Cytoskeleton in Normal and Reactive Human Retinal Pigment Epithelial Cells

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The cytoskeleton of normal and reactive retinal pigment epithelium (RPE) was analyzed immunohistochemically in five light microscopically normal formalin-fixed, paraffin-embedded human eyes enucleated because of orbital tumor and in 44 eyes with a uveal melanoma. In 26 eyes, the RPE overlying the tumor was morphologically normal or atrophic; in 18 eyes, hyperplastic changes were present. Normal RPE cells lacked vimentin, but it was present in 35 of 44 eyes with uveal melanoma. Antibodies that recognize cytokeratins CK8 and CK18 labeled normal and reactive RPE cells in all specimens. Although CAM 5.2 and CY-90 detected RPE cells strongly and quantitatively, clones CK5, KS-B17.2, and pancytokeratin antibody lu-5 reacted weakly and did not label some specimens. Immunoblotting supported the presence of CK8 and CK18 in human RPE. Normal RPE cells did not express other simple epithelial cytokeratins, but both atrophic and hyperplastic reactive RPE cells were labeled with antibodies to CK7 or CK19 in 24 of the 44 eyes. Hyperplastic proliferating RPE cells that formed subretinal membranes reacted positively for α-smooth muscle actin in 13 of 18 eyes. Antibodies to CK8 and CK18 are valuable markers of normal and reactive human RPE cells, but a panel of reagents is necessary to document reactive changes in the cytoskeleton. Acquisition of α-smooth muscle actin by human RPE cells may be related to their ability to form periretinal membranes and contribute to intraocular proliferative diseases. Invest Ophthalmol Vis Sci 32:3178–3186, 1991

The retinal pigment epithelium (RPE) participates in preretinal and subretinal membrane formation; this may complicate several common intraocular diseases.1-5 Unfortunately, RPE cells in such membranes cannot be identified accurately either by light or electron microscopy. They may resemble fibroblasts, myofibroblasts, or macrophages; secrete extracellular substances; and augment contraction of the membrane as a result of reactive changes.1-8 They also may become nonpigmented, and other cell types can phagocytose pigment granules.1-3,8-10 Antibodies to cytokeratins, the intermediate filament type characteristic of most epithelial cells, have been used to identify putative RPE cells in periretinal membranes.4-15

The value of cytokeratins as markers of human RPE is controversial, however, and antibodies to them either have been advocated as a powerful technique of elucidating the presence and distribution of RPE cells in epiretinal membranes9 or opposed as not being an appropriate marker to show such involvement.5 The cytokeratin types normally present in human RPE are still disputed,16-23 and no studies specifically have been done to characterize the cytoskeleton of reactive RPE cells to our knowledge. To clarify these controversies, we studied in detail the cytoskeletal structure of normal human RPE with special reference to changes observed under reactive conditions. For this purpose, RPE cells overlying choroidal melanomas were used as a model of reactive RPE change.24 In this context, the identity of the cells involved is more explicit than in periretinal membranes complicating vitreoretinal diseases.

Materials and Methods

Histologic Specimens

We selected five formalin-fixed, paraffin-embedded normal human eyes enucleated from patients (age range, 49–64 yr; mean, 60 yr) with an orbital tumor and 44 formalin-fixed, paraffin-embedded eyes enucleated because of a malignant uveal melanoma (patient age range, 13–79 yr; mean, 58 yr) from the files of the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital. These patients had not received preoperative radiotherapy or cytostatic drugs. The con-
junctival and corneal epithelia contained populations of cells that were positive for all types of cytokeratin polypeptide tested, providing an internal positive control. Lens epithelium, the ciliary muscle, and smooth muscle of larger blood vessels served as internal positive controls in stainings for vimentin, desmin, and α-smooth muscle actin, respectively.

Sections (5-μm thick) were cut from the specimens and mounted on chromium-gelatin-coated glass slides to ensure tissue adherence (prepared with 0.05 g of potassium chromium (III) sulfate dodecahydrate and 0.5 g of gelatin in 100 ml of distilled water).

