Platelet-derived growth factor is an autocrine growth stimulator in retinal pigmented epithelial cells

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SUMMARY

The retinal pigmented epithelium (RPE) plays a major role in normal and exaggerated retinal wound repair; the latter can result in epiretinal membrane formation and loss of vision. The RPE forms a stable monolayer of highly differentiated cells that proliferates only during wound repair. The mechanism underlying the change to the proliferating phenotype is unknown.

When grown on a plastic substratum, cultured RPE cells mimic the proliferating phenotype in situ; they escape density arrest and proliferate in serum-free medium. In this study, we have demonstrated that a platelet-derived growth factor (PDGF) autocrine loop is involved in RPE growth in serum-free medium, because: (1) RPE cells secrete PDGF into their media and express PDGF receptors; (2) the PDGF receptors on RPE cells are autophosphorylated in serum-free medium and suramin, an agent that displaces PDGF and other growth factors from their receptors, blocks the autophosphorylation; and (3) a neutralizing antibody to PDGF significantly decreases RPE growth in serum-free medium. When a linear scrape is made in an RPE monolayer, the cells migrate and proliferate to fill in the gap mimicking wound repair in situ. Cells along the edge of the scrape show increased expression of PDGF and PDGF-β receptors, and increased staining for proliferating cell nuclear antigen.

Immunohistochemistry and in situ hybridization demonstrate expression of PDGF in ganglion cells and cells of retinal blood vessels. PDGF is not detected in the outer retina or RPE in untreated eyes, but is detected in RPE participating in wound repair, either adjacent to laser burns or underlying retinal detachment. PDGF and PDGF receptors are also expressed in RPE in epiretinal membranes removed during vitreous surgery.

These data suggest that PDGF is an autocrine stimulator of growth in RPE that plays a role in retinal wound repair and epiretinal membrane formation.

Key words: platelet-derived growth factor, retinal pigmented epithelium, proliferative retinopathy
control is achieved would provide important information that could have clinical implications.

In many respects, cultured RPE cells simulate the wound healing phenotype of RPE in situ; they actively proliferate and, under some conditions, fail to density arrest even in the absence of serum (Bryan and Campochiaro, 1986), while under other conditions they stop proliferating and reassure specialized functions that they display in situ (Campochiaro et al., 1991; Campochiaro and Hackett, 1993; Flannery et al., 1990). Understanding the signals responsible for these divergent types of behavior in culture could provide important insights into the wound healing function of RPE and how it might be modulated.

Medium conditioned by RPE stimulates the proliferation of several cell types including RPE (Bryan and Campochiaro, 1986). This provides a possible explanation as to why RPE cells fail to density arrest under some conditions and continue to grow in the absence of serum. We have previously demonstrated that RPE produce PDGF-like proteins, which account for a portion of the mitogenic activity in RPE-conditioned medium (Campochiaro et al., 1989), and that PDGF stimulates the proliferation of RPE (Leschey et al., 1990). In this study, we have tested the hypothesis that PDGF is an autocrine growth stimulator for RPE.

MATERIALS AND METHODS

cDNA probes

A 1.26 kb cDNA fragment coding for human PDGF-A (Betsholtz et al., 1986) and a 2.7 kb fragment coding for human PDGF-B (Johnsson et al., 1984) were provided by Dr Carl Heldin (Stockholm, Sweden). A 4.8 kb ribosomal RNA mouse cDNA was provided by Dr Rama Reddy Guntaka (Columbia, MO). OLIOMTM software (National Biosciences, Inc., Plymouth, MN) was used to select two 20mer oligonucleotides corresponding to unique sequences in murine PDGF-A (upper oligo, 180-199, 5′-AGATCCGGAGCCCTCCAGG-3′; and lower oligo, 738-757, 5′-AAAGACCCGACCGCATTG-3′). The oligonucleotides were synthesized in a model 392 DNA synthesizer (Applied Biosystems, Inc. (ABI), Foster City, CA), deprotected at 55°C overnight, dried, dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and used to amplify cDNA fragments from mouse cDNA by polymerase chain reaction (PCR). The cDNA was synthesized from mouse liver RNA on Dynabeads as described by Rodriguez and Chader (1992). A reaction product of expected size (578 bp) was cloned using the PCR-ScriptTM SK(+) cloning kit (Stratagene, LaJolla, CA) following the manufacturer’s instructions and its veracity was confirmed by fluorescence sequencing. The plasmid clones containing the PDGF-A product were grown in Superbroth containing ampicillin (100 µg/ml) overnight and the plasmid DNA was purified using the Plasmid Midi Kit (Qiagen Inc., Chatsworth, CA). The fluorescence sequencing was performed in a model 370A automated sequencing instrument (ABI) connected to an Apple Macintosh i and ABI’s 373A sequencing software using ABI’s Taq DyeDeoxyTM Terminator cycle sequencing kit. In general, 0.5 pmol of template and 3 pmol of primer were used per sequencing reaction. The reactions were purified using Select-D G-50 columns (5 Prime-3 Prime, Boulder, CO) and electrophoresed in a 6% acrylamide gel.

