Tractional Force Generation by Porcine Müller Cells: Paracrine Stimulation by Retinal Pigment Epithelium

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**PURPOSE.** To examine the ability of retinal pigment epithelial (RPE) cells to modulate Müller cell extracellular matrix contraction through secreted promoters.

**METHODS.** Freshly isolated RPE cells were maintained in continuous culture until the morphologic and immunocytochemical changes associated with myofibroblastic dedifferentiation were complete. Secretory products collected from these cells during extended incubations in serum-free medium and at different stages of dedifferentiation were examined for the ability to promote extracellular matrix contraction by Müller cells. The contributions of specific growth factors to RPE-secreted activity were examined with growth factor-neutralizing antibodies.

**RESULTS.** Secretory products from RPE cells throughout dedifferentiation contained biologically active quantities of Müller cell contraction promoters. Secretory activity increased during extended incubation in serum-free medium and during myofibroblastic dedifferentiation. Growth factor-specific neutralizing antibodies enabled the determination that insulin-like growth factor-1 and platelet-derived growth factor-related proteins were the secreted species to which Müller cells responded. Finally, gene expression of insulin-like growth factor 1 and platelet-derived growth factor A chain by porcine RPE cells was confirmed using reverse transcription-polymerase chain reaction.

**CONCLUSIONS.** RPE cells are a viable source of biologically active quantities of two growth factors that stimulate extracellular matrix contraction by Müller cells. This secretory profile persists for extended periods in an otherwise serum-free environment and is enhanced during myofibroblastic dedifferentiation. (Invest Ophthalmol Vis Sci. 2000;41:529–536)

Proliferative vitreoretinopathy (PVR), a fibrocontractive disorder of the vitreal space, is the leading cause of failure to correct retinal detachment, complicating as many as one third of all repairs.1 The accepted pathogenesis for this disorder begins with the dispersion or recruitment of extravitreal cells into the vitreous cavity and cell proliferation and contraction of vitreal and newly synthesized extracellular matrices into dense tissues.2 The resulting tractional forces can cause retinal folding and detachment, requiring additional corrective surgery. Immunocytochemical studies of contractile scar and observations from tissue culture have implicated a number of extravitreal cells capable of extracellular matrix contraction, including retinal pigment epithelial (RPE) cells, fibroblasts from choroid/sclera, and Müller cells.3–5

Our recent studies of Müller cells revealed that extracellular matrix contraction in vitro is stimulated by two exogenous growth factors, insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF).6 Further, examination of vitreous fluids from patients with PVR indicate that levels of Müller cell contraction-stimulating activity vary with disease severity and that the majority of this activity can be attributed to IGF-1 and PDGF.7 Importantly, this latter study did not determine the origin of the vitreal growth factors detected. IGF-1 and PDGF are present in whole blood serum and account for the majority of the contraction-stimulating activity in this fluid, but evidence of vitreous hemorrhage does not correlate well with levels of contraction-stimulating activity, suggesting a different growth factor origin.8 A more likely source would appear to be cells in the same humoral environment. RPE cells, for example, are reported to produce a number of different growth factors and cytokines, including IGF-1 and PDGF.8 Together, these data constitute strong circumstantial evidence for a paracrine relationship between RPE cells and Müller cells in the progression of PVR.

Despite the data in support of this relationship, a number of important issues remain unresolved. First, there is no direct evidence to indicate that RPE cells secrete these contraction-promoting growth factors in quantities sufficient to drive extracellular matrix contraction by Müller cells. Second, more recent studies indicate that RPE cells harvested from adult tissues are phenotypically unstable in culture, in that the cells progressively dedifferentiate from an epithelial- to myofibroblast-like phenotype.9 The influence of these changes on RPE secretion of Müller cell contraction promoters is not known. Third, the stability of the RPE secretory profile with respect to the humoral environment is uncertain. In those studies exami-
ing growth factor secretion under serum-free conditions, the period examined was 72 hours or less.\(^\text{10–12}\) Does growth factor secretion in biologically active quantities persist during extended periods without the exogenous factors present in serum? Finally, and perhaps most importantly, although RPE cells are known to produce at least two contraction-promoting growth factors, IGF-1 and PDGF, there is no evidence to suggest that these are the only, or even the major promoters of Müller cell contraction synthesized by RPE cells.

