The role played by endocytosis in albumin-induced secretion of TGF-β1 by proximal tubular epithelial cells

Ramaswamy Diwakar,1 Alex L. Pearson,1 Paul Colville-Nash,1 Nigel J. Brunskill,2 and Mark E. C. Dockrell1

1South West Thames Institute for Renal Research, St. Helier Hospital, Carshalton, Surrey, United Kingdom,
2Department of Nephrology, Leicester General Hospital, Leicester, United Kingdom

Submitted 24 February 2006; accepted in final form 27 December 2006

Diwakar R, Pearson AL, Colville-Nash P, Brunskill NJ, Dockrell ME. The role played by endocytosis in albumin-induced secretion of TGF-β1 by proximal tubular epithelial cells. Am J Physiol Renal Physiol 292: F1464–F1470, 2007. First published January 16, 2007; doi:10.1152/ajprenal.00069.2006.—Proteinuria predicts the decline of renal function in chronic kidney disease. Reducing albuminuria has been shown to be associated with a reduction in the rate of decline. Proximal tubular epithelial cells (PTECs), when exposed to albumin, produce matrix proteins, proinflammatory and profibrotic cytokines like TGF-β1. Some of these effects are dependent on endocytosis of albumin by PTECs. However, conditions like diabetic nephropathy, believed to be associated with reduced albumin endocytosis, are associated with interstitial fibrosis. Moreover, megalin, the putative albumin binding receptor in PTECs, has potential signaling motifs in its cytoplasmic domain, suggesting its ability to signal in response to ligand binding from the apical surface of PTECs. Hence, we looked to see whether albumin-induced secretion of TGF-β1 by PTECs is dependent on albumin endocytosis or whether it could occur in the absence of albumin endocytosis. We studied the production of TGF-β1 in two accepted models of PTECs, opossum kidney cells and human kidney cell clone-8 cells, with widely varying degrees of endocytosis. We then studied the effect of inhibiting albumin endocytosis with various inhibitors on albumin-induced TGF-β1 secretion. Our results indicate that albumin-induced TGF-β1 secretion by PTECs does not require albumin endocytosis and therefore the mechanism for the induction of some profibrotic responses by albumin may differ from those required for some of the inflammatory responses. Moreover, we found that albumin-induced TGF-β1 secretion by PTECs is not dependent on its interaction with megalin; receptor-associated protein; simvastatin; albuminuria

END-STATE RENAL DISEASE is an important cause of mortality and morbidity, and the rate of progression of chronic kidney disease to end-stage renal disease correlates best with the degree of interstitial fibrosis (24). The degree of proteinuria predicts the rate of decline of renal function in the setting of a variety of renal diseases (1, 5, 12, 13, 16–17, 22, 30, 43). This suggests the possibility of a causal link between proteinuria and interstitial fibrosis. Albumin is the predominant protein in proteinuric urine, and reduction of albuminuria has been shown to correlate with a reduction in the rate of decline of glomerular filtration rate in renal disease (29).

In keeping with this, albumin has been shown in proximal tubular epithelial cells (PTECs) to induce the production of a variety of proinflammatory cytokines such as RANTES (49) and monocyte chemoattractant protein-1 (MCP-1) (35, 41, 42), extracellular matrix (ECM) proteins such as fibronectin (34) and collagen (44), and profibrotic cytokines such as transforming growth factor-β (TGF-β) (46). TGF-β is a prominent factor in the process of interstitial fibrosis. It modulates the expression of ECM in several cell culture models and is considered a determinant of ECM accumulation in tubulointerstitial fibrosis. The clear relationship between proteinuria and TGF-β has also been shown in biopsy studies (10). Moreover, Johnson et al. (18) have shown that the TGF-β1 secreted by PTECs is of functional importance because it can act on cortical fibroblasts in a paracrine fashion and cause their increased proliferation, thereby contributing to interstitial fibrosis. Hence, blocking albumin-induced production of TGF-β by PTECs may lead to reduced renal damage and potentially retard the progression of renal failure.

