

Short-Term Treatment With Atorvastatin Reduces Platelet CD40 Ligand and Thrombin Generation in Hypercholesterolemic Patients

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Background—Soluble CD40L (sCD40L), a substance that maximally reflects in vivo platelet activation, is increased in patients with hypercholesterolemia. We investigated the relation between sCD40L and platelet CD40L in hypercholesterolemic patients before and after a short-term treatment with atorvastatin.

Methods and Results—Collagen-induced platelet CD40L and plasma levels of sCD40L and prothrombin fragment F1+2, a marker of thrombin generation, were investigated in 30 hypercholesterolemic patients and 20 healthy subjects. Hypercholesterolemic patients were then randomized to either diet (n=15; group A) or atorvastatin 10 mg/d (group B); the aforementioned variables were measured at baseline and after 3 days of treatment. Compared with referents, hypercholesterolemic patients showed higher values of platelet CD40L ($P<0.005$), sCD40L ($P<0.005$), and F1+2 ($P<0.003$). Platelet CD40L was significantly correlated with sCD40L ($P<0.001$), and the latter was significantly correlated with F1+2 ($P<0.001$). The intervention trial showed no changes in group A but a significant decrease in platelet CD40L ($P<0.01$), sCD40L ($P<0.002$), and F1+2 ($P<0.03$) in group B. In vitro studies demonstrated that cholesterol enhanced platelet CD40L and CD40L-mediated clotting activation by human monocytes; also, atorvastatin dose-dependently inhibited platelet CD40L expression and clotting activation by CD40L-stimulated monocytes.

Conclusions—This study shows that, in hypercholesterolemia, platelet overexpression of CD40L may account for enhanced plasma levels of sCD40L and F1+2. Atorvastatin exerts a direct antithrombotic effect via inhibition of platelet CD40L and CD40L-mediated thrombin generation, independently of its cholesterol-lowering effect. (*Circulation*. 2005;111:412-419.)

Key Words: platelet-derived factors ■ platelets ■ thrombin ■ hypercholesterolemia ■ statins

CD40 ligand (CD40L), a member of the tumor necrosis factor family, is a transmembrane protein found on cells of the immune system as well as on endothelial cells, smooth muscle cells, macrophages, and platelets.¹ On interaction with its receptor CD40, CD40L exerts proinflammatory and prothrombotic activity, including increased expression of matrix metalloproteinases, chemokines, cytokines, and tissue factor (TF).¹ The role of CD40L in the pathogenesis of atherosclerosis has been confirmed by the fact that administration to hyperlipidemic mice of an antibody against CD40L reduced atherosclerotic lesions.² CD40L is expressed by platelets on stimulation with common agonists³; it is then cleaved from platelets over a period of minutes to hours, thus generating a soluble form (sCD40L).⁴ It has been calculated that >95% of circulating sCD40L is of platelet origin.⁵ Elevated plasma levels of sCD40L have been found in patients with acute coronary syndrome and in those at risk for cardiovascular events.⁶⁻⁸ There are also converging lines of evidence that patients with hypercholesterolemia show enhanced levels of sCD40L, suggesting that this protein may represent an important promoter of atherosclerotic complications occurring in this setting.⁹ Because sCD40L stems essentially

from platelet CD40L, patients with hypercholesterolemia could be expected to show an upregulation of platelet CD40L; however, there are no clear data in this respect, because both upregulation and downregulation of platelet CD40L have been reported.⁹ Patients with hypercholesterolemia are in an ongoing prothrombotic state, as documented by enhanced TF expression and in vivo thrombin generation.¹⁰ Because CD40L exerts a prothrombotic effect through overexpression of TF,¹¹ it would be reasonable to speculate that in hypercholesterolemia, clotting activation occurs as a consequence of CD40L overexpression. A significant correlation between sCD40L and prothrombin fragment F1+2¹² could support this hypothesis, although sCD40L has not been demonstrated to exert any prothrombotic action to date.¹³

Recent studies have also sought to determine whether statins could influence CD40L expression in patients with hypercholesterolemia: although there are converging data on the efficacy of statins in reducing sCD40L,^{9,14,15} no change in platelet CD40L expression was reported.^{9,12} Also, a previous study suggested that statins could reduce sCD40L plasma levels, independently of their cholesterol-lowering effect.¹⁵

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In the present study, which was conducted in patients with polygenic hypercholesterolemia, we investigated the following elements: (1) CD40L expression in stimulated platelets; (2) the relation between sCD40L and clotting activation; and (3) the effect of a 3-day treatment with atorvastatin on the aforementioned variables compared with diet alone. Finally, we performed *in vitro* studies to evaluate (1) the effect of native LDL cholesterol on platelet CD40L expression; (2) the effect of native LDL cholesterol on CD40L-mediated clotting activation by monocytes; and (3) whether atorvastatin was able to influence the aforementioned parameters.

Methods

Clinical Study

The clinical study was divided into 2 parts. In the first part of the study, we compared 30 patients with polygenic hypercholesterolemia (16 males, 14 females; mean age, 53.4 years) and 20 sex- and age-matched normocholesterolemic subjects (10 males and 10 females; mean age, 52.6 years). Both patients and controls were recruited from the same geographic area and followed a typical Mediterranean diet. None of the patients had clinical evidence of cardiovascular disease (as shown by clinical history, physical examination, or ECG), diabetes mellitus, or hypertension. Patients with hypercholesterolemia had not taken any lipid-lowering agents or antiplatelet drugs in the previous 30 days. Lipid profile, CD40 ligand expression by platelets, sCD40L, and F1+2 were measured.

