Localization of Platelet-Activating Factor Receptor Messenger RNA in the Rat Eye

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**Purpose.** To determine the distribution of platelet-activating factor (PAF) receptor in the rat eye.

**Methods.** Platelet-activating factor receptor messenger RNA (mRNA) expression was evaluated with reverse transcription–polymerase chain reaction (RT-PCR) in RNAs from several rat ocular tissues. Distribution of the expression was determined with in situ hybridization in adult rat eye sections and a 35S-labeled complementary RNA probe synthesized from the rat PAF receptor complementary DNA. In situ hybridization was also done with sections immunostained with OX-42, a microglia marker.

**Results.** RT-PCR revealed that levels of PAF receptor expression were similar among the ocular tissues studied. The in situ hybridization signals were found in the corneal epithelium, iris and ciliary body, and ganglion and microglial cells in the retina.

**Conclusions.** Platelet-activating factor receptor mRNA was ubiquitously expressed in the rat eye with relative concentration in the corneal epithelium, iris and ciliary body, and retinal ganglion and microglial cells.

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) has diverse biologic effects on various cells and tissues despite its initial characterization as a potent activator of platelets.1-3 Platelet-activating factor is also involved in a variety of physiologic and pathologic events in the eye. It is produced in the acetylcholine- or dopamine-treated chick retina,4 photoagulated rabbit retina,5 injured6-7 or ischemic8 rabbit cornea, and several tissues of inflamed rabbit eyes.9,10 It induces release of arachidonic acid and prostaglandins in the rabbit iris11 and cornea12 and induces the expression of plasminogen activator,13 collagenase type I,14 and other matrix metalloproteinases15 in the rabbit cornea. Platelet-activating factor receptor (PAFR) antagonists reduce inflammation16-17 and activities of lipoxygenase18 and cyclooxygenase19 in the injured rabbit cornea. They also inhibit rabbit intraocular pressure increases,20,21 uveitis,22-24 and rat retinal damage.25-27 Specific PAF binding sites are reportedly present in the rabbit iris and ciliary body (ICB)28 and rat retinal membranes.29 Molecular cloning of PAFR complementary DNA (cDNA) in our laboratory30,31 has provided a new tool for investigations into the presence of PAFR in the eye. We32 recently showed that PAFR is expressed predominantly in microglia and moderately in neurons of the rat brain.

In the present study, we attempted to localize PAFR in the rat eye with reverse transcription–polymerase chain reaction (RT-PCR) and in situ hybridization histochemistry. We found that PAFR is expressed in the rat corneal epithelium, ICB, choroid, and retinal ganglion and microglial cells.

**MATERIALS AND METHODS**

**Animals**

Sprague-Dawley rats (adult males) were deeply anesthetized with diethyl ether. Enucleated eyes were dissected, and total RNA was extracted with an RNA extraction kit (Isogen, Nippon Gene, Tokyo, Japan) from the cornea, ICB, retina, and remaining eyecups,
which included the retinal pigment epithelium, choroid, and sclera. Some eyes were embedded in OCT compound and frozen in isopentane at —30°C. We adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Sections (10 μm) were made with a cryostat, thaw-mounted on glass slides coated with poly-L-lysine, and air-dried for 10 minutes at room temperature. The sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 minutes, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine—0.9% NaCl, dehydrated in ethanol of ascending concentrations (70%, 95%, and 100%), and stored at —80°C until use.

RT-PCR was done as follows. First-strand cDNAs were synthesized from 1 μg each of the total RNA samples with 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD), 25 μg/ml oligo-dT, and 0.5 mM dNTP at 70°C for 10 minutes. PCR was done with 1:40 volume of the first-strand cDNA samples, 0.5 μM rat PAFR-specific primers32: CCGC GTGTGAGTGTCTATTA (upstream, 5’ to 3’), and AGGAGGTGATGAAGATGTGG (downstream, 5’ to 3’), 0.2 mM dNTP, 2.5 units of Taq polymerase (Ampli Taq Gold; ABI, Foster City, CA), and the reaction buffer. After incubation at 94°C for 10 minutes, the amplification was done for 30, 33, and 36 cycles (94°C for 1 minute, 55°C for 0.5 minutes, 72°C for 0.5 minutes). The PCR products were electrophoresed in agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The density of the bands on the photograph was quantified with an optical scanner and standardized with that for β-actin in 27-cycle PCR. The nucleotide sequences of the products were determined directly with an automated DNA sequencer (373S; ABI).

