Effects of Retinal Glial Cells on Isolated Rat Retinal Ganglion Cells

Kenji Kashiwagi,1 Yoko Iizuka,1 Makoto Araie,2 Yasuyuki Suzuki,2 and Shigeo Tsukabara1

PURPOSE. The effect of retinal glial cells on retinal ganglion cell (RGC) survival was investigated in cocultures of pure, isolated retinal glial cells with pure, isolated RGCs.

METHODS. RGCs from 2-day-old rats were cocultured for 48 hours, avoiding direct contact between cell types, with either nonconfluent retinal glial cells from 3-day-old rats or confluent retinal glial cells from 3-day-old, 12-day-old, or 1-year-old rats. Survival of RGCs was evaluated by flow cytometry. Amino acids were determined in culture medium. The effects of glutamate antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione and MK801, a nitric oxide (NO) scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (c-PTIO), and an NO synthase inhibitor, N\(^{-}\)-nitro-L-arginine methyl ester (l-NAME), were examined.

RESULTS. Nonconfluent retinal glial cells significantly reduced the survival of small and large RGCs, but confluent retinal glial cells reduced the survival of only small RGCs, regardless of the rat's age at the time of retinal glial cell harvesting. Profiles of some amino acids significantly varied, depending on the culture condition. Cocultures of RGCs with nonconfluent retinal glial cells released significantly more glutamate into the medium than cocultures of RGCs with confluent retinal glial cells or RGCs in pure culture. The glutamate antagonists improved the survival of RGCs cocultured with nonconfluent retinal glial cells, especially when the two were administered in combination, and in the case of large RGCs. c-PTIO and l-NAME, also improved the survival of RGCs cocultured with nonconfluent retinal glial cells.

CONCLUSIONS. Adverse effects of retinal glial cells on the survival of RGCs varied by size of the RGCs and retinal glial cell confluence. Glutamate and NO may be involved in retinal glial cell-related antisurvival effects. (Invest Ophthalmol Vis Sci. 2001;42:2686–2694)

Retinal glial cells are involved in retinal development, protection, and damage of retinal neurons in both physiologic and pathologic situations. The reported effects of retinal glial cells on retinal ganglion cells (RGCs) in culture have been inconsistent among the previous reports. Kitano and Morgan1 and Kawasaki et al.2 reported that culturing RGCs with retinal glial cells significantly improves RGC survival. Heidinger et al.3 found that retinal glial cells prepared from adult rats alleviate cytotoxicity induced by excitotoxic amino acids, but that retinal glial cells prepared from immature rats have no effect on apoptotic retinal neuronal death. Tezel and Wax4 reported that tumor necrosis factor-alpha secreted from glial cells induces apoptosis in cocultured RGCs. Previous studies have investigated the interaction of retinal glial cells with RGCs, either in vivo or in various cultures of mixed retinal cells. Thus, interfering effects of apoptosis and necrosis may have been mediated by the presence of other types of cells. To our knowledge, few studies have quantitatively investigated the interaction of pure cultures of isolated retinal glial cells with pure cultures of isolated RGCs. We used recently established methods to isolate RGCs5 and retinal glial cells6,7 before preparing cocultures of these cells and analyzing RGC survival by flow cytometry.8

Certain amino acids can influence the activity of retinal glial cells and the survival of RGCs. In particular, glutamate exerts important effects on RGC survival. Under developmental or physiologic conditions, glutamate is involved in programmed neuronal death,9–10 while also acting as an excitatory neurotransmitter. Excessive glutamate at postsynaptic sites is thought to damage neurons in pathologic situations.11 Because retinal glial cells transport and metabolize glutamate, alternation in these functions could affect RGC survival. We therefore determined amino acid profiles in the medium of cocultures to examine possible association with RGC survival. We examined the effect of glutamate receptor antagonists on the survival of RGCs cocultured with retinal glial cells.

Nitric oxide (NO), identified as an endothelium-derived relaxation factor,12,13 plays important roles in the nervous system: long-term potentiation, long-term depression, cell proliferation, cell activation, and neuronal death.14 Several studies have indicated that NO in the retina is mainly produced by retinal glial cells.15,16 We investigated the role of NO in the survival of RGCs and in the interrelationship between RGCs and retinal glial cells.

