Synergistic Effect of Platelet-Activating Factor and Tumor Necrosis Factor-α on Corneal Myofibroblast Apoptosis

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PURPOSE. Elimination of myofibroblasts after repair of corneal injury is essential for the maintenance of corneal transparency. In the current study, the role of platelet-activating factor (PAF) in combination with tumor necrosis factor (TNF-α) in corneal myofibroblast apoptosis was explored.

METHODS. Porcine corneal myofibroblasts (PCMs) were obtained from subcultured fibroblasts plated at a low density (5 cells/mm²). Mouse anti-a-smooth muscle actin antibody was used to identify the cell phenotype. Immunofluorescence was performed to localize PAF and TNF-α receptors in those cells. The reactivity of the antibodies was characterized by Western blot analysis. To induce myofibroblast apoptosis, PCMs were treated for 24 to 72 hours with methylcarbamyl-PAF (cPAF, 300 nM), a nonhydrolyzable PAF analogue, TNF-α (20 ng/mL), and TNF-α+cPAF, with or without LAU-0901 (150 nM), a novel PAF antagonist. Apoptosis was assayed by Hoechst 33258 and TUNEL staining and DNA laddering. 6-Diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Images were recorded by fluorescence microscope.

RESULTS. Immunofluorescence with a PAF-receptor (N terminus) polyclonal antibody showed that the receptor was expressed in both plasma and nuclear membranes of myofibroblasts. TNF-α receptor II (TNF-RII) was localized in the cytoplasm, whereas TNF- receptor I (TNF-RI) was found in both cytoplasm and plasma membrane. Treatment with TNF-α for 24, 48, and 72 hours induced apoptosis in 18%, 24%, and 32%, respectively, of the myofibroblasts. Western blot analysis showed expression of single bands corresponding to the molecular weights of the receptors. Treatment with cPAF induced apoptosis in 10%, 18%, and 26% of the cells, respectively. However, treatment with both cytokines induced apoptosis in 42%, 78%, and 86%, respectively, of the cells, demonstrating a synergistic action between PAF and TNF-α. Blocking the PAF receptor with LAU-0901 inhibited the synergistic effect induced by PAF.

CONCLUSIONS. Corneal myofibroblasts express a PAF receptor in the nuclear membrane, and they also express TNF-RI and RII. The synergistic effect on myofibroblast apoptosis by PAF and TNF-α suggests that during corneal stromal wound healing, PAF acting in conjunction with other cytokines could play an important role in eliminating these cells. (Invest Ophthalmol Vis Sci. 2006;47:885–891) DOI:10.1167/iovs.05-0581

The corneal stroma is composed of a mixture of collagen types I, V, VI, and XII, and proteoglycans possessing either keratan sulfate side chains or chondroitin-dermatan sulfate side chains.1–3 It is believed that the highly ordered lamellae and the interrelation of collagens and proteoglycans maintain corneal transparency.4,5 The keratocytes sparsely residing between the lamellae maintain the balance of stromal components. They are normally in a quiescent state, but in response to injury, the cells at the wound periphery become metabolically activated, migrate into the damaged area, and transform to active fibroblasts and myofibroblasts.6–8 These cells proliferate and produce a disorganized and fibrotic extracellular matrix (ECM) to repair the damaged area.6,9 Although both fibroblasts and myofibroblasts contribute to normal wound repair, myofibroblasts play a crucial role in tissue fibrosis, because they produce ECM components at a high rate10,11 and regulate contractile elements that generate the force necessary for wound closure.10–12 Once a wound has been closed, however, myofibroblasts may no longer be necessary. In a completely healed wound, indeed, few if any myofibroblasts are detected.13 Excessive proliferation or persistent presence of these cells has been associated with pathologic fibrosis, contracture, and corneal haze.13–15,16,17 Apoptosis has been suggested to play a central role in the deletion of myofibroblasts during the wound-healing process,17,18,19 but the mechanism by which myofibroblast apoptosis is regulated remains unclear.

