CD40-40L Signaling in Vascular Inflammation*

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Ligation of CD40 in circulating cells or in the vessel wall may promote mononuclear cell recruitment, participate in the weakening of the plaque, and contribute to thrombosis. This process appears to be redox-sensitive, but the precise signaling mechanism by which the interaction between CD40L and its receptor CD40 mediates inflammatory secretion is unclear. Our previous studies have shown that the CD40-CD40L interaction mediates release of reactive oxygen species (ROS) and the current findings demonstrate that in endothelial cells CD40L dose dependently induces intracellular CD40L and MCP1 release in a redox sensitive manner. Pharmacological inhibition of phosphatidylinositol 3-kinase and p38 MAPK as well as adenovirus-mediated inactivation of Akt and p38 MAPK inhibited CD40L effects on endothelial cells. Akt, in particular, appeared to mediate CD40L-induced CD40L synthesis and MCP1 release by endothelial cells in a redox sensitive manner via NFκB activation. In addition, using confocal microscopy, exogenous addition of recombinant CD40L or adenoviral mediated CD40L overexpression was found to stimulate nuclear translocation of NFκB, which was further augmented by Akt overexpression and inhibited by Akt inactivation. These data support a mechanism whereby redox-sensitive CD40-CD40L interactions induce activation of Akt and p38 MAPK, leading to stimulation of NFκB and enhanced synthesis of CD40L and MCP1. Increased CD40L and MCP1 may contribute to the adherence of CD40-positive cells, such as platelets and monocytes, to the vessel wall modulating atherothrombosis.

Thus, the blockade of endothelial cell migration by CD40L may critically affect endothelial regeneration after plaque erosion. This, in turn, may contribute to the development of acute coronary events in patients with high circulating levels of CD40L (2). Platelets are recognized as a major source of soluble CD40 ligand (sCD40L) in the circulation. Soluble CD40 ligand may constitute a molecular link between hypercholesterolemia and prothrombotic states (3). High sCD40L levels are associated with increased expression of adhesion molecules and monocyte chemotactic protein (MCP-1) along with impaired endothelial cell migration and enhanced superoxide generation by monocytes (3). Interestingly, CD40L-induced up-regulation of CD40L expression in human endothelial cells may also influence the progression of proinflammatory reactions, including atherogenesis, through activation of extravasating monocytes (4).

Soluble CD40L and platelets may participate in a self-perpetuating pathogenic loop linking thrombosis and inflammation (5). We have recently reported that sCD40L influences platelet generation of reactive oxygen intermediates (6). However, the signaling pathways linking thrombosis to ROS production in the endothelium are unknown (7). Notably, ROS such as superoxide anions and hydrogen peroxide are transiently produced in response to growth factor and cytokine stimulation. Several lines of evidence also suggest that ligand-induced alterations in cellular redox state participate in downstream signal transduction (8). However, the signal transduction cascades activated by CD40L-induced ROS generation have yet to be defined. In endothelial cells, the phosphatidylinositol 3-kinase/Akt signaling pathway is activated following CD40-40L interaction and confers apoptotic resistance and triggers proangiogenic events, such as proliferation, migration, and vessel-like structure formation (9). Interestingly, high glucose-induced ROS generation has also been reported to activate phosphatidylinositol 3-kinase/Akt and NFκB-related up-regulation of COX-2, which triggers the caspase-3 activity that facilitates human umbilical vein endothelial cell (HUVEC) apoptosis (10). However, the specific role and involvement of ROS-inducible Akt, p38 MAPK, and NFκB signaling following CD40 ligation in endothelial cells has not been reported.