METHODS

All experiments were conducted and all laboratory animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of RGCs

RGCs were isolated by using a previously described two-step panning method.3 Briefly, 2-day-old Sprague-Dawley rats were killed to obtain approximately 60 enucleated eyes for each experiment. Isolated retinas were incubated for 20 minutes in a solution containing papain (5 mg/ml), and dissociated cells were incubated for 5 minutes with rabbit anti-macrophage antibody (Inter-Cell Technologies, Hopewell, NJ). Cell suspensions were treated for 45 minutes in 100-mm petri dishes coated with goat antibody against rabbit IgG long (L) and heavy (H) chains (Southern Biotechnology Associates, Birmingham, AL). Suspensions containing the cells that did not adhere to the petri dish were treated for 1 hour in 100-mm petri dishes coated with anti-Thy 1.1 antibody (from hybridoma T11D7e2; American Type Culture Collection, Rockville, MD).

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2 Supported in part by Grant 11307036 from the Japanese Ministry of Education, Culture, Sports, Science and Technology (KK).

3 Submitted for publication March 27, 2001; revised June 11, 2001; accepted July 6, 2001.

4 Commercial relationships policy: N.

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Cells that adhered to the second petri dish were collected after separation by a 10-minute incubation with 0.125% trypsin and were incubated in 24-well plates.

**Culture Conditions**

Culture plates were coated with 0.1 mg/ml polyornithine (Sigma Chemical, St. Louis, MO) for at least 5 hours. Plates then were additionally coated overnight with 5 μg/ml Engelbreth-Holm-Swarm (EHS)-laminin (Upstate Biotechnology, Lake Placid, NY). Medium developed by Politi et al. for monolayer culture of mixed mouse retinal neurons, as modified for use in this experiment, consisted of Dulbecco’s modified Eagle’s medium (DMEM) with the additions of insulin (1.6 × 10^{-6} M), progesterone (4.0 × 10^{-8} M), selenite (6.0 × 10^{-8} M), transferrin (12.5 × 10^{-6} M), putrescine (2 × 10^{-4} M), hydrocortisone (1.0 × 10^{-7} M), cytidine-5’-diphosphocholine (5.2 × 10^{-6} M), cytidine-5’-diphosphoethanolamine (2.9 × 10^{-6} M), 40 ng/ml each of brain-derived neurotrophic factor and ciliary neurotrophic factor, and 5 μM forskolin. Seeding density was 2 × 10^5 cells/well. Cells were incubated at 37°C in humidified 5% CO₂ and 90% air.

**Identification of Isolated RGCs**

Retrograde Transportation of Tracer. In accordance with previous reports, the retrograde fluorescence tracer 1,1’-diocadecyl-3,3’,3’-tetramethylindocarbozyanine perchlorate (DiI, catalog no. D282; Molecular Probes, Eugene, OR) was used in this experiment. Briefly, eight one-day-old rats were anesthetized by hypothermia, as described by Phifer and Terry. Diffused in dimethylformamide was injected subdurally at 16 to 20 sites covering all areas of the superior colliculi. After 4 days, RGCs from 14 of the 16 eyes were purified and seeded in culture, as described earlier. The two remaining DiI-injected eyes were used to prepare wholemount retinal tissues to ensure that transport into the RGCs of the injected DiI proceeded in a retrograde manner, as previously reported. Wholemount retinal tissue specimens and isolated RGCs were observed using a confocal microscope (TCS-4D; Leika Microsystems, Wetzlar, Germany).

