Endothelin is a potent inhibitor of matrix metalloproteinase-2 secretion and activation in rat mesangial cells

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Endothelin is a potent inhibitor of matrix metalloproteinase-2 secretion and activation in rat mesangial cells. Am J Physiol Renal Physiol 280: F628–F635, 2001.—We examined the effects of endothelin (ET) on the activity of matrix metalloproteinase-2 (MMP-2) in cultured MCs. Addition of the ETA receptor antagonists or neutralizing anti-endothelin antibody into MC cultures marked the secretion and activation of MMP-2. On the contrary, addition of the exogenous ET-1 into MC culture significantly inhibited the synthesis of MMP-2 in both basal and cytokines (tumor necrosis factor-α and interferon-γ) plus lipopolysaccharide-stimulated conditions. Furthermore, pretreatment of cells with exogenous ET-1 obviously prevented cytochalasin D-elicted activation of MMP-2, an effect that was completely abolished by ETA receptor antagonist, FR139317. In addition, ET-1 was found to be able to suppress the expression of membrane type-1 MMP (MT1-MMP) and promote the conversion of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) from cell associated form to secreted form. The addition of recombinant TIMP-2 into the culture abrogated dose-dependently the cytochalasin D-elicted activation of MMP-2. These results suggest that ET is a potent inhibitor of MMP-2 secretion and activation in MCs. These novel findings may help us understand the subtle regulation of the synthesis and activation of MMP-2 in MCs. It also provides us with further insight into the pathophysiological mechanisms involving ET in the regulation of matrix turnover in glomerulus.
diseases with matrix deposition (7,8). Treatment with a specific ET<sub>A</sub> receptor antagonist attenuates ECM accumulation and glomerulosclerosis in several models of kidney injury (5,6). Furthermore, induction of the synthesis of multiple extracellular matrix components via ET<sub>A</sub> receptor by ET in cultured MCs has been reported (18). Because matrix turnover represents both matrix synthesis and degradation, an alternative way of ET action on matrix accumulation might be the inhibition of matrix degradation, via regulation of the synthesis and/or activation of proteolytic enzymes, released by MCs or other cell types. In this respect, the suppressive effects of ET on the activity of fibrinolysis via upregulation of plasminogen activator inhibitor in cultured MCs have been documented (21). In addition, exogenous ET-1 was found to be able to inhibit the activity of collagenase in cultured cardiac fibroblast (19). Conceivably, ET might also be able to modulate the activity of collagenase in cultured cardiac fibroblasts.

**MATERIALS AND METHODS**

**Materials.** Gelatin, lipopolysaccharide (LPS), polyclonal rabbit anti-MMP-2 antibody, anti-tissue inhibitor of metalloproteinase-2 (TIMP-2) antibody, and synthetic human endothelin-1 were obtained from Sigma (St. Louis, MO). Monoclonal antibody to endothelin-1, -2, and -3 was purchased from Biogenesis. BQ-123 and IRL-1038 were from Fujisawa Pharmaceuticals (Osaka, Japan). Recombinant hTIMP-2 was purchased from Fuji Chemical (Toyama, Japan). An MMP-2 activity assay kit was obtained from Amersham Pharmacia (Piscataway, NJ). Microcon and Immobilon polyvinylidene difluoride membranes were supplied by Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Arlington Heights, IL).

**Rat MC culture.** MC isolation and culture were performed as previously described (40,41). In brief, the renal cortices of male Wistar rats (150 g) were homogenized under sterile conditions and passed over three sieves with pore sizes of 75-, 20-, and 7-μm. Glomeruli, which were retained on the 75-μm sieve, were seeded in DMEM containing 20% FCS, 100 U/ml). After three to four passages in DMEM containing 20% FCS, pure MC populations were obtained. MCs were seeded into 75-cm<sup>2</sup> flasks and allowed to grow in 20% FCS-DMEM until 90% confluence; then MCs were thoroughly washed with cold PBS at 4°C. The cells were lysed with RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing 25 μg/ml aprotinin, 2 mM sodium orthovanadate, 25 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 50 mM sodium fluoride for 30 min on ice. Lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C, and protein concentrations were determined by using a Bio-Rad protein assay kit. Equal amounts of cellular lysates or concentrated culture supernatants were separated in 7.5% SDS-polyacrylamide gels and electrotransferred to 0.4-mm-diffusion membrane. The membranes were blocked with 5% BSA in PBS-0.1% Tween 20, pH 7.4, overnight at 4°C. After washing with PBS-0.1% Tween 20, membranes were incubated with either anti-MMP-2 antibody (1:1,000) or anti-TIMP-2 (1:1,000) antibody at room temperature for 1 h.

