

# CD40 Ligand Enhances Monocyte Tissue Factor Expression and Thrombin Generation Via Oxidative Stress in Patients With Hypercholesterolemia

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<b>OBJECTIVES</b>	We tested the hypothesis that CD40 ligand (CD40L) induces a prothrombotic state by enhancing oxidative stress.
<b>BACKGROUND</b>	Patients with hypercholesterolemia show an ongoing prothrombotic state, but the underlying mechanism is still unclear.
<b>METHODS</b>	Circulating levels of the soluble form of CD40L (sCD40L), prothrombin fragment (F1+2, a marker of thrombin generation), and 8-hydroxy-2'-deoxyguanosine (8-OHdG, a marker of oxidative stress) were measured in 40 patients with hypercholesterolemia and in 20 age- and gender-matched healthy subjects.
<b>RESULTS</b>	Patients with hypercholesterolemia showed significantly higher levels of sCD40L ( $p < 0.005$ ), 8-OHdG ( $p < 0.005$ ), and prothrombin F1+2 ( $p < 0.005$ ), as compared with control subjects. Soluble CD40L significantly correlated with 8-OHdG ( $r = 0.85$ , $p < 0.0001$ ) and prothrombin F1+2 ( $r = 0.83$ , $p < 0.0001$ ); a significant correlation between 8-OHdG and prothrombin F1+2 was also observed ( $r = 0.64$ , $p < 0.0001$ ). An <i>in vitro</i> study demonstrated that CD40L-stimulated monocytes from patients with hypercholesterolemia expressed more tissue factor (TF) and prothrombin F1+2 than monocytes from controls; co-incubation of monocytes with either an inhibitor of NADPH oxidase or an inhibitor of phosphatidylinositol-3-kinase significantly reduced CD40L-mediated clotting activation. A marked inhibition of CD40L-mediated clotting activation was also observed in two male patients with hereditary deficiency of gp91 phox, the central core of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Finally, we demonstrated that CD40L-mediated clotting activation was significantly inhibited by vitamin C, a known antioxidant.
<b>CONCLUSIONS</b>	This study indicates that in patients with hypercholesterolemia, CD40L over-expresses TF and increases the thrombin generation rate by an oxidative stress-mediated mechanism that requires the activation of NADPH oxidase. (J Am Coll Cardiol 2005;45:35–42) © 2005 by the American College of Cardiology Foundation

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Epidemiologic and clinical trials have provided clear evidence of a cholesterol role in the occurrence of cardiovascular events (1–3). Accordingly, interventional trials with statins have reduced the risk of atherosclerotic complications in patients with high or average cholesterol levels in their serum (4–6). Among the mechanisms through which cholesterol may facilitate the atherosclerotic complication, the activation of the platelet and clotting system has been suggested to play a pivotal role. An enhanced platelet response to agonists and elevated urinary excretion of 11-dehydro-thromboxane B<sub>2</sub> has been observed in patients with hypercholesterolemia (7). Also, enhanced expression of monocyte tissue factor (TF) and high circulating levels of prothrombin fragment (F1+2), a marker of thrombin generation *in vivo*, suggested an ongoing prothrombotic state in this setting (8). The mechanism accounting for increased

thrombin generation in patients with hypercholesterolemia is still unclear.

The CD40 ligand (CD40L) is a transmembrane protein expressed on the surface of lymphocytes, as well as on the cells of the vascular system, such as endothelial cells, smooth muscle cells (SMCs), and macrophages (9). CD40L is also expressed upon agonist stimulation on platelet surface; then, it is cleaved and circulates as soluble CD40L (sCD40L) (10). It has been calculated that more than 95% of circulating sCD40L originates from platelets (11). Upon interaction with its receptor CD40, CD40L elicits inflammatory and prothrombotic responses that may favor and accelerate atherosclerotic progression (11). In particular, CD40 colocalizes with TF, a glycoprotein of the extrinsic coagulation pathway that converts factor X to Xa (12) on SMCs within the atherosclerotic plaque (13); engagement of CD40 with CD40L induces overexpression of TF in SMCs, endothelial cells, and macrophages (14). Previous studies demonstrated that oxidative stress plays a major role in monocyte expression of TF by promoting nuclear factor (NF)-kappa-B activation (15). On the basis of recent studies showing that CD40L exerts a pro-oxidant effect (16), we speculated that

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**Abbreviations and Acronyms**

F1+2	=	prothrombin fragment
LMWH	=	low-molecular-weight heparin
8-OHdG	=	8-hydroxy-2-deoxyguanosine
PBMC	=	peripheral blood mononuclear cell
PBS	=	phosphate-buffered saline
PI-3-K	=	phosphatidyl-inositol-3-kinase
sCD40L	=	soluble CD40 ligand
SMC	=	smooth muscle cell
TF	=	tissue factor
X-CGD	=	X-linked chronic granulomatous disease

oxidative stress may be involved in CD40L-induced monocyte clotting activation. To explore this hypothesis, we investigated the behavior of sCD40L, oxidative stress, and clotting activation in patients with hypercholesterolemia. In this report, we show, for the first time, that CD40L promotes clotting activation by enhancing oxidative stress.

