RET-PE10: A 61 kD Polypeptide Epitope Expressed Late During Vertebrate RPE Maturation

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Purpose. This study sought to learn more about the mechanisms that determine and maintain the differentiated state of the retinal pigment epithelium (RPE).

Methods. Monoclonal antibodies were raised against human RPE and used in conjunction with other antibodies. Immunocytochemical and biochemical analyses were performed on tissue sections and cells in culture.

Results. An RPE-specific epitope, RET-PE10, has been detected as a 61 kD cytoplasmic polypeptide in a variety of mammalian, amphibian, and avian species. In the rat, RET-PE10 was expressed late in eye development, with a faint initial labelling of the RPE in central regions at postnatal day 9 (PN9) that increased to adult levels and extent of staining by PN14. RET-PE10 expression initially was present in overnight cultures of dissociated rat RPE cells but was lost rapidly from these cultures during the first week. Comparison of the staining patterns of RET-PE10 with those of various cytoskeletal elements suggests that RET-PE10 may be associated with part of the intermediate filament network. Culture of whole eyecups also resulted in a loss of RET-PE10 expression. RET-PE10 expression was normal in eyes of adult rd/rd mutant mice.

Conclusions. RET-PE10 is a late-appearing marker of RPE differentiation. The results also suggest that the maintained expression of RET-PE10 depends upon extrinsic factors but that these do not include maintained contact with Bruch’s membrane or light-induced retinal activity. Invest Ophthalmol Vis Sci. 1993;34:453–462.

The retinal pigment epithelium (RPE) is a monolayer of cells that lies closely apposed to the neural retina. It is a highly differentiated transport epithelium with basal membrane infoldings, tight junctions, and apical microvilli. In the adult, the RPE performs numerous functions that affect the function and survival of retinal cells. Although the mature RPE phenotype is very different from that of the retina, both layers share a common origin from the optic vesicle neuroepithelium. The expression of differentiated characteristics by the RPE can be observed as early as embryonic day 13 (E13) in the rat, when pigment granules and cellular retinaldehyde-binding protein can be detected. Later stages of RPE differentiation have not been well characterized at the molecular level. Also not clear is whether communication between the RPE and the retina is important for differentiation of either layer, although the intimate interaction found in the adult suggests this might occur. Such an idea is supported by the observation that certain nonmammalian RPE retain sufficient developmental plasticity to...
transdifferentiate into a new retina when the original has been removed.7-9

Much less plasticity has been observed in mammalian RPE. Proliferation of RPE continues postnatally in many species but subsides to minimal levels by postnatal day 15 (PN15) in the rat and by 2 yr in humans.3,16,11 RPE cells can be induced to migrate and proliferate after retinal detachment or tears. The proliferation of these “activated” RPE cells and their subsequent accumulation within the vitreous and at the vitreous-retina interface plays a role in some forms of proliferative vitreoretinopathy (PVR). PVR is characterized by the formation of cellular membranes whose traction upon the retina results in recurrent retinal detachment and visual impairment. This is the most common cause of retinal detachment after surgical repair.12

The changes in cellular properties of RPE may be accompanied by changes in molecular properties. When rat RPE cells are dissected and placed in culture, they respond rapidly with increased expression of the neural cell adhesion molecule mRNA and protein.13,14 Because the neuroepithelial cells that differentiate into RPE express N-CAM, this change may reflect a reversion to an embryonic state caused by removal of suppressive factors normally found in the eye.

To learn more about the mechanisms that maintain the differentiated state of the RPE, we have been seeking molecular markers of RPE differentiation. In the present study, we detected an epitope (RET-PE10) on a 61 kD cytoplasmic polypeptide expressed by RPE of many species. In the rat, this epitope is not detectable until the second postnatal week and is rapidly lost by RPE cultures. Only one previously published RPE-specific antigen (9B antigen) is similar to RET-PE10 in that both are shared between species and appear relatively late during development.15 These two antigens are distinct, however, because they differ in their reported molecular weights (42 kD for 9B versus 61 kD for RET-PE10) and in the actual time of first observable staining (PN 6 for 9B versus PN 9 for RET-PE10). Our results, in combination with our previous work, suggest that RPE differentiation may be under continuing active control, and indicate that the RET-PE10 antigen could serve as a marker for the late maturation of RPE.