**Immunohistochemical Staining**

The specimens were deparaffinized in xylene and rehydrated in an ethanol series. When antibodies to intermediate filaments were used, the sections were washed in phosphate-buffered saline (PBS; pH 7.4) and pretreated with 0.4% pepsin (2500 FIP U/g; E. Merck, Darmstadt, Germany) in 0.01 N hydrochloric acid at 37°C for 15 min to reduce background and enhance the intensity of specific staining.18 Endogenous peroxidase activity was destroyed with a 30-min treatment in methanol containing 0.5% hydrogen peroxide. The sections then were incubated with normal horse serum (1:50, Vectastain ABC Elite Kit; Vector, Burlingame, CA) in a moist chamber for 30 min at room temperature. All immunoreagents were diluted with PBS containing 2.0% (w/v) bovine serum albumin (BSA; E. Merck), and the sections were washed for three 10-min changes in PBS between every step.

Primary murine monoclonal antibodies against cytokeratin 4 (1:35, clone 215 B8, lot 12269420-01; Boehringer Mannheim, Mannheim, Germany), an epitope common to cytokeratins 5 and 6 (1:35, clone D5/16 B4, lot 12269820-01; Boehringer Mannheim), cytokeratin 7 (1:32,000 clone LdS68, lot 69F-4800; Sigma, St. Louis, MO),25 cytokeratin 13 (1:500, clone KS-1A3, lot 78F-4806; Sigma),26 three monoclonal antibodies to different epitopes on cytokeratin 18 (1:400, clone CK5, lot 37F-4958; 1:3000, clone CY-90, lot 49F-4815; and 1:500, clone KS-B17.2, lot 128F-4805; all Sigma),26,27 cytokeratin 19 (1:100, clone BA17, lot 109; Dakopatts, Glostrup, Denmark),28 a monoclonal antibody against an epitope common to cytokeratins 8 and 18, mainly recognizing cytokeratin 8 (1:2, clone CAM 5.2, lot M0910; Becton Dickinson, Mountain View, CA),29 and a monoclonal antibody to a conformational epitope common to most cytokeratin polypeptides, including 8, 18, and 19 (1:10, clone lu-5, lot 10968525-01; Boehringer Mannheim)30,31 were obtained commercially. Murine monoclonal antibodies to vimentin (1:750, clone V9, lot 48F-4827),32 desmin (1:400, clone DE-U-10, lot 117F-4809),33 and α-smooth muscle actin (1:8000, clone 1A4, lot 98F-4808)34 also were purchased from Sigma. Incubation with the primary antibodies was done in a moist chamber for 1 hr at 37°C.

Subsequently, the sections were incubated with biotinylated horse anti-mouse IgG antiserum (1:200; Vectastain ABC Elite Kit) and then with the avidin-biotinylated peroxidase complex (reagents A and B, both diluted 1:160, and mixed 30 min before use; Vectastain ABC Elite Kit) in a moist chamber at 37°C for 30 min.

To enable evaluation of the positive immunostaining reaction in pigmented epithelial cells, the peroxidase reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (150 mg in 16 ml of dimethyl sulfoxide and 200 ml PBS containing 0.03% hydrogen peroxide; Sigma) for 10 min at room temperature. This chromogen gives a diffuse or faintly granular dark brown reaction product that is resistant to hydrogen peroxide. Melanin then was bleached by incubating the sections for 18 hr at room temperature in 3.0% (v/v) hydrogen peroxide and 1.0% (w/v) disodium hydrogen phosphate.35 With this procedure, the depigmentation was nearly complete in most specimens, and any residual melanin pigment could be differentiated easily from a true-positive immunoreaction by its yellowish color and coarse granularity. Finally, the specimens were dried cautiously, and coverslips were mounted with Aquamount (BDH, Poole, UK). No immunoreaction was seen in the control sections, in which the primary or secondary antibody or the ABC complex was omitted.

**Gel Electrophoresis and Western Blotting**

Macroscopically normal RPE attached to the choroid away from a medium-sized uveal melanoma was obtained from two eyes immediately after enucleation. The specimen was homogenized in Laemmli’s36 sample buffer, followed by incubation for 5 min in a boiling water bath, centrifugation for 20 min at 12,000 rpm, and storage in frozen aliquots at −20°C.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was done according to Laemmli36 by using 1.5-mm thick 10% slab gels and a Protean II cell (Bio-Rad, Richmond, CA). After SDS-PAGE, the protein bands were transferred electically37 onto a nitrocellulose membrane (pore size, 0.45 μm; Schleicher & Schül, Dassel, Germany) at a constant voltage of 80 V for 3 hr in a Transblot cell (Bio-Rad).