PAF is a potent bioactive lipid cytokine that accumulates rapidly after corneal injury17 and delays epithelial wound healing.18 Treatment of alkali-burned cornea with PAF antagonists prevents corneal perforation.19 PAF stimulates an inflammatory cascade by activating phospholipase A2, thereby releasing arachidonic acid and increasing the synthesis of prostaglandins. It also induces the expression of several genes related to inflammation and wound healing (for review see Ref. 20). An interesting finding has been that PAF receptors are expressed in epithelial cells as well as in keratocytes and myofibroblasts, but not in fibroblasts (He J, et al. IOVS 2003;44:ARVO E-Abstract 877).20

PAF is a strong inducer of apoptosis in corneal keratocytes and enhances UVB-induced apoptosis in corneal epithelial cells.19,21 More recently, we have found that myofibroblasts seem to be more resistant to PAF-induced apoptosis than are keratocytes (He J, et al. IOVS 2004;45:ARVO E-Abstract 1417). This may mean that the signaling through PAF receptors is different in these two cells or that PAF requires activation of other cytokines to complement its action.

TNF-α is a well-known inducer of apoptosis in many cell types.22 It is produced mainly by activated macrophages, lymphocytes, and neutrophils, but can be synthesized by many nonimmune cell types, including corneal cells.23–24 The cellular effects of TNF are mediated by TNF-RI and TNF-RII receptors. Corneal keratocytes and fibroblasts express these two TNF receptors, and TNF-α induces apoptosis in corneal fibroblasts when NF-κB activation is blocked.25 However, there is no information about TNF-α actions in corneal myofibroblasts.
In the present study, the expression of several ECM components and the localization of PAF and TNF-α receptors were examined by immunofluorescence in porcine corneal myofibroblasts. Studies were also performed to investigate the effects of PAF and TNF-α on myofibroblast apoptosis.

**MATERIALS AND METHODS**

Methylcarbaryl PAF (cPAF, 1-alkyl-2n-methylcarbamyl sn-glyceryl-phosphorylcholine, a nonhydrolyzable PAF analogue), rabbit polyclonal anti-PAF-receptor antibody (cat no. 160602), and control peptide (cat no. 160604) were obtained from Cayman Chemical Co. (Ann Arbor, MI); collagenase and 100-bp DNA from Invitrogen-Gibco Corp. (Carlsbad, CA); 4,6-diamidino-2-phenylindole (DAPI), mouse monoclonal anti-c-sMOOTH muscle actin (α-SMA), anti-vimentin (clone Vim-13,2), anti-chondroitin sulfate (clone CS-56), anti-paxillin (Clone PXG-10), anti-vinculin (clone Vin-11-5), anti-collagen type IV (clone Col-94), anti-cellular fibronectin (clone FN-3E2), anti-laminin (clone LAM-89), and TRITC-labeled phalloidin from Sigma-Aldrich (St. Louis, MO); mouse anti-focal adhesion kinase (mAb 2156) from Chemicon International (Temecula, CA); mouse anti-collagen III (Fl7A) antibody (abcam7) and monoclonal anti-keratan sulfate antibody (clone 5-D-4) from Seikagaku (Tokyo, Japan); Hoechst 33258 from Molecular Probes (Eugene, OR); DNA purification kits and TUNEL kits (DeadEnd Fluorometric TUNEL System) from Promega (Madison, WI); mouse monoclonal anti-TNF-receptor 1 (H-5), goat polyclonal anti-TNF-receptor 2 (L-20), control peptide for TFN-RII (sc-1074p), and donkey anti-goat secondary antibody from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-human nuclear membrane antibody from Leinco Technologies, Inc. (St. Louis, MO); and goat anti-rabbit IgG and goat anti-mouse IgG from BD-PharMingen (San Diego, CA). All SDS reagents were from Bio-Rad ( Hercules, CA). Polyvinylidene difluoride (PVDF) membranes were from GE Healthcare (Piscataway, CA); 4,6-diamidino-2-phenylindole (DAPI), mouse monoclonal anti-human nuclear membrane antibody from Bio-Rad Laboratories, Inc. (Hercules, CA); and avidin-biotin peroxidase complex (ABC) kit with DAB chromagen from Vector Laboratories (Burlingame, CA). Primers were synthesized by Operon Technologies (Alameda, CA).抗心磷酯酶抗原(α-SMA)、抗纤维母细胞抗体、抗fibronectin抗体、抗laminin抗体、抗actin抗体、抗vimentin抗体、抗vinculin抗体、抗collagen type IV抗体、抗cellular fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗chondroitin sulfate抗体、抗keratan sulfate抗体、抗paxillin抗体、抗vinculin抗体、抗fibronectin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin抗体、抗collagen III抗体、抗collagen IV抗体、抗fibronectin抗体、抗laminin antibody, to exclude nonspecific staining. As negative controls for DNA ladder was used as a molecular weight marker.

