CD40 ligand increases expression of its receptor CD40 in human coronary artery endothelial cells

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**Background.** Recently, CD40 ligand (CD40L) and its receptor CD40 have been implicated in atherosclerosis. Clinical data showed that elevated CD40L levels are associated with a high risk of cardiovascular events. The aim of this study was to investigate whether CD40L could affect the expression of its membrane receptor CD40 as a feedback mechanism by which CD40L could enhance its functions in human coronary artery endothelial cells (HCAECs).

**Methods.** The HCAECs were treated with human soluble CD40L, and the messenger RNA (mRNA) and protein levels of CD40 were determined by real-time polymerase chain reaction and Western blot analysis, respectively. The specific effect of CD40L was confirmed by a blocking experiment with antibody against CD40L. Involvements of oxidative stress and mitogen-activated protein kinases (MAPKs) were also studied with antioxidant seleno-L-methionine (SeMet) and MAPK inhibitors such as extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor.

**Results.** When HCAECs were cultured with CD40L (5 µg/mL) for 24 hours, CD40 mRNA levels were increased by 79% compared with controls (P < .05). Similarly, Western blot analysis showed an 80% increase in CD40 protein levels (P < .05). The CD40L-induced increase in CD40 mRNA levels were blocked specifically by anti-CD40L antibody. Antioxidant SeMet and specific ERK1/2 inhibitor (PD98059) also effectively blocked CD40L-induced CD40 mRNA increase.

**Conclusions.** These data demonstrate that clinically relevant concentration of CD40L increased the expression of its receptor CD40 in HCAECs. The CD40L-induced upregulation of CD40 may be mediated by oxidative stress and ERK1/2 activation. This study suggests a new mechanism by which CD40L could enhance its biologic functions in the vascular system and contribute to endothelial dysfunction and vascular disease. (Surgery 2006;140:236-42.)

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CD40 ligand (CD40L) is a transmembrane protein of the tumor necrosis factor family that was originally identified on cells of the immune system. The role of CD40L in the immune response involves induction of B-cell proliferation, forma-
The CD40L membrane receptor CD40 could be increased with stimulation of proinflammatory cytokines at the transcriptional level in lymphocytes and other types of cells. In endothelial cells, CD40L can induce the expression of cellular adhesion molecules including vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin. It can also inhibit endothelial cell migration by increasing production of endothelial reactive oxygen species (ROS), and induce human endothelial cell chemokine production and migration of leukocyte subsets. All these data suggest the role of the interaction between CD40L and CD40 in the pathogenesis of atherosclerosis. However, the direct effect of CD40L on its receptor CD40 expression has not been studied. It is not clear whether oxidative stress and mitogen-activated protein kinases (MAPKs) are involved in the effects of CD40L on endothelial cells.

The objective of this study was to investigate whether CD40L could regulate the expression of CD40 in endothelial cells as a positive feedback mechanism by which CD40L could enhance its functions. Furthermore, we determined whether antioxidants such as seleno-L-methionine (SeMet) and specific MAPK inhibitors such as extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor could block the CD40L-induced effects in human endothelial cells. This study may provide new insights into the mechanisms of CD40L interacting with endothelial cells and its potential roles in cardiovascular disease.

MATERIALS AND METHODS

Chemicals and reagents. Recombinant human soluble CD40L was obtained from PeproTech (Rocky Hill, NJ). The endotoxin level in CD40L is less than 0.1 ng/μg (1EU/μg). TriReagent kit, trypsin/EDTA, SeMet, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), rabbit IgG1 (isotype control immunoglobulin), and rabbit IgG (isotype control immunoglobulin), and E-selectin. It can also inhibit endothelial cell migration by increasing production of endothelial reactive oxygen species (ROS), and induce human endothelial cell chemokine production and migration of leukocyte subsets. All these data suggest the role of the interaction between CD40L and CD40 in the pathogenesis of atherosclerosis. However, the direct effect of CD40L on its receptor CD40 expression has not been studied. It is not clear whether oxidative stress and mitogen-activated protein kinases (MAPKs) are involved in the effects of CD40L on endothelial cells.