**Western blots.** MCs were seeded onto 60-mm culture plates and allowed to grow in 20% FCS-DMEM until 90% confluence. MCs were then starved in serum-free DMEM for 2 days, before being stimulated with different agents for various periods of time. The reaction was terminated by washing cells rapidly with cold PBS at 4°C. The cells were lysed by 10.220.33.3 on October 29, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.physiology.org/ by 10.220.33.3 on October 29, 2017

**RT-PCR.** Total RNA was extracted from mesangial cells by using a kit of RNA STAT from Tel-Test B (Friendswood, TX). First-strand cDNA was synthesized by a T-Primed First- Stand Kit from Amersham Pharmacia. PCR were performed and optimized according to standard protocols by using a kit.
Primers used for PCR were custom synthesized (GIBCO-BRL), and the sequences of each primer were as follows:

1. MMP-2, forward, 5'-ATCTGGTGTCTCCCTTACGG and reverse, 5'-GTGCAGTGATGTCCGACAAC;
2. TIMP-2, forward, 5'-CAAAGGACCTGAAGGAC and reverse, 5'-TTGATGCAGGCAAAGAAC;
3. MT1-MMP, forward, 5'-ATTGATGCTGCTCTCTTCTGG and reverse, 5'-TGATGCAGGCAAGAAC;
4. GAPDH, forward, 5'-TGGAGGAGCTGGAAC and reverse, 5'-TATGACACCAACGGATACATT.

The predicted sizes of the amplification products are 150, 182, 348, and 308 bp for MMP-2, MT1-MMP, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively.

Statistical analysis. Statistical analyses were performed by the Student’s t-test. Data are presented as means ± SD. P values of <0.05 were considered statistically significant.

RESULTS

ET is an endogenous inhibitor of MMP-2 secretion and activation. We first examined whether endogenous ET had any effect on the basal secretion of MMP-2 by cultured MCs. For this purpose, a neutralizing monoclonal antibody against ET-1, -2, and -3 was added to MC cultures, and this produced a dose-dependent enhancement of MMP-2 activity, as revealed by zymography (Fig. 1A). The effect was not observed after addition of an irrelevant, isotype-matched control IgG.

ET_A receptor antagonist has been reported to be effective in attenuating ECM accumulation and glomerulosclerosis in several models of kidney injury, as well as in blocking ET-elicited synthesis of ECM in cultured MCs (5, 6, 18). We therefore asked whether ET_A receptor antagonist could also enhance the activity of MMP-2 via blocking of the functional receptor of ET. As depicted in Fig. 1B, incubation of MCs with ET_A receptor antagonist FR139317 resulted in an obvious increase in MMP-2 activity. This action of FR139317 was concentration dependent and most obvious at 24 h (Fig. 1B). A similar effect was observed with another ET_A receptor antagonist, BQ123 (Fig. 1C).

In longer term cultures, there was a spontaneous activation of MMP-2, as revealed by the appearance of an additional band of molecular mass about 62 kDa in the zymogram (Fig. 1). In the presence of ET_A receptor antagonists or anti-ET antibody, this band appeared earlier and clearly increased in intensity (Fig. 1). These results suggest that ET is an endogenous inhibitor of MMP-2 secretion and activation in MCs.

The zymographic data were further confirmed by Western blot by using a specific anti-MMP-2 antibody. As shown in Fig. 2, the neutralizing anti-ET antibody (Fig. 2A) and ET_A receptor antagonist (Fig. 2B) increased the protein secretion of MMP-2 in a dose-dependent fashion.

Exogenous ET-1 inhibits the secretion of MMP-2. If ET is an endogenous inhibitor of MMP-2, addition of exogenous ET-1 into cultures should result in a reduction of MMP-2 activity. As shown in Fig. 3, the addition of exogenous ET-1 into MC culture slightly lowered the basal level of MMP-2 secretion as revealed by zymography (Fig. 3). To clearly demonstrate the inhibitory action of exogenous ET-1 on MMP-2 production, we examined the effects of ET in a system where the activity of MMP-2 was amplified by the addition of different combinations of TNF-α, IFN-γ, or LPS into MC culture. As indicated in Fig. 4, in the presence of
stimulants, the activity of MMP-2 was obviously enhanced, and this enhancement could be partially prevented by pretreatment of MC with exogenous ET-1 (10^{-7} M) (Fig. 4). Because zymography is only a semi-quantitative assay for MMP-2, to further confirm the above results and to accurately quantitate the change of MMP-2 in the presence of exogenous ET-1, additional quantitative assays for MMP-2 were employed. By using an antibody capture assay for MMP-2 activity, we selectively examined the effects of ET-1 on cytokines (INF-γ and TNF-α) plus LPS-stimulated releasing of MMP-2 by MCs. It was found that under the stimulated condition, MCs increased the secretion of MMP-2 more than twofold that of control, whereas treatment of MCs with ET-1 significantly inhibited both the basal and stimulated secretion of MMP-2 (Fig. 5). Very similar results were obtained in Western blot studies by using a specific antibody against MMP-2, as revealed in Fig. 6. Consistent with the reduction of MMP-2 activity and protein secretion, we also found a decreased expression of MMP-2 at mRNA level in the presence of ET under both basal and stimulated conditions. This is reflected by the decreased amount of the amplification product of MMP-2 gene, but not of the product of the housekeeping gene GAPDH in RT-PCR (Fig. 7).

