PAF Produced by Human Breast Cancer Cells Promotes Migration and Proliferation of Tumor Cells and Neo-Angiogenesis

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Platelet-activating factor (PAF), a phospholipid mediator of inflammation, is present in breast cancer tissue and correlates with microvessel density. In the present study, we investigated the biological significance of PAF synthesized within breast cancer. *In vitro*, we observed the production of PAF by two estrogen-dependent (MCF7 and T-47D) and an estrogen-independent (MDA-MB231) breast cancer cell lines after stimulation with vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, tumor necrosis factor, thrombin but not with estrogen, progesterone, and oxytocin. The sensitivity to agonist stimulation and the amount of PAF synthesized as cell-associated or released varied in different cell lines, being higher in MDA-MB231 cells, which are known to be highly invasive. We further demonstrate, by reverse transcriptase-polymerase chain reaction and cytofluorimetry, that all of the breast cancer cells express the PAF receptor and respond to PAF stimulation in terms of proliferation. Moreover, in MDA-MB231 cells PAF elicited cell motility. *In vivo*, two structurally different PAF receptor antagonists WEB 2170 and CV 3988 significantly reduced the formation of new vessels in a tumor induced by subcutaneous implantation of MDA-MB231 cells into SCID mice. In conclusion, these results suggest that PAF, produced and released by breast cancer cells, can contribute to tumor development by enhancing cell motility and proliferation and by stimulating the angiogenic response. (Am J Pathol 2000, 157:1713–1725)

Considerable experimental evidence indicates that the formation of new blood vessels penetrating into solid tumors is required for their growth and metastatic dissemination.^{1,2} In human breast cancer, it was extensively

demonstrated that the tumor vascularization is strictly related to its growth and invasion, providing the basis for the use of the intratumor microvessel density as an independent prognostic marker.³ Several mediators responsible for tumor angiogenesis have been identified in human breast cancer.⁴ Studies on tumor angiogenesis have primarily focused on the role of vascular endothelial growth factor (VEGF), tumor necrosis factor- α (TNF- α), interleukin-8, transforming growth factor- β , basic fibroblast growth factor (bFGF), and tissue factor.⁵ These mediators are likely to be produced from the tumor cells themselves and/or by inflammatory infiltrating cells, such as mast cells and macrophages.^{6,7} Moreover, the simultaneous presence of growth factors and their receptors within breast cancer suggests that autocrine and/or paracrine loops are involved in the stimulation of cell proliferation and angiogenesis in this tumor.⁸

We recently demonstrated that platelet-activating factor (PAF), a phospholipid mediator of inflammation,⁹ that may also trigger angiogenesis,¹⁰ is present in breast cancer tissues and correlates with the tumor microvessel density.¹¹ PAF acts through a specific receptor belonging to the family of seven membrane-spanning domain receptors.¹² The receptor interacts with a G protein that activates phosphatidyl-inositol-specific phospholipase C.¹³ The development of potent PAF-receptor (PAF-R) antagonists allows investigation on the role of this mediator in several pathophysiological conditions.¹⁴

A role for PAF in tumor development has been recently suggested by the spontaneous development of skin tumors in transgenic mice overexpressing PAF-R.¹⁵ Moreover, in a murine model of melanoma, the blockade of PAF-R has been shown to reduce the melanoma cell dissemination into the lung.¹⁶ *In vitro*, PAF induced an autocrine proliferative loop in the endometrial cancer cell line HEC-1A,¹⁷ the migration of Kaposi's cells,¹⁸ and calcium influx in HT29 cells¹⁹ and N1E-115 cells.²⁰ The aim of the present study was to investigate the biological

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significance of inducible PAF synthesis in the invasiveness of breast cancer cells and in the neo-angiogenesis. *In vitro*, we evaluated the production of PAF by two estrogen-dependent breast cancer cell lines (MCF7 and T-47D) and by an estrogen-independent invasive breast cancer cell line (MDA-MB231) in response to proinflammatory, angiogenic, and hormonal stimuli. Moreover, we studied the expression of PAF-R by these cell lines and the effect of PAF on tumor cell motility and growth. *In vivo*, we evaluated, using two structurally unrelated PAF-R antagonist WEB 2170 and CV 3988, the potential role of tumor-synthesized PAF on the angiogenesis occurring in MDA-MB231 cells implanted subcutaneously into SCID mice.

Materials and Methods

Reagents

Synthetic C16 PAF (1-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine) and carbamyl PAF (1-hexadecyl-2-methylcarbamyl-sn-glycerol-3-phosphorylcholine) were 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% bovine serum albumin (BSA) fraction V, low endotoxin, was added immediately before use. CV 3988 was from Takeda Chemical Industries (Kyoto, Japan).²¹ WEB 2170 was obtained from Boehringer (Ingelheim, Germany).²² Silica gel 60F254 thin-layer chromatography plates were obtained from Merck (Darmstadt, Germany). μ Porasil high-pressure liquid chromatography columns were provided from Millipore chromatographic Division (Waters, Milford, MA). RPMI 1640 medium and bovine calf serum were from Life Technologies, Inc. (Grand Island, NY). Polymyxin B, phospholipase A2, phospholipase A1, BSA fraction V (tested for not >1 ng endotoxin per mg), fibronectin, bovine type-I collagen, thrombin, A23187, human recombinant TNF, hepatocyte growth factor (HGF) and bFGF, and FITC-conjugated anti-mouse and anti-rabbit IgG were all purchased from Sigma Chemical Company (St Louis, MO), as well as 17- β -estradiol, progesterone, and oxytocin. The anti-PAF-R monoclonal antibody (mAb) was kindly provided by Marek Rola-Pleszczynski (Sherbrooke, QC, Canada.). The irrelevant isotypic control was from Cedarlane (Hornby, Ontario, Canada). The anti-PAF-R polyclonal antibody (pAb) was obtained from Alexis (San Diego, CA). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Recombinant human VEGF₁₆₅ was obtained from R&D Systems (Abington, UK).

