Controlling Retinal Pigment Epithelium Injury after Experimental Detachment of the Retina

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Purpose. Damage induced by detachment of the neural retina and the retinal pigment epithelium (RPE) can be reduced by dark adaptation. The authors evaluated the influence of the duration of dark adaptation, time of day, and modification of the melatonin-dopamine pathway on acute RPE lesions induced by mechanical detachment.

Methods. BALB/c mice were studied at different times of day and different periods of dark adaptation. Some mice were treated with melatonin or sulpiride, a D2 dopamine receptor antagonist. Enucleated eyes and different saline solutions were used in experiments ex vivo. Retinal detachments in vivo were made by subretinal injections of hyaluronic acid. RPE cell damage was quantitatively evaluated with a dye exclusion procedure, and their viability was tested by preservation of tight junctions in culture. Lectin histochemistry was used to examine the interphotoreceptor matrix (IPM).

Results. Significant propidium iodide (PI) incorporation in RPE cells was detected after ex vivo separation during daytime, but it was very low when detachment took place at night after 24 to 48 hours of dark adaptation. PI exclusion was achieved during daytime after a single hour of dark adaptation when mice were pretreated with melatonin or sulpiride. Reduction of RPE cell damage was accompanied by decreased lectin binding to cone sheaths.

Conclusions. A combination of time of day and length of dark adaptation decreased damage induced by detachment of the retina ex vivo and in vivo. Melatonin or sulpiride could replace these environmental factors. Therefore, melatonin and dopamine pathways might be involved in the control of IPM properties and retina/RPE interactions. (*Invest Ophthalmol Vis Sci.* 2007;48:1348–1354) DOI:10.1167/iovs.06-0964

Artificial separation of the neural retina and the retinal pigment epithelium (RPE) is sometimes performed for specific purposes such as excision of a subretinal membrane, reapplication of a detached and folded retina, or performance of macular translocation surgery.[1,2] Recovery of visual function depends on the reestablishment of functional associations among these layers. Because of the close anatomic interdigitation of photoreceptor outer segments and RPE microvilli, epithelial cells may remain attached to the retina when RPE is mechanically separated. This phenomenon has been used to evaluate neural retina/RPE adhesion strength.[3,4] Attachment also depends on cone sheaths, a specialized region of the interphotoreceptor matrix (IPM) that encases outer and inner segments of cone (but not rod) photoreceptors.[5] Cone sheaths are chemically and structurally distinct from the remainder of the IPM, as revealed by specific binding of peanut agglutinin (PNA) and their structural stability during physical dissociation of the retina.[6,7]

Effects of light exposure,[8] ionic environment, temperature, oxidative metabolism, and hydrostatic and osmotic pressures on retinal adhesion are widely known.[9] Retinal detachment in rabbits is facilitated by Ca^{2+}- and Mg^{2+}-free (CMF) solutions,[10,11] especially after a brief period of dark adaptation.[10] Melatonin is rhythmically synthesized in the retina and acts as a neuromodulator imparting photoperiodic information to the retina.[12–15] This hormone is involved in the regulation of several retinal functions, but its role in retinal detachment has not been previously investigated.

Although we have been able to obtain RPE laminae of high morphologic quality after an hour of dark adaptation, we found that epithelial structure rapidly deteriorated after a few hours in culture. Therefore, we used propidium iodide (PI) to detect submicroscopic damage to RPE cells that occurred before the appearance of overt necrotic or apoptotic signs. This dye-exclusion procedure has been found useful in several tissues, including RPE cells.[16] Here we report observations demonstrating that time of day and duration of dark adaptation affected the viability of detached RPE and that these effects could be reproduced by melatonin. Times and treatments that reduced RPE cell damage also decreased lectin binding to cone sheaths.

Materials and Methods

Male BALB/c albino mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Conditions

Mice were bred under a light/darkness cycle of 12 hours each (7:00 am to 7:00 pm) with maximum illumination levels of 60 lux at the cage floor. At the beginning of experiments, mice were kept in complete darkness between 1 hour and 7 days. Animals were housed in a double enclosure with food and water ad libitum. Feeding and cage cleaning were carried out under a dim red light. One group of animals was submitted to a shifted light cycle (12-hour light/12-hour dark).

Surgical procedures were carried out under chloral hydrate anesthesia (400 mg/kg, intraperitoneal) and bulbar topical application with 0.25% proparacaine (diluted Anestalcon; Laboratorios Alcon, Buenos Aires, Argentina). Melatonin (0.05 mg/kg; Sigma-Aldrich Chemical Co., St. Louis, MO) or the D2 dopamine receptor (D2R) antagonist sulpiride (50 mg/kg; Armstrong-Syncro, Buenos Aires, Argentina) was administered subcutaneously. Control animals received similar volumes of saline solution. Injections and experimental procedures were performed by different researchers.

