Delay of Corneal Epithelial Wound Healing and Induction of Keratocyte Apoptosis by Platelet-Activating Factor

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PURPOSE. To examine the role of the lipid mediator platelet-activating factor (PAF) in epithelial wound healing.

METHODS. A 7-mm central de-epithelializing wound was produced in rabbit corneas, and the tissue was incubated with 125 nM carbamyl PAF (cPAF), an analogue of PAF. Rabbit corneal epithelial and stromal cells were also cultured in the presence of cPAF. Cell adhesion, proliferation, and migration assays were conducted. Apoptosis was assayed by TUNEL staining on preparations of corneal tissue sections and in cells in culture.

RESULTS. Twenty-four hours after injury, 50% of the wounded area was covered by new epithelium, whereas only 30% was covered in the presence of cPAF. At 48 hours, the epithelium completely closed the wound, but only 45% of the original wound was covered in corneas treated with cPAF. Similar inhibition of epithelial wound closure was found with human corneas incubated with PAF in organ culture. Moreover, addition of several growth factors involved in corneal wound healing, such as epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, could not overcome the inhibitory action of PAF in wound closure. Three PAF antagonists, BN50727, BN50730, and BN50739, abolished the effect of PAF. A significant increase in TUNEL-positive staining occurred in corneal stromal cells (keratocytes), which was inhibited by preincubating the corneas with PAF antagonists. However, no TUNEL-positive staining was found in epithelial cells. TUNEL-staining results in cultured stromal cells (keratocytes) and epithelial cells in first-passage cell culture were similar to those in organ-cultured corneas. In addition, PAF caused 35% to 56% inhibition of adhesion of epithelial cells to proteins of the extracellular matrix: collagen I and IV, fibronectin, and laminin. There were no significant changes in proliferation or migration of epithelial cells induced by the lipid mediator.

CONCLUSIONS. The results suggest PAF plays an important role in preventing corneal wound healing by affecting adhesion of epithelial cells and increasing apoptosis in stromal cells. PAF antagonists could be of therapeutic importance during prolonged ocular inflammation, helping to avoid loss of corneal transparency and visual acuity. (Invest Ophthalmol Vis Sci. 2002;43:1422–1428)

Platelet-activating factor (PAF) is a phospholipid cytokine implicated in a wide range of biological and pathologic responses in cells. It plays important roles in inflammation and allergic reactions, as well as synaptic transmission. Most of the actions of PAF are exerted through specific receptors (PAF-R). Previous studies from our laboratory have shown that exposure of rabbit corneas to alkali in vivo triggers the synthesis of PAF. These studies concluded that corneal cells synthesize PAF as early as 30 minutes after injury and that the increased accumulation observed at later times is due in part to the presence of inflammatory cells that arrive at the cornea and actively produce PAF. More recent studies have demonstrated an increase in PAF in human tears during eye closure and after contact lens-induced inflammation. A surface-membrane PAF-R is expressed in corneal epithelium, keratocytes, and endothelial cells. In the corneal epithelium, stimulation by PAF produces multiple responses, including an increase in Ca2+ influx and activation of phospholipase A2 and mitogen-activated protein kinase (MAPK). It also stimulates, at the transcriptional level, cyclooxygenase-2, the inducible prostaglandin synthetase, and in that way prolongs the inflammatory response of the tissue. Another important receptor-mediated action of PAF in the cornea is the selective induction of enzymes involved in the remodeling of components of extracellular matrix (ECM), such as collagenase-1 (MMP-1) and the 92-kDa gelatinase MMP-9, which degrades components of the corneal basement membrane. It also stimulates the expression of urokinase plasminogen activator (uPA), an enzyme that converts plasminogen to plasmin, a widely acting protease that degrades several ECM components. In addition, corneal epithelial injury produces upregulation of PAF-R gene expression, prolonging the aforementioned effects of PAF. These studies suggest that multiple effects exerted by PAF in the cornea influence wound healing in the tissue after injury.