**METHODS**

We performed a cross-sectional study comparing 40 patients with polygenic hypercholesterolemia (21 men and 19 women; mean age 51.6 years) and 20 gender- and age-matched subjects with normal cholesterol levels (11 men and 9 women; mean age 50.4 years). Both patients and control subjects 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, and electrocardiogram), diabetes mellitus, or hypertension. Five patients and seven healthy subjects smoked more than five cigarettes daily. Patients with hypercholesterolemia had not taken any lipid-lowering agents or antiplatelet drugs in the previous 30 days. Blood samples mixed with 0.13 mol/l of sodium citrate (ratio 9:1) were obtained between 8 and 9 AM from patients and healthy volunteers who had fasted for 12 h and provided their informed consent to participate in the study. An aliquot of serum was used to measure lipid profiles.

**Lipid profile.** Serum levels of total cholesterol and triglycerides were determined using an enzyme-based method. High-density lipoprotein cholesterol was measured after phosphotungstic acid/MgCl<sub>2</sub> precipitation of fresh plasma. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.

**Analysis of sCD40L, F1+2, and 8-hydroxy-2-deoxyguanosine (8-OHdG).** Blood samples were immediately centrifuged at 2,000 rpm for 20 min at 4°C, and the supernatant was collected and stored at –80°C until measurement. Plasma levels of sCD40L were measured using a commercial immunoassay (Quantikine CD40 ligand, R&D Systems, Minneapolis, Minnesota). Plasma levels of human prothrombin F1+2 were assayed by an enzyme immunoassay based on the sandwich principle (Enzygnost F1+2, Behringwerke, Marburg, Germany). 8-OHdG was analyzed

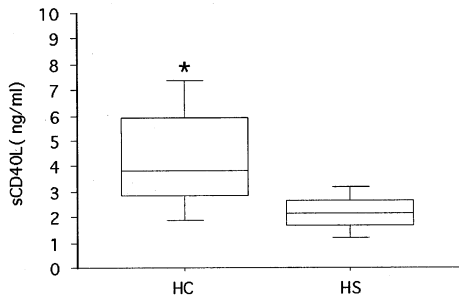
using a competitive enzyme-linked immunosorbent assay (Bioxytech 8-OHdG-EIA, OXIS Health Products, Portland, Oregon) in serum and urine.

**Gp91 phox-deficient patients.** X-LINKED CHRONIC GRANULOMATOUS DISEASE (X-CGD) PATIENT DESCRIPTION: X-linked chronic granulomatous disease, an inherited disorder characterized by the absence or deficiency of phagocyte-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, was diagnosed in two male patients (age 33 and 38 years) by demonstrating the absence or manifest deficiency of oxidase activity in stimulated neutrophils (17). A diagnosis of X-CGD was subsequently confirmed by the mutation analysis of the CYBB gene encoding the gp91 subunit of phagocyte-NADPH oxidase (18). Mutation in Patient #1 was identified as a single-base substitution of guanosine to adenosine at residue 252 in exon 3, resulting in a splicing defect. A deletion of thymine 184 in exon 3 was identified for Patient #2, resulting in a frame shift.

**In vitro study.** MONOCYTE TISSUE FACTOR EXPRESSION AND MONOCYTE-MEDIATED THROMBIN GENERATION. Peripheral blood mononuclear cells were isolated from heparinized venous blood of healthy subjects (n = 4), patients with hypercholesterolemia (n = 4), and gp91 phox-deficient patients (n = 2) using an aseptic technique. Platelets were separated by centrifugation, once at 140 g and twice at 100 g in phosphate-buffered saline (PBS) at room temperature for 10 min. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on lymphoprep (Nyeegard, Oslo, Norway) at 1,200 g for 20 min 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 min at 37°C in a humid atmosphere of 5% CO<sub>2</sub> in petri dishes containing Roswell Park Memorial Institute (RPMI) 1640, supplemented with 2 mmol/l glutamine; lymphocytes (non-adherent cells) were removed by aspiration with a Pasteur pipette and washing of the dishes with warm media. The purified monocyte preparation contained 85% to 95% of monocytes.

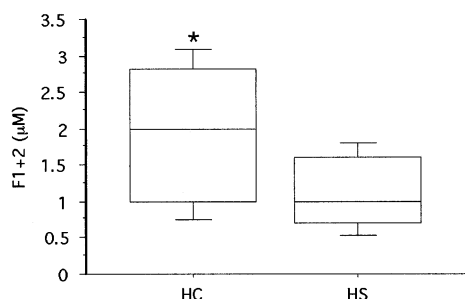
After isolation, cells were washed twice in PBS and preincubated for 1 h at  $2 \times 10^5$  cells/ml in RPMI 1640 at 37°C 5% CO<sub>2</sub> with 50 and 100 μmol/l vitamin C or medium as a control. Cells were then incubated with 50 ng/ml CD40L (recombinant human CD40L/TNFSF5, R&D Systems) for 6 h. At the end of the incubation period, the cells and media were separated by centrifugation (2,000 g × 15 min). The cells were washed with Tris-NaCl buffer (0.1 mmol/l NaCl, 0.1% bovine serum albumin, pH 7.4) then lysed in the same buffer by adding 15 mmol/l n-octyl-beta-D-glycopyranoside at 37°C for 30 min. Cell count and trypan blue exclusion were performed on cell suspensions after washing. The ELISA for measuring TF antigen in cell lysate was performed using a commercial kit (Imubind Tissue Factor ELISA Kit, American Diagnostica Inc., Greenwich, Connecticut). The lower detection limit is ~10 pg/ml. The assay recognizes TF-apo, TF,



