Phagocytosis by Human Retinal Glial Cells in Culture

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Under a variety of pathologic conditions, glial cells of the retina are capable of phagocytosis. Although phagocytosis may play a role in retinal pathobiology, the regulation of the phagocytic activity of retinal glial cells is poorly understood. We used a culture system to study phagocytosis by human retinal glial cells. The cultured cells were obtained from adult postmortem eyes and were immunoreactive to antibodies for glial fibrillary acidic protein and Muller cells. Electron microscopy demonstrated that the glial cells in culture were capable of phagocytosing fragments of retinal cells as well as latex beads. To rapidly quantify phagocytosis, flow cytometry was used to detect glial cells that had internalized fluorescein-labeled microspheres. We found that reducing the extracellular calcium concentration decreased the phagocytic activity of the retinal glia. An inhibitory effect by nifedipine, a calcium channel blocker, on phagocytosis suggests a role for these ion channels in mediating a phagocytic response by retinal glial cells. A possible modulatory action of cyclic AMP was indicated by a decrease in phagocytic activity with exposure to 8-bromo-cyclic AMP. In addition to identifying conditions that reduce phagocytosis, we found that vitamin D3 can stimulate the phagocytic activity of retinal glia. Our experiments establish that phagocytosis by human retinal glial cells can be studied in a culture system and demonstrate that certain molecules can regulate the phagocytic activity of these cells. Invest Ophthalmol Vis Sci 31:1047-1055, 1990

A phagocytic function for retinal glial cells was deduced from histological studies more than 50 yr ago by Friedenwald and Chan. They reported that melanin granules injected into the vitreous cavity of rabbits could be internalized by Muller cells. Additional histologic studies of experimental animals have demonstrated that carbon or copper particles and erythrocyte debris from vitreous or subretinal hemorrhages can be phagocytosed by Muller cells. The presence of copper particles in glia of human eyes with chalcosis is further evidence for a phagocytic function of retinal glial cells. Therefore, a phagocytic activity of retinal glial cells may play a role in the response of the retina to certain pathologic conditions.

Although phagocytosis may be important in retinal pathobiology, knowledge concerning the regulation of the phagocytic activity of retinal glial cells is limited. To learn more about this phagocytic function, we used a culture system to investigate phagocytosis by human retinal glial cells. An initial aim of this study was to establish whether or not human retinal glial cells are capable of phagocytosis in culture. A further goal was to screen for molecules or drugs that can regulate the phagocytic activity of these cells.

Here, we report that human retinal glial cells are capable of phagocytosis in vitro. Using flow cytometry to quantify the phagocytosis of fluorescein-labeled microspheres, we found that the extracellular calcium concentration, a calcium channel blocker, a cyclic AMP analog, and vitamin D3 can affect the phagocytic activity of retinal glial cells.

Materials and Methods

Preparation of Cell Cultures

Cultures of retinal glial cells were prepared using a modification of methods developed by Oka et al and Hjelmeland et al. Postmortem eyes from donors aged 19-88 yr were supplied within 24 hr of death by the Florida Lions Eye Bank. Retinas were removed, exposed to a calcium- and magnesium-free phosphate buffer with 0.1% trypsin (×3 crystallized; Worthington, Freehold, NJ), 0.2% hyaluronidase (Sigma, St. Louis, MO), and 4% chicken serum (GIBCO, Grand Island, NY) for 45 min at 37°C, and then dissociated mechanically in medium A (40% Dulbecco's modified Eagle's medium, 40% Ham's F-12 medium, and 20% fetal bovine serum). Dissociated cells from one eye were added to three 35-mm petri dishes, kept in a humidified environment of 97% air, 3% CO2 at 37°C, and fed with medium A twice per week. Cultures that reached 80-90% confluence were split to three or four new 35-mm petri dishes.
Phagocytosis Assays

Cultures were used when the cells were approximately 50% confluent. Dissociated human retinal cells and cellular debris, polystyrene latex beads (diameter 0.8 μm; Sigma) or fluorescent carboxyl microspheres (diameter 1.75 μm; Polyscience, Niles, IL) were used as objects for phagocytosis. Dissociated retinal cells and cellular debris were prepared as described above with one human retina dissociated in 3 ml medium A and 200 μl of the resulting suspension added to 1.5 ml medium A in each 35-mm petri dish containing a monolayer of retinal glial cells. After 24 hr, the cultures were washed and prepared for transmission electron microscopy. As a control, cultures of comparable ages were not exposed to retinal cells and debris.

