Korean J Physiol Pharmacol
Vol 9: 159-164, June, 2005

Protective Effect of N-Acetylcysteine on Progression of Adriamycin-induced Nephropathy

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Effects of antioxidants on the established nephropathy were investigated. The experimental nephropathy was induced in rats by intravenous injection of adriamycin (2 mg/kg). Six weeks later, when proteinuria was apparent, the rats were supplemented with N-acetylcysteine (NAC, 1 g/kg/day) in drinking water for additional 6 weeks. Glomerulosclerosis score and tubulointerstitial injury index were determined by light microscopy. Expression of transforming growth factor (TGF) β1 and laminin β1 was determined in the renal cortex by reverse transcription-polymerase chain reaction, Western blotting, immunohistochemistry, and immunogold electron microscopy. The adriamycin-induced proteinuria as well as the glomerulosclerosis and tubulointerstitial injury was ameliorated by the treatment with NAC. Adriamycin increased the expression of TGF β1 mRNA and protein, which was ameliorated by NAC. Although the expression of laminin β1 mRNA was increased, adriamycin did not significantly alter that of its protein. These results indicate that antioxidants ameliorate the established nephropathy in association with normalization of overexpressed TGF β1.

Key Words: Nephropathy, N-Acetylcysteine, Glomerulosclerosis, Tubulointerstitial injury, Transforming growth factor β1, Laminin β1

INTRODUCTION

The adriamycin-induced nephropathy represents a progressive glomerular disease (Border et al, 1989). An overexpression of transforming growth factor (TGF) β1 as well as an increased accumulation of extracellular matrix (ECM), including collagen, fibronectin, and laminin, is noted in this model of experimental nephropathy (Tamaki et al, 1994; Manabe et al, 2001). Furthermore, it has been causally related with oxidative stress (Barbey et al, 1989; Van den Branden et al, 2002).

Previous studies examined the effects of antioxidants given concurrently with or shortly after administration of adriamycin (Okasora et al, 1992; Rangan et al, 1999). Therefore, it is unclear whether oxidative stress is also involved in chronic inexorable nephropathy. The present study was undertaken to examine the effects of antioxidants on established nephropathy. The expression of TGF β1 and laminin β1 was determined in the kidney of rats with established adriamycin-induced nephropathy.

METHODS

Animals

Male Sprague-Dawley rats (180~250 g) were used. They were handled according to Guidelines for Laboratory Animals of *The Korean Academy of Medical Sciences*. Experimental group received a single injection of doxorubicin hydrochloride (Adriamycin, 2 mg/kg; Pharmacia & Upjohn, Milan, Italy) via the tail vein in a conscious state. Control group was injected with equal volume of saline. Six weeks later, the adriamycin-treated rats were randomly divided into two groups. They were supplemented with N-acetylcysteine (NAC, 1 g/kg/day; Zambon, Milan, Italy) in drinking water for additional 6 weeks in one group (ADR+NAC) and without in the other (ADR). At weeks 0, 6 and 12, systolic blood pressure was measured by tail-cuff methods without anesthesia, and 24-hr urine was collected in a metabolic cage.

RT-PCR of TGF β1 and laminin β1

Kidneys were quickly removed and stored at -70°C until assessed. Total RNA was isolated from the renal cortex according to the protocols of Ultraspec^{TM} RNA isolation system (Biotecx Laboratories; Houston, TX, USA). The RNA concentration was determined by absorbance at 260 nm. The expression of TGF β1 and laminin β1 was determined by reverse transcription (RT) polymerase chain reaction (PCR). For RT, 1 μg of total RNA was incubated with reverse transcriptase (Gibco BRL; Grand Island, NY, USA; 200 U), RNasin (10 U), dNTP mix (10 mM), dithiothreitol

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ABBREVIATIONS: TGF, transforming growth factor; ECM, extracellular matrix; NAC, N-acetylcysteine; ADR, adriamycin; RT, reverse transcription; PCR, polymerase chain reaction; ROS, reactive oxygen species.

