Cholesterol feeding activates macrophages to upregulate rat mesangial cell fibronectin production

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Abstract

**Background.** Cholesterol feeding has been shown to accelerate the development of glomerulosclerosis in many experimental renal diseases, possibly by promoting the infiltration of macrophages into the glomerulus.

**Methods.** In order to assess whether hyperlipidaemia could directly modulate macrophage function to promote glomerulosclerosis, confluent quiescent mesangial cells were exposed to resident (r) or elicited (e) macrophages, from either control (C) or cholesterol-fed (HC) rats or the conditioned media derived from the various macrophage preparations.

**Results.** All macrophage preparations stimulated mesangial cell fibronectin accumulation over medium alone, but eHC macrophages stimulated significantly greater fibronectin levels. Similarly, all macrophage conditioned media (MPCM) stimulated mesangial cell fibronectin production over medium alone and again the effect was greatest with MPCM derived from eHC macrophages. Proliferation studies using $[^3H]$thymidine incorporation demonstrated that all conditioned media, with the exception of rC, stimulated significant mesangial cell proliferation over control levels. TGF-$eta$ and PDGF, profibrogenic growth factors known to be associated with macrophage infiltration, could not be detected in the MPCM per se. However, they were detected in the culture supernatants of mesangial cells exposed to MPCM and again secretion was greatest from mesangial cells exposed to eHC-MCPM.

**Conclusion.** Monocytes are systemically activated by high serum cholesterol levels so that following maturation to macrophages they elaborate soluble factors that can stimulate mesangial cell fibronectin production, cell proliferation, and growth factor secretion. Hypercholesterolaemia may therefore accelerate glomerulosclerosis not only by increasing macrophage number, but also by upregulating the ability of macrophages to induce pro-sclerotic responses in glomerular mesangial cells.

Key words: fibronectin; glomerulosclerosis; hypercholesterolaemia; macrophages; mesangial cells

Introduction

Experimental evidence suggests that hyperlipidaemia may accelerate the development of glomerulosclerosis following glomerular injury [1]. One potential mechanism by which hypercholesterolaemia may mediate this pathogenetic effect is via an increased infiltrate of glomerular macrophages [2]. The role of the macrophage infiltrate in the progression of glomerular disease has been extensively studied in the puromycin aminonucleoside (PAN) model of nephrosis. It has been demonstrated that manoeuvres that abolish the infiltrate such as X-irradiation [3] or a diet free of essential fatty acids [4] ameliorate the progression of glomerular disease in nephrotic rats. Pharmacologically lowering serum lipid levels in these animals confers protection against the development of glomerulosclerosis [5] whilst superimposition of a high-cholesterol diet increases macrophage number and exacerbates the glomerular injury [2].

Although hyperlipidaemia and increased glomerular macrophage number are known to be correlated with glomerulosclerosis, the precise pathobiological mechanisms involved in this process are not yet fully understood. It has been suggested that a hypercholesterolaemic environment could induce resident glomerular cells to secrete macrophage chemoattractants triggering the initial macrophage infiltrate. Rovin and Tan have demonstrated that mesangial cells secrete the specific macrophage chemoattractant MCP-1 in response to LDL [6], while Rovin et al. have described a lipid-derived chemoattractant expressed by the glomeruli of rats with experimental glomerulonephritis [7]. Macrophages recruited into the glomerulus in this way could, in their turn, go on to elaborate factors involved in initiating the progression to glomerulosclerosis. We have shown, for example, that conditioned medium from thioglycollate-elicited, LPS-stimulated macrophages can promote prosclerotic responses in cultured mesangial cells in a time- and dose-dependent fashion.
Alternatively, the hypercholesterolaemic environment may itself directly affect the monocyte/macrophage phenotype. Diet induced hypercholesterolaemia has been shown to alter several macrophage characteristics in a way that could contribute to lesion formation in atherosclerosis [9]. These include increased adhesion to endothelial cell monolayers and increased secretion of smooth-muscle cell chemoattractants and mitogenic factors. Furthermore, conditioned media from cholesterol-loaded macrophages or macrophages from patients with familial hypercholesterolaemia have been shown to induce 37 and 20% more platelet aggregation respectively than conditioned media from normal subjects [10]. Diamond et al. have demonstrated that peritoneal macrophages from PAN nephrotic and diet-induced hypercholesterolaemic rats are elicited in greater numbers, exhibit a greater degree of phagocytosis, and show a trend towards increased production of thromboxane B2 than control rats. All of these parameters are synergistically increased in macrophages from nephrotic rats on a high-cholesterol diet [11]. It is plausible therefore that similar alterations of glomerular macrophage function in response to hyperlipidaemia could enhance their ability to modulate the progression to glomerulosclerosis.

