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Albumin Induces Cellular Fibrosis by Upregulating Transforming Growth Factor-Beta Ligand and Its Receptors in Renal Distal Tubule Cells

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Abstract Albuminuria is indicative of nephropathy. However, little literature has focused on the role of albumin in renal distal tubule fibrosis. We used a well-defined distal tubule cell, Madin-Darby Canine Kidney (MDCK). Proliferation and cytotoxicity were examined. The conditioned supernatant was collected and subjected to ELISA assay for detection of fibronectin and TGF-β1. Reverse transcription-PCR and Western blot assay were performed to evaluate the expression of mRNA and protein of two types of TGF-β receptors (TbetaR). Flow cytometry assay and phosphotyrosine (pY)-specific antibodies were used to assay the phosphorylation status of TbetaR. We showed that albumin dose dependently (0, 0.1, 1, or 10 mg/ml) inhibited cellular growth in MDCK cells without inducing cellular cytoxicity. In addition, albumin significantly upregulated the secretion of both fibronectin and TGF-β1 at dose over 1 mg/ml. Moreover, 24 h pretreatment of albumin significantly enhanced exogenous TGF-β1-induced secretion of fibronectin. These observations were reminiscent of the implications of TbetaR since TbetaR appears to correlate with the susceptibility of cellular fibrosis. We found that albumin significantly increased protein levels of type I TbetaR (TbetaRI) instead of type II receptors (TbetaRII). In addition, phosphorylation level of TbetaRII of both pY259 and pY424 was significantly enhanced instead of pY336. The novel observation indicates that extreme dose of albumin upregulates TGF-β autocrine loop by upregulating TGF-β1, TbetaRI, and the receptor kinase activity of TbetaRII by inducing tyrosine phosphorylation on key amino residue of TbetaRII in renal distal tubule cells. These combinational effects might contribute to the pathogenesis of renal fibrosis. J. Cell. Biochem. 97: 956–968, 2006.

Key words: albumin; transforming growth factor-β; receptor; fibrosis; diabetic nephropathy; phosphorylation

Proteinuria is a hallmark of end-stage renal disease. The major component present in urine from proteinuria patients is albumin. Despite proteinuria is considered to be a diagnostic marker for renal injury for decades, there are increasing studies showing that albumin per se might play a pivotal role in the pathogenesis of diabetic nephropathy. First, in vivo studies

Abbreviations used: MDCK, Madin-Darby Canine Kidney; FBS, Fetal bovine serum; MEM, minimal essential medium; TGF-β, Transforming growth factor-β; TbetaRI, TGF-β Receptor type I; TbetaRII, type II TGF-β Receptor; cDNA, complementary DNA; PAGE, Polyacrylamide gel electrophoresis.

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and related clinical statistic data have shown that proteinuria is implicated in the pathogenesis (i.e., the induction of renal fibrosis) of inflammatory alternations of the renal tubular interstitium [Burton and Harris, 1996; Ruggenenti et al., 1998]. Second, in vitro studies have demonstrated that albumin is a significant fibrogenic inducer for proximal tubular cells since some proinflammatory and vasoactive factors are significantly upregulated [Zoja et al., 1998; Burton et al., 1999; Yard et al., 2001; Tang et al., 2003]. Thus, proteinuria has recently been suggested as therapeutic target for diabetic nephropathy in these years [Nenigni et al., 1995; D'Amico and Bazzi, 2003].

Of the growth factors affected by albumin, transforming growth factor-beta (TGF-β) is one of the most important factors leading to renal fibrosis. It has been previously shown that albumin activates the synthesis of TGF-β in renal proximal tubular segment [Zoja et al., 1998; Burton et al., 1999]. TGF-β transduces its signal by a pair of transmembrane kinases receptor, known as type I and type II receptors, which are present on many cell types that have been analyzed [Lin et al., 1992; Franzen et al., 1993]. The type I receptors (designated as TbetaRI) do not bind TGF-β in the absence of type II receptors (designated as TbetaRII). The binding of TGF-β1 to TbetaRII results in the recruitment, phosphorylation, and concomitant activation of TbetaRI [Wrana et al., 1992]. The activated TbetaRI in turn phosphorylate and transmit the signal to a family of downstream mediators, termed Smads, resulting in the regulation of target gene expression such as fibronectin [Wrana et al., 1994; Wieser et al., 1995; Souchelnytskyi et al., 1996]. In this respect, both types of TGF-β receptor are essential for transducing the fibrogenic signal of TGF-β and will determine cellular fibrogenic sensitivity. According to Wolf et al. [2004], incubation of mouse proximal tubular cells with albumin leads to an increase in TbetaRII mRNA and protein expression without influencing TbetaRI [Wolf et al., 2004]. This finding shows that proximal tubule cells appear to upregulate its sensitivity to TGF-β under the treatment of albumin. However, little is known about the underlying mechanism in distal tubule cells.

The TbetaRII constitutes a distinct family of transmembrane proteins with a dual specificity kinase, serine/threonine kinase activity [Massague, 1996; Ten Dijke et al., 1996] and tyrosine kinase activity [Wrana et al., 1994; Chen and Weinberg, 1995]). According to Lawler et al. [1997], they observed three phosphotyrosine sites in TbetaRII proteins, locating at kinase subdomain I (Tyr259), subdomain V (Tyr336), and subdomain VIII (Tyr424), respectively. The presence of these tyrosine phosphorylations in TbetaRII proteins suggests a possible autoregulatory role similar to that seen in various other kinases. However, little is known about the distinctive roles of three phosphotyrosines in TbetaRII molecules under albuminuria stress in renal tubule cells.

Tubulointerstitial fibrosis is as important as glomerulopathy in diabetic nephropathy [Ziyadeh and Goldfarb, 1991; Ziyadeh, 1996]. Despite that many studies have been performed on the mesangial or proximal renal tubules, the distal nephron has also been found to be significant as well for several reasons. First, significant changes (e.g., Armanni–Ebstein lesion, hyperplasia, and hypertrophy) in the distal renal tubules are present in diabetes [Rasch, 1984; Ziyadeh and Goldfarb, 1991; Nyengaard et al., 1993; Ziyadeh, 1996]. Second, Na^+,K^+-ATPase is increased in both proximal and distal renal tubule cells [Wald et al., 1993]. Third, urinary excretion of distal nephron markers (e.g., Tamm–Horsfall protein, epidermal growth factor and kinin/kallikrein) is increased in diabetes [Guh et al., 1991; Harvey et al., 1992; Torffvit and Agardh, 1993]. According to our previous studies, distal tubule cells did exert totally different responsive pattern under high ambient glucose treatment by comparison with either mesangial cells or proximal tubule cells [Yang et al., 1998; Chuang et al., 2003]. Moreover, distal tubule segments might be subjected to higher concentration of TGF-β secreted from upstream proximal tubule cells or glomerular segments under diabetic condition since secrected TGF-β from mesangial or proximal tubule may act in a paracrine fashion to affect the biological response at distal tubule cells. Thus, it is essential to explore the effects of albumin on distal tubule cells.