METHODS

All animals used in this study were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies

Primary antibodies used in this study included: SP1, an IgG originally derived against visual cortex homogenates;16 RET-PE2, an antibody recognizing a cell surface determinant on rat RPE;13 TIB 131, an antibody that recognizes a common determinant on most classes of intermediate filaments (ATCC);17 TUB 2.1, an antibody against β-tubulin;18 two antibodies (RET-P2 and P3) against rod photoreceptors; and one against opsin (RET-P1).19

The monoclonal antibody that recognizes RET-PE10 was derived after immunization of BALB/c mice with human RPE. Immunotoxelization of neonatal mouse pups to general human antigens20-20 was used to suppress production of non-RPE-specific clones. Cultured human HeLa and MRC5 fibroblast cells were harvested by treatment with buffered EDTA, and 106 cells per injection were injected at 2 day intervals in inguinal and axillary sites. After five injections, animals were allowed to reach 1 mo of age before RPE immunizations were begun. To prepare antigen for injection, RPE sheets from human cadaver eyes were homogenized in cold phosphate buffered saline (PBS) and centrifuged at low speed (500 × g, 30 min) to remove nuclei. The membranes were resuspended in PBS and stored frozen at −80°C until they were used for immunization. The methods of immunization, fusion, and cloning have been described extensively elsewhere.21,22

After fusion, clone supernatants were screened for RPE-specific antibody using indirect immunofluorescence on formaldehyde-fixed frozen sections of human eyes. Positive colonies were subcloned twice by limiting dilution, weaned from hypoxanthine-aminopterin-thymidine selection, expanded, and used to produce antibody in culture using Nutridoma serum substitute (Boehringer-Mannheim, Indianapolis, IN) instead of serum. The immunoglobulin subclass of RET-PE10 was identified as an IgG2b using a commercially available immunoglobulin isotyping kit (HyClone, Logan, UT).

Tissue Culture

Rat RPE cells were harvested from PN6-16 Long-Evans rats using the method of Mayerson et al.23 Human and bovine RPE cells were harvested using the method of Flood et al.24 Cells were plated at 100 cells/mm² on Lab-Tek 8 chamber slides (Marsh Biomedical, Rochester, NY) that were uncoated or precoated with rat tail collagen or 5 μg/well fibronectin (Collaborative Research, Bedford, MA). Cells were cultured for up to several weeks in 20% FBS/Dulbecco’s modified Eagle’s medium. For some experiments, the culture medium also contained supplements (10 μg/ml insulin and elevated [44 mMol/l] bicarbonate) that have been reported to restore RPE pigment production in vitro.25 Cultured cells were rinsed with Hanks’ balanced salt solution (HBSS) and fixed at room temperature

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Figure 1. Adult RPE of different species expresses RET-PE10 antigen. Phase contrast and fluorescence images of frozen eye sections stained with RET-PE10 (C–F) or with a nonreactive IgG (SP1) as a negative control (B). (A) Phase view, rat eye. (B–F) Fluorescence views of rat (B and C), rabbit (D), bovine (E), and human (F). The labelling in (C) over the outer segments is autofluorescence as judged by comparison with the negative control in (B). Guide to tissue layers: 1, neural retina; 2, RPE; 3, choroid; 4, sclera. (Bar = 20 μm.)

for 30 min with 1% paraformaldehyde in HBSS, pH 7.4.

Whole eyecups were prepared by sterile enucleation and surgical removal of the cornea, iris, lens, and ciliary body. The resulting eyecup, containing retina, RPE, choroid, and sclera, was placed in culture medium for up to 10 days before fixation and sectioning.