Table 1. Oligonucleotide sequences used for PCR

Gene	Primer sequence	References
TGF β1 (294 bp)		
Sense	5'-GGACTACTACGCCAAAGAAG-3'	Fukuda et al, 2001
Antisense	5'-TCAAAAGACAGCCACTCAGG-3'	
Laminin β1 (519 bp)		
Sense	5'-CCAACTGGACCTGGAAGCAGAA-3'	Gene Bank Accession #M15525
Antisense	5'-CCCGTGAACCATTCCTTCCACT-3'	
β-Actin (423 bp)		
Sense	5'-GACTACCTCATGAAGATCCTGACC-3'	Mistry et al, 1998
Antisense	5'-TGATCTTCATGGTGCTAGGAGCC-3'	

(0.1 M), $MgCl_2$ (25 mM), oligo(dT) (0.5 μg/l), and reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] in a final volume of 30 μl at 42°C for 50 min. After inactivation of reverse transcriptase at 72°C for 15 min, 2 μl cDNA was subjected to PCR amplification.

PCR was conducted in a final volume of 20 μl containing 10 pmole of each primer, dNTP mix (250 μM), $MgCl_2$ (1.5 mM), and Taq polymerase (0.3 U) using a thermal cycler (M.J. Research; Watertown, MA, USA). The amplification profile of TGF β1 consisted of 95°C for 45 sec, at 50°C for 45 sec, and at 72°C for 1 min, that of laminin β1 95°C for 1 min, at 59°C for 1 min, and at 72°C for 90 sec, and that of β-actin 94°C for 45 sec, at 56°C for 45 sec, and at 72°C for 90 sec. The final extension was ended with 5 min of elongation at 72°C. Under the conditions performed, RT-PCR allowed semiquantitative evaluation of TGF β1, laminin β1 and β-actin mRNA with 0.1 μg of total RNA each. The amplification cycle was 35, the same in all.

The primers were adopted from those described by previous investigators (Table 1). PCR products were size fractionated by 1.2% agarose gel electrophoresis and visualized under ultraviolet light with ethidium bromide staining. The quantification of cDNA was performed using IMAGER™ & 1D MAIN (Bioneer; Cheongwon, Korea).

Protein preparation and Western blot analysis

The cortex was dissected and homogenized with Polytron homogenizer at 3,000 rpm in a solution containing sucrose (250 mM), EDTA (1 mM), phenylmethylsulfonyl fluoride (0.1 mM), and potassium phosphate buffer (20 mM) at pH 7.6. The homogenate was centrifuged at 1,000×g for 15 min to remove whole cells, nuclei and mitochondria. The supernatant was then centrifuged at 100,000×g for 1 hour. The supernatant was resuspended in homogenizing solution for protein blotting. Protein concentrations were determined using bicinchonic acid assay kit (Bio-Rad; Hercules, CA, USA).

Protein samples (100 μg) were loaded and electrophoretically size-separated with a discontinuous system consisting of 8~12.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane at 40 V for 3 hours. The membranes were washed in Tris-based saline buffer (pH 7.4) containing 0.1% Tween-20 (TBST), blocked with 5% nonfat milk in TBST for 1 hour, and incubated with polyclonal rabbit anti-TGF β1 or polyclonal rabbit anti-laminin β1 diluted 1:750 in 2% nonfat milk/TBST for 2 hours at room temperature. The membranes were then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:1,200) in 2% nonfat milk in TBST for 2 hours. The bound secondary antibody was detected by enhanced chemiluminescence (Amersham; Buckinghamshire, England). The protein levels were determined by analyzing the autoradiogram signals using transmitter scanning videodensitometer (Biomed Instruments; Fullerton, CA, USA).

Glomerular and tubulointerstitial injuries

After blood collection, the kidneys were rapidly taken. The kidneys were fixed in 10% phosphate-buffered formalin and embedded in paraffin blocks. Histologic sections were stained with Masson's trichrome. Slides were examined by light microscopy. Glomerulosclerosis was scored positive when either segmental or global sclerosis was present. The extent of glomerulosclerosis was expressed as a percentage of the total number of glomeruli counted.