Ex Vivo Separation of Neural Retina and RPE

Pharmacologic treatments, enucleation, and separation were performed under dim red light and aseptic conditions. Room temperature was set at 28°C because temperature can modify adhesion between retina and RPE.[3]

After enucleation, the cornea, lens, and vitreous were dissected through perilimbal incision, and the optic nerve stump was removed

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by peripapillar section, allowing detachment of the neural retina without further manipulation. Dissection and separation were made either in Ringer/lactate solution (Fidex, Buenos Aires, Argentina), Hanks balanced saline solution (HBSS) containing 136.9 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.49 mM $MgCl_2$ (6 H_2O), 0.41 mM $MgSO_4$ (7 H_2O), 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 (7 H_2O), 4.17 mM $NaHCO_3$, 5.55 mM glucose, or Ca^{2+} and Mg^{2+} free-HBSS (CMF-HBSS) containing 139.06 mM NaCl, 5.37 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 (7 H_2O), 4.17 mM $NaHCO_3$, and 5.55 mM glucose. The RPE-choroid-sclera cup was immersed in RPMI 1640 media supplemented with 25 mM HEPES and L-glutamine (Gibco, Invitrogen, Carlsbad, CA).

In Vitro Culture

Small disks were obtained from the posterior region of the RPE-choroid-sclera complex using a punch tool made with a nonbeveled 25-gauge needle. They were incubated in the culture medium described above in a humidified atmosphere with 5% CO_2 at 37°C, for 0 to 24 hours.

Retinal Detachment In Vivo

BALB/c mice cared for under normal day/night illumination were used for these experiments. At noon they received subcutaneous injections of melatonin or saline and were immediately transferred to the dark enclosure. At 1:00 pm, they were anesthetized as described. Their pupils were dilated with 5% phenylephrine and 0.5% tropicamide (Fotorretin; Laboratorios Poen, Buenos Aires, Argentina), and the eye was covered with 0.25% carbomer (carboxypolymethylene) drops (Latlas; Atlas, Buenos Aires, Argentina). Procedures were carried out under dim red light except during detachment, which required visual inspection under the surgical microscope. A 30-gauge needle was inserted on the dorsal quadrant, below the limbus, and a 1:1 mix of hyaluronic acid (Hyasol; Bausch & Lomb, Buenos Aires, Argentina) and CMF-HBSS was slowly injected until a detachment covering the dorsal quadrant could be observed. White light was turned on just for the detachment procedure, and eyes were covered immediately afterward. The researcher performing the detachment was blinded to the previous treatment. Mice were euthanatized with chloral hydrate, and eyes were enucleated 15 minutes after detachment. Eyecups were dissected in CMF-HBSS and immediately immersed in culture medium.

Propidium Iodide Uptake

Specimens were incubated in 0.1 μg/mL PI (Sigma-Aldrich) in culture medium. After 15 minutes they were washed 4 times in fresh medium and then fixed for 30 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS). Samples were mounted in glycerol and examined under UV light for evaluation of cell nuclei PI fluorescence (Fluorescence Filter Block, TRITC, excitation 540/25, dichroic mirror 565, barrier filter 605/55; Nikon, Tokyo, Japan) and cytoplasmic autofluorescence (Fluorescence Filter Block, UV-2 A, excitation 330/380, dichroic mirror 400, barrier filter 420; Nikon) to assess continuity of the epithelial lamina.

PI-incorporating cells were scored in whole eyecups. Stained cells (mononucleated and binucleated) were quantified in 16 microscopic fields (64,000 μm^2) covering all quadrants of the posterior eyecup. Six retinas from different animals were used for each experimental point. Comparisons were made with two-way ANOVA with Bonferroni posttests (GraphPad Prism, version 4.00 for Windows; GraphPad Software, San Diego CA; www.graphpad.com).

Zonula Occludens-1 Immunofluorescence

Fixed eyecups or punched disks were incubated overnight with an antibody against the tight junction-associated protein zonula occludens (ZO)-1[17] (Zymed, South San Francisco, CA) and then were labeled with fluorescein-5 isothiocyanate-conjugated goat anti–mouse IgG (FITC; Jackson ImmunoResearch Laboratories, West Grove, PA). Digital images were obtained with a conventional fluorescence microscope (Eclipse 800; Nikon) or with a laser confocal microscope (Laser Scanning System Radiance 2000; BioRad, Hemel Hempstead, UK). Control and treated specimens were examined sequentially on the same settings. Optical sections separated by 0.5 μm were performed in the z-axis, and images were processed (Confocal Assistant Software; BioRad).

Lectin Histochemistry

Mice subjected to different illumination conditions or melatonin treatment were fixed by intracardial perfusion with 4% paraformaldehyde in 0.1 M, pH 7.4, phosphate buffer. Eyes were enucleated, postfixed in the same solution for 1 hour, and cryoprotected using graded sucrose solutions.[18]

Wheat germ agglutinin (WGA) and PNA were used to label the IPM. The former detects membrane surfaces of RPE microvilli and rod- and cone-associated matrices, whereas PNA specifically stains cone sheaths.[6,7] Eyes submitted to different treatments were processed simultaneously to avoid variations in lectin binding. Consecutive cryosections (16 μm) through the central retina were incubated at 37°C with Texas Red-conjugated WGA (1/15; Sigma-Aldrich) or fluorescein-5 isothiocyanate-conjugated PNA (1/10; Sigma-Aldrich) for 30 minutes. Microscopic examination and confocal imaging were performed immediately after incubation.

