Purification of Mammalian Cone Photoreceptors by Lectin Panning and the Enhancement of Their Survival in Glia-Conditioned Medium

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Purpose. In retinal diseases characterized by photoreceptor degeneration, the main cause of clinically significant vision loss is cone, rather than rod, loss. In the present study, a technique was designed to purify cones to make it possible to screen for neuroprotective molecules.

Methods. A suspension of porcine retinal cells was incubated on coverslips coated with the peanut agglutinin (PNA) lectin, which selectively binds to cones. Cones were identified and quantified by using an antibody specific for cone arrestin. Their identity and viability were also assessed by single-cell RT-PCR and patch-clamp recording.

Results. This panning method provided a population of cones that was 80% to 92% pure, depending on the counting strategy used. The panned cells contained both short (S)- and medium/long (M/L)-wavelength opsin cones. The panned retinal cells exhibited the physiological signature of cone photoreceptors and single-cell reverse transcriptase-polymerase chain reaction (RT-PCR) showed that they expressed the cone arrestin mRNA. Most (69%) cone photoreceptors produced neurites and survived for up to 7 days when cultured in a glia-conditioned medium, whereas very few (4%) survived after 7 days in the control medium.

Conclusions. This PNA-lectin–panning method can provide highly pure and viable mammalian cones, the survival of which can be prolonged by glia-conditioned medium. Because PNA lectin binds to cone photoreceptors from various species in both normal and pathologic conditions, this technique should enable the screening of neuroprotective molecules like those released by glial cells and enable the physiological, genomic, and proteomic characterization of cones. (*Invest Ophthalmol Vis Sci.* 2005;46:367–374) DOI:10.1167/iovs.04-0695

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Photoreceptor degeneration results in vision loss in diseases like retinitis pigmentosa and age-related macular degeneration. In these diseases, the main cause of clinically significant vision loss is cone degeneration, rather than rod cell death. Although most of the mutations responsible for retinitis pigmentosa in humans and animal models affect rod-photoreceptor–specific genes, rod apoptosis is followed by secondary cone degeneration.[1,2] People with night blindness can have a normal life, especially in industrialized countries, and can still see satisfactorily, despite the loss of rods.[3] The prevention of cone cell loss is thus a main goal of therapeutic strategies.

Several neurotrophic factors, including fibroblast growth factor 2 (FGF2), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial cell line-derived neurotrophic factor (GDNF), promote photoreceptor cell survival.[4–9] However, secondary cone degeneration has been attributed to the loss of a more specific rod-dependent trophic factor necessary for cone survival.[10] Cultured retinal cells from chick embryos, which are very rich in cones,[11] were used to determine the size of this factor.[12] These chick embryo cell cultures subsequently led to the identification of the first rod-derived cone survival factor (RdCVF1).[13] The absence of glial cells in cultured chick embryo retinal cells suggested that cone survival is not dependent on glial cells.[11]

The identification of other cone survival factors in the human retina has been hampered by the lack of a pure mammalian cone cell culture. Mammalian, including human, rods and cones can survive for weeks in mixed retinal cell cultures.[14,15] However, when photoreceptors are isolated by sectioning the outer retina with a vibratome, rods and cones survive only on a glial feeder cell layer.[16] This approach cannot be used to separate rods from cones but shows that photoreceptor survival is strictly dependent on glial cells. A progressive mechanical dissociation method that generates relatively pure photoreceptor cell cultures (containing 35%–44% cones) from enzyme-treated retinal tissues was used to confirm that such trophic factors as epidermal growth factor (EGF) and FGF2 can delay photoreceptor degeneration.[17]

To characterize mammalian cone photoreceptors and to screen for factors supporting their survival, we designed a lectin-panning procedure that allows the selective isolation and culture of viable adult pig cone photoreceptors. We used the peanut agglutinin (PNA) lectin, which specifically interacts with the cone photoreceptor extracellular matrix in different species,[18–21] to select cones. This PNA-lectin–panning procedure was based on the immunopanning technique first elaborated to purify cells from the immune system and Schwann cells[22] and subsequently extended to the isolation of ganglion cells from the retinas of young rats.[23] The pig retina was used as the source of photoreceptors in this study because it shares many similarities with the human retina.[24] Using lectin-panned cells, we provide evidence that molecules released by glia can directly promote the survival of cone cells.