For experiments using polystyrene latex beads, 100 μl of beads (5 × 10⁶) suspended in Hanks’ balanced salt solution were added to 1.5 ml medium A for each 35-mm culture dish. After 24 hr, the cultures were washed and prepared for transmission electron microscopy.

For experiments using fluorescein-labeled carboxyl microspheres, various numbers of beads were added to 1.5 ml of a serum-free medium, medium B (50% Dulbecco’s modified Eagle’s medium, 50% Ham’s F-12, and 5 μg/ml human transferrin, and 30 nM sodium selenite), in 35-mm dishes containing monolayers of retinal glial cells. After incubation for various times with fluorescein-labeled microspheres, cell cultures were washed six times and removed from the plastic petri dish by exposure to 0.1% trypsin. The cells were centrifuged and resuspended in Hanks’ balanced salt solution containing 1% bovine serum albumin, and if flow cytometry was to be done more than 2 hr later, 2% formaldehyde.

For flow cytometry (FACScan; Becton Dickinson, Towson, MD), cells were identified by combined measurements of wide- and narrow-angle light scatter. These signals were gated to a cytogram for combination of wide-angle light scatter and of the number of microspheres, both control and experimental cultures were matched sister cultures, i.e., from the same donor eye, at the same passage number and at the same level of confluency. For some experiments, monolayers of matched sister cultures were preincubated in medium B with 1 mM ethylene glycol tetraacetic acid (EGTA), 10 μM nifedipine, 1 mM 8-bromo-cyclicAMP, or 10 nM 1,25-dihydroxyvitamin D₃. Preincubation with the vitamin D₃ was for 1 day to give time for the long-term metabolic effects of the secosteroid hormone. After preincubation, fluorescent microspheres (5 × 10⁶/dish) were added. Four hr later, cells were washed and harvested for analysis by flow cytometry.

Immunocytochemistry

Immunocytochemistry with rabbit antibodies to human glial fibillary acidic protein (GFAP; Dako, Santa Barbara, CA) was done on cultures that were washed three times with a phosphate buffer solution and fixed with 70% ethanol at −20°C for 30 min. The staining procedure was performed using the avidin–biotin–peroxidase complex method of Hsu et al. After exposure to 0.5% Triton in phosphate-buffered saline (PBS) for 5 min and to 3% peroxidase for 5 min, cells were immersed in 10% goat serum for 10 min and then exposed for 20 min to rabbit antibodies against GFAP (Kit K507; Dako) or control normal rabbit serum (Dako). After the plates were washed, they were incubated with biotin conjugated goat anti-rabbit IgG (Vector, Burlingame, CA) for 1 hr. The plates were washed, and the avidin–biotin–peroxidase complex (Vector) 1:100 was applied. The plates then were developed in diaminobenzidine. In control experiments, staining was eliminated by substituting normal rabbit serum for the primary antibody.

Immunocytochemistry with an anti-Muller cell monoclonal antibody and control ascites fluid was performed as detailed by Puro et al. Dr. Chi-Chao Chan (National Eye Institute, National Institutes of Health, Bethesda, MD) kindly provided the anti-Muller cell antibody and performed the immunocytochemistry with this antibody on our cultures.

Electron Microscopy

After fixation for 24 hr in 2.5% glutaraldehyde, cultures were dehydrated with passage through...
graded concentrations of alcohol and then embedded in Epon. Specimens were sectioned and double-stained with uranyl acetate and in lead citrate before they were examined with the JEOL 100 CM electron microscope.

Assay of Calcium Concentrations

The concentration of the calcium was determined by colorimetric quantitation using a diagnostic kit (Sigma) and absorption spectrophotometry.  

