

The Proangiogenic Phenotype of Tumor-Derived Endothelial Cells is Reverted by the Overexpression of Platelet-Activating Factor Acetylhydrolase

Sophie Doublier,¹ Monica Ceretto,¹ Enrico Lupia,² Stefania Bravo,¹ Benedetta Bussolati,¹ and Giovanni Camussi¹

Abstract Purpose: We previously reported that human tumor-derived endothelial cells (TEC) have an angiogenic phenotype related to the autocrine production of several angiogenic factors. The purpose of the present study was to evaluate whether an enhanced synthesis of platelet-activating factor (PAF) might contribute to the proangiogenic characteristics of TEC and whether its inactivation might inhibit angiogenesis.

Experimental Design: To address the potential role of PAF in the proangiogenic characteristics of TEC, we engineered TEC to stably overexpress human plasma PAF-acetylhydrolase (PAF-AH), the major PAF-inactivating enzyme, and we evaluated *in vitro* and *in vivo* angiogenesis.

Results: TECs were able to synthesize a significantly enhanced amount of PAF compared with normal human microvascular endothelial cells when stimulated with thrombin, vascular endothelial growth factor, or soluble CD154. Transfection of TEC with PAF-AH (TEC-PAF-AH) significantly inhibited apoptosis resistance and spontaneous motility of TEC. In addition, PAF and vascular endothelial growth factor stimulation enhanced the motility and adhesion of TEC but not of TEC-PAF-AH. *In vitro*, TEC-PAF-AH lost the characteristic ability of TEC to form vessel-like structures when plated on Matrigel. Finally, when cells were injected s.c. within Matrigel in severe combined immunodeficiency mice or coimplanted with a renal carcinoma cell line, the overexpression of PAF-AH induced a significant reduction of functional vessel formation.

Conclusions: These results suggest that inactivation of PAF, produced by TEC, by the overexpression of plasma PAF-AH affects survival, migration, and the angiogenic response of TEC both *in vitro* and *in vivo*.

Neoangiogenesis is crucial for tumor growth and invasion. The neoformed vessels may provide nutrients and access for tumor cells to the circulation thus favoring metastasis (1, 2). Endothelial cells present in tumors have a critical role in the formation of new vessels and their "switch" to an angiogenic phenotype is a hallmark of tumor malignancy.

It is well established that endothelial cells present in tumors differ from normal endothelial cells as they express an activated phenotype and several markers absent or barely detectable in

normal endothelial cells (3–5). It has been recently shown that tumor-derived endothelial cells (TEC) are genetically unstable and are different from normal vessel endothelial cells at the molecular and functional levels (6). We previously showed that TEC derived from human renal and breast carcinomas display increased survival, enhanced motility upon stimulation, and the ability to form vessel-like structures *in vitro* and functional vessels *in vivo* compared with normal endothelial cells (7, 8). These angiogenic properties have been related to the expression of embryonic genes and to the autocrine production of angiogenic growth factors (9, 10).

Platelet-activating factor (PAF) is a 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine mediator of several vascular and inflammatory processes (11–13). PAF possesses multiple biological activities on different cell types bearing the specific receptor (13). PAF physiologic activity is restrained by a powerful enzymatic inactivating system including an intracellular and a plasma PAF-acetylhydrolase (PAF-AH) which is able to catalyze the hydrolysis of sn-2 ester bound to PAF (14). Cleavage of the sn-2-acetyl group abrogates the biological activity of PAF (14). PAF-AH present in plasma is a single polypeptide released mostly by activated macrophages (15). Because PAF is involved in several pathologic processes, the therapeutic effect of the administration of recombinant PAF-AH has been recently tested in several experimental models of inflammation (16). The relevant part of the biological role of

Authors' Affiliations: ¹Department of Internal Medicine, Centre for Molecular Biotechnology and Center of Experimental Research and Medical Sciences, University of Turin and ²Azienda Ospedaliera S. Giovanni Battista di Torino, Turin, Italy Received 2/16/07; revised 5/22/07; accepted 6/19/07.

Grant support: Associazione Italiana per la Ricerca sul Cancro, the Italian Ministry of University and Research COFIN06 and ex60%, Regione Piemonte integrated project A47, the Italian Ministry of Health (Ricerca Finalizzata 02), and Progetto S. Paolo. Dr. S. Doublier is a recipient of a Research Fellowship from the Fondazione Internazionale di Ricerca in Medicina Sperimentale, Torino, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Giovanni Camussi, Dipartimento di Medicina Interna, Corso Dogliotti 14, 10126, Turin, Italy. Phone: 39-633-6708; Fax: 39-663-1184; E-mail: giovanni.camussi@unito.it.

©2007 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-07-0412

PAF has been related to its effect on endothelial cells, which are known to be able to synthesize PAF and respond to its stimulation by increasing vascular permeability and expression of adhesion molecules (12).

It has been described that PAF is involved in angiogenesis because it is synthesized by endothelial cells upon stimulation with several angiogenic growth factors and mediates endothelial cell motility required for the formation of new vessels (17–23). Several studies have sustained the involvement of PAF in tumor growth and vascularization (24–30). PAF has been detected within tumor lesions (24, 25) and it is synthesized by cancer cell lines which may also respond to PAF stimulation (26–28). Transgenic mice overexpressing PAF receptors were shown to develop melanocytic tumors (29). We previously showed that *in situ* PAF inactivation affects the vascularization and growth of experimental tumors (30).

The aim of the present study was to investigate whether an enhanced synthesis of PAF might contribute to the proangiogenic characteristics of human TEC and whether its inactivation might inhibit their angiogenic properties. For this purpose, we first compared the ability of TEC to synthesize PAF with that of normal human microvascular endothelial cells (HMEC), and then we engineered TEC to directly express plasma PAF-AH to inhibit the biological activity of PAF. We therefore evaluated whether the inactivation of PAF affects the survival, motility, and angiogenic properties of TEC both *in vitro* and *in vivo*.

Materials and Methods

Reagents. Synthetic PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; alkyl-PAF C16:0) was obtained from Bachem Feinchemikalien. Carbamyl PAF (1-*O*-palmitol-2-methylcarbamyl-*sn*-glyceryl-3-phosphocholine) was obtained from Sigma Chemical, Co. Stock solutions in chloroform were stored at -20°C until use. The chloroform was then evaporated, and saline containing 0.25% bovine serum albumin (BSA, fraction V; Sigma), with low endotoxins, was added immediately before use. Recombinant human vascular endothelial growth factor (VEGF₁₆₅) was obtained from R&D Systems. Recombinant human soluble CD154 trimeric protein (sCD154) and a cross-linking antibody (enhancer) were from Alexis Biochemicals. Thrombin was from Sigma.

Cell lines. TEC lines used in this study were previously described and characterized (7). Briefly, renal TEC were isolated from six different renal clear-cell carcinomas using anti-CD105 antibodies coupled to magnetic beads by magnetic cell sorting (MACS System, Miltenyi Biotec).

HMECs were obtained from skin specimens of adult healthy individuals undergoing elective surgery for abdominoplasty (31). Dermal sheets were incubated with dispase (50 units/mL; Invitrogen Corporation) for 30 min at 37°C, and the epidermis was removed, and then dermal cellular elements were disaggregated by incubation for 1 h at 37°C in DMEM containing collagenase II (Sigma). After washing in medium plus 10% FCS (Hyclone), the cell suspension was forced through a 40- μ m cell strainer (BD Biosciences) to separate the cell components from aggregates. Endothelial cells were isolated using anti-CD31 antibodies coupled to magnetic beads by magnetic cell sorting using the MACS System. TEC and HMEC lines were maintained in culture in endothelial basal medium, complete medium supplemented with epidermal growth factor (10 ng/mL), hydrocortisone (1 mg/mL), bovine brain extract (all from Cambrex Bioscience), and 10% FCS. The cell lines were characterized as endothelial cells by morphology, positive staining for von Willebrand factor antigen, CD105, CD146, vascular endothelial-cadherin, and

negative staining for cytokeratin and desmin (7). The renal clear cell carcinoma cell line was isolated and characterized as previously described (32).