**FITC-dUTP TUNEL Staining**

If the indicated treatments, cells were fixed in 2% paraformaldehyde solution for 30 minutes at 4°C and permeabilized with 0.3% Triton X-100 solution for 5 minutes on ice. The TdT-mediated FITC-linked dUTP nick-end DNA (TUNEL) labeling was performed according to the manufacturer’s protocol. The cells were counterstained with DAPI for 15 minutes at room temperature and viewed by fluorescence microscope (Eclipse TE 200; Nikon).

**DNA Laddering**

Adherent and floating PCMs were harvested and washed twice in PBS. DNA was extracted with a genomic DNA purification kit, according to the manufacturer’s protocol. Five micrograms of DNA were separated by electrophoresis on a 2.0% agarose gel containing ethidium bromide (100 ng/ml) in 1× TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA; pH 8.3). The fragmentation pattern was visualized under UV light. A 100-bp DNA ladder was used as a molecular weight marker.

**Induction of Apoptosis**

Seven-day cultures were starved overnight in DMEM/F12 containing 0.1% horse serum (HS) and then were treated with cPAF (300 nM), TNF-α (20 ng/ml), or cPAF+TNF-α, with or without LAU-0901 (150 nM) for 24, 48, and 72 hours.

**Hoechst Staining**

Cell cultures in 12-well plates were washed twice with phosphate-buffered saline (PBS) and incubated with 2 μM Hoechst 33258 for 1 hour at 37°C in the dark. After three washes with PBS, the cells were viewed with a fluorescence microscope (Eclipse TE 200; Nikon, Tokyo, Japan). The images were recorded with a digital camera (DXM 1200; Nikon) attached to the microscope and were processed by computer (MetaVue; Universal Imaging Co., Downington, PA). The Hoechst reagent is taken up by the nuclei of the cells, and apoptotic cells exhibit a bright blue fluorescence, whereas living cells are dark blue. The number of cells with nuclear condensation versus normal appearing cells was counted in a blind fashion in 10 different fields clockwise at positions 3, 6, 9, 12 o’clock, and center at low magnification (10×) in two wells and averaged. The percentage of apoptotic cells is expressed as a percentage of total cells counted.

