Upregulation of CD40 and CD40 Ligand (CD154) in Patients With Moderate Hypercholesterolemia

C.D. Garlichs, MD; S. John, MD; A. Schmeißer, MD; S. Eskafi, PhD; C. Stumpf, MD; M. Karl; M. Goppelt-Struebe, PhD; R. Schmieder, MD; W.G. Daniel, MD

**Background**—Hypercholesterolemia, a risk factor for cardiovascular disease, is associated with inflammation and hypercoagulability. Both can be mediated by the CD40 system. This study investigated whether the CD40 system is upregulated in patients with moderate hypercholesterolemia and whether it is influenced by therapy with a hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor.

**Methods and Results**—Fifteen patients with moderate hypercholesterolemia and 15 healthy control subjects were investigated. CD154 and P-selectin were analyzed on platelets and CD40 was analyzed on monocytes before and under therapy with the statin cerivastatin by double-label flow cytometry. Blood concentrations of soluble CD154 and monocyte chemoattractant protein-1 (MCP-1) were evaluated. Our main findings were as follows. Patients with moderate hypercholesterolemia showed a significant increase of CD154 and P-selectin on platelets and CD40 on monocytes compared with healthy subjects. Soluble CD154 showed a nonsignificant trend for higher plasma levels in patients. A positive correlation was found for total or LDL cholesterol and CD154, but not for CD40 on monocytes. The latter was upregulated in vitro by C-reactive protein, which was found to be significantly elevated in patients with moderate hypercholesterolemia. CD154 on platelets proved to be biologically active because it enhanced the release of MCP-1, which was markedly elevated in an in vitro platelet-endothelial cell coculture model and in the serum of patients. Short-term therapy with a HMG-CoA reductase inhibitor significantly downregulated CD40 on monocytes and serum levels of MCP-1.

**Conclusion**—Patients with moderate hypercholesterolemia show upregulation of the CD40 system, which may contribute to the known proinflammatory, proatherogenic, and prothrombotic milieu found in these patients. (Circulation. 2001; 104:2395-2400.)

Key Words: hypercholesterolemia ■ P-selectin ■ atherosclerosis ■ inflammation ■ immune system

Chronic inflammation plays a key role in atherosclerosis and its complications.1 Hypercholesterolemia (HC) as a cardiovascular risk factor is also associated with a chronic inflammatory milieu.2 High cholesterol levels are frequently associated with the induction of cytokines and chemokines, upregulation of endothelial adhesion molecules, and immune reactions against oxidized components on lipoproteins.3 Treating HC with hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) lowers levels of lipids, C-reactive protein (CRP), and proinflammatory cytokines.3

In addition, HC is associated with an imbalance of the hemostatic system. Enhanced levels of thrombin, fibrinogen, and factor VIIc directly correlate with cholesterol levels.5 Furthermore, persistent platelet activation has been reported in HC.5,6 The pathophysiological consequences of platelet activation in HC are not yet fully understood, but it is well established that platelet activation is associated with an enhanced risk of thrombotic complications in different clinical disorders.7 Activated, but not resting, platelets adhere to the intact endothelium and induce inflammatory responses of the endothelium, which substantially contributes to the early steps of atherosclerosis.1,8 In summary, a further evaluation of potential causes for the inflammatory and hemostatic status of HC may have clinical benefit.

Studies have recently supported the emerging role of CD40–CD40 ligand (CD40L or CD154) interactions in atherosclerosis, thrombosis, and inflammation.9 The CD40–CD154 dyad was originally known to be essential in immune reactions and autoimmune diseases.9 In atherosclerosis, disruption of the CD40–CD154 system in LDL receptor or apoE-deficient mice prevents the initiation of atherosclerosis and the progression of established atherosclerotic lesions to more advanced lesions.10 Structurally, CD154 is a transmembrane protein related to tumor necrosis factor-α (TNF-α), and it was originally identified on stimulated CD4+ T cells and,
later, on stimulated mast cells, basophils, platelets,12 and vascular cells like smooth muscle cells.12 The receptor CD40 is constitutively expressed on B cells, monocytes, macrophages, dendritic cells, and endothelial cells.12,13 The engagement of CD40 on endothelial cells or monocytes induces the synthesis of adhesion molecules, proinflammatory cytokines, chemokines, and tissue factor or activates matrix metalloproteinases.10,11 In addition, CD154 is strongly upregulated on platelets constituting a fresh thrombus.11 Thus, the CD40–CD154 system seems to be a critical pathway for local inflammation of the vascular wall and the hemostatic system.