Transfection of TEC lines with PAF-AH. TEC lines were seeded in a T25 flask to reach 90% to 95% confluence and transfected with 8 μ g of plasmidic DNA (pcDNA4/TO/mys-His A, 5.1 kb vector; Invitrogen) expressing human plasma PAF-AH DNA (TEC-PAF-AH; ref. 30) and 20 μ g of LipofectAMINE 2000 (Invitrogen) in DMEM (Sigma) plus 10% FCS without antibiotics according to the protocol suggested by the manufacturer. The construct, PAF-AH cDNA, was introduced in the vector with *Kpn*I and *Xba*I. As a control, cells were transfected with the empty vector (TEC-c). Transfected cells were stably selected by culturing in the presence of 100 μ g/mL of zeocin (Invitrogen) for 6 weeks. Two stable lines of TEC-PAF-AH and TEC-c, characterized as described above and shown to retain endothelial cell markers, were obtained. PAF-AH activity was tested by a radioactive assay based on the hydrolysis of 2-[³H]PAF (NEN Life Science Products; refs. 33, 34). The expression of PAF-AH was tested by Western blot analysis and by reverse transcription-PCR.

Western blot analysis. Cells were lysed at 4°C for 15 min in a lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 0.1% Igepal, 10 mmol/L sodium fluoride, 10 mmol/L sodium PPI, 0.4 mmol/L sodium orthovanadate plus 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, and 100 units/mL aprotinin] and centrifuged at 15,000 \times g. The protein contents of the supernatants were measured by the Bradford method. Aliquots containing 30 μ g of protein per lane of the cell lysates were subjected to 10% SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membrane filters. The blots were blocked with PBS plus 0.5% Tween and 5% BSA. The membranes were subsequently immunoblotted overnight at 4°C with antihuman PAF-AH polyclonal antibody (Cayman Chemical). After extensive washings, the blots were incubated for 1 h at room temperature with peroxidase-conjugated isotype-specific secondary antibody (Santa Cruz Biotechnology), developed with enhanced chemiluminescence detection reagents (Amersham Biosciences) for 2 min, and exposed to X-Omat film (Eastman Kodak).

Reverse transcription-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions; 2 μ g of RNA were reverse-transcribed using oligo(dT) primers and 15 units of reverse transcriptase enzyme (Eppendorf). Two microliters of cDNA were amplified using human PAF-AH mRNA-specific primers: forward 5'-TGTTTCCACTGGGTGATGAA and reverse 5'-TGCCAGTTGCAAAGT-GAAG, and TaqDNA polymerase (Invitrogen). Reactions were done for 30 cycles at a melting temperature of 55°C and analyzed with an ethidium bromide 1.5% agarose gel.

Extraction and quantification of PAF. Cells were equilibrated for 15 min in Tris-buffered Tyrode containing 0.25% delipidized BSA (fraction V), as previously described (17), and incubated at 37°C for the indicated times with the different stimuli in M199 (Sigma) containing 0.25% BSA. The cell pellets were extracted according to a modification of the Bligh and Dyer procedure (35), with formic acid added to lower the pH of the aqueous phase to 3.0. Each individual experiment was done in triplicate. PAF was quantified, after extraction and purification by TLC (60F254 silica gel plates; Merck) and high-pressure liquid chromatography (Porasil column; Millipore-Waters), by aggregation of washed rabbit platelets, as previously reported (17, 36). The biologically active material extracted from cells and supernatants in different experiments was characterized by comparison with synthetic PAF according to the following criteria (37): (a) induction of platelet aggregation by a pathway independent from both ADP and arachidonic acid/thromboxane A₂-mediated pathways; (b) specificity of platelet aggregation as inferred from the inhibitory effect of 5 μ mol/L of WEB2170 or CV3988, two different PAF receptor antagonists; (c) TLC and HPLC behavior and physicochemical characteristics, such as inactivation by strong bases and by PLA₂ treatment, but resistance to PLA₁, acids, weak bases, and 5 min of heating in boiling water.

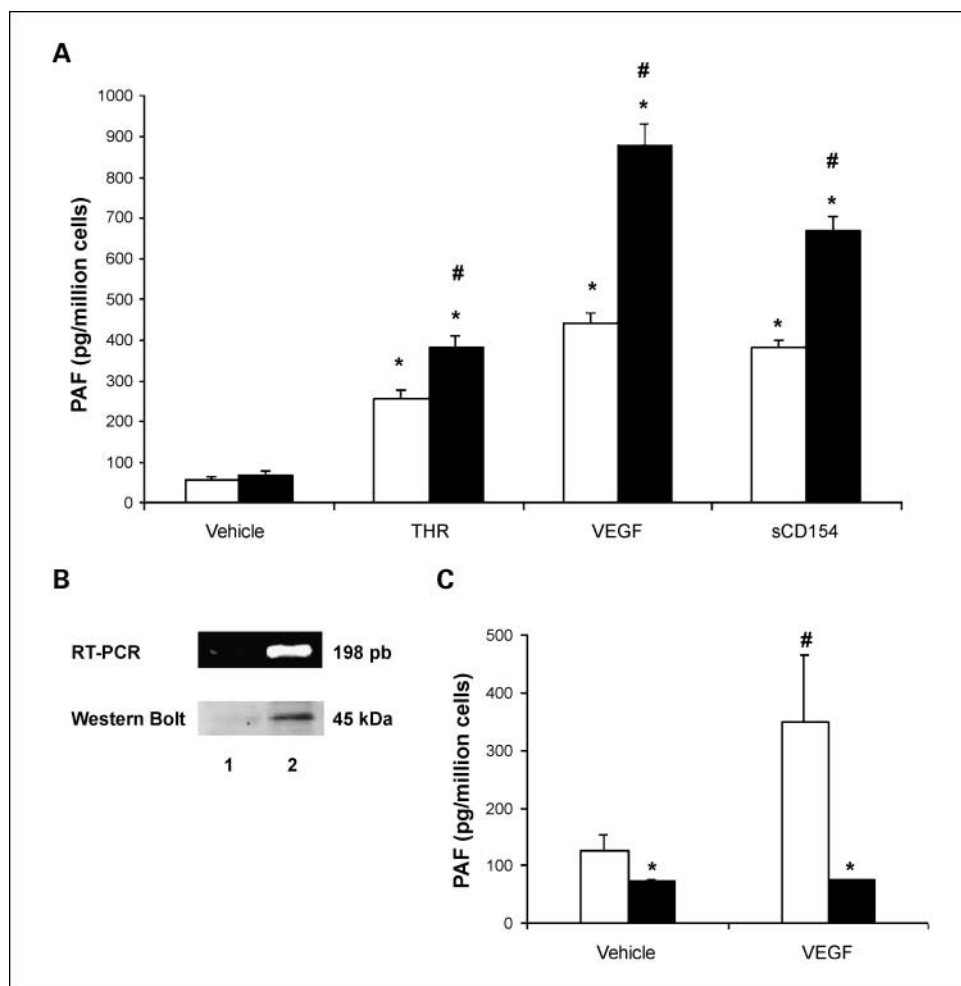


Fig. 1. A, PAF production from 1×10^6 normal HMECs (white columns) and human endothelial cells derived from renal carcinomas (TEC; black columns) stimulated with vehicle alone (M199 containing 0.25% BSA), thrombin (THR, 2 units/mL for 15 min), VEGF (VEGF, 10 ng/mL for 1 h), or soluble CD154 (sCD154, 100 ng/mL for 15 min) at 37°C . PAF was determined by a bioassay of washed rabbit platelet aggregation. Columns, means of three individual experiments; bars, SD. ANOVA with Newman-Keul's multicomparison test was done: *, $P < 0.05$ stimulated HMEC or stimulated TEC versus unstimulated cells; #, $P < 0.05$ stimulated TEC versus stimulated HMEC. **B**, reverse transcription-PCR analysis of PAF-AH mRNA expression and Western blot analysis of PAF-AH protein expression in TEC transfected with the empty vector (TEC-c; 1) and TEC transfected with human plasma PAF-AH cDNA (TEC-PAF-AH; 2). **C**, PAF production from 1×10^6 TEC-c (white columns) and TEC-PAF-AH (black columns) stimulated with vehicle alone (M199 containing 0.25% BSA) or VEGF (10 ng/mL) for 1 h at 37°C . Cell-associated PAF was determined by incorporation of the [^3H]acetate precursor. Columns, means of three individual experiments; bars, SD. ANOVA with Newman-Keul's multicomparison test was done: *, $P < 0.05$ TEC-PAF-AH versus TEC-c; #, $P < 0.05$ TEC-c stimulated with VEGF versus TEC-c stimulated with vehicle alone.

