THE ROLE OF LIPIDS IN THE PATHOGENESIS OF GLOMERULOSCLEROSIS

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Most patients with chronic renal impairment develop progressive glomerulosclerosis and eventually require dialysis. The pathogenesis of this process is poorly understood. This thesis addresses the possibility that hyperlipidaemia initiates and/or aggravates glomerulosclerosis.

The handling of lipoproteins by human glomerular cells was studied by incubating cells cultured from isolated human glomeruli with fluorescent-labelled lipoproteins. It was demonstrated that all cells take up low density lipoproteins (LDL) by receptor-mediated endocytosis. Chemically modified LDL was taken up by macrophages, suggesting that human glomerular 'foam' cells are derived from monocytes rather than intrinsic mesangial cells.

A series of experiments was performed in rats to investigate the effects of manipulating serum lipids on glomerular damage. Attempts to lower serum cholesterol with cholestyramine and synvinolin were unsuccessful. Dietary cholesterol supplementation caused hyperlipoproteinaemia and glomerular lipid deposition, especially of cholesterol and cholesteryl esters. In rats made uraemic and hypertensive by 1/3 nephrectomy, the diet did not affect the severity of proteinuria or glomerulosclerosis over a 7 week period. However, when administered for 21 weeks following unilateral nephrectomy, proteinuria was increased. The number of bone marrow-derived glomerular macrophages was slightly but significantly increased and many became foam cells. These changes were not associated with glomerulosclerosis but there was a significant increase of glomerular size which is known to precede sclerosis.

These results suggest that hyperlipidaemia and glomerular lipid deposition contribute to the development of glomerulosclerosis by stimulating an influx of macrophages which may cause glomerular hypertrophy and injury through the release of factors during phagocytosis. The magnitude of these changes indicates that the effect of lipids is likely to be low-grade and only of importance over a prolonged period of time.
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OF GLOMERULOSCLEROSIS

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A thesis submitted
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to my wife
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DECLARATION

The experimental work described in this thesis was carried out between September 1986 and September 1988 whilst I was employed as Research Assistant in Nephrology (Registrar Grade) at Leicester General Hospital. The post was funded by the National Kidney Research Fund and the Leicester Kidney Patients' Association.

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I was responsible for the design of all experiments and carried out all other experimental work myself. I performed the qualitative and quantitative analysis of all histological samples and biochemical data. I took all the photographs except the electron micrographs and collaborated with the Department of Medical Illustration in their printing and in the production of computer graphics. Mrs. Pam Halford typed the thesis. I am responsible for its final format and any errors contained within it.
The only way that "clear notions upon the subject can be obtained consists in re-examining whether the condition of fatty degeneration is a primary or a secondary one, whether it sets in as soon as the disturbance can be perceived, or whether it does not occur until some other perceptible disturbance has gone before".

RLK Virchow, commenting on the "fatty degeneration of the renal epithelium in Bright's Disease".
A more precise account of fatty metamorphosis. in Chance, Cellular Pathology, 1860.
2nd edition pp 342-366
Gryphon Editions, Birmingham 1860.
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INTRODUCTION

A sad but inevitable fact confronting patients with chronic renal failure is the likelihood that their residual renal function will progressively decline until they require renal replacement therapy. In a recent survey of 108 patients with mild to moderate renal failure attending the Royal Liverpool Hospital, 85 demonstrated a progressive increase in serum creatinine concentration when followed over at least 6 months [1]. Similar trends have been reported by many other authors [1-5]. The decline in function often proceeds despite the original disease process being no longer active. The study of the mechanisms by which this decline occurs and the development of treatments aimed at slowing or arresting it, remains one of the foremost challenges in nephrology.

The histological accompaniment of progressive renal failure is glomerular scarring or sclerosis. A number of similarities can be drawn between the sclerotic lesions that develop in the capillaries of the glomerular tuft and atherosclerotic plaques found in larger vessels. These similarities suggest that common pathogenetic mechanisms may be involved in both lesions. The work contained in this thesis addresses one such possible mechanism; namely that circulating lipids contribute to the damage of glomerular vessels and to the development of glomerulosclerosis.
SECTION I
BACKGROUND
CHAPTER 1
LIPOID METABOLISM AND ITS DISTURBANCE IN RENAL DISEASE.

NORMAL LIPOPROTEIN METABOLISM

Cholesterol and triglyceride are insoluble in water. For them to be transported in the circulation, they must be enclosed within a shell of hydrophilic proteins and phospholipids as lipoproteins. There is a range of these macromolecules composed of varying proportions of cholesterol, cholesteryl esters and triglycerides and bearing different apoproteins on their surface (table 1.1).

The metabolic pathways by which lipoproteins are synthesised and degraded are summarised in Figure 1.1. Dietary fat is taken up by epithelial cells in the small intestine where it is packaged with phospholipids and apoproteins, including apoB-48 [6], to form chylomicrons. These are released into the circulation, where an interchange of apoproteins occurs with the small, high density lipoproteins (HDL). Apoproteins C and E, taken up from HDL [7], are important in the subsequent degradation of chylomicrons. Apoprotein CII activates the enzyme lipoprotein lipase which is situated in the endothelium of peripheral capillaries. This enzyme breaks down the triglyceride contained in chylomicrons, releasing free fatty acids which are taken up by tissues and either for used as an energy source or stored as triglyceride. The excess phospholipids and apoproteins A and C left after the removal of triglyceride are transferred to HDL. The residual chylomicron remnants bind to apoprotein E receptors in the liver and are taken up and catabolised.
Table 1.1 Size, composition and density characteristics of human plasma lipoproteins.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Lipid core</th>
<th>Apoprotein</th>
<th>Average diameter (Å)</th>
<th>Apparent molecular wt. (Da)</th>
<th>Isolation density (g/ml)</th>
<th>Electrophoretic mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>Triglyceride &gt;&gt;cholesterol ester</td>
<td>AI, AII, AIV B-48, C, E</td>
<td>&gt;1000</td>
<td>&gt;50x10^6</td>
<td>&lt;1.006</td>
<td>remain at origin</td>
</tr>
<tr>
<td>VLDL</td>
<td>Triglyceride &gt;&gt;cholesterol ester</td>
<td>B-100, C, E</td>
<td>300-800</td>
<td>10 - 80x10^6</td>
<td>&lt;1.006</td>
<td>pre-β</td>
</tr>
<tr>
<td>IDL</td>
<td>Triglyceride &amp; cholesterol ester</td>
<td>B-100, E</td>
<td>c.300</td>
<td>5 - 15x10^6</td>
<td>1.006 - 1.019</td>
<td>slow pre-β</td>
</tr>
<tr>
<td>LDL</td>
<td>Cholesterol ester &gt;&gt;triglyceride</td>
<td>B-100</td>
<td>220</td>
<td>3x10^6</td>
<td>1.019 - 1.063</td>
<td>β</td>
</tr>
<tr>
<td>HDL₂</td>
<td>Cholesterol ester</td>
<td>AI, AII, C</td>
<td>120</td>
<td>3x10^5</td>
<td>1.063 - 1.125</td>
<td>α</td>
</tr>
<tr>
<td>HDL₃</td>
<td>Cholesterol ester</td>
<td>AI, AII, C</td>
<td>85</td>
<td>1.5x10^5</td>
<td>1.125 - 1.21</td>
<td>α</td>
</tr>
</tbody>
</table>

Taken from:-

i) Disorders of lipid transport. Lewis B.
   In: Oxford Textbook of Medicine, Eds. Weatherall DJ, Ledingham JGG, Warrell DA.

ii) Separation and analysis of lipoproteins by gel filtration.
   Rudel LL, Mazetta CA, Johnson FL. Methods in Enzymology 1986; 129: 46

iii) Lipoprotein receptors and cholesterol homeostasis.
Figure 1.1 The synthesis, transport and catabolism of lipoproteins.
As well as originating in the intestine, lipoproteins are also synthesised in the liver. Cholesterol and free fatty acids are converted into very low density lipoproteins (VLDL), which contain the larger form of apoprotein B, apoB-100 [6]. Following its release into the circulation, VLDL is progressively broken down in a similar way to that described for chylomicrons. It is depleted of triglyceride by lipoprotein lipase on peripheral capillary endothelial cells and the excess apoproteins A and C, cholesterol and phospholipids are taken up by HDL. The residual particles have a density between VLDL and LDL and are designated intermediate density lipoproteins (IDL). Like chylomicron remnants, these are taken up and catabolised by the liver via receptors recognising apoprotein E. Not all IDL is catabolised, however. Approximately 50% is converted into cholesterol-rich low-density lipoprotein (LDL) by lipoprotein lipase on hepatic endothelial cells. This is the principal cholesterol-carrying lipoprotein in man. Approximately one-third of LDL in the circulation is utilised by peripheral tissues (vide infra) and the other two-thirds return to the liver where they are catabolised.

The smallest lipoprotein, HDL, is thought to be important in the reverse transport of cholesterol from peripheral tissues to the liver [8]. HDL is secreted by the small intestine and the liver in a small, discoidal form, designated HDL3. This takes up unesterified cholesterol from peripheral tissues which is then esterified by the plasma enzyme lecithin-cholesterol acyltransferase (LCAT). This enlarges HDL3 to form the spherical, less dense, HDL2.

It is important to remember that, although the classes of lipoproteins are similar, the relative proportions of the different lipoproteins vary considerably between species [9]. In the rat, approximately two-thirds of lipoproteins have α-electrophoretic mobility, i.e. are of high density [10] compared to only one-fifth in man. The apoprotein composition of rat VLDL is also different. Unlike in man, where apoB-100 is the sole apoprotein in VLDL, rat VLDL contains significant amounts of the intestinal apoprotein apoB-48 which is more rapidly catabolised than apo B-100 [11]. Most rat VLDL and
chylomicron remnants are catabolised by the liver and only a small proportion are metabolised to form LDL [12]. These differences in lipoprotein pattern and composition may explain the resistance of this species to the development of atherosclerosis [13,14].
THE UPTAKE OF LIPIDS BY CELLS AND THE ROLE OF LIPOPROTEIN RECEPTORS.

The most important source of energy for peripheral tissues is fatty acids which are transported in the circulation bound to albumin. Since they are lipid soluble, they are able to enter cells by diffusion through the plasma membrane. The concentration gradient that enables diffusion to take place is maintained by the esterification of fatty acids with glycerol within the cell to form triglyceride or by their catabolism via the Kreb's cycle.

Cholesterol is required by peripheral tissues for the synthesis and turnover of plasma membranes. Endocrine glands, such as the adrenals and ovaries, have further cholesterol requirements for the synthesis of steroid hormones. Cholesterol can be obtained either by intracellular synthesis or by the uptake of esterified cholesterol contained in circulating LDL. The mechanism by which LDL is taken up by peripheral tissues has been elucidated by studies on cultured human fibroblasts by Goldstein and Brown [15]. They demonstrated that fibroblasts express a receptor which recognises apoproteins B-100 and E [16]. This receptor has subsequently been isolated, its amino acid structure determined and the genes coding for it sequenced [17]. The receptors are localised in areas of the cell membrane coated with a protein called clathrin. Once LDL is bound to the receptor, these 'coated pits' invaginate and form coated vesicles, by a process of endocytosis (Figure 1.2). The endocytotic vesicles fuse with lysosomes and the LDL is then hydrolysed by lysosomal enzymes. Free cholesterol, released from the breakdown of cholesteryl ester in LDL, diffuses into the cytoplasm where it has three actions. Firstly, the endogenous synthesis of cholesterol is suppressed through down-regulation of the enzyme 3-hydroxymethyl glutaryl-CoA reductase (HMG CoA reductase), which is the rate limiting step of cholesterol synthesis. Secondly, the enzyme that esterifies free cholesterol for subsequent storage, acylCoA acyltransferase (ACAT), is activated [18]. And thirdly, the synthesis of LDL receptor molecules is down-regulated [19]. In this way the level of intracellular cholesterol is homeostatically regulated.
Figure 1.2 Receptor-mediated uptake of LDL and acetylated LDL.
The role of the apo B/E receptors in the uptake of cholesterol by tissues in vivo has been studied by measuring the rate of catabolism of radiolabelled native LDL and comparing it with LDL that has been modified so that it no longer binds to any type of lipoprotein receptor [16]. In the rat [20], native LDL is cleared principally by the adrenal glands and ovaries, which require cholesterol for hormone synthesis. The kidney accounts for 3.2% of the whole-animal clearance of native LDL. LDL modified by methylation is principally cleared by the spleen. In the kidney, only small amounts of methylated LDL are taken up and 83% of LDL uptake by this organ is LDL receptor-dependent.

As well as expressing receptors with high affinity for apoprotein B/E, macrophages [21] and some endothelial cells [22,23,24,25] also express receptors that are specific for LDL that has undergone certain forms of chemical modification [26]. These receptors have also been isolated and characterised [27]. Receptor-bound modified LDL is taken up by endocytosis in the same way as native LDL [28,29]. Unlike the apo B/E receptor, these "scavenger" receptors are not down regulated by the level of intracellular cholesterol (figure 1.2). As a result, progressive accumulation of cholesterol occurs when macrophages are incubated with modified LDL. Eventually lipid vacuoles form within the cytoplasm and the cells take on a "foamy" appearance.

The chemical modification of LDL required for binding to the "scavenger" receptor involves an increase in its negative charge, due to the removal of positive charges from lysine groups in the apoprotein molecule. Common chemical reactions used to modify LDL in vitro include acetylation, maleylation and reaction with malondialdehyde [30]. LDL can also be modified 'biologically' in vitro by incubation in the presence of endothelial cells [31,32], smooth muscle cells [33], neutrophils [38] and macrophages [34]. This biological modification involves peroxidation of LDL by superoxide radicals and is catalysed by iron and copper ions [33,35]. There is evidence that such modification of LDL may also occur in vivo. Low density lipoprotein isolated from inflammatory fluids [36] and from the
arterial intima [36] has been found to be chemically modified in a similar way to that produced in vitro. Extracts from human atherosclerotic aorta are taken up by mouse peritoneal macrophages via specific receptors that are blocked by the polysaccharide fucoidin [37], a known inhibitor of the scavenger receptor.

The uptake of modified LDL in vivo has been studied using fluorescent and radiolabelled chemically-modified LDL [20,27]. When injected into rats, guinea pigs and a mongrel dog, the predominant site of uptake was the hepatic sinusoidal endothelium, followed by the endothelium of the spleen, bone marrow and adrenal glands. No uptake was detected by endothelium of other major organs, including the kidney and hepatic macrophages (Kupffer cells) only accounted for 14% of the total liver uptake. Binding and uptake by hepatic endothelial cells was saturable and of high affinity, indicating that it was receptor-mediated, and degradation of acetoacetoylated LDL, a measure of the rate of uptake, was tenfold greater in hepatic endothelial cells than in aortic endothelial cells.

The distribution of endothelial cells that express scavenger receptors may vary between species. Although Pitas et al. [27] found no uptake by endothelial cells in major organs, Netland et al. [24] demonstrated uptake by the endothelium of liver, brain and testis and by cells in the glomeruli of mice. The glomerular cells involved were described as endothelial cells but were not characterised beyond their anatomical location under light microscopy.

Uptake of modified LDL by macrophages and endothelial cells has been shown to lead to alterations in cellular activity. Incubation of bovine aortic and human umbilical vein endothelial cells with acetylated LDL reversibly reduces the production of platelet-derived growth factor-like protein; a substance that has been shown to stimulate mesenchymal cell growth [39]. Incubation of bovine aortic endothelial cells with malondialdehyde LDL renders them less sensitive to the action of insulin, as measured by the uptake of aminoisobutyric acid [40]. Thirdly, HDL receptor expression by bovine aortic endothelial cells, human fibroblasts and human smooth muscle cells is increased by incubation with acetylated LDL [41]. These effects may be
mediated via an increase in the intracellular cholesterol content following the uptake of modified LDL.

Expression of scavenger receptors on macrophages may be regulated by chemical messengers. When circulating monocytes are incubated in vitro they convert into macrophages over a six days period. The activity of their apo B/E LDL receptors decreases and that of the scavenger receptors increases [42]. Incubation of human macrophages with conditioned culture medium taken from a culture of mature macrophages, increased the expression of scavenger receptors [43]. Conversely, incubation with a lymphokine [42] present in conditioned medium taken from a culture of lymphocytes that had been stimulated with concanavalin A [44], selectively down-regulated both the apo B/E LDL and scavenger receptors on macrophages and prevented the accumulation of cholesteryl ester [44].

In addition to the apoB/E LDL receptor and the scavenger receptor, a third type of receptor has been described that is specific for apoproteins B and E in β-migrating VLDL. βVLDL is isolated from cholesterol-fed animals and is composed of remnant particles from VLDL catabolism [13]. βVLDL receptors have been demonstrated on bovine and rabbit endothelial cells [22], macrophages [45] and foam cells isolated from atherosclerotic rabbit aortas [46]. Since they have an affinity for apoprotein E, the βVLDL receptors on murine macrophages are also able to take up remnants of chylomicron breakdown [47].

Since βVLDL contains apoproteins B and E, it can also bind to the apo B/E LDL receptor, for example on smooth muscle cells [46] and fibroblasts [45]. That there is a receptor for βVLDL distinct from the apo B/E receptor, is suggested by the following evidence. Incubation of macrophages with βVLDL leads to an increase in the cholesterol ester content of cells which is not found following incubation with LDL [45], indicating that the βVLDL receptor is resistant to down regulation by cellular cholesterol [48]. Uptake of βVLDL by endothelial cells [22] and aortic foam cells [46] is not inhibited by excess amounts of native LDL. Uptake of βVLDL has also been demonstrated in macrophages taken from Watanabe rabbits, which congenitally lack the apo B/E LDL receptor [49].
A protein that has a high affinity for βVLDL has been isolated and characterised from mouse peritoneal macrophages [48]. It differs from the apoprotein B/E LDL receptor in having a lower affinity for LDL, a high affinity for βVLDL and apoprotein E, and a lower molecular weight. It is immunologically similar, however, in that it cross-reacts with antibodies raised against the apoB/E LDL receptor [50].
### Table 1.2. Lipid abnormalities in uraemia.

<table>
<thead>
<tr>
<th>Category</th>
<th>Abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids</td>
<td>↑ triglyceride</td>
</tr>
<tr>
<td>Lipid classes</td>
<td>↑ VLDL, ↑ IDL, ↓ HDL</td>
</tr>
<tr>
<td>Lipoprotein composition</td>
<td>↑ triglyceride in VLDL, IDL, LDL</td>
</tr>
<tr>
<td></td>
<td>↑ cholesterol in VLDL, βVLDL</td>
</tr>
<tr>
<td></td>
<td>↓ cholesterol in HDL</td>
</tr>
<tr>
<td>Apolipoproteins</td>
<td>↓ apo A-I, ↓ apo A-II, ↑ apo A-IV</td>
</tr>
<tr>
<td></td>
<td>↑ apo B-48</td>
</tr>
<tr>
<td></td>
<td>or ↑ apo C-II, ↑ apo C-III</td>
</tr>
<tr>
<td></td>
<td>↓ apo E</td>
</tr>
<tr>
<td>Enzymes</td>
<td>↓ hepatic triglyceride lipase</td>
</tr>
<tr>
<td></td>
<td>↓ lecithin-cholesterol acyl transferase</td>
</tr>
</tbody>
</table>

Modified from:-
Ritz E and Bommer J.
Endocrine and metabolic dysfunction in chronic renal failure.
Eds. Schrier RW, Gottschalk CW.
Chapter XIV, p 3136.
ABNORMALITIES OF LIPOPROTEIN METABOLISM IN URAEMIA.

Uraemic hyperlipidaemia in man.

Lactescence of the serum of uraemic patients was reported by Richard Bright in 1827. The abnormalities of lipoprotein metabolism in uraemia described to date are listed in Table 1.2. The most consistent change in serum lipids is an increase in total serum triglyceride concentration [51], resulting in a Fredrickson type IV hyperlipidaemia. Total serum cholesterol levels are usually normal. The distribution of lipoprotein classes is abnormal with VLDL and IDL levels being elevated and HDL levels decreased [52,53,54,55]. The composition of these lipoproteins is also abnormal, VLDL being enriched with cholesterol and LDL, IDL and HDL with triglyceride. Abnormal VLDL bands, designated βVLDL according to their electrophoretic mobility, have been described in uraemic adults [52] and children [54]. These are composed of remnant particles from the breakdown of VLDL and chylomicrons (vide infra).

The profile of apoproteins is abnormal in uraemic patients and this distinguishes the hyperlipidaemia of uraemia from other forms of hypertriglyceridaemia [56]. Consistent with the decrease in HDL, apo AI and AII levels are reduced. Total apo B levels are normal, although the βVLDL particles remaining from chylomicron breakdown contain increased amounts of apo B-48 [57]. Apo CII and CIII levels are increased. Apo E levels are reduced prior to dialysis [56] but are elevated in patients on chronic haemodialysis [57].

An increase in serum triglyceride concentration may be due to an increase in lipoprotein production and/or a reduction in its clearance. Triglyceride production has been measured in uraemic patients using radiolabelled glycerol and has been found to be reduced compared to controls [58]. A correlation normally exists between the rate of VLDL secretion and the plasma triglyceride concentration. This relationship persists in uraemia but for a given level of VLDL secretion, the plasma triglyceride concentration is higher [59]. This, combined with the altered distribution of lipoprotein classes described above, suggests that abnormal lipoprotein interconversion and breakdown is the more important mechanism for the development of
hyperlipidaemia in uraemia.

Hiller et al. observed in 1924 that a fat-rich meal produced a greater rise in post-prandial plasma total fatty acids and lecithin in nephritic-nephrotic patients than in normals [74]. The kinetics of lipoprotein catabolism in uraemia have subsequently been studied in more detail. The clearance of radiolabelled VLDL triglyceride was found to be reduced in patients with non-end stage chronic renal failure by Savdie et al. [60]. Clearance of triglycerides has also been evaluated in the local Leicester population by Russell, Davies and Walls using an intravenous Intralipid fat-tolerance test [61]. The mean rate constant for the removal of Intralipid was reduced in patients both with non-end stage chronic renal failure and on haemodialysis and was inversely proportional to the serum triglyceride concentration. However, there was an overlap of the individual clearance rates with normal controls. In those patients with normal rates of clearance, the level of VLDL synthesis may have been inappropriately high in relation to the rate of catabolism. Variations in diet may explain this overlap since triglyceride synthesis is particularly dependent upon the intake of carbohydrate [58].

The mechanisms underlying the reduction in lipoprotein clearance are unclear. The enzymes responsible for lipoprotein clearance are lipoprotein lipase on extrahepatic endothelial cells and hepatic triglyceride lipase on hepatic endothelial cells. Both enzymes are released into the circulation by heparin and are measured as the post-heparin lipolytic activity (PHLA). The level of PHLA is reduced in uraemia [51] and this has been shown to be due to a selective decrease in hepatic triglyceride lipase levels, with lipoprotein lipase levels being normal [62]. PHLA may not necessarily represent the activity of enzymes within the tissues, however. Low levels of lipolytic activity have been found in adipose tissue taken from uraemic patients [63].

Abnormalities distal to the action of lipoprotein lipase may also exist in uraemia. Walldius et al. have demonstrated an increase in the rate of turnover of fatty acids in uraemia, with the incorporation of fatty acids into adipose tissue being lower in uraemic patients with hypertriglyceridaemia than in those with normal lipid levels [64].
Abnormalities of cholesterol metabolism have been demonstrated in uraemia. Transport of cholesterol away from peripheral tissues may be impaired in patients on haemodialysis due to a decrease in LCAT, the enzyme required for esterification of cholesterol and hence its incorporation into HDL [65]. This abnormality has been investigated in vitro by studying the exchange of cholesterol between cultured human fibroblasts and the culture medium. It has been shown that the normal net transport of cholesterol from the cells into the medium is reversed in the presence of plasma taken from patients on haemodialysis but not from those on chronic peritoneal dialysis [65]. Conversely, abnormalities in the composition of LDL isolated from haemodialysis patients have been shown to be associated with a decrease in the uptake and degradation of LDL by cultured fibroblasts, suggesting an impairment of binding to the LDL receptor [66].

Uraemic hyperlipidaemia in the rat.

Experimental rat models of uraemia show different alterations in lipoprotein metabolism to those described in man. The hyperlipidaemia found in rats made acutely uraemic by removal of nine-tenths of the kidney tissue consists of an increase in both total triglyceride and cholesterol with an increase in cholesterol in both α, pre-β and β-migrating lipoproteins [10]. There is a corresponding increase in both HDL and LDL cholesterol [67] and the ratio of free to total cholesterol is also increased [10].

As in man, impaired clearance of lipoproteins appears to be the more important factor in these changes. The activity of the enzyme regulating cholesterol synthesis, HMG CoA reductase [10], and of the enzyme controlling triglyceride synthesis, acetyl CoA carboxylase [67], are not increased. Triglyceride secretion, measured by inhibiting triglyceride removal with Triton WR-1339, was found to be unchanged compared to sham operated controls [68,67]. Conversely, the half-time for the removal of 3H VLDL in rats was prolonged from 5 to 7 minutes following subtotal nephrectomy [68]. The slowing of lipoprotein clearance is most likely due to a reduction of lipoprotein lipase activity in peripheral tissues. Post-heparin lipolytic activity
is reduced [67] and lipolytic activity in adipose tissue taken from uraemic rats was found to be decreased [69], although only when expressed per number of cells rather than per gram of tissue [67]. Hypercholesterolaemia may be partly due to reduced uptake of LDL by peripheral tissues since preliminary data in uraemic guinea pigs suggest that catabolism of uraemic LDL is slower than normal LDL [70]. Results in the guinea pig may not be applicable to rats, however, since the guinea pig carries cholesterol predominantly in LDL and has different sites of lipoprotein synthesis to the rat [71].

The mechanism of the effect of uraemia on lipoprotein metabolism.

How renal damage and uraemia lead to abnormalities of lipoprotein metabolism in man and experimental animals remains uncertain [72]. The kidney is not important in the synthesis of lipoproteins but does participate in the catabolism of high density lipoprotein [73]. Bilateral nephrectomy in the rat led to an equivalent rise in serum lipids to that induced either by bilateral ureteric ligation [74] or by vesicojugular reinfusion of urine [72], suggesting that it is uraemia itself rather than the loss of parenchymal renal tissue that affects lipid metabolism. Plasma from uraemic patients does not reduce the activity of lipolytic enzymes in vitro [62, 1m51]. However, serum from uraemic rats has been shown to inhibit the lipoprotein lipase activity of adipose tissue [67].

Uraemia may act indirectly via alterations in the hormones that affect lipid metabolism. For example, parathyroid hormone is necessary for normal lipid metabolism in the rat. Hyperparathyroidism, induced by a low calcium diet, led to an increase in serum cholesterol and triglyceride concentrations which was mediated by a reduction in triglyceride clearance [75]. Similarly, parathyroidectomy in intact rats led to a reduction in serum cholesterol and triglyceride. In acute uraemia, following bilateral nephrectomy or bilateral ureteric ligation, parathyroidectomy partially prevented the development of hyperlipidaemia [74]. In chronically uraemic rats with nephrotoxic serum nephritis, parathyroidectomy also reduced cholesterol and triglyceride levels [76] but this was associated with an amelioration
in proteinuria and renal damage and so the improvement in lipid levels may have been a secondary phenomenon. In man, parathyroidectomy did not control hypertriglyceridaemia in uraemic hyperparathyroid patients [77].

It has been suggested that hyperinsulinism in uraemia leads to the overproduction of lipoproteins [67]. However, conflicting results have been reported regarding the relationship between plasma insulin levels and plasma triglyceride concentration. Reaven et al. reported that triglyceride levels were lower than expected for a given level of insulin after a meal [59] whereas Bagdade et al. found an opposite shift [51]. Following transplantation, immunoreactive insulin levels are increased despite cholesterol and triglyceride levels being lower than on dialysis [77]. In uraemic rats, insulin levels are reduced in the presence of hyperlipidaemia [69, 10].

In view of this, and the evidence of a reduction in lipoprotein clearance rather than overproduction of lipoproteins in uraemia, insulin does not appear to be an important mediator of uraemic hyperlipidaemia.
ABNORMALITIES OF LIPOPROTEIN METABOLISM IN THE NEPHROTIC SYNDROME

Hyperlipidaemia is one of the four characteristic features of the nephrotic syndrome, the other three being heavy proteinuria, hypoalbuminaemia and oedema. The pattern of lipoprotein abnormalities found in this condition is different from that in uraemia. Typically, in both man [78] and the rat [79,80], there is an initial elevation in serum total cholesterol concentration. This is due to an increase in the cholesterol in LDL and VLDL, with HDL levels being normal or low [81,82]. There is a smaller increase in phospholipid concentration and serum triglyceride levels remain normal until the degree of proteinuria becomes very severe [82,83]. Abnormalities of the electrophoretic pattern and composition of lipoproteins are quite variable, particularly in man. This is due to the heterogeneous nature of the underlying renal disease, the varying degrees of associated uraemia, variations with the age of patients and the effects of concurrent medication. Even within the same patient, the pattern of abnormalities can vary from day to day [82].

In contrast to the hyperlipidaemia of uraemia, where impaired lipoprotein clearance is the predominant underlying mechanism, increased hepatic production of lipoproteins is an important factor in the development of hyperlipidaemia in the nephrotic syndrome. Studies on isolated liver from nephrotic rats have shown increased production of cholesterol, triglycerides and total fatty acids [79]. This has been confirmed in intact animals [80] and in human nephrotics [84]. Associated increases in the output of apoproteins, particularly apo A-I, apo B and apo E, have also been noted [79].

The hyperlipidaemia that results from the increase in lipoprotein production is exacerbated by a reduction in their catabolism. Early studies in adult nephrotic patients using radio-iodinated lipoproteins demonstrated an impairment in the catabolism of very low density lipoproteins (VLDL) [85]. More recently, Garber et al. have studied the metabolism of VLDL in rats with nephrotic syndrome induced by puromycin aminonucleoside [80]. Metabolism of $^{125}$I VLDL was slowed and there was a decrease in the transfer of phospholipid and apoproteins C and E from VLDL to HDL. This slowing was associated with
a 50% reduction in post-heparin lipolytic activity in nephrotic rats
and there was a similar 50% reduction in the lipolytic activity
released from nephrotic rat livers following perfusion with heparin.

The signal by which lipid metabolism is altered in the nephrotic
syndrome remains unclear. The early studies by Baxter et al. [82]
described an inverse and non-linear relationship between serum albumin
concentration and serum cholesterol, phospholipid and triglyceride
concentrations. The authors concluded that this indicated "no more
than the possibility (sic) that the hyperlipidaemia is caused by
hypoalbuminaemia". Subsequent studies by Baxter showed that nephrotic
hyperlipidaemia could be corrected by infusions of albumin or dextran
[86]. In a study of 20 adults with uncomplicated nephrotic syndrome,
Appel et al. [81] found a significant correlation between plasma
cholesterol concentration and both plasma oncotic pressure and plasma
albumin concentration, suggesting that plasma oncotic pressure was the
signal to increase lipoprotein production. Yedgar et al. have
suggested, however, that changes in plasma viscosity rather than plasma
oncotic pressure may affect lipoprotein synthesis. Studying nephrotic
rats, they demonstrated that the infusion of non-protein
macromolecules, which selectively increased plasma viscosity, reduced
the increased level of VLDL production found in these animals and
lowered serum cholesterol and triglyceride concentrations [87]. It is
not clear how these results apply to man, since no correlation was
found between plasma cholesterol concentration and plasma viscosity in
the study by Appel et al. [81].

The link between proteinuria and decreased lipoprotein catabolism
is likewise unclear. It has been suggested that an activator of
lipoprotein lipase (LPL) may be lost in the urine since urine taken
from rats with nephrotic syndrome induced by puromycin has been shown
to activate LPL [88]. The most potent activator of LPL is apo CII
which is contained in HDL. HDL is normally lost in the urine [89] and
these losses are increased in patients with heavy proteinuria [90] and
the nephrotic syndrome [91,92], especially if the proteinuria is non-
selective [93,94]. However, serum levels of apoprotein CII are not
depressed in nephrotic animals and it is unlikely that this is the
complete explanation of the decrease in lipoprotein catabolism [79].
CHAPTER 2
GLOMERULOSCLEROSIS AND THE GLOMERULAR MESANGIUM.

Glomerulosclerosis, or scarring of the glomerular tuft, is the final stage of glomerular injury [95]. A major part of the scarring process involves expansion of the region of the glomerulus that lies between the capillary loops, the glomerular mesangium. The following chapter briefly describes the normal structure and functions of this region.

The cellular composition of the mesangial region.

Using the light microscope, Zimmerman first described the glomerular mesangium in 1933 [96], but it required the development of the electron microscope for the structure of this region (also referred to as the inter-capillary or centrolobular region) to be described in detail. Previously, it had not been clear whether mesangial cells were distinct in origin or function from endothelial cells. However in 1960, Latta, Mounsbach and Madden described the distinct morphological features of the mesangial cell (figure 2.1) [97]. The cells were localised in the centrolobular region and were characterised by branching processes, which approached capillaries but did not form the border of a capillary lumen. Endothelial cells were closely associated with mesangial cells but did not give off branches into the centrolobular region. Subsequent studies have demonstrated a number of other distinguishing features. Mesangial cells contain actin and myosin [98] and are contractile, both in isolated whole glomeruli [99] and in culture [100]. They are thus comparable to smooth muscle arteriolar pericytes, providing structural support for the glomerular capillary loops. They are active in the regulation of glomerular capillary blood flow [101] and in the clearance of deposited filtration residues [102] (vide infra). They may also be important in the regulation of inflammatory processes within the glomerulus [103].

The smooth muscle-type mesangial cells described above make up the majority of the resident cells in the mesangial region. However, more recently it has been shown that the mesangium is also populated by
Figure 2.1. The structure of the rat mesangium.

Ep = epithelial cell (podocyte)     En = endothelial cell
M = mesangial cell                 RBC = red blood cell
Ma = mesangial matrix              Co = collagen bundles
F = endothelial fenestrations      IC = intercellular channels
IL = inner layer (lamina rara interna)
CL = central layer (lamina densa)
OL = outer layer (lamina rara externa)

(redrawn from [97])
small numbers of cells that express leukocyte common antigen [104,105]. These are resident macrophages that are derived from the bone marrow [106,107]. In the rat, a proportion of these cells are activated in the glomerulus to express immune-associated antigen (Ia Ag), equivalent to human HLA Class II mixed histocompatibility complex (MHC) antigen [105,108]. Infiltrating blood monocytes which express Ia Ag participate in the uptake of macromolecules deposited in the mesangium (vide infra) [109, 110]. In man, the normal glomerulus contains on average two cells expressing leucocyte common antigen per glomerular cross section [104]. These react mainly with antibodies against monocytes and it is likely that they are the equivalent of the resident macrophages in the rat mesangium. Class II MHC antigen is expressed by the glomerular endothelium [108] and therefore it is not possible to distinguish 'Ia-positive' macrophages in the normal human glomerulus. The numbers of monocytes in the glomerulus are increased in proliferative types of glomerulonephritis [111, 104].

Transport of macromolecules within the mesangium.

The mesangial cells are surrounded by an amorphous intercellular substance, the mesangial matrix, which is continuous with the inner less dense layer of the glomerular basement membrane and bounded by the dense layer [97] (Figure 2.1). This matrix contains collagen types IV and V [112], fibronectin [113,114], laminin [112], entactin [102] and heparan sulphate proteoglycan [98,115]. The matrix is permeated by channels between the mesangial cells. Tracer particles can gain access to these channels via fenestrations in the endothelial cells. Overlying the edge of the mesangial region, near to the basement membrane, these are up to 1,600A in diameter. In the more peripheral portions of the capillary wall they are much smaller, on average 100A in diameter [97].

The passage of macromolecules into the mesangial region has been studied in experimental animals using a range of tracer substances. These include inorganic materials such as thorium dioxide, colloidal gold [102] and colloidal carbon [116], polysaccharides such as dextran, iron dextran [98] and polyvinyl alcohol polymers [110],
proteins such as peroxidase, catalase and ferritin [117,118] and aggregated proteins such as albumin [119] immunoglobulin [120] and pre-formed immune complexes [98]. The passage of lipoproteins has not been studied specifically. In their original description, Farquhar and Palade [102] demonstrated that thorotrast (a suspension of thorium dioxide) accumulated in the inner less dense layer of the glomerular basement membrane and in the spongy region of the mesangium, between 1 and 4 hours after administration. After 1 day, particles had concentrated in the mesangium and were found within invaginations of mesangial cell membranes and in cytoplasmic vesicles and vacuoles inside the cells. The cells showed increased numbers of processes (pseudopodia) which extended into the capillary lumen. The phagocytic activity of endothelial cells was negligible compared to that of the mesangial cells. Within 2 to 4 days, the deposits had been cleared from the matrix and were contained in dense cytoplasmic bodies within mesangial cells. Other inorganic and organic tracer molecules have subsequently been shown to be handled in largely similar ways, apart from variations in the time course of their appearance and disappearance [116,119].

A number of factors have been shown to affect the uptake of macromolecules by the mesangium [118]. Clearance of the tracer from the circulation by the mononuclear-phagocyte system (previously known as the reticulo-endothelial system) reduces its availability for uptake by the mesangium [98]. Delivery of material remaining in the circulation to the glomerulus is affected by haemodynamic factors. For example, reduction of the renal blood flow to one kidney by ligation of the aorta between the renal arteries caused a decrease in the uptake of aggregated immunoglobulin G by the underperfused kidney [121]. Conversely, infusion of angiotensin II to normal rats, which caused an increase in blood pressure and the filtration fraction, led to a marked increase in ferritin deposition in the mesangium [121].

The chemical nature of the tracer molecule used has been shown to affect its handling by the mesangium. Batsford et al. have studied the effect of various modifications to ferritin on its uptake by the rat mesangium [118]. Increasing the size of the ferritin molecules
increased their speed of uptake but did not markedly affect their persistence. When ferritin was coupled with glutaraldehyde, rendering it toxic, it was much more readily and extensively taken up and persisted for longer, indicating that the biological activity of the molecule is important. Alteration of the electrical charge on ferritin had only a minor effect, with positively charged ferritin being taken up more rapidly.