The aim of the present study, therefore, was to elucidate at the cellular level whether hypercholesterolaemia could directly modulate macrophage function so as to cause cultured mesangial cells to upregulate the production of the matrix protein fibronectin.

**Subjects and methods**

Unless otherwise stated all chemicals were obtained from Sigma Chemical Company, Poole, Dorset, UK.

**Culture of mesangial cells**

Glomerular mesangial cells were cultured from the glomerular explants of adult Wistar rat kidneys (University of Leicester colony) using standard techniques. The cells were cultured in RPMI 1640 (Gibco, Life Technologies, UK) supplemented with 20% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin and 2 mM glutamine. Cultured cells were characterized as mesangial by their typical stellate fusiform morphology, their positive staining for the Thy-1 antigen and their resistance to the toxic effects of d-valine and PAN.

Mesangial cells were used in passages 2–10. In all experiments they were cultured in 24-well plates (Linbro, ICN Biomedicals Ltd, Bucks, UK.), allowed to grow to confluence, and then rendered quiescent in RPMI medium containing 0.5% FCS for 72 h prior to use.

**Induction of dietary hypercholesterolaemia**

Adult Wistar rats (University of Leicester colony) were fed either a control diet containing 20% casein protein or an identical diet which had been supplemented with 4% cholesterol and 1% cholic acid to induce hypercholesterolaemia. Diets were obtained from ICN Flow, UK. Animals were fed the diet for 14 days after which peritoneal macrophages were isolated and a serum sample obtained for the measurement of cholesterol.

**Preparation of macrophages and macrophage conditioned medium (MPCM)**

 Resident (r) macrophages were isolated from the peritoneal cavity after 14 days on the diet. The peritoneum was lavaged with 20 ml cold serum-free RPMI, the exudate cells were washed and plated at 5 × 10⁶ cells/ml in 25 cm² tissue culture flasks (Linbro). After 2 h incubation at 37°C in a humidified 5% CO₂ 95% air atmosphere, non-adherent cells were removed by washing with Hanks balanced salt solution buffered with 20 mM HEPES. The macrophages were then cultured for a further 48 h in serum-free RPMI to generate the conditioned medium. The MPCM was then harvested and centrifuged for 10 min at 2000 r.p.m. and frozen at −20°C until required. Elicited (e) macrophages were obtained from HC or C rats by injecting 10 ml of 3% thioglycollate broth into the peritoneal cavity 5 days prior to harvesting on day 14. The MPCM was prepared in an identical way.

**Co-culture of macrophages with mesangial cells**

Confluent, quiescent mesangial cells were cultured for 3 days in either medium alone (RPMI containing 0.5% FCS), or in co-culture with r or e macrophages from HC or C rats at a cell density of 2 × 10⁵ cells/ml. Mesangial cell tissue culture supernatants were then centrifuged and frozen for subsequent analysis.

**Culture of mesangial cells in the presence of MPCM**

Confluent, quiescent mesangial cells were exposed to a 50% solution of MPCM (supplemented such that the FCS concentration remained at 0.5%). The cultures were maintained in this medium for up to 7 days. The tissue culture supernatants were harvested and centrifuged for 30 s at 13 000 r.p.m. to remove cell debris, and stored at −20°C for subsequent analysis.

