Soluble CD40 Ligand Impairs the Function of Peripheral Blood Angiogenic Outgrowth Cells and Increases Neointimal Formation After Arterial Injury

Mihail Hristov, MD*; Denis Gümbel, BSc*; Esther Lutgens, MD, PhD; Alma Zernecke, MD; Christian Weber, MD

Background—Recent work has revealed an essential involvement of soluble CD40 ligand (sCD40L) in inflammation and atherosclerosis. We investigated whether sCD40L functionally affects peripheral blood–derived angiogenic early outgrowth cells (EOCs) and neointimal remodeling after arterial injury.

Methods and Results—Besides myeloid and endothelial markers, cultured human EOCs strongly expressed CD40 mRNA and protein. EOC adhesion to fibronectin, fibrinogen, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 under flow conditions, as well as their transmigration toward stromal cell–derived factor-1/H9251, was dose-dependently reduced after preincubation with recombinant human sCD40L for 24 hours. Integrin expression was unaffected by sCD40L, implying that integrin adhesiveness was attenuated. Surface-immobilized CD40L supported much lower adhesion of EOCs than fibronectin. Treatment of EOCs with sCD40L increased superoxide anion production and decreased viability and proliferation. Notably, CD40−/− mice displayed reduced neointima and improved re-endothelialization after carotid wire injury compared with wild-type mice, and therapeutic infusion of control EOCs but not EOCs pretreated with sCD40L attenuated neointimal growth after wire injury in nude mice. Furthermore, neointimal growth was more markedly diminished by infusion of spleen-derived CD40−/− mouse EOCs than by that of wild-type EOCs. Preincubation of wild-type EOCs but not CD40−/− EOCs with sCD40L before their infusion markedly aggravated neointimal formation. Treatment with sCD40L attenuated luminal incorporation of EOCs and accelerated neointimal progression.

Conclusions—Endothelial dysfunction due to persistently elevated plasma levels of sCD40L may be attributable to an impairment of EOC function. Hence, in the context of arterial injury, therapeutic blockade of sCD40L may provide a novel strategy for accelerating endothelial regeneration and attenuating neointimal remodeling.

Key Words: cardiovascular diseases ■ cell adhesion molecules ■ endothelium ■ remodeling ■ restenosis

Atherosclerosis is considered a chronic disease of the arterial wall involving inflammatory, immune, and progenitor cell–related pathophysiological mechanisms that result in atheroma formation entailing acute or chronic tissue ischemia.1–3 The CD40/ soluble CD40 ligand (sCD40L) axis represents an important costimulatory pathway in immune response and has been implicated in multiple inflammatory diseases.4,5 Hence, the role of sCD40L in the initiation, progression, and stability of atherosclerotic plaques has been examined extensively.6 Notably, the interruption of CD40/ sCD40L signaling in apolipoprotein E (ApoE)−/− and low-density lipoprotein receptor (LDLR)−/− mice was associated with a more stable plaque phenotype, as characterized by lipid-poor core, elevated collagen content, and reduced accumulation of T lymphocytes and macrophages.7–9 CD40 belongs to the superfamily of tumor necrosis factor receptor, and soluble sCD40L (also known as CD154) is a member of the tumor necrosis factor superfamily.10 Besides B cells, CD40 is expressed on various cells involved in atherogenesis, namely, endothelial cells, vascular smooth muscle cells, dendritic cells, monocytes, and macrophages.6 In general, activation of the CD40/sCD40L cascade results in secretion of proinflammatory and proatherogenic compounds.11,12

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apoptotic infusion of EOCs has been shown to be associated with endothelial healing after arterial injury and blood flow recovery in ischemic tissue. Clinically, number and functional capacity of circulating (CD133+/CD34+) vascular endothelial growth factor receptor 2 (VEGFR2)+ progenitor cells or in vitro expanded EOCs were differentially affected in diseases involving accelerated arterial remodeling (eg, coronary artery disease [CAD], diabetes mellitus, or cancer). Although some effects of sCD40L on mature endothelial cells have been reported, a potential involvement of this molecule in EOC biology remains to be clarified. Whereas disruption of CD40/sCD40L has been examined with regard to primary atherosclerotic plaque formation and destabilization, systematic evidence in regard to whether the sCD40L could also influence endothelial regeneration and neointimal growth after arterial wire injury is elusive. Hence, we were prompted to investigate in detail the effects of sCD40L on the function of cultured human EOCs and during endothelial recovery and neointimal remodeling after carotid wire injury in CD40+/− mice as well as nudes mice.

