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Homocysteine Modulates the CD40/CD40L System

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Objectives This study evaluated the impact of hyperhomocysteinemia (HHcy) on the CD40/CD40 ligand (CD40L) dyad in vivo and in vitro.

Background Hyperhomocysteinemia is associated with an increased incidence of atherothrombosis, although the molecular mechanisms of this association are incompletely defined. The CD40L pair triggers inflammatory signals in cells of the vascular wall, representing a major pathogenetic pathway of atherosclerosis.

Methods We used a commercially available enzyme-linked immunosorbent assay kit to evaluate circulating levels of soluble (s) CD40L in 24 patients with HHcy and 24 healthy subjects. We also used real-time polymerase chain reaction and flow cytometry to determine expression levels of CD40 and vascular cell adhesion molecule (VCAM)-1 in human umbilical vein endothelial cells (HUVECs) and of CD40L in human platelets.

Results The sCD40L levels were significantly increased in HHcy patients (median [interquartile range] 8.0 [0.7 to 10.5] ng/ml vs. 2.1 [1.9 to 2.3] ng/ml, $p = 0.0001$). Positive correlations were noted between log sCD40L and log homocysteine (Hcy) ($R = 0.68$, $p < 0.0001$) or log sVCAM-1 ($R = 0.41$, $p < 0.005$). Homocysteine significantly stimulated CD40 mRNA expression in HUVECs ($p = 0.033$). Consistently, 24-h exposure to Hcy increased the percentage of CD40-expressing cells ($p = 0.00025$). Homocysteine also significantly enhanced CD40L expression in platelets ($p = 0.025$) to a comparable extent as that of thrombin. Notably, Hcy increased VCAM-1 protein expression induced by CD40L in HUVECs ($p = 0.0046$).

Conclusions The present results uncover a potential molecular target of Hcy, namely the CD40/CD40L dyad. Collectively, they indicate that upregulation of CD40/CD40L signaling may represent a link between HHcy and an increased risk of cardiovascular disease. (J Am Coll Cardiol 2007;49:2182–90) © 2007 by the American College of Cardiology Foundation

Elevated homocysteine (Hcy) concentration, determined by genetic or dietary factors, is recognized as an independent risk factor for cardiovascular disease (1). Homocysteine may promote vascular damage and atherothrombosis by a number of mechanisms, including release of proinflammatory mediators, induction of oxidative and endoplasmic reticulum stress, and activation of apoptotic pathways in vascular cells (2). However, the relative contribution of these processes to the causal relationship between hyperhomocysteinemia (HHcy) and atherothrombosis is still under debate.

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Accumulating evidence supports the involvement of CD40/CD40 ligand (CD40L) signaling in atherosclerosis. Both CD40 and CD40L are expressed by vascular cells, macrophages, and platelets (3,4). The CD40/CD40L engagement on the surface of endothelial cells, smooth muscle cells, or macrophages triggers a potent inflammatory response, characterized by the release of inflammatory cytokines (interleukins 1β, 6, 8, 12) and chemokines (monocyte chemoattractant protein-1), expression of adhesion molecules (E-selectin, vascular cell adhesion molecule [VCAM]-1, intercellular adhesion molecule-1, P-selectin), activation of matrix metalloproteinases, and procoagulant tissue factor (3–7). Antibody blockade or genetic disruption of CD40L in Apolipoprotein-$E^{-/-}$ mice provides direct evidence of the involvement of CD40/CD40L signaling in atherosclerosis progression (8,9).

A soluble form of CD40L (sCD40L) is rapidly released by T cells and activated platelets (10,11). The sCD40L levels are increased in a number of pathological conditions characterized by cardiovascular damage, i.e., unstable angina

(11), acute coronary syndromes (12), hypercholesterolemia (13,14), arterial hypertension (15), and diabetes (16). Thus, measurement of sCD40L is now regarded as an index of platelet activation and inflammatory vascular damage.

Because HHcy is associated with signs of vascular inflammation and platelet activation (17,18), we tested the hypothesis that Hcy may up-regulate the CD40/CD40L system. Here we provide the first evidence of increased sCD40L circulating levels in HHcy patients and of CD40 up-regulation by Hcy in human umbilical vein endothelial cells (HUVECs) and of CD40L in platelets.

Methods

Reagents. Rabbit anti-CD40 and anti-CD40L polyclonal immunoglobulin (Ig) G were from Santa Cruz Biotechnology (Santa Cruz, California). Mouse anti-VCAM-1 (CD106) phycoerythrin-conjugated was from BioLegend (San Diego, California). Anti-rabbit fluorescein-conjugate IgG was from Calbiochem (Milan, Italy). The spin/vacuum total RNA isolation system was from Promega (Milan, Italy). Reagents for real-time analysis were from Applied Biosystems (Milan, Italy). Human thrombin (2,000 NIH U/mg protein); N-2 hydroxyethyl piperazine-N 1-2-ethanesulfonic acid (HEPES); ethylene glycol-bis (b-aminoethyl ether)-N, N, N′, N′,-tetraacetic acid (EGTA); prostaglandin E_1 (PGE_1); DL-homocysteine; L-cysteine; and all other chemicals were purchased from Sigma-Aldrich (Milan, Italy). Thrombin was dissolved in saline at 20 μmol/l (50 U/ml) and stored at −20°C until use.

