Albumin stimulates p44/p42 extracellular-signal-regulated protein kinase in opossum kidney proximal tubular cells

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ABSTRACT

The presence of protein in the urine of patients with renal disease is an adverse prognostic feature. It has therefore been suggested that proteinuria per se may be responsible for the development of renal tubulo-interstitial scarring and fibrosis, and disturbances in tubular cell growth and proliferation. We have used the opossum kidney proximal tubular cell line to investigate the effects of albumin on cell growth. The effect of albumin on cell proliferation was investigated by cell counting and measurement of [3H]thymidine incorporation. We studied the effect of recombinant human albumin on the activity of p44/p42 extracellular-signal-regulated mitogen-activated protein kinase (MAP kinase) using an in vitro kinase assay, and immunoblotting with antibodies against active extracellular-signal-regulated kinase (ERK). The effects of the ERK inhibitor PD98059 were also examined. Recombinant human albumin was found to stimulate proliferation of opossum kidney cells in a dose-dependent manner, with maximal stimulation at a concentration of 1 mg/ml. In addition, recombinant human albumin activated ERK in a time-dependent (maximal after 5 min) and dose-dependent (maximal at 1 mg/ml) fashion. These effects on cell proliferation and ERK activity were inhibited by PD98059, and were not reproduced by ovalbumin or mannitol. The data therefore indicate that albumin is able to stimulate growth and proliferation of proximal tubular cells that is dependent on the ERK family of MAP kinases. The potential importance of this pathway in the development of renal disease is discussed.

INTRODUCTION

Clinical nephrologists recognize the important correlation between the degree of proteinuria and risk of progression of many types of renal disease [1–3]. As such, it is widely observed that patients with significant proteinuria are substantially more likely to develop end-stage renal failure than those without proteinuria. It is also well recognized that in patients with nephrological disease the biochemical parameters of worsening renal function correlate best with histological abnormalities in the renal tubulo-interstitium rather than in the glomeruli, even in primary glomerular disorders [4–6].

The tubulo-interstitial changes seen in progressive
renal failure are characteristic. Tubular atrophy is marked, and inflammatory cells often surround the tubules. The interstitium displays prominent fibrosis and scarring, with deposition of collagen and lipids, and large numbers of fibroblasts are seen [7]. That the kidney has a limited repertoire of response to injury is demonstrated by the observations that these tubulo-interstitial changes are common to most renal diseases and that, when the end-stage kidney is examined, it is generally impossible to determine the cause of the initial renal disease [8].

These observations have led a number of authors to suggest that proteinuria and renal tubulo-interstitial inflammation and scarring may be causally linked [9,10]. This proposal remains controversial, however, the more conventional view being that greater proteinuria simply indicates a more severe renal lesion, which is more likely to progress as a result of this greater severity. Nonetheless, evidence is now beginning to emerge which suggests that albumin, the major protein found in proteinuric urine, may be able to induce phenotypic changes in proximal tubular cells and alter their function in a manner that would produce a pro-inflammatory environment in the renal tubulo-interstitium. When proximal tubular cells are incubated with albumin they produce monocyte chemotactant protein-1 and RANTES (regulated upon activation, normal T cell expressed and secreted) in a dose-dependent manner [11,12]. Using the protein-overload nephrosis model in rats, we have recently demonstrated alterations in the growth and turnover of proximal tubular cells in response to heavy proteinuria [13]. These perturbations in proximal tubular cell turnover are manifest as a combination of both proliferation and apoptosis, and we hypothesized that these proteinuria-induced changes in proximal tubular cell growth and turnover may be responsible, at least in part, for the development of progressive renal scarring in which tubular cell growth is so manifestly disordered. The mechanism of induction of these responses is unknown.

