Partial Characterization of Retina-Derived Cone Neuroprotection in Two Culture Models of Photoreceptor Degeneration

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PURPOSE. To define the nature and estimate the molecular weight range of soluble endogenous retinal trophic activities on cone photoreceptor survival in two models of cone degeneration.

METHODS. Diffusible factors from dissociated retinal cell cultures of 8-day normal-sighted (C57BL/6) mice were tested for cone-survival-promoting activity by two approaches and by using two independent photoreceptor degeneration models. In the first approach, mouse retinal cells were cultured on semi-permeable membranes apposed to dissociated cultures of chick embryo retina. In the second approach, conditioned medium was collected from normal mouse retinal cultures and added to embryonic chicken retina cultures or to retinal explants obtained from 5-week retinal degeneration (rd1) mice. In some experiments, conditioned medium was heated or sequentially fractionated in dialysis tubing with molecular weight cutoffs of 8, 15, and 25 kDa. The number of chicken cones and viability were determined by using morphologic criteria, colorimetric assays, and labeling with antibodies raised against visinin. Mouse cones were counted by differential double immunolabeling with antibodies against rhodopsin (rods)

RESULTS. Coculturing with normal mouse retinal cells delayed cone loss in dispersed embryonic chicken retina, by a maximum of 50% relative to the control. Conditioned medium derived from normal mouse retinas also significantly delayed cone loss in chicken cone cultures by a maximum of 1300%, compared with the control, and by 40% in rd1 mouse retinal explant cultures. The survival activity in conditioned medium was destroyed by heat denaturation, and was partially retained by dialysis with a molecular weight cutoff of 25 kDa in both models.

CONCLUSIONS. These strategies have identified cone-survival-stimulating activities in normal mouse retina, capable of acting across species and enhancing both structural protection and viability. Such molecules may represent candidates for clinical treatment of inherited retinal degeneration. (Invest Ophthalmol Vis Sci. 2003;44:818–825) DOI:10.1167/iovs.01-1144

Retinitis pigmentosa (RP) is a group of inherited photoreceptor (PR) dystrophies displaying extensive clinical and genetic variation, leading to progressive blindness with variable onset. Most identified human forms of RP are caused by defects in proteins restricted to rod PRs.1 Rod PR degeneration caused by a mutation in the β subunit of rod cGMP phosphodiesterase has been described in both human RP and the retinal degeneration 1 (rd1) mouse.2,3 Rods in rd1 mice degenerate rapidly by an apoptotic pathway.4 The remaining cone PRs undergo more prolonged secondary degeneration, leading to an eventual complete loss of vision.5 Treatment with neurotrophic factors such as brain-derived (BDNF), ciliary (CNTF), or glial cell line–derived (GDNF) neurotrophic factor, has been found to protect partially against PR degeneration in several animal models.6–8 Basic fibroblast growth factor (FGF2) has been shown to have direct effects on rod PR survival in vitro,9 mediated entirely through mitogen-activated kinase pathways.10 Previous studies have also demonstrated the existence of endogenous factor(s) influencing cone survival by grafting normal PR-pure layers into the subretinal space of the rd1 mouse. Grafted retinas revealed highly significantly greater numbers of surviving cones (means of 40% more) compared with sham-treated paired control retinas.11,12 An organ culture model of whole retinas from 5-week-old rd1 mice cocultured with primary cultures of mixed cells from postnatal day 8 normal-sighted mice (C57Bl/6) was used to demonstrate the diffusible character of this factor or factors.13 Recent work displayed a similar diffusible trophic activity on rods in retinal explants and dissociated retinal cultures prepared from PRO2HS mutant rhodopsin transgenic mice.14

In this study, we demonstrated that conditioned medium (CM) produced by mouse retinal cell cultures improved cone survival in two different models of cone degeneration, that this soluble survival activity is heat sensitive, and that the molecules involved in this survival have an apparent molecular mass higher than 25 kDa in the rd1 mouse model and appear to consist of several components in the chick cone model.