The severity of tubulointerstitial injury was evaluated semiquantitatively as described previously (Shu et al, 2002). For each sample, 30 cortical fields of Masson's trichrome-stained kidney sections were assessed at a ×20 objective in a blinded manner. The injury was defined as tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. A scale of 0~3 was used according to the area of the tubulointerstitial injury: 0, no injury; 1, less than 25%; 2, 25~50%; and 3, greater than 50%. The extent of injury for each specimen was then calculated as the sum of these scores.

Immunostaining of TGF β1 and laminin β1

Immunostaining of TGF β1 and laminin β1 was carried out on paraffin-embedded tissue sections. The deparaffinized sections were pretreated for antigen retrieval by autoclave heating in citrate buffer (pH 6.0) for 5 min. The endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min, followed by washing in phosphate-buffered saline (PBS). The sections were then immersed in 5% normal goat serum in PBS for 10 min, covered with primary rabbit polyclonal antibodies and incubated overnight at 4°C. The anti-TGF β1 antibody was used at a dilution of 1:100 and the anti-laminin β1 antibody at a dilution of 1:200 (both purchased from Santa Cruz Biotechnology; Santa Cruz, CA, USA). Immunoreactions were

performed using Vectastain peroxidase ABC kit. The antigenic sites were visualized by reacting the sections with a mixture of 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05 M Tris HCl buffer (pH 7.6) for 1 min. The nuclei were stained with hematoxylin.

Immunogold electron microscopy

Kidney tissues were immersed in a solution containing 4% paraformaldehyde, 0.5% glutaraldehyde, 0.3% sucrose in cacodylate buffer (pH 7.4), for 3 hours at room temperature and washed 3 times in cacodylate buffer (5 min each). The samples were dehydrated in a series of graded ethanol. Tissue samples were embedded in LR-White (London Resin; Aldermaston Berks, UK) for 1 hour at 4°C. The resin was polymerized in closed gelatin capsules at 43°C for 48 hours. Ultrathin sections were mounted on Nickel grids. Non-specific reaction was blocked by Tris buffer containing 1% BSA/Tris, 0.05% Tween, and 0.9% NaCl overnight at 4°C. The sections were incubated with rabbit anti-laminin β1 and additionally with colloidal gold (12 nm) labeled goat anti-rabbit IgG (Jackson Immuno Research Laboratories; West Grove, PA, USA). The gold-tagged IgG was used at 1:20 dilutions. Sections were rinsed with water, stained with 2% uranyl acetate, and examined with electron microscope (Hitachi 600; Tokyo, Japan). To exclude non-specific binding, control sections were incubated with the secondary antibody under the same conditions.

Statistical analysis

Results were expressed as means±SEM. The statistical significance of differences between the groups was determined by ANOVA. The value of P less than 0.05 was considered significant.

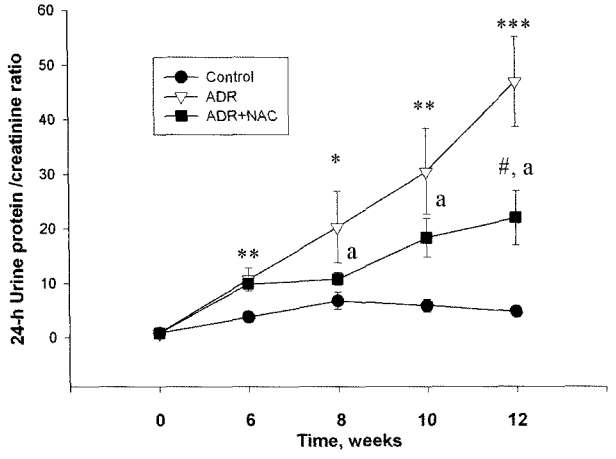

Fig. 1. Urinary protein excretion expressed as the ratio of 24-h urine protein to plasma creatinine in control (n=6), adriamycin-treated [ADR, n=7], and adriamycin- and N-acetylcysteine-treated rats [ADR+NAC, n=7]. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs Control, $^{\#}P<0.05$ vs ADR, ^{a}P=NS vs Control.

RESULTS

Renal function and blood pressure

Adriamycin-treatment progressively deteriorated the renal function and increased the blood pressure, which were ameliorated by the treatment with NAC (Fig. 1, Table 2).