The mitogen-activated protein kinase (MAP kinase) pathways are key components in the transduction of signals leading ultimately to cell growth, proliferation and transformation. These kinase cascades consist of a three-kinase module including a MAP kinase which is activated in turn by a MAP kinase kinase (MEK), which is activated in turn by a MAP kinase [14]. The best characterized MAP kinase cascade results in the activation of extracellular-signal-regulated protein kinases (ERKs) 1/2, and this pathway is activated by many mitogens in many cell types [14]. In the opossum kidney (OK) cell line, ERK is activated by angiotensin II [15]. In view of the potential for the activation of mitogenic pathways in proximal tubular cells by albumin in proteinuric states, we have examined the effect of albumin on the activity of ERK in proximal tubular cells cultured in vitro. The results indicate that albumin is able to stimulate proliferation of proximal tubular cells via ERK, and suggest a possible link between albuminuria and the derangements of proximal tubular cell growth observed in progressive renal scarring.

METHODS

Materials
OK cells were obtained from Dr. J. Caverzasio (University of Geneva, Switzerland), and were used between passages 60 and 87. The peptide ERK substrate representing a portion of the cytoplasmic tail of the epidermal growth factor receptor (RRELVEPLTPSGEAPNQA-LLR) was synthesized by the Protein and Nucleic Acid Chemistry Laboratory, Leicester University. Rabbit polyclonal anti-ERK antisera were from Santa Cruz (Santa Cruz, CA, U.S.A.), and rabbit polyclonal antiphospho-ERK antibodies were from Promega. [3H]Thymidine was from Amersham (Little Chalfont, Bucks., U.K.), and [γ-32P]ATP was from NEN. Protein A–Sepharose CL-4B was from Pharmacia Biotech (Uppsala, Sweden). PD98059 was from Calbiochem (Nottingham, U.K.). Recombumin® yeast recombinant human serum albumin (rHSA) was kindly provided by Delta Biotechnology Ltd. (Nottingham, U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.). Genomic DNA was isolated using a Wizard purification kit (Promega, Madison, WI, U.S.A.), and quantified by spectrophotometry. Cell protein was measured by the Lowry method [15a].

Cell culture
OK cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (1:1, v/v; DMEM/F12) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM l-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in an atmosphere of 5% CO2/95% air. The culture medium was changed three times weekly, and cells were split at confluence approximately every 2 weeks.

[3H]Thymidine incorporation assay
OK cells were grown to ~70% confluence in 24-well plates. Before experiments, OK cells were incubated in serum-free DMEM/F12 for 18 h. For experiments, this medium was replaced by either DMEM/F12 containing 10% (v/v) FCS or serum-free DMEM/F12 containing various concentrations of rHSA. After a further 24 h, 2 μCi of [3H]thymidine was added to each well, and the cells were incubated at 37 °C for a further 2 h. At the end of this period, cells were washed three times with serum-free DMEM/F12, and then incubated with ice-cold 5% (v/v) trichloroacetic acid for 1 h at 4 °C. The trichloroacetic acid was removed and the monolayers were washed
with fresh ice-cold trichloroacetic acid. Then 2 ml of ice-cold ethanol containing 200 μM potassium acetate was added to each well for 5 min. Monolayers were then incubated with 2 ml of ethanol/ether (3:1, v/v) for 2 × 15 min. After drying, cell monolayers were solubilized with 0.1 M NaOH. Samples were then counted for radioactivity in a liquid scintillation analyser (Packard 1900CA Tri-Carb).

Assessment of cell number
To complement the [³H]thymidine data, and in order to definitively confirm that cell numbers were increased after incubation with rHSA, equal numbers of cells were seeded into dishes and grown to 70% confluence. Cells were then incubated in serum-free DMEM/F12 or in DMEM/F12 containing various concentrations of rHSA for 24 h. After incubation, cells were trypsinized and counted using a haemocytometer.