Results

The retinal cells used in this study were obtained from postmortem human eyes and maintained in culture. Cells were classified as glial by their positive immunocytochemical staining with an antibody to glial fibrillary acidic protein (GFAP), a well-established glial marker. Virtually all of the cells stained positively for GFAP (Fig. 1). The presence of 80–100 A intermediate filaments within the cytoplasm of these cells also indicated that they were glia (Fig. 2). In addition, the cells in these cultures also stained positively by immunocytochemistry with a monoclonal antibody to Muller cells (Fig. 3). The controls for the immunocytochemistry studies were negative.

Initial experiments were directed towards establishing whether human retinal glial cells in culture are capable of phagocytosis. After monolayers of human retinal glial cells were exposed to dissociated retinal cells and cellular debris for 24 hr, internalization of subcellular structures could be demonstrated by electron microscopy (Fig. 4). Phagosomes were only rarely seen in cultures not exposed to cellular debris (eg, Fig. 2). In addition to phagocytosing biologic material, glial cells could internalize polystyrene latex beads that had been added to the growth medium (Fig. 5).

Although electron microscopy demonstrated the internal localization of biologic debris and beads, this assay was laborious and difficult for rapid quantification. To screen for conditions that could alter phagocytosis, a rapid quantitative assay was sought. Use of flow cytometry to detect glial cells that contained fluorescein-labeled carboxy microspheres proved to be a relatively quick method for quantifying phagocytosis. Figure 6 shows the flow cytometric pattern before (top) and after a 4-hr (middle) or 24-hr (bottom) exposure to fluorescent microspheres. The low level of fluorescence in cells not exposed to the microspheres represents autofluorescence. Based on the pattern of the top panel in Figure 6, a fluorescent intensity of 51 on the relative scale was used as the threshold for detecting cells with fluorescent beads. For cells that had been exposed to the microspheres for 4 hr, the flow cytometric pattern shows that some glial cells had fluorescence greater than the threshold level. After 24 hr of exposure to the microspheres, there were more cells with fluorescence above the threshold level. The flow cytometric patterns of fluorescent cells after 4 and 24 hr of exposure to the microspheres revealed two initial peaks of cell populations. When the cells from the first peak were examined by fluorescence microscopy, more than 95% of

![Fig. 1. Cultured cells derived from adult human retina and stained with an anti-glial fibrillary acidic protein antibody. Bar = 100 μm.](image-url)
the cells contained one fluorescent bead (Fig. 7A, B). In contrast, when cells from the second peak were examined, about 95% of the cells contained two beads (Fig. 7C, D).

The time course for the percent of glial cells phagocytosing microspheres is shown in Figure 8. These are results pooled from experiments on glial cell cultures from different donor eyes. During the first 6 hr of exposure, there was a rapid increase in the percentage of phagocytosing cells. The percentage increased more slowly over the next 18 hr. Between 24 and 48 hr, there was minimal change in the percentage of phagocytosing cells. The mean fluorescence per cell, which is an indicator of the number of fluorescent microspheres per cell, showed a similar time course (Fig. 9). The rate of phagocytosis in the presence of various numbers of microspheres was assessed in matched sister cultures. Increasing the number of mi-
Fig. 4. Electron photomicrograph of a glial cell 24 hr after being exposed to cellular debris from a dissociated human retina. The arrows point to some of the phagocytosed fragments of retinal cells within the glial cell. Bar = 1 μm.

Fig. 5. Electron photomicrograph of a retinal glial cell 24 hr after polystyrene latex beads were added to the growth medium. Numerous beads (arrows) were found within the cell. Bar = 1 μm.
Fluorescence

Fig. 6. Histograms of fluorescence intensities for retinal glial cells. Monolayers of retinal glial cells either were not exposed to fluorescence-labeled microspheres (top) or were exposed to fluorescent microspheres for 4 hr (middle) or 24 hr (bottom). For each histogram, the flow cytometer measured the relative fluorescence of 5000 cells.

crospheres from $5 \times 10^6$ to $5 \times 10^7$ increased both the percentage of phagocytosing cells (Fig. 10) and the mean fluorescence per cell (Fig. 11).

