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CD40 Ligand Inhibits Endothelial Cell Migration by Increasing Production of Endothelial Reactive Oxygen Species

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Background—The CD40/CD40 ligand system is involved in atherogenesis. Activated T lymphocytes and platelets, which express high amounts of CD40 ligand (CD40L) on their surface, contribute significantly to plaque instability with ensuing thrombus formation, leading to acute coronary syndromes. Because reendothelialization may play a pivotal role for plaque stabilization, we investigated a potential role of CD40L on endothelial cell (EC) migration.

Methods and Results—Stimulation of ECs with recombinant CD40L prevented vascular endothelial growth factor (VEGF)-induced EC migration, as determined by a “scratched wound assay.” In addition, activated T lymphocytes and platelets significantly inhibited VEGF-induced EC migration and tube formation *in vitro*. Because the activation of endothelial nitric oxide (NO) synthase and the release of NO are required for EC migration and angiogenesis, we analyzed the effect of NO. Coincubation with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) did not reverse the inhibitory effect of CD40L on VEGF-induced EC migration and tube formation. In addition, EC migration induced by SNAP was completely inhibited by CD40L. CD40L, however, induced the production of reactive oxygen species and reduced endothelial NO bioavailability. This reactive oxygen species-dependent effect of CD40L stimulation was reversed with vitamin C or N-acetylcysteine.

Conclusions—The activation of the CD40 receptor inhibits EC migration by increasing reactive oxygen species. The blockade of EC migration by CD40L may critically affect endothelial regeneration after plaque erosion and thereby may contribute to the increased risk for development of acute coronary events in patients with high circulating levels of CD40L. (*Circulation*. 2002;106:981-986.)

Key Words: migration ■ ligands ■ endothelium ■ nitric oxide ■ reactive oxygen species

The CD40 receptor (CD40) and CD40 ligand (CD40L/CD154) were originally found to be expressed on B and T lymphocytes and to be involved in T cell-dependent B-cell differentiation and activation.^{1,2} However, recent data indicate that the CD40/CD40L system plays an important role not only in cellular immunity and inflammation but also in the pathophysiology of atherosclerosis.¹ The CD40/CD40L system was shown to be expressed in nonhematopoietic cells, including endothelial cells (ECs), fibroblasts, and smooth muscle cells.³ Ligation of CD40 on these cell types induces a proinflammatory and prothrombotic response, as evidenced by the release of inflammatory cytokines, expression of adhesion molecules, activation of matrix metalloproteinases (MMPs), and procoagulant tissue factor (reviewed in Reference 2). Blockade of CD40L by neutralizing antibodies or genetic disruption of the CD40 ligand in mice prevents the initiation and progression of atherosclerosis and induces a more stable plaque phenotype.⁴⁻⁷

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The clinical sequela of acute coronary syndromes (ACSs) is a result of either plaque rupture or plaque erosion with

concomitant thrombus formation. Activated T lymphocytes and platelets, which express high amounts of CD40L on their surface, contribute significantly to plaque instability with ensuing thrombus formation.² In accordance with this, patients with ACSs exhibit enhanced blood levels of soluble and membrane-bound CD40L.⁸ Moreover, high levels of soluble CD40L were associated with increased cardiovascular events in women,⁹ suggesting that CD40 activation may be involved in the development of ACS.

Endothelial injury and denudation of the endothelial monolayer caused by spontaneous plaque erosion or coronary angioplasty contribute to acute thrombotic events and further progression of the atherosclerotic lesion. The role of CD40/CD40L on reendothelialization is not known. Therefore, we investigated the effect of CD40L on EC migration using an *in vitro* “scratched wound assay.” Recombinant CD40L completely blocked vascular endothelial growth factor (VEGF)-induced EC migration. EC migration was also inhibited by activated platelets or T cells, the biological sources of CD40L. We further elucidated the underlying signaling pathways and demonstrate that CD40L stimulated the pro-

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duction of reactive oxygen species (ROSs) by ECs, which antagonizes endothelial nitric oxide (NO) production. Because EC migration also contributes to angiogenesis, we investigated the effect of CD40L in a human angiogenesis assay and demonstrated a potent inhibition by CD40L.

