The Role of Proteinuria and Proximal Tubular Cells in the Development of Tubulointerstitial Inflammation and Scarring in the Kidney

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by

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The role of proteinuria and proximal tubular cells in the development of
interstitial scarring in the kidney.

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Loss of renal function in chronic kidney disease is correlated with interstitial scarring but the
mechanism for the development of interstitial pathology in glomerular disease is unknown.
Since the quantity of proteinuria, in renal disease, is correlated with the rate of progression
of renal failure it has been suggested that proteinuria may be a cause of progression.
Proteinuria could have this effect by altering proximal tubular cell biology resulting in
inflammation and scarring in the tubulointerstitium. The experiments described in this thesis
investigated alterations in the phenotype of proximal tubular cells in culture following
exposure to proteins.

Growth of proximal tubular cells is known to occur in progressive kidney disease. It was
shown that whilst albumin altered the growth of OK cells, the mixture of proteins present in
proteinuric urine, at the same total protein concentration, had a more marked effect. This
suggested that effects of protein on tubular cell function may not be simply the result of
protein per se but that specific proteins have different effects.

A cell culture model of human tubular epithelial cells (HTEC) grown on membrane supports
was developed. HTEC produced fibronectin, PDGF and MCP-1. Apical exposure of HTEC
to serum proteins increased their basolateral secretion of fibronectin, PDGF and MCP-1.
There was also increased monolayer permeability and increased lactate dehydrogenase release
by HTEC exposed to serum, suggesting toxicity to the cells. The active component of serum
was found in a fraction of molecular weight 43-100kDa. Although the major proteins in this
fraction were albumin and transferrin, these proteins, added alone to the culture medium did
not reproduce the effects of serum.

These studies demonstrated that serum proteins if present in the glomerular ultrafiltrate could
act on proximal tubular cells to alter their phenotype in a way that would promote interstitial
inflammation and scarring.
For my Mother
Acknowledgements

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My greatest thanks are reserved for my wife Katherine, for her love and support, which kept me going throughout and to my daughter Helen who helped keep it all in a sensible perspective.
<table>
<thead>
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<th>Description</th>
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<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ApoF</td>
<td>Apoferritin</td>
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<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic 3’5’ adenosine monophosphate</td>
</tr>
<tr>
<td>DAB</td>
<td>Di-aminobenzidine</td>
</tr>
<tr>
<td>DGM</td>
<td>Defined growth medium</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles Medium</td>
</tr>
<tr>
<td>DMEM:F12</td>
<td>DMEM + Hams F12 nutrient medium (1:1)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FCR</td>
<td>Folin Ciocalteau Reagent</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FNDS</td>
<td>Fibronectin depleted serum</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GMCSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salts solution</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulphonic acid)</td>
</tr>
<tr>
<td>HI</td>
<td>Heat inactivated FNDS</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>LAP</td>
<td>Latency associated peptide</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LFBSA</td>
<td>Lipid free BSA</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCNS</td>
<td>Minimal change nephrotic syndrome</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>NAG</td>
<td>N-Acetyl glucosaminidase</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>NSE</td>
<td>Nonspecific esterase</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAN</td>
<td>Puromycin aminonucleoside</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBSD</td>
<td>Dialysate phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PU</td>
<td>Proteinuric urine</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecasulphate</td>
</tr>
<tr>
<td>SFEM</td>
<td>Serum free experimental medium</td>
</tr>
<tr>
<td>SNGFR</td>
<td>Single nephron GFR</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween TBS</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WB</td>
<td>Washing buffer</td>
</tr>
<tr>
<td>WBN</td>
<td>WB+1% nonidet P40</td>
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CHAPTER 1

INTRODUCTION
1.1 Introduction

Chronic progressive renal failure is a major cause of mortality and morbidity [Burton P 1987] and current estimates would suggest a minimum requirement for renal replacement therapy in the United Kingdom of 80 patients per million population [Feest T 1990]. By investigation of the causes of renal failure it may be possible to identify reversible processes which could lead to treatments that slow the rate of progression of renal disease. Currently apart from those diseases in which immunomodulatory treatment is of proven benefit the therapeutic armoury available to the Nephrologist is limited. The control of hypertension is of importance [Klahr S 1994] and there is now evidence that angiotensin converting enzyme inhibitors have advantages over other antihypertensive agents. This has been shown convincingly in the remnant kidney model of renal failure in the rat [Anderson S 1986] and also in the human with diabetic nephropathy [Lewis E 1993]. In diabetic nephropathy recent evidence has also pointed to the importance of improved glycaemic control in slowing the rate of progression [DCCT research group 1993].

Low protein diets decrease the rate of decline in renal function in animal models [Klahr S 1983, Williams A 1987]. Meta-analysis of controlled trials of low protein diets in humans supported the view that low protein diets reduce the rate of progression [Fouque D 1992]. However a recent, large controlled clinical trial, did not support this hypothesis [Klahr S 1994].

In recent years it has been suggested that common mechanisms contribute to progression in most types of chronic renal failure [Klahr S 1988]. This originated from the observation that once a degree of renal damage has been produced then the progression of kidney failure is inexorable, even if the original injurious process resolves [Walser M 1990]. This is most clearly seen in the subtotal nephrectomy model in the rat [Hostetter T 1981,
Anderson S 1985] in which a single surgical insult of removal of 4/3 to 5/6 of renal tissue produces a continuing progressive loss of kidney function thereafter. Considerable research effort in recent years has concentrated on identifying the processes that underlie this secondary progression of renal failure.

A widely accepted hypothesis is that one cause of progression is an alteration of glomerular haemodynamics [Brenner B 1983]. This hypothesis suggests that the loss of nephrons results in hyperperfusion of the remaining glomeruli and glomerular hypertension, leading to glomerular sclerosis. Hyperlipidaemia [Moorhead J 1982], alterations in renal growth [Fogo A 1988a] and acidosis [Nath K 1985] have also been postulated to contribute to glomerular sclerosis and the progression of renal disease. However, the observation that histologically, interstitial pathology correlates more closely with the degree of renal impairment than does glomerular pathology has focused attention on the tubulo-interstitium as a potential contributor to progression [Schainuck L 1970, Risdon R 1968]. Why interstitial pathology should occur in diseases considered to be primarily of glomerular origin is unknown. One change in the environment of the tubulo-interstitium consequent upon glomerular disease is the exposure of tubular cells to large amounts of medium and high molecular weight proteins, in the tubular lumen, from which they are normally protected by the glomerular barrier. This abnormal exposure to proteins may be damaging and has led to the formulation of the hypothesis on which this thesis is based.

1.2. Hypothesis:

Glomerular disease results in the exposure of tubular cells to proteinuria which alters their phenotype in such a way as to promote interstitial inflammation and scarring in the renal cortex.
1.3 The normal renal cortex

The renal cortex forms the outer layer of the kidney and is approximately 1 cm thick in the normal human adult [Tisher C 1991]. The cortex is composed of glomeruli, proximal and distal tubules, cortical collecting ducts and the space between them known as the interstitium. That part of the cortex excluding the glomeruli is sometimes referred to as the tubulo-interstitium.

1.3.1 The Glomerulus

The glomerulus is composed of a capillary network lined by a thin layer of endothelial cells with a central region containing mesangial cells and their surrounding matrix [Tisher C 1991]. The capillary endothelium is separated by the glomerular basement membrane from the visceral epithelium [Abrahamson D 1987]. The visceral epithelial cells have long cytoplasmic processes (foot processes) which come into contact with the glomerular basement membrane [Arakawa M 1971]. Lining the outside of the glomerulus is a parietal epithelial cell layer which forms Bowmans capsule and between the two epithelial cell layers is the urinary space [Jorgensen F 1966]. The purpose of the glomerulus is to filter the blood passing through the glomerular capillaries. Filtration occurs between the foot processes of the visceral epithelial cells and this gap is referred to as the filtration slit. The glomerular filter has both size and charge selective properties and to cross the filtration barrier a molecule must pass through the fenestrated endothelium of the capillary, the glomerular basement membrane and then the visceral epithelial cell slit diaphragm [Brenner B 1977].

There is now good evidence that the glomerular basement membrane is the principal structure responsible for the permeability properties of the glomerulus [Farquhar M 1981]. Pathological processes which lead to injury to the glomerulus may damage this barrier and
result in the leakage of large proteins into the glomerular ultrafiltrate and hence into the urine where they are detected as proteinuria.

### 1.3.2. The tubulo-interstitium

#### a) The tubules

The proximal tubule begins at the pole of the glomerulus and consists of an initial convoluted portion called the pars convoluta which is a continuation of the parietal epithelium of the glomerulus and a straight portion called the pars recta [Tisher C 1991]. Tight junctions form between the epithelial cells of the proximal tubule and provide a low resistance barrier between the lumen of the tubule and the basolateral intercellular space [Tisher C 1973]. The intercellular space is open toward a basement membrane which separates it from the peritubular interstitium and capillaries [Tisher C 1969].

The luminal or apical surface of the cells has a brush border. Morphometric study of the rabbit proximal tubule has suggested that this brush border increases the surface area of the apical membrane by 36 times [Welling L 1975]. A number of enzymes including alkaline phosphatase, gamma glutamyl transferase, 5'-nucleotidase, aminopeptidase, and Mg"ATPase occur within the brush border [Kinne R 1971]. One major function of the proximal tubule is solute and water reabsorption and approximately 50% of the ultrafiltrate is reabsorbed in this part of the nephron [Tisher C 1991].

Following the proximal tubule the nephron continues as the loop of Henle which occurs within the medulla. The ascending limb of the loop of Henle returns to the cortex and then leads into the distal tubule. The thick ascending limb of the loop of Henle passes close to the glomerulus where it forms a specialised region called the macula densa which is an important component of the renin angiotensin system [Schermann J 1985]. Past the macula
densa the nephron becomes the distal convoluted tubule. The cells of the distal tubule lack the brush border and endocytic apparatus characteristic of the proximal tubule [Tisher C 1991]. The functions of the distal convoluted tubule include sodium and chloride reabsorption and secretion of potassium [Malnic G 1966]. The distal convoluted tubule drains into a connecting segment that joins it to the cortical collecting duct which descends into the medulla on its way to the renal pelvis.

b) The Interstitium

The interstitium is composed of interstitial cells and extracellular matrix. In man the relative volume of the cortical interstitium has been estimated by various studies to be 11.7±5.5% of total cortical volume under the age of 36 increasing to 15.7±3% over this age [Dunnill M 1973, Böhle A 1977, Hestbech J 1977]. The interstitium is surrounded by the basement membranes of Bowmans capsule, the vascular beds and the tubules [Kuncio G 1991]. Common components of the basement membranes are collagen types IV and V, laminin, fibronectin and heparan sulphate proteoglycans [Price R 1987]. Because of the close apposition of basement membranes, in the outer cortex, the interstitium is sparse and not well characterised. However at levels closer to the medulla the interstitium becomes greater in extent. In the rat it has been estimated that just under half the volume of the cortical interstitium is composed of interstitial cells [Bohman S 1980]. These cells include interstitial fibroblasts, macrophages, lymphocytes and dendritic cells [Kaissling B 1994] of which the fibroblasts and dendritic cells are most abundant. Only small numbers of lymphocytes are found and macrophages are very scarce apart from in the connective tissue of large vessels.

Peritubular capillaries run through the interstitial space closely adjacent to their tubules [Bohman S 1974]. The capillary wall is fenestrated where it faces the tubule which may
facilitate solute and water reabsorption [Pederson J 1980]. The interstitial space contains
loose, low density material which has been shown, by immunohistochemistry, to contain types
I and III collagen, fibronectin and proteoglycans in the normal human kidney [Mounier F
1986].

1.4. Pathological changes in interstitial scarring

The final outcome for the interstitium in chronic renal failure is the laying down of
excess extracellular matrix proteins in scarring [Böhle A 1987]. The matrix proteins involved
include collagens type I [Downer G 1988] and III [Adler S 1986] and fibronectin [Vangelista
A 1989]. It is likely that the laying down of the interstitial matrix is the result of a chronic
inflammatory reaction since chronic inflammatory cells are invariably present. In recent years
the leucocyte population of the interstitium in a variety of renal diseases has been
characterised [Boucher A 1986, Kelly C 1991]. T lymphocytes and macrophages account for
the majority of cells and in most cases of human renal disease the lymphocytes predominate.
Most frequently CD4+ and CD8+ T cells are present in equal numbers.

In models of proteinuric renal disease in the rat, such as protein overload proteinuria
[Eddy A 1989] and puromycin aminonucleoside nephrosis [Eddy A 1991], there is a florid
interstitial infiltrate. The macrophages arrive first and predominate throughout. Of the
lymphocytes present the majority are CD8 positive. The significance of the difference in the
distribution of cells between the rat models and human secondary interstitial disease is
unknown.

Once inflammatory cells have been recruited into the interstitium it can be expected
that they will release a cocktail of cytokines which would result in the proliferation of resident
fibroblasts, the chemoattraction of neighbouring fibroblasts and the consequent laying down
of extra-cellular matrix proteins. It has also been suggested that the tubular cells themselves could contribute to the scarring process since they are of mesenchymal origin [Kuncio G 1991] and are capable of producing extracellular matrix. Tubular cells produce collagens types IV and V which are components of the tubular basement membrane [Haverty T 1988]. They are also able to produce components of the scarred interstitium such as collagen types I [Ziyadeh F 1990] and III [Creely J 1988], fibronectin [Viedt C 1995] and heparan sulphate proteoglycans [Humes D 1993]. In addition, Neilson E [1995] has recently suggested that tubuloepithelial cells may be able to transdifferentiate into a fibroblast phenotype in the presence of interstitial inflammation and scarring. They found that a marker which is normally only expressed on fibroblasts, is expressed on the tubuloepithelial cells of rats with interstitial scarring.

1.5 Cell growth in progressive renal disease

Growth of the remaining renal tissue following a reduction in renal mass is a well described clinical phenomenon and has been investigated in animal models of renal disease including partial renal ablation [Arataki M 1926], high protein diets [Leathem J 1945, Brenner B 1982], diabetes mellitus [Seyer-Hansen K 1976, Garcia-Puig J 1981] and ammonium chloride feeding [Lotspeich W 1965]. Organ growth can be the result of hyperplasia (an increase in cell number) or hypertrophy (an increase in cell size). Hyperplasia can be determined experimentally by the uptake of tritiated thymidine into an organ or an increase in total DNA whilst hypertrophy can be determined by an increase in the protein per cell, the protein:DNA ratio or the RNA:DNA ratio [Fine L 1986]. Using these techniques it has been shown that in adult rats following uninephrectomy 80% of kidney growth is due to hypertrophy, the remainder being due to hyperplasia [Johnson H 1969]. In young animals
however, as long as the animal continues to grow, the renal growth following uninephrectomy is primarily by hyperplasia [Sands J 1979, Karp R 1971].

Most components of the nephron have been shown to enlarge in compensatory growth including the glomerulus [Saphir O 1927], the proximal convoluted tubule [Hayslett J 1968], the proximal straight tubule [Fine L 1978] and the collecting tubule [Fine L 1979]. There is a close correlation between the development of glomerular hypertrophy and glomerular sclerosis in animal models [Yoshida Y 1989, Fogo A 1988a] and also in human renal disease [Fogo A 1988b]. The proximal tubule, however, grows out of proportion to the rest of the nephron [Oliver J 1945, Fine L 1985]. The mechanism of compensatory hypertrophy remains uncertain. The 'work' hypothesis in which an increase in single nephron glomerular filtration rate (SNGFR) and increased proximal tubular reabsorption stimulate growth is widely accepted [Fine L 1986]. However this may not be the only factor involved.

Ammonium chloride feeding results in renal hypertrophy which may be due to metabolic acidosis [Lotspeich W 1967], although it has recently been shown that addition of ammonium chloride to rabbit proximal tubular cell cultures causes hypertrophy in the absence of acid medium [Golchini K 1989]. This correlation between cell growth and progression has led to the hypothesis that cell growth is a maladaptive response and under this hypothesis any change in the tubular environment which alters cell growth may contribute to progression of renal failure.

1.6. The importance of interstitial disease in chronic renal failure

It has been known for many years that tubulo-interstitial pathology occurring in chronic renal disease is correlated with renal function. In 1968 Risdon and colleagues investigated 50 patients with persistent glomerulonephritis and showed that the degree of
tubular atrophy was a better predictor of renal function, as determined by plasma creatinine measurement, than were measures of histological changes in glomeruli [Risdon R 1968]. Schairnuck L [1970] examined 70 renal biopsy specimens from a total of 59 patients with a variety of renal diseases. They used semi-quantitative scores of interstitial and glomerular pathology and measured renal function by inulin clearance. There was an inverse correlation between interstitial pathology and renal function whilst the correlation between glomerular disease and renal function was poor.

More recently the prognostic importance of interstitial pathology has been confirmed in a number of renal diseases including membranoproliferative glomerulonephritis type I [Bohle A 1987], membranous glomerulonephritis [Wehrmann M 1989], diabetic nephropathy [Bader R 1980], focal sclerosing glomerulonephritis [Wehrmann M 1990] and Immunoglobulin A nephropathy [Abe S 1989]. Howie A [1990], using an immunohistochemical stain for proximal tubular brush border has shown that loss of brush border is correlated with loss of renal function, measured by the reciprocal of serum creatinine, indicating that changes in tubular cell function may be of importance in this process.

Unlike glomerular pathology it is not immediately obvious why interstitial pathology should result in a decrease in renal function. However since it is known that renal failure can occur in primary diseases of the interstitium [Neilson E 1989], in which the glomeruli are largely intact, there must be a link between changes in the interstitium and glomerular filtration rate (GFR). A number of mechanisms have been proposed. It is possible that damage to the tubules simply obstructs them resulting in increased intratubular pressure which could arrest glomerular filtration [Yee J 1991]. Alternatively a number of vascular effects have been proposed. Expansion of the interstitium may result in increased post glomerular
vascular resistance with the effect of decreasing renal plasma flow and hence GFR [Bohle A 1981, 1991]. Also during the inflammatory and scarring processes the release of vasoactive substances from infiltrating and resident cells may have an effect on post glomerular vascular tone [Nath K 1992]. Such effects on post glomerular vasculature could contribute to glomerular hypertension which has been considered a determinant of progressive renal damage [Brenner B 1983]. An alternative mechanism could be that tubular damage results in impaired reabsorption of sodium in the proximal tubule leading to increased delivery of sodium to the distal tubule. This would decrease GFR through tubulo-glomerular feedback [Bohle A 1987, Wehrmann M 1989]. Thus it is clear that interstitial disease could, by a variety of mechanisms, influence GFR and hence progression of renal failure.

1.7. Evidence for a role of the proximal tubular cell in interstitial scarring

The fact that interstitial inflammation develops in renal disease implies injury to a component of the tubulo-interstitium. As the predominant cell within this part of the kidney it is reasonable to propose that the inflammatory response is orchestrated by the tubular cells. An increasing body of evidence has demonstrated that proximal tubular cells have the necessary armoury to take part in an inflammatory and scarring response. This is not surprising given their origin as mesenchymal cells [Kuncio G 1991].

One of the major features of interstitial inflammation in chronic renal disease is the influx of macrophages (section 1.4). The great majority of the macrophages originate from circulating monocytes rather than from the proliferation of resident macrophages. Thus in puromycin aminonucleoside (PAN) nephrosis, X-irradiation of the kidney, prior to injection of PAN, destroys the resident macrophages but does not decrease the ultimate interstitial macrophage number [Eddy A 1991]. It is known that proximal tubular cells can secrete
agents that attract inflammatory cells (chemoattractants). During protein overload proteinuria, rats excrete into the urine a chemoattractant for monocytes which has been characterised as a novel lipid and it has been shown that rat tubules, *in vitro*, produce this chemoattractant if exposed to bovine serum albumin carrying fatty acids [Kees-Folts D 1994]. The production of this lipid chemoattractant may be the result of the metabolism of the albumin and the consequent release of the fatty acids bound to it [Schreiner G 1995].

Proximal tubular cells also secrete peptides of the chemokine family which are chemoattractant for monocytes. Monocyte chemoattractant protein-1 is produced by human cortical epithelial cells in culture when stimulated with interferon-γ [Schmouder R 1993]. RANTES, a monocyte and lymphocyte chemoattractant, is produced by murine proximal tubular cells in culture and the production is increased by IL-1α and TNFα [Heeger P 1992].

Having attracted inflammatory cells the tubules may interact with them in two ways, firstly by contact with molecules on their surface and secondly by secretion of products into their environment. Proximal tubular cells have been shown to express the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) in glomerulonephritis [Lhotta K 1991, Chow J 1992], human renal allograft rejection [Brockmeyer C 1993] and in a murine model of lupus nephritis [Wuthrich R 1990a]. VCAM-1 is also expressed on proximal tubular cells in human allograft rejection [Brockmeyer C 1993]. MHC class II expression on proximal tubular cells has been described in murine lupus nephritis [Wuthrich R 1989] and human renal allograft rejection [Bishop G 1989].

In cell culture ICAM-1 and MHC class II can be induced on SV40 transformed mouse proximal tubule cells by interferon whilst ICAM-1 alone is induced by TNFα [Jevnikar A 1990]. Evidence for the expression of MHC class II stimulated investigation of the ability of proximal tubule cells to present antigen to lymphocytes. Mouse proximal tubule cells in
culture can present antigen to T cell hybridomas [Hagerty D 1992] and produce a proliferative response. However if the coculture is with a T cell clone rather than a hybridoma then the T cells are induced to undergo anergy which is a state of unresponsiveness to antigen [Singer G 1993]. This suggests that tubular cells do not possess the necessary accessory signals of the 'professional' antigen presenting cell. The role of proximal tubule antigen presentation as a stimulus to inflammation is therefore open to question and the induction of anergy could in fact be a mechanism of inducing tolerance and down regulating inflammation.

In addition to attraction of inflammatory cells which may subsequently initiate a fibrotic reaction it has also been shown that tubular cells themselves are capable of releasing inflammatory cytokines. Frank J [1993] showed tubular cells grown from human kidney biopsies express mRNA for interleukin-6, granulocyte macrophage colony stimulating factor (GMCSF) and platelet derived growth factor-B (PDGF-B). They found higher amounts of mRNA for GMCSF and PDGF-B in tubular cells derived from diseased kidneys than in those derived from normal controls and also in tubular cells derived from fibrotic kidneys in comparison to nonfibrotic kidneys. These authors demonstrated that supernatants from cultured tubular cells stimulated fibroblasts to produce increased amounts of the matrix protein fibronectin. Again they showed a greater effect using supernatants derived from the tubular cells from diseased kidneys than supernatants from the tubular cells of normal kidneys [Frank J 1992].

Transformed mouse kidney proximal tubular cells and human proximal tubular cells in culture have been shown to produce tumour necrosis factor-α when stimulated by interleukin-1α [Wuthrich R 1990b, Jevnikar A 1991, Yard B 1992]. Complement is also manufactured by tubular cells. Northern blots from normal human kidney have shown that the tubulo-interstitium expresses mRNA for the fourth component of complement (C4)
C4 is manufactured by human proximal tubular cells in culture when exposed to interferon-γ [Seelen M 1993]. The third component of complement is manufactured by human proximal tubular cells in culture on exposure to interleukin-2 [Brooimans R 1991].

Recent work has shown that the cytokine transforming growth factor-beta (TGFβ) is of pivotal importance in responses resulting in scarring [Border W 1992]. TGFβ mRNA is expressed in murine proximal tubular cell cultures. The gene and TGFβ bioactivity are upregulated by high glucose concentrations [Rocco M 1992].

It is therefore clear that following a pathological stimulus to tubular cells they can recruit inflammatory cells, interact with them through cytokines and adhesion molecules, stimulate fibroblasts to produce matrix proteins and produce matrix proteins themselves (section 1.4) which could result in chronic inflammation and scarring within the interstitium.

1.8. Possible causes of interstitial pathology in glomerular disease.

i) During glomerular disease it is reasonable to propose that the inflammatory and scarring processes occurring in the glomerulus would result in the release of various cytokines into the urinary space. They could then come into contact with the luminal surface of the tubular cells and could stimulate them to initiate the scarring process in the interstitium [Yee J 1991].

ii) Inflammatory mediators such as cytokines and chemokines may diffuse down the mesangial stalk and gain access to the interstitium where they can attract inflammatory cells. This would provide an explanation for why, in the rat model of anti glomerular basement membrane glomerulonephritis, there is an early perihilar
infiltrate of cells and an impressive periglomerular interstitial infiltrate. It has been suggested that cytokines may diffuse through Bowman's capsule from the inflamed glomerulus. Generalised interstitial inflammation may then occur by spread from the periglomerular area [Lan H 1991].

iii) Oxygen tension within the interstitium of the kidney is known to be less than systemic arterial oxygen tension and hence the highly metabolically active tubular cells are vulnerable to minor changes in oxygen delivery. Fine L [1993] has proposed that glomerular hypertension, which occurs in many models of progressive renal disease, may result in an increased blood pressure being transmitted to the post-glomerular capillaries. The subsequent damage to the capillaries would result in interstitial ischaemia causing inflammation and scarring. Once scarring has been initiated the increase in interstitial volume would increase the distance between tubular cells and peritubular capillaries, further increasing the ischaemic injury. Support for this hypothesis comes from the study of human kidney biopsy specimens in which a loss of post-glomerular capillaries was found associated with interstitial scarring in patients with a variety of glomerular diseases [Böhle A 1981].

iv) Proteinuria, which is an inevitable consequence of glomerular disease, may effect tubular function as described below.
1.9 The role of proteinuria

1.9.1 The Reabsorption of proteins by the proximal tubule

Experimental work in the 1930's identified the proximal convoluted tubule as the site of uptake of proteins filtered by the glomerulus [Gerard P 1934]. In the 1950's differences in the absorption of different proteins was noted [Oliver J 1954]. Intraperitoneal injection of egg white into rats rapidly resulted in formation of intracellular droplets in the proximal tubule, however much larger concentrations and longer periods of time were required for droplet formation following injection of rat serum proteins. This was thought to be due to differences in the rate of metabolism of the proteins by the cells. Since that time, the use of electron microscopy has identified in detail the organelles involved in reabsorption and metabolism of protein from the tubular fluid. This is the apical endocytic pathway which is comprised of endocytic invaginations, endocytic vesicles and vacuoles, dense apical tubules and lysosomes [Christensen E 1991].

Endocytic invaginations occur between the microvilli of the brush border. Immunoelectron microscopy has demonstrated that these areas of the plasma membrane are specialised in that they have a cytoplasmic coat of the protein clathrin [Lin C 1982, Rodman J 1984]. Clathrin coated pits are common to cells which have highly developed endocytic activity such as the macrophage [Aggeler J 1982] and the oocyte [Roth T 1964]. The endocytic invaginations bud to form small endocytic vesicles or the larger endocytic vacuoles [Christensen E 1991]. The vacuoles then fuse with lysosomes which contain enzymes for the digestion of proteins [Straus W 1954, 1956]. Proteins absorbed and transported to lysosomes are rapidly digested to amino acids [Hjelle J 1984, Maunsbach A 1974, Davidson S 1973]. The dense apical tubules are connected to endocytic vacuoles and form a mechanism by which membrane from vacuoles is recycled to the apical plasma membrane [Maunsbach A
Whilst larger proteins are absorbed by endocytosis some smaller peptides, for example glucagon, may be degraded by brush border hydrolases with subsequent reabsorption of their amino acids [Peterson D 1982].

Investigation of albumin reabsorption in the isolated perfused proximal tubule [Park C 1984] and of dextran and lysozyme reabsorption in renal cortical slices [Christensen E 1979] has shown that protein is largely taken up by an adsorptive process with only a small contribution (2%) from nonspecific fluid phase reabsorption. The initial phase of adsorptive endocytosis is the binding of the proteins either directly to the endocytic invaginations or to areas on the microvilli from where they migrate into the invaginations. Using electron microscopy of baby hamster kidney cells in culture it has been shown that following binding of cationic ferritin to anionic sites on the microvilli the anionic sites redistribute into the invaginations carrying the bound protein with them [Grinnell F 1975].

