0031-3998/03/5304-0698
PEDIATRIC RESEARCH
Vol. 53, No. 4, 2003
Printed in U.S.A.

Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinases-1 in Acute Pyelonephritis and Renal Scarring

MILAN CHROMEK, KJELL TULLUS, OLOF HERTTING, GEORG JAREMKO, ADLI KHALIL, YING-HUA LI, AND ANNELIE BRAUNER

Department of Clinical Microbiology, Microbiology and Tumorbiology Center, Karolinska Hospital, Stockholm, Sweden [M.C., O.H., A.K., Y.-H.L., A.B.]; Department of Pediatrics, Comenius University Medical School, Bratislava, Slovakia [M.C.]; Astrid Lindgrens Children's Hospital, Karolinska Hospital, Stockholm, Sweden [M.C., K.T., O.H., Y.-H.L.]; Department of Pathology, Karolinska Hospital, Stockholm, Sweden [G.J.]

ABSTRACT

The aim of the present study was to elucidate the role of matrix metalloproteinase-9 (MMP-9), and its main inhibitor tissue inhibitor of metalloproteinases-1 (TIMP-1), in acute pyelonephritis and the process of renal scarring. Urine samples from 40 children with acute pyelonephritis, 16 children at 6-wk follow-up and 15 children with nonrenal fever were analyzed using ELISA. MMP-9 and TIMP-1 levels were compared with the outcome of pyelonephritis as measured by renal static scintigraphy. A mouse model of acute ascending pyelonephritis was used to localize the sites of production and the kinetics of MMP-9 and TIMP-1 using immunohistochemistry and ELISA. Human renal epithelial A498 cells, primary mesangial cells and monocytic THP-1 cells were stimulated by *Escherichia coli*. MMP-9 and TIMP-1 mRNA was analyzed by reverse transcription-PCR (RT-PCR) and protein production by ELISA. We demonstrate a significant increase of MMP-9 and TIMP-1 in the urine of children with acute pyelonephritis. Both proteins were produced mainly by leukocytes, and TIMP-1 also by resident kidney cells. Cells reacted differently after stimulation by bacteria. In mesangial cells and monocytes a decreased constitutive TIMP-1 production was found, which was in contrast to epithelial cells. Out of 40 children with pyelonephritis, 23 had higher urinary TIMP-1 than MMP-9 levels. These children had significantly more severe changes in both acute and follow-up scintigraphy scans indicating higher degree of acute tissue damage and renal scarring. Thus, our findings suggest an association between TIMP-1 and the process of renal scarring. **(*Pediatr Res* 53: 698–705, 2003)**

Abbreviations

MMP, matrix metalloproteinase
TIMP, tissue inhibitor of metalloproteinases
ECM, extracellular matrix
DMSA, ^{99m}Tc-dimercaptosuccinic acid

Interstitial fibrosis is the final common end-point of progressive renal diseases. Despite its clinical significance, the mechanisms of scar formation in the kidney are still not fully understood. Generally, it is thought that the renal fibrogenesis is induced by the release of chemokines and growth factors by a number of resident cells. Their concentration gradient results in an influx of leukocytes and an increase in the number of interstitial fibroblasts. The induction phase is followed by an increased matrix production and reduced matrix degradation, which add up to a deposition of extracellular matrix (ECM) components (1). However, ECM component deposition does not seem to be the only mechanism in the pathogenesis of scarring. After direct inoculation of *Escherichia coli* into the kidneys of rats, contraction and collapse of the tubulointerstitial parenchyma had a greater influence than new collagen production on final fibrosis (2). In line with that, we have previously shown that mouse kidneys after experimental acute pyelonephritis were smaller compared with those from uninfected animals (3). Likewise, it seems that the end-stage kidney is rather shrunken, not enlarged or full of collagen (4).

The current hypothesis suggests that the normal remodeling and healing is a result of the balance between ECM protein synthesis and degradation (5). Out of a number of matrix-degrading enzymes, gelatinases, matrix metalloproteinase-2 and 9 (MMP-2 and MMP-9) seem to play an important role during inflammation in parenchymal organs. They are specific

Received April 2, 2002; Accepted October 4, 2002.
Correspondence: Annelie Brauner, M.D., Ph.D., Department of Clinical Microbiology, Microbiology and Tumorbiology Center, Karolinska Hospital, S-171 76 Stockholm, Sweden; E-mail: Annelie.Brauner@ks.se
This study was supported by Funds from the Karolinska Institute, Magn. Bergvalls Foundation, Stiftelsen Clas Groschinskys Minnesfond, and Stiftelsen Frimurare Barnhuset. Milan Chromek received financial support from the Swedish Institute and the Socrates/Erasmus exchange program.

DOI: 10.1203/01.PDR.0000057575.86337.CB

mainly for collagen IV, which is the most abundant extracellular matrix component of these organs. Moreover, MMP-9 is produced by neutrophils and macrophages after different stimuli, like LPS (6). Also glomerular epithelial cells, mesangial cells, tubular cells, and fibroblasts have been shown to produce MMP-9 after stimulation by cytokines and growth factors (7–11).

There is an association between MMP production and tissue damage during and after inflammation (12–15). Leukocytes need MMP-s to reach the site of inflammation (16) and mesenchymal cells use MMPs to reorganize and replace wounds in different tissues (17).

It has been suggested that increased production or activity of MMPs is associated with higher tissue degradation and replacement by collagen. Disruption of collagen IV, the main basement membrane component, has also been shown to stimulate epithelial-mesenchymal transformation of renal epithelial cells (1, 18), a proposed mechanism of renal scarring. On the other hand, decreased MMP production or activity can lead to decreased collagen degradation with net accumulation of ECM proteins in the tissue. Finally, MMPs might also play a role in reorganization of primary scar and tissue contraction.

The aim of the present study was to elucidate the role of MMP-9 and its main inhibitor, tissue inhibitor of metalloproteinases (TIMP-1), in acute pyelonephritis and the renal scarring process. To evaluate the role of the MMP-9/TIMP-1 balance in postinfective healing, we have examined urine samples from children with acute pyelonephritis and compared them to the outcome of pyelonephritis measured by renal static scintigraphy. We also wanted to find the sites of production and study the kinetics of MMP-9 and TIMP-1 during acute pyelonephritis using a mouse model and cell culture experiments.