Results

Ex Vivo Separation of Retina and RPE: Effects of Dark Adaptation and Time of Day

Immediately after separation, autofluorescence and ZO-1 immunostaining showed RPE appearing as a continuous epithelial sheet. RPE cells exhibited their normal polygonal shape and were bounded by a continuous thin belt of ZO-1 immunoreactivity. However, intracellular penetration of PI indicated that these preparations had cell membrane damage because PI is excluded by normal cell membranes. Screening experiments with Ringer solution showed that a dye-excluding epithelial lamina could be obtained from animals that underwent enucleation at the beginning of the night phase after 48 hours of constant darkness (Fig. 1). By contrast, PI incorporation was detected in RPEs separated in the daytime even after 7 days of constant darkness. Therefore, we made a quantitative study of PI incorporation into RPE cells after three different durations of constant darkness: 1, 24, and 48 hours. Mice underwent enucleation at 6:00 am, 8:00 am, 1:00 pm, and 8:00 pm. These times of day were chosen to represent the last hour of night phase, the beginning and middle parts of the day phase, and the beginning of the night phase. At each experimental point, separation was performed either in HBSS or in CMF-HBSS.

In specimens separated in HBSS (Fig. 2A), 1 hour of constant darkness resulted in high numbers of PI incorporating cells at every time of day. After 24 hours of constant darkness, a large reduction of PI incorporation was observed in mice that underwent enucleation at 8:00 pm, 1 hour after lights were turned off. After 48 hours of constant darkness, a similar reduction was measured at 6:00 am, 1 hour before lights were turned on. When separation was performed at 8:00 pm, almost no PI-positive nuclei were detected. By contrast, PI incorporation was always very high in RPE specimens separated during daytime, notwithstanding the duration of dark adaptation. Two-way ANOVA indicated significant effects ($P < 0.0001$) of duration of darkness and time of day, with a large and significant interaction between them ($P < 0.0001$). Comparisons by two-way ANOVA and Bonferroni posttests between replicate means by row showed that no significant differences were present between different times of day after just 1 hour of constant darkness. After 24 hours of constant darkness, a sig-

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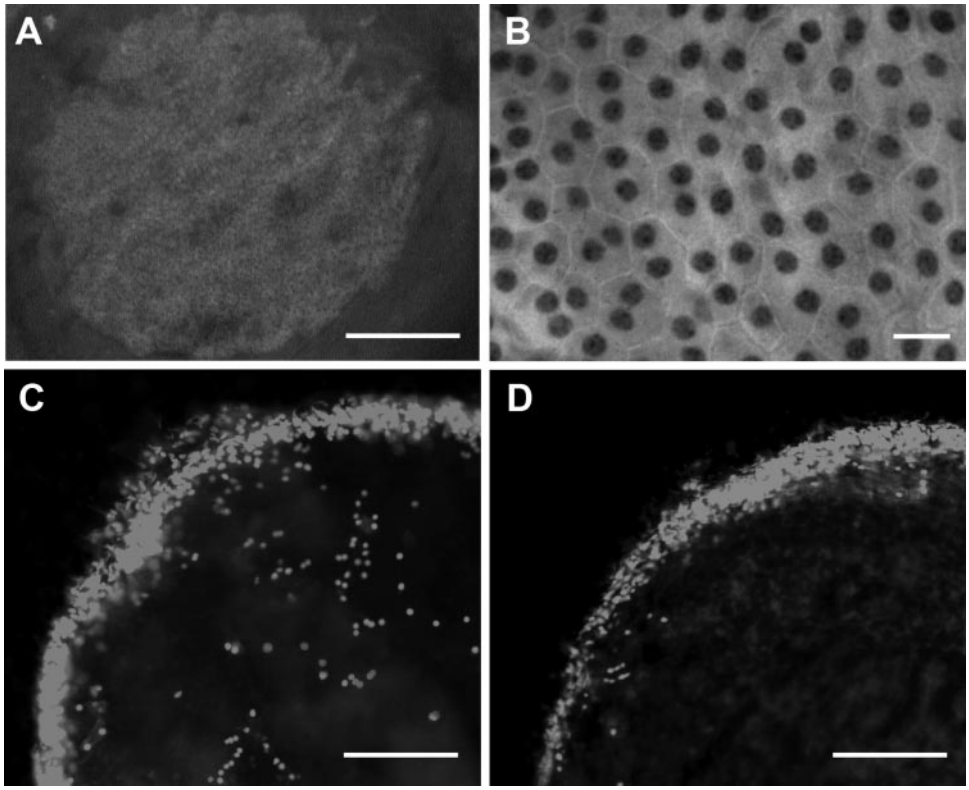

FIGURE 1. RPE disks separated ex vivo. (**A**) Low magnification image of a disk. Autofluorescence shows the continuity of the epithelial lamina. Scale bar, 200 μm. (**B**) A similar disk stained with cresyl violet and viewed under the transmission mode of the confocal microscope shows good preservation of the epithelial cell shape and nuclei. Scale bar, 15 μm. (**C**) RPE disk stained ex vivo with PI. This disk was obtained in Ringer solution at 7:00 pm after only 24 hours of dark adaptation. The outer rims of the stained nuclei reflect dissection trauma, and the epithelial lamina contains several positive nuclei. Scale bar, 100 μm. (**D**) A similar disk obtained at 7:00 pm after 48 hours of dark adaptation. Note the complete absence of fluorescent nuclei within the epithelial lamina. Scale bar, 100 μm.

nificant decrease was observed in PI nuclei density at 8:00 pm ($P < 0.001$), whereas after 48 hours significant differences were observed at 6:00 am ($P < 0.001$) and 8:00 pm ($P < 0.001$).

