Platelet-Activating Factor Mediates CD40-Dependent Angiogenesis and Endothelial-Smooth Muscle Cell Interaction

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The aim of the present study was to investigate whether stimulation of CD40 expressed by endothelial or smooth muscle cells triggers the synthesis of platelet-activating factor (PAF), an inflammatory mediator with angiogenic properties, and whether PAF contributes to CD40-induced neangiogenesis. The results obtained indicate that the interaction of CD40 with soluble CD154 or with CD154 expressed on the membrane of leukocytes (CD154-transfected J558 cells) or of activated platelets, stimulated the synthesis of PAF by endothelial cells but not by smooth cells. The synthesis of PAF triggered by activated platelets was inhibited by a soluble CD40-murine Ig fusion protein that prevents the interaction between membrane CD40 and CD154. Studies with specific inhibitors and evaluation of protein phosphorylation indicated the involvement in PAF synthesis of two intracellular signaling pathways leading to cytosolic phospholipase A2 activation: a phospholipase Cγ-protein kinase C-Raf-p42/p44-mitogen-activated protein kinase (MAPK) and a MAPK kinase-3/6-dependent activation of p38 MAPK. PAF synthesized by endothelial cells after CD40 stimulation was instrumental in the in vitro migration and vessel-like organization of endothelial cells, and in the interaction between endothelial cells and smooth muscle cells, as inferred by the inhibitory effect of two different PAF receptor antagonists, WEB2170 and CV3988. In vivo, blockade of PAF receptors prevented the angiogenic effect triggered by CD40 stimulation in a murine model of s.c. Matrigel implantation. In conclusion, these observations indicate that PAF synthesis induced by stimulation of endothelial CD40 contributes to the formation and organization of new vessels. This may be relevant in the vascular remodeling associated with tumor and inflammatory neangiogenesis. The Journal of Immunology, 2003, 171: 5489–5497.
antagonists, WEB2170 and CV3988. In vivo, the role of PAF in CD40-stimulated neoangiogenesis was evaluated in a murine model of s.c. Matrigel implantation after PAF-R blockade with WEB2170.

Materials and Methods

Reagents
M199, DMEM, and d-valine-modified MEM, BSA fraction V (tested for <1 ng of endotoxin per milligram), poloxymyxin B, PLA₂, PLA₄, EDTA, human thrombin, heparin, trypsin, staurosporine, and FITC-conjugated mouse and anti-rabbit IgG were all purchased from Sigma-Aldrich (St. Louis, MO). FBS and basic fibroblast growth factor (bFGF) were from EuroClone (Wetherby West Yorkshire, UK). Recombinant human soluble (s)CD154 trimeric protein, a cross-linking Ab (enhancer) and CD40-murine Ig (mIg) fusion protein, consisting of the extracellular domain of human CD40 fused to mouse IgG₂a, were from Alexis Biochemicals (San Diego, CA). Agonist rat anti-mouse CD40 was bought from Serotec (clone 3/23, Oxford, U.K.). Fluorescein-conjugated anti-CD40 and anti-CD154 IgG₂a Abs were from EuroClone. Anti-PAF-R polyclonal Abs were obtained from Alexis Biochemicals. Recombinant hirudin, bisindolylmaleimide I, and U73122, were purchased from Calbiochem (La Jolla, CA). 4-Bromodiphenylboron/iodide was from Carlo Erba (Milan, Italy). LY249002 and wortmannin were purchased from Upstate Biotechnology (Lake Placid, NY). Synthetic C₁₆ PAF (1-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine) was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Stock solutions in chloroform were stored at −20°C until use. The chloroform was evaporated, and saline containing 0.25% BSA fraction V, low endotoxin, was added immediately before use. PAF-R antagonist C₅₃₉₈₈₉₉₈ (25) was from Takeda Chemical Industries (Kyoto, Japan). PAF-R antagonist WEB2170 (26) was obtained from Boehringer (Ingelheim, Germany). Silica gel 60F₂₅₄ TLC plates were obtained from Merck (Darmstadt, Germany). μPorous HPLC columns were provided from the Millipore chromatographic division (Waters, Milford, MA). Growth factor-reduced Matrigel was from BD Labware (Bedford, MA). Rabbit polyclonal anti-phospho-p38 mitogen-activated protein kinase (MAPK), anti-phospho-p42/44 MAPK, and anti-phospho PLCy, Abs, and mouse monoclonal anti-β-actin Ab, all used for Western blot analysis, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines
HUVEC were isolated by treatment of human umbilical cord veins with 0.5% trypsin (1 h at 37°C) and cultured with M199 with the addition of 20% FBS, 10 ng/ml bFGF and 100 μg/ml heparin until they reached confluence, as previously described (27). They were used at early passages (II–III). Human SMC were isolated from umbilical cords and identified as described (28). CD154-transfected J558L (29) was kindly provided by Dr. A. Mantovani (Istituto Mario Negri, Milan, Italy).