In the present study, we demonstrate that CD40-CD40L interactions in endothelial cells stimulate CD40L and MCP1 synthesis in a redox-sensitive manner via Akt and p38 MAPK activation. The activation of these specific signaling pathways leads to downstream NFκB activation. This sequence of events
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may contribute to atherosclerotic developments by augmenting recruitment of CD40-positive cells, particularly monocytes, through enhanced synthesis of CD40L and MCP1 in the endothelium. These events may subsequently contribute to other inflammatory conditions associated with high circulating sCD40L including in acute coronary syndrome and diabetes.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Adenoviruses—Carrier-free recombinant CD40L (rsCD40L) was obtained from R&D systems (Minneapolis, MN). Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Phosphatidylinositol 3,4,5-trisphosphate diC8 (a water-soluble PTEN substrate) was procured from Echelon Biosciences (Salt Lake City, UT). The Biomol green reagent kit for phosphate determination was obtained from BIOMOL International (Plymouth Meeting, PA). Unless mentioned otherwise, all chemicals used were obtained from Sigma. HUVECs, endothelial growth medium, and endothelial basal medium were procured from Cambrex (Walkersville, MD). The stable transfectant HEK cell line with an NFκB response element as well as the nuclear extraction kit was obtained from Panomics (Redwood City, CA). The soluble CD40L ELISA kit was obtained from Bender Med Systems (San Bruno, CA). The MCP1 ELISA kit was obtained from R&D Systems (Minneapolis, MN). ELISA kits for phospho-Akt, phospho-p38 MAPK, and phospho-IκBα and phospho-JNK were obtained from Invitrogen. Phospho-p65 ELISA kit was obtained from Cell Signaling Technology (Beverly, MA). TNFα, N-acetylcysteine, N-ethylmaleimide, polyethylene glycol (PEG)-superoxide dismutase, and PEG-catalase were obtained from Sigma. The luciferase assay kit was procured from Promega (Madison, WI). Pharmacological inhibitors, such as LY29004 and SB203580, were purchased from Cayman Chemicals (Ann Arbor, MI). Anti-CD40 antibody was obtained from Beckman Coulter (Fullerton, CA). Anti-phospho-p38 MAPK, anti-phospho-Akt, anti-phospho-p65 and anti-phospho-IκBα, anti-phospho-JNK, and total IκBα antibodies for Western blotting were obtained from Cell Signaling Technology (Beverly, MA). Anti-CD40 ligand antibody, anti-p65 antibody, and anti-IκBα antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-histone H2B antibody was obtained from Imgenex (San Diego, CA), and anti-β-actin antibody was obtained from Novus Biologicals (Littleton, CO). Adenovirus possessing murine CD40L (AdmCD40L) was provided by Dr. Ronald G. Crystal (Weill Medical College of Cornell University, New York) (11). Adenovirus possessing β-galactosidase (Ad-β-Gal), constitutively active Akt (Ad-myr-Akt), and double negative Akt (Ad-dn-Akt) were provided by Dr. Kenneth Walsh (Boston University School of Medicine). Double negative p38 MAPK adenovirus (Ad-dn-p38) was provided by Dr. Janet V. Cross (University of Virginia). The mouse cell line stably expressing CD40 ligand, J558L, was provided by Dr. Alberto Mantovani (Mario Negri Institute for Pharmacological Research, Milan, Italy).

HUVEC Culture—Confluent HUVECs, which had been passaged 2–5 times, were used for experiments. Cells were grown in complete endothelial growth medium in 5% CO2 at 37 °C and switched to serum-free endothelial basal medium 18–24 h before study. Cultures were exposed to thrombin or rsCD40L dissolved in endothelial basal medium for different time periods in the tissue culture incubator. In a subset of experiments, cultures were treated with N-acetylcycteine for 1 h prior to rsCD40L treatment.

For adenoviral infection, HUVECs were infected with the indicated adenovirus (multiplicity of infection = 50) for time periods ranging from 18 to 66 h. Cells were then washed twice with endothelial basal medium and subjected to various treatments. Infection with Ad-β-Gal was employed as a control.

After various treatments, HUVECs were processed for immunolabeling or were lysed in BIOSOURCE (Invitrogen) lysis buffer for analysis of proteins using either ELISA or Western blotting. Alternatively, for the determination of phospho-p65 levels, HUVECs were lysed in non-denaturing lysis buffer supplied by Cell Signaling Technology.

ROS Measurement—Following various treatments, HUVECs were collected by trypsinization, washed, and incubated with 20 μM 2’,7’-dichlorofluorescin diacetate (DCFHDA) for 20 min. Intracellular ROS generation was assessed by flow cytometric monitoring of DCFHDA oxidation in a fluorescence-activated cell sorter instrument (Dako Cytomation). For specific studies, DCFHDA oxidation was also directly monitored for 1 h in a fluorescent microplate reader using excitation and emission wavelengths of 485 and 535 nm.