Cell culture

Human RPE cells were harvested from postmortem eyes obtained from the Old Dominion Eye Bank (Richmond, VA) or the Maryland Eye Bank (Baltimore, MD), by a procedure that has been previously described (Campochiaro et al., 1986). The cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah). Immunohistochemical staining for cytokeratins was performed on RPE cell lines as previously described (Leschey et al., 1990). Cells used in this study were from donors of age 1 day, 48 years and 78 years, and all stained uniformly for cytokeratins.

Immunoblots

RPE cells at passages 2-4 were grown to confluence, extensively washed, and 10 µl of serum-free DMEM with one of the following additions was added to the flasks: (1) no additions; (2) thrombin (3 units/ml; Sigma, St Louis, MO); (3) transforming growth factor-beta (TGF-β, 200 pg/ml; R&D systems, Minneapolis, MN); (4) phorbol myristate acetate (PMA, 100 ng/ml; Sigma); or (5) forskolin (50 µM; Sigma). After 12 or 24 hours, the media were removed, centrifuged, exhaustively dialyzed against 0.5 M acetic acid, and lyophilized. Samples were dissolved in SDS electrophoresis buffer and 50 µl corresponding to 1.5 ml of crude conditioned media were run in immunoblots as previously described (Campochiaro et al., 1989) using an IgG fraction from goat antiserum raised against human PDGF purified from fresh platelets. The characterization of the primary antibody has been previously published; it recognizes PDGF heterodimers and both types of homodimers (Takehara et al., 1987; Flemming et al., 1989).

Two-site ELISA

Two-site ELISA was performed on RPE-conditioned medium that had been concentrated 10-fold by filtration, using a mouse monoclonal antibody raised against PDGF-BB (designated mAb SIS 1; IgG 1, kappa chain; LaRochelle et al., 1989) and the goat polyclonal antibody raised against purified human PDGF (Takehara et al., 1987; Flemming et al., 1989). The monoclonal antibody (50 µg/ml; 10 µg/ml in phosphate buffered saline/0.02% sodium azide (PBS)) was placed in a polystyrene microtiter plate and incubated at room temperature for 5 hours. Following blocking for 1 hour with 4% BSA in PBS, 50 µl of sample was diluted 2-fold in 4% BSA/PBS/Triton X-100/0.1 mM PMSF/10 µg/ml leupeptin and 10 µg/ml pepstatin and placed in the wells. Samples were incubated overnight at room temperature. Plates were washed 4 times with PBS/0.05% Tween-20 (solution A) and 50 µl of the polyclonal anti-PDGF antibody (77 µg/ml in PBS/0.50% Tween-20/1% BSA) was placed in each well. After a 3 hour incubation at room temperature, plates were washed 4 times with solution A and 50 µl of an alkaline phosphatase-conjugated rabbit anti-goat antibody (1:200; Sigma) was placed in each well. After 2 hours, the plates were washed 4 times with solution A and read at 405 nm after addition of 2 mg/ml paranitrophenol phosphate.