In the second part of the study, we tested the hypothesis that atorvastatin could influence platelet CD40L expression and CD40L-mediated clotting activation by a mechanism independent of its cholesterol-lowering effect; thus, only hypercholesterolemic patients were randomized to either a diet (American Heart Association step I diet; $n=15$, 8 males, 7 females) or atorvastatin 10 mg/d ($n=15$, 8 males, 7 females) for 3 days. Lipid profile, CD40 ligand platelet expression, sCD40L, and F1+2 were measured at baseline and after 3 days of treatment.

Blood samples mixed with 0.13 mol/L sodium citrate (ratio 9:1, vol/vol) were obtained between 8 and 9 AM from patients and healthy volunteers who had fasted for 12 hours and had provided their informed consent to participate in the study; an aliquot of serum was used to measure lipid profile. The ethics committee of our university approved the study protocol.

Lipid Profile

Serum levels of total cholesterol and triglycerides were determined with an enzyme-based method. HDL cholesterol was measured after phosphotungstic acid/MgCl₂ precipitation of fresh plasma. LDL cholesterol was calculated according to the Friedewald formula.

Analysis of sCD40L and F1+2

Blood samples were immediately centrifuged at 2000 rpm for 20 minutes at 4°C, and the supernatant was collected and stored at -80°C until measurement. Plasma levels of sCD40 ligand were measured with a commercial immunoassay (Quantikine CD40 ligand, R&D Systems). Intra-assay and interassay coefficients of variation were 7% and 9%, respectively. Plasma levels of human prothrombin fragment F1+2 were assayed by an enzyme immunoassay based on the sandwich principle (Enzygnost F1+2, Behringwerke). Intra-assay and interassay coefficients of variation for this method were 5% and 7%, respectively.

Platelet Isolation From Whole Blood

Blood samples were drawn between 8 and 9 AM without stasis from an antecubital vein with a 21-gauge needle from patients after a 12-hour fast and mixed with 0.13 mol/L sodium citrate (ratio 9:1, vol/vol). Washed platelets were prepared as previously described.¹⁶

Flow Cytometric Analysis of CD40L Expression

CD40L expression on platelet membranes was analyzed with specific fluorescein isothiocyanate-labeled monoclonal antibodies (mAbs; anti-CD40L antibody by Beckman Coulter). In all assays, an irrelevant isotype-matched antibody was used as a negative control.

Twenty microliters of mAb was added to 200 μ L of platelet suspension (2×10^8 /mL) previously fixed with (2%) paraformaldehyde (0.1% bovine serum albumin) and incubated for 60 minutes at 4°C. The unbound mAb was removed by addition of 0.1% bovine serum albumin-phosphate-buffered saline (PBS) and centrifugation at 5000g for 3 minutes (twice). Fluorescence intensity was analyzed on an Epics XL-MCL cytometer (Coulter Electronics) equipped with an argon laser at 488 nm. For every histogram, 50 000 platelets were counted to determine the proportion of positive platelets. Antibody reactivity is reported in arbitrary units obtained by multiplying the number of positive events resulting from platelet stimulation by the mean value of the fluorescence observed when the specific mAb was used and then correcting the values obtained in unstimulated samples treated with the same antibody.

In Vitro Study

Platelet CD40L Expression

Platelets from healthy subjects ($n=4$, 2 males, 2 females; mean age, 50.3 years) and hypercholesterolemic patients ($n=4$, 2 males, 2 females; mean age, 52.2 years) were washed and suspended in fatty acid-free Tyrode's buffer (2×10^8 /mL). Platelet suspensions were incubated with collagen (4 μ g/mL) for 15 minutes at 37°C with and without native LDL cholesterol (100 μ g/mL) or scalar concentrations (0.1 to 10 μ mol/L) of atorvastatin and treated as described earlier to detect CD40L expression on platelet surfaces. Atorvastatin alone did not affect platelet viability, as assessed by the ipotonic shock response test¹⁷ (not shown).

Monocyte TF Expression and Monocyte-Mediated Thrombin Generation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized, venous blood samples of healthy subjects ($n=4$, 2 males, 2 females; mean age, 50.3 years) and hypercholesterolemic patients ($n=4$, 2 males, 2 females; mean age, 52.2 years) by aseptic technique. Platelets were separated by centrifugation, once at 140g and twice at 100g, in PBS at room temperature for 10 minutes. PBMCs were isolated by centrifugation on Lymphoprep (Nyegard Oslo) at 1200g for 20 minutes at 20°C. Monocytes, identified by May-Grunwald-Giemsa staining, were between 16% and 22% (mean, 19%).

Monocytes (adherent cells) were obtained by incubation of PBMCs for 90 minutes at 37°C in a humidified atmosphere of 5% CO₂ in air in Petri dishes containing RPMI 1640, supplemented with 2 mmol/L glutamine; lymphocytes (nonadherent cells) were removed by aspiration with a Pasteur pipette and washing of the dishes with warm medium. The purified monocyte preparation contained 85% to 95% monocytes.

After isolation, cells were washed twice in PBS and preincubated for 1 hour at 2×10^5 cells/mL in RPMI 1640 at 37°C 5% CO₂ with atorvastatin (0.1, 1 and 10 μ mol/L) or medium (as control) and then incubated with 50 ng/mL CD40L (Recombinant human CD40L/TNFSF5, R&D Systems) for 6 hours. In another set of experiments, monocytes were preincubated for 12 hours at 2×10^5 cells/mL in RPMI 1640 at 37°C under 5% CO₂ with native LDL (100 μ g/mL)¹⁸ or medium (as control) and then incubated with 50 ng/mL CD40L for 6 hours. At the end of the incubation period, the cells and media were separated by centrifugation (2000g \times 15 minutes). The cells were washed with Tris-NaCl buffer (0.1 mmol/L NaCl, 0.1% bovine serum albumin, pH 7.4) and then lysed in the same buffer by adding 15 mmol/L *n*-octyl- β -D-glycopyranoside at 37°C for 30 minutes. Cell count and trypan blue exclusion were performed on cell suspensions after washing to evaluate cell viability. Cell viability was 90% at the maximum concentration of atorvastatin used (10 μ mol/L). The ELISA for measuring TF antigen in cell lysate was performed with a commercial kit (Imubind tissue factor ELISA kit, American Diagnostica). The lower detection limit is \approx 10 pg/mL. The assay recognizes TF- α , TF, and TF-factor VII complexes and is designed in such a manner as to prevent any interference from other coagulation factors or inhibitors of procoagulant activity.

