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Letter to the Editor

RECOVERIN EXPRESSION IN THE R28 RETINAL PRECURSOR CELL LINE

Dear Editor:

In vitro studies of photoreceptor cell development have been limited by the availability of pure retinal cultures with unlimited lifespan. To date, human retinoblastoma tumor-derived cell lines have been most commonly used as models for the study of photoreceptor differentiation *in vitro*. The most studied of these include Y79 cells (Kyritsis et al., 1987) and WERI-RB1 cells (DiPolo and Farber, 1995). Such retinoblastoma cells are highly tumorigenic *in vivo* (del Cerro et al., 1993) and often show anomalous gene regulation that differs from that of the normal retina (Fong et al., 1988; DiPolo and Farber, 1995). Therefore, retinoblastoma-derived cells may not always be the best model system for the study of retinal cell growth and differentiation *in vitro* and have serious limitations for *in vivo* experimentation.

As an alternative to transformed, tumorigenic retinal cell lines, this laboratory recently developed a 12S E1A-immortalized retinal cell culture (Seigel, 1996). The 12S portion of the E1A gene contains the immortalizing, but not the transforming functions of the gene (Whyte et al., 1988). The resulting retinal cell line R28 has been propagated *in vitro* for over 100 passages, exhibits nontransformed growth properties, and has a significant glial cell component (Seigel et al., 1996). In this study, we show that R28 cells exhibit immunoreactivity to recoverin, a calcium-binding protein expressed in photoreceptors (Wiechmann and Hammarback, 1993; Wiechmann et al., 1994) and Y79 retinoblastoma cells (Wiechman, 1996), which regulates rhodopsin phosphorylation (Kawamura et al., 1994; De Castro et al., 1995; Senin et al., 1995) and is hypothesized to play a role in cancer- associated retinopathy (Adamus et al., 1993; Polans et al., 1995). Because R28 cells represent a virtually unlimited supply of immortalized, nontumorigenic cells expressing recoverin, the cell line may provide a unique and useful way to study the regulation of recoverin gene expression and its integral role in photoreceptor differentiation and function *in vitro* and *in vivo*.

For all experiments in this study, 12S E1A-immortalized retinal cells derived from PN6 Sprague–Dawley rats were used. Animals were used in accordance with the Association for Research in Vision and Ophthalmology's statement for the Use of Animals in Ophthalmic and Vision Research. Establishment of the cell culture, designated E1A-NR.3, has been described previously (Seigel, 1996). Briefly, a Ψ2 12S E1A replication-defective retroviral vector was used to immortalize retinal tissue, which was then selected for 2 wk on the basis of neomycin resistance. Immortalized cells were maintained at 37° C in a 5% CO_2 incubator under the following culture conditions: Dulbecco's modified Eagle's medium (DMEM +) with 10% calf serum, 1× minimal essential medium (MEM) nonessential amino acids (GIBCO Laboratories, NY), 1× MEM vitamins (GIBCO), 0.37% sodium bicarbonate, 0.058% L-glutamine and 100 μg/ml gentamicin.

The R28 clone derived from a single cell was established by limiting dilution of E1A-NR.3 in 96-well dishes. For cloning, medium was supplemented with 20% calf serum to promote proliferation, as these cells proved difficult to grow singly. Cells went through three rounds of cloning to ensure that the final clonal population was derived from one single cell. Microscopic visualization confirmed the presence of one cell per well.

R28 cells, immortalized by the Adenovirus 12S E1A protein, as well as uninfected P6 retina were analyzed for the expression of E1A and recoverin proteins. Uninfected P6 retinal cells grown *in vitro* for 8 d proved to be reliable immunonegative controls for E1A expression (Fig. 1 *A*), while R28 cells exhibited typical nuclear E1A immunoreactivity in all cells (Fig. 1 *B*). R28 cells and uninfected retinal cells were analyzed for expression of recoverin, a photoreceptor-specific calcium- binding protein, whose developmental appearance in the mouse is at postnatal Day 5 (Stepanik et al., 1993). In uninfected P6 retina, many small round, process-bearing cells showed intense immunoreactivity to anti-recoverin antibody, while large flat cells remained negative (Fig. 1 *C*). Cell staining for recoverin was observed throughout the R28 cell line with less intensity than the positive uninfected P6 retinal cells (Fig. 1 *D*). Western blot analyses confirmed E1A immunoreactivity for R28 cells, its parental culture E1A-NR.3, and a comparable sister cell line R57, whereas uninfected P6 retina remained immunonegative (Fig. 2 *A*). Western blot analyses also confirmed significantly less recoverin immunoreactivity for R28 cells, as compared with uninfected P6 retina, as evidenced by 23-kDa band intensity (Fig. 2 *B*).