Northern blots

Total RNA was harvested by the procedure of Chomczynsk and Sacchi (1987). To harvest RNA from RPE in situ, the anterior segment, vitreous and retina were removed from freshly enucleated eyes and guanidinium lysis buffer was added to eye cups. Under a dissecting microscope, the RPE were brushed from Bruch’s membrane and aspirated leaving Bruch’s membrane intact and assuring isolation of pure RPE RNA. Poly(A)+ RNA was prepared using oligo(dT)-cellulose column chromatography (Badley et al., 1988). Fractionation of denatured RNA on formaldehyde-containing 1.4% agarose gels and blot transfer to Nitran paper (Cuno, Inc., Meriden, CT) was performed as described by Thomas (1983). cDNA probes were labeled with [32P] using the Schleicher and Schuell random priming kit following the manufacturer’s instructions. Hybridization conditions have been published previously (Hackett and Campochiaro, 1993). Washed blots were exposed to XRP film (Kodak, Rochester NY) with an intensifying screen at −80°C and exposure times were varied to stay within the linear range for autoradiographic signals. Blots were stripped and rehybridized with a probe for 18 S ribosomal RNA to control for potential differences in RNA loading.
Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand complementary DNA (cDNA) was prepared from RNA using a Superscript preamplification kit (BRL, Gaithersburg, MD). Reverse transcriptase was excluded from control tubes. Oligonucleotides were prepared in a DNA synthesizer as described above corresponding to nucleotides 1271 through 1290 (5′-CTCCCTCTTG-GTGGGTGTTGGC-3′, upper oligo) and 1603 through 1622 (5′-AGTCCGTGGCTGGTGGAGA-3′, lower oligo) of the coding sequence for the human PDGF-B chain gene, and nucleotides 1950 through 1969 (5′-TGCCCCAGGCCTTTGTTGG-3′, upper oligo) and 2232 through 2259 (5′-TAGCAAGCTTCTCAAGTATA-3′, lower oligo) of the coding sequence for the human PDGF-A chain gene. Using these nucleotides as primers, 1 ng of cDNA as template, and reagents from a GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT), PCR (30 cycles) was performed in a DNA thermal cycler (Perkin Elmer Cetus) using conditions optimized in preliminary experiments. PCR products were cloned and sequenced as described above.

Immunoprecipitations

Cells were washed with 1 mM sodium vanadate and lysed in buffer containing 0.5% Tween-20, 1% NP40, 1% CHAPS, 50 mM HEPES, pH 7.5, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 20 mM sodium pyrophosphate, 5 mM glycerophosphate, 10 µg/mℓ aprotinin, 10 µg/mℓ leupeptin, 10 µg/mℓ pepstatin, 1 mM PMSF, 0.5 mM N-ethylmaleimide, and 25 mM aminocaproic acid. Cell lysates were sonicated briefly and centrifuged at 15,000 g for 30 minutes at 4°C. The supernatants were incubated on a rotator at 4°C with anti-phosphotyrosine antibody bound to agarose (Upstate Biotechnology Incorporated, Lake Placid, NY) for 4 hours. After centrifugation, the pellets were washed and immunoprecipitated proteins were eluted with electrophoresis buffer, resolved by SDS-PAGE, and immunoblotted as described above.

[3H]thymidine incorporation

RPE cells at passage 2-4 were grown to confluence in 24-well plates, placed in serum-free medium for 24 hours, and then incubated in various concentrations of PDGF-AA, PDGF-BB or medium alone. After 18 hours, the cells were pulsed with 2 µCi/mℓ [3H]thymidine (sp. act. 6.7 Ci/mmol; New England Nuclear, Boston, MA) for 2 hours and [3H]thymidine incorporation was measured as previously described (Leschey et al., 1990).

Animal models

C57BL mice were anesthetized and their pupils were dilated. A drop of methylcellulose was placed on a small segment of a coverslip, which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea. A Coherent laser with a slit lamp delivery system was used to deliver 100 burns which was then placed on the surface of the right cornea.