Materials and Methods

Cell Cultures

Retinal cell suspensions were prepared as described previously.[15,25] Briefly, adult pig eyes were obtained from the local abattoir. After rapid immersion in ethanol, the cornea, lens, and vitreous humor were removed. The retina was detached from the eyecup and chopped into small fragments in cold CO_2-independent medium (Invitrogen, Carlsbad, CA). Retinal fragments were incubated for 20 minutes at 37°C in papain solution (1 U/μL; Worthington Bioscience, Freehold, NJ) previously activated by L-cysteine (0.2 mM; Sigma-Aldrich, St. Louis, MO). The enzymatic reaction was stopped by adding 1 mL of a serum-free medium (Neurobasal Medium [NBA]; Invitrogen) supplemented with 2% fetal calf serum (FCS; Invitrogen), and tissue aggregates were eliminated by adding DNase I (30 μL; Sigma-Aldrich). The tissue was then gently shaken. After the retinal fragments were removed, a first cell suspension was isolated. The tissue was then gently ground with a fire-polished Pasteur pipette, and different cell suspensions were collected until the retinal tissue was completely dissociated. The cell suspensions were then centrifuged at 800 rpm for 5 minutes, and the cell pellet was resuspended in the serum free-medium (NBA; Invitrogen) supplemented with B27 (1:50; Invitrogen) and glutamine (2 mM; Invitrogen) (NBA^+).

For cell panning, glass coverslips were placed into Petri dishes (60 mm diameter; Corning Glass Co., Corning, NY) and incubated for 2 hours at 37°C with a goat anti-rabbit IgG directed against PNA lectin (1:100; Sigma-Aldrich) diluted in 2 mL Tris-HCl buffer [50 mM; pH 9.5]. After three washes with warm phosphate-buffered saline (PBS), coverslips were incubated in the Tris-HCl buffer containing PNA lectin (1:40; Sigma-Aldrich). After 2 hours at 37°C, coverslips were again washed with PBS and transferred into 2 mL Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen-Gibco, Grand Island, NY) supplemented with bovine serum albumin (BSA, 0.2%; Fraction V; Sigma-Aldrich). The retinal suspension obtained was subsequently placed on the lectin-panned coverslips at a density of 4×10^5 cells/cm^2 in 24-well culture plates and incubated for 15 minutes, with the plates gently swirled every 5 minutes. Wells were then washed five times with serum-free medium (NBA; Invitrogen) to remove nonadherent cells. Purified cells were finally incubated with either serum-free medium supplemented with glutamine (NBA^+) or conditioned medium obtained from pure retinal Müller glial cell cultures. Purified cones were maintained in culture for 1 week, and the medium was replaced every 2 days with fresh serum free/B27/glutamine or retinal Müller glial (RMG)–conditioned medium.

Müller Glial Cell–Conditioned Medium

Conditioned medium was obtained from purified pig retinal Müller glial (RMG) cells cultured in serum free/B27/glutamine (NBA^+) medium. Müller cells were isolated as described by Guidry[26] from cell suspensions prepared as just described. The total retinal cell suspension was placed on a 10-mL continuous density gradient (0%–50%; Percoll; Pharmacia, Uppsala, Sweden) in normal saline and centrifuged for 5 minutes at 1700 rpm. The middle band, containing the partially purified Müller glial cells, was isolated and diluted in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS. To remove the density gradient, the cell suspension was centrifuged at 800 rpm for 5 minutes. The pellet was resuspended in 1 mL DMEM-10% FCS and centrifuged at 1700 rpm on a second 0% to 50% gradient. The middle band was collected, and the gradient washed out by mild centrifugation. The purified Müller glial cells were finally seeded at a density of 4×10^4 cells/cm^2 and cultured in DMEM-10% FCS in six-well culture plates that had been coated with poly-D-lysine (1:100; Sigma-Aldrich) and laminin (1:200; Sigma-Aldrich). After 24 hours, cells were washed twice with DMEM-10% FCS to remove the remaining gradient and allowed to grow. When the culture reached half confluence, the serum-free/glutamine (NBA^+) was replaced by DMEM-10% FCS. The RMG-conditioned medium was collected every 2 days and immediately added to the lectin-panned cells.

Histology

Cells were stained as described previously.[15,16,25] Isolated pig retinas were fixed in 4% paraformaldehyde (PAF) prepared in 0.1 M PBS (pH 7) for 1 hour and cryoprotected by immersion in sucrose gradients. Retinal sections (8 μm) were prepared using a cryostat and permeabilized with 0.1% Triton X-100 for 5 minutes. Cultured cells were similarly fixed in 4% PAF for 15 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. To prevent nonspecific labeling, cultures, and retinal sections were incubated for 1 hour in a blocking buffer containing 10% goat serum and 2% BSA in 0.01 M PBS. Cells were stained with PNA coupled to Alexa 488 (1:40; Molecular Probes, Eugene, OR), a mouse monoclonal antibody directed against PKCα[27] (1:100, Sigma-Aldrich), a rabbit polyclonal antibody directed against the human cone arrestin[28] (hCAR, 1:20,000 to 1:100,000, QKAVEAEGDEGS), kindly provided by Xuemei Zhu, Bruce Brown, and Cheryl Craft (University of Southern California, Los Angeles, CA) a rabbit polyclonal antibody directed against human S-cone opsin[29] (JH455; 1:10,000–1:20,000), and a rabbit polyclonal antibody directed against human medium/long (M/L)-wavelength cone opsin[30] (JH492; 1:2000–1:5000). JH455 and JH492 were both obtained from Jeremy Nathans (Johns Hopkins University, Baltimore, MD). A BLAST search showed 75% amino acid sequence identity and 91% sequence similarity between the peptide sequence used to generate the hCAR antibody and the pig cone arrestin.