Until now, no study has addressed the potential relationship between HC and the CD40 system. Therefore, the present study was designed to investigate whether the CD40–CD154 dyad is modified in patients with moderate HC with regard to platelets, monocytes, or T cells. In addition, the effects of short-term therapy with cerivastatin were studied with regard to the CD40 system.

Methods

Patients and Controls
Fifteen consecutive patients with moderate HC (all men; mean age, 28.5 ± 4.6 years; LDL-cholesterol >130 mg/dL) and 15 healthy sex- and age-matched normcholesterolemic subjects (mean age, 26.8 ± 2.3 years; LDL-cholesterol <130 mg/dL) were studied between January and November 2000. Patients were on an American Heart Association step I diet, and none of the subjects was taking drugs (including antioxidants or anti-inflammatory drugs) or dietary supplements within the 2 months before and during the study. After overnight fasting and a rest of at least 20 minutes, blood samples were taken for laboratory tests. Blood samples were also taken from the same patients after treatment with cerivastatin (0.3 mg/daily; Baycr) for 3 weeks.

Patients and controls had no clinical evidence of peripheral, cardiovascular, or cerebrovascular atherosclerotic or inflammatory diseases (by clinical history, physical examination, and ECG). Exclusion criteria included renal insufficiency, proteinuria, altered hepatic function, alcohol abuse, and body mass index >25. Patients with diabetes mellitus (fasting blood glucose level >115 mg/dL) and/or treatment with other biochemical parameters was assessed by the Spearman rank correlation test. All values are reported as mean ± 1SD. Statistical significance was set at P<0.05.

Results

The baseline characteristics of HC patients and healthy controls are summarized in Table 1.
CD154 and P-selectin on platelets were significantly increased in formaldehyde-fixed blood samples from HC patients compared with those obtained from control subjects (CD154: 12.6±2.6 versus 8.2±1.4 net mean fluorescence intensity [MFI]; P-selectin: 2.3±1 versus 0.8±0.5 MFI; all P<0.001; Figure 1). This increased expression of CD154 on platelets from HC patients showed a positive correlation with serum levels of total cholesterol (Figure 2), LDL cholesterol (r=0.44, P=0.02), and the LDL/HDL cholesterol ratio (r=0.46, P=0.01) but not with HDL cholesterol (r=0.04, P=0.79) or triglycerides (r=0.01, P=0.86). In contrast, no correlations were found between P-selectin expression and serum lipids (total cholesterol: r=0.36, P=0.13; LDL cholesterol: r=0.34, P=0.14; LDL/HDL cholesterol ratio: r=0.17, P=0.47; HDL cholesterol: r=0.04, P=0.07; triglycerides: r=0.1, P=0.69). Furthermore, the expression of CD154 on platelets did not show a significant correlation with the expression of P-selectin (r=0.23, P=0.3).

Stimulating nonfixed platelets with the agonist ADP increased the expression of CD154 and P-selectin. The magnitude of upregulation of CD154 and P-selectin, however, was significantly less in platelets obtained from HC patients compared with those from healthy controls (Table 2).

The soluble and biologically active form of CD154 showed a nonsignificant trend for higher plasma levels in HC patients (22.8±6.7 versus 28.1±4.2 ng/mL, P=0.06; Figure 3). Correlation analysis found no association between CD154 on platelets and sCD154 (r=0.15, P=0.46).