METHODS

Patients. The study has been approved by the ethics committee of the Karolinska Hospital and informed consent has been obtained from parents of the children. Urine was collected from 40 infants and children presenting in an emergency room with a clinical picture of acute first-time pyelonephritis without symptoms or signs of any other infection. Urine samples were also collected from 16 of the same children at 6-wk follow up and 15 children with nonrenal fever. The median ages (and ranges) in the pyelonephritic group were 6 mo (0–113), follow-up group 13.5 mo (3–118), and nonrenal fever group 8.5 mo (1–38). Routine examinations with blood count, C-reactive protein (CRP), and plasma creatinine were done. Erythrocyturia and leukocyturia were tested semiquantitatively, using Ecur-Test (Boehringer Mannheim, Mannheim, Germany) and recorded as grades 0–3, corresponding to 0, 10–25, 75, and 500×10^6 leukocytes/mL and 0, 10, 50, and 250×10^6 erythrocytes/mL, respectively.

The diagnostic criteria of acute pyelonephritis in our study were: $\geq 10^5$ bacteria/mL urine, body temperature $\geq 38°C$ and CRP ≥ 20 mg/L, in children older than 6 wk. All urinary tract infections were caused by *E. coli.*

^{99m}Tc-dimercaptosuccinic acid scintigraphy (DMSA) was performed within 10 d after admission and 1 y after the acute infection. This method has been shown to detect renal scarring with high sensitivity and specificity (19). ^{99m}Tc-dimercaptosuccinic acid (0.5 MBq/kg body weight, not less than 15 MBq) was given i.v. Static images were recorded in the posterior-anterior and anterior-posterior projections, using a gamma camera (General Electric 400AT, Milwaukee, WI, U.S.A.). Each kidney was divided into three segments (upper pole, middle part, and lower pole). Normal uptake in a segment was given the value 0, and decreased uptake was graded 1–3. From the size and degree of a defect in DMSA uptake, a DMSA score was calculated (20).

Animals. The animal experiments were approved by the ethics committee of the Karolinska Hospital. Bki NMRI female mice were used, 8–10 wk old, weight 20–30 g (Charles River, Uppsala, Sweden), caged 3–7 mice in each group with food and water *ad libitum*. Each mouse was under anesthesia transurethrally catheterized using a soft sterile polyethylene catheter (outer diameter 0.61 mm, inner diameter 0.28 mm, Clay Adams, Becton Dickinson, U.S.A.) with lubricant Glidslem® (Apoteket, Umeå, Sweden). 50 μL of bacterial suspension (10^8 of bacteria/100 μL) or PBS was injected into the bladder. The catheter was immediately removed and the external urethral meatus was coated by collodium (sves 46, Swedish Pharmacopeia). After 6 h collodium was softened and removed using acetone. The mouse kidneys were removed aseptically under anesthesia at 24 h, 48 h, and 6 d, and the mice were then killed by carbon dioxide. After homogenization in mortar homogenizer with PBS the kidneys were centrifuged at $350 \times g$ for 10 min. Supernatants were collected and stored at $-20°C$. Before ELISA analysis the supernatant was centrifuged at $1500 \times g$ for 10 min and passed through a 0.45 μm filter (Pal Gelman Sciences, Ann Arbor, MI, U.S.A.).

For histologic evaluation the kidneys were embedded in paraffin blocks after fixation in 4% buffered paraformaldehyde. From the tissue blocks, 4 μm thick sections were cut. Two animal experiments with 3–5 mice in each group were carried out.

Cells. Human renal epithelial cell line (A498), human monocytic cell line (THP-1) (American Type Culture Collection, Rockville, MD, U.S.A.) and primary human mesangial cells (NHMC) (Clonetics, BioWhittaker, Walkersville, MD, U.S.A.) were used.

A498 were grown in Eagle's minimal essential medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented by 200 mM glutamine (Sigma Chemical Co.), 1.0 mM sodium pyruvate (Invitrogen Life Technologies, U.S.A.), 0.1 mM nonessential amino acids (Life Technologies), and 10% fetal bovine serum Myoclone (Life Technologies). THP-1 monocytes were grown in RPMI 1640 (Sigma Chemical Co.) supplemented by 5 mM mercaptoethanol, 200 mM glutamine, and 10% fetal bovine serum Myoclone. NHMC were grown in RPMI 1640 supplemented by 200 mM glutamine, insulin-transferrin-selenium (Life Technologies), and 10% of fetal bovine serum Myoclone. NHMC were used between passages 5 and 10.

A498 and NHMC were used for the experiments when they formed a confluent layer, while 10^6 of THP-1 cells were added into 1 mL of medium. The experiments were performed with serum-free medium supplemented with gentamicin (40 mg/mL) (21). Three independent cell experiments were done and each sample was analyzed in duplicate or quadruplicate. 10^8 bacteria were added into each well. Cells incubated with medium only served as controls. After incubation in 37°C, 5% CO_2, 95% O_2, and 80% humidity for 0, 6, 24 h the medium was centrifuged (350 × *g*, 10 min) and supernatants were frozen (−20°C) for the ELISA analysis. The cells were then lysed with RNAzol™ (Biotecx Laboratories, Houston, TX, U.S.A.) (200 μL for each well) for the total RNA extraction and the cell suspension stored at −70°C.

Bacteria. *E. coli* CFT O73 was isolated from a patient with pyelonephritis. It expresses type 1, P and S fimbriae and hemolysin, and it induces pyelonephritis in experimentally challenged normal mice (22). The bacteria were grown overnight on cystine lactose electrolyte deficient (CLED) agar at 37°C and in Luria-Bertani broth for 5 h, to reach logarithmic phase of growth, then washed twice in PBS. The bacterial concentration was measured by spectrophotometry at a wavelength of 600 nm and confirmed by viable count on blood agar plates. The same bacterial strain was used in both the animal model and the cell experiments.

