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CD40-dependent Activation of Phosphatidylinositol 3-Kinase/Akt Pathway Mediates Endothelial Cell Survival and *in Vitro* Angiogenesis*

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CD40 has been involved in tumor and inflammatory neoangiogenesis. In this study we determined that stimulation of endothelial CD40 with sCD154 induced resistance to apoptosis and *in vitro* vessel-like formation by human microvascular endothelial cells (HMEC). These effects were determined to be mediated by CD40-dependent signaling because they were inhibited by a soluble CD40-muIg fusion protein. Moreover, apoptosis of HMEC was associated with an impairment of Akt phosphorylation, which was restored by stimulation with sCD154. The anti-apoptotic effect as well as *in vitro* vessel-like formation and Akt phosphorylation were inhibited by treatment of HMEC with two unrelated pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3K), wortmannin and LY294002. CD40 stimulation induced a rapid increase in Akt enzymatic activity that was not prevented by cycloheximide, an inhibitor of protein synthesis. The enhanced Akt activity induced by stimulation of endothelial CD40 was temporarily correlated with the association of CD40 with TRAF6, c-Cbl, and the p85 subunit of PI3K. Expression of negative-dominant Akt inhibited the activation of endogenous Akt through CD40 stimulation, despite the observation that association of CD40 with TRAF6, c-Cbl, and PI3K was intact. The defective activation of Akt abrogated not only the anti-apoptotic effect of CD40 stimulation but also the proliferative response, the enhanced motility, and the *in vitro* formation of vessel-like tubular structures by CD40-stimulated HMEC. In conclusion, these results suggest that endothelial CD40, through activation of the PI3K/Akt signaling pathway, regulates cell survival, proliferation, migration, and vessel-like structure formation, all steps considered critical for angiogenesis.

CD40 is a member of the tumor necrosis factor (TNF)[1] receptor superfamily, which provides activation signals in antigen-presenting cells such as B cells, macrophages, and dendritic cells (1, 2). Among the molecular mechanisms that link immunity to inflammation, the interaction between CD40 and its counterreceptor CD154 has rapidly emerged as a key system in the regulation of vascular pathophysiological processes such as atherogenesis (3, 4), tumor neoangiogenesis (5, 6), and inflammation (2, 8). Under physiological conditions, CD40 is expressed at low levels on endothelial cells but is up-regulated in areas of inflammation (9). Ligation of endothelial CD40 by CD154, either expressed on activated monocytes or T cells (2) or disgorged by platelets upon activation (10), induces production of various inflammatory cytokines and chemokines, procoagulant activity, adhesion molecules, metalloproteinases, and inflammatory mediators (11–14). These mediators have been implicated in the development and progression of atherosclerosis. *In situ* analysis of human atherosclerotic lesions revealed the co-expression of CD154 and CD40 on vascular endothelium and smooth muscle cells (15). Blockade of CD40-CD154 interaction in atherosclerosis was found not only to diminish the formation and progression of mouse atheroma but also to foster changes in lesions associated with plaque destabilization (16, 17). Recently, it has also been shown that the engagement of CD40 on endothelial cells by CD154 induces *in vitro* vessel-like tubule formation and expression of matrix metalloproteinases, two events involved in neovascularization (18). *In vivo*, the stimulation of CD40 triggered neoangiogenesis in mice (6, 19). Moreover, blockade of CD40-CD154 interaction prevented vascularization and tumor growth in an experimental model of Kaposi's sarcoma (6).

CD40 signaling is initiated by receptor oligomerization upon binding the trimeric ligand CD154 (20). CD40 signaling elicits different outcomes in distinct cell types, ranging from proliferation, survival, and differentiation to growth suppression and apoptosis (21, 22), and implies a complex regulation of CD40 signal transduction (23). Indeed, the CD40 cytoplasmic C terminus lacks intrinsic kinase activity and adaptor proteins of the TNF receptor-associated factor (TRAF) family appear to mediate the activation of the CD40 signaling cascade (24–28). It has been recently found that CD40 mediated Akt activation in dendritic and B cells (29). Several studies indicate that Akt-dependent signaling plays a critical role in the regulation of vascular homeostasis and angiogenesis (for review, see Ref.

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[1] The abbreviations used are: TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; WT, wild type; HMEC, human microvascular endothelial cells; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; Ab, antibody; PI3K, phosphatidylinositol 3-kinase; ND, negative-dominant; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; HRP, horseradish peroxidase.

30). Various growth factors, including vascular endothelial growth factor (VEGF) and angiopoietin-1 as well as mechanical stimuli, activate Akt in endothelial cells. However, the effect of stimulation of endothelial CD40 on the activation of Akt and the role of Akt in CD40-induced angiogenesis are unknown.

The aim of the present study was to investigate whether Akt is directly activated by the engagement of endothelial CD40 by its ligand CD154 and whether Akt mediates biological effects relevant for CD40-induced angiogenesis such as cell survival, proliferation, migration, and vessel-like structure formation.

EXPERIMENTAL PROCEDURES

Reagents—ECAF, DMEM, and D-valine-modified DMEM, bovine serum albumin fraction V (tested for not more than 1 ng of endotoxin per mg) were purchased from Sigma Chemical Co. Modified MCDB131 medium was obtained from Invitrogen, FCS was from EuroClone Ltd. (Wetherby West Yorkshire, UK). Recombinant human soluble CD154 trimeric protein (sCD154), a cross-linking Ab (enhancer), and CD40-muIg fusion protein, consisting of the extracellular domain of human CD40 fused to mouse IgG2a, were from Alexis Biochemicals (San Diego, CA).

Mouse monoclonal antibodies specific for TRAF2 (IgG1, H-10) and TRAF3 (IgG1, G-6), polyclonal rabbit anti-TRAF6 (H-274) IgG, polyclonal goat antibody against human CD40, Akt, and phosphorylated Akt (P-Akt), and isotypic control antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-c-Cbl (IgG1) was from BD Transduction Laboratories (San Diego, CA). The polyclonal rabbit antiserum anti p85 subunit of PI3-K (P-85) and active Akt1 were from Upstate Biotechnology (Lake Placid, NY).