Methods

Cell Culture

Pooled human umbilical vein ECs (HUVECs) were purchased from Cell Systems/Clonetics and cultured until the third passage as previously described.¹⁰ CD40 ligand was kindly donated by Immunex (Seattle, Wash) or purchased from Alexis. VEGF was purchased from Calbiochem, S-nitroso-N-acetyl-penicillamine (SNAP) from Alexis, vitamin C from Sigma, N-acetylcysteine (NAC) from Sigma, and anti-CD40L antibody from Calbiochem. HUVECs were exposed to laminar fluid flow (15 dynes/cm²) in a cone-and-plate apparatus as previously described.¹⁰

Scratched Wound Assay

In vitro scratched wounds were created by scraping the cell monolayer with a sterile disposable cell scraper.¹¹ After injury of the monolayer, the cells were gently washed and stimulated. EC migration from the edge of the injured monolayer was quantified by measurement of the distance between the wound edges before and after injury with a computer-assisted microscope (Zeiss) at 5 distinct positions (every 5 mm).

Platelet Preparation and Activation

Platelets were obtained from healthy human subjects as previously described.¹⁰ After resuspension, 1.5×10^8 platelets were incubated with HUVECs and were activated with 0.2 U/mL human thrombin (Boehringer Mannheim). Cell culture dishes were centrifuged at 700g for 2 minutes, and thrombin was neutralized after 5 minutes with 2 U/mL hirudin (Boehringer Mannheim).

T Cell Isolation and Activation

Human mononuclear cells were obtained from the blood of healthy human volunteers by density gradient centrifugation using Biocoll separating solution (density 1.077; Biochrom). CD3⁺-positive T cells were purified from mononuclear cells by positive selection with anti-CD3 microbeads (Miltenyi Biotec) with a magnetic cell sorter device (Miltenyi Biotec). Purity assessed by fluorescence-activated cell sorter (FACS) analysis was >95%.

DAF-2 DA and H₂DCFDA Staining

Cells were stained with the membrane-permeable 4,5-diaminofluorescein diacetate (DAF-2 DA, 10 μmol/L, Calbiochem) or dichlorodihydrofluorescein diacetate (H₂DCFDA; 10 μmol/L; Molecular Probes). The amount of NO or ROSs generated was determined by computer-assisted fluorescence densitometric analysis.^{11,12} For FACS analysis, cells were incubated with DAF-2 DA (10 μmol/L, 3 hours) or DCFDA (10 μmol/L, 30 minutes), washed, and detached with trypsin.¹³ After centrifugation, NO or ROS generation was immediately determined by FACS analysis using a FACScan flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences).

Angiogenesis Assays

The human angiogenesis assay was performed according to the instructions of the manufacturer (Cell Systems/Clonetics). Briefly, human ECs were cocultured with human fibroblasts in a culture matrix and stimulated over a period of 11 days. Cells were fixed and stained with an anti-CD31 antibody and a secondary antibody conjugated with alkaline phosphatase and BCIP/NBT as substrate. Angiogenesis was quantified by measuring tube length with a computer-assisted microscope.

In addition, the formation of tube-like structures was determined with a matrigel assay. HUVECs (1×10^5 cells/mL) were seeded in

endothelial basal medium supplemented with growth factors and 10% FCS on Matrigel basement membrane matrix (BD Biosciences). To investigate the effect of CD40L on VEGF-induced tube formation, HUVECs were cultured in endothelial basal medium supplemented with growth factors and 5% FCS on growth factor-reduced Matrigel (BD Biosciences). The length of tube-like structures was measured by light microscopy after 48 hours in a blinded fashion.

Statistical Analysis

Data are expressed as mean ± SEM from ≥3 independent experiments. Statistical analysis was performed by *t* test. ANOVA was performed for serial analyses.

