Secretion of chemokines and cytokines by human tubular epithelial cells in response to proteins

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Abstract

Background. Chronic interstitial scarring contributes to the progression of renal failure in glomerular disease but its cause is unknown. The development of proteinuria could stimulate tubular cells to release cytokines, chemoattractants and matrix proteins into the interstitium, thus contributing to interstitial disease.

Methods. Polarized human tubular epithelial cells were grown on permeable supports and exposed to serum proteins on their apical surface. The release of tumour necrosis factor \( \alpha \) (TNF\( \alpha \)), platelet derived growth factor (PDGF) and monocyte chemoattractant protein-1 (MCP-1) by the cells was measured using immunoassays.

Results. Under control conditions there was polarized release of PDGF-AB with predominant basolateral secretion (basolateral to apical ratio 4.7 ± 1.6). MCP-1 release was less polarized (ratio 1.7 ± 0.5). TNF\( \alpha \) was not detected. Exposure of the cells to normal human serum proteins on their apical side increased basolateral release of PDGF-AB (1.7 ± 0.4 fold) and MCP-1 (2.4 ± 0.2 fold). Fractionation of the serum showed that this effect on human tubular epithelial cells was reproduced by a fraction of molecular weight 40–100 kDa. The predominant proteins in this fraction were albumin and transferrin but these purified proteins alone did not alter secretion of PDGF-AB or MCP-1.

Conclusion. This data demonstrates that human tubular cells exposed to proteins, which would be filtered in glomerular disease, produce inflammatory mediators with the potential to stimulate inflammation and scarring in the interstitium of the kidney.

Key words: interstitial scarring; monocyte chemo-attractant protein-1; platelet derived growth factor; proteinuria; proximal tubular cell

Introduction

Interstitial inflammation and scarring predict loss of renal function even in those diseases considered to be primarily of glomerular origin [1]. However, the mechanisms resulting in these secondary interstitial changes are unknown. Tubular cells can produce extracellular matrix proteins which could contribute directly to the scarring process, they also have the capacity to manufacture cytokines and chemokines [2]. Therefore a change in tubular cell phenotype causing release of these inflammatory mediators and matrix proteins could result in inflammation and scarring in the interstitium. Glomerular disease changes the environment of the tubular cells in a variety of ways [3,4] including the development of proteinuria [2,5]. The exposure of the apical surface of tubular cells to increased amounts and types of protein from which they are normally protected by an intact glomerular barrier, could be the stimulus that initiates tubulo-interstitial pathology.

Previous work from this laboratory has demonstrated the production of the matrix protein fibronectin and the cytokine platelet derived growth factor (PDGF) by human tubular cells [6]. Using a polarized cell culture model it was shown that apical exposure to serum proteins, as would occur in vivo in proteinuric states, increased basolateral fibronectin and PDGF secretion by these cells. This manuscript describes the use of this culture model to investigate the effects of serum proteins on chemokine production and to investigate the nature of the proteins within serum which alter the behaviour of human proximal tubular cells.

Methods

Materials

Cell culture media were obtained from Life Technologies, UK. Defined medium additives and collagen were supplied by the Sigma Chemical Company, UK. Antibodies for cell characterization were from Dako UK. Permeable membrane supports were purchased from Costar UK. Chromatography columns were from Pharmacia Biotech UK. Tumour necrosis
factor α (TNF-α), PDGF-AB and monocyte chemoattractant protein-1 (MCP-1) assays were purchased from R and D Systems UK.

**Cell culture**

Human tubular epithelial cells (HTEC) were isolated by a modification of the method described by Detrisac et al. as reported previously [6,7]. In brief, the outer cortex was dissected from the normal pole of kidneys which had been removed for treatment of carcinoma or from donor kidneys deemed unsuitable for transplantation. Fragmented cortex was suspended in type II collagenase (1.0 mg/ml) at 37°C for 30 min. The digest was passed through a series of sieves of diminishing mesh size and the glomeruli removed on the top of the 90-μm mesh. The remaining tubular fragments were seeded into 75 cm² flasks which had been coated with bovine collagen type I and adsorbed fetal calf serum proteins.