The charge on proteins is of importance in their initial binding to microvilli. There is competition for uptake between cationic proteins and it has been shown that cationic proteins bind to anionic sites [Cojocel C 1981, Sumpio B 1982]. However despite the negative charge of the plasma membrane anionic proteins such as insulin [Chamberlain M 1967, Rabkin R 1969] and β₂-microglobulin [Peterson P 1969] are efficiently reabsorbed and there is competition for uptake between anionic proteins [Foulkes E 1982, Simonnet H 1988]. Molecular weight and charge are not however the only determinants of efficiency of reabsorption. Insulin and epidermal growth factor are of very similar molecular weight and charge but whilst insulin is almost totally removed from the ultrafiltrate, only 5% of epidermal growth factor is reabsorbed [Nielsen S 1989]. A specific receptor for insulin may be responsible for the avid uptake of this peptide [Rabkin R 1982, Meezan E 1988].

In addition to the adsorptive endocytosis process, experiments on the isolated perfused
proximal tubule have shown that lysozyme [Nielsen J 1986] and insulin [Nielsen S 1988] may be able to cross the tubular cells intact, a process known as transcytosis. This however appears to be a minor route of reabsorption constituting no more than 5% of the total.

Most of the work on proximal tubular handling of proteins has been performed in vitro, using the isolated perfused kidney or the isolated perfused proximal tubule. There is less information on the protein processing of proximal tubular cells in culture. Differences in ultrastructure have been demonstrated between cultured cells and in vivo proximal tubular cells [Christensen E 1991]. It is of particular significance that the apical surface of cultured cells often does not show the typical densely packed brush border of proximal tubular cells, and only express rudimentary projections [Chung S 1982, Chuman L 1982, Wilson P 1985]. However primary proximal tubular cell cultures have been shown to express apical invaginations, vesicles, vacuoles and lysosomes hence possessing the internal protein handling apparatus [Norgaard J 1989].

In the immortalised opossum proximal tubular cell line (OK) it has been shown that uptake of albumin occurs via specific receptor mediated endocytosis as well as by non specific fluid phase uptake. The specific mechanism exceeds the non specific by greater than ten fold [Schwegler J 1991]. However differences in protein handling between cultured cells and isolated perfused tubules have been shown. In OK cells basolateral endocytosis of insulin was five times the amount of apical endocytosis [Rabkin R 1989] whilst in the isolated perfused proximal tubule basolateral uptake of insulin comprised only 15% of cell uptake under physiological concentrations of protein and an even lower proportion at higher concentrations [Nielsen S 1987]. Unlike isolated perfused proximal tubules transcytosis is extensive in cultured rabbit proximal tubular cells [Goligorsky M 1986].

Thus cell culture is a useful method for the study of effects of proteins on tubular
cells, but particularly in quantitative terms care must be taken in comparing results from cell cultures to those obtained in vivo.

1.9.2 Correlations between proteinuria and progression in human renal disease

A causal role for proteinuria in the development of progressive renal failure is suggested by the strong correlation of the rate of progression of chronic renal failure with the quantity of proteinuria in a number of renal diseases. In 40 consecutive patients with a biopsy diagnosis of focal segmental glomerulosclerosis followed from 6 to 16 years, patients presenting with nephrotic syndrome had a worse prognosis than those presenting with less proteinuria [Cameron J 1978]. Similar observations have been made in mesangiocapillary glomerulonephritis type I, where the presence and persistence of nephrotic syndrome predicted renal failure [Cameron J 1983] and in patients with idiopathic membranous nephropathy, where 41% of patients presenting with nephrotic syndrome (followed for a mean of 54.8 months) developed progressive renal failure, compared to none of those presenting with less than nephrotic range proteinuria [Erwin D 1973]. In addition a study of nearly 300 patients with IgA nephropathy demonstrated that proteinuria of more than 1 g/day was an independent variable associated with poor prognosis [D'Amico G 1986]. The development of proteinuria correlates with the development of chronic rejection in transplant kidneys [Massy Z 1996]. The predictive value of the severity of proteinuria on the rate of progression of renal failure has recently been confirmed in a large prospective study of progressive renal failure (the MDRD study) [Klahr S 1994]. Thus the rate of progression of renal failure in a variety of very different primary renal pathologies is predicted by the severity of proteinuria suggesting a potential pathogenetic link between proteinuria and the development of renal scarring.

The significance of proteinuria has recently been supported by the observation that a
reduction in proteinuria accomplished by the use of an ACE inhibitor is associated with a slowing of the rate of progression of renal failure. In this study the beneficial effect of ACE inhibition on progression was only observed in those patients in whom the drug brought about a reduction in the level of proteinuria [Praga M 1994].

1.9.3 Correlations between proteinuria and progression in animal models

Rats with a remnant kidney develop proteinuria, progressive renal impairment and interstitial pathology [Anderson S 1986]. Feeding the animals low protein diets or diets composed of soya rather than casein significantly reduces the amount of proteinuria and abrogates the development of renal failure [El-Nahas A 1983, Williams A 1987]. Similarly treatment of remnant kidney animals with ACE inhibitors reduces the level of proteinuria and lessens the degree of structural damage in the glomeruli and the tubulo-interstitium [Anderson S 1986]. Animals treated with an alternative antihypertensive regime have proteinuria at the same level as controls and develop histological changes similar to controls. In this model therefore the degree of proteinuria, the degree of structural damage and the degree of renal impairment are all closely correlated.

The evolution of interstitial inflammation has been investigated in two models characterised by proteinuria and interstitial disease, namely PAN nephrosis and protein overload nephropathy. A single dose of PAN results in direct toxicity to glomerular epithelial cells and the development of nephrotic range proteinuria. The proteinuria worsens up to 14 days and then returns to normal. Closely related to the time course for proteinuria there is an influx into the interstitium of chronic inflammatory cells including macrophages and T cells [Eddy A 1991]. There is a clear relationship between the degree of the proteinuria and the severity of the interstitial infiltrate and both are reduced by dietary protein restriction.
As the proteinuria develops there is a significant increase in renal mRNA for the chemokine MCP-1 [Eddy A 1993], although neutralising antibodies to MCP-1 did not attenuate macrophage recruitment. This model is also characterised by foci of tubulo-interstitial fibrosis and mRNA levels for genes encoding extracellular matrix proteins, tissue inhibitors of metalloproteinases and transforming growth factor β1 are all increased in association with the development of proteinuria and the interstitial infiltrate. The institution of dietary protein restriction to reduce proteinuria reverses these effects [Eddy A 1994].

Proteinuria can also be induced in rats by intraperitoneal injection of large amounts (5g/day) of bovine serum albumin (protein overload nephropathy). The animals develop heavy proteinuria with no evidence of immune complex deposition in the glomeruli or the interstitium, nor is there evidence of circulating anti-BSA antibodies. Ultrastructural examination of the glomeruli in this model indicates widespread foot process effacement although the cause of this is unknown [Andrews P 1977]. As the proteinuria increases there is an influx of chronic inflammatory cells into the interstitium and an accumulation of extracellular matrix proteins [Eddy A 1989, 1995]. There is a significant increase in renal mRNA for the chemotactic substances MCP-1 and osteopontin [Eddy A 1995]. In the absence of any other explanation it has been proposed that in this model the proteinuria is the injurious agent.

1.9.4 Mesangial toxicity of proteinuria

Whilst most of the evidence for a role of proteinuria in the progression of renal failure relates to effects on the tubulo-interstitium there is some evidence of toxicity of proteinuria to the mesangium of the glomerulus. Accumulation of serum proteins in the glomerular mesangium has been observed in the remnant kidney model [Purkerson M 1976] and PAN
nephrosis [Glasser R 1977]. The consequences of mesangial exposure to lipoproteins have been of particular interest. The apolipoprotein B of LDL and VLDL and apolipoprotein (a) of lipoprotein (a) have been found in glomeruli in proteinuric states [Lee H 1991, Sato H 1993]. *In vitro* LDL interacts with its receptor on human mesangial cells causing cell proliferation [Wheeler D 1990]. In addition LDL promotes the production of the extracellular matrix protein fibronectin by mesangial cells as well as inducing the production of MCP-1 [Rovin B 1993], and PDGF [Grøne E 1992]. Thus LDL promotes a series of cellular events in mesangial cells which may propagate glomerulosclerosis, including the recruitment of macrophages which play a pivotal role in this process [Van Goor 1994]. Once within the mesangium LDL may undergo oxidation either by macrophages or mesangial cells themselves to form oxidised LDL. LDL modified in this way is known to be more cytotoxic to mesangial cells than LDL itself [Wheeler D 1994] and may therefore further promote glomerular damage.

1.9.5 Effects of proteinuria on proximal tubular cell functions

1.9.5.1 Evidence of tubular toxicity *in vivo*

Evidence of *in vivo* tubular toxicity of proteinuria comes from investigation of urinary excretion of two markers of tubular injury. Tubular absorption of lysozyme, which is of low molecular weight and freely filtered by the glomerulus, is a high capacity, low affinity process. The appearance of lysozyme in the final urine is therefore a good indicator of the failure of tubular reabsorption [Sumpio B 1982]. The urinary excretion of lysozyme has been shown to be closely correlated to the degree of albumin excretion in passive Heymann nephritis [Agarwal A 1993]. In human disease treatments which lower albuminuria such as low protein diets or angiotensin converting enzyme inhibition also lower lysozymuria. One
potential criticism of the use of lysozyme as a marker of tubular damage is that in the presence of proteinuria there may be competition for protein reabsorption resulting in increased lysozymuria without tubular damage. N-Acetyl-β-glucosaminidase (NAG) is produced by tubular cells and is released into the urine when they are injured. NAG is a large protein (>125kD) and is not present in the serum in sufficient concentration to suggest its presence in the urine could be due to glomerular filtration. NAG is therefore a useful marker of tubular damage [Guder W 1992]. Excretion of NAG has been shown to correlate with the degree of proteinuria in human glomerular disease [Kunin C 1978, Kind P 1982].

Thus there is evidence for tubular toxicity in the presence of proteinuria. Adverse effects of proteinuria may be the result of the sheer quantity of protein to be reabsorbed or the result of toxicity of particular proteins.

1.9.5.2 Proposed effects due to quantity of protein

i) Proximal tubular lysosomal rupture

It is known that the increased trafficking of protein across the proximal tubular cells as a consequence of proteinuria results in increased lysosomal enzyme activity [Olbright C 1986]. It has been suggested that excessive proteinuria results in leakage of lysosomal enzymes into the cytoplasm of the tubular cell which could result in cell injury [Maack T 1971, Park C 1984].

ii) Ammoniagenesis

Rustom R [1992] has shown that in patients with glomerulonephritis the level of proteinuria is correlated with the level of urinary ammonia. They speculate that this is due to catabolism of the increased amount of reabsorbed proteins. Sodium bicarbonate
administered to rats with a remnant kidney decreases ammoniagenesis and it was found that the rats had a decrease in interstitial damage [Clark E 1991]. It is known that ammonia can activate complement and in these experiments those rats with the sodium bicarbonate supplemented diet had less peritubular deposition of C3 and C5b-9. Once activated complement has a number of proinflammatory effects including chemoattraction by C5a and cell lysis by C5b-9 [Koski C 1983]. In addition C5b-9 in sublytic concentrations has been shown to release cytokines (IL-1 and TNFα) from mesangial cells and could have a similar effect on other cell types [Hänsch G 1992]. C5b-9 also stimulates collagen synthesis in glomerular epithelial cells [Torbohm I 1990].

iii) Exacerbation of hypoxia

Proteinuria may have its effect by exacerbating other pathological processes within the tubulo-interstitium. The normal tubulo-interstitium is relatively hypoxic in comparison to arterial oxygen concentration [Brezis M 1984]. As discussed in 1.7 (iii) tubulointerstitial ischaemia may be exacerbated in glomerular disease. It is possible that in tubular cells that are already stressed by hypoxia the additional energy required to reabsorb and digest large amounts of protein could result in damage to those cells. It has been shown that in experimental ischaemic injury the injection of low molecular weight proteins exacerbates the development of acute tubular necrosis. Renal ischaemia in rats can be produced by clamping of the renal artery for a fixed time or by induction of haemorrhagic shock. In both these models injection of myoglobin, ribonuclease or lysozyme have been shown to worsen the development of acute tubular necrosis [Zager R 1987]. In the haemorrhagic shock model there is a greater depletion of adenine nucleotides in the presence of the low molecular weight proteins which could be the result of the extra work required in reabsorption [Zager R 1991].
There is no reason to suppose that the reabsorption of high molecular weight proteins requires less energy than the reabsorption of low molecular weight proteins.

1.9.5.3 Effects of specific proteins

(i) Albumin

As described in 1.7, rat proximal tubules in vitro produce a powerful lipid chemoattractant for monocytes/macrophages if exposed to bovine serum albumin which carries fatty acids [Kees-Folts D 1994]. Lipid free bovine serum albumin does not have this effect.

BSA carrying fatty acids also has an effect on the growth of the proximal tubular cell line OK cells. Albumin conjugated with oleate increases cell proliferation whilst albumin palmitate inhibits proliferation [Thomas M 1993]. Rat proximal tubular cells in culture if exposed to bovine serum albumin produce the peptide chemoattractant MCP-1 [Harris D 1995]. This however is not altered by delipidation of the BSA.

Two lines of evidence argue against the hypothesis that albumin per se is crucial to the process of interstitial disease and progression. They are renal disease in the anaalbuminaemic rat and minimal change nephrotic syndrome in humans.

The anaalbuminaemic rat has a genetic abnormality which results in failure to manufacture albumin. The normal functions of albumin are performed by alternative proteins such as transferrin. When these animals are given renal disease such as PAN nephrosis [Okuda S 1992] or a remnant kidney [Fujihara C 1991] the levels of proteinuria are much lower than in the equivalent renal disease in normal animals due to the absence of albumin but the animals nevertheless go on to progressive renal failure with the same histological changes as control animals. Although the quantity of proteinuria is less than the controls the animals still have high molecular weight proteinuria which would suggest that the absolute
quantity of proteinuria is not as significant as the types of protein that it contains.

**Minimal Change Nephrotic Syndrome** is characterized by very large amounts of protein, predominantly albumin, leaking through the glomerulus and appearing in the urine. Although there are reports of interstitial infiltrates in minimal change disease [Grčevska L 1992], renal scarring and chronic renal failure are by definition absent. This discrepancy may be explained by the limited duration of the proteinuria in minimal change disease which is usually readily sensitive to steroids, in contrast to progressive renal diseases in which the proteinuria is prolonged. The highly selective nature of the proteinuria in minimal change disease may also be of significance since the urine contains few proteins of higher molecular weight than albumin [Glassock R 1986]. Increased NAG excretion has been shown in minimal change nephrotic syndrome [Kunin C 1978] indicating a degree of tubular damage does occur. The NAG excretion returns to normal when proteinuria decreases in remission. Therefore in minimal change disease the tubular injury is either of insufficient degree to result in long term changes or it is of insufficient duration. An alternative explanation to this paradox may be provided by the observation that the effect of albumin on tubular cell function *in vitro* is dependent on the fatty acid composition of the albumin (as described above). Significantly, urinary albumin in minimal change disease in children has a markedly lower fatty acid content than in patients nephrotic due to other glomerular diseases [Ghiggeri G 1987] and could therefore have a lower potential for initiating injury.

ii) **Lipoproteins**

Abnormalities of lipid metabolism as a result of proteinuria may play a role in the progression of renal disease [Moorhead J 1982, Keane W 1988]. It is known that significant quantities of both high density lipoprotein (HDL) and low density lipoprotein (LDL) appear
in nephrotic urine [Shore V 1982]. In addition albumin which comprises the majority of the filtered load also contains high affinity fatty acid binding sites and is an important lipoprotein [Spector A 1986]. It is clear that tubular cells interact with lipoproteins since lipid laden tubular cells have been found in nephrotic urine [Olson J 1992] and lipid droplets in tubular cells have been demonstrated in renal biopsies from nephrotic patients [Neverov N 1991] as have apoA and apoB [Kashyap M 1979]. A cell culture study demonstrated uptake of HDL and LDL by human proximal tubular cells [Ong A 1994a]. Oxidized LDL or minimally modified LDL both caused cell injury and detachment of cells from the culture plate. Tubular cells themselves may possess LDL oxidising ability and it is postulated that transferrinuria accompanying lipoproteinuria could provide a source of iron to catalyze the oxidative process [Ong A 1994a]. Human tubular cells exposed to HDL have been shown to increase production of endothelin-1 [Ong A 1994b]. Endothelin-1 has effects on the microcirculation [Ko V 1989], fibroblasts [Ong A 1993] and is a monocyte chemoattractant [Achmad T 1992]. Through these effects HDL could influence tubulointerstitial inflammation and scarring.

### Transferrin

Transferrin with a molecular weight only slightly greater than albumin is also filtered in glomerular proteinuria. Urinary transferrin has been proposed as a mediator of tubular toxicity. As fluid passes down the tubule it becomes increasingly acidic. Under these conditions transferrin will release the iron that it carries [Zager R 1993]. Free Fe$^{3+}$ ions are known to be cytotoxic and could injure tubular cells. A role for iron in progressive renal injury is suggested by the demonstration that in rats with nephrotoxic serum nephritis there is a correlation between the quantity of iron excreted in the urine and the degree of tubulo-

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interstitial injury [Alfrey A 1989]. A marked increase in the urinary excretion of iron has been noted in patients with diabetic nephropathy and may be of pathogenetic importance in the progressive renal injury seen in this disease [Howard R 1991]. There is however also some evidence that urinary iron is protective in renal ischaemia [Zager R 1993] and so the effects of iron in vivo are complex.

It has been demonstrated that exposure of tubular cells in culture to transferrin complexed with iron but not transferrin or albumin increases lactate dehydrogenase release and cytosolic levels of the lipid peroxide malondialdehyde [Harris D 1994]. This suggests that reabsorption of transferrin-iron results in release of reactive iron in the proximal tubular cell causing peroxidative injury. It has also been shown that exposure of proximal tubular cells to transferrin results in the upregulation of mRNA to MCP-1 [Harris D 1995].

iv) Complement

As discussed above (section 1.8.5.2ii) the activation of complement by ammonia is a mechanism by which proteinuria could influence interstitial inflammation and scarring. In addition increased glomerular permeability may allow circulating complement components to filter into the tubular fluid. The C5b-9 membrane attack complex has been found in the urine in membranous nephropathy [Schulze M 1991], diabetic nephropathy [Ogradowski J 1991], and focal segmental glomerular sclerosis [Ogradowski J 1991]. In membranous nephropathy this is thought to represent leakage of C5b-9 from active glomerular disease [Schulze M 1991]. In the other conditions however no glomerular deposition of the complex has been detected. It has been shown that the proximal tubular brush border can activate complement via the alternative pathway [Camussi G 1983] and hence urinary C5b-9 may represent activation of filtered complement components by the tubular brush border. Whatever the
mechanism by which this complex arrives in the tubular fluid its presence is a potential cause of cell damage.

v) **Bence Jones Protein**

In myeloma excess production of low molecular weight immunoglobulin light chains which are freely filtered by the glomerulus results in the appearance of protein in the urine in the presence of an otherwise normal kidney. The presence of Bence Jones proteinuria is associated with the development of renal failure [DeFronzo R 1978] although the relationship is not invariable. It is not yet known why some patients with Bence Jones proteinuria develop renal disease whilst others do not [Ledingham J 1990]. Theories as to the cause of renal failure in myeloma have included simple obstruction of the tubules by protein casts although it was noted as long ago as 1968 that the degree of renal failure correlates more closely with tubular atrophy than with presence of casts [Levi D 1968]. Several alterations in tubular function on exposure to light chains have been investigated. *In vivo* 'tubular type' proteinuria occurs in the presence of urinary light chains, indicating proximal tubular dysfunction [Cooper E 1984]. Certain transport functions of rat renal cortex have been shown to be altered by the presence of light chains extracted from the urine. Transport of ammonium and glucose is inhibited [Preuss H 1974] as is the sodium potassium ATPase of rat cortical tubules [McGeogh J 1978]. Sodium dependent alanine and glucose uptake of brush border membrane vesicles from proximal tubular cells have also been inhibited by urinary light chains [Batuman V 1986]. Thus in the presence of a normal kidney some types of Bence Jones Proteinuria have significant effects on tubular cell functions and are known to cause renal failure.
1.10. Conclusion

Chronic progressive renal failure is accompanied by the development of chronic interstitial inflammation and scarring. The cause of these interstitial changes is unknown but it is reasonable to propose that they are due to an insult to the tubulo-interstitium secondary to glomerular disease. One such insult is the development of proteinuria the degree of which correlates with the rate of decline of renal function. Proximal tubular cells which are required to reabsorb proteins in the tubular fluid would be particularly vulnerable to any adverse effect of proteinuria. Given the range of proinflammatory molecules which they express, injured proximal tubular cells would be able to initiate inflammation and scarring within the interstitium. A number of potentially pathological effects of proteins on proximal tubular cells are the subject of current investigation and the experiments described in this thesis were designed to further investigate the mechanisms involved.
CHAPTER 2

METHODS
2.1 Cell culture

Cell culture was carried out in a dedicated cell culture laboratory. Sterile techniques were used in the handling of all culture equipment. A class II laminar air flow cabinet was used. The cells were grown in an incubator at 37°C in an humidified atmosphere of 5% CO₂/95% air. All culture media were purchased from Gibco/Life Technologies. The techniques used for the culture of particular cell types are described in the relevant chapters.

2.2 Protein measurements

Protein concentration was measured in cell samples dissolved in 0.5M sodium hydroxide or 1% nonidet P40 and samples of rat urine and human serum. Lowry assay was used for samples in 0.5M NaOH (section 2.2.1) [Lowry O 1951]. BioRad DC assay, which uses a modified Lowry technique, was used for samples in 1% nonidet and serum (section 2.2.3) and a standard BioRad assay, based on the Bradford dye binding method, was used for samples in urine [Bradford M 1976].

2.2.1 Lowry assay

On the day of the assay 50 parts of Lowry solution A (Appendix 1) were mixed with 1 part of Lowry solution B (Appendix 1), this mixture was called Lowry solution C. Just before use 1 volume of Folin-Ciocalteau reagent (BDH 19058 2P) was diluted with 2 volumes of deionized water (FCR). Standards were prepared using bovine serum albumin (BSA Sigma A7638) dissolved in 0.5M sodium hydroxide in the concentration range 0-500μg/ml. 50μl of appropriately diluted samples or of standards was placed into test tubes. Each sample and standard was measured in duplicate. 12μl of deionized water was added to each tube. At time t=0 600μl of lowry solution C was added to each test tube using a BCL 8000 automatic
pipette. The solutions were mixed using a vortex. At time $t=10$ minutes 60µl of FCR was added and each tube vortexed again. 200µl of each reaction mixture was transferred to one well of a 96 well microtitre plate. At time $t=40$ minutes the optical density was measured using a spectrophotometer (Titertek Multiscan Plus) reading at a wavelength of 660nm.

$OD_{660}$ is directly proportional to protein concentration over this range of standards. A straight line standard curve was constructed and using linear regression the concentration of protein in the samples was calculated from their $OD_{660}$.

### 2.2.2 BioRad protein assay (500-0006)

This assay was purchased from Bio-Rad UK. The assay is based on the colour change of Coomassie brilliant blue G-250 dye in response to different concentrations of protein. Unpublished work by Dr KPG Harris showed that this was the superior technique for measurement of protein in urine samples. BSA standards were diluted in phosphate buffered saline (PBS) (Appendix 1) and used in the concentration range 200-1400µg/ml. 50µl of samples or standards was pipetted into test tubes in duplicate. 2.5ml of dye reagent was added to each test tube and vortexed. 200µl was taken from each tube and transferred to a 96 well microtitre plate. After 5 minutes the optical density was measured at 595 nm. $OD_{595}$ has a linear relationship with protein concentration over this range allowing construction of a straight line standard curve. Sample concentrations were calculated from their $OD_{595}$ using the equation of the line.

### 2.2.3 BioRad DC assay (500-0116)

This assay had the advantage of being compatible with detergents allowing its use in samples dissolved in 1% nonidet P40. Because of its simplicity it was also used for
determination of the protein concentration in serum samples. The method is based on the Lowry reaction [Lowry O 1951]. BSA standards were used in the range of 0.1-2.0 mg/ml. 5µl of samples or standards was pipetted into the wells of a 96 well microtitre plate in duplicate. Sodium dodecasulphate solution and copper tartrate solution, supplied with the assay, were mixed 1:20 and 25µl of this was then added to each well. This was followed by 200µl of a dilute Folin reagent. After incubation for 15 minutes at room temperature the optical density at 710nm was determined. There is a straight line relationship between OD_{710} and protein concentration allowing determination of the protein concentration in the samples.

2.3 Measurement of cellular DNA.

This colourimetric assay of DNA was based on the method of Burton K [1956]. On the day of the assay acetaldehyde solution was prepared by dissolving 25µl of acetaldehyde in 25ml of deionised water to a concentration of 1.6mg/ml. Since acetaldehyde boils at room temperature the bottle and the pipette tip were chilled on ice prior to use. In a fume cupboard, protected from light, 4.00g of diphenylamine (Sigma D2385) was dissolved in 100ml of glacial acetic acid to make the 'diphenylamine reagent'.

The DNA standards were prepared by dissolving 20mg of calf thymus DNA (Sigma D3664) in 50.0ml of 0.005M sodium hydroxide. An equal volume of 20% (w/v) perchloric acid was added which precipitated the DNA. The tubes were then incubated at 70°C for 20 minutes in a water bath to redissolve the DNA which was then present at a concentration of 200µg/ml. Standards in the range of 0-200µg/ml in 10% perchloric acid were then made.

DNA assays were performed on confluent cells in 6 well plates. The cells were washed four times with ice cold 0.9% (w/v) sodium chloride (normal saline) and then stored at -20°C. 600µl of 10% (w/v) perchloric acid was added to each well and the cells scraped
off the plate with the plunger from a 1ml syringe. The suspension of precipitated protein and DNA was transferred to a centrifuge tube. The wells were then washed twice with 300μl of ice cold 10% (w/v) perchloric acid. The tubes were centrifuged at 4000rpm, 0°C for 10 minutes and the supernatant which contained the low molecular weight deoxyribose compounds discarded. The pellet was resuspended in 1200μl of 10% perchloric acid and the DNA hydrolysed by heating at 70°C for 20 minutes. The tubes were chilled on ice to precipitate the protein and then centrifuged at 4000rpm, 0°C for 10 minutes. The supernatant containing the DNA was transferred to another tube. The pellet was dissolved in 1.0ml of 0.5M sodium hydroxide and the protein assayed as described in 2.2.1.

In a darkened fume cupboard 250μl of diphenylamine reagent was added to 250μl of supernatant or standard and mixed by vortex. 50μl of acetaldehyde solution was added to each tube and mixed again. The tubes were incubated in a water bath at 30°C, protected from the light for 16-20 hours. 200μl from each tube was transferred to wells in a 96 well microtitre plate. Absorbance was measured at 710nm and 595nm on the plate spectrophotometer. OD$_{595}$ was subtracted from OD$_{710}$ to eliminate effects due to turbidity of the samples. The result was plotted against the standard concentrations giving a straight line from which the concentrations of the DNA samples could be calculated.

2.4 $^{3}$H-Thymidine incorporation

This measurement of DNA synthesis was used on both OK cells (chapter 3) and human tubuloepithelial cells (HTEC) (chapter 4). The method was adapted from Golchini K [1989] and Greenberg D [1977].
2.4.1 OK cells

OK cells were grown on 6 well plates and prior to measurement of thymidine incorporation the cells were washed three times with Hanks Balanced Salts solution (HBSS) (Life Technologies 24010). $^3$H-thymidine (Amersham TRK 61) was added to Medium 199 (Life Technologies 41150) to produce an activity of 2μCi/ml. 500μl of this incubation medium was added to each well. The cultures were returned to the incubator for 2 hours at 37°C. After 2 hours the incubation medium was removed from the cells and they were washed three times with normal saline. 500μl of Medium 199 containing 0.1mM unlabelled thymidine (Sigma T5018) was then added to each well to compete for non-specific binding. The plates were returned to the incubator for 20 minutes after which the medium was removed and the cells washed with ice cold normal saline. They were then washed five times with 10% trichloroacetic acid and then once more with normal saline and stored at -20°C.

