Disease-activity in ANCA-associated vasculitis
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Renal Expression of Matrix Metalloproteinases in Human ANCA-associated Glomerulonephritis

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ABSTRACT

Background: Expression of matrix metalloproteinases (MMPs) by infiltrating and intrinsic renal cells is increased in inflammatory conditions, and may correlate with disease activity of glomerulonephritis. We analysed renal expression of MMPs, tissue inhibitor of metalloproteinase-1 (TIMP-1) and markers of neutrophil and monocyte infiltration in renal biopsies of patients with active ANCA-associated glomerulonephritis.

Methods: Immunohistochemical expression of MMP-2, -3, -9, TIMP-1, the neutrophil and monocyte derived MMP activators cathepsin G, neutrophil elastase, and myeloperoxidase, and the monocyte marker CD14 was determined in renal biopsies of active PR3-ANCA (n=7) and MPO-ANCA (n=6) associated glomerulonephritis, and in normal renal tissue (n=4). Double-labelling experiments of MMPs and TIMP-1 were performed with MPO and CD68, labelling neutrophils and macrophages.

Results: MMP-2, -3, -9, and TIMP-1 positive cells were detected in ANCA-associated glomerulonephritis in glomeruli with active inflammation (cellular crescents or fibrinoid necrosis), only occasionally in normal appearing glomeruli, and not in sclerotic glomeruli and in the tubulo-interstitium. MMPs and TIMP-1 were predominantly expressed by MPO and CD68 positive cells. In normal renal tissue no expression was detected with the exception of weak mesangial staining for MMP-2. In ANCA-associated glomerulonephritis glomerular MMP-2, -9, and TIMP-1 correlated with glomerular cathepsin G expression, while the number of MMP-9 expressing cells per glomerulus correlated with the percentage of crescentic glomeruli. Tubulo-interstitial expression of MMPs correlated with all markers of neutrophil and monocyte infiltration, and interstitial MMP-9 and TIMP-1 expression correlated with renal function at the time of renal biopsy.

Conclusion: Expression of glomerular and interstitial MMP-2, -3, -9, and TIMP-1 is increased in active ANCA-associated glomerulonephritis and correlates with inflammatory activity.
INTRODUCTION

Anti-neutrophil cytoplasm antibodies (ANCA) directed against proteinase 3 and myeloperoxidase are the hallmark of a group of auto-immune small-vessel vasculitic disorders, such as Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA) and renal limited vasculitis [1, 2]. Many patients with these vasculitic disorders develop renal involvement with necrotizing crescentic glomerulonephritis, characterized by fibrinoid capillary necrosis and cellular crescents. Subsequently these cellular, inflammatory crescents evolve and become acellular and fibrotic leading to permanent loss of functional glomerular volume. The development of crescents is the result of interaction between infiltrating leukocytes, local proliferating cells and changes in the extracellular matrix.

Matrix metalloproteinases (MMPs), the main matrix degrading enzymes, have been implicated in both the inflammatory and fibrotic phase of crescent formation and are upregulated during inflammation and physiological remodelling processes [3]. MMP gene-expression and subsequent production as pro-enzymes is tightly regulated by various controlling mechanisms [4]. The extracellular activation of pro-MMPs is predominantly a two-step process with initial cleavage by a protease, followed by a final cleavage, usually by another MMP [5, 6]. Specialised tissue inhibitors of matrix metalloproteinases (TIMPs) and various other molecules, including $\alpha_2$-macroglobulin, are able to inhibit activated MMPs [7]. During inflammation activated MMPs degrade the extracellular matrix hence promoting trans-basement membrane migration of leukocytes and release of pro-inflammatory products from the extra-cellular matrix. Thus MMPs are likely to have an important pathogenic role in the acute phase of human crescentic glomerulonephritis. To study whether MMPs reflect renal inflammation in active renal ANCA-associated vasculitis we determined intra-renal expression of MMPs, their activators and a specific MMP inhibitor, TIMP-1, and correlated this with histological and functional markers of renal disease.