Other workers have shown that alteration of charge on preformed immune complexes markedly affects their site of deposition within the kidney [122]. A net positive charge favours localisation along glomerular basement membrane, due to binding to the fixed negative charges on heparan sulphate proteoglycan. Conversely, a net negative charge favours deposition in the mesangium with, in some cases, binding to the glomerular basement membrane as well [123].

Once within the mesangial region, a number of tracer molecules have been shown to be phagocytosed by mesangial cells, including ferritin [117], thorotrast, colloidal gold [102], aggregated immunoglobulins [120], polyvinyl alcohol [109] and polystyrene latex [124]. Both the resident smooth muscle-type mesangial cells and infiltrating blood monocyte/macrophages exhibit phagocytic activity [109,110]. The role of these cells in the phagocytosis of deposited immune complexes in vivo is less clear, however [98]. For example, in patients with IgA nephropathy, IgA deposits may be abundant between mesangial cells and within the mesangial matrix but no intracellular deposits are seen [125].

As well as by phagocytosis, tracer macromolecules may be cleared from the mesangium via passage across the glomerular basement membrane into the urinary space and into the efferent glomerular capillaries [126]. Small amounts may pass through the hilum of the glomerular tuft to the juxta-glomerular apparatus [126,109,120,119,117,116] (vide infra) but their exit route thereafter is uncertain. The clearance of macromolecules from the mesangium is not affected by alterations in renal blood flow [121]. Ureteric obstruction markedly impairs removal of aggregated immunoglobulin G from the mesangium [127], suggesting that glomerular filtration is an important factor in mesangial
clearance. However, recent work by Schreiner, Harris et al. [128], has demonstrated that there are marked changes in glomerular and interstitial leukocytes following ureteric ligation, including a decrease in the number of glomerular macrophages. These changes may contribute to the impairment of mesangial clearance.
CHAPTER 3
THE HISTOLOGICAL FEATURES OF GLOMERULOSCLEROSIS.

Focal segmental glomerulosclerosis.

Focal (i.e. occurring in localised regions of the cortex) segmental (i.e. affecting only part of the glomerular tuft) glomerulosclerosis (FGS) is usually thought of as a histological lesion found in some patients with idiopathic nephrotic syndrome. It was first described in 1957 by Rich who noted the presence of obliterative glomerulosclerosis in glomeruli located near the corticomedullary junction of patients with "lipoid" nephrosis [129]. It has subsequently been widely reported and is an important histological indicator of a subgroup of nephrotic patients who tend to have a worse prognosis than those without glomerular lesions under light microscopy [130].

Table 3.1 Conditions in which focal segmental glomerulosclerosis has been reported.

Nephrotic syndrome [130, 131]
IgA nephropathy [132]
Unilateral renal agenesis [133, 134]
Unilateral nephrectomy [135, 136]
Reflux nephropathy [137, 138]
Analgesic abuse [139]
Sickle cell nephropathy [140]
Alport's disease [141]
Diabetes [142]
IV drug abuse [143]
AIDS [143]
Massive obesity [144]
Histological features of FGS have been noted in a range of other renal diseases (Table 3.1) and it seems likely that they represent a non-specific response of the glomerulus to injury [132]. In the subsequent discussion, FGS will be used as an example of the general process of glomerular scarring [112,95], with the implicit assumption that factors found to be important in the development of FGS are likely to be important in the development of the glomerulosclerosis of progressive renal failure.

The main feature of FGS by light microscopy is solidification of one or more lobules of the glomerular tuft [142,145]. This solidification tends to be peripheral and is associated with adherence to Bowman's capsule. The capillary lumina are collapsed and there is an increase in the amount of mesangial matrix, which stains positively with silver stains and the PAS reaction. Visceral epithelial cells are swollen over the sclerotic area. "Foam" cells may be present in the sclerotic areas and within the capillary lumina. Associated with these changes is "hyalinosis". This refers to the accumulation of eosinophilic homogeneous material, which stains positively with the PAS reaction but not with silver stains, within sclerotic areas or on the inner aspect of peripheral capillary loops. As glomerulosclerosis advances, pericapsular fibrosis develops and eventually complete global sclerosis ensues.

Tubular changes follow the glomerular lesions and consist of focal atrophy and 'dropout' of tubules, with associated interstitial fibrosis and infiltration by "foam" cells and chronic inflammatory cells. Interstitial arterioles and small arteries sometimes contain hyalin deposits. These are similar to those that make up the glomerular hyalinosis lesions and there may be continuity between them and hyalinosis of the afferent arteriole [146,147].

Under the electron microscope, the hyalinosis lesions appear as large electron-dense deposits in the sub-endothelial region, with smaller deposits being localised in the paramesangium. The epithelial foot processes are fused and swollen. They may appear to have lifted off the underlying glomerular basement membrane, leaving bare areas (vide infra). Immunofluorescent studies reveal deposits of IgM and C₃.
in sclerotic areas, with weak staining for IgG, C1q and C4 [148]. Fibrin and fibrinogen are absent. No deposits are present in unaffected regions of the glomerulus.

**Experimental focal segmental glomerulosclerosis.**

As in man, focal glomerulosclerosis in the rat is a non-specific response of the glomerulus to injury and results from a variety of experimental insults (Table 3.2).

**Table 3.2.** Experimental rat models of focal segmental glomerulosclerosis.

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<td>Ageing animals</td>
<td>[149, 150]</td>
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<tr>
<td>Unilateral nephrectomy</td>
<td>[151]</td>
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<tr>
<td>Subtotal nephrectomy</td>
<td>[3, 152, 153]</td>
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<tr>
<td>Puromycin aminonucleoside nephrosis</td>
<td>[154]</td>
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<tr>
<td>Adriamycin nephrosis</td>
<td>[155]</td>
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<tr>
<td>Antibody mediated mesangial injury</td>
<td>[156]</td>
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<tr>
<td>Obese Zucker rats</td>
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In a number of strains of rat, the lesions develop spontaneously with ageing [149, 150, 158]. The development of these spontaneous changes can be accelerated by a reduction in renal mass, such as uninephrectomy or subtotal nephrectomy [152,153].

The lesions bear striking similarities to the changes of focal glomerulosclerosis in man, described above. The initial change is hyalinosis, that is the deposition of PAS-positive material in the capillary lumen or wall, particularly of those loops adjacent to the afferent arteriole. Hyalinosis sometimes extends to the mesangium. The material is vacuolated by lipid-filled spaces, and "foam" cells
sometimes obliterate the capillary lumina. Only slight, if any, mesangial cell proliferation is present. The lesions progress by extension of the PAS-positive material into other capillary loops and adhesions form between these loops and Bowman's capsule. Parietal epithelial cells may be hypertrophied. Mesangial matrix material, staining positively with silver stains, is increased and progresses until the whole glomerulus becomes sclerosed. Electron microscopy reveals fusion of the foot processes of visceral epithelial cells which often contain vacuoles. Stripping of the epithelium to leave bare areas of basement membrane have been reported in FGS following puromycin administration but do not appear to be a universal feature of proteinuria [159]. Deposits of IgM are detectable by immunofluorescence in damaged regions of the glomerulus and these are accompanied by complement and small amounts of IgG. No electron dense immune deposits are present. Tubular changes occur secondary to glomerular damage but the extra-glomerular vessels are normal.
CHAPTER 4
THE LINK BETWEEN ABNORMAL MESANGIAL FUNCTION AND GLOMERULOSCLEROSIS.

The effect of glomerular injury on mesangial transport.

Various forms of experimental glomerular injury that subsequently lead to glomerulosclerosis also disturb the passage of macromolecules into and out of the mesangium. For example, colloidal carbon administered to Wistar rats immediately following unilateral nephrectomy [151] and after the chronic administration of puromycin aminonucleoside [160], accumulates in those segments of glomeruli that subsequently develop sclerotic lesions. Similar results have been demonstrated using aggregated immunoglobulin G following puromycin administration [161,162]. Acute mesangial damage and subsequent focal glomerulosclerosis follows the administration of an antibody against the Thy-1 antigen which is expressed on rat mesangial cells [156]. Aggregated proteins and preformed immune complexes, administered between 4 hours and 7 days after the antibody, are deposited in large amounts in the damaged mesangium [156].

The link between the altered handling of macromolecules and subsequent glomerulosclerosis has been emphasised by Grond, Weening and Elema in a comparison of two models of experimental nephrotic syndrome [163]. In rats given puromycin aminonucleoside, 7.8% of glomeruli subsequently developed glomerulosclerosis. Colloidal carbon, infused after the toxin, accumulated within the mesangium in association with areas of sclerosis. In contrast, rats with similar degrees of proteinuria and hypoalbuminaemia following intravenous injection of adriamycin showed no increase in colloidal carbon accumulation in the mesangium and only 0.3% of glomeruli developed sclerosis.

Further evidence for a link is provided by the variation in susceptibility of different rat strains to glomerulosclerosis. Unlike Wistar rats, PVG rats do not develop glomerulosclerosis following unilateral nephrectomy. Correspondingly, colloidal carbon does not accumulate in their glomeruli [164].

The route by which tracer macromolecules are cleared from the mesangium, i.e. via the mesangial stalk to the afferent arteriole, is
similar to the distribution of the hyaline material which is deposited in focal glomerulosclerosis in man (Chapter 3). This suggests that a disturbance of the clearance of endogenous macromolecules may occur during the development of this condition in man, in a similar way to that demonstrated in experimental animals.

These observations do not prove that the accumulation of macromolecules is a causative factor in the development of sclerotic lesions. Such accumulation may merely be a sign that earlier mesangial cell injury has damaged the normal mesangial clearance mechanisms and sclerosis may be a direct and independent response to that injury [165]. However, there is evidence that deposition of macromolecules in the mesangium may itself be injurious to the glomerulus. When iron dextran was administered to normal rats, it persisted within intrinsic mesangial cells for up to three months and was associated with mild but persistent proteinuria [166]. Polystyrene latex [124] and polyvinyl alcohol (PVA) [110] were similarly phagocytosed by intrinsic mesangial cells and caused mesangial hypertrophy and a mild infiltration of the mesangium with monocyte/macrophages. Again there was mild proteinuria although no significant change in glomerular filtration rate occurred. The deposited PVA led to a disturbance of mesangial clearance of subsequently administered colloidal carbon [167] and electron microscopic examination showed that the mesangial intercellular channels were disrupted by polyvinyl alcohol aggregates, suggesting that the accumulation of sufficient amounts of macromolecules may damage the mesangial structure and lead to further deposition. Sclerotic lesions were also seen in the presence of large accumulations of PVA.

Mesangial deposits of macromolecules may act as a nidus for the trapping of antibodies, leading to the in situ formation of immune complexes and further glomerular damage. When administered alone to rats, bovine serum albumin (BSA) was not trapped in the mesangium and immune complexes were not formed with subsequently administered anti-BSA antibody. However, when BSA was bound to latex particles prior to injection, there was a marked increase in their trapping in the mesangium and subsequently administered anti-BSA antibody led to the
formation of immune complexes and the persistence of ingested latex within mesangial cells [124].
CHAPTER 5
LIPID DEPOSITION AS A FEATURE OF GLOMERULOSCLEROSIS.

Lipid deposition is not normally regarded as a prominent feature of glomerular damage. This may be because routine processing of pathological material for histological examination includes treatment of tissue sections with organic solvents which remove lipid. However, in a thorough review of the available literature in 1941, Simonds and Lange [168] produced an extensive list of conditions in which glomerular lipid deposits had been described (Table 5.1). As most of these reports had mentioned fatty changes only in passing, the authors studied material from 76 autopsies on patients with kidney disease, with the specific aim of describing the prevalence and histological appearance of lipid deposition. They found very fine droplets of lipid at the glomerular hilum in approximately 50% of cases of acute and chronic glomerulonephritis. More significant amounts were found in the 30 patients with 'nephrosclerosis' due to hypertension. 21 (70%) had fatty changes in afferent arterioles and 12 (40%) also in the glomeruli. The histological appearances were described as follows: "The fat is in the form of fine or coarse droplets at the periphery of capillary loops that are almost bloodless. Such glomeruli are frequently lobulated so that the deposits of fat form rounded groups, often so compact that it is difficult to determine whether they are intracellular or free in a necrotic mass." "The hyaline scars which occupy the site of glomeruli that have been completely destroyed often contain fat in fine droplets." Figure 5.1 demonstrates examples of these changes in a transplanted kidney which failed after three years due to 'chronic rejection'

Early pathological reports relied on material obtained at post mortem, usually of kidneys at an advanced stage of disease. With the use of percutaneous renal biopsy it became possible to study the early changes of glomerulosclerosis. Prominent deposition of lipid within the lesions of focal segmental glomerulosclerosis and in non-specifically sclerosed glomeruli has been noted [95,169]. In a detailed study of 55 renal biopsies from 33 patients with focal
Table 5.1.

Diseases in Which Fat Has Been Found in the Glomeruli

Toxemias and poisonings:
- Acute yellow atrophy of the liver: Mayer (1922).
- Bichloride of mercury: Heineke (1909); Bohnenkamp (1922).
- Eclampsia: Prym (1910); Segawa (1914); Fahr (1925).
- Phenol poisoning: Peipers (1892).

Metabolic disturbances:
- Beriberi: Segawa (1914).
- Diabetes mellitus: Peipers (1892); Prym (1910); Segawa (1914); Fahr (1920); Lubarsch (1925).
- Exophthalmic goiter: Prym (1914).
- "Arteriosclerosis": Prym (1910); Segawa (1914).

Circulatory disturbances:
- Anemia: Segawa (1914).
- Passive hyperemia: Segawa (1914).
- "Heart disease": Prym (1910).

Other diseases:
- Cirrhosis of the liver: Segawa (1914).
- Jaundice: Prym (1910); Segawa (1914).
- Purpura hemorrhagica: Segawa (1914).
- Malignant tumors: Prym (1910); Segawa (1914).
- Amyloidosis: Tietz (1922); Van Slyke (1930).

Infectious diseases:
- Diphtheria: Nauwerck (1886); v. Kahlden (1893); Segawa (1914); Fahr (1920).
- Dysentery and enteritis: Prym (1910).
- Erysipelas: Segawa (1914).
- Meningitis: Prym (1910); Herzheimer (1916, 1918).
- Peritonitis: Prym (1910); Segawa (1914).
- Pneumonia: Peipers (1892); Gaskell (1911); Segawa (1914).
- Scarlet fever: Löhlein (1904).
- Sepsis: Prym (1910).
- Tuberculosis: v. Kahlden (1891); Prym (1910); Segawa (1914).
- Typhoid fever: v. Kahlden (1893); Gaskell (1911).

Renal diseases:
- Acute glomerulonephritis: Munk (1918); Stern (1924); Volhard (1925); Hückel (1929).
- Chronic glomerulonephritis: Peipers (1892); Löhlein (1905); Prym (1910); Segawa (1914); Volhard (1925); Fahr (1925); Van Slyke (1930); Gray (1933).
- Nephrosclerosis: Prym (1904); Gaskell (1911); Herzheimer (1912, 1916, 1918); Jores (1916); Munk (1918); Tietz (1922); Van Slyke (1930); Gray (1933); Kimmelstiel and Wilson (1936).
- Nephrosis: Löhlein (1918); Munk (1918); Major and Helwig (1925); Fahr (1925); Löwenthal (1927); Bell (1929); Kantrowitz and Klemperer (1931).
- "War nephritis": Herzheimer (1916, 1918); Rochs (1918).
Figure 5.1 Extensive glomerular lipid deposition in a failed renal transplant, removed after 3 years. Oil Red O x 600.
glomerulosclerosis associated with the nephrotic syndrome, Hyman and Burkholder [145] described the appearances of lipid-containing 'foam' cells associated with three stages in the development of these lesions. Initially, occasional endocapillary foam cells were seen associated with increased mesangial matrix material and hyaline PAS-positive deposits. Mesangial cellularity was rarely increased at this stage. As the lesions progressed, foam cells, mesangial matrix thickening and hyaline deposits become more prominent until global glomerulosclerosis supervened with the loss of the original hyaline deposits and foam cells.

The accumulation of lipid within sclerosed glomeruli has also been studied biochemically [171,172]. The cholesterol content of glomerular basement membrane isolated from kidneys with advanced glomerular disease was found to be increased by 150%. A histochemical analysis of glomerulosclerotic lesions in the rat demonstrated the lipid deposits to be mainly composed of free and esterified cholesterol with small amounts of triglyceride and phospholipid [173].
Although the primary concern of this thesis is glomerulosclerosis, a consideration of other renal diseases in which glomerular lipid deposition is prominent may be helpful in defining the role of lipids in glomerular scarring.

**FABRY'S DISEASE (hereditary dystopic lipidosis)**

Fabry's disease is a rare X-linked inherited disorder of the metabolism of glycosphingolipid [174]. It is caused by a deficiency of α-galactosidase A in serum, tissues and urine and results in the accumulation of neutral glycosphingolipid in all tissues, including the kidney. Renal damage is manifested by an impairment of urine concentrating ability, proteinuria, haematuria and lipiduria. Casts and foam cells may be observed in the urine. The number of lysosomes in endothelial, mesangial and glomerular epithelial cells is increased and typical intracellular myeloid bodies are present.

Since this condition is caused by a defect in cellular lipid metabolism, it is unlikely to shed much light on the role of lipoproteins in the damage of metabolically competent glomeruli and will not be discussed further.

**SPONTANEOUS GLOMERULAR LIPOIDOSIS IN DOGS.**

This condition was first reported by Fisher and Fisher in 1954 [175]. In a subsequent survey of normal beagle dogs during routine histological examination for drug safety tests, Zayed et al. [176] found glomerular lesions in 33 out of 775 dogs. In most dogs, only a few glomeruli were affected, although in four animals up to 20% contained lesions. The lesions consisted of foamy eosinophilic changes in a part or the whole of the glomerulus. Special stains showed deposits of lipid and PAS-positive material and there was focal thickening of the glomerular basement membrane. By electron microscopy, the foamy cells had the appearances of altered mesangial
cells, although minor vacuolation was also found in endothelial cells. There was no associated infiltration by inflammatory cells or deposition of electron-dense material. Renal function was normal. The aetiology of this condition remains unclear.

**TYPE III HYPERLIPOPROTEINAEMIA**

Type III hyperlipoproteinaemia is characterised by an increase in the remnants of VLDL catabolism which form a 'broad β' electrophoretic band. It is caused by an inherited defect in apoprotein E which prevents it binding to either the apo B/E or the apo E receptors [371]. The clinical features of the condition are dominated by accelerated atherosclerosis of coronary and peripheral arteries. One case has been reported [170] of a 59 year old woman who also had foam cells in the glomerular mesangium. Her renal function was significantly impaired, with a creatinine clearance of 20ml/minute, but this was most likely due to bilateral renal artery stenoses. The foam cells were not accompanied by sclerotic lesions.

**LECITHIN CHOLESTEROL ACYLTRANSFERASE (LCAT) DEFICIENCY**

Hereditary LCAT deficiency is probably the best example of a primary disorder of lipoprotein metabolism causing renal damage. It was first described in a Norwegian family in 1967 [177]. Characteristically LCAT activity is very low or undetectable in affected patients. Since this enzyme is required for the esterification of cholesterol and its transfer to HDL (see chapter 1) levels of free cholesterol and LDL are raised and HDL is reduced. Abnormally shaped HDL particles have been described and an abnormally high molecular weight form of LDL is commonly found. Patients with the condition develop lipid deposits in many tissues, including the cornea, the arterial endothelium and subendothelium, and the kidney [178]. As expected from a defect in cholesterol esterification, the lipid content of affected tissues consists predominantly of free cholesterol [179]. In atheromatous plaques, for example, only 35% of total cholesterol is esterified, in contrast to 75% in "normal" atheroma. The fatty acid profile of cholesteryl esters in plasma and atheroma is
also different, with the ratio of oleic to linoleic acid (saturated to unsaturated) being 4:1 instead of the normal 1-2:1.

In the kidney, lipid deposits have been demonstrated in the mesangium and the glomerular basement membrane, along with deposits of C₃ [180]. Glomerular foam cells may be prominent [178] but are not invariably present [180]. Mesangial matrix expansion and hypercellularity may accompany the lipid deposits [178].

The earliest clinical feature of glomerular lipid deposition is proteinuria, which is usually first detected in the teenage years. Nephrotic syndrome is not a feature and patients remain normotensive. Glomerular filtration rate remains normal until the fourth or fifth decade when there is a rapid decline leading to renal failure [181]. Following renal transplantation lipid deposition recurs in the donor kidney [179].

The mechanism by which the lipid deposits cause renal failure is unclear. There is no clear correlation between glomerular histological changes, which are present early, and renal function, which does not decline until middle life. The presence of abnormally high molecular weight LDL has been associated with renal damage [182]. Glomerular capillaries in affected kidneys are often occluded by foam cells and this may lead directly to a decline in renal function [179].

**HEPATIC GLOMERULOSCLEROSIS.**

Changes similar to those seen in LCAT deficiency have been described in association with liver disease. In 1959, Bloodworth and Sommers [183] described the results of a study of renal tissue from 100 autopsies on patients with liver cirrhosis. 78% of cases showed changes of glomerulosclerosis, consisting of an increase in the numbers of epithelial and 'endothelial' cells. In more advanced cases, there was a marked increase in PAS-positive mesangial matrix containing numerous nuclei. Some glomerular capillaries were occluded by endothelial cell proliferation and swelling due to lipid vacuolation. Clinically, these lesions were not associated with hypertension, proteinuria or renal failure, nor with fatty change in the liver. They were more commonly found with hyperoestrogenaemia, adrenal cortical
hyperplasia and severe liver failure. Levels of plasma lipids were not reported.

In a subsequent report, Sakaguchi et al. [184] detailed the ultrastructural changes in glomeruli from 24 cases of liver disease. Acute liver damage was associated with a moth-eaten appearance of the mesangial matrix and subendothelial region of the glomerular basement membrane, probably due to the extraction of lipid deposits during the processing procedure. Chronic liver disease was associated with an increase in mesangial matrix with thickening of the glomerular basement membrane and deposition of osmiophilic granules. In patients with proteinuria or the nephrotic syndrome, more marked damage to podocytes was present with deposits of immunoglobulin, probably IgA [185].

Liver damage is associated with a decrease in LCAT activity. Abnormally large molecular weight low density lipoproteins, similar to those present in hereditary LCAT deficiency, have been reported in four patients with liver disease [186] and one with arteriohepatic dysplasia (a failure of bile duct development causing cholestasis) [187]. These changes have been produced experimentally in dogs by the anastomosis of the bile duct to the inferior vena cava [188]. In all these examples, lipid deposition was found in the subendothelial and mesangial regions.

Although the specific lipoprotein abnormalities present in hereditary and acquired LCAT deficiency are not found in focal glomerulosclerosis, these examples do indicate that lipoproteins can be deposited in significant amounts in the glomerular basement membrane and the mesangium. Their presence in the conditions described above does not appear to excite a marked inflammatory response but they are associated with proteinuria, glomerular scarring and, eventually, a decline in renal function.
CHAPTER 7
MECHANISMS OF GLomerular lipid deposition

Although the kidney is not a major organ for the export of fatty acid or sterols to the body, the rate of fatty acid synthesis in the rat kidney per gram of tissue is 50% of that in the liver and that of sterol synthesis is 30% [189]. This synthesis is homeostatically regulated and is reduced in the presence of hyperlipidaemia or when tissue lipid content is increased [189]. For lipids to accumulate therefore, there must be an imbalance between the endogenous synthesis of fatty acids or sterols, their delivery to the glomerulus from the circulation and their utilisation and clearance.

Simonds and Lange [168] noted that glomerular fat deposits accompanied damage to the glomerular capillary loops in patients with nephrosclerosis. Assuming such glomeruli would be ischaemic and anoxic, they suggested that the fatty changes were comparable to those seen in the cardiac muscle of patients with severe anaemia. Such 'fatty change' is a non-specific histological feature seen in many tissues following cellular injury [190]. Normal cells contain small lipid deposits within the endoplasmic reticulum, that are visible by electron microscopy. Under anoxic conditions, the normal oxidative catabolism of fatty acids is impaired and membrane bound lipid deposits accumulate within the cell. If the injury persists, free lipid deposits form and the cytoplasm takes on a 'foamy' appearance, visible under the light microscope.

There is evidence, however, that glomerular lipid deposits are formed by the trapping of circulating lipoproteins within glomerular capillaries, rather than by degenerative fatty change. As discussed in Chapter 4, Grond and Elema noted the association of lipid deposits with the trapping of tracer molecules in the mesangium of rats with glomerulosclerosis [191]. Using an antibody against β lipoprotein, Berger et al. have confirmed that deposits of lipoprotein are present in most areas of human focal glomerulosclerosis and in obsolescent glomeruli, in association with lipid deposits [192]. Hyman and Burkholder have described the appearance of insudated material beneath
damaged glomerular endothelial cells in patients with focal glomerulosclerosis [145].

Lipid may also be transported to the glomerulus inside cells. Fatty degeneration of the liver may lead to the embolisation of lipid-laden liver cells to the kidney [193]. As discussed in more detail in Chapter 10, lipid-laden macrophages may infiltrate the mesangium [109].
CHAPTER 8
THE HYPOTHESIS THAT LIPIDS ARE PATHOGENIC IN GLOMERULOSCLEROSIS.

Simonds and Lange, in their review of glomerular lipid deposition in 1941, ascribed the deposition of fat within glomeruli to ischaemic damage and thereby inferred that these changes were merely the secondary result of glomerular damage [168].

The first authors to propose a primary pathogenetic role for lipid deposits were Wilens, Elster and Baker in 1951 [194]. They studied the prevalence of fat in kidneys taken at necropsy from 21 diabetic patients whose glomeruli showed the changes of 'intercapillary glomerulosclerosis', as first described by Kimmelstiel and Wilson [195]. The specimens were compared with 6 groups of 20 patients with a range of other renal diseases. They found a significantly larger amount of glomerular fat in the cases of intercapillary glomerulosclerosis and showed that the severity of this deposition was proportional to the severity of the sclerotic lesions in most cases. This was in contrast to the changes seen in glomerulonephritis and nephrosclerosis where no close relationship was found between lipid deposition and the severity of glomerular lesions. They noted that whereas hyalinised glomerular tufts were reduced in size, those containing lesions of intercapillary glomerulosclerosis were often normal in size or enlarged and they suggested that "a combination of hyperlipaemia and elevated intraglomerular blood pressure might be responsible for the penetration of lipid-containing materials into the intercapillary substance of the tufts". They went on to compare these changes with those seen in atherosclerosis. "Penetration of lipid substances into the walls of the arteries is probably accelerated in the presence of diabetic hyperlipaemia and hypertension. The process that occurs under similar conditions in the glomeruli may be somewhat analogous."

In a subsequent study of diabetic glomerulosclerosis by Hartroft [196], glomerular lipid deposits were demonstrated to be present in the lumina of glomerular capillaries as well as in the vessel walls and the intercapillary space. He suggested that the intraluminal fat may
either have been embolic in origin or may have precipitated in situ as a lipidic thrombus. He further speculated that the occlusion of glomerular capillaries by this material may be important in initiating the development of the Kimmelstiel-Wilson lesions.

With the better understanding of the normal function of the glomerular mesangium and the effects of glomerular injury on its handling of macromolecules, as discussed in Chapter 4, the hypothesis has evolved that 'overloading' of the mesangium with macromolecules may lead to an increase in the production of mesangial matrix material and hence glomerulosclerosis [197]. Elema et al. noted that lipids were a prominent component of the macromolecules that accumulated in areas of sclerosis and, repeating the comparison between glomerulosclerosis and atherosclerosis made by Wilens, Elster and Baker 31 years previously, suggested that the deposition of lipoproteins in the mesangium may stimulate the sclerotic process [151]. Later the same year, Moorhead et al. put forward a more wide-ranging hypothesis that lipids may be important as nephrotoxins in mediating both glomerular and tubular damage [198]. Since the work for this thesis was commenced, a number of different authors have published reviews reiterating these suggestions [199,200,201,202,203,204]. The following chapter will discuss the evidence supporting the analogy between atherosclerosis and glomerulosclerosis.
Three typical forms of atherosclerotic lesion occur in the arterial intima; the fatty streak, the fibrous plaque and the 'complicated' lesion [205].

The fatty streak is found in children from the age of 10 years, regardless of race, sex or environment. It consists of focal accumulations of small numbers of intimal smooth muscle cells and macrophages which contain and are surrounded by lipid deposits, principally composed of cholesterol and cholesteryl esters [206]. Although the lesions increase in extent with age, they do not cause vascular obstruction.

It seems likely, although is difficult to prove, that fatty streaks progress to form fibrous plaques [206]. These are the characteristic lesions of advancing atherosclerosis. They are made up of three layers. On the surface is a fibrous cap consisting of multiple layers of modified smooth muscle cells and macrophages which contain lipid deposits and are surrounded by basement membrane and proteoglycan. This overlies an accumulation of smooth muscle cells and macrophages which contain lipid deposits and are surrounded by connective tissue. Under this cellular region lies an area of necrotic debris, cholesterol crystals and calcification.

The 'complicated' lesion results from damage to the fibrous plaque by haemorrhage, calcification, cell necrosis and mural thrombosis. It is this lesion that typically causes vascular occlusion.

Table 9.1 summarises the main histopathological features of atherosclerosis and compares them with the equivalent changes found in focal segmental glomerulosclerosis.
Table 9.1  Histopathological similarities between atherosclerosis and glomerulosclerosis

<table>
<thead>
<tr>
<th>Atherosclerosis [205,206]</th>
<th>Glomerulosclerosis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endothelial cell injury</strong></td>
<td>Endothelial cell injury</td>
<td>142</td>
</tr>
<tr>
<td><strong>Smooth muscle cell proliferation</strong></td>
<td>Mesangial hypercellularity</td>
<td>142</td>
</tr>
<tr>
<td><strong>Macrophage infiltration</strong></td>
<td>Macrophage infiltration</td>
<td>-</td>
</tr>
<tr>
<td><strong>Foam cells</strong></td>
<td>Foam cells</td>
<td>145,211</td>
</tr>
<tr>
<td><strong>Increased extracellular matrix</strong></td>
<td>Increased mesangial matrix</td>
<td>142</td>
</tr>
<tr>
<td><strong>Extracellular lipid deposits</strong></td>
<td>Extracellular lipid deposits</td>
<td>172,145,192</td>
</tr>
<tr>
<td><strong>β lipoprotein deposits</strong></td>
<td>β lipoprotein deposits</td>
<td>192</td>
</tr>
<tr>
<td><strong>IgG, C3 deposits [207]</strong></td>
<td>IgM (strong), IgG (weak) C3, C1q, C4 deposits</td>
<td>142</td>
</tr>
<tr>
<td><strong>Cellular debris</strong></td>
<td>Mesangiolyis</td>
<td>142</td>
</tr>
<tr>
<td><strong>Platelet adhesion/thrombosis</strong></td>
<td>Intracapillary thrombosis</td>
<td>142</td>
</tr>
<tr>
<td><strong>-</strong></td>
<td>Podocyte injury</td>
<td>142,211,216</td>
</tr>
</tbody>
</table>
Although the changes occurring in glomerulosclerosis are on a much smaller scale than those in atherosclerosis, there are striking similarities between the two lesions. This suggests that they may be considered as similar "responses to injury" [206,132]. There are also important differences and these will now be discussed further.

**Cellular proliferation.**

Smooth muscle cell proliferation in the atherosclerotic fatty streak and in the cap of the fibrous plaque [205] may be likened to mesangial hypercellularity in glomerulosclerosis. Whereas such proliferation is a prominent feature of atherosclerosis, focal glomerulosclerosis is typically a hypocellular lesion and an increase in mesangial cells is only present in approximately 40% of cases [169]. When present, it consists of a mild diffuse increase in mesangial cells throughout the glomerulus. By electron microscopy these cells are hypertrophied, contain many organelles and occasional lipid deposits and have morphological features of smooth muscle-type mesangial cells.

The significance of mesangial hypercellularity is unclear. Its severity is not directly related to that of the focal sclerotic lesions [145,208]. Hypercellularity without focal sclerosis may be present on an initial biopsy but in a follow-up biopsy, glomerulosclerosis may be present without mesangial hypercellularity. Conversely, hypercellularity may accompany glomerulosclerosis on an initial biopsy but be absent, despite persistent sclerosis, at follow-up [208]. There is evidence, however, that the presence of hypercellularity does indicate a worse prognosis for both subsequent histological changes and eventual renal function. Waldherr [208] noted that up to 70% of nephrotic patients with mesangial hypercellularity later developed focal glomerulosclerosis. Similarly, a study by Shoeneman et al. [209] found that hypercellularity was associated with an increased risk of progression to chronic renal failure.

**Foam cells.**

The hallmark of the atherosclerotic lesion is the lipid-laden 'foam' cell. Once cells become loaded with lipid, their normal
structural features become so distorted that it can be difficult to determine their cellular origin. Traditionally, it was thought that most of these cells were derived from modified smooth muscle cells [205]. However, using specific monoclonal antibody markers, it has been shown that the majority of foam cells in both fatty streaks and advanced lesions are macrophages [210], derived from circulating monocytes [206]. In view of their inflammatory activity, it has been suggested that these cells are primarily involved in the progression of atherosclerotic lesions (Chapter 10) [210].

The evidence that macrophages have an important role in glomerulosclerosis is less clear. Foam cells have been reported in the glomerulus in 45% [169] to 79% [145] of cases of focal segmental glomerulosclerosis associated with the nephrotic syndrome. They may represent one of the earliest features of the focal sclerotic lesion [211] and tend to decrease in numbers as sclerosis progresses [145]. The origin of glomerular foam cells is uncertain. Hyman and Burkholder noted them to be present both within the capillary lumen and within the sclerotic lesion [145]. They concluded that they were either endothelial cells or other cells located in the position of endothelial cells. Verani and Hawkins [211] reported that glomerular foam cells had electron microscopic features of smooth muscle-type mesangial cells, such as myofibrils and cytoplasmic projections. Using an immunoperoxidase stain for muramidase, a marker of mononuclear cells, they did not detect an increase in the overall number of glomerular monocytes. Nevertheless, a few foam cells did stain positively for muramidase and they were unable to exclude the possibility that a proportion of these cells were derived from monocytes. Magil stained 177 renal biopsies with non-specific esterase, a histochemical marker of monocytes [212]. In 26 cases of focal glomerulosclerosis, only small numbers of monocytes were detected. Similarly, 29 cases reported by Serraio et al. [213] and 12 cases reported by Monga et al. [214] revealed no increase in monocytes. Using specific monoclonal antibody markers, no increase in macrophages was detected in 12 cases of focal glomerulosclerosis by Hooke et al. [104]. Atkins et al. have cultured glomeruli from patients with a variety of forms of glomerulonephritis.
In focal glomerulosclerosis, smaller than normal numbers of all cell types, including macrophages, were obtained [215].

It is possible that the accumulation of lipoprotein within the mesangium leads to an infiltration by only small numbers of macrophages but that these are still important in mediating glomerular injury. Cattell et al. studied the clearance of ferritin from the rat mesangium and found that infiltrating macrophages were active in the phagocytosis of this material but that the numbers of macrophages involved were too small to cause hypercellularity by routine light microscopy [109]. One may even speculate that the small numbers of macrophages involved explains the restriction of the sclerotic lesions to segments of the glomerular tuft.

As with mesangial hypercellularity, the pathological and clinical significance of foam cells in focal segmental glomerulosclerosis is unclear. The two abnormalities do not necessarily occur together [145,208], suggesting that macrophages are not involved in stimulating smooth muscle-type mesangial cell proliferation. Foam cells are more commonly associated with persistent nephrotic syndrome, possibly due to the associated hyperlipidaemia [169] and such patients tend to have a worse prognosis [142]. However, foam cells have been reported in patients who have only asymptomatic proteinuria [169]. The small sample of tissue available for study in a biopsy may explain the overall poor correlation between the severity of histological changes and the clinical prognosis [169,142].

Podocytes.

There is no equivalent in atherosclerotic vessels of the glomerular visceral epithelial cells or podocytes. Injury to these cells may be of major importance in the pathogenesis of glomerulosclerosis. Fusion of the podocyte foot processes is the earliest lesion detectable in patients with nephrotic syndrome [142] and more marked podocyte changes may be a feature distinguishing minimal change nephrotic syndrome from focal segmental glomerulosclerosis. Yoshikawa et al. [216] found that podocyte vacuolation was present in the non-sclerotic glomeruli of 21 out of 34
patients with focal glomerulosclerosis, compared to only 5 of 34 patients with minimal change nephrotic syndrome. 11 of the 21 patients with podocyte vacuolation progressed to chronic renal failure, compared to only 1 of the 13 patients with FSGS without vacuoles. The likely importance of podocyte damage in glomerulosclerosis is emphasised by the experimental model of puromycin aminonucleoside-induced nephrosis, in which alterations in glomerular basement charge and irreversible injury to glomerular epithelial cells leads to focal glomerulosclerosis [161].
CHAPTER 10
POSSIBLE MECHANISMS BY WHICH LIPIDS MAY MEDIATE GLOMERULAR DAMAGE.

It is generally accepted that hyperlipidaemia, and in particular hypercholesterolaemia, is an important risk factor in the pathogenesis of atherosclerosis. A number of mechanisms by which lipids may be involved in the pathogenesis of atherosclerotic lesions have been proposed. In view of the histological similarities between atherosclerosis and glomerulosclerosis discussed in Chapter 9, it is possible that one or more of these mechanisms may be involved in the pathogenesis of glomerular damage. These mechanisms will now be discussed.

Alteration in membrane lipids.

