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Inducible Apoptosis-Promoting Activity in Retinal Cell-Conditioned Medium

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Purpose: Apoptosis is implicated in the death of retinal cells during both retinal differentiation and degeneration. We sought to investigate potential diffusible retinal cell signalling factors which may be responsible for this phenomenon.
Methods: 72 hr conditioned medium was collected from death-induced R28 retinal cells undergoing serum-starvation. This conditioned medium was filtered, titrated and added to fresh cultures of R28 cells for 24 hr. Cell death was measured by trypan blue exclusion; apoptosis was evaluated by TUNEL-in situ, DNA gel electrophoresis, and observations of cell morphology.
Results: Apoptotic cell death was at least two-fold greater in retinal cultures which received death-induced cell-conditioned medium. This apoptotic activity in conditioned medium did not appear to be due to nutrient-depletion of the medium, as the control situation (72 hr conditioned medium from resistant, non-dying cells) did not produce the same effect. This diffusible apoptotic activity was heat-labile (56 °C for 45 min).
Conclusions: We have shown for the first time, in our system, that death-induced retinal cells release a diffusible, heat-labile apoptosis-promoting element. Further investigation will be necessary to determine whether this trigger of apoptosis is a toxic metabolite or an "apoptosis-promoting factor."

Apoptosis, or programmed cell death, plays a significant role in retinal degeneration, as well as retinal development. Cell loss in the developing retina has been linked with apoptotic events, and may control the distribution and number of specific cell populations in the mature neural retina (1,2). In animal models of retinal degenerative diseases, including rd/rds mice (3,4), vitiligo mice (5) and other models of retinitis pigmentosa (6), apoptosis has been identified as a pathway for photoreceptor cell death. Apoptosis of photoreceptors has also been seen in human patients after traumatic retinal detachment (7), as well as in experimental retinal detachment in cats (8). Thus, it appears that apoptosis is a common pathway for cell death occurring as part of retinal degenerative processes, as well as in retinal development. Limitations in the amount of retinal material available have slowed progress in the study of retinal apoptotic events on a cellular level. To this end, we developed immortalized retinal precursor cell lines, such as R28, which have been cultured over 100 passages, and express markers consistent with glial, as well as photoreceptor phenotypes, indicative of a pre-committment, precursor cell phenotype (9,10,11).

We can induce apoptosis in R28 cells in vitro by treatment with defined media (12; unpublished data). These metabolic challenges may mimic retinal nutrient deprivation as seen in retinal detachment (7), or the vascular compromise of diabetic retinopathy (13,14). Based on DNA fragmentation analyses and terminal deoxytransferase nick-end labelling (TUNEL)-in situ observations, the R28 retinal cell line undergoes a consistent, density-dependent apoptotic cell death in response to metabolic stress conditions that would provide an excellent model system for further study of apoptotic mechanisms relevant to retinal degeneration (12).

One exciting approach to the control of apoptotic mechanisms presumes that retinal degeneration may involve both intrinsic and extrinsic cellular mechanisms. Our in vitro system is ideal for investigations into the identity of potential environmental diffusible factors. In this report, we present evidence for the existence of a heat-labile, metabolically-inducible apoptosis-promoting activity in retinal cell-conditioned medium.

METHODS

Cell Culture— Immortalized R28 retinal precursor cells, derived from postnatal day 6 Sprague-Dawley rat, were maintained in Dulbecco Modified Eagle's Medium (DMEM) with 10% calf serum (Hyclone, Logan, UT), 1X Minimal Essential Medium (MEM) non-essential amino acids (GIBCO, Gaithersburg, MD), 1X MEM vitamins (GIBCO, Gaithersburg, MD), 0.37% sodium bicarbonate, 0.058% L-glutamine and 100 µg/ml gentamicin. For apoptosis studies, cells were treated with serumless/defined medium (DMEM/F12, 0.64% glucose, 0.148% KCl, 250 mg/ml ascorbic acid, 0.06 ng/ml progesterone, 16 mg/ml putrescine, 0.04 ng/ml sodium selenite, 0.37% sodium bicarbonate, 0.058% L-glutamine and 100 µg/ml gentamicin).

