Article

Protective Effects of Blocking Renin-Angiotensin System on the Progression of Renal Injury in Glomerulosclerosis

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To investigate the protective effects of blocking rennin-angiotensin system (RAS) on the progression of renal injury in glomerulosclerosis, a glomerulosclerosis model was made for SD rats by unilateral nephrectomy and being injected with Adriamycin into caudal vein. The rats with glomerulosclerosis were randomly divided as ten per group into those without further treatment (group D) and those treated with Benazepril (group DB), Losartan (group DL), or sham-operation (group C), respectively. After 6 weeks of administration of Benazepril or Losartan, the mRNA expressions of TGF-β_1, Col IV, Fn, ET-1 and iNOS in renal cortex were measured by RT-PCR. Besides, the expressions of TGF-β_1, ET-1 and iNOS at protein level were detected by Western blotting and the concentrations of Col IV and Fn were analyzed with immunohistochemistry respectively. Results showed that the rats in group D appeared as obvious proteinuria, hypoalbuminemia and hypercholesterolemia, which had a significant difference compared with group C ($p < 0.05$), and most of their mesangiums were detected with cellular proliferation and significant increasing for extracellular matrix. Renal cortex TGF-β_1, Col IV, Fn, ET-1 and iNOS in rats of group D were increased by 3.59, 2.57, 2.21, 2.58 and 3.28 times at mRNA level, and by 2.60, 1.40, 0.75, 1.83 and 2.15 times at protein level, respectively, compared with group C. When the animals were treated with Benazepril (group DB) or Losartan (group DL), however, the biochemical and pathological damages were significantly recovered, and protein expressions of TGF-β_1, Col IV, Fn, ET-1 and iNOS were also significantly diminished ($p < 0.05$). This study suggested that blocking RAS using Benazepril or Losartan can have protective effects on the renal injury in glomerulosclerosis by down-regulating the expressions of TGF-β_1, Col IV, Fn, ET-1 and iNOS. *Cellular & Molecular Immunology*. 2005;2(2):150-154.

Key Words: glomerulosclerosis, rennin-angiotensin system, Benazepril, Losartan

Introduction

The final outcome of the progression of nephrosis is glomerulosclerosis. Therefore the recognition of the related factors and its mechanism are of extreme importance. In recent years, attention has been paid to abnormal activity of renin-agiotensin system in local renal tissue which plays a key role in causing the development of renal pathological changes. Ang II is the most important active factor in renin-angiotensin system and has close relationship with glomerulosclerosis, not only participating in the regulation of intraglomerular hemodynamic changes, but also relating with glomerular mesangial cell proliferation and extracellular matrix formation. The present study suggested that TGF-β_1 of kidney tissue has a close correlation with glomerulosclerosis. TGF-β_1 can stimulate the proliferation of glomerular mesangial cells and the synthesis of extracellular matrix, inhibiting the matrix metalloproteinase, decreasing the degradation of extracellular matrix, which is able to induce the repair, remodeling and sclerosis in the renal tissue under pathological circumstances (1). In addition, over-expressed ET-1 and NO in kidney tissue have positive effects on the renal hemodynamic abnormality (2). In recent years, administration of angiotensin converting enzyme inhibitor (ACEI) Benazepril or angiotensin II-I type of receptor antagonist Losartan can partly improve development of glomerulosclerosis, but related factors and its mechanisms of action on cause of glomerulosclerosis have not been recognized yet. In order to explore the possible protective mechanisms by blocking RAS for treating glomerulosclerosis, the following

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events including how vascular active factors ET-1 and NO influence renal hemodynamics, and what are the relations with TGF-β_1 and extracellular matrix Col IV, Fn at the levels of gene and protein, were observed by applying molecular biological techniques.