PAF synthesis
In standard PAF synthesis assays, 1×10⁵ HUVEC or SMC were maintained for 12 h in DMEM containing 1% FBS. Cells were then equilibrated for 15 min in Tris-buffered Tyrode containing 0.25% delipidized BSA (fraction V), as previously described (15), and incubated at 37°C for the indicated times with the different stimuli. Selected experiments were conducted in the presence of 5 μg/ml polybixin B for 30 min at 37°C to exclude LPS contamination. To obtain CD40 activation, cells were stimulated with recombinant human sCD154 (100 ng/ml). All experiments were performed in the presence of a cross-linking Ab (enhancer, 1 μg/ml). Alternatively, cells were stimulated by a direct coinoculation with 1×10⁶ J558L stably transfected to express CD154 (29), or J558 cells transfected with only the empty vector. In other experiments, HUVEC were stimulated with activated platelet expressing CD154, according to the protocol of Urbich et al. (30). Briefly, platelets were isolated from full blood of healthy human donors and activated with 0.2 U/ml human thrombin for 5 min at 37°C, and then thrombin was neutralized with 2 U/ml hirudin. Platelets (1.5×10⁶) were then coincubated with HUVEC for various times at 37°C. To exclude the effective expression of receptors, the cells were characterized by cytodiagnostic analysis. To block the CD154-induced effects, cells were stimulated with sCD154 in the presence of 20 ng/ml CD40-mulg fusion protein. As positive control, HUVEC were stimulated with 1 U/ml human thrombin. For the inhibition experiments, HUVEC were preincubated for 10 min at 37°C with one of the following chemicals: 10 mM EDTA, 1 μM 4-bromodiphenylboron, 100 nM staurosporine, 50 μM bisindolylmaleimide I, 10 μM U73122, or 10 μM SB203580.

Extraction and quantification of PAF
The supernatants and the cell pellets were extracted according to a modification of the Bligh and Dyer (31) procedure, with formic acid added to lower the pH of the aqueous phase to 3.0. Each individual experiment was performed in triplicate. PAF was quantified after extraction and purification by TLC (60F₂₅₄ silica-gel plates; Merck) by aggregation of washed rabbit platelets, as previously reported (15, 27). The biologically active material extracted from cells and supernatants in different experiments was characterized by comparison with synthetic PAF according to the following criteria (15, 27): 1) induction of platelet aggregation by a pathway independent from both ADP and arachidonic acid/thromboxane A₂-mediated pathways; 2) specificity of platelet aggregation as inferred from the inhibitory effect of 5 μM WEB2170 or CV3988, two different PAF receptor antagonists (25, 26); 3) TLC and HPLC behavior and physicochemical characteristics, such as inactivation by strong bases and by PLA₂ treatment, but resistance to PLA₂, acids, weak bases, and 5 min of heating in boiling water. For the radioactive assay of PAF synthesis, 1×10⁶ HUVEC were incubated in 1 ml of DMEM for 30 min with 30 μCi of [H]acetate before each stimulation (16). The cell pellets were extracted according to a modification of the Bligh and Dyer (31) procedure, and lipids were fractionated by TLC on aluminum-sheet silica-gel plates (silica gel 60F₂₅₄; 0.2-mm thickness; Merck) using a solvent of chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v). The plates were cut into 1-cm sections, and the radioactivity of each section was measured. Radiolabeled [H]PAF (NEN Life Science Products) was used as a standard.

Western blot analysis
After stimulation with sCD154, HUVEC were lysed at 4°C for 15 min in 1× PBS containing 1% Triton X100 (150 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.1% Igepal, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.4 mM sodium orthovanadate, plus 1 mM PMSF, 10 μg/ml leupeptin, and 100 U/ml aprotinin) and centrifuged at 15,000 × g for 1 h. The protein contents of the supernatants were measured by the Bradford method. Aliquots containing 50 μg of protein per lane of the cell lysates were subjected to 8 or 12% SDS-PAGE, according to the molecular mass of the protein of interest, under reducing conditions and electrobotted onto nitrocellulose membrane filters. The blots were blocked with PBS plus 0.5% Tween and 10% BSA. The membranes were subsequently immunoblotted overnight at 4°C with the relevant primary Abs or the irrelevant isotypic controls at the appropriate concentration. After extensive washings, the blots were incubated for 1 h at room temperature with peroxidase-conjugated isotype-specific secondary Abs (Santa Cruz Biotechnology), developed with ECL detection reagents (Amersham Biosciences, Arlington Heights, IL), for 2 min, and exposed to X-Omat film (Eastman Kodak, Rochester, NY).

