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The role of CD40L and VEGF in the modulation of angiogenesis and inflammation

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ABSTRACT

Recently, there has been growing interest in deciphering the role of angiogenesis in the progression of atherogenesis. Importantly, CD40–CD40L interactions are of significant relevance because of their involvement in both angiogenesis and atherosclerotic development. Previously, we have shown that recombinant soluble CD40 ligand (rsCD40L) stimulates auto-inflammatory CD40L synthesis and reactive oxygen species (ROS) generation in vascular cells. In the current study, we demonstrate that redox-mediated CD40–CD40L interaction can enhance vascular endothelial growth factor (VEGF)-induced angiogenesis, endothelial migration, and actin polymerization processes. Interestingly, the addition of exogenous VEGF leads to cleavage of *de novo* CD40L produced in endothelial cells following rsCD40L treatment. Using inhibitor and silencing RNA-based experiments, it was observed that VEGF-induced protease, calpain 2, was responsible for the cleavage of *de novo* CD40L. While our *in vivo* experiments using a matrigel plug assay indicate a VEGF and CD40L induced enhancement of angiogenesis, our studies also identify a novel mechanism by which VEGF can abrogate CD40L-mediated inflammation. Together, these studies reveal a new pathway by which VEGF–CD40L interactions can regulate the angiogenic and inflammatory process depending on the specific environment.

1. Introduction

Angiogenesis is a complex phenomenon that leads to new blood vessel formation and involves endothelial cell migration, proliferation, and differentiation, as well as tube formation. Angiogenesis is the key process involved in normal development and wound repair as well as in ischemic heart and limb diseases, and is stimulated by a variety of growth factors such as vascular endothelial growth factor (VEGF) (Ushio-Fukai, 2006). VEGF, the key angiogenic growth factor, stimulates proliferation, migration, and tube formation in endothelial cells (ECs) (Ushio-Fukai, 2006).

Atherogenesis, on the other hand is a pathobiological process leading to atherosclerosis by three consecutive stages of (i) initiation, (ii) progression, and (iii) rupture of severe narrowing leading to adverse clinical outcome (Herrmann et al., 2006). Atherogenesis is characterized by the process of forming atheromatous plaques in the inner lining (the intima) of arteries and characterized by lipid retention, as well as proteolytic injury and a chronic inflammatory response (Blanco-Colio et al., 2006). The consequences of these processes result in pathological vascular remodeling, stimulating inflammatory cell recruitment, smooth muscle cell proliferation, and fibrosis (Blanco-Colio et al., 2006). These processes are intimately linked with neovascularization and the angiogenesis process (Herrmann et al., 2006). However, the cellular and molecular mechanisms linking these processes, which lead to cardiovascular dysfunction, remain largely unknown.

Evidence suggests that cardiovascular diseases are associated with increased oxidative stress in blood vessels (Ushio-Fukai and Alexander, 2004). Recent reports indicate that ROS play an important role in angiogenesis; however, underlying molecular mechanisms remain unknown (Ushio-Fukai and Alexander, 2004). CD40 ligand stimulates platelet, endothelial and neutrophil ROS generation (Chakrabarti et al., 2005, 2007; Vanichakarn et al., 2008). Soluble CD40 ligand induces endothelial dysfunction in human and porcine coronary artery endothelial cells (Chen et al., 2008). CD40-dependent activation of the phosphatidylinositol 3-kinase/Akt pathway mediates endothelial cell survival and *in vitro* angiogenesis (Deregibus et al., 2003). Presence of hypoxia, as well as HIF (hypoxia inducible factor) and VEGF, has been recently demonstrated in advanced human atherosclerosis (Sluimer et al., 2008). In addition, the HIF pathway is associated with lesion progression and angiogenesis, which suggests its involvement in the response to hypoxia and in the regulation of human intraplaque angiogenesis (Sluimer et al., 2008). Our recent studies showed that hypoxia enhances CD40–CD40L-mediated inflammation in endothelial and monocytic cells (Chakrabarti et al., 2009). Those studies elucidated the role of hypoxia in the CD40–

Abbreviations: sCD40L, soluble CD40 ligand; rsCD40L, recombinant soluble CD40 ligand; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; Ad-β-gal, adenovirus overexpressing beta galactosidase; AdmCD40L, adenovirus over expressing murine CD40 ligand; DCFHDA, dichlorodihydrofluorescein diacetate.

