PATTERN SECRETION OF MATRIX METALLOPROTEINSES AND THEIR BIOLOGICAL TISSUE INHIBITORS BY HUMAN GLOMERULAR MESANGIAL CELLS IN CULTURE

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ABSTRACT

The glomerular mesangial cells (GMC) play a central role in the synthesis and turnover of the glomerular mesangial matrix. The breakdown of the matrix likely depends on the balance between of a variety of proteinases including matrix metalloproteinases and their biological inhibitors secreted by the GMC, and any disturbance in the balance may result in appearance of various pathological states such as glomerulosclerosis. We therefore studied pattern secretion of matrix metalloproteinases (MMPs), MMP-1, MMP-2, MMP-3, MMP-9 and their biological tissue inhibitor of matrix metalloproteinases (TIMPs), TIMP-1 and TIMP-2 by cultured human GMC. We also measured MMP-1/TIMP-1 complex level in the cell culture supernatants. For this purpose, the GMC were incubated under serum-free conditions with medium (RPMI-1640) alone or in combination with TNF-α (30 ng/ml) or phorbol myristate acetate (PMA)(50 ng/ml) for exactly 24, 48 and 72 hours. The above parameters were assayed by established ELISA techniques. Our results showed that the lowest and largest secretions were related to MMP-9 and MMP-2, respectively. The results indicated that the MMPs and TIMPs secretion were increased by TNF-α (MMP-1, MMP-2, TIMP-1 and TIMP-2) and PMA (MMP-2, TIMP-1 and TIMP-2), significantly ($P<0.05$). These results suggest that the GMC can synthesis and release various MMPs and their inhibitors (TIMPs) that, in part, control turnover of extracellular matrix proteins.

Key words: Metalloproteinases, Mesangial cell, Glomerulosclerosis, Matrix

INTRODUCTION

Glomerular mesangial cells (GMC) have different potential roles including: structural support for the glomerulus and specially the capillary loops; generation and turnover of extracellular mesangial matrix; target site for vasoactive agents such as vasoconstrictors and vasodilators; target site for inflammatory mediators, growth factors and cytokines with effect on, for example, local hemodynamics, cell proliferation and matrix turnover; site of production of vasoactive mediators and growth modifying agents; site of production of various growth factors and cytokines such as PDGF, IL-1 and TGF-β; expression of chemokines and adhesion molecules; generation of plasminogen activator and inhibitors; handling of macromolecules such as lipids, immune complexes and advanced glycation endproducts (AGE) (1). Furthermore, the mesangial cells are actively involved in different glomerular diseases including IgA nephropathy, non-IgA mesangioproliferative glomerulosclerosis, membranoproliferative glomerulosclerosis, lupus nephritis, diabetic nephropathy, focal glomerulosclerosis and variants of minimal change disease (2). The key features of these diseases are mesangial cell proliferation and/or mesangial matrix accumulation (3,4). Therefore, the study of factors that affect mesangial cells proliferation and mesangial matrix turnover are important to understand the pathogenesis and to design new therapeutic approaches for glomerular diseases (4). The turnover of the glomerular mesangial matrix is dependent on a balance between its synthesis and degradation, and a change in either of these dynamic parameters will potentially results in morphological and functional changes within the glomerulus (5). Since secretion of glomerular mesangial cell-derived matrix

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metalloproteinses (MMPs) and their biological inhibitors (TIMPs) may play an important role in the turnover of the glomerular extracellular matrix under basal and pathological conditions (6), the study on pattern release of MMPs and TIMPs by human glomerular mesangial cells into cell culture would be necessary. However, since the GMC might be secret the MMPs and/or TIMPs at an undetectable level, the GMC were exposed to stimulator factors, TNF-α and PMA for increasing secretion of MMPs and TIMPs.

MATERIALS AND METHODS

Reagents:

Amphotricin B, Penicillin, streptomycin, phorbol 12-myristate 13-acetate (PMA) and DNA (from Calf Thymus) were purchased from Sigma Chemical Co. (St. Luis, MO, USA). Fetal Calf Serum (FCS) and PBS were from Biochrom KG (Berlin, Germany). The medium RPMI 1640 with L-glutamine was from BioWhitttaer (MD, USA) and human recombinant TNF-α was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Hoechst 33258 were purchased from Fluka (Deisenhofen, Germany). ELISA kits for measurement of the MMPs and TIMPs were purchased from Amersham International Plc (Buckinghamshire, England).

