PDGF-C and -D Induced Proliferation/Migration of Human RPE Is Abolished by Inflammatory Cytokines

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PURPOSE. The role of growth factors and inflammation in regulating retinal pigment epithelial (RPE) function is complex and still poorly understood. The present study investigated human RPE cell proliferation and migration mediated by platelet-derived growth factor (PDGF) and inflammatory cytokines.

METHODS. Human fetal RPE (hfRPE) cells were obtained as previously described. Gene expressions of PDGF isoforms and their receptors were detected using real-time PCR. Protein expression, activity, and localization of PDGFR-α and -β were analyzed by Western blot and immunohistochemistry. BrdU incorporation and wound healing assays were used to test the effects of different PDGF isoforms and inflammatory cytokines on hfRPE proliferation and migration. Annexin-V and phalloidin staining were used to detect apoptosis and the actin cytoskeleton, respectively.

RESULTS. PDGF-C and PDGF-D proteins are expressed in native human adult RPE, and mRNA levels are up to 100-fold higher than PDGF-A and -B. PDGFR-α and -β proteins are expressed in native adult RPE and hfRPE (mainly localized to the apical membrane). In hfRPE, these receptors can be activated by PDGF-CC and -DD. PDGF-CC, -DD, and -BB significantly increased hfRPE proliferation, whereas PDGF-DD, -BB, and -AB significantly increased cell migration. An inflammatory cytokine mixture (TNF-α/IL-1β/IFN-γ) completely inhibited the stimulatory effect of PDGF-BB, -CC, and -DD; in contrast, this mixture stimulated the proliferation of choroidal cells. This inflammatory cytokine mixture also induced apoptosis, significant disruption of actin filaments and zonula occludens (ZO-1), and a decrease in transepithelial resistance.

CONCLUSIONS. These results suggest that proinflammatory cytokines in vivo can inhibit the proliferative effect of PDGF on human RPE and, at the same time, stimulate the proliferation of choroidal cells. They also suggest an important role of proinflammatory cytokines in overcoming local proliferative/wound-healing responses, thereby controlling the development of disease processes at the retina/RPE/choroid interface. (Invest Ophthalmol Vis Sci. 2007;48:5722–5732) DOI:10.1167/iovs.07-0327

The retinal pigment epithelium (RPE) is a monolayer of hexagonal cells located between the sensory neuroretina and the choriocapillaris. It is an important element of the outer blood-retinal barrier and is critical for maintaining the visual cycle, photoreceptor outer segment phagocytosis, and transport of nutrients, ions, and fluid between the distal retina and the choriocapillaris. In diseases such as proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD), RPE cells can reenter the cell cycle and initiate proliferation and migration and secrete extracellular matrix proteins. Breakdown of the blood-retinal barrier can alter the cytokine milieu in the subretinal space and in the vitreous and trigger the activation of RPE cells. In PVR, RPE cells proliferate and migrate to the vitreous cavity along with other types of cells (e.g., glial cells, fibroblasts, macrophages) and form fibrocellular membranes on the retinal surface or in the vitreous. These newly formed membranes eventually contract, resulting in retinal detachment and, if untreated, eventual loss of vision.

PDR is another proliferative ocular disease correlated with the migration and proliferation of RPE cells. In the back of the eye, choroidal neovascularization (CNV) is a clinical hallmark of the “wet” form of AMD, which is the leading cause of irreversible vision loss in the Western world among people older than 65. These newly formed leaky blood vessels eventually penetrate the Bruch membrane and the RPE and lead to the accumulation of blood and serum in the subretinal space, causing detachment of the retina and the formation of disciform scars. In a protective response, the RPE cells proliferate and migrate to envelop the newly formed capillaries.

Numerous investigations have focused on the role of growth factors and cytokines in the regulation of cellular events critical to wound healing. Platelet-derived growth factor (PDGF) belongs to the PDGF/VEGF growth factor family, which are major mitogens for many types of cells, mainly of mesenchymal origin, and thus play an important role in angiogenesis and wound healing. Thus far, four PDGF isoforms—PDGF-A, -B, -C, and -D—have been identified that form homodimers or heterodimers (PDGF-AA, -BB, -AB, -CC, and -DD) through the linking of disulfide bonds. Ligand binding induces PDGFR-α and -β tyrosine kinase receptor dimerization, resulting in three possible combinations, PDGFR-αα, αβ, and ββ, which have different affinities toward different isoforms of PDGF. Results from in vitro and in vivo experiments suggest that PDGFR-α and PDGFR-β can activate distinct signaling pathways.