**Immunofluorescence Staining**

Cells were washed in PBS and fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 minutes at 4°C and permeabilized with 0.3% Triton X-100 solution for 5 minutes on ice. All the procedures were performed at room temperature (RT). After three washes with PBS, the cells were incubated with 10% normal goat serum in PBS containing 0.1% bovine serum albumin (PBS-BSA) for 30 minutes, to block nonspecific binding. Afterward, the cells were incubated for 1 hour with the corresponding primary antibodies at optimal dilutions in PBS containing 1.5% normal goat serum. After they were washed PBS-BSA (three times, 5 minutes each), the cells were incubated with the corresponding secondary antibodies for 45 minutes. For double immunofluorescence, cells were fixed and blocked as just described and incubated with a mixture of primary antibodies followed by a mixture of the secondary antibodies appropriately diluted in PBS containing 1.5% normal goat serum. TRITC-labeled phalloidin was used to stain Factin, and DAPI was used to counterstain the nuclei. For better contrast with green fluorescence, the color of DAPI was changed from blue to red in all merged images, except that in Figure 2C. When localization of PAF and TNF receptors was studied, cells were also treated as above without Triton X-100 (non-permeabilized cells). In all assays, negative controls were prepared using normal mouse IgG (sc-2025; Santa Cruz Biotechnology) or 0.1 M PBS for the primary antibody, to exclude nonspecific staining. As negative controls for PAF-R and TNF-RII antibodies, the primary antibodies were preincubated with the corresponding blocking peptides for 2 hours at room temperature before being added to the cells. In the case of TNF-R1, normal mouse IgG was used to replace the primary antibody as the control because there is no blocking peptide available.
**Determination of Antibody Specificity by Western Blot Analysis**

PCMs grown in culture for 5 days on 100-mm dishes were rinsed twice with PBS and harvested in modified RIPA lysis buffer (50 mM Tris-HCl [pH 7.4]; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/mL each of aprotinin, pepstatin, and leupeptin; 1 mM Na3VO4; and 1 mM NaF). The cell lysates were passed through a 21-gauge needle to shear the DNA, incubated for 45 minutes on ice, and then microcentrifuged at 10,000 g for 10 minutes at 4°C. The supernatant as whole-cell lysate was stored at −20°C until use. Protein was determined by protein assay with BSA as a standard (Bio-Rad).

The specificities of PAF-R and TNF-RI and -RII antibodies were tested by immunoblot analysis. For this purpose, lysate samples containing 50 μg protein were boiled in reducing sample buffer for 5 minutes, loaded on 10% polyacrylamide minigels, run at 100 V, and transferred to PVDF membranes. Biotinylated protein molecular weight standards were applied in one lane of each gel. The cell lysates were passed through a 21-gauge needle to shear the DNA, incubated for 45 minutes on ice, and then microcentrifuged at 10,000 g for 10 minutes at 4°C. The supernatant as whole-cell lysate was stored at −20°C until use. Protein was determined by protein assay with BSA as a standard (Bio-Rad).

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**Statistical Analysis**

Data are presented as the mean ± SD. Comparisons between groups at each time point of treatment were conducted by one-way ANOVA followed by the Tukey test. P < 0.05 was considered statistically significant.

**RESULTS**

**PCM Phenotype Identification and ECM Expression**

To identify cell phenotype in the cultures, we examined the plates for immunofluorescence staining of α-SMA, the specific isoform of actin that is characteristic of myofibroblasts at different times after the cells were seeded. Vim, vimentin; Coll3, collagen type III; Coll4, collagen type IV; KS, keratan sulfate; CS, chondroitin sulfate; FN, fibronectin; LM, laminin; FK, focal adhesion kinase; PAX, paxillin; Vin, vinculin.