Methods:

Isolation of glomerular mesangial cells: Human Glomerular mesangial cells were isolated from nephroctomized patients with kidney tumor. Human mesangial cells were obtained from primary glomerular explants which were isolated using a graded-sieve technique and were plated from culture in RPMI 1640 tissue culture medium supplemented with 20% (V/V) FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotricin B.

Identification of glomerular mesangial cells: The identity of the mesangial cells was confirmed by the following criteria: (1) morphology; (2) survival in a medium containing D-valine substituted for L-valine (excluding fibroblastic cells); (3) resistance to puromycin aminonucleoside (10 μg/mL) but susceptibility to mitomycin C (10 μg/mL) (excluding epithelial cells); (4) presence of receptors specific to angiotensin II and contraction in response to angiotensin II; (5) Positive staining for smooth muscle α-actin; (6) negative staining for factor VIII (excluding endothelial cells) and cytokeratin (excluding epithelial cell).

Culture of glomerular mesangial cells:

The mesangial cells were cultivated in 25-cm^2 flasks and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The cells after four to eight passages were used for this experiment.

Experimental conditions:

After the cells reached 95% confluency, plates were randomly assigned to one of the following experimental conditions: (1) RPMI 1640 medium only; (2) the medium containing PMA (50 ng/mL); (3) the medium containing TNF-α (30 ng/mL); (4) the medium containing dimethyl sulfoxide (DMSO) as solvent of PMA. Before starting of the experiments, the confluent cultures were washed twice with serum-free medium and incubated under serum-free conditions for 24 hours. Subsequently, the above chemicals were added into the medium and the cultures were incubated exactly for 24, 48 and 72 hours. After end of incubation time, the supernatants were collected and centrifuged. The cell-free supernatants were transferred into other clean tubes and stored at less than -80 °C until assay. The cultures were washed with PBS; then, the cells were harvested by trypsination, for assay of DNA content.

Measurement of DNA:

For assay of DNA, the harvested cells were lysed by sonication and then DNA contents were measured using Hoechst 33258 dye, at the wavelengths of 355 nm for excitation and 460 nm for emission, spectrofluorometrically.

Measurement of MMPs and TIMPs:

In this study, the levels of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2 and MMP-1/TIMP-1 complex were assayed by established ELISA techniques, using ELISA kits for MMPs and TIMPs prepared from Amersham International Plc (BIOTRAK™). Then, the concentrations of MMPs and TIMPs were calculated on the basis of the DNA content (ng/μg of DNA).

Statistical analysis:

Results were expressed as the mean ± SEM. The $P<0.05$ was considered to be significant. For

statistical analysis of results by Student *t*-Test and Mann-Whitney *U*-Test, the GrapPad software version 2.01 (GraphPad Software Inc, San Diego, CA) was used.