PDGF-A and -B and their receptors are present in RPE and epi-retinal membranes from patients with PVR and PDR and are elevated in human vitreous of PVR eyes. Although relatively little is known about the effects of PDGF-C and -D on RPE function, recent evidence indicates an important role of PDGF-C and PDGFR-α in PVR and PDGF-D in lens epithelial cell proliferation.

TNF-α, IL-1β, and IFN-γ are three major proinflammatory cytokines that have a broad spectrum of activity in inflammation and wound healing. Previous experiments using confluent monolayers of human fetal RPE (hfRPE) showed that the receptors for these three cytokines are mainly localized to the apical (IL-1β) and basolateral membranes (TNF-α, IFN-γ), and the activation of these receptors significantly alters the polarized secretion of cytokines and chemokines and increased fluid absorption across the RPE monolayer from investigational ophthalmology & visual science, December 2007, Vol. 48, No. 12 — Copyright © Association for Research in Vision and Ophthalmology — Investigative Ophthalmology & Visual Science, December 2007, Vol. 48, No. 12

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The present experiments show that the PDGFRs are expressed and localized to the apical membrane of hRPE. PDGF-C and -D, the major PDGF isoforms expressed in human RPE, induce the phosphorylation of PDGFR and significantly increase proliferation or migration of these cells, which is abrogated by a proinflammatory cytokine mixture (ICM, TNF-α/IL-1β/IFN-γ). These inhibitory effects suggest that proinflammatory cytokines may help regulate proliferative diseases that occur at the retina/RPE/choroid interface.

**Materials and Methods**

**Cell Culture**

The present research followed the tenets of the Declaration of Helsinki and the National Institutes of Health Institutional Review Board. Fetal eyes (gestation, 16–18 weeks) were obtained from an independent source (Advanced Bioscience Resources, Alameda, CA), and adult eyes were obtained from Analytical Biological Services Inc. (Wilmington, DE). RPE and human fetal choroidal cells (hfCHC) were isolated and seeded into tissue culture flasks (Primaria; Becton Dickson, Franklin Lakes, NJ) in culture medium based on MEM-alpha modified medium with additional supplements, as described previously. The culture medium was changed every 3 days, and cells were subcultured by trypsin-EDTA treatment. Passages 1 and 2 were used for all studies.

For PDGFR receptor localization experiments, primary culture of hRPE were seeded onto cell culture inserts at 200,000 cells per well (12 mm diameter inserts, 0.4 µm pore size, polyester membrane; Corning Costar, Corning, NY). Before seeding, the inserts were coated with extracellular matrix from human placenta (10 µg/well; Becton Dickson) and were cured with ultraviolet light for 2 hours.

**Real-Time Polymerase Chain Reaction**

Total mRNA was extracted from native human adult RPE and from confluent monolayers of hRPE (P1) cultured in inserts (RNasy Kit; Qiagen, Valencia, CA). Total RNA (1 µg) was reverse transcribed to cDNA (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA). Real-time PCR was performed using assays (TaqMan; Applied Biosystems, Foster City, CA) for GAPDH, PDGF-A, -B, -C, -D, and PDGF-α and -β (ABI Sequence Detection System7900; Applied Biosystems). The mRNA concentration of each gene is normalized against GAPDH. PCR for each gene was performed in triplicate and repeated using tissues from three different donors.

**Western Blot**

Pigmented hRPE cells (P1) cultured in tissue culture flasks (Primaria; Becton Dickson) for 4 to 8 weeks and native human adult RPE cells were lysed with buffer (RIPA; Sigma-Aldrich, St. Louis, MO) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were centrifuged at 14,000g, and total protein in the supernatants was measured using protein assay (BCA; Pierce Biotechnology, Rockford, IL). Protein (20 µg) was electrophoresed on a 4% to 12% gradient gel (Bio-Tris NuPAGE; Invitrogen) under reducing conditions and was transferred to nitrocellulose membranes (XCell II Blot Module; Invitrogen). Nonspecific binding sites were blocked (BlockingStack T20 [TBS]; Pierce Biotechnology). The membranes were incubated overnight with mouse anti-human monoclonal antibody against PDGFR-α (clone 35248) or -β (clone PR7212; R&D Systems, Minneapolis, MN). After they were washed with TBS, membranes were incubated with goat anti-mouse antibody conjugated with horseradish peroxidase (HRP; Pierce Biotechnology). Immunoreactive bands were visualized using substrate (SuperSignal West Dura Extended Duration; Pierce Biotechnology) and imaged using a gel documentation system (Autochemie; UVP, Upland, CA).