Some of the above-mentioned responses of PTECs to albumin are known to be dependent on albumin endocytosis, e.g., MCP-1 (41) and collagens I, III, and IV (44). However, it is unclear whether this is the case with TGF-β production. This knowledge would be helpful in devising ways of preventing albumin-induced TGF-β production and thus prevent subsequent interstitial fibrosis. Albumin filters through the glomerulus into the tubular lumen and binds to receptors in the luminal surface of PTECs called megalin and cubulin (2, 3, 48). It is then internalized and bound to these receptors. Megalin is a transmembrane receptor, the cytoplasmic domain of which has many potential signaling motifs. Hence, it is possible that some of the effects of albumin on PTECs are via activation of signaling pathways from the luminal surface of PTECs and are either partially or completely independent of its endocytosis. Hence, we undertook the following series of experiments to study the role of albumin endocytosis in the secretion of TGF-β1 by PTECs in response to albumin exposure.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (Sigma-Aldrich, Poole, Dorset, UK) unless otherwise stated.

Cell culture. OK cells, an immortalized PTEC cell line derived from the American opossum (Didelphys virginiana) (20) were grown in DMEM nutrient mixture F-12 HAM (1:1) supplemented with 10% heat inactivated FCS with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine.

Human kidney cell clone 8 (HKC-8) cells were kindly provided by Dr. Lorraine Racusen of Johns Hopkins University (Baltimore, MD). These cells were grown in DMEM-F-12 (1:1) (Invitrogen, GIBCO,
Paisley, UK) with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenite, 20 ng/ml tri-iodo tyronine, 18 ng/ml hydrocortisone, and 5% FCS as supplements (26).

Cells were grown at 37°C in a humidified 5% CO2 atmosphere. Cells were passaged at confluence using trypsin EDTA (Invitrogen) and seeded onto sterile 60-mm Petri dishes. The cells were allowed to grow to confluency with change of media three times per week.

At confluence, the cells were made serum free for 16–24 h before experiments were performed. After the experiments were performed, cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 200 μM PMSF, 2 mM EDTA, 1 mM sodium orthovanadate, protease inhibitor cocktail, and 50 mM sodium fluoride). The lysate was placed on ice for 20 min and then spun at 14,000 rpm for 10 min at 4°C to precipitate any cell debris. Protein concentration of the supernatant was measured with a BCA protein assay kit (Pierce).

Albumin. Rhodamine red X (RRX; excitation peak 550 nm, emission peak 570 nm)-labeled albumin (RRX-albumin) used in quantification of endocytosis was purchased from Jackson ImmunoResearch Laboratories. Fatty acid-free human serum albumin (HSA) was subjected to an endotoxin removal protocol with Bio-Rad AffiPrep polymyxin matrix. Endotoxin levels were determined with limulus amebocyte lysate Endosafe KTA endotoxin assay (Charles River Endosafe). The endotoxin level in all the HSA preparations used was <2.5 EU/ml.

Fluorescence spectrophotometry. Serum-free confluent cells were washed thoroughly and subsequently treated with RRX-albumin (made up in appropriate serum-free media) and incubated at 37°C. At the end of the experiment, endocytosis was stopped by washing the cells with ice-cold PBS. The cells were then treated with ice-cold 0.2 M acetic acid and 2 M NaCl on ice for 10 min to strip any bound but not endocytosed albumin from the cell surface. The cells were washed six times with 2 ml of ice-cold PBS to wash away free RRX-albumin. The adequacy of these washes was ensured by studying the fluorescence of the last wash, which was no greater than background fluorescence. Specific binding was calculated by subtracting nonspecific binding (measured in the presence of excess unlabeled albumin) from the total binding. The cells were lysed and transferred to individual wells of a 96-well plate, and the fluorescence was studied with excitation wavelength of 584 nm and emission wavelength of 612 nm. The degree of fluorescence was corrected for total cellular protein.

Effect of albumin on TGF-β1 secretion. OK, HKC-8, and receptor-associated protein (RAP) (rat recombinant; Progen Biotechnik, Heidelberg, Germany) were used to inhibit albumin endocytosis. The confluent cell cultures were preincubated with EIPA for 15 min and then coincubated with RRX-albumin for 30 min at 37°C. For the simvastatin experiments, the confluent cells were exposed to various concentrations of simvastatin for ~16 h followed by incubation with RRX-albumin for 30 min. In the RAP experiments, the cells were preincubated with RAP for 30 min and then coincubated with labeled albumin for 30 min.

Cytotoxicity assay. CytoTox-ONE homogeneous membrane integrity assay (Promega, Madison, WI) was used to measure lactate dehydrogenase (LDH) release under various conditions as an index of cytotoxicity.