Results

Recombinant CD40L Inhibits EC Migration

To investigate the effect of CD40L on EC migration, HUVECs were incubated with VEGF (50 ng/mL) in the presence or absence of CD40L, and migration was assessed by a scratched wound assay.¹¹ Incubation of HUVECs with human recombinant CD40L dose-dependently inhibited VEGF-induced cell migration (Figure 1A). The inhibitory effect of CD40L was not caused by an increase in EC death, because 0.1 to 5 μg/mL CD40L did not induce EC apoptosis (5 μg/mL CD40L: 112 ± 6% annexin-positive cells compared with control, n=4). Moreover, EC proliferation was not affected by CD40L addition (control: 0.19 ± 0.07 versus CD40L: 0.18 ± 0.08 bromodeoxyuridine incorporation [optical density], n=3). To test whether CD40L generally interferes with EC migration, we used shear stress as an additional important physiological stimulus for EC migration.¹⁴ Interestingly, CD40L did not affect shear stress-induced EC migration (shear stress: 184 ± 14% of control versus shear stress+CD40L: 177 ± 5% of control, n=4). Likewise, basic fibroblast growth factor (bFGF)-stimulated cell migration was not inhibited by CD40L (bFGF: 158 ± 15% of control versus bFGF+CD40L: 159 ± 14% of control, n=4).

Activated T Cells and Platelets Inhibited VEGF-Induced EC Migration

Activated platelets and T cells are the major physiological source for release of CD40L under pathophysiological conditions.^{15,16} Therefore, T cells or platelets were used as a stimulus for CD40 activation in ECs. Platelets were added to the ECs and were activated by thrombin treatment for 5 minutes, which is known to trigger CD40L release. As shown in Figure 1B, VEGF-induced EC migration was significantly abrogated by the addition of activated platelets, whereas unstimulated platelets did not show any effect. Importantly, the inhibition of VEGF-induced EC migration by activated platelets was reversed by neutralizing antibodies directed against CD40L. Control experiments demonstrated that thrombin or hirudin, used to inactivate thrombin, had no effect on EC migration in the absence of platelets (Figure 1B).

Similar results were obtained when phorbol 12-myristate 13-acetate (PMA)-activated CD3⁺-positive T cells were used, which express CD40L on the cell surface. Again, activated T cells significantly inhibited VEGF-induced EC migration (Figure 1C). The inhibitory effect induced by activated T cells was partially dependent on CD40L (Figure 1C).