The time of incubation and dose of MPCM chosen were based on our previous published work in which the dose- and time-dependency of mesangial cell responses to thioglycollate-elicited, LPS-stimulated macrophages from normal rats was defined [8].

**Preparation of cell lysates**

After removal of tissue culture supernatants, cell monolayers were washed with PBS, scraped into 1.0% Nonidet P40 (BDH, Merck, UK.) in wash buffer (PBS containing 0.3 M NaCl, 0.1% Tween 20) and then incubated at 37°C for 2 h. The cell scrapings were then transferred into 2 ml tubes and sonicated for 5 s using a Jencons 50 watt sonicator. The cell lysates were then centrifuged for 30 s at 13 000 r.p.m. before determination of protein concentration and cell-associated fibronectin levels.

**Assays**

Culture supernatants or cell lysates were assayed for fibronectin using an inhibition ELISA as previously described [8].
Total TGF-β (following acid activation), TNF-α and PDGF-AB were assayed using commercially available ELISA assays (TGFβ and TNFα from Genzyme Diagnostics, UK, and PDGF-AB from Amersham, UK) and used according to the manufacturers’ instructions.

Detergent lysed cell monolayer protein was assayed using a commercial BioRad DC protein assay kit using BSA standards (BioRad, UK).

Serum cholesterol was measured using the Beckman Dri-Stat-Reagent Cholesterol-es (Beckman, UK) automated on Vitatron automated sampler (Vital Scientific, UK).

**Determination of cell proliferation**

Cell proliferation was determined by [3H]thymidine incorporation using standard methodology. Briefly, mesangial cells were exposed to conditioned media from r or e macrophages from either C or HC rats. The culture supernatants were removed after 3 days and replaced with fresh medium containing 1 µCi/well of [3H]thymidine (Amersham, UK). The cells were then incubated for a further 24 h after which the supernatants were discarded and the wells rinsed once with PBS; 0.1 mM non-radioactively labelled thymidine in RPMI containing 0.5% FCS was added to each well for 20 min at 37°C. The cells were then washed once with ice-cold PBS, twice with 10% TCA and then once with PBS again. 250 µl of 0.5M NaOH were then added to each well and the plate incubated at 60–70°C for 30 min to dissolve the cell monolayer. 200 µl of cell lysate were added to 4 ml of Ecoscint A scintillation fluid (National Diagnostics, Kimberley Research, UK). The scintillation vials were then counted on an LKB 1219 liquid scintillation counter.

**Statistics**

Two rats from each condition (i.e. rC, eC, rHC, and eHC) were used in each experiment. The mean data represents at least three independent experiments. Each MPCM preparation was assayed on mesangial cells in quadruplicate.

Data are expressed as means ± SEM. Data for cell proliferation, fibronectin and cytokine production in response to MPCMs were corrected for cell protein. For comparison of means between two groups an unpaired t-test was employed. To compare values between multiple groups an analysis of variance (ANOVA) with Bonferroni correction was applied. Statistical significance was defined as P < 0.05.

**Results**

**Serum cholesterol levels**

Serum cholesterol levels confirmed that all animals fed the high-cholesterol diet were significantly hypercholesterolaemic (11.1 ± 0.8 vs 2.2 ± 0.1 mmol/L, P < 0.001) after 14 days on the high-cholesterol diet.

**Effect of co-culture or macrophages with mesangial cells on fibronectin production**

Macrophages (2.5 × 10⁵) from r or e, C or HC rats were directly co-incubated with confluent quiescent mesangial cells for a period of 3 days. All macrophage preparations were able to stimulate fibronectin production over that of medium alone. eHC macrophages induced significantly greater fibronectin levels than the other macrophage preparations. There was no significant difference between rHC and rC macrophage-induced fibronectin levels (Figure 1).

