Clinical research

Patients with familial hypercholesterolemia show enhanced spontaneous chemokine release from peripheral blood mononuclear cells ex vivo

Dependency of xanthomas/xanthelasms, smoking and gender

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Aims Familial hypercholesterolemia (FH) is associated with increased risk of premature atherosclerosis and coronary artery disease (CAD). However, onset of clinically manifested CAD varies widely among patients with heterozygous FH, and we hypothesized that inflammatory mediators such as chemokines could contribute to atherogenesis in these patients.

Methods and results We compared peripheral blood mononuclear cells (PBMCs) from FH patients with an identical mutation with PBMCs from sex- and age-matched healthy controls with respect to spontaneous and oxidized low density lipoprotein (oxLDL)-stimulated release of chemokines. Our main findings were: (1) PBMCs from FH patients spontaneously released significantly higher levels of macrophage inflammatory protein (MIP)-1α, MIP-1β and interleukin (IL)-8, and had a significantly lower oxLDL-stimulatory ratio for MIP-1α and MIP-1β than cells from healthy controls. (2) Spontaneous release of these chemokines correlated positively and stimulatory ratio correlated negatively with plasma concentrations of total and LDL cholesterol. (3) Among FH patients, release of MIP-1α, MIP-1β and IL-8 from PBMCs varied with the presence of xanthomas/xanthelasms, smoking and gender. (4) In vitro studies showed that FH serum but not control serum was able to induce enhanced spontaneous release of chemokines in PBMCs from both FH patients and control subjects.

Conclusions Our data may suggest that a pathophysiological consequence of FH is enhanced chemokine responses, which in turn may promote recruitment and activation of leukocytes within the vessel wall, contributing to atherosclerosis as well as to the different phenotypes in these patients with an identical FH mutation.

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Introduction

Familial hypercholesterolaemia (FH) is caused by mutations in the low density lipoprotein (LDL)-receptor gene, and is associated with increased risk of premature atherosclerosis and coronary artery disease (CAD). However, the onset of clinically manifested CAD varies widely among patients with heterozygous FH, even among carriers of an identical mutation. Hence, the variation seen in the phenotypes among these patients cannot exclusively be explained from their genotype. These findings indicate that factors other than genotype also are of importance for development of atherosclerosis. Hence, the migration of monocytes into the intima is a central step in the early development of atherosclerosis, and extensive infiltration of blood-derived macrophages and T-cells into the vessel wall is seen in the active stages of this disorder. Chemokines or chemotactic cytokines seem to play a major role in this recruitment and activation of leukocytes into atherosclerotic plaque as suggested by several studies in animal models. Raised plasma levels of chemokines have also been reported in CAD patients with particular high levels in those with unstable disease, further supporting a pathogenic role for these cytokines in CAD. One might hypothesize that chemokines also could be involved in the atherogenesis in FH patients possibly contributing to differences in phenotypes between these individuals, but at present few studies have investigated chemokine production in FH. In the present study we investigated the role of chemokines in FH patients by comparing peripheral blood mononuclear cells (PBMCs) obtained from FH patients and sex- and age-matched healthy control subjects with respect to spontaneous and oxidized LDL (oxLDL)-stimulated release of CC- and CXC-chemokines.

Methods

Subjects

Twenty-two adults <75 years of age with FH were recruited at the Lipid Clinic, Rikshospitalet University Hospital, Norway (Table 1). All subjects were diagnosed with definite FH by DNA test. Six of the FH patients had diagnosed premature CAD, defined as myocardial infarction at an age of ≤50. The diagnosis of CAD was confirmed in all these patients by coronary angiography showing ≥1-vessel disease (>75% narrowing of luminal diameter). Sixteen out of 22 patients were on atorvastatin treatment, whereas six were on simvastatin or lovastatin. Prior to blood sampling they had a 4-week washout period without statin treatment to obtain LDL-cholesterol concentrations on ‘baseline levels’. Although we have no information about the effect of the washout period on chemokine concentrations, it is conceivable that no statin-mediated effect on chemokine levels will persist after such a period. Control subjects were 15 healthy sex- and age-matched volunteers recruited among the staff at Rikshospitalet University Hospital, with no history of hypertension, diabetes, CAD or other acute or chronic illness (Table 1). The study complies with the Declaration of Helsinki, and the study protocol was approved by the Regional Committee of Medical Ethics. Informed consent was obtained from all subjects. Venous blood samples were collected after an overnight fast. Plasma and serum samples were collected, divided into aliquots and stored at −80 °C until analysis as previously described. PBMCs was isolated immediately (see below).