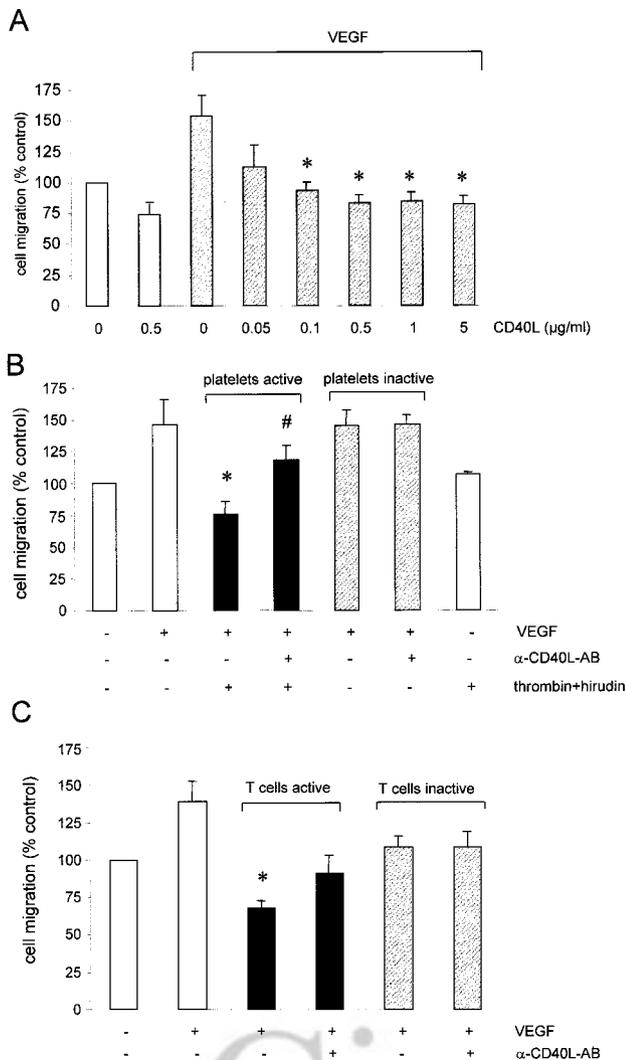


Figure 1. Recombinant CD40L, activated platelets, and CD3⁺ positive T cells inhibit VEGF-induced EC migration. **A**, HUVECs were incubated with VEGF (50 ng/mL) and recombinant CD40L for 24 hours, and cell migration was detected by use of a scratched wound assay. Data are mean ± SEM; n=4; **P*<0.05 vs VEGF. **B**, HUVECs were scraped and incubated with VEGF (50 ng/mL) and anti-CD40L antibody (10 µg/mL). Then 1.5 × 10⁸ platelets were added to HUVECs and activated with 0.2 U/mL human thrombin (Boehringer Mannheim). Cell culture dishes were centrifuged at 700g for 2 minutes, and thrombin was neutralized after 5 minutes with 2 U/mL hirudin (Boehringer Mannheim). Cell migration was detected after 24 hours. Data are mean ± SEM; n=5; **P*<0.05 vs VEGF; #*P*<0.05 vs VEGF+activated platelets. **C**, CD3⁺ positive T cells were activated for 5 hours at 37°C with PMA (5 ng/mL) and washed twice with PBS to remove PMA. Activated T cells were incubated as indicated with anti-CD40L antibody (10 µg/mL) for 30 minutes and added to scratched HUVECs. Cell migration was detected after 24 hours. Data are mean ± SEM; n=5; **P*<0.05 vs VEGF.

In contrast, addition of unstimulated T cells resulted in a minor reduction of EC migration, which was not affected by neutralizing CD40L antibodies (Figure 1C). Thus, CD40 stimulation, induced by either human recombinant CD40L or activated platelets and T cells, inhibits VEGF-induced EC migration.

CD40L Reduces NO and Increases Oxidative Stress

Endothelial NO is critical for VEGF-induced EC migration and angiogenesis.^{11,17} Therefore, we investigated the effect of CD40L on endothelial NO production by using the fluorescence dye DAF-2 DA.¹¹ CD40L reduced basal endothelial NO to an extent similar to that with the NOS inhibitor *N*^G-monomethyl-L-arginine, as assessed by immunofluorescence and FACS analysis (Figure 2, A and B). Furthermore, CD40L prevented VEGF-stimulated increase of NO (data not shown).

NO bioavailability can be reduced by ROS, which can react with NO.¹⁸ Because CD40L increases ROS formation in B cells,¹⁹ we determined ROS formation in ECs in response to CD40L. CD40L time-dependently increased formation of endogenous ROS with maximum levels after 6 hours, as determined by immunofluorescence and FACS analysis using H₂DCFDA (Figure 2, C and D, and data not shown). The levels of ROSs detected after CD40L stimulation are about half of that measured after addition of 500 µmol/L exogenous H₂O₂ for 15 minutes (Figure 2, C and D). The antioxidant vitamin C prevented both the increase in ROSs and the decrease in NO levels, suggesting that the formation of ROSs is causally involved in the reduction of NO (Figure 2).

Next, we tested the functional involvement of CD40L-mediated disturbance of NO and ROSs balance for VEGF-induced EC migration. Therefore, HUVECs were incubated with the exogenous NO-donor SNAP (20 µmol/L), which is known to stimulate EC migration.²⁰ As shown in Figure 3A, CD40L significantly inhibited SNAP-induced cell migration. In addition, SNAP did not reverse the inhibitory effect of CD40L on EC migration (Figure 3A). In contrast, the antioxidants vitamin C and NAC reversed the inhibitory effect of CD40L on VEGF-induced EC migration (Figure 3B). Moreover, H₂O₂ directly prevented EC migration (Figure 3B). These data suggest that CD40L prevents VEGF-induced EC migration by increasing oxidative stress.

Antioxidants Reverse the Inhibitory Effect of CD40 Stimulation on VEGF-Induced Tube Formation

To finally investigate whether the inhibitory effect of CD40L on VEGF-induced EC migration also affects angiogenesis, a human angiogenesis assay was used.²⁰ Cells were incubated with human recombinant VEGF (10 ng/mL) in the presence or absence of human recombinant CD40L (0.5 µg/mL). As shown in Figure 4, A and B, CD40L inhibited basal and VEGF-induced tube formation. Similar effects were detected when angiogenesis was measured in a Matrigel assay (Figure 4, C and D). The inhibition of VEGF-stimulated tube formation was reversed by coincubation of the antioxidants vitamin C or NAC (Figure 4, A and B). In contrast, the NO donor SNAP did not prevent CD40L-induced inhibition of tube formation (Figure 4, A and B).

