Low-density lipoprotein stimulates mesangial cell proteoglycan and hyaluronan synthesis

Ravinder S. Chana*, David C. Wheeler*, Gareth J. Thomas, John D. Williams and Malcolm Davies

University of Wales College of Medicine, Institute of Nephrology, Heath Park, Cardiff, UK

Abstract

Background. Hyperlipidaemia leads to glomerulosclerosis in small mammals and may contribute to progressive renal disease in man. One prominent feature of lipid-induced glomerular injury in animal models is the accumulation of mesangial matrix. These studies were designed to investigate whether low-density lipoprotein (LDL) enhanced mesangial cell (MC) matrix deposition by modulating the production of proteoglycans (PG) and hyaluronan (HA).

Methods. Growth arrested human MC were metabolically labelled with either 50 μCi/ml Na₂[³⁵S]sulphate or 25 μCi/ml [³H]glucosamine and stimulated with LDL (10–100 μg/ml). The radiolabelled PG and HA extracted from the cell layer and the culture medium were isolated, quantified and characterized. Comparison of the PG core proteins synthesized by MC was carried out using Western blot analysis.

Results. LDL stimulation led to a dose- and time-dependent increase in [³⁵S]sulphate incorporation into PG in the culture medium and to a lesser extent in the cell layer. Analysis of the glycosaminoglycan (GAG) chains showed no difference in either their size or charge. Enzyme digestion studies demonstrated that the synthesis of both chondroitin sulphate PG (CSPG) and heparan sulphate PG (HSPG) was enhanced as was the production of the core proteins of versican (a large CSPG), perlecán (a basement membrane HSPG) and to a lesser extent decorin (a small dermatan sulphate PG (DSPG)). An increase in HA synthesis was also demonstrated in [³H]glucosamine labelled cells following LDL stimulation.

Conclusion. LDL selectively enhances the synthesis of specific PG and HA by mesangial cells. Such effects may contribute to the expansion of the mesangial matrix and modify cell-matrix interactions in lipid-induced renal damage.

Key words: glomerulosclerosis, hyaluronan, lipid, low-density lipoprotein, mesangial cell, proteoglycans

Introduction

Although many different disease processes initiate kidney damage, all may lead eventually to a final common pathway characterized histologically by glomerulosclerosis and tubulointerstitial fibrosis [1]. Glomerular lesions similar to those seen in the end-stage kidney can be induced in small mammals by feeding high cholesterol diets whilst in several animal models of kidney disease, correcting the lipid abnormalities that complicate uraemia or heavy proteinuria slows the progression of renal injury [2]. Such observations suggest that abnormalities of lipid metabolism may exacerbate glomerular damage. Histological studies have demonstrated lipoprotein deposition in the glomerular mesangium during the early stages of glomerulosclerosis whilst accumulation of extracellular matrix is a critical event in the development of scarring [3]. Similar changes are observed in the arterial intima in atherosclerosis and it has been suggested that both glomerulosclerosis and atherosclerosis share common pathogenic mechanisms. This analogy is further strengthened by the fact that the cells playing a key role in these scarring processes, namely glomerular MC and vascular smooth muscle cells respectively, are closely related in terms of morphological and functional characteristics. In vitro studies have demonstrated that incubation of vascular smooth muscle cells with low-density lipoprotein (LDL) stimulates production of matrix components thus providing a possible link between lipid deposition and matrix accumulation in the arterial wall [4].

Glomerular mesangial matrix comprises a complex structure of interacting macromolecules which aggregate to form polymers, providing support for cells and influencing their behaviour through cell surface integrin receptors. Analysis of the lesions which characterize glomerulosclerosis in humans reveals that scarred areas contain an excess of the same molecules present in normal matrix, namely collagen, fibronectin, laminin,
glycoproteins and PG [5]. Proteoglycans comprise one or more glycosaminoglycan chains covalently linked to a core protein. These macromolecules accumulate in the early stages of glomerular scarring and are found in abundance in sclerotic lesions. Hyaluronan is a high-molecular weight polysaccharide composed of repeated units of β-1,4 glucuronate and β-1-3-N-acetylgalactosamine. Within matrix, it has a number of important physicochemical functions, acting as a support for cell adhesion and locomotion and mediating cell-matrix interactions through specific binding to CD-44 and the large CSPG versican [6]. Human MC in culture have been shown to synthesise a number of different proteoglycans including versican, biglycan, decorin, and perlecan [7]. Furthermore, these cells secrete HA, which forms large aggregates with MC versican [8].