Methods

Isolation and Culture of EOCs From Peripheral Blood

According to established protocols, EOCs were isolated from citrate/dextran anticoagulated peripheral blood buffy coats of healthy volunteers drawn with approval by the ethics committee of the Medical Faculty at RWTH Aachen University. Mononuclear cells were separated by density gradient centrifugation with Biochrom AG. After they were washed twice in phosphate-buffered saline (PBS), mononuclear cells were resuspended in MV2 (Promocell) and plated on 96-well plates (104 cells per well) for 4 days. Nonadherent cells were removed, and fresh medium was added. At days 5 and 7, adherent cells were detached with Accutase (PAA Laboratories, 5 minutes at 37°C), counted, and subjected to functional analysis. Some EOCs were incubated at day 6 with recombinant human sCD40L (Provitro) at 0.1 or 1 µg/mL for 24 hours. In addition, a short-term treatment of EOCs with sCD40L (1 µg/mL) or Mn2+ (1 mmol/L) for 15 minutes at 37°C was performed at day 7.

Characterization of EOCs by Flow Cytometry

Cultured EOCs at day 7 were detached and resuspended in PBS plus 0.5% bovine serum albumin (BSA) after incubation with fluorochrome-conjugated monoclonal antibodies: anti-CD34-PE, anti-CD45-PerCP, anti-CD14-FITC, and anti-CD16-PE (all BD Biosciences, San Jose, Calif); anti-CD133-APC (Miltenyi Biotec); anti-VEGFR2-PE and anti-Tie2-APC (both R&D Systems, Minneapolis, Minn); anti-CD40L-FITC (Biolegend); and anti-Mac-1/CD11b-PE monoclonal antibody (clone CBRM1/5) and anti-CD31-APC (both eBioscience). Some cells were reacted with monoclonal antibodies against CD40, CD11a, CD11b, and CD49e (all BD Biosciences). After incubation for 30 minutes on ice, cells were washed twice in PBS plus 1% FBS. For the nonconjugated monoclonal antibodies, a secondary antimouse-PE antibody (R&D Systems) was used. Respective control antibodies were matched to isotype and fluorochrome. EOCs were also incubated (1 hour at room temperature) with FITC-conjugated lectin from Ulex europaeus (Sigma, St Louis, Mo). Sample tubes were acquired and analyzed on a FACScantOll flow cytometer with FACS Diva 6.1.2 software (BD Biosciences) by recording 10 000 events within the target scatter population.

Reverse Transcription Polymerase Chain Reaction Analysis of CD40 and CD40L

Total RNA from EOCs was isolated with an RNeasy Kit (Qiagen) and reverse-transcribed into cDNA with the use of Mo-MLV RT (Invitrogen, Carlsbad, Calif). Reverse transcription polymerase chain reaction was performed with following primers: CD40, (forward) 5′-TTCCTTGTTGCAAGCAAGGA, (reverse) 5′-GACCCGAAAGCTT-AGGTG; CD40L, (forward) 5′-GCCAGTCCAGGGCTTTGTG, (reverse) 5′-ATCTTTTTTGACGCCACTGT.

