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CD36 Is a Novel and Potential Anti-Fibrogenic Target in Albumin-Induced Renal Proximal Tubule Fibrosis

Yu-Lin Yang,[1]* Shyh-Horng Lin,[1] Lea-Yea Chuang,[2] Jinn-Yuh Guh,[3] Tung-Nan Liao,[4] Tao-Chen Lee,[5] Wen-Teng Chang,[1] Fang-Rong Chang,[6] Min-Yuan Hung,[7] Tai-An Chiang,[4] and Chien-Ya Hung[8]

[1]Department of Biological Science and Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan
[2]Department of Biochemistry, Kaohsiung Medical University, Kaohsiung, Taiwan
[3]Department of Internal Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[4]Department of Medical Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan
[5]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan
[6]Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan
[7]Graduate Institute of Biological Science and Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan
[8]Department of Food Nutrition, Chung Hwa University of Medical Technology, Tainan, Taiwan

Abstract Albumin is not only a risk factor for diabetic nephropathy (DN), but also a therapeutic target. Hence, scientists have long sought ways to elucidate the interactions between albumin and diabetic renal tubule fibrosis. CD36, a surface receptor for thrombospondin-1, has been reported to interact with latent transforming growth factor-beta1 (TGF-β1) and activate its fibrogenic bioactivity. This study elucidates the interactions between CD36 and renal tubule fibrosis. LLC-PK1 cells were applied to represent renal proximal tubule cells. The expression of CD36 was evaluated by flow cytometry. Fibronectin was assayed by Western blot and enzyme-linked immunosorbent assay (ELISA). Bioactive TGF-β1 was assayed by ELISA. We demonstrated that albumin was shown significantly to inhibit cell growth without affecting hypertrophy status since protein content and cell size remained unaffected under albumin treatment. Moreover, albumin dose-dependently (0, 1, or 10 mg/ml) enhanced the secretion of bioactive TGF-β1 and fibronectin with the upregulation of CD36. Intriguingly, CD36 siRNA, a potent silencer for CD36 effectively suppressed the albumin-induced increase in CD36, TGF-β1, and even fibronectin level. Accordingly, albumin is a pro-fibrogenic factor for proximal tubule cells since albumin per se markedly upregulated the expression of TGF-β1 and fibronectin. Most importantly, CD36 may mediate albumin-induced cellular fibrosis since CD36 siRNA appeared to have anti-fibrosis effects. This work suggests that CD36 is a novel and potential therapeutic target for diabetic renal tubule fibrosis. J. Cell. Biochem. 101: 735–744, 2007.

Key words: CD36; fibrosis; fibronectin; diabetic nephropathy; TGF-beta

Proteinuria is a hallmark of end-stage renal disease. The main component present in urine from proteinuria patients is albumin. Although proteinuria has been regarded as a diagnostic marker for renal injury for decades, an increasing number of studies are showing that albumin per se may be crucial involved a pivotal role in the pathogenesis of diabetic nephropathy. First, in vivo studies and related clinical statistic data have shown that proteinuria is implicated in the

Abbreviations used: FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β; TbetaRI, TGF-β receptor type I; TbetaRII, type II TGF-β receptor; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; siRNA, small interfering RNA; FSC, forward light scatter coefficient.

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*Correspondence to: Dr. Yu-Lin Yang, Director of Department of Biological Science and Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan.
E-mail: tel0955443221@gmail.com

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pathogenesis (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 substantially upregulated [Zoja et al., 1998; Burton et al., 1999; Yard et al., 2001; Tang et al., 2003]. Thus, proteinuria has recently been reconsidered to be as a therapeutic target for diabeic nephropathy in these years [Nenigni et al., 1995; D'Amico and Bazzi, 2003]. Despite these observations, a few works have discussed the underlying mechanisms of albumin-induced renal tubular fibrosis.

CD36 is an 88-kD integral-membrane glycoprotein that is present on renal tubular, glomerular cells and other including platelets, monocytes, endothelial cells, erythroblasts, epithelial cells, and several tumor cell lines. CD36 is a receptor for the extracellular matrix protein thrombospondin (TSP) [Hawighorst et al., 2002; Lagadec et al., 2003; Li et al., 2003; Bastian et al., 2005]. It interacts with TSP-1 via the CSVTCG sequence on TSP-1 [Asch et al., 1992]. Moreover, CD36-TSP-1 interaction is critical to the activation of latent TGF-β and may be involved in initiating and regulating cellular fibrosis [Yehualaeshet et al., 1999, 2000; Susztak et al., 2005]. However, the specific role of CD36 under diabetic stress remains poorly investigated.

This work investigates the role of CD36 using a synthetic CD36 silencing siRNA. This investigation suggests that CD36 is implicated in albumin-induced proximal tubule fibrosis and may act as a novel therapeutic target for treating diabetic renal tubule fibrosis.

