Oxidized Low-Density Lipoprotein Augments and 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Limit CD40 and CD40L Expression in Human Vascular Cells

Uwe Schönbeck, PhD; Norbert Gerdes, BSc; Nerea Varo, PhD; Rebecca S. Reynolds, BA; Daniel B. Horton, BA; Udo Bavendiek, MD; Linda Robbie, PhD; Peter Ganz, MD; Scott Kinlay, MD; Peter Libby, MD

Background—Although CD40 signaling participates in atherosclerosis, links between lipid risk factors and this inflammatory pathway remain obscure. Cardiovascular risk reduction by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) may involve actions beyond lipid lowering, including reduced inflammation. Therefore, this study analyzed whether oxidized low-density lipoprotein (oxLDL) induces CD40/CD40L expression on cells implicated in atherogenesis and whether statins affect their expression in vitro as well as the expression of soluble CD40L (sCD40L) in vivo.

Methods and Results—Treatment of human vascular endothelial and smooth muscle cells and mononuclear phagocytes with oxLDL augmented the basal expression of CD40 and CD40L mRNA and protein. In contrast, cerivastatin, atorvastatin, or simvastatin concentration-dependently diminished the constitutive as well as oxLDL- or cytokine-induced expression of the receptor/ligand dyad, an effect reversed by mevalonate. Patients treated with statins had diminished sCD40L plasma levels compared with untreated control patients (8.3±3.1 ng/mL [n=11] versus 13.1±2.5 ng/mL [n=16], P<0.05), supporting the clinical relevance of the in vitro observations. Platelet-enriched plasma of mice deficient in CD40L showed markedly delayed fibrin clot formation, suggesting a role for the ligand in blood coagulation and supporting the hypothesis that statin-mediated reduction in CD40/CD40L expression might limit thrombosis.

Conclusions—OxLDL may promote expression of CD40 and CD40L in human atheroma. Statins may limit the expression of the CD40 receptor/ligand dyad in two ways, directly as well as through diminished lipoprotein levels. Thus, reduced CD40 signaling may account for some of the statins’ antiinflammatory action. (Circulation. 2002;106:2888-2893.)

Key Words: atherosclerosis • inflammation • statins

Atherosclerosis bears many hallmarks of chronic inflammation,1,2 and inflammatory pathways may prove to be targets for therapy of this prevalent human disease. Recently, we and others implicated the immunomodulatory dyad CD40/CD40L, present on endothelial cells (ECs) and smooth muscle cells (SMCs), macrophages (MØ), T lymphocytes, and platelets within human atheroma,3–6 in aspects of atherogenesis and acute coronary syndromes. The proatherogenic functions of CD40 ligation include augmented expression of matrix metalloproteinases, procoagulant tissue factor, chemo- kines, and cytokines.7–12 Indeed, interruption of CD40 signaling not only reduced the initiation and progression of atherosclerotic lesions in hypercholesterolemic mice in vivo13,14 but also modulated plaque architecture in ways that might lower the risk for causing thrombosis.15 In addition to the 39-kDa cell membrane–associated form, CD40L also exists as a soluble protein, termed sCD40L.17,18 Although lacking the cytoplasmic, the transmembrane, and parts of the extracellular domains, this, the soluble form of CD40L, is considered to possess biological activity.19 Of note, patients with unstable angina express higher sCD40L plasma levels than healthy individuals or patients with stable angina.20 Moreover, we recently demonstrated that elevated plasma concentrations of sCD40L predict risk for future cardiovascular events.20 Although in vitro and in vivo studies established that CD40 signaling participates in atherosclerosis, the initial trigger for CD40/CD40L expression within atheroma remains unknown. This study tested the hypothesis that low-density lipoproteins (LDLs), particularly when oxidized, may furnish such a stimulus.

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) diminish LDL levels and retard...
atherosclerotic events. Recent evidence suggests that some benefits of statins may accrue independently of LDL lowering.\textsuperscript{21–25} Although the possible contribution of such “pleiotropic” effects of statin therapy to clinical benefits have attracted much attention, little is known regarding the molecular pathways by which HMG-CoA reductase inhibitors may limit inflammation.