Cell Cultures—Microvascular endothelial cells (HMEC) were obtained from derma using anti-CD31Ab coupled to magnetic beads, by magnetic cell sorting using the MACS system (Miltenyi Biotec, Auburn, CA). Primary cultures were grown in D-valine-substituted DMEM to avoid fibroblast growth (Sigma).

HMEC were immortalized by infection of primary cultures with a replication-defective adeno-5/SV40 virus as previously described (31). HMEC were characterized as endothelial cells by morphology, positive staining for vWF antigen, CD31, CD105, and the fucosylated receptors for plant lectins (Ulex europeus I and Bandeira Simplicifolia). Cytoplasmic staining was positive for vimentin and negative for cytokeratin and desmin, as previously described (31). HMEC were cultured on ECAF (Sigma)-coated tissue culture plates in modified MCDB131 medium (Invitrogen) supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), bovine brain extract (all from Biowittaker, Walkersville, MD) and 20% FCS.

HMEC Transfection—To obtain HMEC expressing the negative-dominant Akt (ND-Akt), HMEC were transfected with cDNA of K179M Akt1 mutant containing a Myc-His tag at the 3′-end of the Akt1 open reading frame and a substitution of methionine for lysine at residue 179 in pUSEamp plasmid (Upstate Biotechnology). As control cells were transfected with the empty plasmid (WT-Akt). HMEC seeded in 60-mm Petri dishes at a density of 5×10^6 cells per dish in DMEM containing 10% FCS, without antibiotics were transfected using 8 μg/ml DNA and 20 μl of LipofectAMINE 2000 (Invitrogen) according to the protocol suggested by the manufacturer. Transfected cells were stably selected by culturing in the presence of 1 mg/ml geneticin (G418, Sigma). Successful transfection was evaluated by positive immunofluorescence for anti-HIS (C-terminal) antibody (Invitrogen). HMEC were stably transfected up to the 10th passage. Experiments were performed at the 6th–7th passage.

Cytofluorimetric Analysis—For cytofluorimetric analysis, cells were kept for 24 h in DMEM with 10% FCS in the absence of growth factors. Cells were detached from plates with non-enzymatic cell dissociation solution, washed in PBS containing 2% heat-inactivated human serum, and incubated for another 15 min with whole heat-inactivated human serum to block remaining nonspecific sites. Cells were then incubated for 30 min at 4 °C with the appropriate Ab or with the irrelevant control in PBS containing 2% heat-inactivated human serum. The intracellular staining for vimentin, desmin, cytocheratin, and vWF was evaluated in permeabilized cells. Cells (2×10^6) were fixed in 1% paraformaldehyde at 4 °C for 20 min and with the appropriate Ab at 4 °C for 45 min in permeabilizing solution (PBS containing 0.1% saponin, 1% bovine serum albumin, and 0.1% sodium azide). When needing a second-step reagent, cells were stained with fluorescein isothiocyanate-conjugated goat anti-mouse or -rabbit IgG and incubated for another 30 min at 4 °C. Cells were analyzed by FACS (BD Biosciences, Mountain View, CA). 10,000 cells were analyzed for each experimental point.

Assessment of Apoptosis—Apoptosis was evaluated using TUNEL assay analysis (ApopTag Oncor, Gaithersburg, MD). After serum withdrawal or treatment with 0.25 μg/ml vincristine for 24 h in the presence or absence of sCD154 (100 ng/ml) and the enhancer cross-linking antibody (1 μg/ml), cells were suspended in PBS, fixed in 1% paraformaldehyde in PBS, pH 7.4, for 15 min at 4 °C, and then in precooled ethanol/acetic acid (2:1) for 5 min at −20 °C. Cells were treated with terminal deoxynucleotidyl transferase (TdT) enzyme, incubated in an humidified chamber at 37 °C for 1 h, and then with warmed fluorescein isothiocyanate-conjugated anti-digoxigenin for 30 min at room temperature. After washing, samples were mounted in medium containing 1 μg/ml of propidium iodide and the cells analyzed by immunofluorescence.

DNA Fragmentation—Apoptosis is accompanied by fragmentation of DNA (32). To determine the occurrence of DNA fragmentation, total DNA was extracted from unstimulated and stimulated HMEC as previously described (33). The culture medium was removed and centrifuged at $3000 \times g$ for 5 min to collect detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, pH 8, containing EDTA (10 mM) and Triton X-100 (0.5%)) and then pooled with pellets made of detached cells. RNA and proteins were digested using 0.1 mg/ml RNase at 37 °C for 1 h, followed by proteinase K treatment for 2 h at 50 °C. DNA was homogenized by TRI (Sigma) reagent for 10 min at room temperature. After centrifugation, the aqueous phase was gently removed, and the DNA pellet was resuspended with 100% ethanol and centrifuged at 6000 rpm for 5 min at 4 °C. The ethanol was removed, and the DNA pellet was washed twice with a solution containing 0.1 M sodium citrate in 10% ethanol for 30 min at room temperature under agitation and centrifuged at $6000 \times g$ for 5 min at 4 °C. Next, the DNA pellet was suspended in 75% ethanol, shaken for 15 min at room temperature, and centrifuged at $6000 \times g$ for 5 min at 4 °C. The ethanol was removed, and the DNA pellet was briefly air-dried at room temperature. The DNA pellet was dissolved in 8 mM NaOH by slowly passing through a pipette and centrifuged at 10,800 rpm for 10 min at 4 °C. The supernatant was collected and resolved on a 2% agarose gel, stained with 0.5 mg/ml ethidium bromide.

Cell Proliferation Assay—Cells were seeded at 8000 cells/well into 96-well plates in DMEM medium containing 10% FCS and left to adhere. DNA synthesis was detected as incorporation of 5-bromo-2′-deoxyuridine (BrdUrd) into the cellular DNA using an ELISA kit (Roche Applied Science), following the manufacturer's instructions. Briefly, after washing, cells were added with 10 μM BrdUrd, incubated in DMEM without FCS, and stimulated or not with sCD154 (100 ng/ml of sCD154 and 1 μg/ml enhancer cross-linking Ab) or in DMEM plus 10% FCS for 18 h. Cells were then fixed with 0.5 M ethanol/HCl and incubated with nuclease to digest the DNA. BrdUrd incorporated into the DNA was detected using an anti-BrdUrd peroxidase-conjugated mAb and visualized with a soluble chromogenic substrate. Optical density was measured with an ELISA reader at 405 nm.