MATERIALS AND METHODS

Animals

All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Five-week-old C3H/Hen mice homozygous for the retinal degeneration 1 gene (rd1) and 8-day-old C57Bl/6 normal-sighted mice were obtained from Charles River Animal Suppliers (L’Arbresle, France). Fertilized eggs were obtained from Red Label breeding facilities maintained for our laboratory by Couvoirs de l’Est (Willgotheim, France).
Production of the Visinin Antibody

Six-day-old chick embryo retinal total RNA was purified and reverse transcribed into single-stranded cDNA. PCR was used to amplify the complete chick visinin coding region (GenBank accession number M84729) using the following oligonucleotides: 5’-GATGGGGGAAACGC-GCGCGAGGCGTGC-3’ and 5’-TATTATCTGGTGTTGTATTGATG-3’. The PCR product was subcloned in frame into the Smal site of the pGEX-2TK vector (Amersham Pharmacia Biotech, Orsay, France). This construction was introduced into Escherichia coli BL21-competent cells. After induction of fusion protein expression by isopropyl β-D-thiogalactopyranoside (4 hours) at 30°C, cells were lysed by sonication in 1% Triton X-100 in 10 mM dithiothreitol and a cocktail of protease inhibitors (Roche Diagnostics, Meylan, France). The fusion protein was affinity purified on a glutathione Sepharose column (4B, Amersham Pharmacia Biotech) and then cleaved by thrombin. The purified visinin was dialyzed against PBS. A portion of the protein (400 µg) was injected intradermally into two rabbits, and the antiserum was harvested after 2 months and stored at –80°C.

Western Blot Analysis

For Western blot analysis, total proteins from 3-day-old chick cone-enriched cultures were lysed in a buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA, 60 mM KCl, 0.5% NP40, 1 mM dithiothreitol, and a cocktail of protease inhibitors (Roche Diagnostics). Protein (10 µg/lane) was loaded onto a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose sheet (Bio-Rad, Hercules, CA) and then incubated with the following blocking buffer (10% milk in PBS and 0.1% Tween 20) and incubated 1 hour with an equal amount of the primary antibody and secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive bands were visualized using a Biomax light film (Eastman Kodak). Western blot detection system (Amersham Pharmacia Biotech) and then cleaved by thrombin. The purified visinin was dialyzed against PBS. A portion of the protein (400 µg) was injected intradermally into two rabbits, and the antiserum was harvested after 2 months and stored at –80°C.

Mouse Retinal Cell Culture

After enucleation, the retinas of 8-day-old normal-sighted C57B1/6 mice were prepared as described earlier.
X-100; preincubated in PBS containing 0.1% bovine serum albumin, 0.1% Tween 20, and 0.01% sodium azide for 15 minutes to block nonspecific staining; and then incubated with visinin antibodies (10 μg/mL final concentration) in blocking buffer for 2 hours. Coverslips were washed in PBS and incubated with goat anti-rabbit IgG conjugated to Bodipy FL (Molecular Probes, Leiden, The Netherlands), and with 4',6-diamidino-2-phenylindole (DAPI) for 1 hour, washed thoroughly with PBS, mounted, and viewed under a fluorescence microscope (Optiphot 2; Nikon, Tokyo, Japan).

For each coverslip, images of 25 fields observed using a 20× objective were digitized with image analysis software (Visiolab 1000; Biocom, Lyon, France). The total area of the 25 fields represents 1/16 of the total area of the coverslip. The number of visinin-positive cells was then estimated by extrapolating to the entire coverslip. For some experiments, the total number of cells was estimated by counting the DAPI-stained nuclei. The difference between the total number of DAPI-stained nuclei and visinin immunoreactive cells provided the total number of non-visinin-immunoreactive cells.

Conelike cells were counted under an inverted phase-contrast microscope in 1/16 of the total area of a well and extrapolated to the entire surface of the well.