Other experiments showed that the decrease in RPE damage after 48 hours of dark adaptation could be observed since 6:00 pm. The difference between RPE separated during night and during day phases was also evident in mice kept under a shifted light cycle for 2 weeks before the experiment.

After separation of RPE in CMF-HBSS (Fig. 2B), two-way ANOVA also indicated significant effects ($P < 0.0001$) of duration of darkness and time of day, with a large and significant interaction between the two factors ($P < 0.0001$). In these retinas, 1 hour of darkness resulted in high values at every time of day, with PI scores almost identical to those measured in HBSS. After 24 hours of constant darkness, significant decreases of nuclear labeling were observed at 1:00 pm ($P < 0.001$) and 8:00 pm ($P < 0.001$). After 48 hours, a significant decrease was observed at 6:00 am ($P < 0.001$), whereas values remained as low as before at 1:00 pm ($P < 0.001$) and 8:00 pm ($P < 0.001$). Nuclear labeling at 1 pm in CMF-HBSS preparations was significantly different from the same parameter in HBSS preparations after 24 and 48 hours of constant darkness (both $P < 0.001$).

Effects of Melatonin

Because melatonin synthesis in the retina is elevated at night and is reduced during the day,[15] we reasoned that administration of this neurohormone would reproduce the night-phase effect. Melatonin treatment allowed good RPE preparations after just 1 hour of dark adaptation. As shown in Table 1, comparison of samples obtained at 1 pm, after 1 hour of dark adaptation, showed that PI scores in melatonin-treated mice were significantly lower than PI scores in control mice ($P < 0.001$). By contrast, values from melatonin-treated mice were almost identical with those measured in RPEs detached at 8:00 pm after 48 hours of darkness ($P > 0.05$).

Administration of exogenous melatonin can generate rhythms of retinal dopamine.[19] Therefore, we tested the effect of sulpiride, a selective D2R antagonist, on cell membrane damage after mechanical detachment (Table 1). PI incorporation after sulpiride was higher than after melatonin treatment ($P < 0.01$) but still substantially lower than the scores of saline-treated mice that underwent enucleation at the same time of day ($P < 0.001$).

No significant differences were observed between specimens separated in media with and without Ca^{2+} and Mg^{2+}, HBSS, and CMF-HBSS, respectively.

Survival of RPE Cells In Vitro

Punched disks were incubated for 12 or 24 hours in vitro. In disks obtained at 1 pm from mice submitted to a single hour of dark adaptation, the normal polygonal ZO-1 pattern disappeared during the first 3 hours of incubation (Fig. 3A). Similar deterioration was observed in specimens dissected at noon after 24 to 48 hours of dark adaptation. By contrast, disks obtained at 8:00 pm after 48 hours of dark adaptation retained the normal ZO-1 pattern for 12 or 24 hours in vitro (Fig. 3B). These cultures also maintained dye exclusion (data not shown). Identical conservation of dye exclusion and ZO-1 immunostaining pattern was observed in disks obtained at 1 pm, 1 hour after melatonin injection (Fig. 3C).

RPE after Retinal Detachment In Vivo

Treated and control animals were easily detached. Although blinded to previous treatment, the researcher performing detachment could identify melatonin-injected mice by lesser resistance to subretinal injection (19 positive identifications in 25 animals). Animals underwent enucleation 15 minutes after detachment, and almost no manipulation was required to separate retinas in melatonin-treated mice. PI-labeled cells were abundantly found in all areas of control RPEs (Fig. 4). By contrast, in melatonin-treated eyes, PI was incorporated only