It is worth mentioning that, in addition to the obvious increase of MMP-2 secretion, cytokines could elicit the activation of MMP-2 by inducing an additional band of 62-kDa molecular mass in the zymogram and Western blot (Figs. 4 and 6). ET treatment, however, greatly lessened the intensity of this band (Figs. 4 and 6).

Exogenous ET-1 inhibits the activation of MMP-2. MMP-2 is known to be secreted in latent form and must be activated to exert catalytic action. Therefore, it is important to understand the effects and the mechanisms of endothelin on the activation of MMP-2. For

![Fig. 3: Effects of ET-1 on MMP-2 activity. A representative zymographic analysis of MMP-2 activity in culture supernatants of MCs after treatment for 24 h with increasing concentrations of ET is shown. Similar results were obtained from an additional experiment.](image)

![Fig. 4: Suppressive action of ET-1 on stimulant-induced enhancement of MMP-2 activity. MCs either untreated or treated with 10^{-7} M of ET-1 for 45 min were exposed to different combinations of lipopolysaccharide (LPS) (10 μg/ml), tumor necrosis factor-α (TNF-α, 50 ng/ml), and interferon-γ (IFN-γ, 100 U) for 48 h. Culture supernatants were harvested and assayed for gelatinase activity by zymography. Similar results were obtained in 3 additional experiments.](image)

![Fig. 5: Effects of ET-1 on basal and stimulant-induced activity of MMP-2 as revealed by antibody capture assay. MCs treated or without 10^{-7} M of ET-1, were exposed to the mixed stimulant (10 μg/ml LPS, 50 ng/ml TNF-α, and 100 U IFN-γ) for 48 h. Supernatants were collected and assayed for MMP-2 activity by using a commercial kit as described in MATERIALS AND METHODS. Data are expressed as means ± SD (n = 4). Similar results were obtained from an additional experiment. *P < 0.01.](image)

![Fig. 6: Inhibitory effects of ET-1 on the basal and stimulant-induced protein secretion of MMP-2 as indicated by Western blot. A: MCs, with or without treatment with 10^{-7} M of ET-1, were exposed to the mixed stimulant (10 μg/ml LPS, 50 ng/ml TNF-α, and 100 U IFN-γ) for 48 h. Equal amounts of culture supernatants were concentrated and analyzed for the presence of MMP-2 protein by using immunoblotting. B: densitometric analysis of data from A. Values represent the means ± SD of 3 separate experiments and are expressed as percent untreated controls. *P < 0.01.](image)
these purposes, a well-established model of MMP-2 activation induction by cytochalasin D was employed (1, 2). Mesangial cells were exposed to cytochalasin D in the presence or absence of ET-1 for 18 h, and the culture supernatant was then subjected to gel zymography. As indicated in Fig. 8, cytochalasin D elicited the activation of MMP-2, as demonstrated by the appearance of one major band of ~62 kDa. This action of cytochalasin D was dose dependent (Fig. 8B). In the presence of ET-1, the conversion of MMP-2 from the latent to the active form by cytochalasin D was significantly inhibited as revealed by zymography (Fig. 8) and Western blot (Fig. 9). Desitometric analysis of data from four separate experiments by Western blot indicated that the percent active MMP-2 in total MMP-2 was significantly decreased, from 49.5 ± 8.8 in cytochalasin D-treated cells to 34.2 ± 8 in cells pretreated with ET-1 (Fig. 9B). This action of ET-1 could be observed in a wide range of concentrations tested (Fig. 8A) and was most probably mediated by ETA receptors, because ETA receptor antagonist FR139317 almost completely blocked this effect of ET-1 (Fig. 10).