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933440/)
tiation, at different time points of culture. When subcultured fibroblasts were plated at low density in DMEM/F12 containing 5% FBS, an increasing number of cells were \( \alpha \)-SMA-positive from days 1 to 9 (Fig. 1A). Some cells retained the protomyo-fibroblast phenotype, which does not show matured stress fibers, as do differentiated myofibroblasts. In differentiated myofibroblasts, \( \alpha \)-SM actin co-expressed with F-actin in the stress fibers, whereas vimentin, an intermediate filament, expressed differently (Fig. 1B). Myofibroblasts constitutively expressed ECM components such as collagen types III and IV, proteoglycans such as keratan sulfate and chondroitin sulfate, fibronectin, and laminin (Fig. 1B), all of which are presumed to be produced by repair fibroblasts in wound-healing tissues and to play an important role in stromal remodeling.4 – 6,9 The PCMs showed expression of all these components, especially expression of chondroitin sulfate and fibronectin, which appeared as dense dots or patches on the cell surfaces and was also secreted into the medium. When the cells became confluent, the fibronectin formed a network over the cell surface (not shown). The expression of keratan sulfate was less abundant than that of chondroitin sulfate, and its staining appeared as interrupted lines along the filaments within the cytoplasm. The cells also expressed proteins involved in the formation of focal adhesion complexes, including vinculin, paxillin, and focal adhesion kinase (Fig. 1B). In Figure 1B, the last column shows the distribution of vinculin in cells shortly after being seeded (4 hours) and up to 3 days, when the focal adhesion complexes are formed. In a recent report,28 we have also demonstrated that rabbit corneal myofibroblasts express the metalloproteinases (MMP) MT1-MMP, MMP-2, MMP-9, and the inhibitors TIMP-1 and -2, and that PAF triggers a significant increase of these MMPs and its inhibitors. All these findings suggest that these myofibroblasts in vitro have the characteristics of those reported in the stroma during in vivo wound healing.

Localization of PAF Receptor and TNF Receptor in Myofibroblasts

We have previously shown that corneal keratocytes and myofibroblasts express PAF receptor, but fibroblasts do not (He J, et al. IOVS 2003;44:ARVO E-Abstract 877).14,29 The PAF receptor was detected in both plasma and nuclear membranes of PCMs by using a rabbit anti-PAF-receptor (N terminus) polyclonal antibody (Fig. 2A). The plasma membrane staining was clearly visible when the cells were not permeabilized. After permeabilization with 0.3% Triton X-100 for 5 minutes, nuclear membrane staining became evident. Preincubation with blocking peptide did not produce staining (not shown). To confirm the localization further, we stained the cells with an antibody specific for the nuclear membrane (Fig. 2B). To confirm the myofibroblast phenotype, permeabilized cells were double-stained with anti-\( \alpha \)-SMA and anti-PAF-R antibodies, which showed that all the cells stained positive for \( \alpha \)-SMA and that the nuclear membrane stained positive for the PAF-receptor antibody (Fig. 2C).
The specificity of the PAF-R antibody was tested by immunoblot analysis, as described in the Materials and Methods section. As shown in Figure 2D, the PAF-R antibody recognized a single protein band at the expected size, 48 kDa, that was completely eliminated when blots were preincubated with the blocking peptide.

Both TNF-α receptors I and II were expressed in PMFs. In nonpermeabilized cells, there was TNFR-I-positive staining in both cytoplasm and plasma membrane (Fig. 3A). TNFR-II was expressed at a much lower level in a punctate pattern in the cytoplasm of the cells. The specificity of the antibodies was also tested by immunoblot analysis. The TNF-RI monoclonal antibody was expressed as a 55-kDa band and the TNF-RII polyclonal antibody as a 75-kDa band, corresponding to the sizes of the receptors, respectively (Fig. 2B). TNF-RII binding was completely eliminated by preincubation with the corresponding blocking peptide.

**FIGURE 3.** Detection of TNF-α receptors in PMFs. (A) Immunofluorescence for TNF receptors in PMF. Nonpermeabilized cells were immunostained with TNF-RI and TNF-RII antibodies. DAPI was used to counterstain the nuclei (color was changed from blue to red). TNF-RI-positive staining was found in the plasma membrane and cytoplasm; TNF-RII staining was found only in the cytoplasm. Controls with IgG (for TNF-RI) and blocking peptide (for TNF-RII) showed no staining. Magnification ×20. (B) Western blot analysis showed that the anti-TNF-RI and -RII antibodies recognized single bands at the expected sizes of 55 and 75 kDa, respectively.