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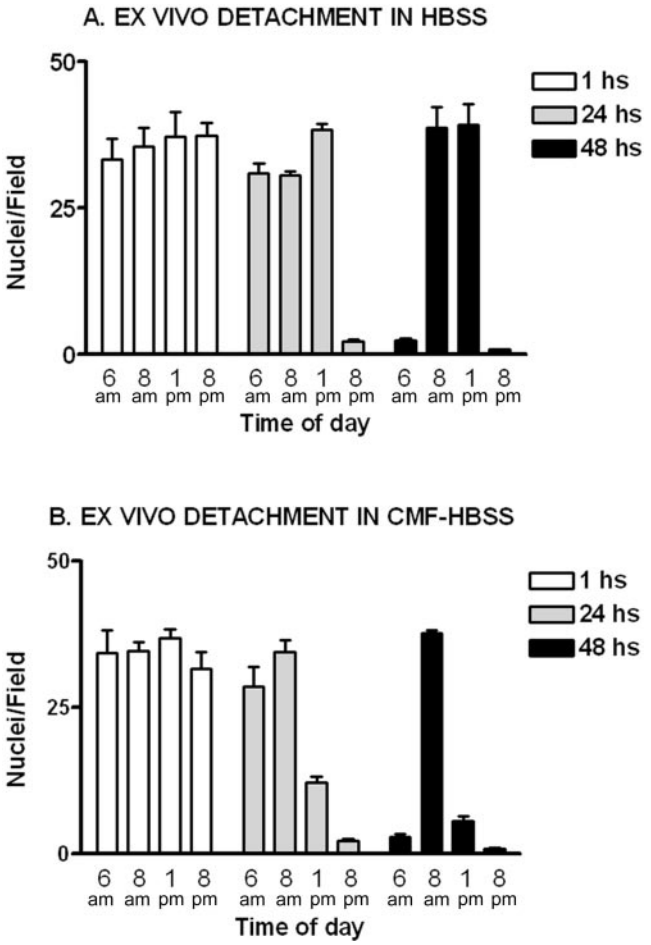

FIGURE 2. Quantitative evaluation of PI incorporation in RPE cells after ex vivo detachment. Bars indicate density of PI labeled nuclei (number of PI labeled nuclei/field) in mice submitted to constant darkness for 1, 24, or 48 hours. For each period of constant darkness, specimens were studied at 6:00 am, 8:00 am, 1:00 pm, and 8:00 pm. (**A**) Separation in HBSS. (**B**) Separation in CMF-HBSS.

by cells surrounding the needle puncture. The rest of the RPE was almost free of PI-labeled cells.

IPM under Different Illumination Conditions and Melatonin Treatment

We hypothesized that changes in RPE injury after separation under different illumination conditions could depend on IPM organization. Therefore, we analyzed changes in the binding of PNA and WGA in mice fixed at 8:00 pm after 1 hour or 48 hours of darkness. Comparisons were made using projections from 10 consecutive 0.5-μm confocal optical sections obtained with the same laser settings. PNA stained the characteristic cone sheaths. Outer and inner segments could be identified, though the latter usually bound more PNA than the former. In retinas fixed after 1 hour of darkness, cone sheaths appeared as a collection of granules strongly stained by PNA. Staining intensity of cone sheaths decreased after 48 hours of darkness (Figs. 5A, 5B). WGA uniformly labeled the outer segment layer, though some highly fluorescent spots appeared close to the inner segment layer. Retinas

TABLE 1. Number of Cells Incorporating PI per Microscopic Field in Mouse RPE Detached from Neural Retina Ex Vivo

	HBSS	CMF-HBSS
1-h darkness, 1 pm	37.24 ± 4.18	37.07 ± 1.82
Melatonin, 1-h darkness, 1 pm	0.86 ± 0.14	1.11 ± 0.08
Sulpiride, 1-h darkness, 1 pm	9.77 ± 0.99	9.74 ± 0.79
48-h darkness, 8 pm	0.85 ± 0.07	0.89 ± 0.11

n = 6 are shown for each experimental point. Melatonin (0.05 mg/kg) or sulpiride (50 mg/kg) was given at noon to mice under basal illumination conditions.