Cytosolic PLA₂ (cPLA₂) assay
The release of calcium-dependent cPLA₂, which exhibits specificity toward arachidonic acid, was measured with an enzymatic assay kit purchased from Cayman Chemicals (Ann Arbor, MI). Briefly, HUVEC stimulated at 37°C for 10 min with sCD154 were detached with a nonenzymatic cell dissociation solution (Sigma-Aldrich), collected, and centrifugated at 1000 × g for 10 min, and then the pellet was sonicated in 1 ml of cold buffer (Tris-HCl containing 50 mM HEPES and 1 mM EDTA, plus 1 mM PMSF, 10 μg/ml leupeptin, and 100 U/ml aprotinin [pH 7.4]). After centrifugation at 10,000 × g for 15 min at 4°C, the supernatants were frozen at −80°C, and an amount was used for the assay kit according to the manufacturer’s protocol.

In vitro cell migration
A total of 1×10⁵ cells/well were plated and rested for 12 h in DMEM containing 1% FBS, and then washed three times with PBS and incubated with DMEM containing 0.25% BSA and the agonists. For inhibition studies, in a selected experimental group, cells were preincubated with WEB2170 (5 μM), or with CV3988 (5 μM) for 10 min. Cell division did not start to any significant degree during the experiments. Cell migration was studied over a 4-h period under a Nikon (Melville, NY) Diaphot inverted microscope with a ×20 phase-contrast objective in an attached, hermetically sealed Plexiglas Nikon NP-2 incubator at 37°C (5). Cell migration was recorded using a cell tracker dye (Invitro Dynamics, Tokyo, Japan). A 1-CD video camera. Image analysis was performed with a MicroImage analysis system (Cast Imaging, Venice, Italy) and an IBM-compatible system equipped with a video card (Targa 2000; True Vision, 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 nucleus of individual cells on each image. The net migratory speed (straight-line velocity) was calculated by the MicroImage software based on the straight-line distance between the
starting and ending points divided by the time of observation (5). Migration of at least 30 cells was analyzed for each experimental condition. Values are given as means ± SD.

In vitro tube formation

In vitro formation of tubular structures (32) and interaction between HUVEC and SMC were studied on growth factor-reduced Matrigel diluted 1/1 in ice with cold DMEM. To evaluate the endothelial-tube formation, HUVEC were washed twice with PBS, detached with 1% trypsin, and seeded (5 × 10^5 cells/well) onto Matrigel-coated wells in DMEM containing 0.25% BSA. Cells were periodically observed with a Nikon inverted microscope, and experimental results were recorded at different times. Image analysis was performed with the MicroImage analysis system (Cast Imaging). To investigate the in vitro interaction between endothelial cells and SMC, HUVEC (1 × 10^6 cells) were labeled with the green fluorescent cell linker PKH26 (2 μM) according to the instructions of the manufacturer (Sigma-Aldrich). Cells maintained the fluorescence staining for several days (33). Fluorescent HUVEC were detached, washed, and seeded (5 × 10^4 cells/well) onto Matrigel-coated wells. HUVEC were then allowed to form spontaneous tubes by overnight incubation in DMEM containing 5% FBS, washed three times with PBS, and cultured with DMEM plus 0.25% BSA. SMC (1.5 × 10^5 cells/well), stained with red fluorescent cell linker PKH26 dye, were detached, washed, and then added to the spontaneously formed endothelial tubes, in the presence of vehicle alone or of sCD154 (100 ng/ml; plus 1 μg/ml enhancer). After 2 h of incubation, the number of SMC associated with endothelial tubes was counted. Pretreatment of SMC with PAF-R antagonist, WEB2170 (5 μM), 10 min before the addition to the formed endothelial tubes, was performed to evaluate whether PAF synthesized by activated endothelial cells mediated HUVEC-SMC interaction.