The rate of thrombin generation by CD40L-stimulated monocytes was evaluated *in vitro* as previously described.¹⁹ For this purpose, a low-molecular-weight heparin-anticoagulated blood sample (ratio 1:10, vol/vol) was taken from healthy volunteers ($n=4$) and hypercholesterolemic patients ($n=4$). The blood was anticoagulated with low-molecular-weight heparin

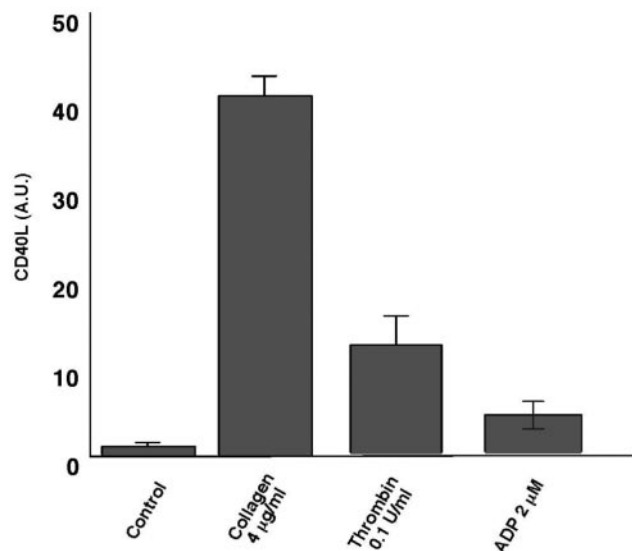


Figure 1. Agonist-dependent platelet CD40L expression in healthy subjects (n=4, 2 males, 2 females; mean age, 50 years). Results are mean±SD. Abbreviations are as defined in text.

(820 U/mL) because this agent effectively inhibits the formation of thrombin in solution but has only a small effect on thrombin generated and bound to surfaces. After isolation, monocytes were washed twice in PBS and preincubated for 1 hour at 2×10^5 cells/mL in RPMI 1640 at 37°C under 5% CO₂ with atorvastatin (0.1, 1, and 10 µmol/L) or medium (as control) and then incubated with CD40L (50 ng/mL) for 6 hours. In another set of experiments, monocytes were preincubated for 12 hours at 2×10^5 cells/mL in RPMI 1640 at 37°C under 5% CO₂ with native LDL (100 µg/mL) or medium (as control) and then incubated with 50 ng/mL CD40L for 6 hours. The medium was removed, and a 1000-mL overlay of heparinized standard plasma was added to each well and incubated at 37°C for 24 hours. After incubation, samples were harvested, centrifuged at 4000g, and assayed for F1+2 generation, calculated as the increase in F1+2 levels compared with the value observed in control samples, which consisted of heparinized plasma added with CD40L. All samples were assayed in duplicate.

Statistical Analysis

Assuming that a short-term (3-day) treatment with atorvastatin would reduce platelet CD40L expression and monocyte tissue factor by 25%, we postulated that the study sample should consist of at least 12 patients for each group ($\alpha=0.05$ and $1-\beta=0.90$).

Comparisons between groups were carried out by 1-way and repeated-measures ANOVA²⁰ and were replicated as appropriate with nonparametric tests, like the Wilcoxon and Kolmogorov-Smirnov (z) test in case of nonhomogeneous variances as verified by Levene's test. MANOVA with a Bonferroni test for multiple comparisons was applied in *in vitro* experiments. The correlation analysis was carried out by Pearson's test. Data are presented as mean±SD. Statistical significance was defined at $P<0.05$. Statistical analysis was performed with SPSS 10.0 software for Windows.

Results

Platelet CD40L, Expression, and Plasma F1+2 in Healthy Subjects and Hypercholesterolemic Patients

To choose the platelet agonist that maximally expressed platelet CD40L, we evaluated the effect of 3 different agonists, namely, ADP, collagen, and thrombin. Because the highest expression was obtained with collagen (Figure 1), all of the experiments in hypercholesterolemic patients were performed with collagen as the platelet agonist.

TABLE 1. Characteristics of Study Participants

	Hypercholesterolemic Patients	Healthy Subjects
Age, y	53±2	52±3
Sex	16 males, 14 females	10 males, 10 females
Smokers, n	3	2
LDL cholesterol, mg/dL	186±13	112±13
Triglycerides, mg/dL	88±18	84±16

Table 1 shows the characteristics of patients and referents. Compared with referents (n=20), patients with hypercholesterolemia (n=30) showed higher expression of platelet CD40L (43.9 ± 13.4 ; 95% confidence interval [CI], 38.8 to 48.9 versus 30.1 ± 5.6 ; 95% CI, 27.6 to 32.5 AU; $P<0.005$; Figure 2A); 11 hypercholesterolemic patients had values >41.3 AU (mean+2SD) versus 1 referent. Compared with referents, patients with hypercholesterolemia showed higher plasma levels of sCD40L (4.3 ± 1.9 ; 95% CI, 3.5 to 5 versus 2.2 ± 0.7 ; 95% CI, 1.8 to 2.5 ng/mL; $P<0.005$; Figure 2B); 19 hypercholesterolemic patients had values >3.6 ng/mL (mean+2SD) versus 1 referent. Compared with referents, patients with hypercholesterolemia showed higher plasma levels of F1+2 (2 ± 0.9 ; 95% CI, 1.6 to 2.3 versus 1.12 ± 0.49 ; 95%