All antibodies were diluted in the blocking buffer. For immunolabeling, cells or retinal sections were incubated with the primary antibodies for 3 hours at room temperature, rinsed several times with PBS, and incubated at 37°C for 1 hour with anti-rabbit or anti-mouse IgGs coupled to Alexa-594 (red emission) or Alexa-488 (green emission; 1:400; Molecular Probes, Inc.). The Alexa 488-coupled PNA and the nuclear marker 4′,6′-diamino-2-phenylindole (DAPI, 1:200; Sigma-Aldrich) were used as secondary antibodies.

Cell Counting and Statistical Analysis

Purity experiments were performed in triplicate, and cell counting was performed on three coverslips for each experiment. Cells were counted under the 40× objective in 30 adjacent fields along the diagonal axes of each coverslip. Results are expressed as the ratio of hCAR-labeled cells to DAPI-stained nuclei and/or cells observed under transmitted light. Fluorescent labeling was observed by microscope (Optiphot 2; Nikon, Tokyo, Japan) under epifluorescence illumination. All images were acquired with a charge-coupled device (CCD) color camera and analyzed on computer (Cool-Snap FX, with Metaview software; Roper Scientific, Inc., Duluth, GA).

The effect of culture medium on cone survival was assessed by analysis of variance (ANOVA) followed, when appropriate, by 2×2 comparisons based on the Newman-Keuls test. The effects of culture medium were determined in triplicate and analyzed on the mean data from four coverslips after counting hCAR-labeled cells in 30 fields under a 20× objective.

Patch-Clamp Recordings

Cones were recorded with the patch-clamp technique in the whole-cell mode in both in situ conditions and after the lectin panning procedure. Recording pipettes were pulled from thin-wall borosilicate glass (TW150F; World Precision Instruments, Sarasota, FL) using a Brown and Flaming–type puller (P-87; Sutter Instruments, San Raphael, CA). Cells were voltage clamped with an RK400 amplifier (Bio-Logic

Science Instruments, Claix, France). Data were acquired and analyzed using the Patchit and Tack software packages (White Perch Software, Somerville, MA), respectively.[31]

For in vitro recordings, the standard perfusing solution contained (in mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 HEPES (pH adjusted to 7.74 with NaOH). This solution was delivered by a general gravity-driven perfusion system (~2 mL/min) at room temperature.

For in situ recordings, the perfusing solution was Ames medium (Sigma-Aldrich). It was continuously bubbled with carbogen (95% O_2 and 5% CO_2) to equilibrate pH. All chemicals were obtained from Sigma-Aldrich.

Retinal Slice Preparation

Small square pieces of fresh pig retina were dissected and flatmounted, photoreceptor side up, on filter paper squares. Retinal sections (100–150 μm thick) were prepared with a razor blade in cold Ames medium. Cells were filled with the sulforhodamine-101 fluorescent dye (Sigma-Aldrich) during the recording and observed under epifluorescence illumination (red emission). Cones were then counterstained with PNA coupled to Alexa 488 (green emission; Molecular Probes).

Cloning of *Sus scrofa* Cone Arrestin

Total pig retina RNA was prepared with extraction reagent (TRIzol; Invitrogen) according to the manufacturer's recommendations. A 922-bp pig cone arrestin fragment was first cloned by PCR amplification using oligonucleotides designed after comparison of various mammalian arrestin sequences. The complete pig arrestin coding sequence (1416 nucleotides) was obtained with the 5′ and 3′ rapid amplification of cDNA ends (RACE) strategy (Invitrogen). The cDNA sequence for *Sus scrofa* arrestin-C (*ARR3* gene) was submitted to the EMBL Nucleotide Sequence Database and registered under accession number AJ564496 (http://www.embl-heidelberg.de/; provided in the public domain by the European Molecular Biology Laboratory, Heidelberg, Germany).