METHODS

Reagents and Materials

Cell culture wells and plates were purchased from Nunclon (Roskilde, Denmark). LLC-PK1 (CCL 34) was purchased from ATCC (Rockville, MD); RPMI1640 and FBS were purchased from Hyclone Labs (Logan, UT). Trypsin (0.25%) was purchased from Hyclone Labs. Penicillin (100 U/ml), 100 μg/ml streptomycin, and 0.4% trypan blue was purchased from Sigma (St. Louis, MO). PBS was purchased from Gibco (NY). BSA was purchased from Amresco (Euclid, OH). Coomassie® Brilliant Blue G-250 dye was purchased from Merck (Germany). Absolute RNA RT-PCR Miniprep Kit was purchased from Stratagene. An advantage RT-for PCR kit was purchased from Clontech (BD, NJ). PVDF paper was purchased from MILLIPORE. SDS was purchased from BioRad Laboratories (Hercules, CA). Quantikine™ TGF-β1 Immunoassay was purchased from R&D (Minneapolis, MN). A Fibronectin ELISA Reagent kit was purchased from Technoclone (Vienna, Austria). The flow cytometry was purchased from FACScan™ (BD Laboratories, Paramus, NJ). The 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). TransIT-TKO Transfection Reagent was purchased from Mirus (Madison, WI).

Manipulation of Conditioned Cells

LLC-PK1 cells were maintained at 37°C in a 5% CO_2/95% air environment incubator and grown in RPMI1640 with 10% heat-inactivated FCS, and 100 U/ml penicillin, and 100 μg/ml streptomycin. As for dose-dependent assay, cells were serum contain for 24 h then incubated in serum free (0.5% FCS) medium at various concentrations (0, 1, or 10 mg/ml) of albumin for an additional 36 h. Conditioned cells were trypsinized and subjected to various analyses.

Cell Culture and Viability Assay

In the cell counting assay, the conditioned cells were trypsinized at 37°C for 10 min. Following centrifugation at 2,200 rpm for 15 min, collected cells were subjected to conventional cell number analysis using a haematocytometer. Cell viability assay was performed as follows. Cells that had been suspended in the culture medium were collected by centrifugation (4,500 rpm). The collected fraction (dead cells) was pooled with that collected from originally attached cells, which were trypsinized and collected by centrifugation at 2,200 rpm for 15 min. Trypan blue (0.4%) was mixed with an equal volume of the cells mixture; then, the cells were counted.

Cell Size and Protein Content Assay

Trypsinized cells were subjected to flow cytometry assay using a FACScan system.

CellQuest software was applied to acquire and analyze these data. The FSC parameter reflected cell size. The protein concentration of each sample was determined by Bradford's method. Briefly, cells were lysed by repeated freezing and thawing. Eight hundred microliters of cell lysate was mixed with 200 μl of Coomassie® Brilliant Blue G-250 dye. Absorbance at a wavelength of 595 nm was determined. The standard curve for protein assay was obtained using a series of known concentrations of exogeneous albumin.

Western Blotting

Western blot was utilized to assay the protein level of fibronectin. Briefly, 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). crude protein lysate (250 μg) was resolved using 10% SDS–PAGE. After protein was transferred to nitrocellulose paper, the blots were probed with a 1:1,000 (v/v) dilution of polyclonal anti-fibronectin primary antibody. Following 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). The blocking procedure was performed by using 10% non-fat milk in TBS-Tween 20 buffer.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA assay was adopted to evaluate the expression of secreted TGF-β1 and fibronectin. Conditioned culture media were collected and centrifuged at 13,000 rpm for 30 min to remove particulate fraction, and the clear supernatant was collected and stored at −40°C until use to evaluate the level of TGF-β1 or fibronectin in the supernatant of cultured cells. A commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit for TGF-β1 (Quantikine™, R&D SYSTEMS, MN) and fibronectin (The Fibronectin ELISA Reagent Kit, Technoclone, Vienna, Austria). The protocol followed 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.

Analysis of Cell Surface CD36 and its mRNA

The expression of cellular surface CD36 was evaluated by flow cytometry. Briefly, the conditioned cells were trypsinized at 37°C for 10 min. After centrifugation at 2,200 rpm for 15 min, cells were washed twice with PBS (in 4% FCS). Then the cells (10^6 cells per ml) were stained by human PE-conjugated CD36-specific antibodies (10 μl/ml, BD Bioscience). After they have been washed with PBS, the cells were subjected to flow cytometry (FACScan) analysis. Moreover, quantitative real-time PCR was used to analyze the expression of CD36 mRNA. The following primer pairs were used: CD36: 5′-GCTCTGGGGCTACAAAGATG-3′ and 5′-TAGGGAGAGATATCG GGCCT-3′. β-actin: 5′-GATGAGATTGGCATGGCTTT-3′ and 5′-CACCTTCA CCGTTCCAGTTT-3′. An assay of CD36 and β-actin was performed as described previously [Susztak et al., 2004].