For the radioactive assay of PAF synthesis, 1×10^6 TEC were incubated in 1 mL of M199 containing 0.25% BSA for 30 min at 37°C with 30 μCi of [^3H]acetate (NEN Life Science Products) before stimulation. Using radioactive acetate as a substrate for PAF synthesis, the amount of PAF detected was newly synthesized. The cell pellets were extracted as described above, and lipids were fractionated by TLC on aluminum sheet silica gel plates (0.2 mm thickness) 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 [^3H -acetyl]PAF (NEN Life Science Products) was used as a standard.

PAF catabolism was assessed by the incubation of 10 nmol/L of [^3H acetyl]PAF with 50 μg of cell lysates for 5 min at 37°C . The reaction was stopped by the addition of one volume of methanol. PAF was further extracted and purified by TLC as described above. The result was expressed as the percentage of PAF remaining, assuming the control (10 nmol/L [^3H acetyl]PAF extracted in 1 mL of methanol and purified by TLC) as 100%.

Cell proliferation and apoptosis assays. Cells were seeded at 8,000 cells/well into 96-well plates in RPMI plus 0.25% BSA. DNA synthesis was detected as incorporation of 5-bromo-2'-deoxy-uridine into the cellular DNA using an ELISA kit (Roche Applied Science), following the manufacturer's instructions. TEC were stimulated with 20 ng/mL of PAF or 10 ng/mL of VEGF.

Apoptosis was evaluated using the terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling assay (ApoTag, Oncor). After treatment with 1 ng/mL of vincristine for 48 h or vehicle alone (medium 199 containing 10% FCS), cells were suspended in PBS

and fixed in 1% paraformaldehyde in PBS (pH 7.4) for 15 min at 4°C followed by precooled ethanol/acetic acid (2:1) for 5 min at -20°C . Cells were treated with terminal deoxynucleotide transferase enzyme and incubated in a humidified chamber for 1 h at 37°C and then treated with warmed FITC-conjugated antidigoxigenin for 30 min at room temperature. After washing, samples were mounted in medium containing 1 $\mu\text{g}/\text{mL}$ of propidium iodide and cells were analyzed by immunofluorescence.

In vitro cell migration and adhesion. Cell motility was investigated both as random motility of cells and as migration of cells into a wound introduced in a confluent monolayer. Random cell motility was investigated with a time-lapse system. A total of 2×10^5 cells were plated in a T25 flask and rested for 12 h in medium 199 containing 1% FCS and then washed thrice with PBS, and incubated with medium 199 containing 1% BSA and the stimuli. Cell division did not start, to any significant degree, during the experiments. Cell migration was studied over a 12-h period under a Nikon Diaphot inverted microscope with a $10\times$ phase-contrast objective in an attached, hermetically sealed Plexiglas Nikon NP-2 incubator at 37°C . Cell migration was recorded by using a JVC 1CCD video camera. Image analysis of at least 30 cells for each experimental condition was done by saving images at 15-min intervals with a MicroImage analysis system (Windows MicroImage, version 3.4; CASTI Imaging) and an IBM-compatible system equipped with a video card (Targa 2000, Truevision). Migration tracks were generated by marking the position of the nucleus of individual cells on each image. The net migratory speed (straight-line velocity) was calculated by the MicroImage software on the basis of the straight-line distance

between the starting and the end points divided by the time of observation.

For the wound-healing assay, cells were grown to confluence on 96-well culture plates. Cell monolayers were allowed to rest for 24 h in medium 199 containing 2% FCS and a "wound" was made under standard conditions by scraping the middle of the cell monolayer with a P10 pipette tip. Floating cells were removed by washing with PBS, and fresh medium 199 containing 1% BSA and the stimuli was added. Cell division did not start, to any significant degree, during the experiments. Before and after a 4-h incubation, cells were observed with a Nikon inverted microscope (Nikon Corporation), and the experimental results were recorded. Cell motility into a wound was measured with the MicroImage analysis system (Windows MicroImage, version 3.4; CASTI Imaging), and was expressed in arbitrary units.

The expression of adhesion molecules was evaluated by cytofluorimetric analysis, done as described (7), using the following FITC-conjugated antibodies directed to β 1-integrin, α 4-integrin (Becton Dickinson), α v β 3-integrin, α 5-integrin, and α 6-integrin (BioLegend). FITC mouse nonimmune isotypic IgG (Dako Cytomation) was used as a control.

Cell adhesion was evaluated on 24-well culture plates previously coated for 6 h with human fibronectin or type IV collagen (20 μ g/mL, Becton Dickinson). Nonspecific adhesion was blocked by incubation

for 2 h with 2% BSA diluted in 1 \times PBS. TEC were detached with trypsin and then incubated for 2 h at 37°C in RPMI plus 0.25% BSA (vehicle) or vehicle with 20 ng/mL of PAF or 10 ng/mL of VEGF in rotating conditions, to prevent cell aggregation. Cells (5×10^4 cells/well) were then added to the wells, and allowed to adhere for 90 min at 37°C. Supernatants were removed and after extensive washings, attached cells in recorded images were counted using a MicroImage analysis system at 20 \times (Windows MicroImage, version 3.4; CASTI Imaging) and expressed as number of cells per microscopic field. Data are \pm SD of three different experiments done in triplicate.

In vitro angiogenesis assay. The assay was done as previously described (7). Briefly, 24-well plates were coated with growth factor-reduced Matrigel (Becton Dickinson) at 4°C and incubated for 30 min at 37°C, 5% CO₂, in a humidified atmosphere. A total of 5×10^4 cells/well were plated on the Matrigel-coated wells and rested for 12 h in medium 199 containing 2% FCS and then washed thrice with PBS and incubated with medium 199 containing 1% BSA and the stimuli. Cells were periodically observed with a Nikon inverted microscope (Nikon Corporation), and experimental results were recorded. The extent of capillary-like structures was measured with the MicroImage analysis system (Windows MicroImage, version 3.4; CASTI Imaging) and was expressed in arbitrary units.

In vivo angiogenesis assay and experiment of tumor coimplantation. Severe combined immunodeficiency (SCID) mice (Charles River, The Jackson Laboratory) were used at 6 to 8 weeks of age. Mice were cared for and handled according to accepted ethical practices. Angiogenesis was assayed as the growth of blood vessels from subcutaneous tissue into a solid gel of basement membrane containing the test samples (38). Briefly, cells were harvested using trypsin solution (Sigma), washed with PBS, resuspended in 200 μ L of endothelial basal medium, and added to 200 μ L of growth factor-reduced Matrigel at 4°C. Cells were injected s.c. into the mid-abdominal region of SCID mice ($n = 5$ for each experimental condition) via a 26-gauge needle and a 1-mL syringe. At day 6, mice were killed, and the plugs were recovered and processed for histology.

To evaluate the involvement of TEC and HMEC cells in tumor growth and vascularization, TEC-c, TEC-PAF-AH, or HMEC were implanted s.c. into SCID mice in combination with renal carcinoma cells (RCC) at a 1:100 ratio (1×10^4 TEC/HMEC to 1×10^6 RCC) and compared with an injection of 1×10^6 RCC alone. After 3 weeks, mice were sacrificed and Matrigel plugs were recovered and processed for histology.

Typically, gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3 μ m) were cut, stained with H&E or with Masson trichrome reaction, and examined under a light microscopy system. Morphometric analysis was done to count vessels that were expressed as a percentage of the area per field. Vessel structures were counted only if they showed a patent lumen with red globuli and/or leukocytes. The vessel area was planimetrically assessed using the MicroImage analysis system (Windows MicroImage, version 3.4; CASTI Imaging). Endothelial cells were detected by immunofluorescence staining with anti-von Willebrand factor antibody (Sigma), antihuman CD31 (hCD31; Becton Dickinson), or antimouse CD31 (mCD31; Abcam) monoclonal antibodies.

