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Thrombin stimulates MMP-9 mRNA expression through AP-1 pathway in human mesangial cells

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KEY WORDS thrombin; gelatinases; glomerular mesangium; nephritis; transcription factor protein AP-1

ABSTRACT

AIM: To investigate the thrombin mediated induction of gelatinase B (MMP-9) in mesangial cells (MC) and the underlying role of activator protein-1 (AP-1). **METHODS**: Cultured human mesangial cells were exposed to thrombin in the presence or absence of hirudin, curcumin, and c-*fos* antisense or sense oligonucleotides. Northern hybridization was employed to assess MMP-9 mRNA expression, and electrophoretic mobility shift assay (EMSA) for AP-1 DNA binding activity. **RESULTS**: The levels of MMP-9 mRNA in the cell treated with different doses of thrombin (500, 1500, and 4500 u/L, respectively) were 1.1, 3.3, and 4.8 times higher than that in the control, respectively. There was also an increase in AP-1 binding activity (3.5, 5.9, and 7.1 fold than that of the control) in accordance with MMP-9 mRNA levels in the presence of thrombin. Hirudin, curcumin, and c-*fos* antisense oligonucleotides could block thrombin-induced expression of MMP-9 mRNA as well as AP-1 binding activity. **CONCLUSION**: Thrombin is a potent stimulator of MMP-9 gene expression in human mesangial cells, and the underlying intracellular events are mediated, at least partly, by AP-1 pathway.

INTRODUCTION

It is well known that certain renal diseases are characterized by intra-glomerular coagulation such as antibasal membrane nephritis, IgA nephritis, and disseminated intravascular coagulation (DIC)-associated renal failure. Thrombin, as a serine protease, not only promotes blood clotting, but also exerts numerous biological effects such as cell proliferation, migration, adhesion, release, and/or synthesis of certain cytokines and growth factors on targeted cells[1]. Under certain pathological situations, glomerular MMPs (matrix metalloproteinases) are often upregulated, and the resulting accelerated matrix breakdown may contribute to the destruction of glomerular architecture as well as abnormal behavior of glomerular cells[2-4]. Gelatinase B (MMP-9) is a member of matrix metalloproteinases and takes part in turnover of extracellular matrix of glomeruli[5]. However, there is no information available about the effects of thrombin on MMP-9 expression in human mesangial cells. Present study was designed to investigate the induction of MMP-9 by thrombin and the underlying role of activator protein-1 (AP-1) in human mesangial cells (MC).

MATERIALS AND METHODS

Materials Thrombin and curcumin were purchased from Sigma, RPMI 1640 from Gibco, gel shift assay kit and TRAzol reagent from Promega. Hirudin was a gift from Prof ZHU Sheng-Geng (Academy of Life Sciences, Beijing University). Primers for MMP-9 and c-*fos* sense and antisense oligonucleotides and probe for gel shift assay were synthesized by SBS biotechnology cooperation.

Cell culture Human glomerular mesangial cells were isolated from three kidneys unsuitable for transplantation, identified according to previous protocols, and maintained in RPMI 1640 supplemented with 15 % fetal calf serum, penicillin (100 000 U/L), and streptomycin (100 mg/L)[6]. Sub-confluent cells between passage 3-5 were used in the following experiments. Deprived of serum for 12 h, the cells were cultured in serum-free medium containing thrombin (500, 1500, 4500 u/L, respectively) in the presence or absence of either hirudin

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(9000 U/L) or curcumin (50 μmol/L). In order to exclude any toxic effects of curcumin and c-*fos* antisense or sense oligonucleotides on mesangial cells, viability tests (trypan blue exclusion and release of lactate dehydrogenase) and cell proliferation assays (MTT) were performed in parallel within the same experimental time span revealing no change in cell viability.

c-*fos* antisense assays Mesangial cells 6×10^6 were pre-incubated with 10 mmol/L phosphothioate modified *fos* antisense (5′- TGC GTT GAA GCC CGA GAA -3′) or sense (5′- TTC TCG GGC TTC AAC GCA -3′) oligonucleotides at 37 ℃ for 4 h according to the previous reports[7]. These nucleotide sequences were complementary to the first 18 bases following the AUG codon of c-*fos* mRNA. They penetrated into the cells without any additional pretreatment. After pre-incubation, cells were washed and treated with thrombin (1500 u/L) at 37 ℃ for 12 h.