HEK-NF-κB Cell Culture—HEK-293 cells stably transfected with translocatable NFκB response element (HEK-NF-κB cells) were cultured in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum, 2 mm L-glutamine, and 100 μg/ml hygromycin. All of the experiments were carried out with cultures that had reached 80% confluence. Cells were incubated in serum-free Dulbecco’s minimum essential medium 18–24 h prior to the addition of the indicated treatments. HEK-NF-κB cells were treated with 5 μg/ml rsCD40L unless otherwise indicated. At predetermined times after various treatments, cells were washed in PBS, and lysates were obtained for luciferase assays. Alternatively, nuclear and cytosolic fractions were isolated using a nuclear extraction kit (Panomics) for Western blot analyses.

In a subset of experiments, J558L cells were added to HEK cell cultures in 24-well plates after mild centrifugation at 100 rpm for 5 min. HEK-NFκB-Luc cells were then co-incubated with J558L cells for 6–7 h prior to luciferase activity assay.

To assay luciferase activity, cells were lysed in a luciferase assay-compatible lysis buffer (Promega). Lysates (20 μl) were incubated with luciferase assay reagent (Promega) in a white-walled opaque microwell plate, and chemiluminescence was measured.

Western Blotting and ELISA—Cell lysate protein concentrations were measured using the micro-BCA protein assay kit (Pierce). Standard Western blot procedures were employed. Briefly, equal amounts of protein (20–50 μg) were electrophoresed on 12% precast polyacrylamide gels (Pierce) for 2 h at 60 V at room temperature. Separated proteins were electrotransferred to polyvinylidene difluoride membrane at 110 V for 2 h at 4 °C. Membranes were then blocked with 5% milk in TBST buffer for 1 h at room temperature, followed by incubation with primary antibody (anti-CD40L antibody, 1:200; phos-
pho-p38 MAPK antibody (Thr(D)^180/Tyr(D)^182), 1:2000; phos-
pho-Akt antibody (Ser(P)^73), 1:1000; phoso-p65 antibody
(Ser(P)^32), 1:1000; phoso-IkBz antibody (Ser(P)^32), 1:1000;
p65 antibody, 1:200; histone H2B, 2 μg/ml; β-actin antibody,
1:5000; phoso-stress-activated protein kinase/JNK antibody
(Thr^183/Tyr^185), 1:1000; JNK antibody, 1:1000 for either1ha t
followed except the cells were incubated with 10
μg/ml CD40Fc (Axxora, San Diego, CA), followed by incubation with 10 μg/ml
anti-human-IgG1 labeled with R-phycoerythrin (Axxora). In
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—PTEN activity in HUVECs was deter-
miered after 18 h of serum starvation followed by exposure to
varying concentrations of rsCD40L for 6 h. PTEN activity was
determined in a manner similar to that described earlier with
some modifications (12). Briefly, following treatment with
rsCD40L, cells were washed with HEPES-buffered saline
(Hanks’ balanced salt solution containing 10 mM HEPES, pH
7.4) and lysed in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM
NaCl, 1% Triton X-100, 0.2% deoxycholic acid, 0.1% SDS, 1 mM
phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leu-
peptin, 5 μg/ml peptatin A) (12). Protein concentrations in the
lysates were determined by a micro-BCA protein assay kit.

For the determination of phosphate release, 4 μl of lysates
were incubated in a total 40-μl volume of assay buffer (100 mM
Tris-HCl, pH 7.5, 250 mM NaCl containing 2 mM dithiothreitol)
supplemented with 40 μM phosphatidylinositol 3,4,5-trisphos-
phate diC8 (12). Samples were incubated for 40 min at 37 °C.
Reaction was stopped by adding 200 μl of BIOMOL green rea-
gent (BIOMOL International, Plymouth Meeting, PA) and
incubated at room temperature for 30 min for color develop-
ment. The absorbance of the samples was measured at 595 nm
in a microplate reader. Lysis buffer was employed as a control
and subtracted from the respective sample values. The amount
of released phosphate was quantified using a standard curve
made with varying phosphate concentrations. Phosphate
released from the samples was normalized with protein con-
tents of the respective lysates.