Data analysis. All data are presented as mean \pm SD. Differences between two groups were analyzed by *t* test. Differences between multiple groups were analyzed by one-way ANOVA in combination with Newman-Keul's multiple comparison test. $P < 0.05$ was considered significant.

Results

We previously isolated and characterized human endothelial cell lines derived from renal carcinomas and showed that they express a proangiogenic phenotype (7). These characteristics include increased resistance to apoptosis induced by vincristine,

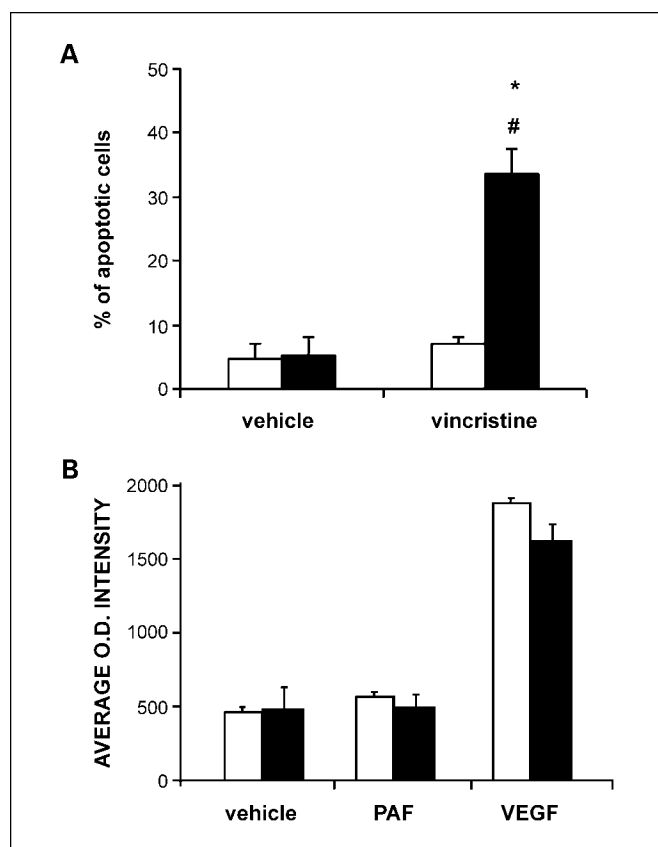


Fig. 2. Effect of PAF inactivation on apoptosis resistance and proliferation of TEC. **A**, apoptosis was evaluated in TEC-c (white columns) and in TEC-PAF-AH (black columns) by the terminal dUTP nick-end labeling assay as a percentage of apoptotic cells after 48 h of treatment with 1 ng/mL of vincristine. Columns, means of three individual experiments; bars, SD. ANOVA with Newman-Keul's multicomparison test was done: *, $P < 0.05$ TEC-PAF-AH stimulated with vincristine versus TEC-PAF-AH stimulated with vehicle alone; #, $P < 0.05$ TEC-PAF-AH stimulated with vincristine versus TEC-c stimulated with vincristine. **B**, proliferation of TEC-c (white columns) and of TEC-PAF-AH (black columns) evaluated 72 h after stimulation with vehicle alone (RPMI + 0.25% BSA) or with 20 ng/mL of PAF or 10 ng/mL of VEGF, as 5-bromo-2'-deoxy-uridine incorporation. Columns, means of three experiments; bars, SD.

enhanced spontaneous and stimulated *in vitro* motility, and a marked ability to form vessel-like structures *in vitro* and *in vivo* compared with normal HMEC.

As shown in Fig. 1A, thrombin (2 units/mL), VEGF (10 ng/mL), and soluble CD154 (100 ng/mL) induced the synthesis of PAF by TEC and HMEC. TEC synthesized significantly higher amounts of PAF than HMEC. To evaluate whether enhanced PAF production could have a role in the proangiogenic phenotype of TEC, we engineered TEC to stably overexpress human plasma PAF-AH to locally inactivate PAF biological activity. As controls, TEC were transfected with the empty vector (TEC-c). As shown by reverse transcription-PCR and Western blot analysis, TEC-PAF-AH expressed high levels of this enzyme which were absent both in parental cells (data not shown) and in TEC-c (Fig. 1B). Using radioactive acetate as a substrate for newly synthesized PAF, we found that the amount of PAF associated with TEC-PAF-AH was significantly lower than that associated with TEC-c both in the basal condition and after stimulation with VEGF (10 ng/mL) for 1 h. VEGF significantly enhanced the production of PAF by TEC-c but not by TEC-PAF-AH (Fig. 1C). The TLC analysis of lipid fractions showed the presence of one main peak of radioactivity that comigrated with synthetic (^3H)C16-PAF (data not shown). Increased PAF inactivation in TEC-PAF-AH was confirmed by experiments in which PAF catabolism was assessed by incubating radiolabeled

PAF with cell lysates. In these conditions, the release of (^3H)acetate from labeled PAF in TEC-PAF-AH lysates was $91 \pm 7.2\%$, and only $20 \pm 5.5\%$ in TEC-c lysates.

To verify the effect of PAF inactivation on apoptosis resistance of TEC, TEC-c and TEC-PAF-AH were incubated with 1 ng/mL of vincristine for 48 h. As shown by terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling analysis, TEC-PAF-AH exhibited a significantly higher sensitivity to apoptosis than TEC-c (Fig. 2A), which was protected from vincristine-induced apoptosis, as previously described (7). In contrast, no significant differences were observed concerning the proliferative response to VEGF between TEC-c and TEC-PAF-AH. This was consistent with the absence of a proliferative effect of PAF on both TEC-c and TEC-PAF-AH (Fig. 2B).

To determine whether the overexpression of PAF-AH could modify the motility profile of TEC, we studied cell motility both by time-lapse recording the random migration assay over a 12-h period and by migration of cells in the wound-healing assay over a 4-h period. In the time-lapse migration assay, baseline spontaneous motility of TEC-PAF-AH was significantly lower in comparison with that of TEC-c (Fig. 3). When cells were stimulated with PAF (20 ng/mL) or VEGF (10 ng/mL), cell motility was enhanced in TEC-c but not in TEC-PAF-AH (Fig. 3C). Further evidence that the response of TEC-PAF-AH