Single-Cell RT-PCR for the Pig Cone Arrestin

Single lectin-panned cells were randomly selected and aspirated into a patch-clamp recording pipette filled with 8 μL of buffer containing (in mM): 140 KCl, 1 $MgCl_2$, 0.5 EGTA, 5 adenosine triphosphate (ATP), and 4 HEPES (pH 7.4). The glass pipette tip was then broken off into a thin-walled PCR tube maintained in ice and containing 40 μL of the reaction mix from a commercial RT-PCR system (SuperScriptIII One-Step; Invitrogen) and the first set of primers (5′-GGGAAACGGGACT-TCGTG-3′ and 5′-GCACAGAAACTCTTCACTTC-3′). Tubes were placed at −80°C until all the samples for one experiment had been collected. Tubes were allowed to thaw slowly on ice and 2 μL of *Taq* polymerase (SuperScriptIII RT/Platinum *Taq* mix; Invitrogen) was added to each sample. The cDNA was synthesized by incubating at 50°C for 30 minutes and denatured at 94°C for 2 minutes. The first PCR consisted of 37 cycles (94°C for 30 seconds, 52°C for 45 seconds, 68°C for 45 seconds) and a final extension at 68°C for 5 minutes. The resultant product was diluted 1:100 and reamplified by *Taq* PCR (Invitrogen) using nested primers (5′-CGTGGACCATGTGGACATG-3′ 5′-AGGTTGA-CAACCATCTGCAG-3′). The second amplification consisted of an initial denaturation step at 94°C for 2 minutes followed by 35 cycles (94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 45 seconds) and a final extension at 72°C for 5 minutes. One fifth of the PCR product was run on an agarose gel and stained with ethidium bromide. All primers were designed from the pig arrestin-C coding sequence (AJ564496) and were carefully chosen to avoid the amplification of genomic DNA and arrestin paralogues. First, the exon–intron junctions in *Sus scrofa* cone arrestin were predicted by alignment with the sequence of cone arrestins from different mammals. Primers for the first PCR were therefore selected in different exons, and the nested PCR reverse primer was designed to overlap predicted exons 5 and 6. The primers contained no less than seven mismatches with sequences located in the homologous regions of *Sus scrofa* S-antigen mRNA.

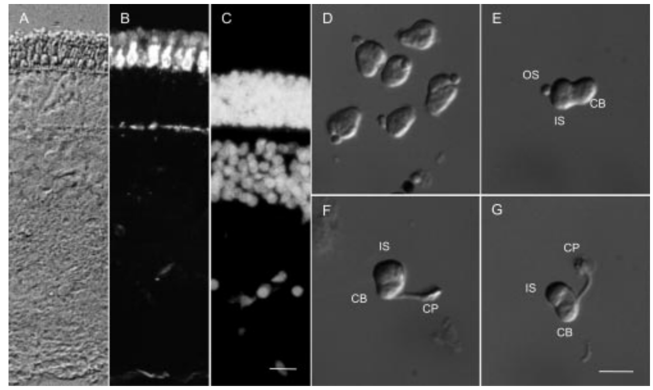

FIGURE 1. PNA-lectin–panned cone photoreceptors from the pig retina. (**A–C**) Localization of PNA lectin binding in a pig retinal section. The section was observed under transmitted light with Nomarski optics (**A**) and by epifluorescence microscopy for PNA lectin labeling (**B**) or DAPI nuclear staining (**C**). (**D–G**) Lectin-panned retinal cells showing the morphologic features of cone photoreceptors, with an outer segment (OS), an inner segment (IS), a cell body (CB) and a cone pedicle (CP). Scale bars, 10 μm.

RESULTS

Lectin Panning of Photoreceptors: Immunohistochemistry

As in other mammalian species,[18–21] PNA lectin binds selectively to the cone photoreceptor extracellular matrix in the pig retina (Figs. 1A–C). We therefore assessed whether PNA could promote the selective adherence of cones to a culture plate, thus making it possible to purify cones. Immediately after the lectin-panning procedure, most cells had the typical morphology of cone photoreceptors, with a short outer segment, an inner segment that was of a size similar to that of the cell body, and a long axon ending with a large pedicle (Figs. 1D–G). Most of these cells were labeled (Fig. 2D) by the human anti-arrestin antibody (hCAR), which labeled the entire cone cell cytoplasm in the pig retina (Figs. 2A–C). Thus, cones can be purified by PNA lectin panning.

Because PNA binds differentially to different populations of cone photoreceptors,[21] we evaluated whether the lectin-panning procedure purifies all cone types. PNA intensely stained the outer and inner segments of cone photoreceptors in the pig retina. It stained their cell bodies located in the outer row of the outer nuclear layer (ONL) more lightly and heavily stained their axons and pedicles (Figs. 1B; 2A, 2E, 2I). The hCAR immunolabeling completely colocalized with the PNA lectin staining, suggesting that both cone photoreceptor cell populations were labeled by PNA in the pig retina (Figs. 2A–C). When sections were immunolabeled with the blue (short wavelength, S) or the red/green (middle- to long-wavelength, M/L) cone opsin antibodies, both types of cone photoreceptor were stained by PNA (Figs. 2E–G, 2I–K). Both S- and M/L-opsin immunopositive cones were present in freshly lectin-panned cells (Figs. 2H, 2L). Hence, PNA lectin panning can select both S- and M/L-cone photoreceptors.

