Leukocyte activation and inflammation in cardiovascular disease

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Secondary prevention with fluvastatin decreases levels of adhesion molecules, neopterin as well as C-reactive protein


Treatment of hypercholesterolaemia with HMG-CoA reductase inhibitors results in an earlier reduction of morbidity and mortality than expected from trials using conventional cholesterol lowering therapies. Possible explanations for this effect include stimulation of angiogenesis, improvement of endothelial function, plaque stabilisation, inhibition of coagulation and/or thrombocyte aggregation and inhibition of the inflammatory response associated with atherosclerosis. We investigated whether statins exert their effects by inhibition of endothelial activation, inflammation and/or monocyte/macrophage activation by measuring plasma levels of soluble cell adhesion molecules, neopterin and C-reactive protein upon treatment with fluvastatin for a period of twelve months in patients with established atherosclerosis and hypercholesterolaemia. Blood samples were taken at baseline, three months and twelve months after starting treatment with fluvastatin 80 mg daily. Upon treatment a reduction of s-ICAM-1 (956.3 ± 123.6 vs. 745.4 ± 127.4 vs. 674.9 ± 70.8 ng/ml, p<0.05) and s-E-selectin (58.6 ± 6.7 vs. 47.0 ± 6.1 vs. 44.9 ± 3.2 ng/ml, p<0.01) was observed. In addition, levels of neopterin decreased, albeit transiently (7.1 ± 0.7 vs. 6.0 ± 0.5 vs. 6.5 ± 0.8 nmol/L, p=0.02), suggesting a reduction in monocyte/macrophage activity. Moreover, we found a decrease in levels of C-reactive protein during follow-up (5.21 ± 2.0 vs. 3.18 ± 0.7 vs. 1.95 ± 0.3 mg/L, p<0.05), compatible with a reduction of inflammatory activity. We conclude that statins have a combined beneficial effect on monocyte/macrophage activity, endothelial function and systemic inflammatory activity.

INTRODUCTION

Atherosclerosis is recognised as a chronic inflammatory disease in which an inflammatory response is the key event that leads to formation of atheromatous plaques, ultimately resulting in vascular occlusion [1]. Endothelial dysfunction is the earliest stage in the atherosclerotic process. It is characterized by increased permeability of endothelial cells, loss of anti-coagulant properties, enhanced leukocyte adhesion due to increased endothelial expression of cell adhesion molecules (CAMs), such as ICAM-1, VCAM-1, P-selectin and E-selectin, and increased vascular tone due to a decrease in NO-production and proliferation of smooth muscle cells [2]. A direct causative factor for the inflammatory response that induces endothelial dysfunction has not yet been identified although important contributions are suggested for oxidised LDL-cholesterol, hyperhomocysteinaemia, infectious agents and auto-immunity [1;3].

Increased expression of CAMs on endothelial cells may result in increased binding of monocytes, T-lymphocytes and thrombocytes, key players in the atherosclerotic process. Endothelial CAMs are known to be shed into the circulation during endothelial cell activation. Elevated levels of soluble CAMs (sCAMs) have been reported in patients with widespread atherosclerosis, but also other diseases in which endothelial activation occurs, e.g. vasculitis. [4-8] Importantly, the relevance of these levels of CAMs is underlined by the fact that s-ICAM-1 is an independent predictor of cardiovascular disease [9].

Hypercholesterolaemia is one of the major risk factors for developing cardiovascular disease and treatment of hypercholesterolaemia with HMG-CoA reductase inhibitors has become a cornerstone in the prevention of cardiovascular disease [10;11]. Whereas non-statin cholesterol lowering therapies such as diet, surgery or therapy with resins showed a significant decrease in cardiovascular mortality after two years of treatment [12-14], statin therapy proved to be beneficial as early as nine months after initiation of therapy [10;11]. This early effect was independent of baseline lipid values [15;16] and has been ascribed to other, non-cholesterol related effects of these drugs, such as
improved endothelial function, plaque stabilisation and/or increased angiogenesis [17-19].