Measurement of MMP-9 and TIMP-1 levels. ELISA kits for total human MMP-9 and TIMP-1 were obtained from R&D systems (Abingdon, UK). The detection limits were 0.36 ng/mL for MMP-9 and 0.12 ng/mL for TIMP-1. The levels of total and active MMP-9 in supernatants from renal tissue were measured by MMP-9 activity assay, specific for mouse (Amersham Pharmacia Biotech, Uppsala, Sweden). The detection limit for MMP-9 was 0.23 ng/mL. MMP-9 and TIMP-1 levels were determined according manufacturer's instructions. The urinary levels of creatinine were analyzed by Jaffé's method. Urinary levels of measured proteins were expressed as protein-creatinine ratios.

Immunohistochemistry. The paraffin embedded sections were first deparaffinized with Bio-Clear (Bio-Optica, Milan, Italy) and rehydrated in graded ethanol. The sections were transferred to 0.01 mol/L citrate buffer (pH 6.0) and heated in a microwave oven for 10 min at 700 W and then allowed to cool for 20 min. After incubation with 2% BSA for 1 h and for 10 min in 3% H_2O_2 in methanol for quenching of endogenous peroxidase activity, the slides were blocked with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, U.S.A.) and incubated overnight at 4°C with the primary antibody solution. Rabbit anti-mouse MMP-9 and anti-TIMP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, U.S.A.) were used and normal rabbit immunoglobulin (Dako, Glostrup, Denmark) served as a negative control. After incubation with goat anti-rabbit biotinylated secondary antibody solution (Vector Laboratories) in a 2% normal goat serum, the slides were incubated for 30 min with Vectastatin® ABC reagent (Vector Laboratories). Between each step the slides were washed in 0.01 M PBS, pH 7.4. After developing with diaminobenzidine tetrahydrochloride (DAB)(Dako) solution for 2 min the sections were washed in double-distilled water and counterstained in hematoxylin. The slides were then rewashed in water, dehydrated by graded ethanol, cleared with Bio-Clear, and mounted using Pertex mounting medium (Histolab Products, Göteborg, Sweden). Using the above-described method MMP-9 and TIMP-1 protein stained brown and the cell nuclei blue. The same slides were also stained with the standard hematoxylin-eosin method where the cell nuclei stained blue and the cell cytoplasm pink or red.

RT-PCR. Total RNA was isolated by the guanidine/phenol method using RNAzol™ B (Biotecx Laboratories) according to the manufacturer's instructions. Reverse transcription of 1 μg RNA was performed using 100 pmol random hexamer primers $pd(N)_6$ (Amersham Pharmacia Biotech, Uppsala, Sweden) and 300U SuperScript RNAse H^- (Life Technologies) with the mixture of 5 × buffer, 10 μmol dNTP, 0.1 mmol DTT, and 60 U RNAsin (Boehringer Mannheim, Mannheim, Germany) in a volume of 20 μL at 45°C for 2 h. cDNA was stored at -70°C until used. Following primers have been used: 5'-MMP-9 AGACCTGAGAACCAATCTCAC, 3'-MMP-9 GGCACTGAGGAATGATCTAA (7), 5'-TIMP-1 GCGGATCCAGCGCCCAGAGGACACC, 3'-TIMP-1 TTAAGCTTCCACTCCGGGCAGGATT (23), 5'-G3PDH TGAAGGTCGGAGTCAACGGATTTGGTC, 3'-G3PDH ATGTGGGCCATGAGGTCCACCAC (24). MMP-9 and TIMP-1 primers were obtained from Innovagen (Lund, Sweden) and the primer for the "housekeeping" gene human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was from CyberGene AB (Huddinge, Sweden). PCR of 2 μL of the cDNA was performed with 0.025 U/μl Taq polymerase (Life Technologies), 2 mM $MgCl_2$, 0.2 mM dNTP, PCR buffer, and 0.5μM of each primer in a final volume of 25 μL in PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, U.S.A.). Numbers of PCR cycles were set after pilot experiments to be in a linear range of the PCR reaction. After 1 min for 95°C, PCR was conducted for 30 (MMP-9), 21 (TIMP-1), and 28 (G3PDH) cycles using the following conditions: 1 min of denaturation at 94°C, 1 min of annealing at 54, 65, and 60°C, respectively, and 1 min of extension at 72°C, followed by a final extension for 5 min at 72°C and cooling to 4°C.

The PCR products (1146 bp, 684 bp and 983 bp long for MMP-9, TIMP-1 and G3PDH, respectively) were separated by electrophoresis on a 1.5% agarose gel (Life Technologies). Following staining with ethidium bromide, the gels were photographed under UV-light using Gel Doc 2000 (BioRad, Hercules, CA, U.S.A.).

Data analysis. The Mann-Whitney U test and Spearman's rank correlation test were used. Differences with p level less than 0.05 were considered as statistically significant.

RESULTS

Significant increase of MMP-9 and TIMP-1 in the urine of children with acute pyelonephritis. Association between TIMP-1 and renal damage and scarring. The urinary MMP-9/creatinine and TIMP-1/creatinine ratios were significantly higher in children with acute pyelonephritis compared with ratios from the same children at 6 wk follow-up ($p < 0.01$, respectively) and also to children with nonrenal fever ($p <$

0.05 for MMP-9, $p < 0.01$ for TIMP-1) (Fig. 1*A*). Out of 40 children with pyelonephritis, 23 had urinary TIMP-1 levels higher than MMP-9 levels with a difference of more than 0.1 ng/mmol (Fig. 1*B*). These children had significantly more severe changes in both the acute and follow-up DMSA scans, indicating a higher degree of acute tissue damage and renal scarring ($p < 0.05$, respectively) (Fig. 1*C*).

