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Less gelatinases is associated with apolipoprotein E accumulation in glomerulosclerosis rats

Tian-Biao Zhou*, Yuan-Han Qin*, Feng-Ying Lei, Li-Na Su, Yan-Jun Zhao and Wei-Fang Huang

Department of Pediatrics, The First Affiliated Hospital of GuangXi Medical University, NanNing, China

*The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

Summary. Background: Gelatinases include matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). The abnormal expressions of gelatinases are implicated in the pathogenesis of extracellular matrix (ECM) accumulation. Apolipoprotein E (apoE) is an important plasma protein in cholesterol homeostasis and plays a key role in the progression of glomerulosclerosis (GS). We conducted this investigation to explore whether gelatinases were associated with the apoE accumulation in the pathological process of GS. Methods: 40 Wistar rats were divided into two groups at random: sham operation group (SHO) and glomerulosclerosis model group (GS); n=20, respectively. The disease of GS was established by uninephrectomy and adriamycin (5 mg/kg) injection. At the end of 13 weeks, the 20 rats in each group were killed and the relevant samples were collected and measured. Results: Serum total protein (TP) and serum albumin (Alb) in GS group were reduced compared to those of the SHO group ($P<0.01$). Compared with the SHO group, values of 24-hour urine total protein (24UTP), 24-hour urine excretion for albumin (24Ualb), blood urea nitrogen (BUN), serum creatinine (Scr) and glomerulosclerosis index (GSI) in GS group were significantly increased ($P<0.01$). The protein of MMP-2 or MMP-9 in the glomerulus, and mRNA expression of MMP-2 or MMP-9 in renal tissue were reduced when compared with those in SHO ($P<0.01$). Protein expressions of apoE, collagen IV (Col-IV), fibronectin (FN), α-smooth muscle actin (α-SMA) and transforming growth factor-ß1 (TGF-ß1) in the glomerulus and expression of apoE mRNA in renal tissue were significantly up-regulated in GS group when compared with those in the SHO group ($P<0.01$). Conclusions: Lower expression of gelatinases is associated with the increased expression of apoE in the glomerulus, and increases the accumulation of ECM and takes part in the pathological change of GS.

Offprint requests to: Yuan-Han Qin: Department of Pediatrics, The First Affiliated Hospital of GuangXi Medical University, NanNing 530021, China. e-mail: yuanhanqin@yahoo.cn

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Introduction

As a family of proteolytic enzymes, matrix metalloproteinases (MMPs) play a major role in the remodelling and homeostasis of the extracellular matrix (ECM) (Mehan et al., 2011; Setz et al., 2011). More than twenty MMPs have been identified and are divided into six sub-groups based on their structure and substrate specificity (collagenases, gelatinases, membrane type MMP, stromelysins, matrilysins, and others) (Hu and Beeton, 2010). Gelatinases, as a key subgroup of matrix metalloproteinases (MMPs), include gelatinase A and gelatinase B which are also called matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), respectively (Duran-Vilaregut et al., 2011). MMP-2 and MMP-9 participate in extracellular protein remodeling (Lorencini et al., 2009; Madro et al., 2009; Belhocine et al., 2010) and gelatinases may cleave collagen IV (Col-IV) (Cortes-Reynosa et al., 2008; Lee et al., 2008) and fibronectin (FN) (Pal et al., 2010). Col-IV and FN are the major components of ECM (Qin et al., 2003; Abe et al., 2011). Accumulation of glomerular ECM may result in glomerulosclerosis (GS) (Zhang et al., 2010). So, the abnormal expression of gelatinases would play an important role in the progression of GS.

Apolipoprotein E (apoE) is a main ligand for the clearance of lipids via low density lipoprotein receptor

and other related receptors (Reilly and Rader, 2006), which plays an important role in systemic lipoprotein metabolism and vascular wall homeostasis (Yue and Mazzone, 2011). Some investigations found that lipids and apoE expressions in renal glomerulus were elevated in some renal diseases, especially in lipoprotein glomerulopathy (Calandra et al., 1981; Saito and Sho, 1995; Deighan et al., 2000; Russi et al., 2009). Lipid deposition in the glomerulus, which can be mediated by apoE, is an important characteristic of nephritic syndrome (Zhou et al., 2011). apoE plays a key role in the progression of GS.