Discussion

The data of the present study demonstrate that activation of the CD40 receptor by human recombinant CD40L inhibits basal and VEGF-induced EC migration, leading to a reduction of reendothelialization in vitro. The inhibitory effect was also achieved when activated platelets or T lymphocytes, which are well-established sources for CD40L,^{15,16} were

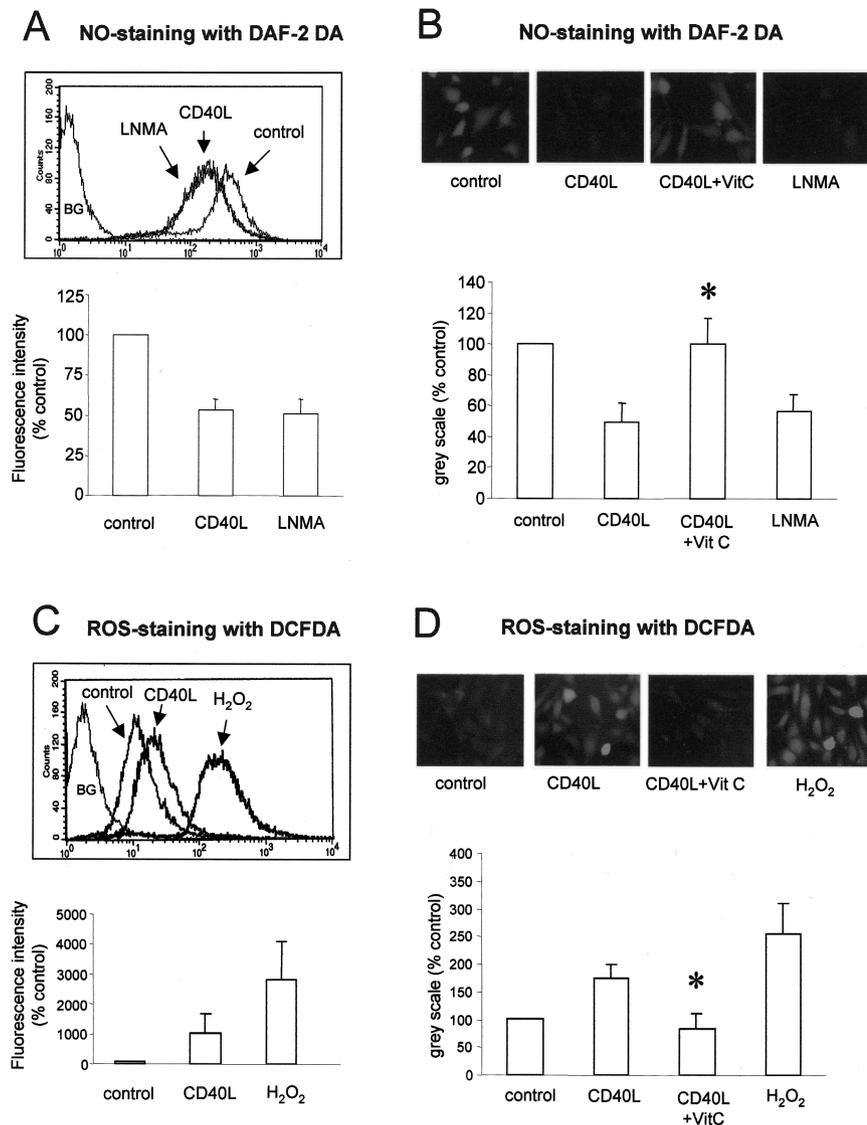


Figure 2. CD40L reduces NO and increases oxidative stress. A and B, HUVECs were incubated with recombinant CD40L (0.5 $\mu\text{g}/\text{mL}$), vitamin C (VitC; 100 $\mu\text{mol}/\text{L}$), or *N*^G-monomethyl-L-arginine (LNMA; 1 mmol/L) for 6 hours. A, Cells were stained with DAF-2 DA (10 $\mu\text{mol}/\text{L}$) for 3 hours. Amount of NO was determined by FACS analysis. Top, Representative histogram. Data are mean \pm SEM; n=4 (bottom). BG indicates background. B, Cells were stained with DAF-2 DA (10 $\mu\text{mol}/\text{L}$) for 5 minutes. Amount of NO was determined by computer-assisted fluorescence densitometric analysis. Top, Representative pictures. Data are mean \pm SEM; n=3; **P*<0.05 vs CD40L (bottom). C and D, HUVECs were incubated with recombinant CD40L (0.5 $\mu\text{g}/\text{mL}$) and vitamin C (100 $\mu\text{mol}/\text{L}$) for 6 hours. H₂O₂ (500 $\mu\text{mol}/\text{L}$; 15 minutes) was used as a positive control. C, Cells were stained with redox-sensitive dye H₂DCFDA (10 $\mu\text{mol}/\text{L}$) for 30 minutes. Amount of ROSs was determined by FACS analysis. Top, Representative histogram. Data are mean \pm SEM; n=3 (bottom). D, Cells were stained with H₂DCFDA (10 $\mu\text{mol}/\text{L}$) for 5 minutes. Amount of ROSs was determined by computer-assisted fluorescence densitometric analysis. Top, Representative pictures. Data are mean \pm SEM; n=3; **P*<0.05 vs CD40L (bottom).

coincubated with ECs. Thus, CD40L released from activated platelets or T lymphocytes at sites of plaque erosion might impair reendothelialization, thereby further aggravating thrombotic events. Indeed, previous clinical studies demonstrated that patients with unstable angina had significantly higher levels of circulating soluble CD40L.