Platelet-Activating Factor (PAF) Induces Corneal Neovascularization and Upregulates VEGF Expression in Endothelial Cells

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PURPOSE. Platelet-activating factor (PAF) is a potent proinflammatory mediator that accumulates in the cornea after injury and induces the expression of genes related to inflammation and wound healing. The current study was conducted to investigate the direct effect of PAF on corneal neovascularization and on the expression of angiogenic growth factors in vascular endothelial cells.

METHODS. Pellets containing carbamyl-PAF (cPAF) were implanted in corneas of wild-type or PAF-receptor (PAF-R)-knockout mice, and the progression of angiogenesis was monitored by microscope. In some experiments, mice were treated with a daily intraperitoneal injection of the PAF-R antagonist LAU8080. Migration assays of human umbilical cord vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMVECs) were performed in a Boyden chamber after addition of various concentrations of cPAF or bovine fibroblast growth factor (FGF-2). Cell proliferation was assessed by fluorescence-binding assay in the presence of cPAF or FGF-2 for 8 days. Vascular endothelial growth factor (VEGF) and FGF-2 expression was studied by RT-PCR and Northern and Western-blot analyses in cells stimulated with cPAF at different concentrations and for different times.

RESULTS. Six days after cPAF pellet implantation, there were new vessels growing from the limbus to the center of the cornea. The PAF-induced neovascularization was significantly reduced in PAF-R-knockout mice and in mice treated with the PAF antagonist. cPAF added to the lower well of the Boyden chamber produced a dose-dependent migration of HUVECs and HMVECs that was inhibited in cells preincubated with LAU8080 or with a VEGF-blocking antibody. In contrast, cPAF did not stimulate proliferation of endothelial cells. cPAF induced VEGF mRNA and protein expression but not FGF-2 expression in HUVECs and HMVECs.

CONCLUSIONS. PAF stimulates corneal neovascularization by a receptor-mediated mechanism. Induction of VEGF expression and stimulation of vascular endothelial cell migration are initial events in PAF-promoted corneal angiogenesis. (Invest Ophthalmol Vis Sci. 2004;45:2915–2921) DOI:10.1167/iovs.04-0128

Angiogenesis, or neovascularization, is a complex, multistep process that includes migration, proliferation, and differentiation of endothelial cells; degradation of the surrounding extracellular matrix; and sprouting of new capillary branches. It plays an important physiologic role in conditions such as embryonic vascular development and wound healing and is activated in pathologic processes such as rheumatoid arthritis, psoriasis, diabetic retinopathy, and growth of solid tumors.1 The growth and migration of vascular endothelial cells are regulated by several growth factors, including basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming growth factors (TGF)-α and -β.2 Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid with very potent inflammatory properties, with biological actions that are mediated through activation of a G-protein–coupled PAF receptor (PAF-R).3 It is produced in tissues in response to injury. Recent studies suggest the involvement of PAF in angiogenesis. Elevated concentrations of PAF have been found in breast carcinomas with high microvesSEL density.4 The angiogenic effects of tumor necrosis factor (TNF)-α and HGF have been linked to synthesis of PAF by macrophages or endothelial cells,5,6 and vascular permeability induced by VEGF is related to increased PAF synthesis.7

Corneal neovascularization is a sequel of several inflammatory diseases of the anterior segment, such as bacterial, fungal, or viral infection. It can also occur after chemical burns and in immune reactions to corneal transplantation and extended contact lens wear. Several factors that trigger neovascularization have been identified in the cornea.8–11 Experiments in our laboratory in which we used a model of inflammatory uveitis have shown that topical treatment with a PAF-R antagonist decreases vascular permeability.12 PAF synthesis increases in cornea after alkali burn, and injury to corneal cells as well as infiltrating inflammatory cells contribute to increased PAF.13 Through its receptor activation PAF increases the expression of metalloproteinase (MMP)-1 and -9 and urokinase plasminogen activator (uPA).13,14 Prostases that are involved in the degradation of the extracellular matrix (ECM) and that facilitate capillary migration during angiogenesis.16

In the current study, we investigated the direct effect of PAF on corneal angiogenesis in vivo by introducing the lipid mediator into a micropocket in the mouse cornea. We also studied the action of PAF on the migration of endothelial cells and on the expression of VEGF and FGF-2, using two models of endothelial cells: human umbilical cord vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMVECs).
METHODS