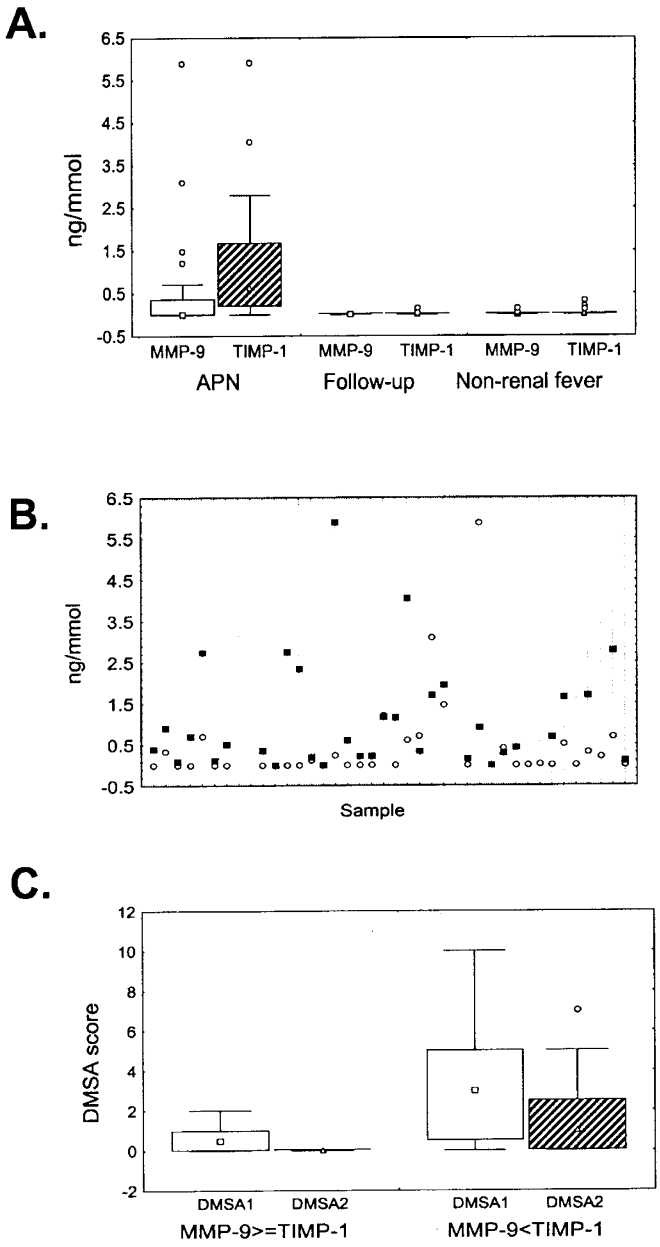

Figure 1. (*A*) MMP-9/creatinine (open box) and TIMP-1/creatinine (hatched box) ratios in the urine of children with acute pyelonephritis (APN) ($n = 40$), at 6 wk follow-up ($n = 16$), and children with nonrenal fever ($n = 15$). Both MMP-9/creatinine and TIMP-1/creatinine ratios were higher in children with acute pyelonephritis compared with the other two groups of children. Data presented as median, box (25% to 75%), and whiskers (nonoutlier minimum and maximum). Circles indicate outliers. (*B*) Individual levels of MMP-9/creatinine (open circles) and TIMP-1/creatinine (filled squares) ratios in the urine of children with pyelonephritis. (*C*) DMSA scores of acute DMSA scans (DMSA1, open boxes) and DMSA scans performed 1 y after acute pyelonephritis (DMSA2, hatched boxes) in children with urinary levels of MMP-9 ≥ TIMP-1 and MMP-9< TIMP-1. Children with MMP-9< TIMP-1 had significantly higher both acute and follow-up DMSA scores. Data presented as median, box (25% to 75%), and whiskers (nonoutlier minimum and maximum). Circles indicate outliers.

A correlation between MMP-9/creatinine ratios and leukocyturia (R 0.55, $p < 0.01$) was seen, likewise there was a correlation between TIMP-1/creatinine ratios and maximal serum CRP (R 0.75, $p < 0.01$), and leukocyturia (R 0.67, $p < 0.01$).

MMP-9 and TIMP-1 produced mainly by leukocytes. TIMP-1 produced also by tubular cells. The total MMP-9 levels in renal tissue homogenates from infected mice were significantly higher in comparison to those from uninfected mice ($p < 0.01$) (Fig. 2). The maximal levels of MMP-9 were found on the 2nd d from inoculation of bacteria with decrease on the 6th d thereafter. Immunohistochemically, both MMP-9 and TIMP-1 were present mainly in the cytoplasm of leukocytes. Moreover, TIMP-1 could be seen also in the cytoplasm of proximal tubular cells of infected kidneys (Fig. 3).

Increased MMP-9/TIMP-1 ratio in mesangial cells and monocytes but not in epithelial cells in response to E. coli. Human THP-1 monocytes produced significantly higher amount of MMP-9 after stimulation with *E. coli* compared with nonstimulated cells ($p < 0.05$). On the other hand TIMP-1 production by the cells was suppressed by *E. coli* ($p < 0.05$), which resulted in a significant net increase of the MMP-9/TIMP-1 ratio ($p < 0.05$) (Fig. 4). Neither mesangial nor epithelial cells produced MMP-9. The TIMP-1 production was suppressed by the *E. coli* stimulation in mesangial ($p < 0.05$) while maintained in epithelial cells. For all the analyzed cells, MMP-9/TIMP-1 mRNA changes had the same pattern as the protein levels.