Immune-complex ERK assay
Confluent OK cells were serum-starved for 24 h and then stimulated with various concentrations of rHSA for various times. In some experiments cells were pretreated with the MEK inhibitor PD98059 prior to stimulation with rHSA. Stimulation with 10% (v/v) FCS was used as a positive control. Following stimulation, monolayers were solubilized in ice-cold lysis buffer (20 mM Tris/HCl, pH 8.0, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 2 mM Na₃VO₄, 20 μg/ml aprotinin, 5 μg/ml leupeptin and 1 mM dithiothreitol). Insoluble material was removed by centrifugation (14000 g for 10 min) and lysates were incubated with anti-ERK-1 antibodies for 90 min at 4 °C. Immune complexes were precipitated using Protein A–Sepharose and washed twice in lysis buffer and twice in kinase buffer (20 mM Hepes, pH 7.2, 20 mM β-glycerophosphate, 10 mM MgCl₂, 1 mM dithiothreitol and 50 μM Na₃VO₄). Immune-complex ERK assays were performed by resuspending immunoprecipitates in a volume of 40 μl of kinase buffer containing 200 μM synthetic kinase substrate peptide, and initiated by the addition of 20 μM [γ-³²P]ATP (2.5 μCi/nmol). Reactions were allowed to proceed for 20 min at 30 °C and were then terminated by the addition 10 μl of 25% (w/v) trichloroacetic acid. Samples were centrifuged (14000 g for 2 min) and equal volumes of supernatant were spotted on to Whatman P81 cation-exchange paper. Papers were washed four times in 0.5% orthophosphoric acid and once with acetone, and then dried and counted for radioactivity by liquid scintillation spectroscopy. To correct for non-specific phosphorylation, the kinase assay was performed in the absence of peptide substrate, and the levels of [³²P]phosphate bound to filters under these conditions were subtracted from those values measured in the presence of substrate. In all experiments, appropriate rHSA vehicle
diluent controls were performed; these did not reproduce the effects of rHSA itself.

Electrophoresis and immunoblotting
Treated cell monolayers were lysed in Laemmli buffer containing 60 mM Tris, pH 6.8, 10% (v/v) glyceral, 2% (w/v) SDS, 100 mM dithiothreitol and 0.01% Bromophenol Blue. Proteins were separated by PAGE and transferred to nitrocellulose. The nitrocellulose membranes were probed with rabbit polyclonal antibodies against active ERK, which recognize only the phosphorylated p42 and p44 forms of ERK. Second antibodies were anti-rabbit peroxidase-conjugated immunoglobulins, and were detected by enhanced chemiluminescence (Amersham).

Data presentation and statistics
Data are presented as means ± S.E.M. For the analysis of differences, unpaired two-tailed Student’s t-tests were performed. Differences were regarded as significant at P < 0.05.

RESULTS
Incubation of OK cells with rHSA in the absence of serum or any other mitogens resulted in a dose-dependent increase in cell proliferation (Figure 1). This effect was evident at a rHSA concentration of 100 μg/ml, at which [³H]thymidine incorporation was increased to 185.9±12.0% of control values. The increase in [³H]thy-

Figure 1 rHSA stimulates [³H]thymidine incorporation by OK cells
Cultured OK cells were incubated with various concentrations of rHSA, and proliferation was measured as [³H]thymidine incorporation. Results are expressed as percentage [³H]thymidine uptake compared with controls incubated with serum-free medium with no additions (Cont). Incubation of OK cells with medium containing 10% (v/v) FCS is depicted as a positive control. Data represent means ± S.E.M. for n≥3 experiments. Significance of differences compared with control: *P < 0.01.
Figure 2  rHSA stimulates proliferation of OK cells
Equal quantities of OK cells were seeded on to dishes. Cell numbers were determined after incubation in serum-free medium (Cont), 10% (v/v) FCS or various concentrations of rHSA. Data represent means ± S.E.M. for n = 3 experiments. Significance of differences compared with control: *P < 0.01.

Figure 3  Stimulation of ERK activity in OK cells by increasing concentrations of rHSA
Cultured OK cells were incubated with the indicated concentrations of rHSA for 5 min, and ERK activity was determined by an immune complex in vitro kinase assay. Results are expressed as fold increase in ERK activity compared with controls incubated with serum-free medium (Cont). Incubation of OK cells with medium containing 10% (v/v) FCS is depicted as a positive control. Data represent means ± S.E.M. for n ≥ 3 experiments. Significance of differences compared with control: *P < 0.01.