Materials and Methods

Setting up the nephrosis model

Thirty SD rats were made into glomerulosclerosis model by unilateral nephrectomy and intravenous injection of Adriamycin: injecting 0.4 ml of 2% sodium pentobarbital into abdominal cavity before operation, injecting Adriamycin (6 mg/kg) into caudal vein, and four weeks later, the ones with urine protein > 50 mg/24 h were taken as rats with nephrosis. They were divided randomly 10 ones per group into glomerulosclerosis group (group D), glomerulosclerosis treated with Benazepril group (group DB), and glomerulosclerosis treated with Losartan group (group DL). The other 10 ones were taken as sham-operation group (group C), injected normal saline into caudal vein. The rats were fed in respective cages, eating and drinking freely. The DB group was given gastric irritation of Benazepril each day (Beijing Novartis Pharma Ltd., 10 mg/kg·d), the DL group was given gastric irritation of Losartan each day (MSD Pharmaceutical Co. Ltd., 40 mg/kg·d), and the groups C and D were given gastric irritation of normal saline of corresponding amount, for 6 weeks totally.

Collection of specimen

Blood and urine specimen were collected before injecting Adriamycin and four weeks later, blood albumin, cholesterol, blood urea nitrogen, creatinine and the excretion amount of 24 hours urine protein were determined, and the kidney was resected, part of which was placed into 10% formaldehyde solution, and the renal cortex separated from the other part was put into liquid nitrogen for frozen saving to be tested.

RT-PCR

Fresh frozen-saved renal cortex (100 mg) was cut to extract total RNA in one-step fluorescein isothiocyanate amino-formamidine-phenol-chloroform. Its purity and content were determined by ultraviolet spectrophotometer, discovering A_{260}/A_{280} in the range of 1.80~2.0 with the content 0.5~1.2 μg/μl. 2 μg of total RNA was taken to be made into cDNA by reverse transcription with oligo(dT), and the reverse transcription Test Kit was the product of Invitrogen Company, enzyme and amortization system required were provided by Shanghai Shengneng Bocai Company. TGF-β_1 sense primer: 5'-CTT CAG CTC CAC AGA GAA GAA CTG A-3'; anti-sense primer: 5'-CAC GAT CAT GTT GGA CAA CTG GTC C-3'. Col IV sense primer: 5'-ATT GGT GGC TCT CCA GGA ATC ACA G-3'; anti-sense primer: 5'-GGT GGT CCG GGG CTA CCC AAC GGT-3'. Fn sense primer: 5'-GCA GCC CAC AGT GGA GTA TGT-3'; anti-sense primer: 5'-TTC TTT CAT TGG TCC GGT CTT-3'. ET-1 sense primer: 5'-AAG ATC CCA GCC AGC ATG GAG AGC G-3'; anti-sense primer: 5'-CGT TGC TCC TGC TCC TCC TTG ATG G-3'. iNOS sense primer: 5'-GTG TTC CAC CAG GAG ATG TTG-3'; anti-sense primer: 5'-CTC CTG CCC ACT GAG TTC GTC-3'. GAPDH was taken as inner reference to monitor RNA amount, each amplified product was dealt by 2% agarose gel electrophoresis and then taken photos under the burdick lamp. The image of amplified strips was analyzed by 2-dimension laser scanner and corrected by GAPDH, indicated with the ratio of absorbance.

Western blotting analysis

The total protein of renal tissue was extracted by tissue splitting solution RIPA, and the concentration of protein was determined by modified Lowry way. Total protein (50 μg) was taken and dealt by gel electrophoresis with 15% SDS-PAGE, then transferred to nitric fibrous membrane. Then it was sealed with TBST 4°C of 5% defatted milk over night. After washing membrane, the polyclonal antibody TGF-β_1, ET-1 or iNOS (Santa Cruza Company, working concentration 1:150) of rabbit-anti-rat was respectively added for hybridization, then anti-rabbit IgG (working concentration 1:150) marked by HRP was used for the hybridization of second antibody, at last immunoblot chemiluminescence reagent was added, with autoradiography.

