Oxidized low-density lipoprotein in experimental focal glomerulosclerosis

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Oxidized low-density lipoprotein in experimental focal glomerulosclerosis. Oxidized low-density lipoproteins (Ox-LDL) have been shown to be involved in the pathogenesis of atherosclerosis. Because of the similarities between atherosclerosis and focal glomerulosclerosis, a study was performed to demonstrate whether Ox-LDL could be detected in the glomeruli in experimental FGS. FGS was induced in 12 rats on a 4% cholesterol-1% choline diet by seven injections of puromycin aminonucleoside over a 10 week period. Eight rats on a normal diet served as controls. Fourteen weeks after the start of the experiment all rats were sacrificed. The test animals showed marked hypercholesterolemia and proteinuria. About 20% of the glomeruli in test animals showed FGS and variable amounts of glomerular lipid were demonstrated. Immunohistochemical staining using five specific monoclonal antibodies against various forms of Ox-LDL showed positive staining of a variable number of glomeruli in the test rats. The staining pattern appeared to be intracellular. Staining with ED1 showed significantly increased numbers of intraglomerular monocytes in the test rats (test vs. control 2.4 ± 1.1 vs. 0.4 ± 0.1 monocytes per glomerulus, P < 0.0001). Control animals showed no segmental sclerosis, no glomerular lipid, and no staining for Ox-LDL. Lipid analysis of isolated glomeruli showed increased cholesterol, increased arachidonic acid and decreased eicosapentaenoic acid in test animals compared to controls. The findings suggest a role for Ox-LDL in the pathogenesis of experimental FGS and support the hypothesis that FGS is analogous to atherosclerosis.

Human primary focal glomerulosclerosis (FGS) is a chronic progressive disease that is often manifested by the nephrotic syndrome, and frequently results in chronic renal failure. The pathogenesis of this disease is uncertain but appears to be multi-factorial [1]. There is considerable experimental and human evidence to suggest the involvement of glomerular capillary hypertension with hyperfiltration [2, 3], intraglomerular monocyte infiltration [4–7], hyperlipidemia and glomerular lipid deposition [8–13], genetic factors [14], and coagulation abnormalities [15, 16] in the development of FGS. Indeed, this constellation of factors has led to the proposal that FGS is analogous to atherosclerosis [8, 17].

Central to the development of atherosclerosis is the involvement of lipid deposition in the walls of arteries, especially low density lipoprotein (LDL) [18]. Recent experimental work suggests that the oxidation of LDL in vessel walls is a critical step in the pathogenesis of atherosclerosis [19]. Apolipoproteins B and E have been demonstrated in the glomeruli of a number of patients with FGS [20, 21]. However, to date there is no evidence that oxidized LDL (Ox-LDL) is present in glomeruli in FGS, either human or experimental.

In view of the putative importance of Ox-LDL in atherosclerosis and the concept of FGS being analogous to atherosclerosis, it is reasonable to hypothesize that Ox-LDL is involved in the pathogenesis of FGS. The purposes of the present study are to demonstrate the presence of Ox-LDL by immunohistochemical means and to perform a biochemical analysis of glomerular lipid in an experimental model of FGS.

Methods

Experimental design

FGS was induced in 12 male, 150 to 200 g Sprague-Dawley rats (Group 1) by seven subcutaneous injections of puromycin aminonucleoside (20 mg/kg; Sigma Chemical Co., St. Louis, Missouri, USA) over a 10 week period according to a previously published protocol [22]. This group was fed a 4% cholesterol-1% choline diet (by weight) (Teklad Premier, Madison, Wisconsin, USA) which has been shown to primarily increase serum total cholesterol (TC) without affecting serum triglycerides (TG), and to exacerbate aminonucleoside-induced FGS [9]. Eight male, 150 to 200 g Sprague-Dawley rats which received seven subcutaneous injections of normal saline over 10 weeks and were fed normal rat chow and water ad libitum served as controls (Group 2). Four weeks after the last injection all the animals were sacrificed by exsanguination under anesthesia. Serum was collected for creatinine (Cr), albumin (Alb), TC, TG, high density lipoprotein (HDL) and LDL, and renal tissue was obtained for histology, immunohistochemistry, histochemistry and glomerular isolation. Prior to sacrifice the animals were housed in metabolic cages for 24 hours and urine for protein excretion was collected.