Quantitative analysis of recoverin expression was compared with known R28 markers IRBP, E1A, and vimentin for entire R28 cell populations. Comparisons for all antigens were made at 20 000 cells/well in the linear range of reaction product for all antibodies (Fig. 3). Results of one representative experiment are shown for each panel. At Passage 35, recoverin was detected at the highest level of the markers examined, with approximately twice the absorbance of the glial marker vimentin and the photoreceptor marker IRBP, and fourfold the absorbance of E1A (Fig. 3 *A*). At Passage 85 (Fig. 3 *B*), recoverin (as well as IRBP, E1A, and vimentin) continued to be expressed to a significant degree. However, there was a generalized decrease in detected antigens across all markers as compared with Passage 35. The percent decreases in 405 nm absorbance from Passage 35 to Passage 85 were as follows: recoverin 36%, IRBP 38%, E1A 20%, and vimentin 50%.

Thus, 12S E1A-immortalized retinal cells (E1A-NR.3 and R28) display characteristics that are consistent with at least photoreceptor and Müller cell phenotypes. In a previous study, we showed that the R28 cell line has a very strong glial component, expressing GFAP, S-100, and vimentin (Seigel et al., 1996). In that study, we were also able to demonstrate simultaneous GFAP/IRBP immunoreactivity within individual cells. Here, we show that because both E1A and