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Conditioned medium was collected from cells grown for 72 hr in serumless/defined medium. The conditioned medium was passed through a 0.22 micron filter to remove dead cells and debris, then stored at -20° C until use. Heat inactivation of conditioned medium was at 56° C for 45 min.

DNA laddering— Evidence of non-random DNA fragmentation was detected by agarose gel electrophoresis of DNA extracts from cells grown in serum-containing and serumless/defined medium. Non-adherent cells in the supernatant, along with trypsinized adherent cells, were utilized for each sample. For each sample of 2 x 10^6 cells, DNA was extracted with the Wizard Genomic DNA extraction kit (Promega, Inc., Madison, WI), and stored in the kit's DNA rehydration buffer at 4° C until use. This extraction procedure did not allow for quantitation of DNA, but based upon cell number, approximately 1.2 µg of each DNA sample was loaded into the wells of a 1% agarose gel, with 0.5 mg/ml ethidium bromide incorporated into both the agarose gel and Tris-acetate buffer. Electrophoresis was carried out for 6 hr at 35V, 12 mA. The agarose gel was visualized and photographed using a 300 nm transilluminator (Hoefer Scientific Instruments, San Francisco, CA).

DNA fragmentation analysis in situ— Detection of DNA fragmentation in situ was visualized with the use of the Apoptag Plus Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, MD) and developed with diaminobenzidine reaction product. Positive cells were counted in ten groups of 100. In some experiments, a 1:6 dilution of 0.4% Trypan blue was added to cell suspensions prior to fixation in order to assess whether cells were living or dead.

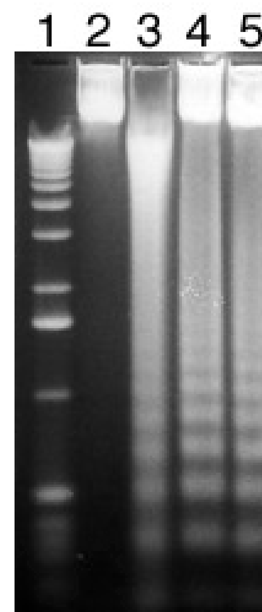

Figure 2. DNA Fragmentation Characteristic of Apoptosis: A Non-quantitative Analysis. DNA fragmentation characteristic of apoptotic cell death can be seen in serumless-defined, death-induced, as well as heat-inactivated death-induced cell cultures, but not in control serum-containing conditions. Lane 1 contains commercial 1 Kb DNA markers. DNA was harvested from cells grown under the following conditions: cells grown under serum-containing conditions (lane 2); cells grown 24 hr in serumless/defined medium (lane 3); cells grown for 24 hr in death-induced cell-conditioned medium (lane 4); cells grown for 24 hr in death-induced cell-conditioned medium which was heat inactivated 56° C for 45 min (lane 5). Approximately 1.2 µg of DNA was loaded per lane, based upon cell number and anticipated yield.