A similar protocol was used in the PDGFR phosphorylation assay. Confluent and pigmented hRPE cells (4–8 weeks in culture) were starved in serum-free medium (SF; MEM-alpha modification medium containing nonessential amino acids and glutamine/penicillin/streptomycin) for 72 hours with media changed every 24 hours. During the last 2 hours, anti-human PDGFR-α blocking antibody (clone 35248) or anti-human PDGFR-β blocking antibody (catalog no. AF385; R&D Systems) was added to the flasks. Cells were placed on ice for 20 minutes, washed with cold PBS, and stimulated with 100 ng/mL PDGF-CC or PDGF-DD in cold PBS for exactly 15 minutes. After washing, cells were lysed using lysis buffer (20 mM Tris [pH 8.0], 157 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Halt; Pierce). Total protein (20–100 µg) was electrophoresed as described for Western blot except that the membranes were incubated with anti-phospho-PDGFR-α (Tyr 762) antibody (catalog no. O7–863), or anti-phospho-PDGFR-β (Tyr 716) antibody (catalog no. O7–021; Upstate, Temecula, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was blotted together with anti-GAPDH antibody (Abcam, Cambridge, MA) and used as loading control. PDGFR-treated or untreated NIH3T3 cell lysate was used as positive and negative control.

**Immunocytochemistry**

**Localization of PDGF Receptors.** Confluent monolayers of pigmented hRPE cells (P1) were cultured in inserts for 6 to 8 weeks and used for PDGFR receptor localization experiments. Before labeling, cultures were washed with PBS (pH 7.4) and fixed with 4% formaldehyde (Ted Pella Inc. Redding, CA) for 30 minutes at room temperature. Cells were permeabilized for 10 minutes with 0.2% Triton X-100 and blocked with a signal enhancer (Image-iT FX; Invitrogen). Antibodies against human PDGFR-α (clone 35248) or -β (clone PR7212; R&D Systems) and ZO-1 (Zymed, South San Francisco, CA) were labeled with different fluorophores (Zenon Alexa Kit; Invitrogen) and incubated with hRPE. Normal mouse serum (Invitrogen) was used as the negative control. Cells were mounted on glass slides with antifade reagent containing DAPI (Prolong Gold; Invitrogen) and were imaged with a fluorescence microscope (Axioplan 2 using Axioscview 3.4 software with ApoTome [Carl Zeiss, Oberkochen, Germany]).

**Actin Cytoskeleton Staining.** To examine cytoskeletal changes in hRPE cells treated with ICM, Factin was stained with fluorescent dye (Alexa Fluor 488; Invitrogen)-conjugated phalloidin. Cells grown in chamber slides (Nalge Nunc International, Naperville, IL) for 4 weeks were incubated with ICM for 48 hours and then fixed with 4% formaldehyde in PBS (pH 7.4) for 10 minutes at room temperature. After washing with PBS, cells were permeabilized and blocked with signal enhancer (Image-iT FX; Invitrogen). Cells were incubated with fluorescent dye (Alexa Fluor 488; Invitrogen) phalloidin and fluorescent dye (Alexa Fluor 555; Invitrogen)-conjugated mouse anti-human ZO-1 antibody. Stained cells were processed as described. As a possible physiological consequence of ICM-induced tight junction change, we also measured the transepithelial resistance (TER) of confluent monolayers using a voltohmmeter (EVOM; World Precision Instruments, Sarasota, FL) before and 24 hours after treatment with ICM.

**Proliferation Assay**

Cell proliferation was analyzed using a bromodeoxyuridine (BrdU) incorporation assay. hRPE cells or hfCHC were plated in 96-well tissue culture plates (Primaria; Becton Dickinson) at a cell density of 2.5 × 10^4 cells/well in complete culture medium containing 5% serum and were allowed to attach for 24 hours. Afterward, cells were starved in SFM. The medium was replaced 24 hours later with a test medium, consisting of SFM and different concentrations of PDGF-AA, -BB, -AB, -CC, and -DD, or of ICM (TNF-α 10 ng/mL, IL-1β 10 ng/mL, and IFN-γ 100 U/mL), or a combination of PDGFs and ICM (recombinant PDGF-AA, -BB, -AB, -CC were obtained from R&D Systems; recombinant core domain of PDGF-CC and -DD were generous gifts from Xuri Li, National
Eye Institute, Bethesda, MD). SFM was used as the negative control, and SFM supplemented with 5% serum was used as the positive control. Cells were incubated for 48 hours before the addition of BrdU labeling solution and then were incubated for another 24 hours. The proliferation rate was measured (Cell Proliferation ELISA BrdU Kit; Roche, Indianapolis, IN). Quadruplicates were used for each condition, and the experiments were repeated at least three times using different tissues. Cell viability was evaluated with a commercial kit (Live/Dead Viability/Cytotoxicity Kit; Invitrogen).