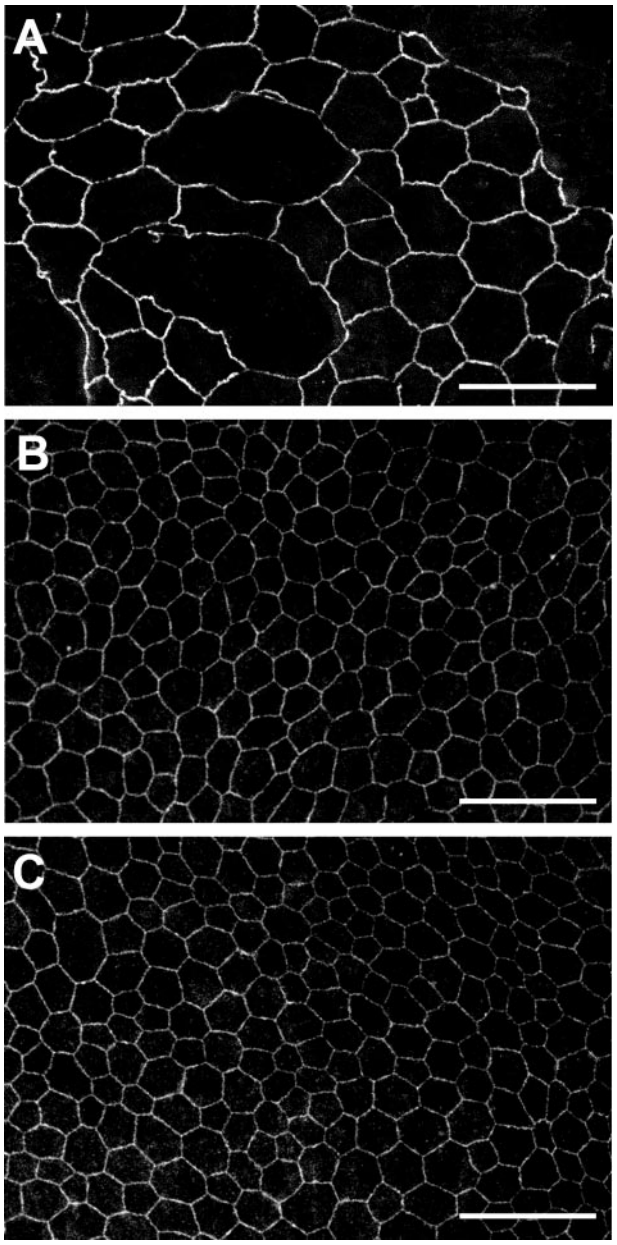

FIGURE 3. RPE specimens obtained under different conditions and immunostained with ZO-1. Images correspond to the projection of all optical sections (0.5 μm) passing through the tight-junction belt. (**A**) Specimen obtained at 1 pm, after 1 hour of dark adaptation, shows extensive damage of the epithelial lamina after incubation in vitro for 3 hours. (**B**) This low-magnification image shows preservation of the normal hexagonal pattern of ZO-1 labeling in a specimen obtained after 48 hours of dark adaptation, at the beginning of the night phase, and incubated in vitro for 12 hours. (**C**) A similar normal pattern was observed in this specimen obtained after melatonin treatment. Note that time of day and illumination conditions were as described in (**A**) and that incubation in vitro lasted 12 hours. Scale bars: (**A**) 25 μm; (**B**, **C**) 50 μm.

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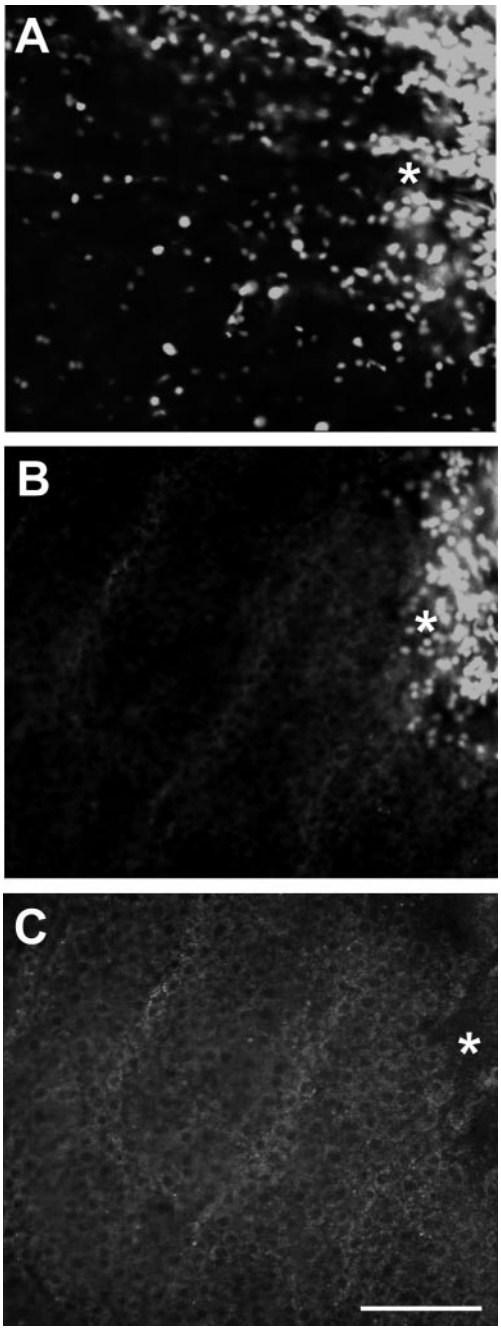

FIGURE 4. RPE whole-mounts from specimens detached in vivo. *Asterisk*: subretinal injection site. (**A**) In a control specimen receiving saline solution and detached after 1 hour of dark adaptation, a large amount of nonexcluding cells can be observed around the injection site and over the detached surface. (**B**) In a specimen receiving melatonin under the same conditions, nonexcluding cells are only found around the injection site. (**C**) Control image to demonstrate structural preservation of the RPE lamina, as shown by autofluorescence. Scale bar for all figures, 50 μm.

fixed after different periods of darkness showed the same staining pattern, but fluorescence became much stronger after 48 hours of darkness (Figs. 5C, 5D).

To study the influence of melatonin on the IPM., mice received injections at noon and remained in a dark environment until they underwent perfusion at 1:00 pm. In saline-injected mice, cone sheaths showed the same lectin-staining pattern observed at 8:00 pm after 1 hour of darkness. After melatonin injection, a decrease of PNA binding to cone photoreceptors could be clearly observed, particularly around outer segments. In these retinas, PNA binding seemed confined to cone inner segments (Figs. 6A, 6B). In control mice, WGA showed an amorphous matrix. Melatonin injection induced a large increase in staining intensity, almost identical to that observed after 48 hours of darkness (Figs. 6C, 6D). Thus, changes in lectin binding detected 1 hour after melatonin injection at noon resembled those observed after 48 hours of constant darkness at 8:00 pm.