Therefore, the present study tested the hypothesis that oxidatively modified LDL induces the expression of CD40/CD40L on cells involved in atherosclerosis, namely human vascular ECs and SMCs, as well as MØ, and that statins can diminish the expression of the receptor/ligand dyad on these cells in vitro and of sCD40L plasma levels in vivo, illustrating a potential novel antiinflammatory pathway of statins.

### Methods

#### Materials

Native and oxidized (5 μmol/L CuSO\(_4\), 37°C, 24 hours) LDLs (TBARS: 0.4 and 8.3 mmol/L MDA/mg protein, respectively) were obtained from Biomedical Technologies, Inc. Human recombinant interleukin (IL)-1β, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) were obtained from Endogen.

#### Cell Isolation and Culture

Human vascular ECs and SMCs were isolated from saphenous veins and cultured as described previously.\textsuperscript{4,26} Mononuclear phagocytes (MØ) were isolated from leukocyte concentrates by density-gradient centrifugation using lymphocyte separation medium (Organon-Teknika) and were cultured (10 days) in RPMI 1640 (BioWhittaker) containing 2% human serum (Sigma).\textsuperscript{26} All cell types were cultured 24 hours before and during the experiment in media lacking serum. Viability of the cultures was determined by trypan blue (Sigma) exclusion count as well as an oligonucleosome formation assay (Cell Death Detection ELISA, Boehringer Mannheim).

#### Reverse Transcription–Polymerase Chain Reaction

Total RNA isolated from cultured EC, SMC, or MØ using RNAzol (Tel-Test) was assessed for purity and yield spectrophotometrically (2100 Bioanalyzer, Agilent Technologies) and was reverse-transcribed (2 μg total RNA; 50 μL reactions) using Superscript II Reverse Transcriptase (LifeTechnologies). Polymerase chain reaction (PCR) was performed for 35 cycles at 95°C (120 seconds), 62°C (120 seconds), and 72°C (180 seconds, 2-second prolongation per cycle) after hot start, using primers for CD40 or CD40L previously described.\textsuperscript{4} Semiquantitative PCR studies using 20, 25, 30, 35, and 72°C (180 seconds, 2-second prolongation per cycle) were monitored at 405 nm for up to 15 minutes. PCR products were analyzed on ethidium bromide–containing 1.3% agarose gels and visualized by UV transilluminatiun. Loading of equal template amounts was verified by reverse transcriptase (RT)-PCR for GAPDH. Mock RT reactions, either lacking reverse transcriptase or using H\(_2\)O as template, demonstrated specificity of the signals obtained.

#### Western Blot

Culture lysates (50 μg total protein/lane) and supernatants were separated by SDS-PAGE and blotted to polyvinylidene difluoride membranes (Bio-Rad) using a semidry blotting apparatus (3 mA/cm\(^2\), 30 minutes; Bio-Rad). Blots were blocked and primary (mouse anti-human CD40 or CD40L; both 1:1000; PharMingen) antibodies were added in 5% defatted dry milk/PBS/0.1% Tween 20. After 1 hour, blots were washed 3 times (PBS/0.1% Tween20) and secondary, peroxidase-conjugated, goat anti-mouse antibody (Jackson Immunoresearch, West Grove, Pa) was added (1 hour). Finally, blots were washed and immunoreactive proteins were visualized using the Western blot chemiluminescence system (NEN). Data were verified by using anti-CD40/anti-CD40L antibodies from Santa Cruz (Santa Cruz, Calif).

#### Flow Cytometry

Human vascular ECs, SMCs, or MØ were washed with ice-cold PBS, harvested by trypsinization, and fixed (PBS/4% paraformaldehyde, 15 minutes). Subsequently, the cells were washed once with PBS/2% BSA before being incubated (1 hour, 4°C) with either buffer alone or FITC-conjugated control IgG, mouse anti-human CD40, or mouse anti-human CD40L antibody (1 μg/mL; Ancell). Finally, cells were washed with PBS/2% BSA and analyzed in a Becton Dickinson FACScan flow cytometer using CellQuest software (Becton Dickinson). At least 20 000 viable cells per condition were analyzed.