**PAF Augments TNF-α-Induced Apoptosis of PMFs**

Previous studies in our laboratory showed that PAF induces apoptosis in both corneal keratocytes and myofibroblasts. Although keratocyte apoptosis occurs very rapidly and at low concentrations of PAF (100 nM), myofibroblasts are more resistant and require a higher (300 nM) PAF concentration (He J, et al. *IOVS* 2003;44:ARVO E-Abstract 877). PMFs were treated with 300 nM PAF, TNF-α (20 ng/mL), or both cytokines for 24, 48, and 72 hours and then stained with Hoechst 33258 for quantitative evaluation of the apoptotic cells. Treatment with PAF produced an average of 10%, 18%, and 26% apoptotic cells at the different time points (Fig. 4). The PAF-receptor antagonist (LAU-0901) completely blocked the effect of PAF. Treatment with TNF-α for 24, 48, and 72 hours induced apoptosis in 18%, 24%, and 32%, respectively, of the myofibroblasts. When both cytokines were combined, there were 42%, 78%, and 86% apoptotic cells, respectively, suggesting a synergistic effect of PAF. If the PMFs were treated with LAU-0901, there was a complete block of the synergistic effect of PAF on myofibroblast apoptosis. To confirm these results further, the cells were treated with the cytokines for 48 hours in DMEM/F12 containing 0.1% HS, and then TUNEL staining for apoptotic cells and immunostaining for α-SMA were performed (Fig. 5). TUNEL staining showed 12% and 28% apoptotic cells after treatment with PAF or TNF-α alone, respectively, whereas 77% apoptotic cells resulted from treatment with PAF in combination with TNF-α, in agreement with the values observed by Hoechst staining and confirming that LAU-0901 inhibits the effect of PAF (Figs. 5A, 5C). The same batch of cells was positive for both α-SMA staining and TUNEL. The bundles of stress fibers positive for α-SMA in the apoptotic cells were obviously degraded and the nuclei fragmented to a greater extent after treatment with than with any other treatment (Fig. 5B, TNF-α + PAF). The PAF antagonist protected against PAF action. When the cells were analyzed by DNA laddering, PAF and TNF-α induced DNA fragmentation (Fig. 5D) that was increased when both cytokines were added together, confirming their synergistic effect. The PAF-R antagonist blocked the effect of PAF on the synergism.

**DISCUSSION**

The differentiation of keratocytes to active fibroblasts (i.e., myofibroblasts) after corneal injury is an important mechanism to produce ECM proteins and regulate contractile elements that generate the forces responsible for wound closure. However, the excessive deposition of fibrotic ECM by the persistent presence of myofibroblasts can lead to scar formation and seriously impair vision. During normal wound healing, myofibroblasts start to disappear after epithelialization is complete. The exact mechanisms responsible for this phenomenon remain unclear. There are in vitro studies suggesting that myofibroblasts can partially revert to fibroblasts or even keratocytes under conditions of serum withdrawal or FGF stimulation; however, more evidence suggests that cytokine-mediated apoptosis may play an important role in the elimination of myofibroblasts from remodeling or wound-healing tissues.