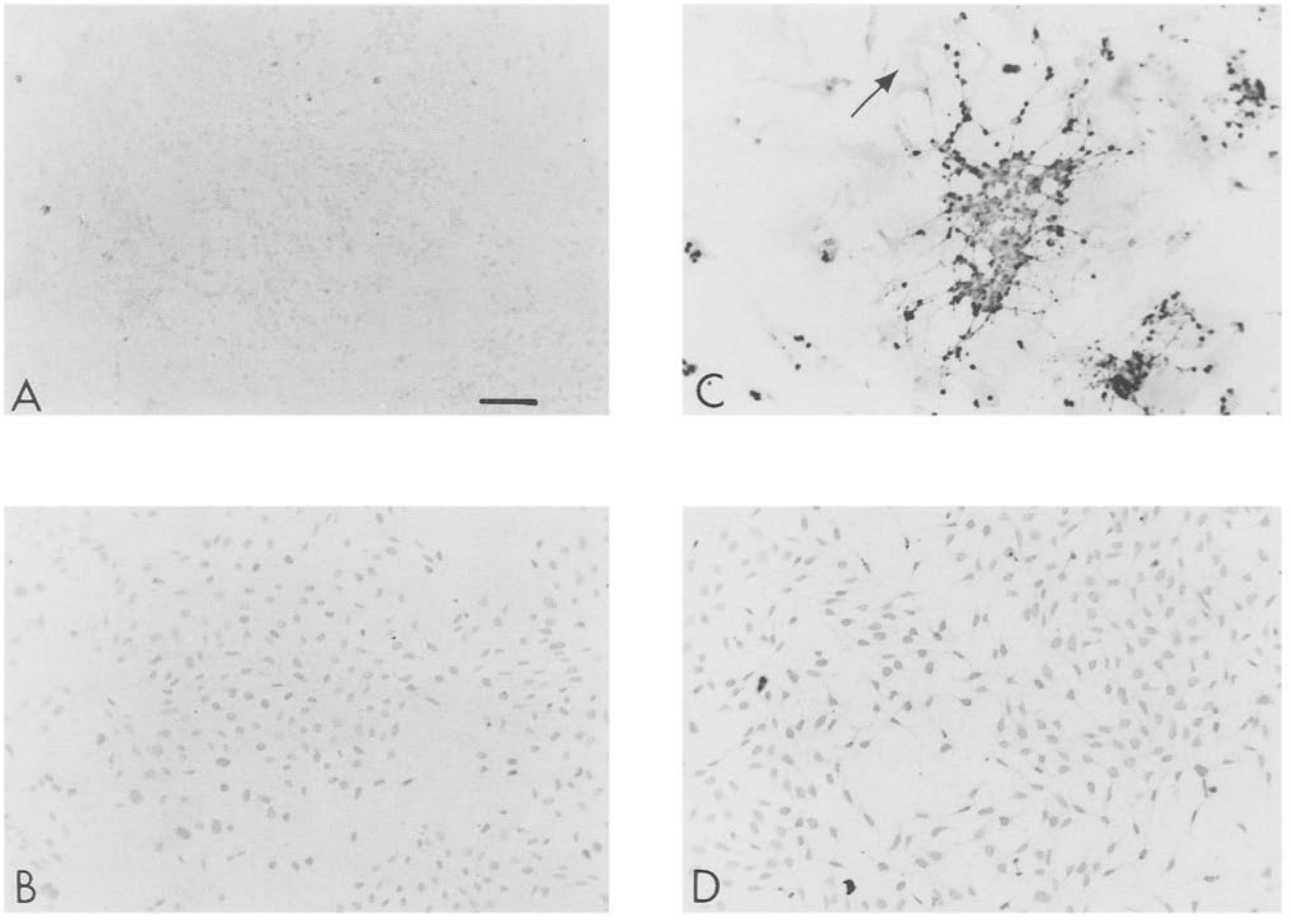

FIG. 1. Cell immunostaining for E1A and recoverin. *A*, P6 retinal cells, 8 d *in vitro*, stained for E1A (photograph underexposed to show the presence of cells); *B*, R28 cells exhibit typical nuclear E1A immunoreactivity in all cells; *C*, P6 retinal cells 8 d *in vitro*, stained for recoverin. Small, process-bearing cells appear most intense, whereas large, flat cells remain immunonegative (*arrow*); *D*, R28 cells express recoverin immunoreactivity in virtually all cells. *Scale bar* = 20 μm. Cells were fixed on poly D-lysine-coated plastic coverslips for 10 min at room temperature in 2% paraformaldehyde, incubated for 1 h with the appropriate primary antibody, rinsed, and incubated with biotinylated goat anti-rabbit or anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) for 60 min. Reaction product was developed with a diaminobenzidine (DAB) kit (Pierce, Rockford, IL). Negative controls consisted of incubations in control serum without primary antibody and were processed simultaneously with experimental cells.

Primary antibodies included rabbit anti-recoverin (Adamus et al., in preparation) at a 1:1000 dilution; rabbit anti- interphotoreceptor retinoid-binding protein (IRBP) (courtesy of Dr. B. Wiggert) (Wiggert et al., 1986) at a 1:750 dilution; E1A (Oncogene Science, Inc., Uniondale, NY) at a 1:100 dilution; and vimentin (Boehringer Mannheim, Indianapolis, IN) at a 1:50 dilution.

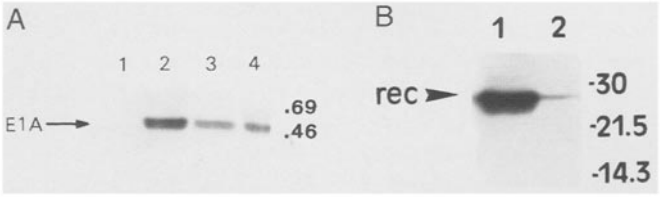

FIG. 2. Fig. 2. Western immunoblot of E1A and recoverin. Ten micrograms of protein per lane was analyzed for immunoreactivity to E1A (*Panel A*) and recoverin (*Panel B*). *Panel A*, *Lane 1*, P6 uninfected retina; *Lane 2*, E1A-NR.3 (parental cell culture of R28); *Lane 3*, R28; *Lane 4*, R57 (a sister clonal cell line of R28). A 1-min ECL chemiluminescent exposure was used for *Panel B*, *Lane 1*, P6 uninfected retina; *Lane 2*, R28. An 11-min ECL chemiluminescent exposure was used for *Panel B*.