On the day of the assay the plates were thawed and then frozen and thawed again to disrupt the cells. 500μl of 0.5M sodium hydroxide was added to each well and the plate incubated at 60-70°C for 20 minutes. Protein was measured in 10μl of the sodium hydroxide digest (section 2.2.1). The remainder of the digest was put into scintillation vials containing Ecoscint A scintillant (National Diagnostics LS 273). 50μl of concentrated hydrochloric acid (SG 1.18) was added to neutralise the sodium hydroxide and the vials vortexed until clear. Background counts were determined in vials containing Ecoscint A only. Scintillation was counted on an LKB 1219 liquid scintillation counter. Background disintegrations per minute (dpm) were subtracted from the dpm of the samples.
2.4.2 HTEC

HTEC grown on 4.7 cm² well inserts (as described in chapter 4) were exposed to 2 μCi/ml of ³H-thymidine in Dulbecco Modified Eagles medium (DMEM Life Technologies 11880) on the apical and basolateral sides of the membrane. After 2 hours incubation at 37°C the cells were washed with normal saline and exposed to 0.1 mM unlabelled thymidine in DMEM for 20 minutes. The cells were washed four times on both sides with normal saline. 300 μl of Washing buffer (WB, Appendix 1) + 1% nonidet P40 (WBN) was added to the apical side of the cells and the plates incubated at +4°C for four hours. The cells were scraped from the surface of the membrane using the plunger of a 1.0 ml syringe and transferred to a 2.0 ml test tube. The membrane was rinsed with a further 200 μl of WBN. The cell suspension was sonicated until the cells were completely dissolved. 250 μl samples of cell solution were added to 4 ml Ecoscint A in scintillation vials, 10 μl of concentrated hydrochloric acid (SG 1.18) was added to increase solubility in the Ecoscint. ³H was counted as described above (section 2.4.1).

2.5 Measurement of the pH of cell culture medium

Cell culture plates were removed from the culture incubator having ensured that the door had not been opened for at least 2 hours to prevent disturbance of the 5% CO₂ atmosphere. The medium was rapidly drawn off the cells using 1 ml syringes which were then plunged into iced water. The pH of the medium was determined on a blood gas analyzer (Ciba-Coming 238).
2.6 Measures of viability of cultured cells.

2.6.1 Trypan blue exclusion

Trypan blue is a dye which is excluded from living cells but is absorbed by non viable cells which then appear blue when viewed by a microscope [Kruse P 1973]. Trypan blue exclusion was performed by different methods for OK cells and HTEC.

i) OK cells

Confluent OK cells were exposed to experimental conditions on 12 well plates. The medium was aspirated from the cells and they were rinsed once with normal saline. Trypan blue (Sigma T6146) (0.2% w/v) solution in HBSS was added to the wells to completely cover the cells. After 60 seconds the trypan blue was aspirated and the cells immediately inspected under an inverted microscope. The number of cells taking up trypan blue was counted in ten high power (x 20 magnification) fields. This method allowed a comparison of viability under the different conditions and by making assumptions as to the total number of cells on a plate a percentage viability could be estimated.

ii) HTEC

HTEC on permeable membrane supports were grown as described in chapter 4. Since cells on the supports were not visible under the inverted microscope the technique described above for OK cells was not suitable and the following alternative approach was developed. After exposure of the cells to experimental conditions they were rinsed with normal saline. 0.25ml of trypsin/EDTA (Life Technologies 45300-019) was added to each well and the cells incubated at 37°C for 5 minutes. 0.25ml of medium containing 10% fetal calf serum was added to stop the trypsin digestion. The cells were dislodged from the support using a sterile
pastern pipette. 500µl of 0.4% (w/v) trypan blue (Sigma T6146) was added to the cell suspension which was then inspected under the inverted microscope and the numbers of viable and non viable cells counted in order that a percentage viability could be calculated. This method had the advantage of providing an absolute measure of viability but the disadvantage that trypsinisation of the cells prior to addition of trypan blue may itself result in loss of viability.

Both methods of trypan blue exclusion removed cells in the medium as a first step and non viable cells which detached from the plate were therefore not counted which could lead to an overestimate of viability.

2.6.2 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a constitutive enzyme of all cells. There is no secretion pathway for this enzyme and hence its release into culture medium is an indicator of damage to cells resulting in loss of integrity of their cell membranes. The release of LDH into culture medium has therefore been used as a measure of loss of cell viability [Wheeler D 1990]. LDH was measured in culture medium using a commercially available method based on the reduction of pyruvate to lactate (Sigma DG1340-k). Cells were prepared for total cellular LDH measurement by addition of 1% Triton X-100 in PBS to the monolayers. The cells were scraped from the supports and transferred to 2ml centrifuge tubes. They were then sonicated until the cells had completely dissolved. LDH was assayed in the cell solution.

0.4ml of 0.194mM NADH in phosphate buffer pH7.5 was added to 10ml of 16.2mM pyruvate solution at room temperature. 625µl of this mixture was placed into a spectrophotometer cuvette. 75µl of sample was added and mixed by inversion. The optical density at 340nm was measured in a spectrophotometer (Cecil CE2040).
The rate of change of OD was determined over 3 minutes and the LDH activity calculated from the following formula:

\[
\text{LDH activity (U/l)} = \frac{\Delta A/\text{min} \times 0.750 \times 1000}{6.22 \times 1 \times 0.075}
\]

\(\Delta A/\text{min}\) = Change in OD per minute

0.750 = total volume (ml)

1000 = conversion to units per litre

6.22 = millimolar absorptivity of NADH at 340nm

1 = light path (1 cm)

0.075 = sample volume (ml)

2.7 Preparation of human serum proteins

Human serum protein was prepared from normal volunteers (age 24-45, six male and eight female) working in the laboratories and on the renal unit at Leicester General Hospital. 20 ml of venous blood taken from each volunteer was allowed to stand until coagulated. The blood sample was centrifuged at 3000 rpm for 10 minutes and the serum removed from the clot. The serum was dialysed by placing 10 ml into semi permeable, cellulose, dialysis tubing (BDH 275/1270/02) with molecular weight cut off of 12-14kD. The tubing was suspended in a bucket containing 2 litres of PBS. This was stirred for four hours and then the PBS exchanged. A total of three exchanges were performed. The protein content of the dialysed serum was measured by Bio-Rad DC assay (section 2.2.3). The serum was then diluted to a protein concentration of 10mg/ml with the PBS which had been used as the dialysate in the final exchange of dialysis (PBSD). The concentration of small molecules in PBSD would have been equal to the concentration of these molecules in the dialysed serum. The diluted dialysed serum was stored at -20°C.
2.7.1 Fibronectin removal from serum

Fibronectin depleted serum (FNDS) was prepared by batch affinity chromatography using a method based on that described by Vuento M [1979]. Serum was collected as described above. 3ml of 0.05M Tris/HCl pH7.5 containing 5mM benzamidine (protease inhibitor) was added to 2ml of serum. 5ml of gelatin conjugated agarose (Sigma G5384) suspended in 0.05M Tris/HCl pH7.5 containing 5mM benzamidine was added. This was mixed for 30 minutes in a rotary mixer. The suspension was centrifuged for 5 minutes at 2000rpm to sediment the agarose and the supernatant removed. An additional 5ml gelatin/agarose was added to the supernatant and mixed for a further 30 minutes. The mixture was again centrifuged and the supernatant harvested (supernatant A). To remove proteins non-specifically bound to the gelatin/agarose it was resuspended in 1M sodium chloride and mixed for 30 minutes. This was centrifuged and the supernatant removed and added to supernatant A. The gelatin/agarose was washed three times in 1M sodium chloride. The pooled supernatants were then concentrated in centrifugal concentrators (molecular weight cut off 3kD) (Amicon 4302) to a protein concentration greater than 10mg/ml by Bio-Rad DC assay. The FNDS was dialysed as described in section 2.7 prior to use. To confirm that fibronectin had been removed from the serum the fibronectin was assayed by ELISA (section 2.8). Untreated serum samples diluted to 10mg/ml total protein contained 17.8±4.9μg/ml of fibronectin. Following fibronectin depletion, samples containing 10mg/ml of total protein contained 0.11±0.05μg/ml of fibronectin representing a 99.4±0.6% depletion.

2.7.2 Fractionation of serum by molecular weight

Fibronectin depleted serum with a protein concentration of 50mg/ml was fractionated by gel filtration using a HiLoad 16/60 Superdex 200 prep grade prepacked column.
(Pharmacia). The fractionation range for globular proteins on superdex 200 is 10-600kD. The column was attached to an HPLC system (Pharmacia). The eluent buffer was NaCl (0.15M) + Na₂HPO₄ (0.05M) pH 7.4. The column was equilibrated with this buffer and a 1.0ml sample of fibronectin depleted serum applied to it. For each serum preparation a total of 100mg of protein was applied to the column in two runs. The protein was eluted at a buffer flow rate of 1.0ml/min. The void volume of 40ml was discarded and the eluted proteins collected in 1.0ml fractions for a further 58ml. The chromatogram for serum is shown in Figure 2.1 demonstrating two major peaks corresponding to albumin and IgG. The 1.0ml fractions were pooled to produce four large fractions. Fraction A contained proteins of molecular weight greater than apoferritin (443kD), Fraction B contained proteins of molecular weight from apoferritin to the trough between the IgG and albumin peaks (molecular weight 100kD), Fraction C was from the trough between IgG and albumin to a position corresponding to the molecular weight of ovalbumin (43kD), Fraction D contained the remainder of the low molecular weight proteins (<43kD) which were not present in sufficient concentrations to be detected by the chromatogram.
Fig 2.1. Gel filtration chromatogram of normal serum. A, B, C and D are pooled fractions as described in the text. The positions of the molecular weight markers are shown: Apoferritin (ApoF), Immunoglobulin G (IgG), Human albumin (ALB) and Ovalbumin (OVA).

Fractions A-D were concentrated using centrifugal concentrators of 3kD molecular weight cut off. Fraction C containing the most protein was concentrated to >10mg/ml. The other fractions were concentrated to the same volume as fraction C. Each fraction was dialysed (section 2.7) against PBS following which fraction C was diluted with PBSD to a final concentration of 10mg/ml. PBSD was then added to the other fractions so that they were in the same final volume as fraction C to ensure that the protein concentration in the fractions was in the same ratio as it was in the original serum, assuming that the loss of proteins due to the fractionation procedure was the same in each fraction. The molecular
weight cut off of the dialysis membrane was 14kD and so the final fraction D contained proteins of molecular weight 14-43kD. All the fractions were stored at -20°C. A representative electrophoresis of the fractions is shown in fig 2.2.

IgG = Human Immunoglobulin G  
Alb = Human Albumin

**Fig 2.2. Electrophoresis of serum fractions by SDS PAGE.** Serum was fractionated into four molecular weight fractions as described (section 2.7.2). (i) All four fractions (A-D) and unfractionated serum (S) were electrophoresed as described in section 2.8 using a 7.5% gel. (ii) Separation of fractions A and B using a 5% gel. The gels were silver stained as described in section 2.9.2.
2.7.3 Ion Exchange Chromatography

Ion exchange chromatography, with a Mono Q (Pharmacia) cation exchanger, was used to analyse the proteins contained in the serum fractions produced above (section 2.7.2). The protein samples were buffer exchanged into low salt buffer A (Appendix 1) by passage through a PD-10 (Pharmacia) buffer exchanger. The protein sample, 25mg in 1.0ml, was loaded onto the column in buffer A. Proteins were eluted from the column using a linear gradient of increasing concentration of buffer B (Appendix 1). Flow rate was 1.0ml/minute the gradient reaching 100% buffer B over 20 minutes. Eluted proteins were detected using a UV spectrophotometer and the chromatogram recorded on a chart recorder (see Fig 6.8).

2.8 Electrophoresis

Analysis of protein samples by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Bio-Rad mini protein II system (165-2940) or for larger gels a BioRad protein II xi system (165-1801).

The glass plates were washed in 1M sulphuric acid, rinsed in deionised water, polished with acetone and then assembled according to the manufacturers (Bio-Rad) instructions. Discontinuous gels were made up of a separating gel (5-7.5%) overlaid by a stacking gel made as described in Appendix 1. Samples were prepared by boiling with non-reducing sample buffer (Appendix 1) for 4 minutes. 30μg of protein was applied to each well of the Mini Protein II gel and 250μg of protein to each well of the Protein II xi gel. Electrophoresis running buffer (Appendix 1) was added to the chamber. Electrical potential was applied to the system at a constant voltage of 200v for the Mini system and constant current of 15mA for the Protein II xi as per the manufactures instructions. The potential was applied until the bromophenol-blue from the sample buffer had traversed the entire length of the gel (40 minutes for minigels and 3-4 hours for large gels).
2.9  Staining of electrophoresis gels

2.9.1  Coomassie Blue

On completion of the electrophoresis the gels were removed from the plates and
immersed in 0.25% Coomassie brilliant blue for 30 minutes. The gel was destained using
several washes with 40% (v/v) methanol/10% (v/v) acetic acid until all the excess dye was
removed.

2.9.2  Silver staining

The protocol described is for gels greater than 1.0mm thick. For thinner gels
(minigels) the incubation times were shorter and are shown in brackets. Following completion
of the electrophoresis the gel was placed in 40% (v/v) methanol/10% (v/v) acetic acid for at
least 60 minutes (30min). The gel was then transferred to 10% (v/v) ethanol/5% (v/v) acetic
acid for 30 minutes (15min) after which this fixative was replaced with fresh 10% (v/v)
ethanol/5% (v/v) acetic acid for a further 30 minutes (15min). The gel was then placed in
an oxidiser solution (BioRad 161-0443), for 10 minutes (5min). The oxidiser was washed
from the gel with deionised water until the gel was completely clear. Silver reagent (BioRad
161-0443) was added for 30 minutes (20min) and then washed off with deionised water for
2 minutes (1min). The colour was developed with several changes of developer solution
(BioRad 161-0443) and development then stopped by immersion of the gel in 5% (v/v) acetic
acid.

2.10  Fibronectin assay

An enzyme linked immunosorbent assay (ELISA) was developed for the measurement
of human fibronectin concentrations in serum, culture medium, and cells dissolved in WB+1%
nonidet P40. A sandwich technique was used and both antihuman fibronectin antibodies were purchased from Sigma. F3648 is a polyclonal antihuman antibody raised in rabbit which has been evaluated for specificity by immunofluorescent labelling of cultured fibroblasts and by immunoblotting. It shows no cross reactivity with laminin, vitronectin, collagen type IV or chondroitin sulphate types A, B or C. F7387 is a mouse IgG1 monoclonal antibody to human fibronectin produced using fibronectin from human plasma. The specificity of both antibodies was evaluated by Western blotting (section 2.11 and Fig 5.1).

Immunoplates (Nunc) were coated using antibody F3648 diluted 1:1000 in carbonate/bicarbonate coating buffer (Appendix 1). 100μl of antibody solution was pipetted into each well of 96 well immunoplates and incubated overnight at +4°C. The plates were washed four times with washing buffer (WB) (Appendix 1) using an automated plate washer (Denley Wellwash 5000). 100μl of WB+2% (w/v) bovine serum albumin (Sigma A2153) was added to each well and the plates incubated for at least 1 hour at room temperature to block the non specific protein binding sites. The plates were washed again as above. The standard curve was formed using eleven fibronectin standards produced by one third dilutions from a concentration of 2000 to 35ng/ml using fibronectin from human plasma purchased from Sigma (F0895). 50μl of standards or appropriately diluted samples were added to each well. The plate was incubated at +4°C overnight. The plates were washed and 50μl of antibody (F7387) diluted 1:500 in wash buffer added to each well. After incubation at room temperature for 1 hour the plates were washed again and 50μl of horseradish peroxidase conjugated anti-mouse-immunoglobulin (Dako 260) (1:1000 in WB) was added to each well. After a further one hour incubation the plates were washed again. 50μl of ELISA substrate solution (Appendix 1) was added to each well and incubated for 10-20 minutes at room temperature. Once the colour had developed sufficiently the reaction was stopped by the
addition of 75µl of 1M sulphuric acid.

The optical density at 492nm was measured using the plate scanner. The OD_{492} was plotted against the log of the fibronectin concentration producing a sigmoid curve see figure 2.3. The steep part of the sigmoid standard curve was between 100 and 1000ng/ml which were used as the limits of detectability of the assay. The inter assay coefficient of variation at the standard concentration 395 ng/ml was 4.9% (data from 36 assays).

Fig 2.3 Representative standard curve of fibronectin ELISA.
2.11 Western Blotting for fibronectin

Protein solutions for Western blotting were first electrophoresed by SDS-PAGE using the Bio-Rad mini protean II system (section 2.8). The 5% gel was soaked overnight in electrophoresis transfer buffer (Appendix 1). Two fibre pads, two filter papers and a nitrocellulose membrane were immersed in transfer buffer. The gel and nitrocellulose membrane were sandwiched together between the filter papers and the fibre pads in a gel holding cassette. The cassette was inserted into an electrophoretic transfer tank (Bio-Rad 170-3930) containing an ice block and the tank was filled with transfer buffer at 0°C. Electric potential was applied at 100 Volts/150mAmps for 1 hour.

The nitrocellulose membrane was removed to a dish containing Tris buffered saline pH7.6 (TBS) (Appendix 1) + 2% BSA and incubated overnight at +4°C or for 2 hours at room temperature. The nitrocellulose membrane was washed three times with TBS+0.1% (v/v) tween 20 (TTBS) and then transferred to a dish containing the primary antibody (F3648 or F7387, see section 2.10) diluted 1/500 in TTBS. After 1 hour the membrane was washed 3 times with TTBS and then transferred to a dish containing horseradish peroxidase conjugated goat antirabbit (Dako P448) or rabbit antimouse (Dako P260) immunoglobulins diluted 1/500 in TTBS. After 1 hour the membrane was washed and immersed in substrate solution (Sigma Fast DAB, D4418 3,3’ diaminobenzidine tetrahydrochloride) until the bands had developed. The membrane was washed in deionised water and then air dried.
2.12 Cytokine and chemokine assays

Platelet derived growth factor (PDGF) AB, Tumour Necrosis Factor α (TNFα), Transforming Growth Factor β1 (TGFβ1) and Monocyte Chemoattractant Protein-1 (MCP-1) were assayed in culture supernatants and serum samples using immunoassays purchased from R and D Systems.

2.12.1 Platelet derived growth factor AB assay

PDGF has two subunits designated as A and B chains and can be either homodimeric (PDGF-AA or PDGF-BB) or heterodimeric (PDGF-AB) [Ross R 1986]. PDGF-AB was assayed using a purchased sandwich enzyme immunoassay. Literature supplied with the assay indicated a coefficient of variation between assays at the mid point of the standard curve of 9.9% and an intra assay coefficient of variation at the mid point of 4.9%. No significant cross reactivity was demonstrable with other cytokines. The 96 well microtitre plates were supplied coated with a monoclonal antibody specific for PDGF-AA. The standards were prepared from recombinant human PDGF-AB in the concentration range 31.25 to 2000pg/ml. 200μl of sample or standard was pipetted into the wells in duplicate. The plate was incubated at room temperature for 2 hours. The wells were washed four times with wash buffer. 200μl of a solution of a polyclonal antibody against human PDGF-BB conjugated to horseradish peroxidase was added to each well and the plate incubated for a further 2 hours at room temperature. The plate was washed four times in wash buffer and then 200μl of the substrate solution was added to each well. The plate was incubated at room temperature for 20 minutes. 50μl of 2M sulphuric acid was added to the wells to stop the reaction. The optical density was measured at 450nm.

To calculate the results the OD of the blank wells was subtracted from the OD
of the standards and samples. The $\log_{10}$ of the OD$_{450}$ of the standards was plotted against the $\log_{10}$ of the concentrations to produce a straight line. The concentrations of the samples were calculated from their OD$_{450}$ using the equation of this line.

### 2.12.2 Tumour necrosis factor $\alpha$ assay

This assay employed a sandwich immunoassay technique. The literature supplied with the assay indicated a coefficient of variation between assays at the mid point of the standard curve of 8.7% and an intra assay coefficient of variation at the mid point of 4.4%. No cross reactivity with other cytokines was demonstrable. The microtitre plate was supplied coated with a mouse monoclonal antibody to human TNF$\alpha$. 200$\mu$l of samples and standards (15.6 - 1000pg/ml of recombinant human TNF$\alpha$) were placed in the wells in duplicate and incubated for 2 hours at room temperature. After washing four times with WB 200$\mu$l of a solution of polyclonal antibody to human TNF$\alpha$ conjugated to horse radish peroxidase was added to each well and incubated at room temperature for 1 hour. After washing the plate the assay was developed as described for PDGF-AB (section 2.12.1).

Plotting $\log_{10}$ of the OD$_{450}$ against the $\log_{10}$ of the concentration of the standards produced a straight line from which the concentration of the samples could be calculated.

### 2.12.3 Transforming growth factor $\beta$, assay

TGF$\beta$ is synthesised as part of a larger precursor molecule. In cells the precursor is cleaved to form the TGF$\beta$ which remains noncovalently complexed to the remainder of the precursor which is called the latency associated peptide (LAP). TGF$\beta$ and LAP are secreted together by the cell. The complex is biologically inactive and not reactive with antibodies to TGF$\beta$ or with the TGF$\beta$ receptor [Jakowlew S 1988]. Therefore in order to assay TGF$\beta$
it was necessary to dissociate the complex by acidification [Spom M 1987].

100μl of 1M hydrochloric acid was added to 0.5ml of sample and incubated at room temperature for 10 minutes. This solution was neutralised with 200μl of 1.2M sodium hydroxide+0.5M HEPES buffer. The activated samples and the standards (31.25-2000pg/ml recombinant TGFβ3) were added to the wells of a microtitre plate which had been coated with recombinant human TGFβ soluble receptor type II and incubated for three hours at room temperature. After washing as described in 2.12.1 a solution containing a polyclonal antibody against TGFβ3, conjugated to horseradish peroxidase, was added to each well and the plate incubated for 1.5 hours at room temperature. The plate was washed again and then developed as described in section 2.12.1. The standard curve was generated from the OD450 using computer software (Titersoft) to produce a four parameter logistic curve fit. Sample concentrations were calculated by the software and corrected for the dilution caused by the activation step.

The TGFβ receptor type II binds TGFβ1, TGFβ3, and TGFβ5, with equal affinity and TGFβ2 at a low affinity [Massagué J 1992]. Specificity of the assay for TGFβ3 was conferred by the antibody against TGFβ3. The literature supplied with the assay indicated no cross reactivity with TGFβ2, 0.24% cross reactivity with TGFβ3, and 0.06% cross reactivity with TGFβ5. The coefficient of variation between assays at the mid point of the standard curve was reported at 3.2% with an intra assay coefficient of variation at the mid point of 5.2%. Significant cross reactivity with other cytokines was not demonstrable.

2.12.4 Monocyte Chemoattractant Protein -1 assay

Like the TNFα assay described above (section 2.12.2) this assay is based on a standard sandwich immunoassay technique. The microtitre plate was supplied coated with a murine
monoclonal antibody against human MCP-1. Appropriately diluted samples and standards (31.25-2000pg/ml) were added for an incubation period of 2 hours at room temperature. After washing a horseradish peroxidase conjugated polyclonal antibody against human MCP-1 was added to the wells for an incubation period of 2 hours. The assay was then developed as described in 2.12.1. A standard curve was created using computer software (Titersoft) to generate a 4 parameter logistic fit, from which the sample concentrations were calculated. Literature supplied with the assay indicated a coefficient of variation between assays at the mid point of the standard curve of 4.8% and an intra assay coefficient of variation at the mid point of 4.9%. There was no significant cross reactivity with other chemokines or cytokines.

2.13 Statistical analysis

The data are presented as means ± 95% confidence limits. When data in two or more groups of equal size were to be compared two way analysis of variance for multiple measures was used. If the groups were of unequal size then one way analysis of variance was used. 95% confidence limits were calculated from the analysis of variance (ANOVA). When ANOVA indicated statistical significance the data and confidence limits were inspected and significant differences accepted between groups in which there was no overlap of the 95% confidence limits. If ANOVA was significant but there was no definitely different group (all the confidence limits overlapped) then a student t test was used to confirm a difference between those groups which appeared most different from inspection of the data. A Bonferroni correction of the significance of the t test was performed according to the number of t tests performed on each data set. Statistical significance was accepted at p<0.05. Data analysis was performed using CSTAT computer software.
CHAPTER 3

THE EFFECT OF ALBUMIN AND PROTEINURIC URINE ON THE GROWTH OF PROXIMAL TUBULAR CELLS
3.1 Introduction

As described in chapter 1 (section 1.5) changes in cell growth, either through hyperplasia or hypertrophy, may be of significance in the progression of renal disease. The experiments described in this chapter investigated whether the growth of the proximal tubular cell line OK cells was altered by exposure to albumin or the mixture of proteins that are present in proteinuric urine.

3.1.1 OK cells

OK cells are an immortalized cell line of proximal tubular origin. They were first isolated in 1978 from the kidney of an American opossum [Koyama H 1978] which was being used for the investigation of X chromosomes. From a whole kidney preparation derived from a single female opossum one colony of epithelial cells became immortalised, survived frozen storage and many subsequent passages. This cell line was designated OK and has subsequently been extensively characterised.

OK cells have a surface parathyroid hormone receptor coupled to cyclic adenosine monophosphate formation [Teitelbaum A 1984]. Malström K [1987] demonstrated that L-glutamate, L-proline, L-alanine and α methyl D-glucoside were all taken up by OK cell monolayers by both sodium dependent and sodium independent pathways. Inorganic phosphate was taken up by a sodium dependent pathway and amiloride sensitive sodium transport was identified [Malström K 1987]. Parathyroid hormone dependent sodium/phosphate cotransport was identified in the apical membrane of OK cells grown on permeable supports [Resishkin S 1990] and parathyroid hormone inhibition of sodium-hydrogen ion antiporter activity has been established [Pollock A 1986]. OK cells have also been shown to transport L-arginine, L-phenyl alanine [Schwegler J 1989] and L-cystine [States B 1990].
These features are all compatible with OK cells being of proximal tubular origin. OK cells are known to endocytose protein [Schwegler J 1991] and they therefore provided a suitable model for the investigation of the effects of proteins on proximal tubular cells.

3.2 Methods

3.2.1 OK cell culture

OK cells were a generous gift to the Leicester Renal Unit Laboratories from Dr J Caverzasio, Geneva, Switzerland. The proximal tubular characteristics of the donated cells had previously been confirmed by Dr A. Bevington in these laboratories by demonstrating sodium dependent glucose and inorganic phosphate transport (unpublished data). Frozen OK cells at passage 65 were defrosted by immersion of the storage tube (containing cells from one confluent 10cm petri dish) in warm water. Once defrosted the cells were transferred into a sterile 30ml container and suspended in 20ml of Hanks Balanced Salts Solution (Life Technologies 24020-091) (HBSS). The cells were centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the cells resuspended in a growth medium consisting of DMEM:F12 (1:1) (Life Technologies 21331-020) with addition of 2.0mM L-Glutamine and 10% fetal calf serum (FCS, Life Technologies) which had been heat inactivated by immersion in a water bath at 56°C for 45 minutes. One vial of stored cells was seeded into a single 10cm tissue culture petri dish in 10ml of growth medium and then placed in an incubator at 37°C in an atmosphere of 5% CO₂/95% air. The growth medium was changed every second day and the cells reached confluence in 4 to 7 days. The cells were used between passages 66 to 75.
3.2.2 OK cell passaging

Confluent 10cm petri dishes of OK cells were washed three times with 10ml of HBSS to remove all of the FCS. 5ml of Trypsin-EDTA (Appendix 1) was added to each petri dish which was then returned to the cell culture incubator for 20 minutes. After the incubation the petri dish was inspected using an inverted microscope to ensure that the cells had detached from the plate. The trypsin-EDTA was inactivated by addition of 10ml of medium containing 10% FCS. The cells were aspirated from the plate using a pasteur pipette and transferred to a sterile container. An additional 10ml of growth medium was added and the cells sedimented by centrifugation at 1000rpm for 5 minutes. The medium was then removed and the cells resuspended in 10ml of growth medium.

