMP-1 and TIMP-2, and α2-M, was found in amyloidotic and non-amyloidotic patients. No particular protease or protease inhibitor was missing or specifically expressed in amyloidotic cases. Immunostaining was not compromised by the use of autopsy material despite a variable degree of autolysis. In each case, all proteases and protease inhibitors were immunolocalized in at least one organ. Therefore, comparison of amyloidotic with non-amyloidotic cases showed no evidence that an exclusive expression, or lack of a specific protease or protease inhibitor, was associated with amyloidosis.

However, there were obvious differences in the spatial distribution patterns of the MMPs and their inhibitors. These can be divided into two categories, i.e., immunostaining not spatially related to amyloid deposits and immunostaining that was spatially related to amyloid deposits. The latter category can be subdivided into three further categories: (1) immunostaining of amyloid, (2) intracellular immunostaining of cells within or immediately attached to amyloid deposits, and (3) interstitial immunostaining in the immediate vicinity of amyloid deposits, as shown diagrammatically in Fig. 3. As a result of this categorization, the expression pattern of proteases and protease inhibitors shows differences between AA- and AL amyloidosis. The most obvious difference was found for immunostaining of amyloid. Only MMP-1, -2, -3, and α2-M were present in AA amyloid deposits, and only TIMP-1, and TIMP-2 were found in deposits of AL amyloid. These differences may be partly explained by the underlying disease and by the precursor proteins. Whereas AA amyloidosis is due to a chronic inflammatory disease with a permanent or recurrent acute phase response, as previously documented for one of the patients investigated here [25], AL amyloidosis is not commonly associated with such an acute phase response. MMPs are selectively expressed inducibly or constitutively by particular cell types. Inflammatory cytokines are potent inducers of some MMPs [16], and SAA increases the secretion of MMP-2 and MMP-3 in synovial fibroblasts [16]. Therefore, it is tempting to speculate that SAA may exert an effect on MMP secretion and concentration in other organs and tissues such as the liver, kidney, and spleen. Thus, the presence of MMP-1, -2,

and -3 in AA amyloid deposits may be due to an increased systemic and/or local production of MMPs in patients with chronic or recurrent acute phase responses.

The deposition of amyloid is associated with remodeling of the interstitial matrix. MMPs play an essential role in the homeostasis of the extracellular matrix and may be involved in tissue remodeling during deposition of amyloid [5]. However, we did not find any MMPs within AL amyloid deposits or any TIMPs within AA amyloid deposits. This may indicate that either MMPs or TIMPs are not an indispensable prerequisite for tissue remodeling during amyloid deposition or that remodeling of the matrix occurs in the interstitium adjacent to the deposits rather than in the center of the deposits. Patients with AA- or AL amyloid showed immunostaining for MMPs and TIMPs in the interstitium adjacent to the deposits.

Since MMP-1 and MMP-3 degrade rabbit acute phase SAA [17], MMPs within amyloid deposits may be involved in the proteolysis of the precursor and/or fibril proteins of AA amyloid instead of tissue remodeling, as was speculated for Aβ amyloidosis associated with Alzheimer's disease [32].

The presence of α2-M in deposits of AA amyloid is difficult to explain. It has previously been described in deposits of AL- [3], Aβ- [29], and dialysis-related Aβ2M amyloidosis [2], but conflicting results have been reported regarding its putative pathophysiological role. α2-M is a negative acute phase reactant, and serum levels decrease in patients suffering from RA [15]. It is also a protease inhibitor for many different proteases, including MMPs. Previous studies have shown that α2-M may inhibit trypsin-mediated degradation of AL amyloid fibrils [8] or, complexed to trypsin, it may promote degradation of fibril proteins, such as Aβ [34]. The putative role of α2-M in AA amyloidosis has not yet been investigated.

Intracellular localization of the proteases and protease inhibitors spatially related to the deposits revealed that many cells may influence amyloid formation and degradation. This influence may not necessarily involve active phagocytosis of fibrils or direct metabolism of the precursor proteins, but it may be indirectly involved in synthesis and secretion of proteases. For instance, if MMP-1, -2, -3, and α2-M in AA deposits were of local origin, then endothelial cells, hepatocytes, histiocytes, renal tubular epithelium, or smooth muscle cells of the vessel walls may serve as a source.

Immunohistochemical localization of proteases and protease inhibitors provides no information about their activities and interaction. For example, MMP-2 can be activated by a membrane-bound molecular complex that involves TIMP-2. However, in this study, we did not find TIMP-2 in deposits of AA amyloid. The antibody used here for immunodetection of MMP-2 does not differentiate between the active and inactive forms, thus its physiological state remains unknown. Immunolocalization of proteases and protease inhibitors merely indicates guilt by association. However, this is the first study to show that MMP-1, -2, and -3 are present in AA amyloid deposits. Further studies are required to investigate the biological significance of MMPs during or after the deposition of AA amyloid.

Acknowledgements This work was supported by grants from the Deutsche Forschungsgemeinschaft (Grant-No. RO 1173/3-1), Bonn Bad-Godesberg, Germany.

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