Hyperlipidaemia leads to a general alteration in the lipid composition of cell membranes. Such changes may affect the structural and functional properties of membranes [217]. Increasing the cholesterol content of a membrane, as occurs in the basement membrane of damaged glomeruli [171], increases its viscosity and may make it less able to withstand haemodynamic injury [206]. The biological properties of cells may also be altered by changes in their membrane composition. Incubation of cultured rabbit aortic endothelial cells with βVLDL taken from cholesterol-fed rats led to a 65% increase in their cholesterol content and was associated with a two-fold increase in the transport of albumin and low density lipoprotein across the endothelial monolayer [218]. Phagocytosis of bacteria and red cells can be stimulated by increasing the unsaturated fatty acid composition of macrophages [217]. Conversely, endocytosis is reduced by increasing membrane fatty acid saturation. Susceptibility of certain cells to complement-mediated and cell-mediated cytotoxicity in vitro is also altered by modification of their fatty acid content [217]. If these effects are extrapolated to the glomerulus, one may speculate that alterations in membrane lipid composition may a) affect the transport of molecules into the peripheral capillary wall or the mesangium, b) disturb their subsequent clearance by mesangial cells and c) modify the susceptibility of glomerular cells to injury.
Lipid-mediated toxicity.

Deposits of lipid may have a directly damaging effect on cells within the glomerulus. The lipid deposits present in the damaged glomeruli of rats with experimentally-induced focal glomerulosclerosis consist predominantly of cholesterol and cholesteryl esters [173]. The finding of β lipoprotein in human glomerulosclerosis [192] suggests that these are also the principal lipids deposited in man. The 'sclerogenic' activity of lipids has been studied by subcutaneous implantation of pure material in rats [219]. Triglycerides and cis-polyunsaturated cholesteryl esters were rapidly absorbed and only slightly sclerogenic. Cholesterol and saturated cholesteryl esters, however, were only slowly absorbed and induced a fibrotic reaction. Oxidation of cholesterol further increased its inflammatory effect [220] (vide infra).

A number of studies have investigated the effects of lipoproteins on cultured cells in vitro. LDL has been shown to inhibit cell proliferation or to cause frank cytotoxicity in a wide range of cells cultured from different species [221]. These include endothelial cells cultured from bovine arteries [222], human umbilical vein endothelial cells [223,224], smooth muscle cells cultured from human aortic media [223] and cultured rat mesangial cells [225]. In a number of studies, the effect of LDL has been found to be biphasic. At concentrations up to 50μg of protein/ml, i.e. comparable to the concentration present in interstitial fluid [224], a stimulatory effect on cell growth was found. At higher concentrations a reversible inhibitory effect [221] or frank cytotoxicity was produced [225]. VLDL, isolated from patients with types III and V hyperlipidaemia, has also been shown to have a toxic effect on human fibroblasts and bovine aortic endothelial cells at relatively low concentrations [226]. The effect of LDL is modified by the conditions under which the cells are grown. The presence of lipoprotein deficient serum [222] and HDL [223,227] in the culture medium, and the use of extracellular matrix as the culture substrate [222], all protect against LDL toxicity. The phase of growth of the cell culture is also important, endothelial cells being protected
against toxicity once they have formed a confluent monolayer [222].

Stimulation of cell proliferation by LDL may indicate an effect on cell division, i.e. a mitogenic effect, or an effect on cell growth, i.e. a nutritive effect. Proliferating cells in vitro require an exogenous source of cholesterol for the synthesis of new plasma membranes [228] since they are unable to synthesise adequate amounts of cholesterol themselves. Fless et al. have demonstrated that only the larger LDL sub-species, which contain greater amounts of cholesterol ester, have a proliferative effect on cultured aortic smooth muscle cells [229]. The stimulatory effect of LDL may therefore only be seen in vitro where the availability of exogenous cholesterol acts as a limiting factor on cell growth. Whether LDL has a direct mitogenic effect on cell proliferation, which could be acting in vivo, remains unclear.

The mechanism of the toxic effect of lipoproteins in vitro is also unclear. LDL toxicity is not dependent on an interaction between LDL and the LDL receptor [230]. Hessler et al. have shown that LDL must be oxidised before it exhibits its toxic effect [231]. The oxidation involves the lipid component of the lipoprotein and occurs spontaneously in vitro. Such oxidation may occur in vivo, since Cathcart et al. have shown that incubation of LDL with monocytes or neutrophils leads to the oxidation of LDL through a free radical-mediated reaction [38]. The resulting oxidised LDL is toxic to proliferating fibroblasts and its toxic effect is proportional to the degree of oxidation achieved. Hydroxycholesterol causes a more severe granulomatous reaction than cholesterol when implanted subcutaneously in rats [220] and in vitro it has been shown to have a toxic effect on cultured smooth muscle cells [220].

On the basis of these results, one may suggest that accumulation of LDL which is subsequently oxidised either by intrinsic mesangial cells [103] or by infiltrating leucocytes (vide infra) may exert a damaging effect on glomerular cells.
Lipid-induced sclerosis.

Fibrosis is a prominent feature in the development of atheromatous plaques and, as mentioned above, cholesterol causes a fibrotic reaction when implanted subcutaneously. One might therefore expect that LDL would have a stimulatory effect on the synthesis of connective tissue elements, such as collagen, by smooth muscle cells in vitro. However, there have been few studies reporting the effects of lipoproteins on collagen synthesis. Using hyperlipidaemic serum from cholesterol-fed rats, Ronnemaa and Doherty [232] demonstrated a 50% increase in the secretion of collagen into the culture medium by rat smooth muscle cells. However, these effects were seen only in cells that had been suspended from the culture plate by digestion and no increase was observed in isolated aortas or adherent cultured smooth muscle cells. Furthermore, the total collagen content of cells and medium together was not significantly increased, suggesting that the hyperlipidaemic serum was increasing collagen release from the cell, rather than overall synthesis. Other workers have also failed to demonstrate a direct effect of lipoproteins on collagen synthesis by smooth muscle [233].

'Cell mediated' lipid toxicity.

Increasing emphasis is currently being put on the role of monocytes and macrophages in the development of atherosclerosis [210]. The earliest stage of atherosclerosis seen in experimental animals with dietary-induced hypercholesterolaemia is the attachment of monocytes to the arterial endothelium [206]. These cells then infiltrate beneath the endothelial layer where they progressively accumulate lipid and become "foam cells".

Macrophages have a number of properties which enable them to clear unwanted deposits of lipoprotein from extravascular tissues (Chapter 1). They produce chemo-attractant factors that recruit other monocytes and cause a focal accumulation of these cells [210]. They secrete lipoprotein lipase which may break down deposits of lipoprotein [234]. They generate free radicals which lead to the modification of LDL by peroxidation. Modified LDL is then taken up by macrophages via
There is evidence that macrophage infiltration causes glomerular damage in man and experimental animals. In some forms of human proliferative glomerulonephritis, a correlation has been found between the severity of monocytic infiltration and proteinuria. In experiments by Holdsworth et al. using a rabbit model of glomerulonephritis, depletion of macrophages by a cytotoxic agent prevented the development of proteinuria and glomerular damage. Reinfusion of isolated macrophages to animals previously depleted of leukocytes led to proteinuria and endothelial cell injury.

This damage may be mediated by a variety of mechanisms. They produce a range of inflammatory mediators that are injurious to tissues including lysosomal hydrolases, proteases and oxygen-free radicals. They secrete cytokines including arachidonic acid metabolites, interleukin I, platelet activating factor, platelet derived growth factor, interferon and tumour necrosis factor which may regulate the activity of other glomerular cells. They secrete complement components and may initiate local coagulation through the expression on their surface of pro-coagulant activity.

Intrinsic smooth muscle-type mesangial cells are not phagocytic in culture but do take up macromolecules by endocytosis. They are also able to produce inflammatory mediators, including reactive oxygen species, platelet activating factor, arachidonic acid metabolites, interleukin I and platelet derived growth factor when studied in culture.

In summary, it is possible that the accumulation of lipoprotein within damaged glomeruli may lead to an infiltration by macrophages. Phagocytosis of the lipid by macrophages and/or by intrinsic mesangial cells may lead to the release of inflammatory mediators which may in turn exacerbate glomerular injury.

Thrombosis.

Thrombosis may be an important component of the response of glomerular capillaries to injury. In experimental models of glomerulosclerosis, local thrombosis has been noted at sites of injury.
and treatment with anticoagulant drugs has been found to retard the progression of glomerular damage [238,239]. Hyperlipidaemia may increase glomerular damage through activation of clotting mechanisms. Dietary supplementation with saturated fat leads to increased blood coagulability [240]. Platelets from patients with familial hypercholesterolaemia have an increased sensitivity to aggregating agents [241]. These effects can be reproduced in vitro by incubating platelets with cholesterol-rich liposomes [242]. When hypercholesterolaemia is associated with the nephrotic syndrome, there is further activation of the clotting mechanisms due to increases in the level of circulating clotting factors and in the adhesiveness and aggregability of platelets [86]. Hypercholesterolaemia may also increase the stimulus to platelet aggregation at sites of vascular injury. Damage to the endothelium reduces its capacity to synthesise prostacyclin (PGI$_2$) which is a potent inhibitor of platelet aggregation. Eldor et al. followed the recovery of prostacyclin synthesis with re-endothelialisation of the rabbit aorta following mechanical injury. In control animals it returned to normal levels over a 10 week period. However, in rabbits fed a cholesterol-supplemented diet causing moderate hypercholesterolaemia, no recovery in prostacyclin synthesis was found [243].

**Immune-mediated lipid toxicity.**

There is some evidence that lipoproteins may act via immune mechanisms to cause vascular damage. Klimov has proposed an autoimmune theory for the pathogenesis of atherosclerosis [207] in which immune complexes are formed between LDL and antibodies against apoprotein B. The complexes are then trapped in the arterial intima and stimulate an inflammatory response. Deposits of apoB-containing lipoprotein, C$_3$ and IgG have been detected in human coronary arteries and in atherosclerotic aortas from experimental rabbits. Circulating anti-apoB antibodies and immune complexes have also been detected in normal adults and in adults with ischaemic heart disease. Deposited immune complexes may be involved in the formation of
foam cells in the arterial intima. Preformed LDL- and VLDL-anti-apoB IgG immune complexes are taken up by macrophages more actively than native LDL. Incubation of macrophages with these complexes in vitro for 3 days leads to the accumulation of lipid and to foam cell formation [244].

The combination of immune deposits and lipid accumulation is also found in glomerular disease. In nephrectomy specimens from patients with non-immunological end-stage kidney disease and in biopsies from patients with focal glomerulosclerosis, deposition of IgM, complement components C1q, C4, 144 and properdin have been regularly detected [148], as has lipid and β lipoprotein [192]. In LCAT deficiency, C3 has been found to accompany the glomerular lipid deposits [180].

One particular condition in which immune-mediated lipid abnormalities have been studied in more detail is that of infection with malarial parasites. Malarial infection in man and experimental animals is associated with abnormalities of lipoprotein metabolism which, in mice, consist of an increase in VLDL and LDL and a decrease in HDL. This is due to a decrease in the activity of lipoprotein lipase in adipose tissue and to an increase in VLDL secretion [245]. In mice infected with Plasmodium chabaudi, Ig-lipoprotein complexes containing anti-lipoprotein antibodies were detected in the serum after 9 days. Complexes were deposited in glomeruli thereafter and apoB could be eluted from the kidney. Immune complexes are found in the glomeruli of humans infected with plasmodia [246]. The antigens involved have not been extensively characterised in man and it is not known whether apoB-containing complexes are present. However, glomeruli from patients with quartern malarial nephrosis contain lacunae in the glomerular basement membrane which look very similar to those found in LCAT deficiency (Chapter 6) [247] and which may have contained lipid material that was removed during processing.

Lipoproteins are known to have important effects on the function of immune cells. Receptors that are different from the apo B/E LDL receptors, have been demonstrated on lymphocytes and have been shown to have a modulating effect on lymphocyte function [248,249]. Accessory cells (i.e. mainly monocytes) are also affected by lipoproteins. Their
ability to activate T lymphocytes is inhibited by preincubation with VLDL or LDL [250].

In summary, there is some evidence that immune mechanisms may interact with lipoproteins in the development of glomerular damage, even when the initial insult is not immunologically mediated. However, this evidence does not permit a definite link to be drawn.
CHAPTER 11
EXPERIMENTAL EVIDENCE THAT LIPIDS ARE IMPORTANT IN THE PATHOGENESIS OF GLOMERULAR DAMAGE.

The role of lipids in the pathogenesis of glomerular damage has been investigated in a range of experimental models. Levels of serum cholesterol and triglyceride have been manipulated by dietary supplementation and drug therapy. The published data will be discussed in two parts according to the direction in which serum lipids have been adjusted.

Evidence that controlling hyperlipidaemia decreases the severity of glomerular damage.

Serum cholesterol and triglyceride concentrations can be reduced in normal rats by dietary modification. Polyunsaturated fatty acids in the form of 12% tuna fish oil led to a 17% fall in serum cholesterol and a 47% fall in serum triglyceride when fed to intact rats [240]. When fed to uraemic rats, polyunsaturated fatty acids lowered serum lipids but had variable effects on glomerular damage [251]. Heifets et al. [252] and Barcelli et al. [251] have demonstrated an improvement in renal function and glomerular damage in rats fed a linoleic acid-rich diet following subtotal nephrectomy. Conversely, Scharschmidt et al. [253] demonstrated a worsening of renal impairment and glomerular damage in nephrectomised rats fed a fish oil supplemented diet. This latter effect occurred despite a significant reduction in serum cholesterol and serum triglyceride concentrations. The effects of dietary polyunsaturated fatty acids on prostaglandin production, and hence on systemic and renal haemodynamics [251], make it difficult to identify the independent role that serum lipids play in these experiments. Nonetheless, the results of Scharschmidt et al. suggest that serum lipids are not of over-riding importance in the development of glomerulosclerosis under these conditions.

A diet based on soya protein rather than casein is associated with a lower serum cholesterol concentration in normal rats [254], due to a reduction in cholesterol absorption from the gut [255,256]. Following
subtotal nephrectomy, Williams and Walls demonstrated that animals fed soya protein had significantly improved renal function and survival, with a reduced percentage of sclerosed glomeruli, compared to animals fed a casein-based diet [257]. This was associated with significantly lower serum triglyceride and cholesterol concentrations in soya-fed animals [254]. These results are compatible with a beneficial effect of lowering serum lipids but it is not possible to separate such an effect from that due to an alteration in haemodynamic factors caused by the different quality of protein ingested.

In an attempt to study the effect of lowering serum lipid levels in isolation, Kasiske, O'Donnell, Garvis and Keane administered the lipid-lowering agents clofibric acid and mevinolin to male Sprague-Dawley rats, by daily subcutaneous injection following one-stage uninephrectomy and two-thirds contralateral renal infarction [258]. Clofibric acid treatment led to a reduction in serum cholesterol concentration at 4-6 and 7-10 weeks after surgery. There was no change in serum triglyceride at 4-6 weeks and no data is given for the second sampling time. Albuminuria was significantly lower at both sampling times and the percentage of glomeruli with focal sclerosis was reduced from 24±5% to 5±2% (p<0.01). Inulin clearance was correspondingly greater in clofibric acid-treated animals. Tail cuff blood pressure was slightly, but not significantly, lower after 4-6 weeks. The animals were not pair-fed and no record of food intake was made. However, body weight was not significantly different.

Unfortunately, a number of important methodological details which are required to ensure comparability between the clofibric acid and control groups are not given in this paper. Firstly, it is not stated whether the control group received vehicle or no injections, they are merely referred to as "untreated" controls. Secondly, the method of allocation of animals to the two treatment groups is not stated. The numbers in the two groups is unequal (control = 10, clofibric acid = 11) but no mention is made of the numbers of animals that died after surgery of before the completion of the experiment. Thirdly, the time of starting the clofibric acid injections after nephrectomy is not stated.
In a separate study, two groups of 6 animals, one "untreated" and the other given s.c. clofibrac acid, underwent micropuncture studies four weeks after nephrectomy. No significant differences in single nephron GFR, plasma flow or glomerular capillary pressure were detected, although in both groups values were higher than in intact controls. These results suggest that the effect of clofibrac acid on glomerulosclerosis demonstrated in the first experiment is not mediated by alterations in glomerular haemodynamics. However, there are a number of differences between the results in the micropuncture study and those in the first experiment. Both groups of animals in the micropuncture study showed much less albuminuria 4 weeks after nephrectomy (control = 7.6±3.2, clofibrac acid = 6.8±1.4mg/24hrs) than did the untreated animals in the first experiment at 4-6 weeks (28.0±7mg/24hrs). Serum cholesterol was higher in the untreated micropuncture animals than in the untreated controls in the first experiment (94.5±6.5 vs 76±4mg/dl). Although serum cholesterol was reduced to 75.7±6.8mg/dl by clofibrac acid in the micropuncture study, this did not reach statistical significance (p=0.07). The failure to detect a significant difference in glomerular haemodynamics in the micropuncture study was therefore associated with a generally smaller effect of clofibrac acid than in the first experiment and it may not be possible to extrapolate with certainty from one to the other.

In a further experiment described in this paper, subcutaneous injections of mevinolin or vehicle were administered to 13 and 15 rats respectively following 5/6 nephrectomy. The mevinolin-treated animals had significantly lower serum triglyceride and cholesterol concentrations and less albuminuria at 4-6 and 7-10 weeks after nephrectomy. Glomerulosclerosis was reduced from 30±3% to 11±2% of glomeruli (p<0.01). Again, there are a number of reservations concerning this experiment. Firstly, one-half of the animals in each group were pair-fed. Despite pair-feeding, body weight was 8.5% lower in the mevinolin-treated group (p<0.05) suggesting the drug had a generally catabolic effect. Secondly, the levels of serum cholesterol and triglyceride in the control animals were markedly higher than in the first experiment (e.g. at 4-6 weeks, triglyceride = 130±9 vs
71±6mg/dl, cholesterol = 132±6 vs 76±4mg/dl). The cause for this wide variation is not given. Thirdly, tail cuff blood pressure was lower in the mevinolin-treated group (169±7 vs 185±5), although this did not reach statistical significance at p<0.05. It is thus possible that the drug was acting via a hypotensive rather than a hypolipidaemic mechanism. Micropuncture studies were not performed in mevinolin-treated animals to investigate this possibility.

Kasiske and Keane have performed similar experiments using obese Zucker rats [259]. This is a strain of rats that is hyperphagic and develops obesity, hyperlipidaemia, insulin resistance, mild glucose intolerance, albuminuria and focal glomerulosclerosis [157,260,261]. Again, both mevinolin and clofibric acid, given to 8 and 7 animals respectively from 8 to 40 weeks of age, led to a reduction in albuminuria, mesangial matrix expansion and focal glomerulosclerosis. This was associated with a reduction in serum cholesterol and triglyceride in mevinolin-treated animals and cholesterol alone in clofibric acid-treated animals. Separate micropuncture studies showed no effect of mevinolin or clofibric acid on glomerular haemodynamics. Animals were fed ad libitum and food intake, measured over a single six day period after 28 weeks on the diet, was not significantly different. Final mean body weight was 10% lower in the mevinolin-treated group and 6% lower in the clofibric acid-treated group but this did not reach statistical significance due to a wide standard error of the mean.

In recent abstracts, Wheeler et al. [262] have reported a significant reduction in proteinuria in obese Zucker rats treated with subcutaneous clofibric acid following unilateral nephrectomy, when compared to pair-fed vehicle-injected controls. Serum cholesterol and triglyceride levels were lowered by clofibric acid, although only cholesterol reached statistical significance. Harris et al. have reported that subcutaneous lovastatin (=mevinolin) preserves renal function and reduces glomerulosclerosis in rats with nephrotic syndrome and severe glomerulosclerosis induced by puromycin and protamine sulphate [263].

In summary, a number of experiments employing a variety of
Experimental models have suggested that control of hyperlipidaemia is associated with amelioration of glomerulosclerosis and renal impairment. However, many of the reports are as yet preliminary and methodological weaknesses are present in others. Further work is therefore required to confirm or refute this suggestion.

Evidence that exacerbating hyperlipidaemia increases the severity of glomerular damage.

The effect of dietary-induced hyperlipidaemia on glomerular damage has been studied in a wide range of animal models. Peric-Golia and Peric-Golia [264] fed male Sprague-Dawley rats diets supplemented with either 3% cholesterol or 3% cholic acid and 3% taurine for up to 80 weeks. All animals on both diets developed hypercholesterolaemia, as did three of the eight on a control diet. All animals with serum cholesterol concentrations above 150 mg/dl, including the three on the control diet, developed aortic intimal plaques and glomerulosclerosis. The cholesterol content of the kidneys and aorta was not increased, despite lipid deposits being present in the glomerular capillary lumina in tissue sections. Fischer et al. [265] studied the effect of a diet supplemented with 4% cholesterol, 4% coconut oil and 1% cholic acid on intact female rats. Animals on the high fat diet developed glomerulosclerosis. In contrast to the results of Peric-Golia, tissue cholesterol content was increased and a significant positive correlation (r=0.842) was found between the systolic blood pressure and the kidney cholesterol content.

Similar experiments have been reported by others in abstract form. Groene et al. [266] fed a diet supplemented with 5% cholesterol and a total of 29% fat to intact and uninephrectomised Wistar rats. After 6 months, the severity of glomerulosclerosis was increased in the fat-supplemented group, especially following uninephrectomy. Interestingly, these effects were only found in male and not in female rats. Kasiske et al. [267] fed 10 week old male intact and uninephrectomised rats a high cholesterol diet. They found an increase in proteinuria and glomerulosclerosis after 15 weeks but, in contrast to Groene, there was no exacerbation of these effects following
uninephrectomy.

As uninephrectomy does not initially cause uraemia, more extensive removal of renal tissue must be performed to study the effect of dietary-induced hyperlipidaemia in uraemic animals. The partial nephrectomy model of chronic renal failure in the rat was used in a series of experiments carried out in the 1930's by Chanutin and others and in an experiment in 1938 [268], they studied the effects of modifying the diet with increasing proportions of dried liver extract. As well as providing increased amounts of protein, this modification increased the cholesterol content of the diet from 0.16% to 1.07%. In intact animals, serum cholesterol concentration was unaltered, except on the 80% dried liver extract diet. However, in nephrectomised animals serum cholesterol was elevated in proportion to the cholesterol content of the diet. Despite continuing the diet for up to 150 days, blood pressure and the urea ratio (a measure of urea clearance) were not worsened on the high liver extract diets. No histological data was provided.

Ritz et al. [269] studied a more severe (8/10) subtotal nephrectomy model in which glomerular filtration rate was reduced by 80-85%. Animals were followed for 25 days and mortality was used as the end point. 25% of animals fed a diet containing 14.5% protein and 3% unspecified fat had died by 25 days whereas none fed 17.5% protein and 20% fat had done so. Since this diet contained more protein as well as fat and a further increase in the dietary protein content increased mortality, the fat supplementation appeared to protect against mortality. Serum lipid levels and further experimental details were not provided in this report. Ando et al. [270] and Drukker et al. [271] have reported, also in abstract form, an increase in proteinuria in rats fed a high fat diet following subtotal nephrectomy.

In view of the incomplete data available in the literature, there is a need for a detailed study of the effect of dietary fat supplementation on the development of glomerular damage in the rat following subtotal nephrectomy.

As described in Chapter 3, glomerulosclerosis in association with
the nephrotic syndrome follows the administration of puromycin aminonucleoside to the rat. Grond, Weening, and Elema [163] have provided evidence that the hyperlipidaemia of the nephrotic syndrome is insufficient alone to cause glomerulosclerosis in these animals. They compared the glomerular changes occurring in animals treated with puromycin with others treated with Adriamycin, which led to equivalent rises in serum cholesterol, serum triglyceride and urinary protein. The incidence of glomerulosclerosis was found to be significantly higher following puromycin (7.8%) than following Adriamycin (0.3%). This suggests that an additional insult to the glomerulus other than hyperlipidaemia is required to initiate sclerosis.

The possibility that glomerular lipid deposits present in puromycin-treated animals may contribute to the sclerotic process has been studied by feeding such rats a fat-supplemented diet. Edwards et al. [272, 273, 274] have reported in abstract form, studies on rats which have received either none, 1 or 2 doses of puromycin administered into the peritoneal cavity. Animals were fed either a control diet or a diet with a high sucrose and lard content. One sucrose-lard-fed group had added halofenate, a clofibrac acid-like drug, added to the diet. The sucrose-lard diet increased the severity of glomerulosclerosis, renal functional impairment and mortality compared to the control diet. Halofenate reduced the hyperlipidaemia in the sucrose-lard-fed animals towards the controls and also reduced the severity of glomerulosclerosis and improved survival.

These preliminary results have been supported by a recently published paper by Diamond and Karnovsky [275]. They studied the effects of a diet supplemented with 4% cholesterol and 1% cholic acid, in rats with chronic nephrotic syndrome induced by a single intravenous dose of puromycin. Animals on the cholesterol diet were significantly more hypercholesterolaemic throughout the 18 week study period and more hypertriglyceridaemic at 4 and 12 weeks. Proteinuria was not different in the cholesterol-fed group until 18 weeks, when there was a dramatic rise. Systolic tail cuff blood pressure was significantly increased at 4 and 18 weeks. Renal function was worse on the cholesterol diet with a significant reduction in glomerular filtration
rate and increase in serum urea at 18 weeks. Glomerular damage was also greater, with the percentage of glomeruli containing foam cells being increased from 17% to 62% (p<0.001). Glomeruli with segmental mesangial proliferation were increased from 44% to 60% (p<0.05) and glomeruli with focal segmental glomerulosclerosis and hyalinosis lesions were increased from 18% to 29% (p<0.05). Intense lipid deposition was detected in sclerotic areas. Specific markers for bone marrow-derived mesangial cells or infiltrating macrophages were not applied, although a subsequent preliminary report by the same workers records an increase in glomerular macrophages in intact and nephrotic animals fed the cholesterol diet [276].

These results demonstrate that hyperlipidaemia exacerbates the development of glomerulosclerosis following intravenous puromycin. The mechanism for this effect is remains unclear. It may be due to a specific toxic effect of cholesterol or triglyceride on glomerular cells or may be mediated by infiltrating macrophages. However, in view of the small but significant increase in blood pressure in the cholesterol-fed animals, a haemodynamically mediated effect cannot be ruled out. Glomerular lipid deposition could have led to occlusion of glomerular capillaries and thus a secondary increase in both systemic blood pressure and in the pressure in those glomerular capillaries still being perfused. These hydrostatic changes may then have led directly to further glomerular injury. A vicious cycle of increasing hyperlipidaemia, lipid deposition, falling GFR and increasing blood pressure would have been created (Figure 11.1). Such a mechanism would have the effect of accelerating the terminal phase of chronic renal failure as the number of functioning nephrons becomes small. This may explain why the marked effect of the diet only commenced after 18 weeks.

Recently published abstracts lend support to the suggestion that the effect of hyperlipidaemia may be mediated by haemodynamic changes. Smitz, Keane et al. have reported that a cholesterol-supplemented diet causes a significant increase in transcapillary hydraulic pressure in intact and uninephrectomised male rats after 4 to 6 weeks [277]. Groene et al. [278] and Fujihara et al. [279] have used enalapril to
lower glomerular capillary pressure. Groene found that this protected intact male Wistar rats from glomerulosclerosis induced by a fat- and cholesterol-supplemented diet. Fujihara studied analbuminaemic rats, which have persistent hypercholesterolaemia. Two weeks following five-sixths nephrectomy, systemic blood pressure and transcapillary hydraulic pressure were higher in analbuminaemic animals than in Sprague-Dawley controls. These were controlled by enalapril and treated animals had preserved renal function, reduced proteinuria and less glomerulosclerosis compared to analbuminaemic controls. This protective effect occurred without any reduction in serum cholesterol concentration.

Cholesterol-supplemented diets have been used in species other than the rat. French et al., in 1967 [280], demonstrated that a 1% cholesterol diet fed to guinea pigs caused glomerulosclerosis associated with mesangial hypercellularity, hypertrophy and lipid deposition. The animals developed haemolytic anaemia on this diet. In a recent study by Al-Shebeb et al. [281], a 2% cholesterol diet caused similar effects to those previously described. Serum creatinine and creatinine clearances were unchanged on the diet but progressive mesangial expansion and glomerular lipid deposition was noted. Using the non-specific esterase reaction, an infiltration of glomeruli by monocytes was detected. These histological changes were further aggravated by a high protein diet. Animals with haemolytic anaemia of a milder degree induced by the drug acetylphenyl hydrazine did not develop glomerular lesions. Comparison between these results and those found in rats is complicated by the differences in lipoprotein metabolism between the two species. For example, guinea pigs have predominantly low density lipoproteins and the distribution of sites of lipoprotein synthesis is more widespread than in the rat [71].

A commonly used species for the study of experimental atherosclerosis is the rabbit. Wellmann and Folk [282] studied the effects of a 1% cholesterol diet administered for 6-12 months in normal and mildly diabetic rabbits. In 30% of normal and 24% of
diabetic animals, glomerular intracapillary foam cell collections were found with mesangial thickening and nodule formation. Interstitial foam cell plaques developed in all cases. The animals did not become uraemic. A preliminary report by Raij *et al.* [283] suggests, however, that severe hyperlipidaemia alone does not necessarily lead to glomerular damage in rabbits. 300 day old Watanabe rabbits were compared with control New Zealand whites. Watanabe rabbits are congenitally deficient in apoB/E LDL receptors and develop severe hyperlipidaemia on a normal diet. Despite this, no glomerular damage was seen in these animals.

The effect of a high fat diet has also been studied in an immunologically-mediated model of glomerular damage. NZBxW mice are a strain that develops spontaneous glomerulonephritis similar to systemic lupus erythematosus. Kelley and Izui [284] fed such mice a diet containing either 4.5% or 51.7% saturated fat. The high fat diet group had higher serum cholesterol and urea levels, greater proteinuria and earlier mortality, with histological changes of accelerated membranous nephritis. Lipid vacuoles were present in the mesangial matrix and within mesangial cells and vacuoles in the glomerular basement membrane were frequently associated with electron-dense deposits. Foam cells were also present in the mesangium and in the glomerular capillary lumina, as well as the interstitium. No differences in auto-antibody titres, immune-complex titres or immunoglobulin levels were detected, suggesting that hyperlipidaemia accelerated the development of nephritis by a local vascular mechanism rather than by an immunological one.
Figure 11.1
Possible pathogenetic mechanisms by which hyperlipidaemia may exacerbate glomerulosclerosis. See text for discussion.
CHAPTER 12
EVIDENCE THAT LIPIDS ARE IMPORTANT IN THE PATHOGENESIS OF GLOMERULAR DAMAGE IN MAN.

A number of studies have investigated factors that may affect the progression of chronic renal failure in man. Proteinuria has been found to be a predictor of poor functional outcome in patients with chronic renal failure [1] and nephrotic syndrome [285]. Since hyperlipidaemia is related to the severity of proteinuria in the nephrotic syndrome, any correlation between serum lipid levels and outcome may be a parallel phenomenon and may not imply a causative link. A recent study by Walser and Ward found a significant correlation between serum triglyceride concentration and the decline in glomerular filtration rate [286]. However, serum triglyceride was correlated with another factor, namely urinary corticosteroid excretion, and so may again have been a parallel phenomenon.

Indirect evidence that similar factors may be involved in the development of glomerulosclerosis and atherosclerosis is provided by a study of autopsy material by Kasiske [287]. Using multiple linear regression analysis, he found that there were significant independent associations between glomerulosclerosis and both age and intrarenal vascular disease, indicating that a significant proportion of the glomerulosclerosis was linked to atherosclerosis. The nature of this relationship is not apparent from this study.

Few studies have been performed in man to investigate the effects of modifying serum lipid levels on glomerular damage. This is partly due to the severe side effects caused by the older lipid-lowering drugs in patients with the nephrotic syndrome.

McKenzie and Kincaid-Smith [288] have reported the use of clofibrate in two patients out of a series of four with hyperlipidaemia and foam cells on renal biopsy. The underlying diagnoses of the two patients were nephrotic syndrome following poststreptococcal glomerulonephritis and chronic renal failure due to chronic glomerulonephritis. Hyperlipidaemia was controlled by clofibrate and in follow-up biopsies, the foam cells were no longer
present. Proteinuria was slightly lower in both patients on clofibrate but serum creatinine was unchanged in the patient with nephrotic syndrome and progression to end stage renal failure continued in the second patient. The authors conclude that glomerular foam cells are "an interesting, but probably unimportant finding in those circumstances where there is systemic hyperlipidaemia and active glomerular damage." However, it is not possible to rule out a contributory role of lipids from the results of treatment in two patients.

Two recent reports have demonstrated successful lowering of serum cholesterol in short-term studies on small groups of adult nephrotic patients with gemfibrozil [289] and lovastatin (=mevinolin) [290]. Urinary protein excretion and serum creatinine levels were not altered by the drugs. The studies did not set out to assess effects on glomerular damage or the progression of renal failure. A longer term study on patients with nephrotic syndrome due to focal glomerulosclerosis using one of these agents would be required to determine an effect of such therapy on these changes.
CHAPTER 13
QUESTIONS RAISED BY THE LIPID HYPOTHESIS.

The preceding chapters have raised a number of questions that need to be answered before the role of lipids in the pathogenesis of glomerulosclerosis can be more fully assessed.

1. Regarding the metabolism of lipoproteins by glomerular cells: -
   (a) Which glomerular cells express lipoprotein receptors and are these apo B/E LDL or scavenger receptors?
   (b) Is the expression of lipoprotein receptors altered under conditions of glomerular damage?

2. Regarding the mechanism by which lipid is deposited within the glomerulus: -
   (a) Are circulating lipoproteins deposited in normal glomeruli?
   (b) Where in the glomerulus are lipoproteins deposited?
   (c) Which cells are involved in the uptake and clearance of lipoproteins from the glomerulus?
   (d) How is the deposition of lipoproteins affected by glomerular injury?

3. Regarding the effects of lipoproteins on glomerular damage: -
   (a) Do all lipid-lowering agents reduce hyperlipidaemia and glomerular damage in uraemic rats?
   (b) Does hyperlipidaemia increase glomerular damage in rats following uninephrectomy and subtotal nephrectomy?

4. Regarding the mechanisms by which lipids may damage glomeruli: -
   (a) Does hyperlipidaemia exacerbate glomerulosclerosis independently of any effects on blood pressure?
   (b) Does hyperlipidaemia cause an infiltration of the kidney by macrophages?
   (c) Does hyperlipidaemia increase the numbers of activated macrophages in the kidney?

The following series of experiments aims to answer these questions.
SECTION II
GLOMERULAR CELL CULTURE
CHAPTER 14
THE DEVELOPMENT OF GLOMERULAR CELL CULTURE.

Organ culture was first carried out in the Strangeways Laboratory in the late 1920s. Early work was largely limited to the maintenance \textit{in vitro} of whole embryo organs which were used for the study of morphogenesis [291]. In 1958, Trowell described the technique of culturing pieces of mature tissue \textit{in vitro} for about one week. His aim was to maintain the normal histological appearance of the tissue and cellular outgrowth was not obtained.

In 1969, Bernik [99] was the first to report the successful maintenance of isolated human glomeruli in culture. These survived for up to 3 months and their viability was demonstrated by rhythmic and synchronous contraction, observed by time-lapse cinematography. Scanty outgrowths of cells were obtained from glomeruli and consisted of two types:- a) large arborised glomerular epithelial cells which were non-motile and showed little division, and b) large motile cells, that frequently changed shape and size and resembled both fibroblasts and macrophages.

Several investigators have subsequently described the properties of a variety of cells cultured from isolated human and animal glomeruli [292-299]. With improved techniques, cells have been encouraged to proliferate so that large numbers can be studied. In most cases, the cells used have remained as primary cultures, i.e. they have a finite life-span and have to be regenerated from fresh tissue. Recently, Striker \textit{et al} have successfully immortalised mouse glomerular cells by genetic transformation with SV40 virus and have produced permanent cell lines [300].

Primary outgrowths from isolated glomeruli contain a mixture of cells originating from the different cell types in the glomerulus. A major hindrance to the development of glomerular cell culture, particularly from humans, has been the difficulty in unequivocally defining the origin of the cells produced. In order to proliferate, the cells must undergo a degree of de-differentiation and in so doing they tend to lose the characteristic features that identified them in
the whole tissue. Conversely, markers that are not expressed in the differentiated state may become manifest when the cells proliferate in culture. The uncertainty of cell identification also makes it difficult to separate reliably pure cell strains, derived from a single glomerular cell type.