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Cell culture. The HCAECs and microvascular endothelial growth medium-2 (EGM-2 MV) were purchased from Cambrex (Walkersville, Md). Cells were used at passage 6 to 8. When HCAECs grew to 80% to 90% confluence in 6-well plates, they were divided into 4 groups. Group I cells were treated with clinically relevant concentration of CD40L (5 μg/mL) for 24 hours. Group II cells were pretreated with antibodies against CD40L (0.5, 2, or 5 μg/mL) or isotype control antibody (rabbit IgG, 5 μg/mL) for 30 minutes before adding CD40L (5 μg/mL) for 24 hours. Group III cells were cocultured with antioxidant SeMet (20 μmol/L) and CD40L (5 μg/mL) for 24 hours. Group IV cells were pretreated with ERK1/2 inhibitor (PD98059, 40 μmol/L) for 1 hour and then cocultured with CD40L (5 μg/mL) for 24 hours. In all groups, endothelial basal medium plus 0.5% fetal bovine serum without additional growth factor was used for the groups of treated cells. Cells cultured in the same medium alone were used as controls.

The HCAECs were cultured in 6-well plates. We initially seeded cells at a density of 5000 cells/cm², which equals 5 × 10⁴ cells per well. Cells were grown in EGM-2 MV for 3 to 4 days until they reached 80% to 90% confluence. At that point, the cell number reached 2 × 10⁶ cells per well. There were no changes in cell viability as examined by microscope observation.

Real-time polymerase chain reaction. Total RNA from HCAECs was isolated with the use of the TriReagent kit according to the manufacturer’s directions. Complementary DNA (cDNA) was generated by reverse transcription (RT) from messenger RNA (mRNA) with the use of the iScript cDNA Synthesis Kit. The iQ SYBR Green SuperMix Kit was used for real-time polymerase chain reaction (PCR) reaction. A master mixture was used to reduce variability in primer and reagent concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control to account for variations in sample loading. Human CD40 and GAPDH primers were designed by Beacon Designer (Bio-Rad Laboratories; Table I) and synthesized by Sigma Genosys (Woodland, Tex). Real-time PCR was performed in an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). The thermal cycle condition used for RT was as follows: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. The condition used for real-time PCR was as follows: 3 minutes at 95°C, 40 repeats of 20 seconds at 95°C, and 1 minute at 60°C. Controls were performed with no RT (mRNA sample only) or no mRNA (water only) to demonstrate the specificity of the primers and the lack of DNA contamination in samples.

Western blot analysis. Total protein was isolated from the HCAECs with the use of the TriReagent
kit and resuspended in 20 μL of 10 mol/L urea. Protein concentration was determined with the use of the Bradford protein assay with bovine serum albumin as the standard. The same amount of endothelial proteins (6 μg) was resolved electrophoretically by 1-dimensional SDS-PAGE (10% polyacrylamide) for approximately 1 hour at 150 V. Subsequently, the gel was equilibrated in transfer buffer (Bio-Rad Laboratories), and the proteins were electrophoretically transferred to the nitrocellulose filter for 2 hours at 100 V. The filter was blocked with the use of 5% nonfat dried milk in TRIS buffer saline (TBS) with 0.05% Tween 20 (TBST) for 1 hour at room temperature. CD40 was detected with the use of a polyclonal rabbit anti-CD40 antibody diluted 1:1000 and monoclonal antibody diluted 1:10000. The CD40 and β-actin primary antibodies were detected with horseradish peroxidase–conjugated IgG secondary antibodies diluted 1:5000. Blots were developed with the use of the ECL-plus kit and analyzed with gel documentation system and analysis software (Alpha Innotech Co, San Leandro, Calif).

Statistical analysis. Data from real-time PCR and Western blot analysis between the treated and control groups were compared with the use of the paired Student t test (2-tailed). Significance was considered for \( P < .05 \). Data were reported as mean ± SEM.

RESULTS

CD40L increases the expression of CD40 in HCAECs. The HCAECs were treated with 5 μg/mL of CD40L for 24 hours. CD40 mRNA and protein levels were detected with the use of real-time PCR (\( n = 3 \)) and Western blot analysis (\( n = 3 \)), respectively. We did not see any change in cell morphology and viability before and after treatment. After treatment, CD40 mRNA levels were increased significantly from 0.0017 in controls to 0.0030 in CD40L-treated groups (79%, \( P < .05, t \) test). Western blot analysis also showed an 80% increase in CD40 compared with controls (\( P < .05, t \) test, Fig 1). To determine the specific effect of CD40L on CD40 expression, we treated HCAECs with CD40L (5 μg/mL) and anti-CD40L antibody (0.5, 2, or 5 μg/mL), or isotype IgG (rabbit IgG1 immunoglobulin, 5 μg/mL) for 24 hours. Anti-CD40L antibody at 5 μg/mL concentration reversed the CD40L-induced increase in CD40 mRNA (\( P < .05, t \) test, Fig 2). Isotype control at a similar concentration showed no effect on CD40L-induced CD40 mRNA increase (Fig 3). These data confirmed that the blocking effect of CD40L was not caused by nonspecific binding between CD40L and other immunoglobulin.