Table 2. Systolic blood pressure (mmHg)

	Control	ADR	ADR+NAC
Week 6	116±2	125±1*	
Week 12	120±3	126±2	119±2#

Data are mean SEM. Numbers of are the same as in Fig. 1. $^*P<0.01$ vs Control, $^{\#}P<0.01$ vs ADR.

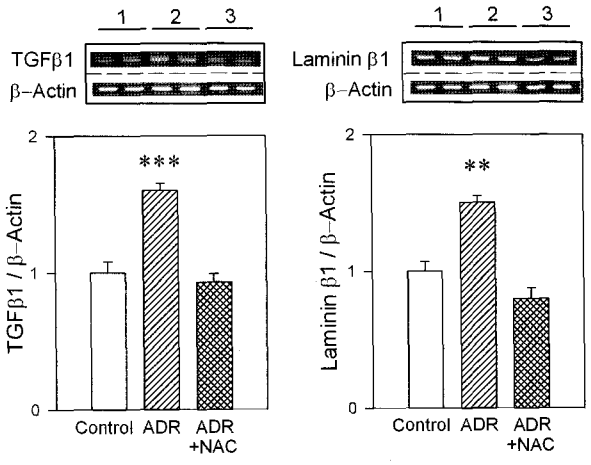

Fig. 2. Expression of TGF β1 mRNA and laminin β1 mRNA in the renal cortex at the end of the study. Groups are the same as in Fig. 1. $^{**}P<0.01$ vs Control, $^{***}P<0.001$ vs Control.

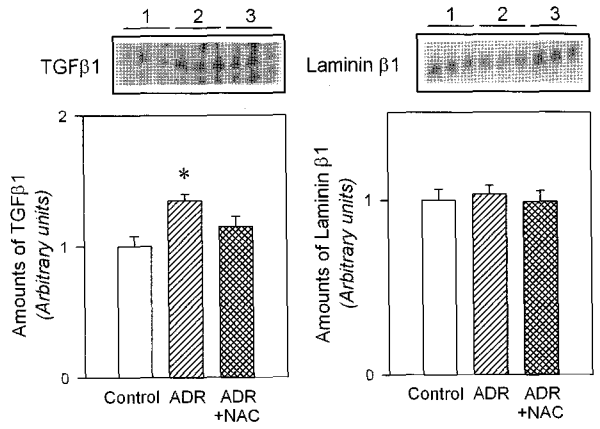

Fig. 3. Western blot analyses of TGF β1 and laminin β1 in the renal cortex at the end of the study. Groups are the same as in Fig. 1. $^*P<0.05$ vs Control.