With the evidence presented above, gelatinases and apoE might take part in the pathologic process of GS. Whether there was an association between gelatinases and apoE, is not elucidated, and there is few report investigating this association. Hwang et al. (2004) found that apoE was a substrate of matrix metalloproteinase-14 (MMP-14) and apoE was cleaved in vitro by MMP-14. Park et al. (2008) confirmed that apoE was a substrate of MMP-14 and also of matrix metalloproteinase-7 (MMP-7) and MMP-2 to a lesser extent. Those two studies might draw a conclusion that MMPs could regulate apoE expression and that apoE might be associated with MMPs. In this context, we hypothesized that gelatinases might be associated with apoE accumulation in the glomerulus of GS. Forty Wistar rats were divided into sham operation group (SHO) and glomerulosclerosis model group (GS) at random, in order to investigate the relationship between gelatinases and apoE in the glomerulus of GS rats.

Materials and methods

Animal model

Forty healthy male Wistar rats (180~200g) were purchased from the Experimental Animal center of Guangxi Medical University, Nanning, China. All the procedures were approved by the animal ethics committee of Guangxi Medical University. The rats were divided into two groups at random: sham operation group (SHO, n=20) and GS model group (GS, n=20). The SHO group was subjected to a sham operation and tail vein injection of 0.9% saline solution alone. GS disease in the GS group was induced by uninephrectomy and a single tail vein injection of adriamycin (Wanle Pharmaceutical Co., Shenzhen, China) at a dose of 5 mg/kg on days 7 after the uninephrectomy treatment. At the end of 13 weeks, the 20 rats in each group were sacrificed. Serum and urine were collected and stored at -20°C, and their renal tissues were collected for histological and molecular biology determination.

Laboratory analysis

All the serum and urine specimens were removed from the refrigerator and balanced to room temperature for detection. 24-hour urine total protein excretion (24UTP) and 24-hour urine excretion for albumin (24Ualb) were measured by the sulfosalicylic acid method. Levels of serum total protein (TP), serum albumin (Alb), blood urea nitrogen (BUN) and serum creatinine (Scr) were determined by the standard enzymatic method.

Renal morphology

Renal tissues were fixed in 10% neutral formaldehyde, and they were dehydrated through a graded ethanol series and embedded in paraffin. 4 μm sections were prepared on a microtome and stained with hematoxylin and eosin (H&E). Renal damage was viewed by light microscopy, and the severity of the renal lesion was defined by the glomerulosclerosis index (GSI). The GSI was counted according to the method of Raij et al. (1984). The severity of the lesion was examined in 100 glomeruli selected at random, graded from 0 to 4 points in accordance with the percentage of morphological changes on each glomerulus (0=0%, 1+=1%-25%, 2+=26%-50%, 3+=51%-75%, 4+=76%-100%). The number of glomeruli showing a lesion of 0 was n_0, of 1+ n_1, of 2+ n_2, of 3+ n_3, of 4+ n_4, respectively. 100 glomeruli were examined independently, and the GSI was obtained by the formula as follows: $GSI=(0xn_0+1xn_1+2xn_2+3xn_3+4xn_4)/(n_0+n_1+n_2+n_3+n_4)= (0xn_0+1xn_1+2xn_2+3xn_3+4xn_4)/100$. The scores obtained by two investigators were averaged.

Immunohistochemical analysis of MMP-2, MMP-9, apoE, Col-IV, FN, α-smooth muscle actin (α-SMA) and transforming growth factor-ß1 (TGF-ß1)