The goal of this study was to extend the available evidence for or against this potential paracrine relationship between Müller and RPE cells. Using an autologous culture system of porcine cells, we examined RPE secretion of contraction-promoting factors using Müller cells on three-dimensional collagen matrices as a target. We systematically examined levels of RPE-secreted activity throughout phenotypic dedifferentiation, defined by changes in the expression of key cytoskeletal proteins. We also examined the stability of the RPE secretory phenotype during extended incubations in an otherwise serum-free environment and established the identity of the major contraction-promoting species.

**METHODS**

**Cell Isolation and Culture**

Porcine Müller cells were isolated from papain and DNase dissociated retina, characterized, and maintained in culture as described previously.\(^\text{6}\) Müller cell cultures between passages 3 and 7 were used for these studies. RPE cells were isolated from porcine eyes by trypsin release, purified, and maintained as described previously.\(^\text{9}\) For these studies, primary RPE cultures (passage 1) were permitted to achieve confluence (2–3 weeks), after which the cells were subcultured weekly. RPE cultures from passages 1 through 6 were used for these studies. Methods used for securing animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RPE-Conditioned Medium**

RPE cultures approaching confluence at passages 1 through 5 were rinsed twice with serum-free medium, and the incubation continued with serum-free Dulbecco’s modified Eagle’s medium (DMEM) containing 1 mg/ml crystalline bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) to reduce nonspecific adsorption of RPE-secreted proteins. After 72 hours, the medium was collected, centrifuged to remove cell debris, and frozen at \(-20^\circ\text{C}\) until use. Using this same procedure, conditioned media were collected every 72 hours for five collections, at RPE passages 1 through 5, without apparent adverse effects on cell viability. This method has been shown to yield medium with serum contamination of less than 0.001%.\(^\text{15}\)

**Contraction Assay**

Native collagen gels with attached Müller cells were prepared as described previously.\(^\text{6}\) As before, matrix contraction was monitored as a function of reduced gel thickness at the central score. Contraction-stimulating activities were assayed and analyzed using methods reported previously.\(^\text{14}\) In this case, the frozen, conditioned medium samples were thawed, centrifuged to pellet insoluble matter, and serially diluted (1:2) with DMEM + BSA to yield a range of dilutions beginning at 50%, each in triplicate. Müller cell contractile responses to these solutions were measured after 24 hours of incubation.

**Growth Factor Neutralization**

Growth factor neutralization experiments with anti-IGF-1 and anti-PDGF (and controls for antibody specificity) were performed, and the results were analyzed as reported previously for human serum and vitreous.\(^\text{6,7}\)

**Immunofluorescence Microscopy**

Porcine tissues were prepared and cryosectioned using a protocol based on that described by Barthel and Raymond with minor modifications.\(^\text{15}\) The posterior poles of porcine eyes, enucleated and dissected as above, were fixed with 2% paraformaldehyde in phosphate buffer (0.1 M Na\(_2\)HPO\(_4\), pH 7.0) for 1 hour at room temperature and overnight at 4°C. Blocks (~1 cm\(^3\)) of the full ocular wall were infiltrated overnight with 30% sucrose in phosphate buffer and stored at \(-70^\circ\text{C}\). For cryosectioning, tissue blocks were thawed, infiltrated for 60 minutes, and then frozen in an embedding medium composed of 2 parts 30% sucrose in phosphate buffer and 1 part HistoPrep (Fisher Diagnostics, Fair Lawn, NJ). Sections (10 \(\mu\)m) were mounted on gelatin-subbed slides, dried at 50°C for 60 minutes, and stored at 4°C.