Release of chemokines from PBMCs

Peripheral blood mononuclear cells were obtained from heparinized blood by isopaque-Ficoll (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) gradient centrifugation within 45 min.

<table>
<thead>
<tr>
<th>Table 1 Characteristics of the study group</th>
<th>FH patients (n=22)</th>
<th>Control subjects (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male (n/n)</td>
<td>8/14</td>
<td>6/9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.9±13.4</td>
<td>42.4±14.0</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.3±4.1</td>
<td>22.1±2.5</td>
</tr>
<tr>
<td>Premature coronary artery disease (n)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Tendon xanthomas/xanthelasms present (n)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Corneal arcus present (n)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Antihypertensive treatment (n)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>9.3±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6±1.0</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>7.6±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9±0.8</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3±0.5</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.3 (0.3–22.1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 (0.4–1.7)</td>
</tr>
<tr>
<td>Lipoprotein (a) (mg/l)</td>
<td>150 (96–787)</td>
<td>159 (&lt;96–648)</td>
</tr>
<tr>
<td>Apolipoprotein B (g/l)</td>
<td>2.0±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>Apolipoprotein A-I (g/l)</td>
<td>1.3±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Homocysteine (µmol/l)</td>
<td>9.5 (6–17)</td>
<td>10.0 (6–13)</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD or median (range).

<sup>a</sup>P<0.02 and

<sup>b</sup>P<0.001 vs control subjects; n indicates number of individuals. LDL-cholesterol was calculated in 20 FH patients.
PBMCs were incubated in flat-bottomed 96-well trays (Costar, Corning Inc., Corning, NY; 2×10^5/ml; 100 µl/well) in medium alone (RPMI-1640 containing 2 mm l-glutamine [Sigma Chemical Co., St. Louis, MO], supplemented with 100 U/ml penicillin and 5% autologous serum), or with oxLDL (final concentration 100 µg/ml). Cell-free supernatants were harvested after culturing for 24 h, divided into aliquots, and stored at −80 °C until analysis.

**Isolation and oxidation of LDL**

Plasma from healthy blood donors, collected in endotoxin-free heparin, was stored in 0.6% sucrose at −80 °C. LDL was isolated by sequential ultracentrifugation and was oxidized in the presence of freshly prepared 5 µM CuSO4 (final concentration) as described previously. Oxidized LDL contained 669±12 nmol lipid peroxides per mg LDL protein; and relative electrophoretic mobility was 4.5±0.2 (n=3). Low-density lipoprotein preparations were free of endotoxin, as determined by the Limulus assay. OxLDL was stored under N2 and used within 2 weeks for stimulation of PBMCs (see above).

**Enzyme immunoassays (EIAs)**

Concentrations of monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, Interleukin (IL)-8, and epithelial neutrophil activating peptide (ENA)-78 were measured by EIA from R&D systems (Minneapolis, MN). To minimize run-to-run variability, all samples from a given patient were analysed in the same microtitre plate.

**Routine laboratory assays**

Standard blood chemistry and lipid parameters was measured in serum/plasma using routine laboratory methods.

**Statistical analysis**

Data are given as means±SD or medians and 25th–75th percentiles when data were skewed. Data from FH patients and control subjects were compared by the Student t test. When the data were not normally distributed (e.g., chemokine data), the data were subjected to logarithmic transformation before the parametric analysis. Pearson’s chi-square test was used for the Lipoprotein (a) data. Associations between variables were tested by Pearson correlation coefficients. The level of statistical significance was set at P<0.05.