HUVEC lysates prepared following rsCD40L exposure were
also subjected to Western blot analysis under denaturing con-
ditions as described above. Transferred blots were probed with
phos-PTEN and PTEN antibodies (Cell Signaling Technol-
ogy, Beverly, MA), both at 1:1000 dilutions, before incubation
with biotinylated goat anti-rabbit IgG in PBS (1:200; Vec-
tor Laboratories, Burlingame, CA) in PBS for 60 min at room tem-
perature and incubated with rabbit anti-p65 antibody (4 μg/ml;
Santa Cruz Biotechnology) in blocking medium for 1 h at room
temperature. Cells were subsequently washed in PBS and incu-
bated with biotinylated goat anti-rabbit IgG in PBS (1:200; Vec-
tor Laboratories) for 45 min at room temperature. Slides were
then washed and incubated with AMCA (7-amino-4-methyl-
coumarin-3-acetic acid)-conjugated streptavidin (5 μg/ml;
Jackson Immunoresearch Laboratories, West Grove, PA) for 60 min.
After additional washing with PBS, slides were incubated with
Oregon green 488 phalloidin (6.6 μM; Invitrogen) for 20
min to visualize actin fibers. Afterward, slides were washed with
PBS, and nuclei were counterstained with propidium iodide (3
μM; Invitrogen) in PBS. The labeled preparation was stored in
PBS protected from light until further examination. All confo-
cal images were captured within 2 h of labeling using two-pho-
ton confocal microscopy as described previously (6). For J558L
cell surface labeling of CD40L antigen, the same procedure
was followed except the cells were incubated with 10 μg/ml CD40Fc
(Axxora, San Diego, CA), followed by incubation with 10 μg/ml
anti-human-IgG1 labeled with R-phycocerythrin (Axxora). In
addition, cell nuclei were stained with Hoescht (0.5 μM; Invitro-
gen), and cells were coverslipped with mounting medium (Vec-
tor Laboratories).

PTEN Activity Assay—PTEN activity in HUVECs was deter-
ned after 18 h of serum starvation followed by exposure to

RESULTS

rsCD40L Induces ROS Generation as well as Redox-depen-
dent CD40L Synthesis and MCP1 Release—CD40-CD40L
interactions are known to elicit proinflammatory responses in
endothelial cells and to enhance production of ROS. Treatment
of HUVECs with 1 μg/ml rsCD40L elicited a marked increase in
ROS, as measured by DCFHDA oxidation (Fig. 1A). This effect
was abolished by co-incubation of cultures with anti-CD40
antibody, indicating that rsCD40L-induced increases in ROS
are mediated by CD40-CD40L interactions.

Exposure of endothelial cells to rsCD40L (Fig. 1B) as well as
to thrombin (Fig. 1C) induced synthesis of CD40L in a dose-de-
pendent manner, as measured by ELISA. To test the hypothesis
that CD40L induces CD40L synthesis via a redox-sensitive
mechanism, we examined rsCD40L-induced CD40L synthesis
in the presence of varying concentrations (0–10 mM) of N-ac-
tetylcyesteine, a thiol-containing antioxidant and scavenger
of reactive oxygen species. Western blot (Fig. 1D) and ELISA (Fig.
analysis of CD40L levels revealed that N-acetylcysteine attenuated CD40L synthesis in a dose-dependent manner.

Release of proinflammatory cytokines, such as MCP1, is a well characterized effect of CD40 ligation (13). Dose-dependent MCP1 release was observed in response to increasing concentrations of rsCD40L (Fig. 1F). Co-treatment with rsCD40L and thrombin further enhanced MCP-1 release (Fig. 1G). Similar to CD40L synthesis, CD40L-induced MCP-1 release was blocked by N-acetylcysteine (Fig. 1H).

CD40L induces redox-dependent NFκB activation—To understand whether CD40-CD40L interactions activate NFκB signaling, we employed a HEK cell line stably transfected with a translucent NFκB response element (HEK-NFκB-Luc cells). Treatment of HEK-NFκB-Luc cells with 2 μg/ml rsCD40L markedly enhanced luciferase activity (Fig. 2A). The nuclear translocation of NFκB p65 was also observed under these conditions (Fig. 2B). These results obtained with recombinant CD40L were verified with biologically active CD40L by employing a cell line stably expressing murine CD40L (J558L cells). J558L cells were labeled with CD40Fc and examined with two-photon confocal microscopy to verify the presence of cell surface CD40L (Fig. 2C). Co-incubation of CD40L-expressing J558L cells with HEK-NFκB-Luc cells led to a significant activation of NFκB, and this effect was dose-dependent (Fig. 2D, ii, p < 0.01). The effect of CD40L on NFκB activation in HEK cells was also examined. As shown in Fig. 2D, levels of phosphorylated NFκB p65 and IκBα in total cell lysates were markedly increased.