RNA isolation and Northern analysis Total cellular RNA was isolated from the cells in different groups by one-step method according to the instruction of TRAzol Reagent (GIBCO). Twenty μg RNA was loaded on 1.0 % agarose gel, fractionated, transferred, and hybridized with [^{32}P]-labeled probes. The probe was a fragment amplified by reverse-transcription polymerase-chain reaction (RT-PCR). Primers used in PCR were as follows: sense 5′-AAG GAT CCG ACT ATG ACA CCG ACC GTC G -3′ and antisense 5′-AAG AAT TCG GCG CCG GTA GGG CTG GTA -3′. The cycling parameters for PCR were as follows: 94 ℃ × 45 s, 58 ℃ × 30 s, 72 ℃ × 45 s, and 35 cycles.

Preparation of nuclear proteins and electrophoretic mobility shift assay (EMSA) The nuclear protein extracts were obtained according to the method reported previously[8,9]. Briefly, cells were rinsed twice with cold PBS, scraped and transferred to microcentrifugation tubes. After centrifugation at 1000 × *g*, cell pellets were suspended in 400 μL of cold buffer A (HEPES 10 mmol/L pH 7.9, $MgCl_2$ 1.5 mmol/L, KCl 10 mmol/L, DTT 0.5 mmol/L, and PMSF 0.2 mmol/L) and left on ice for efficient cell swelling, followed by a centrifugation at 12 000 × *g* for 10 s. Nuclear pellets were suspended again in 40 μL of cold buffer C (HEPES 20 mmol/L pH 7.9, 25 % glycerol, NaCl 420 mmol/L, $MgCl_2$ 1.5 mmol/L, EDTA 0.2 mmol/L, DTT 0.5 mmol/L, and PMSF 0.2 mmol/L) and incubated on ice for 20 min. Cell debris was removed by centrifugation at 12 000 × *g* for 2 min at 4 ℃, and concentrations of the supernatant proteins were determined with Bio-Rad protein assay kit by the Bradford method[10]. Aliquots of the nuclear proteins were stored at −70 ℃ till use.

EMSA was carried out according to a previously reported method with some modifications[9]. The double-strand AP-1 consensus oligonucleotide was end-labeled with gamma-[^{32}P]-ATP by T4 polynucleotide kinase according to the manufacturer's protocols. For binding reaction, the end-labeled AP-1 probe 50 000 cpm was co-incubated with a cocktail of nuclear proteins 20 μg in gel shift binding buffer (Tris-HCl 10 mmol/L pH 7.5, NaCl 50 mmol/L, 4 % glycerol, $MgCl_2$ 1 mmol/L, edetic acid 5.0 mmol/L, and DTT 0.5 mmol/L) and poly (dI-dC) · poly (dI-dC) 50 mg/L at 23 ℃ for 30 min. Probe-AP-1 complexes were resolved on non-denaturing 4 % polyacrylamide gels in 0.5 × TBE buffer at 100 V. Dried gels were subjected to autoradiography at −70 ℃.

Statistics All data were expressed as $\bar{x} \pm s$ and compared with *t* test. Statistical analysis were performed by SAS. For all experiments, a value of $P < 0.05$ was considered significant.

RESULTS

Thrombin stimulated expression of MMP-9 mRNA in mesangial cells Northern hybridization showed that thrombin (500, 1500, and 4500 u/L) dose-dependently enhanced MMP-9 mRNA levels in the mesangial cells during the appointed time course ($P < 0.01$ *vs* control group). The levels of MMP-9 mRNA in the presence of thrombin were 1.1, 3.3, and 4.8 times higher than that in the control. As a specific thrombin inhibitor, hirudin had no effects on MMP-9 mRNA expression in mesangial cells, but could block thrombin mediated induction of MMP-9 mRNA in the cells ($P < 0.05$ *vs* 4500 u/L thrombin group) (Fig 1).

Thrombin promoted AP-1 activity in mesangial cells In the presence of thrombin, mesangial cells exhibited an increased AP-1 DNA binding activity in parallel to the concentration of thrombin. AP-1 DNA binding activity was 3.5, 5.9, and 7.1 fold than that in the control. Hirudin alone did not alter AP-1 activity significantly but could exert a pronounced inhibition on thrombin mediated induction of AP-1-DNA complexes (Fig 2).