Transfection of CD36 siRNA

LLC-PK1 cells were cultured in a medium with 10% fetal calf serum for 1 day. The cells were then transfected by 100 nM of synthetic CD36 siRNA (sense sequence: CUAAGUUGCUGAGACAAGGdTdT, anti-sense: CCUUGUCUCAGCA ACUUAGdTdT), which was purchased from Ambion (Austin, TX). The reverse control of CD36 siRNA is as follows. Sense sequence is 5′-dTdTGGAACAGAG UCGUUGAAUC-3′ and anti-sense sequence is 5′-dTdTGAUUCAACGACUCUGU UCC-3′. Transfecting procedure was performed according to manufacturer's instructions (TransIT-TKO Transfection Reagent, Mirus, Inc.). Cells were mixed with siRNA complex for 24 h, and then subjected to various doses of albumin (0, 1, or 10 mg/ml) in serum-free medium for 36 h.

Statistics

The results were expressed as mean ± SEM. Unpaired Student's *t*-tests were used to compare the two groups. The *P*-value of <0.05 was considered to be statistically significant.

RESULTS

A series of concentrations of albumin to treat renal proximal tubule cells was used to demonstrate the biological effects of albumin. Cells were cultured in 75 cm^2 culture flask for 24 h followed by serum starved (0.5% FCS medium) and albumin treatment (0, 1, or 10 mg/ml) for 36 h. As shown in Figure 1, we showed that albumin significantly inhibited the growth of LLC-PK1 cells, based on the cell number

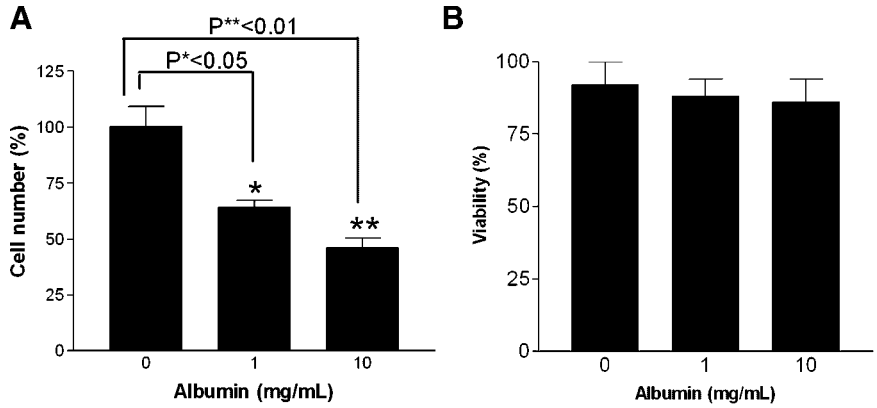

Fig. 1. Effects of albumin on cell proliferation. **A**: Cells were cultured in 75 cm^2 culture flask for 24 h followed by serum starved (0.5% FCS medium) and albumin treatment (0, 1, or 10 mg/ml) for 36 h. Cells were scraped by trypsin-EDTA and cell number analysis assay were performed. **B**: Viability assay was performed as Figure 1A using trypan blue exclusion method. It is evident that albumin dose-dependently inhibited cellular proliferation without affect cellular viability. Data was repeated twice (n = 4) and similar results were obtained. $*P < 0.05$, $**P < 0.01$ versus control (0 mg/ml albumin).

analysis (Fig. 1A). About 50% of growth inhibition was observed when 10 mg/ml of albumin was used by comparison with the control group ($P < 0.01$). Additionally, the growth-inhibitory effect of albumin was not due to the loss of cellular viability, according to the trypan blue exclusion test, as shown in Figure 1B. Hence, albumin per se may have biological effects (growth inhibition) on proximal tubule cells.

The hypertrophic condition for the treatment of albumin was also studied since hyperplasia and hypertrophy are two key features of a diabetic kidney. Bradford protein assay and flow cytometry assay (FSC parameter) were utilized to investigate the hypertrophy. We demonstrated that albumin did not induce cellular hypertrophy according to the results shown in Figure 2.

Fibronectin is one of the most important extracellular matrix proteins that are involved in diabetic nephropathy. According to Weigert et al. [2003] fibronectin is useful in estimating the pathological conditions, especially in the early stage of diabetic nephropathy. Thus, this study investigates the expression of fibronectin under the treatment of albumin. Fibronectin was assayed by Western blot (for intra-cellular fibronectin) and ELISA (for extra-cellular secreted fibronectin). As shown in Figure 3, albumin significantly enhanced the expression of both intracellular and extracellular secreted fibronectin. The observations revealed that