To determine whether CD40-CD40L interactions trigger NFκB activation via a redox-sensitive mechanism, HEK-NFκB-Luc cells were treated with N-acetylcysteine (Fig. 2E) or with a cell-permeable scavenger of hydrogen peroxide (PEG-catalase) or superoxide (PEG-superoxide dismutase; PEG-SOD) (Fig. 2F)
prior to rsCD40L exposure. All treatments blocked rsCD40L-induced NFκB activation (Fig. 2, E and F). Interestingly, pretreatment of rsCD40L with the cysteine cross-linking agent, N-ethylmaleimide, also diminished NFκB activation, indicating that the cysteine residues of CD40L might play a role in CD40L-mediated NFκB activation (Fig. 2G).

rsCD40L-induced NFκB Activation Mediates CD40L Synthesis and MCP1 Release—To understand whether NFκB activation lies upstream of rsCD40L-induced CD40L synthesis, HUVECs were treated with rsCD40L in the presence of the NFκB inhibitor Bay11–7085 and the proteosomal degradation inhibitor MG132. As shown in Fig. 3A, pretreatment of HUVECs with both agents attenuated rsCD40L-induced CD40L synthesis. CD40L synthesis was also inhibited by the presence of the protein synthesis inhibitors puromycin and cycloheximide (Fig. 3A). As shown in Fig. 3B, all of these agents also eliminated MCP1 release, suggesting that CD40L synthesis and MCP1 release are coordinated through a shared signaling mechanism. To further confirm the effectiveness of NFκB inhibitors in the CD40L and MCP1 inhibition, corresponding cell lysates were analyzed for the extent of IκBα and p65 phosphorylation. Serum-starved HEK-NF-Luc cells were pretreated with a 100 or 500 μM concentration of the cysteine alkylating agent, N-ethylmaleimide (NEM) for 30 min. Cells were then pretreated for 4 h with 5 μg/ml rsCD40L, and Western blot analysis of phosphorylated total p65 and IκBα was performed (n = 2).
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**FIGURE 3.** rsCD40L-induced NFκB activation mediates CD40L synthesis and MCP1 release. A, serum-starved HUVECs were incubated with protein synthesis inhibitors (puromycin and cycloheximide, the NFκB inhibitor Bay1–7085, or the proteosomal degradation inhibitor MG132) for 1 h prior to exposure to 2 μg/ml rsCD40L for 6 h. CD40L levels were analyzed using ELISA and were normalized to total protein concentration (*, p < 0.01; **, p < 0.001 versus rsCD40L only; n = 3). B, MCP1 release was determined in HUVECs treated with protein synthesis inhibitors (cycloheximide and puromycin), the NFκB inhibitors Helenalin and Bay1–7085, or the proteosomal degradation inhibitor MG132 prior to rsCD40L exposure (*, p < 0.01; **, p < 0.001; n = 3). C, HUVEC lysates following treatment with rsCD40L in the presence and absence of respective inhibitors were measured for the extent of IκBα phosphorylation by ELISA (*, p < 0.01; **, p < 0.005; n = 3). D, HUVEC lysates prepared after inhibitor treatments were measured for the extent of phosphorylated p65 (NFκB subunit) by ELISA (*, p < 0.02; **, p < 0.001; n = 3).

**FIGURE 4.** CD40L effects on NFκB phosphorylation and nuclear translocation. The effect of dominant negative and constitutively active Akt on rsCD40L-induced CD40L synthesis was also examined. Adenoviral infection with dn-Akt attenuated CD40L synthesis in the presence of rsCD40L, whereas infection with myr-Akt enhanced it (Fig. 4E). Interestingly, Ad-dn-p38 MAPK infection in HUVECs also prevented rsCD40L-induced MCP1 release (Fig. 4F) and concomitant NFκB activation, as evidenced by attenuated p65 phosphorylation (Fig. 4G).