In Vitro Cell Migration—A total of 1×10^5 cells/well were plated and rested for 12 h in DMEM containing 1% FCS, then washed three times with phosphate-buffered saline, and incubated with DMEM containing 0.25% bovine serum albumin in the presence or absence of sCD154 (100 ng/ml) and the enhancer, a cross-linking Ab (1 μg/ml). Cell division did not start to any significant degree during the experiments. Cell migration was studied over a 4-h period under a Nikon Diaphot-inverted microscope with a ×20 phase-contrast objective in an attached, hermetically sealed plexiglas Nikon NP-2 incubator at 37 °C (6). Cell migration was recorded using a JVC-1CCD video camera. Image analysis was performed with a MicroImage analysis system (Casti Imaging srl, Venice, Italy) and an IBM-compatible system equipped with a video card (Targa 2000; Truevision, Santa Clara, CA). Image analysis was performed by digital saving of images at 15-min intervals. Migration tracks were generated by marking the position of the nucleus of individual cells on each image. The net migratory speed (velocity straight line) was calculated with MicroImage software based on the straight line distance between the start and end points divided by the time of observation (6). Migration of at least 30 cells was analyzed for each experimental condition. Values are given as means ± 1 S.D.

In Vitro Tube Formation—In vitro formation of tubular structures (34) was studied on growth factor-reduced Matrigel diluted 1:1 in ice with cold DMEM. To evaluate the endothelial tube formation, HMEC were washed twice with phosphate-buffered saline, detached with 1% trypsin, and seeded (5×10^4 cells/well) onto Matrigel-coated wells in DMEM containing 0.25% bovine serum albumin in the presence or absence of sCD154 (100 ng/ml) and the enhancer, a cross-linking Ab (1 μg/ml). Cells were periodically observed with a Nikon-inverted micro-

scope, and experimental results were recorded at different times. Image analysis was performed with the MicroImage analysis system (Casti Imaging srl).

Akt Kinase Assay—To assay for Akt kinase activity, cells were serum-starved, submitted to different experimental conditions, washed twice in cold phosphate-buffered saline, and lysed in ice with 900 μl of lysis buffer containing 1% Triton X-100, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na_2PP_i, and 1 mM Na_3VO_4 as previously described (33). Equal amounts of lysates (300 μg) were precleared by centrifugation and preabsorbed with protein A-protein G (1:1) agarose slurry. Immunoprecipitation was carried out for 18 h using the immobilized anti-Akt1G1 mAb (Cell Signaling Technology) cross-linked to agarose. Immunoprecipitates were washed three times with lysis buffer and twice with Akt kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$). Kinase assays were performed for 30 min at 30 °C under continuous agitation in kinase buffer containing 200 μM ATP, 1 μg of GSK-3 fusion protein, according to the manufacturer's instructions for the non-radioactive Akt kinase assay (Cell Signaling Technology). Samples were analyzed by Western blot analysis using 12% SDS-polyacrylamide gel and anti-HRP conjugated anti-rabbit Ab and HRP-conjugated anti-biotin Ab (Cell Signaling Technology). Data for the kinase activity are expressed as fold induction with respect to the activity exhibited by control HMEC. In parallel, to assess the level of expression of Akt, the same amounts of immunoprecipitates were submitted by Western blot, using polyclonal goat antibody against human Akt (1 μg/ml) as previously described (33).

Immunoprecipitation and Western Blot Analysis—HMEC cells were lysed at 4 °C for 1 h in a lysis buffer (50 mM Tris-HCl, pH 8.3, containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 units/ml aprotinin) and centrifuged at 15,000 $\times$ *g*. Immunoprecipitation with anti-CD40 polyclonal goat IgG cross-linked to Sepharose-protein A was performed as described (33, 35). The protein contents of the supernatants and of the immunoprecipitates were measured by the Bradford method. Aliquots containing 30 μg of protein per lane of the immunoprecipitates or of the cell lysates were subjected to 10% SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membrane filters. The blots were blocked with 5% nonfat milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl plus 0.1% Tween (TBS-T). The membranes were subsequently immunoblotted overnight at 4 °C with the relevant primary antibodies or the irrelevant isotypic controls at the appropriate concentration. After extensive washing with TBS-T, the blots were incubated for 1 h at room temperature with peroxidase-conjugated isotype-specific secondary antibodies (Santa Cruz Biotechnology), washed with TBS-T, developed with ECL detection reagents (Amersham Biosciences) for 1 min, and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY).

RESULTS

HMEC expressed CD40, as evaluated by cytofluorimetric analysis (Fig. 1*A*). TUNEL assay showed a marked increase in HMEC apoptosis after 24 h of starving, without FCS or treatment with 0.25 μg/ml of vincristine (Fig. 1*B*). Stimulation of CD40 with sCD154 prevented apoptosis induced both by serum deprivation or treatment with vincristine. Soluble CD40-muIg fusion protein inhibited the anti-apoptotic effect elicited by sCD154, preventing the interaction between sCD154 and the CD40 expressed by HMEC (Fig. 1*B*). This result suggests that the anti-apoptotic effect of sCD154 was mediated by the CD40-dependent signaling. Treatment of HMEC with two unrelated PI3K pharmacological inhibitors, wortmannin (0.1 μM) and LY294002 (10 μM) abrogated the anti-apoptotic effect of sCD154, suggesting that this effect was dependent on the activation of PI3K. Wortmannin and LY294002 also inhibited the sCD154-induced *in vitro* cell motility (Fig. 2). The tube formation in Matrigel was absent in unstimulated HMEC cells (Fig. 3*A*). As shown in Fig. 3*B*, sCD154 induced a rapid formation of vessel-like tubular structures of endothelial cells. Soluble CD40-muIg fusion protein inhibited vessel-like formation elicited by sCD154 (not shown). The sCD154-induced *in vitro* vessel-like formation was significantly reduced by treatment with wortmannin and LY294002 (Fig. 3, *C* and *D*).