Modification of lipoproteins

Reactive fatty acid oxidation products (OP) were generated by thermal autoxidation of arachidonic acid or linoleic acid [23]. Ten milligrams arachidonic acid were transferred to a glass vial open to air and heated to 37°C for 72 hours. The yellow-brown products were dissolved in Dulbecco’s PBS with 300 μM EDTA at pH 7.4, and aliquots containing the residue from 0.8 to 1.5 mg
of oxidized fatty acid were added to 1 mg LDL or bovine albumin in 1 ml Dulbecco's PBS containing 300 μM EDTA and 50 μM butylated hydroxytoluene. Irreversible derivatization of proteins occurred within minutes at 20°C; mixtures of protein and oxidation products were washed repeatedly with PBS on Amicon Centricon 30 microconcentrators to remove soluble non-bound materials. Linoleic acid oxidation products were obtained by heating to 100°C for 72 hours. The same procedure that was used with arachidonic acid oxidation products was followed for modification of LDL or albumin with linoleic acid oxidation products. Copper-oxidized LDL was prepared by incubating 200 μg/ml LDL in Dulbecco's PBS containing 5 μM CuSO₄ for 24 hours. This yielded an extensively oxidized LDL with an electrophoretic mobility in agarose about 3.5 times greater than that of native LDL, more than 70% decrease in the content of arachidonic acid and linoleic acid, and derivatization of 40 to 50% of lysine amino groups [23–25]. Malondialdehyde-LDL was prepared essentially as described by Fogelman and colleagues [26]. Malondialdehyde stock solution (0.2 mM) was generated by hydrolysis of 164 μl tetramethoxypropane with 200 μl 12 N HCL. After 1.5 minutes, the mixture was diluted with 4.6 ml 0.1 M sodium phosphate and then pH was adjusted to 6 with 10 n NaOH. Equal volumes of malondialdehyde stock solution and LDL were mixed and incubated at 37°C for three hours, and then dialyzed to remove excess malondialdehyde.

**Production of monoclonal antibodies to modified lipoproteins**

Antibodies against arachidonic or linoleic acid oxidation product-modified LDL were generated by immunizing balb/c mice with 50 μg oxidation product-modified human LDL in Freund's complete adjuvant subcutaneously, and then four or five injections of 50 μg oxidation product-modified human LDL in Freund's incomplete adjuvant at 14-day intervals. Three days before fusion the mice received an intravenous injection of 100 μg oxidation product-modified human LDL via the tail vein. Splenocytes were fused with NS-1 myeloma cells, and hybridomas were selected and cloned according to previously described techniques [27]. To identify clones specific for adducts of oxidation products with lysine residues, and not for human apolipoprotein B, primary screening of hybridoma supernatants was performed by ELISA using by 96-well plates coated with the corresponding oxidation product-modified bovine serum albumin. An identical immunization protocol was used to obtain antibodies against copper-oxidized LDL, except that the immunogen was copper-oxidized human LDL. To select hybridomas specific for oxidized LDL, culture supernatants were doubly screened against native LDL and oxidized LDL. Only clones that produced antibodies reactive against oxidized LDL but not native LDL were retained. Hybridomas were expanded by intraperitoneal injection into pristane-primed balb/c mice. Antibody class and subtype were determined with a commercial ELISA typing kit (Bio-Rad, Richmond, California, USA).

For these studies, five monoclonal antibodies were selected. One (AOP-7) is an IgG₂, antibody that specifically recognizes lysine adducts of arachidonic acid oxidation products. The specificity of this antibody is demonstrated in the competition ELISA shown in Figure 1. Antibody AOP-7 showed partial cross-reactivity with malondialdehyde LDL. LOP-23 is an IgG₃ monoclonal antibody specific for proteins modified by linoleic acid oxidation products (Fig. 1). Antibody AOP-24 is an IgM antibody that recognizes proteins modified with arachidonic acid.
primary MoAb.

MoAb for the primary MoAb and omitting incubation with the tissue LDL during the immunostaining procedure, the endogenous peroxidase-quenching step utilizing H2O2 was performed.

It has been demonstrated that H2O2 with peroxidase can modify LDL in vitro [29]. To avoid potential modification of tissue LDL during the immunostaining procedure, the endogenous peroxidase-quenching step utilizing H2O2 was performed following the application of the secondary antibody when staining for Ox-LDL.

Control studies were performed by substituting an irrelevant MoAb for the primary MoAb and omitting incubation with the primary MoAb.

Histology

Tissue was fixed in methyl Carnoy's solution and embedded in paraffin. Three micron sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff reagent (PAS).