CD40 on monocytes was significantly upregulated in the blood from HC patients compared with controls (5.4±3.3 versus 1.8±0.8 MFI, P<0.02; Figure 4). Expression of CD40 on monocytes did not correlate with the expression of CD154 on platelets in corresponding blood samples from HC patients (r=0.01, P=0.88). In addition, the number of platelet-monocyte aggregates was not different among HC patients and controls (103.9±70.9 versus 115.9±64.8 MFI, P=NS), suggesting that monocyte-platelet interactions through CD40 may not play a prominent role in moderate HC.

Upregulation of CD40-CD154 may enhance the release of MCP-1 from endothelial cells or monocytes in vitro. Accordingly, patients with HC showed higher serum levels of MCP-1 than controls (215.4±74.8 versus 99.7±53.2 pg/mL, P=0.001). Correlation analysis, however, revealed no significant correlation among platelet CD154, sCD154, platelet P-selectin, and serum MCP-1 (CD154: r=0.17, P=0.41; sCD154: r=0.17, P=0.38; P-selectin: r=0.09, P=0.68). Instead, MCP-1 correlated with the magnitude of monocyte CD40 expression (r=0.44, P=0.02). In a coculture model with HUVEC, thrombin (2 U/mL)-activated human platelets enhanced the release of MCP-1 (control, 111±6 pg/mL; versus unstimulated platelets, 260±37 pg/mL; versus stimulated platelets, 492±42 pg/mL, P<0.05).

Table 1. Baseline Characteristics of Hypercholesterolemic Patients and Age- and Sex-Matched Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=15)</th>
<th>Patients (n=15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>26.8±2.3</td>
<td>28.5±4.6</td>
<td>NS</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>100</td>
<td>100</td>
<td>...</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.1±1.3</td>
<td>23.3±1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, n</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Diabetes mellitus, n</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Clinical atherosclerosis, n</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Concomitant medication, n</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>178.5±25</td>
<td>265.7±59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>90.5±22.2</td>
<td>174.3±42.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>90.3±31.1</td>
<td>113.7±22.5</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>52.8±7.6</td>
<td>45.5±9.3</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>
| hs CRP, mg/dL          | 0.4±0.3        | 0.9±0.8        | 0.018

Data are mean±SD. NS indicates nonsignificant; hs, high sensitive.
n=3; Figure 5). Platelet-induced endothelial release of MCP-1 was blocked with a specific monoclonal anti-CD154 antibody but not with the unspecific control antibody (stimulated platelets, 492±42 pg/mL; versus pretreatment with anti-CD154, 293±31 pg/mL; versus pretreatment with MOPC-21, 421±15 pg/mL; Figure 5).

In a short-term experiment, we tried to modulate CD154 and CD40 expression in HC patients by initiating a therapy with cerivastatin (0.3 mg/d for 21 days in 15 HC patients). This treatment resulted in a significant reduction in total cholesterol (265.7±59 versus 215.2±49.8 mg/dL, P<0.02) and LDL cholesterol (174.3±42.4 versus 129.7±35.5 mg/dL, P=0.004). Statin treatment did not affect enhanced platelet CD154 or P-selectin expression or CD154 (CD40: 12.6±2.6 versus 12.8±3 MFI, before versus during therapy, P=NS; P-selectin: 2.3±1 versus 2.1±1.6 MFI, P=NS; sCD154: 28.1±4.2 versus 29.3±6.4 ng/mL, P=NS). However, cerivastatin significantly reduced serum levels of MCP-1 (215.4±74.8 versus 171.4±61.6 pg/mL, P=0.04) and CD40 on monocytes (5.4±3.3 versus 3.3±1.7 MFI, P<0.05). Downregulation of CD40 was confirmed in vitro: TNF-α induced the upregulation of CD40 in the monocyte cell line THP-1 and in HUVEC, whereas pretreatment of cells with cerivastatin and subsequent TNF-α stimulation significantly inhibited TNF-α–induced CD40 expression in both cell types (THP-1 monocytes: basal expression 7.5±2.3 MFI, TNF-α 17.6±4.2 MFI, pretreatment/TNF-α 12.3±3.6 MFI, without versus with statin P<0.05; HUVEC: basal expression 10.8±4.7 MFI, TNF-α 28±4.1 MFI, pretreatment/TNF-α 19±4.9 MFI, without versus with statin P<0.05; n=4; Figure 6).