Extraction and Quantitation of PAF

To investigate the production of PAF from breast cancer cells, two hormone-dependent (MCF-7 and T-47D) and a hormone-independent (MDA-MB231) breast adenocarcinoma cell lines (American Type Culture Collection, Rockville, MD) were used. Cells were equilibrated for 15 minutes in Tris-buffered Tyrode containing 0.25% delipidized

BSA (fraction V), as previously described²³ and incubated at 37°C for the indicated time with the agonists. Selected experiments were conducted in the presence of 5 $\mu\text{g/ml}$ Polymyxin B for 30 minutes at 37°C to exclude lipopolysaccharide contamination. The supernatants and the cell pellets were extracted according to a modification of the Bligh and Dyer procedure,²⁴ 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 thin-layer chromatography and high-pressure liquid chromatography, by aggregation of washed rabbit platelets, as previously reported.^{10,23} The biologically active material extracted from cells and supernatants in different experiments was characterized by comparison with synthetic PAF according to the following criteria:²⁵ 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 $\mu\text{mol/L}$ WEB 2170 or CV 3988, two different PAF-R antagonists; 3) thin-layer chromatography and high-pressure liquid chromatography behavior and physicochemical characteristics, such as inactivation by strong bases and by phospholipase A₂ treatment, but resistance to phospholipase A₁, acids, weak bases, and 5 minutes heating in boiling water.

Cell Transfectant Development and Characterization

The ovarian cancer cells CHO (ATCC) were transfected with a vector containing the neomycin resistance gene only (CHO neo) or with a pRC/RSV vector containing human PAF-R cDNA (CHO PAF-R).²⁶ Transfectants were generated by electroporation at 1,000 μF in 4-mm electroporation cuvettes. Clones were selected for neomycin resistance, isolated for limiting dilution, and screened for expression of PAF-R by using reverse transcriptase-polymerase chain reaction.

PAF-R and PAF-Acetylhydrolase (PAF-AH) mRNA Expression

PAF-R-specific mRNA and PAF-AH mRNA were detected in total RNA extracted from cells by guanidinium thiocyanate phenol-chloroform and precipitated with isopropanol. One μg of RNA was treated with 6 U of RNase-free DNase for 1 hour at 37°C and then for 5 minutes at 94°C : complementary DNA was obtained by using random hexamer primers (Perkin-Elmer Cetus, Norwalk, CT). Reverse transcription was performed at 42°C for 60 minutes; in addition to 1 μg of RNA, the reaction mixture (20 μl) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L MgCl_2 , 1.0 mmol/L dNTPs, 20 U ribonuclease inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus). For PAF-R mRNA detection, cDNA was subjected to 35 cycles of amplification by the polymerase chain reaction in an automated DNA thermal cycler (Perkin-Elmer Cetus) by us-

ing the PAF-R mRNA-specific primer pairs¹⁸ forward: 5' CAC GGG CTC GAG ACC AAC ACA GTG CCC GAC AGT GCT 3'; and reverse: 5' CGC GGG ATC CCG GGT GAC CTG ATG TGC ATC ATT AAT 3'.

The polymerase chain reaction mixture (50 μ l) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 20 pmol of (+) and (–) primers and 2 U thermostable DNA polymerase (Perkin-Elmer Cetus). Times and temperatures for denaturation, annealing, and extension were 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, respectively. Amplification product was analyzed in 2% agarose gels containing 0.5 mg/ml of ethidium bromide. As control, B16 cells (ATCC) untransfected or transfected with the human PAF-R-specific cDNA (kindly provided by Dr. R. D. Ye, La Jolla, CA)¹⁸ were used.

To detect PAF-AH mRNA, cDNA was subjected to 35 cycles of amplification by the polymerase chain reaction by using PAF-AH mRNA-specific primer pairs: forward: 5' TTTTCACTGGCAAGACACATCTTCTTTTGAAGTTC 3'; and reverse: 5' CGTCAAAGTTCTGGTGCCTGAGCCCTTGATTGTA 3'.

Times and temperatures for denaturation, annealing, and extension were 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, respectively. Amplification product was analyzed in 2% agarose gels containing 0.5 mg/ml of ethidium bromide.

PAF Cytofluorimetric Analysis

The presence of the PAF-R on the membrane of breast tumor cells or of a nontumor mammary gland cell line MCF-10A (Michigan Cancer Foundation, Detroit, MI) was evaluated by cytofluorimetric analysis using the murine anti-PAF-R mAb. Cells (2×10^6) were fixed in 1% paraformaldehyde at 4°C for 20 minutes. After washing in phosphate-buffered saline (PBS) containing 2% heat-inactivated human serum and incubation for another 15 minutes with whole heat-inactivated human serum to block remaining nonspecific sites, cells were incubated for 30 minutes with the anti-PAF-R mAb (IgG2a, 1:500 dilution) or with the irrelevant isotypic control, in PBS containing 1% BSA. The intracellular staining for the PAF-R was evaluated in permeabilized cells. Briefly, cells were incubated with the anti-PAFR mAb at 4°C for 45 minutes in permeabilizing solution (PBS containing 0.1% saponin, 1% BSA, and 0.1% Na azide). After appropriate washings, cells were stained by the addition of fluorescein-conjugated goat-anti-mouse IgG antibodies (Sigma). All incubations were performed at 4°C.