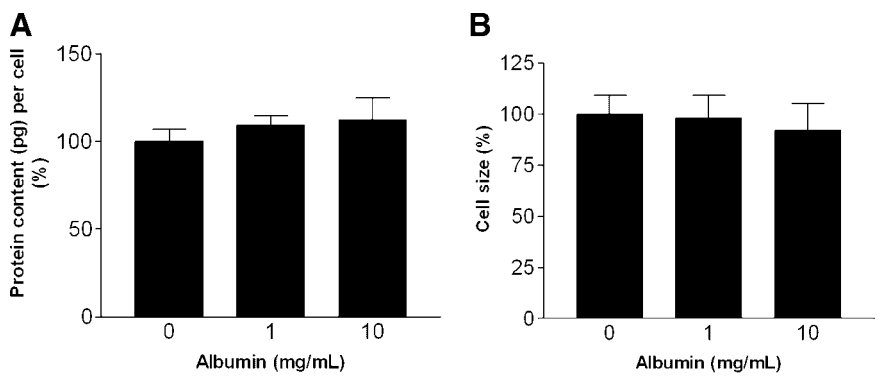

Fig. 2. Effects of albumin on cell hypertrophy status. **A**: Cells were cultured in 75 cm^2 culture flask for 24 h followed by serum starved (0.5% FCS medium) and albumin treatment (0, 1, or 10 mg/ml) for 36 h. Cells were scraped by trypsin-EDTA. Protein analysis (using Bradford method) was performed. **B**: Cell size was examined using flowcytometry assay. It is evident that albumin did not affect cellular hypertrophy. Data was repeated twice (n = 4) and similar results were obtained.

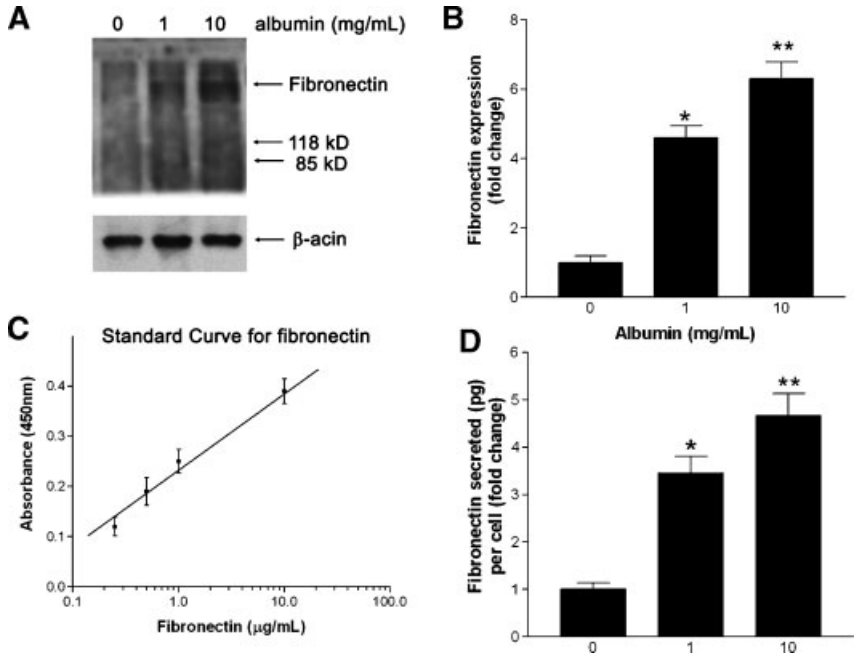

Fig. 3. Effects of albumin on fibronectin expression in LLC-PK1 cells. **A**: Cells were treated with albumin dose-dependently (0, 1, or 10 mg/ml) for 36 h in the presence of 0.5% FCS. LLC-PK1 cells (10^6) were lysed in 200 μl lysis buffer. Twenty microliters of SDS sample was loaded for each lane. SDS–PAGE and Western blotting was performed as described in Methods section. Anti-fibronectin antibodies (1 μg/ml, or anti-actin control antibodies) was hybridized with the membrane for 1.5 h. After a series of washing step, peroxidase conjugated anti-rabbit secondary antibodies (1:2,000, v/v) were added for 1 h. Signals were developed using ECL system. **B**: The expression for fibronectin was normalized to that of β-actin. Results were expressed as mean ± SEM of three observations. **C**: We used different concentrations of exogenous fibronectin as a standard. Quantization of fibronectin was performed according to the instruction of commercial kits. The absorbance (450 nm) of each samples were analyzed by an ELISA reader. **D**: Cells were cultured as in (A). Conditioned culture media were collected and extracellular fibronectin level was assayed by interpolation with the standard curve shown in (C). The results were normalized with the cell numbers in each condition. It is evident that albumin dose-dependently induced the expression of fibronection in LLC-PK1 cells. Data was repeated twice (n = 3) and similar results were obtained. $^*P < 0.05$, $^{**}P < 0.01$ versus control (0 mg/ml albumin).

albumin per se is a fibrogenic risk factor for proximal tubule cells. Taken with the previous results, these observations supported the implications of TGF-β, since TGF-β has been reported to be a powerful growth-inhibitory factor and potent fibrogenic factor for renal proximal cells [Qi et al., 2006]. Hence, the level of bioactive TGF-β was measured according to ELISA assay, as shown in Figure 4. This study reveals that albumin induced significant secretion of TGF-β1, a major form of TGF-β, which is implicated in diabetic nephropathy. Thus, albumin might have pro-fibrotic effects via the upregulation of TGF-β1, which is one of the most pivotal fibrogenic growth factors in diabetic renal fibrosis.