Adhesion Under Flow Conditions

Laminar flow assays were performed as described. In brief, 35-mm culture dishes were coated with fibronectin, fibrinogen (Sigma, both at 10 µg/mL), vascular cell adhesion molecule-1 (VCAM-1) (R&D Systems, at 5 µg/mL), or intercellular adhesion molecule-1 (ICAM-1) (10 µg/mL). The coated dishes were assembled at the lower wall of a flow chamber on the stage of an Olympus IX50 microscope. Control EOCs as well as EOCs pre-treated with sCD40L for 24 hours were detached, resuspended (1.5×106/mL) in HHMC medium (HEPES-buffered Hanks’ balanced salt solution, 1 mmol/L Mg2+/Ca2+50%, 0.5% BSA), kept at 37°C, and perfused at 1.5 dyn/cm2. Some EOCs were incubated with 1 µg/mL sCD40L for 15 minutes before perfusion. Culture dishes were also coated with sCD40L (at 1 µg/mL in PBS) as described, and EOCs preincubated or not with blocking anti-CD40, anti-β2 integrin (CD18), or anti-CD49e antibodies (all at 10 µg/mL; R&D Systems) were perfused. Coating with BSA served as a control. After 4 minutes of perfusion, the number of cells firmly adherent by primary interaction with surface-bound matrix proteins was quantified in multiple fields by analysis of serial images recorded with a JVC 3CCD video camera and JVC SR-DVM700 recorder.

Transmigration Assay

EOCs were treated on day 6 with sCD40L for 24 hours. Transmigration was assessed with the use of 8-µm pore size Fluoroblok inserts (BD Falcon). EOCs (5.0×105) stained with calcein-AM (10 µmol/L) or Mn2+ (1 mmol/L) for 24 hours. Transmigration assays were preincubated or not with anti-CD40, anti-β2 integrin (CD18), or anti-CD49e antibodies (all at 10 µg/mL) at 37°C, and perfused at 1.5 dyn/cm2. Some EOCs were preincubated with blocking anti-CD40 Ab for 30 minutes before sCD40L was added.

Assessment of Cell Viability

EOCs were detached on day 6 after the isolation and seeded on a fibronectin-coated 96-well plate. Medium was removed on the next day, and 100 µL of fresh medium containing or not sCD40L was added for 24 hours. Tests were performed in triplicate, and cell viability was determined with the use of the CellTitre-Blue assay kit (Promega) according to the manufacturer’s protocol. Alternatively, cell viability was assessed by trypan blue exclusion of dead cells after treatment with sCD40L. In some experiments, EOCs were preincubated with blocking anti-CD40 Ab for 30 minutes before sCD40L was added.

Measurement of Intracellular Oxygen Species

Superoxide anions were detected with hydroethidine (Invitrogen). Briefly, EOCs were treated with sCD40L for 24 hours, detached, and incubated with 10 µmol/L hydroethidine for 15 minutes at 37°C after direct analysis by flow cytometry with assessment of ethidium-positive cells.

Proliferation Assay

Cells were detached on day 5 in culture and seeded (4.0×103) on a fibronectin-coated 24-well plate. One day later, cells were incubated...
with sCD40L for 24 hours. Thereafter, medium was removed, and cells were frozen at −80°C. Proliferation was assessed with the use of the CyQUANT kit (Invitrogen) according to the manufacturer’s protocol.

Mouse Model of Carotid Wire Injury

As described previously,27,28 transluminal arterial injury was induced by inserting a 0.36-mm guidewire into the left common carotid artery of C57BL6 mice (wild-type) and CD40−/− mice (n = 6 each). Three weeks after injury, neointimal plaque area was quantified in serial 5-μm cross sections of common carotid arteries within a standardized distance from the bifurcation (0 to 500 μm) stained with Movat hemalum/Orcein-Verhoeff’s hematoxylin. The relative macrophage content was assessed by staining for Mac-2 (CL8942AP, Cedarlane) and the re-endothelialization by staining for CD31 (M-20, Santa Cruz).28 In addition, control EOCs or EOCs pretreated with 1 μg/mL murine sCD40L for 24 hours were labeled with 2.5 μg/mL CM-DiI (Invitrogen) and injected (0.75 × 10^6 in 100 μL PBS) intracardially into athymic NMRI nude mice (Harlan Winkelmann) 24 hours after wire injury (4 mice per group). Additional groups of nude mice (n = 6 each) received intraperitoneally PBS or 4 μg murine recombinant sCD40L (Provitro). In addition, EOCs were isolated from spleen-derived mononuclear cells of wild-type or CD40−/− mice and cultured for 1 week as described.29 Wild-type and CD40−/− EOCs (pretreated or not with 1 μg/mL murine sCD40L for 24 hours) were injected (1 × 10^6 in 200 μL PBS) intravenously into C57BL6 mice 24 hours after wire injury (6 mice per group). At day 7 after wire injury, carotid arteries were harvested by in situ perfusion fixation for immunohistochemical analysis of EOC homing, endothelial recovery, and quantification of neointimal area.23 Animal experiments were approved by local authorities and complied with the German animal protection law.