PATIENTS AND METHODS

Renal biopsy specimens
Renal biopsy specimens were studied from 13 patients with active renal ANCA-associated vasculitis. Samples of normal kidney tissue from patients with renal cell carcinoma ($n=4$) were used as control specimens. Blinded to the clinical status and renal function of the patient at the time of biopsy, renal biopsies were evaluated by light microscopy.
sclerosed, crescentic, and glomeruli with fibrinoid necrosis were counted. Results were expressed as percentage of the total number of glomeruli. Interstitial fibrosis, tubulo-interstitial inflammation, and atrophy were scored semi-quantitatively from 0 to 4 (0=no interstitial fibrosis/inflammation/atrophy; 1=<10%; 2=10-25%; 3=25-50%; 4>50%). Age, ANCA-status, serum creatinine, proteinuria and CRP were recorded from all patients at the time of biopsy. Additionally, data on creatinine-clearance at biopsy, and 1, 3 and 12 months after renal biopsy were collected.

Immunohistochemistry
Frozen 4 mm sections were fixed at room temperature (RT) for 10 minutes in 100% acetone and washed for 5 minutes in phosphate buffered saline (PBS, pH 7.4). The sections were incubated for one hour at RT with the primary antibody diluted in 1% Bovine Serum Albumin (BSA) in PBS (the source, specificity and dilution of the primary antibodies are given in table 1). Next, the slides were rinsed with PBS for 5 minutes. Endogenous peroxidase activity was blocked by incubating the slides in 0.075% hydrogen peroxide/PBS for 30 minutes. Following three washes with PBS the slides were incubated for 30 minutes with peroxidase conjugated secondary antibodies (all from Dako, Glosstrup, Denmark; specific combinations are given in table 1), supplemented with 1% human AB serum. The sections were washed in PBS and peroxidase activity was developed in a freshly prepared solution of 3-amino-9-ethylcarbazol (AEC), containing 0.03% hydrogen peroxide for 10 minutes. Counterstaining was performed using Mayer’s hematoxylin. Control slides, which were incubated in PBS in the absence of the primary antibody, were consistently negative.

Positively stained cells in the glomeruli were manually counted, and interstitial staining was scored semi-quantitatively from 0-4 (0=no staining; 1=occasional positive cells in <10% of interstitium; 2=positive cells in 10-25% of interstitium; 3=positive cells in 25-50% of interstitium; 4=positive cells in >50% of interstitium).

Additional double-labelling experiments were performed on cryostat sections. Briefly, after blocking of endogenous peroxidase, sections were incubated with primary antibodies, monoclonal mouse anti-human CD68, polyclonal rabbit anti-human MPO, and mouse anti-human α-smooth muscle actin (α-SMA) respectively. In contrast to CD14, which predominantly labels monocytes, CD68 is a pan-macrophage marker.
Table 1. Primary antibodies used in immunohistochemistry

| specificity                      | Antibody                                      | supplied by                        | dilution | secondary antibodies             |
|----------------------------------|-----------------------------------------------|------------------------------------|----------|-----------------------------------|
| TIMP-1                           | Monoclonal mouse anti-human TIMP-1            | Oncogene Science, Cambridge, MA, USA | 1:10     | RaMPO/GaRPO/ RaGPO               |
| latent and active MMP-2          | Monoclonal mouse anti-human MMP-2             | Calbiochem, La Jolla, Ca, USA      | 1:20     | RaMPO/GaRPO                      |
| latent and active MMP-3          | Polyclonal rabbit anti-human MMP-3            | Gaubius Institute, TNO, Leiden, The Netherlands | 1:500 | GaRPO/RaGPO/GaRPO                |
| latent and active MMP-9 21       | Monoclonal mouse anti-human MMP-9             | Gaubius Institute, TNO, Leiden, The Netherlands | 1:750 | RaMPO/GaRPO                      |
| neutrophil elastase              | Monoclonal mouse anti-human                   | DAKO, Glosstrup, Denmark           | 1:100    | RaMPO                             |
|                                  | Neutrophil elastase                           |                                    |          |                                   |
| neutrophil cathepsin G           | Polyclonal rabbit anti-human                  | Calbiochem, La Jolla, Ca, USA      | 1:200    | GaRPO                             |
| myeloperoxidase (MPO) CD14       | Monoclonal mouse anti-human MPO               | DAKO, Glosstrup, Denmark           | 1:50     | RaMPO                             |
| in double labelling: CD68        | Monoclonal mouse anti-human CD68              | DAKO, Glosstrup, Denmark           | 1:25     | RaMPO/GaRPO                       |
| myeloperoxidase (MPO) alpha smooth muscle actin (a-SMA) | Polyclonal rabbit anti-human MPO | Clinical Immunology, University Groningen, The Netherlands | 1:100 | SaRPO                        |
| TIMP-1                           | Monoclonal mouse anti-human TIMP-1            | Calbiochem, La Jolla, Ca, USA      | 1:25     | GaM-IgG1FITC                    |
| latent and active MMP-2          | Monoclonal mouse anti-human MMP-2             | Calbiochem, La Jolla, Ca, USA      | 1:25     | GaM-IgG1FITC                    |
| latent and active MMP-9          | Monoclonal mouse anti-human MMP-9             | R&D, Abingdon, UK                  | 1:25     | GaM-IgG1FITC                    |