**Effect of MPCMs on mesangial cell fibronectin levels**

Under basal conditions (culture medium alone) the fibronectin concentration in mesangial cell culture supernatants was typically 132.6 ± 31 ng/µg cell protein. Exposure of mesangial cells to all forms of MPCM resulted in increased supernatant fibronectin levels over those of medium alone (Figure 2A). Conditioned medium derived from eHC macrophages resulted in significantly higher fibronectin levels than observed with conditioned medium from eC, rHC, and rC macrophages. There was no significant difference in fibronectin levels induced by rC and rHC MPCMs.

When cell associated fibronectin levels were assessed in cell lysates, conditioned medium from eHC macrophages stimulated significantly greater fibronectin levels than conditioned medium from eC, rHC and rC macrophages (Figure 2B). This difference was of a greater magnitude than seen in the supernatants.

**Effect of MPCMs on mesangial cell proliferation**

[3H]Thymidine incorporation assays showed that with the exception of MPCM derived from rC macrophages, all conditioned media stimulated cell proliferation over basal levels. However, there appeared to be no significant difference between treatments (Figure 3).

**Detection of TGF-β, TNF-α, and PDGF**

Total TGF-β, TNF-α, and PDGF-AB could not be detected in the conditioned media per se, at least not at the sensitivity of the ELISA assays. However, TGF-β and PDGF-AB were detected in the tissue culture supernatants.
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Ants under control conditions but was found in the tissue culture supernatants of mesangial cells exposed to the various MPCMs. Secretion did not differ between any of the treatment groups (Table 1).

Discussion

The present investigation demonstrates that elicited macrophages are functionally modified by diet-induced hypercholesterolaemia to elaborate factors which stimulate mesangial cell fibronectin production, cell proliferation, and secretion of cytokines and thus corroborates our previous data that macrophage-derived products can increase mesangial cell fibronectin synthesis [8]. This study therefore provides a possible pathobiological mechanism for the adverse effects of hyperlipidaemia on the development of glomerulosclerosis.

Secretion of increased amounts of fibronectin by cultured rat mesangial cells in response to eHC macrophages and conditioned medium derived from eHC macrophages is in accord with the in vivo observations of Ding et al. [12] who demonstrated that glomerular fibronectin gene expression is upregulated in PAN nephrotic and diet-induced hypercholesterolaemic rats in temporal association with the influx of macrophages into the glomerulus. Our data also demonstrate that macrophage–mesangial cell contact is not a prerequisite to effect a sclerotic response and therefore suggest that soluble macrophage derived factors could be responsible.

The observed differences in fibronectin levels could not be accounted for by an increase in cell number since fibronectin levels are expressed as a function of total cell protein.

Cell proliferation is often regarded as a precursor of glomerulosclerosis. In this study, conditioned medium from eHC, eC, and rHC macrophages stimulated \(^{3}H\)thymidine incorporation over control levels although there was no significant difference in proliferation between treatments. Again, the increase in \(^{3}H\)thymidine incorporation was not simply due to an increase in cell number, since expressing c.p.m. as a function of cell protein maintained the differential. This observation concurs with the study of Diamond et al. [13] who demonstrated that the influx of glomerular macrophages in PAN nephrotic rats is associated with proliferation in mesangial areas as evidenced by increased PCNA staining and 3 bromo-2 deoxyuridine incorporation. The authors further showed that dietary manipulation by cholesterol supplementation increased macrophage numbers and upregulated proliferation in mesangial areas. Conversely, an EFAD diet and X-irradiation of cholesterol-fed animals significantly decreased macrophage infiltration and downregulated proliferation.