The membranes were cut into strips and either stained for total protein with amido black (Bio-Rad) or immunostained with the cytokeratin antibodies. For immunostaining, nonspecific binding of protein
was blocked by treating the nitrocellulose strips for 2 hr with 3% (w/v) BSA in PBS, followed by the primary antibody at double the concentrations mentioned previously for 1 hr at 37°C. After washing in PBS, they were incubated with peroxidase-coupled rabbit antiserum to mouse immunoglobulins (1:500, lot 096; Dakopatts) for 30 min at 37°C. The peroxidase reaction was developed with 4 mg of 3-amino-9-ethylcarbazole (dissolved in 1.2 ml of N,N-dimethylaniline; Sigma) in 20 ml of 0.05 M sodium acetate buffer (pH 5.0) containing 0.03% hydrogen peroxide.

Results

Light Microscopy

The five eyes enucleated because of an orbital tumor were morphologically normal under the light microscope. In 22 of the 44 eyes enucleated because of malignant uveal melanomas, the RPE had undergone varying degrees of atrophy with breaks in continuity. In these eyes, the RPE cells, normally cuboidal, had a flattened and elongated shape. In 12 eyes, the RPE reaction was hyperplastic and consisted of alternating layers of proliferating cuboidal or flattened cells and amorphous basement membrane material. In six eyes, the RPE overlying the uveal melanoma showed alternating atrophic and hyperplastic changes in equal proportions. In four eyes, it appeared morphologically normal under the light microscope.

Immunohistochemistry

Normal RPE: Both in morphologically normal human eyes enucleated because of orbital tumors and away from the tumor in eyes containing a uveal melanoma, all antibodies tested that react with CK18 labeled the RPE (Figs. 1A–F, Table 1). The RPE cells were strongly reactive with the monoclonal antibody CY-90 (Figs. 1A, 1C) and CAM 5.2 (Fig. 1B). The monoclonal antibody KS-B17.2, however, gave a much weaker immunoreaction and did not label the RPE at all in 17 of the 49 eyes studied (Fig. 1D); the monoclonal antibody CK5 reacted with a weak-to-moderate intensity with all but three specimens (Fig. 1E). The pancytokeratin antibody lu-5 labeled the normal RPE cells with variable intensity in 39 of the 49 specimens studied (Fig. 1F). Although CY-90 and CAM 5.2 gave a rather uniform cytoplasmic staining in many specimens (Fig. 1A), the three other antibodies generally showed a rim of immunoreaction adjacent to the basal and lateral cell membranes, with the apical and central cytoplasm weakly immunoreactive (Figs. 1D–F). The light microscopically normal RPE did not react with antibodies to the other cytokeratin polypeptides used, even in eyes with a uveal melanoma (Fig. 1G).

A vimentin immunoreaction was not observed in RPE in any of the five normal human eyes studied with monoclonal antibody V9, except in a few cells in the immediate vicinity of the ora serrata. However, in 35 of the 44 eyes enucleated because of a uveal melanoma, the RPE cells away from the tumor reacted positively for vimentin (Fig. 1H). The immunoreaction was generally weak and variable from cell to cell, concentrating adjacent to the basal and lateral cell membranes (Fig. 1H).

In addition to the RPE, a variable population of choroidal cells with long slender cytoplasmic processes and scanty perinuclear cytoplasm reacted positively with CAM 5.2 in 31 and with CY-90 (Fig. 1A) in 42 of the 49 eyes studied. These were often pigmented and distinct from choroidal vascular endothelial cells, some of which also reacted with these antibodies. Other antibodies recognizing CK18 revealed single fibers in three eyes only. Similar choroidal cells reacted positively for vimentin, and some of them also were immunoreactive for α-smooth muscle actin (Fig. 1I).