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CD40L-induced angiogenesis by VEGF expression in endothelial cells and monocytes (Melter et al., 2000; Chakrabarti et al., 2009).

CD40 signaling is reportedly associated with plaque instability (Schonbeck and Libby, 2001) and it has been recently documented that microparticles isolated from human atherosclerotic lesions express CD40L, stimulate endothelial cell proliferation following CD40 ligation, and promote *in vivo* angiogenesis (Leroyer et al., 2008). Microvessels in atherosclerotic plaques provide an alternative pathway for the recruitment of leucocytes in the lesion sites (de Boer et al., 1999). Existence of VEGF in lipid-rich plaques of the vessels and mononuclear cells suggest a possible role of VEGF in microvessel formation (de Boer et al., 1999). Therefore, neovascularisation and adhesion molecule expressions by microvessels at sites of vulnerable lipid-rich plaques may sustain the influx of inflammatory cells and hence, could contribute to plaque destabilization (de Boer et al., 1999). Interestingly, CD40 ligand at inflammatory sites stimulates fibroblasts and tissue monocyte/macrophage production of VEGF, leading to angiogenesis, which promotes and maintains the chronic inflammatory process (Monaco et al., 2004). Further, it has been reported that T cells can play a role in angiogenesis by delivering VEGF to inflammatory sites, and VEGF can augment proinflammatory T cell differentiation, therefore creating a loop involving T cell mediated inflammation and angiogenesis (Mor et al., 2004).

However, it is not known if a CD40L and VEGF interaction plays any role in the angiogenesis and inflammatory process. Therefore, in this study, we have investigated the inflammatory and angiogenic responses of CD40L and VEGF. Our results indicate an additive role of VEGF and CD40L in stimulating angiogenesis via a ROS-related mechanism. Additionally, we identify an effect where VEGF may limit CD40L-mediated inflammation through the induction of the protease, calpain 2. Overall, these studies reveal the existence of a possible interaction by which VEGF and CD40L influence both angiogenesis and inflammation.

2. Materials and methods

Recombinant CD40L (rsCD40L) was obtained from R&D systems (Minneapolis, MN). Human umbilical vein endothelial cells (HUVECs), endothelial growth medium (EGM), and endothelial basal medium (EBM) were obtained from Cambrex (Walkersville, MD). Anti-CD40L antibody was obtained from R&D systems (Minneapolis, MN). Cell permeable calpain inhibitor, E64d, cobalt chloride, and polyethylene glycol-catalase (PEG-Catalase) were obtained from Sigma (St Louis, MO). Human CD40 and CD40L ELISA kits were obtained from Bender Med Systems (Burlingame, CA). DCFHDA, Lipofectamine 2000, and Optimem were obtained from Invitrogen (Carlsbad, CA). Modular hypoxic chamber and gas flow regulator were obtained from Stem Cell Technologies (Vancouver, BC). Smart pool silencing RNAs for CD40 and CD40L were obtained from Dharmacon (Lafayette, CO). Calpain 2-specific siRNA and calpain 2-detecting antibodies were obtained from Cell Signaling Technology (Beverly, MA). BALBc/SCID mice were obtained from Jackson Laboratories (Bar Harbor, ME). Growth factor-reduced Matrigel was obtained from BD Biosciences (San Jose, CA). Cytoselect 96-well cell migration assay kit (8 μM, Fluorometric format) was obtained from Cell Biolabs, Inc. (San Diego, CA). Use of control adenovirus (Ad-β-gal) and adenovirus expressing murine CD40L (AdmCD40L) has been previously described (Chakrabarti et al., 2007).