Statistical Analysis

Data represent medians and interquartile ranges or mean ± SEM values of ≥3 independent experiments and were analyzed by 2-tailed Student t test or ANOVA for normally distributed variables or by nonparametric Mann-Whitney test or Kruskal-Wallis test with post hoc Dunn test (Prism 4.0 software, GraphPad, Calif), as appropriate. Differences with P < 0.05 were considered statistically significant.

Results

Mononuclear cells were isolated from peripheral blood and cultured for 7 days as described.15,19,23,24 These conditions resulted in adherent cells bearing myeloid and endothelial markers, thus corresponding to EOCs. In particular, analysis
by flow cytometry revealed that EOCs were negative for CD133, dimly expressed CD34, but were positive for CD45, CD14, CD16, VEGFR2, Tie2, CD31, and lectin at day 7 after isolation (Figure 1 and data not shown). Furthermore, EOCs strongly expressed CD40 but not CD40L as surface protein, whereas both CD40 and CD40L were detectable at transcript levels (Figure 2A). EOCs also highly expressed the α-chains of the β2-integrins LFA-1 (αL, CD11a) and Mac-1 (αM, CD11b), as well the α5 integrin subunit (CD49e) of the fibronectin receptor VLA-5 (Figure 2B and 2C). In contrast, quiescent EOCs weakly displayed the CD11b neoepitope recognized by CBRM1/5 reporting the activated subpopulation of Mac-1 (Figure 2C).

To investigate whether sCD40L may influence key surface integrins, we performed flow cytometry analysis after treatment of EOCs with sCD40L for 24 hours (Figure 2C). This experiment demonstrated that sCD40L did not significantly affect surface expression of LFA-1 (CD11a), Mac-1 (CD11b), or VLA-5 (CD49e). In addition, treatment with sCD40L for 15 minutes failed to upregulate the Mac-1 activation epitope reported by the monoclonal antibody CBRM1/5, which can be induced, for example, after exposure to Mn2⁺ (Figure 2D).

Next, we investigated effects of sCD40L on adhesion and transmigration. As shown in Figure 3A, treatment of EOCs with sCD40L at 0.1 or 1 μg/mL for 24 hours reduced the adhesion on the VLA-5 ligand fibronectin under physiological flow conditions. Similar results were obtained for adhesion on the Mac-1 ligand fibrinogen, on the combined Mac-1 and LFA-1 ligand ICAM-1, and on the VLA-4 ligand VCAM-1 (Figure 3B to 3D), which could be inhibited by the respective blocking integrin monoclonal antibodies (data not shown). In contrast, short-term incubation of EOCs with sCD40L did not significantly reduce adhesion on these
substrates (Figure 3A to 3D). Because sCD40L has been described as an alternative ligand for VLA-5 and Mac-1 on monocytes but not for VLA-4 and LFA-1, these data indicate that effects of sCD40L on EOCs are not attributable to direct competition for VLA-5 or Mac-1 binding but, given their unaltered expression, rather are due to a general attenuation of integrin adhesiveness. Next, we assessed the adhesion of untreated EOCs under flow conditions on surface-bound CD40L. As shown in Figure 3E, we observed only marginal arrest of EOCs on surface-immobilized CD40L, which was higher than that on BSA (negative control) but much lower than that on fibronectin (positive control). Notably, blocking CD40, CD11b, or CD49e did not significantly reduce this adhesion on surface-bound CD40L, indicating that sCD40L does not appear to serve as a highly relevant adhesive ligand in EOCs.