RαMPO = peroxidase conjugated rabbit anti mouse antibody, GαRPO = peroxidase conjugated goat anti rabbit antibody, RαGPO = peroxidase conjugated rabbit anti goat antibody, GaM-IgG3PO = peroxidase conjugated goat anti mouse IgG3 antibody, GaM-IgG2aPO = peroxidase conjugated goat anti mouse IgG2a antibody, SαRPO = peroxidase conjugated swine anti rabbit antibody, GaM-IgG1FITC = FITC labelled goat anti mouse IgG1 antibody.
Peroxidase conjugated goat anti-mouse IgG3, IgG2a (SBA, Birmingham, USA) or swine anti-rabbit (DAKO, Glostrup, Denmark) antibodies were added and peroxidase activity was developed in diaminobenzidine (DAB). Subsequently, monoclonal mouse anti-human MMP-2, monoclonal mouse anti-human MMP-9 or monoclonal mouse anti-human TIMP-1, respectively, were added. Then, secondary FITC labelled goat anti-mouse IgG1 (Southern Biotechnical Associates) was added. Finally, sections were stained using Mayer’s haematoxylin. In all double-labelling experiments appropriate isotype controls were included, these were consistently negative.

Statistical Analysis
The non-parametric Mann-Whitney test was used to compare clinical and histological data between PR3- and MPO-ANCA positive patients. Using this test renal expression of MMPs, TIMP-1 and markers of neutrophil and monocyte infiltration were compared. Correlations were tested using Spearman’s correlation coefficient. Statistical significance was defined as a two-sided p<0.05.

RESULTS
Clinical and histological characteristics
Clinical and histological features of the included patients are shown in table 2. The median age in the patient group was 63 years (range: 26-86), six patients were MPO-ANCA positive and seven were PR3-ANCA positive, at presentation six patients required dialysis. MPO- and PR3-ANCA positive patients did not differ regarding clinical data. However, the fraction of glomeruli with fibrinoid necrosis differed significantly: the median fraction in PR3-ANCA positive patients was 0.20 (range: 0.11-0.58), whereas in MPO-ANCA positive patients this fraction was 0.07 (range: 0.00-0.56) (p=0.035). Additionally, the fraction of crescentic glomeruli tended to be higher in PR3-ANCA positive patients (0.43 (range: 0.25-1.00)) compared to MPO-ANCA positive patients (0.19 (range: 0.06-0.78)) (p=0.051). Tubulo-interstitial inflammation did not differ between the two groups.

Immunolocalization of MMP-2, -3, -9 and TIMP-1 in normal and nephritic glomeruli
Immunohistochemical staining in renal biopsies of patients with ANCA-associated glomerulonephritis revealed MMP-2 and -3 positive cells in cellular crescents. In addition, MMP-2 and -3 were detected in interstitial, glomerular and periglomerular cells (figure 1).
Table 2. Patient characteristics, renal parameters and histology at the moment of renal biopsy in 13 patients with active ANCA-associated glomerulonephritis.