Apoptosis of HMEC induced by serum deprivation and treatment with vincristine was associated with an impairment of

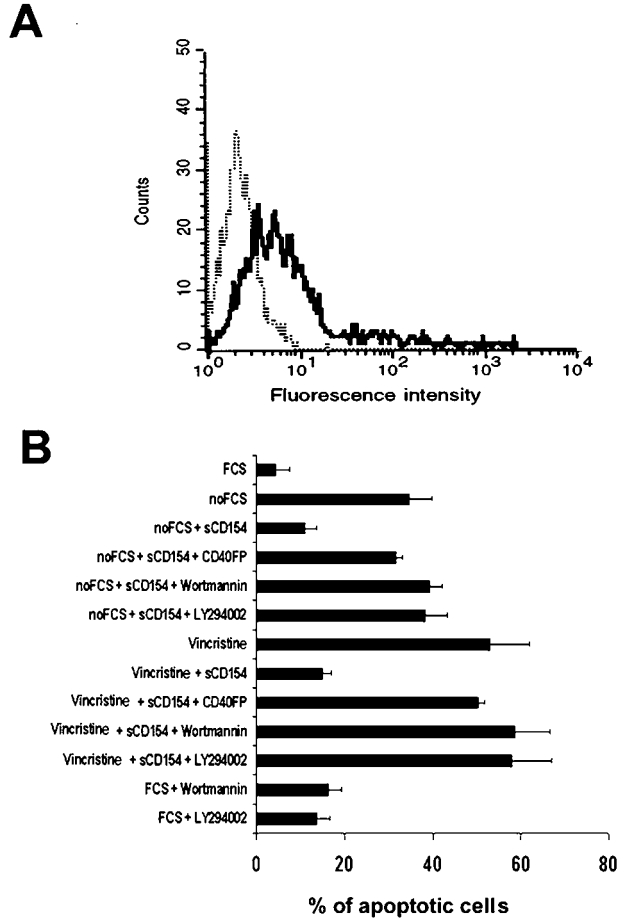

FIG. 1. **Expression of CD40 by HMEC and effect of CD40 stimulation on HMEC apoptosis.** *Panel A*, cytofluorimetric analysis of CD40 expression by HMEC. The figure is representative of three individual experiments. In each experiment the Kolmogorov-Smirnov statistical analysis between anti-CD40 IgG2a mAb (*solid line*) and the isotypic control (*dotted line*) was significant ($p < 0.05$). *Panel B*, apoptosis was evaluated by TUNEL assay as percentage of apoptotic cells after 24-hour serum withdrawal (*noFCS*) or treatment with 0.25 μg/ml vincristine. Where indicated, cells were stimulated with 100 ng/ml of sCD154 (plus 1 μg/ml enhancer) alone or in the presence of 20 ng/ml CD40-muIg fusion protein (*CD40FP*) or of 0.1 μM wortmannin or 10 μM LY294002. As control, cells were incubated in the presence of 10% FCS or with FCS plus 0.1 μM wortmannin or plus 10 μM LY294002. Data are expressed as mean $\pm$ 1 S.D. from three different experiments.

Akt phosphorylation (P-Akt) that was restored by treatment with sCD154 (Fig. 4). This effect was dependent on CD40 stimulation since it was inhibited by the soluble CD40-muIg fusion protein. When PI3K was inhibited by wortmannin and LY294002, the CD40-induced phosphorylation of Akt was significantly prevented.

To evaluate whether Akt enzymatic activity was directly induced by CD40 stimulation, Akt activity was measured in HMEC following incubation with sCD154 for different times. As shown in Fig. 5, *A* and *B*, sCD154 induced a rapid and sustained enhancement of Akt enzymatic activity. This Akt activation was not inhibited by cycloheximide, an inhibitor of protein synthesis, suggesting that a CD40-induced synthesis of secondary mediators is not critical for Akt activation (not shown). It is known that TNF receptor family members, including CD40, transduce signals through TRAF family proteins. In particular an interaction between TRAF6, c-Cbl protein, and PI3K in dendritic cells (29) has been shown. In the present study we evaluated whether endothelial CD40 after stimulation with sCD154 associates TRAF family proteins, c-Cbl, and

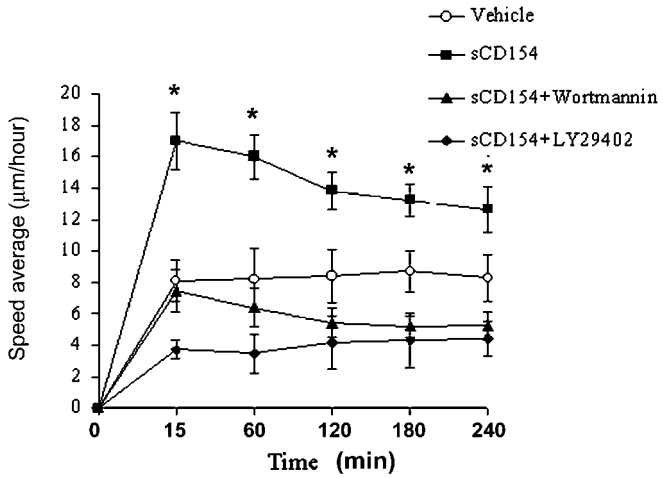

FIG. 2. **Effect of PI3K pharmacological inhibitors on sCD154-induced motility of HMEC.** Time course of HMEC motility (1×10^5 cells) induced by sCD154 (100 ng/ml, plus 1 μg/ml enhancer) in the presence or absence of 0.1 μM wortmannin or 10 μM LY294002. Motility was measured by time-lapse cinematography and digital image analysis as described under "Experimental Procedures." As control, HMEC were incubated with vehicle alone. Results are expressed as means ± 1 S.D. from three individual experiments. Analysis of variance with Newmann-Keul's multicomparison test was performed for sCD154 *versus* control or for sCD154 *versus* sCD154 + wortmannin or sCD154 + LY294002. *, $p < 0.05$.