Next, sCD40L attenuated the transmigration of EOCs and microvascular endothelial cells. For instance, EOC transmigration triggered by CXCL12 gradient was slightly decreased after treatment with sCD40L at 0.1 μg/mL (86.4±14.2% versus control) but was significantly reduced by sCD40L at 1 μg/mL for 24 hours (66.8±10.5% versus control; Figure 4A). Similarly, incubation of microvascular endothelial cells with 0.1 μg/mL sCD40L hardly diminished transmigration toward VEGF (87.3±7.1% versus control), whereas the higher concentration of 1 μg/mL sCD40L resulted in a significant decrease (82.4±8.7% versus control; n=4). We further investigated EOC viability and proliferation after treatment with sCD40L. Incubation of EOCs with 0.1 μg/mL or 1 μg/mL of sCD40L for 24 hours resulted in a significant and dose-dependent decrease in cell viability (Figure 4B). Moreover, treatment of EOCs with 1 μg/mL sCD40L for 24 hours induced an increase in the number of cells positive for trypan blue (25.9±5.3% versus 11.8±3.0%), an effect reversed after preincubation with blocking anti-CD40 Ab (Figure 4C). In parallel to findings on cell viability, we observed a significant reduction in the proliferation rate of EOCs of up to 10% after exposure to sCD40L for 24 hours (Figure 4D). As a potential mechanism underlying impaired proliferation and viability of EOCs, sCD40L increased the generation of intracellular reactive oxygen species (Figure 4E). Thus, sCD40L signaling clearly decreases transmigration, viability, and proliferation of EOCs, possibly by promoting oxidative stress.

To examine whether the aforementioned findings may also have in vivo relevance, we investigated the extent of neointima after carotid wire injury in CD40−/− versus wild-type mice. After 3 weeks of wire injury, CD40−/− mice displayed a markedly reduced neointimal hyperplasia compared with wild-type mice (Figure 5A, C). Luminal lining evidenced by staining for CD31 (Figure 5B) revealed that the deficiency in
CD40 significantly improved re-endothelialization. The neo-intimal macrophage content did not differ between both groups, as assessed by staining for Mac-2 (Figure 5D), indicating that the effect of CD40 deficiency was not related to a modulation of macrophage infiltration. Furthermore, infusion of EOCs derived from CD40<sup>−/−</sup> mouse spleens significantly attenuated the neointimal hyperplasia compared with EOCs from wild-type mice (Figure 5E). In this setting, the preincubation of wild-type EOCs with sCD40L before infusion further aggravated neointimal formation, whereas the benefit of CD40<sup>−/−</sup> EOCs in limiting neointimal formation remained unaffected (Figure 5E).

Additional experiments in nude mice revealed that infused human control EOCs significantly better engrafted into neo-endothelium after wire injury than sCD40L-treated EOCs, as evidenced by colocalization of DiI<sup>+</sup> EOCs and CD31<sup>+</sup> endothelial regions (Figure 6A and 6B). Notably, this was associated with a significantly impaired re-endothelialization in nude mice receiving sCD40L-pretreated human EOCs versus control EOCs (Figure 6C and 6D). Accordingly, intravenous administration of control EOCs but not sCD40L-treated EOCs attenuated neointimal hyperplasia after carotid wire injury without affecting the medial area (Figure 6E). Importantly, intraperitoneal injection of sCD40L itself markedly increased the extent of neointima in nude mice compared with saline, an effect that could partly be rescued by the presence of human EOCs (Figure 6E).