Migration Assay
Migration studies were performed using a modified method described by Murphy et al.²³ hfRPE cells (100 × 10³ cells/well) were seeded into 24-well tissue culture plates (Primaria; Becton Dickson) and were grown for 4 weeks to postconfluence. Cell proliferation was suppressed by incubation with 10 μg/mL mitomycin C (Sigma, Deisenhofen, Germany) for 2 hours before the experiment, and then a denuded area (7 mm in diameter) was made in each well using a custom-designed cell scraper. After three washes, cells were incubated with PDGF-AA, -BB, -AB, -CC, -DD (50 ng/mL), or ICM or combination of PDGF and ICM. SFM was used as the negative control, and SFM supplemented with 5% serum was used as the positive control. After incubation for 48 hours, the cells were fixed with cold methanol at 4°C for 20 minutes and stained with ethidium homodimer-1 (Invitrogen) at room temperature for 40 minutes. Migration was quantified by counting the average number of cells that migrated into the denuded area in 16 microscope fields covering the border of the entire denuded area. Each condition was tested in triplicate, and each assay was repeated at least three times using hfRPE cultures obtained from different tissues.

Apoptosis
An annexin V-FITC apoptosis detection kit (Roche) was used to detect hfRPE apoptosis. For these experiments, hfRPE cells were passaged and cultured in T-25 culture flasks for 3 to 4 days to subconfluence. After treatment with ICM for 24 hours, adherent cells were detached using 0.25% trypsin-EDTA and combined with floating cells. Cells were stained with annexin V-FITC/propidium iodide labeling solution for 15 minutes in the dark. Data were acquired on a flow cytometer (FACSCalibur; Becton Dickinson) and analyzed with software (FlowJo; TreeStar, San Jose, CA).

Statistical Analysis
Data are expressed as mean ± SEM; statistical significance (Student’s t-test, unpaired, two-tailed) was accepted at P < 0.05.

RESULTS
Expression and Activation of PDGF/PDGFR in Human RPE
Multiple PDGF isoforms and PDGF receptors were detected in cultured and native hfRPE cells and in native human adult RPE by microarray analysis (Wang F, et al. IOVS 2006;47:ARVO E-Abstract 2855; and unpublished data). Real-time PCR was used to determine the relative expression levels of all PDGF isoforms and receptors in human RPE (hfRPE and native adult). The results summarized in Figure 1 show that cultured fetal (A) and native adult (B) RPE cells constitutively express high levels of PDGF-C and -D, which were up to 100-fold higher than PDGF-A and -B. PDGF-C and -D are thus the major PDGF isoforms expressed in human adult and fetal RPE. In hfRPE, PDGFR-β is much more abundant than PDGFR-α (~1200-fold). In contrast, the expression levels of PDGFR-α in adult RPE are approximately equal to those of PDGFR-β.

Figures 2A and 2B show Western blot analysis of PDGFR-α and -β in cultured fetal and native adult RPE. Prominent anti-
body-specific bands at 170 to 185 kDa confirm the presence of these two PDGF receptor proteins. PDGF-C and -D protein were also detected in adult human RPE (Fig. 2B, lanes 3 and 4). Activation of PDGF-α and -β by PDGF-C and -D were further tested by phosphorylation experiments. Figures 2C and 2D show representative Western blot analysis of the phosphorylated PDGF receptors α- and β-activated by PDGF-CC and -DD, respectively. Background autophosphorylation of PDGFR-α in serum-free medium (Fig. 2C, lane 2) was increased by pulsing with PDGF-CC (Fig. 2C, lane 2). This effect was significantly inhibited by using blocking antibodies specific for PDGFR-α (Fig. 2C, lanes 3 and 4). Similarly, activation of PDGFR-β using a pulse of PDGF-DD (Fig. 2D, lane 2) was significantly inhibited by using blocking antibodies specific for PDGFR-β (Fig. 2D, lanes 3 and 4).