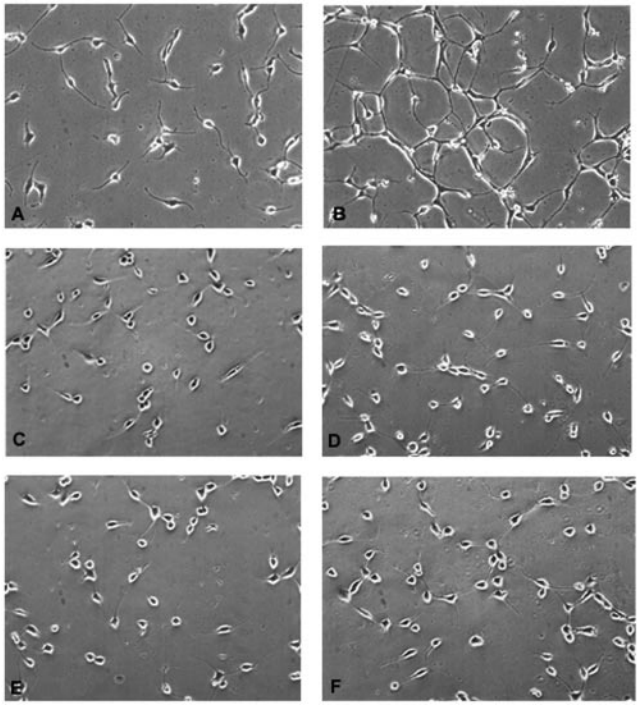

FIG. 3. **Micrographs representative of *in vitro* formation of vessel-like structures by HMEC after CD40 stimulation.** Tube formation by HMEC (5×10^4 cells) plated on growth factor reduced Matrigel (see "Experimental Procedures") was evaluated after stimulation of HMEC for 5 h at 37 °C with vehicle alone (*A*), sCD154 (100 ng/ml, plus 1 μg/ml enhancer) (*B*), or sCD154 (100 ng/ml, plus 1 μg/ml enhancer) in the presence of 0.1 μM wortmannin (*C*) or 10 μM LY294002 (*D*). ND-Akt HMEC were stimulated with vehicle alone (*E*) or sCD154 (*F*). Magnification: ×120.

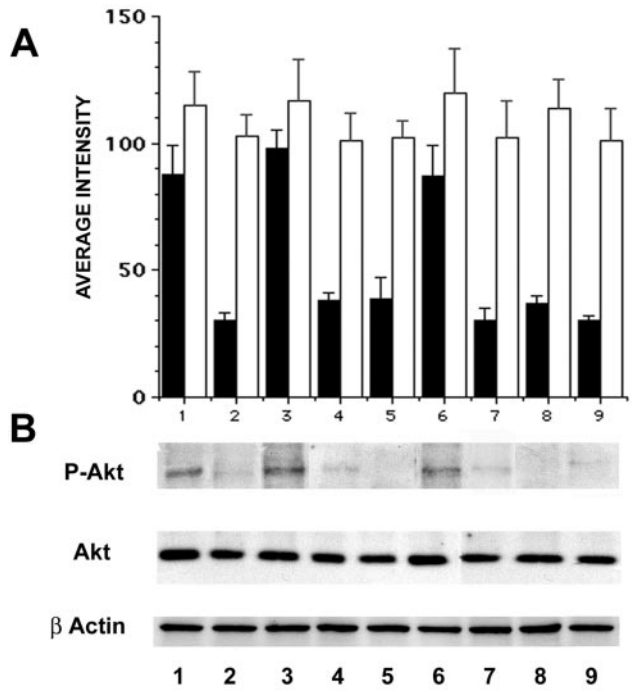

FIG. 4. **Effect of treatment with sCD154 and PI3K pharmacological inhibitors on Akt phosphorylation in HMEC.** Cell lysates (30 μg of protein) were immunoblotted with anti-P-Akt, -Akt, or -β-actin antibodies. *Panel A*, densitometric analysis and *panel B*, representative immunoblot of P-Akt expression (*panel A*, *black column*) and Akt expression (*panel A*, *open column*). *Row 1*, control HMEC cultured in DMEM containing 10% FCS; *row 2*, HMEC serum starved for 24 h; *row 3*, HMEC serum-starved treated with sCD154 (100 ng/ml, plus 1 μg/ml enhancer); *row 4*, HMEC serum-starved treated with sCD154 and 20 ng/ml CD40-muIg fusion protein; *row 5*, HMEC treated with 0.25 μg/ml vincristine; *row 6*, HMEC treated with 0.25 μg/ml vincristine plus sCD154 (100 ng/ml, plus 1 μg/ml enhancer); *row 7*, HMEC treated with 0.25 μg/ml vincristine plus sCD154 (100 ng/ml, plus 1 μg/ml enhancer) and 20 ng/ml CD40-muIg fusion protein; *row 8*, HMEC treated with 0.25 μg/ml vincristine plus sCD154 (100 ng/ml, plus 1 μg/ml enhancer) and 0.1 μM wortmannin; *row 9*, HMEC treated with 0.25 μg/ml vincristine plus sCD154 (100 ng/ml, plus 1 μg/ml enhancer) and 10 μM LY294002. *Panel A*, data are expressed as mean ± 1 S.D. from three different experiments.

the p85 subunit of PI3K. Immunoprecipitation of CD40 followed by Western blotting showed that stimulation of endothelial CD40 induced rapid association of TRAF2 and 6, c-Cbl, and the p85 PI3K subunit (Fig. 5*C*). Such association was temporarily correlated with enhanced Akt activity (Fig. 5*A*). The association of TRAF3 to CD40 was minimal (Fig. 5*C*).