Previous studies have demonstrated that exposure of MC to LDL leads to activation, enhanced proliferation and the synthesis of eicosanoids, chemotactic cytokines, collagen type IV and fibronectin [9]. Oxidation of lipoprotein by the cells modifies its effects and may result in cytotoxic injury [10]. The following experiments are based on the hypothesis that hyperlipidaemia exacerbates renal injury by modifying MC matrix synthesis thereby causing glomerular scarring. In these experiments, the effect of LDL on the synthesis and secretion of PG and HA were investigated. The results demonstrate that activation of cells by lipoprotein stimulation leads to increase production of these matrix components with selective up-regulation of the synthesis of certain PG species.

Methods

Mesangial cell culture and metabolic labelling

Mesangial cell cultures were established from specimens of normal human kidney as previously reported by us [7]. Briefly, human MC were grown in 75 cm² culture flasks (Falcon, Cowley, UK) in RPMI supplemented with 20% foetal calf serum, penicillin (100 i.u./ml), streptomycin (100 μg/ml), insulin (5 μg/ml), sodium selenite (5 μg/ml) and transferrin (5 μg/ml) (RPMI medium) and used between passages 3–9. For labelling experiments the cells were detached with Trypsin/EDTA, washed with medium by centrifugation at 1000 g for 5 min and plated at a density of 2 × 10⁵ cells/well (24-microwell plates: Falcon). After 36 h the cells were growth arrested in serum-free RPMI for 48 h, then metabolically labelled with 50 μCi/ml carrier-free Na₂[³⁵S]sulphate (Amersham, Little Chalfont, UK) in sulphate-low medium in the absence or presence of LDL (0–100 μg/ml) [11]. After labelling, the culture medium was removed, the cells washed twice with PBS and the washes added to the culture medium together with a cocktail of protease inhibitors [11]. The cell layer was extracted with 4% CHAPS, 4 M guanidine HCl in 50 mM sodium acetate, pH 6.0, containing 0.05% sodium azide and protease inhibitors at −20°C overnight. The culture medium and the cell layer were stored at −20°C until used.

For all experiments, parallel cultures were set up in the absence of radiolabel, for the determination of cell numbers. At the termination of each experiment cultures were trypsinized and the detached cells counted using a haemocytometer.

Measurement and characterization of radiolabelled material

The incorporation of [³⁵S]sulphate into macromolecules, the isolation of [³⁵S]-labelled PGs and [³⁵S]-labelled-GAGs and their subsequent analysis by chromatography on a dissociative Sepharose CL 4B column (0.006 × 1.5 m) before and after digestion with chondroitin ABC lyase or heparitinase have been described by us in detail [7,8,12,13].

The measurement of hyaluronan synthesis was undertaken on cells metabolically labelled with 25 μCi/ml d-[³⁵H]glucosamine (specific activity 20 Ci/mMol, Amersham) as previously described [14]. In some experiments cycloheximide (10 ng/ml) or actinomycin (25 ng/ml) was included in the culture medium.

Western blot analysis of human MC PG

Confluent human MC were maintained in serum free RPMI medium in 75 cm² flasks with and without LDL (100 μg/ml). Conditioned medium was harvested after 24 h and unlabelled PG concentrated by DEAE and Mono Q ion exchange chromatography [7]. PG were determined using the dye binding assay of Farndale [15]. Aliquots of the PG (100 μg) were incubated with buffer alone, with 50 mU of proteinase free chondroitin ABC lyase (ICN, Thane, UK) or with a mixture of heparitinase I, II and III [13]. SDS–polyacrylamide gel electrophoresis was carried out using 3–12% gradient gels following the method of Laemmli [16]. The proteins were then transferred to nitrocellulose, incubated with primary antibody and developed using enhanced chemiluminescence (ECL) (Amersham). The antibodies used were rabbit anti-bovine versican (the kind gift of Dr D. Heinegard, University of Lund, Lund, Sweden), rabbit anti-human decorin (LF-30) and anti-biglycan (LF-15) (the kind gift of Dr J. Hassell, Schreiner Hospital for Sick Children, Tampa, Fl, USA).