RESULTS

Matrix metalloproteinases (MMPs) are responsible for the breakdown of extracellular matrix proteins. The activity of these enzymes is inhibited by biological tissue inhibitors such as TIMP-1 and TIMP-2 (3). However, imbalance between MMPs and TIMPs may result in development of various pathological states in different organs such as kidney. Therefore, we studied the release of MMPs and TIMPs by the glomerular mesangial cells (GMC). The cells were exactly incubated for 24, 48 and 72 h under serum free conditions with or without TNF-α, PMA or DMSO as solvent. Then, the conditioned media were collected for assay of MMPs and TIMPs by ELISA technique and the cells were harvested by trypsination for counting the cells and measurement of DNA. Our results show that the GMC secret MMP-1 at a detectable level, time-dependently, and this secretion can stimulate by TNF-α at a significant level ($p<0.05$), whereas PMA can increase the release of MMP-1, but this effect in not significant (figure 1). As shown in figure 2, secretion of TIMP-1 is elevated by TNF-α and PMA, significantly. Our results indicate that the release of MMP-2 and TIMP-2 as TIMP-1 can stimulate by TNF-α and PMA, but these effects are significant only after 24 and 48 hours (See figures 3 and 4). The results show that the ratios of MMP-1/TIMP-1 and MMP-2/TIMP-2 are not affected by TNF-α and PMA except that the effects of TNF-α on MMP-1/TIMP-1 ratio after 24 h incubation (Figures 5 and 6). TNF-α can decrease this ratio significantly ($P<0.05$), because the effect of TNF-α on TIMP-1 is more than on MMP-1, especially after 24 h incubation. The present results show that the GMC secrets MMP-3 (Stromlysin-1) and MMP-9 (Gelatinase B) at detectable levels, but these levels are not affected by TNF-α and PMA (figure 7). In this study, we also measured the concentration of MMP-1/TIMP-1 complex in the supernatants; however, the results show that this index is not affected by TNF-α and PMA (Figure 7). Finally, our results indicate that the GMC secrete MMP-2 more than the other MMPs and TIMPs, normally. The order secretion of MMPs and TIMPs that measured after 72 h incubation is as follow: MMP-2>TIMP-1>TIMP-2>MMP-3>MMP-1>MMP-9. (See figures 1 to 7).

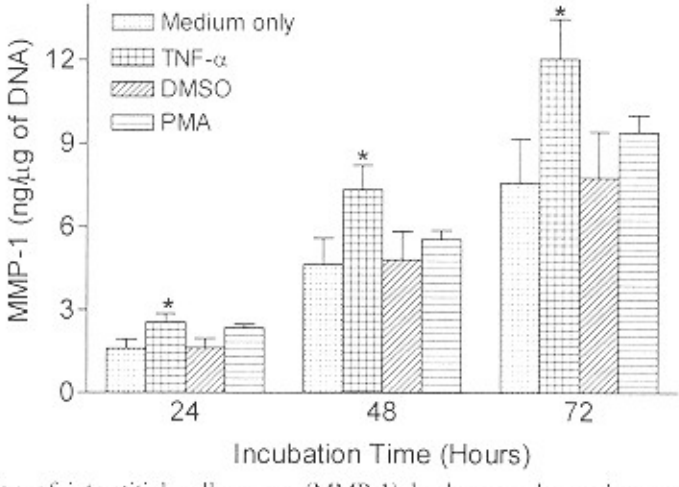

Fig 1. Pattern secretion of interstitial collagenase (MMP-1) by human glomerular mesangial cells into the culture (n=10). The results were shown as mean±SEM. For details, refer to the materials and methods section. *P<0.05: as compared to medium group, evaluated by student *t*-test and Mann-Whitney U-test.

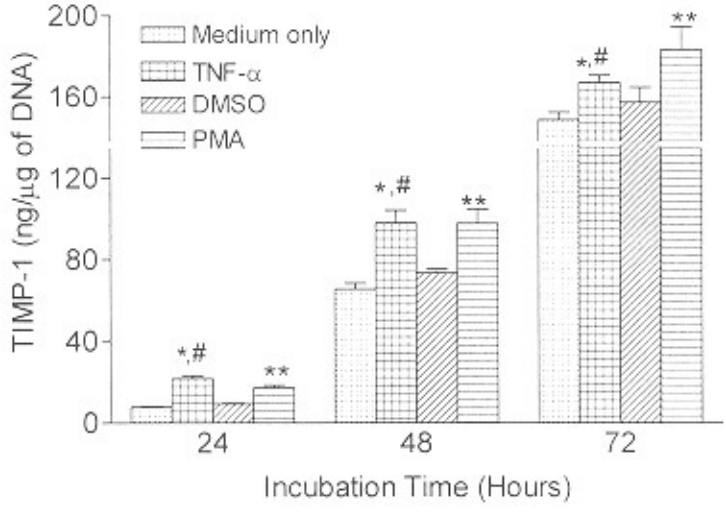

Fig 2. Pattern secretion of tissue inhibitor matrix metalloproteinase-1 (TMIP-1) by human glomerular mesangial cells into the culture (n=10). The results were shown as mean±SEM. For details, refer to the materials and methods section. *P<0.05, #P<0.01: as compared to medium group, evaluated by student's *t*-test and Mann-Whitney U-test, respectively. **p<0.05: as compared to DMSO group, evaluated by student's *t*-test.