It has been well documented that TGF-\(\beta\) plays a pivotal role in the accumulation of extracellular matrix production both in experimental and human renal diseases [14]. Diamond’s group elegantly demonstrated...
an upregulation of TGF-β mRNA expression in glomerular and peritoneal macrophages of PAN nephrotic and diet-induced hypercholesterolaemic rats, suggesting they were the prime cellular source of TGF-β [15]. Observations by others have demonstrated that in adriamycin nephropathy the distribution and morphological appearance of TGF-β-positive cells in glomeruli and interstitium were similar to those of ED1-positive macrophages [16]. In the current study TGF-β, PDGF-AB and TNF-α were not detected in the MPCM, at least not with the sensitivity levels of the assay. Our data did, however, show that macrophages functionally modified by hypercholesterolaemia were able to stimulate a significant increase in secretion of PDGF-AB by mesangial cells. TGF-β was also increased more than fourfold by macrophages derived from hypercholesterolaemic animals, although this did not reach statistical significance. It is thus possible that these mesangial cell derived cytokines are then able to act in an autocrine manner to promote further mesangial cell matrix production. Whilst we have previously shown that exogenous TGF-β and PDGF added individually only produce modest increases in rat mesangial cell fibronectin protein and mRNA levels, TGF-β in combination with PDGF, TNF-α, or IL-1β results in a synergistic upregulation of fibronectin protein to levels comparable with those observed with rHC MPCM in the current study [17]. Furthermore, increased fibronectin levels induced by MPCM from LPS-stimulated macrophages can be partially reduced by a neutralizing antibody to TGF-β [8].

Resident peritoneal macrophages from HC rats did not share the same stimulatory activity as elicited macrophages from HC rats but showed comparable activity with rC macrophages. In the current study, the animals were fed a high-cholesterol diet for only 14 days, a time frame which may not be long enough to have influenced the macrophages already residing in the peritoneum. This would suggest that systemic activation of the monocytes/macrophages by a hypercholesterolaemic environment prior to being elicited into the peritoneum is required for the effect. The factors in hyperlipidaemic serum that induce the change in the functional parameters of macrophages are not known at this stage although one or more of a number of possible factors may be involved. Hypercholesterolaemia induces significant changes in tissue lipid metabolism, including an increase in cholesteryl esters [18]. Previous work has shown that an increase in tissue cholesteryl esters is an early preslesional event in the pathogenesis of atherosclerosis [19] and other investigators have also suggested that cholesteryl esters may be linked to the margination through the vessel wall of circulating monocytes that subsequently become macrophages [20]. Immune complexes containing low-density lipoprotein have been shown to induce a variety of metabolic and functional changes in macrophages such as increased cytokine release, upregulated LDL receptor expression, and increased respiratory bursts [21]. The relevance of these mechanisms in relation to our observations remains to be verified. It is also possible that the observed differences between eHC- and rHC-treated macrophages could be explained by synergistic effects of monocyte elicitation (in this case thioglycollate treatment) with hypercholesterolaemia. The process of macrophage margination through the endothelium is known to upregulate the production of various mediators/factors which could alter the activation state of macrophages. This, in combination with the influences of the hypercholesterolaemic environment may account for the differences in fibronectin stimulating activity observed.

Peritoneal macrophages were used in this study since glomerular macrophages could not be extracted from glomeruli in sufficient numbers to produce an adequately potent conditioned medium. However, it has been previously shown that peritoneal macrophages from diet-induced hypercholesterolaemic rats exhibited alterations in functional parameters [9] and regulation of TGF-β gene expression in peritoneal macrophages was qualitatively similar to that observed in glomerular macrophages [12]. It was therefore felt that this was a suitable model to reflect altered macrophage function in response to raised lipid levels.

In summary, this study demonstrates that hypercholesterolaemia directly modulates macrophage function. These macrophages, via the production of a soluble factor(s), are able to cause an upregulation of mesangial cell fibronectin production, cell proliferation, and growth factor secretion. Activation of macrophages probably occurs systemically, induced by elements contained in the hypercholesterolaemic serum.

These observations suggest that the pathobiological mechanisms whereby hyperlipidaemia accelerates the progression to glomerulosclerosis are a result of not
only increased glomerular macrophage number but also altered macrophage functional parameters.

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