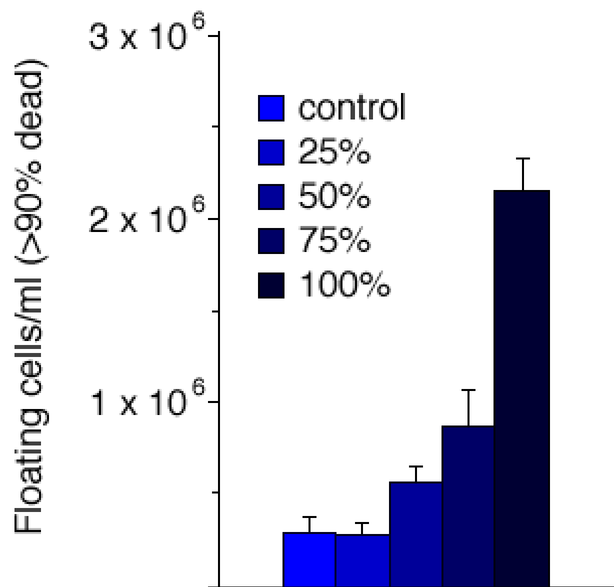

Figure 1. Titration of Cell Death-Promoting Activity of Retinal Cell-Conditioned Medium. 72 hr conditioned medium was collected from dense cultures of cells death-induced under serumless/defined conditions. Titrations of this conditioned medium were added to fresh R28 cultures for 24 hr. Floating cells were collected, counted in hemacytometer squares, and determined to be greater than 90% dead, by trypan blue analysis. Error bars represent standard deviations.

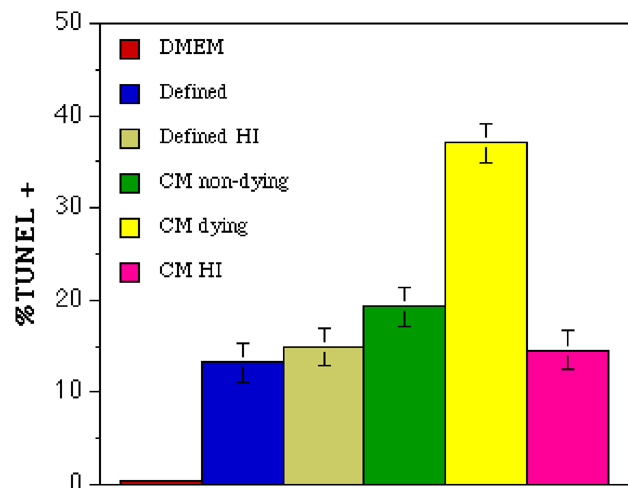

Figure 3. Apoptotic Activity of Retinal Cell-Conditioned Medium. 72 hr conditioned medium was collected from death-induced and non-death-induced R28 cultures. Fresh R28 cultures were treated for 24 hr with serum-containing medium (DMEM); serumless/defined medium (Defined), heat-inactivated serumless/defined medium (Defined HI); conditioned medium from non-dying cells (CM non-dying); conditioned medium from death-induced cells (CM dying); and heat-inactivated conditioned medium from death-induced cells (CM HI). Cells underwent TUNEL in situ analysis and were counted in ten groups of 100. This graph represents one of three separate experiments. Error bars represent standard deviations.

RESULTS

Conditioned medium from death-induced cells promotes cell death— Conditioned medium was collected from the R28 cell line after 72 hr of serumless/defined medium treatment. At 72 hr in serumless/defined medium, TUNEL-positive cells comprise greater than 60% of the population (unpublished data). By five days in serumless/defined medium, all R28 cells die. However, a 72 hr interval was chosen to ensure that there would still be metabolically active cells which could secrete factors into conditioned medium. Conditioned medium from these 72 hr death-induced cells was titrated and added to fresh R28 cultures in order to assess the death-promoting activities of death-induced cell-conditioned medium. A 24 hr timepoint was chosen to minimize background cell death known to be caused by fresh serumless/defined medium, which was used for comparison. At 24 hr, death-induced cell-conditioned medium caused cell death in a concentration-dependent manner (Figure 1). Greater than 90% of these non-adherent