Immunohistochemistry

The avidin-biotin-peroxidase complex procedure (Vector Laboratories, Burlingame, California, USA) for antibody localization was used as previously described [6]. To demonstrate Ox-LDL, liquid nitrogen snap-frozen renal tissue fixed briefly in 4% buffered paraformaldehyde was incubated with mouse monoclonal antibodies (MoAb) specific for various products of oxidation of LDL at a dilution of 1:200. To detect monocytes/macrophages (MO) methyl Carnoy's-fixed paraffin-embedded sections from all animals and snap-frozen sections from four test rats were incubated with ED1 (Serotec, Oxford, UK), a mouse monoclonal antibody specific for rat MO [28]. Human atherosclerotic aorta and rat spleen served as positive controls for the demonstration of Ox-LDL and MO, respectively.

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Glomerular lipid analysis

Approximately 1.5 kidneys per rat were used for glomerular lipid analysis. Glomeruli were obtained by differential sieving of fresh renal cortex with stainless steel grids of varying mesh size (grades 100, 150, and 200 in ascending order). The preparation which was 90 to 95% pure was suspended in Ca2+-free phosphate-buffered saline. Transport and isolation procedures were carried out on ice to prevent the degradation of fatty acids. The number of glomeruli per sample was determined using a hemocytometer. Total lipids were extracted by the method of Folch, Lees and Stanley [30]. The cholesterol concentration was determined enzymatically [31] and phospholipid was assayed from the content of lipid phosphorus [32]. Phospholipids were resolved from other classes by thin layer plate chromatography using petroleum ether/diethyl ether/acetic acid 85:15:3 (vol/vol/vol) as the solvent system. The phospholipids were recovered, the fatty acids (FA) converted to their respective methyl esters and separated, and quantified by gas liquid chromatography [33]. The separation was accomplished using a Varian 3400 gas liquid chromatograph equipped with a 30 m capillary column and a Varian Star Integrator data handling system. The internal standard in all samples was heptadecanoic acid (17:0).

Histochromy

Snap-frozen renal cortex sections were fixed in 10% buffered formalin and stained with oil red O for neutral lipid.

Biochemistry

Serum Cr, Alb, TC, TG, HDL, and LDL and urine protein (U_P) were determined by standard hospital laboratory methods.

Statistics

Results are presented as mean values ± SD. Student’s t-test was used to compare means. A P value of less than 0.05 was considered significant.
Table 1. Mean values (± sd) of measured serum and urine biochemical parameters in Group 1 and 2 rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1</th>
<th>Group 2</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr µmol/liter</td>
<td>41 ± 6</td>
<td>39 ± 4</td>
<td>0.4191</td>
</tr>
<tr>
<td>Alb g/liter</td>
<td>31 ± 2</td>
<td>32 ± 3</td>
<td>0.2872</td>
</tr>
<tr>
<td>U&lt;sub&gt;P&lt;/sub&gt; g/day</td>
<td>0.37 ± 0.19</td>
<td>0.02 ± 0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TC µmol/liter</td>
<td>15.06 ± 6.17</td>
<td>1.66 ± 0.24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TG µmol/liter</td>
<td>2.42 ± 1.05</td>
<td>2.04 ± 1.15</td>
<td>0.4548</td>
</tr>
<tr>
<td>HDL µmol/liter</td>
<td>1.87 ± 0.59</td>
<td>1.21 ± 0.16</td>
<td>0.0063</td>
</tr>
<tr>
<td>LDL µmol/liter</td>
<td>12.09 ± 5.53</td>
<td>0 ± 0</td>
<td>&lt;0.0001</td>
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* P < 0.05 is considered significant (Student’s t-test)

Results

General

Both groups of animals gained weight during the test period. At the end of the experiment the mean body weight of Group 1 was 463 ± 45 g while that of group 2 was 550 ± 19 g, the difference being significant (P < 0.0001). The kidney weights for both groups were comparable (Group 1 = 4.33 ± 1.00 g, Group 2 = 3.65 ± 0.26 g, P = 0.0784).