Immunocytochemical Identification of Isolated RGCs. The primary antibody used for the immunocytochemical study was mouse anti-Cd90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells. The primary antibody used for the immunocytochemical study was mouse anti-CD90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells. The primary antibody used for the immunocytochemical study was mouse anti-CD90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells. The primary antibody used for the immunocytochemical study was mouse anti-CD90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells. The primary antibody used for the immunocytochemical study was mouse anti-CD90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells. The primary antibody used for the immunocytochemical study was mouse anti-CD90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells. The primary antibody used for the immunocytochemical study was mouse anti-CD90 IgG antibody (catalog no. MRC OX-7l; Serotec, Oxford, UK). A staining kit (Vestacat ABC-AP Kit Vector Red SK-5100; Vector, Burlingame, CA) was used to visualize immunoreacted cells with the primary antibody. Briefly, isolated RGCs from 2-day-old rats were placed on a poly-D-lysine- and laminin-coated coverslip (Biocoat Vector, Burlingame, CA) was used to visualize immunoreacted cells.
scribed in the previous report. In brief, the RGCs were divided into four groups according to the intensity of their fluorescence and in regard to cell size: surviving small RGCs and large RGCs, nonsurviving small RGCs and large RGCs. A current flow cytometer system counted the number of cells in each group and automatically calculated the survival ratio of small RGCs and large RGCs.

**Effects of Retinal Glial Cells on RGCs**

To clarify the effects of retinal glial cells on RGCs relative to confluence and rat age at the time of harvesting retinal glial cells, several combinations were prepared: RGCs with nonconfluent retinal glial cells from 3-day-old rats; RGCs with confluent retinal glial cells from 3-day-old, 12-day-old, and 1-year-old rats; and RGCs with blank membrane inserts as control samples.

**Amino Acid Analysis**

Media from the above experiments were collected and kept at −80°C until analysis. To prevent proteins in the samples from interfering with analysis, 5% sulfosalicylic acid was added. Samples were centrifuged at 3000 rpm for 15 minutes, and 20 amino acids were identified in the supernatants by an amino acid analyzer (JLK-500V; Nippon Denshi, Tokyo, Japan), using high-performance liquid chromatography. Because the culture medium contained several amino acids, levels of pre-existing amino acids in the medium were subtracted from raw levels to arrive at levels of cell-releasing amino acids. Because the present assay allowed only the analysis of amino acids in medium from cocultures of retinal glial cells and RGCs without cell–cell contact between the two types of retinal cells, to investigate the changes of amino acids when retinal glial cells and RGCs were cultured under cell–cell contact, retinal glial cells from 3-day-old rats and RGCs sequentially seeded into the bottom of the same 24-well dish, and culture media were subjected to amino acid analysis.

**Effects of Glutamate Antagonists on Cocultured RGCs with Retinal Glial Cells**

An α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-kainic acid (AMPA-KA) receptor-selective antagonist, 30 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an N-methyl-D-aspartate (NMDA) receptor antagonist MK801 (10 μM), or a combination of both was added to the coculture medium. In this experiments nonconfluent retinal glial cells were used. In the control cultures, only the vehicle for CNQX and MK801 was added to the medium of pure cultures of RGCs. According to the results of amino acid profile study, 100 μM glutamate or 100 μM alanine were added to the medium of cocultures of RGCs and confluent retinal glial cells from 3-day-old rats. RGC survival and the duration of evaluation were assessed.

**Statistical Analysis**

ANOVA and the post hoc method were used to compare results of different concentrations of administered compounds. The Mann-Whitney test was used to compare RGC survival rate and amino acid concentrations under various conditions. Significant differences were defined by \( P < 0.05 \). All data are expressed as mean ± SD.

**RESULTS**

**Morphologic Observation and Identification of Isolated RGCs**

Isolated and cultured RGCs were classified morphologically into two subsets: relatively small, round cells and large, spindle-shaped cells. (a) Both small (arrowhead) and large (arrow) isolated RGCs show a high immunopositive rate against anti Thy-1 antibody. (b) No RGCs were stained in the negative control incubated with PBS instead of primary antibody. (c) RGCs in wholemount retinal tissue are incorporated with Dil. (d) Most isolated RGCs incorporated Dil, transported in a retrograde manner as a red-orange hue in their cytoplasm. Scale bar, 10 μm.
Morphologic Observation and Immunohistochemical Identification of Isolated Retinal Glial Cells

The primary culture represented a mixture of retinal glial cells and other cell types. By the third passage, the appearance of the cultured cells became homogenous, and this appearance was sustained through the fifth passage. More than 95% of the third- or fourth-passage cells were immunoreactive with two retinal glial cell-specific antibodies, glutamine synthetase and carbonic anhydrase II. Anti-glutamine synthetase antibody labeled nuclei and the perinuclear cytoplasm. Anti-carbonic anhydrase II antibody showed labeling similar to anti-glutamine synthetase antibody, but this antibody labeled mainly within the nuclei. Anti-GFAP produced either no staining or very faint staining. Therefore, we used third- and fourth-passage cells in the present study. Retinal glial cells seeded on the nitrocellulose inserts reached 60% to 70% confluence in a 48-hour experimental culture.