To selectively stain the intracellular pool of PAF-R, cells were first incubated with the rabbit anti-PAF-R pAb (1:250 dilution) for 30 minutes at 4°C, to saturate the membrane pool of receptors. After fixation, cells were incubated in permeabilizing solution with the monoclonal anti-PAFR mAb and then with the fluorescein-labeled anti-mouse IgG. The stained cells were analyzed on a flow cytometer (Becton-Dickinson). As a positive control, umbilical vein endothelial cells, prepared as previously described,¹⁰ were used. The acquisition was done with 10,000 events

Figure 1. PAF synthesis by breast cancer cell lines. **A:** The basal production of PAF after a 1-hour incubation at 37°C in the absence of stimulation is shown. **B:** PAF production after a 1-hour stimulation at 37°C with 1 μ g/ml A23187. PAF was detected as cell-associated (solid bars) and as released in the supernatant (hatched bars). Data represent the mean $\pm$ SE of five individual experiments.

per sample. The Kolmogorov-Smirnov statistic analysis was performed in each individual experiment.

In Vitro Cell Migration

Cell motility in response to PAF was investigated both as chemotaxis in Boyden's chambers and as random cell movement of adherent cells. Chemotaxis across a polycarbonate filter (8- μ m pore size) was performed as previously described.¹⁰ RPMI medium containing 0.25% BSA and the stimulus or the vehicle alone was placed in the lower compartment of the chamber. Cells (2×10^5), suspended in the same medium, were then seeded in the upper compartment of the Boyden's chamber. In selected experiments, cells were preincubated for 30 minutes at 37°C with the PAF-R antagonists 3 μ mol/L WEB 2170 or 3 μ mol/L CV3988. After 5 hours incubation at 37°C, the upper surface of the filter was scraped with a rubber policeman. The filters were then fixed and stained with Diff-Quick (Harleco, Gibbstown, NJ) and 10 fields at $\times 200$ magnification were counted.

Cell migration of quiescent adherent MDA-MB231 cells or of CHO cells, transfected or not for PAF-R (10^5 cells/

Figure 2. PAF production by MCF-7 (A), T47-D (B), and MDA-MB231 (C) stimulated for 1 hour at 37°C with 20 ng/ml of VEGF, 10 ng/ml of bFGF, 6 U/ml of thrombin, and 10 ng/ml of TNF. PAF was detected as cell-associated (solid bars) and as released in the supernatant (open bars). Data represent the mean $\pm$ SE of five individual experiments.

well in RPMI plus 0.25% BSA) was studied throughout a 4-hour period under a Nikon Diaphot (Tokyo, Japan) inverted microscope with a $\times 10$ phase-contrast objective, as previously described.¹⁸ Cells were kept in an attached, hermetically sealed Plexiglas Nikon NP-2 incubator at 37°C. Cell migration was recorded using a Panasonic, CCTV (Matsushita Communication, Neumünster, Germany) video camera. Image analysis was performed with a Microlmage analysis system (Cast 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 30 minutes of interval. Migration tracks were generated by marking the position of nucleus of individual cells on each image. The net migratory speed (velocity straight line) was calculated by the Microlmage software based on the straight line distance between the starting and ending points divided by the time of observation. Migration of at least 30 cells was analyzed for each experimental condition. Values are given as mean $\pm$ SD. Cell division did not start to any significant degree during the experiments. In selected experiments, MDA-MB231 cells were seeded on plates previously coated with 10 μ g/ml of bovine fibronectin, type I collagen, or reconstituted basement membrane (Matrigel), overnight at 37°C.

Cell Proliferation Assay

PAF-R-positive breast cancer cells and PAF-R-negative COS cells were seeded at 8,000 to 10,000 cells/well into 24-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Stimulation was initiated by addition of different concentrations of carbamyl-PAF or of the PAF-R antagonists WEB 2170 and CV 3988. In the 96-hour experiments, media containing the tested substances was replaced after 48 hours. After 48 or 96 hours of incubation, cells were washed with PBS before addition of 1 ml Hepes (1.19 g/L), MgCl₂ (0.153 g/L) solution plus ZapoglobinR (Coulter Electronics Ltd., Luton Beds, UK). After 10 minutes of incubation at 37°C, cell suspensions were added to 9 ml of NaCl solution with 0.05% formalin in optically clear pots and stored at 4°C until counted. Cell number was determined by triplicate readings per each well of triplicate samples using a Coulter Counter (Coulter Electronics Ltd.). Three experiments were performed in triplicate. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni correction.

MDA-MB231 Xenograft in SCID Mice and Murine Angiogenesis Assay

For the *in vivo* studies, MDA-MB231 cells were implanted subcutaneously into SCID mice (Charles River, Wilmington MA) within growth factor-depleted Matrigel, as previously described.²⁷ The use of Matrigel is necessary for the initial establishment of tumors deriving from this cell line.²⁷ MDA-MB231 cells were harvested using trypsin-ethylenediaminetetraacetic acid, washed with PBS,

Figure 3. Time course of PAF synthesis by MCF-7 (A), T47-D (B), and MDA-MB231 (C) stimulated with 20 ng/ml of VEGF. PAF was detected as cell-associated (●) and as released in the supernatant (□). The numbers are the mean $\pm$ SD of three individual experiments.