The operation was implemented using the streptavidin-peroxidase immunohistochemical method. Renal tissue samples were fixed in 10% neutral formaldehyde, dehydrated with ethanol, and embedded in paraffin. Serial 4 μm sections were collected sequentially on glass slides. The paraffin was removed from the sections with xylene and rehydrated in graded ethanol. In order to retrieve antigenicity from formalin fixation, we incubated the sections for 10 min in 10 mmol/l sodium citrate buffer using a microwave oven. Endogenous peroxidase activity was blocked by further pretreatment with 3% hydrogen peroxide and methanol. Finally, the sections were incubated with antibody against MMP-2 (1/200) (Neomarkers Co., Inc., USA), MMP-9 (1/500) (Chemicon Co., Inc., USA), apoE (1/150) (Bo Ao-Sen, Co., Inc., China), Col-IV (1/100) (Shanghai Changdao, Co., Inc., China), FN (1/100) (Beijing Zhongshan Co., Inc., China), α-SMA (ready - to - use kit) (Shanghai Changdao, Co., Inc., China) and TGF-ß1(1/100) (Wuhan Boshide, Co., Inc., China) overnight at 4°C. The sections were washed thoroughly in phosphate-buffered saline (PBS) solution and incubated with rabbit anti-mouse biotinylated second antibody immunoglobulin (Shanghai Changdao, Co., Inc., China) for 30 min. Finally, the sections were

stained with diaminobenizidine (Maixin Bio, Co., Inc., China). We obtained negative controls by replacing specific antisera with PBS solution. Brownish yellow granular or linear deposits in the cells or matrix were interpreted as positive areas. Semi-quantitative evaluation was performed by computer-assisted image analysis (Leica Co., Germany). The positive staining of MMP-2, MMP-9, apoE, Col-IV, FN, α-SMA and TGF-ß1 was measured at 400-fold original magnification in 100 glomeruli which were selected from coded sections of each rat at random.

Real time reverse transcription polymerase chain reaction to detect the MMP-2, MMP-9 and apoE mRNA expressions in renal tissue

Renal tissue was homogenized and total RNA was extracted with TRIzol (Beijing Tiangen, Co., China). Ultraviolet spectrophotometer measured the absorbance, and agarose gel electrophoresis confirmed that there had been no degradation of RNA by visualizing the 18S and 28S RNA bands under ultraviolet light (An et al., 2010). Primers were designed according to primer design principles by Primer Premier 5.0. The primer sets used were: F 5'-AGCTCCCGGAAAAGATTGAT-3' and R 5'-TCCAGTTAAAGGCAGCGTCT-3' for MMP-2; F 5'-CGCAAGCCTCTAGAGACCAC-3' and R 5'-TGGGGGATCCGTGTTTATTA-3' for MMP-9; F 5'-ACCGCAACGAGGTAAACACCA-3' and R 5'-CGCTCACGGATAGCACTCACA-3' for apoE; F 5'-GCCCCTGAGGAGCACCCTGT-3' and R 5'-ACGCTCGGTCAGGATCTTCA-3' for ß-actin. One microgram total RNA from the renal tissue of each rat was reverse transcribed into cDNA with an ExScript RT reagent kit (Takara Biotechnology, Co., Dalian, China). MMP-2, MMP-9 and apoE were amplified with SYBR Premix Ex Taq (Beijing Tiangen, Co., China). The cycling parameters were denatured at 95°C for 5 s, with annealing at 61°C for 20 s and extension at 72°C for 15 s. A total of 35 cycles were performed within the linear amplification range. Gene expression of ß-actin was also measured in each sample and was used as an internal control for loading and reverse transcription efficiency. The analysis for each sample was performed in triplicate. The average threshold cycle (Ct, the cycles of template amplification to the threshold) was worked out as the value of each sample. The data of fold change was analyzed using $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001): fold change = $2^{-\Delta\Delta Ct}$. For example, the ΔΔCt for MMP-2 mRNA expression in GS group was as follow: $\Delta\Delta Ct_{MMP\text{-}2}=(CT_{MMP\text{-}2,\ GS\ group}-CT_{ß\text{-}actin,\ GS\ group})-(CT_{MMP\text{-}2,\ SHO\ group}-CT_{ß\text{-}actin,\ SHO\ group})$, and the fold change for MMP-2 mRNA expression in GS group was $2^{-\Delta\Delta CtMMP\text{-}2}$.

Statistical analysis

The data are shown as mean ± standard deviation. Independent-Samples T Test was performed to determine the differences between SHO group and GS group, and Pearson's correlation coefficients was used to determine the relationships between the indicators for detection. A value of P<0.05 was considered as significant difference. Statistical analysis was performed using the statistical package for social studies SPSS version 13.0 (SPSS, Chicago, IL, USA).