Analysis Using Flow Cytometry

The seeding density of pure cultures of RGCs was approximately $2 \times 10^5$/well, and the duration to complete evaluation of 10,000 RGCs was approximately 3 minutes. No significant difference in cell population at the time of harvesting and duration for evaluation was observed among samples, except for those RGC cultures in which a high dose of glutamate was added to the culture medium. Two sizes of RGCs were observed by flow cytometry. The ratio of small to large RGCs was approximately 4 to 1 in the control group, similar to that observed microscopically. The fluorescent assay showed very stable fluorescence intensity in all experiments. The RGCs were therefore classified according to the intensity of fluorescence and cell size.

Effects of Retinal Glial Cells on RGCs

The presence of nonconfluent retinal glial cells significantly reduced RGC survival (Fig. 2). Survival of small RGCs was reduced by 13.1% ($P < 0.01$), whereas survival of large RGCs was reduced by 5.9% ($P < 0.01$). The presence of confluent retinal glial cells isolated from 3-day-old, 12-day-old, and 1-year-old rats reduced small RGC survival significantly by 17.4%, 17.0%, and 18.4%, respectively ($P < 0.01$). However, the presence of confluent retinal glial cells obtained from rats of any age did not significantly influence the survival of large RGCs.

Effects of CNQX and MK801

CNQX or MK801 improved the survival of small RGCs by 18.3% and 35.5%, respectively, and improved survival of large RGCs by 45.6% and 19.1%, respectively (Fig. 3). No significant difference in survival enhancement was observed between large and small RGCs with either of the drugs, and the increases in surviving cells fell short of significance. When CNQX and MK801 were administered simultaneously in the culture medium, RGC survival improved more than with administration of either antagonist alone. Although the survival rates of large RGCs improved significantly by 82.4% ($P < 0.01$), survival improvement for small RGCs was only 52.7% ($P = 0.07$).

Amino Acid Profile

The amino acid profile is summarized in Table 1.

Glutamate. The glutamate concentration in medium from coculture of RGCs with nonconfluent retinal glial cells was $288.6 \pm 61.9$ μM, which was significantly higher than the glutamate concentration in media from the coculture of RGCs with confluent retinal glial cells (25.1 $\pm 8.1$ μM), from pure RGCs (19.9 $\pm 3.0$ μM), from pure confluent retinal glial cells (25.1 $\pm 8.1$ μM), from pure RGCs (19.9 $\pm 3.0$ μM), from pure confluent retinal glial cells

![Figure 2. Effects of retinal glial cells on RGC survival. The RGC survival rate is depicted separately for small and large RGCs. Nonconfluent retinal glial cells significantly reduced the survival of small and large RGCs, but confluent glial cells significantly reduced the survival of small RGCs only. The host rat’s age did not influence RGC survival among the cocultures from 3-day-old, 12-day-old, and 1-year-old rats, conf., confluent; nonconf., nonconfluent. *$P < 0.01$, Mann-Whitney test; bar, SD.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933591/ on 06/24/2017)

![Figure 3. Effects of CNQX and MK801 on RGC survival. CNQX or MK801 partially alleviated the deterioration of RGC survival in the presence of nonconfluent glial cells. Concomitant administration of CNQX and MK801 significantly improved the deterioration of the survival of large RGCs in the presence of glial cells compared with the experiments with vehicle control and MK801. *$P < 0.01$, **$P < 0.05$, ‡$P = 0.07$, Mann-Whitney test; bar, SD.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933591/ on 06/24/2017)
### Table 1. Amino Acid Profile