Immunolocalization of PDGF and its receptors

Immunohistochemistry was done as previously described (Vinore et al., 1990, 1993) on cultured cells, sections of mouse eyes, and a series of 23 epiretinal membranes removed from patients during vitreous surgery. The following antibodies were used: (a) a goat polyclonal Ab that recognizes both PDGF-A and PDGF-B (1:50 dilution) (Flemming et al., 1989); (b) a monoclonal Ab that recognizes PDGF-B, but not PDGF-A (1:50 dilution) (LaRochelle et al., 1989); (c) a rabbit polyclonal Ab that recognizes both PDGF-α receptor and PDGF-β receptor (1:250 dilution) (Jensen et al., 1992); (d) a rabbit polyclonal Ab that recognizes PDGF-α receptor, but not PDGF-β receptor (1:250 dilution) (Jensen et al., 1992); (e) a rabbit polyclonal Ab that recognizes PDGF-β receptor, but not PDGF-α receptor (1:250 dilution) (Jensen et al., 1992); (f) a monoclonal Ab directed against PCNA was obtained from Dr Peter Hall (1:20 dilution) (Hall et al., 1990). Each of these Abs was raised against human proteins, but they cross-react with the mouse proteins. For controls, normal rabbit serum or normal goat serum was substituted for primary antibody, or primary antibody was incubated for 1 hour at 4°C with a 10-fold molar excess of purified antigen.

In situ hybridization

Frozen sections were post-fixed in 2% paraformaldehyde, treated with protease K (Sigma) and acetylated with acetic anhydride, after which they were covered with a hybridization mixture consisting of 50% formamide, 5× standard saline citrate (SSC), 2 mg/ml yeast tRNA, 100 μg/ml heparin, 1× Denhardt’s solution, 0.1% Tween-20, 0.1% CHAPS, and 5 mM EDTA, pH 7.2, and incubated for 4 hours at 60°C for pre-hybridization. The pre-hybridization solution was replaced with fresh hybridization solution containing 0.2 μg/ml digoxigenin-labeled antisense or sense riboprobe prepared by in vitro transcription using a Boehringer-Mannheim Genius kit according to the manufacturer’s instructions and the sections were incubated overnight at 60°C in a hydrated chamber. After hybridization, the sections were washed twice for 30 minutes at 65°C in FSC (50% formamide/2× SSC/0.1% CHAPS/50 mM glycine, pH 7.2), and twice for 5 minutes at 60°C in 2× SSC-CHAPS (2× SSC/0.3% CHAPS/50 mM glycine, pH 7.2). Unhybridized RNA was digested in a solution of 4 μg/ml RNase A and 20 units/ml RNase T1 for 35 minutes at 37°C with occasional agitation followed by 5 minutes washes at room temperature in 2× SSC-CHAPS and FSC, and 60°C washes in FSC (30 minutes×2 with gentle agitation), 2× SSC-CHAPS (5 minutes), 0.2× SSC-CHAPS (15 minutes), and PBT with 0.3% CHAPS (5 minutes). The sections were cooled to room temperature and washed 3× 5 minutes with bovine serum albumin (BSA)-saline (PBS with 0.1% Triton X-100 and 2 mg/ml BSA) and placed in BSA-saline containing 40% heat-inactivated lamb serum for 1 hour at 4°C with gentle agitation to block non-specific Ab binding. The solution was replaced with fresh 4°C BSA-saline containing 40% lamb serum, plus a 1:2,000 dilution of sheep anti-digoxigenin Fab Ab conjugated to alkaline phosphatase and the sections were incubated overnight at 4°C. After extensive washing, the tissue was rinsed twice briefly in alkaline phosphatase buffer (100 mM Tris, pH 9.5, with 100 mM NaCl, 50 mM MgCl2, and 0.1% Tween-20) and incubated in this mixture with 18
µl of 75 mg/ml NBT in dimethylformamide, and 14 µl 50 mg/ml BCIP in dimethylformamide for 24-48 hours in the dark at room temperature and then washed with Genius buffer 3 (Boehringer Mannheim) and mounted with Aqua Poly/Mount (Polysciences).

RESULTS

PDGF-B and PDGF-A are expressed in RPE in culture and in situ

As demonstrated in a previous study (Campochiaro et al., 1989), immunoblots using an antibody that recognizes all of the dimer combinations of PDGF-A and PDGF-B demonstrate that RPE-conditioned media contain multiple cross-reacting bands between 36 and 40 kDa (Fig. 1). The addition of thrombin to the conditioning medium results in a relative

Fig. 1. Media conditioned by retinal pigmented epithelial cells (RPE) contain immunoreactive PDGFs. (a) Media were conditioned on RPE for 24 hours in the presence of: no additions (lane 1), phorbol myristate acetate (100 ng/ml, lane 2), transforming growth factor-β (200 pg/ml, lane 3), thrombin (3 units/ml, lane 4), forskolin (30 µM, lane 5), phorbol myristate acetate + forskolin (lane 6), transforming growth factor-β + forskolin (lane 7), or thrombin + forskolin (lane 8). Lane 9 contains 0.1 ng and lane 10 contains 10 ng of PDGF purified from human platelets. Immunoblots were done using goat IgG raised against human PDGF as described in Materials and Methods.

