Development and Characterization of Monoclonal Antibodies Directed against the Retinal Pigment Epithelial Cell

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The retinal pigment epithelium consists of a unicellular layer of neuroepithelial cells that are essential for the maintenance of normal function of the neural retina. In order to evaluate more critically this cell in health and disease, we prepared monoclonal antibodies against human retinal pigment epithelial (RPE) cells. Balb/c mice were immunized with human RPE cells. Spleen cells were fused with myeloma cells and resultant hybridomas were selected for antibody production. Supernatants were assayed by immunoperoxidase on frozen sections of human eye tissues. Two hybrids were cloned and ascites were generated in mice. These IgG antibodies react only with RPE cells and show no cross-reactivity with other cells in the eye or with human brain, kidney, skin, salivary glands, lymphocytes or monocytes. These antibodies recognize cell surface molecules that are highly conserved since they can be found in man, monkey, rat, cow, chicken and frog. SDS gel electrophoresis and immunoblot analysis showed that one of the antibodies reacted with a 42,000 MW polypeptide. Evaluation of the developing rat retina revealed that the epitopes are not detected at birth, are weakly present at day 6 and are highly recognized by day 9. These immunoglobulins will allow us to evaluate RPE cells in disease (proliferation, migration) and to probe the bioregulatory functions (phagocytosis, vitamin A transport) of these cells.

The RPE cells, interposed between the photoreceptors and the choroid, are major regulatory cells in the retina. They are distinguished by the vast array of functions they control. For example, the RPE influences the transport of metabolites between the blood and neural retina, plays a vital role in the rhodopsin cycle, consistently absorbs excess light energy and phagocytizes the photoreceptor outer segments. Since these are critical functions, any disruption in the RPE cells can adversely affect the photoreceptors and vision. In part, alterations in RPE cells may be involved in a variety of ocular diseases such as hereditary and developmental disorders, aging-related degenerations, retinal detachment, light-induced retinopathy and inflammatory eye diseases.

Research on the RPE cell and the photoreceptor cell has progressed rapidly over the last two decades. However, questions remain concerning exactly how these two critical ocular cells develop and function, alone and together, and how their unique working relationship impacts on the visual process in health and disease. Recent developments have provided new technologies, such as monoclonal antibodies, that can now be used to explore both retinal development and degeneration. Although there are numerous anatomical studies on the central events in the maturation of RPE cells, little is known of the underlying molecular changes that trigger and sustain development. Monoclonal antibodies are excellent probes for exploring cellular differentiation and maturation.

In this report, we describe the selection and characterization of two monoclonal antibodies that detect two distinct, evolutionarily highly conserved epitopes on the RPE cell. Studies of postnatal development in the rat retina show that these epitopes appear only after the cells undergo differentiation. That is, the ability of the antibody to recognize RPE cells coincides with the time that RPE cells begin to phagocytize photoreceptor outer segments. These studies suggest that the monoclonal antibodies may be excellent probes for exploring cellular differentiation, development and function.

Materials and Methods

Preparation of RPE Cells

RPE cells were obtained from normal human donor eyes and cow eyes. Briefly, the vitreous and
sensory retina were dissected and removed. The eye cup was rinsed with sterile saline and the RPE cells were exposed to 0.4% trypsin–versene mixture at 37°C for 5 min. Sheets of RPE cells were separated and used in immunization procedures (human RPE) or for SDS gel electrophoresis and immunoblot analysis (cow RPE).

**Immunization and Monoclonal Antibody Development**

Balb/c mice (4–6 weeks old) were initially immunized at multiple sites with human RPE cells emulsified in complete Freund’s adjuvant. The spleen cells from the mouse producing the highest titer of antibody were transferred into five irradiated syngeneic recipients. Immediately after adoptive transfer the animals received RPE cells injected intraperitoneally. Six days after adoptive transfer the spleen cells were fused with the X63-Ag8-653 myeloma cell line. The resulting hybridoma supernatants were screened at 2 and 3 weeks for reactivity with RPE cells by immunoperoxidase assays on frozen human eye sections. The positive cultures were expanded, retested and cloned by limiting dilution on thymocyte feeder layers. The lines obtained were injected into pristane-primed Balb/c mice to obtain ascites. This ascites was used as the source of antibody throughout the study.

