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

Valerio Sanguigni, MD,* Domenico Ferro, MD,† Pasquale Pignatelli, MD,† Maria Del Ben, MD,† Tini Nadia, MD,† Mirella Saliola, PHD,† Roberto Sorge, PHD,* Francesco Violi, MD†

Rome, Italy

OBJECTIVES We tested the hypothesis that CD40 ligand (CD40L) induces a prothrombotic state by enhancing oxidative stress.

BACKGROUND Patients with hypercholesterolemia show an ongoing prothrombotic state, but the underlying mechanism is still unclear.

METHODS 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.

RESULTS 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 in vitro 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.

CONCLUSIONS 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

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_2 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 co-localizes 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

From the *Department of Internal Medicine, University of Rome "Tor Vergata," Rome, Italy; and the †Department of Experimental Medicine and Pathology, University of Rome "La Sapienza," Rome, Italy.

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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/$MgCl_2$ 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_2 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 2×10^5 cells/ml in RPMI 1640 at 37°C 5% CO_2 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,