**Discussion**

Published data on the involvement of CD40(s)CD40L in endothelial cell biology are rather controversial to date. For example, some results revealed that treatment of cultured endothelial cells with 0.1 μg/mL sCD40L exerts antiapoptotic, proliferative, and proangiogenic effects. Similarly, sCD40L induced angiogenesis in vivo via upregulation of VEGF. In contrast, others reported negative effects of sCD40L (0.1 to 10 μg/mL) on endothelial cells in vitro, in particular induction of apoptosis, inhibition of migration, and increased oxidative stress. Nevertheless, studies using CD40L<sup>−/−</sup> and ApoE<sup>−/−</sup> mice have documented stabilization of atherosclerotic plaques, and antibody blockade of CD40L exerted similar effects in LDLR<sup>−/−</sup> mice. Recently, sCD40L has been shown to significantly decrease endothelium-dependent vasorelaxation, an effect related to oxidative stress and reduction of endothelial nitric oxide synthase. Clinical trials have further unveiled that elevated plasma sCD40L was not only present during diabetes mellitus and cardiovascular disease but might be predictive for restenosis and acute ischemic events. Patients with hypercholesterolemia have also shown an upregulation of CD40/sCD40L compared with healthy controls. Conversely, treatment with statins or thiazolidinediones reduced plasma sCD40L. Hence, chronically increased plasma sCD40L has been generally related to endothelial dysfunction and risk for throm-
bosis or restenosis and plaque destabilization. Our in vitro data revealed detrimental effects of sCD40L on EOC function associated with increased generation of intracellular superoxide anions. In agreement with previous results, sCD40L-related endothelial dysfunction may therefore be explained by elevated oxidative stress and may extend to impaired viability and recruitment of EOCs. Other trials have associated reduced EOC counts with diabetes mellitus, the severity of CAD, and the cardiovascular risk score. Elevated levels of sCD40L have been also correlated with the risk for CAD and further predicted ischemic complications. Accordingly, a possible relation of EOCs and sCD40L during CAD requires more attention. However, whether all these events occur in parallel or are mechanismically linked remains an open question. Hence, the present study may provide impetus for further clinical investigations on potential correlation between plasma sCD40L and circulating EOCs in CAD.

In the context of arterial injury, platelets in terms of bridging cells have been reported to support recruitment and endothelial differentiation of progenitor cells, but it has also been shown that activated platelets and platelet-leukocyte aggregates promote atherosclerotic lesions in ApoE−/− mice. Activated platelets release functional sCD40L, which induces expression of adhesion molecules in endothelial cells. Along these lines, not only platelets but also other cells (T lymphocytes, macrophages, activated endothelial and vascular smooth muscle cells) have been shown to secrete sCD40L. During early phases of thrombus formation, re-endothelialization is severely compromised, and the potential positive effects of platelets on EOC recruitment and maturation could be explained by secretion of fibrin, CXCL12, and ligands for CXCR2. Thus, platelets may exert their regulatory function on the homing of EOCs even during the chronic phase, when higher amounts of arresting compounds were dominantly secreted and immobilized. Recently, platelet granules have been found to contain both stimulators (VEGF, basic fibroblast growth factor) but also inhibitors (endostatin, thrombospondin-1) of angiogenesis, which are organized in separate compartments and differentially secreted. In parallel to the release of sCD40L and chemokines, this may further variably influence the balance of platelet-dependent recruitment and differentiation of vascular precursor cells.

It has been shown that platelet-derived sCD40L (eg, after arterial injury) can induce the expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) and secretion of angiogenic chemokines (CCL2 and CXCL8) in endothelial cells. Notably, a maximal secretion of sCD40L was achieved rapidly after platelet activation and gradually decreased. Simultaneous treatment with sCD40L and interleukin-4 also upregulated VCAM-1, thus increasing monocyte and lymphocyte arrest while decreasing the adhe-
sion of neutrophils. Published data further demonstrated adhesion of monocytes on surface-bound CD40L, as well as an interaction of sCD40L with Mac-1 or VLA-5. Our experiments revealed that surface-coated CD40L can support moderate arrest of EOCs in flow. However, this adhesion was much lower than that on fibronectin but was unaffected by blocking CD40, Mac-1, or VLA-5. Moreover, pretreatment with sCD40L for 24 hours but not for 15 minutes significantly impaired EOC adhesion on the integrin ligands fibrinogen, ICAM-1, VCAM-1, and fibronectin, without altering the expression of Mac-1, VLA-4, or VLA-5. These data imply that impaired adhesion of sCD40L-treated EOCs was due to attenuation of intrinsic integrin adhesiveness rather than to direct competition for integrin binding. Thus, sCD40L may serve as an alternative integrin ligand for freshly isolated monocytes but not for cultured EOCs.