DISCUSSION

Our experimental observations demonstrate that ex vivo separation of the neural retina during the illuminated phase of a normal light-darkness cycle induced submicroscopic damage of RPE cells that could be detected by PI incorporation. These findings could be helpful to obtain undamaged RPE cells for primary cell cultures. On the other hand, they also provide new information about RPE–retina interactions.

Signs of cell membrane damage appeared even in RPE preparations in which autofluorescence and ZO-1 immunolabeling indicated good preservation of epithelial architecture. Thus, PI incorporation reflected a more subtle damage than the loss of epithelial continuity and persistence of RPE cells attached to the retina often used to evaluate RPE injury.[3,4] In vitro incubation of PI-incorporating RPE specimens resulted in cell swelling and distortion of the epithelial layer after just 3 hours in vitro, suggesting a necrotic phenomenon.

Changes in the morphology of RPE apical processes and proliferation of detached RPE have been reported during the first 12 hours after in vivo detachment.[20,21] TUNEL labeling after detachment has only shown apoptosis in photoreceptor nuclei.[22,23] Thus, our present findings suggest that RPE proliferation could be a consequence of the initial injury detected by PI incorporation. Given that epithelial cell behavior is modulated by alterations in cell–cell and cell–substrate contacts,[24] death of even a few RPE cells would alter contact interactions, triggering migration, proliferation, and other long-term consequences of detachment-reattachment on the RPE–retina interface.[23,25,26] Detachment could also induce metastable states compatible with both survival and necrotic cell death.[27] Outcome of such metastable states might be a crucial turning point in the evolution of a retinal detachment. Detachment at night after 48 hours of dark adaptation or after melatonin treatment could preserve RPE from injury or influence the outcome of those metastable states.

Highly relevant to understanding RPE–retina interactions is our finding that RPE cell membrane status was shaped by the light history of the animal and the time of day enucleation was performed. When only 1 hour of dark adaptation was allowed, high PI incorporation was present at every phase during the day. By contrast, low PI incorporation was found when separation procedures were carried out at the beginning of the night phase after 24 hours of constant darkness. Moreover, no RPE cell damage could be detected when 48 hours of darkness were allowed. The end of the night phase also favored RPE preservation, but only after 48 hours of dark adaptation. All tested media—Ringer, HBSS, and CMF-HBSS—were equally effective to obtain complete dye exclusion at the beginning of the night phase after 48 hours of dark adaptation. Separation in CMF-HBSS, however, reduced PI incorporation at noon, after both 24 and 48 hours of dark adaptation.

High PI incorporation 1 hour after the onset of light could be related to early morning rod shedding and the associated burst of RPE phagocytosis, which in mice is highest 2 hours after light onset.[28] This is a time of high retinal adhesion, which peaks 3.5 hours after the onset of light.[29]

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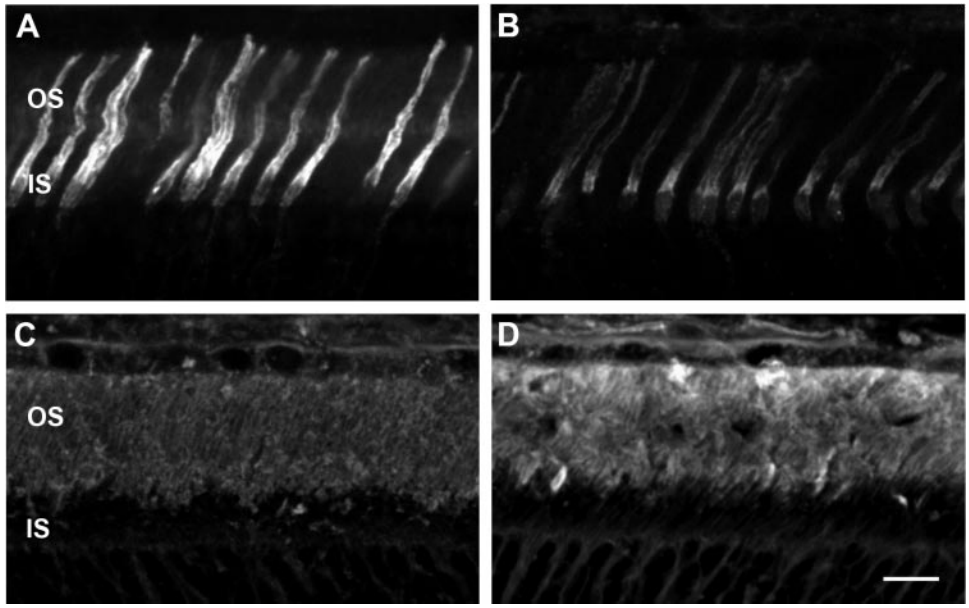

FIGURE 5. Retinal cryosections from animals subjected to constant darkness for 1 hour (**A**, **C**) or 48 hours (**B**, **D**) and fixed 1 hour after the beginning of the night phase. (**A**, **B**) PNA-fluorescent label surrounds individual cone inner and outer segments (IS, OS). Note the decrease in OS labeling in the specimen fixed after 48 hours of darkness. (**C**, **D**) WGA binding shows an amorphous matrix surrounding photoreceptor OS. Comparison of these retinas shows an increase of bound lectin after 48 hours of constant darkness. Scale bar, 15 μm.