Cell proliferation assay

Cells were seeded at 10,000 cells/well into 24-well plates in DMEM containing 10% FCS and allowed to adhere. After starvation for 24 h, cells were incubated with different stimuli. After 24- or 48-h incubation, monolayers were carefully washed, dried, and treated with 0.75% crystal violet in a solution of 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde. After washings, the dye was eluted with 1% SDS in PBS, and the absorbance was read at 595 nm with an ELISA reader. Cell number was determined on the basis of a standard curve obtained with known cell numbers of triplicate samples. All experiments were performed in triplicate.

Cell viability assays

Cell viability, in different experimental conditions, was evaluated by the sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT)-based assay and by the lactate dehydrogenase (LDH) release assay. Cells were cultured in 96-well flat-bottom microtiter plates (Falcon Labware, Oxnard, CA) at a concentration of 5 × 10^4 cells/well. Cell were appropriately stimulated and, at different periods of time, washed and incubated in serum-free DMEM containing 250 μM/μL XTT at 37°C. XTT reduction was monitored by determination of the absorbance values at 620 nm in an automated ELISA reader. LDH activity was measured in the cell-free supernatant using the cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN) and expressed as percentage of Triton X-100 cell lysate.

Murine angiogenesis assay

Female C57 mice were used at 6–8 wk of age. Angiogenesis was assayed as growth of blood vessels from s.c. tissue into a solid gel of basement membrane Matrigel, containing the test sample (34). Matrigel (8.13 mg/ml) in liquid form at 4°C, was mixed with 40 μg/ml agonist rat anti-mouse CD40 (5), or 100 ng/ml sCD154, and injected (0.5 ml) into the abdominal s.c. tissue of mice, along the peritoneal midline. For inhibition studies, in a selected experimental group, a PAF-R antagonist, WEB2170, was added to the Matrigel (5 μM) and to the drinking water (3 mg/kg/day), and was injected s.c. every day (10 mg/kg), for 4 days consecutively. bFGF (10 ng/ml) was used as positive control. At day 7, mice were killed and gels were recovered and processed for histology. The tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections cut at 3 μm and stained with H&E were studied by light microscopy. Vessel area and the total Matrigel area were planimetrically assessed from stained sections, as described by Kibbey et al. (35). Only those structures possessing a patent lumen and containing RBCs were considered vessels (5). Results were expressed as percentage ± SE of the vessel area to the total Matrigel area.

Statistics

Nonparametric statistical analysis was performed by the Kruskal-Wallis test for ANOVA followed by Dunnert’s test for comparison of groups vs one control, and by the Newman-Keuls test for comparison among pairs of groups.

Results

CD40 activation stimulated PAF synthesis by HUVEC but not SMC

Fig. 1 shows the results of experiments performed to investigate whether CD154 induced synthesis of PAF by endothelial cells. The rate of PAF synthesis was determined both by bioassay on washed rabbit platelet aggregation (Fig. 1A) and by incorporation of the [3H]acetate precursor in the PAF molecule (B). Whereas unstimulated HUVEC produce only a minimal amount of PAF, stimulation with sCD154 induced significant PAF synthesis. PAF was also produced by HUVEC incubated with 10 × 10^6 J558L, a murine plasmocytoma stably expressing CD154, but not with the empty transfecant J558 used as control. PAF synthesized by HUVEC after stimulation with sCD154 remained mainly cell associated, because it was undetectable in cell-free supernatants (data not shown). Fig. 1C shows the time course of PAF synthesis by HUVEC after stimulation with sCD154, J558L, and thrombin. PAF synthesis peaked 5 min after stimulation to decrease thereafter. Whereas the synthesis of PAF by thrombin and J558L was transient, the one obtained after stimulation with sCD154 was higher and was sustained up to 60 min.

Because platelets are known to express CD154 after thrombin activation (30), the synthesis of PAF was also studied after incubation of HUVEC with human platelets expressing CD154 upon preincubation with thrombin. Fig. 2A shows the platelet expression of CD154 after 5 min of treatment with 0.2 U/ml thrombin. Platelets, after inhibition of thrombin with 2 U/ml hirudin (30), were incubated with HUVEC. The effective inactivation of thrombin by hirudin was evaluated on washed rabbit platelet aggregation (not shown). Thrombin-activated platelets induced a significant synthesis of PAF by HUVEC (Fig. 2B) with respect to not-activated platelets. The amount of PAF synthesized after stimulation with activated platelets was higher than that synthesized by J558L and comparable with that obtained by HUVEC stimulation with thrombin. The platelet-free supernatant obtained from platelets treated with thrombin and hirudin did not stimulate PAF synthesis, indicating an effective thrombin inhibition. sCD40-muIg fusion protein inhibited the synthesis of PAF induced by thrombin-activated platelets, indicating that sCD40 prevented the interaction between CD154 expressed by platelets and CD40 expressed by HUVEC (Fig. 2B). The time course of PAF synthesis by HUVEC stimulated with CD154-expressing platelets showed a peak at 5 min followed by a sustained synthesis up to 60 min (Fig. 2C).

