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LABORATORY INVESTIGATION

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Photosensitization of retinal pigment epithelium by protoporphyrin IX

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Abstract ● Background: Clinical evidence of injury to the retinal pigment epithelium is an important feature of age-related macular degeneration, but the mechanism of this injury is unknown. Blue-light-dependent activation of the blood-borne photosensitizer protoporphyrin IX is known to produce free radicals which may damage cells and tissues. This study was undertaken to determine the effect of blue light and protoporphyrin IX on retinal pigment epithelial cells in vitro. ● Methods: Third-passage porcine retinal pigment epithelial cells were plated in six-well culture plates at 100 000 cells/well and grown to confluence. Retinal pigment epithelial cells were then incubated in culture media with and without 35 μg/dl protoporphyrin IX and exposed to low intensity (118 $\mu W/cm^2$) blue, blue-free, or full-spectrum white light in an irradiating incubator for 16 h on/8 h off cycles for 7 days. Some of the wells were shielded from light (dark controls). Retinal pigment epithelial cells were examined by light microscopy and were trypsinized and counted after 7 days. ● Results: White light with and without protoporphyrin IX and protoporphyrin IX in dark conditions did not decrease the retinal pigment epithelial cell count significantly. Blue light alone and blue light with protoporphyrin IX decreased the cell count by $22 \pm 4\%$ and $35 \pm 3\%$ compared to the controls, respectively. ● Conclusion: Blue wavelength light without exogenous protoporphyrin IX has a cytotoxic effect on confluent cultures of retinal pigment epithelium, suggesting that endogenous photosensitizers may be present in retinal pigment epithelial cells. Protoporphyrin IX has an additive cytotoxic effect in the presence of blue light, suggesting that this photosensitizer is capable of mediating blue-light-induced retinal pigment epithelial damage. Since protoporphyrin IX is present in blood and tissue fluids, and the retina is chronically exposed to light, protoporphyrin IX-mediated free radical formation may occur in vivo and may play a role in retinal pigment epithelial changes that occur early in the pathogenesis of age-related macular degeneration.

Introduction

Although the pathogenesis of age-related macular degeneration is not known, both clinical and histopathologic evidence suggest that dysfunction of the retinal pigment epithelium occurs early in the course of this disease [17]. Dysfunction of the retinal pigment epithelium may lead to extensive changes in the outer retina and choroid, since healthy retinal pigment epithelium is necessary for the maintenance of both the photoreceptors and the choriocapillaris [3, 4, 10, 16]. Determining possible mechanisms

of the initial injury to the retinal pigment epithelium may extend our understanding of the pathogenesis of age-related macular degeneration.

Protoporphyrin IX is a photosensitizer which is present in erythrocytes and tissue fluids at low concentrations (approximately 50 μg/dl in erythrocytes) [1]. When exposed to short-wavelength (i.e., blue) visible light, protoporphyrin IX generates singlet oxygen and superoxide anions which are toxic to cells and tissues [7]. In vitro and in vivo studies have shown that the retinal pigment epithelium is very sensitive to the toxic effects of blue light [2, 5, 9, 13]. Since the retina is exposed to light, protoporphyrin IX, and oxygen, it is clear that the conditions for a photosensitizing reactions exist in this delicate tissue in vivo. In view of these considerations, we have investigated the effects of blue light and protoporphyrin IX on retinal pigment epithelial cells in vitro.

Materials and methods

Porcine retinal pigment epithelial cells were harvested from fresh eyes obtained from a local slaughterhouse and transported to the laboratory on ice. The excess extraocular tissue was removed and each globe was immersed in betadine solution (Purdue-Federick, Norwalk, Conn.) for 15–20 min for sterilization. Globes were then rinsed three times (5 min per rinse) in calcium- and magnesium-free Hank's balanced saline solution (HBSS; Gibco, Grand Island, N.Y.). Under sterile conditions, a circumferential incision was made in each globe and the anterior segment and vitreous were discarded. Small corneal scissors were used to cut the neurosensory retina around the optic nerve. The neurosensory retina was then removed leaving the retinal pigment epithelial monolayer intact. Each eye cup was then rinsed three times with HBSS and a solution of 0.25% trypsin/0.25% EDTA was added until it covered the bottom. Eye cups were then incubated at 37°C in 95% air/5% carbon dioxide for 15 min. Trypsinized cells were pipetted from the eye cups and added to minimum essential medium with 10% fetal bovine serum (MEM 10; Gibco) and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, fresh MEM 10 was added and the retinal pigment epithelial cells in the pellet were resuspended and spun again a total of three times. Following the final wash, retinal pigment epithelial cells were resuspended in 3 ml of MEM with 20% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 mg/ml streptomycin and plated on a 60-mm^3 disposable tissue culture dish (Falcon, Becton Dickinson Labware, Lincoln, N.J.) and incubated at 37°C in 95% air/5% carbon dioxide. Media was changed every other day until the primary culture reached confluence. The primary culture was serially passaged by splitting 1:5 in MEM with 10% FBS without antibiotics.