Adenovirus-mediated CD40L overexpression in HUVECs activates Akt and p38 MAPK, generates ROS, and mobilizes NFκB in an Akt-dependent manner. To confirm results obtained with recombinant CD40L on p38, Akt, and NFκB signaling, CD40L was overexpressed in HUVECs. As shown in Fig. 5A, infection of HUVECs with murine adenoviral CD40L (AdmCD40L) led to ROS generation. Notably, ROS production was attenuated by Akt and p38 MAPK inhibition with

**FIGURE 5.** CD40L overexpression in HUVECs activates Akt and p38 MAPK, generates ROS, and mobilizes NFκB in an Akt-dependent manner. A, serum-starved HUVECs were incubated with Akt (LY29004 and SB203580, respectively) and p38 MAPK inhibitors (SP600125 and SB203580, respectively) for 1 h prior to exposure to 2 μg/ml rsCD40L for 6 h. HUVECs were then examined by immunofluorescent labeling (Fig. 5B). In addition, nuclear translocation of NFκB p65, as seen by immunolabeling, was readily apparent after rsCD40L treatment. The nuclear movement of NFκB p65 was found to be augmented by Ad-myr-Akt (Fig. 5D). The effect of dominant negative and constitutively active Akt on rsCD40L-induced CD40L synthesis was also examined. Adenoviral infection with dn-Akt attenuated CD40L synthesis in the presence of rsCD40L, whereas infection with myr-Akt enhanced it (Fig. 4E). Interestingly, Ad-dn-p38 MAPK infection in HUVECs also prevented rsCD40L-induced MCP1 release (Fig. 4F) and concomitant NFκB activation, as evidenced by attenuated p65 phosphorylation (Fig. 4G).
Intracellular CD40L content was measured by ELISA (*, p < 0.01; **, p < 0.001; +, p < 0.05; + +, p < 0.01; + + +, p < 0.002; n = 3). B, Western blot shows that N-acetylcysteine attenuates CD40L-induced phosphorylation of proteins as displayed in A. C, HUVECs were infected with control adenovirus (Ad-β-galactosidase) or adenovirus expressing double negative Akt (dn-Akt) or constitutively active Akt (Myr-Akt) for 44 h. Cells were then treated for 4 h with 5 μg/ml rsCD40L. Cell lysates were analyzed by Western blot (n = 2). D, HUVECs were infected (multiplicity of infection = 50) with Ad-β-gal, Ad-dn-Akt, Ad-myr-Akt for 48 h and subsequently treated with recombinant CD40L (2 μg/ml) for 17 h. Cells were then processed for p65 (NF-κB subunit; blue) and actin (green) immunolabeling. Nuclei were counterstained with propidium iodide (red). A, Ad-β-gal; B, Ad-β-gal + rsCD40L; C, Ad-dn-Akt + rsCD40L; D, Ad-myr-Akt + rsCD40L. The arrows indicate the translocation of p65 into the nucleus in samples B and D. The upper quadrants indicate individual color staining. The lower left quadrants indicate overlap of blue and green labeling. The lower right quadrants represent overlap of blue and red staining. The lower middle slot indicates overlap of all three stains (representative image from three separate experiments). E, HUVECs were infected with adenovirus expressing β-galactosidase (control), double negative Akt (dn-Akt), or constitutively active Akt (Myr-Akt) for 42 h. Cells were then treated with 2 μg/ml rsCD40L for 6 h. Intracellular CD40L content was measured by ELISA (*, p < 0.01 versus adenovirus-β-galactosidase-infected sample; n = 4). F, HUVECs were infected with adenovirus (multiplicity of infection = 50) possessing β-galactosidase or double negative Akt or double negative p38 MAPK for 42 h followed by treatment with fresh medium containing rsCD40L for 6 h. MCP1 released in the medium was measured by ELISA (*, p < 0.04; **, p < 0.01; n = 3). G, HUVECs were infected with adenovirus (multiplicity of infection = 50) possessing β-galactosidase or double negative p38 MAPK for 42 h followed by treatment with fresh medium containing 2 μg/ml rsCD40L for 6 h. Lysates were analyzed for p65 phosphorylation by Western blot (n = 3).
enhancing production of reactive oxygen species that act to limit endothelial migration (2). However, the ROS-inducible signaling pathways mediating the effects of CD40L have not been clearly defined. Here, we demonstrate that CD40 ligation in endothelial cells stimulates synthesis of CD40L and MCP1 in a redox-sensitive manner. Moreover, our data reveal that both effects lie downstream of ROS-induced NFκB activation and Akt stimulation.

