Survival and Differentiation of Purified Retinal Ganglion Cells in a Chemically Defined Microenvironment

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Purpose. To develop a fully defined medium and substratum that will support the survival and differentiation of purified retinal ganglion cells (RGCs) from newborn rats, and to evaluate beneficial effects of various hormones.

Methods. RGCs were purified from papain-dissociated retinal cells by a two-stage panning method and cultured in a fully defined medium on a substratum precoated with polyornithine and laminin. RGC purity was assessed by prelabeling RGCs with retrogradely transported horseradish peroxidase and by anti-Thy-1 immunocytochemistry. Various hormones were evaluated for their ability to promote RGC survival by adding them to the culture medium.

Results. Peroxidase labeling yielded purity estimates between 96% and 100%. Within 2 days, two distinct cell types are easily identified: a small cell characterized by thin, unbranched neurites and a large cell characterized by extensively branched neurites. Most small and large cells were round; however, some had elongated cell bodies. Both small and large cells express Thy-1 on their surfaces. Size-histogram analysis of all cells yielded a bimodal distribution. Survival was enhanced by seeding at higher densities and by the addition of hydrocortisone and progesterone to the cultures.

Conclusions. This defined culture system should facilitate further studies on the direct mechanisms regulating retinal ganglion cell survival and differentiation. Invest Ophthalmol Vis Sci. 1994; 35:3640-3648.

Retinal ganglion cell (RGC) atrophy is an important component of the biologic vision loss in several conditions, including glaucoma and Alzheimer’s disease. However, the specific mechanisms associated with this atrophy are poorly understood. Retinal explant cultures and mixed retinal cell cultures containing RGCs have been used to demonstrate a beneficial influence of glial cells for RGC survival, as well as the role of extracellular matrix and adhesion molecules.1-4 However, it is unknown whether these effects were primary responses or were secondary to primary responses of the other cell types present in these culture systems. For example, the differentiation of photoreceptors in mixed retinal cell cultures has been shown to be critically influenced by other retinal cell types also present in the culture.5 Additional uncertainty regarding specific responses of RGCs in these studies arises from the use of undefined biologic fluids, such as serum, in these culture systems.6,7 Hence, a fully defined culture system containing purified mammalian retinal ganglion cells would be useful because it would overcome these limitations.

Recently, a two-step cell-panning procedure was described that isolates RGCs from rat retina.8 Retinal ganglion cell purity in these preparations was greater than 99%. Similar purities using this technique were obtained by a second group of investigators.9 Using this method, purified RGCs have been maintained in vitro in a medium conditioned by exposure to L2 cells.10 Preliminary studies suggest that the L2 cell effect might be mediated by an 18 kD heparin-binding protein.10 Purified RGCs have also been maintained in serum-containing medium.9 The agent(s) in fetal bovine serum necessary for RGC survival have not been identified.
In the present study, we describe a fully defined medium and substratum treatment that supports survival and differentiation of purified RGCs without prior conditioning or the addition of undefined agents. Using this system, we show that RGC survival is promoted by hydrocortisone, progesterone, and insulin. This experimental system should facilitate new investigations into response mechanisms of RGCs to signals in their microenvironment.

MATERIALS AND METHODS

Retinal Ganglion Cell Purification Method

The use of animals in this work was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Newborn Sprague-Dawley rats were killed by quick decapitation, and RGCs were purified from the papain-dissociated retinas as previously described. Briefly, the suspension was first placed in a 100-mm petri dish coated with anti-rat macrophage antibodies (Accurate, Westbury, NY). Cells that did not bind to this dish were transferred to a second dish coated with anti-Thy-1.1 antibodies (from hybridoma T11D7e2; American Type Culture Collection, Rockville, MD). Cells that did not bind to the second dish were removed by repeated washing with Hanks buffered saline, and the bound cells were eluted using 0.125% trypsin. Forty to 50 retinas were used in each experiment. RGC yields typically varied between 0.7% and 1.3% of the original cell number (0.99% ± 0.26%, mean ± SEM; n = 32 purifications).