recoverin are expressed in virtually all R28 cells, it appears that recoverin can coexpress with E1A *in vitro*. Yet, recoverin is expressed to a lesser degree in R28 cells than in uninfected P6 retina. This difference and ensuing decrease in recoverin expression at high passage number, may be due to the proliferative nature of E1A- immortalized retinal cells, which progress through the cell cycle every 24 h (Seigel, 1996). Cell passaging may select for the most proliferative, least mature cells over extended periods in culture. However, this limitation of high cell passage number can be avoided by amplification and freezer storage of lower passage R28 cells.

What accounts for the particular expression pattern of R28 cells? There are several potential explanations. One is that the original retroviral infection may skew the population in one direction or another, depending upon the susceptibility of particular cells to im-

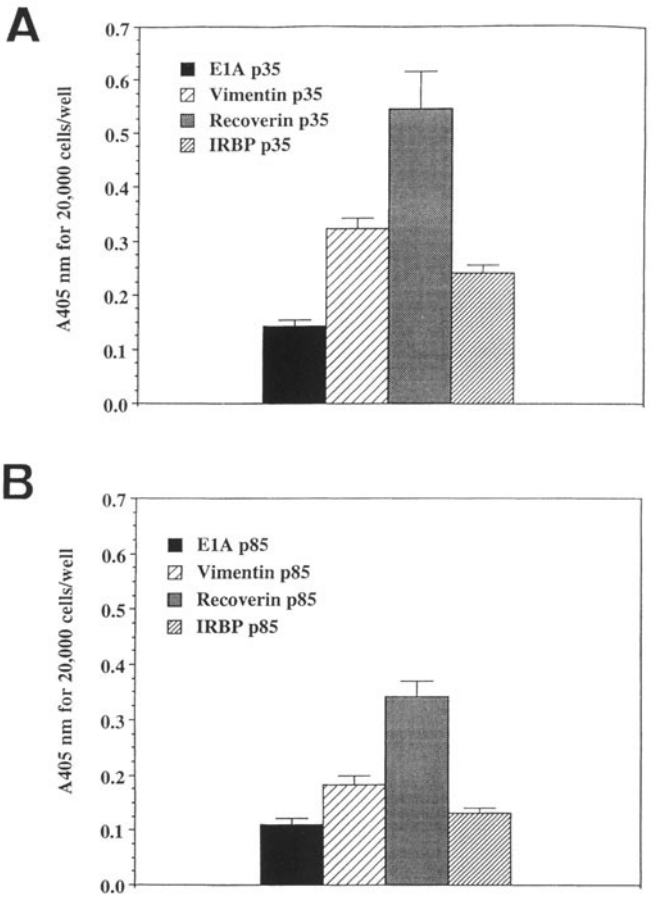

FIG. 3. Relative levels of recoverin expression. R28 cells were grown in 96-well dishes at 5000 to 40,000 cells per well. Cells were fixed in 2% paraformaldehyde and stained for recoverin, interphotoreceptor retinoid-binding protein (IRBP), E1A, and vimentin as per DAB method. To visualize the reaction, a soluble peroxidase substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Vector Laboratories, Burlingame, CA) was used. The colorimetric reaction was read in a Biorad 96-well plate reader at 405 nm. The linear range of the reaction was determined to be 5000 to 40,000 cells per well, and for comparison purposes, 20,000 cells per well were used for determining relative levels of immunoreactivity. Plates were read at 405 nm and blanked against negative controls which received blocking serum instead of primary antibody. Graphs show results of representative experiments of at least three repetitions with a minimum of quintuplicate wells. *A*, R28 cells, Passage 35; *B*, R28 cells, Passage 85. Graphs are shown at the same scale to illustrate differences.

