Effect of Hematoporphyrin Derivative on Rabbit Corneal Endothelial Cell Function and Ultrastructure

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Hematoporphyrin derivative (HpD) is a systemically administered photosensitizing agent that may be of value in the treatment of solid tumors. When corneal endothelial cells were perfused in the specular microscope with HpD and exposed to a 25-W incandescent light at 5 cm (5.5 mW/cm²) there was anatomic disruption of corneal endothelial cells and swelling of the corneal stroma. Perfusion with 0.2 μl/ml (1.0 μg/ml) HpD and 5 min exposure to light resulted in a corneal swelling of 71 ± 4 μm after 3 hr, whereas perfusion with 0.2 μl/ml HpD and a 1-min exposure to light resulted in a corneal swelling of 36 ± 4 μm after 3 hr. Perfusion with 0.2 μl/ml HpD with no light exposure resulted in a corneal swelling of 22 ± 4 μm after 3 hr. Inclusion of 100 μg/ml catalase in the perfusion solution resulted in a significant 38% reduction of the corneal swelling. The inclusion of either 100 μg/ml superoxide dismutase, 15 mM D-mannitol, 5 mM ascorbic acid, 1/4% DMSO, 50 μM EDTA, 50 μM DETAPAC, 10 mM L-histidine, or 1 mM sodium azide did not modify the corneal swelling induced by the photosensitization reaction. Perfusion of corneal endothelial cells with 2 μl/ml (10 μg/ml) HpD and exposure to 25-W incandescent light for 5 min resulted in swelling of mitochondria, the appearance of vacuoles in the cytoplasm, and rapid corneal swelling. The data suggests that corneal endothelial cells can be damaged by hydrogen peroxide generated by the dismutation of superoxide anion produced during the photoreaction. Superoxide anion itself and hydroxyl-free radical do not appear to participate in causing the endothelial cell damage. The role of singlet oxygen remains somewhat unclear. The data suggests that further in vivo studies should be performed to delineate precautions that should be taken to protect the corneal endothelium during photoradiation therapy. Invest Ophthalmol Vis Sci 26:1465-1474, 1985

Photoradiation therapy is a technique currently under investigation for the treatment of solid tumors. Hematoporphyrin derivative (HpD) is a compound that is of value in photoradiation therapy because of its ability to bind selectively to tumor tissue and act as a photosensitizing agent. The tumor cells containing HpD are killed when exposed to light. Hematoporphyrin derivative has been used in the treatment of malignant diseases of the skin, breast, colon, and endometrium, as well as malignant melanoma of the uveal tract.1-4 Hematoporphyrin derivative has also been shown to be effective in killing retinoblastoma cells in tissue culture, and it has been demonstrated that mice hetero-transplanted intraocularly with human retinoblastoma cells accumulate higher levels of HpD than control eyes.5-6 Preliminary work has indicated that HpD photoradiation therapy may be of value for the treatment of iris neovascularization.7

Since the cornea is transparent, certain intraocular structures may potentially be damaged either during photoradiation therapy of intraocular disease processes, or secondary to the entrance of ambient light into the eye when photoradiation therapy is being utilized for tumor management in other parts of the body. It was the purpose of this investigation to determine if corneal endothelial cells were susceptible to damage from an HpD photomediated reaction and what the mechanism and consequences of such a reaction may be on corneal endothelial cell physiology and ultrastructure.

Materials and Methods

Adult albino rabbits weighing approximately 2.5 kg of either sex were killed with an overdose of sodium pentobarbital. The eyes were removed from the animals and the corneas were mounted in a specular microscope.8-11 These techniques conformed to the ARVO Resolution on the Use of Animals in Research. The endothelial surface was perfused with Krebs Ringer bicarbonate solution (KRB) containing 92 mg/l of reduced glutathione and 134 mg/l of adenosine with pO₂ of 155 mmHg at 37°C and 15 mmHg pressure.8-13

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Table 1. Experimental groups: composition of solutions and variations in light exposure of corneas perfused on the endothelial surface with hematoporphyrin derivative (HpD)

