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Inactive matrix metalloproteinase 2 is a normal constituent of human glomerular basement membrane. An immuno-electron microscopic study

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Abstract

Remodelling of the extracellular matrix requires tight control not only of matrix synthesis, but also of matrix degradation. Control of matrix degradation is achieved mainly through the matrix metalloproteinase (MMP) enzymes. In the glomerulus, MMP-2 and MMP-9 are believed to be particularly important, as they have activity against type IV collagen. This study has demonstrated by immuno-electron microscopy that most of the immunoreactivity for MMP-2 in the normal glomerulus is located within the glomerular basement membranes and mesangial matrix. mRNA for MMP-2 is also detectable in normal glomeruli, but the other main gelatinase, MMP-9, could not be localized by immuno-electron microscopy. In the normal glomerulus, it seemed likely that MMP-2 is present in an inactive form. To confirm this, *in situ* zymography was carried out using frozen sections of normal kidney. Baseline activity of normal kidney was relatively weak, but this was dramatically increased by chemical activation of metalloproteinases. The results imply that MMP-2, in an inactive form, is a normal constituent of the extracellular matrix and glomerular basement membranes. Activation would presumably render the matrix 'self-degrading'; membrane-bound MMPs (MT-MMPs) seem particularly likely to be involved in leukocyte penetration of basement membranes in inflammation. Copyright © 2000 John Wiley & Sons, Ltd.

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Introduction

Chronic inflammatory processes usually result in fibrosis. In normal tissues there is a constant slow turnover of extracellular matrix proteins, with constant synthesis and degradation. The development of fibrosis therefore involves modulation not only of the rate of production of extracellular matrix proteins, but also of their rate of destruction [1]. Collagen in the normal glomerulus is almost exclusively of type IV, and in fibrotic processes in the glomerulus the initial accumulation is of type IV collagen, rather than fibrous collagens. Controlled, localized dissolution of basement membranes is also essential to permit leukocyte emigration in inflammation. Control of the synthesis and degradation of type IV collagen is therefore particularly relevant to glomerular disease.

Degradation of extracellular matrix is achieved mainly by the matrix metalloproteinase (MMP) group of enzymes, of which MMP-2 (gelatinase A; 72 kD type collagenase) and MMP-9 (gelatinase B; 92 kD type collagenase) are most active against type IV collagen. Human glomerular mesangial cells, as well as podocytes, have been proved to synthesize and secrete MMP-2 in culture [2,3]. In addition, normal human glomeruli have been shown to produce mRNA for several metalloproteinases and inhibitors [4].

MMP-2, like most collagenases, is secreted from the cells in a latent proenzyme form, which is subsequently activated outside the cell. One of the tissue inhibitors of metalloproteinases (TIMP-2) binds to the pro-MMP-2 molecule and forms a complex. This stabilizes the proenzyme and protects it from undergoing spontaneous activation and autodegradation [5]. Several mechanisms are thought to be capable of activating the proenzyme. Other metalloproteinases are particularly relevant, especially the membrane-bound metalloproteinases (MT-MMPs) [6]. These are expressed on the surface of many infiltrating cells, including leukocytes and malignant cells. This raises the possibility that infiltrating cells might use MT-MMPs to activate latent MMPs, to produce 'auto-digestion' of the extracellular matrix in their immediate vicinity.

This theory assumes that inactive MMP is present within the extracellular matrix. In support, we present the first immuno-electron microscopic localization of MMP-2 in the normal human glomerulus. We have detected MMP-2 and MT-MMP mRNA in isolated human glomeruli, confirming that they can be synthesized locally. Finally, we provide *in situ* zymographic

evidence that the majority of the MMP (gelatinase) activity in the normal glomerulus is in a form that is enzymatically inactive, but capable of activation.

Materials and methods

Immuno-electron microscopy

Normal renal cortical tissue was obtained from a human nephrectomy specimen. Tissue was fixed in 0.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) overnight at 0–4°C. Limited fixation resulted in relatively poor preservation of ultrastructural details but higher concentrations of aldehyde fixatives resulted in loss of antibody binding. The tissue was then dehydrated in graded methanol (50, 70, 90%) for 45 min each at −25°C. Infiltration, polymerization, and embedding into LR gold resin were performed following the steps recommended by the manufacturer (Ted Pella Inc.).