Our results reveal that CD40 ligation leads to both Akt and p38 MAPK activation in HUVECs, as confirmed by enhancement of GSK3 and ATF phosphorylation, respectively. Interestingly, CD40 ligation stimulated ROS generation in an Akt- and p38 MAPK-dependent manner. Akt, in particular, appeared to mediate CD40L-induced NFκB activation, CD40L synthesis, and MCP1 release by endothelial cells, as shown by adenovirus-mediated inactivation of Akt. Our data are supported by the work of Sheu et al. (10), who has described that high glucose-dependent ROS is mediated by phosphatidylinositol 3-kinase/Akt pathway. A recent report by Wang et al. (27) has identified the role of NADPH oxidase-mediated ROS in monocyte/macrophage survival via Akt and p38 MAPK activation. Our results also show that the

FIGURE 5. CD40L overexpression in HUVECs spontaneously activates Akt and p38 MAPK, generates ROS, and mobilizes NFκB in an Akt-dependent manner. A, HUVECs were infected with AdmCD40L or control vector (Ad-β-Gal) for 18 h, and ROS generation was monitored for 1 h by measuring DCFHDA oxidation. Selected samples were pretreated for 1 h with 25 μM LY29004 and 10 μM SB203580 (*, p < 0.005 versus Ad-β-Gal; +, p < 0.005 versus AdmCD40L; n = 4). B, HUVECs were infected with AdmCD40L and/or Ad-β-Gal along with Ad-dn-Akt or Ad-dn-p38 MAPK for 24 h. DCFHDA oxidation was then monitored for 1 h (*, p < 0.001 versus Ad-β-Gal/Ad-dn-Akt/Ad-dn-p38; +, p < 0.002 versus AdmCD40L-infected samples; n = 3). C, HUVECs were infected with the indicated adenovirus for 48 h. Cells were then treated with 1 unit/ml thrombin for 30 min, and MCP1 release was determined. A subset of samples was pretreated with 10 mM N-acetylcysteine for 45 min before thrombin stimulation (*, p < 0.001 versus Ad-β-Gal control; +, p < 0.01 for N-acetylcysteine versus the non-N-acetylcysteine-treated group; **, p < 0.01 for dn-Akt or dn-p38 MAPK versus AdmCD40L-infected samples; n = 3). D, HUVECs were infected with Ad-β-Gal and AdmCD40L for 48 h. One Ad-β-Gal-infected sample was treated with thrombin (1 unit/ml) for 30 min. Total cell lysates were analyzed for CD40L, Akt, and p38 MAPK by Western blotting. E, cells were treated with thrombin (1 unit/ml) for 30 min after a 48-h transfection with Ad-β-Gal and/or AdmCD40L in conjunction with Ad-myr-Akt (constitutively active Akt) or Ad-dn-Akt (inactive Akt). Total lysates were then analyzed by Western blot analysis. F, HUVECs were infected with AdmCD40L, AdmCD40L + Ad-dn-Akt, or AdmCD40L + Ad-dn-p38. Cells were subsequently treated with 1 unit/ml thrombin for 30 min. Equal amounts of proteins were analyzed for Akt and p38 MAPK activity using GSK3 (Akt substrate) and ATF (p38 substrate) phosphorylation. G, HUVECs were infected with AdmCD40L, AdmCD40L, AdmCD40L + Ad-dn-Akt, or AdmCD40L + Ad-dn-p38. Cells were then stimulated with 1 unit/ml thrombin, and p38 MAPK activity was determined. H, HUVECs were infected with AdmCD40L, AdmCD40L, or Ad-dn-Akt for 24 h and then stimulated with 1 unit/ml thrombin for 30 min. Cells were then processed for p65 (NFκB subunit) immunolabeling (blue). Co-labeling for actin (green) was also performed. Nuclei were counterstained with propidium iodide (red) (representative image from two separate experiments).
CD40-40L interaction mediated Akt activation and that ROS generation is accompanied by attenuated PTEN activity. This underscores PTEN vulnerability to oxidative stress. In fact, studies by Flaherty et al. (18) have clearly demonstrated that alveolar macrophages are deficient in PTEN and that this is linked to constitutive activation of Akt. Differentiation-induced increases in base-line ROS contribute to the decreased PTEN protein and increased Akt observed in alveolar macrophages (18).