Frozen Sections
Tissue for cryostat sectioning was obtained from human cadaver eyes, from bovine eyes obtained at a local slaughterhouse, or from laboratory toads, chickens, rabbits, rats, or mice. The rd/rd mutant mice used were 2-mo-old females of the C3H/HEJ strain. Tissue was fixed by immersion for at least 1 hr at 4°C in 4% paraformaldehyde in Dulbecco’s PBS, pH 7.4. The tissue was infiltrated with 30% sucrose in PBS overnight at 4°C, embedded in Tissue Tek (Miles, Elkhart, IN), and frozen. Ten-micron sections were cut and dried onto gelatin-coated slides.

Indirect Immunofluorescence
Tissue sections were incubated with HBSS or PBS containing 5% nonimmune goat serum (NGS; GIBCO/BRL, Gaithersburg, MD) as a blocking agent to prevent nonspecific binding. Cultured cells were incubated with 5% NGS in HBSS, because the presence of divalent cations maintained better cell attachment. The cells were permeabilized with 0.025–0.1% Triton in 5% NGS/HBSS to provide access to intracellular antigens. Tissue or cells were incubated for 1 hr at room temperature or overnight at 4°C.

An IgG that does not specifically stain RPE or retina (SP1) was run on an adjacent section or slide well as a negative control for nonspecific antibody absorption. In some cases, the cells were double-labelled by including rhodamine-labelled phalloidin (5 μg/ml; Sigma) with the primary antibody. The sections/wells were incubated at room temperature with appropriate dilutions in NGS/buffer of monoclonal antibody as ascites or culture supernatant (sections, 1 hour; cells, 30 min), rinsed with buffer, and incubated for 1 hr at room temperature or overnight at 4°C with fluores
cein-conjugated goat anti-mouse IgG (Cappell, Durham, NC or Boehringer-Mannheim) diluted 1:100 in NGS/buffer. Sections were rinsed extensively with buffer, coverslipped in 50% PBS:glycerol, viewed under epifluorescence illumination, and photographed using Kodak (Rochester, NY) T-MAX film.

Immunoblots

RPE homogenates of different species were solubilized in sodium dodecyl sulfate (SDS) sample buffer, run on SDS 5.5–20.0% polyacrylamide gradient gels, transferred to nitrocellulose, and immunoblotted as described previously. Bound primary antibody was visualized using an alkaline phosphatase-conjugated secondary antibody system (Promega, Madison, WI). Nonreactive antibodies of the same immunoglobulin isotypes as the primary antibodies also were run as negative controls.

RESULTS

The RET-PE10 epitope was detected with a monoclonal antibody produced by immunotolerizing BALB/c mice to nonspecific human antigens, followed by immunization with human RPE. The epitope was found in the RPE of a variety of animal species as judged by immunocytochemical labelling of cryostat sections of fixed eyes from rat, rabbit, cow, and human (Fig. 1). In all cases, labelling was located apical and basal to the nucleus, crossing the entire thickness of the RPE, although light microscopy did not allow resolution of staining in apical microvilli. No specific labelling was found in the sclera, choroid, or neural retina. A sharp demarcation was found between the labelled cells of the RPE and the unlabelled cells of the adjacent pigmented layer of the ciliary epithelium. No labelling was observed on sections of other tissues, such as kidney, muscle, or liver (data not shown), suggesting that RET-PE10 is RPE-specific.

**FIGURE 3.** Expression of PE10 and other RPE and photoreceptor antigens during late postnatal development of rat eye. Phase contrast and fluorescence images of rat eye sections stained with a variety of antibodies. From left to right: phase and fluorescent images of negative control antibody SP1; anti-RPE antibody RET-PE2; anti-photoreceptor antibodies RET-P2 and P3; central and peripheral retina stained with anti-RET-PE10. Top row: postnatal (PN) day 9. Second row: PN 11. Third row: PN 13. Bottom row: PN 14. Note similar staining at PN 9/11 versus 13/14 for RET-P3 and RET-PE10. (Bar = 50 μm.)
Immunoblots to detect RET-PE10 were carried out using freshly isolated RPE from toad, chicken, rat, cow, rabbit, and monkey (Fig. 2). In all cases, RET-PE10 appeared as a single band of 61 kD, although the intensity varied from species to species. In particular, chicken and rat were weakest, but the specific band was still discernible above background. A weak band or doublet of lower molecular weight occasionally was observed in chicken, rat, and monkey preparations. Whether these variable and faint bands are truly distinct from the 61 kD antigen or are the result of differences in sample degradation remains to be seen.