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<th>Amino Acid</th>
<th>RGC + Nonconf. 3D G</th>
<th>RGC + Conf. 3D G</th>
<th>RGC + Conf. 12D G</th>
<th>RGC + G on the Same Plate</th>
<th>Conf. 3D G Only</th>
<th>Nonconf. 3D M Only</th>
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<td>Glutamate</td>
<td>19.9 ± 3.0</td>
<td>288.6 ± 61.9</td>
<td>25.1 ± 8.1</td>
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<td>Alanine</td>
<td>5.3 ± 2.8</td>
<td>190.4 ± 45.4</td>
<td>169.8 ± 44.0</td>
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<td>27.4 ± 16.2</td>
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<td>Lysine</td>
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Data are expressed as mean ± SD (n = 4). The profiles of 20 investigated amino acids in the culture medium are shown. Concentrations of glutamate and alanine were significantly changed by confluence with retinal glial cells or by host rat’s age. As a comparison, concentrations of amino acids from medium of cocultured retinal glial cells and RGCs under conditions of cell–cell contact (RGC + G on the same plate). 3D, three-day-old; 1Y, one-year-old; conf., confluent; nonconf., nonconfluent; ND, not detectable; G, retinal glial cell.

(9.1 ± 0.4 μM), or from nonconfluent retinal glial cells (20.4 ± 1.6 μM). Cocultures of RGCs with confluent retinal glial cells from 3-day-old rats yielded a significantly higher concentration than when confluent retinal glial cells in the coculture were obtained from 12-day-old rats (7.9 ± 2.1 μM) or 1-year-old rats (11.1 ± 1.3 μM).

**Alanine.** The alanine concentration in medium from pure RGC cultures was only 5.3 ± 2.8 μM. However, cocultures of RGCs with nonconfluent and confluent retinal glial cells from 3-day-old rats yielded high alanine concentrations: 190.4 ± 45.4 μM and 169.8 ± 44.0 μM, respectively. These concentrations were significantly higher than those associated with RGCs alone, nonconfluent retinal glial cells alone (65.0 ± 4.9 μM), or confluent retinal glial cells alone (88.1 ± 1.7 μM). The concentration of retinal glial cells did not influence alanine concentrations when the cells were cocultured. The amount of alanine in medium from cocultures of confluent retinal glial cells from 3-day-old rats was significantly higher than that obtained when retinal glial cells were harvested from 12-day-old rats (74.5 ± 12.6 μM) or 1-year-old rats (78.6 ± 6.7 μM).

**Other Amino Acids.** The profiles of some other amino acids varied depending on the culture conditions. However, their variances were not remarkable compared with those of glutamate and alanine. Concentrations of eight amino acids were undetectable.

### Effects of c-PTIO and l-NAME on RGCs Cocultured with Retinal Glial Cells

**c-PTIO.** c-PTIO significantly increased the survival rate of cocultured small RGCs by 59.3% and 56.9% at 1 μM and 0.1 μM, respectively. c-PTIO completely blocked the antisurvival effect of cocultured retinal glial cells on large RGCs at all tested concentrations (Fig. 4a). The addition of l-NAME at 10 μM or 100 μM completely blocked the antisurvival effect of cocultured retinal glial cells on small and large RGCs (Fig. 4b).

**Effect of Exogenous Glutamate and Alanine on RGC Survival**

Exogenously administered glutamate showed a concentration-dependent decrease in survival rate and cell population (Figs. 5a, 5b). However, 300 μM glutamate did not influence survival rate and cell population of cocultured RGCs with confluent retinal glial cells (Fig. 5c). Alanine did not influence survival rate, duration, and cell population of pure cultures of RGCs and cocultured RGCs with retinal glial cells.

### Discussion

Purity of RGCs in the present study was confirmed by a consistent recovery rate, morphologic appearance in agreement with previous reports, retrograde labeling, and immunocytochemical identification.18–21,26 Sizes and size distribution of both small and large RGCs, as classified by flow cytometry, were the same as those of RGCs grown in culture dishes and as those described in previous reports.5,8,10,27 Morphologic agreement with the descriptions in previous reports and immunocytochemical results showing positive labeling by anti-glutamine synthetase antibody and anti-carbonic anhydrase II antibody confirmed the purity of the retinal glial cells, and no or very faint labeling by GFAP indicated most of the isolated retinal glial cells, especially those harvested from postnatal rats, were retinal Müller cells.6,7