**Results**

**Characterization of subjects**

Biological characterization of the participants is shown in Table 1. All FH patients had FH-elverum, the most common Norwegian LDL-receptor mutation, with a frequency of 28%. FH-elverum is a splice site mutation, located in the ligand binding domain of the receptor. Compared to healthy controls, FH patients had significantly higher body mass index and significantly higher plasma concentrations of total- and LDL-cholesterol, triglycerides, and apo B, whereas no significant differences were found in high density lipoprotein (HDL)-cholesterol, apo A-I and lipoprotein (a) (Table 1). Furthermore, no significant differences were observed in plasma concentrations of creatinine, urea, total protein, ASAT, ALAT, creatine kinase, high-sensitivity C-reactive protein, folate, and vitamin B12 (data not shown).

**Serum concentrations of chemokines**

There were no significant differences in serum concentrations of MCP-1, MIP-1β and ENA-78 between FH patients (369 pg/ml [218–1170 pg/ml], 175 pg/ml [121–206 pg/ml] and 1230 pg/ml [808–1513 pg/ml], respectively) and control subjects (429 pg/ml [230–727 pg/ml], 129 pg/ml [101–199 pg/ml] and 1257 pg/ml [759–1670 pg/ml], respectively). Furthermore, serum concentrations of MCP-1, MIP-1β and ENA-78 did not correlate with plasma concentrations of total and LDL-cholesterol.

**Release of chemokines from PBMCs ex vivo**

We next examined the release of chemokines from unstimulated and oxLDL-stimulated PBMCs in FH patients and healthy controls after culturing cells for 24 h. To better reflect the in vivo conditions, PBMCs were incubated in medium containing autologous serum (5%) from each individual. Several significant findings were revealed. First, unstimulated PBMCs from FH patients released markedly enhanced levels of MIP-1α, MIP-1β and IL-8 comparing cells from healthy controls (Fig. 1). Second, oxLDL significantly enhanced the release of these chemokines both in FH patients and in healthy controls (Fig. 1), and although the oxLDL-stimulated levels tended to be higher in FH group, this differences did not reach statistical significance (Fig. 1). In contrast, the stimulatory ratio (oxLDL-stimulated/unstimulated) was lower in FH patients compared with healthy controls for both MIP-1α: (3.4 [1–5] vs 4.9 [4–6], P=0.04), MIP-1β (1.7 [1–6] vs 5.8 [3–11], P=0.02), and IL-8: (9.7 [4–101] vs 72 [11–159], P=0.067). As for ENA-78, no significant difference was seen between FH patients and control subjects in either spontaneous or oxLDL-stimulated release (Fig. 1E) or in the oxLDL-stimulatory ratio (2.2 [2–4] vs 1.8 [1–3], respectively).

**Correlations between the release of chemokine and lipid and clinical parameters**

Interestingly, plasma total and LDL-cholesterol correlated positively with spontaneous release of MIP-1α, MIP-1β and IL-8, and total cholesterol correlated negatively with the stimulatory ratio for MIP-1α, MIP-1β and IL-8 (Table 2). No such correlations were observed between cholesterol and release of ENA-78.

While there were no significant differences in the release of chemokines between PBMCs from FH patients with (n=6) or without (n=16) premature CAD (Table 3), PBMCs from FH patients with tendon xanthomas and/or xanthelasmas (n=10) spontaneously released higher amounts of MIP-1α, MIP-1β and IL-8, as well as higher amounts of oxLDL-induced MIP-1α and IL-8 than cells from FH patients without (n=12) this manifestation (Table 3). Furthermore, compared to PBMCs from non-smoking FH patients (n=13), PBMCs from cigarette-smoking FH patients (n=9) spontaneously released higher...
IL-8 levels and had decreased stimulatory ratio for MIP-1α and IL-8 (Table 3). Likewise, PBMCs from smoking healthy controls (n=3) spontaneously released higher levels of MIP-1α, MIP-1β and IL-8 (50 pg/ml [26–305 pg/ml], 83 pg/ml [78–891 pg/ml] and 597 pg/ml [51–2522 pg/ml], respectively), comparing cells from non-smoking (n=12) controls (16 pg/ml [11–22 pg/ml], 15 pg/ml [4–53 pg/ml] and 10 pg/ml [10–999 pg/ml], respectively; P≤0.02 for all). Finally, compared to PBMCs from female FH patients (n=8), the stimulatory ratio for MIP-1α, MIP-1β and IL-8 was lower in cells from male FH patients (n=14; Table 3). No such pattern related to gender was seen in healthy controls. As for ENA-78, we found no relationship with either xanthomas/xanthelasms, cigarette-smoking or gender.