#### Patient Studies

Whole blood (10 mL) was collected in EDTA from 27 patients presenting for coronary arteriography who had at least a 30% stenosis in one coronary artery. The cohort was divided into patients who were or were not treated with any statin at the time of catheterization. Blood was drawn at baseline (0 month) and final follow-up visit (6 months), and plasma was stored at −70°C. All subjects were studied in the fasting state. Written informed consent was obtained from all subjects, and the study was approved by the Human Research Committee of Brigham and Women’s Hospital. Plasma lipids as well as IL-1β, IL-6, TNFα, IFNγ, sVCAM, C-reactive protein, and sCD40L were measured by ELISA (Sigma; Endogen; BenderMedSystems). The two groups did not differ significantly in age, sex, diabetes mellitus, smoking, triglycerides, or HDL (Table). The statin-treated group had significantly lower total cholesterol and LDL, as expected.

#### Ex Vivo Fibrin Clot Formation

Blood was collected from mice by retroorbital bleeding into 0.1 volume of 0.13 mol/L trisodium citrate using noncoated capillary tubes. Platelet-rich plasma (PRP) was prepared by centrifugation (500g, 5 minutes, 20°C), and fibrin clot formation was examined using a modification of a microtiter-plate clot lysis assay described previously.\textsuperscript{27} Clots were prepared with 2.94 μmol/L fibrinogen, 0.24 μmol/L plasminogen, 36 pmol/L t-PA, 3.8 nmol/L thrombin, and 5.3 mmol/L CaCl\(_2\) (all final concentrations). PRP (final concentration of 10% [vol/vol]) was incorporated into clots. Clot formation was monitored at 405 nm for up to 15 minutes.

Characteristics of Study Participants

<table>
<thead>
<tr>
<th>No Statin Therapy</th>
<th>Statin Therapy</th>
</tr>
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<tbody>
<tr>
<td>(n=16)</td>
<td>(n=11)</td>
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<tr>
<td>Age, years</td>
<td>63.1±11.8</td>
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<tr>
<td>Men/women, %</td>
<td>83</td>
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<tr>
<td>Diabetes, %</td>
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<tr>
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<td>Triglycerides, mg/dl</td>
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<td>Total cholesterol, mg/dl</td>
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<tr>
<td>LDL, mg/dl</td>
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<td>IL-6, ng/mL</td>
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<td>TNFα, ng/mL</td>
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<td>IFNγ, ng/mL</td>
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<td>CRP, mg/L</td>
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<tr>
<td>sCD40L, ng/mL</td>
<td>13.1±2.5</td>
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</table>
CD40L expression on either cell type required 4) and CD40L (4.2 ± 0.2 fold; n=3) immunoreactive expression of CD40 (5.1 ± 0.1 fold above nonstimulated control; n=4) and CD40L (4.2 ± 2.2 fold; n=3) immunoreactive protein was achieved with 10 to 30 μg oxDL/mL. Native LDL induced expression of the receptor (2.8 ± 1.1-fold; n=2) and ligand (2.2 ± 0.8-fold; n=2), although to a lesser extent. Furthermore, oxDL concentration-dependently augmented the expression of CD40 and CD40L mRNA in human vascular EC (Figure 1B) or MØ (data not shown). Parallel studies analyzing the expression of GAPDH transcripts demonstrated application of equal amounts of reverse-transcribed mRNA amounts to each reaction and furthermore suggested that oxDL stimulation per se did not affect the RT-PCR.

Statistical Analysis

Data are presented as mean ± SD, and groups were compared using the Student’s t test. A value of P≤0.05 was considered significant.