In Situ Hybridization

In situ hybridization was done as described previously.39 Briefly, an 35 S-labeled complementary RNA probe was made from a PvuII-AvaI fragment (0.9 kb) of the coding region of rat PAFR cDNA31 with a complementary RNA synthesizing kit (MaxiScript; Ambion, Austin, TX). The final radioactivity of the probe solution was adjusted to 10⁵ cpm/μl. After hybridization at 45°C for 18 hours in a humid chamber, the sections were washed in 2 × standard saline citrate with 10 mM β-mercaptoethanol at room temperature overnight, 2 × standard saline citrate with 10 mM β-mercaptoethanol at 60°C for 1 hour, and treated with RNase A (20 μg/ml) at 37°C for 30 minutes in 0.5 M NaCl, 10 mM Tris-Cl, and 1 mM ethylenediaminetetraacetic acid. The final wash was done in 0.1 × standard saline citrate with 10 mM β-mercaptoethanol at 60°C for 1 hour. The sections were dehydrated in ethanol of ascending concentrations (30%, 90%, 100%, and 100%). The air-dried sections were dipped in 1:1-diluted NTB-2 emulsion (Kodak, Rochester, NY), stored for 7 weeks at 4°C, developed, and counterstained with cresyl violet.

In Situ Hybridization After Immunostaining Microglia

Because our previous study30 revealed that microglia predominantly express PAFR messenger RNA in the rat brain, we used the same technique to clarify whether retinal microglia also express the receptor. Monoclonal antimicroglia-macrophage antibody (OX-42; Serotec, Oxford, United Kingdom) was affinity-purified twice with Protein-G columns (Pharmacia, Uppsala, Sweden), because incubation with the crude antibody substantially decreased messenger RNA signals in the subsequent in situ hybridization.

Cryostat sections (10 μm thick) of adult rat eye were fixed serially in 4% paraformaldehyde buffered with 0.1 M phosphate buffer, 50% aqueous acetone, 100% acetone, and 50% aqueous acetone (each for 2 minutes), and blocked with 0.1% nuclelease-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline for 5 minutes at room temperature. Incubation with the purified primary antibody (1:200) was done for 10 minutes at room temperature with 0.1% bovine serum albumin—phosphate-buffered saline. After three brief washes with phosphate-buffered saline, the sections were incubated with biotinylated antimouse IgG antibody (1:200; Vector, Burlingame, CA) with 0.1% bovine serum albumin—phosphate-buffered saline for 10 minutes and subsequently with avidin—biotin—horseradish peroxidase complex (Vectastain Elite; Vector) for 10 minutes according to the manufacturer’s instructions. Immunostaining was visualized with 1 mg/ml diaminobenzidine tetrahydrochloride in phosphate-buffered saline containing 0.02% H₂O₂. The stained sections were then acetylated, dehydrated, and subjected to in situ hybridization as described above. All the immunostaining procedures were done under RNase-free conditions.

RESULTS

The RT-PCR analyses revealed expression of PAFR in all the ocular tissues studied (Fig. 1A). Figure 1B shows the amounts of the PCR products evaluated from the density of the bands. The amounts increased with increasing numbers of amplification cycles, suggesting that PAFR expression levels can be evaluated at least semiquantitatively from the amounts of the PCR products. The expression levels were higher in the retina and other posterior segments, although the differences were small. All the PCR products had nucleotide

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FIGURE 1. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analyses of platelet-activating factor receptor (PAFR) messenger RNA expression in the rat eye. (B) Amounts of RT-PCR products for PAFR. The density of each band was measured and standardized with that for β-actin in 27-cycle PCR. C = cornea; I = iris and ciliary body; R = retina; P = retina-free posterior segment, including retinal pigment epithelium, choroid, and sclera.

sequences identical to those of the corresponding cDNA region for PAFR.

In situ hybridization signals were found in the ICB (Fig. 2A), corneal epithelium (Fig. 2C), ganglion cell and inner nuclear layers of the retina, and choroid (Fig. 2E). The signals were displaced by addition of a 100-fold excess of unlabeled complementary RNA to the probe (Figs. 2B, 2D, 2F), indicating that the hybridization was specific. Figure 3 shows bright-field photomicrographs of the in situ hybridization. The signals were concentrated in the corneal epithelium (Fig. 3A) and diffusely distributed in the ICB (Figs. 3B, 3C, 3D). In the retina, signals were concentrated in the ganglion cells and scattered in the inner nuclear layer (Fig. 3E). In the sections prestained with OX-42, the signals were also detected in the positively stained cells that were found in the inner plexiform layer of the retina (Fig. 4). The signals in these cells were as intense as those in the ganglion cells.