Immunohistochemistry

The renal tissue section was dewaxed regularly, hydrated, hatched 20 min with 0.1% Trypsin at 37°C, and the endogenetic peroxidase was inactived by 1% Hydroperoxide methanol for 8 min, blocked by 10% sheep blood serum, respectively added rabbit-anti-rat Col IV or Fn at room temperature, then dealt with anti-rabbit IgG (Col IV, Fn immunity tissue chemistry kit was the product of Wuhan Boshide Company) marked by HRP, colored by DAB, redyed by brazilin，20 continuous renal cortex sight were observed under microscope. The colored result was classified in half quantitative analysis by stained area and strength in HP.

Pathology

The paraffin section of renal tissue was 2 μm thick, dyed by HE, 20 glomerulus of each specimen were observed in HP double-blindly, scoring by the quantity of mesangial cell, mesangial base, constriction of blood capillary lumen, glomerulosclerosis, crescent and adhesion of saccule. The mean value was calculated as the glomerular assault index. The renal tubule interstitial sights of left-top, right-top, left-bottom, right-bottom and midst of each specimen were observed in LP double-blindly in turn for, scoring by vacuolation of renal tubular epithelial cell, renal tubule dilataltion, renal tubule atrophy, red cell cast, protein cast, interstitial edema, interstitial fibrosis, and interstitial cell infiltration. The mean was calculated as the renal tubule interstitial assault index of this specimen (3).

Statistical analysis

The data shown as mean ± SD, were analyzed by *t*-test and variance using SPSS. A difference was considered significant when *p* value was less than 0.05.

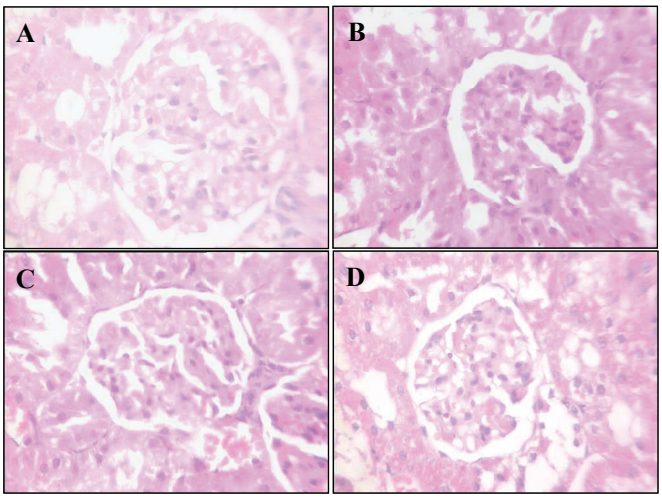

Figure 1. Histopathologic changes in the four groups. (A) Normal glomerulus and renal tubule interstitial structure in C group; (B) Proliferation occurring in the majority of glomerular mesangial cells and extracellular matrix, degeneration of renal tubular epithelial cells, renal interstitial fibrosis, and infiltration of widespread mononuclear cells in D group; (C, D) The pathological changes in DB and DL groups were alleviated remarkably (HE × 400).

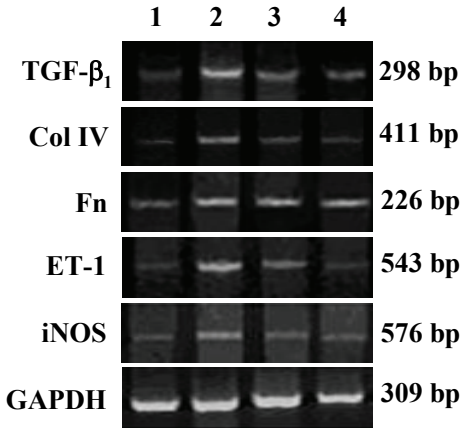

Figure 2. Electrophoretic images of TGF-β_1, Col Ⅳ, Fn, ET-1, iNOS mRNA expressions of renal cortex in all groups. Lane 1, sham-operation group (C group); Lane 2, glomerulosclerosis group (D group); Lane 3, glomerulosclerosis treated with Benazepril group (DB group); Lane 4, glomerulosclerosis treated with Losartan group (DL group).