Results

Biochemical parameters

At the end of week 13, 24UTP, 24Ualb, BUN and Scr values in group GS were markedly higher than those of group SHO (all P<0.01; Fig. 1). The contents of TP and Alb in GS were significantly decreased when compared with those in SHO (each P<0.01; Fig. 1).

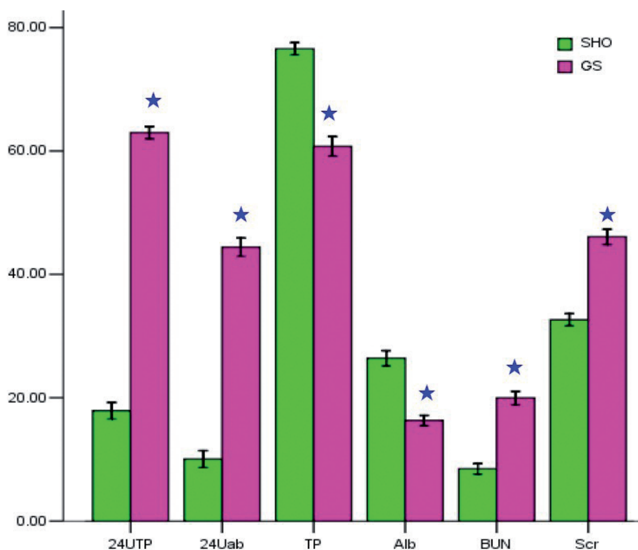
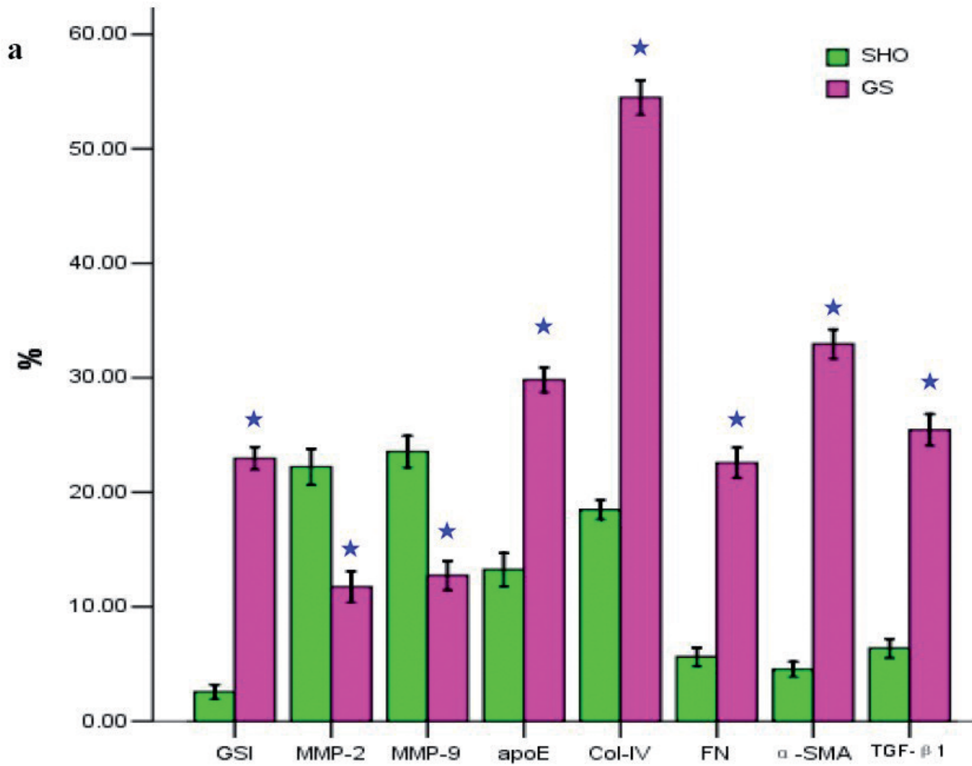


Fig. 1. P<0.01 compared with SHO. 24UTP (mg/24h): 24-hour urine total protein; 24Ualb (mg/24h): 24-hour urine excretion for albumin; TP (g/L): serum total protein; Alb (g/L): serum albumin; BUN (mmol/L): blood urea nitrogen; Scr (μmol/L): serum creatinine. SHO: sham operation group; GS: GS model group.