**Effect of serum on spontaneous release of chemokines from PBMCs ex vivo**

To further explore the finding that PBMCs from FH patients, cultured in the presence of autologous serum,
<table>
<thead>
<tr>
<th></th>
<th>Premature CAD</th>
<th>Xanthomas/xanthelasms</th>
<th>Smoking</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present (n=6)</td>
<td>Not present (n=16)</td>
<td>Present (n=10)</td>
<td>Not present (n=12)</td>
</tr>
<tr>
<td>MIP-1α unstimulated (pg/ml)</td>
<td>106 (28–449)</td>
<td>23 (15–309)</td>
<td>268 (32–499)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 (13–46)</td>
</tr>
<tr>
<td>stimulatory ratio</td>
<td>2.6 (1–5)</td>
<td>4.2 (1–5)</td>
<td>1.8 (1–3)</td>
<td>4.7 (2–6)</td>
</tr>
<tr>
<td>MIP-1β unstimulated (pg/ml)</td>
<td>219 (41–800)</td>
<td>72 (30–518)</td>
<td>298 (135–837)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 (18–189)</td>
</tr>
<tr>
<td>stimulatory ratio</td>
<td>1.0 (0.4–5)</td>
<td>2.4 (1–7)</td>
<td>1.2 (1–2)</td>
<td>3.6 (1–9)</td>
</tr>
<tr>
<td>IL-8 unstimulated (pg/ml)</td>
<td>526 (225–3720)</td>
<td>248 (10–4578)</td>
<td>3521 (423–4926)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 (10–536)</td>
</tr>
<tr>
<td>stimulatory ratio</td>
<td>9.7 (6–57)</td>
<td>15.8 (4–136)</td>
<td>5.7 (3–6)</td>
<td>68 (16–136)</td>
</tr>
</tbody>
</table>

Data are given as median (25th–75th percentiles).

<sup>a</sup>P<sub>≤0.01</sub>.  
<sup>b</sup>P<sub>≤0.05</sub> and  
<sup>c</sup>P<sub>0.058</sub> vs FH patients without this characteristic.  
<sup>n</sup> indicates number of individuals; stimulatory ratio indicates oxLDL-stimulated/unstimulated release of chemokines.
spontaneously released a high level of chemokines, we cultured PBMCs from patients with FH-elyserum (n=3) in the presence of serum from a FH patient or from a control subject. Similarly, PBMCs from control subjects (n=3) were cultured for 24 h under the same conditions. Interestingly, in the presence of serum from the FH patient, PBMCs from FH patients as well as from control subjects spontaneously released enhanced levels of MCP-1 and IL-8, with particular high levels in FH patients (Fig. 2). In contrast, serum from the control subject had no enhancing effect on chemokine release either in FH PBMCs or in control PBMCs (Fig. 2).

Discussion

The present study shows that: (1) PBMCs obtained from FH patients spontaneously released significantly higher levels of MIP-1α, MIP-1β and IL-8, and had significantly lower oxLDL-stimulatory ratio for MIP-1α and MIP-1β than cells from healthy controls. (2) The spontaneous release of these chemokines correlated positively and the stimulatory ratio correlated negatively with plasma concentrations of total and LDL cholesterol. (3) Among FH patients, the release of MIP-1α, MIP-1β and IL-8 from PBMCs varied with the presence of xanthomas/xanthelasms, smoking habits and gender. (4) In vitro studies showed that FH serum but not control serum was able to induce enhanced spontaneous release of chemokines in PBMCs from both FH patients and control subjects. Taken together, our data may suggest that a pathophysiological consequence of FH is enhanced chemokine responses, which in turn may promote recruitment and activation of leukocytes within the vessel wall, contributing to atherosclerosis as well as to the different phenotypes in these patients.