In this study, we elucidate the role of albumin on the biochemical effects of renal distal tubule cells. A novel observation was shown that albumin pretreatment would accentuate the exogenous TGF-β1-induced cellular fibrosis. In addition, we discuss the implication of TGF-β ligand, TbetaRI, and tyrosine phosphorylation

status for TbetaRII under albumin treatment in this study.

EXPERIMENTAL

Reagents and Materials

Cell culture flasks and plates were purchased from Nunclon (Roskilde, Denmark). MDCK (CCL 34) was purchased from ATCC (Rockville, MD), FBS was purchased from Hyclone Labs (Logan, UT). Trypsin (0.25%) was purchased from Hyclone Labs (Logan). Hundred units per milliliter penicillin, 100 μg/ml streptomycin, and 0.4% trypan blue was purchased from Sigma (St Louis, MO). MEM and PBS was purchased from Gibco (NY). Albumin was purchased from Amresco (Euclid, OH), which was certificated to be free of fatty acid. Coomassie® Brilliant Blue G-250 dye was purchased from Merck (Germany). Absolute RNA RT-PCR Miniprep Kit was purchased from Stratagene. Advantage RT-for PCR kit was purchased from Clontech (BD, NJ). Nitrocellulose paper and SDS was purchased from BioRad Laboratories (Hercules, CA). Quantikine™ TGF-β1 Immunoassay was purchased from R&D (Minneapolis, MN). Fibronectin ELISA Reagent kit was purchased from Technoclone (Vienna, Austria). Spectrophotometer was purchased from Beckman DU-64 (Fullerton, CA). RT-PCR used programmable thermal controller MyCycler™ from BioRad Laboratories. Gel Scanner was purchased from Syngene (Frederick, MD).

Manipulation of Conditioned Cells

MDCK cells were maintained at 37°C in a 5% CO_2/95% air environment incubator and grown in minimal essential medium (MEM) with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin, and 100 μg/ml streptomycin. As for dose-dependent assay, cells were serum-contained for 24 h, then incubated in serum-free (0.5% FBS) medium under different concentrations (0, 0.1, 1, or 10 mg/ml) of albumin for additional 24 h. As for time course-dependent experiments, cells were serum-contained for 24 h, then incubated in serum-free (0.5% FBS) medium under different concentrations (0, 10 mg/ml) of albumin for additional 12, 24, 36, or 48 h. Conditioned cells were trypsinized and subjected to different analysis.

Cell culture and viability assay. As for cell counting assay, we trypsinized the conditioned cells at 37°C for 10 min. After centrifugation at 2,200 rpm for 15 min, collected cells were subjected to cell number analysis by conventional technique using hematocytometer. Cell viability assay was performed as following: Cells suspended in the culture medium were collected by centrifugation (4,500 rpm). The collected fraction (dead cells) was pooled with the originally attached cells, which were trypsinized and collected by centrifugation with 2,200 rpm for 15 min. Trypan blue (0.4%) was mixed with equal volume of cells mixture. Cell counting was performed by hematocytometer as described from our previously studies [Guh et al., 1991].

LDH assay for cytotoxicity. Cells were maintained and passaged as described above. The cells were seeded in six-well plates at a density of 2×10^5 cells/well in complete medium and incubated at 37°C under 5% CO_2 overnight. According to previous descriptions, cells were treated with albumin at a range of concentrations (0–10 mg/ml) for 24 h. Supernatant from maintained cells treated with 1% Triton X-100 was regarded as a positive control for maximum lactate dehydrogenase (LDH) release. After 24 h incubation at 37°C under 5% CO_2, the supernatants were collected and centrifuged at 4,500*g* for 5 min to remove contaminating cells, and the level LDH was measured in duplicate by using a cytotoxicity detection kit (Roche, Mannheim, Germany) in a 96-well plate. The cytotoxicity (%) was expressed as 100 × (experimental value – low control)/(high control – low control). Note that supernatant from maintained cells was considered as low control. Supernatant from 1% Triton X-100-treated cells was considered as high control.

Cell Cycle Assay

Conditioned cells were trypsinized and washed twice with PBS. 10^6 cells were subjected to cell cycle analysis using Cycle-test™ kits (Becton-Dickenson Co., Mountain View, CA). Briefly, cells were added with solution A containing high concentration of trypsin at 37°C for 10 min for the destruction of the integrity of cell membrane. Then, we added solution B containing trypsin inhibitor and RNase in order to degrade RNA at 37°C for 10 min. Finally, we used solution C containing propidium iodide to stain the intracellular DNA. The sample was subjected to flowcytometry (FACScan flow cytometer' Becton-Dickenson Co., Paramus, NJ). We used CellFIT™ program

to analyze the G0/G1%, S%, and G2/M% of each sample.

Cell Size and Protein Content Assay

Trypsinized cells were subjected to flow cytometry assay using FACScan system. CellQuest™ software was used to acquire and analyze these data. Forward light scatter coefficient (FSC) was represented to respective cell size. Protein concentration for each sample was determined by Bradford's method. In brief, cells were lysed by repeatedly freezing and thawing. Eight hundred microliters of cell lysate was mixed with 200 μl of Coomassie® Brilliant Blue G-250 dye. Absorbance was determined at 595 nm. Standard curve for protein assay was performed by using a series of known concentration of exogenous albumin.

Western Blotting

We used Western blot assay to evaluate the expression of the protein level for TbetaRI and TbetaRII. In brief, cells were lysed by lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM AEBSF, 0.3 μM Aprotinin,10 μM Bestatin, 10 μM E64, 100 μM Leupeptin); 250 μg of crude protein lysate was resolved by 10% SDS–PAGE. After protein transfer to nitrocellulose paper, the blots were probed with a 1:1,000 (v/v) dilution of polyclonal anti-TGF-β RII and TGF-β RI primary antibody. After hybridization at 37°C, the blots were washed and hybridized with 1:2,000 (v/v) dilution of goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Santa Cruz). Blocking procedure was performed by using 5% defat milk in t-TBS buffer. The signal was generated by adding enhanced chemoluminiscent reagent.

ELISA

We used ELISA assay to evaluate the expression of secreted TGF-β1 and fibronectin. For quantifying TGF-β1 or fibronectin in the supernatant of cultured MDCK cells, conditioned culture media were collected and centrifuged at 13,000 rpm for 30 min to remove particulate; the clear supernatant was collected and stored at −40°C until use. We used a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit for TGF-β1 (Quantikine™, R&D system, MN) or used a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit for fibronectin (The Fibronectin ELISA Reagent Kit, Technoclone, Vienna, Austria). The protocol was according to the manufacturer's description. The absorbance (450 nm) for each sample was analyzed by an ELISA reader. The absorbance for TGF-β1 and fibronectin was assayed and interpolated with the standard curve.