In our study, no significant differences in T cell activation were observed between patients with HC and controls, as measured by CD25 (1.4±0.4 versus 1.4±0.3 MFI, P=NS).

Finally, patients with moderate HC showed significantly elevated levels of CRP within the normal range (Table 1). In vitro, CRP (5 μg/mL) significantly upregulated CD40 on monocytes after 24 hours of incubation (4.7±0.6 versus 6.8±0.3 MFI, P<0.05, n=5). CRP-induced upregulation of CD40 was inhibited by pretreating monocytes with cerivastatin (4.7±0.6 versus 4.4±0.7 MFI, P=NS).

### Discussion

The present study shows CD40–CD154 upregulation in patients with moderate HC. Patients showed a significant increase of CD154 on platelets and CD40 on monocytes, but not on T cells, when compared with sex- and age-matched normocholesterolemic subjects. The soluble form of CD154 showed a nonsignificant trend for higher plasma levels in HC. A positive correlation was found for total or LDL cholesterol and CD154, but not for CD40 on monocytes. The latter was upregulated in vitro by CRP, which was found to be significantly elevated in patients with moderate HC. CD154 on platelets proved to be biologically active because it enhanced the release of MCP-1, which was markedly elevated in an in vitro platelet-endothelial cell coculture model and in the serum of patients. Therapy with a HMG-CoA reductase inhibitor did not interfere with CD154 in our patients, but it

| Table 2. Expression of CD154 and P-Selectin on Platelets Before and After Stimulation with 5 μmol/L ADP |
|-------------------------------------------------|-------------------------------------------------|-----------------|
| Controls (n=15)                                 | Patients (n=15)                                 | P               |
| CD154                                           |                                                 |                 |
| Without ADP                                     | 21.2±6.7                                        |                 |
| ADP                                             | 112.4±76.1†††                                    | *P=0.03 vs controls without ADP. |
| P-selectin                                      |                                                 |                 |
| Without ADP                                     | 11.6±6                                          |                 |
| ADP                                             | 272.9±65.4†††                                    | ††P=0.01 vs controls/patients without ADP. |

Values are mean±SD and are expressed as mean fluorescence intensity (MFI).

Figure 3. Serum levels of soluble CD154 in 15 patients with HC and 15 age- and sex-matched healthy subjects. Circles represent individual measurements (ng/mL); dotted line, mean value of each group.

Figure 4. CD40 on monocytes in 15 patients with HC and 15 age-matched healthy subjects. Circles represent individual measurements (MFI); dotted line, the mean value of each group.
significantly downregulated CD40 on monocytes and serum levels of MCP-1.

Although activation of the CD40 system has been observed in atherosclerosis-related diseases, our findings represent the first in vivo evidence of CD40–CD154 upregulation in patients with asymptomatic HC. These observations represent an additional pathophysiological pathway for the prothrombotic and proatherogenic state in HC.

Several studies have shown that HC is associated with persistent platelet activation in vivo and that membrane-bound and soluble P-selectin levels correlate with LDL cholesterol levels. Our present study confirms platelet activation in moderate HC as well.

Upregulation of CD154 on activated platelets, which has just recently been described, represents a new aspect in HC. P-selectin and CD154 derived from platelets share common features but show biological differences: both can be immediately expressed on the platelets surface on stimulation with platelet activators. However, in contrast to previous reports, CD154 in platelets is not stored in the α-granula (as it is the case for P-selectin), but in the cytosol of platelets, and it follows a different expression pattern on platelet activation compared with P-selectin. In accordance with this, our study did not find a significant correlation between CD154 and P-selectin expression in vivo.