Factors that have been used to identify the four types of glomerular cells are listed in Tables 14.1 - 14.4. For comparison the features of cultured proximal tubular cells are listed in Table 14.5. Reference is made only to human glomerular cell culture to avoid confusion due to species variation. Nonetheless, it will be noted that conflicting results have been reported for a number of features and no single feature has emerged as characteristic for any cell type.
Table 14.1: GLOMERULAR EPITHELIAL CELLS

Morphology [Review 301]

LM: Large, arborised "fried egg" cells forming a monolayer [297, 215, 99, 302, 303.]
EM [297]: peripheral microfilaments and junctional complexes, no bundles of microfilaments.
  smooth surface- occasional cilia and microvilli
  interdigitated cytoplasm
  perinuclear cytoplasm rich in organelles
  prominent rough ER
  no Weibel-Palade bodies

Cytoskeleton

cytokeratin: +ve (regular and evenly distributed) [297]
vimentin: -ve [297], +ve [305]
keratin: +ve [304, 305]
actin: +ve (peripheral) [297]

Receptors

C3b: +ve in vivo [306], +ve in culture [296, 297], -ve in culture [215]
Fc: -ve [215]

Antigens

factor VIIIRAg: -ve [297]
sialoglycoprotein (PHM5): +ve [307]

Products

fibronectin: +ve [297], -ve (PHM13) [309]
collagen type IV: +ve [306]
collagen type I & III: -ve [306]
heparan sulfate: +ve [306]
urokinase: +ve [304]

Enzymes

cyclo-oxygenase: -ve [297]

Behaviour

migrate off glomerulus [215]
slow rate of division [215]
phagocytosis -ve (4u yeast particles) [215]
PAN resistant [297]
trypsin sensitive
Table 14.2  SMOOTH MUSCLE-TYPE MESANGIAL CELLS

Morphology

LM: Stellate, fusiform, elongated cells [297] overgrow to form hills and valleys
EM: microfilament bundles, dense bodies [297]

Cytoskeleton

vimentin: -ve [297]
keraatin: -ve [304]
smooth muscle myosin: +ve [297,336]
actin filaments: +ve [308]

Receptors

C3b: -ve [215,336]
Fc: -ve [215]
PDGF: -ve [297]

Products

fibronectin: +ve [309]
collagen types I,II,IV,(V) [306]
chondroitin-4 and -6 sulfated glycosaminoglycans [306]
urokinase: -ve [304]

Enzymes

cyclo-oxygenase, low activity [297]

Behaviour

rapid proliferation
phagocytosis -ve (4u yeast particles) [215]
trypsin resistant
IL-1 responsive
Table 14.3  BONE MARROW-DERIVED MESANGIAL CELLS

Morphology

small rounded cells [215]

Receptors

C3b: +ve [215]
Fc: +ve [215]

Antigens

Monocyte (PHM2): +ve [335]

Products

fibronectin (PHM13): -ve [309]

Behaviour

motile, only seen with cinemicroscopy [215]
scarce, one per 3-5 glomeruli
phagocytic
? derived from marginated monocytes
Table 14.4  GLOMERULAR ENDOTHELIAL CELLS

**Morphology**

LM: cobblestone monolayer [310]  
like glomerular epithelial cells

EM: tight junctions [310]  
no cilia, few microvilli  
prominent microtubules and intermediate filaments  
microfilaments near cell surface  
subplasmalemmal vesicles  
occasional coated pits  
numerous mitochondria  
Golgi well developed  
one Weibel-Palade body found

**Cytoskeleton**

cytokeratin: -ve [310]  
keratin: -ve  
vimentin: -ve (cf. large vessel endothelium +ve)  
actin: +ve  
tubulin: +ve

**Antigens**

Sialoglycoprotein (PHM5): +ve [311]  
factor VIIIIRAg: +ve (variable size & distribution of granules)

**Enzymes**

angiotensin converting enzyme: +ve [310]

**Behaviour**

first outgrowth from glomeruli  
require PDGF 2ng/ml in 20%FCS
Table 14.5  PROXIMAL TUBULAR CELLS

**Morphology**

| LM: Monolayer with sickle-shaped cells [312,313] |
| EM: dense microvilli [319,313] |
| junctional complexes |
| abundant rough ER and free polysomes |
| cytoplasmic filaments |
| basal cell membrane interdigitations |
| well developed endocytotic apparatus |

**Cytoskeleton**

keratin: -ve [312]

**Antigens**

factor VIIIRAg: -ve [312]

**Products**

fibronectin: -ve [314]

Tamm-Horsfall glycoprotein: -ve

L-fucose: +ve [315,316]

**Enzymes**

g-glutamyl transpeptidase: +ve) [317]

L-leucine aminopeptidase: +ve) greater activity [317]

alkaline phosphatase: +ve) at confluence [312,317]

glucose-6-phosphatase: +ve) [312]

**Behaviour**

'contaminates' glomerular cultures [322]
CHAPTER 15.
THE GROWTH AND CHARACTERISATION OF CELLS FROM ISOLATED HUMAN GLOMERULI.

In view of the difficulties discussed in Chapter 14, a study was made of the behaviour of cells grown from a series of human kidney specimens. An attempt was made:-

a) to define the nature of the cells cultured 
b) to define their optimal culture conditions and 
c) to separate cell strains with distinct morphological features.

Methods

Regular contact was maintained with the Departments of Urology at Leicester General Hospital and Nottingham City Hospital. With the consent of the surgeons and pathologists involved, a 20cm³ piece of the healthy part of kidneys removed for carcinoma was taken immediately after nephrectomy and placed in tissue culture medium on ice.

Glomeruli were isolated from healthy human renal cortical tissue by a sequential sieving technique (Figure 15.1) [303] in a laminar flow hood, using sterile techniques. Details of manufacturers and suppliers of materials are listed in Appendices 1 and 2.

Cortical tissue was finely chopped in 10mls of culture medium and pressed through a coarse steel sieve (Boots tea strainer). The resulting suspension was allowed to settle for 5 minutes (Figure 15.2), the sediment was resuspended in fresh medium and allowed to sediment for a further 5 minutes. Nylon mesh filters were constructed by cutting a cylinder from a 10ml syringe body and attaching a piece of nylon bolting cloth of known pore size to the cylinder with a silicone rubber band. The filters were sterilised by the CSSD at LGH. Sediment was passed initially through a 200µ mesh to remove large tissue fragments and glomeruli were then retained on a 150µ mesh. Retained glomeruli were largely free of capsules, vascular poles and tubular fragments (Figure 15.3). Glomeruli were added to 10ml of culture medium at a surface density of about 10/cm² and left undisturbed for 7-10 days.
Figure 15.1. Isolation of glomeruli from human renal cortex by sequential sieving.
Figure 15.2. Photomicrograph of material from the initial sediment of cortical material. One glomerulus is surrounded by tubular fragments. x 200.

Figure 15.3. Glomeruli retained on the 150μ sieve. x 150.
The culture medium routinely used consisted of:-

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Supplements</th>
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<tbody>
<tr>
<td>RPMI tissue culture medium 1640</td>
<td>tri-iodothyronine 50ng/ml</td>
</tr>
<tr>
<td>foetal calf serum 10%</td>
<td>hydrocortisone 50ng/ml</td>
</tr>
<tr>
<td>penicillin 50u/ml</td>
<td>insulin 5μg/ml</td>
</tr>
<tr>
<td>streptomycin 50μg/ml</td>
<td>transferrin 5μg/ml</td>
</tr>
<tr>
<td>glutamine 2mM</td>
<td>selenite 5ng/ml</td>
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<td>epidermal growth factor 50ng/ml</td>
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</tbody>
</table>

Glomeruli were cultured in uncoated plastic flasks and in 4-well glass or plastic chamber slides at 37°C in humidified air with 5% CO2.

Flasks were fed according to the change in colour of the phenol pH indicator by replacing half of the spent medium with fresh. When the growths became confluent, cells were passaged; i.e. suspended by trypsinisation and split into two new flasks (Appendix 3). Samples of cell strains were preserved under liquid nitrogen for future recovery as described in Appendix 3. Routine care of all cell cultures was supervised by Dr. T. Horsburgh, Department of Surgery, LGH.

A qualitative study of the effects of various modifications of the culture technique was made by visually comparing the growth of cells routinely prepared flasks with flasks prepared from the same kidney in the modified manner.

The viability of isolated glomeruli was assessed by staining with fluorescein diacetate to demonstrate living cells and propidium iodide to demonstrate dead cells (Appendix 3).

The state of preservation of the ultrastructure of isolated glomeruli was studied by transmission electron microscopy (Section III, Appendix X).

The morphology of the cells grown was studied using a phase-contrast invert microscope. Photographs were taken using the attached automatic microscope camera (all by HCR).

An attempt was made to culture glomeruli on Permanox cover slips in order to study cellular ultrastructure by electron microscopy.
<table>
<thead>
<tr>
<th>Reactivity</th>
<th>Code</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular epithelial/ endothelial cells</td>
<td>PHM 5, MCA 117</td>
<td>Serotec Ltd., Unit 22, Bankside, Station Field Industrial Estate, Kidlington, Oxford. OX5 1JD.</td>
</tr>
<tr>
<td>Factor VIIIIR antigen</td>
<td>MCA 127</td>
<td>Monosan, Bradsure Biologicals Ltd., Loughborough.</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>MON HA 6001</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>CK-1</td>
<td>Dako Ltd., 22 The Arcade, The Octagon, High Wycombe, Bucks. HP11 2HT.</td>
</tr>
<tr>
<td>Vimentin</td>
<td>MON F3005</td>
<td>Monosan</td>
</tr>
<tr>
<td>Desmin</td>
<td>MON F3001</td>
<td>Monosan</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>PHM 13 MCA 120</td>
<td>Serotec</td>
</tr>
<tr>
<td>Class I MHC Ag</td>
<td>MCA 111</td>
<td>Serotec</td>
</tr>
<tr>
<td>Class II MHC Ag</td>
<td>HLA DR</td>
<td>Becton Dickinson Ltd., Cowley, Oxford.</td>
</tr>
<tr>
<td>Leucocyte common Ag</td>
<td>MCA 87</td>
<td>Serotec</td>
</tr>
<tr>
<td>Monocyte/macrophage</td>
<td>PHM2, MCA 98</td>
<td>Serotec</td>
</tr>
<tr>
<td>C3b receptor</td>
<td>M710</td>
<td>Dako</td>
</tr>
</tbody>
</table>
Antigen expression by cultured cells was demonstrated using an immunoalkaline phosphatase technique (Miss SL Brown, Department of Surgery, LGH; Section III Appendix 5). Cells were studied in situ by fixing cells grown on chamber slides. Cells from flasks were suspended by trypsinisation and collected on microscope filters using a cytopsin centrifuge. Some cells were studied after fixation in the culture flask and removing the top of the flask with a soldering iron.

Antigen expression by cultured cells was compared with that by cells in frozen sections of normal human kidney cortex. A wide range of monoclonal antibodies was applied and these are listed in Table 15.1. Fluorescent-conjugated lectins and phalloidin were applied as listed in Table 15.2 (HCR). Staining methods are detailed in Appendix 3. Uptake of diI-acetylated LDL was studied as described in Chapter 16.

Table 15.2 Other reagents used for cell characterisation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalogue Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiI-acetylated LDL</td>
<td>E00 902</td>
<td>Biogenesis Ltd., 12 Yeomans Park, Yeomans Way, Bournemouth, England, BM8 OBJ.</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>E00 906</td>
<td></td>
</tr>
<tr>
<td>Lectin from Ulex Europaeus</td>
<td></td>
<td>Sigma Chemical Co., Fancy Road, Poole, Dorset, BM17 7NM.</td>
</tr>
<tr>
<td>(FITC and TRITC labelled) [318]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lectin from Lotus tetragonolobus</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>(FITC and TRITC labelled) [315]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phalloidin</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>(TRITC labelled) [319]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

26 specimens of healthy kidney tissue were obtained from the following sources:

- Leicester General Hospital: 21
- Nottingham City Hospital: 3
- Mansfield District Hospital: 2

The kidneys had been removed for the following reasons:

- Adenocarcinoma: 21
- Transitional cell carcinoma: 4
- Unusable transplant donor: 1

Kidneys were obtained at a rate of 1 every 3 weeks from Leicester General Hospital. The mean age of the patients was 61.5 years (range 36-80).

Cell viability in isolated glomeruli, assessed by vital staining, demonstrated approximately 50% viable cells in freshly isolated glomeruli. Variable degrees of ultrastructural damage were demonstrated by electron microscopy (Figure 15.4). An attempt was made to reduce the damage inflicted on glomeruli during the isolation procedure by pulling the tissue apart rather than pressing it through the coarse steel sieve. However, the yield of glomeruli was significantly reduced and their state of preservation was not significantly improved by this technique.

Glomeruli were prepared on the day the kidney was removed in most cases. However, cells were successfully grown from glomeruli stored for up to 48 hours at 4°C. The best cell yields were obtained from kidneys removed from the younger patients. Nonetheless, satisfactory cells were cultured from all kidneys.

Isolated glomeruli were incubated with collagenase in an attempt to digest off the glomerular epithelial cells for separate culture. However, the cell suspension produced did not yield a cell growth.

In a routine culture, glomeruli adhered to the culture surface after about 7 days. Cells did not grow from glomeruli until they were adherent. Glomerular ultrastructure of non-adherent glomeruli was grossly abnormal after 10 days in culture (Figure 15.5).
Figure 15.4. Electron micrograph of a glomerulus fixed immediately after isolation. Note the extensive damage to both epithelial and endothelial cells. x 6600.

Figure 15.5. Electron micrograph of a glomerulus fixed after 11 days in culture. Capillary loops have collapsed and considerable cell necrosis has occurred. Glomerular epithelial cells have enlarged, lost their foot processes and have developed microvilli.
Capillary loops had collapsed and considerable cell necrosis had occurred. Glomerular epithelial cells had enlarged, lost their foot processes and had developed microvilli [320].

Pre-coating the culture surface poly-L-lysine caused immediate firm adherence of the glomeruli but no cell growth was obtained and the glomeruli died. Glomerular adherence was not improved by pre-coating the surface with plasma or foetal calf serum. Flasks from which mesangial cells had been suspended, leaving extracellular matrix on the culture surface, also did not improve glomerular adherence. Commercially-produced flasks precoated with extracellular matrix did not show increased glomerular adhesion but the morphology of cell outgrowths was altered, cells remaining as a monolayer rather than forming overgrowths.

A number of flasks were coated with serum taken from the same patient as the glomeruli. This did not appear to influence glomerular adhesion or cell growth.

Glomeruli adhered poorly to Permanox cover slips and difficulties were encountered in sectioning areas of adherent cells for electron microscopy. It has subsequently been recommended that glomeruli are more easily cultured on Petri-perm culture dishes (Heraeus) which are suitable for processing and sectioning for electron microscopy (Dr P. Wilson, personal communication).
Cell characterisation
Glomerular epithelial cells

A typical sequence of cell outgrowth was observed with all kidneys. Initially small numbers of large, arborising epithelial-like cells appeared, surrounding the adherent glomeruli (Figure 15.6). After a few days these cells tended to be overgrown by smaller, more rapidly proliferating cells. The two cell types could be readily separated by selective trypsinisation, using a low (0.1%) concentration of trypsin for 20 minutes at room temperature (Figure 15.7). The rapidly proliferating cells were less adherent to the flask and could be removed, leaving the epithelial cells in place. Small monolayers of epithelial cells were grown (Figure 15.8) but the cells were sensitive to full trypsinisation and could not be passaged.

An attempt to culture epithelial cells selectively by using a low concentration (5%) of foetal calf serum was unsuccessful, resulting in a poor growth of all cell types. The growth of these cells could be enhanced, however, by pre-coating the flask with Nu-serum and substituting the culture medium with 10% Nu-serum in place of foetal calf serum. Nu-serum is supplemented with epidermal growth factor, endothelial cell growth supplement and other hormones and has a total protein content approximately 30% of that of foetal calf serum.

The epithelial origin of this cell type was confirmed using monoclonal antibodies. In tissue sections, an antibody against sialoglycoprotein (PHM5) [307,311] predominantly stained glomerular epithelial cells (Figure 15.9). The epithelial cells surrounding glomeruli were also positive for this antibody (Figure 15.10). Antibodies against cytokeratin bound to tubular epithelium but not to the glomerular epithelium (Figure 15.11). These antibodies did not stain the epithelial cell outgrowth. The antibody against the C3b receptor (=CR-1 receptor) bound to glomerular epithelial cells in tissue sections but not to cultured cells.
Figure 15.6  The typical morphological appearances of early outgrowths of glomerular epithelial cells grown on Nu-serum coated plates in medium containing 10% Nu-serum. Phase contrast x 200.
Figure 15.7  Selective trypsinisation of a mixed glomerular outgrowth. Rapidly proliferating cells are less adherent and have rounded off following incubation with 0.1% trypsin for 20 minutes at room temperature. Tightly adherent glomerular epithelial cells remain attached to the flask. Phase contrast x 100.

Figure 15.8  A monolayer of glomerular epithelial cells cultured in medium containing 10% Nu-serum. Overgrowth by rapidly proliferating cells had been prevented by selective trypsinisation. x 200.
Figure 15.9 Frozen section of a normal glomerulus stained with monoclonal antibody against sialoglycoprotein (PHM 5) using the immunoalkaline phosphatase technique. Staining is predominantly on glomerular epithelial cells. x 600.

Figure 15.10 Outgrowth from a glomerulus on a chamber slide stained with monoclonal antibody PHM 5. Glomerular epithelial cells surrounding the glomerulus are positive, staining red. x 100.
It was therefore concluded that these cells were derived from glomerular epithelial cells. They had maintained a degree of terminal differentiation and as a result did not proliferate in culture.

**Proliferating cells**

More rapidly proliferating cells surrounded and overtook the glomerular epithelial cells after about two weeks in culture. They were variable in morphology but two main patterns of growth could be distinguished - those remaining as a monolayer and those that became overgrown in multilayered plaques.

**Monolayer-forming cells:**

A mixture of monolayer-forming cell types were produced. Some were large and formed a loose monolayer similar to that formed by glomerular epithelial cells (Figure 15.12). These cells did not stain with the monoclonal antibody against sialoglycoprotein (PHM5) (Figure 15.13) but did contain cytokeratin (Figure 15.14), indicating an epithelial origin. Their morphology was demonstrated further using phalloidin, a substance that binds to the filamentous actin in cellular microfilaments. These filaments were arranged as isolated lengths across the cell with a ring of filaments around the periphery (Figure 15.15). The cells were shown to be phagocytic by incubation with Dynabeads, 5µ in diameter (Figure 15.16).

It was concluded that these large monolayer cells were derived from glomerular epithelial cells that had dedifferentiated sufficiently to proliferate. In so doing, they no longer produced sialoglycoprotein but reverted to producing intracellular cytokeratin.

Monolayers of smaller, less differentiated cells were also produced. Some of these formed a pavement-like monolayer (Figure 15.17), similar to the morphology of endothelial cells cultured from umbilical veins (Figure 15.18). They did not bind the monoclonal antibody against factor VIII related antigen (von Willebrand factor) [321] or the lectin from Ulex europaeus [318] and could not be characterised further (see Chapter 16).
Figure 15.11 Frozen section of a normal glomerulus stained with the monoclonal antibody against cytokeratin (CK-1). Staining is only present on tubular epithelium and not on the glomerulus. x 400.

Figure 15.12 A monolayer of cells with similar morphological features to glomerular epithelial cells. Phase contrast microscopy x 200.
Figure 15.13  A monolayer of cells adjacent to a glomerulus that has not bound monoclonal antibody against sialoglycoprotein (PHM5). The glomerular epithelial cell attached to the glomerulus is strongly positive, staining red. x 400.

Figure 15.14  'Monolayer' cells stained with the monoclonal antibody against cytokeratin (CK-1). An intracellular lacework of cytokeratin is seen surrounding the nucleus. x 300.
Figure 15.15 'Monolayer' cells stained with TRITC-conjugated phalloidin and viewed by epifluorescence microscopy. Filamentous actin fibres are seen across the body of the cell, with a ring of filaments outlining the periphery. x 400.

Figure 15.16 Phagocytosis of Dynabeads by 'Monolayer' cells. Phase contrast microscopy x 200.
Figure 15.17 A pavement monolayer of cells grown from glomeruli. Phase contrast x 200.

Figure 15.18 Endothelial cells grown from an umbilical vein. Phase contrast x 100. (Courtesy of Dr. T. Horsburgh).
Cultures were sometimes 'contaminated' by tubular epithelial cells [322]. These had a distinctive sickle-shape morphology and tended to form 'domes' when confluent (Figure 15.19). 'Domes' are blisters, formed by the monolayer lifting off the underlying surface, which contain an electrolyte solution that has been transported across the epithelial monolayer [313]. The tubular epithelial cells also bound the lectin from lotus tetragonolobus that binds to L-fucose on tubular epithelium [315].

Figure 15.19 Dome formation by a tubular epithelial monolayer cultured from a preparation of isolated glomeruli. Phase contrast x 20.
Overgrowing 'mesangial' cells:-

After prolonged culture, most flasks became overgrown by rapidly proliferating elongated cells (Figure 15.20). It is generally believed that these are derived from mesangial cells [308] although this is not universally accepted [322] as no unequivocal markers are available. The cells resembled fibroblasts but were less elongated and did not form the typical pattern of fibroblasts on the cultured surface. They were also clearly derived from individual glomeruli. Studies of cell contraction were not attempted [308]. The cells did not phagocytose 0.5µ diameter latex beads nor did they bind antibodies against cytokeratin or sialoglycoprotein.

These cells were principally identified by their growth characteristics. They formed 'hillocks' composed of layers of cells [325]; they were resistant to trypsin, so that they became more dominant in later subcultures; and they showed increased proliferation in the presence of higher concentrations of serum, i.e. 20%.

The morphology of these cells was emphasised by staining for filamentous actin with phalloidin (Figure 15.21). Longitudinal filaments running the length of the cell were seen. The presence of these filaments, however, is not specific to a smooth muscle cell origin and is merely a morphological feature of many cultured cells [324].

Other markers used were unhelpful; antibodies against vimentin, fibronectin and class I MHC antigen bound to all morphological cell types and antibodies against desmin, leucocyte-common antigen and class II MHC antigen bound to none.
Figure 15.20 Outgrowth of elongated, overgrowing 'mesangial' cells from a glomerulus. Phase contrast x 200.

Figure 15.21 'Mesangial' cell stained with TRITC-conjugated phalloidin and viewed by epifluorescence microscopy. Actin filaments run the length of the cell in parallel criss-crossing bundles. x 400.
Macrophages

Small numbers of isolated cells, up to five per glomerular outgrowth, were characterised by their uptake of fluorescent-labelled acetylated LDL (see chapter 16) (Figure 15.2). They had a ruffled cell border and were seen both on the culture surface and on other cell monolayers. They contained prominent cytoplasmic granules. Similar numbers of cells in the outgrowth bound the monoclonal antibody against macrophages (PHM2). Double-labelling studies with diI-acetylated LDL were not performed, however.

Incubation of freshly isolated glomeruli with diI-acetylated LDL demonstrated uptake of fluorescence by monocytes, trapped within glomerular capillary lumina. It was therefore concluded that these cells converted into macrophages and migrated into the culture.

(Uptake of diI-acetylated LDL by other cells, both in vivo and in vitro, is detailed in Chapter 16.)

Conclusions

A number of conclusions can be drawn from the experience gained in glomerular cell culture. Firstly, cells can reliably be grown from glomeruli from all ages of patients and the supply of kidney tissue in a busy general hospital is adequate. Secondly, cells with features of all the cell types present in glomeruli in vivo can be cultured. However, these cells exist in a mixed growth and further work is required to separate successfully cells from different origins into separate cell strains. Furthermore, without specific cell markers, it would not be possible to confirm that such cell strains were not 'contaminated' with other cell types.

Finally, the results emphasise the considerable alteration in cell morphology and behaviour that occurs in the transition from in vivo to in vitro growth and this must be borne in mind when extrapolating from cell culture experiments to the whole animal.
Figure 15.22 A mixed cellular outgrowth from a glomerulus. The upper panel is viewed by phase-contrast microscopy. The lower panel is viewed under fluorescence microscopy and shows the uptake of dil-labelled acetylated LDL by one macrophage in the lower left corner. x 100.
CHAPTER 16
THE UPTAKE OF LIPOPROTEINS BY CULTURED HUMAN GLOMERULAR CELLS.

Introduction

As discussed in Chapter 1, cholesterol is taken up by cells via specific receptors that bind to apoproteins on the outside of lipoproteins. Once bound to the receptor, the lipoprotein is internalised by endocytosis and the cholesterol is liberated by lysosomal enzymes. A variety of lipoprotein receptors have been discovered [325]. The best known of these is the receptor for apoproteins B and E on LDL. This was described by Goldstein and Brown, who studied its behaviour on cultured human fibroblasts [15]. It has subsequently been demonstrated on a number of other cell types. Groene et al. have reported, in abstract form, the existence of receptors for apoproteins B and E on human glomerular epithelial cells. LDL receptors have also been reported on mesangial cells cultured from the rat [225] but not from humans.

As was also discussed in Chapter 1, macrophages and some endothelial cells express 'scavenger' receptors for chemically modified LDL. These are thought to be involved in the progressive uptake of cholesterol by foam cells. To investigate the pathogenesis of glomerular foam cells in man, it would be helpful to determine which cells express either or both of these two types of receptor. Such a study may also provide a further means of characterising cultured human glomerular cells.

The uptake of lipoproteins can be visualised by incorporating a fluorescent probe in the lipoprotein molecule. The probe of choice is 1,1'dioctadecyl 3,3,3'3' tetramethyl indocarbocyanine perchlorate (diII). This is easily incorporated into lipoprotein, does not readily transfer to cell membranes or other unlabelled lipoproteins once it has been incorporated and does not affect receptor binding activity [326]. Once inside the cell, diII is released from the lipoprotein by hydrolysis but is retained in cytoplasmic membrane. Hence cells taking up labelled lipoprotein
become increasingly fluorescent. The use of a qualitative, visual method for demonstrating lipoprotein uptake is particularly suited to mixed cultures since lipoprotein uptake can be directly compared with the morphology of individual cells. Dil can be incorporated into both native and chemically-modified LDL and can be used both \textit{in vitro} and \textit{in vivo} in animals.

The aim of the following experiments is to demonstrate receptor-mediated uptake of lipoprotein by cells cultured from human glomeruli. A number of criteria have to be fulfilled to prove that uptake is indeed receptor-mediated. It must be:--

a) specific, i.e. limited to one form of lipoprotein

b) saturable, i.e. the presence of excess unlabelled lipoprotein should competitively inhibit the binding of labelled lipoprotein to the receptors

c) of high affinity, i.e. uptake should occur with a low concentration of lipoprotein in the medium

d) blocked by known receptor antagonists

e) physiologically regulated

Experiments were therefore carried out to investigate each of these requirements.
Methods

Lipoprotein preparation

Dil-LDL was prepared by Miss Louise Lavender, Department of Clinical Chemistry and Metabolism, Glenfield General Hospital. A fraction of density >1.019 <1.063 containing LDL was prepared from pooled human serum by sequential flotation ultracentrifugation [327] using an MSE Europa 65 ultracentrifuge with a TST 41.14 swinging bucket rotor at 180,000g for 32 hours at 10°C. LDL was dialysed against 3 changes of 0.15M NaCl, 1 mM EDTA pH 7.4 in 50 times its volume, filter sterilised through a 0.22 micron filter and stored at 4°C under sterile conditions. Prior to use LDL was further dialysed against Dulbecco’s phosphate buffered saline (PBS) without calcium or magnesium in 10 times its volume, and filter sterilised.

Dil (Molecular Probes Inc., Oregon, USA.) was dissolved in dimethyl sulfoxide (3mg/ml) and incorporated into LDL by incubation at 37°C for 16 hours [328]. Fluorescent-labelled LDL was isolated by ultracentrifugation, washed by further ultracentrifugation, dialysed as above and stored in the dark at 4°C.

LDL protein content (2-3mg/ml) was measured by the method of Lowry et al. [329] and apoB content (>70% total protein) by immunoturbidimetric assay (Orion Diagnostica, Espoo, Finland). Agarose gel electrophoresis confirmed a pure preparation of LDL whose mobility was unchanged by dil incorporation (Figure 16.1).

Figure 16.1. Agarose gel electrophoretic strip of LDL stained with fat red 7B to demonstrate lipid. Lanes 1 and 3 contain 1µL of unlabelled LDL (2.2mg protein/ml). Lane 2 contains 1 µL of diI-labelled LDL (2.2mg protein/ml).
Lipoprotein deficient serum (LPDS) was prepared from a healthy male fasting donor by ultracentrifugation at 180,000g for 32 hours at 10°C \[t^{18}\]. Plasma of density >1.215 was dialysed as above and then incubated with thrombin (20iu/ml). The clot was removed with an applicator stick and the serum centrifuged at 20,000g for 1 hour at 4°C. Serum was dialysed against Dulbecco's PBS with calcium and magnesium in 10 times its volume, filter sterilised and stored at 4°C prior to use.

Preparation of diI-acetylated LDL

DiI-acetylated LDL was prepared by HCR as follows \[331\]. Equal volumes of LDL in 0.15 M NaCl were added to saturated sodium acetate solution with continuous stirring in an ice water bath. Acetic anhydride was then added at 1μl/5mins over 60 minutes, totalling 12μl, i.e. 1.5 x LDL protein mass. Continual stirring was performed during the addition and for a further 30 minutes thereafter. The resulting solution was dialysed against 12L of 0.15M NaCl, 0.3mM EDTA, pH7.4 buffer at 4°C for 36 hours. The solution was then millipore filtered through a 0.22μ filter.

Protein content was measured as above. Acetylation was confirmed by electrophoresis which showed the increased negative charge by moving the band towards the anode \[331\]. Acetylated LDL was labelled with diI as above.

Commercially produced diI-acetylated LDL was purchased from Biogenesis Ltd.

Uptake of diI-acetylated LDL \textit{in vivo}.

The biological activity of diI-acetylated LDL was tested in rats. One healthy intact rat and one made hypertensive and uremic by \(\frac{1}{3}\) nephrectomy 11 weeks previously were anaesthetised with an intraperitoneal anaesthetic (Chapter 18). The aorta was cannulated with pp10 polyethylene tubing with the tip of the catheter just below the left renal artery. 1.5ml and 0.8 ml of diI-acetylated LDL (270μg protein/ml) was injected into the aorta of the intact and nephrectomised rat respectively. After 20 and 10 minutes
respectively, the animal was sacrificed and organs frozen in liquid nitrogen. 5μ sections were cut and viewed by epifluorescence microscopy.

Uptake of diI-LDL and diI-acetylated LDL in vitro.

To demonstrate uptake of diI-LDL, cells were pre-incubated in RPMI with LPDS for 24 hours to deplete them of cholesterol and maximise receptor expression. The medium was then removed and diI-LDL (7.5μg/ml in RPMI) added through a 0.2μ filter. Incubation was continued for 4 hours at 37°C. The cells were then washed three times with PBS, fixed in 4% formaldehyde in saline for 30 minutes, washed with distilled water and mounted in glycerol gelatin.

To demonstrate endocytosis, cells were pre-incubated in RPMI with LPDS for 24 hours and then cooled to 4°C. The medium was then removed and pre-cooled diI-LDL added as above. Incubation was continued for 4 hours at 4°C to inhibit endocytosis. The cells were then washed 3 times with Hank's balanced salt solution (BSS) + 10mM Hepes at 4°C and either fixed directly at 4°C for 30 minutes and at room temperature for a further 30 minutes, or incubated with warm BSS at 37°C for 1, 3, 10 or 30 minutes before fixing and mounting as above.

To confirm that uptake was receptor-mediated, mixed cultures of cells were incubated at 4°C and 37°C with diI-LDL as above in the presence and absence of 100 fold excess unlabelled LDL and 10 mg/ml preservative-free sodium heparin. The specificity of uptake was confirmed by incubating mixed cultures with diI-acetylated LDL (10mg protein/ml) in RPMI at 4 and 37°C. The effect of increasing intracellular cholesterol concentration on the uptake of diI-LDL was determined by pre-incubating mesangial cells for 48 hours in the presence of 25μg/ml cholesterol + 1μg/ml 25-OH cholesterol dissolved in a final concentration of 2% ethanol in medium containing 20% LPDS. Control cultures were pre-incubated in 2% ethanol in 20% LPDS. The cells were then washed and diI-LDL added as above.

Slides were viewed using a Leitz Dialux 22 epifluorescence
microscope with a rhodamine excitation-emission filter and photographed using a Vario-Orthomat 2 camera with Fujichrome 400 ASA colour slide film or Ilford XP1 400 ASA black and white print film. A 63 x oil immersion fluorescence phase-contrast objective was required to visualise surface binding.

Uptake of diI LDL and diI-acetylated LDL was assessed qualitatively in a total of 19 and 14 separate experiments respectively.

The intensity of fluorescence of surface bound diI-LDL was insufficient to be measured in a conventional fluorimeter. However, incubation of cells at 37°C allowed measurable amounts of diI to be accumulated within cells. To measure uptake, cells were incubated with diI-LDL, washed as above and suspended with trypsin. The trypsin activity was quenched with an equal volume of medium containing 10% foetal calf serum and the suspended cells washed three times in PBS by centrifugation. Fluorescence activity was measured in a Perkin-Elmer fluorescence spectrophotometer 204-A (520nm excitation wavelength, 550nm blocking filter), and activity corrected for background light scattering using a cell-suspension without added diI-LDL. Cell protein content was measured as above. Cultures were performed in quadruplicate and protein estimations in duplicate. Since the expression of LDL receptors on fibroblasts can vary by two to three fold in the same cell strain when studied on different days with different batches of serum [Goldstein, Basu, Brown, 1983] comparison of results was restricted to individual experiments.

Results

Uptake of diI-LDL.

DiI-labelled LDL was accumulated by all cell types when incubated at 37°C (Figures 16.2 - 16.4). Uptake was not uniform throughout the mixed cell growth (Figure 16.3). Differentiated glomerular epithelial cells took up diI-LDL less avidly than mesangial cells (Figure 16.4).
Figure 16.2. Uptake of diILDL by a differentiated glomerular epithelial cell. Same field viewed by phase-contrast (upper panel) and fluorescence microscopy (lower panel), original magnification x 280. Cells were incubated with diILDL (7.5µg protein/ml) for 4 hours at 37°C.
Figure 16.3. Uptake of diI-LDL by mesangial cells. Same field viewed by phase-contrast (upper panel) and fluorescence microscopy (lower panel), original magnification x 70. Cells were incubated with diI-LDL (7.5μg protein/ml) for 4 hours at 37°C. Note the variation in uptake of diI-LDL over the field, with those closest to the glomerulus showing the brightest activity.
Figure 16.4. Uptake of diI-LDL by a mixed growth of differentiated glomerular epithelial cells adjacent to the glomerulus, surrounded by mesangial cells. Same field viewed by phase-contrast (upper panel) and fluorescence microscopy (lower panel), original magnification x 175. Cells were incubated with diI-LDL (7.5 μg protein/ml) for 4 hours at 37°C. Note the much greater uptake of diI-LDL by mesangial cells than by epithelial cells.
Uptake was inhibited by incubation with 100 fold excess unlabelled LDL and 10mg/ml sodium heparin in all cell types when examined by fluorescence microscopy. This was confirmed by measuring the uptake fluorimetrically (Table 16.1).

**Table 16.1.**

Inhibition of uptake of diI-LDL in a mixed population of cells by co-incubation with excess unlabelled LDL and sodium heparin.

<table>
<thead>
<tr>
<th>DiI-LDL (µg/ml)</th>
<th>Inhibitor</th>
<th>Fluorescence activity (U/100µg protein/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i 0</td>
<td>Nil</td>
<td>16.8 ± 0.3 a</td>
</tr>
<tr>
<td>ii 7.5</td>
<td>Nil</td>
<td>83.5 ± 7.4</td>
</tr>
<tr>
<td>iii 7.5</td>
<td>LDL 750µg/ml</td>
<td>19.8 ± 0.9 ab</td>
</tr>
<tr>
<td>iv 7.5</td>
<td>Heparin 10mg/ml</td>
<td>22.0 ± 1.3 ab</td>
</tr>
</tbody>
</table>

a p<0.01 v ii, b p<0.05 v i, Student's t-test

Preincubation of mesangial cells with cholesterol and 25-OH cholesterol to increase intracellular cholesterol content also completely inhibited uptake of diI-LDL (Table 16.2).

**Table 16.2.**

Inhibition of uptake of diI-LDL in mesangial cells by preincubation for 48 hours with free cholesterol and hydroxycholesterol. All preincubation media contained 2% ethanol.

<table>
<thead>
<tr>
<th>Pre-incubation</th>
<th>Incubation diI-LDL (µg/ml)</th>
<th>Fluorescence activity (U/100µg protein /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i 20% FCS</td>
<td>0</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>ii 20% FCS</td>
<td>7.5</td>
<td>11.7 ± 1.7 a</td>
</tr>
<tr>
<td>iii 20% LPDS</td>
<td>7.5</td>
<td>12.4 ± 1.7</td>
</tr>
<tr>
<td>iv 20% LPDS + 20µg/ml cholesterol</td>
<td>7.5</td>
<td>5.8 ± 1.9 b</td>
</tr>
<tr>
<td></td>
<td>+ 1µg/ml 25-OH cholesterol</td>
<td></td>
</tr>
</tbody>
</table>

a p<0.05 v i, b p<0.05 v iii, Student's t-test.
Following incubation at 4°C, diI-labelled LDL was bound to the surface of all cell types. Binding to the small numbers of macrophages could not be studied separately since they required fluorescent labelling to be identified. DiI-LDL binding was localised to discrete areas of the cell surface. On epithelial cells these were distributed in a random pattern (Figure 16.5). On some mesangial cells the binding sites were arranged in linear arrays (Figure 16.6), corresponding to arrangement of intracellular stress fibres visible by phase-contrast microscopy and demonstrated by phalloidin. Surface binding was enhanced after preincubation in 10% lipoprotein deficient serum, in comparison to foetal calf serum. No surface binding of diI-LDL was detectable when cells were incubated in the presence of 100-fold excess unlabelled LDL and was greatly reduced in the presence of 10mg/ml sodium heparin.
Figure 16.5. Binding of diI-LDL to a monolayer of glomerular epithelial cells. Same field viewed by phase contrast (upper panel) and fluorescence microscopy (lower panel), original magnification x 900. Cells were incubated with diI-LDL for 4 hours at 4°C and fixed directly at 4°C. A random array of fluorescent dots is seen. Non-specific fluorescence due to cellular organelles is present around the nuclei.
Figure 16.6. Linear arrays of surface-bound diI-LDL on a mesangial cell. Same field viewed by phase-contrast (upper panel) and fluorescence microscopy (middle panel), original magnification x 900. Cells were incubated with diI-LDL (7.5μg protein/ml) for 4 hours at 4°C and fixed directly at 4°C. The linear arrays are seen to correspond to the longitudinal stress fibres visible on the phase-contrast photomicrograph. A different culture of mesangial cells stained with TRITC-labelled phalloidin (lower panel) demonstrates the stress fibres containing filamentous actin.
The endocytosis of receptor-bound LDL was visualised by incubating cells with diI-LDL at 4°C, washing off unbound diI-LDL and then incubating the cells at 37°C for increasing lengths of time (Figure 16.7). After incubation at 4°C and fixing directly, surface bound diI-LDL was seen (Figure 16.7A). After incubating at 37°C for one minute the fluorescent pattern was unchanged (not shown). After three minutes the fluorescence dots had aggregated to form a smaller number of larger, brighter dots (Figure 16.7B). A similar pattern persisted at ten minutes (Figure 16.7C). After 30 minutes (Figure 16.7D), the number of fluorescent dots was much reduced and diffuse intracellular fluorescence was present, outlining the nucleus, similar to the pattern seen after incubation at 37°C for 4 hours (Figure 16.4).
Figure 16.7. Endocytosis of surface-bound diI-LDL by mesangial cells. Fluorescence microscopy, original magnification x 800. Cells were incubated with diI-LDL (7.5μg protein/ml) for 4 hours at 4°C, washed and then either fixed directly at 4°C (A) or further incubated at 37°C for 3 minutes (B), 10 minutes (C) or 30 minutes (D). Note the progressive internalisation and aggregation of fluorescence activity with the formation of a fluorescent halo around the nucleus after 30 minutes.
Uptake of diI-acetylated LDL.