After 24 hours in culture, lectin-panned cells had lost their outer and inner segments and acquired medium-sized, oval-shaped cell bodies with short neurites (Figs. 3A–C). Most cells were immunopositive for cone arrestin 24 to 48 hours after dissociation (Fig. 3B). When observed under transmitted light,

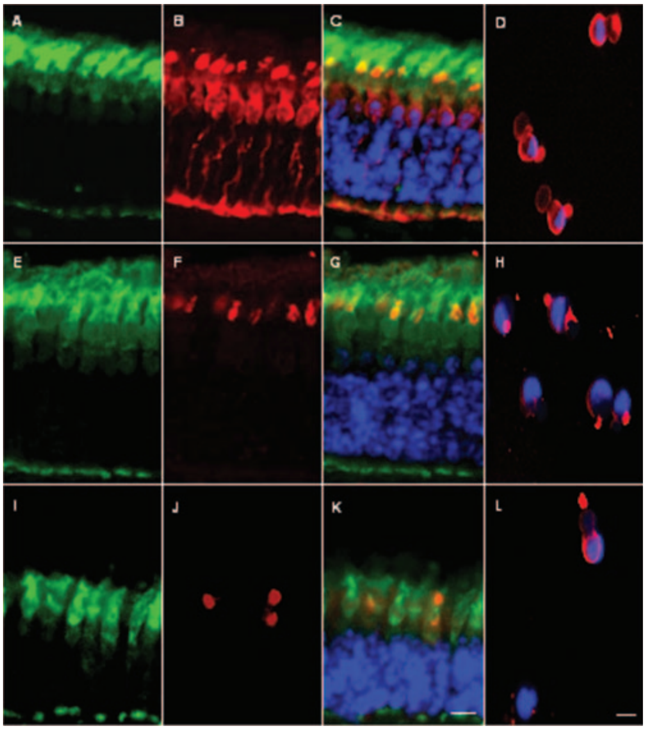

FIGURE 2. Identification of lectin-panned retinal cells using cone photoreceptor markers. **(A–D)** Fluorescence images of the pig ONL showing the PNA lectin labeling **(A)** with respect to the cone arrestin immunostaining **(B, C)**. PNA-lectin–panned retinal cells were immunopositive for cone arrestin **(D)**. **(E–H)** Fluorescence images of the pig ONL showing PNA-lectin labeling **(E)** with respect to M/L-opsin immunostaining **(F, G)**. Several PNA-lectin–panned retinal cells were immunopositive for M/L opsin **(H)**. **(I–L)** Fluorescence images of the pig ONL, showing PNA-lectin labeling **(I)** with respect to S-opsin immunostaining **(K, L)**. Some PNA-lectin–panned retinal cells were immunopositive for S-opsin **(L)**. In merged images **(C, D, G, H, K, L)**, photoreceptor nuclei are *blue* (DAPI staining). Scale bars, 10 μm.

92.57% ± 2.12% (SEM, $n = 9$, Fig. 3D) of cells were positive (Fig. 3A). When cultured cells were labeled with DAPI, 80.2% ± 2.32% (SEM, $n = 9$, Fig. 3D) of cells with DAPI-stained nuclei were positive for cone arrestin (Fig. 3C). The difference between the two counts may be because degenerated cells were not taken into account by the first quantification method. To identify contaminating cells, cultured cells were immunolabeled with the PKCα antibody, which selectively labels rod bipolar cells.[27] We found that rod bipolar cells accounted for 5.58% ± 0.05% (SEM, $n = 3$) of DAPI-stained nuclei. Glial cells, identified by their large DAPI-stained cell bodies, were rarely observed. In conclusion, lectin panning gave rise to a highly pure population of cone photoreceptors (Fig. 3D).

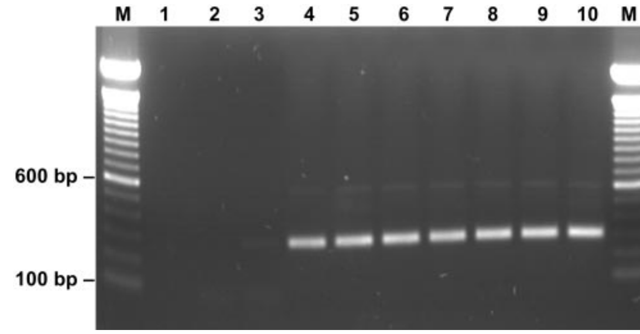

FIGURE 4. Single-cell RT-PCR analysis of PNA-lectin–panned retinal cells. *Lane M*: 100-bp DNA standard marker ladder. *Lanes 1* to *3*: negative controls—samples without reverse transcriptase, RT-PCR on intracellular pipette buffer and extracellular buffer, respectively. *Lanes 4* to *10*: a 291-bp amplified DNA product corresponding to cone arrestin mRNA from seven independent isolated cells collected 3 days after the panning procedure. DNA products were separated on a 1.2% agarose gel and visualized after ethidium bromide staining.