Biochemistry

Table 1 depicts the mean ± sd values for the parameters evaluated in both groups. There was no significant difference in renal function (Cr) between the two groups (Group 1 = 41 ± 6 &micro;mol/liter, Group 2 = 39 ± &micro;mol/liter, P = 0.4191). Urinary protein excretion was significantly greater in Group 1 (U<sub>P</sub> = 0.37 ± 0.19 g/day) than in Group 2 (U<sub>P</sub> = 0.02 ± 0.01 g/day; P < 0.0001). Mean serum Alb for Group 1 was 31 ± 2 g/liter while that for Group 2 was 32 ± 3 g/liter, the difference being insignificant (P = 0.2872). Serum TC and LDL were much higher in Group 1 (TC = 15.06 ± 6.17 mmol/liter, LDL = 12.09 ± 5.53 mmol/liter) than in Group 2 (TC = 1.66 ± 0.24 mmol/liter, LDL = 0.00 ± 0.00 mmol/liter (levels of LDL were too low to be detected by our methods); P < 0.0001) for TC and LDL). Mean TG and HDL were also higher in Group 1 (TG = 2.42 ± 1.05 mmol/liter, HDL = 1.87 ± 0.59 mmol/liter) than in Group 2 (TG = 2.04 ± 1.15 mmol/liter, HDL = 1.21 ± 0.16 mmol/liter) the difference being significant for HDL only (P = 0.0063 for HDL, P = 0.04548 for TG).

Immunohistochemistry

Ox-LDL was demonstrated in glomeruli of the kidneys of all members of Group 1, but in none of the Group 2 animals. The lipid was present only in glomeruli (Fig. 4) and appeared to be intracellular. The distribution of the Ox-LDL corresponded in extent and distribution with the oil red O material. Glomerular staining was positive for all five MoAbs used. The reactions for the two monoclonal IgG antibodies (AOP = 7 and LOP = 23) appeared stronger and more extensive than those for the three monoclonal IgM antibodies (AOP = 24, Ox = 10, Ox = 14). The pattern of reaction with the anti-Ox-LDL MoAbs was granular or punctate in most glomeruli with the reaction product diffusely distributed within what appeared to be cellular cytoplasm (Fig. 4). In some areas one or more small, clear vacuoles coexisted with the reaction product. In occasional glomeruli the reaction product was distributed peripherally around a large vacuole (Fig. 4). The various reaction patterns were very similar to those reported by Rosenfeld et al for Ox-LDL in atherosclerotic aortic lesions in WHHL rabbits [34].

Staining with ED1 showed that the Group 1 animals had a significantly higher mean intraglomerular MO count (2.4 ± 1.1) than did Group 2 rats (0.4 ± 0.1; P < 0.0001, Fig. 5). Most but not all FC were ED1 positive. Comparison of the frozen kidney sections from test rats stained with ED1 to those stained for the oxidized products demonstrated that the localization and distribution of ED1 cells with features of FC appeared to be very similar to that of the oxidized products within glomeruli.

Glomerular lipid analysis

Mean total cholesterol per glomerulus was higher in Group 1 (86.5 ± 60.3 &micro;mol) than in Group 2 (64.9 ± 77.0 &micro;mol), but the

Fig. 3. Focal glomerulosclerosis. The glomerulus shows a relatively early segmental sclerotic lesion at about 9 o'clock. A foam cell is present within the lesion (arrowhead). (Hematoxylin and eosin, ×250)

Fig. 5. Immunohistochemistry for Ox-LDL. A, normal rat kidney; B, group 1 rat. The reaction with anti-Ox-LDL MoAbs was granular or punctate in most glomeruli. (Immunoperoxidase with diaminobenzidine tetrahydrochloride, ×250)
Fig. 4. Oxidized low-density lipoprotein (Ox-LDL) in glomeruli in rats with focal glomerulosclerosis. Immunohistochemical staining of kidney tissue from test rats show intracellular localization of Ox-LDL in glomeruli using monoclonal antibodies AOP-7 (A) and LOP-23 (B). (Immunoperoxidase, ×250).

Fig. 5. Intraglomerular monocytes in focal glomerulosclerosis (FGS). Immunohistochemical staining for rat monocytes with ED1 shows several ED1 + cells (arrowheads) in the glomerulus of a rat with FGS. (Immunoperoxidase, ×250).

difference was not statistically significant. This was probably due to the wide variation in glomerular lipid content demonstrated by oil red O staining and possible glomerular count error. Glomerular phospholipid content was very similar in both groups (Group 1 = 46.5 ± 28.1 μmol/glomerulus, Group 2 = 48.8 ± 44.7 μmol/glomerulus). The ratio of total cholesterol to phospholipid per glomerulus was significantly higher in Group 1 (1.82 ± 0.66) than in Group 2 (1.14 ± 0.50; P = 0.0259).