The process of corneal epithelial wound healing involves three main phases: adhesion to the basement membrane, migration to cover the wound, and cell division. Several studies have described the use of growth factors that are synthesized by cells as molecules that promote corneal wound repair. In addition, interactions between stroma and epithelium have been recognized as important for proper healing, and one of these interactions is the induction of keratocyte apoptosis after epithelial injury. In the present study, we used a corneal organ culture model to mimic in vivo wound healing, which allowed us to investigate how PAF affects wound closure. In our study, PAF was a strong inhibitor of epithelial wound healing, even in the presence of growth factors. Antagonists to the PAF-R blocked this inhibition. To understand the mechanisms involved in the delay of the wound-repair response by PAF, we investigated its effects on epithelial cell adhesion, proliferation, and migration and apoptosis in injured corneal cells.

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Rabbit eyes were obtained from Pel-Freeze Biologicals (Rogers, AR). The eyes were shipped overnight on ice in Hanks’ balanced salt solution (HBSS) containing antibiotics and an antifungal. Human corneas unsuitable for transplantation were obtained from the Southern Eye Bank (New Orleans, LA). Carbamyl PAF (cPAF, a stable agonist of PAF) and lypo-PAF were from Cayman (Ann Arbor, MI). PAF antagonists BN50727, BN50730, and BN50739 were kindly provided by Pierre Braquet (Institut Henri Beaufour, Le Plessis, France). Human recombinant double-chain hepatocyte growth factor (HGF) was a gift from Genentech (San Francisco, CA). Epidermal growth factor (EGF) was obtained from Sigma (St. Louis, MO), human recombinant keratinocyte growth factor (KGF) from Upstate Biotechnology (Lake Placid, NY), a biotinylated chemiluminescence (ECL) protein markers kit from New England BioLabs (Beverly, MA), and collagen I, laminin, fibronectin, and collagen IV from Sigma. A cell proliferation assay kit (CyQuant) was obtained from Molecular Probes (Eugene, OR) and the TdT-mediated FITC-linked diUTP nick-end DNA labeling (TUNEL) reagents from Promega (Madison, WI).

**Materials and Methods**

**Corneal Epithelial Wound Healing**

The in vitro organ culture model was used as previously described. Briefly, the central area of the cornea was gently marked with a 7-mm surgical trephine, and the epithelium was carefully removed with a sterile blade. The wounded corneas were excised with 1- to 2-mm scleral rim and rinsed three times in Dulbecco’s modified Eagle’s medium (DMEM)-F12, containing 1% antibiotic and antifungal solution, and incubated in 12-well plates (Corning Tissue Culture, Boston, MA) at 37°C with 5% CO2-95% air in the presence of 125 nM cPAF dissolved in ethanol. Some corneas were pretreated for 1 hour with 10 μM solutions of PAF antagonists dissolved in dimethyl sulfoxide (DMSO) before the addition of cPAF. Vehicles at the same concentration were added to the medium of control corneas. In some experiments, lypo-PAF, an inactive metabolite of PAF, was added instead of cPAF. Hepatocyte (HGF), keratinocyte (KGF), and epidermal (EGF) growth factors (20 ng/mL) were added at the same time as cPAF. Corneas were removed at different times for tissue sectioning or to determine the degree of wound closure.

**Cell Adhesion, Proliferation, and Migration Assays**

Rabbit corneal epithelial cells (2 × 10⁴ cells/well) were seeded in DMEM-F12 containing 10% bovine serum albumin in the presence or absence of 125 nM cPAF in 48-well plates noncoated or coated with 10 μg/mL collagen I, laminin, fibronectin, or collagen IV. After the coating, all nonspecific sites were blocked with 5% nonfat dry milk. Cells were incubated for 45 minutes, and nonadherent cells were removed by washing the plates with phosphate-buffered saline (PBS). For the proliferation assays, the cells were plated at different cell densities (1,000–10,000/well) in uncoated wells and incubated in DMEM-F12 containing 2.5% fetal bovine serum (FBS), with or without 125 nM cPAF for 5, 24, 48, or 72 hours. Additional experiments were performed in the presence of HGF or EGF (20 ng/mL) with and without PAF, for 24, 48, and 72 hours. The DNA content of the cells present in the wells after adhesion and proliferation experiments was determined by measuring a fluorescent dye (CyQUANT GR; Molecular Probes), which exhibits strong fluorescence enhancement when bound to DNA (excitation wavelength at 485 nm and emission at 536 nm) using the cell proliferation assay kit (CyQuant; Molecular Probes), according to the manufacturer’s instructions. For epithelial cell migration, 2-μL aliquots of cell suspension (2–4 × 10⁴ cells in 50 μL DMEM-F12) were spotted in Petri dishes (six to eight spots/dish), and cells were allowed to settle for a few seconds before a drop of medium was added on each spot. After 2 hours at 37°C, the unattached cells were gently rinsed away with medium, fresh medium was added, and the dishes were incubated overnight. The cells were further incubated with or without 125 nM cPAF. The migration of cells from each spot at different time points was measured with a filar micrometer attached to the microscope.