Results

Biochemical and pathological changes

At the sixth week on experiment, group D presented with the obvious proteinuria, hypoalbuminemia and hypercholesterolemia, which had an obvious difference compared with group C ($p < 0.05$), the blood urea nitrogen and creatinine also elevated. The blood and urine biochemical indices got significant improvement in group DB and DL ($p < 0.05$, Table 1).

The pathological results: most of mesangium had the cellular proliferation in group D and there were significant increase for extracellular matrix, partly there was saccule adhesion, focal segment glomerular sclerosis or the sclerosis of whole renal glomerulus, the narrowing and the obstruction of the capillary vessel lumen, the degeneration of renal tubular epithelial cells, the poly-focal atrophy, partial compensatory hypertrophy, protein casts, renal interstitial fibrosis and the infiltration of the diffusive mononuclear cells. The pathological changes in groups DB and DL were significantly weaker than that in group D and their manifestations were the segmental hyperplasia of the minority of mesangial cells and extracellular matrix. There were seldom saccule adhesion and focal segment sclerosis and there were no stricture for the blood capillary lumen, no renal tubule atrophy, no interstitial fibrosis, but seldom, there were few single nuclear cells infiltration (Figure 1). Half pathological quantitative analysis showed that the glomerular assault index and renal tubule interstitial assault index rose in group D compared with group C, those index in DB and DL group were alleviated remarkably.

Renal cortex TGF-β_1, Col IV, Fn, ET-1, iNOS mRNA and protein expressions

RT-PCR demonstrated that renal cortex TGF-β_1, Col IV, Fn, ET-1 and iNOS mRNA in group D were increased by 3.59,

Table 1. Comparison of biochemical changes among all groups

Group	Urinary protein (mg/24 h)	Serum albumin (g/L)	Serum cholesterol (mmol/L)	Blood urea nitrogen (mmol/L)	Serum creatinine (μmol/L)
C group	16.32 ± 3.75	27.52 ± 4.43	2.08 ± 0.78	5.15 ± 2.57	60.13 ± 8.75
D group	67.38 ± 15.32**	17.53 ± 3.83*	6.63 ± 2.37*	9.68 ± 5.35*	76.57 ± 17.72
DB group	33.87 ± 5.72$^{\triangle}$	26.7 ± 4.6$^{\triangle}$	3.73 ± 1.53$^{\triangle}$	8.32 ± 3.35	68.50 ± 12.45
DL group	28.45 ± 4.36$^{\triangle}$	28.33 ± 4.45$^{\triangle}$	3.45 ± 1.21$^{\triangle}$	6.86 ± 3.09$^{\triangle}$	63.37 ± 13.08

*$p < 0.05$, **$p < 0.01$, *vs* C group; $^{\triangle}p < 0.05$, *vs* D group.

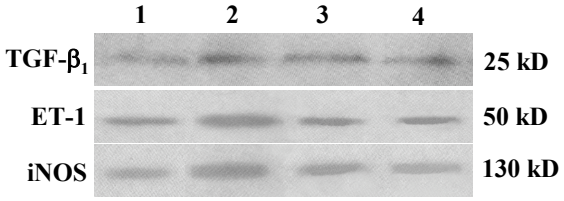

Figure 3. TGF-β_1, ET-1, iNOS protein expressions of renal cortex in all groups. Lane 1, sham-operation group (C group); Lane 2, glomerulosclerosis group (D group); Lane 3, glomerulosclerosis treated with Benazepril group (DB group); Lane 4, glomerulosclerosis treated with Losartan group (DL group).