Uptake of diI-acetylated LDL by rats in vivo followed the pattern previously described [27]. Fluorescence was confined to the sinusoids of the liver (Figure 16.8) and spleen with sparse uptake by the adrenal gland. No uptake was detected in the kidneys of the intact or nephrectomised rat.

Incubation of mixed cell cultures with diI-acetylated LDL demonstrated uptake by small numbers of macrophages as described in Chapter 15. Differentiated epithelial and mesangial cells did not take up diI-acetylated LDL. Some areas of cell outgrowth also took up diI-acetylated LDL but in lesser amounts than macrophages (Figure 16.9). These areas had variable morphologies, usually but not exclusively forming monolayers. Endothelial cells cultured from an umbilical vein took up similar amounts of diI-acetylated LDL. Cells grown from glomeruli did not bind the monoclonal antibody against factor VIII related antigen it could not be confirmed that they were endothelial cells.

Uptake of diI-acetylated LDL was quantified in a preliminary experiment. Uptake was partly inhibited by the presence of excess unlabelled acetylated LDL but was not affected by incubation with fucoidin, an inhibitor of the scavenger receptor [26] (Table 16.3).

Table 16.3 Inhibition of the uptake of diI-AcLDL by a mixed population of cells by excess unlabelled Ac-LDL and fucoidin.

<table>
<thead>
<tr>
<th>DiI-acLDL (U/100µg protein/ml)</th>
<th>Inhibitor</th>
<th>Fluorescence activity (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Nil</td>
<td>5.7</td>
</tr>
<tr>
<td>10</td>
<td>Nil</td>
<td>28.5</td>
</tr>
<tr>
<td>10</td>
<td>AcLDL 200µg/ml</td>
<td>15.5</td>
</tr>
<tr>
<td>10</td>
<td>Fucoidin 1mg/ml</td>
<td>27.5</td>
</tr>
</tbody>
</table>

Data = mean of duplicate experiments.
Figure 16.8. Uptake of diI-acetylated LDL by liver sinusoidal endothelium. Upper panel = phase contrast; lower panel = fluorescence microscopy. Original magnification x 600.
Figure 16.9. Uptake of diI-acetylated LDL by an area of glomerular cell outgrowth. Upper panel = phase contrast; lower panel = fluorescence microscopy, original magnification x 175. Cells were incubated with diI-acetylated LDL (10μg protein/ml) for 4 hours at 37°C.
Discussion

These results have demonstrated a number of features of the uptake of LDL by cultured human glomerular cells.

Binding and uptake were specific for LDL, since diI-acetylated LDL was not bound or taken up by epithelial or mesangial cells. The process was saturable since binding and uptake of diI-LDL was inhibited by excess unlabelled LDL, which competes for binding to the finite number of receptors. Binding was of high affinity, being demonstrated at an LDL concentration of 7.5μg protein/ml, equivalent to a cholesterol concentration of approximately 10^{-5}M. Binding and uptake were inhibited by heparin, which blocks the positively charged site on apoprotein B that is required for receptor binding [332].

Uptake varied in intensity in different areas of the cell outgrowth, with more rapidly proliferating mesangial cells taking up greater amounts of diI-LDL than epithelial cells. Glomerular epithelial cells have previously been shown to have a lower LDL receptor activity as compared to fibroblasts [305]. Since proliferating cells use cholesterol to synthesise new membranes, this is consistent with the uptake of diI-LDL being regulated according to the cells' requirements for cholesterol. In fibroblasts, this feedback effect is governed by the level of intracellular cholesterol [19] and a similar effect was demonstrated in mesangial cells by preincubation with cholesterol (Table 16.2).

Finally, the pattern of surface binding and the time course of internalisation of diI-LDL were typical of receptor-mediated endocytosis, as previously demonstrated in cultured human fibroblasts [333]. Surface binding was restricted to small areas of the plasma membrane, consistent with the receptors being localised in coated pits. On some mesangial cells these were arranged in linear arrays. A similar pattern is seen on fibroblasts and corresponds to the longitudinal stress fibres present in both cell types [334]. Aggregation of bound diI-LDL had occurred after incubation at 37°C for three minutes, consistent with the formation
of endocytotic vesicles which then fuse with lysosomes. After 30 minutes, few fluorescent dots remained and diffuse fluorescence was present around the nucleus, consistent with breakdown of the diI-LDL complex by lysosomal enzymes and the retention of lipid-soluble diI in cytoplasmic membranes.

It is therefore possible to conclude that cultured human glomerular epithelial and mesangial cells do express receptors for LDL and take up this lipoprotein by receptor-mediated endocytosis.

Uptake of acetylated LDL by rats in vivo had the same distribution as previously described [27]. The failure to demonstrate uptake by bone marrow-derived mesangial cells in the kidney may be due to the rapid clearance of acetylated LDL by the liver and spleen.

In vitro, uptake by macrophages was particularly avid. Cells with a morphology similar to endothelial cells cultured from umbilical veins took up diI-acetylated LDL with less avidity. However, the preliminary quantitative experiment showed only partial competitive inhibition by excess unlabelled acetylated LDL and no inhibition at all by the scavenger receptor blocker fucoidin. The dose of fucoidin used was 100x the 50% inhibitory concentration for macrophages [26]. This suggests that uptake of acetylated LDL was mainly occurring by non-specific phagocytosis rather than by scavenger receptors. Further work is required to confirm these results.

On no occasion did typical overgrowths of mesangial cells take up diI-acetylated LDL, indicating that they do not express scavenger receptors.

In summary, it can be concluded that all cell types derived from human glomeruli are able to express apoB/E LDL receptors when grown in culture. Scavenger receptors are apparently restricted to macrophages, although it is possible that some are expressed by culture glomerular endothelial cells.
APPENDIX 1
CHEMICALS USED IN GLOMERULAR CELL CULTURE.

NBL = Northumbria Biologicals, South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL.
Flow = Flow Laboratories Ltd., Woodcock Hill, Harefield Road, Rickmansworth, Herts. WD3 1PQ.
Sigma = Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset BH17 7NH.

<table>
<thead>
<tr>
<th>RPMI 1640</th>
<th>NBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal calf serum</td>
<td>NBL</td>
</tr>
<tr>
<td>Penicillin 5000 Units/ml</td>
<td>NBL</td>
</tr>
<tr>
<td>Streptomycin 5000µg/ml</td>
<td>NBL</td>
</tr>
<tr>
<td>Glutamine 200 mM</td>
<td>NBL</td>
</tr>
<tr>
<td>33'5 Tri Iodo-L-Thyronine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Sigma</td>
</tr>
<tr>
<td>Insulin-transferrin-Na Selenite</td>
<td>Sigma</td>
</tr>
<tr>
<td>media supplement</td>
<td>Sigma</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>Sigma</td>
</tr>
<tr>
<td>NuSerum IV</td>
<td>Flow</td>
</tr>
<tr>
<td>Trypsin (100 mM)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hepes 1M</td>
<td>NBL</td>
</tr>
<tr>
<td>Sodium Bicarbonate (7.5%V/W)</td>
<td>NBL</td>
</tr>
<tr>
<td>Sodium hydroxide 2M</td>
<td>NBL</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hanks BSS</td>
<td>NBL</td>
</tr>
</tbody>
</table>
# APPENDIX 2

## EQUIPMENT USED IN GLOMERULAR CELL CULTURE

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steel sieve</td>
<td>Boots The Chemist Ltd.</td>
</tr>
<tr>
<td>(domestic steel tea strainer)</td>
<td>Nottingham.</td>
</tr>
<tr>
<td>Nylon mesh</td>
<td>Henry Simon Ltd., Special Products Division, PO Box 31,</td>
</tr>
<tr>
<td>(monofilament nylon bolting cloth</td>
<td>Stockport, Cheshire SK3 ORT.</td>
</tr>
<tr>
<td>of various aperture sizes)</td>
<td></td>
</tr>
<tr>
<td>Disposable cell culture ware</td>
<td>Nunc., dist. by Gibco Ltd.,</td>
</tr>
<tr>
<td>50 ml plastic tissue culture flasks</td>
<td>Unit 4, Cowley Mill Trading Est., Longbridge Way,</td>
</tr>
<tr>
<td></td>
<td>Uxbridge, Middx. UB8 2YG.</td>
</tr>
<tr>
<td>Chamber slides</td>
<td>Miles Lab. Inc., dist. by ICN Biomedicals Ltd.,</td>
</tr>
<tr>
<td>Labtek 4 well glass chamber slides</td>
<td>Free Press House, Castle Street, High Wycombe, Bucks.</td>
</tr>
<tr>
<td></td>
<td>HP13 6RN.</td>
</tr>
<tr>
<td>Misc. plastic disposable ware</td>
<td>Bulkstore, Univ. of Leics.</td>
</tr>
<tr>
<td>Petriperm dishes (hydrophilic)</td>
<td>Heraeus Equipment Ltd., Unit 9, Wates Way, Brentwood,</td>
</tr>
<tr>
<td>CM15 9TB.</td>
<td>Essex,</td>
</tr>
<tr>
<td>Incubators</td>
<td>Laboratory &amp; Electrical Engineering Co., Private Road</td>
</tr>
<tr>
<td>LEEC GA3 Automatic CO2 incubator</td>
<td>No. 7, Colwick Industrial Estate, Nottingham NG4 2AJ.</td>
</tr>
<tr>
<td>Flow Automatic CO2 incubator</td>
<td>Flow Laboratories Ltd., Hill, Harefield Road,</td>
</tr>
<tr>
<td>Woodcock</td>
<td>Rickmansworth, Herts. WD3 1PQ.</td>
</tr>
</tbody>
</table>
Laminar Flow Hood
Envair Class II
Microbiological safety cabinet

Envair Ltd., York Avenue,
Haslingden, Rossendale,
Lancs. BB4 4HX.

Centrifuge
Heraeus Christ Bactifuge

VA Howe, 12-14 St. Anne's Crescent, London, SW18 2LS.

Cytospin centrifuge
Cytospin 2

Shandon Southern Products Ltd.,
Chadwick Road, Astmoor, Runcorn,
Cheshire WA17 1PR.

Microscope slides and cover slips

Bulkstore, Univ. of Leicester.

Glycerol gelatin mountant

Sigma Chemical Co. Ltd.

Olympus CK2 phase contrast
invert microscope
PM-10AK automatic exposure camera

Olympus Optical Co. (UK) Ltd.,
2-8 Honduras Street,
London EC1Y OTX.

Leitz Dialux 22
microscope

E. Leitz (Instruments) Ltd.,
48 Park Street, Luton, LU1 3HP.

Leitz 3 lambda ploemak incident light fluorescence unit
Leitz Vario orthomat 2 automatic microscope camera

Microscope objectives:-

- NPL Phaco 10/0.25. 519250
- NPL FL Phaco 25/0.65. 519506
- NPL FL Phaco 40/0.70. 519507
- Fluorescence Phaco Oil 63/1.30 519476
- NPL FL 100/1.32 Oil 519504

Microscope immersion oil
Pan-scan Xtra

Hughes & Hughes Ltd.,
Harlow Wood, Romford, Essex.
Photographic film

Colour slide film: Fuji 400 ASA daylight, Fuji 64 ASA tungsten.
Black and white print film: Ilford XP1 400 ASA, Ilford Pan F 50 ASA,
Kodak T-max 1600 ASA.

Fluorescence spectrophotometer
Perkin-Elmer fluorescence
spectrophotometer No. 204-A.

Ultraviolet spectrophotometer
Pye Unicam SP 1700
APPENDIX 3
METHODS USED IN CELL CULTURE EXPERIMENTS.

Trypsinisation of adherent cell lines
1. Pour off spent medium.
2. Add 5 ml of Hanks BSS without calcium or magnesium.
3. Add 0.5 ml of 100 mM trypsin.
4. Incubate at 37°C for 10 minutes.
5. View cells under phase contrast, aiding detachment by sharp taps to the side of the flask.
6. Add 5 ml of RPMI + 10% foetal calf serum to quench trypsin activity.
7. Aspirate and transfer to universal container.
8. Centrifuge at 1000 rpm for 10 minutes.
9. Pour off supernatant and re-suspend cell pellet in complete culture medium.
10. Transfer cell suspension to new culture vessel as required.
Cryopreservation of cell lines.
1. Make up medium containing RPMI + 10% foetal calf serum + 25 mM hepes + 20% DMSO.
2. Cool medium on ice.
3. Suspend cells by trypsinisation and centrifuge.
4. Pour off supernatant leaving 0.2 mls of medium + cell pellet.
5. Add 0.25 mls of neat foetal calf serum and cool on ice.
7. Add 0.5 mls of DMSO medium to each centrifuge tube and transfer suspension to ampoule.
8. Store ampoule at -70°C overnight.
9. Transfer ampoule to liquid nitrogen store.

Recovery of cryopreserved cell lines
1. Remove ampoule from liquid nitrogen and store on ice.
2. Thaw at 37°C in water bath.
3. Store thawed ampoule on ice to reduce DMSO toxicity.
4. Transfer cells to a conical tube and add 10 mls of RPMI + 10% foetal calf serum medium to wash.
5. Centrifuge at 1000 rpm for 10 minutes.
6. Pour off medium and re-suspend in complete culture medium.
7. Transfer to culture vessel.
**Vital staining method**

1. Dissolve a few μg of fluorescein diacetate in acetone.
2. Add a few drops of the solution to 20ml BSS until the mixture just becomes cloudy.
3. Add a few drops of the mixture to the cell suspension.
4. Incubate for 5 minutes at room temperature.
5. Wash cells by aspirating supernatant and replacing with BSS.
6. Add 20 μl of a 1 mg/ml solution of propidium iodide in water.
7. View under a fluorescence microscope with fluorescein and rhodamine filters.

Results: – live cells stain green with fluorescein diacetate, 
dead cell nuclei stain red with propidium iodide.
Staining of filamentous actin with phalloidin.

1. Fix cells or frozen section in acetone or 10% formalin.
2. Dissolve 0.1mg phalloidin (TRITC or FITC conjugated) in 2ml 1% DMSO in PBS = 50µg/ml final concentration.

N.B. Highly toxic material. Should be handled in a fume cupboard using protective clothing. Contaminated waste to be inactivated in bleach.

3. Incubate cells with phalloidin solution in humid box for 30 minutes at room temperature.
4. Wash in PBS.
5. Mount in glycerol gelatin.
6. View using fluorescein filter (FITC) or rhodamine filter (TRITC).

Staining with lectins

1. Fix cells or frozen section in 10% formalin.
2. Incubate with lectin (1:5 to 1:10 dilution in PBS) for 30 mins at room temperature.
3. Wash in distilled water.
4. Mount in DPX.
**Measurement of Cell Protein Content.**

Protein was measured by the method of Lowry [329].

**Reagent A**  
2% Na$_2$Co$_3$ in 0.1 m NaOH

**Reagent B**  
0.5% CuSO$_4$ in 1% Na/K Tartrate

**Reagent C**  
50ml of A/1.0 ml of B

**Reagent D**  
Folin & Ciocalteu's phenol reagent/water 1:1

Make up a standard curve with crystalline BSA 0-700 μg/ml in duplicate. Add 1ml reagent C to 100 μl of standards and samples at 20 second intervals, vortex mix and stand for 20 mins. At 20 mins add 100μl of reagent D, vortex mix and leave to stand for 30 mins. Measure absorbance on a spectrophotometer at 710 nm and calculate the protein concentration of samples from the standard curve.
SECTION III
ANIMAL EXPERIMENTS
CHAPTER 17
THE RAT SUBTOTAL NEPHRECTOMY MODEL OF CHRONIC RENAL FAILURE.

The following four experiments study aspects of lipid metabolism and glomerular damage in rats following subtotal nephrectomy. Much work has been done by other investigators using this model and a number of factors have been shown to affect its natural history. These need to be taken into consideration when designing and interpreting experiments that involve subtotal nephrectomy.

In 1889, Tuffier first reported the effects of subtotal nephrectomy in dogs. Although he noted renal hypertrophy, he found no change in the elimination of urine or urea. In 1899, Bradford published the results of a series of experiments studying the effects of the removal of progressively greater amounts of renal tissue in dogs [337]. He found that the animals became hypertensive, polyuric and uremic and concluded that the tissues of the body, particularly the muscles, broke down when kidney tissue was markedly reduced. Subsequently, a number of workers performed similar operations on a range of species and produced various combinations of arterial hypertension, cardiac hypertrophy and renal insufficiency. This early work was reviewed in 1932 by Chanutin and Ferris, who were the first to study systematically the effects of partial nephrectomy in the rat [338]. They performed a two-stage procedure, initially ligating the upper and lower poles of the left kidney and then, one week later, removing the whole of the right kidney. The animals developed polyuria, albuminuria, nitrogen retention, renal and cardiac hypertrophy and hypertension. Subsequently investigators have studied similar models [339,3,199].

Factors affecting the natural history of the subtotal nephrectomy model.
Systemic hypertension and glomerular hyperfiltration.

Subtotal nephrectomy in the rat results in systemic hypertension and increases in the glomerular plasma flow rate, the single nephron glomerular filtration rate and the pressure gradient across the glomerular capillary wall in the remaining nephrons [340]. Selective
reduction in glomerular damage, without alteration of the increased glomerular capillary pressure [341], reduces the systemic hypertension, indicating that it is partly a secondary effect of renal injury. Conversely, pharmacological control of systemic hypertension [342], particularly with agents that reduce the transcapillary pressure gradient [343,344], significantly retards the development of glomerular damage and prevents its progression [345]. Largely through the work of Brenner et al., the hypothesis that glomerular hyperfiltration is the major mechanism underlying the progression of glomerulosclerosis has been widely supported [346,2]. Although undeniably important, recent evidence has suggested that it may not be the sole mechanism of injury in the subtotal nephrectomy model. Studies by O'Donnell [347] and Okuda [348] have demonstrated increased severity of proteinuria and glomerulosclerosis, with greater compensatory renal growth, in immature rats compared to mature animals. Glomerular capillary pressure and the transcapillary pressure gradient were not different in the immature animals. These results suggest that there is a link between renal growth and glomerulosclerosis, independent of haemodynamic factors.

Yoshida et al. [349] have demonstrated a correlation between glomerular hypertrophy and subsequent sclerosis. They compared the effects of 2/3 unilateral renal infarction combined with either contralateral nephrectomy or contralateral ureteric diversion into the peritoneal cavity. Both groups of animals developed the same degree of glomerular hyperfiltration, measured by micropuncture study. However, only the group which underwent contralateral nephrectomy developed glomerular hypertrophy and sclerosis. In a separate study [350] they performed serial micropuncture studies of individual glomeruli. No correlation was found between the severity of sclerosis and capillary pressure or single nephron filtration rate in the same glomerulus. They concluded that hypertrophy appeared to be an important step preceding sclerosis but that glomerular hyperfiltration alone was insufficient to induce sclerosis.

Other experiments involving heparin therapy [341] inhibition of thromboxane synthesis [351] and lipid-lowering drugs [259], have also
demonstrated effects on glomerulosclerosis without detectable alterations in glomerular haemodynamics.

Protein intake

The quality and quantity of protein in the diet have marked effects on glomerular damage following subtotal nephrectomy. Numerous investigators have demonstrated a deleterious effect of a high protein diet [199] and Williams and Walls recently showed that animal protein (casein) was more harmful than vegetable protein (soya) [257]. It has been suggested that the deleterious effect of the high protein diet is mediated through an increase in glomerular filtration and flow rates [2]. A reduction in dietary protein intake has been widely advocated as a means of reducing hyperfiltration and preserving renal function in patients with chronic renal failure [2].

Amino acid intake

Dietary supplementation with tryptophan given immediately following 7/8 nephrectomy has been shown to reduce albuminuria but not to improve creatinine clearance or reduce glomerular damage. This effect on proteinuria was not found if the tryptophan was only administered when proteinuria had already developed [352] suggesting that it had an effect on the initiation of glomerular injury but not on its progression.

Carbohydrate and calorie intake

An beneficial effect of calorie and carbohydrate restriction on glomerular injury, independent of protein intake, was suggested by a study of intact rats by Bras and Ross in 1964 [353]. In a recent preliminary report, Tapp et al. [354] have studied the effect of a 40% reduction in sodium and calorie intake. Proteinuria and glomerulosclerosis were reduced further by sodium and calorie restriction than by sodium restriction alone, when compared to animals fed a control 21% protein diet.
Calcium and phosphate

Administration of a phosphate binder to pair-fed rats 30 days after 5/6 nephrectomy, led to a reduction in serum phosphate and a rise in serum calcium [355]. This was associated with a reduction in polyuria, proteinuria, hypercholesterolaemia, tissue calcium content and glomerular damage. The mechanism of this protective effect is not known.

Polyunsaturated fatty acids and prostaglandins

As described in Chapter 11, dietary supplementation with polyunsaturated fatty acids has been found to cause variable effects on glomerular damage. The effects are thought to be mediated via alterations in the balance of prostaglandins, which have important effects on systemic and glomerular haemodynamics [251]. For example, inhibition of the synthesis of thromboxane, a vasoconstrictor and platelet-aggregatory prostaglandin, has been shown to ameliorate renal damage [351].

Anticoagulation

Both Olson [238] and Purkerson [356,239] have demonstrated a protective effect of heparin in the subtotal nephrectomy model. In a recent study from the latter group [341] this effect was shown to occur without an alteration in glomerular haemodynamics. Purkerson has demonstrated that a non-anticoagulant fraction of heparin also reduces glomerular injury following nephrectomy [357]. The effect of heparin therefore may not solely be related to its anticoagulant action.

Genetic factors

There is a wide variation in the susceptibility of rat strains to the development of glomerulosclerosis following subtotal nephrectomy [358]. The reason for these differences is not known. There is also a difference in susceptibility between the sexes, with male rats developing worse lesions and more proteinuria than females [358,359]. Whether these differences are due to differences in sex hormones, food intake or glomerular haemodynamics is unclear. The vulnerability of
male rats is reflected in humans, where a majority of adults with focal glomerulosclerosis are male [358]. Interestingly, this does not apply to children [358].
CHAPTER 18
OPERATIVE METHODS FOR SUBTOTAL NEPHRECTOMY AND UNINEPHRECTOMY.

The operative techniques employed in these experiments were as follows:-

Animals were fed ad libitum prior to surgery. Anaesthesia for subtotal nephrectomy was induced by an i.p. injection of 2.7 ml/kg of a 2:1:1 mixture by volume of sterile water, midazolam (5mgs/ml) and Hypnorm (fentanyl 0.315 mgs/ml, fluanisone 10mgs/ml). For uninephrectomy an inhalational anaesthetic of halothane and oxygen delivered by a Fluotec anaesthetic machine was used. The gas was administered through a nose cone and waste gases were scavenged by a Fluovac and Fluosorber. The abdomen was shaved and the animal secured by autoclave tape to a heated pad.

A laparotomy was performed through a midline incision. For subtotal nephrectomy, the left kidney was exposed and the renal capsule incised at the pelvis. Using blunt dissection with a cotton bud, the renal artery was exposed. A branch of the renal artery usually crossed the renal vein anteriorly at the hilum. This branch was cleaned, lifted with watchmaker's forceps and a 5/0 prolene ligature passed between it and the renal vein. The branch was tied off and the amount of left kidney infarcted was assessed. Other branches were tied as necessary to achieve infarction of 1/3 of the kidney. The right kidney was then exposed and its capsule incised and retracted to preserve the adrenal gland. The hilar vessels and ureter were then tied off together with a 3/0 mersilk ligature and the kidney removed. Haemostatis was ensured and any clots removed from the abdomen. The abdomen was closed with 3/0 mersilk using interrupted stitches to the muscle layer and a subcuticular continuous stitch to the skin.

For unilateral nephrectomy, the right kidney only was mobilised and removed as described above.

For the sham subtotal nephrectomy, both kidneys were exposed as above and cleaned of perinephric fat with a cotton bud.
The intraperitoneal anaesthetic lasted approximately 45 minutes. To retain body heat postoperatively, the unconscious animals were wrapped in silver foil. The inhalational anaesthetic was reversed within minutes and animals were therefore returned directly to their cages.

No perioperative mortality due to surgical error was sustained. Occasional small wound abscesses were detected at the site of sutures but these were drained without further problem. Two animals suffered small ventral hernias through the muscle layer where a suture had loosened. Again, these were not clinically significant.
CHAPTER 19
THE EFFECT OF HOUSING ANIMALS IN METABOLIC CAGES ON FOOD INTAKE.

In order to collect urine samples and to measure food intake accurately, animals must be housed in metabolic cages. These have a metal grill at the base rather than sawdust and have a smaller surface area. Housing rats in these cages may have a stressful effect on the animals and so may alter the variables that are to be measured. To determine whether a period of acclimatisation would provide more representative measurements, food intake and urine output were measured prior to and during a prolonged period of housing in metabolic cages.

Methods
11 sham operated rats housed in individual cages with sawdust bedding were stabilised on a 24% casein diet. They were offered 25g of dry food daily mixed to a paste with 35g water. Daily food intake was measured by subtracting the amount of food paste remaining. Animals were weighed at intervals. At day 0, animals were transferred to individual metabolic cages and continuous 24 hour urine collections were performed for 6 days. Food intake was recorded daily and body weight measured at intervals.

Results
A marked variation in mean daily food intake was found in rats despite being stabilised on the 24% casein diet and acclimatised to normal individual cages (Figure 19.1). Transfer to the metabolic cages caused a slight increase in food intake but this was not outside the normal variation. Body weight did not change significantly throughout the study period. There was no difference in mean daily urine volume during the period in the metabolic cages (Table 19.1).
Figure 19.1. The effect of prolonged housing in metabolic cages on food intake.
Table 19.1. The effect of prolonged housing in metabolic cages on urine output.

<table>
<thead>
<tr>
<th>Day in metabolic cage</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume (ml)</td>
<td>19.2±1.2</td>
<td>16.4±1.2</td>
<td>16.6±1.2</td>
<td>17.2±1.8</td>
<td>19.1±3.1</td>
<td>20.6±3.7</td>
<td>24.7±2.9</td>
</tr>
</tbody>
</table>
Discussion

These results suggest that measurements of food intake during a single 24 hour period in a metabolic cage provide a reliable measure of daily food consumption in non-uraemic animals. Housing animals for more prolonged periods in these cages to allow them to acclimatise does not appear to alter the results and is more inconvenient. This experiment was not repeated with uraemic animals but it is assumed that similar results would have been obtained.
CHAPTER 20
VALIDATION OF INDIRECT ARTERIAL BLOOD PRESSURE MEASUREMENT.

In order to perform repeated measurements of arterial blood pressure in the rat, a non-invasive indirect technique must be used. The method used in these experiments is that developed by Swales and Tange [360]. This technique involves the detection by a photocell of the small differences in light translucency of the tail during systole and diastole. These changes are displayed on an oscilloscope. The tail artery is occluded by inflating a cuff proximal to the photocell which is then deflated by approximately 2mmHg per second. When the deflections first return on the oscilloscope, a simultaneous reading is taken from the mercury manometer attached to the tail cuff.

To ensure that the indirect technique gives valid measurements, direct measurements of intra-arterial pressure were compared with simultaneous readings taken using the tail cuff technique.

In order to obtain a stable trace, the animal must be anaesthetised with ether to abolish muscle movements. Preliminary experiments were therefore also performed to determine the effect of ether on intra-arterial pressure.

**Methods**

4 rats rendered hypertensive by previous subtotal nephrectomy and 4 sham-operated normotensive rats were used in these studies. Animals were anaesthetised using a halothane/oxygen mixture delivered by a Fluotec anaesthetic machine, with waste gases scavenged from a nose cone using a Fluovac and Fluosorber scavenging system (appendix 1). The skin over the back and neck of the animal was shaved and the left carotid artery exposed through a small incision. A subcutaneous tunnel was created through to the back of the neck using a wide gauge needle and a length of PP50 polypropylene tubing, protected by a 3 mm diameter spring steel sheath, was passed through the needle. The catheter was flushed with heparin saline (1U/ml). Two sutures were passed beneath the carotid artery and the distal one tied off. The vessel was occluded by retraction on the proximal tie, a hole made between the sutures and held open with watchmaker's forceps and the artery
cannulated. The catheter was secured with the proximal tie and the skin closed. The catheter was connected by a Clay-Adams connector via a 3-way tap to a blood pressure transducer and oscillograph. The animal recovered consciousness by breathing oxygen alone and was then housed unrestrained in a metal cage.

After a recovery period of approximately 2 hours to ensure a stable reading, the animal was anaesthetised in a perspex box saturated with ether vapour. It was then removed and kept anaesthetised using a nose cone containing cotton wool soaked in ether. A continuous intra-arterial pressure recording was made. To compare intra-arterial and tail cuff measurements, a series of tail cuff measurements were taken and the intra-arterial trace marked simultaneously. Blood pressure was varied by bleeding from the intra-arterial line. At least 3 readings were taken from each animal. Pressures were measured to the nearest 2 mmHg. and the mean arterial pressure was calculated as the diastolic pressure + 1/3 of the pulse pressure.

Results

Ether anaesthesia had a delayed and reversible hypotensive effect, more obvious in hypertensive animals (Figures 20.1 and 20.2).

A highly significant correlation was found between tail cuff blood pressure and mean arterial blood pressure (Figure 20.3). The regression line for the relationship is described by the equation:

\[
\text{tail cuff BP} = 0.96 \times \text{MAP} - 15.8 \text{ mmHg.}
\]
Figure 20.1. The effect of ether anaesthesia on arterial blood pressure in a normotensive rat.
Figure 20.2. The effect of ether anaesthesia on arterial blood pressure in a rat made hypertensive by subtotal nephrectomy.
Figure 20.3. The correlation between tail cuff and mean arterial blood pressures.

\[ r = 0.896 \]
\[ n = 54 \]
\[ p < 0.0001 \]
Discussion

These experiments indicate that ether has a mild hypotensive
effect that becomes more pronounced as the duration of the anaesthetic
increases. To control for this effect in future experiments, the
duration of each anaesthetic was kept to a minimum and the time from
putting the animal in the ether box to the first spontaneous movement
on recovery was recorded. Since blood pressure was restored as the
animal regained consciousness, the last tail cuff reading obtained
prior to recovery from the anaesthetic was taken as the true reading.
To maintain consistent readings, the same two operators were employed
in all experiments, one to read the manometer and the second to judge
the point at which deflections returned to the oscilloscope trace. The
second operator was unable to read the manometer to avoid any bias in
readings.

The correlation between tail cuff blood pressure and mean arterial
pressure remained linear across the range of pressures studied and had
a slope of 1. This demonstrates that comparisons of tail cuff blood
pressure between groups of animals will yield valid comparisons of
intra-arterial pressure. The intercept on the Y axis of -15.8
indicates that tail cuff blood pressure underestimates true mean
arterial pressure by 15.8 mmHg.
CHAPTER 21
THE EFFECT OF SUBTOTAL NEPHRECTOMY ON RENAL STRUCTURE AND FUNCTION.

Introduction
In order to facilitate the design of subsequent experiments, a preliminary experiment was performed to study the effects of subtotal nephrectomy on renal structure and function, blood pressure, serum lipid concentration and survival. As described above (Chapter 17) food intake has an important effect on renal function in the rat. Pair-feeding ensures that food intake is equal between two groups of animals. The effects of this procedure were also studied.

Methods
24 female WAG/ola rats weighing approximately 200g were divided into two groups, matched for body weight. They were housed in individual cages with free access to tap water and fed a 24% casein protein diet containing 0.32 mmols/g sodium, 0.13 mmols/g potassium and 0.5% by weight phosphate (appendix 2). Initially they were fed ad libitum. 25g dry food, mixed with 35g tap water to form a paste, was offered daily and the food remaining the next day was weighed to calculate daily food intake. The animals were weighed weekly. After 2 weeks the animals were placed in metabolic cages for 24 hours, without food but with free access to tap water, and urine collected. They were then anaesthetised with ether and blood was sampled by tail clipping. Two days later, tail cuff blood pressure was measured as described in Chapter 20.

After 4 weeks, animals in Group 1 underwent a 1 1/3 nephrectomy and animals in Group 2 underwent a sham procedure as described in Chapter 18. Thereafter, animals were pair-fed as follows: If the cumulative total of food paste eaten differed by more than 100g between a pair, the animal with the greater intake was offered the weight of food eaten on the previous day by its partner.

Urine and blood samples were taken 4 and 8 weeks after nephrectomy and blood pressure was measured 2, 6 and 10 weeks after nephrectomy as above. Animals were sacrificed when clinically unwell, as manifested by a decrease in food intake and weight loss, poor
condition of the fur and general lethargy. On the death of an animal, its partner was offered 60g of food paste daily. Sham-operated animals were sacrificed after 26 weeks.

At death, the animal was weighed and its heart, liver and kidney(s) removed. The atria and great vessels were removed and blood expressed from the ventricles. Fat was trimmed from the liver and kidney and the organs were weighed. The cardiac and kidney indices were calculated as the ratio of organ to body weight [3].

The kidney was bisected and one half fixed in 10% formalin-saline. Fixed tissue was processed and embedded into wax using an automatic tissue processor (hypercenter II). 2μ thick paraffin-embedded sections were stained with haematoxylin and eosin, PAS reagent and toluidine blue (Appendix 5). The other half kidney was frozen on a CO₂ snowmaker block and sectioned on a cryostat. 5μ thick frozen sections were stained with oil red 0/haematoxylin (Appendix 5). Sections were viewed using a Leitz Dialux 22 microscope (Appendix 1). Serum and urine samples were analysed as described (Appendix 3). Data was analysed by the paired t-test using Oxstat programmes (Medstat Ltd., Nottingham) on an Amstrad PCW 8512 computer and is expressed as mean ± SEM. Graphic design was produced by Cricket graph software on an Apple Mackintosh computer.

Results

Weight gain and survival:-

Ad libitum food intake averaged over a 13 day period prior to operation ranged from 33 to 59g per day. The widest difference of ad libitum food intake between a pair of animals prior to operation was 24g per day. Three animals in each group required food restriction to maintain equal food intake at some stage during the experiment. The two animals with the highest ad libitum intake prior to operation were in Group 1. Following nephrectomy, these animals required prolonged periods of food restriction and their eventual survival was 32 and 42 weeks following nephrectomy.
Group 1 = nephrectomised animals. 
Group 2 = sham operated animals. 
Data = mean ± SEM. 
Statistical significance by paired t-test.
There was an initial drop in food intake and consequent weight loss in both groups following surgery but this was more marked in nephrectomised animals. Thereafter weight gain was significantly lower in nephrectomised animals despite pair-feeding (Figure 21.1).

Two animals died in the first week after nephrectomy. Progressive mortality in the remaining nephrectomised animals occurred after 6 weeks, with a median survival for the group of 13 weeks after surgery. There was no mortality in the sham-operated group.

Blood pressure:
Tail cuff blood pressure was elevated following nephrectomy and rose progressively (Figure 21.2). It was noted in a number of animals that the blood pressure was lower when they were clinically uraemic than at the previous measurement.

Biochemical variables:
Subtotal nephrectomy resulted in uraemia (Figure 21.2), hypercholesterolaemia and hypertriglyceridaemia (Figure 21.3). Serum albumin was slightly lower following nephrectomy but total protein was unchanged (Figure 21.3).

Urine volume and urine protein excretion increased following nephrectomy (Table 21.1). However urine volumes and proteinuria remained low compared to previously published data. When sham operated animals were offered food in the metabolic cages, urine volumes increased tenfold to 24±3 mls/24 hours. No significant contamination of the urine with food was detected.

Organ weights:
Progressive cardiac and renal remnant hypertrophy occurred following nephrectomy (Table 21.2). The final kidney remnant weight equalled the combined weights of the sham operated kidneys (Figure 21.4).
Histology:

No significant glomerular changes were detected in sham operated animals. The kidney remnants taken at death showed widespread glomerulosclerosis, ranging from small segmental lesions to complete sclerosis and obliteration of the glomerulus (Figure 21.5). There was marked tubular dilatation and atrophy, with protein cast formation.

Frozen sections contained deposits of lipid, particularly concentrated in areas of sclerosis (Figure 21.5). There were also scattered deposits of lipid within tubules.
Blood pressure

Urea

FIGURE 21.2. The effect of 1 1/3 nephrectomy on blood pressure and serum urea concentration. (Legend as for Figure 21.1).
FIGURE 21.3. The effect of 1 1/3 nephrectomy on serum total cholesterol, triglyceride, albumin and total protein concentrations. (Legend as for Figure 21.1).
FIGURE 21.4. The effect of 1 1/3 nephrectomy on kidney weight. The kidney on the left was removed at death 10 weeks after nephrectomy. Note the blue suture knot at the renal hilum surrounding a branch of the renal artery and the scar at the lower pole left from the infarcted area. The kidney on the right was taken from a healthy control.

FIGURE 21.5. Photomicrograph of a remnant kidney taken 11 weeks after nephrectomy showing extensive areas of sclerosis compared to a normal glomerulus on the left. Toluidine blue magnified x 1600.
FIGURE 21.6 Photomicrograph of a glomerulus containing extensive lipid deposits in areas of sclerosis, with some tubular lipid deposition. Oil Red O/haematoxylin magnified x 2000.
Table 21.1.
The effect of 1 1/3 nephrectomy on urine volume and 24 hour urinary protein excretion.