Third-passage retinal pigment epithelial cells were plated in six-well culture plates at 100 000 cells per well and grown to confluence, and protoporphyrin IX (35 μg/dl) was added to some of the wells at the beginning of the experiment. Plates were exposed to light at 118 μW/cm^2 in a light irradiating incubator (Lab Light Instruments Model LI 1, Glencoe, Md.) for 16 h on/8 h off cycles for 7 days (total light energy delivered = 47.5 J/cm^2). A blue-light-blocking filter (cut-off at 520 nm), and a blue-light-pass filter (405–470 nm, peak transmission at 450 nm) were used in separate trials. The incubator's halogen light source was equipped with a rheostat that allowed us to keep the light intensity measured at the level of the culture plates constant (i.e. 118 μW/cm^2) at all wavelengths. When no blue-blocking filter was employed, approximately 8–10% of the visible light delivered by this halogen source was blue light between 400 and 450 nm. A light-absorbing black polyurethane box that covered the top surface and three of the four side surfaces of the plates was placed over dark controls that were run concurrently with the light-exposed groups. On day 7, the wells were examined and photographed by phase-contrast microscopy. Retinal pigment epithelial cells were then washed three times in HBSS and then harvested by

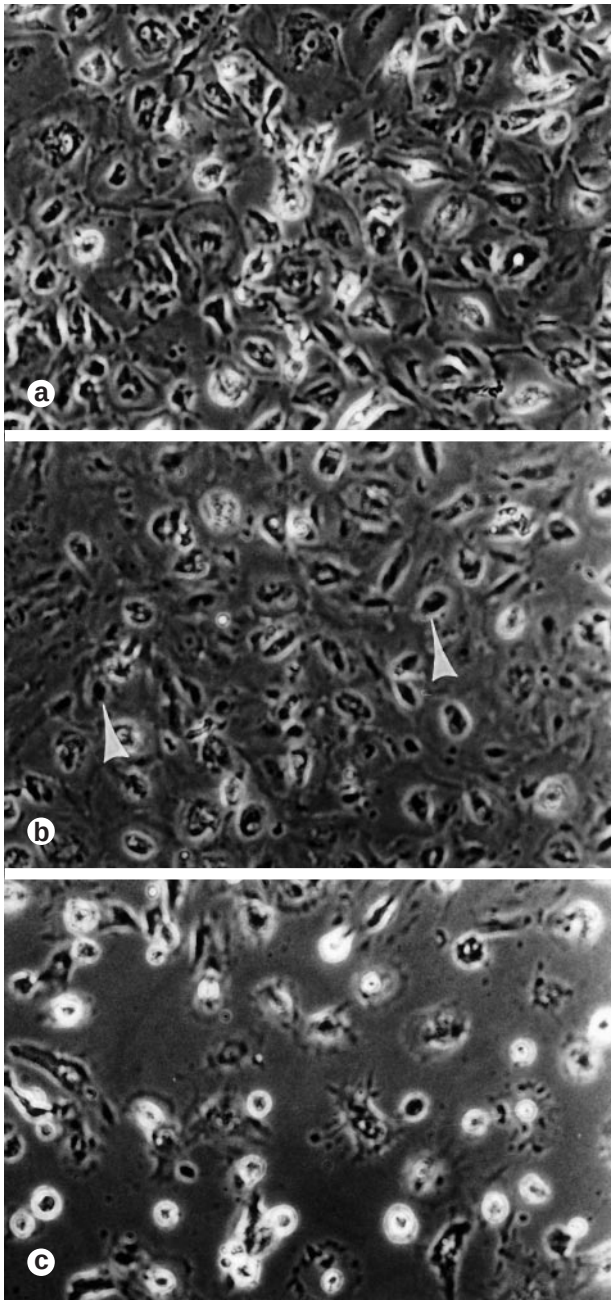

Fig. 1a–c Light microscopy of retinal pigment epithelium in tissue culture. **a** Dark conditions without protoporphyrin IX. **b** Cells exposed to 118 μW/cm^2 of blue light without protoporphyrin IX. There are pyknotic nuclei (*arrowheads*) with some loss of cells from the culture. **c** Cells exposed to 118 μW/cm^2 of blue light with protoporphyrin IX. There is extensive loss of cells from the cultures (×1400)