| Patient | Age (years) | ANCA Dialysis dependent | Serum Creatinine (mmol/l) | Proteinuria (g/24h) | Serum CRP (mg/l) | Glomeruli per biopsy (number) | Normal glomeruli (fraction) | Crescentic glomeruli fibrinoid necrosis (fraction) | Interstitial fibrosis (score) | Tubular atrophy (score) |
|---------|-------------|--------------------------|---------------------------|---------------------|-----------------|-----------------------------|-----------------------------|--------------------------------|----------------------------|-------------------------|
| MPO-group (1-6)* | 67 | | | | | | | | | |
| 1 | 59 | MPO - | 362 | 3.0 | 31 | 18 | 0.29 | 0.19 | 0.07 | 1 | 2 |
| 2 | 69 | MPO + | 482 | 2.9 | 53 | 17 | 0.35 | 0.29 | 0.06 | 1 | 3 |
| 3 | 40 | MPO - | 130 | 4.3 | 9 | 19 | 0.68 | 0.16 | 0.11 | 2 | 0 |
| 4 | 67 | MPO + | 628 | 4.6 | 4 | 22 | 0.00 | 0.23 | 0.09 | 1 | 3 |
| 5 | 73 | MPO - | 223 | 1.8 | 40 | 33 | 0.15 | 0.06 | 0.00 | 1 | 2 |
| 6 | 53 | MPO + | 634 | 3.0 | 105 | 9 | 0.22 | 0.78 | 0.56 | 1 | 1 |
| PR3-group (7-13)* | 55 | | | | | | | | | |
| 7 | 74 | PR3 + | 444 | 4.4 | 212 | 10 | 0.40 | 0.40 | 0.20 | 1 | 2 |
| 8 | 26 | PR3 - | 111 | 2.1 | 33 | 8 | 0.75 | 0.25 | 0.13 | 0 | 0 |
| 9 | 86 | PR3 + | 367 | 1.0 | 108 | 21 | 0.24 | 0.43 | 0.24 | 1 | 2 |
| 10 | 63 | PR3 - | 190 | 2.6 | 115 | 11 | 0.45 | 0.45 | 0.27 | 2 | 0 |
| 11 | 47 | PR3 - | 115 | 2.4 | 282 | 7 | 0.71 | 0.29 | 0.14 | 0 | 0 |
| 12 | 41 | PR3 - | 353 | 7.1 | 214 | 19 | 0.00 | 1.00 | 0.11 | 0 | 1 |
| 13 | 76 | PR3 + | 631 | 1.8 | 17 | 12 | 0.00 | 0.58 | 0.58 | 3 | 1 |

*Data given as median value
MMP-9 positive cells, in contrast to MMP-2 and MMP-3, were only present in glomeruli with active crescents, and in the interstitium. In control specimens only weak MMP-2 staining was found in some mesangial cells, whereas MMP-3 and MMP-9 were absent. TIMP-1 protein was expressed in glomeruli with active crescents and in smooth muscle cells of larger arteries. The latter was also seen in control kidneys (figure 1). Cathepsin G, neutrophil elastase and MPO were found in active crescents and were also present in interstitial inflammatory cells. Cathepsin G, neutrophil elastase and MPO were absent in control kidneys (figure 1). In fibrous crescents staining for MMPs, TIMP-1, cathepsin G, neutrophil elastase, and MPO were virtually absent.

Double-labelling experiments were performed to clarify which cells produced MMP-2, MMP-9 and TIMP-1 in the crescentic glomeruli. MMP-9 and TIMP-1 expressing cells were predominantly MPO-positive, while only a fraction of MMP-2 expressing cells were MPO-positive (figure 2). Additionally, some CD68 positive macrophages produced MMP-2, MMP-9 and TIMP-1 (figure 2). Part of the MMP-2 positive cells were neither MPO, nor CD68 positive, and these cells had the morphological characteristics of glomerular mesangial cells (not shown). In additional double-labelling experiments a substantial part of the MMP-2 positive cells were found to be α-SMA-positive (figure 2).

In general, expression of all enzymes tended to be higher in PR3- as compared to MPO-ANCA associated cases. The differences did, however, only reach statistical significance for glomerular neutrophil elastase and MMP-9 (table 3). The number of positive cells per glomerulus and the interstitial scores for MMP-2, -3, -9 and TIMP-1 are shown in table 3 for controls and patients, categorised according to their ANCA-specificity.