In the present study, by seeding the cells at a low density and adjusting the serum concentration in the medium, we...
can keratan sulfate. Using TGF-
stromal wound sites. Of note, PCMs expressed the proteogly-
thought to be present only in the repair fibroblasts at the
seems to be related to corneal opacity and scarring.4 In fact,
expression of keratan sulfate that we
obtained 100% corneal porcine myofibroblasts, as identified by
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generated (data not shown). The expression of α-SMA and other components of
the ECM indicates that the myofibroblasts used in this study have the typical features of repair fibroblasts in wound-healing
tissues. We used these cells first to investigate the expression of PAF and TNF-α receptors by immunofluorescence. The PAF receptor is a seven-transmembrane G protein-coupled receptor (GPCR) that mediates a diverse array of biological responses to PAF. Earlier studies have shown that the PAF receptor gene is expressed in corneal epithelial cells, keratocytes, and endothelial cells, but not in fibroblasts (He J, et al. IOVS 2003;44:ARVO E-Abstract 877).25 The finding that the transformation of fibroblasts to myofibroblasts leads to the expression of PAF receptor indicates that this expression may be cell phenotype specific. We also report the novel finding of the nuclear localization of PAF receptor in myofibroblasts, indicating a possible role for PAF in stimulating gene transcription through the nuclear membrane. There are a few reports about some GPCRs localized in the nuclear membrane.33,34 Expression of PAF receptor has been described in the nuclei of cerebral microvascular endothelial cells.35 Nuclear localization could be possible through a putative signal at the C-terminus of the PAF receptor that can target the nucleus.35,36 Functionally, a perinuclear receptor for prostaglandin E2 has been shown to activate transcription,35 and, in isolated nuclei of endothelial cells, PAF induces cyclooxygenase-2 gene expression.35 It is tempting to speculate that the recently reported gene induction of MMPs and furin by PAF in myofibroblasts26 implies the activation of a nuclear PAF receptor. Further studies with isolated nuclei are needed, to determine the functions of these receptors.

Although both corneal kerocytes and myofibroblasts expressed the PAF receptor, their responses to PAF-induced apoptosis differed. Studies have shown that in organ- and cell-culture experiments, PAF stimulates apoptosis in keratoctyes and delayed corneal epithelial wound healing.18 Treatment with LAU-0901 significantly reduced keratocyte apoptosis and suppressed the inflammatory response in rabbit models of diffuse lamellar keratitis (DLK)14 and corneal alkali burn.19 Addition of cPAF (300 nM) to the medium of 7-day cultured rabbit kerocytes for 6 hours stimulated more than 60% of the cells to undergo apoptosis, whereas no positive staining was found in myofibroblasts cultured in the same conditions (He J, et al. IOVS 2003;44:ARVO E-Abstract 877). However, when myofibroblasts were incubated with cPAF for 24 to 72 hours, approximately 10% to 30% of the cells were apoptotic.

PCMs expressed both TNF-RI and -II. TNF-RI was more abundantly expressed, which is in agreement with the notion that this receptor is ubiquitous in human tissues and is the major signaling receptor for TNF-α, whereas TNF-RII is mostly expressed in immune cells and mediates limited biological responses. Binding of TNF-α to TNF-RI activates receptor-associated death domain (TRADD) protein, which interacts with Fas-associated death domain (FADD) protein to induce apoptosis. TNF-RII triggering can lead to NF-κB activation, but does not result in cell death.22 Accumulating evidence indicates that the fate of cells that are exposed to TNF-α depends on the balance between proapoptotic and antiapoptotic mechanisms. TNF-α triggers apoptosis in corneal fibroblasts when NF-κB activation is blocked,23 but has little effect on myofibroblasts isolated from a granulation tissue pouch in rat dorsal subcutaneous tissue.37 In the present study, treatment of PCMs with TNF-α induced time-dependent apoptosis, which was more extensive than that with PAF alone, suggesting that TNF-α-induced apoptosis may be cell specific and is signaled via