exposure to 0.25 M trypsin, which was subsequently quenched by adding MEM 10. Cells were counted using a Coulter Counter (Coulter Scientific, Hialeah, Fla.). Experiments were performed on two or three separate occasions with triplicate wells on each occasion.

Results

Light microscopy demonstrates that blue light is toxic to confluent retinal pigment epithelial cell cultures, and this effect is increased in the presence of exogenous protoporphyrin IX (Fig. 1). Figure 1a shows a confluent culture of porcine retinal pigment epithelium in tissue culture maintained in the dark. Exposing the retinal pigment epithelial cells to blue light (118 μW/cm^2) without exogenous protoporphyrin IX for 7 days causes pyknosis (Fig. 1b) with some loss of cells from the cultures. Exposing the retinal pigment epithelial cells to blue light (118 μW/cm^2) with exogenous protoporphyrin IX (35 μGm/dl) for 7 days causes extensive loss of cells from the cultures (Fig. 1c).

Figure 2 summarizes the effects of light and protoporphyrin IX on confluent retinal pigment epithelial cell cultures. Retinal pigment epithelial cell counts are not decreased when cells are exposed to white light or blue-free light for 7 days either with or without exogenous protoporphyrin IX. Cells exposed to blue-free light with protoporphyrin IX, blue-free light without protoporphyrin IX, and dark groups remained confluent (cell counts= $174.4 \pm 4.2\times10^3$, $173.0 \pm 10.7\times10^3$, and $176.8 \pm 3.2\times10^3$, respectively). Blue light decreased the cell counts with and without the addition of exogenous protoporphyrin IX. Retinal pigment epithelial cell counts for wells exposed to blue light without protoporphyrin IX were $138.3 \pm 6.6\times10^3$ (22 ± 4% less than controls). Retinal pigment epithelial cell counts for wells exposed to blue light with protoporphyrin IX were $115.6 \pm 4.9\times10^3$ (35 ± 3% less than controls).

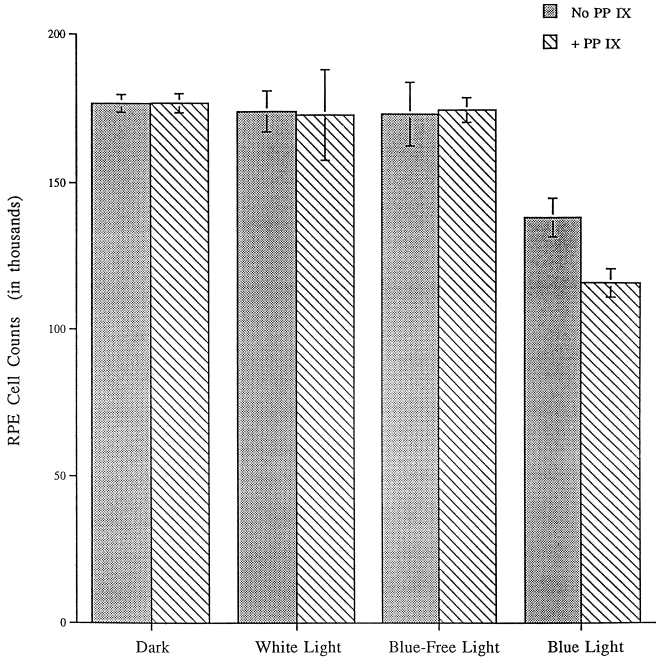

Fig. 2 Retinal pigment epithelial cell counts under different conditions. Retinal pigment epithelial cell counts are not decreased when cells are exposed to white light or blue-free light for 7 days either with or without exogenous protoporphyrin IX. Blue light decreases the cell counts by 22 ± 4% without exogenous protoporphyrin IX and by 35 ± 3% with exogenous protoporphyrin IX