RT-PCR

Total RNA was extracted by Absolute RNA RT-PCR Miniprep Kit (Stratagene®: Catalog #400800) and 2 μg of total RNA was subjected to reverse transcription by Advantage RT for PCR kit (Clontech). PCR reaction was performed by mixing 29.5 μl of H_2O, 4μl of 2.5 mM dNTP, 5μl of 10× reaction buffer, 0.5 μl of 25 mM up primers, 0.5 μl of 25 mM down primers, 10 μl cDNA, and 0.5 μl of DNA polymerase. Amplified PCR products were resolved by 2% agarose gel. Data were analyzed by a scanner. β-actin was used as an internal control. Quality control for RT-PCR was performed as our previous study [Guh et al., 1991; Yang et al., 1998].

Obtained total RNA was extracted by using Absolute RNA RT-PCR Miniprep Kit (Stratagene). Synthesis of complementary first strand cDNA was performed by using QUICK-Clone cDNA kit (BD Laboratories). The RNA concentration was determined with the use of a spectrophotometer (DU-64; Beckman, Fullerton, CA) at wavelengths of 260 nm and 280 nm absorbance. The RNA from the MDCK cells was used as a template for the synthesis of cDNA as follows: 1 μg/μl oligo(dT) (Pharmacia Biotech, Piscataway, NJ) was used as primer for each reverse transcription reaction, 2 μg total RNA and oligo(dT), added to DEPC-H_2O in a final volume of 12.5 μl was heated to 70°C for 2 min and then cooled to 4°C for 5 min (Programmable Thermal Controller MyCycler™; Bio-Rad Laboratories), 4 μl 5× first-strand reverse transcription buffer, 1 μl of 10 mM dNTP, 1 μl of Superscript Reverse Transcriptase (200 U/μl), and 0.5 μl of RNase inhibitor (40,000 U/ml) were added to the messenger RNA and oligo(dT) mixture in a final volume of 20 μl. This mixture was incubated at 42°C for 1 h. The incubation was terminated by heating to 95°C for 5 min and the cDNA was stored at −20°C until used for PCR analysis. The primers used for PCR were derived from the cDNA sequences for TbetaRI and TbetaRII. TbetaRI was amplified using PCR primers (5′-(1268) CGTGCTGACATCTATGCAAT (1287)-3′ and 5′-(1519) AGCTGCTCCATTGGCATAC (1501)-3′), with a resultant

PCR fragment length of 244 bp (GenBank no. L11695), TbetaRII was amplified using PCR primers (5′-(510) TCCACCTGTGACAACCCAGAAA (525)-3′ and 5′-(827) TATGACTAGCAACAAGTCAGG (807)-3′), with a resultant PCR fragment length of 318 bp (GenBank no. M85079).

PCR cDNA amplification was performed with 10 μl of the reverse-transcribed cDNA together with 5 μl of PCR buffer (GIBCO BRL), 4 μl of 2.5 mM dNTP, 0.5 μl of Taq-DNA polymerase, and 0.5 μl of sense and antisense primer (100 μl/ml) added to 29.5μl dd H_2O. Cycles of the following thermocycling conditions were used for TbetaRI (35 cycle): 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s, and for TbetaRII (33cycle): 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The amplified DNA fragments were analyzed by electrophoresis without further purification on 2% agarose gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining.

For the Analysis of Intracellular TbetaRII Phosphorylation

Cells were fixed with 2% formaldehyde for 10 min at 37°C followed by permeabilization with ice-cold 100% methanol for 30 min at 4°C. After washing twice with PBS (in 4% FCS), cells were treated with anti-phosphotyrosine specific TbetaRII goat polyclonal antibodies (1 μg/100 μl in PBS plus 4% FCS) for 15 min at room temperature. Cells were then washed twice with PBS incubated with a fluorescein-conjugated anti-goat antibodies (1:200 dilution, 5 μg/ml) in PBS plus 4% FCS for 15 min at room temperature. Cells were then washed twice and FACScan™ was used to collect the data. 10^5 cells were gated and analyzed with CellQuest™ software.

Statistics

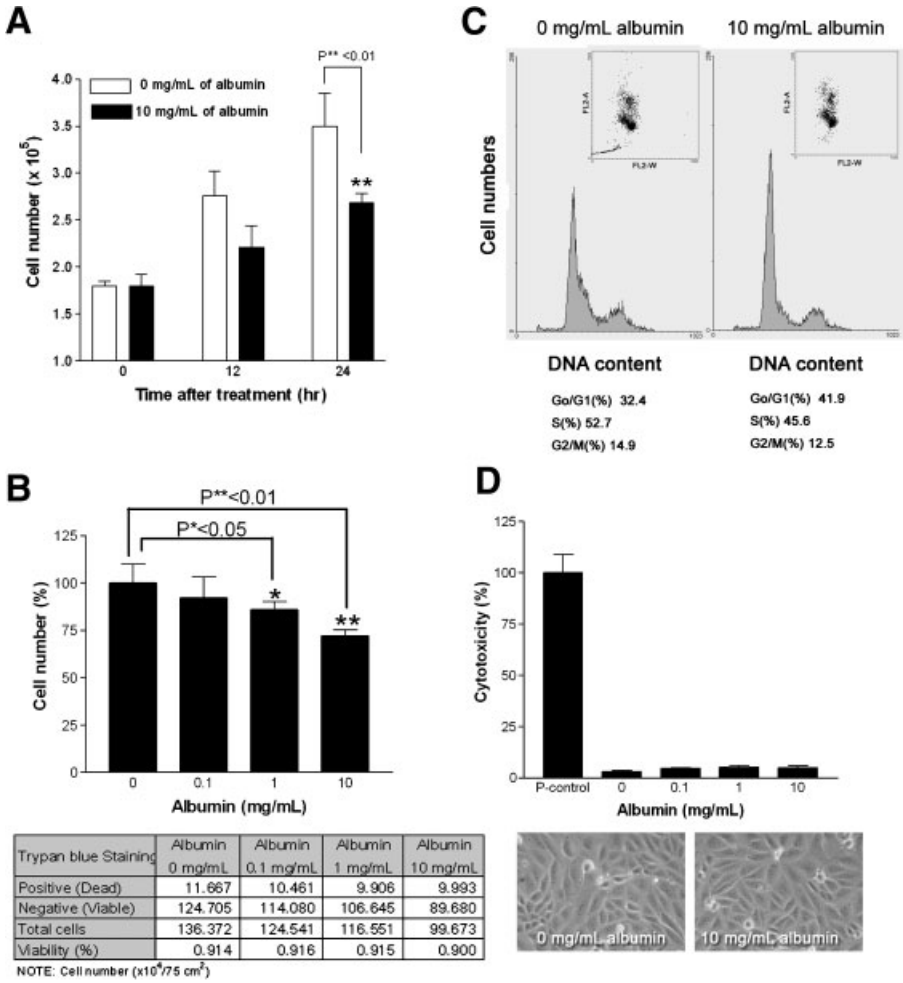
Results were expressed as mean ± SEM. Unpaired Student's *t*-tests were used for comparisons between two groups. *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Since albuminuria is indicative of a nephropathy, researchers are eager to know whether albumin could affect the cell growth of renal tubule cells. As shown in Figure 1A–C, albumin inhibited the proliferation of MDCK cells as reflected by cell number and cell cycle analysis. Ten milligram per milliliter of albumin significantly increased the percentage of Go/G1 from 32.4 to 41.9. In other words, albumin treatment induced growth arrest. In addition, albumin did not affect the cellular viability or induce cytotoxicity (lower table panel of Fig. 1B,D) as reflected by trypan blue exclusion assay and LDH release assay, respectively. There were no significant differences ($P < 0.05$) in LDH release between control group (0 mg/ml) and albumin-treated groups (0.1, 1, or 10 mg/ml). Moreover, the level of LDH release in response to albumin treatment was less than 5% comparing with the LDH release level from triton X-100 treated group.