Effect of albumin on TGF-β1 release: TGF-β1 ELISA. In these experiments, confluent cells in 24-well plates were made serum-free overnight and then treated with 0.1, 1, and 5 mg/ml HSA. The supernatant was collected to assay secreted TGF-β1 in the supernatant was determined by sandwich ELISA (TGF-β1 Emax immunoassay system) according to manufacturer’s instructions (Promega). Briefly, 96-well plates were coated with TGF-β1 coat MAb overnight at 4°C. The plates were incubated with block buffer for 35 min at 37°C. The standards were prepared by serial dilution of a known concentration of TGF-β1. To activate latent TGF-β1 present in the supernatants, all of the samples were acidified with 1 M HCl and then neutralized with 1 M NaOH. After addition of the samples, the plate was serially incubated first with TGF-β1-specific polyclonal antibody and with secondary antibody conjugated with horseradish peroxidase. After each of the incubations, the plates were extensively washed. After addition of a chromogenic substrate (tetramethyl benzidine) for 15 min at room temperature, the reaction was stopped with 1 M HCl. The color change was then measured by recording absorbance at 450 nm. The amount of TGF-β1 in the test solutions is proportional to the color generated. The concentration of TGF-β1 in the supernatant was calculated with the standard curve. The range of biologically active TGF-β1 that can be quan-
REPORTED was in the range of 32–1,000 pg/ml. The amount of secreted TGF-β1 was corrected for total cellular protein.

Statistics. The results are presented as means (SD). Statistical analysis was performed with ANOVA with post-hoc analysis using Tukey’s test.

RESULTS

Albumin induces TGF-β1 production in PTECs (OK cells and HKC-8 cells). HSA induced a concentration-dependent secretion of TGF-β1 at 8 h in OK cells (Fig. 1A). The response plateaued at 1 mg/ml. This effect of albumin was not mimicked by equimolar mannitol, thereby excluding an osmotic effect of albumin as the cause of the TGF-β1 secretion (data not shown). HKC-8 cells showed comparable TGF-β1 secretion at 8 h as OK cells (Fig. 1B).

The results of these experiments were consistent with responses seen previously with primary PTECs (46).

Comparison of albumin endocytosis in OK cells vs. HKC-8 cells. Given the comparable secretion of TGF-β1 by both of the cell types, we studied the endocytosis of albumin by these cells to see whether this was also comparable.

In keeping with existing literature, OK cells demonstrated a time-dependent (Fig. 2) and concentration-dependent (Fig. 3) endocytosis of albumin. The data demonstrate saturation kinetics in keeping with receptor-mediated endocytosis (31). HKC-8 cells showed very little endocytosis of RRX-albumin (Figs. 2 and 3): ~2% compared with OK cells at 0.1 mg/ml concentration (Fig. 3).

The results of these experiments suggested that endocytosis did not play a vital part in the secretion of TGF-β1 by PTECs in response to albumin exposure.

To eliminate the possibility of variable sensitivity of the TGF-β1 ELISA kit to TGF-β1 from various species, we attempted to study the effect of inhibiting albumin endocytosis in PTECs from a single species (the OK cells) on albumin-induced TGF-β1 secretion.

To investigate inhibition of endocytosis on TGF-β1 secretion, the OK cell model was used in the presence and absence of two inhibitors of endocytosis with different mechanisms of action.

Inhibition of albumin endocytosis in OK cells. EIPA, a sodium/hydrogen exchanger-3 inhibitor (225 μM) inhibited...
RRX-albumin endocytosis at 30 min by 70% (Fig. 4). This is comparable to work done previously by others (7). Simvastatin (10 μM), a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, reduced albumin endocytosis by 45% (Fig. 5), as has been shown previously (32, 39).

Inhibiting albumin endocytosis does not reduce albumin-induced TGF-β1 production. Effective concentrations of inhibitors of endocytosis were chosen from the above experiments and utilized in experiments performed to study TGF-β1 secretion. As seen in Figs. 6 and 7, albumin caused a concentration-dependent secretion of TGF-β1. EIPA on its own did not have any effect on the secretion of TGF-β1 and did not reduce albumin-induced TGF-β1 secretion (Fig. 6). There was a trend toward increased TGF-β1 production by albumin in the presence of EIPA, and this reached statistical significance at 1 mg/ml albumin. Similarly, simvastatin did not reduce albumin-induced TGF-β1 secretion (Fig. 7). There was no cytotoxicity (assessed by LDH release) associated with the inhibitors of endocytosis at the concentrations used (data not shown).