Cells attached to coverslips were fixed with 2% paraformaldehyde in 0.1 M Na\(_2\)HPO\(_4\), pH 7.0, for 1 hour at room temperature. These were washed three times with phosphate-buffered saline (PBS; 0.01 M Na\(_2\)PO\(_4\), 0.15 M NaCl, pH 7.4), after which the coverslips were permeabilized by a 10-minute treatment with PBS containing 0.1% Triton X-100.

Cryosections and coverslips were blocked with 20% non-immune goat serum (The Binding Site, Ltd., Birmingham, UK) in PBS for 60 minutes at room temperature. Primary and secondary antibody treatments were for 60 minutes at room temperature using 2% goat serum in PBS with three 5-minute washes in between. Photomicrographs were taken with a Nikon Optiphot (Garden City, NJ) equipped with epifluorescence illumination and phase-contrast optics using a 35-mm camera and T-Max 400 film (Eastman Kodak, Rochester, NY).

**Electrophoresis and Western Blot Analysis**

Extracts of freshly isolated porcine retina and cell cultures were prepared as described previously.\(^\text{16}\) RPE extracts were prepared using the same reagents and methods, except that the extraction cocktail was added directly to the eyecup and gently scraped with a rubber policeman. Extracts were electrophoretically separated, transferred to nitrocellulose, and probed with antibodies using previously reported methods.\(^\text{16}\) The molecular weights reported in the Results section were from the SWISS-PROT online database with Accession numbers cited in the text.

**Reverse Transcription–Polymerase Chain Reaction**

mRNA was isolated from confluent cultures of RPE cells (passage 6) using TRIZOL (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions and stored at \(-70^\circ\text{C}\) until needed. After treatment with Amplification Grade DNase I (Life Technologies), first-strand cDNA synthesis was performed using 1 \(\mu\)g mRNA and the Reverse Transcription
System (Promega, Madison, WI) according to the manufacturer’s instructions. Reactions in a PTC-150 Minicycler (MJ Research, Watertown, MA) were at 42°C for 15 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. Polymerase chain reaction (PCR) was performed on 0.2 μg of first-strand cDNA using the PCR Reagent System (Life Technologies) for 35 cycles at 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 90 seconds. Specific oligonucleotide primers were designed using cDNA sequences from GenBank (Accession numbers M51175, X03420, X03795, and M20488) and manufactured by Synthegen (Houston, TX). Primers for IGF-1 [5′-GGACCTCGAGACCCCTCTTGTGG-3′ and 5′-GGCCGACTTTGCGAGCTTGA-3′] span the exon 3 and exon 4 junction, predicting a product of 209 base pairs (bp). Primers for PDGF-A chain [5′-AGCATCCAGGGCCTCGG-GAC-3′ and 5′-ACTCCACCTTGGCCACCTTGAC-3′] span the exon 1 through exon 5 regions, predicting a product of 492 bp. Products were separated on a 3% agarose gel and visualized using ethidium bromide.

Reagents

Primary antibodies used in this study included monoclonal mouse anti-α smooth muscle actin (αSMA, clone 1A4; Sigma), mouse anti-cytokeratin 18 (clone CY-90; Sigma), mouse anti-β-3 tubulin (clone 5G8; Promega Corp., Madison, WI), mouse anti-vimentin (clone V9; Dako A/S, Glostrup, Denmark), mouse anti-human IGF-1 (clone sm1.2; Upstate Biotechnology, Lake Placid, NY), and rabbit anti-human PDGF (R&D Systems, Minneapolis, MN). Secondary antibodies included horseradish peroxidase-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

RESULTS

Characterization of Porcine RPE Dedifferentiation

We first examined the reactivity of our panel of antibodies against cryosections of normal porcine retina/choroid. Hematoxylin staining revealed that the relevant tissues were present and well preserved (Fig. 1A). In the case of anti-cytokeratin 18, an obvious layer of immunoreactivity was visible in the RPE cytoplasm below the level of the pigment granules (Fig. 1B). In contrast, the RPE cells were nonreactive to anti-αSMA (Fig. 1C). In this case, other cells visible within the field were reactive with the probe, negating concerns about immunoreactivity. Normal RPE cells also were determined to be negative for vimentin and β-3 tubulin (not shown).