DISCUSSION

In the present study we demonstrate for the first time an increase of MMP-9 and TIMP-1 in the urine of children with acute pyelonephritis. Using our animal model and cell culture experiments, we show that during pyelonephritis both of these proteins were produced mainly by leukocytes. Resident renal cells reacted differently after stimulation with bacteria. In

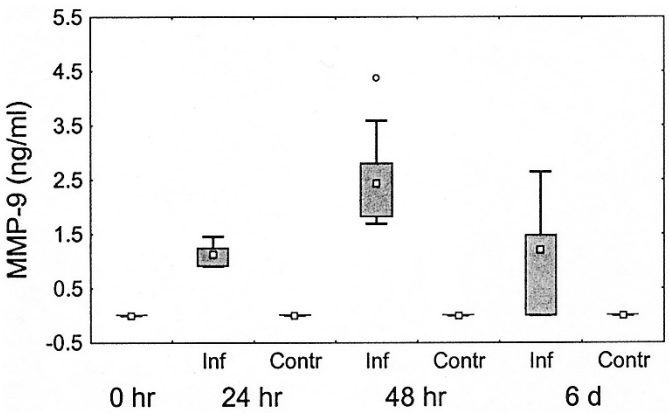

Figure 2. MMP-9 levels in kidney homogenates from infected (Inf) and saline injected uninfected mice (Contr) at 0, 24, 48 h, and 6 d (3–5 mice in each group). Data presented as median, box (25% to 75%), and whiskers (nonoutlier minimum and maximum). The circle indicates an outlier.

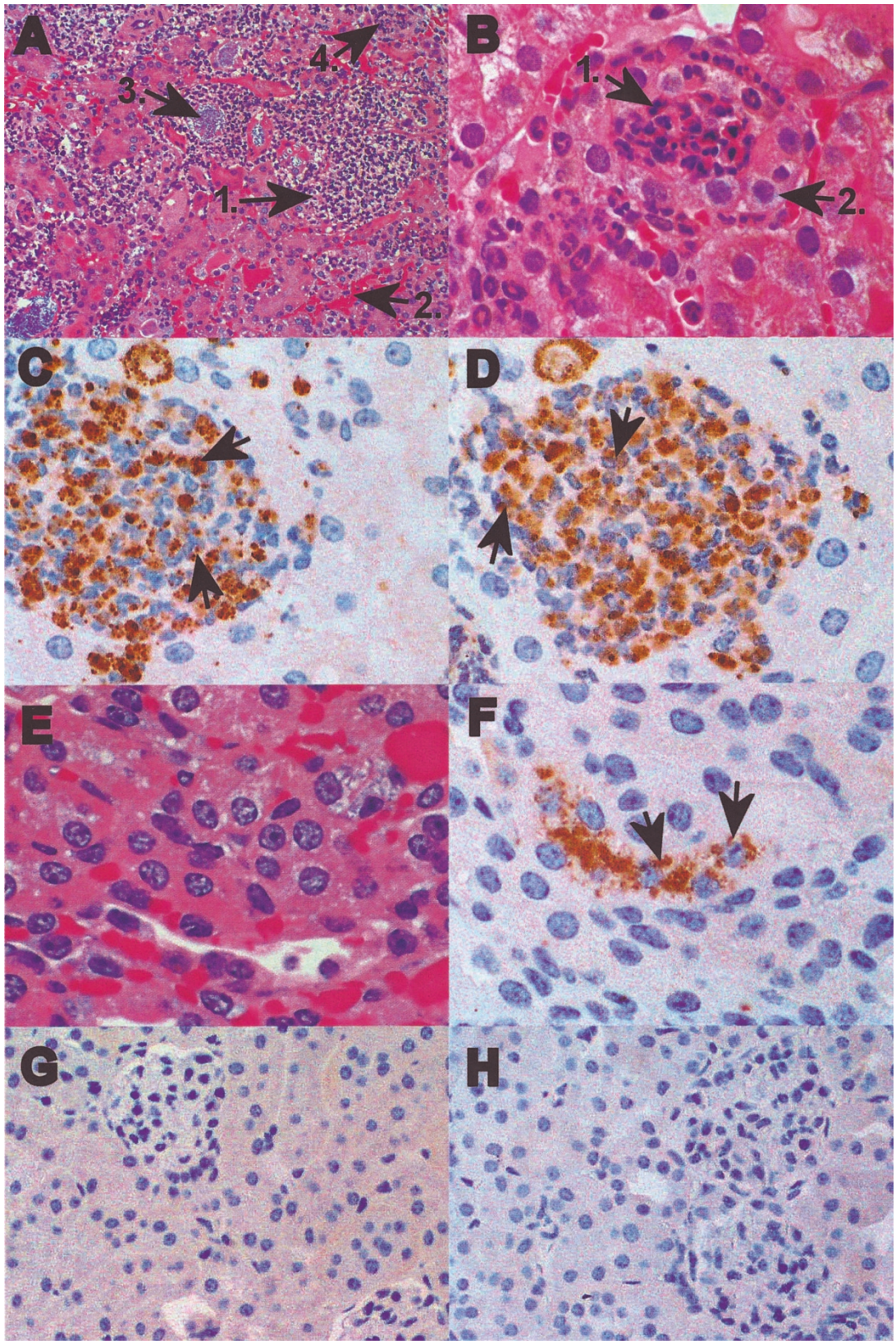

Figure 3. Mouse kidney sections from infected (*A*, *B*, *C*, *D*, *E*, *F*) and uninfected mice (*G*, *H*). Cortical areas of the kidney with the destruction of normal parenchyma and accumulation of leukocytes (A 1.), intercellular bleeding (A 2.), accumulation of bacteria (A 3.), and dilated tubuli full of leukocytes, leukocyte casts (A 4.). Detail of a leukocyte cast (*B*) with polymorphonuclear leukocytes (B 1.) and edematous proximal tubular cells (B 2.). Both MMP-9 (*C*) and TIMP-1 (*D*) are expressed mainly in the cytoplasm of polymorphonuclear leukocytes and extracellularly (*arrows*) in the areas with leukocyte casts. TIMP-1 could be seen also in the cytoplasm of tubular cells (*F*, *arrows*). E shows a corresponding area of the kidney to clarify the morphology of cells and to show the absence of leukocytes in the area. Hematoxylin-eosin staining (*A*, *B*, *E*, nuclei stained blue, cytoplasm pink, erythrocytes red) and immunohistochemical staining of MMP-9 (*C*, *G*, brown) and TIMP-1 (*D*, *F*, *H*, brown) with hematoxylin contrastaining (nuclei stained blue). Magnification 100× (*A*),; 200× (*B*, *C*, *D*, *G*, *H*) and 400× (*E*, *F*). There was no staining in control slides with normal rabbit immunoglobulin instead of primary antibody (not shown).