Figure 4. Expression of mRNA for human PAF-R by untransfected B16 cells (lane 1), PAF-R-transfected B16 cells (lane 2), MDA-MB 231 cells (lane 3), MCF-7 cells (lane 5), and T-47D cells (lane 6), detected by reverse transcriptase-polymerase chain reaction. Lane 4 shows DNA markers.

counted in a microcytometer chamber, and resuspended in DMEM (4×10^6 in $250 \mu\text{l}$ DMEM). Cells were chilled on ice, added to $250 \mu\text{l}$ of Matrigel at 4°C , and injected subcutaneously into the left back of SCID mice using a 26-gauge needle using a 1-ml syringe. For PAF-R inhibition studies, WEB2170 and CV 3988, two structurally different PAF-R antagonists, were added to the Matrigel (final concentration, 250 ng/ml) and to drinking water (3 mg/kg/day), as previously described.²⁸ In selected experiments, VEGF (20 ng/ml) was also added to Matrigel. At day 7, mice (controls, $n = 12$; WEB 2170, $n = 10$; CV 3988, $n = 5$; VEGF, $n = 5$; and VEGF+WEB 2170, $n = 5$) were sacrificed and tumor plugs were recovered and processed for histology. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin, and embedded in paraffin. Sections ($3 \mu\text{m}$) were cut and stained with hematoxylin and eosin or with a Masson trichrome reaction and examined under a light microscope system. Morphometric analysis was performed to count vessels that were expressed as number/ mm^2 . Vessel structures were counted only if showing a patented lumen with red globuli and/or leukocytes. The mean size of vessels was planimetrically assessed using the computing integral area calculation of the Lucia digital system (Nikon UK Limited Instrument Division, Kingston, UK). Endothelial cells in the neoformed vessels were stained with fluorescein-labeled *Griffonia simplicifolia* lectin (Sigma)²⁹ and von Willebrand factor pAb (Sigma) by fluorescence.

The direct angiogenic effect of PAF released from MDA-MB231 cells was evaluated in the murine Matrigel angiogenesis assay.²⁸ PAF, extracted from the supernatant of 1×10^6 unstimulated cells cultured for 8 hours in DMEM containing 0.25% BSA, was resuspended in $50 \mu\text{l}$ saline with 0.25% BSA, added to growth factor depleted Matrigel and injected into mice. Briefly, Matrigel (0.5 ml) was subcutaneously injected into the abdominal tissue of female C57 mice along the peritoneal midline. After 6 days, mice were sacrificed and tumor plugs were recov-

Figure 5. Detection of the membrane and intracellular pools of PAF-R by flow cytometry on MDA-MB231 and endothelial cells. **A:** Membrane staining for PAF-R (dotted line), or membrane and intracellular PAF-R staining (dark line) in MDA-MB231 cells incubated with the anti-PAF mAb (1:500 dilution) or with an irrelevant isotypic control IgG (filled line), as described in Material and Methods. **B:** Selective staining for intracellular PAF-R (filled line) on MDA-MB231 cells incubated with the anti-PAF-R mAb after blockade of membrane receptors with rabbit anti-PAF-R pAb (1:250 dilution). The dark line indicates the total PAF-R detection. **C:** Membrane staining (dotted line), or total staining (dark line) for PAF-R in endothelial cells, used as positive control. Anti-PAF-R mAb or an irrelevant isotypic control IgG (filled line) were used. Three experiments were performed with similar results.

ered and processed for histological analysis of neoangiogenesis, as described above.

Results

PAF Production from MCF-7, T47-D, and MDA-MB231 Cells

In a previous study, we showed that MCF-7 breast cancer cells release bioactive PAF in basal conditions.¹¹ In the present study we compare basal and stimulated PAF synthesis in two estrogen-dependent cell lines (MCF-7 and T47-D) and one estrogen-independent cell line (MDA-MB231). Unstimulated cells produced a small amount of PAF, detectable both as associated to the cell fraction and as released in the supernatant (Figure 1). No significant difference in the amount of PAF produced in basal conditions was observed among these cell lines. In contrast MCF-10A, a nontumor breast cell line did not synthesized PAF (data not shown). Stimulation for 1 hour with calcium ionophore A23187, used as nonspecific inducer of PAF synthesis, increased PAF levels in all of the cell lines (Figure 1). In MCF7 and T-47D cells PAF was detected mainly as cell associated, whereas in MDA-MB231 cells it was almost completely released into the supernatant. A differential sensitivity to stimulation of these cell lines with VEGF, bFGF, thrombin, and TNF was observed. After 1 hour of stimulation with VEGF (20 ng/ml), PAF synthesis and release was detected in all of the tumor cell lines (Figure 2). However MDA-MB231 synthesized and released higher amount of PAF than MCF-7 and T-47D. bFGF (10 ng/ml) stimulated an efficient PAF production by T-47D and MDA-MB231, with a greater release from the latter. Similar results were obtained with HGF (20 ng/ml, data not shown). Concerning VEGF stimulation, we can speculate that neuropilin-1 is the VEGF-receptor involved in PAF production because neuropilin-1 is the only VEGF receptor-isoform expressed by MDA-MB231 cells.³⁰ As shown in Figure 2, thrombin (4 U/ml) induced a PAF production from T-47D and MDA-MB231 cells but not from MCF-7 cells, known to lack the thrombin receptor.³¹ In contrast, stimulation with TNF (10 ng/ml) enhanced PAF synthesis only from MCF-7 cells. No significant enhancement of PAF synthesis, as compared to basal, was detected after stimulation with 100 nmol/L 17- β -estradiol, progesterone, or oxytocin (data not shown). Figure 3 shows the time course of PAF synthesis from MCF-7, T-47D, and MDA-MB231 after stimulation with VEGF (20 ng/ml). PAF was detectable 15 minutes after stimulation and peaked at 1 hour to decrease thereafter. A similar kinetic was also observed after stimulation with bFGF (data not shown).