Renal morphology

Light microscopy was performed to observe the changes of glomerular morphology. Glomerular morphology was normal in sham group (A_1; Fig. 2b). Extensive damage was observed in GS group, such as glomerular hypertrophy, balloon adhesion and wall thickening, degeneration of glomerular epithelial cells and infiltration of widespread mononuclear cells in rats of GS group (A_2; Fig. 2-b). Quantitative analysis showed that the GSI of group GS was much higher than that in the SHO group (P<0.01; Fig. 2-a).



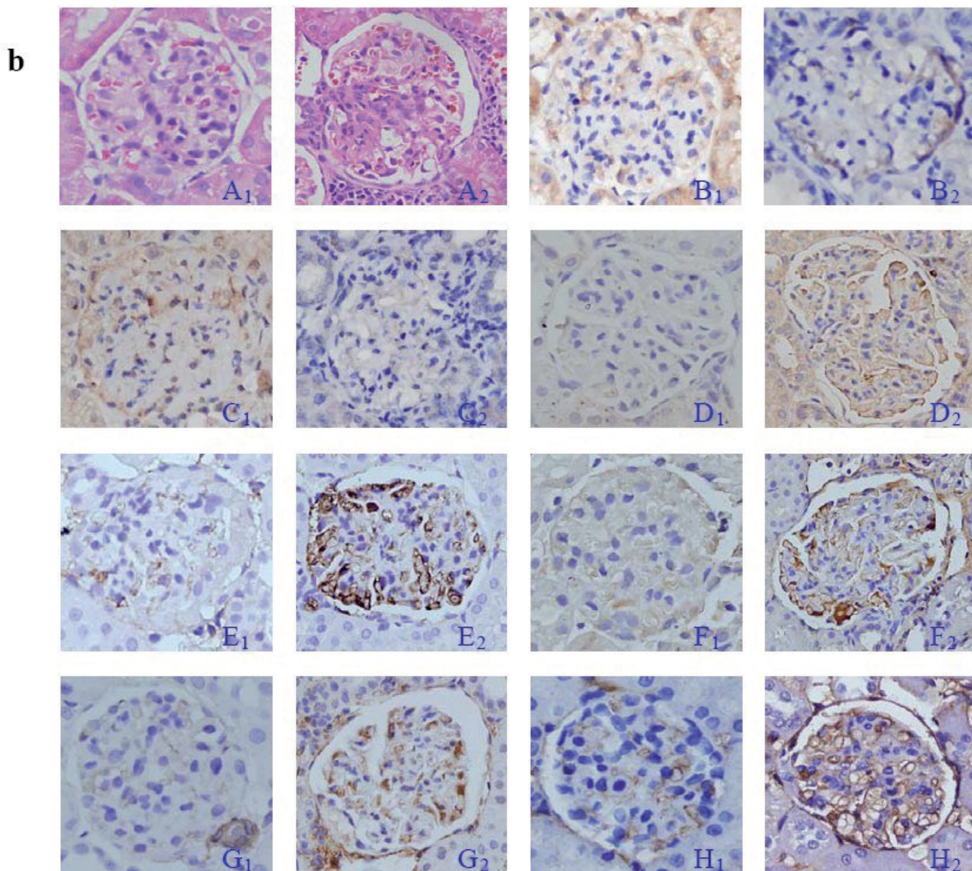


Fig. 2. Tissue parameters of the glomerulus in two groups. **a.** P<0.01 compared with SHO. **b.** Glomerular morphology was normal in sham group (A_1; H&E). Proliferation occurring in the majority of glomerular mesangial cells and extracellular matrix in GS group, and degeneration of glomerular epithelial cells and infiltration of widespread mononuclear cells were shown (A_2; H&E). Representative samples of immunohistochemical staining for glomerular MMP-2 (B_1, B_2), MMP-9 (C_1, C_2), apoE (D_1, D_2), Col-IV (E_1, E_2), FN (F_1, F_2), α-SMA (G_1, G_2) and TGF-ß1 (H_1, H_2) were observed in all groups. Stainings for MMP-2 and MMP-9 in GS group (B_2 and C_2) were markedly alleviated when compared with those in SHO (B_1 and C_1), and the positive staining (in brown) was located in the glomerulus, glomerular endothelial cells, glomerular basement membrane, mesangial cells, and visceral epithelial cells. Positive staining for apoE, Col-IV, FN, α-SMA or TGF-ß1 was strong in most glomerulus, glomerular endothelial cells, glomerular basement membrane, mesangial cells, and visceral epithelial cells in GS group (D_2, E_2, F_2, G_2 and H_2) when compared with those in SHO (D_1, E_1, F_1, G_1 and H_1). GSI: glomerulo-sclerosis index; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; apoE: apolipo-protein E; Col-IV: collagen IV; FN: fibronectin; α-SMA: α-smooth muscle actin; TGF-ß1: transforming growth factor-ß1. SHO: sham operation group; GS: GS model group; H&E: hematoxylin and eosin. x 400