Compared with wild-type mice, CD40−/− mice displayed improved re-endothelialization concomitant with decreased neointimal formation after wire injury of the carotid artery, whereas macrophage content was unaltered. Endothelial recovery after wire injury in nude mice was significantly improved by administration of human control EOCs compared with sCD40L-treated EOCs, in agreement with an impaired incorporation of sCD40L-treated EOCs into the neoendothelium. Accordingly, infusion of EOCs pretreated with sCD40L less markedly reduced neointimal formation, whereas systemic injection of sCD40L clearly increased neointimal hyperplasia compared with saline. Similarly, infusion of CD40−/− mouse EOCs more markedly attenuated neointimal growth after wire injury than wild-type EOCs, an effect that persisted even after pretreatment with sCD40L. Thus, engrafted EOCs may exert homeostatic functions on arterial remodeling after injury, and beyond the direct effects on EOCs and mature endothelial cells, other (eg, inflammatory) mechanisms may contribute to the effects of sCD40L/CD40 on neointimal growth.

Although numerous published articles have used the terminology of endothelial progenitor cells, these cells did not correspond to progenitor cells at day 7 after the isolation from peripheral blood. Their phenotype rather reflected macrophage-like cells expressing endothelial markers, similar to the angiogenic Tie2+/CD14+CD16− myeloid subset. Moreover, cultured EOCs failed to upregulate Mac-1 activity after sCD40L treatment and were negative for surface CD40L, thus implying attenuated inflammatory properties. Taken together and as previously outlined, a reevaluation of the definition for in vitro expanded “endothelial progenitor cells” is urgently required. Nevertheless, and beyond terminological inconsistencies, cells with this phenotype have been largely recognized to reflect endogenous angiogenic capacity and to maintain tissue regeneration after therapeutic infusion.

Collectively, endothelial dysfunction due to continuously elevated levels of plasma sCD40L can be additionally ex-
plained by dysfunction of angiogenic EOCs. Moreover, in the context of secondary arterial healing during atherosclerosis, a therapeutic blockade of sCD40L may provide a promising novel approach for diminishing the neointima, most likely by attenuating inflammation and supporting endothelial regeneration.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Previous work has revealed an essential involvement of soluble CD40 ligand (sCD40L) in atherosclerosis. For instance, interruption of CD40/sCD40L signaling was associated with a more stable plaque phenotype in atherosclerosis-prone mice. Furthermore, clinical trials have unveiled that sCD40L plasma levels are elevated during diabetes mellitus and cardiovascular disease and can predict restenosis and acute ischemic events. Whereas interruption of CD40/sCD40L has mainly been examined with regard to primary atherosclerotic plaque formation and destabilization, systematic evidence as to whether the sCD40L could also influence endothelial regeneration and neointimal growth after arterial injury has been elusive. Thus, we were prompted to investigate the effects of sCD40L on the function of human peripheral blood–derived angiogenic early outgrowth cells (EOCs) and during arterial remodeling after carotid wire injury in CD40-deficient mice, as well as in nude mice. Our data reveal that sCD40L increased generation of intracellular superoxide anions and decreased homing capacity of EOCs. Moreover, CD40-deficient mice displayed reduced neointimal formation and improved re-endothelialization after carotid wire injury compared with wild-type mice, whereas therapeutic infusion of control EOCs but not EOCs pretreated with sCD40L attenuated neointimal growth after wire injury in nude mice. Treatment with sCD40L also attenuated luminal incorporation of EOCs and aggravated neointimal progression. Taken together, the endothelial dysfunction associated with elevated sCD40L plasma levels can be additionally explained by dysfunction of angiogenic EOCs. In the context of secondary arterial healing during atherosclerosis, a therapeutic blockade of sCD40L may provide a novel promising tool to prevent restenosis by attenuating inflammation and supporting endothelial regeneration.
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