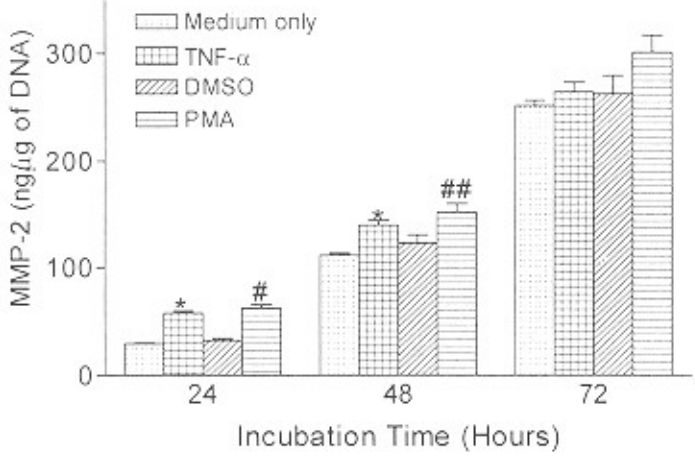

Fig 3. Pattern secretion of gelatinase a (MMP-2) by human glomerular mesangial cells into the culture (n=10). The results were shown as mean±SEM. For details, refer to the materials and methods section. *P<0.001: as compared to medium group, evaluated by student *t*-test and mann-whitney *u*-test. #p<0.001, ##p<0.03: as compared to DMSO group, evaluated by student's *t*-test and Mann-Whitney U-test, respectively.

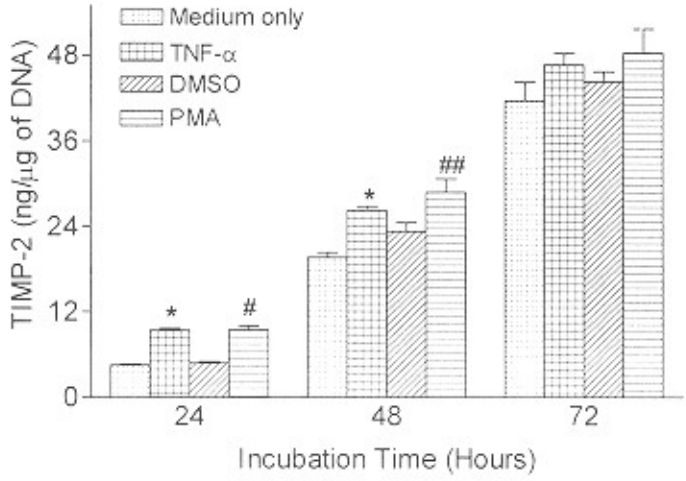

Fig 4. Pattern secretion of tissue inhibitor matrix metalloproteinase-2 (TIMP-2) by human glomerular mesangial cells into the culture (n=10). The results were shown as mean±SEM. For details refer to the materials and methods section. *P<0.001: as compared to medium group, evaluated by student t-test and Mann-Whitney U-test. #P<0.001, ##P<0.05: as compared to DMSO group, evaluated by student's t-test and Mann-Whitney U-test, respectively.

DISCUSSION

A common feature of most chronic forms of glomerulonephritis is the excessive accumulation of multiple glomerular extracellular matrix (ECM) proteins, a process leading to glomerulosclerosis and loss of filtration function (7). As a consequence of its central location within the glomerulus, the intrinsic mesangial cell plays a key role in both synthesis and degradation of the ECM (7). However, the turnover of the glomerular ECM is dependent on a balance between its synthesis and degradation (5). The breakdown of the matrix likely depends on the synthesis and secretion of a variety of proteinases and of these the best candidates are the matrix metalloproteinases (MMPs) that are active in the extracellular milieu (5). Therefore, the study on the secretion of MMPs and their inhibitors, tissue inhibitor matrix metalloproteinases (TIMPs), by mesangial cells could help to understand the pathogenesis of various glomerular diseases.