AP-1 mediated induction of MMP-9 mRNA by thrombin in mesangial cells AP-1 activity was increased in the presence of thrombin, which was consistent with the thrombin mediated induction of MMP-9 mRNA

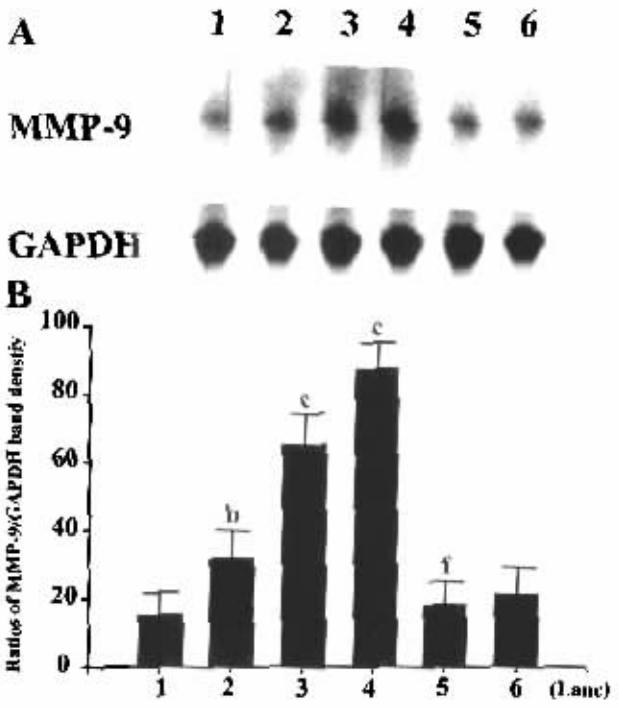

Fig 1. Induction of MMP-9 mRNA in human mesangial cells by thrombin. A shows one of three individual Northern blot assays. B shows a desitometric analysis of MMP-9 mRNA in different groups treated with or without thrombin or hirudin. Lane 1, control; Lane 2-4, 500, 1500, and 4500 u/L thrombin; Lane 5, 4500 u/L thrombin and 9000 u/L hirudin; Lane 6, 9000 u/L hirudin alone. $n = 3$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* 4500 unit/L thrombin.

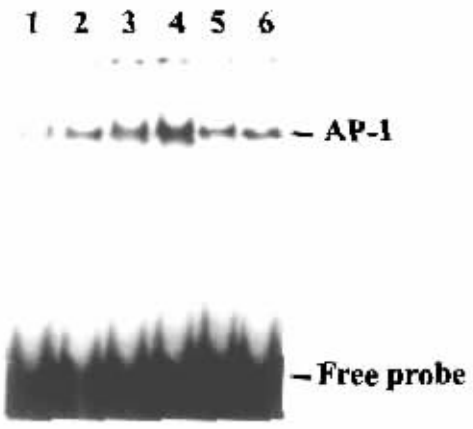

Fig 2. Activation of AP-1 in human mesangial cells by thrombin. Nuclear extracts were prepared from cells treated with or without thrombin or hirudin for 6 h. This figure represents one of three individual experiments. Lane 1, control; Lane 2-4, 500, 1500, and 4500 u/L thrombin; Lane 5, 4500 u/L thrombin plus 9000 u/L hirudin; Lane 6, 9000 u/L hirudin alone.

in mesangial cells. Curcumin, a pharmacological inhibitor of AP-1, significantly suppressed both thrombin-induced AP-1 activity and MMP-9 mRNA expression ($P < 0.01$ *vs* 1500 u/L thrombin group). In order to confirm the involvement of c-*fos* in thrombin mediated induction of MMP-9, c-*fos* sense and antisense oligonucleotides were employed in the present study. During the appointed time cycle, c-*fos* antisense (not sense) oligonucleotides significantly reduced c-*fos* protein levels in the cells (data not shown). c-*fos* antisense oligonucleotides reduced both AP-1 activity and MMP-9 mRNA levels. c-*fos* sense oligonucleotides exhibited no significant effects on AP-1 activity as well as MMP-9 mRNA expression (Fig 3).