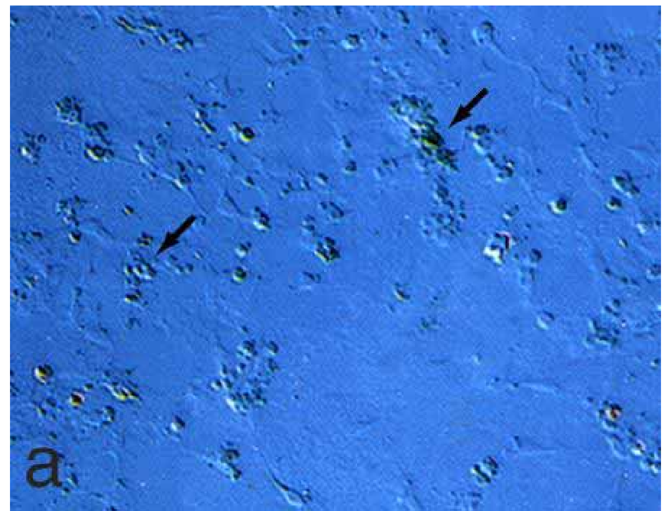

Figure 4a. Morphology of TUNEL-positive cells. Conditioned medium from death-induced cells led to a significant number of TUNEL-positive cells and apoptotic bodies, often in clusters (arrows). Cells were grown for 24 hr.

Figure 4b. Cells grown in serum-containing medium contained virtually no apoptotic cells. Cells were grown for 24 hr.

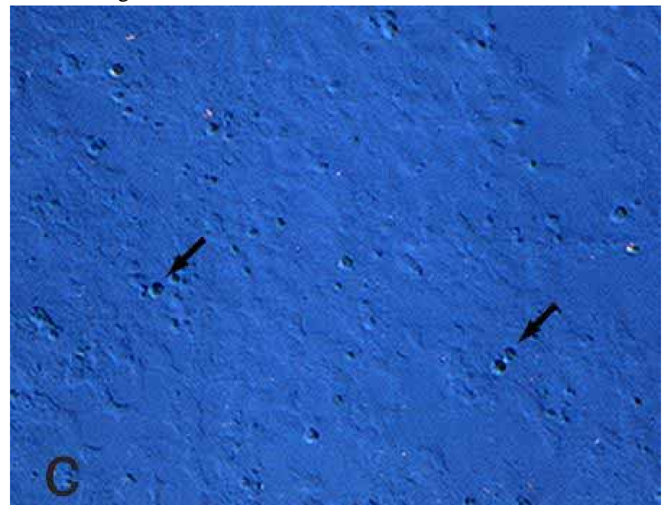

Figure 4c. TUNEL positive cells were present to a lesser degree in serumless/defined medium (arrows) than in death-induced cell conditioned medium. Cells were grown for 24 hr.

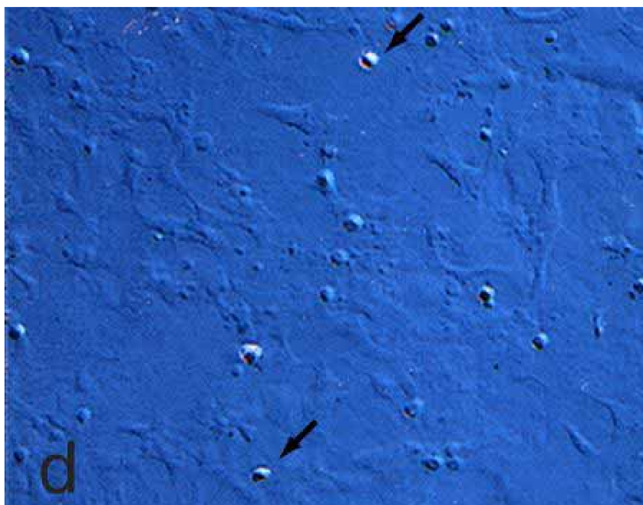

Figure 4d. TUNEL positive cells were present to a lesser degree in heat-inactivated conditioned medium (arrows) than in serum-containing medium. Cells were grown for 24 hr.