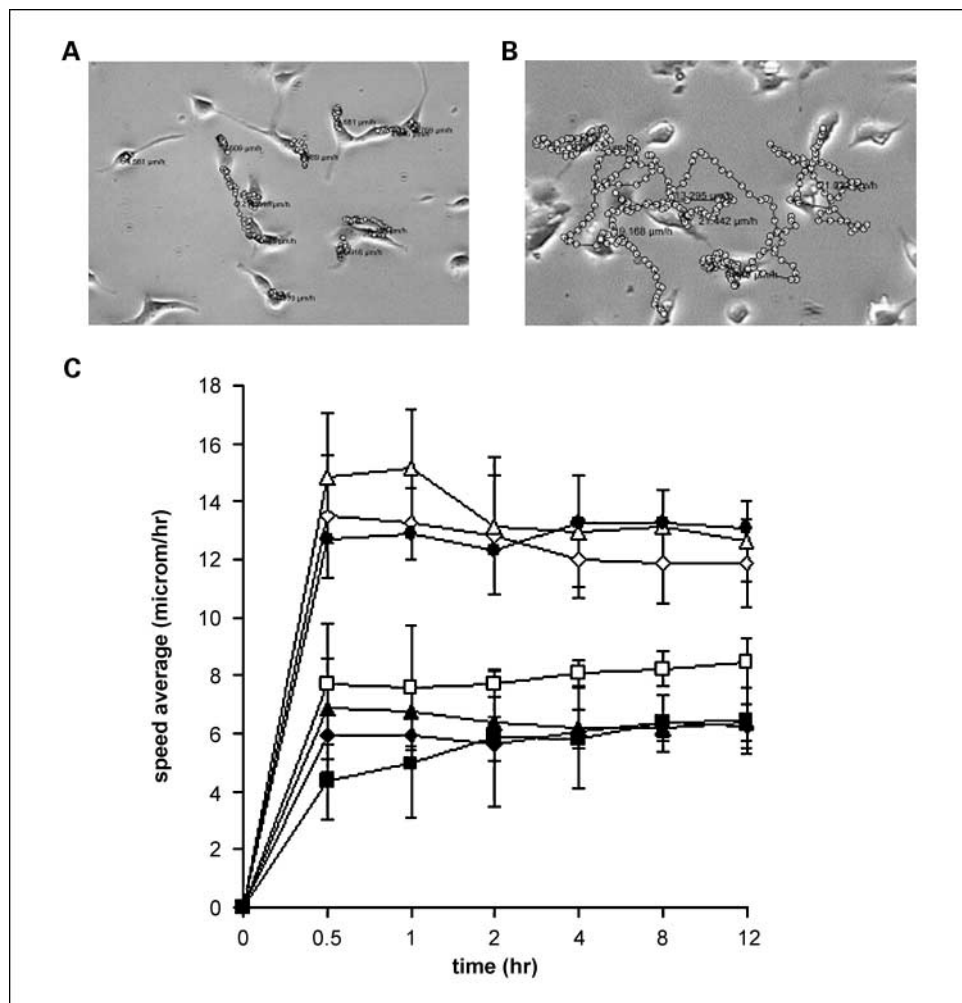


Fig. 3. Effect of PAF inactivation on *in vitro* cell migration. Random cell motility was measured in a time-lapse system. Cells were stimulated with vehicle alone (medium 199 containing 1% BSA), PAF (20 ng/mL), VEGF (10 ng/mL), or carbamyl-PAF (10 ng/mL) at 37°C and cell migration was studied over a 12-h period. Image analysis was done by saving images at 15-min intervals. *A* and *B*, representative micrographs of time-lapse analysis of TEC-PAF-AH (*A*) and TEC-c (*B*) stimulated with PAF. Migration tracks were generated by marking the position of the nucleus of individual cells in each image. *C*, average speed of TEC-c stimulated with vehicle alone (□), PAF (◇), or VEGF (△), and TEC-PAF-AH stimulated with vehicle alone (■), PAF (◆), VEGF (▲), or C-PAF (●). Points, means of three individual experiments; bars, SD.

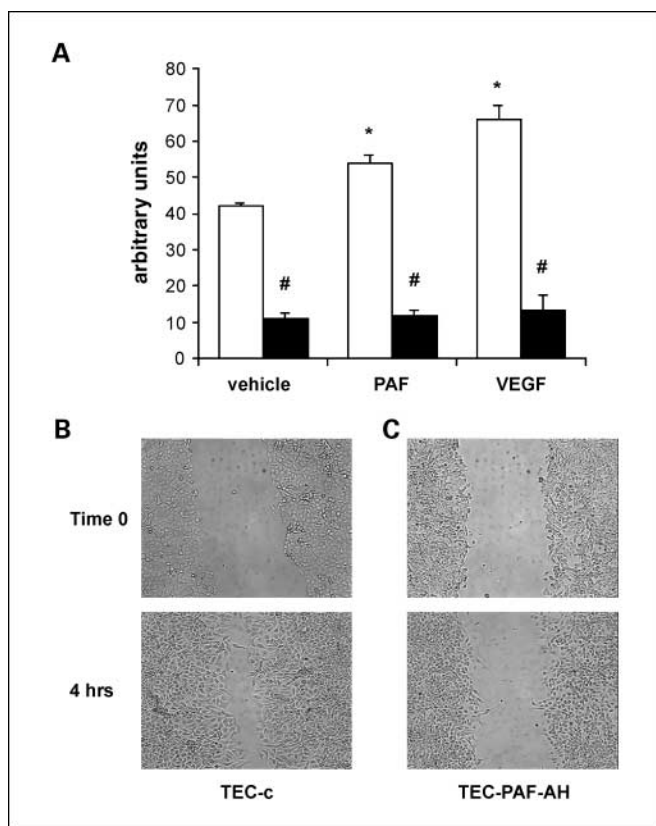


Fig. 4. Effect of PAF inactivation on *in vitro* cell migration. Motility was measured as the migration of cells into a wound introduced in a confluent monolayer. Cells were stimulated with vehicle alone (medium 199 containing 1% BSA), PAF (20 ng/mL), or VEGF (10 ng/mL) at 37°C. Image analysis was done by saving images before and after 4 h of incubation with the stimuli. **A**, results expressed in arbitrary units represent the migration into a wound of TEC-c (white columns) and TEC-PAF-AH (black columns) stimulated with vehicle alone, PAF, or VEGF. Columns, means of three individual experiments; bars, SD. ANOVA with Newman-Keul's multicomparison test was done: *, $P < 0.05$ TEC-c stimulated with PAF and VEGF versus TEC-c stimulated with vehicle alone; #, $P < 0.05$ TEC-PAF-AH versus TEC-c. **B** and **C**, representative micrographs of TEC-c and TEC-PAF-AH submitted to wounds before and after 4 h of stimulation with PAF.

was due to the inactivation of exogenous PAF, or of PAF synthesized after stimulation with VEGF, was provided by experiments using synthetic carbamyl-PAF. Carbamyl-PAF is a PAF isoform insensitive to the hydrolysis by plasma PAF-AH (39). When carbamyl-PAF was used to stimulate TEC-PAF-AH, the motility was similar to that of TEC-c stimulated with PAF or VEGF (Fig. 3C).

In the wound-healing assay, baseline spontaneous motility of TEC-PAF-AH was significantly lower than that of TEC-c (Fig. 4). Incubation with PAF (20 ng/mL) or VEGF (10 ng/mL) for 4 h significantly enhanced the motility of TEC-c (Fig. 4A). In contrast, when TEC-PAF-AH were stimulated with PAF or VEGF, the motility of the cells did not change from basal conditions (Fig. 4A).

In addition, we evaluated other variables related to cell migration, such as integrin expression and cell adhesion. No differential expression of $\beta 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ integrins were observed between TEC-PAF-AH and TEC-c both in the basal condition and after stimulation with 20 ng/mL of PAF (data not shown). In contrast, PAF stimulation of TEC-c significantly enhanced the expression of $\alpha v\beta 3$ (Fig. 5A), whereas no effect was observed on TEC-PAF-AH. Moreover, TEC-PAF-AH stimu-

lated with PAF or VEGF, at a variance of TEC-c, did not show a significant increase in adhesion to collagen and fibronectin (Fig. 5B).

Taken together, these results suggest that PAF-AH overexpression into TEC modifies PAF-dependent cell migration and adhesion, possibly related to the abrogation of PAF-induced $\alpha v\beta 3$ integrin up-regulation.

To determine whether the overexpression of PAF-AH could modify the *in vitro* proangiogenic properties of TEC, we compared TEC-PAF-AH and TEC-c for the ability to organize in vessel-like structures when plated on Matrigel. Under basal conditions, TEC-c formed an extensive network of ring-like structures within 4 h. This property was significantly reduced in TEC-PAF-AH (Fig. 5). Stimulation with PAF (20 ng/mL) and VEGF (10 ng/mL) for 4 h significantly increased the number of vessel-like structures in TEC-c. In contrast, when TEC-PAF-AH were stimulated with PAF or VEGF, the number of vessel-like structures did not change from basal conditions (Fig. 5).

To evaluate the involvement of PAF in the angiogenic properties of TEC *in vivo*, TEC-c and TEC-PAF-AH were injected s.c. within diluted Matrigel in SCID mice. After 6 days, plugs were recovered and processed for histologic analysis. We previously showed that TEC, at a variance of HMEC, spontaneously organized in microvessels containing blood cells (7). This ability was maintained by TEC-c whereas the overexpression of PAF-AH in TEC-PAF-AH was associated with a significant reduction of functional vessel formation (Fig. 6).