Cell Membrane Localization of PDGF Receptors

Through immunohistochemistry, we used monolayers of hRPE cells grown to confluence in inserts to localize PDGFR-α and -β. Figure 3 shows the immunofluorescence staining for PDGFR-α and -β: nuclei are stained with DAPI (blue), the tight junction marker ZO-1 is stained in red, and PDGFR-α and -β are stained in green. The middle part of each panel is an en face view of the monolayer shown as a maximum intensity projection through the z-axis. It shows a uniform hexagonal pattern of ZO-1 (red), typical of epithelia. PDGFR-α (green) appears as punctuate staining visible throughout the cell (Fig. 3A). The top and right side of each panel is a cross-section through the z-plane of multiple optical slices obtained using ApoTome (Carl Zeiss). ZO-1 (red) serves as a tight junction marker separating the apical and basolateral sides of the epithelial cells. Nuclei (blue) are located close to the basal side and serve as a marker to help define basal localization. In the cut view through the z-axis, the cells appear to form a single layer, with PDGFR-α (green) located predominantly on the apical side. Similarly, Figure 3B shows apical localization of PDGFR-β (green). The intensities of the PDGFR-β and PDGFR-α staining were normalized in Figure 3 for ease of RPE cell visualization. The presence of these receptors in hRPE suggests the possibility of paracrine or autocrine signaling at the RPE. These data are also consistent with a recent report that PDGFR/VEGF receptor ligands are accumulated in the apical extracellular space in Drosophila epithelial tissues, which has been shown to be important for actin cytoskeleton regulation.24
Effects of PDGF Isoforms on hfRPE Proliferation

Cultured hfRPE cells were incubated in the presence of recombinant human PDGF-AA, -BB, -AB, -CC, and -DD in serum-free medium for 72 hours, and the rates of proliferation were quantified using BrdU. Figure 4 summarize proliferation responses for a range of PDGF isoforms and concentrations. PDGF-AA (approximately 12.5 ng/mL) had a stimulatory effect of approximately 17% compared with the serum-free medium ($P < 0.001$), whereas cells incubated with 5% serum (positive control) increased proliferation more than 100%. Smaller but still significant proliferation was also detected over a wider range, from 2 to 100 ng/mL ($P < 0.05$). PDGF-BB shows dose–response significant stimulatory effects from 6 to 100 ng/mL; the maximum stimulatory effect of approximately 35% occurred at 100 ng/mL ($P < 0.01$). PDGF-AB is less potent, producing a small but significant effect only at 50 ng/mL ($P < 0.001$). Interestingly, newly discovered PDGF isoforms—PDGF-CC and PDGF-DD—have a much stronger proliferative effect on hfRPE cells, 142% and 92% ($P < 0.001$), respectively. PDGF-CC and PDGF-DD are thus the major PDGF isoforms affecting hfRPE proliferation.

Effects of PDGF Isoforms on hfRPE Migration

We examined the migration of hfRPE cells under the influence of different PDGF isoforms using a wound-healing model. Results in Figure 5 summarize the effectiveness of each isoform at a concentration of 50 ng/mL. PDGF-AA and -CC produced small but not statistically significant increases in migration relative to the SFM negative control (=100). In contrast, 50 ng/mL PDGF-BB, -AB, or -DD significantly stimulated hfRPE cell migration, and the stimulatory effects for PDGF-BB, -AB, and -DD were approximately 97% ($P < 0.05$), 101% ($P < 0.001$), and 86% ($P < 0.001$), respectively.

Inflammatory Cytokines Inhibit hfRPE Proliferation and Migration

Proliferation experiments summarized in Figure 6A show that PDGF-BB, -CC, and -DD (50 ng/mL) significantly stimulated BrdU incorporation compared with SFM control (=100%) and that these effects were completely abolished by the ICM. ICM also significantly inhibited BrdU incorporation stimulated by 5% serum ($P < 0.01$). Figure 7 summarizes a series of migration experiments showing that 50 ng/mL PDGF-BB significantly stimulated hfRPE migration that was completely blocked by the presence of ICM ($P < 0.001$). ICM also significantly inhibited hfRPE migration stimulated by 5% serum ($P < 0.001$). In striking contrast, adding ICM to hfCHC caused a significant increase in cell proliferation compared with SFM control (100%) and ICM had no significant inhibitory effect on 5% serum-stimulated cell proliferation (Fig. 6B). Cell viability was evaluated in all these experiments. Percentages of viable cells were greater than 85% in every examined group (not shown),
including SFM control. Viability was not significantly reduced after incubation with PDGF isoforms or ICM.