A post-embedding immunogold labelling technique was performed on ultrathin sections to detect MMP-2 and TIMP-2. Monoclonal primary antibody (Oncogene, Calbiochem.) was applied in a dilution of 1 : 20 for MMP-2 in phosphate buffered saline (pH 7.4) and incubated overnight at 4°C. The sections were washed in several changes of buffer and incubated with gold-conjugated antibodies (particle size 10 nm; British Biocell International) for 1 h at a dilution of 1 : 10 in Tris buffer (pH 8.2, 20 mM). Grids were then rinsed in buffer, then distilled water and briefly counterstained in uranyl acetate and lead citrate. Examination of sections was with a Philips CM100 transmission electron microscope.

Controls omitting the primary antibody were consistently negative.

RT-PCR

To confirm the expression of relevant mRNA species in human glomeruli, glomeruli were plucked by hand from the cut surface of normal renal cortex, removed incidentally during nephrectomy for renal cell carcinoma. Glomeruli were also obtained from the surfaces of fresh renal biopsies from patients being investigated for microscopic haematuria without proteinuria. We used only cases where subsequent investigation with light microscopy, immunocytochemistry, and electron microscopy showed no abnormality. In all, seven glomeruli were used, from one nephrectomy and three normal biopsies.

mRNA from single glomeruli was isolated using Dynabeads (Dynal Inc.) as described previously [4]. Bead-linked cDNA was produced by reverse transcription using AMV reverse transcriptase (Promega), followed by PCR amplification, as described previously [4]. The primer sequences are given in Table 1; primers were synthesized by Gibco BRL (Paisley, UK) and were biotinylated at source. Detection of PCR product was achieved by gel electrophoresis and independently by ELISA [7]; specificity of the product was confirmed by the molecular weight on electrophoresis and by binding of the specific biotinylated probe in the ELISA. Appropriate negative controls were included [4].

In situ zymography

Normal human renal cortex was obtained from the uninvolved pole of a nephrectomy performed for carcinoma. The tissue was frozen in 3 mm cubes by dropping into freezing isopentane. Frozen sections were cut onto gelatin-coated slides.

The zymography method was a modification [8] of that of Galis *et al.* [9]. Sections were washed in phosphate buffered saline (pH 7.6). They were then were treated with 20 mM EDTA (as an MMP inhibitor), or phosphate buffered saline (pH 7.6), or 1 mM 4-aminophenylmercuric acetate (APMA, an MMP activator [10]) in dimethyl sulphoxide, each for 30 min at room temperature. All sections were thereafter processed as a single batch. They were drained and blotted dry before being dipped in Kodak NTB-2 photographic emulsion, diluted 3 : 8 in water, at 45°C. The slides were placed on a cold plate at 4°C for 20 min to gel and then placed in a humidified chamber at 25°C for 24 h. They were then processed conventionally with Kodak D-19 developer and Kodak fixative and dried.

Differences between the three groups of slides were obvious, but for confirmation the light transmission through ten glomeruli was measured using a digital image analysis system, with an untreated specimen and one treated with APMA. Representative images are presented, having been taken and printed under identical conditions.

Results

Immuno-electron microscopy

Ultrastructural examination of normal human renal glomeruli labelled for MMP-2 demonstrated a positive reaction for this enzyme mainly in the mesangial matrix and in the basement membrane, with very low background elsewhere (Figure 1). In the basement membrane the distribution of gold particles seemed to be mainly in the subendothelial areas, but the labelling was clearly within the substance of the basement membrane itself. The gold particles were more numerous in the mesangial areas than in the basement membrane. All glomeruli examined showed the same pattern. Cell cytoplasm and all extracellular spaces other than the matrix were negative.

RT-PCR

The presence of mRNA for MMP-9, MT-MMP-1, TIMP-1, and TIMP-2 in lysates of normal glomeruli was confirmed by bands of appropriate molecular weight on gel electrophoresis (Figure 2) and by ELISA.

Table 1. Primer and probe sequences

PCR primers	
MMP-2 [16,17] 5′	ATTGATGCGGTATACGAGGC
MMP-2 3′	GGCACCCTTGAAGAAGTAGC
MMP-9 5′	TTCTACGGGCCACTACTGTGC
MMP-9 3′	CGCCCAGAGAAGAAGAAAAG
MT1-MMP 5′	TGCCCAATGGAAAGACCTAC
MT1-MMP 3′	TGATGATCACCTCCGTCTCC
TIMP-1 [18] 5′	TGGGGACACCAGAAGTCAAC
TIMP-1 3′	CAGGGGATGGATAAACAGGG
TIMP-2 [19] 5′	AACGACATTTATGGCAACCC
TIMP-2 3′	ACCTGTGGTTCAGGCTCTTC
ELISA probes	
MMP-2	CTCCAGAATTTGTCTCCAGC
MMP-9	GTTGCAGGCATCGTCCACCGGACTCAAAGG
MT1-MMP	CATCCAGAAGAGAGCAGCATCAATCTTGTC
TIMP-1	GTAGTGATGTGCAAGAGTCC
TIMP-2	TCTATATCCT TCTCAGGCCC

Bands for MMP-2 were weaker and were not detected in three glomeruli, though the more sensitive ELISA method showed the presence of small amounts of MMP-2 mRNA in every glomerulus studied.