Figure 6. PAF induces MDA-MB231 cell motility. **A:** Chemotaxis of MDA-MB231 cells stimulated in Boyden's chamber with vehicle alone (shaded column) or various doses of PAF (solid columns). Preincubation of the cells (2×10^6 /ml) with $3 \mu\text{mol/L}$ PAF-R antagonists WEB2170 (hatched columns) inhibited the chemotactic effect of PAF. Figure shows data of one experiment performed in triplicate (mean $\pm$ SD). Four different experiments were performed with similar results. **B:** Time-lapse analysis of the motility of MDA-MB231 cells and of CHO cells transfected with the PAF-R (CHO-PAF-R) or with the empty vector (CHO-neo). Results are expressed as the percent increase in cell motility after a 2-hour stimulation with 10 nmol/L PAF (solid column) compared to the unstimulated cells. WEB 2170 ($3 \mu\text{mol/L}$; hatched columns) inhibited the migratory effect of PAF on MDA-MB231 and CHO-PAF-R cells. The results are the mean $\pm$ SD of three different experiments.

PAF-R Expression

Reverse transcriptase-polymerase chain reaction analysis revealed that mRNA encoding PAF-R was expressed by MCF7, T-47D, and MDA-MB231 (Figure 4). The expected cDNA fragment of 394 bp was amplified from total RNA extracted from breast cancer cells. Analysis of mRNA from B16 cells transfected with human PAF-R-specific cDNA, used as positive control, but not from untransfected cells, displayed an identical amplification product (Figure 4). Parallel cytofluorimetric experiments were performed to evaluate the differential PAF-R protein expression on the breast cancer cell lines. All of the cell

Table 1. Gradient-Dependent Analysis of PAF-Induced Migration of MDA-MB231 Cells

Lower chamber	Upper chamber		
	Vehicle	1 nmol/L PAF	10 nmol/L PAF
Vehicle	23.6 $\pm$ 4.02	31.2 $\pm$ 3.8	40.8 $\pm$ 6
PAF 1 nmol/L	62.7 $\pm$ 11.4	73 $\pm$ 14.68	160 $\pm$ 18.1
PAF 10 nmol/L	80.1 $\pm$ 13.7	146 $\pm$ 23.9	76.5 $\pm$ 24.4

Migration assay was performed in the Boyden chamber by adding PAF in the upper and/or lower compartments of the chamber to establish positive, negative, or absent gradient across the filter barrier. Cells that migrated after 6 hours of incubation to the lower surface of the filter were counted. Numbers are the mean $\pm$ SD of eight fields counted in a representative experiment out of three.

lines showed expression of the PAF-R on the cell membrane. MCF-7 and MDA-MB231 cells exhibited higher positivity (mean fluorescent intensity: 17.06 ± 3.2 and 24.0 ± 3.5 , respectively; 2.7 ± 0.8 control IgG; $P < 0.001$ versus control in $n = 3$ experiments) than T-47D cells (mean fluorescent intensity: 11.7 ± 1.2 anti-PAF-R antibody, 3 ± 1.2 control IgG; $P < 0.001$ versus control in $n = 3$ experiments). MCF-10A, a line of immortalized nontumor breast cells, did not express membrane PAF-R staining (data not shown). Recently, Ihida and colleagues³² demonstrated the presence of the PAF-R both on the plasma membrane and in the endosomal compartment of endothelial cells. We therefore investigated the presence of the intracellular PAF-R in the cancer cell lines. Permeabilized MDA cells showed an increased staining because of the presence of both the membrane and the intracellular PAF-R (Figure 5). A positive intracellular staining was also observed in MCF-7 cells and not in T-47D cells (data not shown). No staining was detected using an isotype control antibody. Moreover, to prevent cell surface reactivity, MDA cells were pretreated with a rabbit anti-PAF-R pAb before permeabilization and staining with the anti-PAFR mAb. Preincubation with the pAb reduced the observed staining for the receptor, showing the presence of a specific pool of intracellular receptors in MDA cells (Figure 5). Similar results were obtained using endothelial cells, as positive control (Figure 5). mRNA for PAF-AH, the main enzyme responsible for PAF catabolism, was not detected in MCF7, T-47D, and MDA-MB231 after reverse transcriptase-polymerase chain reaction amplification (data not shown).

PAF-Induced MDA-MB231 Cell Movement

Cell motility of breast cancer cells was studied both by directional migration assay in a Boyden chamber and by time-lapse recording migration assay of adherent cells. In the directional migration assay, PAF induced a dose-dependent chemotaxis on MDA-MB231 cells that was maximal at the dose of 10 nmol/L PAF (Figure 6). Preincubation with the PAF-R antagonist WEB 2170 ($3 \mu\text{mol/L}$) completely inhibited the chemotactic effect of PAF, thus providing evidence for the receptor-dependency of this effect (Figure 6). Similar results were obtained with another PAF-R antagonist, CV 3988 ($3 \mu\text{mol/L}$) ($86 \pm 4\%$ reduction of the migratory effect induced by 10 nmol/L

Figure 7. Micrographs representative of time-lapse analysis of MDA-MB 231 cell motility. Cell motility was performed by digital saving at 30 minutes of intervals. Migration tracks (original magnification, $\times 120$) were generated by marking the position of nucleus of individual cells of each image. **A:** Basal motility in unstimulated MDA-MB231 cells. **B:** Enhanced cell motility induced by 10 nmol/L PAF. **C:** Stimulation of MDA-MB231 cells with PAF in the presence of the PAF-R antagonist WEB 2170 (3 μ mol/L) inhibited cell motility.