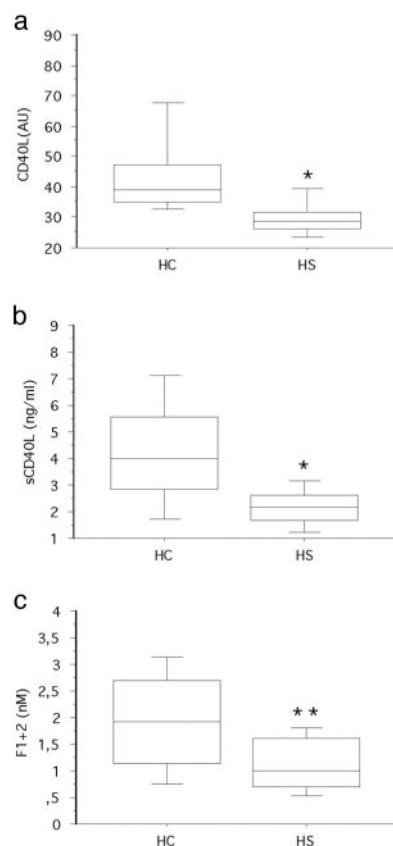


Figure 2. Platelet CD40L expression (a), sCD40L (b), and prothrombin F1+2 (c) plasma levels in hypercholesterolemic patients (HC, n=30) and healthy subjects (HS, n=20). Repeated-measures ANOVA: $P<0.001$, HC vs HS; $**P<0.005$ HC vs HS. Results are mean±SD. Box plots depict median and 95% CIs; whiskers represent minimum and maximum values. Abbreviations are as defined in text.

TABLE 2. Lipid Profile Before and After 3 Days of Treatment

	Diet Only	Atorvastatin
LDL cholesterol, mg/dL		
Before	185±14	187±12
After	185±16	182±13
Triglycerides, mg/dL		
Before	88±22	87±14
After	88±18	86±17

CI, 0.8 to 1.3 nmol/L; $P<0.003$; Figure 2C); 13 hypercholesterolemic patients had values of >2.1 nmol/L (mean+2SD) versus 0 referents. Platelet CD40L expression was significantly correlated with sCD40L ($r=0.87$, $P<0.001$) and F1+2 ($r=0.75$, $P<0.001$), and sCD40L was significantly correlated with F1+2 ($r=0.86$, $P<0.001$). Platelet CD40L was also significantly correlated with LDL cholesterol ($r=0.58$, $P<0.05$). In healthy subjects, platelet CD40L expression was significantly correlated with sCD40L ($r=0.79$, $P<0.001$) and F1+2 ($r=0.58$, $P<0.01$), and sCD40L was significantly correlated with F1+2 ($r=0.67$, $P<0.001$). Platelet CD40L was also significantly correlated with LDL cholesterol ($r=0.73$, $P<0.001$). In hypercholesterolemic patients, platelet CD40L expression was significantly correlated with sCD40L ($r=0.82$, $P<0.001$) and F1+2 ($r=0.68$, $P<0.001$), and sCD40L was significantly correlated with F1+2 ($r=0.85$, $P<0.001$). Platelet CD40L was also significantly correlated with LDL cholesterol ($r=0.82$, $P<0.001$).

Effect of Atorvastatin on Platelet CD40L and F1+2 in Hypercholesterolemic Patients

At baseline, patients randomized to diet alone (group A) and those randomized to diet plus atorvastatin (10 mg/d, group B) had similar values of platelet CD40L, sCD40L, and F1+2. Both groups did not show any changes in lipid profile after 3 days of treatment (Table 2). In group A (n=15), no changes in platelet CD40L (44.5 ± 12.3 ; 95% CI, 37.7 to 51.4 versus 43.3 ± 14.8 ; 95% CI, 35 to 51.4 AU) sCD40L (4.2 ± 2 ; 95% CI, 3.1 to 5.3 versus 4.3 ± 2 ; 95% CI, 3.2 to 5.4 ng/mL), and F1+2 (2 ± 1 ; 95% CI, 1.4 to 2.6 versus 1.9 ± 0.7 , 95% CI, 1.5 to 2.4 nmol/L) were observed (Figure 3). In group B (n=15), a significant decrease in platelet CD40L (46.3 ± 14.6 ; 95% CI, 38.2 to 54.5 versus 32.2 ± 6.4 ; 95% CI, 28.6 to 35.8 AU; $P<0.01$), sCD40L (4.1 ± 1.9 ; 95% CI, 3 to 5.2 versus 3 ± 1 ; 95% CI, 3 to 5.2 ng/mL; $P<0.002$), and F1+2 (2 ± 1.1 ; 95% CI, 1.4 to 2.7 versus 1.4 ± 0.4 ; 95% CI, 1.2 to 1.6 nmol/L; $P<0.03$) was observed within and between groups (A and B; Figure 3) after 3 days of atorvastatin. In group B, before-after treatment changes in platelet CD40L and sCD40L ($r=0.66$, $P<0.01$), sCD40L and F1+2 ($r=0.87$, $P<0.001$), and CD40L and F1+2 ($r=0.55$, $P<0.05$) were significantly correlated.