The absence of genomic DNA contamination was confirmed by the negativity of the -RT controls (data not shown).

In situ zymography

Normal, untreated glomeruli showed only weak gelatinase activity, as demonstrated by the faint pallor of the developed emulsion over the glomerulus in Figure 3a. Inhibition of metalloproteinase activity by EDTA treatment prior to digestion resulted in a reduction in this activity, though not complete elimination (Figure 3b). In contrast, prior activation with APMA resulted in a dramatic increase in gelatolytic activity (Figure 3c). It is therefore evident that most of the gelatinase activity in the normal glomerulus is present in an inactive form.

This was confirmed by the image analysis; mean light transmission per glomerulus (on an arbitrary scale) was 12.4 for normal glomeruli vs. 45 after APMA activation (95% limits 10.4–14.4 and 31.2–58.8; $p < 0.00005$).

Discussion

These results suggest that MMP-2 is an integral component of the normal glomerular basement membrane. Incorporation of *active* MMP-2 in the glomerular basement membrane would result in destruction of its components, but ultrastructurally the membrane appears normal. Hence the MMP-2 that we are detecting is either present in such small amounts that it results only in the normal low rate of turnover of the basement membrane, or it is in an inactive form.

In situ zymography cannot provide resolution at an ultrastructural level, nor can it distinguish between the various enzymes which are capable of degrading gelatin. It is acknowledged that one cannot assume that the relationship between degradation of the emulsion and enzyme activity is linear [8]. Nevertheless, the value of this technique lies in its ability to indicate the degradative activity of gelatinases in a tissue at a specific location. The activity may bear no relationship to the amount of gelatinase present, or its rate of synthesis, because of the various mechanisms by which metalloproteinase activity is controlled. The fact that EDTA does not completely eliminate gelatinase activity raises the possibility that a little activity is derived from non-metalloproteinase enzymes in the tissues. The possibility that the simple process of freezing and sectioning the tissues might have led to artefactual activation of some protease cannot be excluded. However, reversible inactivation of proteases by this procedure seems unlikely, so the dramatic increase in gelatolytic activity which is induced by APMA clearly indicates that gelatinases are present in renal glomeruli predominantly in an inactive form.

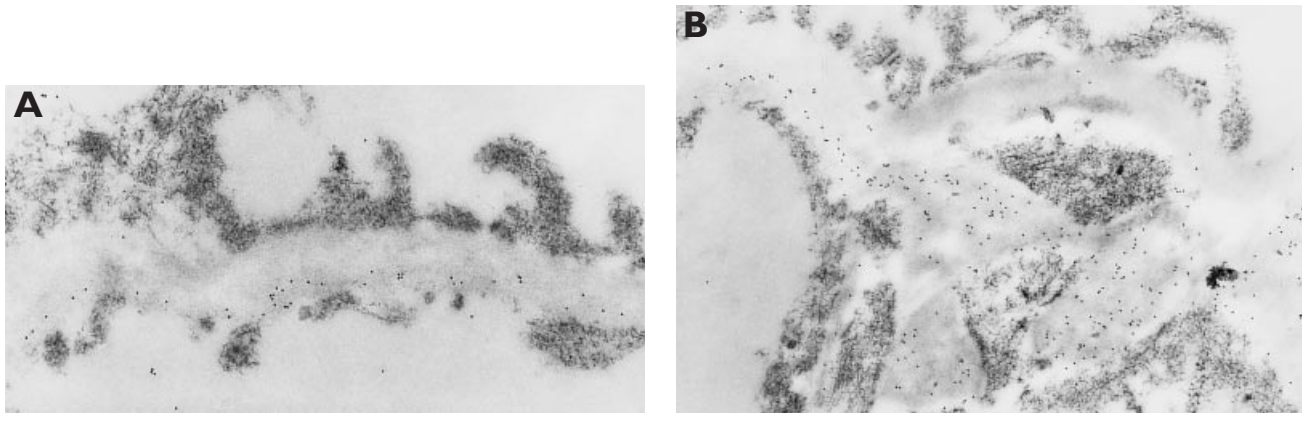

Figure 1. Immuno-electron microscopy for MMP-2. (A) Gold particles can be seen in the glomerular basement membrane, principally in the subendothelial zone. Urinary space is at the top, capillary lumen below. (B) Gold particles also locate over the mesangial matrix, but not in cell cytoplasm or extracellular space (capillary lumen at left). Gold particles can also be seen over the subendothelial basement membrane (left), as in A, but are much less prominent where the basement membrane overlies the mesangium (top)