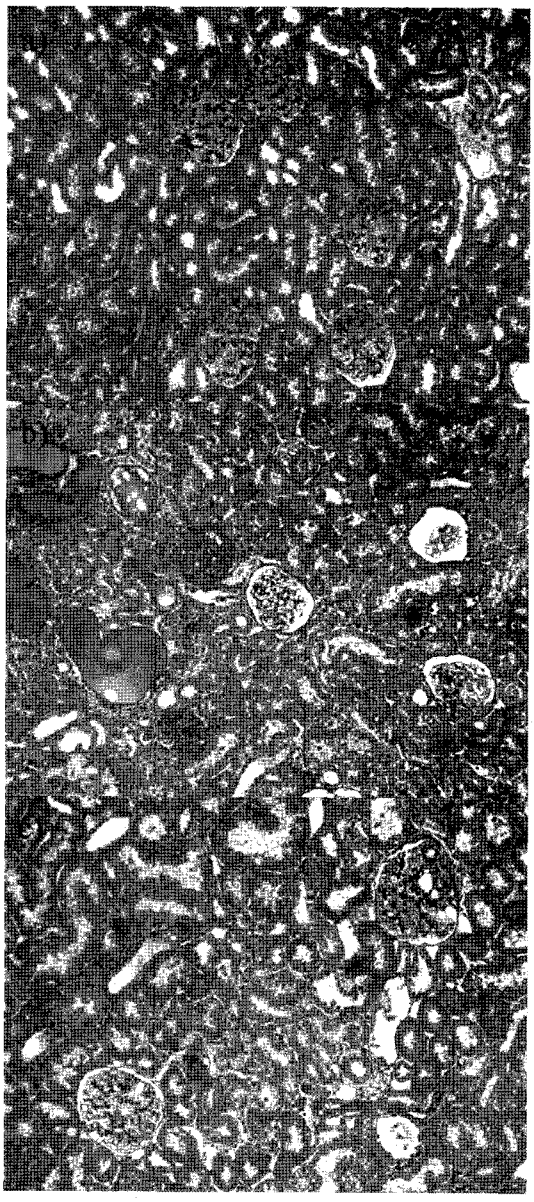

Fig. 4. Histologic findings of renal cortex stained with Masson's trichrome (×200). (a) Control. (b) Adriamycin-treated: there are notable glomerular and interstitial changes including global or segmental glomerulosclerosis, tubular atrophy or dilation, and interstitial fibrosis or thickening of basement membrane. (c) Adriamycin- and N-acetylcysteine-treated: there are minimal glomerular change and focal tubulointerstitial changes.

Expression of TGF β1 and laminin β1

RT-PCR revealed a marked increase of TGF β1 mRNA expression following the treatment with adriamycin, which was normalized by the treatment with NAC (Fig. 2). Accordingly, the expression of TGF β1 protein was markedly increased following the treatment with adriamycin, which was again normalized by NAC (Fig. 3).

The expression of laminin β1 mRNA was also markedly increased by the adriamycin-treatment, which was normalized by NAC (Fig. 2). However, its protein expression

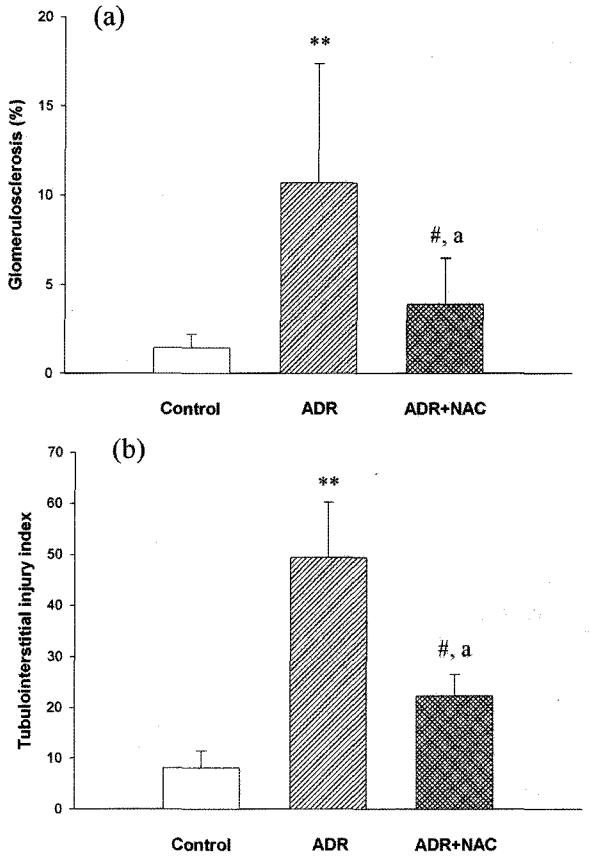

Fig. 5. Glomerulosclerosis score and tubulointerstitial injury index at the end of the study. Groups are the same as in Fig. 1. $^{**}P<0.01$ vs Control, $^{\#}P<0.05$ vs ADR, ^{a}P=NS vs Control.

was not significantly altered by the treatment with adriamycin (Fig. 3).

Glomerular and tubulointerstitial changes

Fig. 4 illustrates glomerular and tubulointerstitial changes following the treatment with adriamycin. There were collapse or obliteration of the capillary lumen and enlargement of the mesangial area, hyaline deposition in the glomerular epithelium and adhesion of the glomerular tuft to Bowman's capsule. The renal tubules showed focal atrophic and ballooning changes. The tubular interstitium showed fibrotic changes of variable degrees and thickening of basement membrane. However, the degree of glomerulosclerosis and tubulointerstitial injury was ameliorated by NAC (Fig. 5).