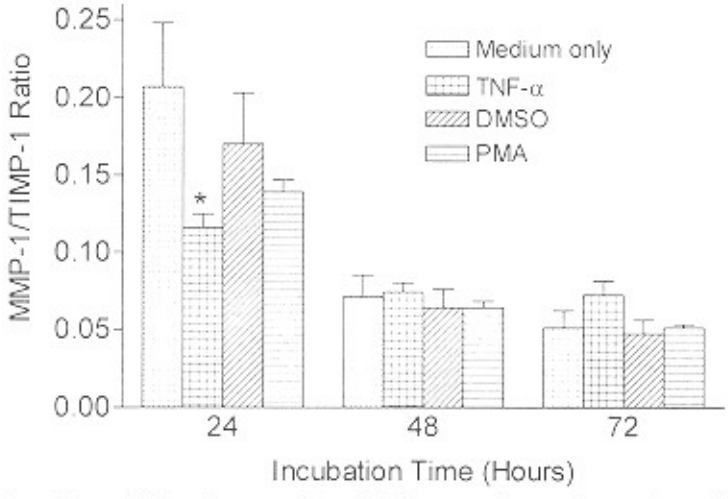

Fig 5. Pattern secretion of interstitial collagenase/tissue inhibitor matrix metalloproteinase-1 (MMP-1/TIMP-1) ratio by human glomerular mesangial cells into the culture (n=10). The results were shown as mean±SEM. For details, refer to the materials and methods section. *P<0.05: as compared to medium group, evaluated by student *t*-test.

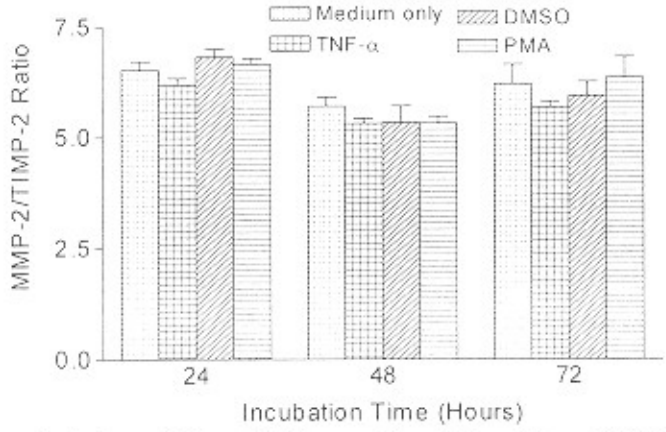

Fig 6. Pattern secretion of gelatinase A/tissue inhibitor matrix metalloproteinase-2 (MMP-2/TIMP-2) ratio by human glomerular mesangial cells into the culture (n=10). The results were shown as mean±SEM. For details, refer to the materials and methods section. There were no significant differences between the groups.