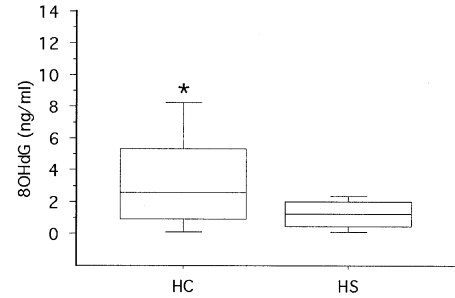
**Figure 1.** Plasma levels of soluble CD40 ligand (sCD40L) in patients with hypercholesterolemia (HC) and healthy subjects (HS). \* $p < 0.0005$  for HC versus HS.

and TF-VII complexes and is designed such that there is no interference from other coagulation factors or inhibitors of procoagulant activity.

The thrombin generation rate by CD40L-stimulated monocytes was evaluated in vitro, as previously described (19). For this purpose, a low-molecular-weight heparin (LMWH) anticoagulated blood sample (ratio 1:10) was taken from healthy volunteers ( $n = 4$ ) and patients with hypercholesterolemia ( $n = 4$ ). The blood was anticoagulated with LMWH (820 U/ml), because this agent effectively inhibits the formation of thrombin in solution but has only a small effect on thrombin generated at and bound to surfaces. After isolation, monocytes were washed twice in PBS, then incubated ( $2 \times 10^5$  cells/ml in RPMI 1640 at  $37^\circ\text{C}$  5%  $\text{CO}_2$ ) with scalar concentration (5 to 50 ng/ml) of CD40L for 6 h. In another set of experiments, monocytes were pre-incubated for 1 h with diphenyliodonium (DPI) (15  $\mu\text{mol/l}$ ) (NADPH oxidase inhibitor), eicosatetraenoic acid (ETYA) (35  $\mu\text{mol/l}$ ) (lipooxygenase inhibitor), wortmannin (0.1  $\mu\text{mol/l}$ ) (phosphatidylinositol-3-kinase [PI-3-K] inhibitor), vitamin C (50 and 100  $\mu\text{mol/l}$ ), or medium as a control before the stimulation with CD40L. The medium was removed and 1,000 ml overlay heparinized standard plasma was added to each well and incubated at  $37^\circ\text{C}$  for 24 h. After incubation, samples were harvested, centrifuged at 4,000  $g$ , and assayed for prothrombin F1+2 generation, calculated as the increase in prothrombin 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.



**Figure 2.** Plasma levels of prothrombin fragment (F1+2) in patients with hypercholesterolemia (HC) and healthy subjects (HS). \* $p < 0.0005$  for HC versus HS.



**Figure 3.** Plasma levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) in patients with hypercholesterolemia (HC) and healthy subjects (HS). \* $p < 0.005$  for HC versus HS.

Endotoxin levels, measured in all reagents according to a commercially chromogenic substrate test (Kabi Diagnostica, Stockholm, Sweden), as previously described (20), were  $<10$  pg/ml.

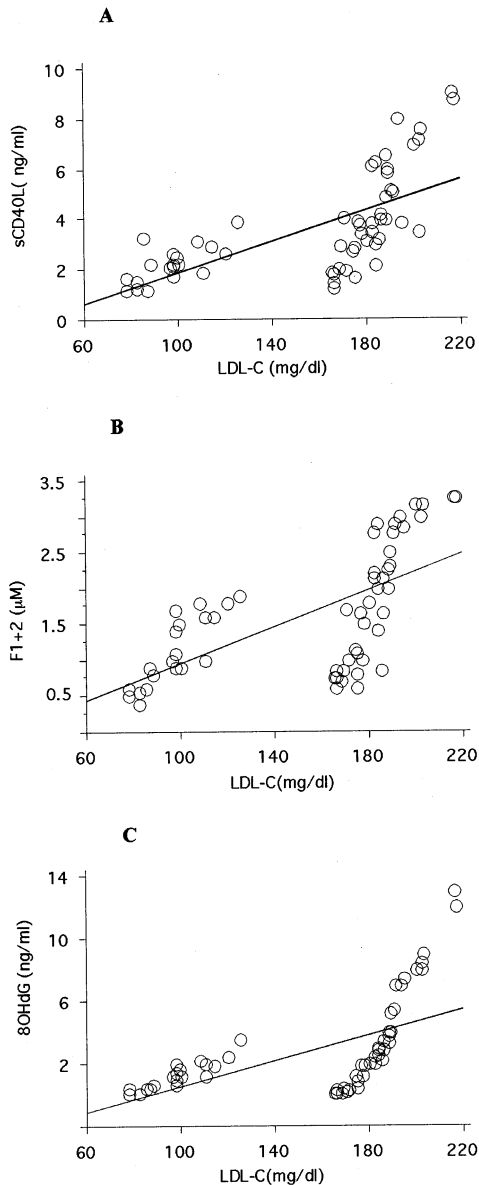
**Statistical analysis.** The comparisons between variables for the two independent samples were carried out using the Student  $t$  test. The two-sample Kolmogorov-Smirnov ( $Z$ ) test was used only in case of non-homogeneous variances, as verified by Levene's test. The analysis was carried out by using Pearson correlation. Data were presented as the mean value  $\pm$  SD. Statistical significance was defined at  $p < 0.05$ .