Immunostaining of TGF β1

Immunostaining of TGF β1 showed focal minimal reactions in the glomerular basement membrane and Bowman's capsule, and no reactions in the tubulointerstitium in control. On the contrary, the treatment with adriamycin significantly increased its immunostaining in the glomerular basement membrane, Bowman's capsule and tubulointerstitium, the degree of which was markedly decreased by NAC (Fig. 6).

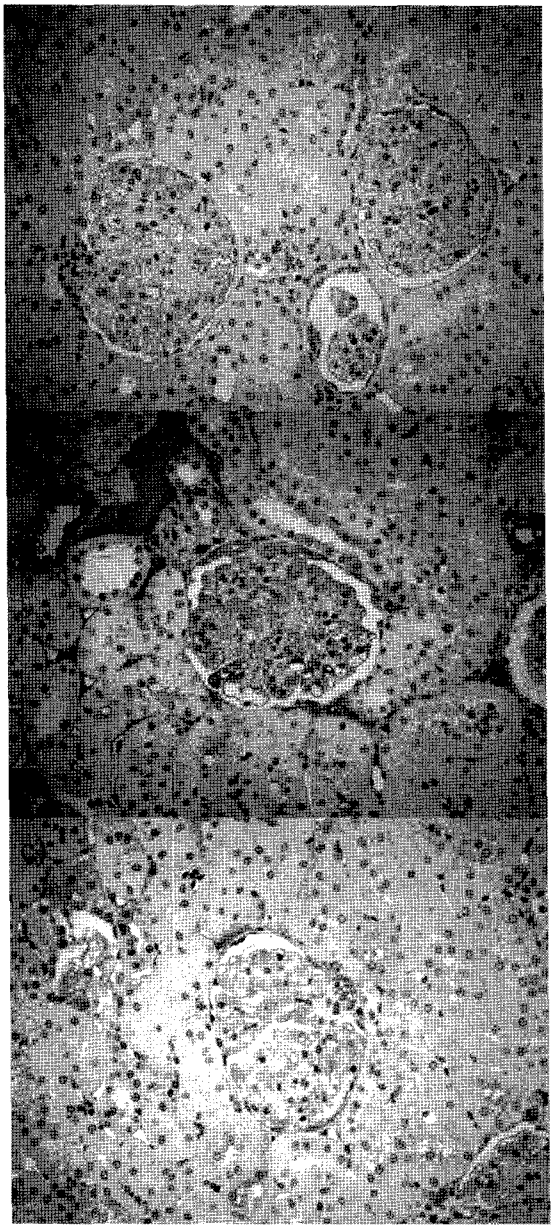

Fig. 6. Immunostaining of TGF 1 in the renal cortex. (a) Control rats show focal minimal reactions in glomerular basement membrane and Bowman's capsule, and negative reaction in the tubulointerstitium. (b) Adriamycin-treated rats show positive staining of TGF β1 in the glomerular basement membrane, Bowman's capsule and tubulointerstitium. (c) Treatment with N- acetylcysteine markedly decreased the adriamycin-induced increase of TGF β1 expression.

Immunogold electron microscopy

Gold-labeling for anti-laminin β1 appeared in the cytoplasm of podocytes, mesangial cells, and epithelial cells of proximal convoluted tubules, but not in the glomerular basement membrane and the tubular epithelial basement membrane in control rats. Gold particles for anti-laminin β1 were not affected by either adriamycin alone or combined with NAC (Fig. 7).