Since the process of phagocytosis in other types of cells may be affected by extracellular calcium levels, we examined the effect of this ion on phagocytosis by human retinal glial cells (Fig. 12). Reducing the extracellular calcium level from 1.2 to 0.2 mM decreased the percentage of phagocytosing cells and the mean fluorescence per cell ($P < 0.05$ by the chi-squared test for each of three experiments).

The finding that phagocytosis by human retinal glial cells is at least partially dependent on extracellular calcium led to an examination of the effect of a calcium channel blocker, nifedipine (Fig. 12). In the presence of 10 μM nifedipine, there was a reduction both in the mean percentage of phagocytosing cells and in the mean fluorescence per cell. These were significant changes ($P < 0.05$ by the chi-squared test in each of three experiments).

Because cyclic AMP analogs have been reported to affect phagocytic activity of various cell types, the

Fig. 7. Combined phase-contrast and fluorescence photomicrographs of retinal glial cells sorted by flow cytometry after exposure to fluorescent microspheres for 4 hr. Cells were from the same experiment as shown in the histogram of the middle panel of Figure 6. Flow cytometry was used to select cells with relative fluorescence of 51–112 (corresponding to the first peak of labeled cells in the histogram) or 113–199 (corresponding to the second peak of cells). (A, B) Cells from the first peak. (C, D) Cells from the second peak. Arrows point to fluorescent microspheres.
Fig. 8. Time course for the percentage of cells phagocytosing fluorescent microspheres. Retinal glial cells with fluorescence greater than autofluorescent levels (greater than 51 on the scale shown in Fig. 6) were classified as having phagocytosed at least one microsphere. The means with standard errors are shown. Each point was derived from at least four experiments using cells from different donors. For each experiment, $5 \times 10^6$ microspheres were added in 1.5 ml medium B to each 35-mm culture dish containing a monolayer of glial cells. Cells were analyzed by flow cytometry after various periods of exposure to the fluorescent microspheres.

effect of 8-bromo-cyclic AMP on phagocytosis by retinal glial cells was assessed (Fig. 12). The mean percentage of phagocytosing cells and the mean fluorescence per cell were reduced with exposure to 1 mM 8-bromo-cyclic AMP ($P < 0.05$ by the chi-squared test in each of five experiments).

We also examined the effect of vitamin D$_3$, which can induce phagocytic activity in leukemic cells. Exposure of retinal glial cells to 10 nM 1,25-dihydroxyvitamin D$_3$ increased both the percentage of phagocytosing retinal glial cells and the mean fluorescence per cell (Fig. 12, $P < 0.05$ by the chi-squared in each of three experiments).

Fig. 9. Time course for the mean fluorescence per cell. The bars show standard errors. These data are from the same experiments as those in Figure 7.

Fig. 10. Effect of the addition of various numbers of microspheres to the culture medium on the percentage of phagocytosing cells. The experimental paradigm was as noted in the legend of Figure 8 except that a set of matched sister cultures was used.

**Discussion**

The results show that human retinal glial cells in culture are capable of phagocytosis and that the phagocytic activity of these cells can be altered by certain molecules in the extracellular environment.

Retinal glial cell phagocytosis and its regulation are of interest since it appears likely that the phagocytic activity of glia may play a role in retinal pathobiology. For example, retinal glial cells in vivo can remove from the extracellular space particles of carbon, copper, and melanin as well as fragments of red blood cells from vitreous or subretinal hemorrhages. In addition, the ability to phagocytose particles and to express class II antigens makes retinal glial cells candidates as antigen-presenting cells in certain immunopathologic disorders.