Results

Oxidized LDL Induces Expression of CD40 and CD40L in Human Vascular Cells

Oxidatively modified LDL (oxLDL) concentration-dependently enhanced the faint constitutive expression of CD40 and CD40L protein in human vascular ECs (Figure 1) and MØ (data not shown). Augmentation of basal CD40/CD40L expression on either cell type required ≥1 μg oxDL/mL, as shown here for EC (Figure 1A). Maximal expression of CD40 (5.1 ± 1.1-fold above nonstimulated control; n=4) and CD40L (4.2 ± 2.2 fold; n=3) immunoreactive protein was achieved with 10 to 30 μg oxDL/mL. Native LDL also induced expression of the receptor (2.8 ± 1.1-fold; n=2) and ligand (2.2 ± 0.8-fold; n=2), although to a lesser extent. Furthermore, oxDL concentration-dependently augmented the expression of CD40 and CD40L mRNA in human vascular EC (Figure 1B) or MØ (data not shown). Parallel studies analyzing the expression of GAPDH transcripts demonstrated application of equal amounts of reverse-transcribed mRNA amounts to each reaction and furthermore suggested that oxDL stimulation per se did not affect the RT-PCR.

HMG-CoA Reductase Inhibitors Diminish the Expression of CD40 and CD40L on Human Vascular Endothelial and Smooth Muscle Cells and Macrophages In Vitro

Exposure to HMG-CoA reductase inhibitors concentration-dependently diminished the expression of both CD40 and CD40L in human vascular ECs, SMCs, and MØ. At concentrations ≥2 nmol/L, cerivastatin diminished the constitutive as well as IL-1β/TNFα/IFNγ-induced expression of the receptor in ECs (Figure 2). Maximal inhibition was achieved at 10 to 50 nmol/L cerivastatin. Mevalonate reversed the diminished expression of CD40 and CD40L by statins. Parallel studies using oxDL as a stimulus or SMCs or MØ as the cell type yielded similar results (data not shown). To determine whether the modulation of the CD40/CD40L expression extends to other statins, parallel experiments used atorvastatin or simvastatin. These HMG-CoA reductase inhibitors similarly reduced CD40/CD40L expression but required higher concentrations (100 nmol/L) (Figure 2). Development of the Western blots with a mouse anti-human GAPDH antibody, providing similar band intensities across the blots, verified equal loading among the lanes and also suggested that statin treatment per se did not affect Western

Figure 1. LDL induces the expression of CD40 and CD40L on human vascular ECs. Protein extracts (50 μg) (A) or total RNA preparations (B) obtained from human vascular EC stimulated (A, 24 hours; B, 4 hours) with the respective concentrations of oxDL, native LDL (30 μg/mL), or IL-1β/TNFα/IFNγ (I/T/I; 10/50/30 ng/mL) were analyzed by Western blotting (A) or RT-PCR (B) for the expression of CD40 or CD40L protein or transcript, respectively. RT-PCR for GAPDH demonstrated application of equal amounts of total RNA to all reaction. ECs from at least 3 different donors yielded comparable data.

Figure 2. HMG-CoA reductase inhibitors diminish the expression of immunoreactive CD40 and CD40L in extracts of human vascular ECs. Protein extracts (50 μg) obtained from unstimulated ECs (−) or IL-1β/TNFα/IFNγ-stimulated ECs (10/50/30 ng/mL; 24 hours) (+), cultured in the presence or absence of the respective concentrations of cerivastatin, atorvastatin (100 nmol/L), simvastatin (100 nmol/L), or mevalonate (100 μmol/L), were analyzed by Western blotting for expression of CD40 or CD40L. Cell lysates from at least 3 different donors yielded comparable data.