Mouse Corneal Micropocket Assay

C57BL/6 mice (25–30 g) and PAF-R-knockout mice were used according to a protocol approved by the Institutional Animal Care and Use Committee of the LSU Health Sciences Center and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The PAF-R gene-deficient mice were generated as previously described17 and intercrossed for at least seven generations to obtain the C57BL/6 strain. The offspring were genotyped at 4 weeks of age for the PAF-R, using RT-PCR as detailed.18

Slow-release pellets containing 500 ng carbamyl-PAF (cPAF), a non-hydrolyzable analogue of PAF (Cayman Chemical Co., Ann Arbor, MI) and 0.1% BSA in PBS with 12% polyhydroxyethyl-methacrylate (Hydron; Interferon Sciences, New Brunswick, NJ) in ethanol were prepared in sterile conditions and allowed to dry for 30 minutes under a hood. The eyes were anesthetized by aspiration of a mixture of 2-bromo-2-chloro-1,1-trifluoroethanol containing 0.01% thymol (halothane, VIP 300; Butler, Dublin, OH) into which a mixture of NO2/O2 was bubbled. The eyes were irrigated with saline solution and antibiotics and then topically anesthetized with 1 drop 0.5% proparacaine. Corneal micropockets were created in both eyes under an operating microscope with a modified von Graefe knife, as previously described.19 The pellets were implanted in the micropockets 1 mm away from the limbus, and daily observations of the implants were made by slit lamp. Six days later, corneal photomicrographs were made. Vessel length and clock hours of circumferential neovascularization were scored in anesthetized animals observed by microscope. The arbitrary scale of 1+ equals 0.25 mm of vessel length from the limbal area, and 1+ equals 1 clock hour of the entire cornea divided into 12 hours counting from the center relative to the line of the pellet was used. The sums of the two parameters were used as arbitrary units.20

HVECs and HMVECs were treated with cPAF (1–100 nM) or PAF (100 nM) was denatured at 65°C for 10 minutes with 2.5 U RNase inhibitor and 0.5 μg Oligo(DT) primer (Invitrogen-Gibco, Grand island, NY). First-strand cDNA was synthesized using 100 U MMLV reverse transcriptase (Invitrogen-Gibco), 4 mM dNTP, and 10 mM dithiothreitol (DTT). The mixture was heated at 37°C for 60 minutes. PCR was performed using a 1:10 dilution of cDNA and 1.5 U Taq DNA polymerase in a 50-μL reaction mixture containing 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.01% Triton X-100), 2.5 mM MgCl2, 0.2 mM of each dNTP, and 0.4 μM primers. Primers were synthesized by the ISU Core Laboratories. Human VEGF primers were: upstream 5'-GAA-AGT-GTG-AAG-ATG-GAT-GTC-3' and downstream 5'-CGA-TCC-TGC-TTG-ATC-ACC-TGG-3'; and downstream 5'-GAA-GTG-GTC-TGC-CTG-3' and upstream 5'-TGA-GGC-GGT-GTG-GAG-3'. Primers for PAF-R were: upstream 5'-TAA-GCC-GTC-TGC-3' and downstream 5'-GAA-GTG-GTA-GAC-3'. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5'-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3' and downstream 5'-TCC-CTT-TGG-GCG-CAG-3'. Primers for FGF-2 were: upstream 5’-TAT-AAC-GCG-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Primers for PAF-R were: upstream 5’-TAC-AAC-GGC-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5’-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3’ and downstream 5’-TCC-CTT-TGG-GCG-CAG-3’. Primers for PAF-R were: upstream 5’-TAC-AAC-GGC-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5’-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3’ and downstream 5’-TCC-CTT-TGG-GCG-CAG-3’. Primers for PAF-R were: upstream 5’-TAC-AAC-GGC-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5’-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3’ and downstream 5’-TCC-CTT-TGG-GCG-CAG-3’. Primers for PAF-R were: upstream 5’-TAC-AAC-GGC-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5’-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3’ and downstream 5’-TCC-CTT-TGG-GCG-CAG-3’. Primers for PAF-R were: upstream 5’-TAC-AAC-GGC-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5’-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3’ and downstream 5’-TCC-CTT-TGG-GCG-CAG-3’. Primers for PAF-R were: upstream 5’-TAC-AAC-GGC-CTG-CAG-GCA-3’ and downstream 5’-GAA-GTG-GTC-TGC-CTG-3’. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as control for RNA/cDNA loading were: upstream 5’-CCA-CCC-AGT-GCA-AAT-TCC-ATG-GCA-3’ and downstream 5’-TCC-CTT-TGG-GCG-CAG-3’.