**Correlation between MMPs and TIMP-1 versus cathepsin G, neutrophil elastase, MPO and CD14**

The relation between expression of MMPs and their neutrophil derived activators, cathepsin G, neutrophil elastase and MPO, was studied. The interstitial expression of MMP-3 and MMP-9 correlated significantly (p<0.01) with CD14 as marker of monocyte-infiltration and all neutrophil derived proteinases (table 4). The interstitial expression of MMP-2 correlated with MPO (p<0.01), cathepsin G (p<0.05), and CD14 (p<0.01), but not with the number of cells expressing neutrophil elastase. In the glomeruli, the number of MMP expressing cells did not correlate with neutrophil elastase and MPO (see table 4). CD14 was not detected in the glomeruli. Only MMP-2 correlated significantly (p<0.05) with the glomerular expression of cathepsin G. In contrast, the number of TIMP-1 expressing cells in the glomeruli correlated with all markers of neutrophil infiltration in the glomerulus (p<0.01).
Figure 1. Immunohistochemical staining for Cathepsin G (A,B), MMP-2 (C,D), MMP-3 (E,F), MMP-9 (g,h) and TIMP-1 (I,J) on control (A,C,E,G,I) renal tissue and tissue from patients with necrotizing crescentic glomerulonephritis (B,D,F,H,J). Cathepsin G staining was absent in control kidneys (A) and abundantly present in solitary cells (arrow) in glomeruli and interstitium in necrotizing glomerulonephritis (B). MMP-2 was weakly stained in the glomerular mesangium from control kidneys (C). Increased expression was observed in glomerular crescents (outlined), interstitial and glomerular cells from patients with necrotizing glomerulonephritis (D). MMP-3 and MMP-9 were absent in control kidneys (E,G) and markedly upregulated in glomerular crescents (arrows) from patients with necrotizing glomerulonephritis (F,H). TIMP-1 was expressed in smooth muscle cells (arrows), but not in glomeruli and in the interstitium from control kidneys (I), whereas TIMP-1 was expressed in glomeruli (arrows) and in the interstitium from patients with necrotizing glomerulonephritis (J).
Figure 2. Double immunostaining for MMP-2 (B,H,N), MMP-9 (D,J) and TIMP-1 (F,L) with MPO (A,C,E), CD68 (G,I,K), and a-SMA (M) respectively. Arrows in the figures indicate double-labelled cells. A minority of MMP-2 producing cells were MPO-positive (A,B). MMP-9 (C,D) and TIMP-1 producing cells (E, F) were predominantly MPO-positive. Some, but not all, glomerular and peri-glomerular CD68-positive cells co-localised with MMP-2 (G,H), MMP-9 (I,J) and TIMP-1 (K,L). A considerable number of MMP-2 producing cells were a-SMA-positive (M,N).
Table 3. Glomerular and tubulo-interstitial expression of matrix metalloproteinase (MMP)-2, -3, -9, tissue inhibitor of metalloproteinases (TIMP)-1, cathepsin G, myeloperoxidase, neutrophil elastase-in renal biopsies of 13 patients with active ANCA-associated glomerulonephritis.

|                       | Number of positive glomerular cells | Tubulo-interstitial score |
|-----------------------|------------------------------------|---------------------------|
|                       | MPO-ANCA (n=6)                     | PR3-ANCA (n=7)            | MPO-ANCA (n=6) | PR3-ANCA (n=7) |
| MMP-2                 | 2.3 (0.9-3.8)                      | 3.6 (2.6-4.0)             | 2 (1-4)        | 3 (1-4)        |
| MMP-3                 | 2.0 (1.0-2.8)                      | 1.8 (1.0-3.4)             | 1.5 (1-3)      | 3 (1-4)        |
| MMP-9                 | 0.0 (0.0-0.3)                      | 0.3 (0.0-5.4)             | 0 (0-1)        | 1 (0-2)        |
| TIMP-1                | 0.8 (0.0-1.4)                      | 1.6 (0.1-3.5)             | 0.5 (0.0-2.0)  | 1 (0-3)        |
| Cathepsin-G           | 2.1 (1.0-3.5)                      | 3.5 (1.3-13.0)            | 2.5 (1-3)      | 3 (1-4)        |
| Myeloperoxidase       | 4.6 (0.6-6.8)                      | 3.9 (0.8-21.3)            | 1 (1-2)        | 2 (1-3)        |
| Neutrophil elastase   | 0.3 (0.0-1.5)                      | 5.0 (0.4-14.4)            | 1 (0-1)        | 2 (0-3)        |

Data given as median value (range) for the group of the mean values per biopsy (glomerular) or the score (tubulo-interstitial)

a p=0.007 compared to MPO-ANCA

b p=0.03 compared to MPO-ANCA

Table 4. Correlation coefficients of the relation between matrix metalloproteinase (MMP)-2, -3, -9, and tissue inhibitor of metalloproteinases (TIMP)-1 expression in glomeruli and tubulo-interstitium with cathepsin G (Cath G), myeloperoxidase (MPO), neutrophil elastase (HNE), and CD14 as markers of inflammatory cell infiltration in renal biopsies of 13 patients with active ANCA-associated glomerulonephritis.