Figure 3. HMG-CoA reductase inhibitors diminish the expression of CD40 and CD40L in human vascular ECs. Total RNA preparations were obtained from unstimulated ECs (−), IL-1β/TNFα/IFNγ-stimulated ECs (10/50/30 ng/mL), or oxDL-stimulated ECs (30 μg/mL) (+, 6 hours) (B), cultured in the absence or presence of cerivastatin or mevalonate (100 μmol/L), and were applied to RT-PCR analysis for CD40, CD40L, or GAPDH. Products obtained from mock RT reactions (w/o RT) and total RNA obtained from PMA-stimulated (50 ng/mL, 6 hours) peripheral blood mononuclear cells (PBMC) were applied for control purposes. Cells obtained from 3 different donors yielded comparable data.
CD40L mRNA expression in human vascular ECs. Human vascular ECs were incubated with IL-1β/TNFα/IFNγ (10/50/30 ng/mL; 24 hours) in the absence or presence of various concentrations of cerivastatin and were studied for the expression of CD40 (left) or CD40L (right) by FACS analysis (solid histograms; compared with isotype control, dotted line). At least 20,000 viable cells were analyzed for each staining. Three independent experiments using cells of different donors yielded comparable results. 

HMG-CoA reductase inhibitors, moreover, diminished CD40 and CD40L mRNA expression in human vascular EC (Figure 3) and MØ (data not shown) stimulated either with proinflammatory cytokines (IL-1β/TNFα/IFNγ) (Figure 3A) or oxLDL (Figure 3B). Atorvastatin or simvastatin yielded similar results (data not shown). Mevalonate reversed the diminished expression of CD40 and CD40L transcripts (Figure 3). Parallel studies analyzing the expression of GAPDH transcripts demonstrated application of equal amounts of reverse-transcribed mRNA to each reaction and furthermore suggested that treatment with statins per se did not affect the RT-PCR.

In accord with the findings for whole-cell lysates, cerivastatin concentration-dependently diminished the cell-surface expression of both CD40 and CD40L on human vascular EC (Figure 4) as well as MØ (data not shown). Notably, the cerivastatin concentrations required for minimal and maximal reduction in CD40 and CD40L surface expression resembled those observed for whole-cell lysates in the Western blot as well as the RT-PCR studies. All 3 statins acted similarly but required different concentrations to achieve equal reduction in CD40/CD40L expression (cerivastatin > simvastatin > atorvastatin).

Because CD40L can also be shed as a biologically active soluble form, sCD40L, we tested whether treatment of EC, SMCs, and MØ with HMG-CoA reductase inhibitors affected the secretion of sCD40L. Indeed, cerivastatin treatment markedly lowered sCD40L concentrations in culture (Figure 5). Combined, RT-PCR, Western blot, and FACS studies suggest that oxLDL induces and statins diminish the cell surface expression or release of CD40 or CD40L by regulating gene activity rather than intracellular translocation. 

**HMG-CoA Reductase Inhibitors Diminish sCD40L Plasma Levels in Humans**

To assess the potential clinical relevance of these in vitro findings, we performed a pilot study to determine whether treatment of patients with HMG-CoA reductase inhibitors diminished sCD40L plasma levels. Plasma of statin-treated atherosclerotic subjects had significantly lower levels of sCD40L compared with nontreated patients (8.3 ± 3.1 ng/mL [n = 11] versus 13.1 ± 2.5 ng/mL [n = 16]; P < 0.05). The individual sCD40L levels did not vary significantly if the treatment status of the patient at baseline and 6-month follow-up did not change (Figure 6A). However, sCD40L plasma levels at 6-month follow-up decreased markedly in patients changed to statin treatment (13.1 ± 5.74 ng/mL versus 5.21 ± 2.36 ng/mL [n = 4]; P < 0.05) (Figure 6B). Plasma levels of IL-1β, IL-6, TNFα, IFNγ, and sVCAM-1 did not change with statin treatment. Plasma concentrations of C-reactive protein were lower in the statin-treated group, although the difference did not achieve statistical significance (Table).