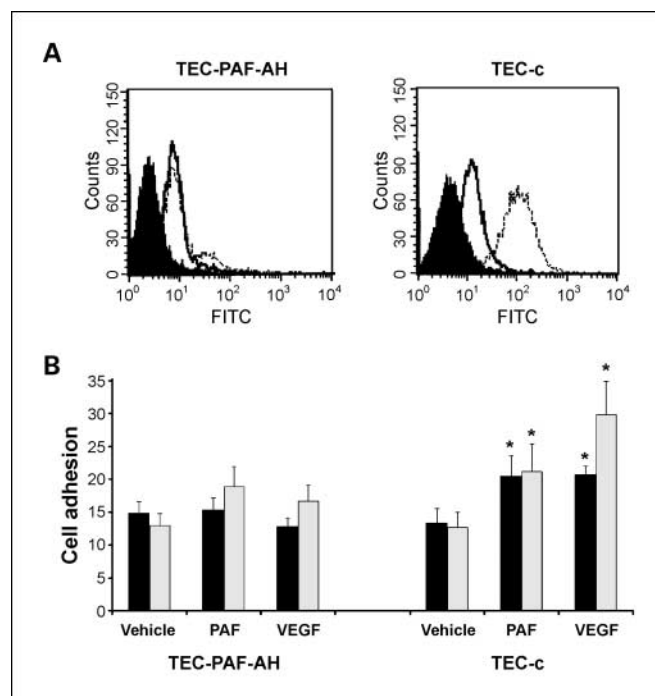


Fig. 5. Effect of PAF inactivation on integrin expression and cell adhesion. **A**, effect of PAF (20 ng/mL; 2 h at 37°C) on the expression of $\alpha v\beta 3$ integrin was evaluated by cytofluorimetric analysis in TEC-PAF-AH and TEC-c. TEC-c, but not TEC-PAF-AH, showed an increase in the expression of $\alpha v\beta 3$ integrin after PAF stimulation (dotted line) with respect to unstimulated cells (dark line). The filled area is the isotopic control. **B**, TEC adhesion to fibronectin (grey columns) or type IV collagen (black columns) after 2 h of stimulation with vehicle alone (RPMI + 0.25% BSA), PAF (20 ng/mL), or VEGF (10 ng/mL), as described in Materials and Methods. Columns, means of three individual experiments; bars, SD. ANOVA with Dunnett multicomparison test was done: *, $P < 0.05$ TEC-c stimulated with PAF and VEGF versus TEC-c stimulated with vehicle alone.

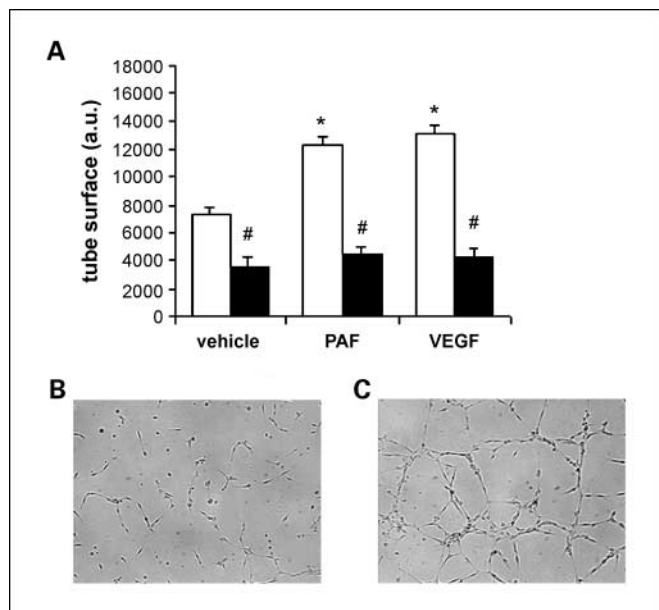


Fig. 6. *In vitro* angiogenesis. **A**, TEC-c and TEC-PAF-AH were plated on growth factor – reduced Matrigel-coated wells and stimulated with vehicle alone (medium 199 containing 1% BSA), PAF (20 ng/mL), or VEGF (10 ng/mL) for 4 h at 37 °C. **A**, results expressed in arbitrary units represent the extent of vessel-like structures in TEC-c (white columns) and TEC-PAF-AH (black columns). Columns, means of three individual experiments; bars, SD. ANOVA with Newman-Keul's multicomparison test was done: *, $P < 0.05$ TEC-c stimulated with PAF and VEGF versus TEC-c stimulated with vehicle alone; #, $P < 0.05$ TEC-PAF-AH versus TEC-c. **B** and **C**, representative micrographs of the uncompleted organization of TEC-PAF-AH in vessel-like structures on Matrigel (**B**) and the complete network of vessel-like structure formed by TEC-c (**C**) after 4 h of incubation with PAF.

To evaluate the role of TEC in tumor vascularization and growth, we did coimplantation experiments in SCID mice with RCC within diluted Matrigel at a ratio of 1:100 (Fig. 7). TEC significantly enhanced the incidence of implanted tumors whereas HMEC were ineffective (Table 1). In coimplantation experiments of RCC and TEC-PAF-AH, the vascularization derived from human TEC as well as murine endothelial cells, detected with antihuman and antimouse CD31 was significantly reduced with respect to TEC-c (Table 1), suggesting that PAF inactivation by PAF-AH impaired the formation of new vessels. However, no significant differences in the incidence and growth of the implanted tumors was observed between TEC-PAF-AH and TEC-c (Table 1).

Discussion

The results of the present study show that the proangiogenic phenotype of TEC is mediated, at least in part, by an enhanced production of PAF. In addition, we found that a strategy based on the local inactivation of PAF by PAF-AH resulted in the inhibition of the angiogenic behavior of TEC.

Endothelial-stimulating factors may promote tumor growth and invasiveness by supporting neovascularization of tumor tissue. PAF, a well-known vascular inflammatory agent, has been shown to induce angiogenesis through stimulation of endothelial migration (36, 40). Moreover, the angiogenic effect of PAF has been related to the induction of metalloproteinase synthesis from endothelial cells (41). We have previously studied the effect of PAF stimulation *in vitro* on Kaposi's sarcoma cells (27) and breast cancer cells (28) which produce

PAF and express PAF-R. Although PAF was able to enhance motility in both tumor cell types, in breast cancer cells, it also induced proliferation. An indirect evidence for the role of PAF in tumor vascularization was obtained in an *in vivo* model in which breast cancer cells were implanted s.c. in Matrigel containing a PAF-R antagonist (28). In this model, neoangiogenesis elicited by tumor cells was inhibited. It has been