The synthesis of PAF by SMC stimulated with sCD154 was also studied. By cytofluorimetric analysis, SMC expressed an amount of CD40 comparable with HUVEC (data not shown). However, SMC did not synthesize a significant amount of PAF after stimulation of CD40 (unstimulated SMC, 15 ± 2.5 pg per 1 × 10^6 cells; sCD154-stimulated SMC, 17 ± 3.5 pg per 1 × 10^6 cells).

Intracellular mechanisms of sCD154-induced PAF synthesis

To determine the intracellular pathway by which CD40 activation induced PAF synthesis in endothelial cells, we pretreated HUVEC with a range of selective inhibitors and investigated their effect on sCD154-induced PAF synthesis. Pretreatment of HUVEC with the specific inhibitor of PLCγ (U73122) and protein kinase C (PKC) (staurosporine and bisindolylmaleimide) and of p38 MAPK (SB203580) induced a significant inhibition of sCD154-induced
PAF synthesis (Fig. 3A). As shown in Fig. 3B, sCD154 stimulation of HUVEC induced phosphorylation of PKC, and of p42/44 MAPK, indicating the activation of the cascade involving PLC, PKC, Raf, MAPK kinase, and p42/44 MAPK (36). Indeed, the inhibitory effect on PAF synthesis of U73122 was associated with inhibition of phosphorylation of PKC, and p42/44 MAPK. The
phosphorylation of p42/44 MAPK was also inhibited by bisindolylmaleimide, an inhibitor of PKC. In addition, the involvement of a second cascade implicating p38 MAPK was indicated by phosphorylation of p38 MAPK induced by sCD154 and by the inhibition of PAF synthesis by SB203580 (Fig. 3, A and B). We recently found that CD40 stimulation activates the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent survival pathway (37). We therefore tested the effect of two different inhibitors of PI3K, LY294002 and wortmannin, on PAF synthesis induced by sCD154. We did not find any significant effect of PI3K inhibitors on PAF synthesis (Fig. 3A). Moreover, 4-bromodiphenacylbromide and EDTA, which inhibit the activation of cPLA2, which is involved in the phospholipid remodeling pathway of PAF synthesis, also inhibited the production of PAF (Fig. 3A). As shown in Fig. 3C, sCD154 was able to activate the cPLA2, which is downstream to the activation of both p42/44 MAPK and p38 MAPK pathways, as indicated by the inhibitory effect of bisindolylmaleimide, U73122, and SB203580. Trypan blue exclusion and LDH release experiments indicate that, in similar conditions, the inhibitors used had no toxic effect on HUVEC (data not shown).

**sCD154-stimulated motility of HUVEC**

Cell motility of HUVEC was studied by time-lapse recording migration assay (Figs. 4 and 5). Unstimulated cells were first measured for 5 min at 37°C with 100 ng/ml sCD154 (plus 1 μg/ml enhancer) alone or after preincubation for 10 min at 37°C with 10 mM EDTA or specific inhibitor of PKC (100 nM staurosporine and 50 μM bisindolylmaleimide), of PLCγ (10 μM U73122), of PLA2 (1 μM 4-bromodiphenacylbromide), or of PI3K (10 μM LY294002 or 0.1 μM wortmannin). Data represent means ± SD of three individual experiments. ANOVA with Dunnett’s multicomparison test was performed between sCD154 alone vs treatment with inhibitors (ε, p < 0.05). B, Western Blot analysis representative of phosphorylation of PLCγ (p-PLCγ), p44/42 MAPK (p-p44/42 MAPK), and p38 MAPK (p-p38 MAPK) after treatment of HUVEC with sCD154 alone or with specific inhibitors (lane 1, untreated; lane 2, 100 ng/ml sCD154 (plus 1 μg/ml enhancer); lane 3, sCD154 plus 10 mM EDTA, lane 4, sCD154 plus 50 μM bisindolylmaleimide; lane 5, sCD154 plus 10 μM U73122; lane 6, sCD154 plus 10 μM SB203580; and lane 7, sCD154 plus 1 μM 4-bromodiphenacylbromide (see Material and Methods)). Three experiments were performed with similar results. C, cPLA2 activity measured in cell lysates after treatment with sCD154 alone or in the presence of selective inhibitors: 10 mM EDTA; 50 μM bisindolylmaleimide; 10 μM U73122; 10 μM SB203580; and 1 μM 4-bromodiphenacylbromide (4-Br). Data are representative of mean ± SD of three individual experiments.