Assessment of Purification Efficiency

Newborn rat pups were anesthetized using hypothenia as described by Phifer and Terry, and 30% aqueous horseradish peroxidase (type VI; Sigma, St. Louis, MO) within a glass micropipet was injected subdurally at 12 to 16 sites across the exposed superior colliculi surface using a Picospritzer II microinjector (General Valve, Fairfield, NJ). Injections were restricted to the superior colliculus because pilot attempts to inject other retino-recipient nuclei in neonates were often fatal. Seven to 10 pups were injected for each experiment. After 4 days, to allow for attachment, the cultures were seeded in triplicate at 550 cells/mm². Cultures were incubated at 37°C in a humidified atmosphere containing 10% CO₂ and 90% air. Cell cultures were terminated by fixation with 37°C in a humidified atmosphere containing 10% CO₂ and 90% air. Cell cultures were terminated by fixation with 2% formaldehyde in phosphate-buffered saline.

Quantitative Evaluation of Cell Survival

Cell survival was assessed by counting the number of cells present in 10 successive 200× microscope fields using phase optics (total assay area = 0.306 mm²). Each culture condition in each experiment was replicated in triplicate. Means and standard deviations of the scores were calculated. Where statistical comparison was made between two experimental conditions, the unpaired Student's t-test was used. Multiple comparisons among several conditions were made using the Student–Newman–Keuls test.

Immunocytochemical Characterization

After fixation with 2% formaldehyde, the cultures were washed with phosphate-buffered saline, treated with 10% goat serum to block nonspecific staining, and incubated with monoclonal anti-Thy-1.1 (TN-26; Sigma; diluted 1:100) for 90 minutes. The cultures were then washed, exposed to rhodamine-conjugated goat anti-mouse IgG–IgM (Cappel, Durham, NC; diluted 1:40), washed, and mounted in gelvatol.

Size–Shape Histogram Analysis

Cultures were seeded in triplicate at 550 cells/mm² and fixed after 2 days in vitro. Using a video camera (NC 670-M; Dage–MTI, Michigan City, IN), the phase image of the cultured cells was projected onto the
screen of a video monitor (VM 4512A; Sanyo Electric, Compton, CA). A stage micrometer was used to determine that the final magnification was 840X. The dimensions of the first 300 cells in each well as they appeared on the screen of the video monitor were measured using an electronic digital caliper (Digimatic CD-6B; Mitutoyo, Tokyo, Japan). This caliper can measure accurately to ±0.02 mm. For round cells, the diameter was measured. For elongated cells, both the long- and short-axis dimensions were obtained. Means and ratios of these long- and short-axis measurements were determined.

Based on the magnification factor, the screen measurements were converted to the actual size of the cells in micrometers. The resulting cell dimensions were sorted into histogram bins incremented by 1.0 μm, using Excel 4.0 (Microsoft, Seattle, WA). Means and standard deviations of the triplicate scores for each bin were calculated. This experiment was repeated twice.

Differentiation Stability Assessment

The culture surface in 16-mm culture wells was lightly scored with an asymmetric grid pattern using a sharpened tungsten needle and then coated with polyornithine and laminin. After seeding purified RGCs at 550 cell/mm², specific fields within the cultures were photographed once every 12 hours during the second, third, and fourth days after seeding, and once per day on the fifth through the ninth days after seeding. The score marks guided the return to the same fields at each photography session. Total area photographed was 61,000 μm². Each cell photographed at each time point was scored as round phase-bright cell, small cell, large cell, or dead. Round phase-bright cells were also present in the cultures.

FIGURE 1. Phase image of purified RGCs from neonatal rats after 8 hours (A), 2 days (B), 4 days (C), and 11 days (D) in vitro. Two distinct cell types were observed: a small cell (S) with a phase dark cell body and typically with one or two short, thin, unbranched neurites; and a large cell type (L) with a visible nucleus and branched neurites that eventually can become extensive. Dead large cells (D) can be recognized by their granular cytoplasm. Magnification, X260. Bar = 40 μm.