Patients, genotyping, and measurements. We selected 24 subjects carrying the 5,10 methylene tetrahydrofolate reductase (MTHFR) C677T genotype with HHcy (>15 μmol/l) and 24 age- and gender-matched subjects, also expressing the MTHFR C677T polymorphism, but with normal homocysteinemia (<15 μmol/l). Exclusion criteria were represented by a recent history of thrombotic events (<6 months), pregnancy or delivery in the previous 6 months, hypercholesterolemia, diabetes, current medication for birth control or hormone replacement therapy, and recent use of aspirin, ticlopidine, clopidogrel, anti-inflammatory drugs, vitamin supplements, or anticoagulant agents. Among the 48 patients, 21 (44%) had clinical evidence of vascular disease, in particular, angina pectoris (n = 4), myocardial infarction (n = 3), transient ischemic attack (n = 2), stroke (n = 1), and peripheral artery disease (n = 2). Nine patients had suffered deep venous thrombosis or pulmonary embolism. Patient characteristics are summarized in Table 1.

Subjects were studied as outpatients after a 12-h fast. Blood samples were obtained in the morning. Informed consent was obtained from each subject after approval of the protocol by the local institutional ethics committee.

Analysis of the MTHFR C677T mutation was carried out by digestion with the restriction enzyme *Hinf* I. The section of the gene containing the mutation was amplified by polymerase chain reaction as described previously (19).

Fasting plasma total Hcy (the sum of free and protein-bound forms plus cysteine-homocysteine mixed disulfide) was measured in 51 ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood samples immediately refrigerated to prevent in vitro total Hcy formation. Plasma was stored at −80°C. The total Hcy was measured using the Imx Hcy assay (Abbott Park, Illinois). Plasma sCD40L and sVCAM-1 levels were measured using specific immunoassays (R & D Systems) according to the instructions of the manufacturer. Intra-assay and inter-assay coefficients of variation were <6%.

Abbreviations and Acronyms

BSA = bovine serum albumin
CD40L = CD40 ligand
EDTA = ethylenediaminetetraacetic acid
Hcy = homocysteine
HEPES = N-2 hydroxyethyl piperazine-N 1-2-ethanesulfonic acid
HHcy = hyperhomocysteinemia
HUVECs = human umbilical vein endothelial cells
Ig = immunoglobulin
MFI = mean fluorescence intensity
MTHFR = 5,10 methylene tetrahydrofolate reductase
NF = nuclear factor
PBS = phosphate-buffered saline
s = soluble
VCAM = vascular cell adhesion molecule

Cells. Human umbilical vein endothelial cells were isolated from umbilical cords obtained from randomly selected healthy mothers delivering at the Chieti University Hospital, using 0.1% collagenase at 37°C. Cells were grown on 1.5% gelatin-coated plates in medium Dulbecco's modified Eagle's medium (D-MEM)/M-199 (50:50) supplemented with 20% heat-inactivated fetal calf serum, 10 μg/ml heparin, 50 μg/ml endothelial cell growth factor (ECGF), 50 mg/ml penicillin/streptomycin in 5% CO_2 at 37°C, and used within 4 passages. Before treatment, HUVECs were made quiescent with D-MEM/M-199 (50:50) medium supplemented with 1% bovine serum albumin (BSA) and 50 μg/ml ECGF for 20 h. Homocysteine was delivered to cells in D-MEM/F12 (50:50) with 2% fetal calf serum and ECGF.

For platelet isolation, blood was collected from healthy volunteers who had not received any medication for at least 2 weeks. Platelet-rich plasma was prepared by centrifugation at 200*g* for 15 min. Platelets were isolated by centrifugation at 1,100*g* for 15 min, after addition to platelet-rich plasma of 1 μmol/l PGE_1. The pellet was suspended in HEPES-tyrode containing 1 μmol/l PGE_1 and 5 mmol/l EGTA and centrifuged at 1,100*g* for 10 min. Platelets were suspended with HEPES-tyrode buffer containing 1 mmol/l Ca^{2+} at the concentration of 1×10^8/ml.

Real-time polymerase chain reaction. Total cellular RNA was extracted using the SV total RNA Isolation System (Promega). Polyadenosine RNA was reverse-transcribed for 60 min at 42°C with StrataScript II (50 U/ml) (Stratagene, Milan, Italy). Real-time measurements of CD40 were carried out using the Assay-on-Demand Hs00374176 from Applied Biosystems, in the ABI PRISM 7900 HT apparatus, according to the instructions of the manufacturer.