Since CD36 is implicated in the transition of latent TGF-β to its active form [Yehualaeshet et al., 1999, 2000; Susztak et al., 2005], the possible interactions between CD36 and albumin-induced cellular fibrosis are of interest. RNAi has been established to be a powerful tool for gene silencing. The role of CD36 in albumin-induced cellular fibrosis is herein elucidated using synthetic CD36 siRNA. As shown in Figure 5, CD36 siRNA is an ideal tool for specifically suppressing the expression of CD36 in renal proximal tubule cells. Evidently, CD36 mRNA synthesis is significantly (about 90% inhibition) and dose-dependently (0, 25, 50, 100 nM) suppressed in LLC-PK1 using synthetic CD36 siRNA. Moreover, the silencing process is specific to the CD36 mRNA sequence since administration with a reverse sequence of CD36 siRNA showed no silencing effects. Hence, CD36 siRNA was applied in the following experiments.

The following experiments were to demonstrate the role of CD36 in albumin-induced renal fibrosis. As shown in Figure 6A, we found that albumin treatment markedly enhanced the expression of CD36 assayed by flow cytometry.

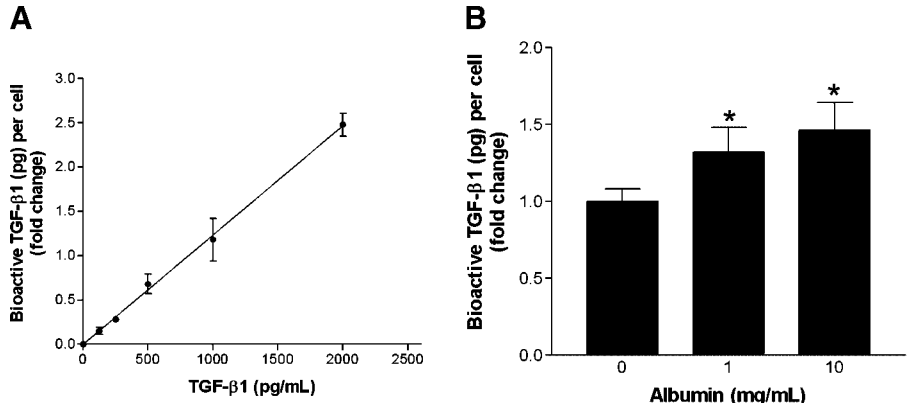

Fig. 4. Effects of albumin on the level of secreted bioactive TGF-β1. **A**: To establish the standard curve for TGF-β1 ELISA assay, we used exogenous TGF-β1 (from 0 to 2,000 pg/ml) as a standard. The absorbance (450 nm) of each samples were correlated with the concentration of TGF-β1. Linear regression was performed as above ($r = 0.998$). **B**: LLC-PK1 cells was dose-dependently (0, 1, or 10 mg/ml) treated with albumin for 36 h. Conditioned media were collected and supernatant TGF-β1 was assayed and interpolated with the standard curve shown in (A). The secreted level for each experimental condition was normalized with the cell numbers in each condition. Data was repeated twice ($n = 4$) and similar results were obtained. It is evident that albumin induced an increase in the secretion of TGF-β1 in LLC-PK1 cells. $^*P < 0.05$ versus control (0 mg/ml albumin).

Thus, we hypothesized that albumin-induced TGF-β1 activation and cellular fibrosis may have proceeded by upregulating the expression of CD36, transforming the latent form of TGF-β1 into activated form. A synthetic CD36 siRNA was used to investigate the albumin-induced biological effects to establish the above hypothesis. As shown in Figure 6B, CD36 siRNA efficiently suppressed the expression of CD36 by flow cytometry assay. These results indicated that synthetic CD36 siRNA is a powerful and ideal tool for silencing CD36 and is helpful for elucidating the roles of CD36 in albumin-induced cellular fibrosis. Moreover, a question exists regarding whether administering CD36 siRNA (inhibits OR suppresses) the albumin-induced increase in ether fibronectin and TGF-β1 level. As shown in Figure 7, it is evident that CD36 siRNA efficiently suppressed albumin-induced increase in the secreted level of both fibronectin and TGF-β in LLC-PK1 cells. This result suggests that CD36 involves albumin-induced renal proximal tubule fibrosis. These results represent the first and novel demonstration that CD36 may be a potent therapeutic target in diabetic nephropathy. Figure 8 presents the overall situation. Since the inhibition of cell surface CD36 protein may reduce binding with extracellular thrombospondin-1, which is a physiological activator for fibrogenic growth factor TGF-β, modulating the expression of CD36 regulated the activation status of TGF-β1 and the cellular fibrosis condition in renal tubule cells.