Idine incorporation was maximal at 1.0 mg/ml rHSA (290.8 ± 12.5% of control values). Increased incorporation of thymidine into DNA was accompanied by an increase in cell number (Figure 2). Again, this effect was maximal at an incubated rHSA concentration of 1 mg/ml, at which cell numbers increased by approx. 3-fold compared with controls. Although increases in both protein and DNA were significantly greater in the cells cultured with rHSA, protein/DNA ratios remained unchanged, indicating that, while cell number increased, hypertrophy did not occur (results not shown).

In addition, incubation with rHSA stimulated the activity of p44/p42 ERK in OK cells in a dose-dependent manner (Figure 3). This effect was maximal at 1 mg/ml rHSA, at which concentration ERK activity was stimulated 2.1 ± 0.4-fold above that in control cells incubated with serum-free medium containing no additives. The stimulation of ERK activity by rHSA was rapid. Significant activation was seen after 1 min of incubation of OK cells with 1 mg/ml rHSA, and was maximal after 5 min of incubation with 1 mg/ml rHSA, at which time ERK activity was increased 264 ± 33.8% over controls (Figure 4). Thereafter ERK activity declined, reaching baseline levels by 30 min of incubation with rHSA.

Activation of ERK by 1.0 mg/ml rHSA was also assessed by lysing cells after incubation, and immunoblotting using an antibody which recognizes only the phosphorylated activated form of p44/p42 ERK. In this way we were able to confirm stimulation of ERK, with maximal activity after 5 min (Figure 5).

In order to determine whether rHSA-stimulated OK cell proliferation is dependent on ERK activity, cells were
pre-incubated with PD98059, an inhibitor of ERK kinase. At a concentration of 5 μM, PD98059 inhibited rHSA-stimulated ERK activity after a 20 min pre-incubation (results not shown). This manoeuvre also completely inhibited rHSA-stimulated OK cell proliferation, as measured by [3H]thymidine incorporation and increasing cell numbers (Figure 6).

In order to exclude a non-specific nutrient-type effect of protein on cell growth and proliferation, OK cells were incubated with the structurally dissimilar and irrelevant protein ovalbumin at various concentrations. Ovalbumin treatment was incapable of stimulating ERK activity in OK cells (Figure 7), and did not result in cell proliferation at any of the concentrations used. These experiments with ovalbumin suggested that ERK stimulation is not due to an osmotic effect of protein incubation, and this conclusion is strengthened by the observation that increasing concentrations of mannitol were also unable to stimulate OK cell proliferation (results not shown) or ERK activity (Figure 7).

DISCUSSION

The search for the mechanisms underlying chronic renal tubulo-interstitial inflammation and scarring has led to the accumulation of a considerable literature. Despite this, a unifying hypothesis is still lacking. Most recently the powerful association of proteinuria with an adverse prognosis for any given renal disease has encouraged several groups of workers, including ourselves, to examine the effects of protein on proximal tubular cell function. The most abundant protein moiety in proteinuric urine is albumin, and hence we chose to study the effects of rHSA on proximal tubular cell biology. We have demonstrated previously that albumin binds to high- and low-affinity binding sites in OK proximal tubular cells, and that it is subsequently taken up into the cells by receptor-mediated endocytosis [16,17]. Indeed, binding and uptake of albumin by OK cells results in stimulation of a kinase cascade including phosphatidylinositol 3-kinase and pp70^56 kinase [19]. We postulated that this effect might have profound physiological and pathophysiological implications for kidney tubular cell function.

The present study extends our work on signal transduction pathways stimulated by albumin in the kidney proximal tubule. The results clearly demonstrate that albumin is mitogenic when added to serum-starved proximal tubular cells in culture. Proliferation of cells occurs via stimulation of the p44/p42 ERK family of MAP kinase enzymes. Albumin was shown to stimulate
the activity of ERK, as determined using two distinct techniques, and the proliferative effect of albumin was abolished by PD98059, an inhibitor of ERK activation. Stimulation of ERK by albumin is not related to changes in osmolality, and does not appear to be a non-specific protein effect, as the irrelevant unrelated protein ovalbumin failed to reproduce the mitogenic and ERK-stimulating effects of rHSA.