Isolation of LDL

LDL (density range 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of human plasma collected from healthy volunteers and stored under nitrogen at 4°C for up to 4 weeks [10,17]. The preparation was free from endotoxin as determined by the Limulus reaction and remained in an un-oxidized form under these storage conditions. For use in tissue culture the lipoprotein preparations were dialysed against 0.15 M NaCl, pH 7.4, containing 0.3 mM EDTA and sterilised by passage through a 0.22 μm filter (Millipore, Harrow, UK).

Statistical analysis

Statistical analysis was performed using a Mann–Whitney unpaired single-tailed test or by analysis of variance (ANOVA). Results are expressed as mean ± 1 standard deviation. P<0.05 was taken as significant.
Results

Effect of lipoproteins on the incorporation of $[^{35}S]$sulphate into glycosaminoglycans

To investigate the effect of lipoproteins on the synthesis of PG by human MC, confluent quiescent cells were metabolically labelled with $[^{35}S]$sulphate in the presence of native LDL (range 0–100 μg/ml). Over the concentration and time periods studied in these experiments there were no changes in cell number. The results showed a dose-dependent increase in the amount of $^{35}$S-labelled PG extracted from the cell layer and culture medium (Figure 1a). As lipoprotein concentration increased, a relatively greater proportion of labelled proteoglycan was secreted into the conditioned medium as opposed to being retained in the cell layer. Thus when compared to control cells grown in RPMI alone (100%), exposure to 50 μg/ml and 100 μg/ml LDL for 24 h led to a 238.0% (±44.4%, P < 0.001; n = 5) and 426.5% (±28.5%, P < 0.001; n = 5) increase in secreted $^{35}$S-labelled PG respectively. In contrast, at the same concentrations, LDL increased cell-associated $^{35}$S-labelled PG by only 140.6% (±25.1%, P < 0.001; n = 5) and 197.9% (±20.4%, P < 0.001; n = 5).

Exposure of cells to 50 μg/ml LDL from 6 to 48 h led to a linear increase in total (secreted plus cell associated) $^{35}$S-labelled PG (Figure 1b). At all time-points, more $[^{35}S]$sulphate was incorporated into macromolecules synthesized by LDL-stimulated cells than by control cells labelled in medium alone (Figure 1b).

Analysis of the $^{35}$S-labelled glycosaminoglycans and PGs

Analysis of the $^{35}$S-labelled glycosaminoglycans by gel chromatography and anion-exchange chromatography indicated that the enhanced levels of $^{35}$S-labelled PG extracted from treated cells was not accounted for by either elongation or increased sulphation of the GAG chains. Selective enzyme digestion studies, however, showed that with respect to the culture medium, there was a 5-fold increase in both the large CSPG (versican) ($K_v$ 0.16) and small CSPG (decorin and biglycan) ($K_v$ 0.5) above control levels (Figure 2 panel a and Table 1). In addition a single HSPG species ($K_v$ 0.36) was increased 3-fold (Figure 2 panel b). With regards to the CL, of particular note was a 5-fold increase in a large CSPG (Figure 2 panel c). In addition there was a 2-fold increase in HSPG ($K_v$ 0.3) when cells were stimulated with lipoprotein (Figure 2 panel d). LDL had no major effect on the material eluting between $K_v$ 0.6 to 0.9, which has previously been shown to contain single GAG chains and their degraded product [7].