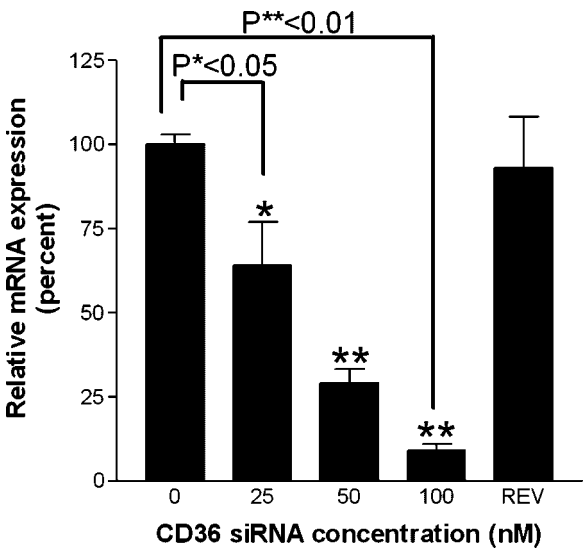

Fig. 5. CD36 mRNA synthesis is suppressed in LLC-PK1 using CD36 siRNA. Relative CD36 mRNA abundance was determined by quantitative real-time PCR in LLC-PK1 cell line treated with dose-dependent CD36 siRNA (0, 25, 50, 100 nM) for 24 h following maintenance of cells in complete medium for 36 h. Bars represent mean ± SEM of three experiments. Numbers on top of bars indicate significant *P*-values of experimental groups relative to 0 nM CD36 siRNA. It is evident that CD36 mRNA synthesis is significantly suppressed in LLC-PK1 using CD36 siRNA instead of reverse sequence of CD36 siRNA (designated as "REV"). Thus, CD36 siRNA is a suitable tool applied in *CD36* gene silencing.

DISCUSSION

CD36 is a transmembrane protein of the class B scavenger receptor family and is involved in

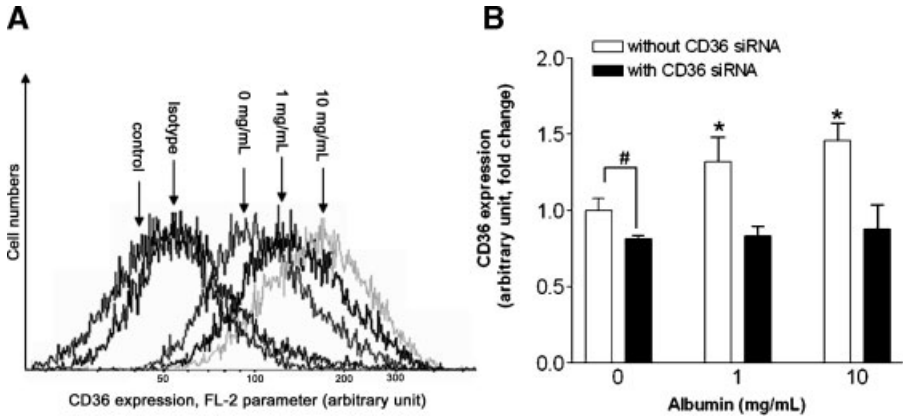

Fig. 6. Effects of albumin on the expression of CD36 in LLC-PK1 cells. **A**: Cells were cultured in 25 cm^2 flask in 10% FBS supplemented media for 1 day, followed by serum starvation (0.5% FCS) for 36 h in the presence of different concentrations of albumin (0, 1, or 10 mg/ml). Cells were stained by human PE-conjugated CD36-specific antibodies (10 μl per 10^6 cells). Cells (10^4) were gated and analyzed by flow cytometry. Data was expressed by histogram (X axis: FL-2 intensity representative for the level of CD36). Experiments were repeated twice. Isotype control was performed by using non-immune IgG. **B**: Cells were cultured in medium with 10% fetal calf serum 1 day, then transfected by synthetic CD36 siRNA (100 nM). The cells were then subjected to various doses of albumin (0, 1, or 10 mg/ml) in serum-free medium for 36 h. Cells were stained and analyzed as above procedures. Data was expressed by mean FL-2 (i.e., CD36). Experiments were repeated twice (n = 4) and similar results were obtained. It is evident that CD36 siRNA per se may markedly suppress the expression of CD36 protein ($^{\#}P < 0.05$). Additionally, CD36 siRNA would significantly attenuate albumin-induced increase in CD36 expression in LLC-PK1 cells. $^{*}P < 0.05$ (0 mg/ml albumin).

multiple biological processes [Febbraio et al., 2001]. CD36 is broadly expressed and may interact with multiple extracellular ligands, including thrombospondin-1 (TSP-1), long-chain free fatty acids (FFAs), modified (oxidized) low-density lipoprotein (ox-LDL), advanced glycation end (AGE) products, and collagens I and IV [Febbraio et al., 2001]. Hyperglycemia-induced synthesis of CD36 protein in macrophages has been associated with increased uptake of ox-LDL by macrophages and foam cell formation in atherosclerotic lesions in people with diabetes [Febbraio et al., 2000, 2001; Griffin et al., 2001]. While diabetic cardiovascular complications are closely linked epidemiologically with albuminuria and diabetic nephropathy (DN), a role for CD36 in DN and renal pathophysiology has not to our