Reactive RPE: In addition to a strong immunoreactivity with CY-90 (Fig. 2A) and CAM 5.2 (Figs. 2B, 3A, Table 1) in all specimens, in 8 of the 22 cases, in which the RPE overlying the melanoma was predominantly atrophic (Figs. 2C–D), and in 16 of 18 cases, in which it was at least partially hyperplastic (Figs. 3C–D), a population of RPE cells also reacted positively with the monoclonal antibodies Ld68 to CK7 (Figs. 2C, 3C), BA17 to CK19 (Figs. 2D, 3D), or both. The monoclonal antibody KS-B17.2 to CK18 often reacted more intensely with reactive than with normal RPE cells. Monoclonal antibody CK5 to CK18 labeled only a population of reactive RPE cells. The pancytokeratin antibody lu-5 gave a variable and generally weak immunoreaction in a population of reactive RPE cells (Fig. 3B). In addition, CK13 was detected in some flattened cells with KS-IA3 in two cases, in which the RPE reaction was of the atrophic type (Fig. 2E). The other cytokeratin polypeptides tested were absent from all specimens.

The monoclonal antibody V9 to vimentin reacted uniformly with atrophic (Fig. 2F) and hyperplastic RPE cells (Fig. 3E), the positive immunoreaction being distributed throughout the cytoplasm. In all 26 specimens, in which the RPE overlying the uveal melanoma was atrophic or morphologically normal, the RPE did not react with monoclonal antibody IA4 to α-smooth muscle actin. However, in 13 of the 18 eyes, with areas of hyperplastic RPE cells overlying the tumor, many of these reactive cells were immunoreactive using this antibody (Fig. 3F). The reactive RPE cells were negative for desmin in all eyes, regardless of the type of reactive change.
Fig. 1. Cytoskeleton in normal retinal pigment epithelium (RPE) (immunoperoxidase staining). (A) Mab CY-90 against CK18 gives a strong immunoreaction throughout the cytoplasm of RPE cells. Note a positively reacting choroidal cell with a long slender process (arrowhead) unassociated with vascular elements. (B) The strong immunoreaction obtained with mab CAM 5.2 against CK8 and CK18 concentrates in basal and lateral cytoplasm. (C) Cytokeratin bundles outline the periphery of tangentially cut cuboidal RPE cells. The nuclei and larger bleached melanin granules impart a finely vacuolated appearance to the less intensely immunopositive central cytoplasm. (D) Mab KS-B17.2 to CK18 reacts weakly with the basal and lateral peripheral cytokeratin bundles. (E) Mab CK5 to CK18 gives an identical reaction pattern. (F) Mab lu-5 against a conformational epitope common to most cytokeratins labels the RPE cells with a moderate intensity. (G) No positive immunoreaction for CK19 is obtained with mab BA17 in normal RPE (arrowhead). (H) Mab V9 to vimentin labels the peripheral basal and lateral cytoplasm in RPE cells. Note variation in reaction intensity from cell to cell. (I) Mab against α-smooth muscle actin does not label RPE cells (arrowhead) but reacts intensely with long slender cytoplasmic processes in the choroid (double arrowhead). Original magnifications: A, B, ×300; C, ×425; D-I, ×300.

One eye with a uveal melanoma contained a preretinal membrane consisting of a single layer of flat elongated cells, which did not contain any pigment (Fig. 3G). This membrane was intensely positive for CK18 with CY-90 (Fig. 3H) and CAM 5.2, and for vimentin with V9. Some of the cells also reacted with BA17 to CK19 and 1A4 against α-smooth muscle actin.

Western Blotting
The presence of a 52-kilodalton (kD) polypeptide corresponding to CK8 (Fig. 4, Lane 4) and a 45-kD polypeptide compatible with CK18 (Fig. 4, Lane 3) in human RPE was substantiated by western blotting with CAM 5.2 and CY-90, respectively. The epitopes recognized by KS-B17.2 and CK5 were below detection level in the two specimens studied (Fig. 4, Lanes 5, 6). Other types of cytokeratin were not detected in RPE cells by immunoblotting with LdS68, KS-1A3, or BA17 (Fig. 4, Lane 7).