<table>
<thead>
<tr>
<th></th>
<th>weeks after operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-op n=12</td>
</tr>
<tr>
<td>Urine vol. ml/24 hr.</td>
<td>NephX 2.9±0.4</td>
</tr>
<tr>
<td></td>
<td>Sham 3.1±0.4</td>
</tr>
<tr>
<td>Urine protein mg/24 hr.</td>
<td>NephX 0.3±0.07</td>
</tr>
<tr>
<td></td>
<td>Sham 0.4±0.09</td>
</tr>
</tbody>
</table>

* p < 0.05, **** p < 0.0001 paired t-test v sham operated group

Table 21.2
The effect of 1 1/3 nephrectomy on heart and kidney weight.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart (mg) Body (g)</td>
<td>2.98±0.08****</td>
<td>5.13±0.21</td>
</tr>
<tr>
<td>Kidney(s) (mg) Body (g)</td>
<td>7.02±0.29</td>
<td>7.41±0.55</td>
</tr>
</tbody>
</table>

**** p < 0.0001 paired t-test
Discussion

The results of subtotal nephrectomy in this experiment are similar to those reported by many previous workers [3,338,339]. However, this experiment has highlighted a number of important points to be considered in designing subsequent experiments:-

1) Mortality occurs at two stages following nephrectomy. Some animals die in the immediate post-operative period, presumably from acute renal failure [338]. Those that survive this insult enter a stage of chronic uraemia and develop progressive glomerulosclerosis and hypertension leading to death. The progressive mortality after 6 weeks means that the number of animals available for study is reduced in prolonged experiments. If animals that do not survive the duration of the experiment are excluded, serious biases are liable to be introduced into the results. Experiments designed to mimic human chronic renal failure should therefore be timed to coincide with the period of chronic uraemia, should include adequate numbers of animals initially and should be ended before significant numbers of animals develop terminal renal failure.

2) There is a wide range of spontaneous daily food intake between animals, presumably reflecting differing levels of activity and energy requirements. Pairing animals by body weight alone does not allow for this. In maintaining an equal food intake between a pair of animals by pair-feeding, one of the two may be chronically starved. This may markedly retard the development of renal failure following nephrectomy. In other words, pair feeding may not equalise the effective protein load on a pair of animals if their metabolic requirements are unequal. It is preferable to allow ad libitum feeding and have sufficiently large numbers of animals in each group to ensure an equal mean food intake. This is only possible, however, when the two diets are equally palatable to the rats.

3) In the original experiments by Chanutin and Ferris [338] food was withheld in the metabolic cage. In this experiment, fluid intake and urine output was much reduced when animals were not offered food whilst
in the metabolic cage. A reduction in urinary volume may mask differences in urinary protein loss. In view of this effect, fasting prior to blood sampling (as is required for lipid analysis) should be performed on a separate day to the urine collection. Unfortunately, this rules out the possibility of calculating creatinine clearance, since simultaneous urine and blood samples are required for this.
CHAPTER 22
THE EFFECT OF CHOLESTYRAMINE ON HYPERLIPIDAEMIA AND RENAL FUNCTION FOLLOWING SUBTOTAL NEPHRECTOMY.

Introduction
As discussed in chapter 11, a number of experiments have suggested that the use of lipid-lowering agents, in particular clofibrac acid, is associated with a reduction in glomerulosclerosis and renal impairment. Clofibrac acid is contraindicated in renal impairment in man due to the development of myositis [361]. It was therefore decided to study the effects on glomerular damage of a lipid-lowering agent that would be suitable for use in man.

Cholestyramine is an effective agent for the control of familial hypercholesterolaemia and hypercholesterolaemia due to the nephrotic syndrome [362]. It acts by binding to bile acids in the gut and preventing their reabsorption by the small intestine. Increased bile acid synthesis is required to compensate for these losses. The uptake of lipoproteins by the liver is thus increased to provide cholesterol for bile acid synthesis. In the following experiment, cholestyramine has been administered to rats following subtotal nephrectomy and its effects on the development of hyperlipidaemia and glomerular damage have been studied.

Methods
24 female WAG/ola rats weighing approximately 200g were divided into two groups, matched for body weight. One group was fed the 24% casein diet and the other a 24% casein diet with 2% cholestyramine substituted for cellulose (appendix 2). Pure compound was supplied as a generous gift by Bristol-Myers Co. Ltd., Uxbridge. Both diets were initially fed ad libitum. Blood pressure, urine samples and blood samples were collected after 2 weeks, as previously described. After 4 weeks, both animals underwent a $1\frac{1}{3}$ nephrectomy, the operator being unaware of the dietary allocation of the animals. Following the operation, the animals were pair-fed as in Chapter 21. Blood samples were taken at 5 and 9 weeks after nephrectomy and blood pressure was measured 3, 6 and 8 weeks after nephrectomy.
In order to confirm that cholestyramine was effective in increasing bile acid turnover, the excretion of a radioactive bile acid tracer was measured as follows. 10 weeks after nephrectomy, both groups were fed a 24% casein diet for 2 days to clear the intestine of cholestyramine. The animals were then fasted for 5 hours and 3.7 kBq SeHCAT (tauro-23-[\(^{75}\)Se]selena-25-homocholic acid, Amersham International Plc, Aylesbury, U.K.) dissolved in 1ml 2% ethanol was administered by gavage. Fasting was continued for a further 4 hours, during which time total body radio activity was measured using an uncollimated gamma camera located 15 cms above a perspex restrainer. Energy analysers were set at 130 keV and 270 keV with a 20% window. Settings were standardised with a \(^{75}\)Se source at the beginning and the end of each session to allow for variations in counting sensitivity. Background measurements were taken before, during and after each counting session. Two counts of 100 seconds duration were taken per animal and values were corrected for sensitivity and background activity. Animals were then fed their allocated diets as before and counting was repeated 1, 2, 5, 8 and 12 days after the administration of SeHCAT.

Serum and urine were analysed as described (Appendix 3). Data was analysed as in Chapter 21.

Results
Weight gain and survival:

Weight gain was not significantly different in the two groups (Figure 22.1). Three animals in the control diet group died due to severe anorexia in the three weeks following nephrectomy. After 7 weeks, animals developed terminal uraemia at the same rate in both groups. Median survival was 96 days on the control diet and 111 days on cholestyramine.
FIGURE 22.1. The effect of cholestyramine on body weight following 1 1/3 nephrectomy. Data = mean ± SEM.
Blood pressure:-

The cholestyramine-treated group was significantly more hypertensive at 6 and 8 weeks after nephrectomy (Figure 22.2).

Biochemical variables:-

Serum urea was increased on cholestyramine prior to nephrectomy, when the animals were being fed *ad libitum* (Figure 22.2). After nephrectomy, when the animals were being pair-fed, serum urea was not significantly different.

Serum cholesterol was not significantly different from cholestyramine prior to nephrectomy (Figure 22.3). Following nephrectomy, serum cholesterol was higher on cholestyramine, this being statistically significant at 5 weeks. Serum triglyceride concentration was not significantly different before or after nephrectomy.

Serum albumin was reduced following nephrectomy, particularly at 9 weeks. There was no difference in the cholestyramine-treated group. Total protein was also not significantly different on cholestyramine (Figure 22.3).

Bile acid excretion:-

Excretion of SeHCAT followed an exponential decay in both groups (Figure 22.4). Significantly greater amounts of radioactivity were retained in animals fed cholestyramine. The half-life of elimination of radioactivity was reduced from 3.2 to 1.1 days.
FIGURE 22.2. The effect of cholestyramine on blood pressure and serum urea concentration following 1/3 nephrectomy.
Serum total cholesterol mmol/L

Serum triglyceride mmol/L

Serum albumin g/L

Serum total protein g/L

* p<0.05

FIGURE 22.3. The effect of cholestyramine on serum total cholesterol, triglyceride, albumin and total protein concentrations following $1 \frac{1}{3}$ nephrectomy.
FIGURE 22.4. The effect of cholestyramine on the excretion of SeHCAT.
Discussion

In rats made uraemic by subtotal nephrectomy, bile acid excretion was significantly increased by cholestyramine. The half-time for excretion of SeHCAT on the normal diet is comparable to previously reported values [363]. The half-time on cholestyramine of 1.1 days is significantly longer than the normal intestinal transit time of the rat of about 0.17 days [363] suggesting that SeHCAT was absorbed into the enterohepatic circulation and not simply bound to cholestyramine in the intestinal lumen and excreted directly.

However, cholestyramine was ineffective in chronically reducing serum cholesterol or triglyceride despite the increased bile acid losses. Serum cholesterol concentration and blood pressure were in fact significantly higher in the cholestyramine-treated group. This may be due to a bias introduced by the pairing of animals. Three animals in the control diet group died of uraemia in the three weeks following nephrectomy and this removed three less uraemic animals from the cholestyramine-treated group.

The lack of effect of cholestyramine suggests that de novo cholesterol synthesis by the liver, rather than lipoprotein uptake, was increased to compensate for the increased cholesterol requirements for bile acid production. Similar results have been found in intact rats [364,365]. The difference between these results and those obtained in man [366] is reflected in the higher activity of cholesterol synthetic enzymes found in the rat compared to other species [367].

The subsequent experiment investigates the possibility of using an inhibitor of the cholesterol synthetic enzyme HMG CoA reductase to reduce serum cholesterol concentration.
CHAPTER 23
THE EFFECT OF SYNVINOLIN ON SERUM LIPID CONCENTRATIONS IN THE INTACT RAT.

Introduction
In the previous experiments, cholestyramine failed to lower serum cholesterol concentration in both intact and uraemic rats. Synvinolin is an effective agent for the control of hypercholesterolaemia in man, both when familial in origin or secondary to the nephrotic syndrome [290,362]. It acts by inhibiting the enzyme HMG CoA reductase, the rate limiting step for cholesterol synthesis. It is readily absorbed from the gut in an inactive form and is broken down in the liver to release the active metabolite [Dr. D. Glover, MSD, Personal Communication]. The effect of dietary administration of this agent was therefore studied in intact rats.

Methods
16 female WAG/ola rats weighing approximately 200g were divided into two groups of 8, paired for body weight. Both groups were initially fed a standard 24% casein diet ad libitum as before. After one week blood was taken from the tail vein after an overnight fast. The diet for group 2 was thereafter changed to a 24% casein diet to which synvinolin (supplied as a generous gift by Merck, Sharpe and Dohme Ltd., Hoddesdon, Herts.) had been added at 150mg/kg dry food weight. The drug was added to the diet by RHM Research Ltd. using a commercial mixer to ensure an even distribution within the diet. After 14 days, blood samples were repeated after an overnight fast.

Results
Food intake was not affected by the addition of synvinolin. The mean daily intake was 42.0 ± 1.7g of wet food per day (mean ± SEM) before synvinolin and 40.3 ± 1.7g of wet food per day on synvinolin. The mean ingested dose of synvinolin was therefore 12.6 mgs/kg body weight.
No change in serum total cholesterol or triglyceride concentrations was detected after two weeks on synvinolin (Table 23.1).

Table 23.1. The effect of synvinolin on serum lipid concentrations in intact rats. Data = mean ± SEM.

<table>
<thead>
<tr>
<th>Total cholesterol mmol/L</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.53±0.10</td>
<td>2.42±0.14</td>
</tr>
<tr>
<td>Synvinolin</td>
<td>2.44±0.3</td>
<td>2.50±0.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triglyceride mmol/L</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.54±0.03</td>
<td>0.42±0.03</td>
</tr>
<tr>
<td>Synvinolin</td>
<td>0.62±0.04</td>
<td>0.60±0.06</td>
</tr>
</tbody>
</table>

Discussion

The lack of effect of synvinolin on serum lipid concentrations in normal rats has been confirmed by the manufacturers [Dr. D. Glover, MSD, Personal Communication] and has been reported using the related compound mevinolin [368]. Trials by the manufacturers have shown that the drug is well absorbed from the gut and the dose employed in this experiment is approximately three times the therapeutic dose in man. It was not possible to confirm that adequate dosing had been achieved in this experiment since circulating drug levels cannot currently be measured. They also would have been difficult to interpret since the drug is only in an active form in the liver.

McCune and Jurin have reported that mevinolin does reduce hypercholesterolaemia in obese Zucker rats but has no effect on their lean litter mates [368]. This was a short-term study lasting five days and previous workers have failed to demonstrate a significant drop in serum cholesterol when mevinolin was administered over a longer period [368]. Synvinolin administered orally over a ten week period to obese Zucker rats following unilateral nephrectomy caused no change in
serum lipid concentrations [Dr. D. Wheeler, Royal Free Hospital, Personal Communication].

The mechanism of the rat's resistance to lipid-lowering agents has been studied by Tanaka et al. [365]. They found an eight-fold increase in the levels of HMG CoA reductase following administration of 5% cholestyramine in the diet. When the diet was supplemented with both 5% cholestyramine and mevinolin (in a dose of approximately 200 mgs/kg body weight per day), HMG CoA reductase levels were massively increased to a peak of 133 times the basal activity in control animals. This was all due to an increase in the mass of enzyme in the liver rather than in its state of activation.

As discussed in Chapter 11, Keane et al. have reported that subcutaneous injections of mevinolin led to a reduction in serum cholesterol concentration in rats following subtotal nephrectomy [258]. In the light of the rat's resistance to the chronic lowering of serum cholesterol as described above, an alternative explanation of these results should be considered. As discussed in Chapter 1, uraemia leads to a reduction in lipoprotein clearance and consequently to hyperlipidaemia. Reduction in renal damage and uraemia by whatever means will inevitably lead to a reduction in serum cholesterol levels. Control of hypercholesterolaemia by lipid-lowering drugs in uraemic animals may therefore be the secondary effect of a reduction in renal damage rather than the direct result of the drug on lipid metabolism. Glomerular damage may be reduced by the drug through a separate mechanism from its effects on lipid metabolism. Wheeler has found that when synvinolin is administered subcutaneously, severe skin ulceration results which proves fatal in some animals (Dr. D. Wheeler, Personal Communication). This appears to be an effect of the drug itself, as various vehicles administered alone had no effect. These lesions will inevitably reduce food, and hence protein, intake and may thus reduce glomerulosclerosis.

Subcutaneous clofibric acid has also been shown to be
associated with a reduction in glomerulosclerosis (Chapter 11). It does not cause ulceration when administered subcutaneously (Dr D Wheeler, personal communication). However, it may still influence glomerular damage through actions other than those on lipid metabolism. For example, it has been shown to reduce glucose intolerance, have a vasopressin-like action and may affect fibrinolysis [369].

In view of these difficulties, further experiments using lipid lowering agents were not carried out and efforts were concentrated on studying the effects of dietary cholesterol supplementation on the development of glomerular damage.
CHAPTER 24
THE EFFECTS OF DIETARY CHOLESTEROL SUPPLEMENTATION IN THE RAT FOLLOWING SUBTOTAL NEPHRECTOMY.

Introduction
The hypothesis that hyperlipidaemia and glomerular lipid deposition are pathogenetic factors in the development of glomerulosclerosis can be tested by increasing the amount of lipid in the plasma and tissues of animals developing glomerular damage. Since cholesterol is accepted as being the damaging lipid in atherosclerosis, the intention in the following experiment was to selectively increase the levels of this lipid by dietary cholesterol supplementation. When such a diet is fed to a range of animal species, an increase in cholesterol-rich low density lipoproteins results. These are composed particularly of remnants from the breakdown of chylomicrons and VLDL [13,370]. The pattern of lipoproteins produced mimicks that seen in Fredricksen type III hyperlipidaemia in man, a condition associated with accelerated atherosclerosis [170,371].

The following experiment was designed to maximise the chances of demonstrating lipid-induced glomerular damage. A strain of rat was used that develops a large rise in serum lipids on a cholesterol-rich diet [372]. Cholesterol was added to a diet based on casein. This has been shown to accentuate the effect of cholesterol feeding compared to soya protein [373,374], due to an associated reduction in the metabolism of VLDL [375]. Cholic acid was added to ensure that the cholesterol was absorbed [376]. Rats are generally resistant to dietary hyperlipidaemia and previous cholesterol-rich diets have had to include the anti-thyroid drug thiouracil (which inhibits cholesterol excretion in the bile) to be effective [377].

The subtotal nephrectomy model combines the effects of hypertension and uraemia. Uraemia causes a reduction in lipoprotein clearance (Chapter 1) and accentuates the hyperlipidaemic effect of dietary fat [74]. Hypertension and
hyperlipidaemia act synergistically in the rat to cause atherosclerosis [378,379]. Therefore, this model may be expected to amplify any effect of lipids in mediating glomerular damage.

As discussed in Chapter 21, dietary modification was performed during the phase of chronic renal impairment, following the induction of renal injury, in order to mimic progressive renal damage in man. Pair-feeding was not performed to avoid any bias due to selective starvation. A close record was therefore kept of dietary food intake to ensure an equivalent protein load in both groups.

**Methods**

Studies were performed on 40 female WAG/ola rats weighing approximately 200g, housed and fed as before. At week -4, i.e. 4 weeks before commencement of the cholesterol supplemented diet, a subtotal nephrectomy was performed as described in Chapter 18. At week -1, a 24 hour urine collection was performed, with animals allowed free access to food and water. The following day, the tail cuff blood pressure was measured as described in Chapter 20. The animals were then starved overnight and blood sampled by tail clipping.

Thirty-two surviving animals were divided into two groups of 16 and pairs of animals were matched for the severity of proteinuria measured at week -1 (Figure 24.1). At week 0, group 2 was changed to a 24% casein diet supplemented with 4% cholesterol and 1% cholic acid in place of cellulose. Group 1 continued on the control diet. The diets contained identical sodium (0.32mmol/g), potassium (0.13mmol/g), phosphorus (0.5%), vitamin and trace element contents (Appendix 2) and were fed *ad libitum*. At weeks +1, 3 and 5, blood pressure measurement and urine and blood sampling were repeated.

At week 6, the animals were placed in metabolic cages for 24 hours and urine collected in tubes containing 0.5ml 50mMol EDTA in 150mMol NaCl, pH 7.4. Urine from groups 1 and 2 were pooled separately, passed through a 0.22μ filter and concentrated x 600 at 4°C using lyphogel granules (Gelman Sciences Ltd.,
Northampton, U.K.). Concentrated samples were separated by agarose gel electrophoresis (Corning Medical Ltd., Palo Alto, CA, U.S.A.). Gels were stained with fat red 7B for lipid.

Animals were sacrificed in matched pairs when terminally uraemic or at week 7, using i.p. pentobarbitone. The heart ventricles, liver and the remaining kidney were weighed.

**Tissue lipid analysis:**

The kidney was bisected coronally. One half was processed for tissue lipid analysis as follows. Excess fat was removed and the tissue stored at between -20°C and -70°C until analysed. It was then freeze dried for 36 hours in an Edward's Modulyo. The dry tissue was weighed in a screw necked vial and 10mls of 2:1 v/v chloroform:methanol solvent was added [380]. The tissue was finely chopped and left overnight at 4°C. The following day the tissue was macerated with a ground glass tissue grinder and sonicated for 15 minutes. The sample was then centrifuged and the supernatant aspirated into a clean tube. The remaining tissue was washed with 1ml of solvent, centrifuged and the supernatant added to the previous extract. 2ml deionised water was added to the extract. The suspension was vortex mixed and centrifuged at 4,000 rpm for 5 minutes. The upper phase was aspirated and discarded and the lower phase was dried down under nitrogen at 55°C on a heated block. The lipid extract was redissolved in 2ml chloroform by vortex mixing and divided into two 1ml aliquots. The extract was dried under nitrogen as above and stored at -20°C before further analysis.

To one set of vials was added 800μl of isopropranol. 10μl samples of this solution were assayed for total cholesterol, free cholesterol and total triglyceride using enzymatic kits (Appendix 3). Standard curves were constructed using pure cholesterol and triolein dissolved in isopropranol, with an isopropranol reagent blank. The remaining extract was applied to a silica gel thin layer chromatography plate (Merck) with a Hamilton syringe. Marker solutions of free cholesterol and cholesterol palmitate
were also applied. Plates were placed in chromatography tanks containing an 80:20:1 v/v mixture of N-hexane:diethyl ether:glacial acetic acid. When saturated, plates were dried and stained with iodine vapour in a separate tank. The free cholesterol and cholesteryl ester bands were scraped into test tubes and the lipid extracted by washing twice with 2ml of chloroform. The chloroform extracts were dried and the lipid redissolved in 200 μl of isopropranol. Samples were analysed for total cholesterol as above. An isopropranol blank for this assay was prepared from TLC plate scrapings that had been soaked in the chromatography solvent.

To the other set of vials was added 1ml of isopropranol and samples were analysed for phospholipids using an enzymatic kit (Appendix 3). The aqueous standard supplied with the kit was used with an isopropranol reagent blank.

The percentage recovery of pure cholesterol and triglyceride by the extraction procedure was measured in triplicate using measured amounts of pure substance in place of the dried kidney tissue. The percentage extraction and recovery of total cholesterol, free cholesterol, triglyceride and phospholipid from tissue was estimated using lyophilised reference serum and a sample of normal human serum. Known amounts of serum were processed in the same way as kidney tissue. The original amount of lipid present in the fresh serum and in an aqueous solution of the lyophilised serum was measured using the enzymatic methods and isopropranol standards as above. The percentage recovery of lipid in the extract was then calculated.

Histological methods:-

5μ thick frozen sections from the other half-kidney were stained with Mayer's haematoxylin/oil red 0 to demonstrate lipid (VL Ross-Gilbertson, Department of Pathology, Leicester General Hospital; Appendix 5) and with polyclonal anti-human β lipoprotein antibody using an immuno-alkaline phosphatase technique (Appendix 5; SL Brown, Department of Surgery, L.G.H.).
Formalin-fixed tissue was processed, embedded in paraffin wax and 2μ sections stained with toluidine blue (V.L.R-G). All glomeruli in one frozen section (c.100 glomeruli) and one fixed section (c. 200 glomeruli) per animal were counted using an eyepiece graticule. Frozen sections contained fewer glomeruli due to the difficulty in cutting complete coronal sections. Glomeruli were scored for lipid deposition and glomerulosclerosis respectively, on a scale of 0 (none) to 3 (complete obliteration). Sclerosis was defined as widening of the mesangium by toluidine blue-positive material (Figure 24.9). Frozen sections were examined by phase-contrast microscopy to delineate glomeruli. Frozen sections were counted twice by one observer (HC Rayner) in a random order and fixed sections were counted once each by two observers (H.C.R and V.L.R-G) and the mean scores were calculated. The observers were unaware of the dietary group of the sections. Samples of renal cortex were processed for transmission electron microscopy (PM Wells-Jordan, Department of Pathology, Leicester Royal Infirmary; Appendix 6).

Serum and urine were analysed as described in Appendix 3. An aliquot of fresh serum was added to one drop of 50 mMol EDTA in 150 mMol NaCl, pH 7.4 and stored at 4°C for separation by electrophoresis as above.

For comparison, two groups of four intact animals (not sham-operated) were fed the control and cholesterol-supplemented diets. Blood pressure, blood samples and urine collections were taken after 1, 3 and 5 weeks on the diets as above. Organs were weighed and kidney sections prepared as above.

Data was analysed statistically by Wilcoxon matched-pairs signed-rank test, Spearman rank correlation and multiple linear regression using Oxstat programs (Medstat Ltd., Nottingham, U.K.). Data from the intact animals was not tested statistically due to the small numbers. Biochemical data and organ weight ratio are expressed as the mean ± SEM and the histological scores as the median and range.
Figure 24.1.
The design of the experiment to study the effect of dietary cholesterol and cholic acid supplementation on rats following subtotal nephrectomy.
Results

Survival: There were no preoperative deaths. 7 animals died by week -1 with severe anorexia. Between week 0, when the cholesterol diet was commenced, and week 7, 4 animals on the control diet and 3 on the cholesterol diet became terminally uraemic and were killed with their matched partners, leaving 10 matched pairs to be sacrificed at week 7 (Figure 24.1).

Food intake and weight gain (Figure 24.2): Following nephrectomy, there was a decrease in food intake that recovered before the diets were changed. The cholesterol-supplemented diet was less well tolerated for the first week but thereafter intake of the two diets was not significantly different. Body weight paralleled these changes, increasing equally on the two diets after a post-operative fall, until week 5. Two terminally uraemic animals on the control diet were killed during week 5. Their two partners killed at the same time were not uraemic. The remaining animals on the cholesterol diet were therefore biased towards uraemia and this may explain their subsequent weight loss. No animals became oedematous.

Blood pressure: Blood pressure rose progressively after subtotal nephrectomy on the control diet but was not significantly different on the cholesterol diet (Table 24.1). Intact animals remained normotensive. Tail cuff blood pressure was 95.0 ± 3.9 mmHg after 5 weeks on the control diet and 101.5 ± 3.6 mmHg on the cholesterol diet.

Biochemical changes: In nephrectomised animals on the control diet, serum triglyceride and total cholesterol concentrations were elevated compared to intact animals and rose progressively during the experiment (Figure 24.3). On the cholesterol diet, both serum triglyceride and total cholesterol were significantly elevated, with total cholesterol reaching a peak after 3 weeks on the diet. HDL cholesterol was significantly lower on the cholesterol diet (Table 24.1) and a β-migrating electrophoretic band was very prominent (Figure 24.3).

Serum urea rose following nephrectomy (intact animals = 5.3 ± 0.3
mmol/L, Table 24.1.) After one week on the cholesterol diet, serum urea was significantly lower than on the control diet, probably due to the initial depression of food intake on changing to this diet. Thereafter, there was no significant difference between the two groups.

Serum albumin fell following nephrectomy (intact animals = 36.0 ± 0.77 g/L, nephrectomised animals at week -1 = 32.4 ± 0.4 g/L) and remained depressed throughout the experiment (control diet group at week 5 = 31.6 ± 1.2 g/L). There was no significant difference on the cholesterol diet (cholesterol diet group at week 5 = 32.8 ± 0.5 g/L).

Serum total protein was significantly increased on the cholesterol diet due to the higher concentration of lipoprotein (control diet group at week 5 = 66.2 ± 0.9 g/L, cholesterol diet = 72.2 ± 1.1 g/L, p<0.01).

Urine protein was not detectable in intact animals on either diet. Following nephrectomy, urinary protein excretion increased but was not significantly different on the cholesterol diet (Table 24.1).

Urine electrophoresis demonstrated α-migrating bands on both diets but a β-migrating band was barely detectable (Figure 24.4). No lipid was detectable in urine from intact animals.

**Linear regression analysis:**

Significant correlations were found between serum total cholesterol and triglyceride concentrations and 24 hour urinary protein excretion. The equations describing these relationships are given in Table 24.2 and are shown graphically, with their 95% confidence limits [381], in Figure 24.5. The gradient of the regression lines for both cholesterol and triglyceride was significantly increased on the cholesterol diet.

Multiple linear regression analysis was performed to investigate the effect of time on these correlations. The results are given in Table 24.2. Although there were alterations in the intercept on the Y axis at weeks +3 and +5, the correlations between serum lipid and urinary protein excretion remained highly significant when these time co-variates were included.

**Organ weights (Table 24.3):** Cardiac and renal hypertrophy occurred
equally following nephrectomy on the two diets, the remaining kidney equalling the combined weight of the two kidneys in intact controls. Cardiac and renal hypertrophy were significantly correlated with the mean blood pressure (Table 24.7). The livers from animals on the cholesterol diet were enlarged and yellow due to fatty change.

Kidney lipid content:— The percentage recovery of lipids from samples of pure substance, lyophilised reference sera (Precinorm UBS and Precipath UBS; BCL) and normal human serum are shown in Table 24.4. Complete recovery of pure cholesterol and triglyceride was achieved. However, recovery of cholesterol, triglyceride and phospholipid from lyophilised serum and cholesterol alone from normal human serum was incomplete. Previous preliminary experiments had yielded similar recovery fractions and these were not improved using an extraction technique based on hexane-isopropanol solvent [382].

Kidney total cholesterol content was measured using a two-stage enzymatic method; cholesteryl esterase released free cholesterol from cholesteryl esters, then cholesterol oxidase generated a coloured product with free cholesterol. Total cholesterol was significantly increased on the cholesterol diet (Table 24.5.). Free cholesterol was measured by a one-stage enzymatic method that only used the second cholesterol oxidase step of the total cholesterol assay. Kidney free cholesterol content was also significantly increased on the cholesterol diet but for each dietary group, the free cholesterol content was higher than the measured total cholesterol content. This discrepancy was investigated as follows:—

a) The analysis of quality-control sera gave satisfactory results using both the free and total cholesterol assays.

b) Extracts of lyophilised reference sera and normal human sera were assayed using the free and total cholesterol assays. The percentage of total cholesterol present as free cholesterol was 68% by this method. The gross discrepancy in free cholesterol compared to total cholesterol levels was thus only found in the extracts of kidney tissue.

c) Extracts of serum were then separated by thin layer chromatography. The free and esterified cholesterol factions were assayed separately
using the total cholesterol assay alone. By this method, the mean percentage of free cholesterol was 47%. The free cholesterol assay thus gave only slightly higher values for free cholesterol in the extract of serum (68%) than were measured by the total cholesterol assay following TLC separation of the extract (47%).

Extracts of kidney lipid were separated by thin layer chromatography and the percentage of total cholesterol that was esterified was calculated (Table 24.5). The percentage of esterified cholesterol was increased significantly on the cholesterol diet. Kidney triglyceride and phospholipid contents were unchanged.

The kidney total cholesterol content was significantly correlated with the last-measured serum cholesterol concentration ($r_s = 0.6053$, $p<0.001$).

**Histology** :- Intact animals on the cholesterol diet showed scattered deposits of lipid in glomeruli with occasional foam cells. No glomerulosclerosis was present on either diet.

From nephrectomised animals, 13 of the 16 pairs of frozen sections were technically adequate for counting. Similar numbers of glomeruli in each dietary group were counted for lipid (control diet median = 86 (range 62-227), cholesterol diet = 125 (71-169)) and for glomerulosclerosis (control diet = 209 (93-320), cholesterol diet = 183 (146-320)). Intra- and inter-observer reliability for the scoring of glomerular lipid deposition and sclerosis respectively was assessed by Spearman rank correlation (lipid $r_s = 0.9167$ $p<0.0001$, sclerosis $r_s = 0.6816$ $p<0.0001$).

Lipid deposition was significantly increased on the cholesterol diet, (Figure 24.6), all degrees of deposition being increased (Table 24.6). The percentage of glomeruli containing lipid deposits was significantly correlated with the last-measured serum cholesterol concentration (Figure 24.7).

Deposits of β lipoprotein were detected within areas of sclerosis by immuno-histochemistry and were present in greater amounts on the cholesterol diet (Figure 24.8).

Electron microscopy of tissue from intact animals fed the
cholesterol diet demonstrated the presence of lipid vacuoles within the extracellular matrix, particularly in the paramesangial region (Figure 24.9) and within mesangial cells. The size of the electron-lucent spaces in the matrix was 60nm (600A), i.e. comparable to the size of a βVLDL molecule (300-800A, Chapter 1). Deposition in animals fed the cholesterol diet following subtotal nephrectomy was similarly distributed but in greater amounts than in intact animals (Figure 24.10). Myofibrils and hemi-desmosomes were present within lipid-containing cells and lipid accumulation did not appear to be restricted to a sub-population of mesangial cells.

An example of glomerulosclerosis and associated lipid deposition is shown in Figure 24.11. The percentage of glomeruli with areas of sclerosis was lower on the cholesterol diet and significantly fewer glomeruli showed moderate or severe changes (Table 24.6). The percentage of glomeruli containing areas of sclerosis was correlated with the mean tail-cuff blood pressure and the last-measured urinary protein excretion (Table 24.7).
Table 24.1. Effect of dietary cholesterol supplementation on tail cuff blood pressure, serum HDL cholesterol and urea concentrations and urinary protein excretion in rats following subtotal nephrectomy, performed at week -4. Cholesterol supplementation was commenced at week 0.

<table>
<thead>
<tr>
<th>Week</th>
<th>Tail cuff blood (mmHg)</th>
<th>HDL cholesterol (mmol/L)</th>
<th>Serum urea (mmol/L)</th>
<th>Urinary protein (mg/24hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=16)</td>
<td>(n=14)</td>
<td>(n=13)</td>
<td>(n=13)</td>
</tr>
<tr>
<td>Week -1</td>
<td>173.3±6.3 C</td>
<td>2.35±0.11</td>
<td>13.5±0. C</td>
<td>49.3±9.6</td>
</tr>
<tr>
<td>Week +1</td>
<td>184.5±5.6 E</td>
<td>3.10±0.26</td>
<td>13.3±1.4</td>
<td>71.7±12.0</td>
</tr>
<tr>
<td>Week +3</td>
<td>182.2±6.6</td>
<td>2.84±0.24</td>
<td>14.8±1.7</td>
<td>57.8±7.4</td>
</tr>
<tr>
<td>Week +5</td>
<td>191.8±8.9</td>
<td>4.23±0.48</td>
<td>13.5±2.2</td>
<td>72.3±10.2</td>
</tr>
</tbody>
</table>

C v E * p<0.05, ** p<0.01, *** p<0.001 Wilcoxon signed-rank test
C = Control diet, E = Cholesterol supplemented diet.
Data = mean ± SEM.
Table 24.2. Correlations between serum total cholesterol and triglyceride and 24 hour urinary protein excretion. The regression line is described by the equation \( Y = MX + C \), where \( Y \) = serum lipid concentration and \( X \) = 24 hour urinary protein excretion. The effect of the time of sampling on the regression line (from multiple linear regression analysis) is given in the last column. No significant effect was detected at week +1 on slope or \( Y \) intercept.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>Regression slope (M)</th>
<th>( Y ) intercept (C)</th>
<th>n</th>
<th>p</th>
<th>Effect of time of sampling on regression line</th>
<th>Week +3</th>
<th>Week +5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum total cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>0.600</td>
<td>0.02</td>
<td>2.87</td>
<td>73</td>
<td>&lt;0.0001</td>
<td>Intercept +0.87 p=0.0003</td>
<td>Intercept +1.19 p=0.0001</td>
<td></td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>0.672</td>
<td>0.09</td>
<td>2.56</td>
<td>40</td>
<td>&lt;0.0001</td>
<td>Intercept +4.01 p&lt;0.0001</td>
<td>No significant change</td>
<td></td>
</tr>
<tr>
<td><strong>Serum triglyceride</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>0.544</td>
<td>0.01</td>
<td>0.42</td>
<td>73</td>
<td>&lt;0.0001</td>
<td>No significant change</td>
<td></td>
<td>No significant change</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>0.678</td>
<td>0.02</td>
<td>0.42</td>
<td>40</td>
<td>&lt;0.0001</td>
<td>Intercept +1.49 p&lt;0.05</td>
<td>No significant change</td>
<td></td>
</tr>
</tbody>
</table>
Table 24.3. Ratios of organ weight (mg) to total body weight (g) at sacrifice. The kidney ratio represents the combined weight of two kidneys in intact animals and the weight of the remaining left kidney in nephrectomised animals.

<table>
<thead>
<tr>
<th></th>
<th>Intact animals</th>
<th>Nephrectomised animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control diet</td>
<td>Cholesterol diet</td>
</tr>
<tr>
<td>Heart</td>
<td>3.01 ± 0.10</td>
<td>3.12 ± 0.17</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.59 ± 0.2</td>
<td>6.98 ± 0.32</td>
</tr>
<tr>
<td>Liver</td>
<td>33.90 ± 1.08</td>
<td>44.39 ± 2.48</td>
</tr>
</tbody>
</table>

Control v Cholesterol diet ** p<0.01 Wilcoxon signed-rank test.

Data = mean ± SEM.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure substance</td>
<td>101%</td>
<td>112%</td>
<td>-</td>
</tr>
<tr>
<td>Precinorm UBS (lyophilised)</td>
<td>47%</td>
<td>21%</td>
<td>68%</td>
</tr>
<tr>
<td>Precipath UBS (lyophilised)</td>
<td>49%</td>
<td>22%</td>
<td>70%</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>47%</td>
<td>108%</td>
<td>96%</td>
</tr>
</tbody>
</table>
Table 24.5. The effect of cholesterol feeding on kidney lipid content. Free cholesterol was measured using an enzymatic method. % esterified cholesterol was calculated from fractions of free and esterified cholesterol, separated by thin layer chromatography. See text for discussion.

<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol (μmols/g dry wt)</th>
<th>Free cholesterol (μmols/g dry wt)</th>
<th>Esterified cholesterol (% of total)</th>
<th>Total triglyceride (μmols/g wt)</th>
<th>Total phospholipids (μmols/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet (n=16)</td>
<td>29.2 ± 0.8</td>
<td>48.3 ± 1.4</td>
<td>7.5 ± 0.4</td>
<td>39.4 ± 4.5</td>
<td>73.1 ± 1.7</td>
</tr>
<tr>
<td>Cholesterol diet (n=16)</td>
<td>47.7 ± 3.3****</td>
<td>59.1 ± 2.0****</td>
<td>14.5 ± 2.1**</td>
<td>35.1 ± 4.1</td>
<td>76.6 ± 1.8</td>
</tr>
</tbody>
</table>

** p<0.01, **** p<0.0001 v control diet, Wilcoxon signed-rank test.
Data = mean ± SEM
Table 24.6. Histological changes following subtotal nephrectomy. Percentage of glomeruli containing any changes (i.e. grade 1, 2 or 3) and moderate or severe changes (grade 2 or 3) are shown as the median and range.

<table>
<thead>
<tr>
<th>Lipid deposition.</th>
<th>Percentage of glomeruli with</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grade 1, 2 or 3 lipid</td>
<td>grade 2 or 3 lipid</td>
<td></td>
</tr>
<tr>
<td>Control Diet</td>
<td>21.0% (0-62)</td>
<td>6.5% (0-28)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol Diet</td>
<td>60.0% (14-82)</td>
<td>18.5% (1-67)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glomerulosclerosis.</th>
<th>Percentage of glomeruli with</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grade 1, 2 or 3 sclerosis</td>
<td>grade 2 or 3 sclerosis</td>
<td></td>
</tr>
<tr>
<td>Control Diet</td>
<td>29.6% (0-54.3)</td>
<td>11.03% (0-31.5)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>11.7% (0-56.0)</td>
<td>0.88% (0-31.2)</td>
<td></td>
</tr>
</tbody>
</table>

Control v Cholesterol diet * p<0.05 Wilcoxon signed-rank test
Table 24.7. Spearman rank correlation matrix for the percentage of glomeruli containing sclerosed segments against mean tail cuff blood pressure*, last-measured 24 hour urinary protein excretion, heart weight/body weight ratio and kidney weight/body weight ratio.