The number of cells was counted using a haemocytometer and additional growth medium added to produce 13x10⁶ cells/ml. The cell suspension was added to the culture plates as follows: 10ml per 10cm petri dish, 2ml per well of a 6 well plate and 1ml per well of a 12 well plate. The growth medium was replaced with fresh medium on Monday, Wednesday and Friday each week. Previous experience in the laboratory had shown that this density of cells produced rapid growth and confluent monolayers within 1 week.

3.2.3 Preparation of rat proteinuric urine (PU)

Proteinuric urine was obtained from rats rendered nephrotic using puromycin aminonucleoside (PAN) [Eddy A 1991]. Fifteen female Wistar rats were housed in the animal house at Leicester General Hospital for one week to acclimatise. PAN (Sigma P7130) was diluted with sterile normal saline to a concentration of 10mg/ml. Ten rats were given a single intravenous injection of PAN (5mg/100g body weight) into the tail vein [Olson J 1981]. Five rats were given control injections of normal saline. Twelve days after the injection, at the
time of peak proteinuria [Eddy A 1991], the rats were individually placed in metabolic cages for urine collection over 24 hours. The proteinuric urines were pooled as were the control urines and then dialysed against phosphate buffered saline as described in 2.7. The protein concentration of the proteinuric urine was 14mg/ml by Bio-Rad assay (section 2.2.2). This was diluted using the dialysate PBS (PBS-D) from the final dialysis exchange to a concentration of 10mg/ml (PU). The urine from the controls was diluted by the same amount as the proteinuric urine. The urine and a sample of final dialysate was stored at -20°C.

3.2.4 Experimental protocols

OK cells grown on 6 or 12 well plates were used for the following experiments:

Exp 1) The effect of bovine serum albumin on the growth of subconfluent OK cells
Exp 2) The effect of bovine serum albumin on the growth of confluent OK cells
Exp 3) The effect of proteinuric urine on the growth of confluent OK cells

Proximal tubular epithelial cells exist in vivo in confluent monolayers and would therefore be modelled best in vitro using confluent cells. Phenotypic differences have been demonstrated between subconfluent and confluent cells [Scheinman S 1988]. Work in this laboratory (Dr A Bevington) has suggested that confluent OK cells may be relatively oxygen starved due to their high density. This has also been reported in the proximal tubular cell line LLC-PK1, in which rocking the cultures improves oxygenation and differentiation of the cells [Sahai A 1989]. It was, therefore, decided to compare confluent and subconfluent cultures in the investigation of cell growth in the presence of protein.
3.2.4.1 Experiment 1: The effect of bovine serum albumin on the growth of subconfluent OK cells

OK cells at passage 65 to 75 were transferred to six 6 well plates in growth medium at the density described above. On day 2 the following experimental media were prepared:

A) Medium 199
B) Medium 199 + 0.1 mg/ml bovine serum albumin
C) Medium 199 + 1.0 mg/ml bovine serum albumin
D) Medium 199 + 10.0 mg/ml bovine serum albumin
E) Medium 199 + 20mM ammonium chloride

Medium 199 (Life Technologies 31150-022) contains a physiological concentration of glucose and OK cells had been shown to survive without serum in this medium for up to eight days (Dr A Bevington). 30 ml of medium D was produced by addition of 300 mg of bovine serum albumin (BSA, essentially globulin free, Sigma A7638) to 30ml of Medium 199. This was sterilised by filtration through a filter of 0.22μm pore size. 27 ml of Medium 199 was put into sterile containers labelled B and C. 3ml was transferred from D to C and mixed followed by transfer of 3ml from C to B, thus producing serial dilutions by a factor of 10.

Ammonium chloride was used as a positive control that is known to cause hypertrophy of tubular cells [Golchini K 1989]. 2M ammonium chloride was prepared in nanopure water and sterile filtered. This solution was stored at -20°C. 300μl of 2M ammonium chloride was added to 30ml of Medium 199 in a sterile universal to produce medium E.

After removal of the growth medium from the subconfluent cells and washing three times with HBSS, 2.0ml of media A - E were added to each of the wells on the 6 well plates.
The plates were returned to the incubator for 72 hours. On day 5 three plates were used for measurement of total cell protein (section 2.2.1) and DNA (section 2.3) and three plates were used for measurement of tritiated thymidine uptake (section 2.4)

3.2.4.2 Experiment 2 The effect of bovine serum albumin on the growth of confluent OK cells

On day one, twelve 6 well plates were set up as described in experiment 1 and an additional two 12 well plates were set up for viability studies. Growth medium was changed on day 3 and day 5 and the cells reached confluence by day 8. At confluence the medium on the cells was changed to those described in experiment 1 for 6 of the plates. For the other 6 plates the protocol was as in experiment 1 except that the bovine albumin used was essentially lipid free, essentially globulin free BSA (Sigma A0281). Incubation with experimental media was for 72 hours after which half the plates were used for measurement of tritiated thymidine incorporation and the remainder for protein and DNA measurements.

Since albumin avidly binds many molecules an experiment was performed to investigate the possibility that the effect of albumin was not a direct effect on the cells but instead, the albumin bound and inactivated a component of the medium which was important for cell growth. BSA (0-10mg/ml) was added to Medium 199 and then removed from it, together with anything that it had bound, by centrifugal filtration through filters with a molecular weight cut off of 25kD (Amicon CF25). The filtered medium was then sterile filtered prior to addition to confluent OK cell cultures as previously described.

Viability was assessed on two 12 well plates using trypan blue exclusion under the various conditions as described in section 2.6.1.
3.2.4.3 Experiment 3  The effect of proteinuric urine on the growth of confluent OK cells

OK cells were passaged onto six 6 well plates as described in experiment 1. After 8 days the medium was changed to the following:

A) 18 ml Medium 199 + 2.0ml PU (final protein concentration 1.0mg/ml)
B) 18 ml Medium 199 + 0.2ml PU + 1.8ml PBSD (protein concentration 0.1mg/ml)
C) 18 ml Medium 199 + 0.02ml PU + 1.98ml PBSD (protein concentration 0.01mg/ml)
D) 18 ml Medium 199 + 2ml PBSD (control)
PU = proteinuric urine (10mg/ml protein) (section 2.3)
PBSD = PBS from the final dialysate exchange (section 2.7)

After 72 hours the growth of the cultures was measured as described in experiment 1.

To compare effects of proteinuric urine with those of control urine 4 x 6 well plates were set up. After 8 days the medium was changed to the following:

A) 18 ml Medium 199 + 2 ml PBSD
B) 18 ml Medium 199 + 2 ml PU
C) 18 ml Medium 199 + 0.2 ml PU + 1.8 ml PBSD
D) 18 ml Medium 199 + 2 ml CU
E) 18 ml Medium 199 + 0.2 ml CU + 1.8 ml PBSD
F) 18 ml Medium 199 + 2 ml CU + 2 mg BSA
G) 18 ml Medium 199 + 0.2 ml CU + 1.8 ml PBSD + 0.2 mg BSA
CU = control urine

These conditions were applied to confluent OK cells for 72 hours after which the tritiated thymidine incorporation was measured (section 2.4).

Since the rat urine was likely to be contaminated with lipopolysaccharide (LPS) it was important to investigate any possible effects of LPS on the growth of OK cells. Therefore
confluent OK cells on 6 well plates were exposed to Medium 199 containing lipopolysaccharide (from E Coli serotype 026:B6 Sigma L8399) \((10^{-5}, 10^{-6} \text{ or } 10^{-7} \text{ g/ml})\) for 72 hours prior to measurement of tritiated thymidine incorporation as previously described.

3.3 Results

3.3.1 Experiment 1

One day after passaging to the 6 well plates the OK cells formed numerous colonies of epithelial cells. The effect of addition of bovine serum albumin to the cultures, for 72 hours, on the protein and DNA contents of the cultures, is shown in Table 3.1.

<table>
<thead>
<tr>
<th>BSA concentration (mg/ml)</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/well (µg)</td>
<td>132±8</td>
<td>126±8</td>
<td>133±8</td>
<td>136±8</td>
</tr>
<tr>
<td>DNA/well (µg)</td>
<td>14.6±0.8</td>
<td>*16.2±0.8 *16.5±0.8</td>
<td>13.6±0.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. Protein and DNA contents of subconfluent OK cell cultures exposed to BSA. The results are from five experiments performed in triplicate and are expressed as means±95% confidence limits. The protein contents were not significantly different. DNA contents, ANOVA p<0.01. * significantly different to control and 10.0mg/ml.

Whilst the protein content of the wells was not significantly different the DNA content was increased by 0.1mg/ml and 1.0mg/ml of BSA in the medium. 10mg/ml of BSA did not alter DNA content compared to control. The tritiated thymidine results, which reflect DNA synthesis, showed an increase on exposure to 1.0mg/ml of BSA, compared to control, whereas 0.1mg/ml and 10mg/ml had no effect (Fig 3.1). Figure 3.2 shows the protein to DNA ratios. 20mM ammonium chloride, a known hypertrophic stimulus, increased the protein to DNA
ratio as did exposure to 10.0mg/ml of BSA. The increase in the protein to DNA ratio on exposure to 10mg/ml BSA was due to a small (and nonsignificant) rise in the protein content of the wells combined with a small (and nonsignificant) fall in the total DNA content of the wells (Table 3.1).

It was noted that addition of 10.0mg/ml of BSA to the culture medium brought about a change in the colour of the pH indicator (phenol red). The pH of the culture medium was therefore measured as described in section 2.5. There was no difference in the pH of the medium under the different conditions and the colour change in the presence of BSA was likely to have been the result of BSA binding the indicator.

3.3.2 Experiment 2

Exposure to 10.0mg/ml of BSA increased the total protein content of confluent OK cell cultures but there were no differences in the DNA content (Table 3.2).

<table>
<thead>
<tr>
<th>BSA concentration (mg/ml)</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/well (µg)</td>
<td>439±24</td>
<td>413±24</td>
<td>442±24</td>
<td>*506±24</td>
</tr>
<tr>
<td>DNA/well (µg)</td>
<td>49.3±3.3</td>
<td>46.6±3.3</td>
<td>47.2±3.3</td>
<td>47.6±3.3</td>
</tr>
</tbody>
</table>

Table 3.2. Protein and DNA contents of confluent OK cell cultures exposed to BSA. The results are from five experiments and are expressed as means±95% confidence limits. The DNA contents were not significantly different. Protein contents, ANOVA p<0.01. * significantly different to control, 0.1 and 1.0mg/ml.
Despite the fact that DNA content was not changed the tritiated thymidine incorporation of confluent OK cells increased on exposure to BSA (Fig 3.3). There was a significant increase compared to control on exposure to 0.1mg/ml with a further increase on exposure to 10.0mg/ml. This therefore contrasts with the results using subconfluent cells in which 10.0mg/ml of BSA was not a stimulus to proliferation. As a result of an increased protein and a nonsignificant fall in DNA, the protein to DNA ratio increased on exposure to 10.0mg/ml of BSA (Fig 3.4).

To show that the cells withstood the experimental conditions the cell viability was assessed by trypan blue exclusion. There were no differences in viability under any of the conditions used. An estimate of the percentage viability calculated from the number of cells on a confluent plate at time of passaging indicated 94% viability.

It was possible that the effect of albumin was due to the binding of a component of the medium rather than a direct effect of albumin on the cells. This was investigated by adding BSA to the medium and then removing it, together with anything that it had bound, by filtration. Table 3.3. shows that there was no effect of the filtered medium on total protein, DNA, tritiated thymidine incorporation or protein to DNA ratio of OK cells, indicating that the previously described effects of albumin were due to a direct action on the cells.
<table>
<thead>
<tr>
<th>BSA concentration (mg/ml)</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/well (µg)</td>
<td>539±67</td>
<td>569±67</td>
<td>547±67</td>
<td>554±67</td>
</tr>
<tr>
<td>DNA/well (µg)</td>
<td>52.9±2.5</td>
<td>53.7±2.5</td>
<td>53.3±2.5</td>
<td>53.8±2.5</td>
</tr>
<tr>
<td>Protein:DNA (µg/µg)</td>
<td>10.3±1.3</td>
<td>10.6±1.3</td>
<td>10.3±1.3</td>
<td>10.4±1.3</td>
</tr>
<tr>
<td>H-thymidine (dpm x1000)</td>
<td>50.7±13.4</td>
<td>57.0±13.4</td>
<td>60.3±13.4</td>
<td>59.1±13.4</td>
</tr>
</tbody>
</table>

Table 3.3. Effects on the growth of confluent OK cells of incubation with medium conditioned with BSA from which the albumin had been removed by filtration. The results are from three experiments and are expressed as means±95% confidence limits. No significant differences were observed.

To determine whether the lipid which is carried on albumin contributed to its effects on growth, the effect of exposure to lipid free BSA (LFBSA) was compared to that of lipid replete BSA. No significant changes were found in the protein or DNA content of the OK cell cultures (Table 3.4).

<table>
<thead>
<tr>
<th>LFBSA concentration (mg/ml)</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (µg/well)</td>
<td>524±47</td>
<td>515±47</td>
<td>517±47</td>
<td>591±47</td>
</tr>
<tr>
<td>DNA (µg/well)</td>
<td>44.9±2.2</td>
<td>44.1±2.2</td>
<td>44.9±2.2</td>
<td>43.8±2.2</td>
</tr>
</tbody>
</table>

Table 3.4. Protein and DNA contents of confluent OK cell cultures exposed to LFBSA. The results are from five experiments and are expressed as means±95% confidence limits. No significant differences were observed in either the protein or DNA contents of the wells.
Despite the lack of effect on total DNA there was an increase in tritiated thymidine incorporation on exposure to 0.1 and 1.0 mg/ml of LFBSA. As with exposure of subconfluent cells to BSA 10.0 mg/ml of LFBSA did not alter thymidine uptake by confluent OK cells compared to control. Similar to BSA there was an increase in the protein to DNA ratio on exposure to 10 mg/ml of LFBSA (21±14% increase with LFBSA, 23±10% with BSA) (Fig 3.6). This was the result of a nonsignificant rise in protein content combined with a nonsignificant fall in DNA content (Table 3.4).

3.3.3. Experiment 3

The aim of this experiment was to investigate the effect of the complex mixture of proteins present in proteinuria and to compare that with the effect of albumin alone. Proteinuric urine was produced from the puromycin aminonucleoside nephrosis model in the rat. The range of proteins present in the urine was demonstrated by the electrophoresis shown in figure 3.7. Since it was likely that the urine used in these experiments was contaminated with lipopolysaccharide (LPS) it was important to investigate whether LPS could affect the growth of OK cells. There was no effect of LPS (10^{-7} - 10^{-4} g/ml) added to the culture medium for 72 hours on the tritiated thymidine incorporation by OK cells.

OK cells were exposed to medium containing pooled proteinuric urine at a protein concentration of 1.0, 0.1 or 0.01 mg/ml or to medium containing the same volume of PBS/D to ensure that an equal dilution of the medium was produced. Proteinuric urine at these concentrations did not significantly alter the total protein content of the cultures (Table 3.5). The DNA content, however, was increased by exposure to 0.1 mg/ml of proteinuric urine (Table 3.5).
### Table 3.5. Protein and DNA contents of confluent OK cell cultures exposed to proteinuric urine.

The results are from five experiments and are expressed as means±95% confidence limits. The protein contents were not significantly different. DNA contents, ANOVA p<0.01. * significantly different to control.

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>PBSD (0)</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/well (µg)</td>
<td>516±36</td>
<td>574±36</td>
<td>561±36</td>
<td>536±36</td>
</tr>
<tr>
<td>DNA/well (µg)</td>
<td>49.0±1.9</td>
<td>49.6±1.9*</td>
<td>52.8±1.9</td>
<td>51.7±1.9</td>
</tr>
</tbody>
</table>

Consistent with the DNA results the tritiated thymidine uptake was markedly increased by 0.1 mg/ml of proteinuric urine. Exposure to 1.0 mg/ml of proteinuric urine also increased tritiated thymidine compared to control but to a lesser extent than 0.1 mg/ml (Fig 3.8). There was no difference in the protein to DNA ratio under the various conditions (Fig 3.9).

It was possible that the final urine contains proteins which had been added to it distal to the tubules. To control for this possibility the proliferative effect of proteinuric urine was investigated in comparison to the effect of normal rat urine. The results are shown in figure 3.10. The disintegrations per minute of tritiated thymidine incorporation in this experiment were lower than those previously seen because of the use of a different batch of tritiated thymidine. Analysis of variance indicates that there was an increase in tritiated thymidine incorporation on exposure to control urine. The increase previously seen on exposure to 0.1 mg/ml of nephrotic urine was confirmed in this experiment. At this concentration the thymidine incorporated on exposure to nephrotic urine was significantly greater than that on exposure to control urine. Since there was an effect on tritiated thymidine incorporation of control urine, it was possible that the greater effect of nephrotic urine compared to albumin...
alone was due to a combination of the effect of control urine and the effect of albumin. However, control urine + 0.1 mg/ml of BSA did not increase tritiated thymidine incorporation over control urine alone and hence the effect of nephrotic urine could not be accounted for only by the presence of albumin and other components of the proteinuric urine must have had an important effect.

3.4. Discussion

Alterations in cell growth may be a maladaptive response to decreased renal mass and contribute to the progression of renal disease (section 1.5). These experiments investigated the effects on cell growth of proteins that are present in proteinuria. The first series of experiments described in this chapter were performed on subconfluent OK cells, because they are less likely to be hypoxic than confluent cells. The disadvantage of this model is that many of the cells are not in contact with their neighbours and consequently have a proliferative phenotype. As expected the subconfluent cells had a higher baseline tritiated thymidine uptake than the confluent cells. In subconfluent cells there was an increase in proliferation on exposure to 1.0 mg/ml of BSA but not on exposure to 10.0 mg/ml. BSA at 10 mg/ml resulted in hypertrophy of the cells.

The confluent cell culture model more closely represents the in vivo state of proximal tubular cells although the possibility of effects due to hypoxia need to be considered. As expected the tritiated thymidine incorporation of confluent cells was less than that of subconfluent cells. The tritiated thymidine incorporation increased up to and including exposure to 10 mg/ml of BSA. In contrast, exposure of cells to lipid free BSA resulted in increases in tritiated thymidine incorporation at 0.1 and 1.0 mg/ml but not at 10.0 mg/ml, results which were similar to those with subconfluent cells. Exposure of OK cells to
proteinuric urine also produced this biphasic response in thymidine incorporation. The response to proteinuric urine was considerably more marked than that to BSA and occurred at a lower concentration (0.1mg/ml). It is difficult to produce a single hypothesis to explain these changes in thymidine incorporation. The loss of the proliferative effect of BSA on subconfluent cells and proteinuric rat urine on confluent cells at the higher protein concentrations could be the result of the high levels of thymidine incorporation in these models. Subconfluent cells have high background thymidine incorporation and proteinuric urine had a marked effect, which could have resulted in stimulation of the growth of the cells to the point that by 72 hours a component of the medium that was essential for growth had been exhausted and hence thymidine incorporation at that time point was decreased. The biphasic response to LFBSA however occurred at a lower level of thymidine incorporation and this may have been the result of the albumin scavenging fatty acids in the culture medium so that they would not then be available for the formation of cell walls and hence proliferation would be prevented. These explanations are however speculative.

Similarly to subconfluent cells 10mg/ml of BSA and LFBSA resulted in the hypertrophy of confluent OK cells. It is unlikely that the increase in protein to DNA ratio was simply due to excess BSA taken up by the cells into the endosomal/lysosomal compartment, rather than true hypertrophy for two reasons. Firstly the increase in protein to DNA ratio was the result of a decrease in total DNA in combination with an increase in total protein. Secondly an increase in cell protein of 15% (as seen in confluent cells exposed to 10mg/ml BSA) would require an additional 75µg of protein/µl of cell water (assuming 500µg cell protein/µl of cell water [Kletzein R 1975, Bennett S 1994]) which would require the absorption of 7.5µl of medium containing 10mg/ml of protein for every 1µl of cell volume. This would be unlikely. The highest achievable protein concentration using rat proteinuric
urine was 1.0 mg/ml and no hypertrophy was demonstrated in this experiment.

In the interpretation of this data it is important to consider some caveats. The experimental design used materials obtained from a number of different species. The cells were from the opossum, the albumin was bovine and the urine was from the rat. It would be possible, for instance, for rat albumin to have a different effect to bovine albumin. OK cells come from an immortalised cell line and by definition their growth is altered compared to normal proximal tubular cells and interpretation of changes in that growth should therefore be viewed with caution. Although tritiated thymidine incorporation is an accepted method for the measurement of DNA synthesis, thymidine is not exclusively incorporated into DNA in the cell and increased DNA synthesis does not necessarily result in mitosis.

The appropriate range of protein concentrations to be used in vitro to investigate effects of proteinuria in vivo is uncertain. Other investigators have used concentrations up to 10mg/ml of individual proteins that are present in the serum in lower concentrations than albumin [Ong A 1994a, Harris D 1995]. However micropuncture studies have demonstrated a protein concentration in the tubular fluid in nephrotic syndrome in rats as low as 0.07mg/ml [Landwehr D 1977]. This emphasises the significance of the experiments which showed effects of rat proteinuric urine at a protein concentration of 0.1mg/ml. In trying to model the effects of proteinuria it should be noted that progression of renal disease is a long term process occurring over months and years and it may therefore be necessary to use high concentrations of proteins in short term culture to reproduce the cumulative effects of long term exposure to proteinuria in vivo. The development of hypertrophy in response to 10mg/ml of BSA could therefore be of relevance to the effects of proteinuria.

The conclusions from this series of experiments are that proteins can alter the growth of the proximal tubular cell line, OK cells. The effects on subconfluent and confluent cells
were similar. Proliferative effects of BSA were observed at a lower concentration of protein than the hypertrophic effects. The mixture of proteins present in proteinuric urine had a more marked effect on proliferation and at a lower concentration than did BSA. At higher concentrations the proliferative effect of albumin and proteinuric urine was lost and the mechanism for this is uncertain. These experiments therefore provide evidence that the proteins present in proteinuric urine may alter the behaviour of proximal tubular cells and could therefore influence the development of progressive renal disease. Although albumin is the major protein in glomerular proteinuria other components of proteinuric urine may have significant effects on tubular cell functions.
Fig 3.1. *The effect of bovine serum albumin on the uptake of tritiated thymidine by subconfluent OK cells.* Subconfluent OK cells were exposed to bovine serum albumin in the concentrations shown for 72 hours. Tritiated thymidine uptake was measured as described in the text and recorded as disintegrations per minute (dpm). The results are from 6 experiments with each condition applied in triplicate. Results were analysed by two way analysis of variance. Error bars represent 95% confidence limits. ANOVA p<0.01. * greater than control (0mg/ml), 0.1mg/ml and 10.0mg/ml.
Fig 3.2. The effect of bovine serum albumin on the protein to DNA ratio of subconfluent OK cells. Subconfluent OK cells were exposed to bovine serum albumin in the concentrations shown or to 20mM ammonium chloride, for 72 hours. Protein and DNA were measured as described in the text and the protein to DNA ratio calculated. The results are from 5 experiments with each condition applied in triplicate. BSA results were analysed by two way ANOVA and ammonium chloride results were compared separately to control by two way ANOVA. Error bars represent 95% confidence limits. BSA ANOVA p<0.01. * greater than control (0 mg/ml), 0.1mg/ml and 1.0mg/ml, ** Ammonium chloride p<0.005 compared to control (0mg/ml).
Fig 3.3. The effect of bovine serum albumin on the uptake of tritiated thymidine by confluent OK cells. Confluent OK cells were exposed to bovine serum albumin in the concentrations shown for 72 hours. Tritiated thymidine uptake was measured as described in the text. The results are from 5 experiments with each condition applied in triplicate. Results were analysed by two way ANOVA. Error bars represent 95% confidence limits. ANOVA p<0.01. ** greater than control (0 mg/ml), 0.1 mg/ml and 1.0 mg/ml. * greater than control (0 mg/ml).
**Fig 3.4.** The effect of bovine serum albumin on the protein to DNA ratio of confluent OK cells. Confluent OK cells were exposed to bovine serum albumin in the concentrations shown or to 20mM ammonium chloride, for 72 hours. Protein and DNA were measured as described in the text and the protein to DNA ratio calculated. The results are from 5 experiments with each condition applied in triplicate. BSA results were analysed by two way ANOVA and ammonium chloride results were compared separately to control by two way ANOVA. Error bars represent 95% confidence limits. BSA ANOVA p<0.05. * greater than control (0mg/ml). Ammonium chloride was not significantly different to control.
Fig 3.5. The effect of lipid free bovine serum albumin on the uptake of tritiated thymidine by confluent OK cells. Confluent OK cells were exposed to lipid free bovine serum albumin in the concentrations shown for 72 hours. Tritiated thymidine uptake was measured as described in the text. The results are from 5 experiments with each condition applied in triplicate. Results were analysed by two way analysis of variance. Error bars represent 95% confidence limits. ANOVA p<0.01. * greater than control (0mg/ml) and 10.0mg/ml.
Fig 3.6. The effect of lipid free bovine serum albumin on the protein to DNA ratio of confluent OK cells. Confluent OK cells were exposed to lipid free bovine serum albumin in the concentrations shown or to 20mM ammonium chloride, for 72 hours. Protein and DNA were measured as described in the text and the protein to DNA ratio calculated. The results are from 5 experiments with each condition applied in triplicate. BSA results were analysed by two way ANOVA and ammonium chloride results were compared separately to control by two way ANOVA. Error bars represent 95% confidence limits. BSA ANOVA p<0.01.

* greater than control (0mg/ml), 0.1mg/ml and 1.0mg/ml. Ammonium chloride was not significantly different to control.
Fig 3.7 *Electrophoresis of urine from rats with puromycin aminonucleoside nephrosis.* Urine was collected from rats 12 days after a single injection of puromycin aminonucleoside (5mg/100g body weight). SDS PAGE was performed (section 2.8.) and the gel stained with coomassie blue (section 2.9.1). Lane 1 represents proteinuric urine, lane 2 rat albumin standard and lane 3 molecular weight standards.
Fig 3.8. *The effect of dialysed proteinuric rat urine on the uptake of tritiated thymidine by confluent OK cell.* Confluent OK cells were exposed to dialysed proteinuric urine in the concentrations shown. PBSD represents medium to which dialysate phosphate buffered saline had been added as a control. The results are from 5 experiments each performed in triplicate. The results were analysed by two way analysis of variance. The error bars represent 95% confidence limits. ANOVA p<0.01. ** greater than control (PBSD), 0.01mg/ml and 1.0mg/ml, * greater than control (PBSD).
Fig 3.9. The effect of dialysed proteinuric rat urine on the protein to DNA ratio of confluent OK cell. Confluent OK cells were exposed to dialysed proteinuric urine in the concentrations shown. PBSD represents medium to which dialysate phosphate buffered saline had been added as a control. The results are from 5 experiments each performed in triplicate. The results were analysed by two way analysis of variance. The error bars represent 95% confidence limits. No significant differences were shown.
3.10. The effect of control urine □, nephrotic urine □ and control urine with addition of bovine serum albumin ■ on the tritiated thymidine uptake of confluent OK cell cultures. Confluent OK cells were exposed to PBSD, nephrotic urine at a protein concentration of 0.1 mg/ml (U-0.1) or 0.01 mg/ml (U-0.01), control urine at an equivalent dilution or diluted control urine with added bovine serum albumin at a concentration of 0.1mg/ml (U-0.1) or 0.01 mg/ml (U-0.01). The results are from 3 experiments performed in triplicate. The results for each type of urine were analysed separately by two way analysis of variance and 95% confidence limits determined. Urine at the dilution U-0.01 had no significant effect on tritiated thymidine incorporation. Control urine U-0.1 significantly increased thymidine incorporation (p<0.01). The effect of the three conditions at U-0.1 were compared by ANOVA. This showed that nephrotic urine increased thymidine incorporation to a greater level than control urine (p<0.01) and addition of BSA to control urine did not reproduce the results of nephrotic urine.
CHAPTER 4
THE DEVELOPMENT OF A HUMAN PROXIMAL TUBULAR CELL CULTURE MODEL FOR INVESTIGATION OF THE EFFECTS OF PROTEINURIA
4.1. Introduction

Although it is known that alterations in cell growth, such as those described in chapter 3, occur in progressive renal disease the mechanisms by which they may induce interstitial inflammation and scarring are not known. Further investigation of the hypothesis therefore required the development of a model in which cellular functions more specifically related to the development of inflammation and scarring could be investigated. The OK cell line used in the experiments in chapter 3 was derived from the opossum. Specific antibodies required for further investigation were not available in this species or in the alternative immortalized proximal tubular cell line LLCPK₁. The use of primary cell culture had the advantage of a more differentiated phenotype, and the choice of a species for which a greater number of analytical tools were available.