Inflammatory Cytokines Induce hfRPE Apoptosis

The inhibitory effect of ICM on hfRPE might have been caused in part by apoptosis. This possibility was examined using an annexin V-FITC/propidium iodide staining assay for apoptotic and necrotic cells. Figure 8 compares hfRPE in control and treated with ICM for 24 hours. Flow cytometry data show that after ICM treatment, the total number of apoptotic cells (late + early) increased more than twofold, from 3.8% (control) to 8.4% (ICM). The ICM-induced apoptosis in hfRPE could contribute to the inhibitory effect of the inflammatory cytokine mixture on hfRPE proliferation and migration.
Inflammatory Cytokines Induce Actin Cytoskeleton Disruption

In confluent, well pigmented monolayers of hRPE (6–8 weeks in culture), the F-actin filaments were mainly uniformly distributed around the periphery of the cell as a dense band; this distribution was not affected by the presence or absence of ICM (not shown). In contrast, relatively young polymorphic cells (2 weeks or less in culture) show a less uniform distribution of cytoskeletal elements that are clearly visible throughout the cell. Figure 9 shows confluent monolayers (2 weeks in culture) whose F-actin filaments are stained with phalloidin (green), junctional complexes are stained with ZO-1 (red), and nuclei are stained with DAPI (blue). Figures 9A–C show three Z sections from the apical side (4.2 μm) to the basolateral side (7.8 μm) of the control monolayer. Figures 9D–F show three similar sections from another confluent monolayer treated with ICM for 48 hours.

Compared with control (Figs. 9A–C), ICM treatment (Figs. 9D–F) caused a decrease of dense circumferential bands and an increase of diffuse distribution of F-actin filaments throughout the cytoplasm. The usual hexagonal distribution of ZO-1 localization was also significantly altered after incubation with ICM for 48 hours. ZO-1 displayed disorganization similar to that of F-actin and was diffusely distributed throughout the cytoplasm. This ICM-induced disruption of the hRPE cytoskeleton, also observed in two other experiments, might have contributed to the inhibitory effect of ICM on hRPE. Relative monolayer integrity was also estimated by measuring changes of TER. In control cell culture medium (SFM), TER of hfRPE monolayers was 907 ± 46 Ω·cm² and remained essentially constant for 24 hours (883 ± 46 Ω·cm²). In contrast, incubation with ICM for 24 hours decreased TER by 48%, from 843 ± 34 Ω·cm² to 441 ± 31 Ω·cm² (n = 4; P < 0.05).

**DISCUSSION**

The present experiments were conducted using a well-characterized model of confluent monolayers of primary hRPE cultures that exhibit many of the morphologic, biochemical, and physiological properties of adult mammalian and human RPE. The apical membrane localization of PDGFR-α and -β is, to our knowledge, the first demonstration of the polarized expression of these two receptors on RPE. We also show that PDGF-C and PDGF-D are the most abundant isoforms in native adult and cultured fetal human RPE and that they induce the phosphorylation of PDGF receptors and significantly affect the proliferation or migration of hRPE. In addition, PDGF-BB significantly increased the proliferation of hRPE cells, whereas PDGF-BB and -AB significantly increased cell migration.

It is thought that PVR is amplified by certain inflammatory factors, and anti-inflammatory drugs have been used to treat PVR. However, the present experiments show that a proinflammatory cytokine mixture (ICM), consisting of TNF-α, IL-1β, and IFN-γ, abrogated completely the stimulatory effect of PDGF on hRPE proliferation and migration (Figs. 6, 7). This mixture also significantly inhibited the effect of serum-positive (5%) control. These effects were not accompanied by ICM-induced cell necrosis but rather by hRPE cell apoptosis and disruption of the cytoskeleton along with tight junction integrity (Fig. 9), concomitant with a decrease in TER.

**Constitutive Expression of PDGF and PDGFR in Human RPE**

Ligand-receptor affinities for all known members of the PDGF family are summarized in a schematic diagram (Fig. 10). The present data show that PDGFR-α and -β are expressed in hRPE cells, which are mainly localized to the apical membrane (Figs. 2, 3) and can be activated by PDGF-CC and -DD (Figs. 2C, 2D). The relatively high abundance of PDGF-C and -D suggests an important role of these two PDGF isoforms in RPE physiology or pathophysiology. The relatively high expression of PDGFR-β compared with PDGFR-α and the absence of a stimulatory effect of PDGF-AA (Figs. 4, 5) suggest an important role for PDGFR-β in the activation of the signaling pathways leading to RPE migration and proliferation. PDGFR-α expression is high in adult tissue compared to hRPE, which may be a peculiarity of the culturing process or a pathophysiological component of the donor tissue, or it may be part of a developmental expres-
sion pattern. The latter possibility suggests a significant role for PDGFR-α in the physiology and pathology of the adult eye.