| Glomerular expression* | Tubulo-interstitial expression** |
|------------------------|----------------------------------|
|                        | Cath G  | MPO   | HNE  | Cath G  | MPO   | HNE  | CD14 |
| MMP-2                  | 0.59a   | 0.39  | 0.52 | 0.62a   | 0.79b | 0.54 | 0.80b |
| MMP-3                  | 0.38    | 0.51  | 0.15 | 0.84b   | 0.80b | 0.81b | 0.85b |
| MMP-9                  | 0.65a   | 0.46  | 0.51 | 0.73b   | 0.83b | 0.84b | 0.78b |
| TIMP-1                 | 0.74b   | 0.85b | 0.72b| 0.48    | 0.50  | 0.77b| 0.51 |

* expression scored as the mean number of positive cells per glomerulus

** expression scored as semi-quantitative score

a p<0.05

b p<0.01
Correlation between MMPs, TIMP-1 and renal histology
To study whether expression of MMPs and TIMP-1 reflected active inflammation, correlation with renal histology was tested. The number of MMP-9 positive cells in the glomeruli correlated significantly (r=0.60; p < 0.05) with the percentage crescentic glomeruli. In PR3-ANCA positive patients the number of crescentic glomeruli tended to be higher (p=0.051), and significantly more MMP-9 positive cells were present (p=0.007) than in MPO-ANCA positive patients. There were no correlations between numbers of positive cells for MMP-2, -3 and TIMP-1 in the glomeruli and glomerular damage, as reflected by the percentage of normal, sclerosed, crescentic glomeruli and glomeruli with fibrinoid necrosis. The interstitial expression of MMP-2, -3, -9 and TIMP-1 did not correlate with the histologically classified grades of interstitial fibrosis, and tubular atrophy (data not shown). In contrast, interstitial expression of MMP-9 (r=0.87, p<0.0001) and TIMP-1 (r=0.66, p<0.05) correlated significantly with tubulo-interstitial inflammation, whereas interstitial expression of MMP-2 (r=0.43, p=0.17) and MMP-3 (r=0.57, p=0.06) did not.

Correlation with renal function
The interstitial expression of MMP-9 (r=-0.59, p<0.05) and TIMP-1 (r=-0.60, p<0.05) correlated with the creatinine clearance at the moment of biopsy. Also interstitial neutrophil elastase (r=-0.62, p<0.05), tubular atrophy (r=-0.59, p<0.05) and the percentage of normal glomeruli (r=0.68, p<0.05) correlated with the clearance, while the percentage of sclerosed glomeruli (r=-0.50, p=0.08) and tubulo-interstitial inflammation (r=-0.54, p=0.06) did not correlate significantly with the clearance at the moment of biopsy. The glomerular expression of MMP-2, -3, -9 and TIMP-1 did not correlate with the clearance. During follow-up clearance was recorded at 1, 3 and 12 months. The renal expression of matrix metalloproteinases and TIMP-1 did not correlate with clearance during follow-up. In contrast, the percentage of normal glomeruli (r=0.64; p<0.05) and sclerosed glomeruli (r=-0.71; p<0.01) correlated with the creatinine clearance at 12 months after biopsy.

DISCUSSION
In the present study, MMP-2, MMP-3, MMP-9, and TIMP-1 were detected in glomeruli and tubulo-interstitium in renal biopsies of patients with active ANCA-associated necrotising crescentic glomerulonephritis, while expression was virtually absent in controls. In addition, expression of MMPs and TIMP-1 was mainly found in glomeruli with cellular crescents and/
or fibrinoid necrosis, but not in unaffected or sclerotic glomeruli, and expression correlated with markers of neutrophil and monocyte infiltration. Also, tubulo-interstitial expression of MMPs and TIMP-1 correlated with markers of neutrophil and monocyte infiltration, and correlated with renal function at the moment of renal biopsy. These findings suggest that expression of these MMPs and TIMP-1 represents active inflammation in ANCA-associated necrotising crescentic glomerulonephritis and can potentially be useful in assessing activity of the inflammatory process. Especially as double staining experiments showed that MPO-positive infiltrated neutrophils produced MMP-2, MMP-9 and TIMP-1; in contrast, fewer CD68-positive macrophages were found to produce MMPs and TIMP-1. These results correspond with the findings of Urushihara et al who also showed that many neutrophils, but not macrophages, produced MMP-9 in various glomerulonephritides [8].