Flow cytometry allowed for examination of 10,000 RGCs at a speed of approximately 3 minutes per sample. Cells were classified automatically by size and fluorescence intensity. We believe that the present method evaluates these cells much more objectively and accurately than previous methods.2,18,27 It is possible that currently available methods of evaluating RGC survival, including ours, would fail to reliably detect necrotic or destroyed cells.5 Chloromethylfluorescein diacetate used in the present study and in prior studies to evaluate RGC survival can differentiate apoptotic cells from healthy cells by detecting early changes of plasma membrane permeability.
ability. However, 5-chloromethylfluorescein diacetate does not identify necrotic cells and destroyed cells well. Therefore, we analyzed the duration of the evaluation of 10,000 RGCs. The total number of RGCs among cultures was also compared under a microscope. These two analyses were used as supplemental parameters to evaluate RGC survival in addition to flow cytometric evaluation. The duration of RGC evaluation and the number of RGCs were approximately 3 minutes and 1200 cells/mm², which was quite similar among the flow cytometric analyses. Only pure cultures of RGCs showed a longer duration and smaller number of RGCs in culture, when they were treated with a high dose of glutamate. Therefore, by combining these data, we can conclude that the current assay reliably evaluates the effects of retinal glial cells on RGC survival.

Retinal glial cells are capable of both neuroprotective and neurotoxic activity, depending on the biological context and the maturity of the retinal glial cells. In the present study, we focused on the diffusible interaction between retinal glial cells and RGCs, because when these two types of cells are cultured together, RGC survival cannot be accurately analyzed by flow cytometer and because the current coculture system is suitable for investigating the effects of molecules showing the diffusible interaction, such as NO and glutamate. The age of the rat at the time of harvesting cocultured retinal glial cells was not associated with RGC survival, although some amino acid profiles in the culture media differed by rat age. However, the influence of retinal glial cells on RGCs also varied, depending on size of the RGCs. The effects of retinal glial cells on RGC survival and the involvement of glutamate on these effects were relatively small but significant in the present study. Because the investigated culture conditions were relatively mild, resulting in a relatively small change in RGC survival, we believe that these data may represent the relationship between retinal glial cells and RGCs in vivo. It is possible that confluent retinal glial cells hinder the diffusion of molecules through the culture insert due to the deposition of extracellular matrix. However, we found that even confluent retinal glial cells did not fully cover the surface of the insert and that there was no difference in amino acid profile between medium from the inner chamber and that from the interspace between the inner and outer chambers.

We observed that survival of RGCs simultaneously cocultured under conditions of cell–cell contact with nonconfluent retinal glial cells from 3-day-old rats seemed to be reduced in comparison with survival of RGCs in pure cell culture (data not shown) and that the concentration of glutamate in the medium was more than 500 μM. Although in this preliminary experiment we evaluated RGC survival by means of subjective observation of the amount of fluorescence using 5-chloromethylfluorescein diacetate, these results indicate that retinal glial cells exert neurotoxicity under some conditions in spite of cell–cell interaction. Therefore, the effects of retinal glial cells were variable, depending on their confluence and on the size of the RGCs. Nonconfluent retinal glial cells, are thought to decrease RGC survival, especially that of small RGCs, at least under the present experimental conditions.

Our results showed that retinal glial cells exert neurotoxic effects on purified RGCs in some situations, which is inconsistent with several previous studies that showed retinal glial cells' neuroprotective effects on RGCs. These differences could be due to several factors, including differences in culture conditions, the design of the experiments, and the types of assay used to determine RGC survival. The media used in the previous reports contained serum or dialyzed FBS, which cannot eliminate effects from unknown factors on the results. In some studies, including that of Kitano and Morgan, RGCs...
were cultured with other types of retinal neurons in a layer of retinal glial cells. Although among the previous studies only Kawasaki et al. used pure, isolated RGCs, their medium contained dialyzed FBS and they evaluated RGC survival by means of a subjective comparison of axon lengths with the cell body diameter, using relatively small numbers of cells.