Fig. 11. Effect on the mean fluorescence per cell of various numbers of fluorescent microspheres. Data are from the same experiment as in Figure 10.
To study the phagocytic activity of human retinal glial cells, we used a culture system. A culture system enabled us to control the exposure to phagocytic targets; to manipulate the extracellular ionic composition; to test systematically the effects of known concentrations of pharmacologic agents; and to do experimental studies on human retinal glial cells. Evidence is good that the cells in the culture system used here are, in fact, glial cells. Nearly all the cells showed positive immunoreactivity to antibodies for glial fibrillary acidic protein, a well-established glial marker. In addition, our ultrastructural studies (Fig. 2) helped to confirm these cells were glia. Since virtually all the cells reacted positively with a monoclonal antibody specific for Muller cells (Fig. 3), the cells in this culture system were likely Muller cells.

Our electron microscopic findings demonstrated that human retinal glial cells in culture are capable of phagocytosing biologic and nonbiologic material. To quantitate phagocytosis rapidly, flow cytometry was used to detect glial cells that had internalized fluorescent microspheres. The measurement of microsphere internalization by retinal glial cells is most likely an assay of a nonspecific phagocytic activity rather than a molecularly selective function. Relatively nonspecific phagocytosis is likely to occur in vivo under pathophysiologic conditions, since Muller cells can internalize carbon, copper, melanin, and hemoglobin from the extracellular space. Whether there is a specific phagocytic target for Muller cells in the normal retina has yet to be determined.

In this study, the effect of various molecules on the phagocytic activity of retinal glial cells was examined. We found that reducing the extracellular concentration of calcium inhibited, but did not completely block, phagocytosis. A partial dependence on extracellular calcium is found also for the phagocytic activity of other types of cells. From studies of macrophages and neutrophils, it has been suggested that phagocytosis is stimulated by an influx of extracellular calcium into the cell and a release of calcium from intracellular stores. Although additional studies on phagocytosis by human retinal glial cells are needed to detect an intracellular release of calcium, the findings presented here indicate that extracellular calcium plays a significant role in the phagocytic activity of these glial cells.

There are a variety of possible mechanisms for extracellular calcium to enter glial cells. Previous electrophysiologic studies on retinal glial cells of rats and salamanders indicated that these cells have ion channels that can allow calcium to enter. Our finding of an inhibition in phagocytosis in the presence of nifedipine, a calcium channel blocker, suggests a role for these ion channels in the phagocytic activity of retinal glial cells. The fact that the decrease in phagocytosis with 10 μM nifedipine was less than the reduction in low calcium suggests that calcium may enter the glial cells also via nifedipine-insensitive channels or other mechanisms.

In addition to examining the role of extracellular calcium in phagocytosis, we tested the possibility that the addition to the growth medium of a soluble analog of cyclic AMP could alter phagocytosis. Our finding that 8-bromo-cyclic AMP reduced phagocytosis by the retinal glial cells is consistent with the observation by Lodin et al that a cyclic AMP analog can depress phagocytosis by cultured brain astrocytes. Although a modulatory role for cyclic AMP is suggested, the mechanism by which cyclic AMP analogs reduce phagocytosis remains unknown.

Another molecule tested for an effect on phagocytosis was 1,25-dihydroxyvitamin D₃, the active form of vitamin D. This molecule was of potential interest since it can induce phagocytic activity in leukemic cells and can stimulate calcium transport in certain cells. The possibility that vitamin D₃ may play a role in the eye is suggested by the presence of vitamin D receptors in the retina. We found that vitamin D₃ stimulated the phagocytic activity of human retinal glial cells. The mechanism for this effect on glial phagocytosis is not known. Of possible significance are studies indicating that vitamin D can directly affect the membranes of certain types of cells to enhance an influx of calcium. Further studies are required to determine if the vitamin D₃-induced increase in glial phagocytosis involves a change in
calcium influx. In addition to direct membrane effects, gene-dependent actions also must be considered, since many effects of vitamin D involve a change in gene expression.31

In summary, the regulation of the phagocytic activity of human retinal glial cells can be studied in a culture system. With this model system, molecules that can increase or decrease phagocytosis have been identified. Additional studies are needed to determine if a pharmacologically induced change in the phagocytic activity of retinal glial cells in vivo could enhance a clearing of intraocular hemorrhages, the removal of foreign particles, or the response of the eye to immunologic challenges.

Key words: phagocytosis, Muller cells, calcium, vitamin D, cyclic AMP

References