**FIGURE 3.** Effect of selective inhibitors on PAF synthesis, protein phosphorylation, and cPLA2 activation. A, PAF synthesis induced by HUVEC (1 × 10⁶ cells) stimulated for 5 min at 37°C with 100 ng/ml sCD154 (plus 1 μg/ml enhancer) alone or after preincubation for 10 min at 37°C with 10 mM EDTA or specific inhibitor of PKC (100 nM staurosporine and 50 μM bisindolylmaleimide), of PLCγ (10 μM U73122), of PLA2 (1 μM 4-bromodiphenacylbromide), of p38 MAPK (10 μM SB203580), or of PI3K (10 μM LY294002 or 0.1 μM wortmannin). Data represent means ± SD of three individual experiments. ANOVA with Dunnett’s multicomparison test was performed between sCD154 alone vs treatment with inhibitors (ε, p < 0.05). B, Western Blot analysis representative of phosphorylation of PLCγ (p-PLCγ), p44/42 MAPK (p-p44/42 MAPK), and p38 MAPK (p-p38 MAPK) after treatment of HUVEC with sCD154 alone or with specific inhibitors (lane 1, untreated; lane 2, 100 ng/ml sCD154 (plus 1 μg/ml enhancer); lane 3, sCD154 plus 10 mM EDTA, lane 4, sCD154 plus 50 μM bisindolylmaleimide; lane 5, sCD154 plus 10 μM U73122; lane 6, sCD154 plus 10 μM SB203580; and lane 7, sCD154 plus 1 μM 4-bromodiphenacylbromide (see Material and Methods)). Three experiments were performed with similar results. C, cPLA2 activity measured in cell lysates after treatment with sCD154 alone or in the presence of selective inhibitors: 10 mM EDTA; 50 μM bisindolylmaleimide; 10 μM U73122; 10 μM SB203580; and 1 μM 4-bromodiphenacylbromide (4-Br). Data are representative of mean ± SD of three individual experiments.

**FIGURE 4.** Micrographs representative of time-lapse analysis of HUVEC motility performed by digital saving at 15-min intervals. Migration tracks (magnification, ×120) were generated by marking the position of nucleus of individual cells in each image (see Materials and Methods). A, HUVEC stimulated for 4 h at 37°C with vehicle alone. B, HUVEC stimulated with sCD154 (100 ng/ml; plus 1 μg/ml enhancer). C, HUVEC stimulated with sCD154 in the presence of WEB2170 (3 μM).
and remained sustained for the whole period of observation (Fig. 5B). To investigate the role of PAF, we incubated the cells with two different PAF receptor antagonists, WEB2170 and CV3988, both of which significantly reduced the endothelial motility induced by sCD154 (Figs. 4C and 5), suggesting that PAF mediates, at least in part, the motogenic effect of sCD154. Cell viability, evaluated by an XTT-based assay, was 96 ± 3% on vehicle-treated cells, 96 ± 4.7% on WEB2170-treated cells, and 95 ± 4.3% on CV3988-treated cells. Moreover, the release of LDH in different cell conditions was always <1%.

sCD154 stimulated in vitro formation of vessel-like structures and interaction between HUVEC and SMC

As shown in Figs. 6, A and B, and 7A, sCD154 stimulated the organization of HUVEC plated on Matrigel with the formation of vessel-like tubular structures. PAF-R antagonist WEB2170 reduced tube formation triggered by stimulation of endothelial CD40 (Figs. 6C and 7A). We also evaluated whether in vitro interaction between endothelial cells and SMC was triggered by PAF synthesized by HUVEC after activation of the CD40/CD154 pathway. HUVEC, stained with green fluorescent PKH2 dye, were allowed to spontaneously form tubes by overnight incubation, and then SMC, stained with red fluorescent PKH26 dye, were added in the presence of vehicle alone or sCD154. After 2 h of incubation, the number of SMC associated with endothelial tubes was counted. As shown in Figs. 6, D and E, and 7B, sCD154 enhanced the interaction between HUVEC and SMC. Pretreatment of SMC with the PAF-R antagonist, WEB2170, before the addition to endothelial tubes, inhibited the interaction between HUVEC and SMC (Figs. 6F and 7B). This result suggests that PAF synthesized by HUVEC mediates, at least in part, their interaction with SMC. Treatment with WEB2170 reduced tube formation below the levels seen with vehicle alone as well as the basal interaction between HUVEC and SMC (Fig. 7). This was not due to a toxic effect of PAF-R antagonists, because vitality, evaluated by the XTT method, was >95%. Indeed, basal proliferation of HUVEC, which is not influenced by PAF (14) was unaffected by WEB2170 (untreated HUVEC, 8,118 ± 11,017; 10 nM PAF-treated cells, 7,950 ± 1,213; 3 μM WEB2170, 7,920 ± 952; 10 nM PAF plus 3 μM WEB2170, 8,010 ± 1,012). A possible explanation is that the basal production