Freshly isolated RPE cells, seeded onto coverslips, also were characterized using the same set of antibodies at varying times after introduction into culture. Phase-contrast microscopic examination after 14 days revealed substantial cell proliferation (Fig. 1D). Still epithelioid with respect to cell–cell contacts, cells at the borders of the foci possessed little pigment and were of lower density than those near the center. The morphologic and pigmentary changes coincided with loss of cytokeratin 18 expression (Fig. 1E, same field as 1D). Interestingly, the immunoreactive zones in these proliferating cultures were considerably larger than the pigmented region, suggesting that proliferating cells continued to express cytokeratin 18 for some period. Finally, at this culture stage, occasional cells near the margins of the proliferating groups contained αSMA-positive stress fibers (Fig. 1F). Of note, vimentin expression was evident in the entire cell population, whereas no cells reacted with the probe against β-3 tubulin (not shown).

Subculture and maintenance of these cells completed the trends observed at 14 days. Passage 3 cells were of mixed morphologies, including large, flat, fibroblastic cells and smaller polygonal cells (Fig. 1G). The population was negative for cytokeratin 18 (Fig. 1H, same field as 1G). αSMA expression was apparent in a large proportion of the cells, but varied in intensity (Fig. 1I). Passage 5 cells were morphologically large and flat (Fig. 1J), negative for cytokeratin 18 (Fig. 1K, same field as 1J), and positive for αSMA (Fig. 1L). Vimentin expression remained high through passage 5, and as before, the cells were negative for β-3 tubulin (not shown).

Western blot analyses performed on tissue and cell culture extracts confirmed the changes in protein expression for the population as a whole. Volumes of cell culture extract loaded were adjusted to produce vimentin [molecular weight (mw) 54 kDa; P08670] staining of the same visual intensity because expression of this protein changed least between passages 1 and 5. The lanes containing freshly isolated (normal) RPE cells received 5 μl of the 2 ml extract per lane. Normal RPE extract was negative for anti-vimentin reactivity, whereas all culture extracts from passages 1, 3, and 5 contained prominent reaction products (Fig. 1M, lanes 0, 1, 3, and 5, respectively). In contrast, anti-cytokeratin 18 (mw 48 kDa; P05783) produced an intense reaction product in the normal RPE extract that was diminished in passage 1 and was absent in passage 3 and 5 cultures (Fig. 1N). Normal RPE cells were negative for αSMA (mw 42 kDa; P03996), but in this case the reaction product increased with passage number (Fig. 1O). β-3 tubulin (mw 51 kDa, Q13509) was undetectable in the RPE extracts at any stage, but was detected in retinal extracts (not shown).

RPE Secreted Contraction-Stimulating Factors

To examine the capacity of RPE secretory products to stimulate extracellular matrix contraction, Müller cells attached to collagen gels were incubated in 50% RPE-conditioned medium, medium containing BSA alone, or 5% FBS as a positive control. Cells exposed to serum progressively reduced gel thickness to approximately 80% within 24 hours (Fig. 2A). Cells in 50% RPE-conditioned medium reduced gel thickness by more than 50%, whereas those in BSA alone reduced the gel by approximately 10%. The dose–response profile obtained after 24 hours indicated that stimulation was dose-dependent (Fig. 2B).

The morphology of Müller cells exposed to RPE secretory products reflected differences in cell activity. FBS-stimulated cells were well spread within 2 hours with blunt processes from which lines of tension radiated (Fig. 3A). Cells incubated in 50% RPE-conditioned medium were morphologically similar to serum-stimulated cells (Fig. 3B), whereas cells incubated in medium containing BSA alone remained rounded and without evidence of active matrix contraction (Fig. 3C).