The medium from cocultures of RGCs and nonconfluent retinal glial cells showed more than a 10-fold increase in con-

![Figure 5. Effects of exogenous glutamate and alanine on RGC survival. Glutamate decreased survival (a) and cell population (b) in pure RGC cultures in a concentration-dependent manner, but 300 μM glutamate did not influence survival and cell population of cocultured of RGCs with confluent glial cells (c). *P < 0.01, †P < 0.05. ANOVA and the post hoc method were used to compare among different concentrations of administered compounds. The Mann-Whitney test was used to compare other combinations. conf., confluent; nonconf., nonconfluent; Glu, glutamate.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933591/)
centration of glutamate over that of the control samples. This increase may represent an upregulation of glutamate synthesis, an acceleration of glutamate release from retinal glial cells and/or RGCs, or the limited capacity of retinal glial cells to remove glutamate from the extracellular space. Retinal glial cells, especially Müller cells regulate the extracellular concentration of glutamate by transport through glutamate transporters, especially the L-glutamate-L-aspartate transporter and by the properties of glutamate synthase. Further studies are needed to clarify why the concentration of glutamate in the medium was drastically changed in the coculture conditions. According to cell maturity, retinal glial cells vary in their ability to express particular receptors and enzymes. Heidinger et al. have reported that adult retinal glial cells cocultured with retinal neurons triple in glutamate synthase activity, whereas neonatal retinal glial cells do not show this increase. Therefore, retinal glial cells under proliferative conditions may express fewer receptors and possess limited enzyme activity, which may exert neurotoxic effects of retinal glial cells on RGCs, as observed in the present study. These changes in signaling and metabolism may partially explain age- and confluence-dependent differences in the glutamate concentrations observed in our experimental cocultures.

The mechanism of the neurotoxic effects of excessive glutamate is not fully understood. Kitano and Morgan reported NMDA receptor-mediated neurotoxicity. Otori et al. reported damage from glutamate to RGCs in pure cultures; AMPA-KA receptor-mediated neurotoxicity was involved additionally in this process. Dugan et al. reported that retinal glial cells could rescue cortical neurons from NMDA-mediated neurotoxicity while enhancing AMPA receptor-mediated neurotoxicity. In the present study, CNQX or MK801 alone modestly improved survival of RGCs in the presence of nonconfluent retinal glial cells, whereas coadministration of both glutamate receptor antagonists showed an enhanced ability to improve RGC survival. These results indicate that glutamate is importantly involved in RGC survival through the NMDA and the AMPA-KA receptors.

Under pathologic conditions, glutamate is released in excess from retinal glial cells through the glutamate transporter. Dreyer et al. reported high levels of glutamate in the aqueous humor and in the vitreous body of patients with glaucoma and also from in that of monkeys with experimental glaucoma. In the present study, exogenously administered glutamate showed a dose-dependent reduction of cell survival in pure cultures of RGCs. However, a potentially toxic amount of glutamate showed a dose-dependent reduction of cell survival in glaucoma. In the present study, exogenously administered glucose medium was drastically changed in the coculture conditions. These results indicate that glutamate is importantly involved in RGC survival through the NMDA and the AMPA-KA receptors.


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simple administration of alanine may not affect RGC survival in this study.

In the present study, the survival of small RGCs was improved only somewhat by coconcomitant administration of CNQX and MK801, whereas that of large RGCs was greatly improved by concomitant administration of these compounds. These results indicate that small and large RGCs respond differently to glutamate. We have reported that large and small RGCs show different responses to neurotrophic factors and also to chemical compounds. The current results may support the hypothesis that large RGCs are more sensitive than small RGCs in glaucoma, in which the extracellular concentrations of glutamate may be increased. Our results showed that c-PTIO and L-NAME inhibit retinal glial cell-related anti-survival effect, which indicates that NO may be deeply involved in retinal glial cell-associated RGC death. The presence of NO synthases in retinal glial cells has been reported. Release of NO from retinal glial cells is responsible for retinal neuronal cell death in vitro, suggesting a neurotoxic role for NO in retinal inflammatory or degenerative diseases characterized by activation of retinal glial cells. The present study indicates that glial cells are deeply involved in retinal development under physiological situation and that those also play a key role in some pathologic situations. Because the RGCs in the study were harvested from neonatal rats and therefore were still developing, additional studies in adult rat RGCs are necessary to further investigate cell interactions under several pathologic conditions.

References