Protein expressions of MMP-2, MMP-9, apoE, Col-IV, FN, α-SMA and TGF-ß1 in the glomerulus

Immunohistochemical staining for MMP-2, MMP-9, apoE, Col-IV, FN, α-SMA and TGF-ß1 was performed. The staining for apoE, Col-IV, FN, α-SMA and TGF-ß1 was markedly enhanced in the majority of glomerulus, glomerular endothelial cells, glomerular basement membrane, mesangial cells and visceral epithelial cells of GS rats when compared that in SHO group (D_1, E_1, F_1, G_1 and H_1 for SHO; D_2, E_2, F_2, G_2 and H_2 for GS, Fig. 2b; all P<0.01, Fig. 2a). However, in the GS group, the glomerular staining for MMP-2 or MMP-9 was markedly reduced compared to that in group SHO (B_1 and C_1 for SHO; B_2 and C_2 for GS, Fig. 2b; each P<0.01, Fig. 2a).

mRNA expressions of MMP-2, MMP-9 and apoE in renal tissue

The mRNA expressions of MMP-2 and MMP-9 in GS group were markedly reduced when compared to those in SHO (each P<0.01; Fig. 3). However, the apoE mRNA expression of renal tissue in the GS rats was notably increased when compared with that of SHO rats (P<0.01; Fig. 3).

Correlation analysis

Correlation analysis between MMP-2 or MMP-9 protein and apoE, Col-IV, FN, α-SMA, TGF-ß1 protein or GSI in the glomerulus was conducted in our investigation. Significant negative correlation was observed between MMP-2 protein and apoE, Col-IV, FN, α-SMA, TGF-ß1 protein or GSI (r=-0.827, r=-0.901, r= -0.865, r=-0.769, r=-0.872, r=-0.885; each P<0.01), and negative correlation between MMP-9 protein and apoE, Col-IV, FN, α-SMA, TGF-ß1 protein or GSI (r=-0.853, r=-0.816, r= -0.794, r=-0.855, r=-0.906, r=-0.893; each P<0.01) was also found. Interestingly, there was a positive correlation between apoE protein and Col-IV, FN, α-SMA, TGF-ß1 protein or GSI (r=0.901, r=0.893, r= 0.842, r=0.826, r=0.932; each P<0.01).

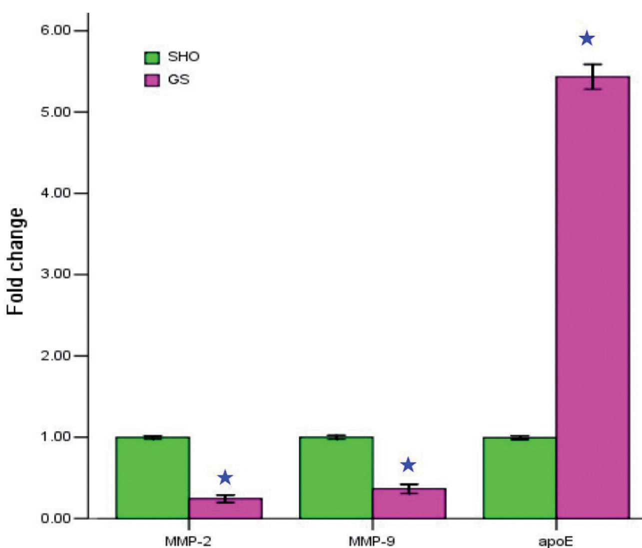


Fig. 3. P<0.01 compared with SHO. MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; apoE: apolipoprotein E. SHO: sham operation group; GS: GS model group.