Monocytes are culprit cells in atherosclerotic plaque formation. Binding of monocytes to dysfunctional endothelium requires activation of these monocytes. Recently, it was shown that patients with coronary artery disease have higher levels of neopterin, a marker of monocyte/macrophage activation [20]. Importantly, it was demonstrated that statins may influence monocyte function as was found in in vitro studies [17;21;22].

In the present study, we tested the hypothesis that statins may influence endothelial activation and/or monocyte/macrophage activation, by measuring levels of soluble CAMs, neopterin, and C-reactive protein, a systemic marker of inflammation, in patients with stable atherosclerotic disease and hypercholesterolaemia during treatment with a HMG-CoA inhibitor.

**Materials and Methods**

**Patients and controls**

A group of ten consecutive patients who were referred to our outpatient clinic for atherosclerosis and lipid disorders were asked to participate in an open label study. Patient inclusion was from May 1997 to April 1998.

All patients had established atherosclerosis. This was defined as clinically manifest cardiovascular and/or cerebrovascular disease, as diagnosed by their referring physician. All patients had stable cardiovascular disease, baseline measurements were taken at least 3 months after their last cardiovascular event. Furthermore, they had untreated hypercholesterolaemia, defined as a fasting serum cholesterol level > 5.5 mmol/L. None of the patients or controls had hypertension, diabetes mellitus or concurrent infectious, inflammatory or malignant disease. All patients were treated with fluvastatin 80 mg daily. Patients were seen every 3 months during a follow-up of 1 year. A group of 28 age and gender-matched healthy blood donors served as controls.

**Methods**

Overnight fasting venous blood was obtained following non-traumatic venepuncture. Total cholesterol, HDL-cholesterol and triglycerides were determined according to routine laboratory techniques. LDL-cholesterol was calculated using the Friedewald formula.

For other measurements, blood was collected in EDTA tubes and centrifuged, and plasma samples were stored immediately after collection at –20°C until further analysis. s-ICAM-1 and s-E-selectin levels were measured by sandwich ELISA, using a commercially available kit (Bender MedSystems, Boehringer Ingelheim, Austria). CRP levels were measured by ELISA, as previously described [23]. Neopterin levels were determined using a commercially available ELISA (Brahms Diagnostica, Berlin, Germany). All measurements were performed in duplicate.

**Statistics**

Differences in baseline levels of s-ICAM-1, s-E-selectin, CRP and neopterin between patients and healthy controls were tested by a Wilcoxon 2-sample test because of skewed distribution. A random effects model for repeated measurements was used.

**Table 1.**

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>9/1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>24.3 ± 3.0</td>
</tr>
<tr>
<td>Smoking</td>
<td>2/10</td>
</tr>
<tr>
<td>Concomitant medication</td>
<td></td>
</tr>
<tr>
<td>Fibrate</td>
<td>1/10</td>
</tr>
<tr>
<td>Salicylates</td>
<td>6/10</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>2/10</td>
</tr>
<tr>
<td>beta blockers</td>
<td>5/10</td>
</tr>
<tr>
<td>Diuretics</td>
<td>1/10</td>
</tr>
<tr>
<td>Medical history</td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>5/10</td>
</tr>
<tr>
<td>PTCA</td>
<td>1/10</td>
</tr>
<tr>
<td>CABG</td>
<td>2/10</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>1/10</td>
</tr>
<tr>
<td>Ischaemic stroke</td>
<td>2/10</td>
</tr>
</tbody>
</table>
### Table 2. Levels of s-ICAM-1, s-E-selectin, neopterin and CRP in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3 months</th>
<th>12 months</th>
<th>P-value (Bonferroni)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-ICAM-1</td>
<td>6.78 ± 0.1</td>
<td>6.50 ± 0.1</td>
<td>6.46 ± 0.1</td>
<td>0.009*</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>6.47 ± 0.1</td>
<td>605 (393-1749)</td>
<td>710.5 (347-1059)</td>
<td>0.03</td>
</tr>
<tr>
<td>s-E-selectin</td>
<td>58.6 ± 3.7</td>
<td>47.0 ± 3.7</td>
<td>44.9 ± 3.7</td>
<td>0.006*</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>61.6 ± 3.5</td>
<td>55 (21-80)</td>
<td>44 (30-59)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Neopterin</td>
<td>1.92 ± 0.1</td>
<td>1.74 ± 0.1</td>
<td>1.81 ± 0.1</td>
<td>0.02**</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>1.71 ± 0.1</td>
<td>5.7 (9.4-3.8)</td>
<td>6.1 (12.8-4.3)</td>
<td>0.06*</td>
</tr>
<tr>
<td>CRP</td>
<td>1.21 ± 0.2</td>
<td>0.94 ± 0.2</td>
<td>0.57 ± 0.2</td>
<td>0.03*</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0.69 ± 0.2</td>
<td>2.0 (1.1-6.3)</td>
<td>1.9 (0.8-3.0)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6.44 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>4.6 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>4.5 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.8 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