Lectin Panning of Photoreceptors: Single-Cell RT-PCR

Individual cells were collected by using a patch-clamp recording pipette at different times after panning (0, 3, and 5 days) and single-cell RT-PCR was used to examine their expression of a cone-specific gene (Fig. 4). Each experiment included several controls. For detection of possible genomic contamination, no reverse transcriptase was applied to one single-cell sample (Fig. 4, lane 1). We ensured that there was no contamination by extracellular mRNA by performing single-cell RT-PCR on intracellular pipette buffer and perfusing solution (Fig. 4, lanes 2 and 3, respectively). No signal was present in these three negative controls. Conversely, the expected 291-bp product, corresponding to the cone arrestin fragment, was observed in most of the single-cell RT-PCR samples. On day 0, all cells (12/12) were positive for cone arrestin mRNA, compared with 92.86% on day 3 (13/14) and 83.3% on day 5 (10/12).

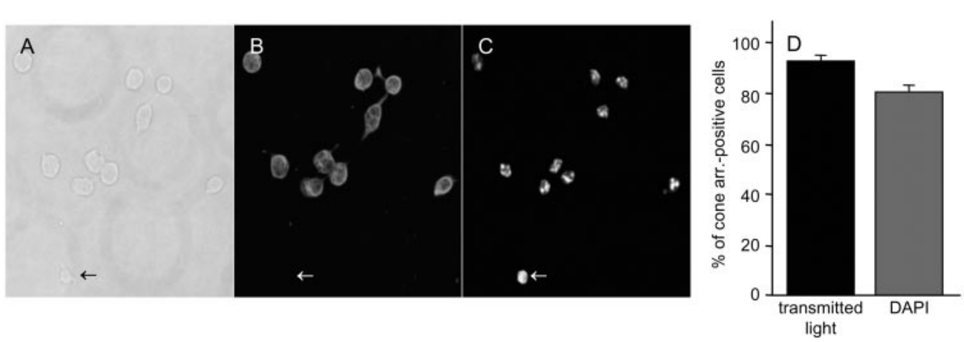

FIGURE 3. Quantification of cone photoreceptors in PNA-lectin–panned retinal cells. After 2 days in culture, isolated cells were counted under transmitted light **(A)** and under epifluorescence illumination after labeling with the anti-hCAR antibody **(B)** and after staining with the nuclear marker DAPI **(C)**. A cone-arrestin–immunonegative cell is present in the observed field **(A–C,** *arrows***)**. **(D)** Percentage of cone-arrestin–positive cells among all cells observed under transmitted light or with respect to the total number of nuclei labeled by DAPI. Scale bar, 10 μm.

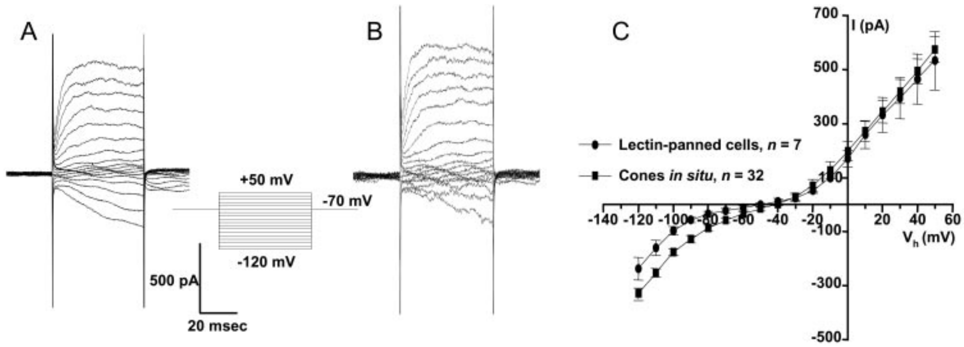

Figure 5. Identification of PNA-lectin–panned cone photoreceptors according to their electrophysiological signature. (**A**, **B**) Responses to voltage steps in a cone photoreceptor in situ (**A**) and in a lectin-panned cell (**B**). Cells were voltage-clamped at −70 mV and submitted to voltage steps in 10-mV increments ranging from −120 to +50 mV. (**C**) Averaged *I-V* curves of cone photoreceptors in situ and PNA-lectin–panned cells. Note the similarities between voltage step responses in the cells from the two preparations.

Intrinsic Electrophysiological Homogeneity of Cultured Cells

As each retinal cell type has its specific electrophysiological signature, we used the patch-clamp technique in the whole-cell configuration to compare the current–voltage responses of lectin-panned cone photoreceptors with those of cones in situ (Fig. 5A). Cone photoreceptors showed a slowly activating inward current at hyperpolarizing potentials (V_h = -70 mV, I_{max} = −58 ± 9 pA; V_h = −120 mV, I_{max} = −329 ± 19 pA, n = 32), peaking within 50 ms. With a standard intracellular solution, the total current reversed near −40 mV. Small sustained outward currents were recorded at depolarizing potentials (V_h = +50 mV, I_{max} = −575 ± 47 pA, n = 32) and were linear above −20 mV. When retinal cells isolated by lectin panning were recorded after the purification procedure, they expressed voltage-gated conductances (Fig. 5B) similar to those of cone photoreceptors in situ except for a slight decrease in the whole-cell conductance recorded at very hyperpolarized potentials (Fig. 5C). Recordings were obtained from three different cultures. These results confirmed that lectin-panned retinal cells are viable and have the same physiological features as cone photoreceptors.