Fibronectin is one of the most important extracellular matrix proteins implicated in diabetic nephropathy. According to Kanauchi et al. [1995], fibronectin is useful in estimating pathologic conditions, especially the early stage of diabetic nephropathy. Thus, we hereby investigate the interactions of fibronectin with albumin. As shown in Figure 3, albumin treatment statistically increased the secretion of fibronectin in conditioned culture media especially in higher dose (over 1 mg/ml). Thus, the observations suggested that albumin per se is a fibrogenic factor for distal tubule cells as well. Moreover, these effects were reminiscent of the implication of TGF-β1 since TGF-β1 was reported to induce significant growth arrest and secretion of fibronectin in MDCK cells according to others and our previous study [Yang et al., 1998].

Thus, we wonder if TGF-β1 mediated albumin-induced secretion of fibronectin since TGF-β1 per se is a potent fibrogenic factor. According to TGF-β1 secretion assay shown in Figure 4, we demonstrated that albumin induced significant secretion of TGF-β1 under extreme dosage (over 1 mg/ml). Thus, higher concentration of albumin in renal distal segment might induce significant secretion of TGF-β1, which is one of the most pivotal fibrogenic growth factors for diabetic renal fibrosis as previously reported [Yang et al., 1998]. This notion appears consistent with our previous studies since we showed that albumin would not statistically affect the expression of TGF-β1 under the concentration of 0, 0.1, 0.4 mg/ml [Yang et al., 2004]. Combining with the current data, we demonstrated that albumin-induced secretion of TGF-β1 and fibrobronectin was observed only under extremely higher dosage of albumin treatment.



Trypan blue Staining	Albumin 0 mg/mL	Albumin 0.1 mg/mL	Albumin 1 mg/mL	Albumin 10 mg/mL
Positive (Dead)	11.667	10.461	9.906	9.993
Negative (Viable)	124.705	114.080	106.645	89.680
Total cells	136.372	124.541	116.551	99.673
Viability (%)	0.914	0.916	0.915	0.900

NOTE: Cell number (x10^4/75 cm^2)

Fig. 1. Effects of albumin on cell proliferation and cytotoxicity. **A**: Cells were cultured in six-well plate. Cells were serum-starved (0.5% FBS) and incubated in different concentrations (0 or 10 mg/ml) of albumin for indicated time point (0, 12 h, 24 h). Cells were trypsinized and subjected to hemocytometry assay as described in Methods. It is evident that albumin treatment suppressed the growth curve in MDCK cells ($P^{**} < 0.01$) **B**: Cells were cultured in 75 cm^2 culture plate. Cells were trypsinized and subjected to hemocytometry assay (upper graph). In addition, trypsinized cells were pooled with cells in the supernatant of each conditioned media and subjected to trypan blue extrusion test (**lower table panel**). It is obvious that extreme concentration of albumin dose dependently decreased cell proliferation without affecting cellular viability in MDCK cells. $P^{*} < 0.05$ and $P^{**} < 0.01$ versus control (0 mg/ml of albumin). Results were expressed as mean ± SEM of three independent experiments performed in duplicate. **C**: 10^6 cells treated with albumin (0 or 10 mg/ml) were subjected to cell cycle analysis using Cycletest^{TM} kits. CellFIT^{TM} program was used to analyze the G0/G1%, S%, and G2/M% of each sample. Results were repeated three times and similar results were obtained. It is evident that albumin treatment induced growth arrest. **D**: Cells were treated with albumin (0–10 mg/ml) for 24 h. After 24 h incubation, the supernatants were collected and subjected to LDH assay. The cytotoxicity (%) was expressed as 100× (experimental value – low control)/(high control – low control). Supernatant from maintained cells was considered as low control. Supernatant from 1% Triton X-100 treated cells (designated as P-control) were considered as high control. Results were expressed as mean ± SEM of three independent experiments performed in duplicate. Lower panel showed that albumin did not affect the integrity of culture cells.

In addition to the induction of TGF-β1, we would like to elucidate the other pro-fibrogenic effects exerted by albumin. As shown in Figure 5, we found that albumin pretreatment (1 or 10 mg/ml) significantly accentuated exogenous TGF-β1 (5 ng/ml)-induced secretion of fibronectin. We considered that the effects might be due to the implication of TGF-β receptors since albumin-treated culture medium, which might contain albumin-induced secretion of endogenous TGF-β, was totally replaced with serum-free medium. Thus, albumin pretreatment might upregulate or modulate the expression of TGF-β receptor.

To demonstrate the possible implication of TGF-β receptors, two main types of TGF-β

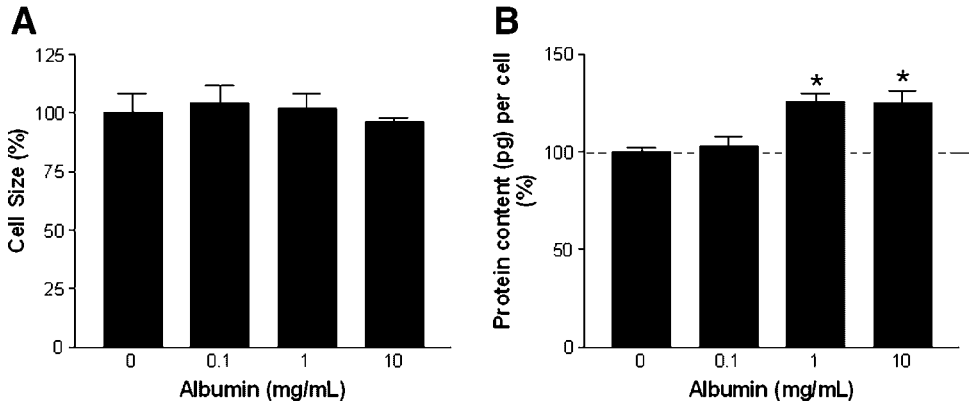


Fig. 2. Effects of albumin on cellular hypertrophy status. Cells were cultured in 75 cm^2 flask. Cells were serum-starved (0.5% FBS) and incubated in different concentration (0, 0.1, 1, or 10 mg/ml) of albumin for 24 h. **A**: Cells were trypsinized and 5×10^5 cells were subjected to flow cytometry assay. Data were acquired by CellQuest software as described in Methods. Note: Forward light scatter coefficient (FSC) was regarded as cell size. **B**: As demonstrated in (A), 2×10^5 cells were homogenized by repeated freezing and thawing. Protein content was determined by Bradford's method as described in Method section. It is obvious that extreme concentration (i.e., over 1 mg/ml) of albumin appears to enhance cellular protein content without affecting cell size in renal distal tubule cells. $P^* < 0.05$ versus control (0 mg/ml of albumin). Results were expressed as mean ± SEM of three independent experiments performed in duplicate.