RPE-Secreted Contraction-Stimulating Activity During Dedifferentiation

The stability of this secretory activity was examined by comparing the contraction-stimulating activity of five serial collections of medium conditioned by passage 3 RPE cells over a period of 15 days. Regression analyses of dose-
response profiles generated after 24-hour incubation suggested lengthy exposure to serum-free medium results in generally increased levels of secreted activity (Table 1A). Similar assay of the third conditioned medium collection from RPE at passages 1 through 5 suggested that media-specific activity decreased dramatically between passages 1 through 4, after which it stabilized at approximately half that of passage 1 (Table 1B). Because the phenotypic changes we observed also include a substantial decrease in overall cell density, these values also were normalized to counts of trypsin-released cells from these cultures, revealing that activity per cell increases rather than decreases with dedifferentiation.

FIGURE 2. Kinetics and dose response of extracellular matrix contraction by Müller cells stimulated with RPE-secreted promoters. Müller cells attached to collagen gels were incubated in varying dilutions of RPE-conditioned medium, 3% FBS or medium with 0.1% BSA. The kinetics of matrix contraction by cells incubated in 5% FBS (closed circles), 50% RPE-CM (open circles), or DMEM + BSA (open squares) are presented in (A). The dose–response profile achieved with RPE-conditioned medium after 24 hours is presented in (B). The data presented represent the means and SDs obtained from triplicate cultures under each condition. Using paired Student’s t-tests to compare control and experimental samples, significant differences were observed at ≥2 hours of incubation (A, P < 0.02) and conditioned medium concentrations ≥1.6% (B, P < 0.01).

FIGURE 3. Morphologies of Müller cells contracting collagen matrices under the conditions described in the legend to Figure 2. Phase-contrast photomicrographs were taken of Müller cells after 2 hours of incubation on collagen matrices in DMEM containing 3% FBS (A), 50% RPE-conditioned medium (B), or medium containing BSA alone (C). Magnification, ×190.

FIGURE 1. Indirect immunofluorescence localization of cytokeratin 18, and αSMA in porcine retina/choroid and RPE cultures. Cryosections of porcine retina/choroid (A through C) and RPE cultures from passage 1 (D through F), passage 3 (G through I) and passage 5 (J through L) were stained with hematoxylin (A), visualized by phase-contrast (D, G, J), and probed with monoclonal anti-cytokeratin 18 (B, E, H, K) or monoclonal anti-αSMA (C, F, I, L). Primary antibodies were detected with a rhodamine-conjugated secondary antibody. Magnification: (A through C) ×300, (D, E, and G through L) ×50, (F) ×200. Detergent-extracted proteins from freshly isolated RPE cells (lane 0). RPE cultures at passage 1, 21 days (lane 1), passage 3 (lane 3), and passage 5 (lane 5) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with monoclonal antibodies against vimentin (M), cytokeratin 18 (N), and αSMA (O). Primary antibodies were detected with a horseradish peroxidase–conjugated secondary antibody and diaminobenzidine. The asterisks indicate the positions of prestained molecular weight standards including (from the top) 85, 50.2, and 34.2 kDa.
PDGF and IGF-1 Contraction-Stimulating Activity Secreted by RPE Cells

The contributions of IGF-1 and PDGF to RPE-secreted activity were determined in assays in which Müller cells attached to collagen gels were incubated in 25% RPE-conditioned medium from passage 3 cells and varying concentrations of neutralizing antibodies specific for PDGF and IGF-1. Data obtained after 24 hours indicated that both antibodies caused marginal reductions in stimulatory activity (Fig. 4A). Addition of polyclonal anti-PDGF reduced the Müller cell contractile response from 56% to 36%, whereas monoclonal anti–IGF-1 resulted in a slightly larger reduction in response from 58% to 20%.

To quantify the contribution of each growth factor to RPE-secreted activity, Müller cells attached to collagen gels as above were incubated in serial dilutions of RPE-conditioned medium to which a constant amount of anti-PDGF (20 μg/ml), anti–IGF-1 (20 μg/ml), both antibodies, or an equivalent volume of PBS were added. The data obtained after 24 hours of incubation indicated that the addition of either and both antibodies substantially decreased the contraction-stimulating activity of the conditioned medium (Fig. 4B). Regression analysis of these profiles indicated that anti-PDGF and anti–IGF-1 neutralized 63% and 54% of the RPE-secreted activity, respectively, and together reduced the activity by 96% (Table 1C).