The cells were grown in DMEM:F12 with the addition of 25 mM HEPES buffer, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), tri-iodo-thyronine (4 pg/ml), hydrocortisone (36 ng/ml), benzyl penicillin (100 IU/ml) and streptomycin (50 μg/ml). After 10–14 days the cells reached confluence and were subcultured. At the second passage the cells were transferred into 6-well plates containing polycarbonate permeable membrane supports of 3.0 μm pore size, coated with bovine collagen type I. They were grown in the medium described above with the addition of 5% fetal calf serum. The apical and basolateral media were thus separated by the confluent monolayer of cells and could be sampled independently. We have previously demonstrated that cells isolated and grown in this way stain for cytokeratin but not factor VIII related antigen and produce cAMP in response to parathyroid hormone confirming the cells to be of predominantly proximal tubular origin. In addition the cells form a polarized uniform monolayer with a very low permeability to albumin [6].

**Serum preparation**

Venous blood was collected from normal volunteers and allowed to coagulate. The serum was separated and dialysed against a phosphate buffered saline. The protein concentration in the serum was measured using a BioRad DC protein assay and the serum samples were diluted to a total protein concentration of 10 mg/ml. The serum was stored at −20°C until used.

Since serum complement components are biologically active the contribution of complement to effects of serum was tested by heat inactivation of complement at 56°C for 30 min. The effect of 10⁻⁶ M endotoxin added to the apical culture medium was used to control for possible contamination of the serum during processing.

**Serum fractionation**

The dialysed serum proteins were fractionated by molecular weight using a Superdex 200 gel filtration column (Pharmacia). One milliliter of serum containing 50 mg of total protein was added to the column and eluted using a 0.15 M NaCl/0.05 M Na₂HPO₄ pH 7.2 buffer. Four molecular weight fractions were collected: (A) >440 kDa, (B) 440–100 kDa, (C) 100–40 kDa and (D) <40 kDa. Proteins in fraction A were sufficiently large that they would only be minimally filtered even in severe glomerular damage. Proteins in fraction B would be filtered in a non-selective proteinuria. Fraction C contained proteins which would be filtered in selective proteinuria and fraction D contained proteins that would be filtered by a normal glomerulus. The greatest concentration of proteins occurred in fraction C, which was adjusted to a final protein concentration of 1.0 mg/ml in the cell cultures. The other fractions were collected in an equal volume and diluted identically to fraction C, thus ensuring that the protein concentrations were in the same ratio to fraction C as they were in unseparated serum (A: 0.075±0.009 mg/ml; B: 0.457±0.005 mg/ml; C: 0.007±0.005 mg/ml; D: 0.009±0.005 mg/ml).

To further characterize components of serum fractions they were subjected to ion exchange chromatography using a Mono Q cation exchanger (Pharmacia UK). Protein samples were buffer exchanged into Bis-Tris-Propane (6.25 mM) pH 7.5 and loaded onto the column. Proteins were eluted using a linear gradient of increasing concentration of sodium chloride (0.35 M) in Bis-Tris-Propane (6.25 mM) pH 9.5 and detected using a UV spectrophotometer.

**The effect of serum proteins on cytokine production**

Once the cells on the permeable supports formed confluent monolayers the medium was changed to DMEM (containing a physiological concentration of glucose) with addition of 25 mM HEPES, hydrocortisone (36 ng/ml), benzyl penicillin (100 IU/ml) and streptomycin (50 μg/ml). After 24 h in this medium, the media bathing both the apical and basolateral surfaces of the cells were changed. The apical medium contained the serum proteins (1.0 mg/ml), serum protein fractions, albumin (1.0 mg/ml), transferrin (0.05 mg/ml) or an equivalent volume of phosphate buffered saline as a control. Fresh serum-free medium was placed on the basolateral side ensuring that the fluid in the apical and basolateral compartments were level so that there was no hydrostatic pressure gradient across the membrane. The apical and basolateral media were sampled after 48 h and stored at −70°C for cytokine assays. Following removal of the medium, 1% nonidet P40 was added to the wells and the cells scraped off the membrane. They were dissolved using sonification. Total cell protein was measured in the cell solution using BioRad DC assay.