The upper values are means (± SEM), the lower values are medians (range).  
* P-value for the entire study period; ** P-value for first 3 months; # P-value for baseline comparisons.  
n.s. not significant. * Means derived from nlog-transformed values.

To analyse differences in time for s-ICAM-1, s-E-selectin, CRP, neopterin and lipid levels. To obtain normally distributed parameters, a (natural) logarithmic transformation of s-ICAM-1, CRP and neopterin was performed. Normality was then tested with the Shapiro-Wilk test. The differences in time for each variable were corrected for baseline characteristics. A Bonferroni correction was applied to correct for repeated measurements. All data are expressed as mean ± SEM unless otherwise indicated. For better comprehension the data mentioned in the abstract and results section are the original values; table 2 contains log-transformed values as used for statistics and medians plus ranges. Correlations between changes in levels of cholesterol and levels in inflammatory markers after 12 months were calculated using Spearman’s Rho.

**RESULTS**

**Patients**

Patient characteristics are presented in table 1. None of the patients reported progression of the vascular disease, i.e. no hospitalization, changes in medication or progression of complaints occurred. Medication was well tolerated and no side effects were reported.

**Lipids**

Serum levels of total cholesterol (figure 1.) and LDL-cholesterol in patients decreased after 3 months of treatment with fluvastatin and remained unchanged for the remainder of the study (-28%±7.7 and -38%±11.4 respectively). Levels of HDL-cholesterol and serum triglycerides were unaffected.

**s-ICAM-1**

At baseline, levels of s-ICAM-1 were significantly higher in patients than in the healthy controls (956.3 ± 123.6 ng/ml vs. 693.2 ± 50.8 ng/mg, P <0.05). After 3 months of therapy, levels of s-ICAM-1 declined to values comparable with levels found in healthy controls; they declined even further after 12 months (956.3 ± 123.6 ng/ml vs. 745.4 ± 127.4 ng/ml vs. 674.9 ± 70.8 ng/ml, P <0.001; figure 2.).
s-E-selectin
s-E-selectin levels did not differ between patients and controls at the beginning of the study (58.6 ± 6.7 ng/ml vs. 61.6 ± 3.9 ng/ml, P = n.s.). However, after 3 months s-E-selectin levels in patients were significantly lower, decreasing further after 12 months (58.6 ± 6.7 ng/ml vs. 47.0 ± 6.1 ng/ml vs. 44.9 ± 3.2 ng/ml, P <0.01; figure 3).

C-reactive protein
C-reactive protein at baseline was no higher in patients than it was in the control group (5.21 ± 2.0 mg/L vs. 3.18 ± 0.9 mg/L, P = 0.10). During treatment, CRP levels in patients declined significantly after 12 months (5.21 ± 2.0 mg/L vs. 3.18 ± 0.7 mg/L vs. 1.95 ± 0.3 mg/L, P <0.05; figure 4).