Migration Assay

Chemotactic migration assays of HVECs and HMVECs were performed in Boyden chambers. Polycarbonate filters (8-μm pore size; NeuroProbe, Inc., Gaithersburg, MD) were coated with 0.1% gelatin overnight at 4°C. EB or EGM supplemented with 0.5% FBS overnight and then into medium without serum followed by treatment with cPAF (1-100 nM) for different times. In some experiments, 100 nM PAF (Cayman Chemical Co.) was added. The PAF antagonist LAU8080 (10 μM) was added 1 hour before addition of PAF. Total RNA was extracted as described previously21 for RT-PCR and Northern blot. Proteins were also extracted for Western blot analysis.

Northern Hybridization

HVECs and HMVECs were treated with different concentrations of cPAF for different times depending on the experiment. RNA was isolated from cells as described.22 and 15 μg/lane of total RNA was denatured, electrophoresed, and transferred to a membrane (Hybond N+; Amersham Pharmacia Biotech, Buckinghamshire, UK). Hybridization was performed for 18 hours at 42°C in a buffer containing 5× SSC, 1% sodium dodecyl sulfate (SDS), 1× Denhardt solution, 50% formamide, 100 μg/mL denatured salmon sperm DNA, and 2 to 4×10^{6} cpm/3 to 8 ng/mL.21-22 Labeled human VEGF probe. The probe was prepared from purified RT-PCR products using a DNA-purification kit (Wizard PCR prep; Promega, Madison, WI). The VEGF PCR product...
PAF-induced corneal neovascularization. (A) Photographs of mouse corneas at 6 days after implantation of 12% polyhydroxyethylmethacrylate (Hydron; Interferon Sciences) pellets containing 0.1% BSA in PBS (control), 500 ng cPAF alone, or followed by intraperitoneal treatment with the PAF antagonist LAU8080 (30 μg/g body weight) once a day. The rightmost frame depicts a PAF-R-knockout mouse with a cPAF implant. (B) Angiogenic response at two different doses of cPAF, after treatment with LAU8080 and in PAF-R-knockout mice. Data are the mean ± SEM. Numbers above the bars represent the number of corneas with angiogenesis divided by the number of total corneas for each condition.

**Western-Blot Analysis**

HUVECs and HMVECs were treated with cPAF for 24 hours. Fifty micrograms of protein from cell lysates was loaded on 9% SDS-PAGE gels followed by Western transfer to nitrocellulose membranes (Hybond ECL; Amershams Biosciences). The membranes were blocked with 5% nonfat milk in PBS (control), 500 ng cPAF alone, or followed by intraperitoneal treatment with the PAF antagonist LAU8080 (30 μg/g body weight) once a day. The rightmost frame depicts a PAF-R-knockout mouse with a cPAF implant. (B) Angiogenic response at two different doses of cPAF, after treatment with LAU8080 and in PAF-R-knockout mice. Data are the mean ± SEM. Numbers above the bars represent the number of corneas with angiogenesis divided by the number of total corneas for each condition.

**RESULTS**

PAF induced extensive neovascularization in mouse corneas. The newly formed vessels reached the pellet implant by day 6 (Fig. 1A). The formation of new capillaries from the limbus toward the pellet was evident in all the animals implanted with 200 ng or more cPAF, and significant neovascularization (P < 0.05) was seen with 500 ng cPAF (Fig. 1B). Lower concentrations of PAF (1–100 ng) did not produce a significant response (data not shown). cPAF at higher concentrations (1 μg) with 5 U heparin in the pellet produced only a weak response in 25% of the treated animals, indicating that the response is decreased in the presence of heparin. We also tested the presence of 500 ng cPAF with sulcrate (sucrose octasulfate-aluminum complex; Sigma-Aldrich) in the pellet. Under these conditions, there was 100% response in treated animals, but this was not different from results obtained without sulcrate. Only 1 of 6 animals that received no cPAF in the pellet showed weak neovascularization (Fig. 1B). Eighty-six percent of the animals that received daily intraperitoneal injection of the PAF antagonist LAU8080 did not show angiogenesis. This route of administration was very effective and allowed treatment of the animals once a day. Vehicle treatment alone did not inhibit PAF-induced neovascularization in six mouse corneas. In addition, there was a very significant reduction in corneal neovascularization produced by cPAF in PAF-R-knockout animals (P < 0.001) compared with wild-type animals (Figs. 1).