Primary proximal tubule cell cultures have been developed from the rat [Vinay P 1981, Boogaard P 1990], the rabbit [Chung S 1982] and from the human [Detrisac C 1984, Kempson S 1989]. Human cells had the advantage of greater relevance to human disease. A large number of human cells could be obtained from a single kidney preparation and the cells could be passaged several times [Detrisac C 1984] thus overcoming the restriction in availability of kidneys. For these reasons a human proximal tubular cell model was used.

Proximal tubular cells in vivo are polarised having an apical surface with a brush border facing the tubular lumen and a basolateral surface in contact with a basement membrane which faces the interstitium. In proteinuria, filtered proteins interact with proximal tubular cells on their apical surfaces whilst they may influence interstitial inflammation and scarring from the basolateral side. A model to investigate the effect of proteinuria on interstitial pathology therefore requires independent access to the apical and basolateral surfaces of the tubular cells. Recently a technique has been developed in which cells are
grown on permeable membrane supports which insert into cell culture dishes. These have been shown to increase growth and differentiation of proximal tubular cells [Handler J 1986]. The immortalised tubular cell line LLCPK1 expressed an apical sodium dependent hexose transporter [Mullin J 1980] when grown on supports and transports glucose from the apical to the basolateral side [Misfeldt D 1981]. OK cells grown on permeable supports have been used to investigate differences in the binding of insulin to the apical and basolateral membranes [Rabkin R 1989].

Rabbit proximal tubular cells on permeable supports express an apical brush border and develop an appropriate electrical resistance and transepithelial voltage [Bello-Reuss E 1986]. They also demonstrate transepithelial transport of hydrogen ions, decreasing the pH of the apical medium and increasing the pH of the basolateral medium [Ford S 1990]. Rat proximal tubular cells on permeable supports also express apical microvilli suggestive of a brush border and transport sodium in an apical to basolateral direction [Schelling J 1992]. The growth of human proximal tubular cells on permeable supports had not been extensively studied. They have however been shown express morphological polarity with formation of apical microvilli and develop an appropriate transepithelial potential difference [Blackburn J 1988]. It was therefore decided to develop a model of human proximal tubular cells grown on permeable supports to investigate the effects of apical exposure to proteins.

4.2 Methods

4.2.1 Primary Cell Culture

The method used was based on that of Detrisac C [1984]. Two sources of human renal tissue were used. The majority of the kidneys were from patients undergoing nephrectomy for the treatment of renal cell carcinoma. The second source was from kidneys...
removed as potential transplants which were considered unsuitable for transplantation for technical reasons. Kidneys for transplantation were perfused with preservation solutions and stored on ice until use which was within 24 hours of the nephrectomy. Normal kidney tissue from therapeutic nephrectomies was removed in the operating theatre and transferred to a sterile container and covered with Hanks balanced salts solution (HBSS) with the addition of benzyl penicillin (100 IU/ml) and streptomycin (50μg/ml) at +4°C.

The kidney was transferred to a tissue culture cabinet and the renal capsule was stripped using sterile forceps. The outer cortex was removed and diced using a sterile scalpel blade. The diced tissue was placed in 30ml universal containers, suspended in HBSS and centrifuged at 1000rpm for 5 minutes at +4°C. The HBSS was aspirated and this washing process was repeated three times. The tissue was transferred to a 60ml sterile pot containing 30mg of collagenase type II in 30ml of HBSS. With the lid loosely applied (to allow gas exchange) the container was placed in the tissue culture incubator. The mixture was stirred with a sterile stir bar for 30 minutes.

The collagenase reaction was stopped by addition of 10ml of HBSS containing 40mg/ml BSA. The digested kidney tissue was washed twice with HBSS and then added to the top of a sieve of 500μm mesh size. The tissue was forced through the sieve using the plunger from a 10ml syringe. It was then passed successively through 250μm and 90μm sieves. The glomeruli were retained on top of the 90μm sieve whilst the tubular fragments passed through and were collected. The tubular fragments were washed twice with HBSS before seeding into four prepared 75cm² flasks.

The flasks were prepared by coating with collagen type I and fetal calf serum. 50μg/ml of bovine collagen type I (Sigma C8919) solution was prepared in 0.1M acetic acid. 7.5ml was placed in each flask to produce 50μg collagen/cm². The flasks were left in the
culture cabinet without lids overnight to allow evaporation of the acetic acid and adherence of the collagen to the plastic. The collagen solution was aspirated and the flasks washed three times with HBSS. Heat inactivated fetal calf serum was then added to each plate in sufficient quantity to cover the bottom of the flasks which were then incubated at +4°C overnight. The fetal calf serum was removed by aspiration and the flasks washed three times with HBSS before storage at +4°C until use (within 1 month).

The cells were grown in the following medium: DMEM:F12 with the addition of 25mM HEPES buffer, recombinant human epidermal growth factor (10ng/ml), insulin (bovine) (5µg/ml), transferrin (human, iron free) (5µg/ml), sodium selenite (5ng/ml), tri-iodo-thyronine (4pg/ml), hydrocortisone (36ng/ml), benzyl penicillin (100IU/ml) and streptomycin (50µg/ml). (This medium was called defined growth medium, DGM). The medium was changed after 3 days and then on alternate days until confluent monolayers were achieved in 10 to 14 days.

Once the cells reached confluence they were passaged. The medium was removed from each flask and the cells washed three times with calcium and magnesium free HBSS (Life Technologies 14170). 5ml of trypsin/EDTA solution (Appendix 1) was added to each flask which were then incubated at 37°C for 5 minutes. The reaction was stopped by addition of 5µg soy bean trypsin inhibitor (Sigma T9003). Detachment of the cells was aided by tapping the flasks on the palm of the hand and any cells that remained attached were removed by scraping with the tip of a pipette. The cells from each flask were divided into three new flasks coated as described above and grown in the same medium as used in the primary culture. These reached confluence by 7 days and were passaged again in the same way. The second passage cells were transferred to permeable supports for experiments, to culture plates for characterisation of cAMP response to hormones, or onto microscope slides by cytospin for immunocytochemistry. Some cells were passaged a third and fourth time into 75cm²
flasks to investigate the effects of passage on morphology and phenotype.

A group of cells were stored frozen following the first passage. After passaging the
cells from one 75cm² flask were transferred to 1.0ml of DGM containing 10% (v/v) FCS +
10% (v/v) dimethyl sulphoxide (Sigma D2650). This was transferred into a cryogenic vial
which was placed into a cell freezing chamber containing isopropanol. The chamber was
placed in a freezer at -70°C for four hours. The vials containing frozen cells were then
transferred to a liquid nitrogen containing cell freezer.

4.2.2 Growth on permeable supports

Figure 4.1 demonstrates the arrangement by which the cells were grown on permeable
membrane supports. The single description in the literature of human proximal tubular cells
grown in this way used Millicell filters (Millipore) coated with collagen type I. To study the
growth of the cells on supports three different membranes were used. 1) Millicell-HA inserts
of 0.45μm pore size and made from a mixed cellulose ester membrane (Millipore PIBLA
03050). 2) Falcon inserts of 3.0μm pore size and made from polyethylene tetraphthalate
(Falcon 3092). 3) Costar inserts of 3.0μm pore size and made of a polycarbonate membrane
(Costar 3414). The falcon inserts had the advantage that eells growing on them were visible
by light microscopy whereas cells on the alternative inserts were not visible and had to be
stained before they could be visualised.

Two alternative matrices were used for coating the inserts. All the reports in the
literature in which human tubular cells were grown in culture used dishes that were coated
with collagen type I. Cell-Tak (Collaborative Biomedical Products 40240) is an alternative
matrix which has previously been used for the growth of rat proximal tubular cells on
permeable supports [Chen T 1989]. Cell Tak Cell and Tissue adhesive is a formulation of
polyphenolic proteins extracted from Mytilus Edulis (marine mussel) [Waite J 1983] and comprises the key components of the glue which the mussel uses to anchor itself to rocks.

Experiments were performed in which the defined medium was supplemented with 5% or 10% fetal calf serum (Advanced Protein Products). There are several reports in the literature demonstrating growth of human cortical epithelial cells in medium containing fetal calf serum (FCS) in amounts between 3% [Wilson P 1985] and 10% (v/v) [Kempson S 1989].

Prior to experiments the medium was changed to one in which the growth factors were not added in order that the cells became quiescent. The medium used was DMEM (Life Technologies 11880) which contains a physiological concentration of glucose (5.6mM) since high concentrations of glucose, such as occur in DMEM:F12 (17.5mM), have been shown to stimulate matrix protein and TGFβ production by proximal tubular cells [Ziyadeh F 1990, Rocco M 1992]. The serum free experimental medium (SFEM) consisted of DMEM with addition of hydrocortisone (36ng/ml), benzyl penicillin (100IU/ml) and streptomycin (50μg/ml). Hydrocortisone was used as it has been shown to improve the differentiation of rabbit proximal tubular cells in primary culture [Rocco P 1990].

4.2.3 Coating permeable well insert supports

a) Collagen type I

A 60% (v/v) ethanol solution was made using absolute alcohol and sterile water. Type I collagen solution (0.1% w/v in 0.1M acetic acid) was mixed with this in the ratio 1 part collagen to 3 parts 60% ethanol. 400μl of this mixture was added to each insert in a 6 well plate. The plates were kept uncovered in the culture cabinet for 4 hours or overnight until the solution had completely evaporated. The inserts were washed three times with HBSS and stored at +4°C until use (within 1 week).
b) Cell Tak Cell and Tissue Adhesive

Cell Tak solution (40μg/ml) was made up in 0.1M sodium bicarbonate pH 8.0. 400μl of the coating solution was then added to the inserts in 6 well plates and allowed to stand at room temperature for 30 minutes. The inserts were washed three times with HBSS and stored at +4°C until use (within 1 week).

4.2.4 Staining of cells on permeable supports

In order to visualise the cells on the supports they were stained using Giemsa. The growth medium was removed from the cells and they were washed with PBS. Sufficient methanol was placed in the wells to ensure that the cell monolayers were completely covered. They were incubated at room temperature for 15 minutes. Giemsa stain (BDH 35014 4M) was diluted 1:10 with buffered deionised water pH 6.8. Diluted stain was added to the inside and outside of the insert to cover the cells which were then incubated for 15 minutes at room temperature. The stain was removed with several rapid rinses of buffered water (pH 6.8) and then the insert was allowed to stand in buffered water for a several minutes. The inserts were removed from the wells and air dried. The permeable membrane was cut from the supporting plastic using a scalpel blade and placed on a microscope slide for mounting in Xam (BDH 36119) and inspection by light microscopy.

4.2.5 Cell characterisation

The following characterisation studies were carried out on each of the cell preparations used.

a) Morphology

Morphology of the cells was examined using an inverted microscope.
b) Immunostaining of cytopsins

Cytokeratin, epithelial membrane antigen and factor VIII related antigen (Factor VIII Rag) were detected using immunocytochemistry. Cytokeratin and epithelial membrane antigen are expressed by epithelial cells and factor VIII RAg is expressed by endothelial cells. Since factor VIII RAg should be negative on tubular epithelial cells a positive control was used which was cultured human umbilical vein endothelial cells (HUVEC, a generous gift from Dr Bryan Williams, Department of Medicine, Leicester).

Following passage cells suspended in HBSS were spread on microscope slides using a Shandon Cytospin 2. The cells were fixed on the slides by immersion in a 1:1 mixture of methanol and acetone for 90 seconds and then washed twice with Tris buffered saline pH7.6 (TBS, Appendix 1). 50μl of primary antibody diluted in TBS was added to the cells in the following dilutions:

- 1:50 mouse monoclonal anti human cytokeratin (Dako M821)
- 1:100 mouse monoclonal anti human epithelial membrane antigen (Dako M613)
- 1:20 mouse monoclonal anti human factor VIII related antigen (Dako M616)

The slides were incubated for 1 hour in a humidified box at room temperature. They were washed twice with TBS pH7.6 prior to addition of 50μl of the secondary antibody which was an alkaline phosphatase conjugated rabbit antimouse immunoglobulin (Dako D314) (1:50 in TBS). This was incubated for 1 hour at room temperature and then washed three times with TBS pH7.6. During this incubation the preprepared alkaline phosphatase substrate solution (Appendix 1) was defrosted. This contained levamisole for the inhibition of endogenous kidney alkaline phosphatase [Van Belle H 1972]. 2mg of Fast Red TR salt (Sigma F1500) was added to 2ml of substrate solution and vortexed thoroughly until dissolved. The solution was filtered.
50µl of substrate solution was added to each slide and incubated for 20 minutes before washing twice with TBS pH7.6. The cells were counter stained with haematoxylin for 30-60 seconds and then washed with running tap water. The slides were mounted in Aquamount (BDH 36086) and examined by light microscope.

c) α-Naphthyl acetate esterase (non-specific esterase)

This enzyme is present in proximal tubular cells but not in glomerular epithelial cells and therefore provides a useful distinguishing marker [Detrisac C 1984]. Slides produced by cytocentrifugation were fixed by immersion in citrate/acetone/formalin fixative (Appendix 1) for 30 seconds and vigorously agitated for 5 seconds. The slides were rinsed for 60 seconds in running tap water and then placed in a Coplin jar containing preprepared staining solution (Appendix 1). The jar was incubated at 37°C for 30 minutes. The slides were rinsed under running tap water for 2 minutes and then counterstained with haematoxylin for 2 minutes. They were washed with tap water and the slides mounted using Aquamount for examination by light microscope.

d) Electron Microscopy

To investigate the morphology of the cells on the inserts they were examined by electron microscopy which was performed by Mrs E. Roberts in the electron microscopy section of the department of Biology, University of Leicester. Cells, at second passage were grown on well inserts as previously described. Having reached confluence, after 7 days in culture, the medium was changed to serum free experimental medium (section 4.2.2). After 48 hours the medium was aspirated from the cells and they were washed twice with HBSS. The inserts were then immersed in a fixative containing 2.5% glutaraldehyde (Appendix 1).
and were transferred to the electron microscopy laboratory. Scanning and transmission electron microscopy was performed by the standard techniques used in the laboratory.

c) Cyclic AMP production in response to hormones

Cyclic AMP response to vasopressin and parathyroid hormone was determined by standard method [Kempson S 1989]. At second passage the cells were transferred to 12 well plates which had been coated with type I collagen as described above. They were grown in medium containing 5% FCS and reached confluence after 7 days. They were then transferred to serum free experimental medium for 24 hours. The medium was removed and the cells rinsed twice with HBSS. Three conditions were tested:

i) SFEM + 5mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma I7018)

ii) SFEM + 5mM IBMX + 10^{-7}M parathyroid hormone fragment 1-34 (Sigma P3796)

iii) SFEM + 5mM IBMX + 10^{-7}M arginine vasopressin (Sigma V5501)

IBMX is a phosphodiesterase inhibitor used to prevent the breakdown of cAMP [Montague W 1970]. The concentration of PTH and AVP used is known to cause maximal stimulation of cAMP production in human proximal and distal tubular cells respectively [Van Der Biaet I 1994]. 1ml of the media was added to each well and the plate returned to the culture incubator for 1 hour. The media were aspirated from the wells and discarded. 500µl of 0.01M hydrochloric acid was added to each well to extract the cellular cAMP [Wang X 1993]. The plate was left on ice for 30 minutes. The acid was then removed from the cells and stored at -70°C until assayed (a maximum of two weeks). The cells were washed with PBS and 500µl of 0.5M sodium hydroxide was added to each well. After several hours at +4°C the cells had fully dissolved and the protein content of the cell solution was assayed by BioRad DC assay (section 2.2.3).
Cyclic 3'5' adenosine monophosphate was assayed by radioimmunoassay (Amersham RPA 509). Standards were prepared in the range 25-1600fmol in 100µl. Cell extracts were diluted with a 0.05M acetate buffer pH5.8 to provide cAMP concentrations in the range of the standards. 100µl of samples and standards was pipetted into assay tubes in duplicate. 100µl of 125I-cAMP was added followed by 100µl of rabbit anti succinyl cAMP serum. After thorough mixing the tubes were incubated at +4°C for 3 hours. 500µl of the second antibody (donkey anti rabbit serum) was added to each tube and the mixture incubated for 10 minutes at room temperature. The tubes were centrifuged at 1500g for 10 minutes. The supernatant was discarded. The radioactivity was counted in each tube on a gamma scintillation counter. A standard curve was constructed and the sample concentrations read directly from the graph.

4.2.6 Permeability of the monolayers

In order to use this cell culture model to investigate the effects of proteins presented to the apical surface of tubular cells it was important to ensure that proteins added to the apical medium did not cross the monolayer into the basolateral medium. The monolayer permeability to proteins was measured using radiolabelled albumin. An increased permeability of epithelial monolayers, to small molecules, can be used as a measure of injury to the monolayer [Kroshian V 1994] and therefore the permeability to radiolabelled inulin was determined.

4.2.6.1 Permeability to labelled human albumin

Cells were grown on permeable membrane supports in 6 well plates as described above. After 7 days growth the medium was changed to SFEM (section 4.2.2). After a further 24 hours the medium was replaced with fresh SFEM and 10µl of 125I labelled human
albumin (Amersham IM.17P: 20mg/ml human albumin 1.85MBq/ml) was added to the medium bathing the upper surface of the cells. After 24 and 48 hours the apical and basolateral media were sampled. 50μl of medium was counted using a gamma counter. The percentage of albumin added to the apical medium which crossed to the basolateral medium was determined. Some albumin added to the apical medium would be endocytosed by the cells and then metabolised and released into the basolateral medium. To differentiate between leakage between the cells of the monolayer and the metabolic pathway, intact protein was precipitated by the addition of trichloroacetic acid (TCA) to the medium to achieve a final concentration of 10% (w/v). The precipitate was removed by centrifugation and the radioactivity remaining in the medium counted to quantify that portion of total activity that could be explained by endocytosis and metabolism of the albumin.

4.2.6.2 Monolayer permeability to inulin

Cells were grown on the permeable membrane supports for 7 days. The medium was then changed to SFEM to achieve quiescence. After 48 hours exposure to experimental conditions the permeability to inulin was measured. The apical medium was replaced by 1.0ml SFEM containing 0.02MBq/ml of 14C-inulin carboxylic acid (Amersham CFA 399) whilst the basolateral medium was replaced by 1.5ml of SFEM alone ensuring that the levels of fluid in the apical and basolateral medium were equal so that there was no hydrostatic pressure gradient. After 2 hours in the cell culture incubator the media were removed. 250μl of each medium was added to 4ml of Ecoscint A in a scintillation vial and the activity counted on a liquid scintillation counter.

The permeability of the monolayer to inulin was calculated as the flux of inulin per unit time relative to the concentration gradient for inulin and the surface area of the
monolayer using the following formula:

\[
P = \frac{\text{total basolateral counts}}{(\text{apical counts/ml} \times 4.7 \times 2) - \text{basolateral counts/ml}} \text{ cm/hr}
\]

4.7 is the surface area of the monolayers in cm\(^2\)

2 is the experiment time in hours

4.3 Results

4.3.1 Primary cell culture

The cells grown using these techniques were designated as HTEC (human tubular epithelial cells). Following collagenase digestion and sieving the material which passed through the 90\(\mu\)m sieve was examined using the inverted microscope. The suspension contained tubular fragments of varying sizes, single cells and a few fragmented glomeruli. After 3 days in culture the fragments had attached to the coated plates and cells were explanting from them. Single cells did not appear to attach. After 7-10 days a confluent monolayer of epithelial cells was achieved although occasional cells could be seen with a morphology consistent with fibroblasts or mesangial cells. After a further 3 to 4 days dome formation was seen (Fig 4.2) which is a feature of transporting epithelia [Detrisac C 1984, Kempson S 1989]. Following the first and second passages HTEC maintained epithelial morphology. The fibroblast like cells were not detectable after the first passage. After the third passage there was poor attachment and poor growth of cells in serum free medium and they failed to reach confluence. Confluent monolayers were achieved using medium containing 5\% fetal calf serum. From third passage onwards there were changes in the morphology of HTEC which became larger and more elongated suggesting a degree of dedifferentiation. Dome formation was seen after the second passage but not after subsequent passages.
Following storage and freezing of the cells in liquid nitrogen they would not attach to coated 75cm² flasks in defined medium. Grown in the presence of 5% FCS in the medium, the cells were enlarged and of similar morphology to that seen in unfrozen cells in the later passages.

4.3.2 Characterisation studies

a) Immunocytochemistry

The staining of HTEC from all 10 kidney preparations used was the same and is summarised in table 4.1 and demonstrated in figures 4.3 - 4.5. More than 99% of cells were cytokeratin positive and factor VIII Rag negative compared to 100% FVIII Rag positivity in the HUVECS. 80±5% of cells were epithelial membrane antigen positive. These characteristics demonstrate that the cells were of epithelial origin and were not contaminated by endothelial cells. They are compatible with those previously described for human proximal tubular epithelial cells [Detrisac C 1984].

<table>
<thead>
<tr>
<th></th>
<th>HTEC</th>
<th>HUVEC</th>
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<tbody>
<tr>
<td>Cytokeratin</td>
<td>Positive (&gt;99%)</td>
<td>Negative (100%)</td>
</tr>
<tr>
<td>Epithelial Membrane Antigen</td>
<td>Positive (80±5%)</td>
<td>Negative (100%)</td>
</tr>
<tr>
<td>Factor VIII Rag</td>
<td>Negative (100%)</td>
<td>Positive (&gt;99%)</td>
</tr>
<tr>
<td>Non specific esterase</td>
<td>Positive (&gt;99%)</td>
<td>N/A</td>
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Table 4.1. Immunostaining and histochemistry of HTEC compared to HUVEC.
b) **Non specific esterase (NSE) activity**

Non specific esterase activity was positive in more than 99% of HTEC (Fig 4.6).

c) **Cyclic 3'5' adenosine monophosphate production**

The cAMP results are shown in figure 4.7. The data was log transformed to equalise the variances between the groups and two way ANOVA was performed on the transformed data. There was a significant increase in cAMP on exposure to vasopressin but a greater increase on exposure to parathyroid hormone. AVP increased cAMP by a mean of 3.8 fold with a range between experiments of 1.02 to 10.72 fold. PTH increased cAMP by a mean of 12.3 fold with a range of 3.7 to 21.1 fold. In every cell preparation tested there was a greater increase in cAMP produced in response to parathyroid hormone than in response to vasopressin.

The characteristics described are those of tubular epithelial cells. The response to arginine vasopressin suggests that there may have been some distal tubular cells present but the predominant response to parathyroid hormone suggests that the majority of cells were of proximal tubular origin.

**4.3.3 Cell growth on well inserts**

When the cells were grown in defined medium containing no fetal calf serum few cells attached to any of the types of insert and subsequent growth was poor. By the third day the cells were detaching from the inserts. However in the presence of fetal calf serum satisfactory growth was achieved with no difference between those grown in 5% and 10% FCS. Figures 4.8 and 4.9 show cells grown for 7 days in medium containing 5% FCS stained with Giemsa. The results for Millicell filters and Falcon filters were indistinguishable and only the cells on
Falcon filters are shown. Using Falcon or Millicell filters coated with collagen type I initial attachment of cells was poor and subsequent growth limited such that large holes remained in the monolayers after 10 days (Fig 4.8A). When Falcon or Millicell filters were coated with Cell Tak the initial adhesion was improved and growth continued, however as shown in Fig 4.8B the cells did not form smooth monolayers but tended to overlap. Cells grown on Costar filters coated with collagen type I or Cell Tak readily attached to the inserts and grew to achieve uniform confluent monolayers (Fig 4.9). Best results were achieved when the number of cells added to the inserts following passage was in the ratio $1 \text{cm}^2$ of confluent flask to $2 \text{cm}^2$ of permeable membrane.

4.3.4 Electron microscopy

Figure 4.10 shows transmission electron microscopy of confluent cells growing on Costar inserts coated with collagen type I. The cells formed a polarised monolayer with the nucleus situated toward the side of the cell in contact with the permeable support. The apical surface of the cells had scanty, small, microvillus projections with numerous vacuoles beneath the surface. In defined areas the microvilli were more prominent (Fig 4.11A) although they did not form the dense brush border typical of proximal tubule cells in vivo. The microvilli were readily visualised by scanning electron microscopy of the apical surface of the monolayer (Fig 4.11B).

4.3.5 Permeability to albumin

The permeability to albumin of monolayers grown on Costar supports and coated with type I collagen, was very low. At 24 hours $0.48 \pm 0.10\%$ of $^{125}$I added to the apical medium had crossed to the basolateral medium. By 48 hours this was $0.94 \pm 0.25\%$. Of the basolateral
counts 56.9±7.8% were removed from the medium by precipitation with 10% TCA. This represents albumin leaking between cells.

4.3.6 Permeability to inulin

The permeability of the monolayers grown on Costar supports and coated with type I collagen, to inulin was 1.3±0.3 x 10^{-5} cm/hr. This is similar to the permeability of mouse proximal tubular monolayers, that has previously been described, of 1.2±0.4 x 10^{-5} cm/hr [Kroshian V 1994].

4.4 Discussion

The characteristics of HTEC were the same in each kidney preparation and consistent with those described in the literature for cells prepared by similar methods. The morphology of the cells was consistent with a transporting epithelium. The absence of dome formation and altered morphology after the third passage and after frozen storage suggested a degree of dedifferentiation. Stored cells were therefore not used in experiments. The immunocytochemistry confirmed the epithelial nature of HTEC. Most of the glomerular epithelial cells were removed by the 90μm sieve. This was confirmed by positive staining for non specific esterase. HTEC produced large amounts of cAMP on exposure to parathyroid hormone, a characteristic of proximal tubular cells. The smaller response on exposure to arginine vasopressin would suggest that there may also have been some distal tubular cells present. In each experiment there was a greater response to PTH than to AVP indicating that proximal tubular cells predominated.

To achieve confluent monolayers it was necessary to use medium containing 5% fetal calf serum. There were significant differences in the degree of attachment and growth of
HTEC when seeded onto different membrane supports coated with different matrices. The polycarbonate membrane manufactured by Costar produced a uniform monolayer of polarised cells. Type I collagen and Cell Tak were equally good for achieving tight monolayers on the polycarbonate membrane. Since collagen type I is likely to be closer in composition to the tubular basement membrane in vivo this was the matrix chosen for the experiments.

The HTEC model used for further studies was as follows:

Up until the second passage HTEC were grown in defined growth medium (DGM) consisting of: DMEM:F12 containing 25mM HEPES buffer, epidermal growth factor (10ng/ml), insulin (5μg/ml), transferrin (5μg/ml), sodium selenite (5ng/ml), hydrocortisone (36ng/ml), benzyl penicillin (100IU/ml) and streptomycin (50μg/ml).