The MMPs have been firmly linked to inflammation. Pro-inflammatory cytokines such as IL-1 and TNF-α have been shown to induce transcription and expression of several MMPs in both inflammatory cells such as neutrophils, monocytes and intrinsic renal cells, while the anti-inflammatory and profibrotic cytokine TGF-β is an important downregulator of MMP expression and enhances expression of MMP-inhibitors such as TIMP-1 [4, 9, 10]. In addition, an essential step in the activation of MMPs, which are secreted as inactive proenzymes, is the cleavage by proteases derived from activated inflammatory cells, especially neutrophils and monocytes. In experimental glomerulonephritis increased expression of MMPs is associated with disease activity and infiltration by inflammatory cells. Recently, gene expression of MMP-2 and the membrane bound MT1-MMP, which is an important activator of MMPs, was shown to correlate in different stages with inflammation and renal damage in a longitudinal murine anti-GBM glomerulonephritis model [11]. Moreover, in another model treatment with an MMP-inhibitor targeting elevated levels of MMP-2 and MMP-9 expression in anti-Thy1.1 nephritis diminished histological damage and proteinuria [12]. However, in an accelerated model of anti-GBM nephritis MMP-9-deficient mice showed more severe disease than control mice [13]. This suggests that MMP-9 not only causes glomerular damage, but might also protect glomeruli against injury, or promote glomerular recovery, after initial damage.

In human renal disease increased glomerular expression of MMP-9, but not of MMP-2, has been reported in IgA nephropathy, mesangial proliferative glomerulonephritis and lupus nephritis [8]. In addition, elevated plasma levels of MMP-2, MMP-3, MMP-9 and of TIMP-1 have been found in some of these diseases, while elevated urinary levels of MMP-2 and MMP-9 have been found in diabetic nephropathy [14-16]. In human rheumatoid arthritis elevated plasma levels of different MMPs, most often MMP-3, have been found and
correlated with disease activity [17]. Data on expression of MMPs and TIMP-1 in human crescentic glomerulonephritis have, to our knowledge, not been published. As our study involved only a limited number of patients, all with active glomerulonephritis at the time of renal biopsy, but none with previous, but currently inactive, renal disease we can not be certain that expression is not increased in the latter situation. However, expression of MMPs and TIMP-1 clearly co-localised with histological markers of active glomerulonephritis, i.e. in crescentic glomeruli and within the tubulo-interstitial infiltrates, while normal or sclerotic glomeruli were negative.

As has been suggested by others based on morphological data, our data, despite the limited number of biopsies, do suggest a difference in inflammation according to ANCA antigenic specificity [18]. In line with these morphological data, higher expression of inflammation associated proteins in PR3- as compared to MPO-ANCA associated glomerulonephritis was found, although only for glomerular neutrophil elastase and MMP-9 this difference reached statistical significance. Since our data suggest that MMPs and TIMP-1 expression is limited to active lesions of ANCA-associated glomerulonephritis and may reflect inflammatory activity, determination of plasma or urinary levels may be of potential value in assessing renal disease activity in these diseases and should be studied. Whether expression of MMPs or TIMPs in the acute or recovery stage of active ANCA-associated glomerulonephritis or other forms of glomerulonephritis also may have prognostic significance on long term follow up has to be studied. Given the important role of TGF-β, and probably other pro-fibrotic cytokines such as CTGF in regulating expression and activation of MMPs and TIMPs, longitudinal assessment may be of value in predicting long term outcome in glomerulonephritis and other progressive renal diseases. Increased serum and urinary levels of TIMP-1 and the matrix protein tenascin have been described in patients with renal function impairment due to different diseases, but have not been correlated to renal inflammation and fibrosis or changes of renal function during follow up [19]. Likewise, cross-sectional data in patients with a kidney transplant have correlated renal TIMP-1 mRNA expression with interstitial fibrosis 6 months post transplant, but not to serum and urinary levels of TIMP-1 or renal function during follow up [8, 20].

In conclusion, increased renal MMP-2, -3, -9 and TIMP-1 expression reflected the inflammatory process in ANCA-associated glomerulonephritis, in particular MMP-9 expression correlated with active glomerular lesions and tubulo-interstitial inflammation. Establishing a link between the increased inflammation related renal expression and elevated serum or urinary levels of MMPs and TIMP-1 will be a first step required to test whether monitoring of MMP and TIMP-1 levels has potential value as non-invasive marker of renal disease activity in ANCA-associated and other forms of glomerulonephritis.
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