The investigations using animals adhered to the ARVO Resolution on the Use of Animals in Research.

**Immunoperoxidase Procedure**

eyes were obtained from human (eye bank donors), monkey (Macaca mulatta), cow, rabbit, rat, mouse, chicken and frog. Human brain, kidney, skin and salivary glands were obtained from autopsy specimens.

Four micrometer-thick serial frozen sections were cut and placed on gelatinized slides. Immunocytochemical evaluation of these sections was performed by the avidin-biotin-peroxidase (ABC) complex techniques. Slides were fixed for 5 min in acetone, washed in Tris-buffered saline (pH 7.6) and immersed for an additional 10 min in 5% normal horse serum. Slides were overlaid with appropriate mouse monoclonal antibodies (1:50 to 1:1000 dilution) or with irrelevant (unrelated) monoclonal antibody (Leu 4) (Becton-Dickinson, Oxnard, CA) which served as the negative control. After 1 hr incubation in a moist chamber, the slides were washed in Tris-buffered saline and then overlaid with biotin-conjugated horse anti-mouse IgG (1:200) (Vector Lab, Burlingame, CA). This was followed by another 1 hr incubation in a moist chamber, after which slides were washed in Tris-buffered saline and overlaid 45 min with a 1:100 dilution of avidin-biotin-peroxidase complexes. The slides were washed again in Tris-buffered saline and developed in diaminobenzidine-8% nickel sulfate-3% hydrogen peroxide solution. The slides were then counterstained with methyl green (1% in methanol), dehydrated, cleared and mounted.

Since the frog eyes were so heavily pigmented the staining procedure had to be altered. Instead of using diaminobenzidine, which results in the positive black stain, 3-amino-9-ethylcarbazole was used, which results in a positive red stain.

**Immunofluorescent Staining**

Cryostat sections were air-dried and fixed in cold acetone for 10 min. Slides were then rehydrated in PBS for 15 min and preincubated with normal goat serum for 30 min at 35°C in a humidified chamber. Monoclonal antibodies (1:50 to 1:1000 dilution) were applied for 1 hr in a humidified chamber. Slides were washed three times in PBS for 10 min followed by incubation with fluorescein-conjugated goat anti-mouse IgG for 30 min. Slides were washed three times in PBS, mounted and viewed in a fluorescent microscope.

**Immunoblot Analysis**

Cow RPE cells at a concentration of $1 \times 10^7$ cells were boiled for 3 min before loading onto polyacrylamide gel (10% SDS gels). The gels were electrophoresed for 3 hr at 30 mA. Proteins were transferred to nitrocellulose strips by electroblotting overnight. The monoclonal antibodies were incubated with the nitrocellulose strips for 2 hr. The strips were washed and $^{125}$I-labeled rabbit anti-mouse IgG was then added for an additional 2 hr incubation. The blots were washed, dried, and then incubated with x-ray film.

**Results**

**Antibody Isolation**

Injection of whole human RPE cells into Balb/c mice and subsequent fusion of spleen cells with myeloma cells yielded many hybrid clones that secreted antibodies that bound to cells within the retina, choroid and sclera. Two hybridoma-secreted antibodies were designated 9B and 15B. Both antibodies recognize RPE cells in the immunoperoxidase assay. These hybridomas were cloned and ascites fluid was prepared from each hybridoma. Characterization of the antibodies showed that 9B and 15B were both of the IgG3 subclass.
Table 1. Reactivity of two monoclonal antibodies prepared against human RPE cells

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<th>Human tissue</th>
<th>Reactivity of monoclonal antibodies</th>
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<tr>
<td></td>
<td>9B</td>
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<tr>
<td>Eye</td>
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<td>Anterior segment</td>
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<td>Posterior pole</td>
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<td>Retina</td>
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Although the monoclonal antibodies reacted with RPE cells in frozen tissue sections, the antibodies failed to react with tissues fixed in 10% formalin.