To determine the time course of RET-PE10 expression and to compare this with the expression of other RPE and photoreceptor molecules, sections of eyes from rats of various postnatal ages were labelled with appropriate antibodies (Fig. 3). The earliest age that RET-PE10 could be detected in central retina was PN9, with the faint staining at these early ages most clearly visualized on tangential sections through the RPE layer (Fig. 4). Fluorescent staining of such sections at this age was concentrated within a portion of the RPE cell close to the apical region and appeared to show an association with the plasma membrane. By PN11, RET-PE10 expression in central retina was stronger and the first signs of RET-PE10 expression were observed in peripheral retina (Fig. 3). By PN15, expression was found in all regions of the RPE, with adult levels of expression reached 1 day later. This late expression is in striking contrast to the expression of a number of other RPE and rod photoreceptor molecules. The cell surface epitope on RPE that is recognized by antibody RET-PE2 is expressed during embryonic development, and it was found throughout the RPE at all of the ages examined in the present study. The rod outer segment protein recognized by RET-P2 was first detected at PN5. Between PN9 and PN14, as shown in Figure 3, the extent of its expression increased with the elongating outer segments. Its intensity also increased. The epitope recognized by antibody RET-P3 is detectable on the surface of rod cell bodies. It was barely detectable in a few cells at PN9. The number of positive cells and the level of expression increased until staining of the entire outer nuclear layer was strongly positive, at PN15.

RET-PE10 was detected in cultured rat and human RPE cells, but only when the fixed monolayers had been permeabilized with detergent (Figs. 5 and 6). The labelling given by RET-PE10 antibody was strongest in the perinuclear region of the cells, whereas the nucleus itself was unlabelled. Although a few pigmented cells were unlabelled in the human culture tested (see arrows in Figs. 6C, D), their different morphology and scarcity suggest they probably were contaminating cells from the choroid, neural retina, or vasculature.

Because RET-PE10 was located internal to the cell membrane, and because it showed a slightly fibrillar pattern in cultured cells, its staining was compared
with that of various known cytoskeletal components. Double-labelling experiments were conducted using RET-PE10 and a phalloidin/rhodamine conjugate that binds actin (Fig. 7). The distribution of actin filaments contrasted with that of RET-PE10: Actin microfilaments were distributed throughout the cell, especially concentrated around the cell periphery, whereas RET-PE10 was not found at all in the cell periphery. RET-PE10 staining also differed from that of tubulin (Figs. 8A, B) in that tubulin was usually asymmetrically distributed to one side of the cells and did not show the perinuclear concentration of RET-PE10 (Figs. 8E, F). Comparing RET-PE10 labelling with that using an antibody against a highly conserved portion of intermediate filament proteins (TIB 131) showed some similarity in staining (Figs. 8C, D). Intermediate filaments in these overnight cultures were concentrated in the perinuclear region and did not extend to the far periphery of the cells. Thus, it is possible that RET-PE10 is an epitope on intermediate filaments. The RET-PE10 epitope diminished until it was undetectable, after 72 hr in culture, regardless of whether the cells

FIGURE 6. Newly isolated human RPE cells express RET-PE10 antigen. Cultured human RPE (A, C; first passage, 20 hr in vitro), lightly permeabilized and stained with SP1 (A, B) or RET-PE10 (C, D). Note arrows that indicate unstained cells. (A, C) Phase image. (B, D) Fluorescent image. (Bar = 50 μm.)