The neurites on the small cells typically were thin and numbered one or two. Large cell neurites were thicker than those extending from the small cells, were occasionally branched, and were often tipped by large growth cones.

On the fourth day in vitro, the neurites on the large cells often were thicker, more branched, and longer (Fig. 1C). The small cells, for the most part, still had one or two thin neurites. By day 11, many of the surviving large cells had developed numerous long branched neurites (Fig. 1D). The small cells in the 11-day-old cultures typically had three or four unbranched neurites.

Purity of Isolated Retinal Ganglion Cells

After injection of the superior colliculus with horse-radish peroxidase and the subsequent isolation of RGC using the two-stage panning method, it was observed that 86.53% ± 3.47% of the cells contained...
defined microenvironment for retinal ganglion cells

Figure 2. Bright-field image of RGCs purified from a 4-day-old rat that had received injections of horseradish peroxidase into the superior colliculus 1 day after birth. These cells were maintained in vitro for 4 hours and then were reacted with peroxide and diaminobenzidine. Magnification, ×375. Bar = 40 μm.

demonstrable peroxidase activity (n = 6; Fig. 2). Immunocytochemistry showed that both large and small cells in 2-day-old cultures were uniformly labeled with anti-Thy-1.1 antibodies (Fig. 3).

Cell Survival

Cell survival was assessed in parallel triplicate cultures generated from the same suspensions that were seeded at 250 cells/mm² and 500 cells/mm². Parallel cultures from this experiment were fixed 8 hours, 24 hours, 2 days, and 4 days after initiation. In both the low-density and the high-density cultures, the number of cells bearing neurites increased to 124 ± 19 cells/mm² and 300 ± 62 cells/mm², respectively, during the first 2 days. Between the second and fourth days in vitro, neurite-bearing cell densities declined to 72 ± 17 cells/mm² and 143 ± 62 cells/mm² (Fig. 4A).

During the first 2 days, the large neurite-bearing cells were approximately 70% of the total neurite-bearing cells (Fig. 4B). This proportion dropped to approximately 60% after 4 days in vitro. The differences between the mean proportions of large cells in the low- and high-density cultures were insignificant at each of the four time points examined.

Size–Shape Histogram Analysis

Cell measurements of the first 300 cells in each of 3 wells incubated for 2 days in vitro showed that 30% ± 6% of the cells were small cells and 70% ± 7% were large cells, confirming the results described above. Overall, 82 ± 8% of these cells were round and 18% ± 5% of the cells were elongated. Specifically, the proportions of round–small, elongated–small, round–large, and elongated–large cells were 25 ± 5%, 4 ± 1%, 57 ± 3%, and 14 ± 4%, respectively. The ratio of the long axis to the short axis was less than 3.1, within 74 ± 11% of the small elongated cells and within 71 ± 15% of the large elongated cells.

Histogram analysis showed bimodal distributions among both the round and the elongated cells (Fig. 5). The peaks for the round cells were at 4.5 and 7.5 μm, and the peaks for the mean elongated cell dimensions were at 3.5 and 8.5 μm. The overlap in the dimension distributions of the small and large round cells was 3.2 ± 2.2%. In the case of elongated

Figure 3. (A) Immunocytochemical staining of 2-day-old cultures using anti-Thy-1.1 antibody. Note that both the large and small cells are labeled. (B) Control prepared without primary antibody. Magnification, ×600. Bar = 20 μm.
FIGURE 4. (A) Density of neurite-bearing RGCs that have extended at least one neurite in cultures fixed during the first 4 days in vitro. Parallel cultures were seeded at 250 cells/mm² and 500 cells/mm² from the same cell suspension, and triplicates from each density group were fixed 8 hours, 1 day, 2 days, and 4 days later. The proportion of total neurite-bearing cells that were large cells is shown in (B). Similar results were obtained in a duplicate experiment.