Role of Glia in Cone Photoreceptor Survival

We have reported that Müller glial cells are essential for the survival of adult pig photoreceptors isolated by vibratome sectioning of the retina.[16] To assess whether glial neurotrophic factors are necessary for the survival of cultured cones, we cultured PNA-lectin–panned cones in RMG-conditioned medium. After 24 hours in culture, no significant difference was observed in the number of cone photoreceptors between conditioned and control media (data not shown). In contrast, a major difference was observed between the two media after 4 days in culture (Figs. 6A, C). The number of cone-arrestin–positive cells decreased by 69.41% ± 8.97% in control conditions (SEM, n = 3), whereas it remained stable in the RMG-conditioned medium (2.59% ± 9.44% decrease SEM, n = 3; Fig. 6E). After 1 week in vitro, the survival effect conferred by the RMG-conditioned medium was even more pronounced (Figs. 6B, 6D). The number of cone photoreceptors decreased by 96.48% ± 0.65% (SEM, n = 3) in control conditions, whereas it decreased by only 31.10% ± 8.13% in RMG-conditioned medium (SEM, n = 3; Fig. 6E). Hence, cultured glial cells release molecules that promote the survival of cultured cones.

We observed no significant morphologic differences between PNA-lectin–panned cells that had been incubated for 24 hours in RMG-conditioned medium or in control medium. In both cases, cells had a rounded appearance with medium-sized cell bodies, sometimes showing short neurites (data not shown). However, after 4 days in culture, the number of cells developing neurites was higher in RMG-conditioned medium than in serum free/B27/glutamine (NBA^+) medium (Figs. 6A,

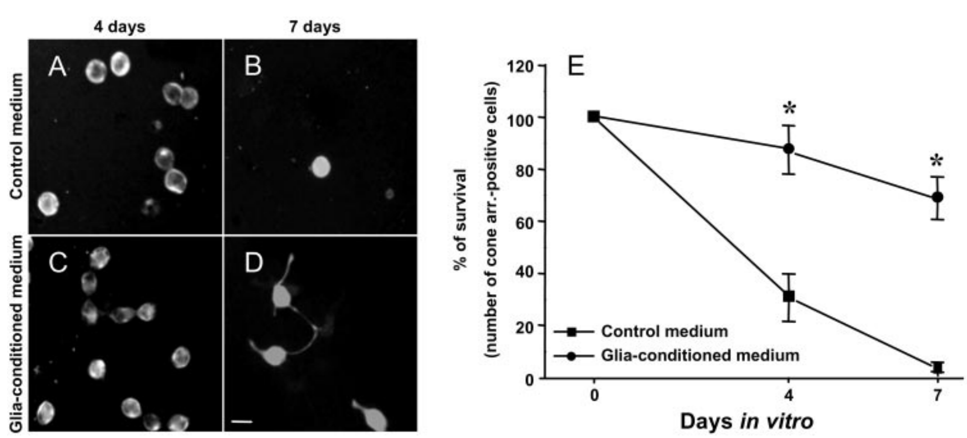

Figure 6. Effect of glial cells on cone survival. (**A–D**) Cone-arrestin–positive cells cultured in the absence (control medium, **A**, **B**) and presence of RMG-conditioned medium (**C**, **D**) for 4 (**A**, **C**) or 7 (**B**, **D**) days. Note the presence of long neurites in PNA-lectin–panned cone photoreceptors cultured for 7 days in the presence of RMG-conditioned medium. (**E**) Quantification of cone survival after incubation in control medium and the RMG-conditioned medium in vitro. Cone survival was assessed by counting cone-arrestin–immunopositive cells at different times. At 4 and 7 days, the number of cone photoreceptors was significantly higher in the RMG-conditioned medium than in the control condition (*$P < 0.001$). Scale bar, 10 μm.

6C). These processes were still observed in cone photoreceptors cultured for 1 week in glia-conditioned medium (Fig. 6D). Thus, RMG cells may release diffusible factors that promote both adult cone photoreceptor survival and outgrowth in vitro.