PAF). MCF-10A that did not express membrane PAF-R cells did not migrate in response to PAF (number of cells/field: vehicle alone = 1.5 ± 0.7 ; 10 nmol/L PAF = 1.3 ± 0.4). To discriminate between the chemotactic and the chemokinetic effect of PAF on MDA-MB231 cells, migration studies were performed in the presence of PAF on both sides of the Boyden chamber. As shown in Table 1, the migratory effect of PAF was related to the dose of agonist in use rather than to its gradient, suggesting a chemokinetic effect of this mediator. The chemokinetic effect of PAF on MDA-MB231 cells was also suggested in time-lapse migration assay, in which PAF (10 nmol/L) was directly added to the plated cells (Figure 6B and Figure 7B). The dependency on PAF-R stimulation was indicated by the inhibitory effect of WEB 2170 on MDA-MB231 cells and by the requirement of PAF-R transfection

in the CHO cells, used as control because of not expressing PAF-R (Figure 6B and Figure 7C). In the time-lapse migration assay of adherent MDA-MB231 cells, baseline spontaneous cell motility was minimal, never exceeding 1 μ m/hour. PAF (10 nmol/L) induced a rapid increase of scattered cell motility that was maximal 1 hour after stimulation and sustained up to 4 hours (Figure 8). As control, cells were exposed to PAF in the presence of 3 μ mol/L WEB 2170, which completely abrogated the cell motility (Figure 8). Absence of response was also observed when PAF was replaced by same amount of lyso-PAF, the inactive de-acetylated metabolite of PAF (data not shown). To evaluate the role of different substrates in the PAF-dependent motility, MDA-MB231 cells were seeded on plates previously coated with fibronectin, collagen, or reconstituted basement membrane (Matrigel), and then stimulated with PAF. No significant difference was observed in the average speed of the cells onto plastic in comparison to that observed onto the different matrix components (Figure 8).

Effect of PAF on Cell Proliferation

The role of endogenously produced PAF in breast cancer cell proliferation was evaluated by blocking its biological activity with two chemically different PAF-R antagonists: WEB 2170 and CV 3988. Moreover, the effect of exogenous synthetic carbamyl-PAF on cell growth was investigated. This PAF isoform was chosen because it is not unsensitive to hydrolysis by serum PAF-AH. Both WEB 2170 (3 μ mol/L) and CV 3988 (3 μ mol/L) reduced the growth of the breast carcinoma cell lines (Figure 9). This inhibitory effect was significant after 96 hours of incubation except for the T-47D cells that exhibited a significant reduction of proliferation already after 48 hours (Figure 9). WEB 2170 and CV 3988 did not affect on the growth of MCF-10A and COS cells, which lack PAF-R, indicating the specificity of the observed effect. The proliferative role of PAF in breast carcinoma cells was confirmed by the synthetic carbamyl-PAF. Carbamyl-PAF (10 nmol/L) significantly increased proliferation of MCF-7 and MDA-MB231 cells, effect that was abrogated by the concomitant addition of 3 μ mol/L WEB2170 (Figure 10).

Effect of PAF Blockade on Vessel Formation in MDA-MB231 Implanted in SCID Mice

PAF, extracted from the supernatant of MDA-MB231 breast tumor cells cultured for 8 hours without additional stimuli, induced an angiogenic effect when injected into mice within Matrigel (MDA-MB231: 8.4 ± 1.9 vessels/ mm^2 ; $n = 4$, control = 0.5 ± 0.12 vessels/ mm^2 ; $n = 4$). In contrast, the lipid extract from MCF-10A cells, that did not contain a significant amount of PAF activity, failed to stimulate an angiogenic response when injected into mice (1.5 ± 0.25 vessels/ mm^2 , $n = 3$). To study the role of PAF, produced *in vivo* by implanted MDA-MB231 cells, in tumor vascularization, MDA-MB231 cells were injected subcutaneously in SCID mice within Matrigel containing 250 ng/ml WEB 2170 or CV3988, two structurally unre-

Figure 8. Motility of MDA-MB231 cells seeded onto plastic (A), fibronectin (B), Matrigel (C), or type-I collagen (D) measured by time-lapse analysis. Quiescent cells were stimulated with vehicle alone (●), 10 nmol/L PAF (▲), or 10 nmol/L PAF plus 3 μ mol/L WEB 2170 (■). Results are expressed as mean $\pm$ SD of three separate experiments.

lated PAF-R antagonists, or the vehicle alone. In the treated group, WEB 2170 and CV 3988 (3 mg/kg/die) were also added to the drinking water. Mice were sacrificed after 6 days and the whole tumor area was morphometrically analyzed for vascularization. In the presence of WEB 2170 or CV 3988, the number of newly formed canalized vessel was significantly reduced as compared to the control (Table 2). In untreated tumors many small vessels and few large aneurismatic structures were present (Figure 11A). These large structures were mainly absent in tumors treated with WEB 2170 (Figure 11B) or CV 3988. However, because of the high number of small vessels and the relative low number of aneurismatic structures present in untreated MDA-MB231 tumors, no statistical difference in the mean size of the vessels was

observed in respect to PAF antagonist-treated tumors (Table 2). The reduction in the vascular network in MDA-MB231 tumors treated with PAF antagonist (Figure 11D) versus the untreated (Figure 11C) was also detectable by the endothelial staining with fluorescein-labeled *G. simplicifolia* lectin. The extent of macrophages and polymorphonuclear cell infiltration was also reduced in mice treated with the PAF-R antagonists. Moreover, we evaluated the involvement of PAF in the angiogenic effect of VEGF added to the MDA-MB231 tumor in SCID mice. Addition of VEGF (20 ng/ml) significantly enhanced the tumor vascularization (Table 2). The inhibition of PAF activity with WEB 2170 induced a statistically significant, although partial, reduction of the enhancement in neoangiogenesis because of the VEGF addition (Table 2).