A low dose of melatonin during the day phase reduced PI incorporation, after ex vivo detachment, to the same levels of the night phase in 48-hour dark-adapted mice. The shielding effect of melatonin during in vivo experimental detachment indicated that our observations were not derived from ex vivo conditions. Melatonin is involved in the control of photoreceptor disc shedding and phagocytosis, probably through a circadian oscillator entrained by the daily light-dark cycle.[30,31] Melatonin synthesis is activated only when retinas are submitted to darkness during the last hours of the day or during the night.[13] Therefore, the time pattern of PI incorporation in detached RPE might partially reflect an intrinsic circadian rhythm because the high PI incorporation at 1 pm with a low PI incorporation at night persisted even after 7 days of constant darkness, and the time of maximal RPE dye incorporation could be reversed by light-phase shifting.

Some melatonin effects are mediated by receptors in dopaminergic neurons,[13,32] and stimulation of D2Rs mimics light on several retinal rhythmic phenomena.[33,34] Partial reproduction of the light history effects on RPE cell membranes by a selective D2R antagonist supports the involvement of a melatonin–dopamine pathway in adhesive interactions between neural retina and RPE.

Melatonin might also act independently of the circadian oscillator. This hormone is a well-known antioxidant and free radical scavenger[35] that protects human RPE cells against ischemic[36] and oxidative stress,[37] and it can reduce cardiac infarct size[38] and the effects of experimental proliferative vitreoretinopathy.[39] These results are usually attained after prolonged treatment[37] or the administration of 4- to 10-mg/kg doses of melatonin,[38,39] whereas much smaller doses (0.05 mg/kg) for just 1 hour suffice to induce RPE dye exclusion.

Rod and cone IPM components were affected by illumination conditions and melatonin injection. Cone sheath glycoconjugates probably play a major role in retinal adhesion by forming a molecular bridge between the neural retina and the RPE.[40] The correspondence between the decrease of PNA binding after certain illumination conditions (or melatonin injection) and the decrease of detectable RPE injury supports this function of cone sheaths. Moreover, the differences between PI incorporation at 1 pm, after separation in HBSS or CMF-HBSS, could be related to the small number of cone sheaths remaining attached to the RPE after irrigation with CMF solutions.[11] Most important, the almost immediate effect of melatonin suggests that properties of the neural retina–RPE interface can be rapidly modified by this hormone. Several genes in the rat retina and the RPE are regulated by a

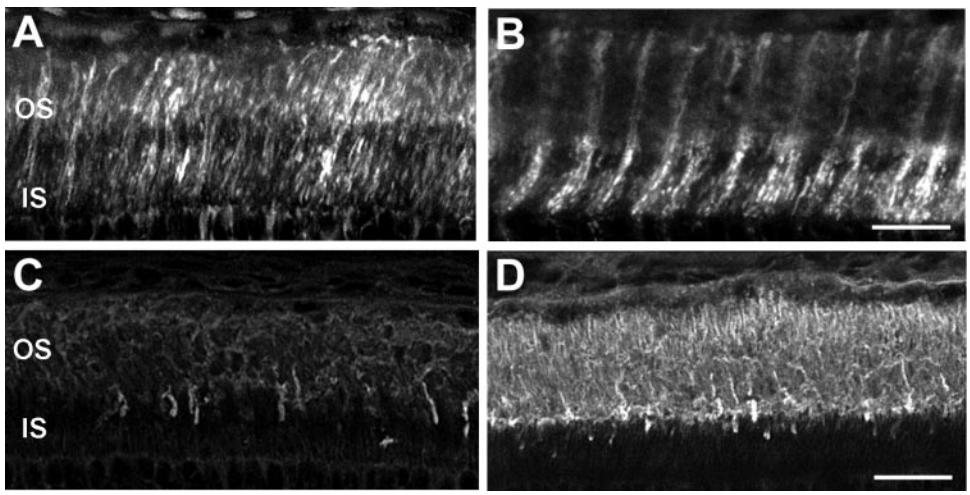

FIGURE 6. Confocal images of retinal cryosections fixed at 1 pm, after 1 hour of dark adaptation. (**A**, **C**) Specimens received saline injection at the beginning of dark adaptation. (**B**, **D**) Specimens received melatonin injection. (**A**, **B**) In the control retina, bound PNA labeled individual cone sheaths. Decreased staining intensity of cone sheaths could be observed after melatonin injection. (**C**, **D**) Bound WGA shows an amorphous matrix surrounding photoreceptor OS. Binding was much stronger after melatonin injection. Note that differences between these retinas are similar to those shown in Figure 5. Scale bar, 25 μm.

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single melatonin injection,[41] but their relationship to IPM synthesis is still unknown.

Even though our observations provide evidence of experimental detachment only in mice, it is tempting to speculate about their possible significance for human pathology. Benefits could perhaps ensue from the application of dark adaptation, time of day, and time of melatonin treatment to programmed retinal surgery. On the other hand, the association of retina–RPE adhesion with a melatonin–dopamine pathway could imply a relationship with central serous chorioretinopathy, a condition that has been connected to stress and sympathomimetic agents.[42]

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