2.57, 2.21, 2.58 and 3.28 times respectively, compared with group C. After administration of Benazepril or Losartan, TGF-β_1 mRNA expressions of renal cortex were decreased 46%, 53%, Col IV mRNA decreased 46%, 54%, Fn mRNA decreased 42%, 51%, ET-1 mRNA decreased 51%, 58%, and iNOS mRNA decreased 40%, 47%, respectively. There were significant differences by the variance analysis in TGF-β_1, Col IV, Fn, ET-1 and iNOS mRNA among the four groups ($p < 0.01$).

Compared with sham-operation group, the protein levels of TGF-β_1, Col IV, Fn, ET-1 and iNOS in glomerulosclerosis group were increased 2.60, 1.40, 0.75, 1.83 and 2.15 times respectively. In the treatment groups with Benazepril or Losartan, TGF-β_1 protein expressions in renal cortex were decreased 55%, 59%, Col IV decreased 43%, 48%, Fn decreased 34%, 36%, ET-1 decreased 31%, 37%, and iNOS decreased 44%, 58%, respectively. There was significant difference by the variance analysis on TGF-β_1, Col IV, Fn, ET-1 and iNOS proteins among the four groups ($p < 0.01$).

There were significant differences in TGF-β_1, Col IV, Fn, ET-1 and iNOS mRNA and protein expressions between D and C group, also among DB group, DL group and D group ($p < 0.05$). Besides iNOS protein had an obvious difference ($q = 3.23$, $p < 0.05$), there was no significant difference between DB and DL group (Table 2, Figures 2 and 3).

Discussion

Extracellular matrix plays an important role in maintaining the normal renal tissue structure, function, cell growth, and differentiation. It is in a dynamic equilibrium of unceasing metabolism renewing and degradation remodeling. When the dynamic equilibrium is disturbed, the excessive deposition of extracellular matrix Col IV, Fn is the main reason leading to glomerulosclerosis (4).

Renal hemodynamics is regulated by the dilation or contraction status of the renal arteriole. When the dilation of afferent arteriole is larger than the dilation of efferent arteriole in the glomerulus, hyperfiltration and hyperperfusion appear. NO can diffuse into smooth muscle cells to activate soluble guanosine monophosphate cyclase and to stimulate the production of 3',5'-cyclic guanosine monophasphate, and then dilate the vascular smooth muscle, moreover has more dilatant effect on afferent arteriole than on efferent arteriole (5). NO comes mainly from L-arginine in the body, and is produced through catalysis of iNOS, as a signal transduction molecule easy to diffuse and its half-life is only several seconds, so that it is difficult to be tested in the tissue. Kashem (6) found that iNOS can reflect the actual NO content under pathological circumstances, so the expression of iNOS was determined to reflect the changes of NO in this

Table 2. Comparison of TGF-β_1, Col IV, Fn, ET-1, iNOS mRNA and protein among all groups

Groups (n = 10)	TGF-β_1		Col IV		Fn	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
C group	0.17 ± 0.06	0.032 ± 0.008	0.14 ± 0.03	9.78 ± 3.08	0.14 ± 0.03	14.32 ± 5.14
D group	0.78 ± 0.15**	0.115 ± 0.012**	0.50 ± 0.12**	23.47 ± 6.27*	0.45 ± 0.10*	26.75 ± 8.42*
DB group	0.42 ± 0.11$^{\triangle}$	0.052 ± 0.010$^{\triangle}$	0.27 ± 0.06$^{\triangle}$	13.45 ± 5.25$^{\triangle}$	0.26 ± 0.06$^{\triangle}$	18.55 ± 5.74$^{\triangle}$
DL group	0.37 ± 0.07$^{\triangle\triangle}$	0.047 ± 0.006$^{\triangle}$	0.23 ± 0.05$^{\triangle}$	12.26 ± 4.78$^{\triangle}$	0.22 ± 0.05$^{\triangle}$	17.23 ± 3.68$^{\triangle}$