receptors (TbetaRI and TbetaRII) were examined. As shown in Figure 6, we found that albumin did not statistically affect the mRNA level of both types of TGF-β receptor according to RT-PCR analysis. However, the protein level of TbetaRI (rather than TbetaRII) was significantly enhanced according to the Western blot assay shown in Figure 7. Thus, albumin might increase cellular sensitivity to exogeneous TGF-β1 by increasing TbetaRI protein synthesis in MDCK cells. Despite the protein level of TbetaRII was not affected by albumin, there still exist possibilities that the activity of TbetaRII was modulated instead. According to studies shown by Lawler et al. [1997], receptor kinase activity of TbetaRII is closely associated with there phosphotyrosine amino acid residue in TbetaRII molecule. Thus, we investigated the level of phosphorylation status of three tyrosine amino residues (259, 336, and 424,

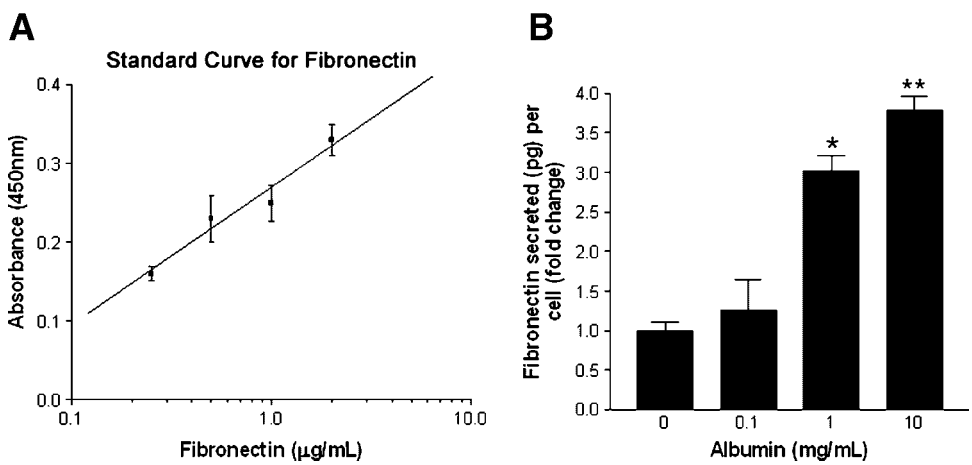


Fig. 3. Effects of albumin on the expression of fibronectin. **A**: We used different concentrations of fibronectin as a standard. Quantitation of fibronectin was performed according to the instruction of commercial kits. The absorbance (450 nm) of each sample was analyzed by an ELISA reader. **B**: MDCK cells were cultured in serum-free medium (0.5% FBS) under the treatment of albumin (0, 0.1, 1, or 10 mg/ml) for 24 h. Conditioned culture media were collected and fibronectin level was assayed by interpolation with the standard curve shown in (A). The results were normalized with the cell numbers of each experiment. Data were repeated twice (n = 4) and similar results were obtained. It is evident that albumin significantly induced an increase in the secretion of fibronectin in MDCK cells particularly under extreme dosage (i.e., over 1 mg/ml). $P^* < 0.05$, $P^{**} < 0.01$ versus control (0 mg/ml of albumin).

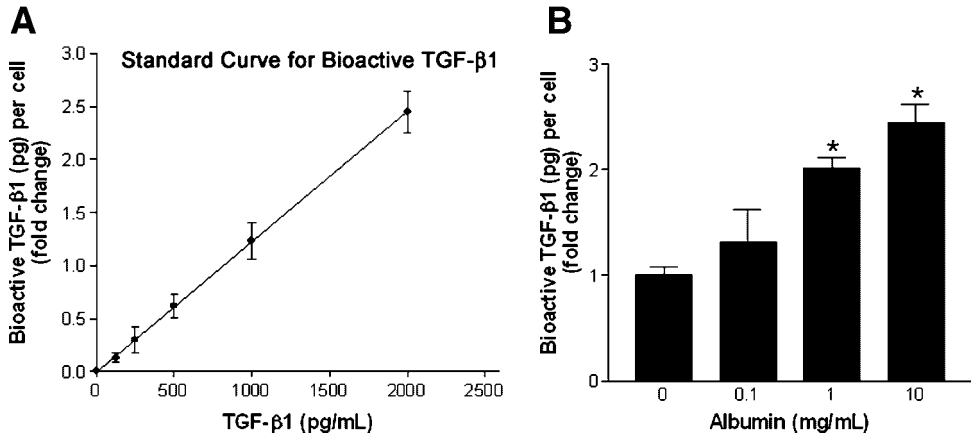


Fig. 4. Effects of albumin on the level of secreted bioactive TGF-β1. **A**: To establish the standard curve for TGF-β1 ELISA assay, we used exogeneous TGF-β1 (from 0 to 2,000 pg/ml) as a standard. The absorbance (450 nm) of each sample was correlated with the concentration of TGF-β1. Linear regression was performed as above. **B**: MDCK cells was dose-dependently (0, 0.1, 1, or 10 mg/ml) treated with albumin for 24 h. Conditioned culture media were collected and supernant TGF-β1 was assayed and interpolated with the standard curve shown in (A). The secreted level for each experimental condition was normalized with cell numbers. Data were repeated twice (n = 4) and similar results were obtained. It is evident that extreme dose of albumin induced an increase in the secretion of TGF-β1 in MDCK cells. $P^* < 0.05$ versus control (0 mg/ml of albumin).

respectively) in TbetaRII. According to Figure 8, we found that albumin dose-dependently and time course-dependently increased pY259 and pY424 without affecting the level of pY336. The novel observations indicate that extreme dose of albumin per se modulated the tyrosine phosphorylation status of TβRII. And this might affect the biological activity of TβRII and its following signaling pathway.