### Immunohistochemical Labeling and Counting of rd1 Retina

Retinas were fixed overnight in 4% paraformaldehyde at 4°C, permeabilized, and incubated 30 minutes in blocking buffer, as described earlier. Retinas were then incubated for 2 hours with rho-4D2 monoclonal antibody18 and arrestin polyclonal antibodies in blocking buffer, washed, and incubated with goat anti-mouse IgG/Fluorophore (Bodipy FL; Molecular Probes) or with goat anti-rabbit IgG/Texas red, respectively (10 μg/mL each antibody; Molecular Probes) for 2 hours. Retinas were washed and flatmounted in PBS/glycerol (1:1) with the PRs uppermost, and examined under the microscope as described earlier. The number of labeled cells was estimated in the flatmounts by using a stereological method,19 which had previously been adapted to quantification of PR cells.15 In this case, 300 sampled, nonoverlapping 1,825-μm² fields, determined in a systematic random fashion to cover the whole retinal surface, were digitized. Double labeling involved double digitization in the same field. Cells counts were performed on two ×300 fields viewed on a computer screen by using an unbiased counting frame. The total number of PRs (arrestin-immunopositive rods and cones) and rod cells (opsin-immunopositive) in the entire retina was then estimated by normalizing to the entire retinal surface. The difference between the total number of arrestin- and opsin-immunopositive cells provided the total number of surviving cones.

### Statistical Analysis

Significant levels were obtained from the paired or unpaired Student's t-test when experimental groups implied two treatments. In experimental groups with several treatments, significant levels were obtained from the Kruskal-Wallis or Friedman test followed by the Newman-Keuls multiple comparison test, whenever the results of the Kruskal-Wallis or Friedman test were significant. The level of significance was set at P < 0.05.

Initially, in the dialysis experiment with rd explants, paired series were used in which treated retinas were matched to CM-treated retinas from the same mouse. Because no difference was found in the number of cones in CM-treated retina of each paired group and for more clarity in the text and figures, all cone counts in CM-treated retinas were pooled to be used as one group.

### Results

#### Effect of Normal Mouse Retina on Chick Embryo Retinal Cone Survival

Embryonic chicken retinal cells were cultured either alone or with dissociated normal mouse retinal cells, separated by a semipermeable membrane. Conelike cells were identified by morphologic features: a bipolar shape with prominent oil droplet (Fig. 1A). Control cultures in CDM showed equal survival at 1 and 3 days in vitro and thereafter constant progressive degeneration throughout the culture period (Fig. 1B). Cell survival in cocultures was identical with survival in the control during the initial 3 days, but thereafter the cocultures showed better cell survival, with 30% more surviving cone cells at 5 days and 50% more at 9 days, compared with the control (Fig. 1B). The assessment of cell viability was made spectrophotometrically by quantification of reduced MTT, which was produced by all viable cells and revealed an essentially identical profile (Fig. 1C).

In experiments involving addition of CM, except at day 1, the overall number of cells was much lower than in cocultures, but quantification of surviving conelike cells showed statistically significant increases in the presence of CM compared with the control at all times in vitro, by both morphologic counting (Fig. 2A) and viability assay (Fig. 2B).

#### Characterization of Antibodies Raised against Visinin

A rabbit polyclonal antibody was raised against recombinant visinin, a cone-specific calcium-binding protein.20,21 Immunolabeling of ED6 chick embryonic sections revealed selective staining of the outer retina (Figs. 3A, 3B), and ED17 retinal sections showed specific labeling within the PR outer segments and synapses (Figs. 3C, 3D). Chick conelike cells in vitro were invariably strongly stained by visinin antibodies, whereas multipolar neurons in the same culture were unlabelled (Figs. 3E, 3F). Mouse and rat retina sections were not labeled (data not shown). The specificity of visinin antibody was tested by Western blot analysis of total chick protein extracted from 3-day-old retinal cultures. A single band at ~24 kDa was detected in anti-serum– but not preimmune serum–treated membrane (Fig. 3G).

#### Conditioned Medium Promotes Cone PR Survival

Activity of CM was tested on chick embryo cone-enriched cultures and explants of rd1 mice (C3H/HeN). In chick cone-enriched–cell cultures, counting of visinin-immunopositive cells after 5 days in vitro confirmed and extended the results of the counting and viability assays, showing that the number of cone cells was an average of 1.6-fold (significantly) higher in treated cultures than in the control (Fig. 4A), whereas no significant difference was seen in the number of visinin-immunoreactive cells incubated in CDM versus CM (Fig. 4B).

