Generation and Characterization of Monoclonal Antibodies Specific for the Retinal Pigment Epithelium

Peiguo Chu and Gerald B. Grunwald

Although both the neural retina and the retinal pigment epithelium (RPE) arise as neighboring portions of the embryonic optic cup, these two tissues follow very different developmental pathways. In order to obtain probes for the analysis of RPE development from its earliest divergence from the neural retina to late stages of differentiation, we have developed a panel of monoclonal antibodies which recognize antigens specific to the RPE. These probes have been applied to an immunohistochemical analysis of RPE development. The results indicate that the RPE is antigenically distinct from the neural retina even before the onset of overt differentiation. In addition, the RPE layer of the retina becomes further subdivided antigenically as its distinct anterior and posterior derivatives develop. These antibodies will be useful markers in the analysis of RPE development.

During the course of ocular development, invagination of the optic cup gives rise to a double layered neuroepithelium which becomes the neural retina (NR) and the retinal pigment epithelium (RPE). Although this dramatic differentiation process has long been a model system in studies of tissue induction, differentiation, and transdifferentiation, the molecular mechanisms which control the differentiation of these two tissues and which set them on very different developmental pathways remain obscure. Since the appearance of final differentiated properties such as pigmentation may be several steps removed from the primary events which affect RPE development, the current studies were undertaken in order to develop probes for the identification and analysis of RPE differentiation markers which could be used to study RPE development prior to the onset of overt differentiation and pigmentation. Markers of this type might also prove useful in the analysis of retinal dystrophies that involve the RPE.

One method that has been successfully employed to identify early markers of tissue and cell differentiation is the production of monoclonal antibodies against the tissue of interest and the application of these antibodies to histochemical and biochemical analysis of differentiation. Indeed, one of the first applications of this approach was in the retina; the development and the cellular and molecular diversity of this ocular tissue has been extensively and successfully studied by this method. Our own studies of retinal development have used the monoclonal antibody approach to identify differentiation antigens whose expression correlates with the development of retinal stratification and synaptogenesis. These studies have led to the identification of several different types of molecules which may play a role in retinal histogenesis.

The current report describes the generation and analysis of several monoclonal antibodies which recognize differentiation markers which appear very early and very specifically in the developing RPE of the embryonic chick.

Materials and Methods

These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Embryo and Tissue Preparation

Fertilized White Leghorn Chicken eggs were obtained from Shaw Hatcheries (Oxford, PA) and maintained in a humidified incubator at 38°C. For embryos of 4 days or less incubation, the stage of development was determined according to Hamburger and Hamilton. Later developmental stages...
Fig. 1. Immunocytochemical analysis of RPE differentiation antigen expression during development. Tissues were dissected and processed for indirect immunofluorescent histochemistry as described in Materials and Methods. The panels represent sections of tissues obtained from 3-day (A–E), 10-day (F–J), and 21-day (K–O) embryos or from whole mounts of RPE tissue obtained from 10-day embryos (P–T). Tissues were stained with monoclonal antibodies 3C10 (B, G, L, Q), 3G11 (C, H, M, R), 1D5 (D, I, N, S) and 1H2 (E, J, O, T), and were examined by phase contrast (A, F, K, P) or fluorescence (B–E, G–J, L–O) and (Q–T) microscopy (X130).

were defined in terms of days of incubation. Up until the 4th day of development, whole embryo heads were dissected from the surrounding blastoderm and trunk, whereas after 4 days of development the eyes were dissected from the head.

Immunological Reagents and Procedures

Monoclonal antibodies were prepared as described in the accompanying paper. Briefly, BALB/c mice were immunized by four weekly intraperitoneal in-
jections of 10^7 RPE cells from 10-day chick embryos. Three days after the last immunization, spleen cells were fused with P3-X63-Ag8 myeloma cells, and growing hybridomas were screened for antibody production by immunohistochemistry. Hybridomas were subcloned two times prior to use in these experiments. Indirect fluorescence immunohistochemistry was done as described in the accompanying paper. Briefly, embryonic heads (which were embedded first in gelatin [7.5% w/v in phosphate-buffered saline] for ease of manipulation) or whole eyes of older embryos were embedded in OCT compound (Miles Scientific, Naperville, IL) and snap-frozen in liquid nitrogen. Sections were cut at 10 µm on a Histostat Freezing Microtome (American Optical, Buffalo, NY). The sections were thaw-mounted on clean glass slides, rehydrated, and blocked with Tris-buffered saline (TBS; 0.01 M Tris, pH 7.5, 0.15 M NaCl) with 5% normal goat serum (NGS) and 1 mM CaCl₂ for 15 min, and then incubated with hybridoma supernatant containing monoclonal antibodies for 1 hr at room temperature. Control sections received no primary antibodies. The sections were then washed three times with TBS-CaCl₂ and incubated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse secondary antibodies (Fisher Scientific, Pittsburgh, PA) at a 1:40 dilution in TBS-CaCl₂ for 30 min at room temperature. After three washes in TBS-CaCl₂, the sections were mounted in Elvanol (polyvinyl alcohol; Hoechst) and viewed on a Nikon microscope equipped for epifluorescence microscopy. For experiments in which tissues were stained as whole mounts, the RPE was dissected from 10-day chick embryo eyes and stained in suspension as for mounted sections.