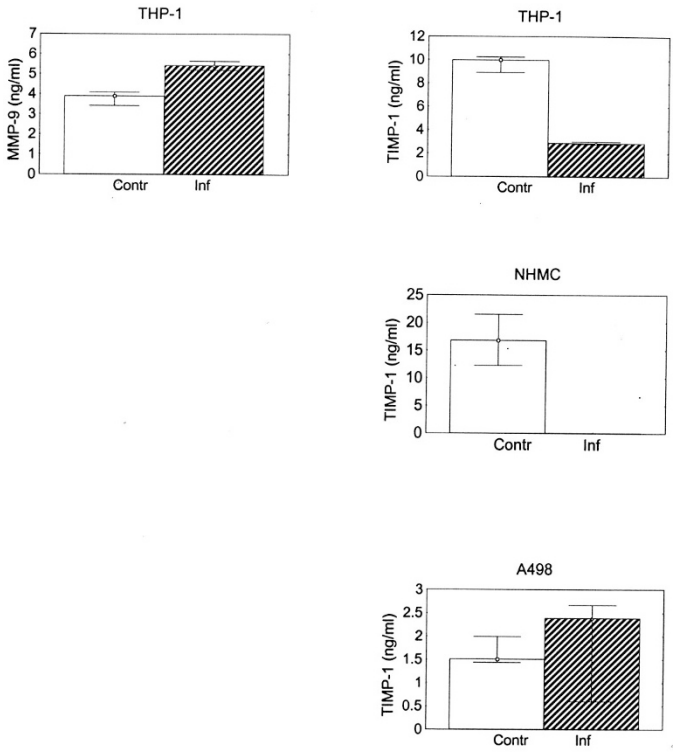

Figure 4. THP-1 human monocytes, normal human mesangial cells (NHMC), and human renal epithelial cells (A498) stimulated by *E. coli* CFTO73 for 24 h. MMP-9 (*left*) and TIMP-1 production (*right*). Open bars show protein levels from unstimulated cells (Contr), hatched bars from cells after stimulation (Inf). Incubation by *E. coli* caused increased expression of MMP-9 and decreased expression of TIMP-1 in monocytes. No MMP-9 protein production was observed in either mesangial or epithelial cells (data not shown). Stimulation by *E. coli* suppressed constitutive production of TIMP-1 by mesangial cells with no significant change on epithelial cells. Protein levels are expressed as median and range from three experiments.

mesenchymal cells, TIMP-1 production was decreased while maintained in tubular cells, probably as a protective mechanism during inflammation. Moreover, significantly more severe acute tissue damage and renal scarring was found in children with urinary levels of TIMP-1 higher than MMP-9.

Acute pyelonephritis is one of the most common serious infections in infancy and early childhood. Without prompt and accurate treatment, it may lead to permanent damage to the kidneys. Postinfective renal scarring can lead to loss of renal function and is still a worldwide major cause of end-stage renal failure (25). An imbalance between MMP-9 and TIMP-1 has been suggested to be important in the pathogenesis of a number of diseases (15, 26–29). Up-regulation of MMP/TIMP expression in renal tubular epithelial cells and renal fibroblasts has been demonstrated in both human and murine polycystic kidney disease (30–32), diabetic nephropathy (26), and membranous nephropathy (27). On the contrary, MMP-9/TIMP-1 down-regulation has been shown in rat models of streptozocin-induced diabetic nephropathy (28) and bromethylamine-induced nephropathy (33), and in a mouse model of renal scarring due to overload proteinuria (34). Increased urinary levels of MMP-9 and TIMP-1 in our study suggest that these proteins play a role also in acute pyelonephritis.

Out of 40 children with pyelonephritis, 23 had urinary TIMP-1 levels higher than the corresponding MMP-9 levels. These children had significantly more severe changes in both acute and follow-up DMSA scans indicating higher degree of acute tissue damage and renal scarring. This result seems to be contradictory to the hypothesis about an association between MMP-9 and a large destruction of the tissue as the prerequisite of replacement by collagen and subsequent scar development. One possible explanation of our findings can be that the measured levels did not reflect the "induction phase" of renal scarring but the "phase of matrix synthesis" which is characterized by decreased gelatinolytic activity with accumulation of collagen. Another speculation might be that urinary levels might not always correspond to renal levels of the protein. Similarly urinary leukocyte levels do not always reflect tissue concentration of leukocytes. Leukocyte "entrapment" in renal tissue, not high urinary leukocyte levels, was associated with renal scarring (35, 36). And finally, it cannot be ruled out that "low-grade" prolonged inflammation, probably accompanied by lower MMP-9/TIMP-1 ratio, is a greater risk for renal scarring than "high-grade" shorter inflammation. MMP-9 is known to be able to digest the precursors of IL-1β and TNF-α so it could be speculated that low levels of MMP-9 might promote the persistence of inflammation.

On the other hand, our findings support the hypothesis about an association between TIMP-1 and renal fibrosis. TGF-β, a pro-fibrotic agent, has been shown to induce TIMP-1 expression. These results imply that a link between TGF-β and TIMP-1 might be of importance in the pathogenesis of renal scarring (33, 37, 38).

Both MMP-9/creatinine ratios and TIMP-1/creatinine ratios in our study correlated with the degree of leukocyturia. Anyway, the MMP-9/TIMP-1 ratio does not seem to be a mere reflection of the leukocyte load since TIMP-1/creatinine ratios correlated with DMSA changes, which was not the case of leukocyturia. TIMP-1 in the urine may thus originate also from epithelial cells and leukocytes in renal tissue and not only from cells present in the urine.

In the present study, using an animal model and cell experiments we show that the main producers of MMP-9 and TIMP-1 during pyelonephritis are leukocytes, while TIMP-1 can be produced also by renal tubular and mesenchymal cells. MMP-9 was found in the kidneys of infected mice but not in kidneys from uninfected short time obstructed ones. The maximal levels of MMP-9 were found on the 2nd d from inoculation of bacteria with decrease on the 6th d thereafter.