A difference in the number of cone cells was detected in the rd1 explants incubated in CM for 7 days versus control rd1 mouse explants incubated in CDM. As illustrated in Fig. 5A, the average number of cones in 5-week-old rd1 mice in control explants was 44,462 ± 2,170, whereas the average number of cone cells in CM was 57,803 ± 1,909. These results confirm that soluble activity from mouse retinal cells exerts a trophic effect on rd1 mouse cone PRs by slowing degeneration.

#### Fractionation of Cone-Survival–Promoting Activity

To determine the apparent molecular weight of this soluble activity, CM was dialyzed in tubing of three molecular sieves. No loss of survival activity was observed after molecular sieving. The number of cone in explants cultured in fractionated medium (>8, >15, and >25 kDa) was not significantly different from that cultured with undialyzed CM, but was significantly more than the number of cone in explants incubated with CDM alone (Fig. 5A).
In chick cone cell cultures, survival-promoting activity decreased as the molecular sieve size increased. Loss of survival activity was approximately 60% after elimination of less than the 15-kDa and less than 25-kDa molecular species, but significant differences in visinin-immunopositive cell numbers in dialyzed CM were still observed compared with chick cone PRs cultured in CDM alone (Fig. 5B).

Relation of Heat Sensitivity to Survival Activity of CM

To partially define the nature of cone-survival–promoting factors, CM was heat-inactivated by boiling and tested on rd1 explants (Fig. 6A) or on chick cone cell cultures (Fig. 6B). The stimulatory effect was abolished after heat denaturation, suggesting that thermolabile molecules mediate the survival activity.

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DISCUSSION

The major focus of this study was to analyze further the interactions between rods and cones by comparing potential neuroprotective effects in two independent models of cone degeneration. Cone-survival–promoting activities were reliably and reproducibly detected under both experimental paradigms and using a number of different procedures. Taken together,

**FIGURE 3.** Specificity of visinin antibodies. (A, C) Nomarski and (B, D) immunofluorescence images. Visinin immunoreactivity is shown in retinal sections from (A, B) embryonic day (ED)6 chick embryos and (C, D) ED17 chick embryos. ED17 images show strong labeling by visinin antibodies of PR outer segments and synapses. (E, F) Labeling of chick cells after 3 days in vitro, showing specific staining of conelike cells (arrows) but not multipolar neurons (arrowheads). (G) Specificity of the visinin antibody was confirmed by Western blot of the preimmune serum (control) and antiserum against 3-day chick embryo retinal protein extract. Lane a: antiserum, lane b: preimmune serum.

**FIGURE 4.** CM from normal mouse retinas, compared with CDM. (A) promoted survival of immunocytochemically identified cones in chick retinal cultures, but (B) did not promote survival of non-visinin-immunoreactive cells. Data are the mean ± SEM of results in three independent cultures performed in triplicate wells. *Significant difference from control at P < 0.05 by Student’s paired t-test.
The data indicate that molecules liberated by normal mouse retina are capable of delaying cone death across species and at different developmental stages, and improve parameters of structural integrity, biochemical expression, and viability. Such soluble molecules, available extracellularly and operating through diffusion, have already been described in rod PR development, promoting either survival or inhibition of differentiation (for review, see Levine et al.22). To the best of our knowledge these are the first data to characterize the biochemical nature and molecular weight range of cone-survival-promoting molecules. The different experimental approaches suggest cone-survival-promoting factors to be thermolabile and to belong to different molecular weight ranges in the case of promoting chick cone-survival, whereas mouse cone-survival-promoting factors are more than 25 kDa molecular mass.

The embryonic chicken retinal monolayer model developed by Adler and Hatle23 contains up to 80% cone cells in the absence of intercellular contact and non-neuronal cells under defined conditions (low-density seeding and chemically defined medium). Increasing cell death occurs during maintenance in defined media, presumably through apoptosis induced by deprivation of trophic factor.23 This hypothesis is reinforced by qualitative observations during cell counting in our experiments. Dead or dying cells were more often found shrunk, rather than swollen, with DAPI-stained, degraded nuclei—two major morphologic criteria allowing preliminary discrimination between apoptosis and necrosis. This simplified system has already been used to clarify partially the trophic activity in bovine interphotoreceptor matrix25 and to display the effects of retinoic acid, retinol, and 11-cis retinaldehyde as survival-promoting agents.26 The rd1 mouse presents a useful pathologic model, because the retinal degeneration in this model is due to the same mutation in the β subunit of the cGMP-dependent phosphodiesterase as one form of human RP. Explant tissue culture of whole retinas from 5-week-old rd1 mice, which at this age contain few (<0.02%) remaining rods but numerous surviving cones, is appropriate for testing the effects of soluble factors, because it allows a more controlled...
and reproducible environment for testing PR interactions than in vivo. Estimating the number of cone cells by the difference between arrestin-immunoreactive cells and Rho+ID2-immunoreactive cells was thought to be more appropriate in these experimental conditions on account of the harsher treatments (frequent medium replacement), which would damage cells, especially the peanut agglutinin-immunoreactive cone sheath. Moreover, previous studies found no significant differences between the present results and previously published peanut lectin immunolabeling, with the same ratio between the number of cones in control and cocultured explants.¹⁴