There are three main subgroups of MMPs (8): interstitial collagenases, stromelysins and gelatinases. Three distinct collagenases have been identified including interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13), which specifically cleave the triple helix of collagens type I, II and III (9). Stromelysins, of which three have been described such as stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) that degrade laminin, fibronectin, and non-fibrillar collagens (types IV and V collagen) and stromelysin-3 (MMP-11) that cleave serpin (10). Finally, two type of gelatinases have been identified, a 92-kD type IV collagenase (gelatinase B or MMP-9) (11) produced mainly by neutropils and macrophages and a 72-kD type IV collagenase (gelatinase A or MMP-2) (12) produced mainly by connective tissue and mesangial cells (6, 13). MMP-2 cleave the type IV and V collagens and fibronectin but MMP-9 cleave the type IV and V collagens only (10). The expression of MMPs is regulated by a wide variety of compounds including inflammatory cytokines such as interleukin-1 (14), growth factors such as TGF-β (15) as well as the tumor-promoting agent PMA (16). The second level of control of MMPs activity is achieved through the secretion of specific biological inhibitors (TIMPs), of which two have been described (17, 18). These inhibitors (TIMP-1 and TIMP-2) inhibit active MMPs by binding in a 1:1 molar ratio to form tight non-covalent complexes. A more complex interaction exists between TIMP-2 and MMP-2 in which the native inhibitor binds selectively to the latent proenzyme in a region distinct from its inhibitory binding site. TIMP-1 has a similar association with MMP-9 (19). These inhibitors are also highly regulated by cytokines and growth factors. Thus, the net amount of matrix layed down in tissue depends on the relative concentration of active enzyme and natural inhibitor (5). Therefore, in this study, we investigated the pattern secretion of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 by human GMC into cell culture and concentration of MMP-1/TIMP-1 complex into the supernatants. Our results demonstrated that GMC can secret the above MMPs and TIMPs at detectable levels and the release of some of them may affect by TNF-α and/or PMA (Refer to Results section). The role of MMP-1, MMP-3 and MMP-9 secreted by GMC is not exactly clear in the glomerulus. The present results showed that GMC secret MMP-2 at a high level. This may be described the central role of GMC in different glomerular diseases such as IgA nephropathy, membranoproliferative glomerulosclerosis, variants of minimal change disease and focal sclerosis (3). Lovett et al. and Marti et al. have recently demonstrated a close temporal linkage between mesangial cell activation (augmented proliferation and synthesis of ECM proteins) and the enhanced secretion of the 72-kD gelatinase A (MMP-2) in a model of immune complex-mediated glomerulonephritis (20, 21). The linkage between cellular activation and enhanced MMP-2 expression suggested that a primary action of this enzyme might be a direct effect upon the cells, with subsequent induction of the inflammatory phenotype (13). MMP-2 acts not only as a matrix- degrading enzyme but also directly stimulates growth by glomerular

mesangial cells with assumption of an inflammatory phenotype characterized by increased synthesis of prosclerotic interstitial collagens (22). Our results indicated the GMC secretes TIMP-1 and TIMP-2 at detectable concentrations. These results indicate that GMC are capable of controlling the activity of its own secreted enzymes (MMPs) (7). However, the values of MMPs and TIMPs secreted by GMC and the ratios of MMP-1/TIMP-1 and MMP-2/TIMP-2 may affect by some of agents including TNF-α, PMA, cyclosporine and tacrolimus and pathological states (4, 23). Finally, the more secretion of TIMPs can inhibit the degradation of ECM by MMPs, and then deposit the ECM resulting in glomerulosclerosis. Therefore, the balance between MMPs and TIMPs has an important role in developing of glomerular damages.

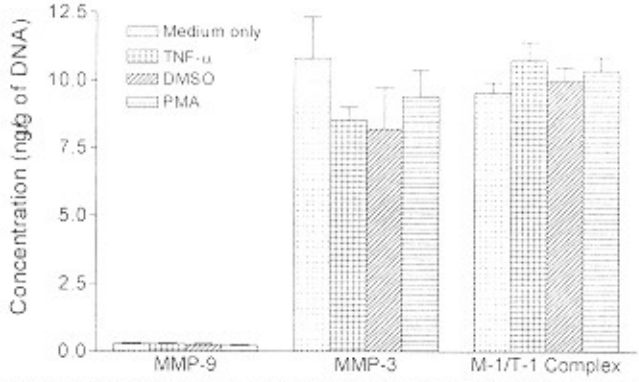

Fig 7. Pattern secretion of MMP-3 and MMP-9 by human glomerular mesangial cells and MMP-1/TIMP-1 (M-1/T-1) complex level as ng/µg of DNA into the culture after 72 h incubation (n=10). The results were shown as Mean ± SEM. For details, refer to the materials and methods Section. There were no significant differences between the groups.