\( r_s \) = Spearman rank correlation coefficient.

<table>
<thead>
<tr>
<th>% glomerulosclerosis</th>
<th>mean tail cuff BP</th>
<th>last 24hr Uprotein</th>
<th>heart wt body wt</th>
<th>kidney wt body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>% glomerulosclerosis</td>
<td>( r_s = 1.0 )</td>
<td>( r_s = 1.0 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean tail cuff BP</td>
<td>( r_s = 0.360 )</td>
<td>( r_s = 1.0 )</td>
<td>( p = 0.043 )</td>
<td></td>
</tr>
<tr>
<td>last 24hr Uprotein</td>
<td>( r_s = 0.564 )</td>
<td>( r_s = 0.511 )</td>
<td>( r_s = 1.0 )</td>
<td>( p = 0.003 )</td>
</tr>
<tr>
<td>heart/body ratio</td>
<td>( r_s = 0.614 )</td>
<td>( r_s = 0.533 )</td>
<td>( r_s = 0.622 )</td>
<td>( r_s = 1.0 )</td>
</tr>
<tr>
<td>kidney/body ratio</td>
<td>( r_s = 0.783 )</td>
<td>( r_s = 0.428 )</td>
<td>( r_s = 0.735 )</td>
<td>( r_s = 0.659 )</td>
</tr>
</tbody>
</table>

* The correlation with the last-measured tail cuff blood pressure was less significant than with the mean since blood pressure tended to be lower in clinically uraemic animals [338].
Figure 24.2.
Changes in food intake and body weight following subtotal nephrectomy. Both groups were fed the control diet until week 0. Group 2 was changed to the cholesterol-supplemented diet from week 0.
Figure 24.3.
The effect of cholesterol supplementation on serum triglyceride and total cholesterol concentrations in intact and uraemic rats. Prediet samples were taken from animals stabilised on the control diet, one week before the diets were changed. Nephrectomised animals underwent subtotal nephrectomy three weeks before the prediet samples. Statistical significance refers to cholesterol diet v control diet in nephrectomised animals.
Figure 24.4.
The effect of cholesterol supplementation on serum and urinary lipid electrophoretic patterns.
Lane 1 = serum from a subtotally nephrectomised animal fed the control diet.
Lane 2 = serum from a subtotally nephrectomised animal fed the cholesterol supplemented diet.
Lane 3 = concentrated urine pooled from group 1, fed the control diet.
Lane 4 = concentrated urine pooled from group 2, fed the cholesterol supplemented diet.
Figure 24.5.
Correlations between serum cholesterol and triglyceride concentrations and 24 hour urinary protein excretion in animals fed the control and cholesterol supplemented diets. 95% confidence intervals are indicated by the broken lines.
Figure 24.6.
Photomicrograph of a frozen section of kidney from a rat fed the cholesterol diet, stained with Oil Red O. Extensive lipid deposition is demonstrated in three glomeruli, with patchy deposits in tubules. Oil red O/haematoxylin, magnification x 400.

Figure 24.7.
Correlation between percentage of glomeruli containing lipid deposits and the last-measured serum cholesterol concentration (normalised logarithmically). Data from the two dietary groups has been combined to calculate the correlation coefficient.
Figure 24.8.
Photomicrograph of a frozen section of kidney from a rat fed the cholesterol diet, stained with polyclonal anti-β lipoprotein antibody using an immuno-alkaline phosphatase technique. Heavy staining is present in three glomeruli, particularly in areas of sclerosis. Magnification x 800.

Figure 24.9.
Electron micrograph of the paramesangial region of an intact rat fed the cholesterol diet. Electron-lucent spaces of 60nm diameter are seen on the mesangial side of the basement membrane, between mesangial cell processes. Normal podocyte foot processes fill the upper part of the field. (Magnification x 26,000.)
Figure 24.10.
Transmission electron micrograph of a glomerulus from an animal fed the cholesterol supplemented diet for 7 weeks following subtotal nephrectomy (x 5200). Small lipid vacuoles are present in the paramesangial region (*) and within mesangial cells (M). Extensive epithelial cell foot process fusion has occurred (Ep). C = capillary lumen. U = urinary space, E = endothelial cell.

Figure 24.11.
Photomicrograph of a paraffin section of kidney from a rat fed the cholesterol diet, stained with toluidine blue. Two glomeruli are shown. A large segment of one is sclerosed and contains lipid vacuoles and large foam cells. The overlying Bowman's capsule is thickened and adherent. Magnification x 800.
Discussion

Following subtotal nephrectomy, animals on the control diet developed hyperlipidaemia. As discussed in Chapter 1, this is due to a reduction in lipoprotein clearance. As expected, uraemic animals showed a greater increase in serum lipids on the cholesterol diet compared to intact animals. In uraemic animals, the degree of hyperlipidaemia increased linearly with increasing proteinuria, suggesting that the reduction in lipoprotein clearance was proportional to the severity of renal damage. The gradient of this correlation was significantly increased on the cholesterol diet. This is compatible with the increase in secretion of lipoproteins on the diet not being matched by an increase in their clearance.

Hyperlipidaemia on the cholesterol diet consisted mainly of β-migrating lipoproteins. Levels of serum HDL cholesterol were lower on the cholesterol diet when measured by the precipitation technique. As discussed in detail in the next experiment, results using this method may not be reliable. However, a similar reduction in the concentration of HDL on a cholesterol-rich diet have been demonstrated using sequential ultracentrifugation to separate the lipoproteins [374].

The cholesterol content of kidney tissue was estimated using a standard lipid extraction technique [380]. The percentage of cholesterol and triglyceride extracted from lyophilised plasma was surprisingly low and differed from that obtained from fresh human serum. This was not due to losses of substance during the procedure as the recovery of pure lipid was complete and the percentage of lipid that was recovered differed between the three types of lipid measured. In his original description of the technique, Folch claimed to achieve complete lipid extraction from tissues [380]. He supported this claim by failing to extract further amounts of lipid from the residual tissue that was left after an initial extraction procedure. He did not measure the total lipid content of the tissue using another method. It is thus possible that some lipid is tightly bound within the tissue or serum and cannot be extracted by the organic solvent. When the enzymes contained in the assay kits are applied to the reconstituted serum, these lipids may be released and become available for measurement.
Although incomplete, an equal percentage of each lipid was extracted from the two types of lyophilised reference sera and one may therefore conclude that the measurements of kidney lipid content reflect the relative proportions of lipid present in the tissues of the two dietary groups.

Overall kidney cholesterol content was significantly increased on the cholesterol diet and was strongly correlated with the serum cholesterol concentration. Cholesterol feeding causes a decrease in the percentage of circulating cholesterol that is esterified (Chapter 26) and suppresses endogenous cholesterol synthesis in the kidney [189]. The increase in the percentage of tissue cholesterol that is esterified therefore suggests that circulating free cholesterol is taken up and esterified within kidney cells. Despite significant hypertriglyceridaemia, there was no increase in kidney triglyceride or phospholipid content, indicating that the uptake and storage of cholesterol was selective.

The discrepancy between measurements of free cholesterol using the enzymatic assay and the TLC separation method remains unexplained. Since it was only found in extracts of tissue and not serum, it must be assumed that there was a substance present in the tissue extracts that produced a colour reaction with the free, but not with the total, cholesterol assay.

Deposition of lipid in glomeruli measured histologically was significantly increased in the cholesterol-fed group and increased in proportion to the rise in serum cholesterol. β-migrating lipoproteins were not detected in the urine and the positive binding of anti-β lipoprotein antibody confirmed that they were being trapped in glomeruli. Electron microscopy confirmed both intra- and extracellular lipid deposition. The size of the lipid spaces in the mesangial matrix was compatible with them having been occupied by individual βVLDL macromolecules.

Cells containing lipid vacuoles had features of intrinsic, smooth muscle type mesangial cells. However, it is not possible to definitely identify the origin of mesangial cells from morphological criteria alone (Chapter 26).
Despite the accumulation of 'atherogenic' lipoprotein in glomeruli and the marked increase in overall kidney cholesterol content, neither hypertension, uraemia, proteinuria nor glomerulosclerosis were significantly increased on the cholesterol-supplemented diet. The apparent decrease in the percentage of glomeruli with severe degrees of sclerosis on the cholesterol diet may be an artefact caused by the presence of large foam cells within sclerotic areas. These spaces reduce the percentage cross sectional area occupied by widened mesangium and therefore decrease the grade of severity of sclerosis recorded.

These results indicate either that the accumulation of cholesterol-rich lipoproteins within the glomerulus does not affect glomerulosclerosis or that the effect that they exert is too small to be detected in this experimental model. As discussed in the introduction, this experiment was designed to maximise the 'atherogenic' risk factors for glomerular damage. Due to the progressive mortality of the uraemic animals, it was not possible to continue the experiment beyond 11 weeks after nephrectomy. The possibility that prolonged hyperlipidaemia may aggravate glomerulosclerosis was therefore investigated in a further experiment, in which a less severe reduction in renal mass was performed, allowing prolonged survival (Chapter 26).
CHAPTER 25
THE EFFECT OF LDL ON THE PRECIPITATION OF LIPOPROTEINS IN THE HDL ASSAY.

Introduction

The definitive quantitation of the different density classes of lipoproteins requires their separation by ultracentrifugation. A more convenient method for the estimation of the concentration of high density lipoprotein relies on the selective removal of low density lipoprotein by precipitation. The residual 'HDL' cholesterol in the supernatant is then measured. The commercially-produced kit employed in the present experiments uses manganese-phosphotungstate as the precipitating agent. The kits are designed for use with human lipoproteins and it is necessary to confirm their applicability to rat serum. Furthermore, it is important to determine whether the presence of abnormal lipoproteins, such as those induced by cholesterol feeding, alters the precipitation of lipoproteins.

Dietary-induced hyperlipoproteinaemia was mimicked in vitro by adding purified human LDL to normal rat serum. Samples of normal control and LDL-supplemented rat serum were then assayed for HDL. If the assay gives reliable measurements of HDL, the measured concentration should not have been altered, after allowing for the dilutional effect of adding LDL.

Methods

Fresh rat serum, pooled from healthy control animals, was divided into five 300µL samples. 100µL of purified human LDL (protein content 8 mgs/ml, prepared by sequential flotation ultracentrifugation as detailed in Chapter 16) was added to a further five 200 µL samples. The initial total cholesterol concentration was measured using an enzymatic colorimetric method (BCL Ltd.). Samples were then assayed in duplicate for HDL. Manganese phosphotungstate precipitating agent was added and the samples centrifuged for 30 minutes at 4000 rpm at room temperature. The supernatant, containing unprecipitated 'HDL', was aspirated and assayed for total cholesterol concentration as above.
Results

Total cholesterol concentration was increased from 3.22 ± 0.02 mmols/L (mean ± SEM) to 8.33 ± 0.07 mmols/L by the addition of LDL. Following precipitation, the residual total cholesterol concentration in the supernatant of control samples was 2.65 ± 0.04 mmols/L. However, in samples with added LDL the residual cholesterol concentration was reduced to 0.53 ± 0.01 mmols/L. Allowing for the dilutional effect of the added LDL, the measured serum 'HDL' concentration in these samples was 0.80 ± 0.02 mmols/L (p<0.001, paired t-test).

Discussion

These results indicate that the addition of human LDL to rat serum, in similar amounts to those produced by dietary cholesterol supplementation, leads to a lowering of 'HDL' levels when measured by the manganese-phosphotungstate precipitation technique. This is probably due to the trapping of HDL within clumps of precipitated LDL. A similar effect may well occur with samples containing high levels of rat β lipoprotein induced by dietary cholesterol supplementation. The manganese-phosphotungstate precipitation technique therefore may not provide reliable measurements of rat HDL concentrations under these conditions.
CHAPTER 26
THE EFFECTS OF DIETARY CHOLESTEROL SUPPLEMENTATION IN THE RAT FOLLOWING UNILATERAL NEPHRECTOMY.

Introduction
As discussed in Chapter 24, it is possible that hyperlipidaemia and glomerular lipid deposition have a low-grade detrimental effect on glomerulosclerosis that did not become apparent in the short-term experiment that employed the subtotal nephrectomy model. A further experiment was therefore performed using the same dietary manipulation as in experiment 4 introduced after unilateral rather than subtotal nephrectomy. Following unilateral nephrectomy, rats do not become hypertensive or uraemic but they do develop glomerulosclerosis, in association with the deposition of macromolecules in the mesangium, over a period of 4-6 months [151].

In the previous experiment, a diet containing a relatively high sodium content was used. To reduce the possibility of hypertension developing in the current experiment, a more highly refined 24% casein diet, from which sodium had been removed by dialysis, was used.

In experiment 4, it had not proved possible to determine the type of cell that was principally responsible for the uptake of lipid in the mesangium. Hence in this experiment, monoclonal antibody markers were used to identify the nature of glomerular foam cells. In view of the possibility that hyperlipidaemia may lead to a cellular infiltration of the glomerulus as discussed in Chapter 10, the number of cells staining with these antibodies was quantified. Since the histological damage to glomeruli is much less severe following uninephrectomy than following subtotal nephrectomy, a computerised image analysis system was used to quantify the amount of mesangial matrix present in glomerular sections.

Methods
Studies were performed on 36 female WAG/ola rats weighing approximately 200g. A 24% casein diet was offered as a paste (45g food:42.5g water) containing 50g dry food every two days. At week -5, i.e. 5 weeks before the commencement of the cholesterol-supplemented diet, tail cuff blood pressure was measured as described in Chapter 20.
Figure 26.1 The experimental protocol.
Three days later the animals were placed in metabolic cages with free access to food and water and a 24 hour urine collection was performed. The animals were then starved overnight and blood was sampled by tail-clipping. Daily food intake measurements proved unreliable due to the scattering of food powder within the cage. Food intake was therefore assessed whilst animals were in the metabolic cages by measuring urinary electrolyte excretion. Animals were weighed weekly.

At week -3, a unilateral nephrectomy was performed as described in Chapter 18. Two weeks later (week -1), urine collection and blood sampling was repeated. The four animals with the highest levels of urinary protein excretion at week -1 were discarded and the remaining 32 animals were divided into two groups of 16, matched for the severity of proteinuria (Figure 26.1).

At week 0, group 2 was changed to a 24% casein diet supplemented with 4% cholesterol and 1% cholic acid in place of cellulose. 50g dry food was offered as a paste (45g food:35g water to achieve the same consistency as the control diet) every two days. Group 1 continued on the control diet. The diets contained identical sodium (control 0.14 mmols/g, cholesterol 0.13 mmols/g), potassium (control and cholesterol diets 0.12 mmols/g), phosphorus (0.5%), vitamin and trace element contents (Appendix 2) and were fed ad libitum. Blood pressure was measured at weeks +2, 7 and 20. Urine collections were performed at weeks +2, 7, 11, 15 and 19. Blood samples were taken at weeks +2, 7 and at sacrifice at week 21.

At week 21 animals were sacrificed as follows. Following overnight starvation, animals were weighed and anaesthetised with ether. The thorax was opened and the heart punctured with a 23 gauge butterfly needle. Approximately 1ml of blood was aspirated and transferred to a 5ml potassium EDTA tube. As much blood as possible was then aspirated from the heart and thoracic cavity and allowed to clot at room temperature. The remaining kidney, heart ventricles and liver were trimmed of fat and weighed. The kidney was bisected and half was fixed in 4% formaldehyde solution in saline. Sections of cortex from the other half were placed in a special fixative to retain
unesterified cholesterol (Appendix 5). Further slices were mounted on
cork in OCT compound (Tissue-Tek) and snap frozen in pre-cooled
isopentane in liquid nitrogen. Rings of abdominal aorta from two
animals on each diet were placed in the special fixative for EM.

Histological methods:-

Formalin-fixed tissue was processed and embedded in paraffin wax.
3μ sections were taken from all blocks at one session and stained by
PAS without counterstain, at one staining session (Appendix 5).

Frozen sections of cryopreserved kidney were stained with the
following antibodies:-- monoclonal antibodies against rat leucocyte
common antigen (OX-1), rat Ia antigen common determinant (OX-6) and rat
macrophages (ED-1). Some sections were also stained with a polyclonal
antibody against human β lipoprotein. Sections were also stained for
lipid with oil red O (Appendix 5; L. Ward, Dept. of Pathology, LRI).

Tissue in special EM fixative was processed into Epon resin for
transmission electronmicroscopy (Appendix 5; PM Wells-Jordan,
Department of Pathology, LRI).

Histological quantitation - morphology:-

Paraffin-embedded sections stained with PAS were examined using
Seescan image processing equipment (Seescan Ltd., Equipment Unit 9, 25
Gwydir Street, Cambridge, CB1 2LG. Department of Pathology, Queen's
Medical Centre, Nottingham) as follows:-

The section was viewed with a 25 x objective. Illumination was
adjusted for optimal contrast on the computer monitor. An area of the
slide away from the tissue section was viewed and a reading of
background illumination taken. This would then automatically be
subtracted from each field. The kidney section was scanned and a
glomerular cross-section containing a definite glomerular hilum
(irrespective of its overall sectional area) was identified. The
'frame area' to be analysed was delineated around the inside of
Bowman's capsule using the computer mouse. The threshold for the
detection of positive staining by the computer was then adjusted to
register only the glomerular mesangium (Figure 26.2).
Figure 26.2 A simulation of the measurement of glomerular and mesangial areas using a digital computer image. The glomerular area is delineated in green. The threshold for the detection of a positive image by the computer has been adjusted to include only the mesangium, indicated on the overlay in red.
Glomerular mesangium was defined as that area of the capillary tuft with an intense PAS reaction, which radiated out from the glomerular hilum and which did not form a complete outline of capillary loops. The area of mesangium was then measured. For each glomerulus, the percentage of the glomerular frame area occupied by mesangium was calculated.

A pilot study was carried out to determine the optimal number of glomerular sections to be measured. The mean glomerular and mesangial areas were found not to alter significantly between 20 and 50 glomeruli counted. Therefore 20 glomeruli per section were counted sequentially. Reproduceability was assessed by recounting 4 sections at the end of the analysis and calculating the mean percentage difference between the two counts. Kidney sections were examined in a random order with the observer unaware of their dietary group.

Unfortunately, the presence of glomerular foam cells tended to 'unblind' the sections. This was unavoidable. A strict adherence to the sequential counting of glomeruli was maintained, however. With practice, an assessment of the percentage of the glomerular area occupied by the mesangium could be made by eye. As a result, the initial measurement of the mesangial area was reassessed in a small number of glomerular sections that initially gave aberrant readings.

Histological quantitation - immunocytochemistry:

The numbers of cells giving a positive reaction with monoclonal antibodies OX-1 and OX-6 were counted as follows:

Sections were examined in a random order with the observer unaware of the dietary group. Glomeruli were initially identified using phase-contrast microscopy. It was not possible to identify glomerular sections containing a hilum, hence only sections of large diameter were assessed. 20 glomeruli were counted per section as this was the maximum number available in every specimen. Separate counts were made of the number of positively staining cells within the glomerular tuft and immediately outside Bowman's capsule, surrounding the glomerulus. Only areas of staining that were clearly associated with a cell nucleus were accepted.
Unfortunately, not all sections stained with ED-1 were technically adequate for counting due to damage to the tissue caused by repeated freezing and thawing. Therefore a qualitative assessment only of staining with this antibody was made.

A sample of EDTA anticoagulated blood was analysed by a Coulter counter (Dept. of Haematology, LGH).

Serum and urine were analysed as described in Appendix 3. An aliquot of fresh serum was separated by electrophoresis as in Chapter 24, and compared with samples of normal and hyperlipidaemic human serum.

Data was analysed statistically by the paired t-test and linear correlation using Oxstat programmes. Data was expressed as the mean ± SEM.

Results

Food intake and survival:–

Food intake, measured by urinary sodium and potassium excretion every 4 weeks, was not significantly different on the cholesterol-supplemented diet (Table 26.1). Body weight tended to be greater on the cholesterol diet due to some animals becoming obese. This did not reach statistical significance (Figure 26.3). Three animals died under ether anaesthesia at week 7. Their partners were killed at the same time. All other animals remained healthy throughout the experiment. Animals not completing the experiment were excluded from the histological analysis.
Table 26.1. The effect of dietary cholesterol supplementation on urine volume and electrolyte excretion following unilateral nephrectomy.

<table>
<thead>
<tr>
<th></th>
<th>2 wks preop</th>
<th>-1</th>
<th>+2</th>
<th>+7</th>
<th>+11</th>
<th>+15</th>
<th>+19</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine Volume ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>13.8±1.8</td>
<td>13.6±1.9</td>
<td>10.6±1.2</td>
<td>12.7±2.0</td>
<td>13.5±1.2</td>
<td>16.6±2.2</td>
<td>17.9±3.9</td>
</tr>
<tr>
<td>E</td>
<td>11.4±1.2</td>
<td>14.0±1.5</td>
<td>8.5±1.1</td>
<td>10.4±1.2</td>
<td>11.2±1.1</td>
<td>10.7±1.2*</td>
<td>11.5±1.0</td>
</tr>
</tbody>
</table>

| **24 hour Urine sodium mol** |          |     |     |     |     |     |     |
| C                        | 1.42±0.09  | 1.54±0.09 | 1.34±0.08 | 1.56±0.09 | 1.67±0.09 | 1.77±0.07 | 1.65±0.06 |
| E                        | 1.40±0.11  | 1.61±0.13 | 1.11±0.08 | 1.41±0.10 | 1.61±0.09 | 1.52±0.14 | 1.63±0.08 |

| **24 hour Urine potassium mol** |       |     |     |     |     |     |     |
| C                        | 1.31±0.07  | 1.33±0.06 | 1.24±0.07 | 1.43±0.09 | 1.67±0.10 | 1.76±0.07 | 1.59±0.06 |
| E                        | 1.36±0.11  | 1.39±0.11 | 1.01±0.07 | 1.26±0.08 | 1.53±0.10 | 1.48±0.12 | 1.43±0.08 |

*p<0.05 paired t-test.  C = control diet group, E = cholesterol diet group.
Figure 26.3 The effect of cholesterol supplementation on body weight following unilateral nephrectomy.
**Blood pressure:**

Animals became mildly hypertensive following unilateral nephrectomy (preop = 102 ± 2.4 vs postop = 111.5 ± 1.7 mmHg, p<0.01). Blood pressure was unchanged on the cholesterol diet (Table 26.2.).

**Table 26.2.** The effect of cholesterol supplementation on tail cuff blood pressure following unilateral nephrectomy.

<table>
<thead>
<tr>
<th>Weeks after uninephrectomy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+1</td>
<td>+6</td>
</tr>
<tr>
<td><strong>BP (mmHg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>111.5±1.7</td>
<td>108.0±2.2</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>107.9±1.8</td>
<td>110.6±2.2</td>
</tr>
<tr>
<td><strong>Anaes. (mins)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>167.6±11.4</td>
<td>176.9±11.4</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>157.4±4.8</td>
<td>148.6±3.5</td>
</tr>
</tbody>
</table>

**Biochemistry:**

As demonstrated in Chapter 24, cholesterol feeding resulted in a prominent β-migrating electrophoretic band (Figure 26.4). There was a progressive increase in serum total cholesterol and triglyceride concentrations (Figure 26.5) and the serum phospholipid concentration measured at week 21 was increased (Table 26.3). The percentage of serum cholesterol circulating as free cholesterol was also increased (Table 26.3).

Serum albumin concentration was significantly lower on the cholesterol diet at week 21, at which time urinary protein excretion was highest (Figure 26.6). Serum total protein concentration was increased on the cholesterol diet, in association with the increased concentration of lipoproteins (Figure 26.6). Serum urea concentration was unaltered by unilateral nephrectomy or cholesterol feeding (Table 26.4). Serum glucose concentration was mildly, but statistically significantly, increased on the cholesterol-supplemented diet at week 21 (Table 26.4). There was no difference in serum sodium or potassium concentrations at any time.
Figure 26.4 Agarose gel electrophoresis of serum stained with fat red 7B to detect lipid.
Lane 1 = normal human serum.
Lane 2 = serum from a rat on the control diet following unilateral nephrectomy.
Lane 3 = serum from a rat fed the cholesterol-supplemented diet following unilateral nephrectomy.
Lane 4 = human VLDL isolated by ultracentrifugation.
Lane 5 = whole plasma from a patient with mixed hypertriglyceridaemia.
The amount of lipoprotein applied to the gel was not standardised and this may slightly alter the relative positions of the bands.
Figure 26.5  The effect of dietary cholesterol supplementation on serum total cholesterol and triglyceride concentrations following unilateral nephrectomy.
Table 26.3. Percentage of serum total cholesterol present as free cholesterol and serum phospholipid concentration after 21 weeks on the cholesterol diet. Samples had been stored for 3 months at -20°C prior to analysis.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Cholesterol diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=13</td>
<td>n=12</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% free cholesterol</td>
<td>31.6 ± 2.0</td>
<td>43.0 ± 1.7****</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phospholipid</td>
<td>2.98 ± 0.22</td>
<td>4.16 ± 0.27**</td>
</tr>
</tbody>
</table>

** p = 0.01, **** p = 0.0001 paired t-test

Table 26.4 The effect of cholesterol supplementation on serum urea and glucose concentrations following uninephrectomy.

<table>
<thead>
<tr>
<th></th>
<th>2 wks preop n = 16</th>
<th>-1  n = 16</th>
<th>+2  n = 16</th>
<th>+7  n = 13</th>
<th>+21 n = 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea C mmol/L E</td>
<td>5.9±0.2</td>
<td>6.9±0.3</td>
<td>6.3±0.3</td>
<td>5.4±0.3</td>
<td>5.7±0.2</td>
</tr>
<tr>
<td></td>
<td>6.2±0.3</td>
<td>6.0±0.4</td>
<td>7.4±0.4</td>
<td>5.5±0.3</td>
<td>6.2±0.4</td>
</tr>
<tr>
<td>Glucose C mmol/L E</td>
<td>6.5±0.3</td>
<td>5.5±0.2</td>
<td>6.0±0.3</td>
<td>4.3±0.2</td>
<td>6.4±0.3</td>
</tr>
<tr>
<td></td>
<td>6.4±0.3</td>
<td>6.0±0.2</td>
<td>6.4±0.3</td>
<td>4.5±0.2</td>
<td>7.3±0.3*</td>
</tr>
</tbody>
</table>

* p<0.05 paired t-test
Figure 26.6  The effect of dietary cholesterol supplementation on serum albumin and total protein concentrations following unilateral nephrectomy.
Urine volume was slightly greater on the control diet towards the end of the experiment, reaching statistical significance at 15 weeks (Table 26.1). Urinary protein excretion rose progressively over the 22 weeks following unilateral nephrectomy on the control diet (Figure 26.7). Two weeks after starting the cholesterol diet, mean urinary protein excretion was increased and this difference was maintained throughout the subsequent weeks. Due to the wide standard deviation of these measurements, these differences did not reach statistical significance at $p<0.05$.

![Figure 26.7](image)

**Figure 26.7** The effect of dietary cholesterol supplementation on urinary protein excretion following unilateral nephrectomy.
Haematology:

Blood taken from animals on the cholesterol diet had a greater tendency to clot in the EDTA tube; 5 of the 13 samples on the cholesterol diet clotted compared to 1 of 13 on the control diet (\(\chi^2 = 3.47\), p>0.05). There were no significant differences in any haematological variable on the cholesterol diet (Table 26.5).

Table 26.5. Coulter analysis of blood taken at sacrifice after 21 weeks on the cholesterol diet.

<table>
<thead>
<tr>
<th></th>
<th>Control diet n=12</th>
<th>Cholesterol diet n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC x10^9/L</td>
<td>4.01 ± 0.45</td>
<td>4.70 ± 0.50</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>71.7 ± 3.3</td>
<td>70.1 ± 1.4</td>
</tr>
<tr>
<td>% Granulocytes</td>
<td>16.0 ± 2.6</td>
<td>17.4 ± 1.5</td>
</tr>
<tr>
<td>% Monocytes</td>
<td>12.4 ± 1.3</td>
<td>12.5 ± 0.7</td>
</tr>
<tr>
<td>RBC x10^12/L</td>
<td>8.0 ± 0.1</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>Hb g/dl</td>
<td>14.8 ± 0.2</td>
<td>14.5 ± 0.3</td>
</tr>
<tr>
<td>Hct %</td>
<td>40.7 ± 0.5</td>
<td>42.2 ± 1.2</td>
</tr>
<tr>
<td>MCV fl</td>
<td>51.2 ± 0.1</td>
<td>51.5 ± 0.3</td>
</tr>
<tr>
<td>Platelets x10^9/L</td>
<td>1087 ± 35</td>
<td>1084 ± 68</td>
</tr>
</tbody>
</table>
There was no increase in heart/body weight ratio on the control or cholesterol diets (Table 26.6) and these values did not differ from normal intact animals in Chapter 24 (heart(mg)/body(g) = 3.01 ± 0.10). Renal hypertrophy occurred equally on the control and cholesterol diets compared to an initial ratio for one kidney of 3.3 (intact animals in Chapter 24). The liver was markedly enlarged on the cholesterol diet and showed extensive fatty change.

Table 26.6. Organ weight ratios following uninephrectomy after 21 weeks on the cholesterol diet.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>Cholesterol diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=13</td>
<td>n=13</td>
</tr>
<tr>
<td>Heart (mg) Body (g)</td>
<td>2.93 ± 0.06</td>
<td>3.01 ± 0.13</td>
</tr>
<tr>
<td>Kidney (mg) Body (g)</td>
<td>5.02 ± 0.15</td>
<td>5.14 ± 0.24</td>
</tr>
<tr>
<td>Liver (mg) Body (g)</td>
<td>26.31 ± 0.82</td>
<td>41.40 ± 1.93</td>
</tr>
</tbody>
</table>

**** p<0.0001 Paired t-test
Histology:

Frozen sections stained with Oil Red O demonstrated segmental lipid deposition in almost all glomeruli of rats on the cholesterol diet. There was also patchy tubular deposition in many animals (Figure 26.8). No lipid deposition was detected in animals on the control diet. Deposition of β lipoprotein was confirmed by positive staining with anti-β lipoprotein antibody in animals on the cholesterol diet.

Figure 26.8 Frozen section of kidney from a rat on the cholesterol diet stained with Oil Red O, demonstrating segmental glomerular lipid deposition and extensive lipid deposits in the tubular epithelium. Magnified x 1100.
Paraffin-embedded sections from animals on the control diet showed no significant glomerular capillary damage or sclerosis. In animals on the cholesterol diet, glomeruli contained segmental clusters of foam cells associated with capsular adhesions (Figure 26.9).

Figure 26.9  Paraffin-embedded section of a kidney from a rat on the cholesterol diet stained with PAS, demonstrating a segmental cluster of foam cells associated with a capsular adhesion. Magnified x 1100.
Computerised image analysis revealed a significant increase in the mean area of mid-glomerular sections (Table 26.7). There was a corresponding increase in the area of mesangium. This resulted in there being no change in the percentage of the glomerular area occupied by the mesangium (Figure 26.10).

**Table 26.7.** The effect of cholesterol supplementation for 21 weeks following unilateral nephrectomy on glomerular and mesangial areas.

<table>
<thead>
<tr>
<th></th>
<th>Glomerular Sectional area $\mu^2$</th>
<th>Mesangial Sectional area $\mu^2$</th>
<th>Percentage Mesangial area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>11127±440</td>
<td>1502±82</td>
<td>13.6±0.7</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>13109±393**</td>
<td>1812±69*</td>
<td>14.0±0.5</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data = mean ± SEM  * p < 0.05  ** p < 0.01 paired t-test

These increases were considerably greater than would be expected from experimental error, as measured by the mean percentage difference between four repeated counts (Table 26.8).

**Table 26.8.** Comparison of the percentage change in glomerular and mesangial area with the variation between repeated counts of 4 slides.

<table>
<thead>
<tr>
<th>% difference between diets n= 13</th>
<th>Glomerular Sectional area</th>
<th>Mesangial Sectional area</th>
<th>Percentage Mesangial area</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.4%</td>
<td>18.7%</td>
<td>2.7%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% difference between counts n = 4</th>
<th>Glomerular Sectional area</th>
<th>Mesangial Sectional area</th>
<th>Percentage Mesangial area</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4%</td>
<td>6.4%</td>
<td>6.3</td>
<td></td>
</tr>
</tbody>
</table>
Figure 26.10  The effect of dietary cholesterol supplementation on glomerular sectional area, mesangial area and the percentage of the glomerular section occupied by mesangium. Data = mean ± SEM.
Highly significant correlations were found between the last-measured 24 hour urinary protein excretion and both mean glomerular sectional area and mesangial area (Figure 26.11).

**Figure 26.11** The correlations between 24 hr urinary protein excretion and the mean glomerular sectional area (upper panel) and the mean mesangial area (lower panel).
Electron microscopy:-

Sections of glomeruli from animals on the cholesterol diet demonstrated extensive electron-lucent spaces in the subendothelial region, particularly adjacent to the glomerular mesangium. Foam cells, containing massive lipid vacuoles, were found in the mesangium. These had slightly darker nuclear chromatin and appeared to have displaced the intrinsic mesangial cells to one side (Figure 26.12). There was no obvious widening of the mesangial matrix or glomerular basement membrane in association with the lipid deposits or the foam cells.

Sections of aorta were examined from two animals on each diet. No lipid deposits or foam cells were detected in either group.

Figure 26.12  Electron micrograph of the mesangial region of a rat fed the cholesterol-supplemented diet for 21 weeks following unilateral nephrectomy. Note the large foam cell with dark nuclear chromatin, apparently displacing downwards the intrinsic mesangial cell with a lighter-staining nucleus. The foam cell has cytoplasmic processes protruding into adjacent capillary lumina. A platelet is adjacent to the endothelial cell body in the upper capillary loop. Extensive electron-lucent spaces are present in the mesangial matrix. Mag. x 4000.
Immunocytochemistry:-
Foam cells in glomeruli from animals on the cholesterol diet stained positively with the monoclonal antibody against leucocyte common antigen (OX-1) (Figure 26.13) and with that against macrophages (ED-1). A proportion of the foam cells also stained positively with the monoclonal antibody against Ia antigen (OX-6) (Figure 26.14). The number of OX-1 positive cells was significantly increased on the cholesterol diet when expressed both as cells per glomerular section (Table 26.9) and when corrected for the mean glomerular sectional area (Table 26.10). There was also a slight increase in the number of OX-6 positive cells per glomerulus on the cholesterol diet but this was not statistically significant (Tables 26.9 & 10).

The numbers of leucocyte common antigen-expressing and Ia antigen-expressing cells immediately outside Bowman's capsule, were increased on the cholesterol diet (Table 26.9). These increases remained statistically significant for both antigens when corrected for the mean glomerular sectional circumference, calculated from the mean glomerular sectional area for each slide (Table 26.10). Many periglomerular cells stained positively with the antibody against macrophages (ED-1) but they could not be quantified due to poor tissue preservation.
Figure 26.13  Photomicrographs of frozen sections stained with the monoclonal antibody against leucocyte common antigen (OX-1). The upper panel shows a section from a rat fed the control diet. The lower panel shows one from a rat fed the cholesterol diet for 21 weeks following unilateral nephrectomy. Foam cells in the cholesterol-fed animal are positive for OX-1. Magnification x 1100.
Figure 26.14  Photomicrographs of frozen sections stained with the monoclonal antibody against immune-associated (Ia) antigen (OX-6). The upper panel shows a section from a rat fed the control diet. The lower panel shows one from a rat fed the cholesterol diet for 21 weeks following unilateral nephrectomy. A foam cell staining positively with the antibody is adjacent to two foam cells that have not stained. Magnification x 1100.
Table 26.9. The effect of cholesterol supplementation on the numbers of leucocyte common antigen (OX-1) and la antigen (OX-6)-expressing cells per glomerular section, present within the glomerular tuft (intraglomerular cells) and immediately surrounding Bowman's capsule (periglomerular cells).

<table>
<thead>
<tr>
<th></th>
<th>Intraglomerular cells</th>
<th>Periglomerular cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OX-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>3.15±0.30</td>
<td>4.13±0.27</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>5.43±0.67**</td>
<td>5.85±0.51**</td>
</tr>
<tr>
<td><strong>OX-6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>2.92±0.29</td>
<td>2.98±0.36</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>3.96±0.33</td>
<td>4.95±0.37**</td>
</tr>
</tbody>
</table>

*p < 0.05, **p = 0.01 paired t-test.

Table 26.10. The effect of cholesterol supplementation on the numbers of leucocyte common antigen (OX-1) and la antigen (OX-6)-expressing cells, per 10,000 μ² of glomerular sectional area and per 100μ of glomerular sectional circumference.

<table>
<thead>
<tr>
<th></th>
<th>Intraglomerular cells/10,000μ²</th>
<th>Periglomerular cells/100μ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OX-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>2.86±0.28</td>
<td>1.10±0.06</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>4.10±0.44*</td>
<td>1.44±0.12*</td>
</tr>
<tr>
<td><strong>OX-6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>2.64±0.26</td>
<td>0.80±0.09</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>3.04±0.27</td>
<td>1.22±0.09*</td>
</tr>
</tbody>
</table>

*p < 0.05 paired t-test
Discussion

In contrast to the previous experiment, unilateral nephrectomy did not result in uraemia or hypertension and apart from three deaths due to ether anaesthesia, animals remained healthy for 24 weeks following the operation. Equal amounts of the cholesterol and control diets were eaten and there was no significant difference in blood pressure or serum urea concentration on the cholesterol diet.