**Cytokine assays**

TNF-α, PDGF-AB and MCP-1 were measured in the medium samples and in the serum by commercially available ELISA. The limits for detection were 31–2000 pg/ml for the PDGF-AB, and MCP-1 assays; and 15–1000 pg/ml for the TNF-α assay. The normal human serum contained PDGF-AB at a concentration of 0.3±0.1 ng/ml in 1.0 mg/ml of serum. Therefore in samples of apical medium to which serum had been added the concentration of PDGF-AB in the serum sample was subtracted from that in the medium to determine the amount which had been secreted by the cells. The concentration of MCP-1 in 1.0 mg/ml of normal serum (3.1±0.5 pg/ml) was at least three orders of magnitude less than the amount produced by the cells and was therefore disregarded. TNF-α was not detectable in the serum samples.

**Statistics**

The results are expressed as mean±standard error of the mean. Statistics were performed using analysis of variance. Where statistical significance was demonstrated the groups
were compared using Student’s t-tests with a Bonferroni correction. \( P<0.05 \) was taken as significant.

**Results**

Results were obtained using cells from five different kidney preparations all of which were exposed to serum, three were exposed to serum fractions and four to albumin and transferrin. A total of 10 different serum samples were used. When two different serum samples were used on cells from a single kidney preparation this was taken as an \( n \) of 2.

It has previously been shown that HTEC secrete PDGF-AB into both the apical and basolateral media under control conditions [6] and this was confirmed in the current experiments. The ratios of apical to basolateral secretion are shown in Table 1 which demonstrates that the secretion of PDGF-AB was polarized with a higher concentration in the basolateral medium. The maintenance of a concentration gradient suggests that the permeability of the cell monolayer to PDGF-AB is low. In the presence of apical serum the secretion of PDGF-AB remained polarized with a basolateral to apical ratio of 3.4 ± 0.3 which was not significantly different to controls.

The concentrations of MCP-1 in the apical and basolateral media were not significantly different under control conditions or on exposure to serum proteins (controls: 3.80 ± 0.46 ng/ml apical, 3.64 ± 0.41 ng/ml basolateral; serum exposed: 10.0 ± 0.7 ng/ml apical, 8.1 ± 1.0 ng/ml basolateral). However, the total amounts of MCP-1 in the apical and basolateral media were different as a result of the difference in volume of apical and basolateral medium. This is reflected in the apparent polarity of secretion of MCP-1 shown in Table 1.

The effect of apical exposure to 1.0 mg/ml of serum proteins on the basolateral secretion of PDGF-AB and MCP-1 by HTEC is shown in Table 2. Basolateral secretion of PDGF-AB increased by 1.7 ± 0.4-fold (\( P<0.01 \)) similar to that previously reported [6]. Basolateral secretion of MCP-1 increased by 2.4 ± 0.2-fold (\( P<0.001 \)). TNF\( \alpha \) secretion was not detected in either the apical or basolateral media either under control conditions or when HTEC were exposed to apical serum.

To control for the possibility of endotoxin contamination during production of the serum the cell mono-

Table 1. The ratio of basolateral to apical secretion of PDGF-AB and MCP-1, after 48 h culture, under control conditions or on exposure to 10 mg/ml of serum proteins in the apical medium (results from five kidney preparations and 10 serum samples). Ratios >1.0 indicate predominant basolateral secretion

<table>
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<th>Control no protein</th>
<th>Apical serum 1.0 mg/ml protein</th>
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<tbody>
<tr>
<td>PDGF-AB</td>
<td>4.7 ± 1.6</td>
<td>3.4 ± 0.7</td>
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<tr>
<td>MCP-1</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.3</td>
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layers were exposed to apical medium containing 10\(^{-5}\) M lipopolysaccharide. This had no effect on the production of PDGF-AB or MCP-1 by HTEC. Heating serum to 56°C for 30 min to inactivate complement components had no effect on its ability to increase production of PDGF-AB or MCP-1.

To further define which component within serum was responsible for the stimulated production of PDGF-AB and MCP-1 by HTEC, the serum was fractionated into four molecular weight fractions. Each fraction was added to the apical medium on HTEC. Figure 1 shows the effect of the fractions on secretion of PDGF-AB. The 1.3 ± 0.1-fold increase in PDGF-AB secretion produced by serum in this series of experiments was reproduced by fraction C (1.4 ± 0.2-fold increase). There was also a small (1.2 ± 0.1-fold) but statistically significant increase in PDGF-AB secretion in response to the high molecular weight fraction A (>440 kDa). Serum fraction C contained only very small amounts of PDGF-AB (0.01 ± 0.005 ng/ml) and therefore diffusion of added PDGF-AB across the monolayer could not have accounted for the increased amount of basolateral PDGF-AB. There were no significant effects of fractions B or D compared to control.