**Assessment of Apoptosis**

**Preparation of Corneal Tissue Sections.** Control and treated corneas were gently washed with cold PBS, flattened by radial cuts, and bisected (total of eight sections). The tissue was fixed overnight in 10% formaldehyde and embedded in paraffin wax, and 5-μm thick sections were prepared. Afterward, the sections were dewaxed with xylene and rehydrated through a graded series of ethanol to water. These sections were washed with PBS, fixed in 4% methanol-free formaldehyde at room temperature, treated with proteinase K (20 μg/mL) for 15 minutes, and washed twice with PBS.

**FITC-dUTP TUNEL Staining.** The cells were fixed in 4% formaldehyde solution for 25 minutes at 4°C and permeabilized with 0.2% Triton X-100 solution for 5 minutes on ice. The TdT-mediated FITC-linked dUTP nick-end DNA (TUNEL) labeling of the corneal epithelial cells, fibroblasts, keratocytes, or tissue sections was performed using the apoptosis detection system, according to the manufacturer’s protocol. The cells were counterstained with propidium iodide (1 μg/mL) for 15 minutes at room temperature. The stained cells were viewed under a confocal microscope. The apoptotic cells labeled green and the normal cells red. The images were captured with a chilled 5-charge-coupled device (CCD) color camera (Hamamatsu, Hamamatsu City, Japan) and digitized by computer (Photoshop; Adobe, San Diego, CA).
Corneal Epithelial Wound Healing Promoted by Growth Factors

Several growth factors are known to promote corneal wound healing. 14-17 We investigated whether PAF is capable of inhibiting wound repair in the presence of some of these growth factors. Corneas were injured and incubated for different times in the presence of two paracrine growth factors, HGF and KGF, whose receptors have been demonstrated in the epithelium, 21 or in the presence of EGF, an autocrine and paracrine growth factor that stimulates corneal wound healing. 15 In our in vitro model, 24 hours after the injury an increase in epithelial wound healing occurred in corneas incubated with growth factors (Fig. 2). In the presence of HGF, there was a significant decrease compared with control corneas in the uncovered area, from 500 ± 20 to 340 ± 16 AU (n = 4, P < 0.01). A similar decrease in the wound area was observed in the presence of EGF, with an average of 310 ± 33 AU (n = 4, P < 0.01), and KGF, with an average of 370 ± 28 AU (n = 3, P < 0.01, data not shown). However, the wound closure of the corneas incubated with growth factors in the presence of PAF was significantly inhibited. The addition of the PAF antagonist BN50739 blocked the inhibition of PAF in the presence of growth factors (Fig. 2). Significant delay in wound closure caused by PAF in the presence of growth factors was also observed 48 hours after the injury (data not shown).

Corneal Epithelial Cell Adhesion

Because PAF is a plethoric agent involved in many different actions, we investigated whether it affects some parameters linked directly to the process of wound healing: adhesion, migration, and proliferation. To investigate whether PAF affects the attachment of epithelial cells, we examined epithelial cell adhesion in the presence of different ECM components present in the cornea. When the four ECM proteins were incubated without cPAF for 48 hours, there was substantial inhibition of epithelial wound closure compared with medium without serum and growth factors. After 24 hours, approximately 50% of the wounded area was covered by epithelium—that is, if the initial wound area represented 1000 arbitrary units (AU), the wounded area uncovered by epithelium was 500 ± 20 AU (n = 6 experiments, with the average of three to four corneas in each experiment). A 7-mm central epithelial wound was covered almost completely in approximately 48 hours in the organ-culture conditions used in these experiments (Fig. 1A).