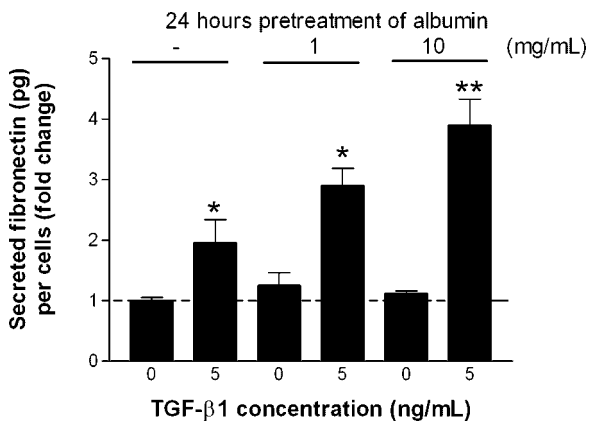


Fig. 5. Effects of albumin pretreatment on exogenous TGF-β1-induced secretion of fibronectin. MDCK cells were pretreated with albumin (0, 1, or 10 mg/ml) in serum-free medium (0.5% FBS) for 24 h. Then, the culture media was removed and replaced with fresh serum-free MEM medium (0.5% FBS) for another 24 h with or without the treatment of exogenous TGF-β1 (5 ng/ml). Conditioned culture media was collected and fibronectin level was assayed by interpolation with the standard curve shown in Figure 3A. The results were normalized with the cell number of each experiment. Data were repeated twice (n = 4) and similar results were obtained. It is evident that exogenous TGF-β1-induced significant secretion of fibronectin in MDCK cells. Moreover, albumin pretreatment accentuated these effects in a dose-dependent manner. $P^* < 0.05$, $P^{**} < 0.01$ versus control (0 mg/ml of albumin).

DISCUSSION

This study investigated whether albuminuria may affect the expression of TGF-β receptors on cultured renal distal tubule cells. We showed that albumin pretreatment significantly enhanced exogenous TGF-β1-induced secretion of fibronectin. Moreover, we demonstrated that albumin upregulated both the level of secreted TGF-β1 and the protein expression of TbetaRI (not TbetaRII). In addition, the tyrosine phosphorylation status of TbetaRII was modulated by albumin as well. Since TbetaRII is primarily engaged in the initial binding of TGF-β, an increased receptor autophosphorylation may result in the amplification of the TGF-β effects on tubular cells. These novel findings suggested that albumin per se exerts its fibrogenic effects by enhancing TGF-β autocrine loop (i.e., simultaneously inducing TGF-β secretion and TGF-β receptor).

Renal lesions of diabetes mellitus exist not only in the glomeruli and proximal tubules but also in the distal tubules. Thus, there are increasing studies focusing on the role of distal tubule cells in diabetic nephropathy including our previous studies [Yang et al., 1998, 2004; Chuang et al., 2003]. Like mesangial

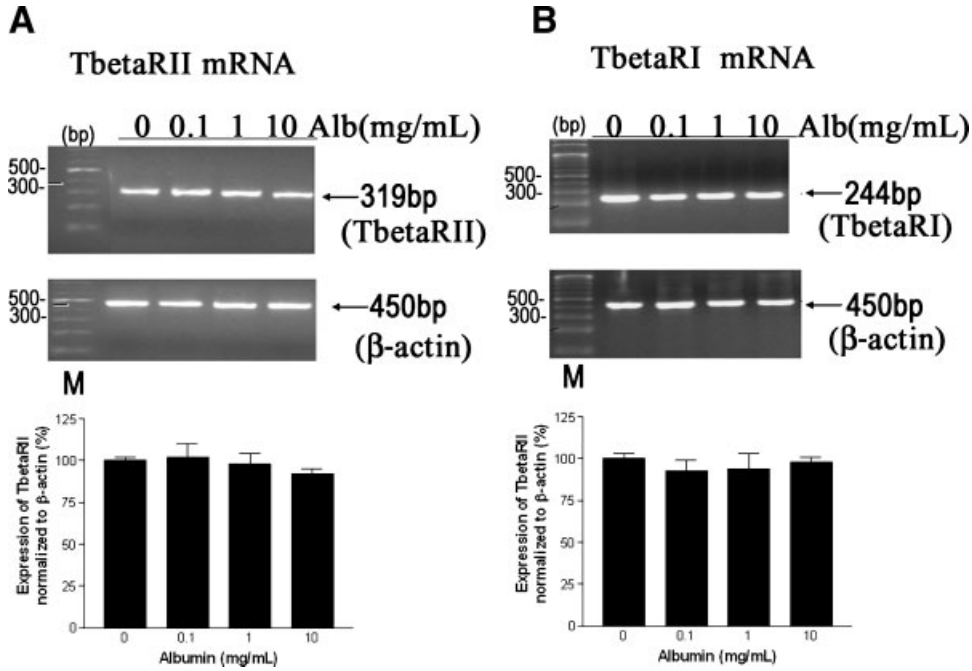


Fig. 6. Effects of albumin on the mRNA expression of both types of TGF-β receptor. Cells were cultured in 75 cm^2 flask. Cells were serum-starved (0.5% FBS) and incubated in different concentrations (0, 0.1, 1, or 10 μg/ml) of albumin for 24 h. Total RNA was extracted and 2 mg of total RNA was subjected to reverse transcription by Advantage RT-for PCR kit (Clontech). Polymerase chain reaction for both types of TGF-β receptor and b-actin was performed as described in Methods section. Amplified products (Tbeta RII 319 bp; TbetaRI 244 bp; b-actin 450 bp) were resolved by 2% agarose gel. Intensity was analyzed by a laser densitometer. β-actin was used as an internal control. It is evident that high albumin concentration did not affect the mRNA level of either TbetaRII or TbetaRI mRNA level. Experiments were repeated three times and similar results were observed. M: DNA 100 bp ladder marker; Alb: albumin.