Antioxidant SeMet reverses CD40L-induced CD40 upregulation in HCAECs. To investigate whether oxidative stress could play a role in CD40L-induced upregulation of CD40, we included antioxidant SeMet in the experiments. The HCAECs were cocultured with SeMet (20 μmol/L) and CD40L (5 μg/mL) for 24 hours; CD40 mRNA levels were determined by using real-time PCR. The CD40L-induced increase in CD40 mRNA was significantly blocked by SeMet compared with the CD40L-alone group (\( P < .05, t \) test, Fig 3). Thus, CD40L could upregulate CD40 expression by a mechanism related to oxidative stress in HCAECs.

ERK1/2 inhibitor effectively blocks CD40L-induced CD40 mRNA increase. Since CD40L has been shown to induce activation of MAPK ERK1/2 in activated dendritic cells,12 we investigated, in a current study, whether ERK1/2 could be involved...

Table I. Sequence details of individual pairs of primers

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<thead>
<tr>
<th>Gene</th>
<th>GenBank No.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CD40</td>
<td>NM_001250</td>
<td>5'-CCCTGTCCTCACCTGCGATGG-3'</td>
<td>5'-CATGCAGTGATTCTTGAG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AF017079</td>
<td>5’-TGTACCCAAACTGCTTGCC-3’</td>
<td>5’-GGCATGACTGTTGTCATGAG-3’</td>
</tr>
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GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.
in CD40L-induced upregulation of CD40 in HCAECs. Activation of ERK1/2 is also sensitive to oxidative stress, which was observed in the effect of CD40L in HCAECs. We included specific ERK1/2 inhibitor PD98059 in the experiments. The HCAECs were pretreated with PD98059 (40 µM) for 1 hour, followed by coculture with CD40L (5 µg/mL) for 24 hours. PD98059 effectively reversed CD40L-induced CD40 mRNA increase (*P < .05, n = 3, t test). Isotype control and other concentrations of CD40L antibody showed no effects on CD40 mRNA levels. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA.

**DISCUSSION**

In the current study, we have tested our hypothesis that CD40L may upregulate expression of its receptor CD40, thereby enhancing its biologic functions in human endothelial cells. Our findings demonstrate that recombinant human soluble CD40L at the clinically relevant concentration induces overexpression of CD40 in HCAECs. This overexpression is confirmed at both mRNA and protein levels. In addition, an antioxidant and a specific inhibitor of ERK1/2 effectively block this CD40L-induced upregulation of CD40, suggesting the potential molecular mechanisms are involved in oxidative stress and the ERK1/2 signal transduction pathway. Interaction between CD40L and CD40 may contribute to the development of cardiovascular disease.

Since the first report in 1984, CD40L/CD40 interaction has gained increasing interest. CD40 was
originally discovered in immunohistochemistry when an antibody was used to detect a 50-kDa protein on the B-cell surface.13 Further studies showed B-lymphocyte differentiation and survival are closely related to the ligation of CD40L. Recent studies have shown the link between elevated plasma level of CD40L and cardiovascular events.4 However, the underlying mechanisms of CD40L-induced endothelial dysfunction are still unclear. In the current study, new findings indicated that CD40L at the concentrations relevant to the plasma levels of patients with high cardiovascular risk can induce overexpression of CD40 in HCAECs. As a consequence of CD40L binding to its receptor CD40, several inflammatory processes could be initiated.3 The induction of a series of cell adhesion molecules including VCAM-1, ICAM-1, and E-selectin,14-16 was among the first biologic consequence reported for the CD40L/CD40 interaction. Besides, CD40 can also mediate induction of chemotactic mediators such as interleukin 8 and monocyte chemotactic protein-1 in endothelial cells,5 mediate expression of extracellular matrix–degrading activity referred to the enhanced expression of matrix metalloproteinases in monocytes,17,18 and induce procoagulant activities on human vascular cells.19 All of these CD40L-mediated vascular activities suggest that CD40L may play a very important role in the progression of atherosclerosis and other vascular diseases. CD40L-induced upregulation of CD40 could enhance its biologic functions in the vascular system.

Reactive oxygen species are the key mediators for vascular inflammation and atherogenesis.20,21 This observation is further supported by the observed impairment of vascular functions and enhanced atherogenesis in animal models that have deficiencies in internal antioxidant enzymes.22 To confirm whether ROS is involved in the action of CD40L in HCAECs, we demonstrated that antioxidant SeMet could effectively block CD40L-induced overexpression of CD40. SeMet is the major component of dietary selenium, for which the recommended daily allowance by the US Food and Drug Administration is 50 µg per day. SeMet has potent antioxidative effects by the mechanism of intramolecular transsulfuration reaction to form selenocysteine, which increases activities of internal antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase.23 Recently, we have demonstrated that SeMet can effectively block lysophosphatidylcholine-induced oxidative stress and endothelial dysfunction in porcine coronary arteries.24 Thus, CD40L-induced oxidative stress may be one of the molecular mechanisms of CD40L-induced endothelial dysfunction. Accordingly, antioxidant therapy may become an effective strategy to control CD40L-involved vascular dysfunction and disease.