Our findings with rsCD40L-mediated effects could also be extended to thrombin-stimulated, AdmCD40L-infected HUVECs, in which a significant mobilization of NfκB was observed. The finding that CD40L-induced phosphorylation of p65, IkBα, Akt, and p38 was reversed by N-acetylcysteine indicates that this is a redox-mediated signaling mechanism. Our results diverge from those of Ha et al. (28), who showed that ROS generation precedes NfκB and p38 MAPK activation. In the same study, TRAF3 (tumor necrosis factor receptor-associated factor 3) was shown to participate in the production of ROS elicited by CD40 by association with p40phox, a cytosolic subunit of nicotinamide adenine dinucleotide phosphate oxidase (28). However, B lymphocytes of tumor origin were employed in that study, and the actions of CD40L in these cells are possibly distinct from those in primary endothelial cells.

It has been recently reported that thrombin stimulation may lead to endothelial Akt and p38 MAPK activation (7). Specifically, it was demonstrated that thrombin rapidly increases ROS production and activation of p38 MAPK and phosphatidylinositol 3-kinase/Akt in an endothelial cell line. This, in turn, led to up-regulation of p22phox, which was accompanied by a delayed increase in ROS generation and enhanced proliferation. This study suggested a positive feedback mechanism whereby ROS, possibly generated by the NADPH oxidase, leads to elevated levels of p22phox and sustained ROS generation, as is observed in endothelial dysfunction (7). A role for p38 MAPK in neutrophil oxidative burst has also been reported (29). Specifically, fMLP-induced neutrophil oxidase activation is almost completely eliminated by p38 MAPK inhibitors, regardless of the priming agent (29). The potential contribution of CD40-CD40L in these signaling pathways is unknown, and our results, obtained with exogenous CD40L as well as intracellular overexpression of CD40L, indicate the presence of a common pathway that culminates in the activation of Akt and p38 MAPK, ROS generation, and NfκB activation. Activation of NfκB then leads to enhanced CD40L synthesis and MCP1 generation.

Consistent with our results, it has been demonstrated that NfκB participates in the recruitment of neutrophils and lymphocytes after thrombin stimulation of endothelial cells (30). More recently, it has been shown that pretreatment of HUVECs with rapamycin, an inhibitor of mTOR, augmented thrombin-induced ICAM-1 expression (31). Inhibition of mTOR using rapamycin promoted, whereas overexpression of mTOR inhib-
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...thrombin-induced transcriptional activity of NFκB, an essential regulator of ICAM-1 (intercellular adhesion molecule-1) transcription (31). These results, taken with those presented here, indicate a common modality for thrombin- and CD40L-induced effects, which involve activation of NFκB.

Here we have employed N-acetylcysteine as well as N-ethylmaleimide to demonstrate that a redox-sensitive mechanism is involved in CD40-CD40L-mediated NFκB activation. The potential effectiveness of N-acetylcysteine, an effective ROS scavenger, in attenuating activation of Akt, p38 MAPK, and NFκB implicates a redox-mediated mechanism. Additionally, we demonstrate a dose-dependent JNK activation by rsCD40L in the present study. Interestingly enough, rsCD40L-mediated JNK activation was found to be a redox-sensitive phenomenon, since N-acetylcysteine effectively blocked its activation. Exposure to ROS or glutathione depletion has been implicated in the regulation of JNK and p38 MAPK activation (32). p38 MAPK/MSK1 (mitogen- and stress-activated protein kinase 1) and JNK have been recently reported to up-regulate heme oxygenase (stress response protein) mRNA in hydrogen peroxide-treated cardiac cells (24). Overall, our results indicate that CD40-40L-induced oxidative stress is responsible for NFκB activation and phosphorylation. Recent studies also indicate that post-translational modifications of NFκB, particularly acetylation and phosphorylation, play an additional role in the activation of this transcription factor (33, 34). Four different serine and phosphorylation, play an additional role in the activation. This implies that redox-sensitive cysteine residues of dsCD40L may be involved in sensing and transducing changes in cellular redox status caused by the generation of ROS and oxidized thiols (38). In addition, common mechanisms underlie the sensitivity of cytokines to redox changes, such as proximity to polar and charged groups. Moreover, signal transduction is initiated via conformational changes that are conferred by the formation of disulfide and cyclic sulfoxidime covalent bonds, sulfinic acids, and sulfonic acids (38).

In conclusion, our results show that CD40-40L interaction in endothelial cells stimulates a redox-sensitive signaling mechanism, leading to enhanced synthesis of endothelial CD40L and MCP1 via activation of Akt and p38 MAPK and NFκB. Further studies will determine whether CD40L-induced CD40L synthesis leads to CD40-positive leukocyte or platelet adhesion and monocyte recruitment by enhanced MCP1 generation.

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