Discussion

We designed a procedure to purify cone photoreceptors based on the knowledge that PNA binds selectively to their extracellular matrix. The incubation of a pig retinal cell suspension on PNA-coated coverslips promoted the selective adherence of both S and M/L cone photoreceptors. The selected cone population was approximately 90% pure according to cone-specific staining experiments and single-cell RT-PCR. When lectin-purified cells were incubated with Müller glial cell–conditioned medium, cone photoreceptor survival increased from 4% to 69% after 1 week in culture. This study not only provides a new model for the screening of neuroprotective molecules promoting cone photoreceptor survival, but it also opens up new possibilities for the characterization of cone photoreceptors in normal and pathologic conditions.

Cone Photoreceptor Cell Sorting

Cone photoreceptor cell cultures were first obtained as glia-free monolayers from the chick embryo retina and used to investigate retinal cell differentiation.[11,32] Other methods for the purification of photoreceptors have also been developed, including isolation of the outer retina by vibratome sectioning[33] or laser dissection.[34] The photoreceptors isolated by these methods survive for only a few days in culture when prepared from young rat retinas[35] or from embryonic human retinas.[34] However, adult isolated photoreceptors require a glial feeder layer to survive.[16] Recently, a progressive mechanical dissociation method of isolating photoreceptors from enzyme-treated retinas was developed. This method also provided cultures enriched in photoreceptors (95%) containing 35% to 45% cone photoreceptors.[17] The technique described in our study generated a 92% pure population of adult differentiated cone photoreceptors. Cone cell identity was verified by their electrophysiological signature and by molecular markers, such as cone arrestin and cone opsins, detected either at the mRNA or protein level by single-cell RT-PCR or immunohistochemistry, respectively. Thus, unlike the other techniques described to date our lectin-panning procedure allows the selective purification of cone photoreceptors.

PNA is classically considered to be a valuable marker of the extracellular matrix domain surrounding cone photoreceptor outer and inner segments.[18–21] However, depending on the species, the lectin labeling is not always identical in S and M/L cones.[21,36] The cone spectral sensitivity may thus reveal different compositions of cone extracellular matrix domains. In the ground squirrel, for instance, S-cone labeling is more intense than that of L cones,[37,38] whereas in the *Dace* fish and *Xenopus* retinas, PNA identifies L cones selectively.[36,39] In the primate retina, both S and M/L cones are labeled around their outer and inner segments, with an additional weak staining around their cell body and at their cone pedicle.[21,40] These observations are generally confirmed in most mammalian species.[19,41] In the pig retina, both S and M/L cones were PNA labeled and purified (Fig. 2). Cone photoreceptor labeling is observed in normal and pathologic conditions.[42] During the secondary degeneration of cones, cone cells can indeed be identified and quantified by PNA labeling in the *rd1* mouse retina either freshly isolated[42,43] or after explant culture.[10] Further studies are therefore needed to determine whether our lectin-panning method can be generalized to all mammalian species and to different animal models of human diseases.

Glial Neuroprotection of Cone Photoreceptors

Cultured chick embryo cone photoreceptors can survive in the absence of glial cells that are not differentiated at the time of the culture.[11] In contrast, glial cells appear to be very important for the survival of neonatal and adult mammalian rod photoreceptors.[14,44] The absolute glial dependence of both rods and cones was demonstrated when photoreceptors isolated by vibratome sectioning of the outer retina were found to survive only on a glial feeder layer.[16] This glial cell dependence of photoreceptors was further supported in mixed retinal cell cultures and animal models of photoreceptor degeneration in which neurotrophic factors like BDNF, GDNF, and CNTF were found to be neuroprotective of photoreceptors, despite the absence of functional receptors on photoreceptors, but their presence on glial cells.[45–47] The neurotrophic rescue of photoreceptors seems to rely on the neurotrophic-factor–mediated activation of Müller cells, which allows the release of secondary factors that are able to act directly on photoreceptors.[47–49] Another potential explanation for indirect protection of photoreceptor cells by Müller cells involves the cell contact–mediated mechanism. In vitro, Müller cells promote neurite extension in both adult mammalian rod photoreceptors and ganglion cells.[14,44,50] Experiments with RMG-conditioned medium clearly showed that cone photoreceptor survival does not require contact with retinal Müller cell but may be supported by the diffusible trophic molecules released by these glial cells.

Retinal glial cells synthesize many growth factors and cytokines.[47,51,52] The notion that glial cells synthesize trophic factors for retinal cells is consistent with the survival and outgrowth of retinal ganglion cells in the presence of a glia-conditioned medium.[50] It is also in agreement with the requirement of Müller cell activation for the BDNF-, GDNF- and CNTF-mediated neuroprotective effects on photoreceptors. Future studies should therefore focus on the isolation and characterization of such glia-derived trophic molecules.

Conclusions

We have developed a method to isolate cone photoreceptors that could facilitate the genomic and proteomic characterization of cones in both normal and pathologic conditions. Future studies will be focused on extending the lectin-panning method to species such as mouse and rat, for which both transcriptome and proteome data are available. The demonstration that cone photoreceptor survival is dependent on glial cells opens up new perspectives in the search for cone neuroprotective molecules.

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