Role played by megalin in albumin-induced TGF-β1 production. Megalin, a large transmembrane receptor at the surface of PTECs, has been shown to bind albumin and is thought to be involved in albumin endocytosis. The cytoplasmic tail of megalin has potential signaling sequences, which raise the interesting possibility that megalin may initiate signaling cascades in response to binding of ligands to its extracellular domain. To study the role played by megalin in albumin-induced TGF-β1 secretion, we chose to use RAP, a known inhibitor of binding of albumin to megalin (3, 48).

RAP tended to inhibit RRX-albumin endocytosis in a dose-dependent fashion, with ~45% inhibition at 0.08 μM. A significant inhibition of 63% was achieved by 0.25 μM RAP (Fig. 8). However, RAP did not have any effect on TGF-β1 secretion on its own, nor did it affect albumin-induced TGF-β1 secretion (Fig. 9). This suggests that megalin was not involved in albumin-induced TGF-β1 secretion.

Role played by TGF-β receptor in albumin-induced TGF-β1 secretion. In light of the above results suggesting that megalin is not involved in albumin-induced TGF-β1 secretion, we investigated an alternative receptor-mediated pathway. It is known that TGF-β can induce its own production by acting through TGF-β II receptor (23). Moreover, in vascular endothelial cells, albumin is known to bind to and induce TGF-β receptor II signaling (33). Hence, we studied the role of TGF-β receptor transactivation in the secretion of TGF-β1 on albumin exposure in PTECs. SB-431542 (an activin receptor-like kinase 4, 5, and 7 inhibitor; Ref. 14) has been shown to inhibit a variety of effects of TGF-β1 (11, 21).

SB-431542 at 10 μM concentration did not have any effect on albumin-induced TGF-β1 secretion (Fig. 10). This result was duplicated in HKC-8 cells, where 10 μM SB-431542 was

![Fig. 5. Effect of simvastatin on RRX-albumin endocytosis in OK cells. Confluent quiescent cells were preincubated with indicated concentrations of simvastatin for 16 h followed by coincubation with 0.1 mg/ml RRX-albumin for 30 min. Results are means of 3 experiments (SD). *P < 0.05 compared with 0 μM simvastatin.](http://ajprenal.physiology.org/)

![Fig. 6. Effect of EIPA on HSA-induced TGF-β1 secretion in OK cells. Confluent quiescent cells were preincubated with 225 μM EIPA for 15 min followed by coincubation with appropriate concentrations of HSA for 8 h. Results are means of 3 experiments (SD). *P < 0.05; ***P < 0.001.](http://ajprenal.physiology.org/)

![Fig. 7. Effect of simvastatin on HSA-induced TGF-β1 production in OK cells. Confluent quiescent cells were preincubated with 10 μM simvastatin for 16 h followed by coincubation with appropriate concentrations of HSA for 8 h. Results are means of 3 experiments (SD). *P < 0.05; **P < 0.01.](http://ajprenal.physiology.org/)
ROLE OF ENDOCYTOSIS IN ALBUMIN INDUCED TGF-β1 SECRETION

We have demonstrated that endocytosis of HSA is not a requirement for albumin-induced TGF-β1 secretion by two distinct approaches. We have compared TGF-β1 secretion in two cell lines with very different degrees of endocytosis. TGF-β1 production was comparable in both of these cell lines. To confirm this finding, we inhibited albumin endocytosis with two different inhibitors, EIPA (6, 7), which acts by preventing the recycling of receptors back to the apical surface, and simvastatin (32, 39), which inhibits the prenylation of GTP-binding proteins, in a cell model that shows good albumin endocytosis (OK cells) (31). The results confirmed that receptor internalization is not required for the secretion of TGF-β1. This is in agreement with the data of Zafiriou et al. (47), which suggests that, despite pioglitazone’s effect of increasing albuminuria is accompanied and to a certain extent contributed to by reduced tubular albumin endocytosis (36–38). In this setting, the production of classical inflammatory cytokines like MCP-1 by PTECs, an endocytosis-dependent phenomenon (41), would not be enhanced. We, however, see progressive renal fibrosis and decline in renal function, which is reversed on reducing albuminuria (29).

The lack of inhibition of albumin-induced TGF-β1 secretion in the presence of inhibitors of albumin endocytosis EIPA and simvastatin suggests that the signaling pathways involved in this process are operative from the surface of PTECs.