In Vitro Study

Effect of Cholesterol on Platelet CD40L

Compared with unstimulated platelets, collagen induced a significant increase in CD40L expression; a further increase was observed when collagen-stimulated platelets were added with native LDL cholesterol (100 μ g/mL; MANOVA, $P<0.001$; Figure 4A). No

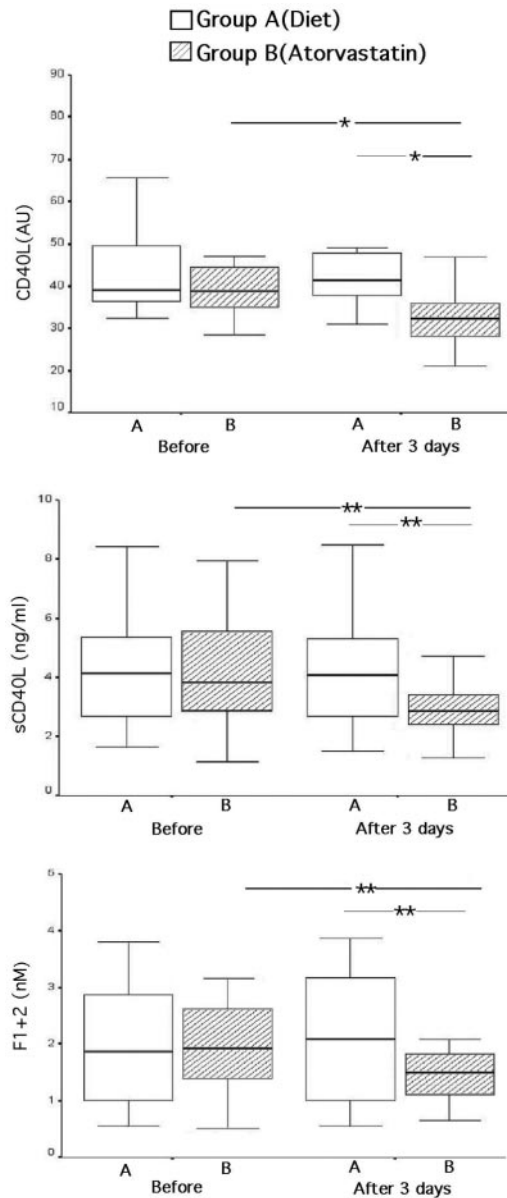


Figure 3. Platelet CD40L expression, sCD40L, and prothrombin F1+2 plasma levels in group A (diet only, n=15) and group B (atorvastatin 10 mg/d, n=15) patients before (A) and after (B) 3 days of either treatment. ●Repeated-measures ANOVA: CD40L, * $P<0.01$: group B, before vs after 3 days; group B vs group A after 3 days; sCD40L and F1+2, ** $P<0.05$: group B, before vs after 3 days; group B vs group A after 3 days. ●Results are given as mean±SD. ●Box plots depict median and 95% CIs; whiskers represent minimum and maximum values. Abbreviations are as defined in text.

change in CD40L was observed in platelets treated with native LDL cholesterol alone (not shown).

Effect of Cholesterol on CD40L-Induced Clotting Activation

Compared with unstimulated monocytes, CD40L-stimulated monocytes showed a significant increase in TF and F1+2; further increases in monocyte TF (MANOVA, $P<0.005$; Figure 4B) and F1+2 (MANOVA, $P<0.005$; Figure 4C) were observed when monocytes were added with native LDL cholesterol (100 μ g/mL). No change in clotting activation was observed in monocytes added with native LDL cholesterol alone (not shown).

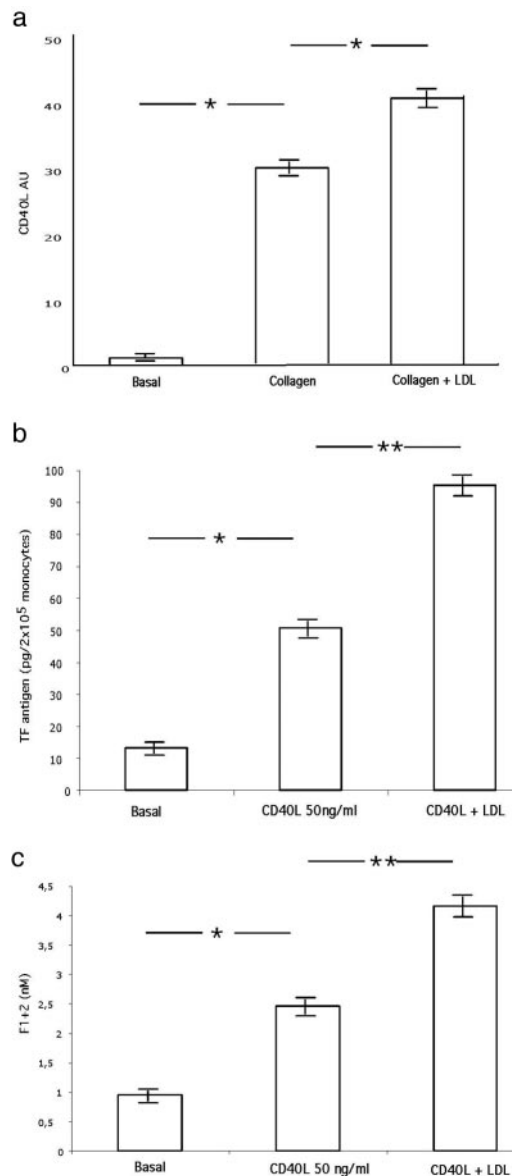


Figure 4. Effect of native LDL cholesterol (100 μg/mL) on CD40L platelet expression in collagen-stimulated platelets (a) and on TF (b) and F1+2 (c) in CD40L-stimulated monocytes (n=4 healthy subjects, 2 males, 2 females; mean age, 50.3 years). MANOVA, * $P < 0.001$, ** $P < 0.005$. Results are mean \pm SD.