FIGURE 5. Effect of PAF receptor antagonists on sCD154-induced motility of HUVEC. A, Motility of HUVEC stimulated for 4 h at 37°C with sCD154 (100 ng/ml; plus 1 μg/ml enhancer) or PAF (10 ng/ml) in the presence or absence of WEB2170 (3 μM) or CV3988 (5 μM). Motility was monitored by time-lapse analysis and measured as described in Material and Methods. Results are expressed as means ± SD of three individual experiments. ANOVA with Newman-Keuls multicomparison test was performed for sCD154 or PAF vs control (*, p < 0.05); sCD154 vs sCD154 plus WEB-2170 and sCD154 plus CV3988 or PAF vs PAF plus WEB-2170 and PAF plus CV3988 (**, p < 0.05). B, Time course of HUVEC motility induced by sCD154 in the presence or absence of WEB2170 (3 μM). Results are expressed as means ± SD of three individual experiments. ANOVA with Dunnett’s multicomparison test was performed for sCD154 vs sCD154 plus WEB-2170 (*, p < 0.05).

FIGURE 6. Micrographs representative of in vitro formation of vessel-like structures by HUVEC and interaction between HUVEC and SMC. Tube formation by HUVEC (5 × 10⁴ cells) plated on growth factor-reduced Matrigel (see Materials and Methods) was evaluated after stimulation 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 3 μM WEB2170 (C). The interaction between HUVEC and SMC was evaluated using HUVEC labeled with green fluorescent cell linker PKH26 plated overnight on Matrigel. After spontaneous tube formation, SMC (1.5 × 10⁴ cells), labeled with red fluorescent cell linker PKH26, were added in the presence of vehicle alone (D) or sCD154 (E). F, SMC were pretreated for 10 min with WEB2170 (3 μM) and then added to HUVEC in the presence of sCD154. Magnification, ×120.
The in vivo angiogenic effect of CD40 engagement and the potential role of PAF were studied in the murine model of Matrigel s.c. implantation. CD40 engagement induces neoangiogenesis (Fig. 8B) with respect to controls (A). Fig. 8D shows the morphometric analysis of neoangiogenesis induced both by the agonist rat anti-CD40 mAb and sCD154 within Matrigel, 7 days after implantation. PAF-R antagonist WEB2170 inhibited development of neoformed vessels, suggesting that PAF synthesized during the neoangiogenic process contributes to CD40-induced vessel formation (Fig. 8, C and D).

Discussion

The results of the present study indicate that the activation of CD40 induced the synthesis of PAF by endothelial cells. In vitro, it was found that PAF synthesized by HUVEC after stimulation with the CD40 ligand, CD154, is instrumental in the migration and vessel-like organization of endothelial cells, and in the interaction between endothelial cells and SMC. In vivo, blockade of PAF-R prevented the angiogenic effect triggered by CD40 stimulation.

Among the molecular mechanisms that link the immunity to the inflammation, the CD40-CD154 interaction has rapidly emerged as a potential key system, particularly involved in vascular disease processes. In physiological conditions, CD40 is expressed at low levels on endothelial cells, but it is up-regulated in areas of inflammation (8, 38, 39). Recently, CD154 has also been found to be expressed by endothelial cells as well as by SMC, platelets, and macrophages within the atherosclerotic plaque (1).