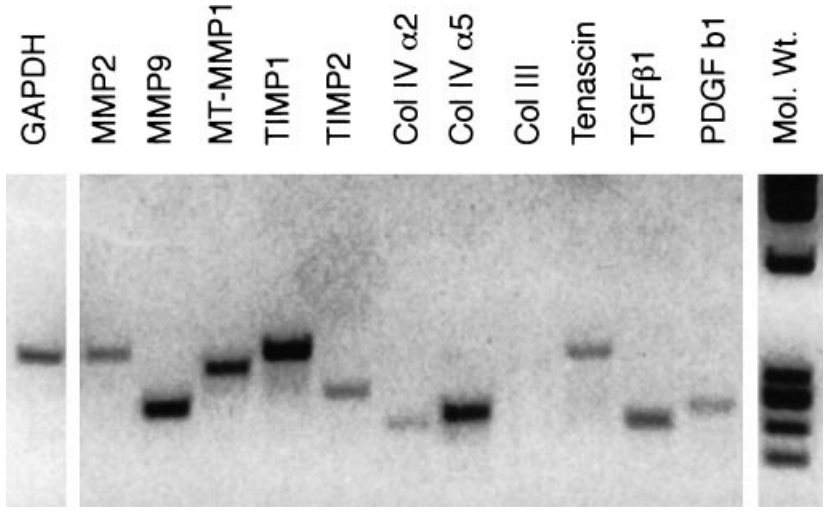

Figure 2. RT-PCR of mRNA from a single glomerulus

The RT-PCR results clearly demonstrate that normal human glomeruli produce mRNA for MMP-2, MMP-9, MT-MMP, TIMP-1, and TIMP-2. MMP-2 is believed to be the main source of gelatinase activity in the glomerulus under normal, physiological conditions. Taken together, these results show that the MMP-2 is predominantly located in the glomerular basement membranes and imply that most of it is in an inactive form. The relatively low levels of mRNA for MMP-2 suggest that the protein probably has a relatively long half-life, as one might expect if it is embedded in the basement membranes in an inactive form.

MMP-2 is secreted as a proenzyme, which may then be activated by a variety of methods, including proteolytic cleavage (reviewed in ref. 10). The association of MMP-2 with TIMP-2 is involved in regulating the enzyme activity [5]. Pre-MMP-2 is known to bind to various extracellular matrix components *in vitro* [11]. Localisation of MMP-2 with the extracellular matrix has been reported previously, but only at light microscope level, and in an inconsistent manner; indeed, it was suggested that this was a consequence of a disease process rather than a normal feature [12]. Our immuno-electron microscopy results suggest that MMP-2 is localized within the normal glomerular basement membrane. Accordingly, we propose that at least some MMP-2 is present in the glomerular basement membrane in an inactive form. This, together with its apparent activation by APMA, suggests that it is in the proenzyme form in this location.

Unlike several other MMPs, MMP-2 activation is not plasmin-mediated. MMP-2 activation is reported to occur selectively at the cell surface [13]; it has been proposed that membrane-type MMPs (MT-MMPs) are mainly responsible for this activation [14]. TIMPs are generally regarded as being involved in the suppression of MMP activity [15], but paradoxically it has been reported that binding of TIMP-2 to MMP-2 facilitates the activation of MMP-2 by MT-MMP [14].

If, as these results suggest, pro-MMP2 is an integral part of the glomerular basement membrane, it would follow that an inflammatory cell need only express a relatively small amount of MT-MMP at its surface in order to activate the basement membrane's own autocatalytic mechanism of self-destruction. If TIMP-2 is needed in the reaction between MT-MMP and MMP-2, it is available in the surrounding extracellular fluid. This TIMP-2 may also be involved in limiting the activity of activated MMP-2 away from the surface of