2.1. ELISA assays

HUVEC cell lysates, stored in aliquots at −70 °C, were thawed on ice before the measurement of CD40L by ELISA, in a similar manner as described previously (Chakrabarti et al., 2007). CD40 was measured by an ELISA kit supplied by Bender Med Systems, according to the manufacturer's instructions.

2.2. siRNA-based inactivation of CD40, CD40L and calpain2

CD40 and CD40L siRNA transfection: 0.583 μl 100 μM CD40 or CD40L siRNA were mixed with 146 μl optimem. In another tube, 2.92 μl of lipofectamine was mixed with 146 μl optimem. Each tube containing siRNA and lipofectamine was incubated for 5 min at room temperature and then mixed together and incubated for 20 min to allow complex formation. Mixtures were then added to respective endothelial cells containing 600 μl endothelial total growth medium (EGM). The final siRNA concentration used was 65 nM. Cells with siRNA were incubated for 48–72 h, and then treated with recombinant CD40L for 6 h following a 3 h serum starvation. HUVEC cells were washed with cold PBS and collected by scraping in the presence of lysis buffer. siRNA-based inactivation of calpain 2 was carried out as described above for CD40 and CD40L, except that the final siRNA concentration used was 50 nM.

2.3. ROS measurements using DCFHDA oxidation

HUVECs were serum-starved for 3 h after 42 h of CD40 siRNA transfection. Cells were treated with $CoCl_2$ (hypoxic) or rsCD40L for 6 h and were labeled with 50 μM DCFHDA for 30 min, washed, and then fluorescence was measured in a microplate reader (Molecular Devices) following excitation at 485 nm and emission at 535 nm as described earlier (Chakrabarti et al., 2007).

2.4. Actin fiber staining and endothelial morphology

Endothelial actin fiber morphology was verified by oregon green phalloidin staining. Briefly, HUVECs following defined treatments were washed, fixed, permeabilized and labeled with oregon green phalloidin as described earlier (Chakrabarti et al., 2007). Images were captured either in a two-photon or in a Nikon Fluorescence Microscope (Chakrabarti et al., 2007, 2009).

AdmCD40L (adenovirus expressing CD40L) induced alteration of endothelial morphology and vessel structure was monitored following siRNA-based CD40 inactivation. Thus, HUVECs were incubated with CD40 siRNA for 42 h as described in Section 2.2. Following siRNA treatment, cells were infected with AdmCD40L (MOI = 50) or control adenovirus Ad-β-Gal (MOI = 50) for 30 h. Endothelial cells were labeled with fluorescent dye calcein-AM for 1 h, washed and then fluorescent microscopic and bright field images were captured in a similar manner as described earlier (Chakrabarti et al., 2009)

2.5. Western blot

Cell lysates were prepared after described treatment of HUVEC cells. Briefly, HUVECs were washed twice with PBS, and lysis buffer (Invitrogen) was added to the plate, as described (Chakrabarti et al., 2007, 2009). Cells were collected using a cell scraper and kept on ice with occasional vortexing for 30 min. Finally, after centrifugation at 14,000 rpm in a microcentrifuge (10 min at 4 °C), supernatants were collected and stored at −70 °C. Lysate protein concentrations were determined by Pierce Micro BCA assay kit (Chicago, IL). Equal amounts of total protein (30–50 μg) were separated on 12% polyacrylamide protein gels (Pierce, Rockford, IL). Proteins were transferred to a PVDF membrane and then blocked with 5% milk in TBST buffer. For detection of CD40L, membranes were incubated overnight with 2 μg/ml anti-CD40L antibody (R&D systems), followed by incubation with a biotinylated antimouse secondary antibody (1:1000, Vector Laboatories). Finally, the blots were incubated with streptavidin-HRP (1:1000, Vector Laboratories). To detect calpain 2, blots were incubated with anti-calpain 2 antibody (1:1000, Cell Signaling Technology) and developed with anti-rabbit secondary antibody. To verify equal amounts of protein loading, the same blots were stripped and incubated with antimouse beta actin antibody