These monoclonal antibodies, 9B and 15B, do not react by immunoperoxidase staining with frozen sections of brain, kidney, skin, salivary gland, lymphocytes and monocytes. These studies highlight the specificity of the antibody-binding site in that the antibodies react only with the RPE cell within the eye and lack reactivity with any other tissue tested.

Species Reactivity of Monoclonal Antibodies

Eyes from a variety of vertebrates were frozen, sectioned and reacted with the two monoclonal antibodies, 9B and 15B, and with an irrelevant monoclonal antibody (Leu 4). Both the 9B and 15B monoclonal antibodies reacted with RPE cells from human, monkey, cow, rabbit, rat, mouse, chicken and frog eyes. In each case the antibodies reacted with RPE cells and not with other ocular cells. In contrast to this reactivity, the two controls (Leu 4 and mouse ascites) failed to react with any ocular cells. These studies show that the monoclonal antibodies recognize a highly conserved epitope on the RPE cells.

The reactivity of the antibodies is most evident in the rat because we selected the albino Lewis rat in which the RPE cells are devoid of their brown pigment (Fig. 2). These conditions were also ideal to evaluate possible cross-reactivity with the ciliary epithelium, which is contiguous with the RPE. As is shown in Figure 3, the monoclonal antibodies identify RPE cells but do not stain the ciliary epithelial cells.
Due to the yellow autofluorescence of the RPE cell granules, the immunofluorescent assay could not be used to evaluate these antibodies. However, the Lewis rat eye, which is devoid of autofluorescence, could be examined by this technique. When Lewis rat RPE were treated with the monoclonal antibodies, 9B and 15B and evaluated by the immunofluorescent procedure, the typical green fluorescent pattern was observed only at the RPE cell layer (Fig. 4).

Molecular Size

Since studies to determine the molecular size of the epitopes identified by monoclonal antibodies 9B and 15B required large quantities of RPE cells, we selected freshly collected cow RPE cells for these experiments. SDS gel electrophoresis and immunoblot analysis on cow RPE cell proteins revealed that monoclonal antibody 9B reacted with a 42,000 MW
Fig. 3. Immunoperoxidase analysis of a frozen section of a Lewis rat eye. (a) Tissue incubated with an irrelevant monoclonal antibody (anti-T cell). (b) Tissue stained with monoclonal antibody 9B (1:100 dilution). Arrow indicates the specific black staining on the RPE cells. Ciliary body is devoid of staining. Photographed at ×100 magnification.

Fig. 5. SDS gel electrophoresis and immunoblot analysis of cow RPE cells. The proteins were separated on SDS polyacrylamide gels and electrophoretically transferred onto a nitrocellulose sheet and incubated with monoclonal antibody RPE 9 and RPE 13. Lane 1, IgG (control). Lane 2, monoclonal antibody 9B. Lane 3, monoclonal antibody 15B.

Fig. 4. Immunofluorescent staining of a frozen section of the posterior pole of a Lewis rat eye. (a) Rat eye viewed under light microscopy. (b) Rat eye incubated with 9B antibody followed by incubation with fluorescein conjugated goat anti-mouse IgG. Arrow indicates positive staining of the RPE cells. (c) Rat eye incubated with an irrelevant monoclonal antibody (anti-T cell) followed by incubation with fluorescein conjugated goat anti-mouse IgG. Arrow indicates negative reaction. Photographs at ×100 magnification.
Fig. 6. RPE cell maturation studies with monoclonal antibodies. Frozen sections of 1-, 6- and 9-day-old Lewis rat eyes were evaluated. Day 1 is represented on the top row across, day 6 on the middle row across and day 9 in the bottom row across. H & E staining is shown in (a, d, g). Immunoperoxidase staining is shown in (b, c, e, f, h, i). Lack of staining with an irrelevant monoclonal antibody (anti-T cell) is shown in (b, e, h). Anti-RPE cell monoclonal antibody does not react with RPE cells at day 1 (c), reacts weekly at day 6 and reacts strongly by day 9. Arrows point to RPE cells.
polypeptide (Fig. 5). In contrast, monoclonal antibody 15B failed to react in the Western blot analysis, most probably due to the inability of this epitope to withstand SDS gel electrophoresis.