## RESULTS

**In vivo study.** Subjects with hypercholesterolemia had higher values of total cholesterol (275.33 vs. 171.12 mg/dl,  $p < 0.0005$ ) and LDL cholesterol (183.9 vs. 97.7 mg/dl,  $p < 0.0005$ ) than control subjects. Soluble CD40L plasma levels were significantly higher in patients with hypercholesterolemia than in control subjects ( $4.18 \pm 2.07$  ng/ml vs.  $2.60 \pm 0.7$  ng/ml,  $p < 0.0005$ ) (Fig. 1); 21 patients with hypercholesterolemia (53%) versus one control subject had values above 3.74 ng/ml (mean + 2 SD of control subjects). The prothrombin F1+2 plasma levels were significantly higher in patients with hypercholesterolemia than in control subjects ( $1.91 \pm 0.89$  nmol/l vs.  $1.12 \pm 0.49$  nmol/l,  $p < 0.0005$ ) (Fig. 2); 17 patients with hypercholesterolemia (43%) versus no control subject had values above 1.86 nmol/l (mean + 2 SD of control subjects).

The 8-OHdG plasma levels were significantly higher in patients with hypercholesterolemia than in control subjects ( $4.74 \pm 3.36$  ng/ml vs.  $1.25 \pm 0.92$  ng/ml,  $p < 0.005$ ) (Fig. 3); 16 patients with hypercholesterolemia (40%) versus one control subject had values above 3.04 ng/ml (mean + 2 SD of control subjects). In a subgroup of 20 patients with hypercholesterolemia (10 men and 10 women, mean age 50.8 years), urinary excretion of 8-OHdG was  $14.8 \pm 5$  ng/mg creatinine and significantly correlated with serum levels ( $r = 0.894$ ,  $p < 0.0001$ ).

Low-density lipoprotein cholesterol was significantly correlated with sCD40L ( $r = 0.62$ ,  $p < 0.0001$ ) (Fig. 4A), prothrombin F1+2 ( $r = 0.60$ ,  $p < 0.0001$ ) (Fig. 4B), and 8-OHdG plasma levels ( $r = 0.59$ ,  $p < 0.0001$ ) (Fig. 4C).

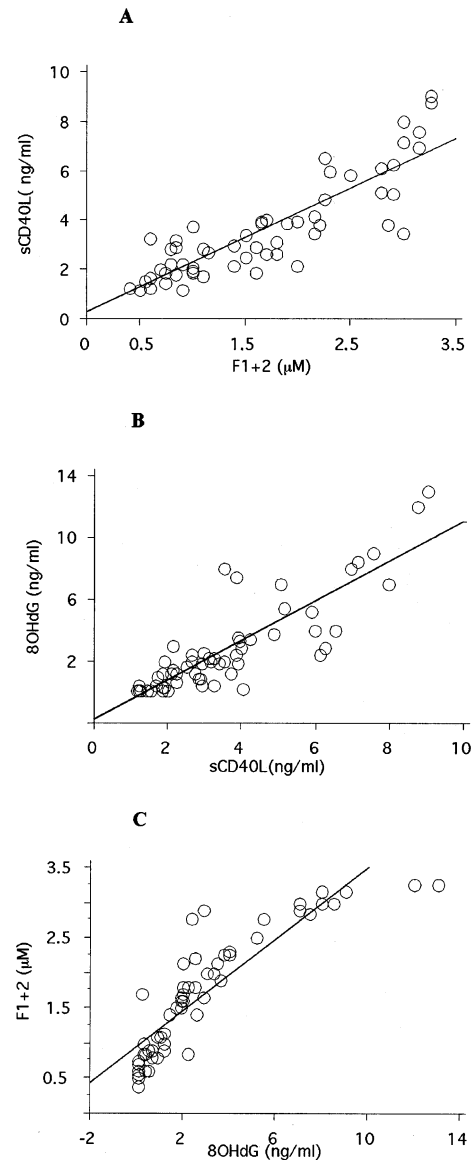


**Figure 4.** (A) Correlation between low-density lipoprotein cholesterol (LDL-C) and soluble CD40 ligand (sCD40L). (B) Correlation between LDL-C and prothrombin fragment (F1+2). (C) Correlation between LDL-C and 8-hydroxy-2-deoxyguanosine (8-OHdG).

Soluble CD40L was significantly correlated with prothrombin F1+2 ( $r = 0.83$ ,  $p < 0.0001$ ) (Fig. 5A) and 8-OHdG plasma levels ( $r = 0.85$ ,  $p < 0.0001$ ) (Fig. 5B). Prothrombin F1+2 plasma levels were significantly correlated with 8-OHdG plasma levels ( $r = 0.64$ ,  $p < 0.0001$ ) (Fig. 5C).

