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

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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.4 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.6 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, and vascular cells like smooth muscle cells. The receptor CD40 is constitutively expressed on B cells, monocytes, macrophages, dendritic cells, and endothelial cells. 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. In addition, CD154 is strongly upregulated on platelets constituting a fresh thrombus. 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 regarding the CD40 system. Therefore, the present study was designed to investigate whether the CD40–CD154 system seems to be a critical pathway for local inflammation of the vascular wall and the hemostatic system.

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### 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; Bayer) 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 a hypoglycemic agent), hypertension (systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, and/or antihypertensive treatment), and smokers were excluded. The local Ethics Committee approved the study, and the patients provided written, informed consent.

#### Blood Sampling Protocol

Peripheral venous blood was drawn into blood collection tubes containing sodium citrate. Citrated blood samples were either centrifuged (190g for 10 minutes at room temperature) to obtain platelet-rich plasma or immediately fixed with 1% formaldehyde (1:1, v:v). Noncitrated blood was immersed in melting ice and allowed to clot for 1 hour before centrifugation (1500g for 10 minutes at 4°C). The supernatant was stored at −80°C until analysis. Samples were thawed only once.

#### Immunofluorescence

The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD154 (B-B29, mouse IgG₂a), anti-CD62P (FITC, AK-4, mouse IgG₂a), mouse IgG (FITC), phycoerythrin (PE)-conjugated anti-CD61 (PM6, mouse IgG), and anti-CD4 (PE; RFT-4, mouse IgG) from Dianova, and anti-CD40 (FITC, 5C3, mouse IgG), anti-CD14 (PE; M5E2, mouse IgG), and anti-CD25 (FITC; M-A251, mouse IgG) from PharMingen.

Platelet immunostaining was performed as previously described. Fixed blood was diluted 1:200 with PBS and incubated with the antibodies for 30 minutes at room temperature. In some experiments, we used platelet-rich plasma to stimulate platelets with ADP (Sigma). Platelets (20 000 per μL) were stimulated with 5 μmol/L ADP for 10 minutes at room temperature. Thereafter, the cells were diluted to 2500 per μL and incubated with antibodies (anti-CD154, anti-CD62P, and anti-CD61) for a further 30 minutes at room temperature. A total of 10 000 cells was measured by flow cytometry (FACS Calibur, Becton Dickinson) within 2 hours after sampling and was analyzed by CellQuest Software (Becton Dickinson). Platelets were identified by gating on CD61-PE positivity and their characteristic light scatter. The platelet population evaluated was ≥98% positive for CD61.

To evaluate CD40 on monocytes, fixed blood was diluted 1:5 and incubated with anti-CD40 and anti-CD14 for 30 minutes at room temperature. To determine T-cell activation, fixed blood was incubated with anti-CD25 and anti-CD4. Erythrocytes were removed by adding 2 mL of FACS lysis solution (PharMingen) for 10 minutes at room temperature. Leukocytes were washed twice with PBS and fixed in 1% formaldehyde in PBS. Monocytes were identified by gating for CD14-positive cells and T cells by gating for CD4-positive cells.

Platelet-monocyte aggregates were measured as previously described. Briefly, 100 μL of citrate-anticoagulated blood was diluted with HEPES-Tyrode’s buffer (1:2). Samples were incubated with anti-CD61 or an isotype-matched control. After 30 minutes of incubation at room temperature, 1 mL of FACS lysis solution was added for 10 minutes to lyse erythrocytes before analysis by flow cytometry. Platelet-monocyte aggregates were identified by gating on the monocyte population.

#### Culture of Human Umbilical Vein Endothelial Cells and Monocytes and Activation by Platelets, TNF-α, or CRP

Cell culture of human umbilical vein endothelial cells (HUVEC) and coculture of endothelial monolayers with platelets were performed as described previously. HUVEC were left untreated or were incubated for 10 minutes at 37°C with α-thrombin–stimulated platelets. For blocking engagement of CD40 on HUVEC, anti-CD154 (IgG₁, PharMingen) or MOPC-21 (isotype control; both 10 μg/mL) were added to some portions of α-thrombin–stimulated platelets before incubation with HUVEC. Thereafter, the supernatants of coculture experiments were analyzed for monocyte chemoattractant protein 1 (MCP-1; see below). To investigate the effect of cerivastatin on CD40 expression, HUVEC and the monocyte cell line THP-1 were stimulated for 24 hours with 500 U/mL TNF-α (Sigma) or 5 μg/mL CRP (human serum, high purity, Calbiochem). Additional samples of HUVEC or THP-1 were pretreated for 24 hours with cerivastatin (1 μmol/L) before stimulation with TNF-α or CRP. Thereafter, cells were incubated with FITC-conjugated anti-CD40 and finally analyzed by flow cytometry.

#### Measurement of Soluble CD154 and MCP-1

Serum MCP-1 levels, plasma sCD154 levels, and the supernatants of cultured HUVEC (MCP-1) treated with platelets were analyzed using commercially available ELISAs (sCD154: detection limit, 95 pg/mL; Bender MedSystems; MCP-1: detection limit, 5 pg/mL, R&D) according to the manufacturers’ instructions.

#### Statistics

The data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. Comparisons between groups were made with the Mann-Whitney U test. The differences between baseline and posttreatment values were analyzed with the Wilcoxon signed-rank test. The association of measurements with other biochemical parameters was assessed by the Spearman rank correlation test. All values are reported as mean±SD. 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=0.4, 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.20 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,
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 (CD154: 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 normcholesterolemic 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...
Figure 5. CD154 on activated platelets induces endothelial cells to secrete MCP-1. In the experiments described in Methods, supernatants of HUVEC were analyzed after 20 minutes of coin-cubation with platelets for MCP-1 by using ELISA. *P = 0.02 vs activated platelets, +P = NS vs activated platelets. mAb indicates monoclonal antibody.

significantly downregulated CD40 on monocytes and serum levels of MCP-1.

Although activation of the CD40 system has been observed in atherosclerosis-related diseases,10,16 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.6,5 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.11 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 platelets activators. However, in contrast to previous reports,11 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.17 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 form16 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.18

The activation of monocytes in HC has been reported previously.19 The adhesion of monocytes to endothelium is crucial in atherosclerosis, 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,20 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,21 but not in moderate HC. MCP-1 release may be the result of CD40 engagement on endothelial cells11 or on monocytes.22 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.23 However, our observation about similar numbers of platelet-monocyte aggregates in patients and controls suggests that monocyte activation via CD154 on platelets plays a minor role in moderate HC.

Upregulation of CD40 on monocytes in patients with moderate HC was not caused by TNF-α and interleukin-1, because serum levels did not differ significantly between the groups (data not shown). However, significantly elevated CRP levels in moderate 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.

Figure 6. Basal and TNF-α (500 U/mL)–induced CD40 expression on monocytes (A) and HUVEC (B). Pretreatment for 24 hours with cerivastatin (1 μmol/L) significantly reduced TNF-α–induced CD40 upregulation.
observed in cerivastatin-treated patients. Therefore, down-regulation 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-lipid or anti-platelet drugs may prove to downregulate CD154. Thus far, an inhibitory potential for CD154 expression on platelets has been shown for prostaglandin E1 and sodium nitroprusside. Nevertheless, cerivastatin effectively interrupted signal transduction by downregulating CD40 in endothelial cells and monocytes, and thereby prevented CD40-mediated proatherogenic or prothrombotic actions. Hence, interference with the CD40 system can be understood as an integral part of the anti-inflammatory activity of cerivastatin.

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
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