Discussion
The cytoskeleton of normal RPE varies between different species. Avian RPE contains vimentin as its only intermediate filament type. Cytokeratins are found in addition to vimentin in bovine and
Table 1. Antigenic profile of normal and reactive human retinal pigment epithelium (RPE)*

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<th>KS-B17.2 CK18</th>
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* Immunopositive/negative specimens: CK = cytokeratin, Vim = vimentin, Des = desmin, and aSMA = a-smooth muscle actin. Clones 215 B8 to CK4 and DS/16 B4 to CK5/6 did not react with RPE cells.

† Except with antibodies CAM 5.2 and CY-90, only a population of cells was immunopositive.

Guinea pig RPE. In contrast, antibodies to vimentin do not label rat, frog, and monkey RPE cells, although cytokeratins can be detected in them and in mice and rabbits. With one exception, most authors have not obtained a convincing positive reaction for vimentin in human RPE either in fetal or adult eyes obtained at autopsy. In our study, vimentin was not detected in RPE of normal eyes enucleated because of orbital tumors. However, in at least 37 of the 44 eyes containing a uveal melanoma, morphologically normal RPE away from the tumor showed variable peripheral cytoplasmic immunoreactivity for vimentin, and most flattened or hyperplastic RPE cells directly overlying it reacted strongly for vimentin throughout their cytoplasm with this technique.

In monkeys, RPE cells accumulate vimentin after experimental retinal detachment, and they slowly lose it after retinal reattachment. Apparently, RPE cells may acquire this antigen as a reactive response to various intraocular diseases; this would explain why the RPE of human eyes enucleated because of retinoblastoma were positive for vimentin. Vimentin also accumulates in human, monkey, and bovine RPE.

Fig. 2. Cytoskeleton in atrophic retinal pigment epithelium (RPE) overlying a choroidal melanoma (mel) (immunoperoxidase staining). (A) Mab CY-90 to CK18 reacts strongly with flattened RPE cells. There is a recent break in RPE and Bruch's membrane, through which the tumor extends (between arrowheads) into the subretinal space. (B) Flattened RPE cells react uniformly with mab CAM 5.2 to CK8 and CK18. (C) Several atrophic RPE cells (arrowheads) react with mab LdS68 against CK7. (D) Flattened RPE cells are labeled with mab BA17 to CK19. (E) A few cells within the atrophic RPE (arrowheads) show immunoreactivity for CK13 with mab KS-1A3. (F) The flattened RPE cells (arrowhead) and neoplastic melanoma cells are strongly immunopositive for vimentin with mab V9. Original magnifications: A, ×250; B–F, ×300.
cells in culture, and caution must be exercised before classifying positive cells found in periretinal membranes as mesenchymal or fibroblastic.

Although normal human RPE often reacts positively for cytokeratins in eyes obtained at autopsy, others have been unable to detect them in similar material. Antibodies recognizing CK8 and CK18 labeled the normal RPE in all eyes we studied. The intensity of the positive reaction, however, was dependent on the antibody used. The pan-cytokeratin antibody lu-5 that recognizes a conformational epitope common to most cytokeratins, including CK8 and CK18, often did not react with human RPE. Other antibodies that detect CK18, such as LP34, also did not react with human RPE in previous studies. Reactive RPE overlying uveal melanomas continued to express CK18, and probably CK8, as judged by their uniform immunopositivity with CY-90 and CAM 5.2. The monoclonal antibody KS-B17.2 often reacted particularly strongly with hyperplastic RPE cells. This may be a result of similar changes in epitope availability that possibly caused preferential labeling of proliferating RPE cells by RGE 53. Antibodies against other types of cytokeratin did not label human RPE cells.