Dietary cholesterol supplementation following unilateral nephrectomy caused similar changes in plasma lipoproteins as were seen following 1/3 nephrectomy. A marked increase in β-migrating lipoprotein was found, associated with an increase in the percentage of total cholesterol circulating as free cholesterol. Triglyceride and phospholipid concentrations were also increased.

Cholesterol feeding resulted in large amounts of lipid being deposited in the glomerular mesangium. This was associated with an increase in urinary protein excretion that became evident after only two weeks. Although not statistically significant at the p<0.05 level, the increase in urinary protein excretion was consistent throughout the experiment.

After 21 weeks on the diet, glomeruli from animals on the cholesterol diet were significantly larger than those from animals on the control diet. The increase in glomerular size correlated with the severity of urinary protein excretion. The glomerular mesangium increased in size in proportion to the increase in size of the whole glomerulus, indicating that no significant glomerulosclerosis was present. The increase in glomerular size was independent of the generalised compensatory renal hypertrophy that occurred equally on the two diets following unilateral nephrectomy.

The principal cells involved in the uptake of lipid within the mesangium were bone marrow-derived macrophages and there was a small but significant increase in the number of these cells in the glomerular tuft on the cholesterol diet. This was associated with a similarly small but significant increase in the number of periglomerular mononuclear leucocytes. The identity of all of these cells is not certain but many of them were macrophages. The increase
in cell numbers both within and surrounding glomeruli was not due to an increase in the numbers of circulating leucocytes on the cholesterol diet.

The preferential uptake of lipid by macrophages, rather than by intrinsic smooth muscle-type mesangial cells, is compatible with their expression of lipoprotein receptors. As demonstrated in Section II, smooth muscle-type mesangial cells express LDL receptors that are down-regulated when the level of intracellular cholesterol is increased. They do not express 'scavenger' lipoprotein receptors. Conversely, macrophages express receptors for \( \beta \text{VLDL} \), the predominant lipoprotein present in the cholesterol-fed animals, and these receptors are not down-regulated by increases in intracellular cholesterol [46].

The expression of immune-associated (Ia) antigen was used as a marker of macrophage 'activation'. Glomerular lipid deposition did not cause a significant increase in the number of activated macrophages in the glomerular tuft. There was a small but significant increase in Ia antigen-expressing cells immediately surrounding the glomerulus but some of these cells may have been lymphocytes.

There are two possible mechanisms which may have mediated the effects of cholesterol supplementation on glomerular size and cellular composition. The increase in glomerular size may have been due to an increase in glomerular capillary hydraulic pressure on the cholesterol diet. Uninephrectomy leads to an increase in glomerular capillary pressure through a decrease in afferent arteriolar resistance [349]. In a recent abstract, Schmidt et al. reported a significant further increase in glomerular capillary pressure 4-6 weeks after unilateral nephrectomy, in male rats fed a cholesterol-supplemented diet [277]. The mechanism for this further rise was not given. In the present experiment, there was no increase in systemic blood pressure on the cholesterol diet which could have led to an elevated glomerular capillary pressure. As discussed in Chapter 4, macromolecules are partly cleared from the mesangium via the efferent arteriole. It is thus possible that mesangial lipid deposition leads to an increase in efferent arteriolar tone which would explain the rise in glomerular capillary pressure.
Recent work by Yoshida et al. discussed in Chapter 17, has shed doubt on the link between glomerular hyperfiltration and subsequent hypertrophy and sclerosis. Their results have confirmed that glomerular hypertrophy is closely associated with subsequent glomerulosclerosis. However, they have found no correlation between the size of the glomerulus and its capillary hydraulic pressure [383]. They have also disassociated pressure and sclerosis by comparing two models of renal injury in which there were equivalent increases in glomerular capillary pressure. Glomerular hypertrophy and sclerosis were only found in rats in which a kidney had been removed and were not found when its function was deleted by ureteric diversion but the tissue was left in place [349].

An alternative explanation for the effects of cholesterol supplementation in the current experiment is that the deposition of lipid in the mesangium stimulates growth of the glomerulus, independent of any alteration in glomerular pressure. Growth may be mediated either by a direct effect of lipid on intrinsic smooth muscle-mesangial cells or by the release of growth factors by infiltrating macrophages.

Cultured mesangial cells have been shown to proliferate in the presence of low concentrations of low density lipoprotein [225]. However, it is difficult to extrapolate from the effects of lipoproteins in culture to in vivo conditions. In culture, the availability of cholesterol in the culture medium is a limiting factor to the growth of cells [228]. In contrast, most cholesterol required by peripheral tissues can be synthesised in situ and the availability of exogenous lipoprotein is unlikely to be a limiting factor on growth [15].

In rats fed the cholesterol diet, foam cells were associated with glomerular capsular adhesions, suggesting that the phagocytosis of lipid by macrophages leads to the release of toxic substances that damage surrounding cells. Macrophages have been shown to secrete a wide range of potentially toxic products, including lysosomal hydrolases and oxygen free-radicals [234]. They also secrete a number of growth factors, including platelet-derived growth factor, fibroblast
growth factor and epidermal growth factor [234]. The possible release of such products in this experiment was not associated with immune 'activation' of macrophages, as indicated by the expression of immune-associated (Ia) antigen.

A further factor that may exacerbate the inflammatory effect of macrophages is the activation of clotting mechanisms by hypercholesterolaemia as discussed in Chapter 10. Blood appeared to be hypercoagulable and the electron micrograph in Figure 26.12 happens to include a platelet that is adjacent to an endothelial cell overlying a mesangial foam cell. Platelets are known to be important producers of platelet-derived growth factor [234].

The increase in periglomerular leucocytes on the cholesterol diet may be due to a general increase in interstitial leucocytes, resulting from lipid deposition in tubules. It is also possible that the phagocytosis of lipid by increased numbers of intraglomerular macrophages leads to the release of chemoattractant factors that cause a clustering of leucocytes around Bowman's capsule [384]. These cells may play a role in the development of capsular fibrosis which is associated with progressive glomerular scarring (Chapter 3).

The results of this experiment are consistent with previously reported work studying the clearance of exogenously administered macromolecules from the mesangium. Deposition of exogenous material has been shown to be associated with low levels of proteinuria and an infiltration by small numbers of bone marrow-derived macrophages [107, 109, 110, 166]. No significant changes in whole kidney glomerular filtration rate or plasma flow were found. Hence, it may be valid to generalise from this experiment and hypothesise that 'overload' of the mesangium with various endogenous macromolecules exacerbates the development of glomerulosclerosis.
APPENDICES
APPENDIX 1
MATERIALS USED IN ANIMAL EXPERIMENTS

Anaesthetics

Anaesthetic ether (diethyl ether)  May & Baker Ltd., Dagenham, Essex.

Halothane anaesthetic (Fluothane)  ICI Plc., Pharmaceutical Divn., Alderley Park, Macclesfield, Cheshire.

Anaesthetic machine
Fluotec
Cyprane Ltd., Keighley, England

Fluovac
International Market Supply, 183 Crompton Road, Macclesfield, Cheshire. SK1 8EH.

Fluosorber
Shirley Aldred & Co. Ltd., Medical Equipment Division, Worksop, Notts. S80 3EY.

Hypnorm  (Fentanyl base 0.20mg/ml Fluanisone 10.0 mg/ml)  Janssen Pharmaceutical Ltd.
Dist. By Crown Chemical Co. Ltd., Lamberhurst, Kent.

Hypnovel  (Midazolam 5 mg/ml)  Roche Products Ltd., P.O.Box 8, Welwyn Garden City, Herts. AL7 3AY.

Pentobarbitone forte  (Pentobarbitone sodium 200mgs/ml)  Veterinary Drug Co. Plc., York.

Surgical materials

Alcide ABQ sterilising solution  Lab. Care Precision Ltd., Upper Rucinge, Ashford, Kent. TN26 2PJ

Shaver  Oster Corpn., Milwaukee, Wisconsin
Small animal clipper

Cautery  Down Brothers and Mayer and Phelps Ltd., Mitcham, Surrey
7 inch burner
Surgical sutures
5/0 prolene W8710 round bodied 13 mm needle
3/0 mersilk W502 cutting needle
16 mm needle

Thackray
Ethicon Ltd., UK., Supplies Dept., L.G.H.
Ethicon Ltd. U.K.

Blood pressure measurement
Tail cuff
Photoelectric Pletysmograph
Oscilloscope
Model F.0859 016.
Intra-arterial
Tubing
PP50 (800/110/200/100)
PP10 (800/110/100/100)
Oscillograph
Washington MD2 2 Channel curvilinear
FC118 Coupler
EM750 blood pressure transducer

Medical Physics Dept.,
Leicester Royal Infirmary.
Farnell Electronic Components,
Canal Road, Leeds LS12 2TU.
Portex. Dist. by Hawsell Ltd.,
Homerton St., Cambridge
CB2 2NZ.
Palmer Bioscience, Harbour Est.,
Sheerness, Kent. ME12 1RZ.

Other items
Metabolic cages
Model for rats > 300g
Tecniplast Gazzada s. a r.l.
1 21020 Buguggiate (VA), Italy

Fume cupboard.
Astecair 4000 H filtration fume cupboard
Astec Environmental Systems Ltd.,
31 Lynx Crescent, Weston Industrial Estate, Weston-Super-Mare, Avon,
BS24 9DJ.
Weighing balances
   Mettler H31
   Sartorius 1401

Gallenkamp, Bilton Road
West, Loughborough, Leics. LE11 0TR.

Balance Consultancy Systems Ltd.,
35 Harford Street, Trowbridge,
Wiltshire

Avery domestic balance
W.& T. Avery Ltd., Smethwick, Warley,
West Midlands. D66 2LP.
APPENDIX 2

ANIMAL DIETS

Analysis of dietary composition.

The animal diets were supplied by RHM Research and Engineering Ltd., High Wycombe, Bucks. (They no longer provide this service.) The dietary composition listed overleaf was supplied by the manufacturers and was determined according to the weight of each substance added.

The ionic content of the diet per gram of food for sodium and potassium was analysed by Mr. Nigel Godfrey (Dept. of Medicine, Leicester University) as follows:-

Quadruplicate 3g aliquots of dried food were ashed overnight in a crucible at 500°C. 10mls of 1 M HCl was added and the suspension was centrifuged at 1000 rpm for 15 minutes. The sodium and potassium concentrations in the supernatant were analysed in a flame photometer and the concentrations of these ions per g of dry food were calculated.
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Control diet</th>
<th>Cholestyramine diet</th>
<th>Cholesterol diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% by weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
</tr>
<tr>
<td>Premix</td>
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<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Casein BDH</td>
<td>24.0</td>
<td>24.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Solka Floc</td>
<td>8.0</td>
<td>6.0</td>
<td>3.0</td>
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<tr>
<td>Maize starch</td>
<td>53.5</td>
<td>53.5</td>
<td>53.5</td>
</tr>
<tr>
<td>Caster sugar</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Ca.Hyd-O-Phosphate</td>
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<td>1.4</td>
<td>1.4</td>
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<tr>
<td>Magnesium carbonate</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
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<tr>
<td>Potassium chloride</td>
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<td>0.95</td>
<td>0.95</td>
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<tr>
<td>Cholestyramine</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
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<tr>
<td>Cholic acid</td>
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<td>0</td>
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## Fatty Acid Analysis

Results are % of total fatty acids

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<tr>
<th></th>
<th>Control</th>
<th>Cholesterol-supplemented diet</th>
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<tbody>
<tr>
<td>C10:0</td>
<td>Decanoic Acid</td>
<td>0.4</td>
</tr>
<tr>
<td>C12:0</td>
<td>Lauric Acid</td>
<td>0.6</td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic Acid</td>
<td>2.0</td>
</tr>
<tr>
<td>C15:0</td>
<td>Pentadecanoic Acid</td>
<td>0.6</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic Acid</td>
<td>15.0</td>
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<tr>
<td>C16:1</td>
<td>Palmitoleic Acid</td>
<td>0.4</td>
</tr>
<tr>
<td>C17:0</td>
<td>Heptadecanoic Acid</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic Acid</td>
<td>4.0</td>
</tr>
<tr>
<td>C18:1</td>
<td>Oleic Acid</td>
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<tr>
<td>C18:2</td>
<td>Linoleic Acid</td>
<td>44.3</td>
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<tr>
<td>C18:3</td>
<td>Linolenic Acid</td>
<td>1.8</td>
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<td>C20:0</td>
<td>Arachidic Acid</td>
<td>0.4</td>
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<tr>
<td>C20:1</td>
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<td>0.3</td>
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<tr>
<td>C22:0</td>
<td>Behenic Acid</td>
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<tr>
<td>C24:0</td>
<td>Lignoceric Acid</td>
<td></td>
</tr>
<tr>
<td>Others</td>
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<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>100</strong></td>
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APPENDIX 3
BIOCHEMISTRY

Blood samples were allowed to clot at room temperature and serum was separated by centrifugation. Samples were stored at 4°C until analysed, usually within one week of sampling. Serum electrolyte concentrations were measured with a flame photometer. Other biochemical measurements were performed using commercial assay kits as listed using a Vitalab 100 autoanalyser. Adjustments were made to sample and reagent volumes to suit the low volume of serum available. Alternate samples were assayed from each dietary group to avoid any bias due to assay drift during the run. Absorbance values were corrected for a reagent blank with each run and then calibrated against a single aqueous standard or reference serum sample. Quality control reference samples were assayed at the beginning and end of each run. Any run in which the reference sample fell outside the control range was discarded. Samples were not routinely run in duplicate due to the low volume of serum available.

Between batch and within-batch quality control coefficients of variation are listed below.

- Flame photometer: Corning Medical & Scientific Ltd., Corning 480, Halstead, Essex, CO9 2DX.
- Autoanalyser: Vital Scientific Ltd., Vitatron SPS, Huffwood Trading Est., Unit 14, Partridge Green, W. Sussex, RH13 8AU.
- Sample Tubes: Sarstedt Ltd., 68 Boston Road, Sarstedt microtube 72.692, Beaumont Leys, Leicester LE4 1AW.
- Microcentrifuge tubes: Boehringer Corporation (London) Ltd., Boehringer Mannheim House, Bell Lane, Lewes, E. Sussex, BN7 1LG.
**BIOCHEMICAL ASSAY METHODS**

**Suppliers**
Boehringer Corporation Ltd., (BCL), Boehringer Mannheim House, Bell Lane, Lewes, E. Sussex, BN7 1LG.
Beckman Instruments Inc., Progress Road, Sands Industrial Estate, High Wycombe, Bucks. HP12 4JL.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Sample volume (µl)</th>
<th>Method</th>
<th>Manufacturer</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>5</td>
<td>C-system, CHOD-PAP</td>
<td>Beckman</td>
<td>Precipath UBS (BCL)</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>10</td>
<td>Enzymatic colorimetric</td>
<td>Boehringer</td>
<td>Precilip (BCL)</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>50</td>
<td>Phosphotungstate &amp; magnesium chloride</td>
<td>Boehringer</td>
<td>Precipath UBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total triglyceride</td>
<td>5</td>
<td>GPO-PAP</td>
<td>Boehringer</td>
<td>Precipath UBS</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>5</td>
<td>Enzymatic colorimetric</td>
<td>Boehringer</td>
<td>Precilip (BCL)</td>
</tr>
<tr>
<td>Serum total protein</td>
<td>10</td>
<td>Biuret</td>
<td>Beckman</td>
<td>Precipath UBS</td>
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<tr>
<td>Urine total protein</td>
<td>50</td>
<td>Tetrabromophenol blue [385] In house</td>
<td></td>
<td>Aqueous</td>
</tr>
<tr>
<td>Albumin</td>
<td>5</td>
<td>Bromcresol-green</td>
<td>Beckman</td>
<td>Precipath UBS</td>
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<td>Urea</td>
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<td>Urease GLDH</td>
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<td>Hexokinase GOD-PAP</td>
<td>Boehringer</td>
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</table>

Precipath UBS and Precilip are reference serum samples.
### Between-batch quality control for period 6/11/86 to 16/9/88

(HDL only from 15/7/87)

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<tr>
<td></td>
<td>mmol/L</td>
<td></td>
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<tr>
<td>Total cholesterol</td>
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<td>2.53 - 3.21</td>
</tr>
<tr>
<td>HDL cholesterol</td>
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<td>0.96 - 1.32</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.19</td>
<td>1.07 - 1.31</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.05</td>
<td>5.45 - 6.65</td>
</tr>
<tr>
<td>Urea</td>
<td>8.67</td>
<td>7.46 - 9.88</td>
</tr>
<tr>
<td>Total protein g/L</td>
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<td>57.1 - 67.1</td>
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<tr>
<td>Albumin g/L</td>
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<tr>
<td>Urine protein</td>
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### Within-batch quality control

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<th>control range</th>
<th>value</th>
<th>CV% n=16</th>
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<td>3.06</td>
<td>2.63 - 3.49</td>
<td>3.04</td>
<td>0.8</td>
</tr>
<tr>
<td>mmol/L</td>
<td></td>
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<tr>
<td>Free cholesterol</td>
<td>2.67</td>
<td>2.30 - 3.04</td>
<td>2.74</td>
<td>0.6</td>
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<tr>
<td>mmol/L</td>
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<tr>
<td>HDL cholesterol</td>
<td></td>
<td>Pooled rat serum</td>
<td>2.65</td>
<td>5.1</td>
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<td>mmol/L</td>
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<tr>
<td>Triglyceride</td>
<td>1.29</td>
<td>1.16 - 1.42</td>
<td>1.31</td>
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<tr>
<td>Phospholipid</td>
<td>5.96</td>
<td>5.24 - 6.68</td>
<td>6.10</td>
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<tr>
<td>mmol/L</td>
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<td></td>
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<tr>
<td>Glucose</td>
<td>6.66</td>
<td>5.99 - 7.33</td>
<td>6.51</td>
<td>1.0</td>
</tr>
<tr>
<td>mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>9.90</td>
<td>9.00 - 10.80</td>
<td>10.7</td>
<td>1.5</td>
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<tr>
<td>mmol/L</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>63.0</td>
<td>58.0 - 68.4</td>
<td>62.5</td>
<td>2.6</td>
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<tr>
<td>g/L</td>
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</tr>
<tr>
<td>Albumin</td>
<td>31.0</td>
<td>27.9 - 34.1</td>
<td>29.1</td>
<td>1.7</td>
</tr>
<tr>
<td>g/L</td>
<td></td>
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</tbody>
</table>
APPENDIX 4

MATERIALS USED IN TISSUE LIPID ANALYSIS

Sonicator Kerry

Tissue grinders Gallenkamp

Screw necked vials F.B.G. Trident
(aluminium rather than Supplied by Richardson of Leicester waxed cork cap inserts) Ltd., Evington Valley Road, LE5 5LJ.

Sample concentrator heater Fisons Scientific Equipment Division, Techne SC-3 Bishop Meadow Rd., Loughborough, Leics. LE11 ORG

Chromatography tank BDH Ltd., Fourways, Carlyon Industrial 306/6420/00 Estate, Atherstone, Warwicks CB91 BAG

Chromatography plates BDH Ltd.
E. Merck ART 5721 plastic base silica gel

Applicator syringe Hamilton Fisons
100μL volume, point style 3
APPENDIX 5

HISTOLOGY

Chemicals used in Chapter 24

Oil Red 0
  colour index 26125  BDH

Toluidine blue
  colour index 52040  BDH

Mayers haematoxylin
  colour index 75290  Difco, Central Avenue, E.Molesey, Surrey.

XAM neutral medium
  improved white mountant  Gurr., supp. BDH.

Apathy's mounting medium
  (Highman's modification)  BDH

Xylene  Infrachem

Formaldehyde solution 36%  Evans Medical Ltd., Greenford, Middx.

OCT compound (Tissue-Tek)  Raymond A Lamb,
  6, Sunbeam Road, London NW10 6JL.

Equipment at Leicester General Hospital

Cryostat  Slee Medical Equipment Ltd. London.

Microtomes  Anglia Scientific Ltd., Cambridge.

Automatic tissue processor  Shandon Southern Products Ltd.,
  Chadwick Road, Astmoor, Runcorn, Cheshire WA17 1TR.

Tissue-tek embedding systems  Shandon Southern Products Ltd.
METHODS

Periodic Acid Schiff reaction.

Ingredients:
- Basic fuchsin (for Feulgen and Gomori) 1g.
- Distilled water 200 mls.
- Potassium or sodium metabisulphite 2g.
- Concentrated hydrochloric acid 2 mls.
- Decolourising charcoal 0.2g.

Reagent preparation:
Heat distilled water to boiling and add the basic fuchsin. Mix and cool to 50°C. Add metabisulphite, mix and cool to room temperature. Add the hydrochloride acid and leave overnight in the dark at room temperature. Next day, add charcoal and mix for 1-2 minutes. Filter and store at 4°C in a dark container. (Solution should be discarded when colour changes from pale yellow to pink.)

Staining technique:
1. Rehydrate section in water.
2. Apply 1% of periodic acid for 5 minutes.
3. Wash in distilled water.
4. Apply Schiff solution for 10 minutes.
5. Wash in running tap water for 15 minutes.
6. Counterstain with Mayer's haematoxylin for 30 seconds.
7. Blue in Scott's alkaline tap water solution.
8. Dehydrate in alcohol.
10. Mount in XAM.
Toluidine-Blue
Preparation:
Dissolve 1g toluidine blue in a 1% solution of sodium tetraborate.

Staining technique:
1. Rehydrate section in water.
2. Apply toluidine blue for 2 minutes.
3. Wash briefly in 70% alcohol.
5. Mount in XAM.

Oil Red 0 neutral lipid stain.
Preparation:
Dissolve 0.2-0.5% oil red 0 in isopropanol. Warm solution in 56°C water bath for 1 hour. Cool and store. Working solution is prepared immediately prior to use as follows:- 30 mls of Oil Red 0 in isopropanol are added to 20 mls of distilled water. The mixture is left for 10 minutes and filtered through filter paper prior to use.

Scott's alkaline water
Preparation: Dissolve 7g of sodium bicarbonate and 40g of magnesium sulphate in 1.2 litres of tap water. Add a few crystals of thymol.

Mayer's haematoxylin
Preparation:
1. 2g haematoxylin are dissolved in 10 mls of 99% alcohol (solution A.)
2. 100g of aluminium potassium sulphate are dissolved in 2 litres of water (solution B.)
3. Mix solution A. with solution B.
4. Add in order: 0.4g of sodium iodate.
   0.4g of citric acid.
   100g of chloral hydrate.
5. Filter before use.
**Oil red 0**

Staining technique used in experiment 4:
1. Rinse frozen section in 70% alcohol.
2. Stain with dye for 15 minutes.
3. Rinse briefly with 70% alcohol.
4. Strain in Mayer's haematoxylin for one and a half minutes.
5. Wash.
6. Blue in Scott's alkaline water.

Staining technique used in experiment 6:
1. Cut frozen sections at 8μm and store overnight at -20°C.
2. Place negative control in a 50:50 solution of chloroform:methanol for 1 hour at 60° C to delipidise.
3. Rinse negative and positive sections in 60% triethyl phosphate.
4. Stain in Oil Red 0 solution for 20 minutes.
5. Differentiate in 60% triethyl phosphate until delipidised control is macroscopically colourless.
6. Wash well in H2O and counterstain in Mayer's haematoxylin for 3 minutes or in toluidine blue.
7. Wash well and 'blue' in Scott's Tap Water substitute.
8. Mount in aquamount.
Immunohistochemistry

**Anti-human polyclonal antibody**

<table>
<thead>
<tr>
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<th>Supplier</th>
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<tr>
<td>ß lipoprotein</td>
<td>Biogenesis Ltd., 12, Yeomans Park, (BPO 718)</td>
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<td>Yeomans Way, Bournemouth, BH8 OBJ</td>
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**Anti-rat monoclonal antibodies**

<table>
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<tr>
<td>Leucocyte common Ag</td>
<td>Sera-lab Ltd., Crawley Down, Surrey, RH104FF</td>
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<tr>
<td>(OX-1; MAS 026p)</td>
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<tr>
<td>Macrophages/monocytes</td>
<td>Serotec Ltd., 22 Bankside, Station</td>
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<tr>
<td>(ED-1; MCA 341)</td>
<td>Approach, Kidlington, Oxford, OX5 1JE</td>
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<tr>
<td>Ia antigen (common determinant)</td>
<td>Sera-lab Ltd.</td>
</tr>
<tr>
<td>(OX-6; MAS 043p)</td>
<td></td>
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</tbody>
</table>

**Indirect immuno-alkaline phosphatase staining technique.**

1. Fix in acetone or acetone:methanol 80:20.
2. Block non-specific protein binding sites with 1% human serum albumin in phosphate buffered saline (PBS) for 15 minutes at room temperature.
3. Incubate with primary mouse anti-human monoclonal antibody for 45-60 minutes. Wash in PBS for 5 minutes three times.
4. Incubate with secondary goat anti-mouse antibody, conjugated with calf intestinal alkaline phosphatase, for 30 minutes. Wash in PBS as in No.3 with last wash containing levamisole to block endogenous alkaline phosphatase.
5. Incubate with naphthol AS-BI phosphate, fast red TR salt and levamisole for 30 minutes.
6. Counterstain with Mayer's haemalum for 15 minutes.
7. Mount in glycerol gelatin.
8. For negative controls omit primary antibody.
**Immunoperoxidase staining technique**

**anti-β lipoprotein antibody**
1. Cut frozen sections at 8 μ and store overnight at -20°C.
2. Fix sections in acetone for 10 minutes.
3. Block sections in 3% H₂O₂ in methanol for 30 minutes at room temp.
4. Wash in water and then rinse in Tris buffered saline/bovine serum albumin.
5. Place in normal swine serum (1:20) for 10 minutes.
6. Place in anti-β lipoprotein antibody overnight (neat) at room temp.
7. Wash in TBS/BSA for 20 minutes.
8. Place in rabbit anti-goat peroxidase-conjugated (1:25) for 30 minutes at room temperature.
9. Wash in TBS/BSA for 20 minutes.
10. Place in swine anti-rabbit peroxidase-conjugated (1:50) for 30 minutes at room temperature.
11. Wash in TBS/BSA for 20 minutes.
12. Develop sections for peroxidase using DAB for 5 minutes.
13. Wash in water. Counterstain in Mayer's haematoxylin or toluidine blue.
14. Dehydrate, clear and mount in Xam.

Negative control omits stage 6 and is left in tris-buffered saline.

**OX-1, OX-6 and ED-1 antibodies**
1-4 as above.
5. Place in normal rat serum for 10 minutes at room temperature.
6. Place in primary antiserum at room temperature overnight (OX-1 = 1:10; OX-6 = 1:20; ED-1 = 1:2000 dilution).
7. Wash in TBS/BSA for 20 minutes.
8. Place in rabbit anti-mouse peroxidase-conjugated (1:40) for 30 minutes.
9-14 as above.
TRANSMISSION ELECTRON MICROSCOPY

Processing method used in subtotal nephrectomy experiment (Chapter 24).

Cubes of tissue were fixed in 3% w/v glutaraldehyde for at least 4 hours, washed in 0.2M cacodylate buffer, block-stained in 1% w/v osmium tetroxide for 1 hour and dehydrated through graded alcohols. The tissue was immersed in propylene oxide for 20 minutes before embedding in epoxy resin (Emix, Emscope Ltd., U.K.) and curing at 60°C for at least 16 hours. 90nm sections were collected on copper grids, stained with uranyl acetate and lead citrate and examined on a Jeol 100 CX TEM/SCAN electron microscope.

Method used for unilateral nephrectomy experiment (Chapter 26).

Tissue was fixed in a special fixative which formed an insoluble complex of free cholesterol with digitonin, in an attempt to reduce losses of lipid from the tissue during processing [386]. The fixative was prepared according to the following method:-

Three separate solutions were prepared:
A - 2.5ml of 50% glutaraldehyde added to 2.5ml of 0.2M cacodylate/HCl buffer at pH 7.2;
B - 1g of paraformaldehyde heated in 22.5ml of distilled water to 60°C, stirred vigorously until the solution became almost clear, 2-3 drops of N NaOH added to clear and the mixture cooled;
C - 0.1g of digitonin dissolved in 22.5ml of 0.2M cacodylate/HCl buffer at pH 7.2, boiled carefully until the solution cleared and then cooled.

Solutions A, B and C were combined and added to 0.025g of CaCl₂.

Cubes of tissue were fixed for 18 hours at room temperature and washed in 0.2 M cacodylate buffer, block-stained in 1% w/v osmium tetroxide for 1 hour and dehydrated through graded alcohols. Tissue was then immersed in a 50% alcohol:Epon monomer mixture overnight, transferred to neat Epon resin monomer for 8 hours and thence into activated resin. The resin was cured at 60°C for 18 hours. Sections were collected, stained and examined as above.
SECTION IV

SUMMARY AND CONCLUSIONS
CHAPTER 27
THE ROLE OF LIPIDS IN THE PATHOGENESIS OF GLOMERULOSCLEROSIS

Before making an assessment of the role of lipids in the pathogenesis of glomerulosclerosis, the main points raised in the preceding chapters will be summarised.

Abnormalities of lipid metabolism often accompany renal disease. Deposition of lipid within damaged glomeruli is also common and may be underestimated if not looked for specifically.

A comparison of the histological features of atherosclerosis and glomerulosclerosis yields some interesting similarities and these suggest that similar 'responses to injury' may be occurring in both lesions. However, there are also important differences between these lesions, for example the relative scarcity of macrophages in areas of glomerulosclerosis compared to atherosclerotic plaques.

Research directed at determining the pathogenesis of atherosclerosis has revealed a wide variety of mechanisms by which lipids could be involved in the development of glomerulosclerosis. Nonetheless, the key questions remain:

Does lipid deposition occur merely as a secondary phenomenon following glomerular damage due to another cause?

or

Does the presence of lipid within the glomerulus provoke further glomerular injury and so worsen glomerulosclerosis?

These questions have been investigated in a series of experiments. In the rat, renal damage was shown to cause secondary hyperlipidaemia in proportion to the severity of renal damage. Two types of lipid-lowering agent were used in an attempt to control this hyperlipidaemia but neither proved effective. These results emphasise the impossibility of proving that lipids have a primary role in the development of glomerulosclerosis by the use of lipid-lowering drugs. Any beneficial effect they may have on glomerular injury need not necessarily be mediated through the control of hyperlipidaemia.
Two rat models of glomerulosclerosis induced by renal ablation were used to study the effects of supplementing the diet with cholesterol. Cholesterol feeding was shown to have no detrimental effect on the development of glomerulosclerosis in rats which were hypertensive and uraemic following the removal of 2/3 of functioning renal tissue. This was despite a significant increase in circulating and tissue levels of cholesterol. These results suggest that any effect of hyperlipidaemia in causing glomerular injury is likely to be less significant than the effect of hypertension.

When cholesterol was fed to rats for a more prolonged period following the removal of half the functioning renal tissue, glomerular lipid deposition was associated with a worsening of glomerular damage. This was manifested by increased proteinuria and a significant increase in glomerular size, a known precursor of glomerulosclerosis. These changes were not the result of an increase in systemic blood pressure.

This last experiment also enabled the mechanisms by which lipid deposits are dealt with in the glomerulus to be investigated. Deposits of lipid were taken up by bone marrow-derived macrophages that had infiltrated glomeruli in small but significant numbers. The phagocytosis of lipid was associated with damage to surrounding cells, shown by capsular adhesions, but was not accompanied by a local increase in mesangial matrix production. Phagocytosis of lipid did not lead to 'immune activation' of macrophages as demonstrated by the expression of immune-associated antigen.

The effects of lipid deposition may have been mediated by two mechanisms. Vascular resistance of the afferent or efferent arterioles may have been altered on the cholesterol diet, causing an increase in glomerular capillary pressure. This may directly damage glomerular capillaries and may further increase the deposition of macromolecules in the mesangium. Alternatively or in addition to this, the phagocytosis of lipid by macrophages may have led to the release of factors which stimulated glomerular hypertrophy.

In combination with work already reported in the literature, these results suggest that the deposition in the mesangium of endogenous macromolecules such as lipid, over a prolonged period, may be important
in the eventual development of glomerular hypertrophy and sclerosis. Once injury has occurred, clearance of this material from the mesangium will be impaired and progressive accumulation will result. Hence, damage caused by the presence of material deposited in the mesangium will perpetuate a cycle of progressive glomerulosclerosis.

**Extrapolation from the rat to man.**

Direct extrapolation from the rat to man may not be valid due to the differences that exist in normal lipid metabolism. For example, most lipoproteins in the rat are α- rather than β-migrating. Cholesterol feeding increased the proportion of β-migrating lipoproteins to a level similar to that present as LDL in man. However, the β lipoproteins formed are abnormal, being composed of the remnants of VLDL breakdown, and are known to be more readily deposited in tissues than LDL.

Both uraemia and the nephrotic syndrome are associated with an impairment of the clearance of lipoproteins. In uraemia this predominantly leads to an increase in serum triglyceride. In rats, hypertriglyceridaemia did not lead to an increase in triglyceride content of the kidney. Conversely, nephrotic syndrome leads to an increase in cholesterol-rich lipoproteins and this suggests that lipid deposition similar to that produced in the rat is more likely to be seen in patients with glomerulosclerosis associated with the nephrotic syndrome.

Familial hypercholesterolaemia, type III hyperlipoproteinaemia and familial deficiency of lecithin-cholesterol acyl transferase (LCAT) are nature’s experiments for investigating the effects of prolonged hypercholesterolaemia on the kidney. In the familial hyperlipidaemias, any effects in the kidney are overshadowed by accelerated atherosclerosis. In LCAT deficiency, renal failure takes 30-40 years to develop, suggesting that any direct effect of lipids on glomeruli is low-grade in severity. Nonetheless, it is possible that this low-grade effect may be amplified in the presence of pre-existing glomerular damage.

The results of the experiments on cultured human glomeruli suggest
that macrophages are most likely to be the principal cells involved in the clearance of lipid from the human as well as the rat mesangium. Smooth muscle-type mesangial cells only expressed LDL receptors and not 'scavenger' receptors and hence are not equipped to accumulate cholesterol.

In the rat, significant glomerular hypertrophy occurred with only a small increase in the number of glomerular macrophages. The lack of a significant increase in the number of macrophages in human glomerulosclerosis may therefore not exclude the possibility that they are involved in the development of these lesions. Indeed, it is tempting to suggest that the segmental nature of the sclerotic lesions is the result of local macrophage activity.

Possible directions for future experiments.

A number of questions are posed by the results described in these experiments, in particular concerning the precise mechanism of the effect of hyperlipidaemia and lipid deposition on glomerular hypertrophy.

(a) What is the mechanism of the acute increase in urinary protein excretion present after only two weeks? Are macrophages already loaded with lipid and/or present in increased numbers at this time and is there an associated increase in glomerular size?

(b) How do these changes relate to glomerular capillary hydraulic pressure measured by micropuncture study?

(c) Does depletion of macrophages from the rat protect it from the effects of hyperlipidaemia?

(d) What is the sequence of events that occurs when dietary cholesterol supplementation is stopped? How rapidly is lipid cleared from the mesangium? Does its clearance lead to a resolution in glomerular hypertrophy and cellular infiltration?

(e) Is the behaviour of macrophages affected by loading with lipid? For example, is the production of superoxide radical or interleukin 1 affected in vitro?
(f) Is the production of inflammatory substances by isolated whole glomeruli affected by prior lipid deposition in vivo? For example, does the supernatant from cultures of such glomeruli lead to the proliferation of cultured smooth muscle-type mesangial cells?

Investigation of the importance of lipids in glomerulosclerosis in man will centre around the effects of new lipid-lowering drugs in patients with steroid-resistant nephrotic syndrome. If these drugs prove to be well tolerated, a long-term study of their use in these patients would be justified from the point of view of possible protection against atherosclerosis as well as their potential effects on glomerular damage. Such a trial would be a daunting task, requiring multicentre cooperation to recruit sufficient numbers of patients.

In the meantime, one can make some limited recommendations on the management of patients with renal disease on the basis of these experiments. It would seem advisable not to exacerbate hypercholesterolaemia by excessive amounts of dietary saturated fat and cholesterol. This is particularly relevant to uraemic children, where calorie supplements are required to promote growth. Otherwise, attention should be focussed on a factor known to be important in causing glomerular damage, namely hypertension.


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THE ROLE OF LIPIDS IN THE PATHOGENESIS OF GLOMERULOSCLEROSIS

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Most patients with chronic renal impairment develop progressive glomerulosclerosis and eventually require dialysis. The pathogenesis of this process is poorly understood. This thesis addresses the possibility that hyperlipidaemia initiates and/or aggravates glomerulosclerosis.

The handling of lipoproteins by human glomerular cells was studied by incubating cells cultured from isolated human glomeruli with fluorescent-labelled lipoproteins. It was demonstrated that all cells take up low density lipoproteins (LDL) by receptor-mediated endocytosis. Chemically modified LDL was taken up by macrophages, suggesting that human glomerular 'foam' cells are derived from monocytes rather than intrinsic mesangial cells.

A series of experiments was performed in rats to investigate the effects of manipulating serum lipids on glomerular damage. Attempts to lower serum cholesterol with cholestyramine and synvinolin were unsuccessful. Dietary cholesterol supplementation caused hyperlipoproteinaemia and glomerular lipid deposition, especially of cholesterol and cholesteryl esters. In rats made uraemic and hypertensive by $\frac{1}{3}$ nephrectomy, the diet did not affect the severity of proteinuria or glomerulosclerosis over a 7 week period. However, when administered for 21 weeks following unilateral nephrectomy, proteinuria was increased. The number of bone marrow-derived glomerular-macrophages was slightly but significantly increased and many became foam cells. These changes were not associated with glomerulosclerosis but there was a significant increase of glomerular size which is known to precede sclerosis.

These results suggest that hyperlipidaemia and glomerular lipid deposition contribute to the development of glomerulosclerosis by stimulating an influx of macrophages which may cause glomerular hypertrophy and injury through the release of factors during phagocytosis. The magnitude of these changes indicates that the effect of lipids is likely to be low-grade and only of importance over a prolonged period of time.