Serum albumin is a sticky molecule and is able to bind a variety of serum components, such as lipids and drugs. To facilitate our studies we have used yeast rHSA. This preparation is devoid of all known ligands, and in particular contains no serum-derived components which may have a mitogenic or stimulatory effect in cell culture experiments. Thus rHSA is the most pure preparation of human albumin available, and we are therefore confident that the phenomena observed in these studies are due to rHSA and not to an unidentified molecule bound to albumin.

The emergence of albumin as a signalling molecule challenges the conventional view of the role of this protein in physiology. Traditional dogma holds that albumin is a relatively benign and inert molecule, serving predominantly to exert osmotic pressure within the circulation. The present study does not support this view, and nor does the work of other authors who have also documented unexpected properties of albumin in a number of biological systems. Albumin in the circulation is able to modulate vascular permeability, possibly via changes in the intracellular Ca\(^{2+}\) concentration [20,21]. Furthermore, when cultured endothelial cells arestarved of serum, rapid apoptosis results. Albumin acts as a survival factor in this situation, abrogating the apoptotic response to serum starvation [22]. Interaction of albumin with its binding protein gp60 in bovine pulmonary microvascular endothelial cells not only precedes transcytosis, but also activates an intracellular kinase cascade, resulting in phosphorylation of a number of proteins on tyrosine residues [23]. Based on the observation that albumin induces changes in the intracellular Ca\(^{2+}\) concentration in astrocytes, other authors have proposed a pathophysiological role for albumin in cerebral scarring after breakdown of the blood–brain barrier, such as occurs in cerebral haemorrhage [24,25].

With regard to the kidney, proteins including albumin have been shown to exert diverse effects on proximal tubular function. Albumin and urine from proteinuric rats stimulate proximal tubule hypertrophy and hyperplasia [26]. The hypertrophic response is only observed after a 24 h incubation with albumin, but not after 24 h as documented in the present study. Cytokine expression is also enhanced by exposure of proximal tubular cells to albumin, with production of both monocyte chemoattractant protein-1 and RANTES being stimulated in a manner dependent on the activation of nuclear factor \(\kappa B\) [11,12,27].

In vivo, proteinuria induces both proliferation and apoptosis of the proximal tubular epithelium [13], although the precise mechanisms underlying these effects are obscure. One possibility is that interaction of albumin with proximal tubular cells causes both cell proliferation and the chemoattraction of inflammatory cells into the renal tubulo-interstitium, and that apoptosis occurs as a result of this developing inflammatory response, with tubular atrophy being the net result. Albumin may therefore act as a ‘double-edged sword’, on the one hand stimulating proliferation and growth of proximal tubular cells, but on the other hand precipitating an inflammatory environment in the kidney which leads ultimately to tubular atrophy and renal failure.

Measurement of the proximal tubular concentration of albumin in humans is obviously not possible. Rodent studies suggest that, in health, an albumin concentration of 20–30 µg/ml is likely to be found in the proximal tubular fluid. Induction of nephrosis results in up to a 100-fold increase in the albumin concentration prevailing in the proximal tubule [28–30]. Thus the ERK-stimulatory effect of albumin demonstrated in the present study is not likely to be observed at physiological concentrations of albumin in the proximal tubule, but may have a potentially important influence on proximal tubule cell growth in the setting of pathophysiological proteinuria.

Elucidation of the mechanism of stimulation of ERK by albumin is a key issue that requires further study. The signalling mechanisms underlying the development of the pro-inflammatory phenotype of proximal tubular cells have so far not been determined. However, the potential role of ERK activation in the regulation of cytokine expression in the kidney merits further investigation.

In summary, therefore, we have demonstrated that albumin can activate the ERK family of MAP kinase enzymes in the kidney proximal tubule. This activation results in cell proliferation, and may be important in the development of the typical growth abnormalities observed in the proximal tubules of diseased kidneys.

ACKNOWLEDGMENTS
We are grateful to the Wellcome Trust for their support of this work. N.J.B. holds a Wellcome Trust Advanced Fellowship.

REFERENCES
Received 28 June 1999; accepted 16 November 1999

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