Figure 2 shows the effect of each of the fractions on the basolateral secretion of MCP-1. 1.0 mg/ml of unfractonated serum increased MCP-1 secretion by an amount (2.5 ± 0.3-fold) that was reproduced by fraction C (2.4 ± 0.2-fold) with no significant effect of fractions A, B or D.

**Table 2.** The total amount of PDGF-AB and MCP-1 secreted by HTEC into the basolateral medium over 48 h under control conditions and on exposure to 1.0 mg/ml of serum proteins in the apical medium (results from five kidney preparations and 10 serum samples)

<table>
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<th>Control no protein</th>
<th>Apical serum 1.0 mg/ml protein</th>
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<tbody>
<tr>
<td>PDGF-AB</td>
<td>1.4 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>MCP-1</td>
<td>13.0 ± 1.2</td>
<td>30.6 ± 3.0</td>
</tr>
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![Fig. 1. Basolateral PDGF-AB secretion by HTEC on apical exposure to serum fractions. HTEC were exposed to serum fractions (A, B, C, D), unfractionated serum (S) or control conditions (0) in the apical medium. After 48 h PDGF-AB was assayed in the basolateral medium. The results are from three kidney preparations and five serum samples. *\( P<0.01 \), **\( P<0.05 \) compared to control.](image-url)
Production of PDGF and MCP-1 by HTEC exposed to protein

Fig. 2. Basolateral MCP-1 secretion by HTEC on apical exposure to serum fractions. HTEC were exposed to serum fractions (A, B, C, D), unfractionated serum (S) or control conditions (0) in the apical medium. After 48 h MCP-1 was assayed in the basolateral medium. The results are from three kidney preparations and five serum samples. *P < 0.001 compared to control.

Fig. 4. Basolateral MCP-1 secretion by HTEC on apical exposure to albumin and transferrin. HTEC were exposed to human albumin (ALB) or partially iron saturated human transferrin (TF), unfractionated serum (S) or control conditions (0) in the apical medium. After 48 h MCP-1 was assayed in the basolateral medium. The results are from four kidney preparations. *P < 0.001 compared to control.

Fraction C (which contained proteins of molecular weight 40–100 kDa) was analysed by ion exchange chromatography. This demonstrated that albumin and transferrin were the proteins present in greatest concentration. Each of these proteins was added individually to the apical medium bathing HTEC cultures and the response compared to whole serum. Pure human albumin (Sigma) was added at a concentration of 1.0 mg/ml and pure partially iron saturated human transferrin (Sigma) was added at a concentration of 0.05 mg/ml which was the concentration of transferrin found in fraction C. The effect of these proteins on basolateral secretion of PDGF-AB and MCP-1 is shown in Figures 3 and 4, respectively. Neither albumin nor transferrin were able to reproduce the effects of serum.

Discussion

The cause of interstitial scarring in glomerular disease is unknown. One hypothesis is that tubular cells exposed to proteinuria release matrix proteins, cytokines and chemoattractants into the interstitium promoting inflammation and scarring [2,5]. To investigate this, a polarized model of human proximal tubular cells grown on permeable membrane supports has been developed. These monolayers secrete fibronectin in increased amounts following exposure to serum proteins [6], a mechanism that could directly contribute to interstitial scarring. Proximal tubular cells could also stimulate scarring by release of the fibroblast chemoattractant PDGF. Inflammation might be provoked by tubular release of cytokines such as TNFα or macrophage chemoattractants such as MCP-1. An interstitial infiltrate of macrophages is a prominent feature of human proteinuric renal diseases [8,9] and in animal models the macrophage infiltrate has been correlated with the quantity [10,11] of proteinuria.

MCP-1 is produced by human proximal tubular cells when stimulated by cytokines [12].