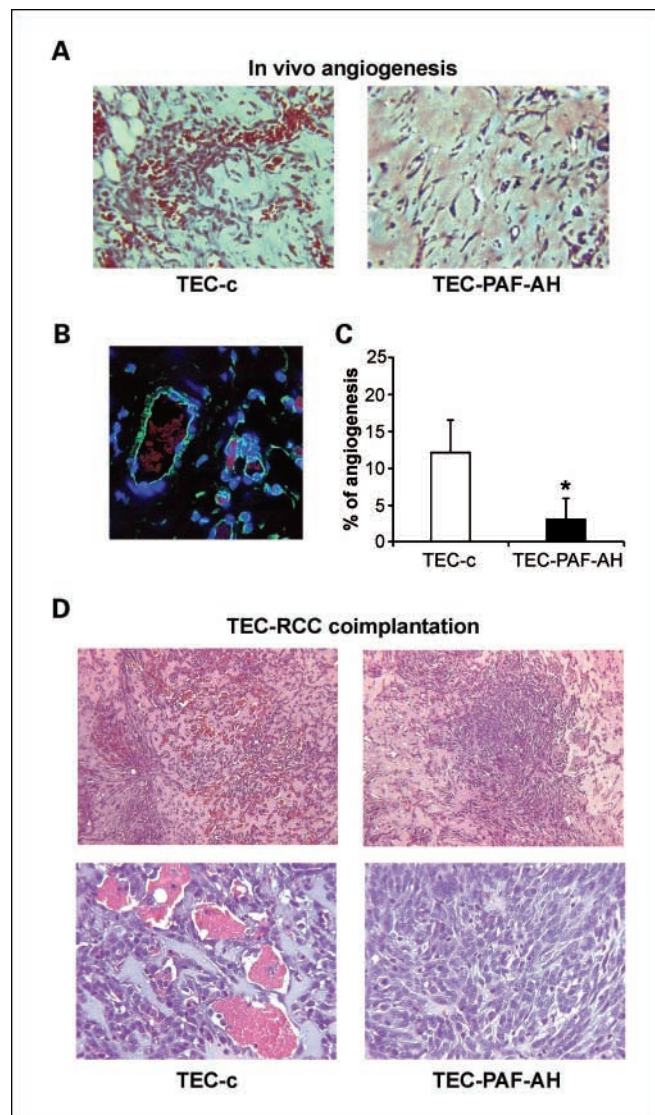


Fig. 7. *In vivo* angiogenesis. TEC-c and TEC-PAF-AH were implanted s.c. in SCID mice within growth factor – reduced Matrigel and the formation of organized vascular structures was evaluated after 6 d. **A** and **B**, representative micrographs of the formation of canalized vessels in mice implanted with TEC-c (**A**) and in mice implanted with TEC-PAF-AH (**B**). **C**, immunofluorescence staining for von Willebrand factor of vessels induced by implantation of TEC-c. **D**, quantitative analysis of neoformed vessels was done on histologic sections and results were expressed as the percent mean \pm SD of the vessel area per field. Vessel structures were counted only if they showed a patent lumen with red cells and/or leukocytes. t test was done between TEC-c and TEC-PAF-AH (*, $P < 0.05$). **D**, representative micrographs of the morphologic aspects of tumors which developed 3 wks after coimplantation of TEC-c or TEC-PAF-AH with a RCC line at a ratio of 1:100. Tumors which developed after coimplantation of RCC with TEC-c showed intense vascularization with the presence of large intratumor vascular lacunae containing RBC (original magnifications: top micrograph, $\times 100$; bottom micrograph, $\times 250$). In contrast, tumors which developed after coimplantation of RCC with TEC-PAF-AH showed a scarce vascularization; small vessels are seen only around the tumor (original magnifications: top micrograph, $\times 100$; bottom micrograph, $\times 250$).

Table 1. Experiments of coimplantation of a RCC line with TEC or HMEC

	RCC alone	RCC + HMEC	RCC + TEC-c	RCC + TEC-PAF-AH
Tumor incidence	0/10	0/6	6/6	6/6
Tumor volume (mm ³)	—	—	75 ± 22	68 ± 18
Tumor blood vessel density	—	—	15.2 ± 6.9	2.8 ± 1.1

NOTE: RCC (1×10^6 cells) alone or with HMEC, TEC-c or TEC-PAF-AH (1×10^4 cells) were injected s.c. in diluted Matrigel in SCID mice and the appearance of tumors was evaluated after 3 wks. Tumor microvessel density was assessed by counting intratumoral human and mouse CD31-positive vessels in $\times 20$ magnification fields. Fifty microscopic fields were analyzed for each experimental condition.

recently shown that estrogens, which are involved in the development of breast cancer, induced the production of angiogenic factors via a mechanism involving PAF-mediated nuclear factor- κ B activation (22). The essential role of PAF-induced nuclear factor- κ B activation has also been shown in macrophage-induced angiogenesis (42). Macrophages have been implicated in tumor angiogenesis via the capacity to produce many potent angiogenic factors. PAF-induced nuclear factor- κ B activation is a common upstream pathway leading to the production of such factors (42). The involvement of PAF in tumor growth, vascularization, and metastasis has been suggested in different tumor models (29, 43, 44). When PAF activity was suppressed in the intratumor environment of human KS and murine melanoma by stably transfecting plasma PAF-AH into tumor cells, the growth of tumors obtained by implantation in mice was inhibited (30).

We previously showed that endothelial cells derived from human renal and breast carcinomas display an embryonic phenotype with an increased survival and angiogenic properties compared with normal endothelial cells (7, 8). This was confirmed by the present results indicating that TEC, but not normal endothelial cells, favored the engraftment of a renal tumors in SCID mice. These properties might be related to the expression of embryonic genes and to the autocrine production of angiogenic growth factors (7–10). In the present study, we show that TECs were able to synthesize, upon stimulation, a significantly enhanced amount of PAF compared with normal HMEC. The inactivation of TEC synthesized PAF by overexpression of human plasma PAF-AH was found to significantly change the functional characteristics of TEC. The inactivation of PAF, obtained by PAF-AH transfection, restored the sensitivity to apoptosis, reduced cell motility and adhesion, and the *in vitro* and *in vivo* angiogenic properties of TEC. As far as the role of PAF in apoptosis, results in the literature are conflicting. Some studies in line with our results showed that PAF can inhibit apoptosis. Bazan et al. showed that signaling by lipids, such as PAF, prostaglandin E₂, or neuroprotectin D₁, regulate critical events essential for neuronal survival (45). Moreover, PAF was shown to up-regulate antiapoptotic factors in a melanoma cell line through nuclear factor- κ B activation (46). However, other

studies have reported that PAF could stimulate apoptosis (13, 47). A partial explanation of this discrepancy can be found in the dose of PAF used and in the cell types studied (13).

The profound effect in angiogenic potential of TEC obtained through local inactivation of PAF may be explained by the peculiar nature of this mediator. Indeed, PAF is considered a short-range inflammatory mediator that physiologically triggers the adhesion and migration of leukocytes tethered on the surface of activated endothelial cells (11, 12). This phospholipid is synthesized within minutes after stimulation by a number of inflammatory and proangiogenic mediators, such as tumor necrosis factor- α (17), hepatocyte growth factor (48), and VEGF (19). Because its rapid inactivation is guaranteed by the presence of both plasma and intracellular PAF-AH, it is conceivable that most of the activity of this phospholipid is exerted within defined areas of cell-to-cell contact where inhibitors are excluded. This is also consistent with the fact that several cell types retain the produced PAF on their surface and they may therefore stimulate PAF-R-expressing cells only during cell surface interaction (11, 13). In turn, PAF itself may induce the expression of several angiogenic factors and chemokines, including basic and acidic fibroblast growth factors, placental growth factor, VEGF and its specific receptors flk-1, hepatocyte growth factor, keratinocyte-derived chemokine, and macrophage-inflammatory protein 2; thus, sustaining and amplifying the inflammatory or angiogenic process (49). Therefore, in TEC, PAF might play a role in the amplification of the autocrine loop which sustains the activated angiogenic program (7). The results of the present study show that PAF-AH, by enhancing the local inactivation of PAF, interfered with this loop which sustains the angiogenic properties of TEC. Moreover, coimplantation experiments of TEC-PAF-AH and RCC indicated that local inactivation of PAF impairs tumor vascularization. However, PAF inactivation did not significantly affect TEC-induced tumor growth, suggesting that factors other than PAF may be released from TEC (7) and might favor the engraftment and expansion of tumor cells.

In conclusion, the present study provides direct evidence that PAF contributes to the proangiogenic phenotype of TEC, suggesting a key role of PAF in tumoral neoangiogenesis.

References

- Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;1:27–31.
- Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002;29:15–8.
- Garlanda C, Dejana E. Heterogeneity of endothelial cells. Specific markers. *Arterioscler Thromb Vasc Biol* 1997;17:1193–202.
- Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissue and tumors. *Annu Rev Immunol* 2000;18:813–27.
- Miebach S, Grau S, Hummel V, Rieckmann P, Tonn JC, Goldbrunner RH. Isolation and culture of microvascular endothelial cells from gliomas of different WHO grades. *J Neurooncol* 2006;76:39–48.
- Hida K, Hida Y, Amin DN, et al. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res* 2004;64:8249–55.
- Bussolati B, Deambrosio I, Russo S, Deregibus MC, Camussi G. Altered angiogenesis and survival in human tumor-derived endothelial cells. *FASEB J* 2003;17:1159–61.