Effect of Atorvastatin on Platelet CD40L

Because these data suggested that atorvastatin reduced CD40L by a mechanism that was independent of its cholesterol-lowering effect, we investigated whether it had a direct influence on platelet CD40L expression. Incubation of platelets from healthy subjects or hypercholesterolemic patients with atorvastatin showed a significant decrease in platelet CD40L expression (MANOVA, $P < 0.005$; Figure 5A and 5B). This effect was dependent on the concentration of atorvastatin in both patients and controls, as demonstrated by comparison of dose-response curves for hypercholesterolemic patients ($r = 0.82$, $P < 0.001$) and healthy control subjects ($r = 0.92$, $P > 0.001$; Figure 5C).

Effect of Atorvastatin on CD40L-Induced Clotting Activation

Finally, we investigated in vitro whether atorvastatin was able to influence the prothrombotic effect of CD40L in a thrombin-

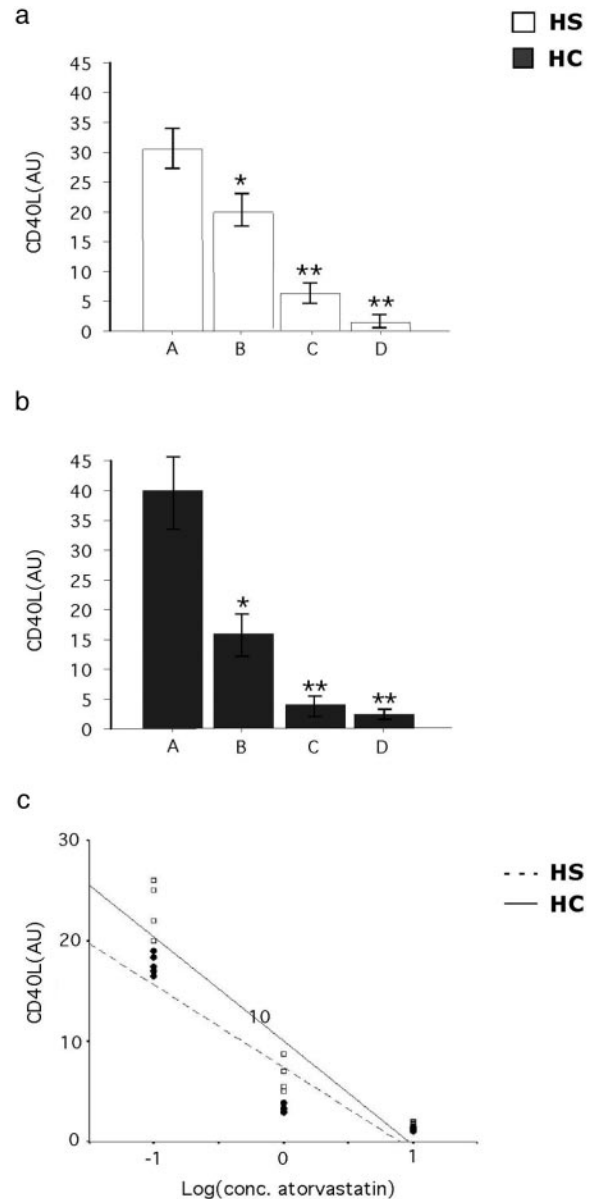


Figure 5. CD40L expression by collagen-stimulated platelets (A) and collagen-stimulated platelets together with 0.1 μmol/L (B), 1 μmol/L (C), or 10 μmol/L (D) atorvastatin in healthy subjects (HS; n=4, 2 males, 2 females; mean age, 50 years) (a) and hypercholesterolemic patients (HC; n=4, 2 males, 2 females; mean age, 53 years) (b). c, Dose-response curves for HC vs HS subjects. MANOVA with Bonferroni test: * $P < 0.001$, B vs A; ** $P < 0.005$, C vs A and D vs A. Results are mean \pm SD. Abbreviations are as defined in text.

generating system. This study showed that incubation of human monocytes with CD40L induced a significant increase in F1+2 compared with unstimulated monocytes in both healthy subjects and hypercholesterolemic patients (MANOVA, $P < 0.005$); this effect, however, was more marked in patients than in controls, as documented by the higher rate of thrombin generation in hypercholesterolemic patients (Figure 6A). Similar findings were obtained when TF expression by unstimulated and CD40L-stimulated monocytes was investigated (MANOVA, $P < 0.005$; Figure 6B). Incubation of monocytes with atorvastatin dose-dependently inhibited the rate of thrombin generation (Figure 7A and 7B) and TF expression