Following the second passage the medium used was DGM + 5% (v/v) fetal calf serum.

Experiments were performed in a serum free medium (SFEM) consisting of: DMEM (5.6mM D-glucose) with addition of hydrocortisone (36ng/ml), benzyl penicillin (100IU/ml) and streptomycin (50μg/ml).

The cells were grown on Costar polycarbonate permeable membranes of 3.0μm pore size and coated with type I collagen.

Under these conditions the monolayers obtained were virtually impermeable to albumin allowing less than 2% of albumin added to the apical medium to leak to the basolateral medium over 48 hours and had a permeability to inulin similar to that previously described for healthy mouse proximal tubular monolayers. They also had appropriate polarised morphology on electron microscopy.
Fig 4.1. *The experimental layout for the growth of human tubular cells on permeable membrane supports*
Fig 4.2. The morphology of first passage HTEC grown on plastic plates coated with collagen type I and adsorbed fetal calf serum proteins (x 20). Dome formation (arrows) indicates a transporting epithelium.
Fig 4.3. Cytospins of HTEC (A) and HUVEC (B) stained for cytokeratin using an alkaline phosphatase immunostaining technique. Red indicates positive staining.
Fig 4.4. Cytospins of HTEC (A) and HUVEC (B) stained for epithelial membrane antigen using an alkaline phosphatase immunostaining technique. Red indicates positive staining.
Fig 4.5. Cytospins of HTEC (A) and HUVEC (B) stained for factor VIII RAg using an alkaline phosphatase immunostaining technique. Red indicates positive staining.
Fig 4.6. Cytospin of HTEC stained for non specific esterase. Black indicates positive staining.
Fig 4.7. The production of cAMP by HTEC in response to arginine vasopressin (AVP) or parathyroid hormone (PTH). HTEC were exposed to $10^{-6}$M AVP or $10^{-6}$M PTH for 1 hour. Cellular cAMP was extracted with hydrochloric acid and assayed by radioimmunoassay. The results are from 7 kidney preparations. Because the variance in each group was different the data was log transformed. ANOVA $p<0.001$ on the transformed data. The error bars represent 95% confidence limits. * statistically greater than IBMX. ** statistically greater than IBMX and AVP.
Fig 4.8. The growth of HTEC on Falcon (polyethylene tetrathalate) permeable membrane supports coated with (A) collagen type I or (B) Cell Tak. HTEC were stained with Giemsa. Arrows indicate areas of heaping up of cells grown on Cell Tak coated supports. Large holes can be seen in the monolayers grown on collagen I coated supports. Indistinguishable results were obtained using Millipore (mixed cellulose ester) supports.
Fig 4.9. The growth of HTEC on Costar (polycarbonate) permeable membrane supports coated with (A) collagen type I or (B) Cell Tak. Stained with Giemsa.
Fig 4.10. *Transmission electron microscopy of HTEC grown on permeable membrane supports (x 5500)*
Fig 4.11. Transmission (A) and scanning (B) electron microscopy of the apical surface of HTEC grown on permeable membrane supports. Magnification A: x 16500 and B: x 6000.
CHAPTER 5

PRODUCTION OF FIBRONECTIN BY CULTURED HUMAN TUBULAR EPITHELIAL CELLS AND THE EFFECT OF SERUM PROTEINS
5.1 Introduction

As discussed in chapter 1 proximal tubular cells can produce a number of matrix proteins which contribute to the tubular basement membrane but which may also contribute to interstitial scarring. Fibronectin production has recently been described from human tubular epithelial cells exposed to TGFβ1 [Viedt C 1995]. The experiments described in this chapter were designed to characterise the production of fibronectin by human tubular epithelial cells grown on permeable membrane supports and to determine the effect of apical exposure of the cells to serum proteins.

A damaged glomerulus leaks proteins into the ultrafiltrate. The leakage of proteins is determined largely by their size although charge also has an important effect (section 1.9.1). It was not possible to obtain sufficient quantities of tubular fluid and therefore an alternative model for the proteins to which tubular cells are exposed was required. Proteinuric urine, as used in chapter 3, was considered but has the disadvantage that proteins may be added, removed or altered in their passage through the urinary tract. It was therefore concluded that the best model to use would be a dilute solution of serum proteins.

5.2 Methods

5.2.1 Fibronectin production by HTEC and the effect of passaging

Human tubular epithelial cells were grown as described in section 4.2.1. Following the second, third and fourth passages HTEC were transferred to Costar well inserts coated with collagen type I. The cells were fed on alternate days with defined growth medium + 5% FCS (section 4.4). After 7 days the cells had reached confluence. The medium was then changed to serum free experimental medium (SFEM, section 4.4). After 24 hours to achieve quiescence of the cells, the apical and basolateral media were replaced with fresh SFEM. The
volumes of the apical (2.0 ml) and basolateral (3.0 ml) media were such that there was no hydrostatic pressure gradient across the cells. After 24 hours, samples (0.5 ml) of the apical and basolateral media were collected. After 48 hours all the medium was aspirated from the apical and basolateral sides and the media were stored at -20°C. The cells were then rinsed three times with HBSS and dissolved in WB+1% nonidet P40 as described in section 2.4.2. Total cell protein was measured in the cell solution as described in section 2.2.3.

Fibronectin was measured in the stored media by ELISA (section 2.9). The specificity of the antibodies used in the ELISA, for fibronectin, in the conditioned medium, was evaluated by Western blotting (section 2.11) following concentration of the medium by a factor of 10 using Minicon concentrators (Amicon 9031).

The effect of passaging HTEC on the permeability of the monolayers to albumin was determined as described in section 4.2.6.1.

5.2.2 The effects on HTEC of apical exposure to serum proteins

Serum proteins were obtained from normal controls, fibronectin depleted, dialysed and stored at a concentration of 10 mg/ml as described in section 2.7 (fibronectin depleted serum: ENDS). Second passage HTEC were transferred to Costar well inserts coated with collagen type I and grown as described above (5.2.1). After 24 hours quiescence in SFEM the basolateral medium was replaced with 3.0 ml of fresh SFEM and the apical medium was replaced with 2.0 ml of medium prepared as follows:

<table>
<thead>
<tr>
<th>Protein concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) 9 ml SFEM + 1 ml PBSD (section 2.7)</td>
</tr>
<tr>
<td>B) 9 ml SFEM + 0.1 ml ENDS + 0.9 ml PBSD 0.1 mg/ml</td>
</tr>
<tr>
<td>C) 9 ml SFEM + 1.0 ml ENDS 1.0 mg/ml</td>
</tr>
</tbody>
</table>
After 24 hours and 48 hours samples of the apical and basolateral media were collected as described above (5.2.1) and analysed as follows:

i) Fibronectin was measured by ELISA (section 2.9).

ii) Lactate dehydrogenase release into the culture medium was measured (section 2.7.2) as a marker of cytotoxicity.

After aspiration of the culture medium at 48 hours the inulin permeability (section 4.2.6.2) and the tritiated thymidine incorporation (section 2.4.2) of the monolayers was measured over a 2 hour incubation. The LKB 1219 liquid scintillation counter was able to distinguish between the counts of $^{14}$C-inulin and $^{3}$H-thymidine in the medium. The cells were then rinsed with HBSS and dissolved in WB + 1% nonidet P40 as described in section 2.4.2.

The cell solution was used for:

i) Measurement of thymidine uptake (section 2.4.2)

ii) Total cell protein by BioRad DC assay (section 2.2.3)

iii) Assay of cell associated fibronectin (section 2.9).

On separate plates of HTEC cultures, run in parallel, the following were determined:

i) The permeability of HTEC monolayers to $^{125}$I-human albumin in the presence and absence of apical FNDS (1.0mg/ml) (section 4.2.6.1).

ii) Cell viability by trypan blue exclusion (section 2.7.1b).
5.3 Results

5.3.1 The effect of passage on fibronectin production by HTEC

In unstimulated HTEC fibronectin was detectable (>100ng/ml) in the apical medium after 24 hours in three out of the 5 kidney preparations at passage 2. In the single kidney preparation subcultured beyond passage 2 fibronectin was not detectable in the apical medium after 24 hours at passage 3 but was detectable at passage 4 (4.40±0.970 μg/μg cell protein). Fibronectin was detectable in the basolateral medium at 24 hours and in the apical and basolateral media at 48 hours in all the kidney preparations at passage 2. HTEC at passages 3 and 4 secreted greater amounts of fibronectin into the apical and basolateral media than HTEC at passage 2 (Figs 5.1 and 5.2). There was also increased cell associated fibronectin at passage 4 (Fig 5.3).

The concentration of fibronectin in the basolateral medium was greater than that in the apical medium, i.e. the secretion of fibronectin was polarised. A measure of the polarity of secretion is given by the ratio of basolateral:apical secretion after correcting for the different apical and basolateral volumes. The polarity of fibronectin secretion reduced with increasing passage. At the second passage 2.9±0.6 times more fibronectin was secreted basolaterally than apically whilst by the fourth passage the ratio was 2.1±0.3 (p<0.05). Passaging the cells also reduced their ability to maintain a tight monolayer. Whilst at the second passage 1.1±0.3% of apical albumin leaked to the basolateral medium over 48 hours, at the third passage this was 2.4±0.6% (p<0.01 compared to P2) and by the fourth passage the leak had increased to 5.0±1.25% (p<0.01 compared to P3).

Despite the increased permeability it is unlikely that a leakage of fibronectin across the monolayer accounted for the apparent decrease in polarity of fibronectin secretion at the fourth passage. The leak of fibronectin is likely to be less than albumin since it is of greater
molecular weight (443kD vs 67kD for albumin) and even a 5% leakage of fibronectin over 48 hours would not entirely account for the decreased ratio of apical to basolateral fibronectin. The change in polarity with passage is therefore a genuine change to the cells phenotype.

Since passage altered the morphology of HTEC (section 4.3.1) and also altered the integrity of the monolayer and the fibronectin secretion it was decided that all subsequent experiments would be performed using cells from the second passage only.

5.3.2 The production of fibronectin by unstimulated second passage HTEC

There was considerable variation in the amount of fibronectin produced by different kidney preparations as shown in Table 5.1. For this reason when comparing effects of different experimental conditions it was ensured that the conditions were compared to controls run in parallel on the same kidney preparation. The replicates within each kidney preparation were acceptably reproducible, for a biological system, as shown by the coefficients of variation in Table 5.1.

<table>
<thead>
<tr>
<th></th>
<th>Mean µg/mg cell protein</th>
<th>Minimum µg/mg cell protein</th>
<th>Maximum µg/mg cell protein</th>
<th>Between replicate CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours apical</td>
<td>0.98</td>
<td>0</td>
<td>1.82</td>
<td>23.7%</td>
</tr>
<tr>
<td>24 hours basolateral</td>
<td>4.12</td>
<td>0.88</td>
<td>9.15</td>
<td>24.0%</td>
</tr>
<tr>
<td>48 hours apical</td>
<td>2.74</td>
<td>0.76</td>
<td>4.21</td>
<td>15.7%</td>
</tr>
<tr>
<td>48 hours basolateral</td>
<td>10.29</td>
<td>2.57</td>
<td>36.25</td>
<td>20.0%</td>
</tr>
<tr>
<td>48 hours cell associated</td>
<td>4.45</td>
<td>0.498</td>
<td>13.15</td>
<td>21.1%</td>
</tr>
</tbody>
</table>

Table 5.1. The range of fibronectin production between different kidney preparations and the coefficient of variation (CV) of the three replicates in each experiment.
Secretion of fibronectin from all of the kidney preparations used was polarised and the ratio of basolateral:apical fibronectin secretion is shown in Table 5.2. There was no statistical difference between the polarity at 24 and 48 hours.

<table>
<thead>
<tr>
<th></th>
<th>0 mg/ml</th>
<th>1.0 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>(4.2±0.7)</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td>48 hours</td>
<td>3.8±0.8</td>
<td>3.5±0.4</td>
</tr>
</tbody>
</table>

**Table 5.2.** The ratio of basolateral to apical fibronectin secretion of HTEC grown on permeable membrane supports. The results are from 5 kidney preparations and 13 separate serum samples each in triplicate. The errors represent 95% confidence limits. At 24 hours in unstimulated HTEC from 2 kidney preparations, fibronectin was not detectable in the apical medium and the results from these wells were therefore excluded. There were no significant differences at the two time points or on addition of FNDS.

### 5.3.3 Western blot for fibronectin in HTEC conditioned medium

To ensure that the fibronectin ELISA was detecting fibronectin and not breakdown products of fibronectin or other proteins in the media, Western blots were performed using concentrated conditioned culture medium. The mouse monoclonal antibody (Sigma F7387) detected the fibronectin standard which produced a number of bands of similar molecular weight. This antibody did not detect fibronectin in the conditioned medium presumably because of the low concentration of fibronectin present. The rabbit polyclonal antibody (F3648) detected fibronectin in the standard and also in apical and basolateral conditioned media at the same molecular weight as the standard (Fig 5.4). No other proteins were detected by this antibody. It can therefore be concluded that the rabbit polyclonal antibody is specific for fibronectin and that the ELISA must therefore also be specific.
5.3.4 The effects on HTEC of apical exposure to serum proteins

An initial experiment was performed investigating the effect of 0.1 mg/ml and 1.0 mg/ml of apical FNDS on second passage HTEC from a single kidney. Four separate serum samples from normal controls were used. Addition of 0.1 mg/ml of FNDS to the apical medium did not significantly alter fibronectin secretion into the basolateral medium at 24 hours or into the medium on either side of the cells at 48 hours. There was, however, an increase in fibronectin secretion into the apical medium at 24 hours (0.41±0.24 μg/mg cell protein in controls, 1.03±0.24 μg/mg cell protein on apical exposure to 0.1 mg/ml serum). Addition of 1.0 mg/ml of FNDS to the apical medium increased fibronectin secretion into the apical and basolateral media at both 24 and 48 hours and for this reason subsequent experiments concentrated on the effect of 1.0 mg/ml serum protein.

The results of exposure of second passage HTEC to 1.0 mg/ml of FNDS are shown in Figures 5.5 and 5.6. The results are from 5 kidney preparations and serum samples from 13 normal controls. Apical exposure to 1.0 mg/ml FNDS significantly increased fibronectin secretion, by 3.34±1.34 fold apically and 2.53±0.39 fold basolaterally at 24 hours and by 2.29±0.25 fold apically and 2.36±0.28 fold basolaterally at 48 hours. The polarity of fibronectin secretion was not significantly altered by apical exposure 1.0 mg/ml of FNDS (Table 5.2). Cell associated fibronectin (measured in four kidney preparations with 10 serum samples) was not altered by FNDS (4.45±0.81 μg/mg cell protein in controls, 4.75±0.91 μg/mg cell protein on exposure to serum). To ensure that the effect of FNDS on fibronectin production by HTEC was not the result of a change to the serum during the process of fibronectin depletion an experiment was carried out using serum (1.0 mg/ml) in the apical medium which had not been fibronectin depleted. In this experiment basolateral secretion of fibronectin on exposure to serum increased by 1.87±0.50 fold at 24 hours (p<0.01) and by
1.54±0.40 fold at 48 hours (p<0.01). Thus unchanged serum had a comparable effect on basolateral fibronectin secretion to FNDS.

The permeability of the monolayers to albumin and inulin was increased by 1.0 mg/ml apical FNDS. Figure 5.7 demonstrates increased permeability to labelled albumin on exposure to FNDS at both 24 and 48 hours (p<0.01). Despite this the albumin permeability remained low with a mean of less than 2% leakage from apical to basolateral medium over 48 hours in the presence of FNDS. The inulin permeability of the monolayers also increased by 2.36±0.56 fold (p<0.01) on exposure to FNDS (Fig 5.8).

Increased lactate dehydrogenase was released into the culture medium on exposure to FNDS (Fig 5.9). Because it is possible that the increase in LDH could reflect increased total cellular LDH without a change in the number of injured cells the results were confirmed in an experiment using one kidney preparation and four serum samples by measuring LDH released as a fraction of total cellular LDH. In controls the fraction released was 0.05±0.01 which increased to 0.08±0.01 on apical exposure to FNDS (p<0.001). Total cellular LDH decreased on apical exposure to FNDS (4.69±23IU/mg cell protein in controls, 4.31±0.23IU/mg cell protein on exposure to 1.0mg/ml apical FNDS p<0.05). Therefore the increased LDH release into the medium is an indication of cellular toxicity. The viability of the cells as measured by trypan blue exclusion did not change. Under control conditions 96.1±2.4% of HTEC were viable at the end of the experiments compared to 95.0±1.4% in cells exposed to FNDS.

Tritiated thymidine incorporation by HTEC was determined as a measure of cell proliferation. There was a small but significant increase in tritiated thymidine incorporation on exposure to FNDS (Fig 5.10). This indicates increased proliferation of cells although the total cell protein was not altered by exposure to FNDS (0.81±0.04 mg/well in controls, 0.82±0.06 mg/well on exposure to 1.0 mg/ml of apical FNDS).
5.4. Discussion

This series of experiments demonstrated that HTEC can produce the matrix protein fibronectin and human proximal tubular cells would therefore be able to contribute to the increase in interstitial matrix which characterises interstitial scarring in chronic progressive glomerular disease. Moreover fibronectin secretion was polarised with the greater amount of fibronectin being secreted basolaterally i.e. in the direction of the interstitial compartment. The pattern of fibronectin production was altered by increasing the number of passages. Baseline production increased substantially in the fourth passage cells compared to second passage cells. There was also a decrease in the degree of polarity of fibronectin secretion. This may have been influenced by an increase in permeability of the monolayers however this is unlikely to account entirely for the decreased difference in apical and basolateral fibronectin concentration in the fourth passage. In view of these differences and the changes in morphology described in chapter 4 it was decided that further experiments should be performed on second passage cells only.

Addition of 0.1mg/ml of FNDS to the apical medium only affected fibronectin secretion into the apical medium at 24 hours. 1.0mg/ml of FNDS, however, consistently increased fibronectin production by HTEC into both the apical and basolateral media whilst the polarity of secretion was maintained. Interestingly however the cell associated fibronectin was not altered raising the possibility that there may be different mechanisms of control for secretion into the medium and association with the cells. In the context of the in vivo tubular cell there may therefore be different mechanisms of release of fibronectin into the basement membrane (cell associated) or into the interstitial space (secreted fibronectin).

Many cells require the presence of serum in the medium to thrive in cell culture. Indeed HTEC needed the presence of 5% fetal calf serum to grow on the permeable supports.
It could therefore be argued that the presence of human serum simply improved the culture conditions so that the cells made fibronectin more actively. It was with this in mind that the polarized culture conditions were designed since tubular cells in vivo are not exposed to serum proteins on their apical side and effects of apical proteins are therefore more likely to be of pathological significance. To further address this issue two measures of cell viability were used. It would be expected that if the cells were in improved conditions then their viability would improve whereas trypan blue exclusion demonstrated similar, low numbers of non-viable cells in the presence and absence of serum. Trypan blue exclusion is not a sensitive method for detecting differences in viability and so in addition the release of lactate dehydrogenase into the medium was measured. There is no normal method for secretion of lactate dehydrogenase by cells and hence any LDH in the medium must have originated from injured cells. LDH release increased in the presence of apical serum suggesting that far from improving the culture conditions the serum was exerting a cytotoxic effect. A degree of cytotoxicity would also explain why the monolayers become more permeable to albumin and inulin on exposure to serum. Changes in permeability have previously been used to indicate toxicity to monolayers [Kroshian V 1994].

Exposure of HTEC to serum resulted in an increase in tritiated thymidine incorporation. The mechanism of this is not certain although it could be a response to increased cell death and consequent loss of contact inhibition.

In summary these experiments indicated polarised secretion of the matrix protein fibronectin by HTEC and increased secretion on exposure to apical serum proteins at 1.0 mg/ml. The increased secretion was associated with increased permeability of the monolayers and increased release of LDH into the medium.
Fig 5.1. *The effect of passage number on the constitutive basolateral secretion of fibronectin by HTEC after 24 hours.* HTEC at passages 2, 3 and 4 (P2, P3 and P4) were grown on collagen coated permeable membrane supports. At confluence they were changed to SFEM. The basolateral medium was sampled at 24 hours and the fibronectin concentration measured. The results are from four experiments at each passage performed using a single kidney preparation. ANOVA p<0.01. Error bars represent 95% confidence limits. ** statistically greater than P3 and P2, * statistically greater than P2.
Fig 5.2. The effect of passage number on the constitutive secretion of fibronectin by HTEC after 48 hours. HTEC at passages 2, 3 and 4 (P2, P3 and P4) were grown on collagen coated permeable membrane supports. At confluence they were changed to SFEM. The apical (i) and basolateral (ii) media were sampled at 48 hours and the fibronectin concentration measured. The results are from four experiments at each passage performed using a single kidney preparation. ANOVA p<0.01 for (i) and (ii). Error bars represent 95% confidence limits. ** statistically greater than P3 and P2, * statistically greater than P2.
Fig 5.3. The effect of passage number on the constitutive cell associated fibronectin in HTEC cultures after 48 hours. HTEC at passages 2, 3 and 4 (P2, P3 and P4) were grown on collagen coated permeable membrane supports. At confluence they were changed to SFEM. After 48 hours the cells were dissolved as described in the text and the cell associated fibronectin measured. The results are from four experiments at each passage performed using a single kidney preparation. ANOVA p<0.01. Error bars represent 95% confidence limits. * statistically greater than P3 and P2.
Fig 5.4. Western blotting of HTEC conditioned media using rabbit polyclonal antihuman fibronectin. Lane 1 fibronectin standard (Sigma F0895), Lane 2 apical medium from HTEC culture in the presence of 1.0mg/ml apical FNDS, Lane 3 basolateral medium from HTEC culture in the presence of 1.0mg/ml apical FNDS.
Fig 5.5. *The secretion of fibronectin into the medium by second passage HTEC after 24 hours.* HTEC were exposed to 1.0mg/ml of FNDS in the apical medium as described in the text. The apical □ and basolateral □ media were sampled at 24 hours for fibronectin assay. The results are from five kidney preparations and 13 normal serum samples. Error bars represent 95% confidence intervals. * p<0.001 compared to the appropriate control.
Fig 5.6. The secretion of fibronectin into the medium by second passage HTEC after 48 hours. HTEC were exposed to 1.0 mg/ml of FNDS in the apical medium as described in the text. The apical □ and basolateral □ media were sampled at 48 hours for fibronectin assay. The results are from five kidney preparations and 13 normal serum samples. Error bars represent 95% confidence intervals. * p<0.001 compared to the appropriate control.
Fig 5.7. *The effect of apical serum on the permeability of HTEC monolayers to albumin.*

HTEC were exposed to 1.0mg/ml of FNDS as described in the text. \(^\text{125I}\) labelled albumin was added to the apical medium. The amount of \(^\text{125I}\) crossing the monolayer into the basolateral medium was determined at 24 hours and 48 hours. Error bars represent 95% confidence limits. * \(p<0.01\) compared to control.
Fig 5.8. The effect of apical serum on the inulin permeability of HTEC monolayers at 48 hours. HTEC were exposed to 1.0mg/ml of FNDS as described in the text. The monolayer permeability to labelled inulin was measured after 48 hours. The results are from 4 kidney preparations and 11 separate serum samples. Error bars represent 95% confidence limits. * p<0.01 compared to control.
Fig 5.9. *The effect of apical serum on the release of Lactate Dehydrogenase by HTEC.* HTEC were exposed to 1.0mg/ml of FNDS as described in the text. Lactate dehydrogenase activity was measured in the apical and basolateral medium after 48 hours. Apical and basolateral LDH were added to obtain total LDH release. The results are from 5 kidney preparations and 13 separate serum samples. Error bars represent 95% confidence limits. *p<0.05 compared to control.
Fig 5.10. The uptake of tritiated thymidine by HTEC following apical exposure to serum proteins. HTEC were exposed to 1.0mg/ml FNDS for 48 hours as described in the text. Tritiated thymidine incorporation was measured over a 2 hour incubation. Thymidine uptake into the cells is presented as disintegrations per minute (DPM) corrected for total cell protein. Error bars represent 95% confidence limits. * p<0.05 compared to control.
CHAPTER 6
CHARACTERISATION OF THE INCREASED
FIBRONECTIN SECRETION BY HTEC IN
RESPONSE TO SERUM
6.1. Introduction

In chapter 5 it was shown that HTEC increased apical and basolateral fibronectin secretion, increased LDH release and increased monolayer permeability when exposed to 1.0μg/ml of FNDS in the medium bathing their apical surface. The following questions arise:

(i) The production of fibronectin was polarised, but is the effect of FNDS different if added to the basolateral as compared to the apical medium?

(ii) Could the effects of FNDS be the result of endotoxin contamination due to the manipulation of the serum in non-sterile conditions?

(iii) Serum contains complement components which are known to be biologically active [Kosci C 1983, Torbohm I 1990, Hansch G 1992]. Complement in serum is usually heat inactivated prior to addition to cell cultures. Could the effects of FNDS be due to the presence of complement?

(iv) Serum is a complex mixture of proteins with a broad range of molecular weights. The lowest molecular weight proteins in plasma are freely filtered by the glomerulus whilst the highest molecular weight proteins may not be filtered in significant amounts even by a diseased glomerulus [Hardwicke J 1970]. Can the effect of serum be localised to a particular molecular weight band?

(v) Serum contains a number of cytokines and growth factors, do these influence the matrix protein production by HTEC? The role of cytokines in the production of fibronectin by HTEC is discussed in chapter 7.
6.2 Methods

HTEC were grown on permeable membrane supports as described in chapter 4. Once confluent, after 7 days growth, they were transferred to serum free experimental medium (SFEM) (section 5.2.1.) for 24 hours. The medium was then replaced with SFEM containing the experimental conditions as follows:

6.2.1 Experiment 1

A) Apical: SFEM + 10% (v/v) PBSD
   Basolateral: SFEM + 10% (v/v) PBSD

B) Apical: SFEM + 1.0mg/ml FNDS
   Basolateral SFEM + 10% (v/v) PBSD

C) Apical: SFEM + 10% (v/v) PBSD
   Basolateral SFEM + 1.0mg/ml FNDS

D) Apical: SFEM + 1.0mg/ml FNDS
   Basolateral: SFEM + 1.0mg/ml FNDS

6.2.2 Experiment 2

FNDS was heat inactivated (HI) by heating to 56°C for 30 minutes in a water bath prior to addition to the culture medium and the effect of lipopolysaccharide (LPS) on the production of fibronectin by HTEC was also investigated.

The experimental conditions were as follows:

A) Apical: SFEM + 10% (v/v) PBSD
B) Apical: SFEM + 1.0mg/ml FNDS
C) Apical: SFEM + 1.0mg/ml HI
D) Apical: SFEM + LPS 1.0µg/ml + 10% (v/v) PBSD

All basolateral media were SFEM alone.
6.2.3 Experiment 3

Four fractions were derived from fibronectin depleted serum by gel filtration chromatography as described in 2.8.2 (fraction A molecular weight >443kD, fraction B 100-443kD, fraction C 43-100kD, fraction D 14-43kD). The fractions were added to the medium bathing the apical side of the culture. The concentration of protein in medium containing fraction C was adjusted to 1.0mg/ml. The concentrations of the other fractions were calculated to be the same relative to fraction C as that found in serum (Table 6.1). The responses were compared to those on exposure to 1.0 mg/ml of non fractionated fibronectin depleted serum (FNDS).

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>FNDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.081±0.028 mg/ml</td>
<td>0.463±0.078 mg/ml</td>
<td>1.0 mg/ml</td>
<td>&lt;0.025 mg/ml</td>
<td>1.0 mg/ml</td>
</tr>
</tbody>
</table>

Table 6.1. Protein concentrations in the apical culture medium following addition of molecular weight fractions. Fractions: (A) >443kD, (B) 100-443kD, (C) 43-100kD, (D) 14-43kD, (FNDS) unfractionated fibronectin depleted serum.