The changes in cholesterol/phospholipid ratio were associated with significant changes in the FA composition of the glomerular phospholipids (Table 2). Within the family of saturated FA, 16:0 was higher, although not significantly so, and 18:0 significantly lower in Group 1 animals (P = 0.0003). The major mono-unsaturated FA, 18:1, was increased in Group 1 (12.8 ± 0.3%) compared to controls (7.8 ± 0.5%; P < 0.0001). In the series of n-6 and n-3 FA, Group 1 rats had significantly lower levels of the dietary essential FA 18:2n-6 (Group 1 = 20:4 n-6 (Group 1 = 27.4 ± 0.1%, Group 2 = 22.1 ± 1.9% of FA in Group 1 and 22.1 ± 1.9% of FA in Group 2, the difference being significant (P < 0.0001). The changes in phospholipid FA composition with decreased n-3 FA, especially eicosapentaenoic acid (20:5n-3), and increase in n-6, such as arachidonic acid (20:4n-6) resulted in a much higher C20 + 22

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suggested that these molecules may cause endothelial injury have been shown to be cytotoxic [47, 48], and it has been chemotaxis by inducing monocyte chemoattractant protein 1 chemotaxis by inducing monocyte chemoattractant protein 1 MO [43] and an inhibitor of macrophage motility [44], and thus [40, 41] and vice versa [42]. Ox-LDL is a chemoattractant for shown to increase the adhesiveness of endothelial cells for MO [38, 39]. Ox-LDL has been "scavenger pathway" receptor distinct from the one responsi-

n-6/n-3 ratio in Group 1 rats (17.8 ± 1.7) than in Group 2 animals (3.1 ± 0.5; P < 0.0001).

### Discussion

The significance of the finding of oxidatively modified epipithes suggesting the presence of Ox-LDL in glomeruli in experimental FGS is uncertain. There is considerable evidence suggesting an important role for Ox-LDL in the pathogenesis of atherosclerosis [19]. MO have been shown to play a key part in the development of the fatty streak, the early lesion of atherosclerosis [35–37]. In animals fed an atherogenic, cholesterol rich diet, there is arterial intimal infiltration by MO which take up native LDL [38, 39]. Ox-LDL has been shown to increase the adhesiveness of endothelial cells for MO [38, 39]. Ox-LDL has been demonstrated that the foam cells in the early atherosclerotic lesions, and to both intimal foam cells and smooth muscle cells in the transitional lesions [34]. This closely corresponds to the findings of the present study in that the great majority of the glomerular lesions were relatively early and the oxidatively modified epitopes were observed exclusively within the cytoplasm of some glomerular cells, most of which were phenotypically foam cells. The intracellular accumu-

In WHHL rabbits the oxidatively modified protein is largely confined to the cytoplasm of intimal MO-derived foam cells in the early atherosclerotic lesions, and to both intimal foam cells and smooth muscle cells in the transitional lesions [34]. This is consistent with the observations that uptake of Ox-LDL by cultured rat mesangial cells and mouse peritoneal macrophages stimulates series 2 eicosanoid production [54, 56], and that administration of an antioxidant, probucol, to cholesterol-fed rats inhibits elaboration of these particular eicosanoids [57]. A possible explanation for the increased arachidonic acid might be to compensate for the altered membrane fluidity that could result from increased membrane cholesterol and/or decreased n-3 long chain fatty acids.

A high cholesterol diet has been shown to exacerbate experimental FGS [9, 12, 58]. While the precise mechanisms for this have not been completely elucidated, several factors such as increased intraglomerular MO infiltration [9, 52, 59] which has been shown to mediate, at least in part, glomerular injury in one model of FGS [13], hypercholesterolemic-induced hyperviscosity [60], and increased glomerular capillary pressure (Pcap) [12] have been implicated. The results of the phospholipid fatty acid composition analysis in the present study are relevant to the latter mechanism. Several experimental studies suggest an association between hypercholesterolemia and glomerular levels of series 2 eicosanoids such as PGE₂ and TXB₂ [57, 61, 62]. In one of these investigations the cholesterol-associated eleva-

In conclusion, the results of this study suggest that Ox-LDL...
is present in glomeruli in experimental FGS and is largely localized to foam cells, many of which were observed in segmental sclerotic lesions. While this observation does not prove a pathogenetic role for Ox-LDL in this model of FGS, it does significantly strengthen the concept of FGS being analogous to atherosclerosis.

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