**Deficiency of CD40L Prolongs Time of Coagulation of Murine Blood Ex Vivo**

In light of recent data implicating CD40L in thromboembolic complications and the statin-mediated lowering of plasma sCD40L levels shown above, we additionally tested the hypothesis that diminished expression of CD40L affects blood coagulation. Indeed, platelet-rich plasma from CD40L-
deficient mice showed delayed coagulation in a fibrin clot formation assay compared with wild-type mice preparations (Figure 7), supporting a role for CD40L in blood coagulation. These observations on the role of CD40L in blood coagulability provide a pathway by which reduction in CD40 signaling via HMG-CoA reductase inhibitors might reduce thrombotic complications of atherosclerosis.

Discussion

Clinical benefits in patients with average or below-average LDL levels and reduced cardiovascular risk independent of the degree of LDL lowering in a consistent series of previous clinical trials have highlighted the potential clinical relevance of the putative "pleiotropic" effects of statins. In addition to their lipid-lowering effects, numerous clinical and experimental studies have suggested antiinflammatory pathways of statins, such as diminished expression of chemokines, major histocompatibility complex II molecules, matrix-degrading enzymes, and the procoagulant tissue factor as well as the augmented expression of nitric oxide. Moreover, treatment of Watanabe heritable hyperlipidemic rabbits with HMG-CoA reductase inhibitors diminishes the expression of numerous proatherogenic inflammatory mediators in vivo. Although statins lowered lipids only modestly in these rabbits lacking LDL receptors, these in vivo observations could not conclusively distinguish the degree to which effects on lipoproteins account for the antiinflammatory effects observed. The present report provides evidence for a novel antiinflammatory pathway by which statins may act both dependently and independently of lipid lowering. Three members of this drug class—cerivastatin, atorvastatin, and simvastatin—significantly diminished the constitutive as well as cytokine-induced expression of CD40 and CD40L protein and transcript in cell types implicated in atherosclerosis, namely human vascular EC, SMCs, and MØ, arguing for a lipid-lowering independent function of statins. However, the identification of oxLDL as an inducer of CD40 and CD40L in these cell types additionally suggests that statins might affect CD40/CD40L expression, at least in part, also via their lipid-lowering properties. Of note, previous studies have colocalized oxLDL with CD40 and CD40L within early human atherosclerotic lesions, a finding in accord with our hypothesis that oxLDL provides an initial signal for the expression of the CD40 receptor/ligand dyad in atherosclerotic plaques.

The pilot observation that patients treated with statins have diminished levels of sCD40L indicates the potential clinical relevance of the present in vitro observations. Several cell types might give rise to sCD40L. Platelets release sCD40L on ligation of the thrombin receptor in vitro as well as on thrombus formation in vivo. However, as suggested by our own and other previous studies, other cell types, including EC, MØ, and T lymphocytes, might also generate sCD40L. It remains unknown whether the lowering of plasma sCD40L levels results from lipid-lowering or non-lipid-lowering properties of statins or a combination of both mechanisms. Notably, a recently published study also reported diminished expression of surface CD40 on freshly isolated peripheral blood monocytes obtained from statin-treated hypercholesterolemic subjects. This study found no differences in plasma sCD40L levels in subjects with different statin treatment status, contrasting with our observations. These differences might be attributable to the more prolonged treatment (6 months versus 3 weeks) in our study. Moreover, the higher baseline of plasma sCD40L levels in the study by Garlits et al may reflect serum generation, which could enhance sCD40L levels via release from platelets and other circulating cell types, a process that might mask modulations in sCD40L levels by statins.

The functional consequences of enhanced plasma levels of soluble CD40L still remain uncertain, because the biological activity of this form is controversial. However, the present observation that CD40L-deficient platelet-rich plasma clots more slowly than preparations from wild-type mice suggests that CD40L, in its membrane-bound or soluble form, modulates thrombosis. The finding that CD40L can...
activate platelets by functioning as an $\alpha_{\text{IIb}}\beta_3$ ligand additionally supports this hypothesis.\textsuperscript{15}

In summary, the present study identifies oxLDL as a potential inducer of the CD40/CD40L dyad on cell types implicated in atherogenesis and provides novel insights into the antiinflammatory action of HMG-CoA reductase inhibitors.

Acknowledgments

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References


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