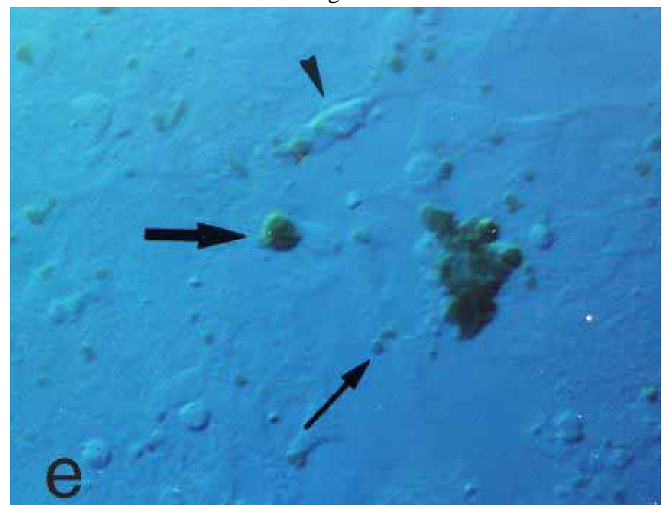

Figure 4e. A higher magnification of cells grown in the presence of death-induced cell conditioned medium to illustrate TUNEL positive (arrows) and negative (arrowhead) cells which were counted in Figure 3. Cells were grown for 24 hr.

cells appeared dead, as evaluated by trypan blue exclusion assay.

Conditioned medium-induced cell death is apoptotic, and not due to nutrient depletion— Further analysis was necessary in order to determine whether this cell death observed was due to apoptosis. In a non-quantitative experiment, DNA gel electrophoresis of adherent and non-adherent cell DNA demonstrated the non-random DNA laddering characteristic of apoptosis, except in the serum-containing control condition (Figure 2). It was also important to address the question of nutrient depletion as a contributing factor in conditioned-medium induced cell death. Therefore, additional conditioned medium was tested: 72 hr conditioned medium from the closely-related parental culture, E1A-NR.3, from which the R28 cell line was derived. Death-resistant E1A-NR.3 cells did not exhibit significant cell death during treatment with serumless/defined medium. Conditioned medium from non-dying cells was used as a control for nutrient depletion. 72 hr conditioned media from non-dying and death-induced cells were compared with serumless/defined conditions and standard serum-containing conditions. TUNEL-in situ analysis was then used to quantitate the number of adherent apoptotic cells. As can be seen in Figure 3, conditioned medium from death-induced cells caused the greatest degree of TUNEL positivity at 24 hr. Conditioned medium from non-dying cells caused cell death at levels comparable to fresh serumless/defined medium at 24 hr. In addition, heat inactivation (56° C for 45 min) appeared to reduce the apoptosis-promoting activity of death-induced cell conditioned medium to levels comparable with serumless/defined medium. (As an additional control, heat inactivation of fresh serumless/defined medium had no effect on the extent of apoptosis, also shown in Figure 3).

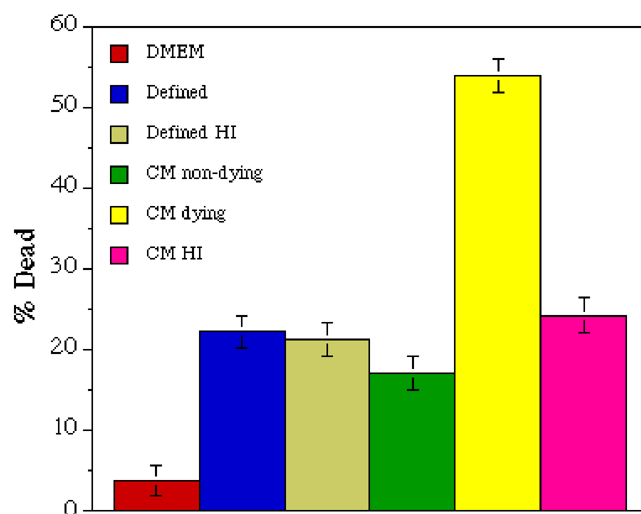

Figure 5. Analysis of Cell Death: Adherent & Non-Adherent Cells. The same conditions in Figure 3 were analyzed by trypan blue analysis to determine the percentage of dead cells in the entire population (to include both adherent and non-adherent cells). 72 hr conditioned medium was collected from death-induced and non-death-induced R28 cultures. Fresh R28 cultures were treated for 24 hr with serum-containing medium (DMEM); serumless/defined medium (Defined), heat-inactivated serumless/defined medium (Defined HI); conditioned medium from non-dying cells (CM non-dying); conditioned medium from death-induced cells (CM dying); and heat-inactivated conditioned medium from death-induced cells (CM HI). Error bars represent standard deviations.