**Results**

Monoclonal Antibodies Recognize RPE-Specific Differentiation Antigens

Monoclonal antibodies produced in mice immunized with embryonic chick RPE cells were screened by indirect immunocytochemistry on sections of embryonic and hatchling chick eyes (Fig. 1). Four hybridomas were obtained which retained positive and specific binding to the RPE after subcloning. Antibody binding to the RPE occurs prior to the onset of pigmentation (Figs. 1A–E); is detected at high levels in 10-day embryos (Figs. 1F–J); and diminishes by the time of hatching (Figs. 1K–O). The antigens detected with antibodies 3C10 and 1D5 are not detected in the RPE of the hatched chick, while those antigens detected with antibodies 3G11 and 1H2 are still detected at that time. At all developmental stages tested, the binding of all four antibodies is specific to the RPE, except for certain stages when 1H2 was observed to bind to the neural retina. In order to deter-

![Fig. 2. Immunocytochemical analysis of the onset of RPE antigen expression during early development. Whole embryo heads were dissected and processed for indirect immunofluorescence histochemistry as described in Materials and Methods. The panels represent fluorescent micrographs of tissues obtained from embryos at 70 hr (A–D), 75 hr (E–H), and 80 hr (I–L) of development, and stained with monoclonal antibodies 3C10 (A, E, I), 3G11 (B, F, J), 1D5 (C, G, K), and 1H2 (D, H, L) (×100).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933153/)
Fig. 3. Immunocytochemical analysis of the earliest expression of antigen 3C10. The head of a 70-hr embryo was dissected and processed for indirect immunofluorescence histochemistry as described in Materials and Methods. The panels represent phase contrast (A) and fluorescence (B) micrographs showing the localization of monoclonal antibody 3C10 binding to the primitive RPE. le, lens; oc, optic cup; and de, diencephalon. The box in (A) marks the region shown in (B). Arrows indicate the region of the RPE where antigen 3C10 is first detected (A, x30; B, x150).

mine if any of the antigens are expressed on the cell surface, whole mounts of 10-day embryo RPE tissue were stained with the four antibodies. Only one, 3C10, reacted with whole RPE tissue, yielding a pattern of antibody binding which highlights the borders between RPE cells (Fig. 1Q). None of the other antibodies bound to intact RPE tissue (Figs. 1R-T), despite strong binding to sectioned RPE tissue. This suggests that the antigen recognized by antibody 3C10 is associated with the surface of RPE cells, whereas the other antigens are likely to be intracellular. To date, no immunologic crossreactivity has been detected with tissues from adult humans, rabbits, mice, or rats (data not shown).

Differentiation Antigens Are Expressed Early, Sequentially, and Uniquely in the RPE

To ascertain the time of onset of differentiation antigen expression in the RPE, tissues were dissected...
from closely spaced stages of developing embryos and examined by indirect immunofluorescent histochecmistry for antibody binding. As early as 70 hr of development, binding of antibody 3C10 was first detectable (Fig. 2A); there was some non-RPE-specific binding of antibody 1H2 (Fig. 2D); and binding of the others was undetectable (Figs. 2B, C). By 75 hr, binding of 3C10 was much stronger (Fig. 2E) and binding of 1H2 was now detected in a RPE-specific fashion, whereas binding of 3G11 and 1D5 was still undetectable (Figs. 2F, G). By 80 hr of development, 3G11 bound strongly (Fig. 2J), whereas 1D5 was barely detectable (Fig. 2K). These results indicate that while differentiation markers 3C10, 3G11, and 1D5 are specifically expressed in the RPE, each antibody is directed against a unique determinant whose expression is under independent temporal regulation. Specific expression in the RPE was observed both at the onset and at later times of antigen expression. This was demonstrated for antibody 3C10 in sections of whole heads of 70-hr embryos. The onset of expression was detected only in the RPE (Fig. 3). Even at later stages of development, such as the 4-day embryo, antibody binding was specific to the RPE layer of the eye, and extended fully to the anterior margin of the optic cup (Fig. 4). In this section, it is evident that most antibody binding is located at the basal portion of the RPE where it rests on Bruch’s membrane.