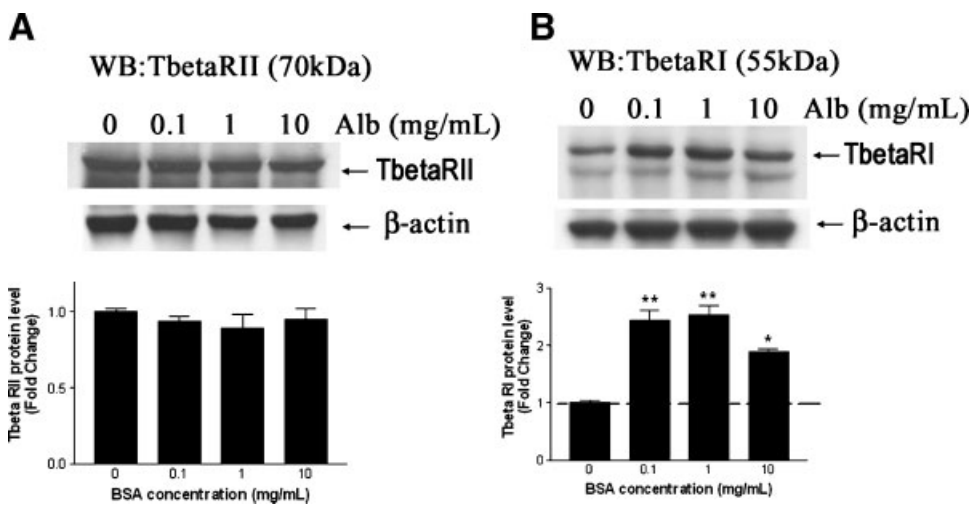


Fig. 7. Effects of albumin on the protein expression of both types of TGF-β receptor. Cells were cultured in 75 cm^2 flask. Cells were serum-starved (0.5% FBS) and incubated in different concentrations (0, 0.1, 1, 10 mg/ml) of albumin for 24 h. Protein was extracted and 250 μg of protein lysate was subjected to 10% SDS–PAGE analysis. After protein transferring to nitrocellulose paper, the blots were probed with 1:1,000 (v/v) dilution of polyclonal anti-TbetaRII (**A**) or anti-TbetaRI (**B**) primary antibody. After hybridization at 37°C, the blots were washed and hybridized with 1:2,000 (v/v) dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody. Blocking procedure was performed by using 5% defat milk in t-TBS buffer. Signals were captured by adding enhanced chemoluminiscent reagent. b-actin was used as a loading control. It is evident that high concentration of albumin (designated as Alb) does significantly increase TbetaRI protein level. Experiment was repeated three times and similar results were observed. $P^* < 0.05$, $P^{**} < 0.01$ versus control (0 mg/ml of albumin).

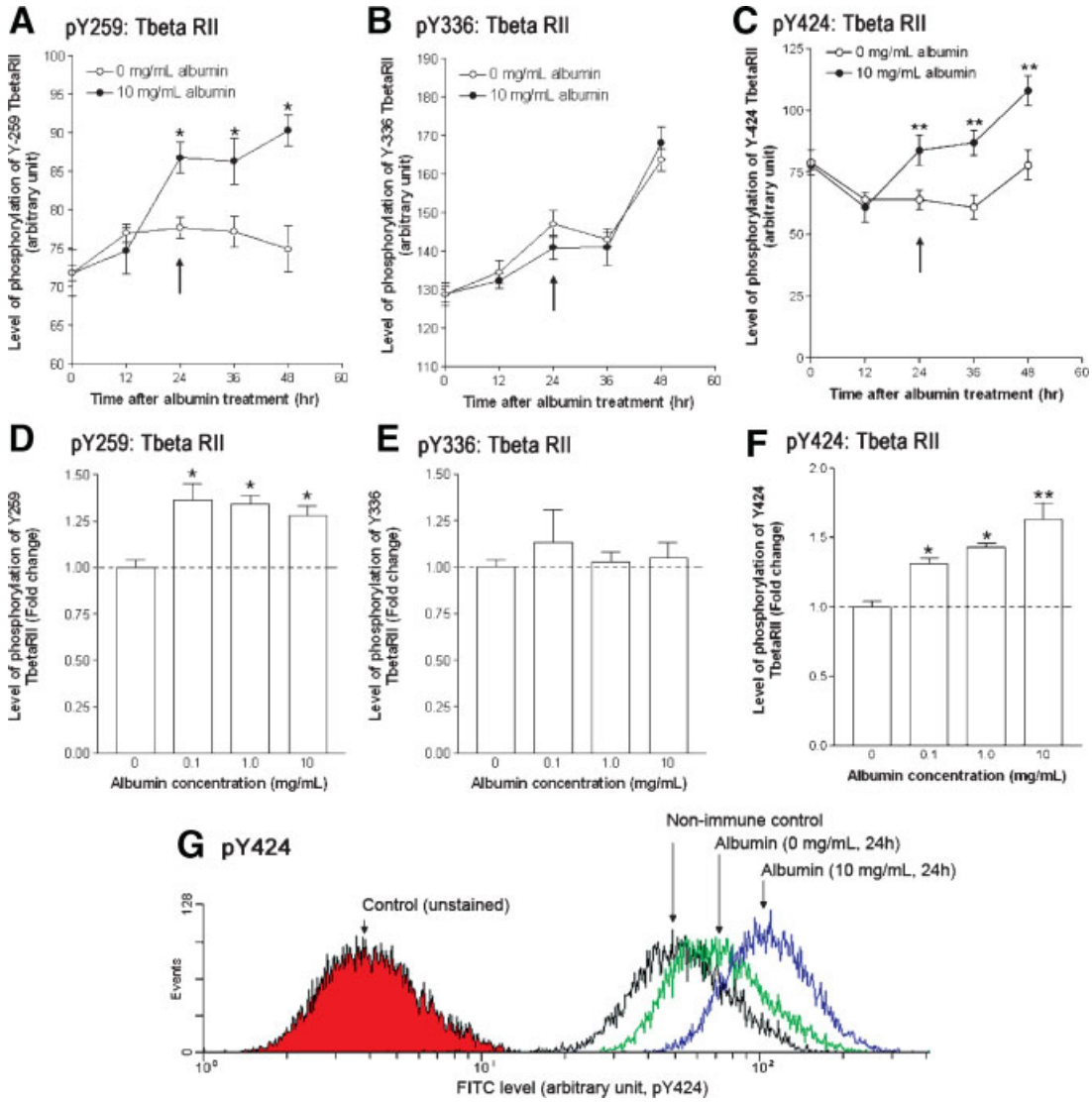


Fig. 8. Effects of albumin on tyrosine phosphorylation of TbetaRII in MDCK cells. Cells were cultured in 75 cm^2 flask. **A–C**: Cells were serum-starved (0.5% FBS) and incubated in either 0 or 10 mg/ml albumin for indicated time point (0, 12 h, 24 h, 36 h, or 48 h). Cells were trypsinized and 5×10^5 cells were subjected to flow cytometry assay using phosphotyrosine-specific TbetaRII antibodies (for pY259, pY336, and pY424, respectively). Data were acquired by CellQuest software as described in Methods. The results were expressed as mean ± SEM of three independent experiments performed in duplicated. Note: Arrow designated the time point applied by dose response experiments. **D–F**: As shown in previous studies, cells were cultured in different concentration (0, 0.1, 1, or 10 mg/ml) of albumin for 24 h. Phosphorylation level was examined by the same approach as (A). **G**: The histogram from (F) was shown here with 0 or 10 mg/ml of albumin concentration with unstained control and non-immune control (i.e., primary antibodies with non-immune IgG instead). It is obvious that albumin appeared to enhance the phosphorylation level of Y259 and Y424 (dose- and time course-dependent) instead of Y336. For (A–C), $P^* < 0.05$ versus control (0 mg/ml of albumin at respective time point). For (D–F), $P^* < 0.05$ versus control (0 mg/ml of albumin). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or proximal tubule cells, distal tubule cells were found to act abnormally either in cell growth status or in cellular hypertrophy condition under the diabetic stimuli (hyperglycemia or advanced glycation end products). Here in this study, we provide another evidence that albumin exposure appeared to be implicated in distal tubular hypertrophy since albumin significantly induced an increase in protein content and a decrease in cell numbers as shown in Figures 1 and 2. These observations were totally different with renal proximal tubule cells [Lee et al., 2003]. Thus, it is evident that exposure to albumin might affect the growth condition of renal tubule cells. And these observations might be the reason why the progression of renal

disease correlates strongly with the degree of albuminuria.