Fig. 2. Cultured retinal pigmented epithelial (RPE) cells contain mRNA for PDGF-B. RPE cells were incubated for 24 hours in: medium alone (lane 1), thrombin (3 units/ml, lane 2), dibutyryl cAMP (1mM, lane 3), thrombin + dibutyryl cAMP (lane 4), forskolin (50 µM, lane 5) or thrombin + forskolin (lane 6), and then RNA was isolated. Northern blots were done as described in Materials and Methods, loading 20 µg of total RNA in each lane and using a 2.7 kb cDNA coding for human PDGF-B.

Fig. 3. Cultured RPE and RPE freshly isolated from human eyes contain mRNA for PDGF-B and PDGF-A demonstrated by RT-PCR. (a) RT-PCR was done using oligonucleotides coding for unique sequences in the PDGF-B gene and 1 µg of RNA isolated from RPE from a postmortem adult human eye (lane 1), RPE cultured from two different human donors (lanes 2 and 3), or two cultures of human umbilical vein endothelial cells (lanes 4 and 5). A φX174 ladder is located to the left of lane 1. (b) RT-PCR was done using oligonucleotides coding for unique sequences in the PDGF-A gene and 1 µg of RNA isolated from retina (lane 1) or RPE (lane 2) from a postmortem adult human eye, cultured human RPE (lane 3), or cultured human RPE incubated with 3 units/ml of thrombin for 24 hours (lane 4). A 100 base pair ladder is located to the left of lane 1.
increase in the immunoreactivity (lane 4), while conditioning in the presence of PMA or TGF-β does not cause a significant increase. Forskolin, which increases intracellular cyclic AMP, decreases the constitutive production of PDGF immunoreactivity (lane 5) and decreases the thrombin-stimulated PDGF immunoreactivity (lane 8).

The concentration of PDGF-BB in RPE-conditioned medium was determined with a two-site ELISA using a monoclonal antibody that recognizes PDGF-BB and a polyclonal antibody that recognizes all PDGF dimers. Media conditioned for 24 hours on RPE from two different donors contained 29 and 15 pg/ml. Of the mediators tested, only thrombin caused a substantial increase in the concentration of PDGF-BB in conditioned medium (Table 1).

A northern blot of total RNA from RPE cells using cDNA corresponding to a portion of the coding region of the gene for PDGF-B demonstrates a 3.7 kb transcript that is significantly increased when the RPE are incubated with thrombin (Fig. 2, lane 2). This transcript was not increased in cells treated with TGF-β or PMA and the thrombin-induced increase was blocked by forskolin (lane 6). A northern blot using a cDNA for PDGF-A chain failed to detect any transcripts in 20 µg of total RNA harvested from RPE cells, but demonstrated faint 2.9 and 2.3 kb transcripts in lanes loaded with 10 µg of poly(A)^+ RNA prepared from RPE cells; there was no definite modulation by thrombin or forskolin (not shown). A northern blot using 10 µg of poly(A)^+ RNA from RPE cells and 32p-labeled riboprobes synthesized from a cDNA coding for a portion of the murine PDGF-A gene demonstrated the same 2.9 and 2.3 kb transcripts (not shown).

Using oligonucleotides coding for unique sequences in the genes for PDGF-A or PDGF-B, RT-PCR was done using RNA harvested from cultured RPE or RPE freshly isolated from human eye bank eyes. Reaction products of expected size (PDGF-A, 309 bp; PDGF-B 350 bp) were obtained using RNA from cultured RPE or freshly isolated RPE (Fig. 3) and their identity was confirmed by sequencing. No PCR products were obtained in the absence of reverse transcriptase, indicating that RNA, not genomic DNA, served as template. RNA from human adult retina demonstrated a strong signal for PDGF-A

Table 1. Concentration of PDGF-BB in media conditioned by RPE in the presence of various mediators