In the present study, both experimental models were validated as functional assays for studying cone survival under the same experimental conditions (i.e., coculturing with, and CM from, dissociated cells of young, normal-sighted mouse retinas). Both of these techniques have been used to study interactions between retinal pigmented epithelial cells and PRs.²⁵,²⁶

Under the experimental conditions tested, chick cone-enriched cell cultures appear to be a more sensitive assay for testing paracrine activities, because effects on cones were greater in this system than in the rd1 mouse model. The chick cone-enriched model appears thus to be a general cone survival assay that allows the testing of the cocktail of molecules contained in the CM, with or without specificity of the survival effect. The mouse rd1 explant model, in contrast, allows the targeting of specific survival factor(s) for cones in a degenerating system.

Survival effects on cones in rd1 mouse explants were conserved after dialysis at different molecular weight cutoffs, suggesting that active factor(s) have an apparent molecular weight higher than 25 kDa. However, chick cone cultures showed rather different behavior, with CM survival activity being partially reduced after molecular partition, suggesting existence of at least two molecular species. We postulate the existence of several proteins with distinct effects that promote cone survival in the two different paradigms. Survival effects in monodispersed chick embryo retinal cell cultures must operate in a direct manner on cone PR, because all contact-mediated cell-cell interactions were prevented. In theory, neighboring cell types may still release additional soluble factors, but the low cell density used in these experiments argues against such a possibility. Induction of survival may also necessitate stimulation of several pathways within the same cell. In rd1 mouse retinal explants, which retain a high degree of organization, however, survival could result more from stimulation of other cells that in turn secrete putative cone-survival–promoting factors. Comparing results between these two different experimental models does not provide a simple explanation, but demonstrates the complementarity of such a dual approach. Thanks to the enrichment of cones and their progressive degeneration in relation to the duration of culturing, the experimental model of chick cone cell culture is a convenient but simplified representation of the cone-degeneration phenomenon in eye disease, allowing rapid further evaluation of candidate factors for cone survival. However, the limits of the pertinence of the model must be borne in mind: Cone survival factors are screened with primary cultures of avian retinal cells, not with a pathologic model of cone degeneration. The rd1 mouse model is superior to the latter in its potential for clinical application, because it represents a spontaneous pathologic animal model of human RP, allowing testing of wound hypotheses applied to the study of cone survival. However, the low proportion of cones in the mouse retina (3%) and the intensive labor involved in stereologic counting in evaluating the survival effect represent alternative difficulties.

Taking into account the apparent molecular weights of the survival-promoting activities obtained with CM from normal mouse retinal cell cultures, potential known endogenous candidates are platelet-derived growth factor (25–29 kDa), osteogenic protein I/bone morphogenetic protein 7 (30–38 kDa), and pigment epithelium-derived factor (50 kDa). These factors are expressed in the mammalian retina²⁳ and influence neuronal survival and differentiation.²⁹,³³,³⁵ Neurotransmitters could equally be a source of candidate molecules. α-Adrenergic agonists have been shown to increase endogenous expression of FGF and protect PR in a specific and selective manner.³³,³⁴ We are currently investigating the possible presence of these substances in CM.

In conclusion, these findings suggest the existence of retinaderived diffusible factors influencing cone PR. They open new therapeutic pathways that it is hoped will allow the significant limitation of functional deficits related to retinal degeneration, without directly treating the original abnormality.

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References