The Expression of Differentiation Antigens is Distinct in Anterior and Posterior Regions of the RPE

Since it was observed that certain antibodies no longer bind to hatching chick tissues, we tested to see if this transient binding was consistent throughout the RPE layer at this stage of development. When the binding of antibodies to the anterior and posterior regions of the eye was compared, it was observed that whereas antibodies 3C10 and 1D5 no longer bind to the posterior RPE layer, binding could still be observed to the RPE layer of the ciliary body (Figs. 5 A, C, I, K). In contrast, antibody 3G11, which still binds to the posterior RPE in hatched chicks, does not bind to the RPE layer of the ciliary body (Figs. 5B, J). Antibody 1H2, which still binds weakly to the RPE of hatched chicks, also binds to the RPE layer of the ciliary body (Figs. 5D, L). Thus, spatial as well as temporal differences of expression of the antibody-defined differentiation markers are observed, which again indicates that each antibody is likely directed against distinct antigenic determinants.

Discussion

The temporal patterns of expression of the monoclonal antibody-defined differentiation antigens described in this study are summarized in Figure 6. During normal development of the chick eye, the primary optic vesicle forms from the diencephalon at stages 10–12 (33–50 hr). At the end of stage 15 (45–50 hr) the optic vesicle invaginates to form the double layered optic cup, with the outer layer established as the primordial RPE. RPE precursor cells continue to proliferate rapidly until about 72 hr of development when there is a rapid withdrawal of cells from the mitotic cycle, but pigment accumulation does not begin in earnest until 24 hr later.
The results of the current study indicate that the RPE and NR layers of the optic cup are antigenically distinct prior to the onset of overt differentiation and expression of neuronal and pigmented characteristics. Through the use of monoclonal antibodies recognizing RPE differentiation antigens, the immunohistochemical studies described here show that coincident with the rapid withdrawal of RPE precursor cells from the mitotic cycle, we are able to detect the initial expression of differentiation antigens which distinguish the RPE from other ocular tissues, including the NR. This initial expression of differentiation antigens occurs while the RPE is still several cells thick; the flattening of the RPE into a monolayer does not begin until after the fourth day of development.4

The initial expression we detect is towards the posterior pole, consistent with the general observation that differentiation of the optic cup derivatives proceeds in a posterior-anterior fashion. It is of interest that the monoclonal antibodies bind initially and most strongly to the basal region of the RPE, since this is the region in contact with the ectomesenchymal cells which condense to form the choroidal and scleral layers of the eye. It is possible that the restricted distribution of antigens reflects a function related to the basal region of the cells, such as contact with the basal lamina of Bruch's membrane. Alternatively, the basal appearance of the antigens may reflect inductive interactions between the RPE cells and the surrounding ectomesenchymal cells. In any case, localized expression of antigens by the most basal cells of the RPE at the early multilaminar stage suggests that the RPE cells are not homogeneous.

Monoclonal antibodies directed against the developing rat and human RPE have been described,20,21 as have monoclonal antibodies against adult bovine and rat RPE.22,23 Our preliminary experiments indicated that the monoclonal antibodies we have used for immunohistochemistry in the current report, unlike the 2A10 monoclonal antibody described in the accompanying paper,17 are not suitable for western blotting; other means will have to be employed to obtain information as to the structure of the former differentiation antigens. It is clear, however, that at least one of these antigens, that detected by antibody 3C10, is a cell surface molecule, since the antibodies were shown to bind to the surface of intact RPE tissue.

The function of these differentiation antigens is completely unknown at this time, but it is unlikely that they all are related to melanogenesis, since at least one is a cell surface molecule; since the expression of all precede the appearance of melanin; and since most are expressed at highest levels during the development of the RPE and are expressed little if at all in the mature RPE. This last observation suggests that these antigens may play a role specifically during development of the RPE. Further experiments will be required to determine the biochemical identity and functional role of these molecules. Even in the absence of such information, the monoclonal antibodies described in the current paper will be useful tools for the analysis of ocular development.

Key words: retinal pigment epithelium, differentiation, ocular development, monoclonal antibodies, RPE specific antigens
References


9. Trisler GD, Schneider MD, and Nirenberg M: A topographic gradient of molecules in retina can be used to identify neuron position. Proc Natl Acad Sci USA 78:2145, 1981.