FIGURE 7. Comparison of actin and RET-PE10 staining show dissimilar staining patterns. Cultured rat RPE (A; 1st passage, 20 hr in vitro) double labeled with rhodamine-conjugated phalloidin (B) and RET-PE10 (C). (A) Phase image. (B, C) Fluorescent image. (Bar = 10 μm.)
FIGURE 8. Comparison of antibody labelling shows that RET-PE10 staining differs from that of tubulin but does resemble intermediate filament staining. (A, C, E) Cultured rat RPE labeled with anti-tubulin (B), TIB 131 anti-intermediate filament (D), or anti-RET-PE10 (F). (A, C) Phase image. (B, D) Fluorescent image. (Bar = 10 μm.)

were cultured on plastic, collagen, or fibronectin. TIB 131 staining of cultured RPE intensified with increasing time in culture (not shown).

To determine whether the loss of RET-PE10 expression might be due to disruption of the interaction between RPE cells and nearby basal or apical influences, such as Bruch's membrane or rod photoreceptors, two experiments were carried out. In the first, eyecups were maintained in culture, fixed, sectioned, and labelled with antibodies detecting either photoreceptor or RPE antigens (Fig. 9). A variety of photoreceptor antigens, such as RET-P2, were detected, indicating that this layer had survived the culture. RET-PE2 antigen also was brightly labelled, indicating that the RPE layer remained intact. No RET-PE10 antigen was labelled, indicating that the altered environment of the eyecup culture led to a disappearance or masking of this epitope.

FIGURE 9. Culture of whole rat eyecups indicates that RET-PE10 antigen is lost from RPE that have maintained their normal basal and apical contacts in vitro. (A, C, E, G) Staining of cultured rat eyecup sections for SP1 (B), RET-PE2 (D), RET-PE10 (F) or RET-P2 (H). (A, C, E, G) Phase image. (B, D, F, H) Fluorescent image. Some autofluorescence is detectable over the photoreceptor outer segments, but this is easily distinguishable from the specific labelling of the RPE with RET-PE2 (D) and from the photoreceptors with RET-P2 (H). (Bar = 20 μm.)
FIGURE 10. Lack of rod outer segments in mutant mice does not prevent expression of RET-PE10 antigen. Staining of C3H rd/rd mouse eye sections (A, C) for SP1 (B) or RET-PE10 (D). (A, C) Phase image. (B, D) Fluorescent image. (Bar = 50 μm.)

For the second set of experiments, we tested whether a continued interaction with rod outer segments was necessary for RET-PE10 expression by examined eyes of rd/rd mice in which the rods had fully degenerated. As shown in Figure 10, expression of RET-PE10 was unaltered in the mutant.

**DISCUSSION**

RET-PE10 has been defined with a specific monoclonal antibody as an epitope on a 61 kD cytoplasmic polypeptide that was found in the RPE of a wide array of species. Immunocytochemical labelling of fixed cultured cells suggested that RET-PE10 was filamentous or cytoskeletal. The distribution of RET-PE10 showed some differences between intact RPE sheets and cultured RPE cells, possibly reflecting a change in the organization of the cytoskeleton. Based on the patterns of labelling given by reagents that recognize different classes of cytoskeletal filaments, it seems likely that RET-PE10 is associated with intermediate filaments. Several studies have examined the expression of distinct isoforms of intermediate filament proteins by RPE, focusing especially on vimentin and the cytokeratins, intermediate filament isoforms that are known to be commonly employed by various types of epithelia.\(^{29-30}\) These studies have shown that RPE of different species do not all share a similar complement of intermediate filament proteins. For example, cytokeratins 8 and 18 are present in bovine and human RPE\(^{28,29}\) but not in chick RPE.\(^{30}\) Because RET-PE10 is present in all of the species examined, it is unlikely to be one of these cytoskeletal proteins, but still may be associated with some portion of the intermediate filament network. It is interesting that on first appearance, RET-PE10 may be concentrated close to the cell margins, as suggested by Figure 3, whereas staining of adult and cultured cells is strongest in central regions of the cells. Further work using ultrastructural and biochemical methods is needed to clarify whether RET-PE10 is an integral component of the filaments, an associated protein, or a post-translational modification, such as phosphorylation.