⁸ Moreover, elevated concentrations of CD40L in apparently healthy women are associated with a significantly increased risk of future cardiovascular events.⁹ Particularly in women, ACSs are more likely to be secondary to plaque erosion.²¹ EC migration is essential to reendothelialize denuded plaque areas to prevent thrombus formation. Therefore, one may speculate that endothelial regeneration might be disturbed by high levels of CD40L, which may contribute to increased risk for the development of acute coronary events. Interestingly, CD40L did not affect EC migration stimulated by laminar flow. Endothelial injury occurs predominantly in plaque surface areas with low or turbulent blood flow.²² Therefore, laminar flow may prevent the inhibitory effect of CD40L on reendothelialization and thereby protect these areas from extensive plaque erosion.¹⁰ Thus, CD40L may particularly affect the ECs within regions prone to plaque erosion.

Our data further revealed that CD40L blocks VEGF-induced angiogenesis. These data are in contrast to previous studies, which demonstrate that CD40L stimulates MMP and VEGF expression and promotes vascularization.^{23–25} The reason for these discrepancies is unclear. To rule out a potential influence by the CD40L used, we tested recombinant CD40L provided by 2 different sources. Moreover, activated T cells and platelets were also shown to prevent VEGF-induced EC migration in a manner that was at least partially prevented by neutralizing CD40 antibodies. In addition, the experimental set-up was varied to test whether the time point of incubation might have affected the inhibitory potential of CD40L. However, CD40L potently blocked VEGF-induced EC migration when added 1 hour before or after VEGF stimulation (data not shown). Previous studies demonstrated that CD40L increased MMPs, which contribute to the stimulation of cell migration and improvement of angiogenesis.^{23,26} Interestingly, under our experimental conditions (medium with 10% FCS), CD40L did not stimulate MMP3 release. In contrast, a potent 6-fold increase in MMP3 release was detected when cells were serum-depleted before