mortalization infection on postnatal day 6. The only cells susceptible to immortalization are still proliferative, (Cone et al., 1988) and hence, conditions favor the immortalization of mitotic precursor cells. Mitoses can be detected in the rat retina during development until P7; at P6 the majority of dividing cells appear to be destined for the photoreceptor lineage (Grun, 1982). However, it is clear that individual precursor cells may lead to multiple phenotypes (Turner and Cepko, 1987; Turner et al., 1990). Late developmental photoreceptor markers, such as S-Antigen and rhodopsin, may depend upon quiescence for enhanced expression, whereas the immortalization/growth stimulation process itself may lead to more immature phenotypes (Berlingieri et al., 1993; Boulukos and Ziff, 1993). To promote expression of postmitotic markers or to enhance recoverin-fjexpression to levels seen in uninfected retina, treatment of cells with inhibitors of cell division may be required, and such experiments are now ongoing, including dibutyryl cAMP/sodium butyrate treatment which has been shown to enhance recoverin expression in Y79 retinoblastoma cells (Wiechman, 1996).

Potential applications for immortalized retinal cells which express recoverin are numerous at both the cellular and molecular levels. The parent culture of the R28 cell line (E1A-NR.3) has been used as a system for the study of IRBP promoter gene regulation and found to be comparable to normal retina (Fei et al., 1996). In addition, the parental cells have been useful for studies of recoverin antibody effects in an *in vitro* model of cancer-associated retinopathy (Adamus et al., 1997; Machnicki et al., 1996). Recoverin-expressing R28 cells can readily be used for studies of gene regulation, promoter characterization, and long-term transfection studies not possible with short-lived primary retinal cultures, as well as developmental retinal transplantation studies not possible with retinoblastoma-derived cells.

In summary, we have shown that the R28 cell line expresses recoverin along with IRBP, consistent with a photoreceptor cell lineage. The photoreceptor elements, along with strong glial components, support the hypothesis of a precursor cell origin for R28 cells. We are currently conducting both *in vitro* and *in vivo* studies to determine the full spectrum of their differentiation potential. Further study and identification of nontumorigenic retinal cells such as R28 which express recoverin will continue to elucidate the function and regulation of this protein in the retina.

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REFERENCES

Adamus, G.; Guy, J.; Schmied, J., et al. Role of anti- recoverin antibodies in cancer-associated retinopathy. Invest. Ophthalmol. Vis. Sci. 34:2626–2633; 1993.

Adamus, G.; Machicki, M.; Seigel, G. Apoptotic retinal cell death induced by antirecoverin antibodies of cancer-associated retinopathy. Invest. Ophthalmol. & Vis. Sci. 38:283–291; 1997.

Berlingieri, M.; Santoro, M.; Battaglia, C., et al. The adenovirus E1A gene blocks the differentiation of a thyroid epithelial cell line, however, the neoplastic phenotype is achieved only after cooperation with other oncogenes. Oncogene 8:237–248; 1993.

Boulukos, K.; Ziff, E. Adenovirus E1A proteins disrupt the neuronal phenotype and growth factor responsiveness of PC12 cells by a conserved region-1 dependent mechanism. Oncogene 8:237–248; 1993.

Cone, R.; Grodzicker, T.; Jaramillo, M. A retrovirus expressing the 12S adenoviral E1A gene product can immortalize epithelial cells from a broad range of tissues. Mol. Cell. Biol. 8:1036–1044; 1988.

De Castro, E.; Nef, S.; Fiumelli, H., et al. Regulation of rhodopsin phosphorylation by a family of neuronal calcium sensors. Biochem. Biophys. Res. Comm. 216:133–140; 1995.

del Cerro, M.; Seigel, G. M.; Lazar, E. S., et al. Intraretinal transplantation of Y79 cells into rats: a useful in vivo model of human retinoblastomas. Invest. Ophthalmol. Vis Sci. 34:11–24; 1993.

DiPolo, A.; Farber, D. Rod photoreceptor-specific gene expression in human retinoblastoma cells. Proc. Natl. Acad. Sci. USA 92:4016–4020; 1995.