<table>
<thead>
<tr>
<th>Mediator</th>
<th>RPE-1 (pg/ml)</th>
<th>RPE-2 (pg/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>29</td>
</tr>
<tr>
<td>Thrombin, 3 units/ml</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>PMA, 100 µg/ml</td>
<td>ND</td>
<td>30</td>
</tr>
<tr>
<td>TNF-α, 200 pg/ml</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>TGF-β, 200 pg/ml</td>
<td>17</td>
<td>34</td>
</tr>
</tbody>
</table>

RPE cells from two different donors (RPE-1 and RPE-2) were grown to confluence and then incubated in serum-free medium for 24 hours in the presence or absence (control) of the agents listed above. The concentration of PDGF BB was measured by two site ELISA as described in Materials and Methods. ND, not detectable.
Fig. 6. Immunohistochemical staining for PDGF and PDGF receptor in wounded RPE cultures. (a) Using an antibody that recognizes both PDGF-A and PDGF-B, all cells are positive, but many cells along the edge of the scrape (top) demonstrate intense cytoplasmic staining (alkaline phosphatase; ×200). (b) Using an antibody that recognizes PDGF-β receptor, but not PDGF-α receptor, all cells are positive, but many of the cells along the edge of the scrape (left) show intense cytoplasmic staining (immunoperoxidase - AEC; ×50). (c) Wounded cultures in which normal rabbit serum was substituted for primary antibody are unstained (alkaline phosphatase; ×400). (d and e) Incubation of anti-PDGF serum with PDGF-AA and PDGF-BB for 1 hour results in a marked decrease in staining of RPE cells (d) compared to staining with the same dilution of anti-PDGF serum incubated with medium alone (e).
2465PDGF is an autocrine stimulator of RPE growth (Fig. 3b, lane 1), but a signal for PDGF-B could not be detected.

**Cultured RPE possess PDGF-α receptors and PDGF-β receptors that mediate autocrine growth stimulation**

ImmunobLOTS of cultured RPE cell homogenates using an antibody that recognizes both types of PDGF receptors show a cross-reacting band at 180 kDa, the size of full-length receptors, and a smaller band at 160 kDa, the size of immature receptors (Fig. 4a). Fig. 4a demonstrates autophosphorylation of RPE PDGF receptors in serum-free medium that is increased by pulsing with 0.1 ng/ml or 10 ng/ml of PDGF-AA or PDGF-BB. Immunoprecipitation of cell lysate from NIH 3T3 cells stimulated with 100 ng/ml of PDGF shows a corresponding band at 180 kDa (Fig. 4a, lane 7), while human umbilical vein endothelial cells (for which PDGF is not mitogenic) treated in the same way showed no immunoprecipitated proteins. Immunoprecipitations with antibodies that specifically recognize PDGF-α or PDGF-β receptors demonstrate autophosphorylation of both receptor types on RPE in serum-free medium and in both instances the phosphorylation is eliminated by a 24 hour incubation with suramin, an agent that displaces PDGF from its receptors (Fig. 5b). Both PDGF-AA and PDGF-BB also stimulate [H]thymidine incorporation in RPE cells (Fig. 4a, lane 7). This suggests that both PDGF-α and -β receptors are present on RPE and their basal phosphorylation represents autocrine stimulation due to endogenous production of PDGF. This possibility was also explored by incubating RPE in 50 µg/ml of goat anti-PDGF IgG or nonimmune IgG. The anti-PDGF IgG substantially blocked the increase in RPE cell number that occurred in serum-free medium over 5 days (percentage decrease from control was 56.5±7.0, n=3), while the nonimmune IgG had no effect (5.6±8.5% increase from control, n=3), resulting in a statistically significant difference of P<0.01 by Student’s paired t-test.

**PDGF and PDGF-β receptor expression is enhanced in wounded RPE cultures**

Immunohistochemical staining for PDGF and PDGF receptors was done 24 hours after a 2 mm linear scrape was made through RPE monolayers. There was faint staining of all cells for PDGF, with more intense staining along the edge of the scrape or in subconfluent areas (Fig. 6a). A similar staining pattern was seen for PDGF-β receptor (Fig. 6b), and there was co-localization of increased staining for PDGF-β receptor with proliferating cell nuclear antigen adjacent to the scrape (not shown). Normal serum controls were negative (Fig. 6c) and preabsorption of the antibodies with purified antigen eliminated PDGF staining (Fig. 6d and e) and markedly reduced PDGF receptor staining.