Albumin per se appears to be a fibrogenic factor for either renal proximal tubule or distal tubule cells since Stephan et al. have demonstrated that albumin stimulates the accumulation of extracellular matrix (ECM) proteins by proximal tubular epithelial cells through a post-transcriptional mechanism. In addition, addition of TGF-β-specific neutralizing antibody failed to alter ECM protein levels after albumin treatment, indicating that the albumin-induced increase in ECM in proximal tubule cells is TGF-β-independent [Stephan et al., 2004]. Similarly, in this study we showed that albumin per se induced significant secretion of both the level of TGF-β1 and fibronectin in distal tubule cells as shown in Figures 3 and 4. Moreover, in distal tubule cells, albumin-induced increase in the secretion of fibronectin appears to be TGF-β dependent since TGF-β-neutralizing (15 μg/ml) antibodies significantly attenuated albumin-induced increase in fibronectin level [unpublished observations]. These observations indicate that exposure of tubular cell to albumin leads to the synthesis of ECM proteins, suggesting that albumin may be a contributing factor to the progression of renal tubule fibrosis in proteinuric states. The notion was further demonstrated from Figure 5 in this study since we showed that 24 h pretreatment of albumin significantly increased exogeneous TGF-β1-induced secretion of fibronectin in renal distal tubule cells. In other words, albumin treatment enhances both TGF-β1 secretion and the cellular susceptibility to TGF-β1 (i.e., the expression of TGF-β receptors) in distal tubule cells. These findings give strong evidences that albunuria is not only a diagnostic marker for diabetic nephropathy, but also a therapeutic target for renal fibrosis.

TGF-beta has been considered as a fibrogenic growth factor for decades [Piguet et al., 1990], many literatures have suggested that the expression level of TGF-beta receptors appears to correlate with the susceptibility of cellular fibrosis. Our previous studies have also demonstrated that regulating the expression of TGF-beta type II receptor would affect the expression fibronectin. Moreover, upregulation of type II TGF-beta receptor induced by high glucose might contribute to renal distal tubular fibrosis [Yang et al., 1998]. More importantly, TGF-beta type II receptors have recently been considered as one of the therapeutic targets for ECM accumulation. Kasuga et al. [2001] showed that anti-type II TGF-beta receptor antibody ameliorated ECM accumulation in anti-Thy-1 nephritis. Nakamura et al. [2004] showed that RNA interference targeting transforming growth factor-beta type II receptor suppresses tissue fibrosis. Thus, regulating transforming growth factor-beta type II receptor appears to be closely linked with the expression of cellular fibrosis. That's the reason why the ability of albumin to enhance TGF-beta induced-secretion of fibronectin (as shown in Fig. 5) was reminiscent of the implication of TbetaR.

We have demonstrated previously that distal renal tubule cells exhibit a completely different responsive patterns compared with mesangial cells or proximal renal tubule cells [Yang et al., 2004]. Similarly, we here showed that distal tubule cells acted differently comparing with the other renal segments under the treatment of albumin. According to Wolf et al. [2004], albumin upregulated the protein expression of TbetaRII in renal proximal tubule cells. In this study, we found that albumin did not affect the expression (either mRNA or protein) of TbetaRII in renal distal tubule cells. However, protein level of TbetaRI and tyrosine phosphorylation status of TbetaRII were modulated instead (Figs. 7 and 8). These observations indicate that exposure to albumin appeared to render renal tubule cell susceptible to cellular fibrosis since both types of TGF-β receptors were significantly regulated. Despite how albumin may modulate the tyrosine phosphorylation status remains currently unclear, recent studies demonstrate that albumin uptake in tubular cells induces the formation of reactive oxygen species (ROS) and also activates certain signal transduction pathways such as the STAT/Jak2 system, which can modulate tyrosine phosphorylation in signal transduction process [Morigi et al., 2002; Nakajima et al., 2004]. Further studies beyond the scope of the present work are pivotal to investigate how such a tubular uptake of albumin could then activate tyrosine phosphorylation of TbetaRII.

The type I and type II receptors cooperate in signal transduction, and both receptor types are required for full responsiveness to TGF-β [Feng et al., 1995]. Thus, both types of TGF-β receptor were evaluated in this study. According to the observations shown by Lawler et al. [1997], they have demonstrated that the TbetaRII (as a dual

specificity kinase) autophosphorylates not only on serine and threonine but also on tyrosine residues. However, the cytoplasmic domains of the type II receptor are constitutively ligand-independently phosphorylated on serine and threonine [Chen and Weinberg, 1995; Lawler et al., 1997]. Thus, we hypothesized that tyrosine phosphorylation might be more correlated with the activity of TbetaRII and deserved more intensive investigation.

Tyr259 is conserved among the type II TGF-β receptors from different species. Replacement of this tyrosine by phenylalanine decreased the kinase activity. Since Tyr259 is in the ATP-binding site of the kinase, and phosphorylation of residues in this region is known to be an important factor in the inhibition of kinase activity of cyclin-dependent kinases [Atherton-Fessler et al., 1994; Kornbluth et al., 2004]. The notion is compatible with what we have observed in this study (Fig. 8A, D). Thus, albumin-induced phosphorylation of Tyr259 in TbetaRII might be essential for the activity of this kinase receptor.

Phosphorylation of Tyr336 in TbetaRII appear to be insignificant for its activity since Lawler et al. [1997] showed that replacement of Tyr336 by phenylalanine did not affect the kinase activity of the TbetaRII. Similarly, we found that albumin dose statistically does not affect the phosphorylation level of Tyr336 comparing with control (Fig. 8B,E). Despite phosphorylation of Tyr336 was increased from time 0 to time 48 h. This might be correlated with the background serum-free starvation stress.

Finally, according to the study shown by Lawler et al., replacement of Tyr424 residue by phenylalanine strongly decreased the kinase activity of the type II receptor, indicating that phosphorylation of Tyr424 appears to be essential for its activity. The finding is consistent with our results shown in Figure 8C,F since albumin significantly induced the phosphorylation of Tyr424. This tyrosine is located two amino acids upstream from the signature sequence APE in kinase subdomain VIII. The sequence between subdomain VII and the APE sequence represents a target for regulatory phosphorylations in several kinases and can function as an activation loop [Taylor and Radzio-Andzelm, 1994]. Thus, this study is a pioneer demonstration showing the correlation of two phosphotyrosine residue (i.e., pY424 and pY259) with increased activity of TbetaRII and the associations with diabetic renal fibrosis.

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