6.2.4 Experiment 4

The protein composition of the active fraction was analysed by ion exchange chromatography (section 2.7.3). The major components of the active fraction were albumin and transferrin (Fig 6.8). The role of these two proteins was therefore investigated in the following experiment:

A) Apical: SFEM + 10% (v/v) PBSD
B) Apical: SFEM + 1.0mg/ml FNDS
C) Apical SFEM + 1.0mg/ml human albumin (essentially globulin free, Sigma A8763).
D) Apical SFEM + 0.05mg/ml human transferrin (partially iron saturated Sigma T8158).
In all four experiments the apical and basolateral media were sampled at 24 and 48 hours and stored at -20°C prior to analysis. The following were determined:

i) Fibronectin in the medium and associated with the cells as described in section 2.10.
ii) Total cell protein as described in section 2.2.3
iii) Inulin permeability of the monolayers as described in section 4.2.6.2
iv) Lactate dehydrogenase in the medium and for one kidney preparation in the cells as described in section 2.7.2.

v) Tritiated thymidine uptake by the cells as described in section 2.4.2.

6.3. Results

6.3.1 Experiment 1

Fibronectin secretion by HTEC remained polarised whether stimulated by FNDS apically, basolaterally or on both sides. Fibronectin secretion increased when HTEC were exposed to 1.0 mg/ml FNDS in the apical or the basolateral medium. The increase was not significantly different whether the serum was added to the apical or the basolateral side. However addition of FNDS to both the apical and basolateral medium, at the same time, increased fibronectin secretion by a greater amount than addition to the apical side alone (Figs 6.1 and 6.2). At 48 hours the effect of apical + basolateral FNDS on the apical secretion of fibronectin was greater than addition of FNDS to either apical or basolateral side alone (Fig 6.2i).
6.3.2 Experiment 2

Table 6.2 shows that heat inactivation of serum did not significantly alter its ability to increase fibronectin secretion by HTEC. LPS had no significant effect on secretion of fibronectin into the apical or basolateral media compared to control indicating that the effects of serum were not due to LPS contamination.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>FNDS</th>
<th>HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours a</td>
<td>0.18±0.25</td>
<td>0.54±0.57</td>
<td>2.47±0.518</td>
<td>2.68±0.49</td>
</tr>
<tr>
<td>24 hours b</td>
<td>4.38±1.12</td>
<td>5.96±1.64</td>
<td>12.80±1.54</td>
<td>13.16±2.53</td>
</tr>
<tr>
<td>48 hours a</td>
<td>2.49±0.31</td>
<td>2.37±0.35</td>
<td>8.40±2.06</td>
<td>9.07±2.05</td>
</tr>
<tr>
<td>48 hours b</td>
<td>19.80±2.77</td>
<td>19.94±2.64</td>
<td>38.22±9.08</td>
<td>33.39±9.71</td>
</tr>
</tbody>
</table>

Table 6.2. The effect of endotoxin on the fibronectin secretion of HTEC and the effect of heat inactivation on the ability of serum to stimulate fibronectin secretion. HTEC grown on membrane supports were exposed to 1.0µg/ml of lipopolysaccharide in the apical medium or to control serum (FNDS 1.0mg/ml) or to serum which had been heat inactivated (HI 1.0mg/ml). Results are expressed as micrograms of fibronectin produced/mg of cell protein (± 95% confidence limits). LPS results were from three kidney preparations each in triplicate. Heat inactivation results were from three kidney preparations and five serum samples each in triplicate.

6.3.3 Experiment 3

6.3.3.1 The effect of serum fractions on secretion of fibronectin by HTEC

The effect of serum fractions on fibronectin secretion by HTEC is shown in Figures 6.3. and 6.4. At both 24 and 48 hours fraction C reproduced the stimulation of fibronectin secretion into the basolateral medium caused by unfractionated FNDS. Apical fibronectin
secretion in the presence of fraction C was significantly increased at 48 hours but was less than in the presence of FNDS. At 48 hours there was a small but significant increase in apical secretion on exposure to fraction A (Fig 6.4i). There was no effect of fractions B or D.

6.3.3.2 The effect of serum fractions on inulin permeability

The monolayer permeability to inulin increased on exposure to unfractionated serum and fraction C but not to fractions A, B, or D. The increase due to fraction C was not significantly different to that due to unfractionated FNDS (Fig 6.5).

6.3.3.3 The effect of serum fractions on lactate dehydrogenase release

The lactate dehydrogenase released into the medium by HTEC is shown in Figure 6.6. LDH release was increased by fraction C and unfractionated serum. There was no effect of fractions A, B or D. As described in chapter 5, exposure to apical FNDS decreased total cellular LDH (section 5.3.4), but in this experiment no significant differences were found in total cellular LDH on exposure to any of the fractions. This may have been due to the small number of samples used in this data (2 serum samples on a single kidney preparation). However since total LDH did not increase with fraction C and hence the increased release of LDH on exposure to this fraction did reflect increased toxicity to the cells.

6.3.3.4 The effect of serum fractions on the tritiated thymidine incorporation

The tritiated thymidine incorporation results are shown in Figure 6.7. FNDS produced a small and non significant increase in tritiated thymidine incorporation in this set of experiments although a significant increase due to FNDS was previously shown (section 5.3.4). Fraction C significantly increased tritiated thymidine incorporation above controls.
6.3.4. The effects of albumin, fatty acid free albumin and partially saturated transferrin

Experiment 3 demonstrated that the effects of FNDS were reproduced by a serum fraction of molecular weight 43-100kD. The protein composition of this fraction was analysed by ion exchange chromatography and the chromatogram is shown in Figure 6.8. Two major peaks were detected corresponding to transferrin (molecular weight 76kD) and albumin (molecular weight 67kD). To investigate whether one of these proteins altered fibronectin secretion, commercially purchased albumin and transferrin were added to the apical medium. The transferrin concentration in the serum and fraction C samples (measured by the department of biochemistry, Glenfield Hospital NHS Trust, Leicester) was 0.055±0.010 mg/mg total protein and therefore the effect of addition of 0.05mg/ml of transferrin, to the apical medium was investigated. Partially iron saturated transferrin (30-60% saturated) was used as this most closely resembles normal circulating transferrin.

Figures 6.9. and 6.10. show the effect of albumin on fibronectin secretion. There was no significant increase in basolateral fibronectin secretion in the presence of apical albumin. At 48 hours albumin increased apical fibronectin secretion but by significantly less than FNDS. Addition of transferrin to the apical medium had no effect on the fibronectin secretion by HTEC compared to controls (Table 6.3).
Control Transferrin (0.05mg/ml) FNDS (1.0mg/ml)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Transferrin (0.05mg/ml)</th>
<th>FNDS (1.0mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours a</td>
<td>1.43±0.34</td>
<td>1.32±0.34</td>
<td>1.99±0.34</td>
</tr>
<tr>
<td>24 hours b</td>
<td>3.99±1.04</td>
<td>4.00±1.04</td>
<td>7.63±1.04</td>
</tr>
<tr>
<td>48 hours a</td>
<td>3.28±0.89</td>
<td>3.41±0.89</td>
<td>6.34±0.89</td>
</tr>
<tr>
<td>48 hours b</td>
<td>6.21±1.11</td>
<td>4.63±1.11</td>
<td>12.02±1.11</td>
</tr>
</tbody>
</table>

Table 6.3. The effect of transferrin on the fibronectin secretion by HTEC. The table shows the fibronectin secreted in µg/mg cell protein (±95% confidence limits). There was no significant differences between control and transferrin. The results are from two kidney preparations and were performed in triplicate. a=apical, b=basolateral

6.4. Discussion

It could be argued that the most accurate model to represent the in vivo state of a tubular cell would be one in which the basolateral surface of the cell was in contact with a dilute concentration of plasma proteins. However the precise composition of the extracellular fluid of the kidney interstitium is not known. Interstitial fluid protein concentrations have been measured between 0.3% (w/v) in oedema fluid and 5.6% (w/v) in the interstitium of the liver [Sterling K 1951]. The effect of apical serum was investigated in cells which were also exposed to basolateral serum. The concentration used in the basolateral medium was the same as that used in the apical medium (0.1% w/v). Fibronectin secretion increased to a similar extent when serum was added to either the apical medium or the basolateral medium. However when serum was added to the apical side of cells in addition to the basolateral side, the increase in fibronectin secretion was greater than when serum was added to one side only.
There are a number of possible interpretations of this data. The apical and basolateral effects could occur through different mechanisms which separately increased fibronectin secretion. For instance, the basolateral effect could be the result of improved nutrition of the cells which in vivo receive their nutrition from the basolateral surface. The additional effect of the apical medium could then represent an adverse effect equivalent to an effect of proteinuria.

The apical and basolateral effects could have occurred through the same mechanism. If endocytosis were important then it is of significance that an abnormally large proportion of cellular endocytosis of proteins may occur through the basolateral surface of cultured tubular cells. Rabkin R [1989] investigating insulin uptake by OK cells grown on permeable membranes found that basolateral internalisation was five times greater than apical internalisation. In contrast in the isolated perfused proximal tubule basolateral internalisation of insulin does occur but is minor (15%) compared to apical internalisation [Nielsen S 1987]. Such an abnormality in uptake of other proteins by HTEC might explain the response to basolateral serum. Thus although HTEC grown on permeable supports are polarised the polarity of particular cellular functions such as protein uptake may not be precisely the same as in vivo.

Because of these difficulties in interpretation of the significance of different apical and basolateral effects it was decided to pursue the hypothesis by investigating which of the components of serum had the effect on fibronectin production using addition of protein to the apical medium only. Tubular cells are not normally exposed to large amounts of protein in the tubular fluid and so any effects of serum proteins presented to their apical surface are of potential pathological significance.

The addition of serum fractions to the apical medium demonstrated that the effect of
FNDS on basolateral fibronectin secretion was almost entirely reproduced by apical exposure to fraction C. Effects on apical secretion may be more complex as the effect of fraction C was less than that of FNDS and in addition fraction A at 48 hours caused a small but significant increase in apical fibronectin secretion.

In chapter 5 it was noted that FNDS increased permeability of the monolayers to inulin, increased LDH release into the medium and increased tritiated thymidine incorporation by HTEC. Each of these effects was reproduced by fraction C. Ion exchange chromatography of fraction C demonstrated that the dominant proteins in this fraction were albumin and transferrin. Apical albumin alone was not able to reproduce the effect of FNDS in increasing fibronectin secretion. Transferrin at the concentration present in serum and fraction C had no significant effect on HTEC.

The cause of the increased secretion of fibronectin, increased permeability to inulin, increased LDH release and increased tritiated thymidine incorporation by HTEC exposed to apical serum was therefore localised to a fraction of molecular weight 43-100kD. Proteins of this molecular weight would not normally appear in large amount in the glomerular ultrafiltrate but would be present following glomerular disease resulting in proteinuria. These proteins would also be filtered in a selective proteinuria such as occurs in minimal change nephrotic syndrome. This data does not, therefore, explain the lack of interstitial scarring seen in that condition. The lack of an effect of albumin at the same protein concentration as FNDS indicates that the effect is not simply due to overload of the tubular cells with protein but is due to a specific biologically active component of serum. It may be explained by one of the many proteins that fall into this molecular weight band but are present at low concentration. Alternatively it may require the presence of a combination of proteins or be due to a molecule which is carried by albumin but is removed by the process of commercial manufacture.
Fig 6.1. *Secretion of fibronectin on exposure to apical, basolateral or apical and basolateral serum after 24 hours.* Fibronectin depleted serum (1.0 mg/ml) was added to the apical (a), basolateral (b) or apical and basolateral (a+b) media in HTEC cultures, PBS was added to controls (0). Fibronectin was measured in the apical (i) and basolateral (ii) media after 24 hours. Results are from a single kidney preparation using three different serum samples in triplicate. Statistical analysis compared fibronectin secreted in (a), (b) and (a+b). ANOVA (i) p<0.05, (ii) p<0.001. Error bars represent 95% confidence limits. *statistical difference compared to (a).*
Fig 6.2. *Secretion of fibronectin on exposure to apical, basolateral or apical and basolateral serum after 48 hours.* Fibronectin depleted serum (1.0 mg/ml) was added to the apical (a), basolateral (b) or apical and basolateral (a+b) media in HTEC cultures, PBSD was added to controls (0). Fibronectin was measured in the apical (i) and basolateral (ii) media after 48 hours. Results are from a single kidney preparation using three different serum samples in triplicate. Statistical analysis compared fibronectin secreted in (a), (b) and (a+b). ANOVA (i) p<0.001, (ii) p<0.05. Error bars represent 95% confidence limits. *statistical difference compared to (a) **statistical difference compared to (a) and (b).
Fig 6.3. *Secretion of fibronectin following exposure to serum fractions after 24 hours.* Serum was fractionated by molecular weight, into four fractions (A, B, C, and D). Fractions were added to the apical medium and compared to the effect of adding unfractionated serum (FNDS). Fibronectin was measured in the media after 24 hours. i) apical medium, ii) basolateral medium. The results are from two kidney preparations using five serum samples, each condition was applied in triplicate. ANOVA (i) p<0.01, (ii) p<0.01. Error bars represent 95% confidence limits. * statistical difference compared to control (0).
Fig 6.4. *Secretion of fibronectin following exposure to serum fractions after 48 hours.* Serum was fractionated by molecular weight into four fractions (A, B, C, and D). Fractions were added to the apical medium and compared to the effect of adding unfractionated serum (FNDS). Fibronectin was measured in the media after 48 hours. i) apical medium, ii) basolateral medium. The results are from two kidney preparations using five serum samples, each condition was applied in triplicate. ANOVA (i) $p<0.01$, (ii) $p<0.01$. Error bars represent 95% confidence limits. * statistical difference from control (0), ** statistical difference from control (0) and C.
Fig 6.5. The effect of serum fractions on the inulin permeability of HTEC monolayers. After 48 hours exposure to the serum fractions (A, B, C and D) or unfractionated serum (FNDS) the inulin permeability of the monolayers was determined as described in the text. The results are from two kidney preparations and five serum samples each in triplicate. ANOVA p<0.01. Error bars represent 95% confidence limits. * statistical difference from control (0).
Fig 6.6  
*The effect of serum fractions on the release of lactate dehydrogenase into the medium of HTEC cultures.* After 48 hours exposure to the serum fractions (A, B, C and D) or unfractionated serum (FNDS) the lactate dehydrogenase (LDH) in the apical and basolateral medium was measured and added to provide total LDH released. The results are from two kidney preparations and five serum samples each in triplicate. ANOVA p<0.01. Error bars represent 95% confidence limits. * statistical difference from control (0).
**Fig 6.7.** *The effect of serum fractions on the uptake of tritiated thymidine by HTEC.* After 48 hours exposure to the serum fractions (A, B, C and D), or unfractionated serum (FNDS) the uptake of thymidine by HTEC was determined as described in the text. The results are from two kidneys and three serum samples each in triplicate. ANOVA $p<0.01$. Error bars represent 95% confidence limits. * statistical difference from control (0).
Fig 6.8. Ion exchange chromatogram of fraction C.
Sample: 25mg fraction C in 1.0ml buffer A
Column: Mono Q (Pharmacia)
Flow rate: 1.0ml/min
Buffer A: 6.25mM bis-Tris propane pH7.5
Buffer B: A+0.35M NaCl pH9.5
Gradient: 0-100% B in 20 min
Fig 6.9. The effect of albumin on the fibronectin secretion by HTEC at 24 hours. Human albumin (alb, 1.0 mg/ml) was added to the apical medium and compared to the effect of fibronectin depleted serum (FNDS) or control (0). Apical (i) and basolateral (ii) media were sampled at 24 hours for measurement of fibronectin. The results are from four kidney preparations performed in triplicate. ANOVA p<0.01 for (i) and (ii). Error bars represent 95% confidence limits. * statistically different to FNDS not different to control (0).
Fig 6.10. *The effect of albumin on the fibronectin secretion by HTEC at 48 hours.* Human albumin (alb, 1.0 mg/ml) was added to the apical medium and compared to the effect of fibronectin depleted serum (FNDS) or control (0). Apical (i) and basolateral (ii) media were sampled at 48 hours for measurement of fibronectin. The results are from four kidney preparations performed in triplicate. ANOVA p<0.01 for (i) and (ii). Error bars represent 95% confidence limits. * statistically less than FNDS but greater than control (0), ** statistically less than FNDS not different to control (0).
CHAPTER 7

THE PRODUCTION OF CYTOKINES BY HTEC AND
THEIR ROLE IN THE STIMULATION OF
FIBRONECTIN SECRETION BY SERUM
7.1. Introduction

Serum contains a number of cytokines which may influence the production of extracellular matrix proteins. In particular serum contains significant amounts of transforming growth factor \( \beta \) (TGF \( \beta \)) [O'Connor-McCourt M 1987] and platelet derived growth factor (PDGF) [Ross R 1986]. Although, in general, cytokines are of low molecular weight, and are not therefore likely to occur in serum fraction C, it was important to investigate the possibility that they were involved in the increase in fibronectin production by HTEC on apical exposure to FNDS.

As described in chapter 1 proximal tubular cells have been shown to produce a variety of cytokines which may be of significance in interstitial inflammation. Tumour necrosis factor \( \alpha \) (TNF \( \alpha \)) is an important inflammatory cytokine and is produced by human proximal tubular cells [Wuthrich R 1990b]. TGF\( \beta \) is thought to be crucial to many scarring processes and mRNA for TGF\( \beta \) is expressed in murine proximal tubular cell cultures [Rocco M 1992]. PDGF which is also produced by proximal tubular cells, can increase matrix protein production and is an important fibroblast chemoattractant [Frank J 1992]. The production of these cytokines by HTEC in response to serum proteins would provide a mechanism by which proteinuria could influence the development of interstitial inflammation and scarring. In addition the release of cytokines such as TGF\( \beta \) or PDGF, by HTEC, into the culture medium could result in autocrine stimulation to increase fibronectin production by the cells.

An important event in interstitial inflammation is the development of a macrophage infiltrate (section 1.4). This is likely to be the result of the release of chemoattractants and a likely source of these is the tubular cells. Monocyte chemoattractant protein-1 (MCP-1) is an important peptide chemoattractant specific for monocytes and it is known to be produced by human proximal tubular cells [Schmouder R 1993].
The experiments described in this chapter address the questions of the role of cytokines present in serum in the production of fibronectin, the production of cytokines and chemoattractants by HTEC when exposed to serum and whether those cytokines could act autocrinally to increase fibronectin production by HTEC.

7.2. Methods

7.2.1. The cytokines in serum

Using commercially available assays PDGF-AB (section 2.12.1) and TGFβ, (section 2.12.3) were measured in the fibronectin depleted serum and in the serum fractions (A-D) produced as described in section 2.8.2.

7.2.2. Cytokine and chemokine production by HTEC

Apical and basolateral conditioned medium from experiments 2, 3 and 4, described in chapter 6, were collected after 48 hours exposure to HTEC and then stored at -70°C. The effect on cytokine production by HTEC of exposure to FNDS, heat inactivated FNDS, serum fractions (A, B, C and D), albumin and transferrin could thus be determined. PDGF-AB (section 2.12.1), TNFα (section 2.12.2), TGFβ1 (section 2.12.3) and MCP-1 (section 2.12.4) were measured in this medium using commercially available assays.

7.2.3. Investigation of an autocrine role for PDGF in stimulation of fibronectin production by HTEC

Second passage HTEC were grown on collagen coated permeable membrane supports in 12 well plates in an identical fashion to that described for growth on 6 well plates (chapter 4). The growth medium was changed on alternate days and confluence was reached by day
7. The medium was changed to SFEM for 24 hours after which the experimental conditions were applied as follows:

s) apical SFEM + 10% PBSD
basolateral SFEM

t) apical SFEM + 1.0mg/ml serum
basolateral SFEM

u) apical SFEM + 10% PBSD + 5.0µg/ml anti PDGF
basolateral SFEM

v) apical SFEM + 1.0mg/ml serum + 5.0µg/ml anti PDGF
basolateral SFEM

w) apical SFEM + 10% PBSD
basolateral SFEM + 5.0µg/ml anti PDGF

x) apical SFEM + 1.0mg/ml serum
basolateral SFEM + 5.0µg/ml anti PDGF

y) apical SFEM + 10% PBSD + 1.0ng/ml PDGF-AB
basolateral SFEM

z) apical SFEM + 10% PBSD
basolateral SFEM + 1.0ng/ml PDGF-AB

---

1 Goat polyclonal anti-PDGF (R and D AB-23-NA) is a PDGF neutralising antibody, 5.0µg/ml of which neutralises 50% of the activity of 10ng/ml of natural human PDGF (manufacturers information).

2 The PDGF (R and D 222-AB) was recombinant human PDGF-AB. The concentration of PDGF-AB used (1.0ng/ml) was greater than the maximum concentration found in HTEC cultures exposed to apical FNDS after 48 hours (0.896ng/ml).
After 48 hours the apical and basolateral media were removed and stored at -20°C prior to fibronectin measurement. The cells were dissolved in WB+1% nonidet P40 (section 2.4.2) and total protein measured by Bio Rad DC assay (section 2.2.3).

7.3. Results

7.3.1 The cytokines in serum

Table 7.1 shows the concentrations of PDGF-AB and TGFβ, that were present in the apical culture medium following the addition of FNDS or serum fractions.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>FNDS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGFβ, (ng/ml)</strong></td>
<td>0.34±0.22</td>
<td>0.09±0.02</td>
<td>nd</td>
<td>nd</td>
<td>0.46±0.24</td>
</tr>
<tr>
<td><strong>PDGF-AB (ng/ml)</strong></td>
<td>nd</td>
<td>nd</td>
<td>0.01±0.005</td>
<td>0.17±0.04</td>
<td>0.31±0.10</td>
</tr>
</tbody>
</table>

Table 7.1  *The concentrations of Transforming Growth Factor β, and Platelet derived growth factor-AB in the apical medium containing FNDS or serum fractions.* Errors represent 95% confidence limits. A, B, C and D are the serum fractions. FNDS is 1.0mg/ml fibronectin depleted serum. 

nd= not detectable

As expected PDGF-AB is predominantly found in the low molecular weight fraction D which had no effect on fibronectin production by HTEC (section 6.3.3.1) with a lesser amount in the active fraction C. TGFβ, was predominantly found in the high molecular weight fraction A with lesser amounts in fraction B. This is because TGFβ, in serum is bound to α2-macroglobulin (725kD) [O'Connor-McCourt M 1987]. Thus the increase in fibronectin secretion on apical exposure to serum fraction C was not explained by TGFβ, or PDGF-AB added with the serum. It is possible however that the small increase in production
of apical fibronectin on exposure to fraction A at 48 hours (section 6.3.3.1) was the result of
the presence of TGFβ, which is known to upregulate fibronectin gene expression in human
proximal tubular cells [Viedt C 1995]. TGFβ that is bound to α2 macroglobulin is however
inactivated [O'Connor-McCourt M 1987] and would have to be released from the carrier
molecule prior to having a biological effect.

7.3.2 Cytokine production by HTEC

7.3.2.1 TGFβ and TNFα secretion by HTEC

The lower limit of detectability of the TGFβ, assay was 31 pg/ml. TGFβ, was not
detectable in the apical or basolateral medium of control HTEC or in the basolateral medium
from those cells exposed to apical FNDS after 48 hours culture. No excess of TGFβ, was
detected in the apical medium of HTEC that were exposed to apical FNDS, over that which
was added with the serum. TNFα was not detectable in either apical or basolateral medium
in the controls or on addition of serum.

7.3.2.2 PDGF secretion by HTEC

PDGF-AB was found in the apical and basolateral media of HTEC cultures under
control conditions. As with fibronectin secretion there was considerable variation in PDGF-
AB secretion by different kidney preparations (range 0.38ng/mg cell protein to 3.08ng/mg cell
protein, mean 1.37±0.51ng/mg cell protein after 48 hours). Figure 7.1. shows the effect of
addition of 1.0mg/ml FNDS to the apical medium on the secretion of PDGF-AB, by HTEC,
into the apical and basolateral media. Apical FNDS increased apical PDGF-AB secretion by
2.11±0.84 fold and basolateral PDGF-AB secretion by 1.70±0.37 fold. Similar to fibronectin
the secretion of PDGF-AB was polarised with predominant basolateral secretion. As shown
in Table 7.2, basolateral secretion was 4.7±1.6 times greater than apical secretion under control conditions and 3.4±0.7 times greater in cells exposed to apical serum. The degree of polarity on exposure to serum was not significantly different to controls.

Heat inactivation of serum did not alter its ability to increase PDGF-AB secretion and LPS added alone did not alter PDGF-AB secretion compared to control (Table 7.3). The basolateral production of PDGF-AB by HTEC exposed to the serum fractions is shown in Figure 7.2. The increase in basolateral secretion of PDGF-AB on exposure to FNDS was reproduced by apical exposure to serum fraction C. Despite the fact that fraction C was predominantly composed of albumin and transferrin (Fig 6.8) these proteins used at concentrations comparable to those in fraction C (section 6.3.4) had no effect on basolateral PDGF-AB secretion (Tables 7.4 and 7.5).

7.3.2.3 MCP-1 secretion by HTEC

MCP-1 was secreted by HTEC into both the apical and basolateral medium. There was no significant difference between the concentration of MCP-1 in the apical medium compared to the basolateral medium under control conditions or in the presence of serum. Because of the difference in volume of the apical and basolateral media the total MCP-1 in the basolateral medium exceeded that in the apical medium. There are two possible explanations for these results. MCP-1 secretion may be polarised in which case the degree of polarity was less than that of PDGF (Table 7.2) and Fibronectin (Table 5.2). Alternatively the small size of MCP-1 (8kD) could have resulted in a significant leak of MCP-1 across the monolayer thus equalising the concentrations on either side. In view of this second possibility it was not possible to come to any conclusions about the true polarity of MCP-1 secretion by HTEC.
Similar to fibronectin secretion and PDGF secretion the basolateral MCP-1 under control conditions was very variable (range 7.2 to 20.4 ng/mg cell protein, mean 11.8 ± 3.3 ng/mg cell protein). Exposure of HTEC to FNDS in the apical medium increased basolateral MCP-1 by 2.4 ± 0.4 fold and apical MCP-1 by 3.20 ± 1.43 fold (Fig 7.3). The ability of serum to increase MCP-1 secretion was not altered by heat inactivation of the serum and was not reproduced by LPS (Table 7.3). The effect of addition of the serum fractions to the apical medium on the basolateral MCP-1 is shown in Fig 7.4. The increase in basolateral MCP-1 on exposure to apical FNDS was reproduced by apical exposure to serum fraction C with no effect of fractions A, B or D. As for fibronectin and PDGF secretion, albumin (Table 7.4) and transferrin (Table 7.5) did not significantly alter basolateral MCP-1.

7.3.3 The role of PDGF in the increase in fibronectin secretion on exposure to serum

PDGF-AB and fibronectin secretion were both increased by exposure to FNDS and serum fraction C raising the possibility that serum increased PDGF secretion which then acted autocrinally to increase fibronectin secretion. To investigate this possibility HTEC were exposed to apical serum in the presence of anti PDGF neutralising antibodies in the apical or basolateral medium. In addition recombinant human PDGF-AB was added to HTEC cultures to determine whether it increased fibronectin production. The results are shown in Figure 7.5. Anti PDGF antibodies whether added to the apical or basolateral medium did not themselves alter apical or basolateral fibronectin secretion by HTEC (conditions 'u' and 'w' compared to control 's'). Anti PDGF neutralising antibodies, added in excess to either the apical or the basolateral medium, did not prevent the apical and basolateral increase in fibronectin secretion by HTEC following apical exposure to FNDS ('v' compared to 'u' and 'x' compared to 'w'). Recombinant human PDGF-AB when added to either the apical or the basolateral medium did
not increase fibronectin secretion by HTEC (‘y’ and ‘z’ compared to ‘s’). Together with the lack of effect of serum fraction D, which contained PDGF, this evidence does not support an autocrine effect of PDGF to stimulate fibronectin secretion by HTEC.