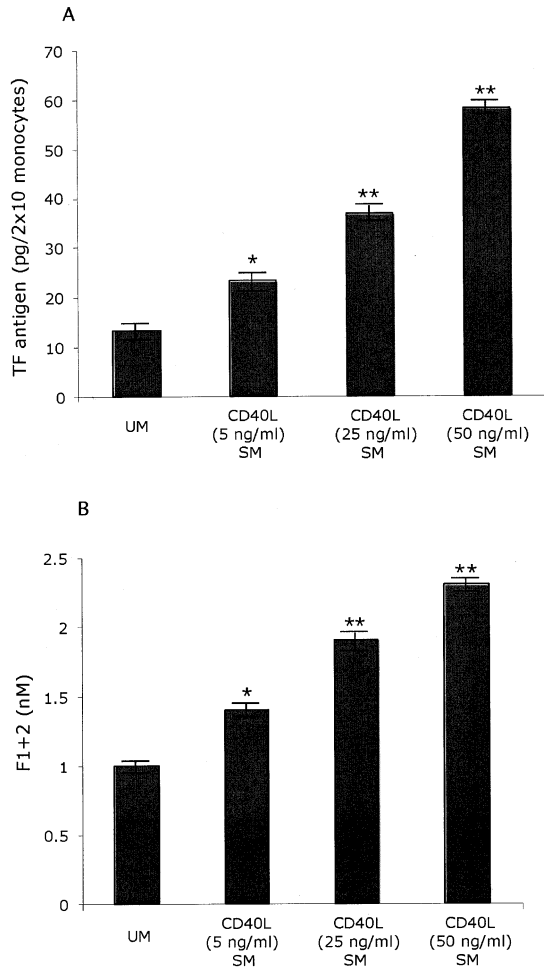
**In vitro studies. CLOTTING ACTIVATION BY CD40L.** In the first part of the in vitro study, we investigated if CD40L, at a concentration relatively close to that found in human circulation, was able to up-regulate the monocyte expression of TF. This experiment demonstrated that CD40L (5 to 50 ng/ml) as low as 5 ng/ml enhanced TF and thrombin generation; a higher rate of clotting activation was found by increasing CD40L concentration (Fig. 6).

Next we investigated whether monocytes from four



**Figure 5.** (A) Correlation between soluble CD40 ligand (sCD40L) and prothrombin fragment (F1+2). (B) Correlation between 8-hydroxy-2-deoxyguanosine (8-OHdG) and sCD40L. (C) Correlation between prothrombin F1+2 and 8-OHdG.

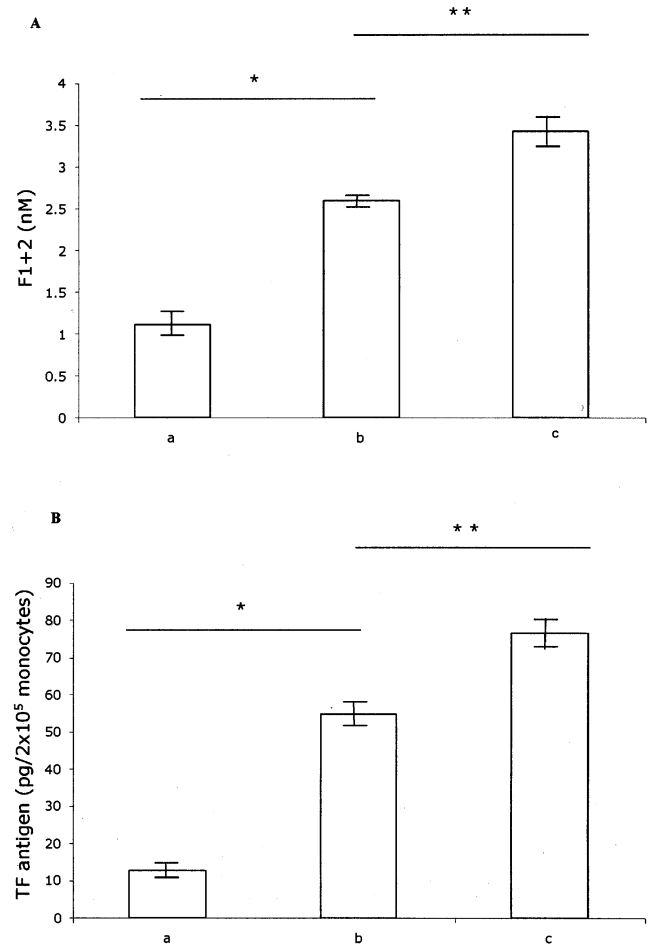
healthy subjects (2 men and 2 women, ages 44 to 58 years) and four patients with hypercholesterolemia (3 men and 1 woman, ages 42 to 59 years) differently expressed TF and prothrombin F1+2 upon stimulation with CD40L. Compared with unstimulated monocytes, CD40L enhanced the rate of thrombin generation in both healthy subjects and patients with hypercholesterolemia (Fig. 7); however, a higher concentration of prothrombin F1+2 was found in patients compared with healthy subjects. The increased formation of prothrombin F1+2 was likely to be due to monocyte TF overexpression, as CD40L-stimulated monocytes produced more TF compared with unstimulated monocytes (Fig. 7); in this case, higher monocyte TF expression was detected in patients compared with healthy subjects.