In the present study we show that PBMCs from FH patients cultured in the presence of autologous serum spontaneously released higher levels of chemokines accompanied by a decrease in the stimulatory response to oxLDL, as assessed by stimulatory ratio, compared with PBMCs from healthy controls. Such a pattern may suggest in vivo activation of these cells in FH patients, and if such enhanced release of chemokines also exists within the vessel wall it may clearly reflect important pathogenic processes in these individuals. Thus, migration and activation of leukocytes is an important event in the development of atherosclerosis, and chemokines may play a crucial role in this process. Accordingly, enhanced expression of MCP-1, IL-8, MIP-1α and MIP-1β has been found in human coronary atheroma. Furthermore, intimal macrophages with enhanced expression of the IL-8 receptor (i.e. CXCR2) have been reported in murine and human atherosclerotic lesions. Also, knockout mice lacking the MCP-1 receptor (i.e. CCR2) have reduced progression of atherosclerosis.

Recently, enhanced gene expression of MIP-1α, MIP-1β and their corresponding receptors CCR1 and CCR5 was found in PBMCs from CAD patients compared to cells from healthy control subjects. Interestingly, MIP-1α and MIP-1β co-localize in atherosclerotic plaques, and are expressed by T-cells in macrophage/foam cell-rich areas of the plaque, suggesting cross-talk between T-cells and macrophages through chemokine signalling, resulting in movement of macrophages to the appropriate inflammatory areas. Moreover, studies in CCR1 knockout mice indicate the involvement of MIP-1α in the pathogenesis of allograft atherosclerosis. MIP-1β has been shown to increase the activity of tissue factor, a potent initiator of coagulation, suggesting that this chemokine could mediate both inflammatory and thrombotic responses within an atherosclerotic plaque. Our findings in the present study further support a possible role of these mediators in the atherogenesis of FH patients.

The specific underlying molecular defect of the LDL-receptor gene may contribute to the phenotypic variance seen in the onset of CAD in patients with heterozygous FH. In the present study we selected participants with identical gene mutation (FH-elyserum). In spite of this, there were large individual variations in total and LDL-cholesterol levels as well as in release of chemokines.
Interestingly, we found that FH serum, but not serum from healthy controls, were able to enhance the release of chemokines in PBMCs in both FH patients and controls. Moreover, the highest spontaneous release of chemokines and the lowest oxLDL-stimulatory ratio was found in PBMCs from FH patients with the highest serum levels of total and LDL-cholesterol. However, despite the use of FH serum, PBMCs in FH patients still had a higher chemokine secretion than cells in healthy individuals, suggesting that the cellular factor also contribute to the enhanced chemokine release of FH patients. Nevertheless, these data may suggest that lipid loading of the cells could explain some of the inflammatory responses observed. Moreover, these findings in these ‘transgenic’ individuals further support a link between lipids and inflammation, and support the notion that LDL- and in particular oxLDL-cholesterol may be important stimuli for the inflammatory response in atherosclerotic disorders.

Among FH patients PBMCs from smokers spontaneously released higher levels of chemokines and had decreased oxLDL-stimulatory ratio compared non-smoking FH patients. Similar differences appeared between smokers and non-smokers in the control group, suggesting a link between smoking and enhanced chemokine levels as a more general phenomenon. Furthermore, in FH patients, but not in healthy controls, men had significantly decreased oxLDL-stimulatory ratio for MIP-1α, MIP-1β and IL-8 comparing FH women. We were unable to show any differences in markers of inflammation among those with clinically manifest premature CAD compared to those without. This could be due to too low number of CAD patients (n=6) to provide adequate power in this sub-study. However, PBMCs from FH patients with tendon xanthomas/xanthelasms had enhanced spontaneous release of chemokines compared to those without tendon xanthomas/xanthelasms.

Taken together, these data show that MIP-1α, MIP-1β and IL-8 are associated with established risk factors for CAD and suggest that these chemokines may be additional factors contributing to accelerated atherosclerosis as well as to the different phenotypes in this group of high-risk patients with identical FH mutation.

Acknowledgements

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References