Groups (n = 10)	ET-1		iNOS	
	mRNA	Protein	mRNA	Protein
C group	0.12 ± 0.05	10.37 ± 3.75	0.07 ± 0.03	0.82 ± 0.08
D group	0.43 ± 0.14**	29.34 ± 6.18**	0.30 ± 0.11**	2.58 ± 0.14**
DB group	0.21 ± 0.12$^{\triangle}$	20.37 ± 5.83$^{\triangle}$	0.18 ± 0.06$^{\triangle}$	1.44 ± 0.10$^{\triangle}$
DL group	0.18 ± 0.08$^{\triangle}$	18.62 ± 6.76$^{\triangle}$	0.16 ± 0.04$^{\triangle}$	1.08 ± 0.09$^{\triangle\triangle\#}$

Note: After 6 weeks of treatment with Benazepril or Losartan, the mRNA expressions of TGF-β_1, Col IV, Fn, ET-1 and iNOS in renal cortex were measured by RT-PCR, the protein levels of TGF-β_1, ET-1 and iNOS proteins were detected by Western blotting, and the concentrations of Col IV and Fn were analyzed with immunohistochemistry respectively. There were significant differences by the variance analysis in TGF-β_1, Col IV, Fn, ET-1 and iNOS mRNA and protein expressions among the four groups ($p < 0.01$). *$p < 0.05$, **$p < 0.01$, *vs* C group; $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$, *vs* D group; $^{\#}p < 0.05$, *vs* DB group.

study. The results showed that the expression of iNOS was quite weak in the sham-operation group and elevated obviously in glonerulosclerosis group, which indicated that iNOS was activated only under pathological circumstances. After the administration of Benazepril or Losartan, iNOS gene and protein expressions of renal tissue were decreased significantly. Therefore, the over-expression of NO might be the main factor in the initiation of hyperperfusion and hyperfiltration in the early stage of glomerulosclerosis, at the same time, the compensative over-expression of ET-1 accelerated the extracellular matrix proliferation.

ET-1 could promote expression of TGF-β_1 through ETa-receptor, and TGF-β_1 could promote synthesis of ET-1 too. It had been reported that ET-1 and Ang II could synergize and activate each other in the regulation of glomerular function, matrix formation and cell growth (7). Ang II is the most important active factor in reninangiotensin system and has close relationship with glomerulosclerosis (8). It was reported that the increase of "shearing stress" led to the increase of excretion TGF-β_1 of the mesangial cells, which might be mechanism of high pressure inside glomerulus stimulating the synthesis of TGF-β_1 (9). TGF-β_1 can stimulate the proliferation of glomerular mesangial cells, the synthesis of extracellular matrix, inhibit the matrix metalloproteinase, and decrease the degradation of extracellular matrix, which induces the repair, remodeling and sclerosis in the renal tissue under pathological circumstances (1).

In the study, TGF-β_1, ET-1 and iNOS of glomerulosclerosis group at the levels of gene and protein increased, in accordance with the expressions of extracellular matrix Col IV and Fn. After the administration of Benazepril or Losartan, proteinuria, hypoalbuminemia and hypercholesterolemia and renal function improved significantly, hyperplasia of the mesangial cells and matrix were relieved. There is only Ang II-I type receptor and no Ang II-II type receptor in kidney of mature rats and the human, so renal local Ang II takes effects mainly by Ang II-I type receptor mediation (10). Losartan is an Ang II-I type receptor antagonist, whose effects is more notable than another medicine though the effects of both of treatment groups were similar in our study, which might be relevant to its dosage and specificity of its receptor level. However, its mechanism still need be observed and studied further.

In conclusion, angiotensin converting enzyme inhibitor (ACEI) Benazepril or angiotensin II-I type of receptor antagonist Losartan could improve development of glomerulosclerosis. The elevated mRNA and protein expressions of TGF-β_1, Col IV, Fn, ET-1 and iNOS in renal cortex were involved in the progression of renal injury in glomerulosclerosis. Thus, the direct regulation of Ang II activation by blocking RAS might provide a novel therapeutic approach to attenuating renal damage in glomerulosclerosis.

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