**Figure 6.** Expression of tissue factor (TF) (A) and generation of prothrombin fragment (F1+2) (B) in monocytes taken from healthy subjects (2 men and 2 women, mean age 50 years) and stimulated with scalar doses of CD40 ligand (CD40L) (5 to 50 ng/ml). UM = unstimulated monocytes; SM = stimulated monocytes. \* $p < 0.001$  for CD40L (5 ng/ml) SM vs. UM. \*\* $p < 0.0001$  for CD40L (25 ng/ml) SM versus UM and CD40L (50 ng/ml) SM versus UM.

**Oxidative stress and clotting activation by CD40L.** A previous study demonstrated that CD40 ligation enhances the production of reactive oxidant species via activation of NADPH oxidase and PI-3-K (21,22). Incubation of human monocytes with an inhibitor of NADPH oxidase induced a significant decrease of CD40L-mediated TF expression and thrombin generation (Fig. 8); TF and thrombin generation were also inhibited when monocytes were incubated with a PI-3-K antagonist (Fig. 8). Conversely, no changes in clotting activation were observed when monocytes were incubated with ETYA, an inhibitor of 5-lipoxygenase (Fig. 8).

To further explore the role of NADPH oxidase on CD40L-mediated clotting activation, we repeated the experiments in two male patients who had X-CGD, a hereditary deficiency of gp91 phox, the central core of NADPH oxidase. We demonstrated that, compared with controls, the gp91 phox-deficient patients had much lower expression of TF and thrombin generation, thus reinforcing



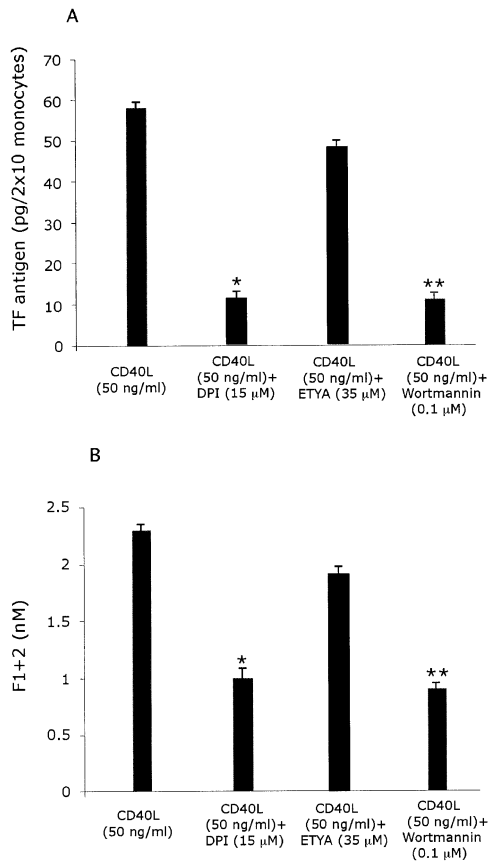
**Figure 7.** Generation of prothrombin fragment (F1+2) (A) and expression of tissue factor (TF) (B) by unstimulated monocytes (a) and CD40 ligand (CD40L)-stimulated monocytes taken from healthy subjects (b; n = 4) or patients with hypercholesterolemia (c; n = 4). \* $p < 0.0005$  for b versus a. \*\* $p < 0.003$  for c versus b.

the hypothesis that NADPH oxidase has a key role on CD40L-mediated clotting activation (Fig. 9).

In order to investigate whether vitamin C, a known antioxidant molecule, influenced CD40L-mediated clotting activation, the experiments were repeated in the presence or absence of this vitamin. Co-incubation of CD40L-stimulated monocytes with vitamin C-induced dose-dependent inhibition of thrombin generation (F:75;  $p < 0.0001$ ) (Fig. 10) and TF expression (F:54;  $p < 0.0001$ ) (Fig. 11). The inhibition of monocyte-dependent prothrombin F1+2 and TF formation was observed in both healthy subjects and patients with hypercholesterolemia.

## DISCUSSION

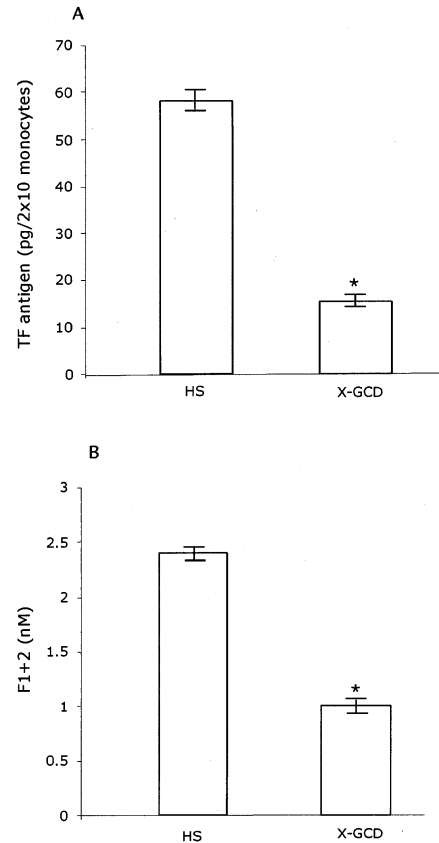
Thrombophilia is a frequent occurrence in patients with hypercholesterolemia and can precipitate cardiovascular events in this setting. Thrombophilia is basically characterized by platelet hyperactivation and up-regulation of TF with enhanced thrombin generation (7,8). CD40L may represent an important link between platelet activa-