**PDGF expression is enhanced in wounded retina and RPE**

In normal human or mouse retina, there is staining for PDGF around blood vessels; detergent treatment intensifies staining around blood vessels and unmasks specific staining in the nerve fiber layer and inner plexiform layer (Fig. 7a). A similar staining pattern was seen for PDGF-β receptor (Fig. 7b), and there was co-localization of increased staining for PDGF-β receptor with proliferating cell nuclear antigen adjacent to the scrape (not shown). Similar staining patterns were seen for PDGF-A and PDGF-B, and both were increased in the inner plexiform layer and nerve fiber layer (Fig. 7c). Normal serum controls were negative (Fig. 7d) and preabsorption of the antibodies with purified antigen eliminated PDGF staining (Fig. 7e and f) and markedly reduced PDGF receptor staining.

**PDGFs and PDGF-R are expressed in RPE in epiretinal membranes**

RPE cells are a major component of epiretinal membranes, scar tissue that forms on the retina and leads to loss of vision. Epiretinal membranes removed during vitreous surgery were immunohistochemically stained for PDGF and PDGF receptors. Many cells in epiretinal membranes stain for PDGF and/or PDGF receptors and many of the cells can be identified as RPE cells by the presence of pigment (Fig. 8f and g and Fig. 9) and immunohistochemical staining for RPE markers (not shown).
Fig. 8. (a) In situ hybridization with antisense riboprobes for PDGF-A demonstrates PDGF-A mRNA (blue-violet reaction product) in the nerve fiber layer of mouse retina, corresponding to localization of PDGF protein by immunohistochemistry (×400). (b) RPE-associated PDGF-A mRNA is demonstrated adjacent to a laser burn (arrows; ×1000). (c) Laser-treated retina from the same mouse shown in (b), incubated with sense rather than antisense probe, is negative throughout the retina and at sites of laser burns (arrows; ×200). (Digoxigenin-labeled probes without counterstain; blue violet reaction product.) (d) Immunohistochemical staining for PDGF demonstrates blue-violet reaction product in cells adjacent to two laser burns (arrows; ×400). (e) RPE-associated PDGF is visualized along the apical side of the RPE under a retinal detachment (alkaline phosphatase; ×400). (f) Pigmented cells in an epiretinal membrane occurring after retinal reattachment show positivity for PDGF-b receptor (red; ×1000). (g) The same membrane shown in (f) shows pigmented cells with no staining when normal rabbit serum is substituted for primary antibody.
2467PDGF is an autocrine stimulator of RPE growth (shown). Controls were negative (Figs 8g and 9c), demonstrating the specificity of the immunoreaction.

DISCUSSION

Cultured RPE cells express PDGF-A, PDGF-B, PDGF-α receptor and PDGF-β receptor. The co-expression of PDGF and its receptors has been demonstrated in several cell lines, but only in simian sarcoma virus (SSV)-transformed 3T3 cells (Fleming et al., 1989) and Schwann cells (Eccleston et al., 1990) has such co-expression been rigorously demonstrated to mediate autocrine growth stimulation. As is true for SSV-transformed cells, autophosphorylation of PDGF receptors in RPE occurs in serum-free medium and is blocked by suramin, which displaces PDGF from its receptors. Also, an antibody to PDGF partially inhibits the growth of RPE in serum-free medium. These data indicate the presence of an autocrine loop involving PDGF in cultured RPE.

Growth regulation in cultured RPE may occur in part due to modulation of the PDGF autocrine loop. This is suggested by the in vitro model of wound repair in which a scrape is made in an RPE culture in the absence of serum. Cells along the edge of the scrape show increased expression of PDGF and PDGF-β receptor, along with increased labeling with PCNA, indicating that upregulation of PDGF and its receptors occurs in the proliferating fraction of cells and could be involved in repair of the defect. Cells in subconfluent areas of the culture also show increased expression of PDGF and PDGF-β receptor, suggesting that cell contact may play a role in modulation of the autocrine loop.