Previously in the same experimental model, we have shown prolonged increase of TGF-β mRNA as well as pro-inflammatory cytokines after short time obstruction only (22). However, others have demonstrated that longer time of obstruction was needed to induce renal fibrosis (39). MMP-9 and TIMP-1 are known to be produced by leukocytes and also by a number of resident kidney cells stimulated with cytokines and growth factors (7–11, 22). In a rat model of unilateral ureteral obstruction, MMP-9 was detected mainly in the cytoplasm of tubular cells (29). We found a correlation between urinary MMP-9 and TIMP-1 levels and leukocyturia in children with acute pyelonephritis. These results are in line with

our cell experiments and immunohistochemistry where leukocytes seem to be the main source of MMP-9 and TIMP-1 during pyelonephritis.

Monocytes stimulated with *E. coli* showed increased MMP-9 and decreased TIMP-1 production. To our knowledge this is the first evidence suggesting that bacteria suppress TIMP-1 production. We have found a similar pattern of TIMP-1 production in mesangial cells but conversely renal tubular cells maintained production of TIMP-1. MMP-9 was detected neither in epithelial nor mesangial cells. All observed events after stimulation with bacteria seem to result in increased MMP-9/TIMP-1 ratio in monocytes and mesangial cells but not in epithelial cells.

For the cell experiments, we used only 24-h incubation with bacteria due to decreased cell viability after longer stimulation (data not shown). It has been shown previously that prolonged stimulation of mesangial cells and epithelial cells by cytokines and growth factors was needed to induce MMP-9 production (9, 10, 40). However, our findings are in line with the immunohistochemistry results where MMP-9 was seen only in the cytoplasm of leukocytes.

Renal tubular cells have important functions in the metabolism and homeostasis. Therefore TIMP-1 production may be a protection against digestion by leukocytes and mesenchymal cells during inflammation. Similarly, greater amounts of TIMP-1 mRNA were found in mouse corneas after *Pseudomonas aeruginosa* infection. Moreover, anti TIMP-1 treatment was associated with loss of epithelium within the central cornea (41).

CONCLUSIONS

We conclude that MMP-9 and TIMP-1 are produced during acute pyelonephritis. They are both expressed in professional immune cells as well as in resident renal cells, although apparently different kidney cells play different roles in the process of inflammation and healing. Interestingly, it seems that increased MMP-9/TIMP-1 is not a factor inducing renal scarring. Therefore possible treatment attempts trying to inhibit MMP-9 activity may not be beneficial for the patient. On the contrary, scarring was associated with decreased MMP-9/TIMP-1 ratio, which may favor an accumulation of collagen. Further understanding of the protease/antiprotease system in the kidney is needed to allow us to influence the outcome of renal diseases.

Acknowledgments. We thank Denis Stygar and Lena Sahlin for antibodies and technical advice concerning immunohistochemistry.