Our data demonstrates that polarized human tubulo-epithelial cells produce both PDGF-AB and MCP-1 but TNFα was not detected. PDGF-AB secretion was polarized with predominant basolateral secretion. The concentrations of MCP-1 in apical and basolateral media were equivalent but following correction for volume of medium the total basolateral MCP-1 was significantly more than apical MCP-1. This ratio was, however, less than that for PDGF. As a small peptide (8 kDa) MCP-1 may have leaked across the cell culture monolayer reducing the concentration difference but significant apical secretion of MCP-1 is consistent with the finding of MCP-1 in urine of patients with glomerular disease in an amount correlated with the amount of proteinuria [13]. Clearly urinary MCP-1 might also originate from the glomerulus.

Basolateral PDGF-AB and MCP-1 secretion were increased by exposure to apical serum proteins. MCP-1 secretion was more than doubled but the increase in PDGF-AB secretion was more modest. Whilst under basal conditions tubular cells in vivo will produce little PDGF there was significant production in the control cell cultures which might blunt the apparent response to proteins. It is also difficult to translate the significance of total levels of release of a cytokine into a cell...
culture medium back to the *in vivo* state in which the concentration immediately local to the secreting cell is of greatest importance. The biological significance of this level of increased PDGF-AB release is therefore uncertain.

Coagulation of blood to produce serum results in release of cytokines which might contribute to the effects on the cell cultures but would not be present in the normal glomerular ultrafiltrate. Since the glomerular ultrafiltrate is inaccessible, it is not possible to produce a perfect model of the proteins to which tubular cells are exposed in proteinuric states. Our approach was to further define the components of serum which were active within the system in order to exclude an effect of released cytokines. Exogenously added endotoxin did not reproduce the effects indicating that contamination during serum production was not responsible. Inactivation of complement components by heating did not alter the ability of serum to stimulate PDGF-AB and MCP-1 release. The effect of serum was reproduced by a fraction of molecular weight 40–100 kDa, a range of proteins that would be expected to be filtered in glomerular disease but not by a normal glomerulus [14]. Other fractions had no effect on MCP-1 secretion although there was a small increase in PDGF-AB secretion from cells exposed to the high molecular weight fraction A. This effect is of doubtful significance given that proteins of this size are not filtered in large amounts. Most serum cytokines such as PDGF are of low molecular weight and are found in fraction D [6], which had no effect on HTEC, and not in fraction C.

The predominant serum proteins of molecular weight 40–100 kDa are albumin and transferrin. Purified human albumin (1.0 mg/ml) and partially iron saturated transferrin (0.05 mg/ml) did not reproduce the effects of serum. Wang et al. [15] demonstrated upregulation of mRNA for MCP-1 in rat proximal tubular cells exposed to bovine transferrin at high concentrations (1–8 mg/ml). We used partially iron saturated transferrin at a low concentration to most closely represent the transferrin found in the original serum samples. Wang et al. [15] also showed that bovine albumin at a concentration of 0.5 mg/ml increased mRNA for MCP-1 in rat proximal tubular cells. However, to demonstrate an increase in MCP-1 protein production by polarized cells 10 mg/ml of bovine albumin was required. Using LLC-PK1, cells Zoja et al. [16] showed increased release of the chemokine RANTES following exposure to 10 mg/ml of BSA whereas 1.0 mg/ml of BSA had no detectable effect. The lack of effect of pure human albumin (1.0 mg/ml) indicates that albumin *per se* was not responsible for the observed effects of serum in this human system and also shows that the effect of serum was not simply a non-specific effect of a large quantity of protein.

Until the precise agent within serum which caused the observed effects is determined it will not be possible to be certain of its presence in the glomerular ultrafiltrate. The effect of the active serum fraction may require a combination of proteins. Alternatively a molecule carried by the protein rather than the protein *per se* may be important for its biological activity. It has been shown that the effect of bovine albumin on OK cell growth (a proximal tubular cell line) is dependent on the type of fatty acids carried by the albumin [16]. Whilst the human albumin used in our experiments was not delipidated it is possible that other molecules carried by the albumin are significant.

Purification of proteins may alter their structure or remove carried molecules which could explain the discrepancy between the effect of serum proteins and the purified components.

This work has demonstrated that HTEC can produce MCP-1 and PDGF-AB and that secretion is increased by apical exposure to proteins that are likely to be filtered in glomerular disease but not by a normal glomerulus. This could provide an explanation for the development of secondary interstitial scarring in glomerular disease.

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