In this study, we did not measure ROS levels directly in cultured cells. CD40L can induce overproduction of ROS in human umbilical vein endothelial cells detected by staining with the dye H2DCFDA.10 The same study also showed that the antioxidant vitamin C prevented both the CD40L-induced increase of ROS. How CD40L regulates ROS production, however, is not clear. It could activate ROS-generating enzymes such as reduced nicotinamide adenine dinucleotide phosphate oxidase or downregulate intracellular levels of antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase. Mitochondrial functions also contribute to the ROS production. Future studies on sources of ROS production in CD40L-treated cells are warranted.

MAPKs are important signal transduction molecules that regulate cell growth, migration, and differentiation in response to various extracellular stimuli.25 Three major subfamilies of structurally related MAPKs have been identified in mammalian cells, which are termed p44/42 MAPK, ERK1/2, p38 MAPK, and Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs). Many MAPK pathways are still unknown. For instance, the MAPK pathways that couple endothelial activation from receptor levels to the expression of several key genes such as CD40 are largely unclear. In the present study, we demonstrated that ERK1/2 inhibitor PD98059 effectively blocked CD40L-induced upregulation of CD40 in HCAECs, indicating ERK1/2 is involved in the signal transduction pathway in the interaction between CD40L and CD40 in human endothelial cells.

Although our data indicate that ERK1/2 activation may be involved in CD40L-induced upregulation of CD40 in endothelial cells, we did not measure the levels of phosphorylated ERK1/2 and other types of MAPKs in the HCAECs after CD40L treatment. Future investigation of these MAPK pathways is warranted and may provide a better understanding of the mechanisms of CD40L actions effect.

Regulation of CD40 expression is complicated. In this study, we observed that the antioxidant SeMet decreased CD40 mRNA levels, whereas ERK1/2 inhibitor did not affect CD40 mRNA levels in endothelial cells without CD40L treatment. These interesting findings indicate that the cellular ROS level is critical to maintain or regulate CD40 expression. Low levels of cellular ROS could de-
increase CD40 expression. The MAPKs are important signal transduction molecules in response to many chemical and environmental stimuli. Without any stimulation, these MAPKs normally remain in the inactivated (unphosphorylated) state. Thus, it is not surprising that, in this study, ERK1/2 inhibitor did not affect expression of CD40 mRNA in the cells without CD40L treatment, possibly because of the inactivated state of ERK1/2. Although MAPKs are sensitive to cellular levels of ROS, how ROS regulate MAPK activity is largely unknown.

The significance of CD40L-induced upregulation of its receptor CD40 in endothelial cells is not well understood yet. We speculate that it could be a positive feedback mechanism by which CD40L could enhance its actions in endothelial cells such as upregulation of cell adhesion molecules and downregulation of endothelial nitric oxide synthase. These effects may depend on the CD40L-CD40 interaction. On the other hand, CD40 may have many other functions that are independent of the CD40L-CD40 interaction. Thus, CD40L-increased levels of CD40 could enhance both functions of CD40L, and/or CD40 in endothelial cells.

In this study, we chose one concentration and one incubation time of CD40L treatment on the basis of recent reports in the literature. In HUVEC, CD40L at 5 μg/mL can inhibit VEGF-induced endothelial cell migration and angiogenesis after 24 hours. This effect appears to involve the generation of ROS. The concentration of CD40L (5 μg/mL) used in this study is relevant to clinical plasma concentrations in patients with aggressive vascular diseases. In this study, we did not test multiple concentrations and incubation times mainly because of the high cost of commercially available CD40L, which could be a limitation of this study.

In summary, our study demonstrates that a clinically relevant concentration of CD40L increases expression of its receptor CD40 at both mRNA and protein levels. CD40L-induced oxidative stress and ERK1/2 activation may be the underlying mechanisms. These data support the hypothesis that CD40L may have new mechanisms to enhance its biologic effects in the vascular system through upregulation of its cellular receptor. Antioxidant therapy or blocking of the signal transduction pathways of CD40L-induced endothelial dysfunction may become effective strategies to control vascular disease that is associated with inflammatory mediators including CD40L.

REFERENCES
19. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to