7.4. Discussion

Three issues were addressed by this chapter. Firstly do the cytokines present in serum affect fibronectin secretion, secondly can HTEC produce cytokines or chemoattractants which could stimulate interstitial inflammation and scarring and thirdly could the production of these cytokines act autocrinally to increase fibronectin secretion. The lack of effect of the low molecular weight fraction D which would contain most of the cytokines and growth factors argues against an important effect for these in the increased production of fibronectin secretion by HTEC exposed to FNDS.

Proximal tubular cells have been shown to produce PDGF, TGFβ, TNFα and MCP-1 in response to a variety of stimuli (section 1.7). The experiments described above showed that HTEC grown on permeable supports constitutively produced PDGF-AB and MCP-1. Secretion of PDGF-AB, like fibronectin was polarised and predominantly basolateral. The polarity of MCP-1 secretion could not be determined because the concentrations of MCP-1 were not significantly different in the apical and basolateral medium raising the possibility that MCP-1 may have leaked across the monolayer and masked any polarity of secretion. It is of interest to note that MCP-1 has been detected in the urine in kidney disease [Rovin B 1995] suggesting that MCP-1 may be secreted in a significant amount into the tubular lumen (i.e. apically). TGFβ1 and TNFα were not produced in detectable amounts by HTEC either under control conditions or when exposed to apical serum.

FNDS in the apical medium increased apical and basolateral secretion of PDGF-AB and MCP-1. The increase was not reproduced by LPS or reduced by heat inactivation of the
serum. Fractionation of FNDS showed that, similarly to fibronectin, the increase in PDGF-AB and MCP-1 secretion was reproduced by exposure to fraction C. Despite the fact that the major proteins in this fraction are albumin and transferrin these proteins, if added alone to the apical medium, did not significantly alter PDGF-AB or MCP-1 secretion.

The cytokine most likely to increase fibronectin secretion would be TGFβ which has been shown to increase fibronectin mRNA expression by human proximal tubular cells [Viedt C 1995]. However serum fraction C which caused the greatest increase in fibronectin secretion did not contain detectable levels of TGFβ. HTEC did not themselves produce TGFβ; and hence this cytokine was not involved in the increase in fibronectin secretion by HTEC.

PDGF-AB was produced in response to fraction C so that although it was present in serum fraction D, which did not increase fibronectin secretion, it was possible that PDGF produced by HTEC could act autocrinally to increase fibronectin secretion. PDGF neutralising antibodies did not reduce the ability of serum to stimulate fibronectin secretion. Furthermore recombinant human PDGF-AB added to the medium, at a concentration greater than that found in the serum stimulated cultures, did not stimulate fibronectin secretion. Therefore although secretion of PDGF may be of importance to the development of scarring in the interstitium there was no evidence that it acts autocrinally in this cell culture system to increase fibronectin secretion by HTEC.

In summary HTEC produced MCP-1 and PDGF-AB in increased amounts when exposed to apical serum proteins. As with fibronectin the response to serum was reproduced on exposure to a fraction of molecular weight 43-100kD which contains a range of proteins, which would be filtered in glomerular disease. However like fibronectin this effect was not reproduced by albumin or transferrin which are the major serum proteins in this molecular weight range.
Table 7.2  The ratio of basolateral to apical secretion of PDGF and MCP-1. After 48 hours in experimental conditions the apical and basolateral medium were sampled for measurement of PDGF-AB and MCP-1. The ratio of total amount in the apical medium to the total amount in the basolateral medium is shown which takes account of the different apical and basolateral volumes. Error bars represent 95% confidence limits. FNDS=1.0mg/ml fibronectin depleted serum.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FNDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AB</td>
<td>4.7±1.6</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.7±0.5</td>
<td>1.3±0.3</td>
</tr>
</tbody>
</table>

Table 7.3. The basolateral secretion of PDGF and MCP-1 in response to LPS and the effect of heat inactivation of serum. Following 48 hours apical exposure to the experimental conditions PDGF-AB and MCP-1 were assayed in the basolateral medium. The results are expressed with 95% confidence limits. The LPS results are from three kidney preparations each in triplicate and the heat inactivation results are from three kidney preparations and five serum samples each in triplicate. Results are shown a mean±95% confidence limits. FNDS=1.0mg/ml fibronectin depleted serum. HI=1.0 mg/ml heat inactivated FNDS.
Table 7.4. The effect of apical exposure to albumin on the basolateral secretion of PDGF and MCP-1. HTEC were exposed to control conditions, human albumin (1.0 mg/ml) or FNDS (1.0 mg/ml) for 48 hours. PDGF-AB and MCP-1 were assayed in the basolateral medium. The results are from four kidney preparations each performed in triplicate. Results are shown as means±95% confidence limits.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Albumin (1.0 mg/ml)</th>
<th>FNDS (1.0 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AB (ng/mg cell protein)</td>
<td>1.03±0.24</td>
<td>1.23±0.24</td>
<td>1.80±0.24</td>
</tr>
<tr>
<td>MCP-1 (ng/mg cell protein)</td>
<td>12.4±6.5</td>
<td>14.1±6.5</td>
<td>25.1±6.5</td>
</tr>
</tbody>
</table>

Table 7.5. The effect of apical exposure to transferrin on the basolateral secretion of PDGF and MCP-1. HTEC were exposed to control conditions, human transferrin (0.05 mg/ml) or FNDS (1.0 mg/ml) for 48 hours. PDGF-AB and MCP-1 were assayed in the basolateral medium. The results are from two kidney preparations each performed in triplicate. Results are shown as the mean±95% confidence limits.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Transferrin (0.05 mg/ml)</th>
<th>FNDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AB (ng/mg cell protein)</td>
<td>1.16±0.50</td>
<td>1.57±0.50</td>
<td>1.97±0.50</td>
</tr>
<tr>
<td>MCP-1 (ng/mg cell protein)</td>
<td>17.3±5.0</td>
<td>17.0±5.0</td>
<td>34.0±5.0</td>
</tr>
</tbody>
</table>
Fig 7.1.  *PDGF-AB secretion by HTEC exposed to 1.0 mg/ml fibronectin depleted serum (FNDS) in the apical medium.*  HTEC were exposed to 1.0 mg/ml of serum in the apical medium for 48 hours. PDGF-AB was assayed in the apical and basolateral media. PDGF added in the serum was subtracted from the apical concentration. The results are from five kidney preparations and 13 serum samples.  □ Apical medium, □ basolateral medium.  ANOVA p<0.001 for apical and basolateral results. Error bars represent 95% confidence limits. * statistical difference from control (0).
Fig 7.2. Basolateral PDGF-AB secretion by HTEC on apical exposure to serum fractions. HTEC were exposed to serum fractions (A, B, C, D), unfractionated serum (FNDS) or control conditions (0) in the apical medium. After 48 hours PDGF-AB was assayed in the basolateral medium. The results are from two kidney preparations and five serum samples. ANOVA p<0.001. Error bars represent 95% confidence limits. * statistical difference compared to control (0).
Fig 7.3. MCP-1 secretion by HTEC exposed to 1.0 mg/ml fibronectin depleted serum (FNDS) in the apical medium. HTEC were exposed to 1.0 mg/ml of serum in the apical medium for 48 hours. MCP-1 was assayed in the apical and basolateral media. The results are from five kidney preparations and 13 serum samples. □ Apical medium, ■ basolateral medium. ANOVA p<0.001 for apical and basolateral results. Error bars represent 95% confidence limits. * statistical difference from control.
Fig 7.4.  *Basolateral MCP-1 secretion by HTEC on apical exposure to serum fractions.*

HTEC were exposed to serum fractions (A, B, C, D), unfractionated serum (FNDS) or control conditions (0) in the apical medium. After 48 hours MCP-1 was assayed in the basolateral medium. The results are from two kidney preparations and five serum samples. ANOVA p<0.001. Error bars represent 95% confidence limits. * statistical difference compared to control.
Investigation of the role of platelet derived growth factor in the stimulation of fibronectin secretion by HTEC exposed to serum. HTEC grown on permeable supports in 12 well plates were exposed to the following conditions:

- s) control
- t) apical serum (1.0 mg/ml)
- u) control + apical anti PDGF
- v) apical serum + apical anti PDGF
- w) control + basolateral anti PDGF
- x) apical serum + basolateral anti PDGF
- y) apical PDGF
- z) basolateral PDGF

After 48 hours the medium was removed for measurement of fibronectin. (i) apical medium, (ii) basolateral medium. The results are from two kidney preparations and four serum samples. Error bars represent 95% confidence limits. * p<0.05, ** p<0.01, *** p<0.001 compared to the appropriate control by two way ANOVA.
Fig 7.5
CHAPTER 8

DISCUSSION
It has been known for many years that interstitial inflammation and scarring are characteristics of renal histology in chronic progressive renal failure [Risdon R 1968, Bohle A 1977]. The degree of interstitial pathology has been demonstrated to be a better predictor of decreased renal function than glomerular pathology [Schainuck L 1970]. However it is not known why interstitial pathology occurs in conditions thought to be primarily of glomerular origin. As discussed in the introduction to this thesis (section 1.8.) a number of hypotheses have been proposed to explain this phenomenon including the spread of inflammation from the glomerulus as a result of the leakage of inflammatory mediators into the interstitium [Lan H 1991], or into the tubular fluid [Yee J 1991], interstitial hypoxia as a result of decreased post glomerular blood flow [Fine L 1993] or an effect of proteinuria on tubular cell function. It may be that a number of different mechanisms are involved in what is likely to be a multifactorial process. For instance tubular cells using energy on the uptake and metabolism of proteins may be more vulnerable to hypoxia than tubular cells not effected by proteinuria.

Proteinuria was suggested as a cause of progression of renal failure following the observation that the rate of progression of renal disease correlates with the quantity of proteinuria (section 1.9.2) in many human renal diseases and in animal models of renal disease (section 1.9.3). In addition, angiotensin converting enzyme (ACE) inhibitors which reduce the rate of decline of renal function also reduce proteinuria in the remnant kidney model in the rat [Anderson S 1986]. In humans, ACE inhibitors reduced the rate of progression of renal failure only in those patients in which they also reduced proteinuria [Praga M 1994]. Correlative data of this type raises the possibility of a pathogenic role for proteinuria but cannot rule out the possibility that proteinuria is merely a marker of the severity of glomerular disease.

Proximal tubular cells are vulnerable to any effects of proteinuria because they
reabsorb proteins from the tubular fluid (section 1.9.1) and they have been shown to produce a number of mediators and surface markers which may initiate and propagate an inflammatory response (section 1.7). Proximal tubular cells may also be an important source of the interstitial matrix proteins that are laid down in a scarred interstitium (section 1.4). It has even been suggested that tubular cells may transdifferentiate into a fibroblast phenotype in response to renal damage [Neilson E 1995].

The experiments in this thesis were designed to investigate whether proteins that are present in proteinuria have direct effects on proximal tubular cells which could initiate tubulointerstitial pathology. The initial experiments used an immortalised proximal tubular cell line (OK cells) which had the advantage of ease of availability and reproducibility. The effect of proteins on the growth of these cells was investigated since hypertrophy and hyperplasia of kidneys accompany development of chronic renal failure in compensatory growth following reduction of renal mass (section 1.5). Exposure of OK cells to a high (10mg/ml) concentration of bovine serum albumin resulted in hypertrophy. At lower concentrations (0.1-1.0mg/ml), proliferation was demonstrated. The proliferation that was observed on exposure to 10mg/ml of albumin was prevented if fatty acid free albumin was used.

The mixture of proteins present in the urine from nephrotic rats produced a marked proliferation of OK cells at a concentration of 0.1mg/ml which is similar to that observed in the proximal tubular fluid of nephrotic rats [Landwehr D 1977]. A higher concentration of proteinuric urine (1.0mg/ml) produced a lesser proliferative response. Biphasic proliferative responses to proteins were therefore observed with proteinuric urine and fatty acid free albumin on confluent OK cells and with fatty acid replete albumin on subconfluent OK cells. The loss of the proliferative responses at a high concentration of protein may be due to
exhaustion of a component of the medium that is required for growth. This exhaustion could be either a result of the scavenging of lipid by fatty acid free albumin or a result of the high levels of proliferation that occurred in subconfluent cells and confluent cells stimulated by proteinuric urine. It is difficult to conclude that high concentrations of albumin are frankly toxic to OK cells as fatty acid replete albumin caused increasing levels of proliferation up to 10.0mg/ml. A variety of effects on growth of OK cells were therefore demonstrated at different concentrations of proteins.

Interpretation of these results highlights one of the problems inherent in using in vitro techniques to investigate effects in chronic disease states. The relationship between the degree of a pathological stimulus and time is unknown. A small stimulus may have a significant effect over a prolonged time in vivo but no detectable effect in a few days in vitro. It is possible however that a supraphysiological stimulus over a short period in vitro reproduces the effect of a lesser stimulus over a prolonged period in vivo. The protein concentration in the tubules of nephrotic rats has been reported as low as 0.07mg/ml [Landwehr D 1977]. This would suggest that the proliferative response of OK cells to a low concentration of proteinuric urine is of greatest significance. However over the months and years that it takes to develop chronic interstitial scarring of the kidney the hypertrophy seen in the presence of 10mg/ml of albumin could play an important role in the biology of the tubular cells. An additional factor to be taken into account in the interpretation of these experiments is that tubular cells in culture have only a rudimentary brush border (section 1.9.1) and the uptake of protein by these cells may be reduced compared to the normal. They may therefore require exposure to a higher protein concentration in vitro to produce intracellular effects similar to conditions in vivo.

Opossum kidney cells provide a good model for proximal tubular cell functions but
their relevance to human kidney disease is questionable. In addition because of the unusual species involved more sophisticated investigation of OK cell functions was not possible. Therefore an in vitro human model was developed for further investigation of the hypothesis.

Human tubular epithelial cells (HTEC) were grown on permeable membrane supports. They were characterised to be predominantly of proximal tubular origin although it is likely that there were also distal tubular cells present shown by the small cAMP response to vasopressin (section 4.3.2.c).

It has previously been shown that human tubular cells grown on supports express appropriate electrical polarity [Blackbum J 1988]. Scanning electron microscopy of HTEC showed that they expressed microvilli on their apical surface although these did not form a well differentiated brush border. HTEC secreted fibronectin and PDGF predominantly in a basolateral direction. However secretion was not exclusively basolateral and it is not certain whether the small amount of apical secretion was the result of dedifferentiation or is a normal function of tubular cells. Fibronectin is detectable in the urine of patients with renal disease and could be of tubular origin [Gwinner W 1993]. MCP-1 secretion was apparently less polarised although it is possible that this was an artifact due to the leakage of this small peptide across the monolayer. MCP-1 is also found in the urine in renal disease [Rovin B 1995]. It can therefore be concluded that HTEC grown on membrane supports are appropriately polarised although it is uncertain whether the degree of polarity is identical to that of in vivo cells.

Since tubular cells in vivo are in contact with extra cellular fluid containing some serum proteins the best cell culture model for proteinuria may be one in which HTEC were in contact with basolateral serum and the effect of additional apical serum then determined. Addition of 1.0mg/ml of serum to the apical medium of HTEC when the basolateral medium
also contained 1.0mg/ml of serum resulted in increased fibronectin production compared to basolateral serum alone. This additional effect was however small and only reached statistical significance at one time point. Addition of serum to the basolateral medium itself increased fibronectin production compared to controls.

Proximal tubular cells in culture are known to differ from \textit{in vivo} cells in their handling of proteins. Studies in OK cells grown on permeable membrane supports have shown that the polarity of insulin uptake was reversed compared to \textit{in vivo} with greater basolateral uptake than apical uptake [Rabkin R 1989]. In the isolated perfused rabbit proximal tubule basolateral uptake of albumin constituted only 6% of the corresponding luminal uptake [Bourdeau J 1973]. An abnormally large basolateral uptake in this culture system could account for the basolateral effect of serum on fibronectin secretion. Alternatively the apical and basolateral effects of serum may occur through different mechanisms. To evaluate these possibilities further investigation is required of the protein handling by HTEC which was not addressed in this thesis. The effects of serum on HTEC described here could be the result of the uptake of proteins by the cells or could be a receptor mediated event at the surface of the cells which does not involve endocytosis.

Because of the variation in the behaviour of HTEC with increasing passage, all of the experiments were carried out using second passage cells. Despite this there was considerable variation in the baseline fibronectin, PDGF and MCP-1 production of the different kidney preparations although the degree of response to serum was similar in each case. Addition of 0.1mg/ml of serum to the apical medium of HTEC cultures had little effect on fibronectin production and it was therefore decided to concentrate on the effects of 1.0mg/ml.

Apical serum increased secretion of fibronectin, PDGF and MCP-1 whilst the polarity of secretion was unchanged. TNF$\alpha$ and TGF$\beta_1$ secretion were not detected. Serum is a
requirement of many cell culture systems and indeed HTEC required serum to produce confluent monolayers on the membrane supports. It could therefore be suggested that the addition of serum to the medium merely improved the culture conditions with a resultant increase in secretory products from the cells. This was addressed firstly by the development of the polarised system since the apical surface of tubular cells is not normally exposed to large amounts of serum proteins except in glomerular disease. It was further investigated by assessing markers of cell toxicity. If the cell culture conditions improved in the presence of serum then less toxicity would be expected. The permeability of the monolayer to albumin and inulin increased on apical exposure to serum as did the release of lactate dehydrogenase into the culture medium. Both of these markers would suggest increased cell injury rather than improved culture conditions.

Serum is a complex mixture of proteins and for further investigation the serum was divided into four molecular weight fractions: fraction A (>443kD), fraction B (100-443kD), fraction C (43-100kD) and fraction D (14-43kD). Fraction D therefore contained proteins that are likely to be filtered by a normal glomerulus and would contain most of the cytokines and growth factors that are present in serum. Fraction C proteins would not be filtered in large amount except in glomerular disease but would be filtered in a selective proteinuria such as occurs in minimal change nephrotic syndrome (MCNS). Fraction B proteins would be filtered by a glomerulus which had been severely damaged resulting in a nonselective proteinuria. The filtration of proteins in fraction A, even by a highly damaged glomerulus, is likely to be low because of their large size. It should be noted however that molecular weight is not the only determinant of glomerular filtration of a protein which is also influenced by the charge carried by the protein [Brenner B 1977].

All of the effects of serum on HTEC were reproduced by fraction C although there
There are three possible explanations for an effect of fraction C in the absence of an effect of albumin or transferrin. The effect of fraction C may be due to another biologically active protein which is present in a low concentration in serum. Secondly the effect of fraction C could require a combination of two or more proteins or thirdly the effect of fraction C may be due to a molecule which is carried by albumin but which is removed in the commercial preparation of pure albumin.

Because the active fraction of serum had a molecular weight 43-100kD the findings presented here cannot provide an explanation for the lack of interstitial scarring in MCNS which typically produces a selective proteinuria. As pointed out in section 1.9.5.3. MCNS is associated with evidence of tubular injury. The lack of interstitial scarring may simply be due to the short duration of this condition which is sensitive to corticosteroid treatment. An
alternative explanation is that if the effect is due to a molecule carried by a protein then that carriage may be modified in MCNS. There is precedent for this hypothesis since the fatty acid composition of the albumin in the urine of children with MCNS is known to be altered [Ghiggeri G 1987]. Since fatty acid replete albumin had no effect on fibronectin, PDGF or MCP-1 production by HTEC the fatty acid component was not of significance in these experiments. However alterations to molecules carried by albumin in minimal change nephrosis may not be confined to fatty acids.

Summary

The data in this thesis has shown a number of effects of proteins on the biology of tubular cells. A polarised human tubular cell model was developed to investigate the effects of proteins applied specifically to the apical surface of the cells as would occur in proteinuria. It was demonstrated that HTEC grown on permeable membranes produced the matrix protein fibronectin, the cytokine PDGF and the chemokine MCP-1 all of which may be of importance in the development of interstitial inflammation and scarring. On apical exposure to serum proteins HTEC increased their basolateral secretion of fibronectin, PDGF and MCP-1 which could provide a mechanism by which proteinuria initiates or worsens interstitial pathology. Moreover apical serum proteins were shown to be toxic to HTEC monolayers. The effects of serum on HTEC were isolated to a fraction of molecular weight 43-100kD which would be filtered in glomerular disease. The precise component of this fraction which has the effect on HTEC is yet to be determined but its effects could not be reproduced by pure albumin or transferrin when added alone.

There is now accumulating evidence that tubular cells, through interactions with chronic inflammatory cells and fibroblasts, can influence the development of interstitial
inflammation and scarring and this thesis has provided further evidence to suggest how that process may be stimulated by proteinuria. The currently available evidence is summarised in Figure 8.1.
Fig 8.1 Interactions between proteinuria, tubular cells, macrophages and fibroblasts
APPENDIX 1

SOLUTIONS
Solutions were made up in deionized water unless otherwise stated

**Alkaline Phosphatase Substrate Solution**

Naphthol AS-mx phosphate (Sigma N4875) 0.02 g
in 2ml N,N dimethyl formamide (Sigma 90-10)

Added to: 98ml 0.1M Tris HCl pH 8.2
+ 0.1ml 1M levamisole (Sigma L9756).

**Electron Microscopy fixative**

Glutaraldehyde 2.5% (v/v)
Formaldehyde 2% (v/v)
Sodium phosphate buffer 0.1M pH7.4

**Electrophoresis running buffer**

Tris base 3g/l
Glycine 14.40g/l
SDS 1g/l

**Electrophoresis sample buffer**

Deionised water 3.8ml
0.5M Tris-HCl, pH 6.8 1.0ml
Glycerol 0.80ml
10%(w/v) SDS 1.6ml
0.05% bromophenol blue 0.4ml
### 7.5% Electrophoresis separating gel

Deionised water & 4.85ml \\
1.5M Tris-HCl pH 8.8 & 2.50ml \\
10\%(w/v) SDS & 0.10ml \\
Acrylamide/Bis 37.5:1 (30% stock) & 2.50ml \\

The above mixture was degassed for >15 minutes

10\%(w/v) ammonium persulphate & 0.05ml (made fresh daily) \\
TEMED & 5.00μl \\

Alternative percentage gels were made by varying the amount of acrylamide/bis and water.

### Electrophoresis stacking gel

Deionised water & 6.10ml \\
0.5M Tris-HCl pH 6.8 & 2.50ml \\
10\%(w/v) SDS & 0.10ml \\
Acrylamide/Bis 37.5:1 (30% stock) & 1.30ml \\

Degas >15 minutes

10\%(w/v) ammonium persulphate & 0.05ml \\
TEMED & 0.01ml \\

### ELISA plate coating buffer

Sodium bicarbonate 0.378 g/100ml (45mM) \\
Sodium carbonate 0.053g/100ml (5mM) \\
pH 9.6
ELISA substrate buffer

1,2-Phenylenediamine dihydrochloride 8mg (4 tablets) in 3ml
Disodium hydrogen orthophosphate 12 H₂O 0.67M
Citric acid 0.33M pH 5.0
immediately before use 5μl of 30% hydrogen peroxide added

Ion exchange chromatography buffers

Buffer A
Bis Tris Propane (6.25mM) 1.76 g/l
Adjusted to pH7.5 with 1M HCl

Buffer B
Bis Tris Propane 6.25mM
Sodium chloride (0.35M) 20.3g/l
Adjusted to pH9.5 with 1M HCl

Lowry solution A
Anhydrous Na₂CO₃ 20.00 g/l
NaOH pellets 4.00g/l
K₂Na-tartrate.4H₂O 0.2g/l
made up in deionized water and stored at +4°C

Lowry solution B
CuSO₄.5H₂O 5.00g/l
made up in deionized water and stored at +4°C
α-Naphthyl acetate esterase staining

Staining solution
Sodium nitrite 0.1M 1.0 ml
Fast Blue Base solution 1.0 ml
stand for 2 minutes then add to 40 ml deionised water prewarmed to 37°C
Tris maleate 1.0 M pH 7.6 5.0 ml
α-Naphthyl acetate (12.5 mg/ml in methanol) 1.0 ml

Citrate-acetone-formaldehyde fixative (CAF)
Citric acid 18 mM 25 ml
Acetone 65 ml
Formaldehyde 37% 8 ml

Phosphate Buffered Saline (PBS)
NaCl 8.00g/l 137mM
Na₂HPO₄ 1.15g/l 8mM
KCl 0.20g/l 2.7mM
KH₂PO₄ 0.20g/l 1.5mM

Tris Buffered Saline pH 7.6
Tris base 60.56 g/l
NaCl 85.2 g/l
pH adjusted using 1M HCl.
1.5M Tris HCl pH 8.8
Tris base  18.15g/100ml
pH corrected with 1M HCl

0.5M Tris HCl pH 6.8
Tris base  6.5g/100ml
pH corrected with 1M HCl

Trypsin-EDTA
Trypsin  0.5g/l
EDTA  0.2g/l
made up in modified Pucks Saline A
Life Technologies: 45300-019

Washing Buffer (WB)
NaCl  28.00g/l
Na₂HPO₄  1.15g/l
KCl  0.20g/l
KH₂PO₄  0.20g/l
Polyoxyethylene-sorbitan monolaurate (Tween 20) 1ml/l
Western Blot Transfer Buffer

Tris Base 3.03g/l (25mM)
Glycine 14.4g/l (192mM)
Methanol (high grade analytical reagent) 20% (v/v)
APPENDIX 2

SUPPLIERS
Advanced Protein Products
Unit 18H, Premier Partnership Estate,
Brierley Hill, West Midlands, DY5 3UP

Amicon Ltd
Upper Mill, Stonehouse,
Gloucs GL10 2BJ

Bio Rad Labs Ltd
Bio Rad House, Maylands Ave,
Hemel Hempstead, Herts, HP2 7TD

Collaborative Biomedical
Products/Falcon/Beckton Dickinson Ltd
Between Towns RD, Cowley,
Oxford, OX4 3LY

Dako Ltd
16 Manor Courtyard, Hughenden Ave,
High Wycombe, Bucks, HP13 5RE

Millipore UK Ltd
The Boulevard, Blackmoor Lane,
Watford, WD1 8YW

Pharmacia Biotech
23 Grosvenor Rd, St Albans,
Herts, AL1 3AW

Sigma Chemical Co Ltd,
Fancy Rd, Poole,
Dorset, BH17 7NH

Amersham Int Plc
Amersham Place, Little Chalfont,
Bucks, HP7 9NA

BDH (Merck Ltd)
Hunter Boulevard, Magna Park,
Lutterworth, Leics, LE17 4XN

Ciba-Corning Diagnostics
Colchester Rd, Halstead,
Essex CO9 2DX

Costar UK Ltd
10 The Valley Centre, Gordon Rd,
High Wycombe, Bucks, HP13 6EQ

Gibco/Life Technologies
Ichinnan Business Park,
Renfrewshire, PA4 9RF

National Diagnostics
Unit 4, Fleet Business Park,
Hull, HU13 9LX

R and D Systems
4-10 The Quadrant, Barton Lane,
Abindon, Oxon, OX14 3YS
APPENDIX 3

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK
MANUSCRIPTS


ABSTRACTS:


3. Burton CJ, Harris KPG and Walls J. The production of fibronectin by cultured proximal tubular cells is increased by serum proteins. UK Renal Association spring meeting, Edinburgh 1994. (poster presentation)


5. Burton CJ, Walls J, Harris KPG. Human cortical epithelial cells (HCEC) secrete PDGF and fibronectin on apical exposure to proteins. UK Renal Association spring meeting, Newcastle upon Tyne 1995. (poster presentation)


9. Burton CJ, Walls J, Harris KPG. Apical exposure of tubular cells to proteins: a stimulus to interstitial inflammation and scarring?
European Kidney Research Forum, Bergamo, 1996
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Maunsbach AB, Christensen EJ (1974): Lysosomal accumulation and digestion of b2-microglobulin in rat kidney tubules. IRCS 2: 1737

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Teitelbaum AP, Strewler GJ (1984): Parathyroid hormone receptors coupled to cyclic adenosine monophosphate formation in an established renal cell line. Endocrinology 114: 980-985


Welling LW, Welling DJ (1975): Surface area of brush border and lateral cell walls in the rabbit proximal nephron. Kidney Int 8: 343-348