Discussion

Understanding the detailed mechanisms that contribute to the regression of GS is most important for developing new strategies to treat chronic kidney disease. Over-depositing of ECM such as FN and Col-IV is an important characteristic of GS. α-SMA is a specific marker for myofibroblasts and takes part in the development and progression of GS (Danilewicz and Wagrowska-Danielwicz, 2009; Kaouthar et al., 2009). Of all the cytokines and growth factors involved, TGF-ß1 plays the most important role and the increase of TGF-ß1 expression is closely correlated with the development of GS (Wang et al., 2007; Adan et al., 2010). TGF-ß1 is known to be one of the major mediators that lead to GS by inducing the productions of α-SMA and ECM (Col-IV and FN) in the glomerulus (Yutaka et al., 2002; Wang et al., 2007; Hakki et al., 2009). So, FN, Col-IV, α-SMA and TGF-ß1 are important indicators to evaluate the grade of glomerulosclerotic lesions for GS. In this investigation, those indicators were detected.

In renal tissue, apoE is mainly synthesized by mesangial cells under normal physiologic condition (Lin et al., 1986). Some investigations found that apoE expression in renal glomerulus of renal disease group was markedly elevated when compared with that in the normal group (Calandra et al., 1981; Deighan et al., 2000; Russi et al., 2009). The deposition of lipids in the glomerulus, as an important pathogenetic factor, is involved in glomerular sclerosis and leads to progression of glomerular disease. However, the role of apoE is complicated. In this context, one of the aims of this study was to determine whether apoE expression was correlated with the protein expression of Col-IV, FN, TGF-ß1 or α-SMA in the development of GS.

In this investigation, we found that the expression of apoE in the glomerulus could reflect the seriousness of GS in rats. The protein expressions of Col-IV, FN, α-SMA and TGF-ß1 in the glomerulus were markedly increased when compared with those in controls. The apoE protein accumulation in the glomerulus of GS group was also notable when compared with that in SHO. The correlation analysis was performed to explore the association between apoE protein and Col-IV, FN, α-SMA, TGF-ß1 protein or GSI in the glomerulus, and significantly positive correlation was observed between apoE protein and Col-IV, FN, α-SMA, TGF-ß1 protein

or GSI. The expression of apoE mRNA in renal tissue of GS rats was also elevated when compared with that in control group. We speculated that the mechanism for why the expression of apoE (mRNA and protein) in glomerulus of GS rats increased was as follows: In the nephrotic syndrome rats, hyperlipoidemia was a very common phenomenon, and the lipids passed through the broken glomerular basement membrane (GBM) and deposited in the renal capsule (we conducted the Oil red O to detect the lipid deposit in the glomerulus, and found that the lipid deposit of GS was markedly increased when compared with that of SHO). In this process, apoE mediated the lipids to combine with the mesangial cells, visceral epithelial cells and so on. The increased lipids passed the GBM and more apoE were needed for clearance of lipids. So, as a negative reflection, the apoE expression (mRNA and protein) in the glomerulus was increased. On the other hand, the increased apoE would also mediate more lipids to deposit in the glomerulus, which could induce the increased production of reactive oxygen species (ROS) (Farooqui and Farooqui, 2011; Obulesu et al., 2011) and could increase ECM accumulation in the glomerulus. The generation and degradation for apoE might be unbalanced (generation of apoE was increased and degradation of apoE was reduced), and the clearance of apoE might be impaired in our observation. As the result mentioned above, the impairment of apoE clearance might be responsible for inducing the pathological change of GS.

Adriamycin (doxorubicin) is a potent available antitumor agent (Mukhopadhyay et al., 2009), which can reduce the ability of invasion for tumor cells by reducing the expression of MMPs (Zhang et al., 2004; Pastorino et al., 2010). Over-expression of MMPs is an important indicator which reflects the ability of invasion for tumor cells. The adriamycin induced GS model in rats is an important animal model for the investigation of GS at present. Duan et al. (2010) reported that adriamycin injection in rats could decrease the expression of matrix metalloproteinase-1 (MMP-1) in renal tissue in rats. Hayashi et al. (2010) conducted a study in a mouse model of adriamycin-induced GS and found that the expression of MMP-2 was down-regulated when compared with that in control group. In our study, we found that the expressions of MMP-2 and MMP-9 (mRNA, protein and activity) in the glomerulus were reduced in adriamycin-induced GS rats. The results were similar to those of Duan et al. (2010) and Hayashi et al (2010). However, few study mentioned the change of MMP-9 in the glomerulus of adriamycin-induced GS rats.