To understand better how PAF regulates the behavior of vascular endothelial cells, we examined the effects of PAF and of the PAF antagonist on migration of two kinds of endothelial cells: HUVECs and HMVECs. Both cells express the PAF-R (data not shown) and cPAF induced their migration in a dose-dependent manner (Fig. 2A). The number of cells migrating across the filter with 10 nM cPAF was similar to that when 10 ng/mL FGF-2 was used as a positive control. cPAF at 100 nM produced a greater migration, and the same concentration of PAF was even more active. cPAF added to the upper chamber failed to induce migration. When cPAF was used at a concentration of 100 nM in the lower chamber and cells were preincubated for 1 hour with different concentrations of the PAF antagonist LAU8080 in the upper chamber, migration of the cells was completely inhibited with 10 μM LAU8080. In the absence of cPAF, LAU8080 did not affect the migration (Fig. 2B). The migration of the cells was dependent on preincubation of the cells with LAU8080. When PAF antagonist was added to the lower chamber along with cPAF, there was no inhibition of migration.

To evaluate whether cPAF had some effect on cell proliferation, different concentrations of cPAF were added to HUVECs or HMVECs and the cells were incubated for 4, 6, or 8 days. As shown in Table 1, cPAF did not affect the proliferation of the HUVECs or HMVECs. Under similar conditions, 10 ng/mL FGF-2 induced a significant proliferation at 4 and 8 days.

VEGF is expressed in vascularized corneas. We investigated whether PAF induces the expression of the angiogenic factors VEGF and FGF-2 in endothelial cells. RT-PCR analysis revealed the expression of a 541-bp product corresponding to the VEGF165 isoform and a 408-bp product corresponding to the VEGF121 isoform (Figs. 3A, 3B) in both HUVECs and HMVECs. When the cells were stimulated with 100 nM cPAF for different times, there was a significant increase in VEGF expression as early as 4 hours in HUVECs and at 8 hours in HMVECs, with a peak expression at 24 hours in HUVECs and at 12 hours in HMVECs. cPAF increased the gene expression of both isoforms, with VEGF165 being the most activated (Figs. 3A, 3B). No changes in GAPDH were noticed. When both isoforms were analyzed together, the data revealed a 4.5-fold increase in VEGF mRNA expression compared with the control in HUVECs 24 hours after cPAF stimulation and a 3.6-fold increase in HMVECs at 12 hours after PAF stimulation. The PAF-R antagonist LAU8080 at 10 μM completely inhibited the effect of PAF, but did not affect the basal expression of VEGF at any time. The effect was selective for VEGF, and different doses of cPAF (1, 10, 100 nM) at different times (1, 6, 12, 24,
or 48 hours) did not stimulate FGF-2 mRNA expression. Figure 3C illustrates one experiment using 100 nM cPAF.

The RT-PCR results for VEGF gene expression were confirmed by Northern blot analysis (Fig. 4). HUVECs stimulated with 100 nM cPAF for different times showed a 3.9-kb band corresponding to the size of the VEGF mRNA. There were marked increases in the band intensity at 12 and 24 hours, coinciding with the times obtained by RT-PCR. In addition, preincubation with LAU8080 blocked the VEGF induction pro-
duced by cPAF. GAPDH did not change with the treatment (Fig. 4A). Different concentrations of cPAF were also examined in HUVECs and HMVECs for 24 and 12 hours, respectively. In both endothelial cell types, increases in gene expression were obtained with 10 nM cPAF. There was a 3.9-fold increase compared with the control, with 100 nM cPAF in HUVECs (Fig. 4B). No differences between the effects of 10 and 100 nM cPAF were observed in HMVECs (Fig. 4C).

The gene expression induced by cPAF was translated into upregulation of VEGF protein that was detected by Western blot in both HUVECs and HMVECs (Fig. 5). A 42-kDa protein (top band) that was recognized by the antibody corresponding to the VEGF 

165 isoform was increased after 100 nM cPAF treatment for 24 hours. According to the manufacturer of the antibody, the lower band could be due to recognition of another VEGF isoform (probably VEGF 

121) or to the presence of a glycosylated protein. No other bands were seen on the gel.

To assess the contribution of PAF-induced VEGF synthesis on HUVEC migration, the cells were preincubated for 2 hours with an antibody to VEGF that blocks all VEGF isoforms and then were treated with 100 nM cPAF. Under these conditions, the effect of PAF on migration was abrogated (Fig. 6), suggesting that migration of vascular endothelial cells is linked to PAF-induced VEGF synthesis.