The present experiments demonstrate the expression of all PDGF isoforms in human RPE (Fig. 1). Previous studies have shown that at least some of these isoforms are secreted from the RPE.17,55 PDGF may therefore provide autocrine stimulation that plays an important role in retinal wound repair and epi-retinal membrane formation. Consistent with this notion, PDGFR-α and PDGFR are expressed in RPE from epi-retinal membranes removed during vitreous surgery.17 However, only low levels of PDGFR-α are expressed on human adult cultured RPE cells; no PDGFR-β was detected by real-time PCR or immunohistochemistry from an earlier report.35 These differences in PDGF and PDGFR expression in these cultured human RPE cells could be attributed to a variety of methodological differences, such as different cell origin (fetal vs. adult), different cell purities, different stages of development, and differences in cell culture conditions. PDGFR-β expression can change markedly depending on culture properties, such as cell density, attachment of cell to matrix, presence of serum, and the differentiation status of cells.34

**Effects of PDGF Isoforms on hrRPE Proliferation and Migration**

In previous studies, we defined confluent hrRPE cells in terms of cell polarity, relatively high transepithelial resistance (approximately 500 Ω·cm²) and potential (approximately 3 mV), and high expression levels of a variety of tight junction proteins, including claudin-19,22 itself a hallmark of high transepithelial resistance.35,36 Figures 4 and 5 show that PDGF-AA, which can only activate PDGFR-α, has no significant effect on hrRPE migration and only a small effect on cell proliferation. In contrast, PDGF-BB, a universal ligand for all three receptors (Fig. 10), shows strong stimulatory effects for the proliferation and migration of hrRPE cells. This latter conclusion is supported by several previous reports using different culture conditions in human fetal and adult RPE cells.17,37 These results with PDGF-AA and -BB, taken together, indicate that PDGFR-α provides no significant input into hrRPE proliferation or migration.

PDGF-AB increased hrRPE migration by almost a factor of 2 compared with control but had no effect on proliferation except at 50 ng/mL (Figs. 4, 5). The very different effect of PDGF-AB on hrRPE proliferation and migration suggests a dependence of these two signaling pathways. Compared with PDGF-AA, which can only activate PDGFR-α, PDGF-AB can bind and activate PDGFR-α and PDGFR-β (Fig. 10). This comparison suggests that the strong stimulatory effect of PDGF-AB on hrRPE migration is mainly mediated through the PDGFR-β pathway.

PDGF-CC, a more recently discovered member of the family,38,39 has the same receptors as PDGF-AB (Fig. 10) but, in striking contrast, stimulated proliferation by more than twofold at some concentrations and had no significant effect on hrRPE migration (Figs. 4, 5). This result suggests that separate mechanisms are involved in hrRPE cell proliferation and migration. The strong effect of PDGF-CC on the proliferation of the hrRPE cells, at low and high concentrations, is unexpected given that the expression level of PDGFR-α, the major receptor used by PDGF-CC, is much lower than that of PDGFR-β, leading to limited availability of PDGFR-α or -β. However, the proliferative effect of PDGF-CC could be triggered by receptors or mediators other than PDGFR-α. Another new member of this family, PDGF-DD, maximally stimulated proliferation and migration by 86%, which is comparable to the effects produced by PDGF-BB. PDGF-CC and -AB activate the same receptors, but PDGF-AB only affects migration whereas -CC only affects proliferation. Given the specificities summarized in Figure 10 and the relatively large abundance of the PDGFR β subunit (Fig. 1), we can hypothesize that the stimulatory effects of PDGF-CC and -AB are mainly mediated through the PDGFR-αβ signaling pathway.

**ICM Abolishes PDGF-Induced hrRPE Proliferation and Migration**

The data summarized in Figures 6A and 7 clearly show that ICM completely abolished PDGF-BB-/-CC-/-DD-induced pro-
liferation and PDGF-BB-induced migration and, in addition, significantly inhibited the serum-induced migration and proliferation of hRPE by more than twofold. The strong inhibition effect of TNF-α, IL-1β, and IFN-γ on the migration and proliferation of hRPE cells may be explained in part by the interaction of the intracellular signaling pathways stimulated by these three cytokines. In other systems, IL-1β and TNF-α induce gene expression by activating NF-κB, whereas IFN-γ induces gene expression by activating STAT1. In preliminary experiments (not shown), we have found that the inhibition of proliferation by IFN-γ alone is significantly greater than the effect of ICM, suggesting that these pathways are interactive and perhaps counteract each other in their ability to inhibit proliferation.