Immunohistochemistry in normal mouse retina demonstrates PDGF in ganglion cells and in vessel walls, and in situ hybridization shows PDGF-A mRNA in ganglion cells. This is in good agreement with a previous study (Mudhar et al., 1993) that localized PDGF-A to ganglion cells and PDGF-B to vessel walls in developing and adult rat retina. PDGF-A was also demonstrated in the RPE of the rat and we detected PDGF-A and PDGF-B mRNA in RPE from human eye bank eyes by RT-PCR. Our inability to detect PDGF-A mRNA in normal mouse RPE by in situ hybridization could be because our technique with digoxigenin-labeled probes is less sensitive than the technique using 35S-labeled probes (Mudhar et al., 1993). Another possibility is that there is a species difference in the basal expression of PDGF in RPE; we also failed to detect PDGF-A in normal mouse RPE by immunohistochemistry. However, after laser or retinal detachment, two situations in which there is onset of proliferation in the normally quiescent RPE, immunohistochemistry and in situ hybridization with digoxigenin-labeled probes demonstrated PDGF-A in the RPE. These data suggest that growth regulation of RPE in situ may also occur in part due to modulation of the PDGF autocrine loop.

A role for modulated expression of PDGF in growth regulation in RPE is consistent with the purported role of PDGF elsewhere in the body. PDGF may be involved in regulation of cell growth and differentiation during development (Rappolee et al., 1988; Mercola et al., 1988, 1990) and it has been implicated in tissue repair in adult animals. PDGF promotes the formation of granulation tissue (Sprugel et al., 1987), increases the strength of healing wounds (Pierce et al., 1989), and shortens the time required for healing (Pierce et al., 1991). Altered expression of PDGF and PDGF receptors has been implicated in repair of traumatic skin injury (Antonaiides et al., 1991), synovitis (Rubin and Terracio, 1988), and vascular injury (Majesky et al., 1990). The controlled reversible expression of PDGF and PDGF receptors induced by injury may function to transiently stimulate cellular migration and proliferation during many types of wound repair, including retinal wound repair involving the RPE.

There is also ample evidence implicating altered expression of PDGF and/or their receptors in several diseases in which there is exaggerated wound repair resulting in scarring,

Fig. 9. Immunohistochemical staining for PDGFs and their receptors in epiretinal membranes. (a) PDGF-positivity in a pigmented cell of an epiretinal membrane removed from a diabetic patient with a traction-rhegmatogenous retinal detachment and proliferative vitreoretinopathy (center; ×200). (b and c) Staining for PDGF in an epiretinal membrane removed from a patient with retinal detachment and proliferative vitreoretinopathy (b) is markedly decreased by incubation of primary antibody with PDGF-AA and PDGF-BB (c). (d) PDGF-α receptor is demonstrated in cells of an epiretinal membrane removed from a patient with retinal detachment and proliferative vitreoretinopathy (×400).
including atherosclerosis (Wilcox et al., 1988; Ross et al., 1990), rheumatoid arthritis (Reuterdau et al., 1991; Remmers et al., 1991), scleroderma (Gay et al., 1989) and pulmonary fibrosis (Martinet et al., 1987). Proliferative vitreoretinopathy is a disease of exaggerated wound repair in which there is excessive proliferation and migration of the RPE and retinal glia. In epiretinal membranes removed during vitrectomy for proliferative vitreoretinopathy, many cells expressed PDGF or PDGF receptors, and many of the PDGF positive cells were identified as RPE. Thus, upregulation of the PDGF autocrine loop in RPE may play a role in epiretinal membrane formation, which is analogous to scarring elsewhere in the body.

There are many growth factors in addition to PDGF that could play a role in proliferative disorders that result in retinal scarring and loss of vision. RPE cells produce many growth factors and when RPE conditioned medium is run on a PDGF antibody affinity column, most but not all of its chemotactic and mitogenic activities are removed (Campochiaro et al., 1989), suggesting that other factors also contribute to these activities. But there are many situations in which other growth factors exert their proliferative effects indirectly through PDGF (Raines et al., 1989; Gay and Winkles, 1990; Leaf et al., 1986; Ikeda et al., 1991). Even if there are several growth factors that act independently, interruption of the action of one may be sufficient to significantly alter the scarring process. Therefore the identification of PDGF as a contributor to retinal scarring has potential clinical implications, because it provides useful targets for future investigations aimed at identifying specific therapies.

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