DISCUSSION

Previous reports showed that urinary thrombin activity was increased in patients with mesangial proliferative glomerulonephritis[11]. It is well known that thrombin plays a central role in blood clotting and exerts lots of cellular biological effects on certain cells, especially renal resident cells[1], which might accelerate progression of some glomerular diseases through the over-deposition of fibrin in glomeruli or activating its receptors on the cell membrane.

Imbalance between matrix synthesis and degradation results in matrix remodeling of many organs, consequently leading to organ sclerosis and failure. MMP-9 is a member of Zn^{2+} endopeptidase superfamilies involved in the extra-cellular matrix turnover and remodeling[5]. An *in vitro* study reported that human mesangial cell grown either in elevated glucose (25 mmol/L) medium or on glycated IV collagen-coated plates expressed high levels of MMP-9 and this may contribute to glomerular matrix remodeling in diabetic patients[12]. Glomerular mesangial cells can also express MMP-9 in response to the pro-inflammatory cytokine interleukin-1 beta (IL-1β)[13]. In rats with passive Heymann nephritis, there was a major increase in MMP-9 mRNA and protein within the visceral epithelial cells, which correlated with the period of proteinuria associated with this model, suggesting that a causal relationship may exist between MMP-9 expression and changes in glomerular capillary permeability[14]. Present study demonstrated that thrombin dose-dependently promoted MMP-9 mRNA transcription in cultured human mesangial cells. Specificity of the effect of thrombin in mesangial cells was proved by suppression of hirudin.

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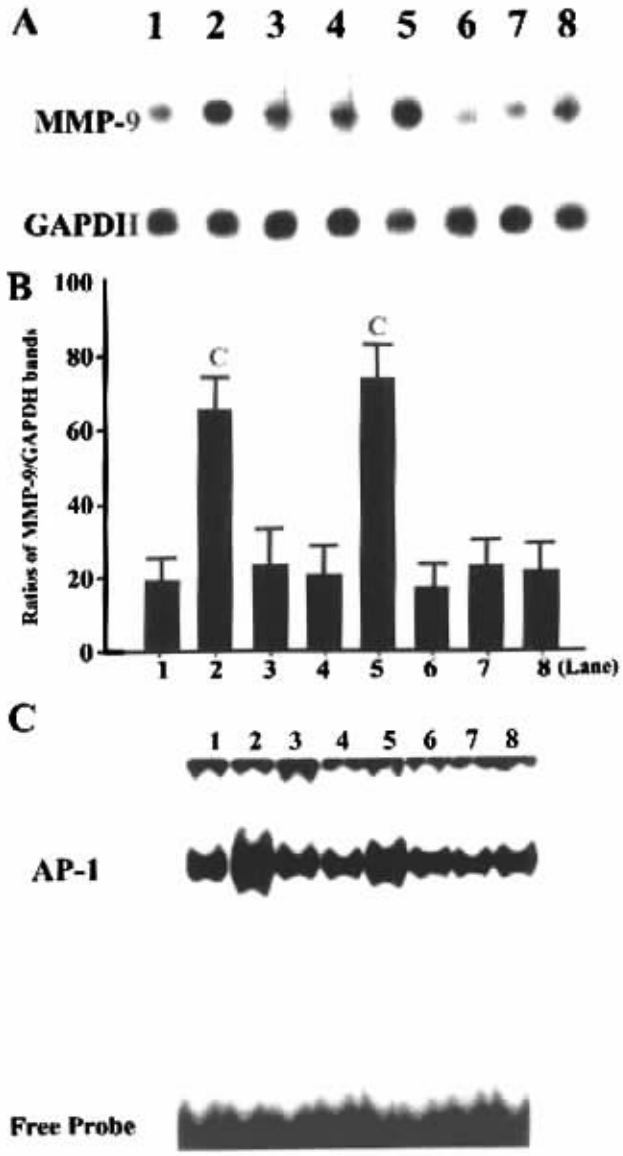