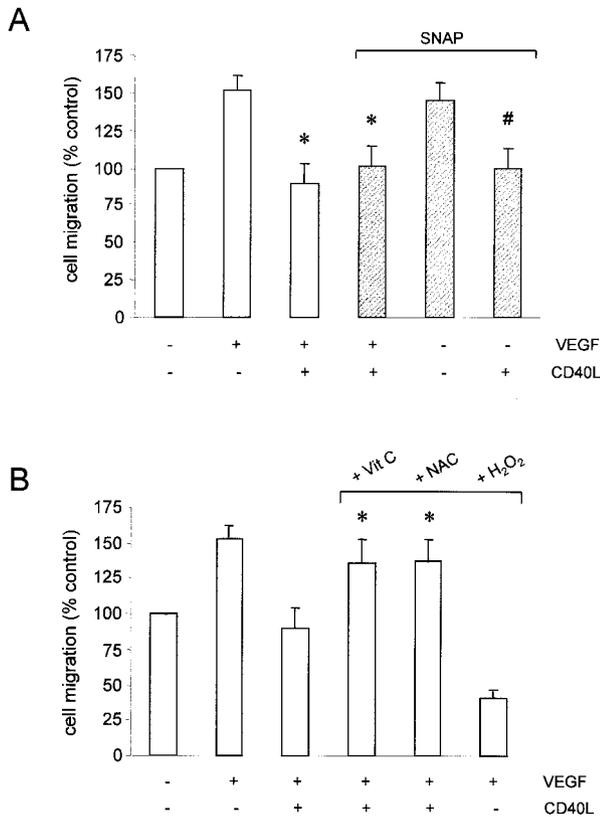


Figure 3. Antioxidants reverse the inhibitory effect of CD40 stimulation. **A**, HUVECs were incubated with VEGF (50 ng/mL), SNAP (20 μ mol/L), or CD40L (0.5 μ g/mL). Cell migration was detected after 24 hours with a scratched wound assay. Data are mean \pm SEM; $n=4$; * $P<0.05$ vs VEGF; # $P<0.05$ vs SNAP. **B**, HUVECs were incubated with VEGF (50 ng/mL), CD40L (0.5 μ g/mL), vitamin C (Vit C; 100 μ mol/L), NAC (200 μ mol/L), or H₂O₂ (200 μ mol/L) for 24 hours. Data are mean \pm SEM; $n=3$; * $P<0.05$ vs VEGF+CD40L.

and during CD40L stimulation (data not shown). This experimental set-up was used in the studies, which demonstrated a proangiogenic effect of CD40L.²³ Therefore, one may speculate about a context-dependent action of CD40L. Interestingly, similar controversial findings are reported for other cytokines, such as tumor necrosis factor- α , which elicits proangiogenic and antiangiogenic responses.²⁷

The mechanism by which CD40L inhibits VEGF-induced EC migration and angiogenesis appears to involve the generation of ROSs. Stimulation of the CD40 receptor increased intracellular formation of ROS with concomitant reduction of NO. The antimigratory and antiangiogenic effects of CD40L were reversed by antioxidants, suggesting that CD40L-induced increase in ROSs mediates the inhibitory effects of CD40L. In contrast, exogenous NO donors did not prevent CD40L-mediated inhibition. Therefore, one may speculate that the generation of ROSs is sufficient to inactivate NO and thereby prevents NO-dependent EC migration. This is consistent with previous findings that endothelial NO is essential for both VEGF-induced cell migration and angiogenesis.^{11,17} Furthermore, stimulation of CD40 has previously been shown to induce the generation of ROSs in B lymphocytes.¹⁹ Likewise, ROSs are mediators for other related receptors