In order to evaluate the relevance of Akt in the biological activities elicited by CD40 stimulation in HMEC, we developed negative-dominant HMEC for Akt by transfecting the cells with Akt1 cDNA containing the mutation of lysine 179 to methionine. Expression of ND-Akt has been shown to interfere with the activation of endogenous Akt1 (36, 37). Fig. 6 (*A* and *B*) compares Akt activity after CD40 stimulation with sCD154 in ND-Akt and in WT-Akt HMEC, transfected with an empty vector as control. At variance with the WT-Akt HMEC, ND-Akt HMEC did not display enhancement of Akt activity, despite the observation that the pathway of CD40-dependent PI3K activation was intact. Indeed, no differences in the association of CD40 with TRAF6, c-Cbl, and PI3K was observed after stimulation with sCD154 (Fig. 6*C*). As shown in Fig. 7, the anti-apoptotic effect of CD40 stimulation was abrogated in ND-Akt but not in WT-Akt HMEC. ND-Akt showed an enhanced apoptosis also in basal conditions. In addition, the proliferative response of HMEC to CD40 stimulation observed in WT-Akt, was impaired in ND-Akt cells (Fig. 8*A*). Moreover, the *in vitro* formation of vessel-like tubular structures by CD40-stimulated HMEC plated on Matrigel was reduced in ND-Akt cells (Fig. 3, *E* and *F*), suggesting a role of Akt activation in the coordinate migration of endothelial cells. Indeed, as shown by the time-lapse analysis of HMEC motility, WT-Akt exhibited an en-

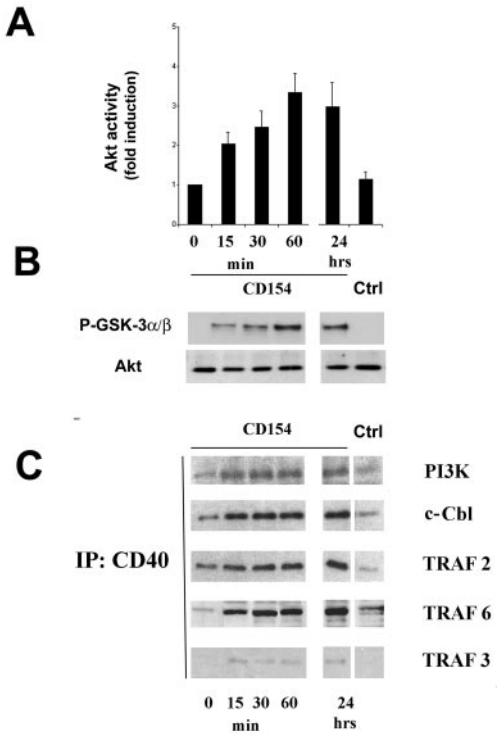

FIG. 5. **Time course of Akt kinase activity and association of PI3K, c-Cbl, and TRAFs with CD40 upon sCD154 stimulation of HMEC.** HMEC were treated for the indicated times with sCD154 (100 ng/ml, plus 1 μg/ml enhancer) and lysed. As control (*Ctrl*), cells were treated with vehicle alone. Kinase reactions and Western blot analysis were performed in anti-Akt immunoprecipitates from the corresponding lysates, as described under "Experimental Procedures." Akt activity was assessed using GSK-3α/β as substrate for phosphorylation (P-GSK-3α/β). *A*, densitometric analysis of Akt kinase activity expressed as fold increase with respect to unstimulated cells (mean ± 1 S.D. from three different experiments). *B*, representative Western blot analysis showing P-GSK-3α/β generation and specific bands detected by the anti-Akt antibody. *C*, after treatment of HMEC with sCD154 (100 ng/ml, plus 1 μg/ml enhancer) for the indicated times, CD40 was immunoprecipitated from cell lysates. The immunoprecipitates were probed with antibodies to PI3K, c-Cbl, TRAF2, TRAF6, and TRAF3. Data are representative from three independent experiments.

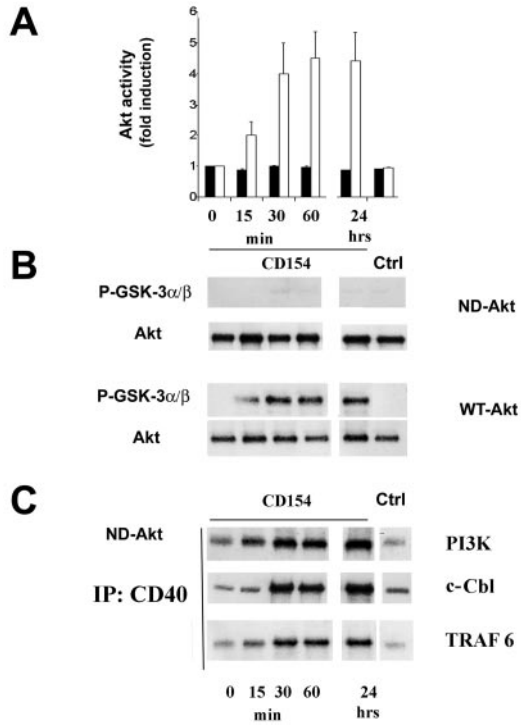

FIG. 6. **Comparison of Akt kinase activity and association of PI3K, c-Cbl, and TRAF-6 with CD40 upon sCD154 stimulation in ND-Akt and WT-Akt HMEC.** Akt-negative-dominant HMEC were generated by transfecting the cells with Akt1 cDNA containing the mutation of lysine 179 to methionine. Control WT-Akt were transfected with the empty vector. ND-Akt and WT-Akt HMEC were treated for the indicated times with sCD154 (100 ng/ml, plus 1 μg/ml enhancer) and lysed. Kinase reactions and Western blot analysis were performed in anti-Akt immunoprecipitates from the corresponding lysates, as described under "Experimental Procedures." *A*, densitometric analysis of Akt kinase activity was expressed as fold increase with respect to unstimulated cells (mean ± 1 S.D. from three different experiments). ND-Akt, *dark column*; WT-Akt, *open column*. *B*, representative Western blot showing P-GSK-3α/β generation and specific bands detected by the anti-Akt antibody in ND-Akt and WT-Akt HMEC. *C*, after treatment of ND-HMEC with sCD154 (100 ng/ml, plus 1 μg/ml enhancer) for the indicated times, CD40 was immunoprecipitated from cell lysates, and the immunoprecipitates were probed with antibodies to PI3K, c-Cbl, and TRAF6. Data are representative of three independent experiments.

hanced motility after stimulation with sCD154 compared with control whereas ND-Akt did not (Fig. 8*B* and Fig. 9).