The last possibility is supported by the observation that RET-PE10 was no longer detectable in RPE after 3 days of culture, even though intermediate filaments still were present. The loss of this epitope could have been the result of loss of contact with Bruch's membrane, loss of interaction with retina, loss of a circulating hormone or other serum factor, or a shift in cell activities from differentiated functions to migration and division. Because eyecup cultures lost RET-PE10 expression without detachment from Bruch's membrane, this is not likely the determining factor. Similarly, these cultures showed no sign of RPE migration and proliferation, suggesting that a change to a proliferative state is not essential for loss of RET-PE10 expression. In these cultures, the retina was present, alive (as judged by the well-maintained expression of a number of retinal membrane molecules), and in some areas in continued contact with apical RPE. Nevertheless, staining for such photoreceptor antigens may not be an adequate measure of retinal health in vitro, and without more functional measures we cannot exclude the possibility that a factor released from normal retina is necessary for maintained expression of RET-PE10. Because mature rd/rd mouse eyes, which had lost all rod photoreceptors, continued to express RET-PE10, any such retinal factor must have come from other retinal cell types and must not have depended upon light-induced retinal activity. Insulin can be eliminated from the list of candidates for such a trophic factor, because its presence in the medium does not induce RET-PE10 expression in RPE cultures (data not shown).

RET-PE10 was expressed late in eye development. It was first detected in central regions at PN9. This is approximately 4 days later than photoreceptor outer segments begin to form in the apposing retinal layer. Outer segment-specific antigens such as RET-P2 also can be detected earlier (PN5) than RET-PE10, as can the protein and mRNA for a number of components of the visual transduction apparatus, such as transducin and cyclic guanosine monophosphate-phosphodiesterase.\(^{31-33}\) However, not all known photoreceptor antigens are expressed before RET-PE10. As shown in Figure 3, a rod cell body surface molecule, RET-P3,
also is expressed with a similar time course to RET-PE10. RET-P3 shows some center to periphery gradient of expression, but this is partially masked by heterogeneity of expression by the rods at any location. Only at PN13 was the complete outer nuclear layer positive for RET-P3, and by this time, RET-P3 was present even in peripheral retina.

Because of the similarity in time course of expression between RET-P3 in rods and RET-PE10 in RPE, we considered the possibility that their expression requires some interaction between the two layers. The rd/rd mutant produces post-mitotic rod photoreceptors, but these never develop complete outer segments. Signs of degeneration can be found before the stage at which RET-PE10 is expressed, assuming the developmental expression is similar in mouse and rat. Thus, it is unlikely that rod photoreceptors in rd/rd mice provide an inductive signal for RET-PE10 expression in the adjacent RPE. A few surviving cone photoreceptors have been observed in animals up to 1 yr old, so the possibility exists that some cones still were present and able to supply the factors needed to sustain RET-PE10 expression.

The idea that the differentiated phenotype of RPE cells is under continuous regulation was suggested in our earlier studies. In the embryonic eye, the cell adhesion molecule N-CAM is expressed by both layers of the optic cup. As the RPE differentiates, N-CAM expression is lost. When the RPE cells are plated in monolayer culture, N-CAM polypeptide and mRNA are re-expressed within 24 hr. Pigment granules also are lost from cultured RPE but can be induced by growing the cells in medium supplemented with bicarbonate and insulin. In our studies, however, this treatment did not down regulate N-CAM expression or up regulate RET-PE10 expression, suggesting that different regulatory mechanisms may be involved.

Further studies are needed to determine how RPE cells regulate expression of specific antigens such as NCAM and RET-PE10. Understanding the molecular mechanisms involved in maintaining the differentiated state of RPE also will be important in devising ways to regulate RPE phenotype and thus prevent proliferation and migration of these cells in human disease.

Key Words
cell culture, development, intermediate filaments, monoclonal antibodies, retinal pigment epithelium.

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