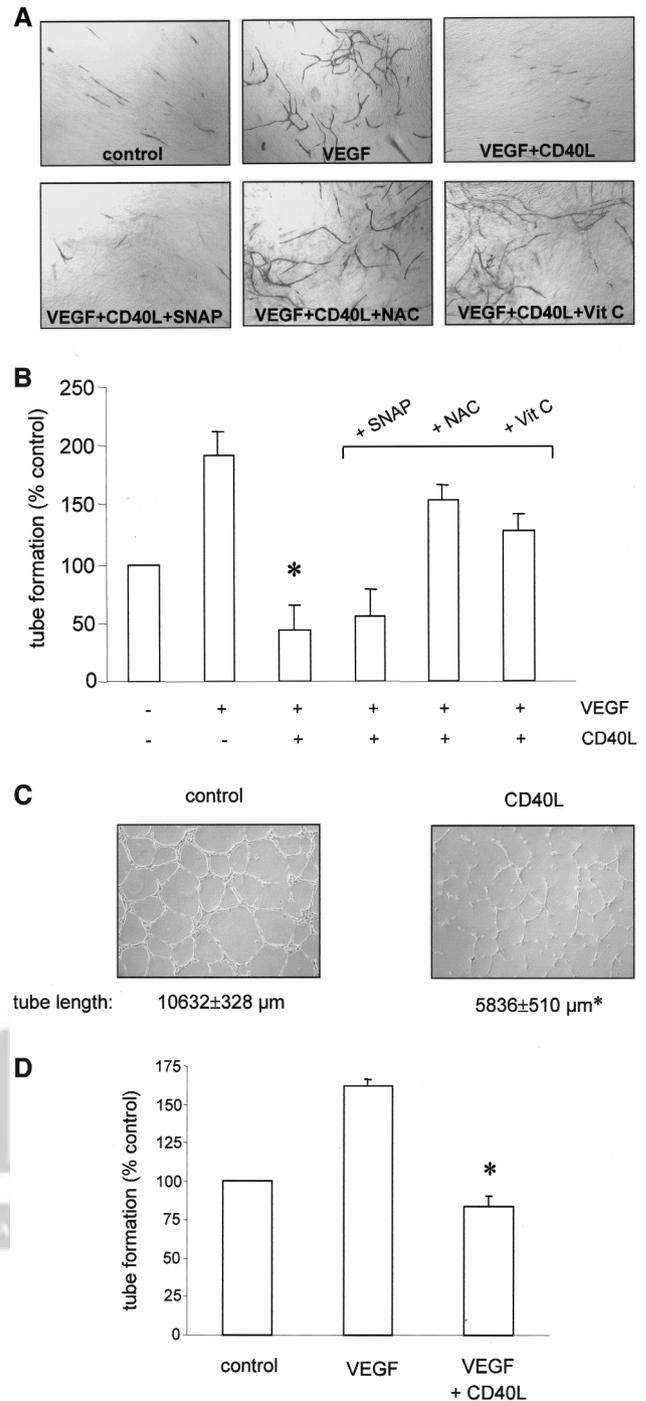


Figure 4. CD40L inhibits VEGF-induced tube formation. **A** and **B**, A human angiogenesis assay was used to measure tube formation. Cells were stimulated with VEGF (10 ng/mL) with or without CD40L (0.5 μ g/mL) in presence or absence of SNAP (20 μ mol/L), NAC (200 μ mol/L), or vitamin C (Vit C; 100 μ mol/L). **A**, Representative pictures. **B**, Angiogenesis was quantified by measuring tube length with a computer-assisted microscope. Data are mean \pm SEM; $n=3$; * $P<0.05$ vs VEGF. **C**, HUVECs were incubated with or without CD40L (0.5 μ g/mL) on matrigel basement membrane matrix, and length of tube-like structures was measured in a blinded fashion by light microscopy after 48 hours. Data are mean \pm SEM; $n=3$; * $P<0.05$ vs control. **D**, HUVECs were incubated with VEGF (50 ng/mL) in presence or absence of CD40L (0.5 μ g/mL) on growth factor-reduced matrigel, and length of tube-like structures was measured by light microscopy after 48 hours in a blinded fashion. Data are mean \pm SEM; $n=3$; * $P<0.05$ vs VEGF.

belonging to the tumor necrosis factor receptor family.^{28,29} Therefore, the CD40 receptor might activate similar intracellular pathways.

ROSs play a critical role in vascular biology. Whereas initially ROSs were expected to exert mainly toxic and proapoptotic effects, increasing evidence suggests that they also act as important physiological signaling molecules.³⁰ In line with these conflicting reports, ROSs also exert a double-edged role in angiogenesis signaling. Consistent with our data, various studies reported that ROSs inhibit EC migration and angiogenesis.³¹ However, ROSs may also be critical for EC migration,^{32,33} angiogenesis,³⁴ and cell proliferation.³³ The magnitude and the extent of ROS formation may underlie these discrepancies. Thus, a promigratory involvement of ROSs on EC migration was detected within 1 to 5 hours after wounding.³² In contrast, CD40L-stimulated ROS formation is long lasting, starting after 5 hours of incubation.

In conclusion, the present study demonstrates that activation of CD40 inhibits both EC migration and angiogenesis. The inhibitory effect of CD40L was not related to the induction of apoptosis or the blockade of cell cycle progression. The blockade of EC migration by CD40L may critically affect endothelial regeneration after injury induced by plaque erosion and thereby contribute to the increased risk for development of acute coronary events in patients with high circulating levels of CD40L.

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