The activation as well as activity of MMPs are strictly regulated by endogenous inhibitors, delineated as TIMPs. MMP-2 activity in particular is controlled by TIMP-2 through a direct protein-to-protein interaction (10). We therefore studied the effects of ET on the secretion and expression of TIMP-2 by MCs. As demonstrated in Fig. 11, treatment of MC with cytochalasin D inhibited dose-dependently the secretion of TIMP-2 into the medium, whereas the treatment increased the level of cell-associated TIMP-2 (Fig. 11). Conversely, ET exerted an exactly opposite effect and partially counteracted the action of cytochalasin D on TIMP-2 redistribution. The increment of the soluble form of TIMP-2 by ET could contribute to the inhibition of MMP-2 activation, because the direct addition of recombinant TIMP-2 into the culture suppressed concentration-dependently the cytochalasin D-elicited activation of MMP-2 (Fig. 12).

Because MMP-2 is considered to be activated on the cell surface by the membrane type 1 matrix metallo-
proteinase (MT1-MMP) (1, 20), we also examined the effects of ET on the mRNA expression of MT1-MMP by using semi-quantitative RT-PCR. As indicated in Fig. 13, cytochalasin D obviously increased the levels of the amplification product of MMP-2 and MT1-MMP in RT-PCR, compared with the respective nontreated controls. On the contrary, ET exerted an opposite effect under both basal and cytochalasin D-stimulated conditions. Interestingly, neither cytochalasin D nor ET had any influence on the expression of TIMP-2 (Fig. 13). As an internal control, the expression of the housekeeping gene GAPDH was not altered.

DISCUSSION

The suppressive action of exogenous ET-1 on collagenase activity has been previously reported in cardiac fibroblasts (19). However, the role of endogenous ET on collagenase activity as well as on the activation of collagenase and the underlying mechanisms implicated has not been fully examined so far. Here, we reported several novel findings related to the regulation of MMP-2 by ET in cultured MCs. First, ET was demonstrated to be an endogenous inhibitor of MMP-2 secretion and activation in cultured MCs. Second, ET was found to be able to inhibit the cytokines (IFN-γ and TNF-α) plus LPS-elicited production of MMP-2. Third, ET had the ability to block the conversion of pro-MMP-2 to active MMP-2, possibly via suppressing the MT1-MMP expression and increasing the secreted form of TIMP-2.

Addition of ET receptor antagonists into MC cultures caused an obvious increase of MMP-2 activity into the medium. Similar effects were produced by neutralizing anti-ET monoclonal antibody. The results suggest that the action of ET receptor antagonists was through blocking of the function of endogenous ET, rather than a direct induction of MMP-2 expression. Besides the obviously augmented activity of pro-MMP-2 in zymography, the amount of activated MMP-2 was also increased by ETA receptor antagonists or by the neutralizing anti-ET antibody. This was reflected by the earlier appearance as well as the enhanced intensity of the 62-kDa band, representing activated MMP-2. These results clearly indicate that ET is a potent endogenous inhibitor of both MMP-2 secretion and activation in rat MCs. This idea is further strengthened by the fact that exogenous ET was able to inhibit the synthesis and activation of MMP-2 in both basal and stimulated conditions. It should be noted that the accumulation of the main substrate of MMP-2, collagen IV, in cultured rat mesangial cells in the presence of ET, has been previously reported (18). Furthermore, production of ET by MC (36) and the autocrine actions of ET on MC behavior have also been well documented (23, 24).

The inhibitory action of exogenous ET on basal MMP-2 secretion as detected by zymography was marginal, whereas by using Western blot or quantitative MMP-2 activity assay, a 30% reduction of MMP-2 was found; the discrepancy of these results may reflect the different sensitivities of the assay systems employed. Zymography is only a simple, semi-quantitative assay for MMP-2. It is worth mentioning that the detection and comparison of MMP-2 activity in this study were based on the equal volume of culture supernatants. The possible influence of increased MC numbers under ET stimulation was not taken into account. Because ET is a potent mitogen for MCs and there was a constant increase of MC numbers in the presence of ET (data not shown), the actual suppressive effect of ET on MMP-2 activity is on a cell-for-cell basis, greater than that expressed by the data.