**Figure 8.** Expression of tissue factor (TF) (A) and generation of prothrombin fragment (F1+2) (B) by monocytes taken from healthy subjects (n = 4) and stimulated with CD40 ligand (CD40L) (50 ng/ml) or CD40L plus diphenyliodonium (DPI) (15 μmol/l), eicosatetraenoic acid (ETYA) (35 μmol/l), and wortmannin (0.1 μmol/l). \*p < 0.0005 for CD40L (50 ng/ml) + DPI (15 μM) versus CD40L (50 ng/ml). \*\*p < 0.0001 for CD40L (50 ng/ml) + wortmannin (0.1 μM) versus CD40L (50 ng/ml).

tion and TF expression. A previous study has demonstrated that, upon activation, platelets induce TF overexpression in whole blood, a phenomenon that was significantly inhibited by an antibody against CD40L (23). In patients with hypercholesterolemia, CD40L is up-regulated on the platelet surface (24) and may therefore represent an important stimulus for TF expression and clotting activation. The significant correlation between sCD40L and prothrombin F1+2, as shown by the present and previous study (25), could indirectly support this hypothesis but not prove it, because sCD40L has not been shown to elicit cell activation.

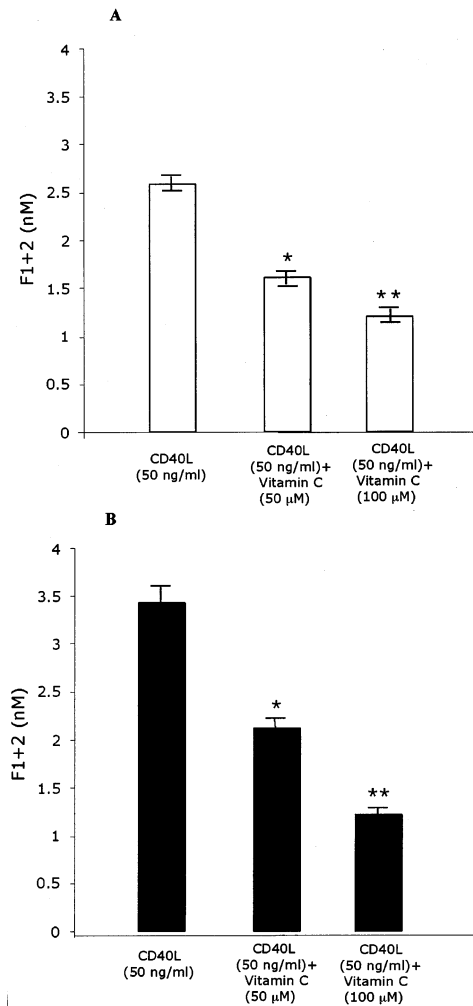
To explore this issue, monocytes from patients and healthy subjects were incubated with CD40L to determine whether a different response to CD40L could account for the enhanced thrombin generation rate observed in patients with hypercholesterolemia. The first novel finding of this study is that CD40L, used in a concentration relatively close to that found in our patients, exerted a higher procoagulant effect in patients with hypercholesterolemia than in control subjects; such a difference was probably due to a higher



**Figure 9.** Expression of tissue factor (TF) (A) and generation of prothrombin fragment (F1+2) (B) by 50 ng/ml CD40 ligand-stimulated monocytes taken from hereditary gp91 deficient-patients (X-GCD; four different determinations) or from four healthy subjects (HS) matched for age. \*p < 0.001 for X-GCD versus HS.

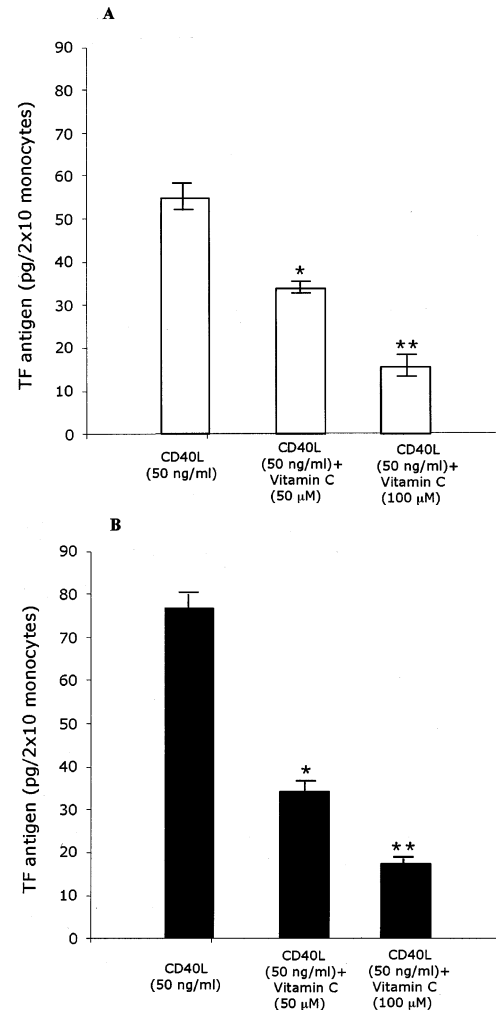
monocyte expression of TF detected in patients compared with controls.