When injured rabbit corneas were incubated with 125 nM cPAF for 24 to 48 hours, there was substantial inhibition of epithelial wound closure compared with corneas incubated without cPAF (Fig. 1A). Data expressed as average ± SD of n experiments showed that the wound area uncovered by epithelium in corneas incubated with cPAF was 700 ± 30 AU (n = 6) at 24 hours and 450 ± 15 AU (n = 4) at 48 hours. In the presence of PAF antagonist BN50727 or BN50730 the inhibition of wound healing by PAF was blocked, suggesting that PAF actions are mediated by its receptor. Lyso-PAF, a biologically inactive metabolite of PAF, was not effective in inhibiting wound closure, compared with PAF (Fig. 1A), and 85% to 90% of the wounded area was covered by 48 hours. PAF also produced a significant inhibition of wound healing in human corneas incubated for 48 hours in the same conditions (Fig. 1B).
attachment of epithelial cells to different ECM proteins \((P < 0.001, \text{ compared with cells without PAF})\). In contrast, no significant differences compared with HGF or EGF.

**Apoptosis in Cultured Corneal Stromal Cells**

Apoptosis in corneal keratocytes occurs in human corneas after mechanical removal of the epithelium, a common procedure before excimer laser photorefractive keratectomy.\(^\text{22}\) We examined the possibility of inhibition of wound healing by PAF, owing to increased apoptosis in corneal cells surrounding the injury. For these experiments, corneas were incubated as before in the presence of cPAF, with or without PAF antagonists, in organ culture for 24 and 48 hours. The corneas were then sectioned, and TUNEL staining for apoptotic cells was performed. A significant number of TUNEL-positive keratocytes were found in the stromal area of uncovered wound and in the area surrounding the wound in corneas treated with PAF for 24 hours (Fig. 4). A few positively stained cells were observed in control corneas or corneas treated with cPAF in the presence of BN50739. Forty-eight hours after treatment, there was still intense TUNEL-positive staining in stromal cells of corneas treated with cPAF, but very low staining was observed in corneas incubated in the absence of PAF or in the presence of PAF plus PAF antagonist. No apoptotic cells were found in the epithelial cell layer of corneas incubated with cPAF, even when observed at higher magnification. To corroborate these findings, we also studied the ability of PAF to induce apoptosis in cultures of corneal epithelial cells and keratocytes (Fig. 5). Cells were starved (incubated in serum- and growth factor-free medium) overnight and then incubated with 125 nM cPAF for 6 hours. The lipid mediator was not able to induce apoptosis in epithelial cells under these conditions (Fig. 5E). Even when the cells were incubated for 24 hours with 10 times higher concentrations of cPAF, no apoptosis was found in epithelial cells (data not shown). However, between 70% and 75% (means of several fields counted in three individual experiments) of keratocytes in cultures incubated with cPAF showed TUNEL-positive staining (Fig. 5B). The antagonist BN50730 (4 \(\mu\)M) blocked PAF-induced apoptosis in keratocytes (Fig. 5C). However, when we cultured corneal fibroblasts (which are transformed from keratocytes), we found that they did not undergo apoptosis in the presence of PAF (data not shown).

**DISCUSSION**

When the epithelium is injured, the cornea responds by synthesizing several growth factors and cytokines that regulate the repair of the tissue.\(^\text{14–17,23}\) PAF synthesis also increases in response to injury and inflammation.\(^\text{2,4}\) Although PAF is known to exert multiple effects on corneal epithelial cell metabolism,\(^\text{6–13}\) its involvement in the wound-repair process is not well understood. In the current studies PAF inhibited corneal epithelial wound repair by a receptor-mediated mechanism. To the best of our knowledge this is the first report that describes the inhibitory effect of this biologically important molecule on wound healing. There is a previous report describing an increase in skin wound healing with topical application of PAF in a rat surgical incision model.\(^\text{24}\) The investigators suggest that PAF, through macrophage recruitment and activation, increases the wound’s strength and promotes wound healing in that model.\(^\text{25}\) The cornea, due to its avascular properties, can have different wound-healing dynamics, without some of the cellular elements present in vascularized tissue, such as platelets. In our study, inhibition of wound closure in...
the cornea by PAF persisted, even in the presence of growth factors, indicating the ability of PAF to suppress growth factor-promoted wound healing. Our recent studies have shown that HGF and KGF increase the expression of PAF-R during wound healing13; hence, under conditions that favor strong and sustained inflammation (e.g., infection or viral diseases, deficiencies of the tear film, trauma), increases in PAF and PAF receptor synthesis could be an important mechanism in delay of the repair process.