Figure 4 illustrates the morphology of the TUNEL-positive cells counted in Figure 3. Conditioned medium from death-induced cells led to a significant number of TUNEL-positive cells and apoptotic bodies in R28 cultures within 24 hr (Figure 4a). This can be seen in comparison with the control cells in Figure 4b which contained virtually no apoptotic cells. TUNEL-positive cells were present at a reduced level in other cases, including serumless/defined (Figure 4c), and heat-inactivated conditioned medium (Figure 4d). In Figure 4e, a higher magnification shows that TUNEL positive cells included full-size cells, as well as smaller apoptotic bodies.

Loss of cell adherence in conditioned medium— It is clear from the fields shown in Figure 4a that there was significant loss of adherent cells in cultures treated with death-induced cell-conditioned medium, which might mask the extent of cell death shown in Figure 3, due to detachment of dying cells which escaped TUNEL analysis. We addressed this by revisiting the number of non-adherent cells floating in the medium, which were seen to be greater than 90% dead as measured by trypan blue analysis (Figure 1). The number of dead floating cells/ml in the death-induced cell conditioned medium was at least 3-fold greater than fresh defined medium. This 3-fold difference in the number of non-adherent dead cells was even greater than the two-fold difference in the number of TUNEL positive adherent cells between the two conditions. As seen in Figure 5, when measured by trypan blue analysis, the differences between the percentage of dead cells (total of both adherent and non-adherent) under test conditions remained comparable to the TUNEL analysis shown in Figure 3. Despite significant loss of cell adherence, the majority of cells remain attached in death-induced conditioned medium at the 24 hr timepoint.

DISCUSSION

From these results, it is evident that death-induced R28 cells generate an apoptosis-promoting activity, which is heat-labile. This activity does not appear to be solely due to nutrient-depletion of the medium, as the control situation (72 hr conditioned medium from closely related non-dying cells) does not produce the same effect. Heat inactivation would also suggest against nutrient depletion, and in favor of a conditioned medium component which is neutralized by heat.

What is the nature of the cell signalling which results in apoptotic cell death? Apoptosis-promoting activity measured in our system does not depend upon cell-cell contact, but would appear to be a diffusible component released by our death-induced retinal cultures. This could be in the form of a novel apoptosis-promoting factor, or alternatively, a toxic metabolite released by death-induced cells. In our paradigm, we cannot distinguish between the possibility that the apoptosis-promoting activity is released by dying cells, or by cells not yet dying in an attempt to clear dying cells from the system as a survival strategy (hence our reference to "death-induced cell

conditioned medium"). In either case, information about the triggers of retinal cell apoptosis would have many health-related implications. In human retinal degenerative disease, apoptosis is directly linked to retinal cell death as it occurs in both retinal detachment (7) and retinitis pigmentosa (15). Apoptosis is also strongly suspected as the cause of photoreceptor cell loss in other blinding, degenerative retinal diseases, including diabetic retinopathy (16,17) and macular degeneration (18). A better understanding of the triggers of retinal cell apoptosis may help identify cellular pathways and signals which would aid in the development of therapeutic strategies for all of these retinal degenerative diseases.

In a fibroblast cell culture system (19,20), intercellular induction of apoptosis was seen in transformed fibroblasts when exposed to conditioned medium from TGF-β-treated non-transformed fibroblasts. In that paradigm, apoptotic activity was blocked by γ-irradiation, colchicine treatment, and thymidine treatment. In those studies, apoptosis-promoting activity was also seen to be diffusible, but dependent upon proliferation of the TGF-β-treated non-transformed cells. In our system, apoptosis-promoting activity appears to be diffusible, yet released by metabolically challenged, death-induced retinal cells.

Naturally, much more work needs to be done in order to identify the precise nature of this inducible apoptosis-promoting activity in retinal cell line-conditioned medium. Results from these and future studies may offer insight into cell-mediated signals of apoptosis.