Afterward, we investigated whether CD40L enhanced clotting system activation by oxidative stress, which is an important intracellular signaling for TF expression. In fact, antioxidants have been reported to inhibit lipopolysaccharide-induced transcriptional and post-transcriptional activation of macrophage TF (26,27). Furthermore, human monocytes exposed to copper-induced oxidative stress showed an enhanced expression of TF, which was also inhibited by antioxidants (28). In this study, we measured plasma levels of 8-OHdG, a reliable marker of oxidative stress (29), and found that, in accordance with a previous study (30), patients with hypercholesterolemia showed enhanced oxidative stress. We also found a significant correlation between 8-OHdG plasma levels and prothrombin F1+2 plasma levels, suggesting that oxidative stress could be implicated in clotting system activation. This finding is consistent with a previous study showing a significant correlation between prothrombin F1+2 plasma levels and urinary excretion of isoprostanes, another marker of oxidative stress, in patients at risk of thrombotic complications (31). Previous studies have provided evidence that CD40L exerts a pro-oxidant effect via a mechanism involv-



**Figure 10.** Generation of prothrombin fragment (F1+2) by monocytes stimulated with CD40 ligand (CD40L) (50 ng/ml) and CD40L-stimulated monocytes added with 50 or 100 μmol/l of vitamin C in healthy subjects (open bars; n = 4) (A) and patients with hypercholesterolemia (solid bars; n = 4) (B). \*p < 0.003 for CD40L (50 ng/ml)-stimulated monocytes added with 50 μmol/l of vitamin C versus CD40L (50 ng/ml)-stimulated monocytes. \*\*p < 0.002 for CD40L (50 ng/ml)-stimulated monocytes added with 100 μmol/l of vitamin C versus CD40L (50 ng/ml)-stimulated monocytes.

ing NADPH oxidase (16,21,22). Ha and Lee (22) demonstrated, in particular, that CD40 ligation uses NADPH oxidase for the production of reactive oxidant species and requires the activity of PI-3-K. Our data confirm and extend these data showing that inhibition of NADPH oxidase and PI-3-K markedly reduces CD40L-mediated clotting activation by human monocytes. The role of NADPH oxidase was further reinforced by experiments conducted in gp91 phox-deficient patients, who showed a marked decrease of TF and thrombin generation of CD40L-stimulated monocytes compared with control subjects. Finally, we investigated whether vitamin C, a known antioxidant, influenced CD40L-mediated clotting activation. To test this hypothesis, CD40L-induced monocyte-dependent clotting activation was measured with or without adding vitamin C; the inhibition of TF and thrombin generation obtained in monocytes incubated with vitamin C



**Figure 11.** Expression of tissue factor (TF) by monocytes stimulated with CD40 ligand (CD40L) (50 ng/ml) and CD40L-stimulated monocytes added with 50 or 100 μmol/l of vitamin C in healthy subjects (open bars; A) and patients with hypercholesterolemia (solid bars; B). \*p < 0.003 for CD40L (50 ng/ml)-stimulated monocytes added with 50 μmol/l of vitamin C versus CD40L (50 ng/ml)-stimulated monocytes. \*\*p < 0.002 for CD40L (50 ng/ml)-stimulated monocytes added with 100 μmol/l of vitamin C versus CD40L (50 ng/ml)-stimulated monocytes.

supports the suggestion that oxidative stress may be implicated in clotting system activation by CD40L.

Cross-sectional and prospective studies have shown an increase of sCD40L in patients with acute coronary syndromes, and sCD40L is predictive of future cardiovascular events in patients with and without cardiovascular events (32-35).

The fact that CD40L exerts a prothrombotic effect via oxidative stress represents novel information that adds further insights into CD40L's role in the progression of atherosclerosis. Our findings suggest that, at the site of the atherosclerotic lesion, CD40L-induced oxidative stress could, on the one hand, facilitate accumulation of oxidized LDL within macrophages and, on the other hand, enhance thrombogenicity of the atherosclerotic plaque.

An intriguing implication of this study is that antioxi-

dants may be used to modulate CD40L prothrombotic activity. In fact, the *in vitro* study demonstrated that CD40L-induced clotting system activation could be inhibited using concentrations of vitamin C that may be achieved in humans after supplementation (36). This hypothesis should be investigated by an *ad hoc* study.

**Conclusions.** This study suggests that clotting system activation in patients with hypercholesterolemia may be mediated by CD40L. This effect seems to be due to enhancement of oxidative stress via a NADPH oxidase-dependent mechanism, thus suggesting a novel mechanism accounting for CD40L thrombogenicity.

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