CD40 stimulation on endothelial cells plays an important role in the phenotypic modulation of the endothelium to an activated state. CD40 was shown to induce expression of proteinases such as collagenase and stromelysin on human monocytes/macrophages, and of collagenase, stromelysin, gelatinase B, and activated gelatinase A on vascular smooth muscle and endothelial cells that may induce plaque destabilization (1, 11, 40). It has been recently shown that inhibition of CD40 signaling limits evolution of established atherosclerosis in mice (9, 10). Ligation of endothelial CD40 by CD154, either expressed on activated monocytes or T cells, or disorgended from platelet granules after activation, stimulated the production of various inflammatory cytokines by endothelial cells (1, 41–44). Activated platelets, in particular, may have a role in vascular injury, because CD154, which is cryptic in unstimulated platelets, is rapidly expressed on the surface after activation. This may allow the interaction of platelets with endothelial cells via the CD154-CD40 system. Moreover, the surface-expressed CD154 is rapidly cleaved with generation of a soluble CD154, which remains trimeric and biologically active (45). In addition, a soluble form of CD154 released from the surface of tumor cells (13) may contribute to endothelial activation in tumor angiogenesis. We recently found that stimulation of endothelial CD40 triggers neoangiogenesis in vivo, and that its inhibition limits neoangiogenesis and allows apoptotic regression in an experimental model of tumor neoangiogenesis (5).

In the present study, we demonstrate that both sCD154 and membrane CD154 expressed by leukocytes (J558L) or by activated platelets, trigger the synthesis of PAF, a phospholipid mediator of inflammation (17, 18). The amount of PAF synthesized after incubation with J558L was considerably lower than that obtained after incubation with activated platelets. This may depend on the ratio of membrane surface and exposed CD154 per cell and by the number of stimulating cells. The amount of PAF synthesized by HUVEC after stimulation with activated platelets was comparable with that of thrombin, which is considered one of the more efficient stimuli for PAF synthesis by endothelial cells. Therefore, activated platelets are the best candidates for stimulation of PAF synthesis by endothelial cells in vascular injury. Indeed, activated platelets have been suggested as one of the main effector cells in CD40 stimulation in several pathological conditions (45). In endothelial cells, PAF synthesis is known to be mediated via a remodeling pathway in which membrane phospholipids are converted by a PL_A2 into lyso-PAF, which is then acetylated by the acetyl-CoA:lyso-PAF acetyltransferase to form PAF (17, 18). It has been recently found that, in bovine aortic...
endothelial cells, the mechanism of VEGF-induced PAF synthesis involves two intracellular signaling pathways leading to PLA2 activation: a PLCγ-PKC-Raf-p42/p44 MAPK and a MAPK kinase-3/6-dependent activation of p38 MAPK (36). In the present study, we found, using specific inhibitors, that sCD154-induced PAF synthesis by HUVEC depended on both of these pathways. We recently found that CD40 stimulation on endothelial cells activated the PI3K/Akt survival pathway (37). Because it was reported that PI3K plays a negative regulatory role in VEGF-induced PAF synthesis, we tested the effect of two different inhibitors of PI3K, LY294002 and wortmannin. However, we did not observe an enhancement of PAF synthesis after PI3K blockade followed by sCD40 stimulation.

PAF was previously shown to contribute to the angiogenic properties of several polypeptide mediators including VEGF (16). The role of PAF in this contest is possibly mainly related to its motogenic properties that are instrumental in the coordinate migration and in the interaction between endothelial cells required for the formation of new vessels (14–16). In the present study, we demonstrate that PAF synthesized after stimulation of endothelial CD40 contributed to the in vitro migration and organization of endothelial cells in vessel-like structures and to the in vivo angiogenesis in a murine model of s.c. Matrigel implantation. PAF has been implicated in several inflammatory processes involving angiogenesis, including atherosclerosis (46, 47). In the present study, we provide the first evidence that PAF may be synthesized after engagement of CD40, which has been recently suggested a key signaling pathway in atherosclerosis (2, 3). The synthesis of PAF within the atherosclerotic plaque may amplify the vascular injury by recruiting inflammatory cells and stimulating neoangiogenesis, which may contribute to plaque instability. In addition, we found that PAF also mediated the interaction between endothelial cells organized in tubes and SMC. In these experimental settings, we found that PAF-R blockade inhibited the incorporation of SMC within the endothelial tubes. SMC were unable to synthesize PAF after CD40 stimulation. However, SMC were found to express PAF-R (48, 49) and to proliferate after PAF stimulation (50). The observation that PAF-R antagonism prevented the incorporation of SMC within the endothelial tubes suggests that PAF synthesized by endothelial cells acts on the recruitment of SMC.

In conclusion, these observations provide further evidence for a role of CD40/CD154 in vascular remodeling during inflammation and link this property to the synthesis of PAF by endothelial cells.

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