Fei, Y.; Seigel, G. M.; Liou, G. I. The use of an E1A- immortalized retinal culture to study the regulation of the interphotoreceptor retinoid-binding protein gene. Invest. Ophthalmol. Vis. Sci. 37:S337 abstract; 1996.

Fong, S.; Balakier, H.; Canton, M., et al. Retinoid-binding proteins in retinoblastoma tumors. Cancer Res. 48:1124–1128; 1988.

Grun, G. The development of the vertebrate retina: a comparative survey. New York: Springer-Verlag; 1982.

Kyritsis, A.; Tsokos, M.; Chader, G. Behavior of human retinoblastoma cells in culture. In: Osborne, N.; Chader, G., ed. Progress in retinal research. Vol. 6. Oxford: Pergamon Press; 1987:245–273.

Kawamura, S.; Cox, J.; Nef, P. Inhibition of rhodopsin phosphorylation by nonmyristoylated recombinant recoverin. Biochem. Biophys. Res. Comm. 203:121–127; 1994.

Machnicki, M.; Seigel, G. M.; Adamus, G. Evidence for the pathogenic effect of anti-recoverin antibodies in cancer- associated retinopathy. Invest. Ophthalmol. Vis. Sci. 37:S535 abstract; 1996.

Polans, A.; Witkowska, D.; Haley, T., et al. Recoverin, a photoreceptor-specific calcium-binding protein is expressed by the tumor of a patient with cancer-associated retinopathy. Proc. Natl. Acad. Sci. USA 92:9176–9180; 1995.

Seigel, G. M. Establishment of an E1A-immortalized retinal cell line. In Vitro Cell. Dev. Biol. 32:66–68; 1996.

Seigel, G. M.; Mutchler, A. L.; Imperato, E. L. Expression of glial markers in a retinal precursor cell line. Mol. Vis. 2:96001; 1996.

Senin, I.; Zagarov, A.; Alekseev, A., et al. N-myristoylation of recoverin enhances its efficiency as an inhibitor of rhodopsin kinase. FEBS Lett. 376:87–90; 1995.

Stepanik, P.; Lerious, V.; McGinnis, J. Developmental appearance, species, and tissue specificity of mouse 23-kDa, a retinal calcium-binding protein (recoverin). Exp. Eye Res. 57:189–197; 1993.

Turner, D.; Cepko, C. A common progenitor for neurons and glia persists in the rat retina late in development. Nature 328:131–136; 1987.

Turner, D.; Snyder, E.; Cepko, C. Lineage-dependent determination of cell type in the embryonic mouse retina. Neuron 4:833–845; 1990.

Whyte, P.; Ruley, H.; Harlow, E. Two regions of the adenovirus early region 1A proteins are required for transformation. J. Virol. 62:257–265; 1988.

Wiechman, A. Recoverin in cultured human retinoblastoma cells: enhanced expression during morphological differentiation. J. Neurochem. 67:105–110; 1996.

Wiechmann, A.; Akots, G.; Hammarback, J., et al. Genetic and physical mapping of human recoverin: a gene expressed in retinal photoreceptors. Invest. Ophthalmol. Vis. Sci. 35:325–331; 1994.

Wiechmann, A.; Hammarback, J. Expression of recoverin mRNA in the human retina: localization by in situ hybridization. Exp. Eye Res. 57:763–769; 1993.

Wiggert, B.; Lee, L.; Rodrigues, M., et al. Immunochemical distribution of interphotoreceptor retinoid binding protein in selected species. Inv. Ophthalmol. Vis. Sci. 27:1041–1049; 1986.

Gail M. Seigel[1] Grazyna Adamus
Aimee L. Mutchler Eileen L. Imperato-Kalmar

Department of Neurobiology and Anatomy
University of Rochester
School of Medicine and Dentistry
601 Elmwood Avenue
Rochester, New York 14642 (G. M. S., A. L. M., E. L. I.-K.)
R. S. Dow Neurological Sciences Institute
1120 N.W. 20th Avenue
Portland, Oregon 97209 (G. A.)

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[1]To whom correspondence should be addressed.