Regarding simvastatin, our data show interesting differences from those of Kim et al. (19), who found that lovastatin reduced TGF-β1 mRNA synthesis and protein secretion in cultured rat mesangial cells in response to glucose. However, studies in cultured heart cells have previously shown that statins upregulate TGF-β1 mRNA production, TGF-β receptor II expression, and TGF-β signaling (25). Hence, it is possible that the effect of statins on TGF-β1 production is either cell type specific or is dependent on the agonist.

We studied the role of megalin in albumin-induced TGF-β1 production. RAP binds to megalin with high affinity and prevents albumin binding and hence endocytosis of albumin (3, 48). The data again showed that endocytosis is not a requirement for albumin-induced secretion of TGF-β1. Interestingly, the data also suggest that albumin-induced secretion of TGF-β1 is not via megalin signaling. The concentration of RAP used in our experiments caused a significant increase in LDH release at 8 h compared with that shown in control. However, this amounted to only 3% of positive control. Moreover, RAP did not affect basal TGF-β1 secretion, indicating that this degree of cytotoxicity does not result in measurable TGF-β1 release. Consequently, we do not believe that cell death-induced TGF-β1 release masked an inhibition of the effects of albumin by RAP.

TGF-β1 has been shown to induce its own production by acting through the TGF-β II receptor (23). Moreover, previous evidence has shown the induction of TGF-β II receptor signaling by albumin (33). Hence, we studied the role of TGF-β receptor activation in the secretion of TGF-β1 on albumin exposure in PTECs. Our data suggest that TGF-β receptor does not play a part in albumin-induced TGF-β1 secretion at 8 h of albumin exposure. However, this does not rule out the involve-
ment of TGF-β receptor II at later time points by the action of the secreted TGF-β1 by autoinduction (23). This is particularly relevant because albumin has been shown to upregulate TGF-β type II receptors in PTECS from 12 h onward (45).

One of the attractive alternative receptors for albumin-induced TGF-β1 production is the EGF receptor (EGFR). Recent data suggest the involvement of EGFR in albumin-induced signaling events (27).

There was a trend toward increased albumin-induced TGF-β1 secretion when endocytosis was inhibited (best seen with EIPA when used with 1 mg/ml albumin). Further work needs to be done to validate this finding. Interesting parallels can be seen with receptors such as EGFR. It has been shown that inhibition of endocytosis of EGFR using a conditional dynamin mutant resulted in increased rather than reduced cell proliferation in response to EGF (40). If this were true with albumin-induced TGF-β1 secretion, it becomes theoretically possible that the TGF-β1 secreted in response to albumin by PTECs can potentially act by positive feedback where albumin induces the secretion of TGF-β1, which then inhibits albumin endocytosis (8), leading to further secretion of TGF-β1. A further positive feedback loop in the setting of proteinuric renal disease becomes a possibility because long-term exposure of PTECs to protein reduces albumin endocytosis (9), and this could potentially increase TGF-β1 secretion. It is noteworthy that HKC-8 cells, a well-characterized PTEC line, display such a limited capacity for albumin endocytosis. The precise reason for this is unknown. This is despite the expression of megalin in these cells (data not shown).

Albumin has traditionally been believed to cause renal injury secondary to excessive tubular absorption by PTECs, abnormal accumulation of these proteins in the endolysosomes resulting in generation of reactive oxygen species, and activation of signaling sequences such as NF-κB, resulting in the release of inflammatory cytokines into the interstitium (28). Drumm et al. (4) have shown that sodium/hydrogen exchanger-dependent endocytosis induces an increase in NF-κB activity in PTECs, which is inhibited by EIPA. By showing that albumin-induced TGF-β1 secretion is not dependent on endocytosis, we thus provide evidence for the first time that this commonly accepted mechanism of action of albumin on PTECs is not the only way in which albuminuria can potentially result in progressive renal fibrosis. Hence, emphasis must be placed on reducing glomerular leakage of albumin rather than attempting to reduce albumin uptake by PTECs from the tubular lumen.

ACKNOWLEDGMENTS

We thank Dr. Jonathan Kwan and Dr. Michael Bending for valuable suggestions and without whose energy the SW Thames Institute for Renal Research would not exist.

GRANTS

This work was made possible by a generous grant from the South West Thames Kidney Fund.

REFERENCES


22. Locatelli F, Marcelli D, Comelli M, Alberti D, Graziani G, Bucciante G, Redaelli B, Giangrande A, Northern Italian Cooperative Study Group T. Proteinuria and blood pressure as causal components of pro-