Taken together, our evidence suggests that simple epithelial-type cytokeratins CK8 and CK18 are the major intermediate filaments in normal human RPE. Polypeptides compatible with this cytokeratin pair were also detected by immunoblotting in samples from two eyes containing a uveal melanoma, and their equivalents have been found by immunoblotting in RPE preparations of bovine and rat origin. Antibodies against CK18 differed in their ability to react with the 45-kD polypeptide corresponding to CK18. A similar observation was made with antibo-

**Fig. 3. Cytoskeleton in hyperplastic retinal pigment epithelium (RPE) overlying a choroidal melanoma (mel) and forming subretinal membranes (srm) (immunoperoxidase staining).** (A) A multilayered subretinal membrane on top of flattened RPE cells (arrowhead) and remnants of normal choroid (ch) overlying a uveal melanoma are uniformly positive with mab CAM 5.2 to CK8 and CK18. (B) Compare with the weaker and weaker immunoreaction obtained with the pan-cytokeratin antibody lu-5 in the same specimen. (C) Reactive RPE cells within a thick subretinal membrane are strongly positive with mab LdS68 against CK7. (D) Similar reaction pattern is obtained with mab BA17 to CK19. (E) Hyperplastic RPE cells uniformly positive for vimentin. (F) Hyperplastic RPE cells react positively with mab 1A4 to a-smooth muscle actin. (G) A thin preretinal membrane (arrow) overlies the nerve fibre (nfl) and ganglion cell (gel) layers. Note total absence of pigmentation (PAS stain). (H) In addition to the RPE cells (arrowheads), the preretinal membrane (arrow) is intensely positive for CK18 with mab CY-90. Original magnifications: A-F, x250; G, x425; H, x210.
Fig. 4. Western blotting of SDS-PAGE-separated polypeptides from crude homogenates of human choroid and retinal pigment epithelium. Lane 2: Amido black staining for total protein. Lanes 3-7: Immunoblotting for cytokeratin polypeptides. Mab CY-90 (Lane 3) reveals a 45-kD polypeptide corresponding to the relative mobility of CK18, and CAM 5.2 (Lane 4) reacts with a single 52-kD polypeptide compatible with CK8. Mabs KS-B17.2 (Lane 5) and CK.5 (Lane 6) against CK18, as well as mab BA17 (Lane 7) against CK19 have not detected any polypeptides in this specimen. Molecular weight standards: (Lane 1; top to bottom) phosphorylase B (92.5 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21.5 kD).

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1 2 3 4 5 6 7

CK8

CK18

It is possible that other, unrelated types of cytokeratin may be present in reactive RPE cells because an immunoreaction to CK13, normally expressed in stratified epithelia only, was detected in a few cells. In one study, a bewildering array of polypeptides, thought to correspond to cytokeratins CK5, CK6, and CK14 through CK16, in addition to other unidentified bands, were found by immunoblotting in cultured human RPE cells. However, CK13 was not detected in these cultured cells. The identity of the choroidal cells that reacted positively for cytokeratins CK8 and CK18 is unknown, but they did not appear to participate in subretinal membrane formation in any specimen.

Although both morphologically normal and atrophic, apparently nonproliferating RPE cells were negative for α-smooth muscle actin, most proliferating RPE cells that formed subretinal membranes were immunopositive. This observation suggests that metaplastic RPE acquired contractile properties pertinent to the growth of the subretinal membrane, a phenomenon that might be important in proliferative vitreoretinopathy and other intraocular proliferations in which RPE has been implicated. Previously, actin has been detected in most periretinal membranes and derived cell cultures, but no correlation to their contractility was found with antibodies recognizing all actin types. This is understandable because other isolectric forms of actin predominate in RPE cells. Antibodies to α-smooth muscle actin will provide a much more specific tool for such studies.

In conclusion, the selection of cytokeratin antibodies is of paramount importance in studies of RPE cells. Although some authors advocate broadly reacting polyclonal or monoclonal antibodies, these seldom detect optimally any particular cytokeratin type. Negative results with the broad-spectrum cytokeratin antibody LP3413-15 are probably related to the fact that it does not detect CK8 and CK19, major components of normal and reactive RPE, respectively. Polyclonal antisera often react poorly with CK18. In our study, the monoclonal antibody CY-90 against CK18 was the most reliable reagent to detect normal and reactive human RPE cells. Only by using a panel of
antibodies to different cytokeratin types and epitopes can changes in their expression, which may underlie important pathobiologic properties, be recorded. When judiciously used, antibodies to cytoskeletal components are powerful tools to study basic pathologic changes in diseases of the RPE.

Key words: intermediate filaments, smooth muscle actin, retinal pigment epithelium, subretinal membranes, malignant uveal melanoma

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References