The mechanisms by which ET exerts its effect on the activity of MMP-2 appear to be both complicated and
multiple. ET could act by inhibiting the mRNA and protein expression of MMP-2, as indicated in this study by RT-PCR, Western blot, and the specific antibody capture assay. It could also control the activation of MMP-2 by regulating the expression and/or activation of molecules involved in MMP-2 activation. In this respect, we demonstrated that ET was able to suppress the mRNA expression of MT1-MMP and to enhance the proportion of the secreted form of TIMP-2. The increased level of TIMP-2 in the culture medium may contribute to the suppression of MMP-2 activation by ET. This is supported by the observation that the addition of recombinant T2M-2 into medium concentration dependently inhibited the cytochalasin D-elicited activation of MMP-2. A previous study has reported that ET has the ability to upregulate plasminogen activator inhibitors and suppress the activity of fibrolysis in MCs (21). Several studies have suggested a close interaction between MMPs and the plasminogen/plasmin system. For example, a role of plasmin in the activation of latent MT1-MMP, which in turn could activate latent MMP-2, has been proposed (33). Addition of plasmin into MC cultures can lead to the conversion of latent MMP-2 into active MMP-2 (3, 4). Thus it is likely that part of the action of ET on MMP-2 activation could be a secondary phenomenon, resulting from its effects on the plasminogen/plasmin system.

Mesangial cells have both ET<sub>A</sub> and ET<sub>B</sub> receptors. The available data support the concept that ETs act on MC via two different receptors (23). In this study, we demonstrated that ET<sub>A</sub> receptor antagonists poteniated the secretion and activation of MMP-2 in MCs, indicating that the ET<sub>A</sub> receptor is responsible for the regulation of MMP-2 activity. The complete blockade of the suppressive effects of ET on cytochalasin D-elicited activation of MMP-2 by the ET<sub>A</sub> receptor antagonist FR139317, provided additional evidence supporting this conclusion. Several investigators have reported that ET induces mesangial matrix synthesis via ET<sub>A</sub> receptor (18). Furthermore, ET<sub>A</sub> receptor antagonist treatment of rats with various kidney diseases reduces the glomerular deposition of ECM proteins compared with untreated controls (5, 6, 17). In addition, the suppressive action of exogenous ET-1 on collagenase activity in cultured cardiac fibroblasts has also been demonstrated to be mediated by ET<sub>A</sub> receptors (19). Thus it seems likely that ET acts on matrix turnover via ET<sub>A</sub> receptors.

It is worth noting that some of the actions of ET on matrix synthesis in MCs are reported to be mediated by TGF-β (18). TGF-β itself has proved to be a potent inhibitor in ECM degradation in cultured MCs (3). Therefore, it can be assumed that the effects of ET on MMP-2 activity might also be via TGF-β. However, most of the previous studies have implicated TGF-β as a promoter rather than an inhibitor of MMP-2, releasing in MCs as well as in other cell types (26, 30, 34). Thus the exact role of TGF-β in ET-induced suppression of MMP-2 activity remains to be addressed.

What are the potential in vivo pathophysiological implications of this study? As a predominant MMP involved in the degradation of major components of the GBM and mesangial matrix, MMP-2 plays an important role in the turnover of ECM in the renal glomerulus. The production of MMP-2 under steady-state conditions has been reported. Studies on renal biopsies demonstrated the presence of small amounts of MMP-2 in the normal glomerulus (14). The expression of MMP-2 in quiescent mouse, rat, and human mesangial cell lines has also been shown (25, 27, 28). Physiological levels of ET, secreted by MCs as well as other cell types within the glomerulus, may negatively regulate the activity of MMP-2, keeping its proteolytic potential within acceptable limits. Interestingly, one of the substrates of MMP-2 is reported to be big ET-1. MMP-2 cleaves big ET-1 to yield a novel and potent vasoconstrictor, ET-1 (15). It is highly possible that the auto-regulatory loop between MMP-2 and ET might play an important role in the regulation of vascular responses. Under inflammatory conditions, ET expression is upregulated by a variety of cytokines, including IL-1, TNF-α, and IFN-γ (22, 24). The enhanced level of ET might, in turn, counteract the action of these cytokines on MMP-2 activity, thus lessening the abnormal degradation of ECM and damage to glomerular structures. On the other hand, in diseases characterized by the accumulation of glomerular extracellular matrix, such as diabetic glomerulosclerosis, the increased expression of ET and decreased activity of MMP-2 have been reported (8, 9, 16, 37). Suppression of the activity of the protein-degrading enzymes by ET could contribute to glomerulosclerosis. In this context, augmentation of MMP-2 activity by ET receptor antagonists may be one of the important mechanisms by which such agents act therapeutically to slow progressive glomerulosclerosis.

In conclusion, our results demonstrate that ET is a potent inhibitor of MMP-2 secretion and activation in MCs. This finding may help us understand the subtle regulation of the synthesis and activation of MMP-2 in MCs. It also provides us with further insight into the pathophysiological mechanisms involving ET in the regulation of matrix turnover in glomerulus.

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