REFERENCES

1. Muller GA, Zeisberg M, Strutz F 2000 The importance of tubulointerstitial damage in progressive renal disease. Nephrol Dial Transplant 15:76–77
2. Hewitson TD, Darby IA, Bisucci T, Jones CL, Becker GJ 1998 Evolution of tubulointerstitial fibrosis in experimental renal infection and scarring. J Am Soc Nephrol 9:632–642
3. Khalil A, Tullus K, Bakhiet M, Burman LG, Jaremko G, Brauner A 2000 Angiotensin II type 1 receptor antagonist (losartan) down-regulates transforming growth factor-beta in experimental acute pyelonephritis. J Urol 164: 186–191.
4. Heptinstall R 1992 End-stage renal disease. In: Heptinstall R (eds) Pathology of the kidney. Little, Brown and Company, Boston, pp 713–778
5. Eddy AA 2000 Molecular basis of renal fibrosis. Pediatr Nephrol 15:290–301
6. Ravanti L KV 2000 Matrix metalloproteinases in wound repair (Review). Int J Mol Med 6:391–407
7. Martin J, Eynstone L, Davies M, Steadman R 2001 Induction of metalloproteinases by glomerular mesangial cells stimulated by proteins of the extracellular matrix. J Am Soc Nephrol 12:88–96
8. Martin J, Steadman R, Knowlden J, Williams J, Davis M 1998 Differential regulation of matrix metalloproteinases and their inhibitors in human glomerular epithelial cells in vitro. J Am Soc Nephrol 9:1629–1637
9. Liu Y, Rajur K, Tolbert E, Dworkin LD 2000 Endogenous hepatocyte growth factor ameliorates chronic renal injury by activating matrix degradation pathways. Kidney Int 58:2028–2043
10. Eberhardt W, Beeg T, Beck KF, Walpen S, Gauer S, Bohles H, Pfeilschifter J 2000 Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells. Kidney Int 57:59–69
11. Johnson DW, Saunders HJ, Baxter RC, Field MJ, Pollock CA 1998 Paracrine stimulation of human renal fibroblasts by proximal tubule cells. Kidney Int 54:747–757
12. Paemen L, Jansen PM, Proost P, Damme JV, Opdenakker G, Hack E, Taylor FB 1997 Induction of gelatinase B and MCP-2 in baboons during sublethal and lethal bacteriaemia. Cytokine 9:412–415
13. John A, Tuszynski G 2001 The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. Pathol Oncol Res 7:14–23
14. Lalancette M, Aoudjit F, Potworowski EF, St-Pierre Y 2000 Resistance of ICAM-1-deficient mice to metastasis overcome by increased aggressiveness of lymphoma cells Blood 95:314–319
15. Leib SL, Leppert D, Clements J, Tauber MG 2000 Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. Infect Immun 68:615–620
16. Osman M, Tortorella M, Londei M, Quaratino S 2002 Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases define the migratory characteristics of human monocyte-derived dendritic cells. Immunology 105:73–82
17. Pilcher BK, Wang M, Qin XJ, Parks WC, Senior RM, Welgus HG 1999 Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. Ann NY Acad Sci 878:12–24
18. Song W, Jackson K, McGuire PG 2000 Degradation of type IV collagen by matrix metalloproteinases is an important step in the epithelial-mesenchymal transformation of the endocardial cushions. Dev Biol 227:606–617
19. Shanon A, Feldman W, McDonald P, Martin DJ, Matzinger MA, Shillinger JF, McLaine PN, Wolfish N 1992 Evaluation of renal scars by technetium-labeled dimercaptosuccinic acid scan, intravenous urography, and ultrasonography: a comparative study. J Pediatr 120:399–403
20. Tullus K, Fituri O, Linne T, Escobar-Billing R, Wikstad I, Karlsson A, Burman LG, Wretlind B, Brauner A 1994 Urine interleukin-6 and interleukin-8 in children with acute pyelonephritis, in relation to DMSA scintigraphy in the acute phase and at 1-year follow-up. Pediatr Radiol 24:513–515
21. Hedges SR, Bjarnadottir M, Agace W, Hang L, Svanborg C 1996 Immunoregulatory cytokines modify Escherichia coli induced uroepithelial cell IL-6 and IL-8 responses. Cytokine 8:686–697
22. Khalil A, Brauner A, Bakhiet M, Burman LG, Jaremko G, Wretlind B, Tullus K 1997 Cytokine gene expression during experimental Escherichia coli pyelonephritis in mice. J Urol 158:1576–1580
23. Janowska-Wieczorek A, Marquez LA, Matsuzaki A, Hashmi HR, Larratt LM, Boshkov LM, Turner AR, Zhang MC, Edwards DR, Kossakowska AE 1999 Expression of matrix metalloproteinases (MMP-2 and -9) and tissue inhibitors of metalloproteinases (TIMP-1 and -2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. Br J Haematol 105:402–411
24. Aguilar-Santelises M, Rottenberg ME, Lewin N, Mellstedt H, Jondal M 1996 Bcl-2, Bax and p53 expression in B-CLL in relation to in vitro survival and clinical progression. Int J Cancer 69:114–119
25. Wassner SJ, Baum M 1999 Chronic renal failure. Physiology and management. In: Barratt TM, Avner ED, Harmon WE (eds) Pediatric Nephrology. Lippincott Williams & Wilkins, Baltimore, pp 1155–1182
26. Zaoui P, Cantin JF, Alimardani-Bessette M, Monier F, Halimi S, Morel F, Cordonnier D 2000 Role of metalloproteases and inhibitors in the occurrence and progression of diabetic renal lesions. Diabetes Metab 26(suppl 4):25–29
27. McMillan JI, Riordan JW, Couser WG, Pollock AS, Lovett DH 1996 Characterisation of a glomerular epithelial cell metalloproteinase as matrix metalloproteinase-9 with enhanced expression in a model of membranous nephropathy. J Clin Invest 97:1094–1101
28. Baricos WH 1995 Chronic renal disease: do metalloproteinase inhibitors have a demonstrable role in extracellular matrix accumulation? Curr Opin Nephrol Hypertens 4:365–368
29. Gonzalez-Avila G, Iturria C, Vadillo-Ortega F, Ovalle C, Montano M 1998 Changes in matrix metalloproteinases during the evolution of interstitial renal fibrosis in a rat experimental model. Pathobiology 66:196–204
30. Nakamura T, Ushiyama C, Suzuki S, Ebihara J, Shimada N, Koide H 2000 Elevation of serum levels of metalloproteinase-1, tissue inhibitor of metalloproteinase-1 and type IV collagen, and plasma levels of metalloproteinase-9 in polycystic kidney disease. Am J Nephrol 20:32–36
31. Rankin CA, Suzuki K, Itoh Y, Ziemer DM, Grantham JJ, Calvet JP, Nagase H 1996 Matrix metalloproteinases and TIMPS in cultured C57BL/6J-cpk kidney tubules. Kidney Int 50:835–844

32. Rankin CA, Itoh Y, Tian C, Ziemer DM, Calvet JP, Gattone VH 1999 Matrix metalloproteinase-2 in a murine model of infantile-type polycystic kidney disease. J Am Soc Nephrol 10:210–217
33. Mo W, Brecklin C, Garber SL, Song RH, Pegoraro AA, Au J, Arruda JA, Dunea G, Singh AK 1999 Changes in collagenases and TGF-beta precede structural alterations in a model of chronic renal fibrosis. Kidney Int 56:145–153
34. Eddy A 2001 Role of cellular infiltrates in response to proteinuria. Am J Kidney Dis 37:S25–29
35. Hang L, Frendeus B, Godaly G, Svanborg C 2000 Interleukin-8 receptor knockout mice have subepithelial neutrophil entrapment and renal scarring following acute pyelonephritis. J Infect Dis 182:1738–1748
36. Hang L, Haraoka M, Agace WW, Leffler H, Burdick M, Strieter R, Svanborg C 1999 Macrophage inflammatory protein-2 is required for neutrophil passage across the epithelial barrier of the infected urinary tract. J Immunol 162:3037–3044
37. Duymelinck C, Dauwe SE, De Greef KE, Ysebaert DK, Verpooten GA, De Broe ME 2000 TIMP-1 gene expression and PAI-1 antigen after unilateral ureteral obstruction in the adult male rat. Kidney Int 58:1186–201
38. Engelmyer E, van Goor H, Edwards DR, Diamond JR 1995 Differential mRNA expression of renal cortical tissue inhibitor of metalloproteinase-1, -2, and -3 in experimental hydronephrosis. J Am Soc Nephrol 5:1675–1683
39. Chevalier RL, Thornhill BA, Wolstenholme JT, Kim A 1999 Unilateral ureteral obstruction in early development alters renal growth: dependence on the duration of obstruction. J Urol 161:309–313
40. Norman JT, Lewis MP 1996 Matrix metalloproteinases (MMPs) in renal fibrosis. Kidney Int Suppl 54:S61–63
41. Kernacki KA, Barrett R, Hazlett LD 1999 Evidence for TIMP-1 protection against P. aeruginosa-induced corneal ulceration and perforation. Invest Ophthalmol Vis Sci 40:3168–3176