**DISCUSSION**

A sequela of corneal angiogenesis is reduction of visual acuity that, in severe cases, could lead to blindness. However, the pathogenesis of corneal neovascularization and the molecular events that trigger the condition are not well known. The arachidonic acid metabolite 12 (R)-hydroxyeicosatrienoic acid (12-HETrE) induces corneal neovascularization mediated in part by the induction of VEGF. Lipid hydroperoxides also induce retinal, choroidal, and corneal neovascularization. Corneal neovascularization is due to an increase in the synthesis of VEGF and tumor necrosis factor-α. In the current study, we present evidence that the potent inflammatory mediator PAF is an angiogenic factor in the cornea. We demonstrated that the action of PAF in the cornea is mediated by activation of its own receptor because an antagonist injected intraperitoneally blocked the corneal angiogenesis caused by PAF, and PAF did not cause corneal angiogenesis in PAF-R-knockout mice. Previous studies have shown that PAF is a mediator of angiogenesis induced by TNF and that PAF cooperates with other angiogenic factors, such as VEGF, acidic and basic FGF, and HGF in inducing vascular development in Kaposi’s sarcoma. Corneal neovascularization is dependent on inflammatory cells that arrive at the cornea as well as on various corneal cells that, when injured, synthesize PAF. In fact, we

**Table 1. Effect of cPAF in HUVEC and HMVEC Proliferation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>2 Days</th>
<th>4 Days</th>
<th>8 Days</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HUVECs</td>
<td>HMVECs</td>
<td>HUVECs</td>
</tr>
<tr>
<td>Control</td>
<td>3.0 ± 0.7</td>
<td>4.6 ± 1.0</td>
<td>4.2 ± 1.2</td>
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<tr>
<td>cPAF</td>
<td></td>
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<tr>
<td>0.1 nM</td>
<td>2.5 ± 1.0</td>
<td>4.0 ± 0.8</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>1 nM</td>
<td>2.9 ± 1.0</td>
<td>4.2 ± 0.2</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>10 nM</td>
<td>3.1 ± 1.0</td>
<td>4.4 ± 0.8</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>100 nM</td>
<td>3.2 ± 1.0</td>
<td>4.2 ± 0.4</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>FGF-2 10 ng/mL</td>
<td>4.0 ± 1.0</td>
<td>5.9 ± 1.0</td>
<td>6.8 ± 1.3**</td>
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Cell proliferation was assessed at the indicated times. The results are expressed in relative fluorescence and are the average ± SD of six experiments. Significant difference with FGF-2 compared with the control: *P ≤ 0.05; **P ≤ 0.01.
showed that in an alkali-burn model, PAF synthesis increases due to the arrival of infiltrating cells. The PAF receptor also recognizes oxidative fragments derived from phosphatidylcholine that can be synthesized during inflammation; therefore, some of the effects of lipid hydroperoxides on neovascularization could be through the activation of the PAF receptor. The finding that the angiogenic activity of PAF does not require heparin is in agreement with previous studies using PAF in a subcutaneous sponge implant in mice and suggests that the action of this bioactive lipid is not related to a proliferative action that can be exerted by other heparin-dependent growth factors.

Endothelial cell migration and proliferation are also essential to angiogenesis, and two models of endothelial cells, HUVECs and HMVECs, were used in the present study. Both expressed PAF-R, as demonstrated by RT-PCR and confirmed by sequencing (data not shown). PAF stimulates directional migration of HUVECs and HMVECs in vitro by acting on the

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933229/)

**FIGURE 3.** VEGF mRNA, but not FGFR2 mRNA, expression was up-regulated by PAF and inhibited by LAU8080. RT-PCR analysis of the expression of VEGF in HUVECs (A) and HMVECs (B) at different times after incubation with 100 nM cPAF. LAU8080 (10 μM) was added 1 hour before PAF. (C) RT-PCR analysis of FGFR2 expression at different times after incubation with 100 nM cPAF in HUVECs. GAPDH was used as a housekeeping-gene control for similar loading. VEGF was expressed as two different bands, a 541-bp band that corresponded to VEGF165 isoform, and a 408-bp band corresponding to the VEGF121 isoform. The experiment was repeated three times with similar results.