Finally, to confirm RPE gene expression of IGF-1 and PDGF, we examined the cells for presence of IGF-1 and PDGF message. This was particularly important in the case of PDGF because the anti-PDGF–neutralizing antibody was polyclonal rather than monoclonal. RNA isolated from passage 6 RPE cells served at all antibody concentrations encoding the two growth factors. Electrophoretic separation and staining with ethidium bromide revealed amplification products of the predicted size for IGF-1 of 209 bp (Fig. 5, lane 1) and PDGF-A chain at 492 bp (Fig. 5, lane 2).

**DISCUSSION**

The goal of this study was to examine the potential for a paracrine relationship between RPE and Müller cells involving secreted contraction-promoting growth factors. Analysis of the data obtained from the tissue culture studies yielded four novel conclusions in support of this relationship. First, RPE cells in culture synthesize and secrete sufficient quantities of biologically active contraction-promoting growth factors to induce extracellular matrix contraction by Müller cells. Second, the secretory phenotype appears to be stable with respect to exogenous stimulation, in that removing the cells from serum for as long as 2 weeks resulted...
in an overall increase rather than decrease in secretory activity. Third, RPE secretory activity is not diminished with respect to cell dedifferentiation. The levels of activity secreted per cell increase as the cell population shifts toward the myofibroblast phenotype. Fourth, the majority of the RPE-secreted activity can be ascribed to IGF-I and PDGF, negating the possibility of substantial contribution by other, yet undescribed factors. In short, to the extent that this relationship can be examined with a tissue culture system, a paracrine relationship between RPE and Müller cells in the progression of fibrocontractive disease is within the RPE repertoire.

Interestingly, this relationship was tested directly in studies described more than a decade ago by Peters and colleagues. It was reported that intravitreal injections of rabbit Müller cells alone resulted in the formation of only limited connective tissue densities. Furthermore, that which did develop formed soon after injection but did not progress. However, when Müller cells were co-cultured with modest numbers of RPE cells, the initial fibrocontractive response was substantially greater and the pathology was progressive. These results confirmed both the need for an exogenous promoter to drive Müller cell participation and that this can be provided by RPE cells in the same humoral environment. It is also important to point out that other ocular cell types synthesize these growth factors. IGF-I, for example, is produced by retinal microvascular endothelial cells and pericytes.

Several other observations made during this study also warrant discussion. Human RPE cells in culture are reported to express β-3 tubulin, but this protein was undetected in porcine cultures. Also, we have not observed in porcine RPE cultures, the small fusiform RPE phenotype reported by McKay and Burke. The same isolation and culture techniques used in this study yielded heterogeneous populations of human RPE cells that were, in part β-3 tubulin positive, and of the fusiform phenotype (unpublished observations). On the surface, this suggests that there may be important species-related differences between porcine and human RPE cells. However, the growth characteristics and morphologic and cytoskeletal changes we observed do resemble those reported for fetal human RPE cells. These differences may arise from the ages of the tissue used, because the porcine RPE cells were isolated from prepubescent animals, whereas human RPE cells are most often isolated from older humans.

Our conclusions regarding RPE biosynthesis IGF-I and PDGF are generally consistent with studies of human RPE cells. An interesting caveat emerged from the study by Campochiaro and colleagues, suggesting that RPE cells respond to endogenously produced PDGF in an autocrine fashion. Whether or not endogenously produced PDGF or IGF-I serves to sustain or modulate the biosynthesis of the other is unknown. Another recent study with implications in this area examined intraocular fibroproliferative responses in pig, after retinotomy and intraocular injections of different promoters. The severity of the fibroproliferative response observed in response to PDGF was significantly enhanced when the injection was supplemented with plasma, leading the authors to conclude that a plasma-derived factor acted in synergy with PDGF. Inasmuch as plasma contains biologically active quantities of IGF-I, this could have accounted for the more robust response. In sum, there is ample evidence to implicate PDGF in the pathogenesis of PVR but considerably less in support of a causative role for IGF-I. These issues will only be resolved by additional experimentation.

References