Fig 3. Inhibition of thrombin-induced MMP-9 mRNA expression and AP-1 activation by curcumin and c-*fos* antisense oligonucleotides. A shows one of three individual Northern analysis of MMP-9 mRNA expression. B shows a desitometric analyses of MMP-9 bands in different groups. $n = 3$. $\bar{x} \pm s$. C shows the effects of curcumin or c-*fos* antisense oligonucleotides on thrombin induced activation of AP-1. Nuclear protein 60 μg was used in this experiment. Lane 1, control; Lane 2, 1500 u/L thrombin alone; Lane 3, 1500 u/L thrombin plus 50 μmol/L curcumin; Lane 4, 50 μmol/L curcumin alone; Lane 5, 1500 u/L thrombin plus 10 mmol/L c-*fos* sense oligonucleotides; Lane 6, 10 mmol/L c-*fos* sense oligonucleotides alone; Lane 7, 1500 u/L thrombin plus 10 mmol/L c-*fos* antisense oligonucleotides; Lane 8, 10 mmol/L c-*fos* antisense oligonucleotides alone. $^cP < 0.01$ *vs* control.

It is reported that thrombin can stimulate AP-1 activation in mesangial cells, and AP-1 activity is increased in glomeruli in experimental glomerulonephritis[15,16]. Our results also demonstrated that AP-1 activity was enhanced by thrombin in a concentration-dependent manner and the activity of AP-1 was consistent with the levels of MMP-9 mRNA expression in the cells exposed to thrombin. As a specific inhibitor of thrombin, hirudin suppressed both AP-1 activity and MMP-9 mRNA expression induced by thrombin. In order to ensure the role of AP-1 in the thrombin mediated induction of MMP-9 mRNA, curcumin and c-*fos* antisense oligonucleotides were used to suppress AP-1 activity in the present study. c-*fos* antisense oligonucleotides rather than c-*fos* sense oligonucleotides could reduce c-*fos* protein level in the cell (data not shown). However, both c-*fos* antisense oligonucleotides and curcumin were effective to lower thrombin mediated induction of AP-1 DNA-binding activity as well as MMP-9 mRNA transcription in mesangial cell. These data suggest that AP-1 is essential for thrombin mediated induction of MMP-9 gene expression in mesangial cell.

In conclusion, present research revealed that thrombin stimulated MMP-9 gene expression at both transcriptional and translational level and AP-1 mediated, at least partly, this process in mesangial cell. This might help to further elucidate pathological mechanism involved in the injuries induced by thrombin in renal tissues.

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凝血酶通过 AP-1 信号途径刺激人肾小球系膜细胞表达 MMP-9 mRNA

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关键词 凝血酶; 明胶酶; 肾小球系膜; 肾炎; 转录因子AP-1

目的: 利用体外培养人肾小球系膜细胞观察凝血酶对系膜细胞表达 MMP-9 mRNA 的诱导作用及其可能的细胞内信号传导机制. **方法**: 体外分离并培养人肾小球系膜细胞, 以不同浓度凝血酶(500, 1500 和 4500 u/L)刺激后采用 Northern 杂交及凝胶阻滞试验(EMSA)分别观察 MMP-9 mRNA 表达水平及转录因子 AP-1 活性变化; 同时观察了凝血酶特异性抑活物(水蛭素)、AP-1 药理阻断剂(curcumin)和 c-*fos* 反义寡核苷酸对凝血酶上述作用的影响. **结果**: Northern 分析表明凝血酶可以剂量依赖性地提高人系膜细胞表达 MMP-9 mRNA, 与对照组相比, 分别提高 1.1, 3.3 和 4.8 倍; EMSA 实验结果发现凝血酶在促进 MMP-9 mRNA 表达的同时, 系膜细胞 AP-1 的结合能力也相应地提高, 与对照组相比分别增加 2.5, 4.9 和 6.1 倍, 与 MMP-9 mRNA呈平行性变化; 凝血酶抑活物水蛭素可明显地抑制凝血酶上调 MMP-9 mRNA及 AP-1 的 DNA 结合活性; 同时 AP-1 药理阻断剂 curcumin 和 c-*fos* 反义寡核苷酸在有效抑制 AP-1 结合活性的同时, 也明显地抑制 MMP-9 mRNA 表达水平. **结论**: 凝血酶在体外培养的人肾小球系膜细胞具有上调 MMP-9 mRNA 表达的作用, 转录因子 AP-1 介导了凝血酶的这一作用.

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