8. Grange C, Bussolati B, Bruno S, Fonsato V, Sapino A, Camussi G. Isolation and characterization of human breast tumor-derived endothelial cells. *Oncol Rep* 2006;15:381–6.
9. Fonsato V, Buttiglieri S, Deregibus M C, et al. Expression of Pax2 in human renal tumor-derived endothelial cells sustains apoptosis resistance and angiogenesis. *Am J Pathol* 2006;168:706–13.
10. Bussolati B, Grange C, Bruno S, et al. Neural-cell adhesion molecule (NCAM) expression by immature and tumor-derived endothelial cells favors cell organization into capillary-like structures. *Exp Cell Res* 2006;312:913–24.
11. Prescott SM, Zimmerman GA, McIntyre TM. Platelet-activating factor. *J Biol Chem* 1990;265:17381–4.
12. Montrucchio G, Alloati G, Camussi G. Role of platelet-activating factor in cardiovascular pathophysiology. *Physiol Rev* 2000;80:1669–99.
13. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. Platelet-activating factor, a pleiotropic mediator of physiological and pathological processes. *Crit Rev Clin Lab Sci* 2003;40:643–72.
14. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. Platelet-activating factor acetylhydrolases. *J Biol Chem* 1997;272:17895–8.
15. Tjoelker L W, Wilder C, Eberhardt C, et al. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature* 1995;374:549–53.
16. Karasawa K, Harada A, Satoh N, Inoue K, Setaka M. Plasma platelet activating factor-acetylhydrolase (PAF-AH). *Prog Lipid Res* 2003;42:93–114.
17. Montrucchio G, Lupia E, Battaglia E, Bussolino F, Emanuelli G, Camussi G. Tumor-necrosis factor α -induced angiogenesis depends on “*in situ*” platelet-activating factor biosynthesis. *J Exp Med* 1994;180:377–82.
18. Brizzi MF, Battaglia E, Montrucchio G, et al. Thrombopoietin stimulates endothelial cell motility and neo-angiogenesis by a platelet-activating factor-dependent mechanism. *Circ Res* 1999;84:785–96.
19. Montrucchio G, Lupia E, Battaglia E, et al. Platelet-activating factor enhances vascular endothelial growth factor-induced endothelial cell motility and neoangiogenesis in a murine Matrigel model. *Arterioscler Thromb Vasc Biol* 2000;20:80–8.
20. Del Sorbo L, Arese M, Giraudo E, et al. Tat-induced platelet-activating factor synthesis contributes to the angiogenic effect of HIV-1 Tat. *Eur J Immunol* 2001;31:376–83.
21. Russo S, Bussolati B, Deambrosio I, Mariano F, Camussi G. Platelet-activating factor mediates CD40-dependent angiogenesis and endothelial-smooth muscle cell interaction. *J Immunol* 2003;171:5489–97.
22. Seo KH, Lee HS, Jung B, et al. Estrogen enhances angiogenesis through a pathway involving platelet-activating factor-mediated nuclear factor- κ B. *Cancer Res* 2004;64:6482–8.
23. Maliba R, Lapointe S, Neagoe PE, Brkovic A, Sirois MG. Angiopoietins-1 and -2 are both capable of mediating endothelial PAF synthesis: intracellular signalling pathways. *Cell Signal* 2006;18:1947–57.
24. Bussolino F, Arese M, Montrucchio G, et al. Platelet-activating factor produced *in vitro* by Kaposi's sarcoma cells induces and sustains *in vivo* angiogenesis. *J Clin Invest* 1995;96:940–52.
25. Montrucchio G, Sapino A, Bussolati B, et al. Potential angiogenic role of platelet-activating factor in human breast cancer. *Am J Pathol* 1998;153:1589–96.
26. Maggi M, Bonaccorsi L, Finetti G, et al. Platelet-activating factor mediates an autocrine proliferative loop in the endometrial adenocarcinoma cell line HEC-1A. *Cancer Res* 1994;54:4777–84.
27. Biancone L, Cantaluppi V, Boccellino M, et al. Motility induced by human immunodeficiency virus-1 Tat on Kaposi's sarcoma cells requires platelet-activating factor synthesis. *Am J Pathol* 1999;155:1731–9.
28. Bussolati B, Biancone L, Cassoni P, et al. PAF produced by human breast cancer cells promotes migration and proliferation of tumor cells and neo-angiogenesis. *Am J Pathol* 2000;157:1713–25.
29. Ishii S, Nagase T, Tashiro F, et al. Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. *EMBO J* 1997;16:133–42.
30. Biancone L, Cantaluppi V, Del Sorbo L, Russo S, Tjoelker LW, Camussi G. Platelet-activating factor inactivation by local expression of platelet-activating factor acetylhydrolase modifies tumor vascularization and growth. *Clin Cancer Res* 2003;9:4214–20.
31. Bussolati B, Assenzio B, Deregibus MC, Camussi G. The proangiogenic phenotype of human tumor-derived endothelial cells depends on thrombospondin-1 downregulation via phosphatidylinositol 3-kinase/Akt pathway. *J Mol Med* 2006;84:852–63.
32. Bruno S, Bussolati B, Grange C, Collino F, Graziano ME, Ferrando U, Camussi G. CD133+ renal progenitor cells contribute to tumor angiogenesis. *Am J Pathol* 2006;169:2223–35.
33. Tjoelker LW, Wilder C, Eberhardt C, et al. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature* 1995;374:549–53.
34. Blank ML, Hall MN, Cress EA, Snyder F. Inactivation of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine by a plasmaacetylhydrolase: higher activities in hypertensive rats. *Biochem Biophys Res Commun* 1983;113:666–71.
35. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–7.
36. Camussi G, Montrucchio G, Lupia E, et al. Platelet-activating factor directly stimulates *in vitro* migration of endothelial cells and promotes *in vivo* angiogenesis by a heparin-dependent mechanism. *J Immunol* 1995;154:6462–501.
37. McManus LM, Woodard DS, Deavers SI, Pinckard SI. PAF molecular heterogeneity: pathobiological implications. *Lab Invest* 1993;69:639–48.
38. Passaniti A, Taylor RM, Pili R, et al. A simple, quantitative method for assessing angiogenesis and anti-angiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 1992;67:519–28.
39. O'Flaherty JT, Redman JF, Schmitt JD, et al. 1-O-Alkyl-2-N-methylcarbonyl-glycerophosphocholine: a biologically potent, non-metabolizable analog of platelet-activating factor. *Biochem Biophys Res Commun* 1987;147:18–24.
40. Bernatchez PN, Tremblay F, Rollin S, Neagoe PE, Sirois MG. Sphingosine 1-phosphate effect on endothelial cell PAF synthesis: role in cellular migration. *J Cell Biochem* 2003;90:719–31.
41. Ko HM, Kang JH, Choi JH, Park SJ, Bai S, Im SY. Platelet-activating factor induces matrix metalloproteinase-9 expression through Ca(2+)- or PI3K-dependent signaling pathway in a human vascular endothelial cell line. *FEBS Lett* 2005;579:6451–8.
42. Seo KH, Ko HM, Choi JH, et al. Essential role for platelet-activating factor-induced NF- κ B activation in macrophage-derived angiogenesis. *Eur J Immunol* 2004;34:2129–37.
43. SY Im, Ko HM, Kim JW, et al. Augmentation of tumor metastasis by platelet-activating factor. *Cancer Res* 1996;56:2662–5.
44. Melnikova VO, Mourad-Zeian AA, Lev DC, Bar-Eli M. Platelet-activating factor mediates MMP-2 expression and activation via phosphorylation of cAMP-response element-binding protein and contributes to melanoma metastasis. *J Biol Chem* 2006;281:2911–22.
45. Bazan NG. The onset of brain injury and neurodegeneration triggers the synthesis of docosanoid neuroprotective signaling. *Cell Mol Neurobiol* 2006;26:899–911.
46. Heon Seo K, Ko HM, Kim HA, et al. Platelet-activating factor induces up-regulation of antiapoptotic factors in a melanoma cell line through nuclear factor- κ B activation. *Cancer Res* 2006;66:4681–6.
47. Matsuzawa A, Hattori K, Aoki J, Arai H, Inoue K. Protection against oxidative stress-induced cell death by intracellular platelet-activating factor-acetylhydrolase II. *J Biol Chem* 1997;272:32315–20.
48. Camussi G, Montrucchio G, Lupia E, Soldi R, Comoglio PM, Bussolino F. Angiogenesis induced *in vivo* by hepatocyte growth factor is mediated by platelet-activating factor synthesis from macrophages. *J Immunol* 1997;158:1302–9.
49. Negro Alvarez JM, Miralles Lopez JC, Ortiz Martinez JL, Abellan Alemmam A, Rubio del Barrio R. Platelet-activating factor antagonists. *Allergol Immunopathol* 1997;25:249–58.