Discussion

Our results demonstrate that blue light is cytotoxic to porcine retinal pigment epithelial cells without the addition of exogenous protoporphyrin IX, and this effect is increased when exogenous protoporphyrin IX is added to the cultures. Protoporphyrin IX has minimal effect on confluent retinal pigment epithelial cell cultures in the dark or in the presence of blue-free or white light. This is not surprising, since the maximum absorption wavelength of protoporphyrin IX is 405 nm, and protoporphyrin IX has less light absorption at wavelengths transmitted by the blue-free filter (> 520 nm). The mechanism of cytotoxicity caused by blue light and protoporphyrin IX involves the generation of reactive oxygen species such as superoxide anion and singlet oxygen, which are known to damage cells and tissues. Previous workers have shown that protoporphyrin IX produces more of these reactive oxygen species when exposed to blue light than when exposed to either yellow or red light of equal intensity [8]. Carotenoids, which are singlet oxygen scavengers, may offer some protection against species generated in photosensitizing reactions involving protoporphyrin IX.

We have shown that blue light alone is cytotoxic to confluent retinal pigment epithelial cultures in the absence of exogenous protoporphyrin IX. Previous studies have also demonstrated the cytotoxic effects of blue light on the retinal pigment epithelium without exogenous photosensitizers. Dorey et al. [5] demonstrated that cell growth was inhibited by exposure of retinal pigment epithelium to cycled blue light at 650 μW/cm^2 delivered for 6 h each day for 3 days (total energy = 42 J/cm^2). Crockett and Lawwill [2] demonstrated that blue light causes damage to confluent retinal pigment epithelial cultures after 2 h exposure at 20 000 μW/cm^2 (total energy = 144 J/cm^2). Our study was designed to mimic some of the conditions present in vivo by using confluent cell cultures and employing lower light intensity (118 μW/cm^2), and longer durations of cycled light exposure than in these previous studies (16 h on/8 h off for 7 days). Under our conditions, a total energy of 47.5 J/cm^2 of blue light was sufficient to cause retinal pigment epithelial cytotoxicity. This is lower than the total energy required for cytotoxicity determined by Crockett and Lawwill [2]. This finding suggests that chronic, cyclical low-level light exposure may be more toxic to retinal pigment epithelium than acute exposure of short duration.

Chronic exposure to low levels of blue light may play a significant role in light-induced retinal pigment epithelial damage in vivo, since the human retina is exposed to much lower levels of ambient light (on the order of 0.5 J/cm^2) [13]. Chronic low-level light exposure may play a role in the pathogenesis of age-related macular degeneration, a chronic degenerative process that develops over years to decades. Epidemiological evidence suggests an association between history of blue light exposure and neovascular age-related macular degeneration [14, 15]. Furthermore, in an animal model of protoporphyric mice, blue light exposure accelerated retinal degenerative changes in mice with elevated blood protoporphyrin IX levels [8]. Our demonstration that protoporphyrin IX increases blue light-mediated damage to the retinal pigment epithelium suggests that this mechanism may play a role in the pathogenesis of age-related macular degeneration.

The mechanism of retinal pigment epithelial toxicity produced by blue light alone probably involves the generation of reactive oxygen species mediated by endogenous photosensitizers. Crockett and Lawwill [2] showed that the threshold energy for blue light-mediated retinal pigment epithelial damage in vitro was reduced 10-fold when the oxygen concentration was increased from 20% to 95%. Recently, lipofuscin, the intracellular pigment found in senescent retinal pigment epithelium, has been shown to generate reactive oxygen species when exposed to blue light [6, 12]. The possibility of other endogenous photosensitizers in retinal pigment epithelial cells cannot be excluded.

We investigated a possible role for protoporphyrin IX because it is a naturally occurring photosensitizer that has previously been shown to produce potentially toxic reactive oxygen species. Protoporphyrin IX is the most abundant porphyrin in human blood, at concentrations of 50 μgm/dl [1]. Since the retina is a metabolically active tissue that is chronically exposed to focused light, circulating protoporphyrin IX may participate in photosensitizing reactions that lead to the production of reactive oxygen species. Protoporphyrin IX is confined to the intravascular spaces in vivo, but blue-light exposure may also lead to the breakdown of the blood-retinal barrier at the level of the retinal pigment epithelium [11]. Under such conditions protoporphyrin IX may find its way to the retinal pigment epithelium and participate in photosensitizing reactions. The light-dependent generation of reactive oxygen species from protoporphyrin IX could contribute to retinal pigment epithelial injury caused by blue light exposure.

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