RPE Cell Maturation Studied with Monoclonal Antibodies

Although there are numerous anatomical studies on the central events in the maturation of RPE cells, little is known of the underlying molecular changes that trigger and sustain development. Monoclonal antibodies are excellent probes for exploring cellular differentiation and maturation. We therefore initiated studies to monitor the sequential appearance of the RPE cell epitopes during maturation of the Lewis rat eye.

At birth, the rat RPE cell already has the histologic appearance of the RPE cell found at later stages of development. However, when 1-day-old rat tissue was examined immunocytochemically with 9B and 15B monoclonal antibodies, no immunoreactivity was detected (Fig. 6). When 6-day-old tissue was examined, a weak but definite immunoreactivity was observed. Strong immunoreactivity was noted in 9-day-old eye tissue. This sequence of events coincides with the time that photoreceptors begin to elongate outer segments and to the time that RPE cells begin to phagocytize. These studies show that the epitopes recognized by the two monoclonal antibodies are detected in the more terminally differentiated RPE cell at a time when this cell begins to perform one of its most important functions, phagocytosis.

Discussion

In this study, we report the development and characterization of two monoclonal antibodies directed against the RPE cell. Both of these antibodies appear to be specific for the RPE cell. They show no cross-reactivity with other cells in the eye or with cells in a variety of other tissues. Moreover, these RPE cell epitopes appear to be highly conserved since they can be found not only in a variety of mammalian species but also in avian and amphibian species.

The two monoclonal antibodies react solely with RPE cells but appear to react with different epitopes. Monoclonal antibody 9B reacts with a 42,000 MW polypeptide, while the epitope recognized by 15B fails to withstand the SDS treatment. This is a phenomenon observed with a variety of proteins, such as the MHC class II antigens.

The exact location of these epitopes on the RPE cell in situ cannot be clearly distinguished by immunoperoxidase or immunofluorescent staining. Whether or not the epitopes are on the apical or basal surface of the cell will require immunoelectronmicroscopic examination.

Since these antibodies detect epitopes present solely on RPE cells they provide us with the unique opportunity to evaluate a variety of aspects of RPE cell development and function. We therefore initiated studies to elucidate the importance of the epitopes recognized by the antibodies in cellular differentiation. Monoclonal antibodies are invaluable in this respect since they can be used to locate molecules as well as to monitor and quantify their expression during development. When 1-day-old rat tissue was examined immunocytochemically no reactivity was detected. However, a gradual but definite increase in immunoreactivity was observed when tissue taken from 6- and 9-day-old rat retinas was examined. This sequence of events coincides with the time that photoreceptors begin to elongate outer segments and to the time that RPE cells begin to phagocytize. These findings are encouraging and suggest that the antibodies recognize and bind their determinants only after the cells have begun terminal differentiation. Studies are now in progress to localize these epitopes and to more directly relate their expression to specific events in cellular maturation and function.

The RPE cell is not only an important factor in normal retinal development and function but is also viewed as an important cell in numerous retinal pathologic processes. Depending upon the condition, the cell may respond to insult by degenerating, proliferating and/or migrating. Studies are in progress to identify alterations in RPE cellular epitopes and to correlate these changes with retinal diseases in man and animals.

Key words: RPE, monoclonal antibodies

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References