Western blot analysis of the proteoglycan core proteins

To confirm the selective synthesis of versican, biglycan, decorin and possibly perlecan, Western blotting analysis was performed on the culture medium using antibodies that recognize the core proteins of these PG. Anti-versican antibody recognized 2 core proteins (~M, 380 and 400 × 10^3) which were increased three-fold in the presence of 100 μg/ml LDL (Figure 3a compare lanes 2 and 4). Using anti-perlecan antibody the two 2 core proteins (~M, 260 and 300 × 10^3) identified were also increased (3.5-fold) in the presence of the LDL (Figure 3b lanes 2 and 4). The core protein of decorin (Figure 3c) was also increased (2-fold) but there was no apparent change in biglycan (Figure 3d). These findings suggest that LDL selectively increased the synthesis of specific proteoglycans by mesangial cells.
Effect of LDL on HA synthesis by MC

A further series of experiments was conducted to determine whether LDL also enhanced the synthesis of hyaluronan. At a concentration of 100 μg/ml and after 24 h incubation, LDL increased the incorporation of [3H]glucosamine into HA by 202.6% (± 67.6%, P<0.05; n = 5) of control. Approximately 60% of the newly synthesized hyaluronan was secreted and stimulation of MC with lipoproteins did not alter the ratio of secreted to cell-associated molecules. The inclusion of cycloheximide (10 ng/ml) or actinomycin (25 ng/ml) in the culture medium did not alter the basal synthesis of [3H]hyaluronan, however the increased synthesis caused by the inclusion of LDL was inhibited by 80% and 60% respectively.

Discussion

These studies demonstrate that stimulation of human MC with LDL enhances proteoglycan production in the absence of cell proliferation. This was shown to be the result of increased de novo synthesis of versican, perlecan and decorin but not biglycan. LDL also enhanced hyaluronan production by a mechanism that probably involved increased synthetic enzyme activity.

LDL stimulation did not alter the hydrodynamic size of secreted or cell-associated PG or that of their constituent glycosaminoglycan chains. Furthermore, all GAGs had similar charge densities to those produced by the control cells. These results strongly suggest that the increased incorporation of radiolabelled sulphate into secreted and cell-associated macromolecules was the result of enhanced de novo synthesis. Selective digestion of labelled macromolecules followed by Western blotting studies confirmed these findings by demonstrating increased production of the core proteins of versican (a large CSPG) and perlecan (a basement membrane HSPG). A less marked effect on DSPG was noted with a small increase in decorin but not in biglycan. Our data therefore suggest that the various proteoglycans synthesized by MC may be differentially regulated. This conclusion is in agreement with other studies that show TGF-β uniquely affects the metabolism of decorin and biglycan, while IGF-1 appears to selectively increase CSPGs [18,19]. In addition recent reports show that high glucose culture conditions mainly decrease heparan sulphate rather than dermatan/chondroitin sulphate [20].

The roles of different PG synthesized by MC are poorly understood making it difficult to predict the

Fig. 2. Sepharose CL-4B chromatography of MC PG. MC were metabolically labelled with [35S]sulphate for 24 h either in the absence (○) or presence (●) of 100 μg/ml LDL. Aliquots of the 35S-labelled PG in the culture medium (panels a & b) and cell layer (panels c & d) were incubated with either heparitinase (a & c) or chondroitin ABC lyase (b & d) and the remaining CSPG and HSPG respectively chromatographed on a dissociative Sepharose CL 4B column. The excluded (V₀) and total volumes (Vₜ) of the columns are indicated with arrows.
Table 1. The effect LDL on the distribution of proteoglycans

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[35S]sulphate dpm/10^3 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSPG CM CL DSPG CM CL HSPG CM CL DS GAG CL HS GAG CL</td>
</tr>
<tr>
<td>Control</td>
<td>20  28  106  32  52  42  23  32</td>
</tr>
<tr>
<td>100 μg/ml LDL</td>
<td>110  133  522  22  161  91  14  43</td>
</tr>
</tbody>
</table>

Mesangial cells were labelled with [35S]sulphate and the proteoglycan populations separated as shown in Fig. 2. The large CSPG that elutes just after the column V₀ has been previously identified as versican, the small DSPG consists of decorin and biglycan and the HSPG is in part perlecan.