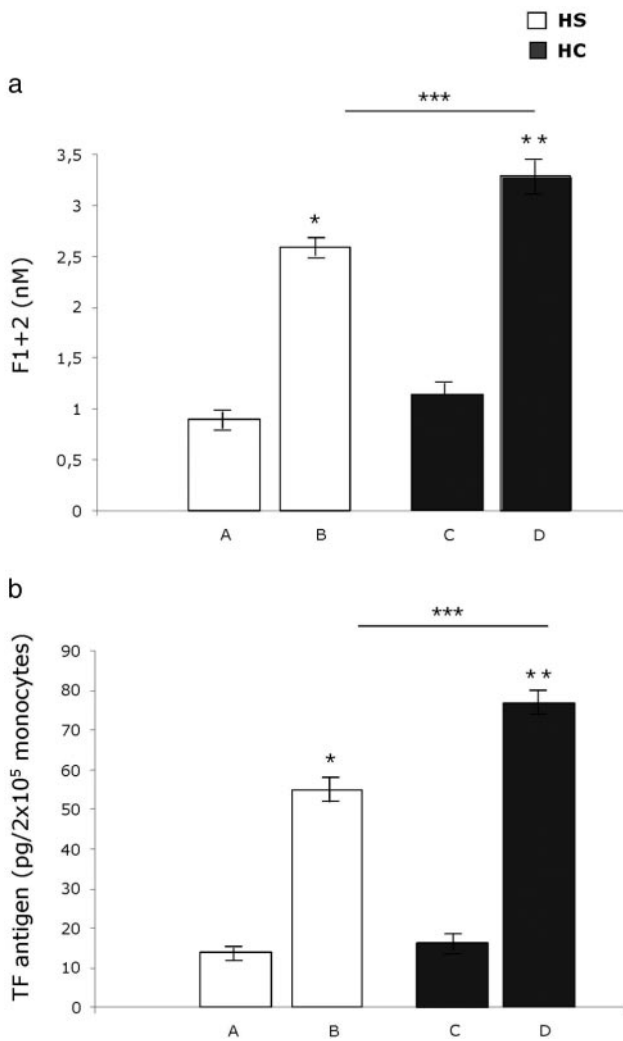


Figure 6. Prothrombin F1+2 (a) and TF expression (b) by unstimulated monocytes (A and C) and monocytes stimulated with CD40L (50 ng/mL; B and D) in healthy subjects (HS; n=4, 2 males, 2 females; mean age, 50 years) and hypercholesterolemic patients (HC; n=4, 2 males, 2 females; mean age, 53 years). MANOVA: * $P < 0.003$, B vs A; ** $P < 0.001$, D vs C; *** $P < 0.005$, D vs B. Results are mean \pm SD. Abbreviations are as defined in text.

(Figure 7D and 7E) by monocytes (MANOVA, $P < 0.05$). This effect was demonstrated in both patients and controls, as demonstrated by comparison of dose-response curves of hypercholesterolemic and healthy subjects (Figure 7C and 7F; for TF and F1+2, $r = 0.97$, $P < 0.001$, and $r = 0.87$, $P < 0.001$, respectively).

Discussion

This study provides the first evidence that in hypercholesterolemia, platelet CD40L is overexpressed and is significantly correlated with sCD40L, thus suggesting that enhanced levels of sCD40L are likely to reflect platelet CD40L upregulation.

Cholesterol, CD40L, and Clotting Activation

The significant correlation between serum cholesterol and platelet CD40L suggests a role for cholesterol in enhancing platelet CD40L overexpression. We also sought to investigate whether CD40L has an influence on clotting system activation. The experiments con-

ducted in monocytes from healthy subjects and hypercholesterolemic patients demonstrated that CD40L, at a concentration close to that observed in the peripheral circulation, was able to increase TF and prothrombin fragment F1+2, with more marked effect in patients with hypercholesterolemia.

Because these findings suggested a role for cholesterol in enhancing platelet CD40L expression and clotting activation, we performed further experiments to support this hypothesis. Our study provides the first evidence that cholesterol is implicated in the overexpression of platelet CD40L elicited by agonist; also, we demonstrated that CD40L-induced clotting activation is amplified in monocytes coincubated with cholesterol. Both findings provide a novel insight into the atherothrombotic role of cholesterol and warrant further studies to identify the mechanism accounting for such effects.

Atorvastatin and Platelet CD40L

Because CD40L has a key role in the initiation and progression of atherosclerosis, we investigated whether statins, that are potent inhibitors of atherosclerotic progression,^{21–23} could influence platelet CD40L expression. Although our data suggest that cholesterol plays a major role in enhancing platelet CD40L expression, it is also possible that statins influence CD40L expression independently of their principal mechanism of action. In one study, Semb et al¹⁵ randomized 100 patients with familial hypercholesterolemia to either atorvastatin or simvastatin and demonstrated a significant decrease in sCD40L that was independent of the cholesterol-lowering effect of these agents. To further investigate this issue, we studied platelet CD40L and sCD40L before and immediately after 3 days of atorvastatin treatment; using the same approach, we had previously found no changes in the lipid profile of hypercholesterolemic patients treated with a statin.²⁴ Compared with diet-assigned patients, atorvastatin-treated patients demonstrated a significant and parallel decrease of platelet CD40L and sCD40L, suggesting that atorvastatin inhibits sCD40L by downregulating platelet CD40L through a mechanism that is independent of its cholesterol-lowering effect. This hypothesis was supported by an in vitro study, which demonstrated that atorvastatin dose-dependently inhibited collagen-induced CD40L expression; it should be noted that inhibition of platelet CD40L expression was observed at atorvastatin concentrations as low as 0.1 $\mu\text{mol/L}$, which may be achieved in the peripheral circulation of subjects treated with 5 to 20 mg/d atorvastatin.²⁵

Atorvastatin and Clotting Activation

Another novel finding of the present study was an early reduction in the levels of prothrombin fragment F1+2, suggesting that atorvastatin has an inhibitory effect on activation of the coagulation cascade. At least 2 mechanisms may account for this finding. One mechanism may be related to atorvastatin-induced CD40L downregulation, which could be responsible for reduced TF expression and perhaps a lower thrombin generation rate. Another mechanism may involve direct interference of the drug with the intracellular signaling responsible for TF expression. This hypothesis is in accordance with previous studies showing that statins inhibit nuclear factor NF- κ B, which has a key role in TF expression by monocytes^{26–28}; however, this hypothesis should be wisely considered because inhibition of clotting activation was achieved with relatively high concentrations (1 $\mu\text{mol/L}$) of atorvastatin.