3. Differentiation Stability in Long-Term Cultures

In view of the ongoing maturation and cell death in these cultures, it is possible that cells switch from one category to the other either as they mature or as they die. To investigate this possibility, specifically identified microscope fields were repeatedly photographed between 2 and 9 days after cultures were seeded. These photographs were compared to determine if any cell had switched from small to large or vice versa. The ease of identifying specific cells in the photographic series is illustrated in Figure 6.

On the second day in vitro, 56 of 148 cells were large cells, 44 were small cells, and the remainder were round-phase bright cells. During the observation period, three of the round phase-bright cells became large cells and seven became small cells. Thirty-eight cells either remained round-phase bright cells throughout the observation period or changed from round phase-bright cells to dead cells without differentiation. During the observation period, 28 (47%) of the large cells died and 18 (35%) of the small cells died. None of the cells that became large cells was found to change from large cell to small cell, nor did any small cell become a large cell. In addition, dead cells were the same size or larger than when they were alive (Figs. 6B and 6C).

4. The Role of Hormones

The 2-day survival of RGCs in medium containing progesterone, insulin, and hydrocortisone was compared to parallel cultures in various media in which one or more of these hormones was not present. Of all the media considered, the survival rate was greatest in the medium that contained all three hormones (Fig. 7A). The decrease of survival in medium lacking insulin was not significant. In contrast, survival in media lacking progesterone, hydrocortisone, or both progesterone and hydrocortisone was significantly less than in media where both these hormones were present. The small and large cells appear to have responded similarly in these experiments, because the minor differences in the proportion of large cells among the surviving neurite-bearing cells in each of the experimental media were not significant (Fig. 7B).

5. DISCUSSION

The in vitro results show that purified RGCs can survive and differentiate in a fully defined microenvironment. Within the first 48 hours, differentiating cells can be categorized as either small phase-dark cells with a few unbranched neurites or large phase-medium cells with several branched neurites. These characteristics appear to be stable because small cells were not observed to become large cells during repeated examinations of the same microscope fields between 2 and 11 days in vitro, nor were large cells observed to become small cells. Thus, the increase in neurite-
FIGURE 5. Distributions of the diameters of small and large round cells, as well as of the means of short- and long-axis measurements of small and large elongated cells expressed as percentage of total cells. Data represent means and standard deviations of measurements from 300 cells analyzed in each of three parallel cultures.

bearing cells over the first 2 days in vitro most likely reflects cells with various delays in the initial production of neurites. In contrast, the decline in such cells between days 2 and 4 most likely reflects the gradual cell death in the cultures.

A high purity of RGCs obtained in the present study is indicated by our observation that 86.5% ± 3.5% of cells purified from retinas prelabeled by horseradish peroxidase injections into the superior colliculus contained the label. Previous studies have shown that among albino rat retinal ganglion cells that project to either the superior colliculus or the lateral geniculate nucleus, 12.1% of the contralaterally projecting RGCs and 15.6% of the ipsilaterally projecting RGCs do not project to the superior colliculus.15,16 The ratio of uncrossed to crossed visual fibers is approximately 10:1,17 and the proportion of RGCs projecting to the accessory optic system is approximately 1.5%.18 Thus, the proportion of RGCs with projections to the superior colliculus in the adult rat can be estimated to be approximately 86% ((1 - 0.15)(1 - [(0.09)(0.156) + (0.91)(0.121)]) = 0.86). Adjusting the calculations for the additional 35% loss of ipsilaterally projecting RGCs during developmental cell death19 shifts the estimate to 85%. Because a small population of neonatal rat RGCs have retino-retinal projections, and because these are eliminated during developmental cell death,20,21 it is possible that this figure is a slight overestimate. Hence, our observation that 86.5% ± 3.5% of purified cells contain horseradish peroxidase after superior colliculus injections suggests that RGC purity in the present study is between 96% and 100%. This is further supported by our observation of uniform staining of the cultured cells by anti-Thy-1 immunocytochemistry22 and is consistent with the observations of others who have previously assessed this method.8,9