Fig. 3. Western blot analysis of the PG core proteins. MC was cultured in the absence or presence of 100 μg/ml LDL and PG isolated from culture medium. Aliquots of the PG from cultures with medium alone (lanes 1 and 2) or stimulated with LDL (lanes 3 and 4) were incubated with buffer alone (lanes 1 and 3) or with either chondroitin ABC lyase (panels a, c & d) or heparitinase (panel b) (lanes 2 and 4) and subjected to gradient SDS PAGE. Western blots were generated with anti-sera raised to (a) versican, (b) perlecan, (c) decorin and (d) biglycan. The 64 × 10^3 Mr band in panel a (lanes 1–4) represents BSA present in the medium and immunoidentified by the anti-bovine versican antisera.

pathophysiological consequences of LDL stimulation. One possibility is that increased proteoglycan production will promote entrapment of lipid within the glomerular mesangium. CSPG bind apo-B-containing lipoproteins in the presence of calcium to form insoluble complexes, a process that may contribute to the development of atherosclerotic lesions [21]. It is possible that similar interactions occur in the glomerulus. Previous studies have shown that versican and decorin/biglycan extracted from human MC conditioned medium form insoluble complexes with LDL in the presence of calcium [9]. The binding, optimal at 30 mM Ca²⁺, was abolished by pre-treatment of PG fractions with chondroitin ABC lyase demonstrating the critical importance of GAG side chains. Thus, increased production of PG by MC following exposure to LDL may promote further lipid accumulation in the glomerular mesangium, thereby setting up a cycle of progressive scarring.

Matrix accumulation resulting from LDL stimulation may also assist cytokine accumulation within the mesangium. For example, decorin binds TGF-β, which in turn has been implicated as a mediator of increased proteoglycan production, and perlecan immobilizes basic fibroblast growth factor [18,22]. Such interactions may modulate exposure of MC to the biological effects of these mediators. Additional mechanical effects of increased proteoglycan accumulation include changes in matrix charge, which may modulate filtration of plasma proteins and disruption of the normal interactions between various matrix components and cells [23].

LDL also enhanced hyaluronan production by MC. More than 60% of this glycosaminoglycan was secreted into the medium and lipoprotein stimulation did not alter the ratio of secreted to cell-associated molecules. Furthermore, inclusion of transcriptional and protein synthesis inhibitors blocked the effects of LDL suggesting that the lipoprotein modulates enzymes involved in hyaluronan synthesis. Apart from playing a critical role in the organization and structure of extracellular matrix, hyaluronan also regulates cell migration, adhesion and proliferation [6]. Although the role of hyaluronan in the mesangium has not been fully investigated, recent studies suggest that this glycosaminoglycan interacts with glomerular cells via specific receptors and binds to large versican PG to form aggregates [8]. These interactions may play an
important role in glomerular cell migration and proliferation. Thus by enhancing HA synthesis, lipoproteins might modulate the interaction between matrix components and MC.

It is well established that the precise nature of extra cellular matrix exerts a stringent control on the phenotype of normal MC. Our results also suggest that LDL may indirectly affect MC growth. Unmodified LDL has a biphasic effect on human MC growth [10]. At low concentrations (less than 50 μg/ml) it enhances cell proliferation as determined by [3H]thymidine incorporation, whereas at 100 μg/ml it has no effect and if anything inhibits cell growth. The present results that are based on cell number therefore confirm our earlier finding that at 100 μg/ml LDL is not a mitogen for human MC. They also indicate that LDL induced synthesis of versican and hyaluronan is independent of cell proliferation. This conclusion is difficult to reconcile with preliminary in vitro data, which demonstrated that rat MC proliferation induced by serum is accompanied by increased production of CSPG [24]. Vascular smooth muscle cells stimulated to divide by PDGF also show increased PG synthesis, which is mainly accounted for by a large CSPG [25]. At the present, however, it is uncertain whether CSPGs such as versican play a direct or indirect role in the control of cell growth.

In conclusion, this study demonstrates that LDL stimulates the de novo synthesis of chondroitin sulphate, dermatan sulphate, heparan sulphate PG and hyaluronan by cultured human MC. This increase in PG synthesis was due to enhanced production of versican, perlecan and decorin, but not to biglycan. Thus, upregulation in proteoglycan and hyaluronan synthesis resulting from lipoprotein stimulation is likely to contribute to the extracellular matrix expansion within the glomerulus. Furthermore, selective increases in certain proteoglycan species may lead to an imbalance of matrix components and adversely modify normal cell-matrix interactions.

Acknowledgements. This study was supported by the Baxter Healthcare Extramural Grant Program and by the Kidney Research Foundation for Wales.

References


Received for publication: 25.5.99
Accepted in revised form: 31.8.99