DISCUSSION

In the present study we demonstrated that resistance to apoptosis and the *in vitro* vessel-like formation elicited in endothelial cells after CD40 stimulation were dependent on the activation of Akt. This activation was triggered by PI3K that associated with CD40 after its ligation by sCD154.

The stimulation of endothelial CD40 plays an important role in the phenotypic modulation of the endothelium to an activated state (10). CD40 was shown to induce expression of collagenase and stromelysin on human monocytes/macrophages, and of collagenase, stromelysin, gelatinase B, and activated gelatinase A on vascular smooth muscle and endothelial cells (11–14). Ligation of endothelial CD40 by CD154, either expressed on activated monocytes or T cells or disgorged from platelet granules after activation, stimulated the production of various inflammatory cytokines by endothelial cells (11, 13). Moreover, it has been reported that surface-expressed CD154 is rapidly cleaved with generation of a circulating soluble CD154, which remains trimeric and biologically active (10). A soluble form of CD154 released from the surface of tumor cells (38) may contribute to endothelial activation in tumor angiogenesis. *In vivo* stimulation of endothelial CD40 was shown to trigger neoangiogenesis and its inhibition limited neoangiogenesis and allowed apoptotic regression in an experimental model of tumor neoangiogenesis (6).

In the present study we found that stimulation of endothelial CD40 with sCD154 prevented apoptosis induced both by serum deprivation or treatment with vincristine and induced motility and vessel-like formation by HMEC. These effects were mediated by CD40-dependent signaling because it was abrogated by preventing the interaction between sCD154 and CD40 by a soluble CD40-muIg fusion protein. In addition, we found that apoptosis of HMEC was associated with an impairment of Akt phosphorylation, which was restored by sCD154. The anti-apoptotic effect of sCD154 as well as cell motility, vessel-like formation, and Akt phosphorylation were inhibited by treatment of HMEC with two unrelated pharmacological inhibitors of PI3K, wortmannin and LY294002, suggesting that the effect was dependent on the activation of this kinase.

PI3K/Akt is one of the central pathways involved in survival signaling (39). Several receptors, including those for VEGF (40), IGF-1 (41), and IL-3 (42), transmit survival signals through these pathways. PI3K activation catalyzes the transfer of a phosphate group from ATP to the D3 position of phosphatidylinositol (PI), thus generating 3′-phosphatidylinositol phos-

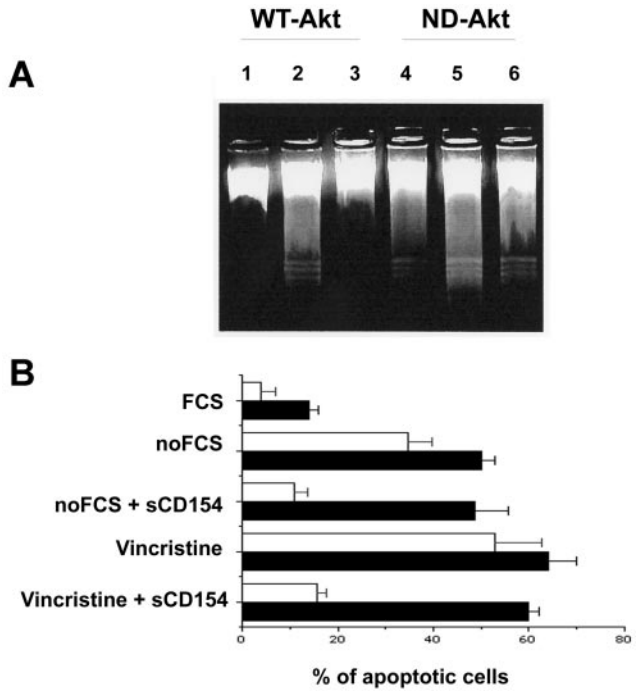

FIG. 7. **Abrogation of anti-apoptotic effect of CD40 stimulation in ND-Akt HMEC.** *A*, DNA fragmentation was detected after DNA extraction and electrophoresis on 2% agarose gel. *Row 1*, control WT-Akt HMEC cultured in DMEM containing 10% FCS; *row 2*, WT-Akt HMEC serum-starved for 24 h; *row 3*, WT-Akt HMEC serum-starved treated with sCD154 (100 ng/ml, plus 1 μg/ml enhancer); *row 4*, control ND-Akt HMEC cultured in DMEM containing 10% FCS; *row 5*, ND-Akt HMEC serum-starved for 24 h; *row 6*, ND-Akt HMEC serum-starved treated with sCD154 (100 ng/ml, plus 1 μg/ml enhancer). *B*, apoptosis of HMEC evaluated by TUNEL assay after 24 h incubation with different stimuli (see "Experimental Procedures"). ND-Akt (*dark column*) and WT-Akt (*open column*) HMEC were incubated with 10% FCS, without FCS (*noFCS*), without FCS plus sCD154 (100 ng/ml, plus 1 μg/ml enhancer) or with 0.25 μg/ml vincristine or 0.25 μg/ml vincristine plus sCD154 (100 ng/ml, plus 1 μg/ml enhancer). Data are expressed as mean ± 1 S.D. from three different experiments.

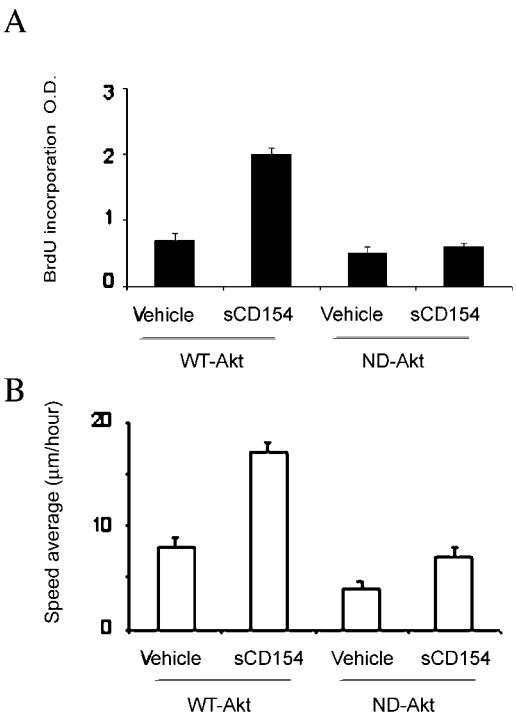

FIG. 8. **Reduction of proliferation and motility induced by CD40 stimulation in ND-Akt HMEC.** *A*, proliferation was evaluated by BrdUrd incorporation in ND-Akt and WT-Akt HMEC after 18-hour stimulation with vehicle alone or sCD154 (100 ng/ml, plus 1 μg/ml enhancer). *OD*, optical density. *Panel B*, cell motility, measured by time-lapse cinematography and digital image analysis, was evaluated after 4 h of stimulation with vehicle alone or sCD154 (100 ng/ml, plus 1 μg/ml enhancer) in ND-Akt and WT-Akt HMEC. Data are expressed as mean ± 1 S.D. from three different experiments.