Our results showed that PAF induced significant apoptosis in keratocytes, both in organ- and cell-culture experiments. Apoptosis in superficial keratocytes has been identified as an early event after epithelial injury,18 and it has been suggested as an initiator of the epithelial wound-healing process in the cornea.22 In the presence of PAF, the persistent apoptosis in keratocytes in the wounded area could lead to disturbance in the dynamics of epithelial and keratocyte interactions, resulting in delayed wound healing. In a normal wound-healing

FIGURE 4. PAF induced apoptosis in keratocytes of injured cornea in organ culture. Corneas were incubated without PAF (control), 125 nM cPAF, and 125 nM cPAF plus 10 μM of the PAF antagonist BN50739 (BN) for 24 hours. Corneal sections were prepared and stained for TUNEL-positive labeling. Arrows: borders of the epithelial debridement. The experiment was repeated three times with similar results.

FIGURE 5. PAF caused apoptosis in cultured stromal cells (keratocytes), but not in corneal epithelial cells. Stromal (A–C) and epithelial (D, E) cells were cultured. Cells were starved overnight and then incubated without (A, D) or with (B, E) 125 nM cPAF for 6 hours. (C) The PAF antagonist BN50730 (4 μM) was added to cultured stromal cells before cPAF. The experiment was repeated three times with similar results.
situation, several hours after an epithelial injury, keratocytes are transformed into fibroblasts that migrate to the wound site and actively release factors, such as cytokines and growth factors, that are important in the process of wound healing. Fibroblasts do not express PAF-R, and we found that PAF did not produce apoptosis in fibroblasts. Our results suggest that the significant delay in epithelial wound closure is in part due to the action of PAF in increasing keratocyte apoptosis at the site of injury. This increase could hamper the migration of fibroblasts to the wound to start the healing process.

Another interesting finding is that PAF did not cause apoptosis in epithelial cells, even at higher concentrations. Instead, it reduced the ability of the cells to attach to different ECM proteins. This result supports the hypothesis that the actions of PAF are cell specific. The addition of PAF significantly reduces corneal epithelial cell attachment to basement membrane components, such as laminin-1 and collagen type IV. PAF also reduces the attachment of these cells to ECM proteins normally synthesized by fibroblasts (fibronectin and collagen type I). However, in cell cultures, PAF did not inhibit cell migration of individual corneal epithelial cells. Cell migration in vivo is thought to result from the coordinated regulation of both cell- cell and cell-ECM interactions. Thus, a reduction of cell-ECM attachment by PAF could disrupt this equilibrium, resulting in a dominance of cell-cell attachment and a cessation of migration. Because the corneal epithelial cells close to an exposed wound site move as a sheet of cells, PAF may have a greater effect on restricting cell movement in vivo than in organ culture than in isolated cells. Earlier studies from our laboratory have shown that PAF activates matrix metalloproteinase (MMP)-9, an enzyme that degrades several components of the basal lamina necessary for the attachment of epithelial cells, as well as uPA, a protease that regulates the activity of other metalloproteinases. Recent studies from our laboratory have shown that PAF produces a strong imbalance between the expression of MMP-9 and its tissue inhibitor (TIMP-1). PAF-stimulated activation of MMPs may be an additional factor in reducing attachment of epithelial cells, leading to the delay in wound healing.

In conclusion, the significant inhibition of corneal epithelial wound healing by PAF seems to involve several molecular changes affecting different cells of the cornea. These changes, acting in a concerted manner, could strongly impair the repair of the cornea after severe inflammation. Prolonged ocular inflammation is known to cause visual impairment and corneal scarring. In these cases, PAF antagonists could be of great therapeutic value in speeding wound healing and maintaining the transparency of the cornea.

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References