Neopterin
A trend towards higher neopterin levels in the plasma of patients than in healthy controls was seen at baseline (7.10 ± 0.7 nmol/L vs. 5.71 ± 0.3 nmol/L, P = 0.067). Initially, neopterin levels in patients decreased after 3 months of fluvastatin treatment (7.10 ± 0.7 nmol/L vs. 5.96 ± 0.5 nmol/L, P = 0.02), but this difference had disappeared after 12 months of treatment (6.45 ± 0.8 nmol/L; figure 5).

Correlations
No correlations were found between changes in cholesterol over 12 months and changes in s-ICAM-1 (r = -0.04, P = n.s.), s-E-selectin (r = 0.16, P = n.s.), neopterin (r = 0.06, P = n.s.) or CRP (r = -0.26, P = n.s.) during the same period.

DISCUSSION
In this prospective, open label-intervention study in patients with established atherosclerosis and hypercholesterolaemia we demonstrate a reduction in markers of endothelial cell activation (s-E-selectin, s-ICAM-1) as in C-reactive protein after short- and long-term treatment with fluvastatin, an HMG-CoA reductase inhibitor.

Figure 1-5. Intra-individual changes in levels of cholesterol, s-ICAM, s-E-selectin, CRP, and neopterin in patients during the study. The broken line represents the mean changes.
In addition, neopterin levels were transiently reduced after short-term treatment, suggesting a decrease in monocyte/macrophage activity in these patients. As was previously reported [7;8;24;25], levels of s-ICAM-1 are elevated in patients suffering from cardiovascular disease complicated by hypercholesterolaemia. Importantly, we demonstrated a reduction of s-ICAM-1 during HMG-CoA reductase inhibition. This reduction in levels of ICAM-1 did not correlate with cholesterol reduction and may, therefore, be considered a pleitropic effect of the statin. Since ICAM-1 is expressed on endothelial cells as well as on monocytes and macrophages, we also measured endothelial-specific and monocyte/macrophage-specific markers in our patients.

It is well established that statins have beneficial effects on endothelial cell function [2]. Our observations that levels of s-E-selectin, which is predominantly present on activated endothelial cells, decreased during HMG-CoA reductase inhibition is in line with this observation. Previously, Hackman et al. [24] also found decreased s-E-selectin levels during treatment with HMG-CoA reductase inhibitors. We hypothesize that therapy with statins results in increased nitric oxide (NO) production by activation of endothelial NO synthetase which, in turn promotes angiogenesis and inhibits inflammation [19]. Kureishi et al. [26] recently demonstrated that HMG-CoA reductase inhibitors may activate endothelial cell NO production by stimulating the protein kinase Akt, a multifunctional regulator of cell survival, growth and glucose metabolism.

Apart from the effects of the HMG-CoA reductase inhibitor fluvastatin on endothelial cells, we observed that neopterin levels were lowered in the first 3 months as well, implying that the lowering of s-ICAM-1 in this phase may be attributed in part to a decrease in monocyte/macrophage activity. This is in accordance with prior in vitro studies on monocyte/macrophage activity upon treatment with HMG-CoA reductase inhibitors [17;22;27]. Our findings contrast, however, with earlier findings of Gottsater et al. [28], who failed to detect differences in neopterin levels during treatment with fluvastatin. This difference may be explained by the fact that much lower doses were used in their study than in ours. Furthermore, we found that the effect on neopterin levels was a short-term effect of the medication; during longer follow-up periods, neopterin levels tended to increase again.

Finally, we found that CRP levels also declined during fluvastatin treatment, as previously described by the CARE investigators [29]. Baseline CRP levels, however, were not statistically different from those in healthy controls.

In summary, we demonstrated that an HMG-CoA reductase inhibitor, fluvastatin, influences parameters of monocyte/macrophage activation and endothelial activation. Our findings suggest that the inflammatory response in atherosclerotic vascular disease can be modulated by this form of therapy and that a decrease in monocyte/macrophage activation and a decrease in endothelial cell activation can be induced in patients with established atherosclerosis and hypercholesterolaemia by the HMG-CoA reductase inhibitor fluvastatin.

REFERENCES