REFERENCES

1. Schlondorff, D. (1996) Roles of the mesangium in glomerular function. Kidney Int. 49: 1583-1585.
2. Turck, J., Pollock, A.S., Lee, L.K., Marti, H.P., Lovett, D.H. (1996) Matrix metalloproteinase-2 (gelatinase A) regulates glomerular mesangial cell proliferation and differentiation. J Bio. Chem. 271: 15074-15083.
3. Johnson, R.J., Lida, H., Alpers, C.E. (1991) Expression of smooth muscle cell phenotype by rat mesangial cells. J. Clin. Invest. 87: 847-858.
4. Eitner, F., Westerhuis, R., Burg, M. (1997) Role of interleukin-6 in mediating mesangial cell proliferation and matrix production in vivo. Kidney Int. 51:69-78.
5. Martin, J., Knowlden, J., Davies, M., Williams, J.D. (1994) Identification and independent regulation of human mesangial cell metalloproteinases. Kidney Int. 46:877-885.
6. Nagase, H. (1994) Matrix metalloproteinases. Kidney Contrib. Nephrol. 107: 85-93.
7. Marti, H.P., Mcneil, L., Davies, M., Martin, J., Lovett, D.H. (1993) Homology cloning of rat 72 kDa type collagenase: cytokine and second-messenger inducibility in glomerular mesangial cells. Biochem. J. (Great Britain) 291: 441-446.
8. Woessner, J.F. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5:2145-2154.
9. Gross, J., Harper, E.D., McCroskery, P.A., Highberger, J.H., Corbett, C., Kang, A.H. (1974) Animal collagenases, specificity of action and structure of the substrate cleavage site. Biochem. Biophys. Res. Commun. 61: 605-612.
10. Brown, P.D., Giavazzi, R. (1995) Matrix metalloproteinase inhibition: a review of anti-tumor activity. Ann. Oncol. 6: 967-974.
11. Huhtala, P., Tuuttila, A., Chow, L.T., Lohi, J., Keski-Oja, J., Tryggvason, K. (1991) Complete structure of the human gene for 92 kD type IV collagenase. J. Biol. Chem. 266: 16485-16490.
12. Matrisian, L.M. (1992) The matrix-degrading metalloproteinases. Bioassays 14: 455-463.

13. Turck, J., Pollock, A.S., Lee, L.K., Marti, H.P., Lovett, D.H. (1996) Matrix metalloproteinase 2 (gelatinase A) regulates glomerular mesangial cell proliferation and differentiation. J. Biol. Chem. 271: 15074-15083.
14. Katsura, M., Ito, A., Hirakawa, S., Mori, Y. (1989) Human recombinant interleukin 1α increases biosynthesis of collagenase and hyaluronic acid in cultured human chorionic cells. FEBS Lett. 244: 315-318.
15. Salo, T., Lyons, J.G., Rahemtulla, F., Birkedal-Hansen, H., Larjava, H. (1991) Transforming growth factor β1 upregulates type IV collagenase expression in cultured human keratinocytes. J. Biol. Chem. 266:11436-11441.
16. Salo, T., Turpeeniemi-Hujanen, R., Tryggvason, K. (1985) Tumor-promoting esters and cell proliferation stimulate secretion of basement membrane (Type IV) collagen-degrading metalloproteinase by human fibroblasts. J. Biol. Chem. 260:8526-8531.
17. Dochery, A.J.P., Lyons, A., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J.R. (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 318: 66-69.
18. Stetler-Stevenson, W.G., Brown, P.D., Onista, M., Levy, A.T., Liotta, L.A. (1990) Tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem. 265:13933-13938.
19. Parsons, S.L., Watson, S.A., Brown, P.D., Collins, H.M., Steele, R.J.C. (1997) Matrix metalloproteinases. Br. J. Sur. 84:160-166.
20. Lovett, D.H., Johnson, R.J., Marti, H.P., Martin, J., Davies, M., Couser, W. (1992) Structural characterization of the mesangial cell type IV collagenase and enhanced expression in a model of immune complex-mediated glomerulonephritis. Am. J. Pathol. 141: 85-98.
21. Marti, H.P., Lee, L., Kashgarian, M., Lovett, D.H. (1994) Transforming growth factor-beta 1 stimulates glomerular mesangial cell synthesis of the 72-kD type IV collagenase. Am. J. Pathol. 144: 82-94.
22. Steinmann-Niggli, K., Lukes, M., Marti, H.P. (1997) Rat mesangial cells and matrix metalloproteinase inhibitor: inhibition of 72-kD IV collagenase (MMP-2) and of cell proliferation. J. Am. Soc. Nephrol. 8: 395-405.
23. Hampel, G., Hosseini, R., Jung, K. (1999) Different effects of cyclosporine and tacrolimus on the activation of mesangial metalloproteinases and their inhibitors. Transplant. Proc. 31: 2757-2758.