In contrast to the present findings, earlier studies have indicated that TNF-α, IL-1β, and IFN-γ stimulate the proliferation and migration of adult cultured human RPE. This difference suggests that fetal and adult RPE cells may be functionally different, but other possibilities may explain the difference. Figure 6B shows that ICM stimulates the proliferation of hfCHCs, suggesting that previous work using adult human RPE might have been affected by choroidal contamination. A combination of choroidal and RPE cells may provide a more realistic model of the signals that regulate the retina/RPE/choroid interface. In the early inflammatory stages of AMD, the RPE barrier seems intact, perhaps because the disease-induced inflammation prevents the RPE from proliferation/migration. At the same time, choroidal cell proliferation can occur, as suggested by the data in Figure 6B, and may contribute to choroidal neovascularization that eventually breaks through Bruch membrane and the RPE.

IFN-γ, one of the ICM components, is a lymphokine and a potent activator of monocytes and macrophages mainly produced by activated T and NK cells. The biological effects of IFN-γ include induction of an antiviral state of cells, inhibition or enhancement of major histocompatibility complex antigen expression, and other immune regulatory activities. However, the ability of IFN-γ to inhibit the mitogenic activity of regulatory growth factors PDGF, EGF, and FGF has been demonstrated in fibroblasts. It has also been shown that IFN-γ exhibits antiproliferative effects on some other cell types, such as endothelial cells and transformed cell lines. Although IFN-γ is normally associated with inflammatory processes, the present data support the notion that IFN-γ has a dual role in that it can also inhibit processes normally associated with inflammation, such as proliferation and migration.

ICM-Induced Apoptosis and Cytoskeleton Disruption in hRPE

The annexin-V data summarized in Figure 8 indicate that the ICM-induced inhibition of proliferation and migration might have been associated with hRPE apoptosis. Activation of NF-κB through the TNF-α signaling pathway can induce apoptosis in a wide variety of cell types. An early report indicates that cultured adult human RPE are resistant to TNF-α-induced apoptosis. In the present experiments (Fig. 8), the ICM more than doubled the percentage of apoptotic cells, from 3.78% (control) to 8.36%. This increase undoubtedly contributed to the ICM-induced inhibition of hRPE proliferation and migration.

It seems likely that hRPE migration and proliferation are partly mediated by the cytoskeleton. Actin is a major component of the cytoskeleton-forming microfilaments and, together with microtubules and intermediate filaments, help mediate cell movement. Figure 9 shows that ICM disrupted hRPE cytoskeleton and changed the distribution of ZO-1 in the junctional complexes. The cytoskeleton is important in mediating the migratory response of human RPE cells induced by PDGF. These ICM-induced changes in cytoskeleton presumably contribute to the inhibition of migration and proliferation of actively dividing hRPE. In vivo RPE cells are fully matured with intact tight junctions that presumably would not be affected by ICM, as observed in our 6- to 8-week cultures. After disease-induced disruption of the epithelial syncytium (e.g., in PVR), RPE cells divide; in this case, the ICM may provide therapeutic benefit.

Nitric oxide (NO) production in hRPE is another possible mechanism for ICM-induced inhibition of RPE proliferation. Previous reports have demonstrated that the cytokine mixture used in the present experiments can induce the production of NO in cultured adult human RPE cells. In addition, NO elevation inhibits proliferation of cultured bovine RPE. Against this hypothesis is a previous report showing that adult human cultured RPE (passages 2–6) treated with TNF-α 200 U/mL, IL-1β 100 U/mL, IFN-γ 200 U/mL plus lipopolysaccharide (LPS) 10 U/mL had no effect on RPE proliferation, despite elevated NO levels. This possible contribution of NO on the ICM-induced inhibitory effect need to be critically tested in hRPE.

Physiological Implications

Recent studies have emphasized the role of the RPE in the innate and the adaptive immune systems. The present findings expand the RPE repertoire by showing that PDGF-α and -β are mainly localized to the apical membrane facing the neural retina, suggesting important roles of the PDGF ligands in the functional status of these cells. Indeed, we further showed that PDGF-CC and -DD can strongly induce RPE proliferation and migration and therefore played major roles in the elaboration or reduction of inflammation at the retina/RPE interface. It is striking to observe that hRPE migration and proliferation induced by PDGFs can be abrogated by a set of common inflammatory mediators (TNF-α, IL-1β, and IFN-γ) that also significantly inhibit the stimulatory effects of serum. In addition, the ICM-induced disruption of ZO-1 and the reduction in TER could allow increased leukocyte traffic across the RPE, possibly through the paracellular pathway. Finally, the present results suggest that PDGF/PDGF, RPE-specific cytokines, and their receptors, located on the apical and basolateral membranes, could provide specific targets for therapeutic interventions for alleviating inflammatory diseases.

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