Figure 9. Effect of two different PAF-R antagonists WEB 2170 (3 μ mol/L) and CV 3988 (3 μ mol/L) on proliferation of PAF-R-positive MCF-7 (A), T47-D (B), MDA-MB231 (C) cells, and PAF-R-negative COS cells (D). Results are expressed as mean $\pm$ SD of four separate experiments performed in triplicate. One-way analysis of variance was performed between cell in the presence of 10% fetal calf serum alone and cells treated with 10% fetal calf serum plus WEB 2170 or CV 3988 (*, $P < 0.05$).

Discussion

Previous studies have shown that certain tumor cells express the PAF-R, however the significance of the PAF/PAF-R system in tumor biology remains to be defined. The present study demonstrates that human breast cancer cells not only express PAF-R, but also actively synthesize PAF after stimulation with several polypeptide mediators that are known to be expressed within the breast tumor *in vivo*. Moreover, we demonstrate a dual potential role of PAF synthesized by breast cancer cells: an autocrine effect on tumor cells as PAF stimulates their motility and proliferation and a paracrine effect on endothelial cells as PAF synthesized by cancer cells contributes *in vivo* to tumor angiogenesis.

PAF is a phospholipid mediator with multiple activities that is produced by inflammatory and endothelial cells.²⁵ It has been shown that PAF is also produced by human gastric³³ and endometrial¹⁷ adenocarcinoma cell lines. Previous studies demonstrated the presence of PAF in breast cancer tissue.^{11,34} PAF detected within this tumor may either derive from the infiltrating inflammatory cells or from the tumor cells.

In the present study, we demonstrate that human breast cancer cells, from two estrogen-dependent and one estrogen-independent cell lines, synthesize PAF after stimulation with angiogenic and proinflammatory stimuli, such as VEGF, bFGF, HGF, TNF, and thrombin. The more effective stimuli for PAF synthesis and release in MDA-MB23 cells were VEGF and bFGF. The observed

Figure 10. Effect of carbamyl-PAF (10 nmol/L), in the presence or absence of WEB 2170 (3 μ mol/L), on cell proliferation. MCF-7 (black columns), T47-D (dark hatched columns), and MDA-MB231 (open columns) cells were incubated for 96 hours in the presence of 10% fetal calf serum. The results are expressed as percent variation of cell number in comparison with unstimulated cells. Results are expressed as mean $\pm$ SD of four separate experiments performed in triplicate. One-way analysis of variance was performed between cells alone and cells treated with carbamyl-PAF (*, $P < 0.05$).

different cell sensitivity to agonist stimulation possibly reflects a differential receptor expression for the agonists by the breast cancer cell lines. Moreover, differences were seen among the cell lines in the amount of PAF that remains cell associated and that is released. Although PAF associated with cells may account only for an autocrine stimulation, PAF released in the extracellular environment may influence the function of tumor and endothelial cells in a paracrine way, thus supporting a potential role of PAF in tumor cell invasion. MDA-MB231, which are the most invasive breast tumor cells, showed higher ability to release PAF than MCF-7 and T-47D, suggesting a correlation between PAF release and malignancy. This contention is also supported by the inability of a nontumor breast cell line MCF-10A to synthesize and release PAF. Moreover, the expression of PAF-R was more pronounced in MDA-MB231 than in the MCF-7 and T-47D and was absent in MCF-10A. Cytofluorimetric studies using permeabilized and nonpermeabilized cells demonstrated the presence of a membrane and an intracellular pool of receptors. This observation is consistent with a recent demonstration that an intracellular pool of PAF-R is present in the endosomal compartment of endothelial cells.³² Although the functional significance of these intracellular receptors is currently unknown, it has

been suggested that they could represent a physiological intracellular reserve for mobilization to the cell surface. Alternatively, they could transduce intracellular signals. PAF-R is expressed by several human tumor lineages, such as epidermoid carcinoma (A431 cells),³⁵ endometrial cancer cell line HEC-1A,¹⁷ stomach cancer cell line (JR-St cells),³⁶ N1E-115 neuroblastoma cells,²⁰ Kaposi's sarcoma cells,³⁷ and HT29 colon carcinoma cells.¹⁹ However, little is known on the role of PAF-R in tumor biology.