The bimodal distributions of purified RGC dimensions and the minimal overlap in these distributions supports the view that small and large cell types represent distinct RGC subsets. Interestingly, the differentiation of the purified ganglion cells in the present study is similar to that of similarly purified RGCs seeded on polylysine and laminin and maintained in a serum-containing medium.9 Within the first 44 hours, it was observed that large cells produced several short neurites. Although it was not noted, examination of Figure 5 in this report shows both small and large cells. The small and large neurons were simultaneously immunopositive for Thy-1 and neurofilament protein. Because only RGCs among retinal neurons express both Thy-1 and neurofilament protein,22,23 this provides additional support that both the small and large cells in the present study are RGCs.

In 3-day-old rat retinas impregnated by the Golgi cell staining method, the RGCs are readily grouped into smaller unistratified cells and larger type I/II cells.24 More recent studies using di-I labeling of 1-day-old rat RGCs classified cells into three groups, based on their structure. Two groups contained cells with mean somatic diameters of approximately 12 μm, and one contained cells with a mean somatic diameter of 14.3 μm.25 Thus, the small and large cells seen in the purified RGC cultures may correspond to the smaller and larger RGCs observed in intact newborn rat retinas. Similarly, the presence of some RGCs in vitro with elongated cell bodies is consistent with the observation that a portion of RGCs in vivo have elongated cell bodies.26 Further investigation will be required to confirm these possible associations.
During the first few days in vitro, the extension of neurites is relatively limited. However, after a week in vitro, the neurites of the large cells often become long and branched. Although the final appearance is the same, this represents slower growth than has been observed in rat RGC neurites extending from retinal explant cultures. The result raises the possibility that the RGCs alter their microenvironment in the cultures. The survival study results support this view because doubling the density of neurons seeded in the cultures from 250 cells/mm² to 500 cells/mm² resulted in a threefold increase in survival at day 2 in vitro. A possible candidate for such modifications is TAG-1, a molecule that appears to be secreted by growing axons and to promote axonal growth. Further work will be required to assess such possible modifications.

The observation that elimination of insulin has a minimal effect on purified RGC survival is striking in light of the observation that it appears to promote survival and differentiation of many central and peripheral neuron types in vitro. RGC survival under certain circumstances is critically dependent on coculturing with glial cells and, because the supportive effect of the glial cell can be modulated by insulin, it is possible that insulin, or an insulin-like growth factor, facilitates RGC survival in vivo indirectly.
through neighboring glial cells. Recent evidence suggests that this may be the case for spinal motor neurons and hypothalamic neurons. However, a direct effect of insulin on RGCs cannot be ruled out because insulin receptor has been identified in monkey RGCs in situ. In addition, although the concentration of insulin tested was several times higher than that of the insulin receptor in other cell systems, the minimal effect may reflect rapid degradation of insulin in the medium.

The dramatic promotion of RGC survival in response to hydrocortisone and progesterone is interesting because the response of other neurons to steroids has been reported to be variable. Depending on the neuron type, glucocorticoids have been observed either to facilitate survival in the presence of stress or to mediate neuronal cell death. In vivo, elevated systemic progesterone has been linked with increased motoneuron survival after facial nerve damage. Thus, it will be of interest to determine in future studies if these steroids merely facilitate survival during trauma associated with retinal dissection to generate the cultures or if there is a continued requirement for steroids in long-term cultures.

In conclusion, the present experimental system should facilitate future investigations into the role of molecular signals within the cellular microenvironment in the survival and differentiation of RGCs. The availability of purified cells also should facilitate biochemical analysis of signal transduction and possible gene activation, as well as elucidation of the mechanism of glial-mediated contributions to survival. Such experiments may provide new leads for unraveling the mechanism of retinal ganglion cell death in glaucoma and other degenerative conditions.

**Key Words**
retinal ganglion cell, cell purification, cell culture, defined microenvironment, glaucoma

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**References**