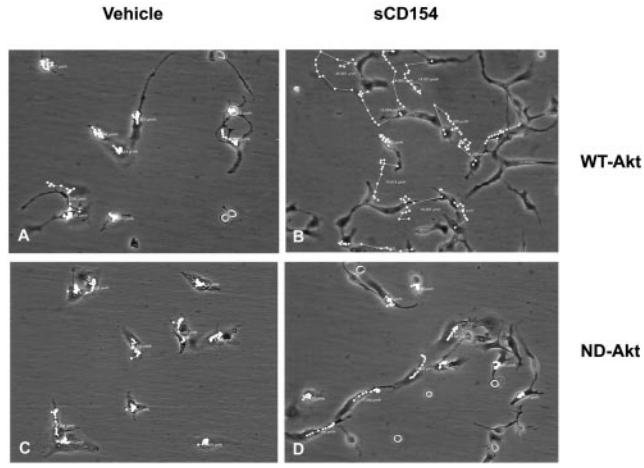

FIG. 9. **Micrographs representative of time-lapse analysis of WT-Akt and NT-Akt HMEC motility after stimulation with sCD154.** Motility was performed by digital saving at 15-min intervals. Migration tracks (magnification: ×120) were generated by marking the position of the nucleus of individual cells in each image (see "Experimental Procedures"). WT-Akt (*A* and *B*) and ND-Akt (*C* and *D*) HMEC were stimulated for 4 h at 37 °C with vehicle alone (*A* and *C*) or with sCD154 (100 ng/ml, plus 1 μg/ml enhancer) (*B* and *D*).

phates (43). 3′-Phosphatidylinositol phosphates serve as binding sites for proteins that possess a pleckstrin homology domain such as Akt. The binding of Akt to 3′-phosphatidylinositol phosphates results in its translocation from cytosol to plasma membrane and phosphorylation of threonine 308 and serine 473 residues. Phosphorylation of threonine 308 and membrane localization depend on the activation of a phosphatidylinositol-dependent kinase-1 that also contains a pleckstrin homology domain (44). Several studies have shown that Akt is the major effector of PI3K survival signaling (39, 45, 46).

In the present study we found that a rapid increase in Akt enzymatic activity was induced by CD40 stimulation. Although, CD40 is known to stimulate the synthesis of cytokines that may activate Akt (19), we found that Akt activation was rapid and independent from protein synthesis, suggesting a direct effect of CD40 stimulation.

CD40 is a member of the TNF receptor family, which lacks intrinsic enzymatic activity but is linked to intracellular signaling cascades through TRAF proteins (24–28). Activation of CD40-dependent signaling pathways is thought to be mediated primarily by recruitment of several members of the TRAF protein family to the multimerized CD40 cytoplasmic domain (23). The CD40 cytoplasmic domain contains a membrane-proximal site that binds TRAF6 and a membrane-distal site that binds TRAF1, TRAF2, and TRAF3 (24, 25). We found that TRAF6 and TRAF2 co-precipitated with CD40 after stimulation of HMEC with sCD154. No significant enhancement of TRAF3 binding to CD40 was observed after sCD154 stimulation of endothelial cells. It has been described that TRAF3 up-regulation by shear stress abrogates CD40 signaling in endothelial cells (47). In contrast, the binding of CD40-TRAF2 domain has been associated with the activation of transcription factors and of apoptosis-regulating proteins (7, 48–52). Recently it has been found that in dendritic cells TRAF6 in concert with c-Cbl mediates the binding and the activation of PI3K

by TNF receptor family members (29). In the present study we found that the enhanced Akt activity induced by stimulation of endothelial CD40 was temporarily correlated with the association of CD40 with TRAF6, c-Cbl, and the p85 subunit of PI3K. c-Cbl, a cytoplasmic adapter molecule implicated in the negative regulation of signaling from a variety of tyrosine kinase receptors, has been recently identified as a positive modulator of the TNF receptor superfamily (29). Information on the critical role of c-Cbl in the interaction of PI3K and in the subsequent activation of Akt was obtained in c-Cbl negative-dominant B cells (29). In this context, it has been proposed that c-Cbl recruited PI3K by favoring association with TRAF6.

In order to evaluate the relevance of Akt activation to biological activity dependent on the stimulation of endothelial CD40, we developed Akt negative-dominant cells by transfecting HMEC with Akt1 cDNA containing the mutation of lysine 179 to methionine (37). Expression of ND-Akt has been shown to interfere with activation of the endogenous Akt1, suggesting that it displaced endogenous Akt from critical protein-protein interactions (36, 37). CD40 stimulation in ND-Akt HMEC failed to enhance Akt activity, despite the observation that the association of CD40 with TRAF6, c-Cbl, and PI3K was intact. The defective activation of Akt abrogated not only the antiapoptotic effect of CD40 stimulation but also the proliferative response, the *in vitro* formation of vessel-like tubular structures, and the enhanced motility of HMEC.

In conclusion, these results suggest that the PI3K/Akt signaling axis was activated by endothelial CD40 stimulation and regulated multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and vessel-like structure formation.

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CD40-dependent Activation of Phosphatidylinositol 3-Kinase/Akt Pathway Mediates Endothelial Cell Survival and *in Vitro* Angiogenesis

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