In our study, membrane-bound and the biologically active soluble form of CD154 closely correlated with total and LDL cholesterol. This observation corresponds to in vitro experiments in which native LDL and lysophosphatidic acid significantly induced surface upregulation of CD154 on platelets (data not shown). Therefore, the interaction of circulating lipids with platelets may result in CD154 upregulation and may thus initiate and maintain the proinflammatory milieu observed in HC.

The activation of monocytes in HC has been reported previously. The adhesion of monocytes to endothelium is crucial in atherogenesis, and statins have been shown to reduce monocyte activation and adhesive properties, which may contribute to the clinical benefit of HMG-CoA reductase inhibitors in atherosclerosis, independent of their cholesterol-lowering effects. Our study showed a highly upregulated CD40 expression in patients with rather moderate HC.

Because CD40 engagement on monocytes induces many procoagulant and proatherosclerotic effects, we propose the CD40 pathway as being crucial for the pathophysiology of HC. In our patients, the CD40 upregulation on monocytes paralleled the elevation of MCP-1, an important chemokine that attracts specific leukocytes to sites of inflammation. Thus far, elevated MCP-1 levels have been shown only in severe HC, but not in moderate HC. MCP-1 release may be the result of CD40 engagement on endothelial cells or on monocytes. Evidence for a monocyte-derived MCP-1 release in our patients may be drawn from the fact, that upregulation and downregulation of CD40 on monocytes and MCP-1 levels positively correlated before and after statin therapy. CD40 engagement on monocytes may occur through platelet-monocyte interactions, because monocytes rapidly adhere for prolonged periods of time to activated platelets that display P-selectin.

In accordance with previous studies, cerivastatin significantly reduced TNF-α and interleukin-1, because serum levels did not differ significantly between the groups (data not shown). However, significantly elevated CRP levels in severe HC proved to upregulate CD40 on monocytes in vitro, an effect that was blocked in vitro by pretreating monocytes with cerivastatin. However, induction by CRP may represent one, but probably not the only, mechanism for the increase of CD40 on monocytes found in HC.

The relevance of MCP-1 release via CD40 engagement is further supported by our findings that pretreatment of endothelial cells or monocytes with cerivastatin resulted in a significant downregulation of cytokine-induced CD40, an effect that may explain the reduction of MCP-1 serum levels.
observed in cerivastatin-treated patients. Therefore, downregulation of CD40 by statin therapy could have an additional pathophysiological benefit in HC with regard to the other procoagulant and proatherosclerotic effects mediated by CD40 on endothelial cells or monocytes.

Cerivastatin did not modulate CD154 or P-selectin in our study, although total and LDL cholesterol significantly declined. Possible explanations for this phenomenon may be the short period of treatment in our patients or a drug specific effect. Longer treatment periods with cerivastatin or other anti-atherosclerotic agents may be necessary to conclude on the potential benefit of CD154 inhibition in HC.

Besides platelets and monocytes, T cells can be activated in HC. T cell activation and the T cell help autoantibody production correspond to cholesterol levels. In our patients with moderate HC, we did not observe the activation of T lymphocytes, indicating that T cell–derived CD154 does not promote inflammation in this setting. In addition, the observed nonsignificant trend of elevated levels of sCD154 in patients with HC does not originate from T cells, because these would need activation for rapid release of sCD154.

In conclusion, we have shown that moderate HC is associated with an activation of the CD40–CD154 system that can partially be downregulated by cerivastatin. Because the upregulation of this system is involved in the pathogenesis of atherosclerosis, we propose that activation of the CD40–CD154 system may create a proinflammatory and prothrombotic milieu, aggravating the development of atherosclerosis in moderate HC.

References

Upregulation of CD40 and CD40 Ligand (CD154) in Patients With Moderate Hypercholesterolemia


Circulation. 2001;104:2395-2400
doi: 10.1161/hc4501.099312

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/20/2395

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/