DISCUSSION

PAFR in the Eye

In this study, we demonstrated that PAFR messenger RNA is ubiquitously expressed in ocular tissues with relative concentration to the corneal epithelium, ICB, and ganglion cell and inner nuclear layers of the retina, microglia, and choroid. In situ hybridization is currently the only method available for localizing

FIGURE 2. Dark-field photomicrographs showing platelet-activating factor receptor messenger RNA signals in iris and ciliary body (A,B), cornea (C,D), and posterior segment (E,F) in the absence (A,C,E) or presence (B,D,F) of an excess of unlabeled probe. AC = anterior chamber; PC = posterior chamber; GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; Ch = choroid. Scale bars = 100 μm.
PAFR, because immunohistochemistry has given non-specific staining with the antibodies raised against PAFR. The PAFR-expressing tissues and cells were the sites of the PAF-related events in the eye, suggesting that these events are mediated, if not exclusively, by activation of PAFR. However, there are reports of unidentified intracellular PAF binding sites and their involvement as transcription factors in induction of several molecules. We are currently studying PAF effects on various tissues of PAFR-deficient mice. Platelet-activating factor receptor is expressed ubiquitously in various tissues and cells (reviewed by Izumi and Shimizu), particularly in the leukocytes and brain microglia. This is a cell-surface receptor coupled with at least two types of heterotrimeric guanosine 5′-triphosphate-binding proteins (Gi and Gq), and its diverse signaling cascades include phosphoinositide turnover, increases in intracellular calcium concentration, and activation of mitogen-activated protein kinase and PISK kinase. Further studies should be directed at identifying which of these pathways mediate each PAF-related event in ocular tissues.

**Ocular Surface**

We found PAFR expression in the corneal epithelium. This finding appears relevant to involvement of PAF in corneal disorders; however, PAF reportedly induces expression of several proteinases in the corneal epithelium through the intracellular binding sites. We thus need to characterize the distinct roles of PAFR and the putative intracellular PAF binding sites in the PAF-related events in the cornea.

Platelet-activating factor reportedly causes inflammatory responses in the conjunctiva. We found mild in situ hybridization signals in the conjunctiva and subconjunctiva (data not shown). However, because these signals were not completely displaced by an excess of the unlabeled probe, we could not determine PAFR expression in these tissues.

**Iris and Ciliary Body**

We found moderate PAFR expression in the ICB, which is compatible with the report of specific PAF binding in these tissues. The expression was diffuse and was found in both the stroma and epithelia, although the pigmented and unpigmented epithelia could not be distinguished in the albino rats we used. There is evidence that arachidonate-related compounds, including prostaglandins, leukotrienes, and PAF, play distinct roles in inflammation of the anterior uvea. Although PAFR antagonists prevent intraocular pressure elevation in eyes treated with laser or prostaglandin, the effects of PAF on aqueous humor production are unknown.

**Retina**

Platelet-activating factor has diverse effects on the central nervous system, including modulation of excit-
FIGURE 4. Bright-field photomicrograph showing platelet-activating factor receptor messenger RNA signals in microglia in inner plexiform layer of retina. The section was immunostained with OX-42, a microglia marker, before in situ hybridization. Scale bar = 10 μm.

Platelet-activating factor receptor expression in retinal ganglion and microglial cells. The signals in the inner nuclear layer suggest PAFR expression in bipolar cells. However, further identification will require combined use of cell markers and a non-radioactive probe for PAFR messenger RNA or a PAFR antibody. Expression of PAFR in these cells is in accordance with our previous report that PAFR is expressed in neurons and microglia of the brain.32 Platelet-activating factor receptor expression in retinal ganglion cells may result in neuronal death of rat retinal ganglion cell culture by PAF treatment.45 Interestingly, PAF functions in the nervous system in association with N-methyl-D-aspartate (NMDA) receptors. In the acquired immunodeficiency syndrome, antagonists of NMDA receptors reduce the production and neurotoxicity of PAF. In retinal ischemia, neuronal cell death involves both PAFR and NMDA receptors.47 However, the pathophysiological relevance of PAFR in microglia is not well understood. Microglia are related to macrophages and are activated in various conditions, particularly in immune responses and injury. Activated microglia produce cytokines and other factors and phagocytose degenerating or apoptotic cells (for review, see Barron46). Platelet-activating factor may be one of the key activators of retinal as well as brain microglia. To understand the roles of PAF in the retina, we should next identify the source of PAF in the retina, outputs of PAFR activation in the retinal ganglion and microglial cells, and functions of PAF as a neurotransmitter and modulator.

Choroid
Platelet-activating factor receptor was expressed in the posterior as well as anterior uvea. We do not know whether angiogenesis of the choroid involves PAF. Nevertheless, it involves macrophages and microglia,39-41 which abundantly express PAFR and metalloproteinases.53-55 It is an open question whether PAF and PAFR have some roles in the pathogenesis of choroidal neovascularization.

Further Perspectives
Platelet-activating factor receptor activation is probably involved in a variety of both physiological and pathologic events in ocular tissues. Our present findings warrant further studies on the localization and evaluation of PAFR expression in normal and diseased human eyes. Molecular dissection of PAF-related events in the eye is underway using PAFR-transgenic and -deficient mice.

Key Words
eye, in situ hybridization, platelet-activating factor, receptor

References
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