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933229/)

**FIGURE 4.** Northern-blot analysis of VEGF. (A) HUVECs were stimulated with 100 nM cPAF for different times or pretreated with 10 μM LAU8080 for 1 hour. (B) HUVECs and (C) HMVECs incubated with different concentrations of cPAF for 24 and 12 hours, respectively. The size of the VEGF mRNA in the gels was 3.9 kb. The experiment was repeated three times with similar results.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933229/)

**FIGURE 5.** PAF increased VEGF protein in endothelial cells. (A) Western-blot analysis of VEGF in HUVECs treated with different concentrations of cPAF. (B) PAF (100 nM) also stimulated the expression of VEGF in HMVECs. P is recombinant VEGF (10 ng), used as positive control. Top band: 42-kDa VEGF165 isoform; bottom band: may be variant glycosylation of the protein or recognition by the antibody of another VEGF isoform. The experiment was repeated three times with similar results.
PAF-R without affecting cell proliferation. The migration of the endothelial cells was dependent on a chemotactic effect of PAF, since addition of PAF to the upper well of the Boyden chamber failed to increase the transmembrane migration of HUVECs and HMVECs. Previous studies using PAF antagonists suggest that PAF enhances migration but not proliferation of HUVECs by an indirect effect of VEGF that activates the synthesis of PAF. One important event in angiogenesis is the proteolysis of components of the ECM. Degradation of the ECM is essential for the migration of endothelial cells through a basement membrane that is disrupted by the action of MMPs. In corneal epithelium PAF induces the expression of MMP-1, and -9, and uPA and promotes a significant imbalance in favor of MMP-9 rather than its inhibitors, tissue inhibitor of metalloproteinases (TIMP)-1 and -2. Therefore, the induction of MMPs, PAF may contribute to corneal neovascularization by enabling vascular endothelial cell migration through corneal tissue after disrupting ECM components and releasing sequestered angiogenic factors.

In addition, we present evidence that PAF stimulates the expression of VEGF mRNA and protein in both HUVECs and HMVECs, suggesting that PAF may modulate some of the functions of endothelial cells by upregulating VEGF expression. The action of PAF is selective, and FGF-2 expression is not stimulated by PAF. Increased expression of VEGF and its receptors Flt-1 and KDR occurs in endothelial cells of vascularized cornneas. The VEGF<sub>165</sub> isoform binds to the neuropilin-1 receptor and potentiates the interaction of the growth factor with its KDR receptor in endothelial cells, whereas VEGF<sub>121</sub> does not. The formation of juxtacrine-like complexes among VEGF<sub>165</sub>, neuropilin-1, and KDR can also be found between endothelial cells and adjacent epithelial cells. Our recent studies suggest that PAF decreases the attachment of rabbit corneal epithelial cells. Apparently, VEGF also stimulates the tyrosine phosphorylation of several proteins. These findings raise the possibility that PAF, directly or through VEGF, regulates endothelial cell motility and adhesion. In our studies, we found that PAF stimulated the expression of both VEGF<sub>165</sub> and VEGF<sub>121</sub> isoforms. VEGF type A contains four different isoforms: VEGF<sub>165</sub>, VEGF<sub>121</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub>. VEGF<sub>165</sub> is the predominant form found in most cells and can be secreted, although a significant proportion remains bound to the cell surface. VEGF<sub>121</sub>, in contrast, is thought to be a diffusible protein. In our study, neutralizing VEGF with an antibody that recognizes all the isoforms prevented PAF-induced migration of HUVECs, suggesting that migration is linked to expression of one of the two VEGF isoforms. The finding that PAF-induced VEGF production did not stimulate endothelial cell proliferation is puzzling. One possibility is that the amount of VEGF produced after PAF stimulation is below the threshold needed to induce proliferation in these cells. Future experiments in our laboratory will address these questions. Although in the present study we did not investigate the mechanisms by which PAF stimulates VEGF gene expression, our recent work has shown that monkey choroid retinal endothelial cells exposed to hypoxia regulate VEGF expression in part through NF-κB-mediated cyclooxygenase (COX)-2 expression and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis. PAF is an inducer of COX-2 expression and PGE<sub>2</sub> synthesis in corneas.

In summary, our results suggest that after corneal inflammation, PAF synthesis stimulates angiogenesis by inducing a chemotactic effect on the endothelial cells and activating the degradation of ECM components in the tissue. PAF triggers vascular endothelial cell migration by stimulating the expression of VEGF in endothelial cells. All these effects are blocked by LAU8080, which raises the possibility of the therapeutic use of PAF antagonists in the management of inflammatory corneal neovascularization after chemical burns and corneal transplantation.

References