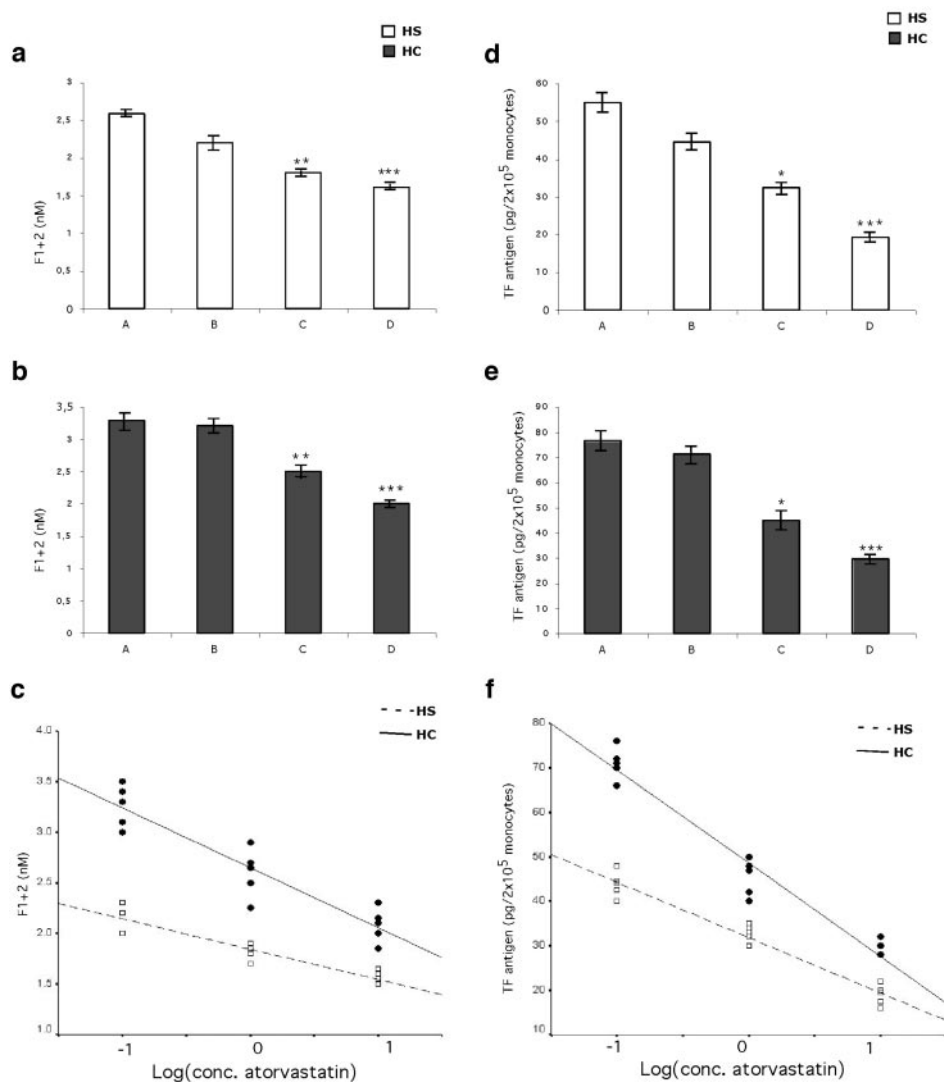


Figure 7. Prothrombin F1+2 (a and b) and TF (d and e) generation by monocytes stimulated with CD40L (50 ng/mL, A) and CD40L-stimulated monocytes added with 0.1 μ mol/L (B), 1 μ mol/L (C), or 10 μ mol/L (D) atrovastatin in healthy subjects (HS; n=4, 2 males, 2 females; mean age, 50 years) and hypercholesterolemic patients (HC; n=4, 2 males, 2 females; mean age, 53 years). Dose-response curves for HC versus HS subjects (c and f). MANOVA with Bonferroni test: * P <0.01, C vs A; ** P <0.02, C vs A; *** P <0.005, D vs A. Results are mean \pm SD.

Atrovastatin and Coronary Artery Disease

The effect observed on platelet CD40L and F1+2 as early as after 3 days of treatment suggests that atrovastatin has an immediate antiinflammatory and antithrombotic action that may prove useful in cases of plaque instability. In fact, inflammation with the ensuing activation of platelets and the coagulation cascade is a typical feature of vulnerable plaque and triggers coronary thrombus growth. So far, statins have been mainly used in patients with stable coronary syndromes or in those at risk of cardiovascular disease to retard atherosclerotic progression. Atrovastatin's early antiinflammatory and antithrombotic effect could suggest the use of statins even in patients with acute coronary syndromes; in these patients, timely halting of plaque instability could retard thrombus growth and perhaps have a favorable effect on clinical outcome. This concept is in agreement with the results of a recent trial in patients with acute coronary syndromes, in whom atrovastatin was shown to reduce cardiovascular events as early as 30 days after starting the

treatment.²⁹ Our findings may also explain the significant reduction of myocardial necrosis observed after 7 days of atrovastatin treatment in patients undergoing percutaneous coronary angioplasty.³⁰

In conclusion, we provide evidence that in hypercholesterolemia, platelet CD40L is upregulated and is likely responsible for enhanced sCD40L. Overexpression of CD40L has important implications for activation of the clotting system and may represent a novel mechanism accounting for the thrombotic events occurring in hypercholesterolemia. The inhibition of platelet CD40L expression and CD40L-mediated thrombin generation provides new insight into the antiatherothrombotic action of atrovastatin and may open new paths to the use of statins in the prevention of cardiovascular disease.

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