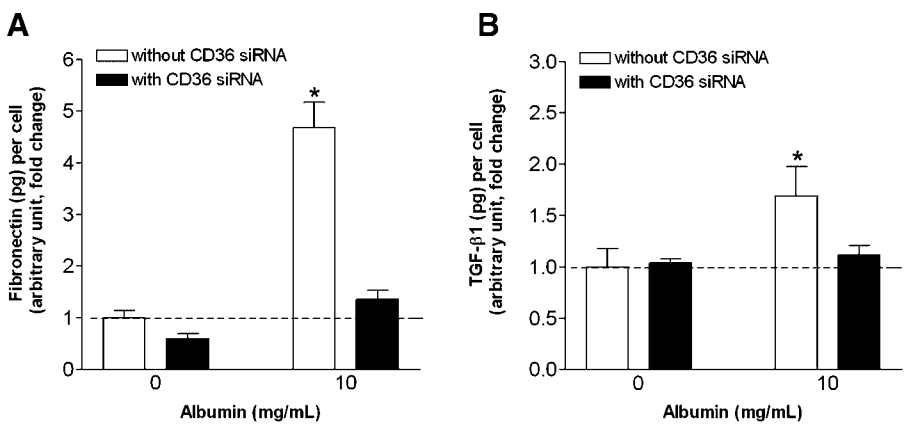

Fig. 7. Effects of CD36 siRNA on albumin-induced secretion of fibronectin and bioactive TGF-β1 in LLC-PK1. LLC-PK1 cells were cultured in 25 cm^2 flask in 10% FBS supplemented media for 1 day. After transfection with 100 nM of CD36 siRNA (for 24 h), cells were grown under serum starvation (0.5% FBS) for 36 h in the presence of different concentrations of albumin (0, 1, or 10 mg/ml). Conditioned medium was collected and directly subjected to ELISA assay for detection of fibronectin (**A**) and bioactive TGF-β1 (**B**) as described in Methods. Data was expressed by mean value of fibronectin or TGF-β. Experiments were repeated twice (n = 4). It is evident that CD36 siRNA efficiently suppressed albumin-induced increase in the level of fibronectin and TGF-β in LLC-PK1 cells. $^{*}P < 0.05$ versus control (0 mg/ml albumin).

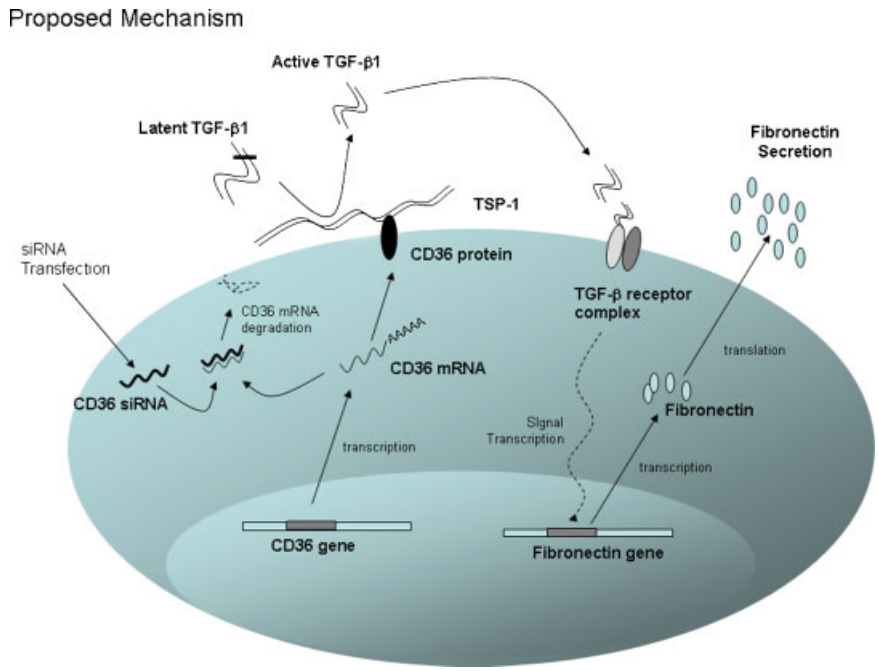

Fig. 8. Proposed mechanism for the interactions between CD36 and fibronectin in renal proximal tubule cells. Latent TGF-β1 from extracellular matrix can be transformed into bioactive form by interacting with CD36 complexing with extracellular TSP-1. Active TGF-β1 can in turn bind with two types of TGF-β receptors (designated as ''TGF-β receptors complex'') followed by a complex signal transduction process (e.g., Smads). Some gene (e.g., fibronectin) is upregulated and then expressed in a secreted form. Since CD36 is essential in the activation of latent TGF-β, suppressing the expression of CD36 by siRNA did inhibit and lower the level of bioactive TGF-β1 and the expression level of fibronectin.

knowledge been described to date. This work investigated the role of CD36 in albumin-induced renal tubule fibrosis. Blocking the expression of CD36 by siRNA markedly suppressed the upregulation of TGF-β1 and fibronectin (both intracellular and extracellular). Thus, we hereby propose that CD36 may be closely implicated in albumin-induced fibrosis in renal proximal tubule cells. This work is a novel demonstration of CD36 as a therapeutic target for treating renal tubule fibrosis.