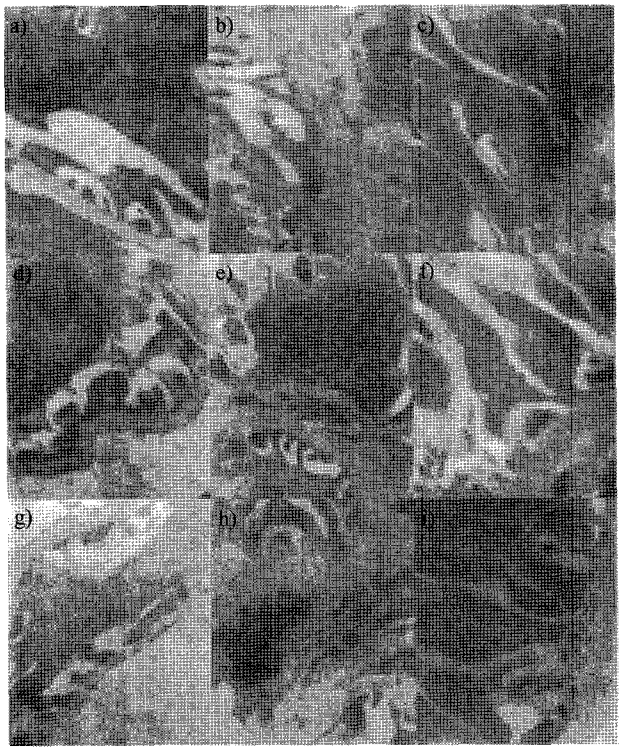

Fig. 7. Localization of gold-labelled anti-laminin β1 antibody. Control rats show a few gold particles in the cytoplasm of podocyte (a) and mesangial cell (b), and in the epithelial cell of proximal convoluted tubule (c). The labeling of laminin β1 was not changed in adriamycin-treated rats in the cytoplasm of podocyte (d), mesangial cell (e) and the epithelial cell of proximal convoluted tubule (f). Nor was it altered in the adriamycin- and N-acetylcysteine-treated rats in the cytoplasm of podocyte (g), mesangial cell (h) and the epithelial cell of proximal convoluted tubule (i) ($\times$30,000).

DISCUSSION

The present study demonstrated that NAC ameliorated the adriamycin-induced proteinuric nephropathy. Accordingly, the glomerulosclerosis and tubulointerstitial injury was ameliorated by NAC. NAC was also shown to return the TGF β1 mRNA and protein expression to the control level. The increased TGF expression may be causally related with structural changes of glomeruli and tubulointerstitia, leading to a progressive nephropathy.

As a critical component of glomerular basement membrane, the expression of laminins may play an important role in various glomerular diseases (Horikoshi et al, 1999). Indeed, the total content of laminin along with other ECM components in the cortex have been reported to increase 3-fold at week 10 and 10-fold at week 28 in rats with adriamycin-induced nephropathy, which was associated with progressive glomerulosclerosis and tubulointerstitial fibrosis (Manabe et al, 2001). However, the expression of laminin β1 proteins was not significantly altered in the present study. It has been demonstrated in anti-Thy 1.1 nephritis model that glomerular deposition of laminin and type IV collagen is increased by day 7 and declines thereafter (Sasaki et al, 1999). Therefore, the expression of laminin β1 proteins may have been increased at an initial

stage before the administration of NAC in the present study.

Adriamycin reacts with molecular oxygen, generating reactive oxygen species (ROS) in the kidney (Shah, 1989). The oxidative stress due to continued ROS stimulation increases cell proliferation, ECM synthesis, inflammatory cell infiltration, and apoptosis (Nath et al, 1998). NAC has a protective effect against oxygen radical damage by its direct scavenging properties and the ability to modulate intracellular glutathione levels (Aruoma et al, 1989). However, NAC failed to completely reverse the proteinuria despite its distinct effects on glomerular and tubulointerstitial injuries in the present study. A recent study suggests that NAC has vasodilatory properties and blocks the expression of vascular-cell adhesion molecule 1 and the activation of nuclear factor-κB in glomerular mesangial cells (Khachigian et al, 1997). Therefore, the renoprotective effects of NAC appears to be multifactorial. Among others, increased protein flux itself through the glomerular capillary barrier could injure glomeruli and tubulointerstitia.

In summary, the present study demonstrated that antioxidants had renoprotective effects in association with suppression of overexpressed TGF β1 in established nephropathy, suggesting a role of ROS in the progression of established nephropathy. The renal scarring with glomerulosclerosis and tubulointerstitial lesion and the enhanced expression of TGF β1 may be related to an accumulation of ROS.

ACKNOWLEDGEMENT

This work was supported by Research Grant from The Korean Society of Nephrology (2001, Baxter). The authors thank Dr Jung Kyun Kwon (Electron Microscope Lab, Hanyang University College of Medicine) for his technical assistance.

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