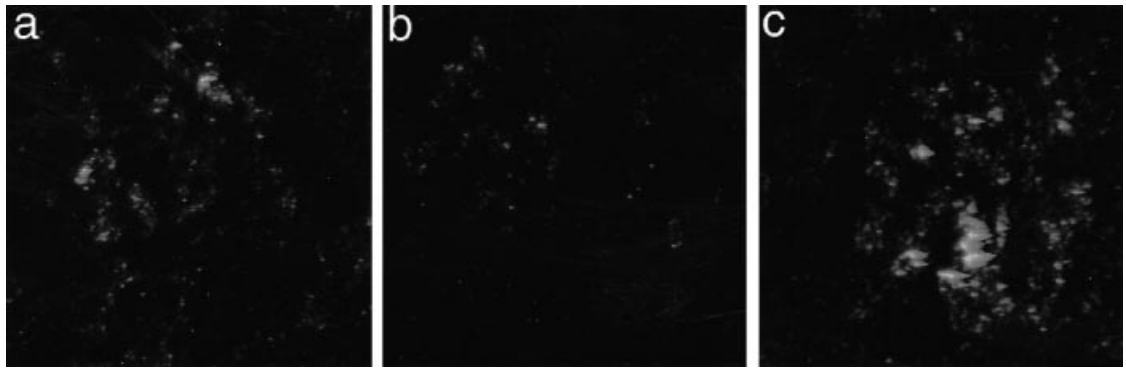

Figure 3. *In situ* zymography of glomeruli in normal renal cortex (a), after inhibition of MMP activity by EDTA (b), and after MMP activation with APMA (c)

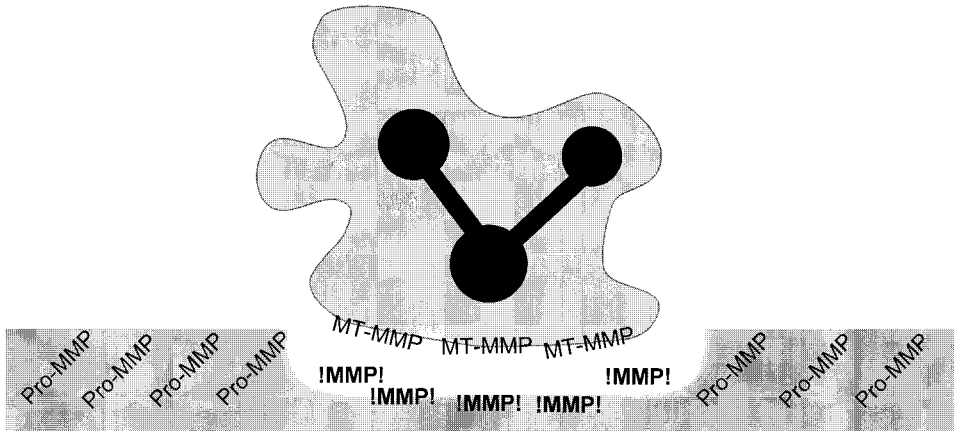

Figure 4. A possible mechanism for inflammatory cell penetration of the basement membrane. If, as our results suggest, inactive matrix metalloproteinase (Pro-MMP) is an integral part of the normal basement membrane, then if the membrane-type metalloproteinase (MT-MMP) which is known to be at the surface of inflammatory cells is brought into direct contact with the basement membrane, one might expect very localized activation of the integral basement membrane metalloproteinase (!MMP!) with consequent localized dissolution of the basement membrane

the leukocyte. Consequently, apposition of a leukocyte expressing surface MT-MMP against a basement membrane will result in a hole in the basement membrane which is precisely tailored to facilitate the passage of the leukocyte (Figure 4). The basement membrane in the glomerulus is unusually thick and therefore lends itself to the present study. However, if this is a general feature of basement membranes elsewhere in the body, then this would be an important aspect of inflammatory cell biology, and possibly of relevance to early tumour invasion.

Even in the absence of inflammation or neoplasia, basement membranes in stable adult tissues show a slow rate of normal turnover. Degradation is closely matched to synthesis, by mechanisms which are not understood; understanding this matching is likely to be important for understanding those disease processes which result in excessive accumulation (or excessive degradation) of matrix components. Our demonstration that MT-MMP mRNA is synthesized by normal glomeruli could, of course, result from blood leukocytes being trapped within the capillary loops, but it was found in every glomerulus that we studied. It therefore raises the intriguing speculation that intrinsic glomerular cells might also use this mechanism to control the degradation of the basement membranes to which they are adjacent. The same cells would then control synthesis and degradation. Dissolution of an area of basement membrane would presumably detach MT-MMP at the cell surface from the underlying matrix, resulting in a reflex decrease in matrix degradation: an intrinsic 'negative feedback' control.

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