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REFERENCES

1. Gregory CY, Bird AC. Cell loss in retinal dystrophies by apoptosis—death by informed consent! Br J Ophthalmol 1995; 79:186-190.

2. Young RW. Cell death during differentiation of the retina in the mouse. J Comp Neurol 1984; 229:362-373.

3. Chang GQ, Hao Y, Wong F. Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. Neuron 1993; 11:595-605.

4. Portera-Cailliau C, Sung CH, Nathans J, Adler R. Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. Proc Natl Acad Sci U S A 1994; 91:974-978.

5. Smith SB, Bora N, McCool D, Kutty G, Wong P, Kutty RK, Wiggert B. Photoreceptor cells in the vitiligo mouse die by apoptosis. TRPM-2/clusterin expression is increased in the neural retina and in the retinal pigment epithelium. Invest Ophthalmol Vis Sci 1995; 36:2193-2201.

6. Wong P. Apoptosis, retinitis pigmentosa, and degeneration. Biochem Cell Biol 1994; 72:489-498.

7. Chang CJ, Lai WW, Edward DP, Tso MO. Apoptotic photoreceptor cell death after traumatic retinal detachment in humans. Arch Ophthalmol 1995; 113(7):880-886.

8. Cook B, Lewis GP, Fisher SK, Adler R. Apoptotic photoreceptor degeneration in experimental retinal detachment. Invest Ophthalmol Vis Sci 1995; 36:990-996.

9. Seigel GM, Mutchler AL, Adamus G, Imperato-Kalmar EL. Recoverin expression in the R28 retinal precursor cell line. In Vitro Cell Dev Biol Anim 1997; 33:499-502.

10. Seigel GM. Establishment of an E1A-immortalized retinal cell culture. In Vitro Cell Dev Biol Anim 1996; 32:66-68.

11. Seigel GM, Mutchler AL, Imperato EL. Expression of Glial Markers in a Retinal Precursor Cell Line. Mol Vis 1996; 2:2 <http://www.emory.edu/molvis/v2/seigel>.

12. Seigel GM, DiPaola M, Imperato-Kalmar EL. Apoptosis of retinal precursor cells in vitro. Keystone Symposium, Durango, CO, January 1997.

13. Mandarino LJ. Current hypotheses for the biochemical basis of diabetic retinopathy. Diabetes Care 1992; 15:1892-1901.

14. Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. J Clin Invest 1996; 97:2883-2890.

15. Lane SC, Jolly RD, Schmechel DE, Alroy J, Boustany RM. Apoptosis as the mechanism of neurodegeneration in Batten's disease. J Neurochem 1996; 67:677-683.

16. Chihara E, Matsuoka T, Ogura Y, Matsumura M. Retinal nerve fiber layer defect as an early manifestation of diabetic retinopathy. Ophthalmology 1993; 100:1147-1151.

17. Schellini SA, Gregorio EA, Spadella CT, Machado JL, de-Moraes-Silva MA. Muller cells and diabetic retinopathy. Braz J Med Biol Res 1995; 28:977-980.

18. Xu GZ, Li WW, Tso MO. Apoptosis in human retinal degenerations. Trans Am Ophthalmol Soc 1996; 94:411-430.

19. Bassler D, Brauns HD, Bauer G. Role of proliferation for intercellular induction of apoptosis. Exp Cell Res 1997; 232:349-352.

20. Langer C, Jurgensmeier JM, Bauer G. Reactive oxygen species act at both TGF-beta-dependent and -independent steps during induction of apoptosis of transformed cells by normal cells. Exp Cell Res 1996; 222:117-124.

Erratum— 30 Dec 1997: In the caption of Figure 4c, the original sentence: "TUNEL positive cells were present to a lesser degree in serumless/defined medium (arrows) than in serum-containing medium." was corrected to "TUNEL positive cells were present to a lesser degree in serumless/defined medium (arrows) than in death-induced cell conditioned medium."