**Figure 4.** Effect of PAF and TNF-α on PCM apoptosis. Seven-day cultures were starved overnight in DMEM/F12 containing 0.1% HS and then treated with cPAF (300 nM), TNF-α (20 ng/mL), and cPAF plus TNF-α with or without LAU-0901 (150 nM) for 24, 48, and 72 hours. Apoptotic cells were detected by Hoechst 33258 staining. The data are averages ± SD of percentages of apoptotic cells with respect to total cells counted in a blind fashion in 10 different fields of two wells. The results are representative of two separate experiments. Significant differences (*P < 0.05, one-way ANOVA, post hoc Tukey test) were found compared with *untreated, **PAF or TNF-α alone, ***PAF+TNF-α, and ΔPAF.
TNF-RI. Although myofibroblasts have an important role in the generation of contractile forces that are necessary for wound closure, their continued presence may result in corneal scarring and opacity.\textsuperscript{12,13} There are no experimental reports on how myofibroblasts are eliminated. The most interesting and important finding in this study is the synergistic action between PAF and TNF-\alpha that stimulates apoptosis in myofibroblasts. This combination induced more than 75\% of the cells to undergo apoptosis by 48 hours, as demonstrated by TUNEL and Hoechst staining.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{TUNEL staining and DNA laddering of PCMs treated with PAF and TNF-\alpha. Seven-day cultured cells were treated with cPAF (300 nM), TNF-\alpha (20 ng/mL), and TNF-\alpha+cPAF for 48 hours. The PAF-receptor antagonist LAU-0901 was added in some cultures. (A) TUNEL staining was performed to detect apoptotic cells. DAPI was used to stain the nuclei (color was changed from blue to red to obtain better contrast). (B) Immunofluorescence with \textalpha-SMA confirms that the cells are myofibroblasts. Representative images show that the bundles of stress fibers composed of \textalpha-SMA in the apoptotic cells were degraded and the nuclei fragmented to a greater extent after treatment with TNF-\alpha plus PAF than with any other treatment. The ratios between the number of TUNEL-positive cells and total cells counted in 10 different fields of two wells are expressed as the mean percentage \pm SD of total cells counted. Significant differences ($P < 0.05$, one-way ANOVA, post-hoc Tukey test) were found compared with *untreated; **PAF or TNF-\alpha alone; ***PAF+TNF-\alpha; and \Delta PAF. Magnification: (A) \times 10; (B, C) \times 20. (D) DNA was obtained from cells with the different treatments, and DNA fragmentation was analyzed by gel electrophoresis. Lane M: 100-bp ladder. The experiments were repeated three times with similar results.}
\end{figure}
Studies have described interactions between TNF-α and PAF during the pathogenesis of inflammatory and infectious diseases. Synergistic or reciprocal relationships between TNF-α and PAF have been shown in animal models of endotoxin-induced shock, nephrosis, HIV-1-associated dementia, and ischemic bowel necrosis. At the cellular level, TNF-α can induce the synthesis and release of PAF in macrophages and augment the expression of the PAF-receptor gene in human monocytes. Conversely, PAF can stimulate TNF-α production by those cells. In the present study, the synergistic increase in apoptosis was through PAF-receptor stimulation, since it was inhibited by LAU-0901. At present the mechanisms activate apoptosis. PAF is rapidly formed after corneal injury, and suggests a convergence of postreceptor signaling pathways that activate apoptosis. PAF is rapidly formed after corneal injury, and suggests a convergence of postreceptor signaling pathways that activate apoptosis. PAF is rapidly formed after corneal injury, whereas TNF-α is released from epithelial cells after infection or dry eye.

Myofibroblast apoptosis commonly has been thought to occur at the end of the wound-healing process, long after corneal inflammation has disappeared, but this may not always be the case. Recently, in rabbit models of DLK and corneal alkali burn, apoptosis of stromal cells, including myofibroblasts, was found in all stages of wound healing, as detected by TUNEL staining. Increased myofibroblast apoptosis could be a defensive response induced by the cytokines after stromal injury to decrease opacity and scar tissue formation. Alternatively, prolonged inflammation could impair wound contraction and healing of the cornea.

In summary, our studies have shown for the first time that corneal myofibroblasts express TNF-α receptors and a PAF nuclear receptor. Both PAF and TNF-α induce time-dependent apoptosis in myofibroblasts and, more importantly, there is a synergistic relationship between the two cytokines. Further studies are needed to investigate the mechanisms by which these cytokines exert this synergistic effect on myofibroblast apoptosis.

References

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