Interestingly, Hwang et al (2004) and Park et al (Park et al., 2008) confirmed that apoE was a substrate of MMPs. Whether there is an association between gelatinases and apoE in the pathological change of GS, there hasn't any report. So, this study was performed to investigate the relation. In our study, protein expressions of MMP-2 and MMP-9 were reduced in the glomerulus of GS group compared to those in control group, and apoE expression in the glomerulus in GS group was up-regulated when compared with that of control group. Furthermore, significantly negative correlation was observed between MMP-2 or MMP-9 protein and apoE protein in the glomerulus in GS rats. We speculated the mechanism as follows: adriamycin administration reduced the expressions of MMP-2 and MMP-9 (mRNA and protein). The lower expression of MMP-2 and MMP-9 in the glomerulus could only degrade a small part of apoE which expressed in the glomerulus. So, apoE accumulation was observed in the glomerulus (generation of apoE was increased and degradation of apoE was reduced), which could mediate the lipids to deposit in the glomerulus. The accumulation of lipids could induce the generation of ROS (Farooqui and Farooqui, 2011; Obulesu et al., 2011). Overall expression of ROS could increase the production of TGF-ß1 (Zhou et al., 2009; Lin et al., 2010), which led to the development of GS by increasing the production of α-SMA and ECM (Col-IV and FN) in the glomerulus (Amara et al., 2010; Finnson et al., 2010; Diao et al., 2011). In conclusion, there might be an association between genatinases and apoE.

In our study, we found that lower expression of genatinases was associated with apo E expression. However, there were some limitations in our study. Whether there was a signal pathway between genatinases and apoE, it was not fully elucidated, and our investigation did not illustrate this mechanism. In order to clarify the effects of genatinases on apo E expression, interventions to the gelatinases in vivo (inhibitors such as doxycycline (Neto-Neves et al., 2011; Zeng et al., 2011) or tolylsam (Van Hul and Lijnen, 2011)) are necessary. Furthermore, cell culture (such as mesangial cell, podocyte, endothelial cell and so on) in vitro and transfection with small inhibitory RNA of MMP-2, MMP-9 or apoE to decrease the MMP-2, MMP-9 or apoE gene expression might be needed in future to investigate whether there was a signal pathway between MMP-2 or MMP-9 and apoE in the pathological change of GS.

TP, Alb in GS group were reduced compared to those of the SHO group in our study. Values of 24UTP, 24Ualb, BUN, Scr and GSI in GS group were significantly increased when compared with those in the SHO group. Those indicators reflected that the rat model in our study was suffering from nephrotic syndrome and the model of GS rats induced by adriamycin was successful. So, the conclusion from our research was robust to some extent.

In conclusion, the lower expression of MMP-2 or MMP-9 was associated with apoE accumulation in the glomerulus, which up-regulated the expression of TGF-ß1, α-SMA and ECM and induced the occurrence of GS. Whether there is a signal pathway between MMP-2 or MMP-9 and apoE in the progression of GS, it is not fully elucidated at present. Considering the widespread interest in MMP-2, MMP-9 and apoE and their interactions in the field of GS, our investigation might

have significant influence on future research for GS. However, more studies are needed to explore the possible signal pathway and molecular mechanisms, and confirm the role of gelatinases and apoE in the pathogenesis of GS in the future.

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Author contributions: Conceived and designed the experiments: Tian-Biao Zhou. Performed the experiments: Tian-Biao Zhou, Yuan-Han Qin and Feng-Ying Lei. Analyzed the data: Tian-Biao Zhou, Yan-Jun Zhao and Wei-Fang Huang. Contributed reagents/materials/analysis tools: Yuan-Han Qin. Wrote the paper: Tian-Biao Zhou

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