In this study, we evaluated whether the engagement of PAF-R may activate cell functions relevant for tumor progression or invasion, such as proliferation and motility. We obtained both direct and indirect evidence for a stimulatory role of PAF in breast cancer cell proliferation. The inhibition of PAF-R engagement was achieved using two structurally different PAF-R antagonists.^{21,22,28,38} WEB2170 is a tetrazepinoic benzodiazepine with a specific PAF-R-binding activity.²² In a previous study we demonstrated that WEB2170, but not a control nonhetrazepinoic benzodiazepine, specifically inhibited PAF-induced neovascularization *in vivo*.³⁹ CV3988 is a structural analogue of PAF, which has been shown to specifically inhibit both *in vitro*⁴⁰ and *in vivo*⁴¹ PAF activity by competitive binding with PAF-R.^{21,28,38} Blockade of PAF-R using both WEB2170 or CV3988 significantly reduced proliferation of MCF-7, T-47D, and MDA-MB231 cells, but not of MCF-10A and COS cells, that do not express PAF-R. Stimulation of breast cancer cells with carbamyl-PAF, which is not inactivated by serum acetyl-hydrolase, induced an increase in cell proliferation. The role of PAF in cell proliferation and tumor formation is further supported by studies in transgenic mice overexpressing PAF-R. These mice exhibited an increased local cell growth in the skin, characterized by dermal and epidermal hyperthickening with dermal melanosis and, in some aged mice, a spontaneous development of melanocytic tumors.¹⁵

We also observed that PAF stimulates direct migration of MDA-MB231, but not of MCF-10A or COS used as PAF-R-negative controls. The analysis of PAF-induced motility indicates that movement is not triggered by a gradient of PAF, but is rather dependent on a chemokinetic effect of this mediator. This effect is dependent on PAF-R engagement as shown by the inhibitory effect of two chemically different PAF-R antagonists. Moreover, transfection of PAF-R in COS cells was shown to induce a PAF-dependent motility. The mitogenic activity of PAF has been previously demonstrated in several cell types,

Table 2. Effect of PAF-R Inhibition with WEB 2170 or CV 3988 on MDA-MB231 Tumors Implanted Subcutaneously in SCID mice, as Described in Material and Methods

Treatment	Vehicle	WEB 2170	CV 3988	VEGF	VEGF + WEB 2170
No. of animals	12	10	5	5	5
No. of vessels/mm ²	15.6 $\pm$ 1.8	7.4 $\pm$ 0.6*	5.3 $\pm$ 1.22*	29.4 $\pm$ 7.8*	19.3 $\pm$ 3.5†
Vessel size (μ m ²)	1287 $\pm$ 240	1619 $\pm$ 311	1033 $\pm$ 223	1820 $\pm$ 206	495 $\pm$ 164†

The number of vessels/mm² and the size of vessels were evaluated by morphometric analysis. Analysis of variance with Newman-Keuls multicomparison test was performed among vehicle *versus* vehicle plus PAF-R antagonists or *versus* VEGF treatment (*, $P < 0.05$) and among VEGF *versus* VEGF plus WEB 2170 (†, $P < 0.05$).

Figure 11. Role of PAF-R blockade in the vascularization of MDA-MB231 tumors implanted subcutaneously in SCID mice within Matrigel. Light microscopy representative of a tumor from an untreated mouse (**A**) and from a mouse treated with WEB 2170 (**B**). Original magnification, $\times 250$. Control tumor presents several canaliculated vessels and some aneurismatic structures containing red blood cells and leukocytes. The number of vessels is markedly reduced in a tumor from a treated mouse. Original magnification, $\times 250$. **C** and **D**: Fluorescence micrograph showing an articulate vascular network in MDA-MB 231 tumors as detected by fluorescein-labeled *G. simplicifolia* lectin. In contrast, few vessels are detectable in a tumor from a mouse treated with 3 $\mu\text{mol/L}$ WEB 2170. Original magnification, $\times 200$.

including leukocytes, Kaposi's sarcoma cells, and endothelial cells.^{25,37} Additional evidence for a role of PAF in regulating cell motility stems from the observation of a defect of PAF-AH in Miller-Dieker lissencephaly, a human brain malformation characterized by abnormal neuronal migration.⁴² The role of PAF in tumor progression has been recently addressed in an experimental model of melanoma. In this model, the production of PAF was correlated with the formation of pulmonary metastasis.¹⁶ In addition, PAF mediated the metastasis promoting effect of TNF and interleukin-1,¹⁶ further supporting its function as mediator of the biological activities of these cytokines.⁴³ In breast cancer we can envisage a function for PAF as a common mediator of several angiogenic, growth, and migratory factors, involved in the amplification of signals triggering tumor growth, invasion, and recruitment of endothelial cells.

Recent studies indicate that PAF may trigger angiogenesis and contribute to the angiogenic effect of certain polypeptide mediators such as TNF,²³ HGF,⁴⁴ and VEGF.⁴¹ PAF triggers angiogenesis both by stimulating the recruitment of endothelial cells and by inducing the expression of several angiogenic factors and chemokines including basic and acidic FGF, placental growth factor, VEGF and its specific receptor flk-1, HGF, KC, and macrophage inflammatory protein 2.³⁸ We previously

demonstrated a positive correlation between PAF levels and microvessel density in breast cancer.¹¹ Herein we demonstrate the direct involvement of PAF in new vessel formation in a model of breast cell-induced cancer based on subcutaneous injection of MDA-MB231 cells in SCID mice. In this model blockade of PAF-R both by WEB2170 and CV3988 induced a significant reduction in vessel density. Moreover, we found that the increased angiogenesis obtained by addition of VEGF to MDA-MB231 implanted in SCID mice was significantly inhibited by a PAF-R antagonist. This result suggests that PAF produced within the tumor plays a role in tumor vascularization by amplifying the effect of angiogenic cytokines *in vivo*.

These findings imply a dual role for PAF produced and released by cancer cells. PAF, in concert with polypeptide mediators, may have an autocrine action on tumor cells by promoting their motility and proliferation and a paracrine action on endothelial cells by stimulating the development of new vessels.

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