The renal lesions of diabetes mellitus exist not only in the glomeruli but also in the region of proximal tubules region. Thus, an increasing number of studies focusing on the role of renal tubule cells in diabetic nephropathy including our previous studies [Yang et al., 1998, 2004; Chuang et al., 2003; Liu et al., 2006]. Like mesangial cells, proximal tubule cells have been found to act abnormally either in cell growth status or in cellular hypertrophic conditions under the diabetic stimuli (hyperglycemia or advanced glycation end-products). This study provides further evidence that albumin exposure is involved in the regulation of growth of proximal tubular cells since albumin decreased cell numbers as shown in Figure 1A. Moreover, albumin per se appears to be a fibrogenic factor for renal tubule cells since albumin was discovered to induce significant secretion of both the level of TGF-β1 and fibronectin in proximal tubule cells, as shown in Figures 3 and 4. Hence, exposure to albumin affects the growth and alters the biological effects in renal proximal tubule cells. These observations may explain why the progression of renal disease is strongly related to the degree of albuminuria.

Fibronectin and alpha1(I) collagen are common fibrotic markers which are usually applied in renal-fibrotic experimental model [Tan et al., 2006]. However, based on our unpublished observation, albumin did not statistically affect the level of alpha1(I) collagen in LLC-PK1 cells. Thus, in this study, we use fibronectin as the cellular fibrotic marker instead. Albumin-induced increase in the secretion of fibronectin and TGF-β in renal proximal tubule cells appears to be CD36-dependent as CD36 siRNA markedly attenuated the albumin-induced increase in fibronectin level and TGF-β (Figs. 6 and 7). These observations indicate that the

exposure of renal tubule cells to albumin lead to the synthesis of ECM proteins, indicating that albumin may contribute to the progression of renal tubule fibrosis in proteinuric states. These findings are strong evidence that CD36 may mediate albunuria-induced diabetic renal fibrosis. This study suggests that CD36 may be considered to be a therapeutic target for renal fibrosis.

In Figure 7A, it seems that there was a CD36 siRNA effect (visually) on the downregulation of fibronectin in the absence of albumin treatment. However, this observation is statistically insignificant based on our data. CD36 siRNA may exert its fibrosis-antagonizing effects only under the albumin treatment since fibrotic condition (i.e., fibronectin expression) was markedly induced in the presence of albumin rather than in the absence of albumin (as shown in Fig. 7). Additionally, the time-point issue may be another critical factor affecting the interpretation of data since the cells which were transfected with CD36 siRNA (for 24 h) were grown under the treatment of albumin for additional 36 h. Thus, the CD36 levels in siRNA-transfected cells may decrease and then graduately restore. Actually, in our routinely control experiments, the inhibition of CD36 mRNA starts from 12 h after transfection of CD36 siRNA and reaches maximum inhibition after 24-h transfection of CD36 siRNA. The inhibition time period was lasting at least 72 h after the transfection of CD36 siRNA.

Diabetes mellitus and chronic kidney disease involve the secretion of urinary albumin into renal tubule filtrate and urine. Predictors of albumin excretion rate (AER) abnormalities could provide earlier indices of diabetic nephropathy risk [Steinke et al., 2005]. Moreover, albumin modified by Amadori-glucose adducts (designated as glycated albumin) has been linked to the mid- to late-stage development of diabetic nephropathy. According to Susztak et al. [2005] CD36 is an essential mediator of proximal tubular apoptosis in human diabetic nephropathy. They found that CD36 expression was induced by glucose in proximal tubular epithelial cells, and increased CD36 mediated glycated albumin-induced apoptosis in proximal tubular cells. However, this study finds that CD36 mediates albumin-induced proximal tubule cell fibrosis instead of apoptosis. Hence, we hypothesize a three-step procedure for CD36-induced diabetic tubular epithelial degeneration and fibrosis. First (in the short-term), hyperglycemia induces the upregulation of CD36 in proximal tubule cells [Susztak et al., 2005]. Second (in the mid-term), albumin induces the upregulation of CD36 concomitantly with the tubule fibrosis (Figs. 3, 4, and 6). Third (in the long-term), glycated albumin induces the upregulation of CD36 concomitantly with the renal proximal tubule apoptosis (degeneration) [Susztak et al., 2005]. Taken together, the upregulation of the expression of CD36 appears to be closely associated with the renal tubule dysfunction and modulating the expression of CD36 may be an alternative approach to treat renal tubule fibrosis.

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