<table>
<thead>
<tr>
<th>µl/ml HpD</th>
<th>N</th>
<th>Minutes of exposure to 25 W light at 5 cm (5.5 mW/cm²)</th>
<th>Light</th>
<th>Additional additives to HpD solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>6</td>
<td>1</td>
<td>(-)</td>
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<tr>
<td>0.2</td>
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<tr>
<td>2.0</td>
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<td>2.0</td>
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<tr>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>1 µg/ml catalase</td>
</tr>
<tr>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>100 µg/ml catalase</td>
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<tr>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>500 µg/ml catalase</td>
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<tr>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>100 µg/ml SOD*</td>
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<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>15 mM D-mannitol</td>
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<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>5 mM ascorbic acid</td>
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<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>1/4% DMSO†</td>
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<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>50 µM EDTA§</td>
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<td>0.2</td>
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<td>50 µM EDTA§</td>
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<td>0.2</td>
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<td>50 µM DETA§</td>
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<tr>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>10 mM L-histidine</td>
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<tr>
<td>0.2</td>
<td>6</td>
<td>5</td>
<td>(+)</td>
<td>1 mM sodium azide</td>
</tr>
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</table>

* Superoxide dismutase
† Solution also contained 15 mM sucrose to maintain osmotic equivalency with the paired cornea which was perfused with HpD +15 mM D-mannitol.
‡ dimethyl sulfoxide
§ ethylenediaminetetraacetic acid
|| diethylenetriaminepentaacetic acid
† Solution also contained 10 mM sucrose to maintain osmotic equivalency with the paired cornea which was perfused with HpD + 10 mM L-histidine.

Silicone oil was placed on the epithelial surface to prevent evaporation. After a 1-hr stabilization period and the recording of corneal thickness, the room lights were shut off and paired experimental and control corneas were perfused in the dark with hematoporphyrin derivative (Photofrin Medical; Cheektowaga, NY). Liquid HpD (5 mg/ml) was added to KRB and concentrations of HpD were expressed as µl HpD/ml KRB. In order to facilitate complete replacement of the KRB solution in the chambers with HpD and additives, corneas were perfused rapidly with 4 ml of the solution for 4.5 min upon initiation of perfusion with HpD. Subsequently the perfusion rate was reduced to 1 ml/hr for the remaining 3 hr. The composition of perfusion solutions as well as variations in light exposure are shown in Table 1. Following the introduction of HpD into the specular microscope perfusion chamber, photoradiation therapy was accomplished by exposure of the corneas to a 25-W incandescent light at a distance of 5 cm for variable time periods (Table 1). Photometric measurements were performed with a model IL-1500 Research Radiometer (International Light; Newburyport, MA). Irradiance was determined to be 5.5 mW/cm² using a spectrally flat filter. A petri dish filled with water was placed between the bulb and the cornea to absorb heat generated by the lamp.14

Early in the investigation, it was determined that exposure of corneal endothelial cells that were being perfused with a solution containing HpD underwent an anatomic and physiologic alteration following exposure to the specular microscope light. For that reason, all experiments were performed totally in the dark, and a specular microscope reading of corneal thickness was taken only at the end of each experiment. Therefore the first reading was taken prior to introduction of HpD into the perfusion chamber, and the last reading was taken at the end of the perfusion period, which was 3 hr unless otherwise noted. The change in corneal thickness was determined at the end of each experiment, and the mean and standard error of the mean (SEM) of the change in corneal thickness were determined. Comparison of the change in corneal thickness of experimental and control groups was determined utilizing the Student’s t-test. A minimum of six corneas were used for each experimental and each control group.

Tissues were prepared for either electron microscopy or the determination of intracellular glutathione using previously described methods.15,16

Results

Corneal Swelling as a Function of Concentration of Hematoporphyrin Derivative

Corneas perfused with 2 µl/ml (10 µg/ml) HpD and exposed to light for 5 min swelled 314 ± 16 µm in 3 hr, compared to corneas perfused with 0.2 µl/ml (1 µg/ml) HpD and exposed to light for 5 min which swelled 71 ± 4 µm, P < 0.05 (Fig. 1). Corneas perfused with 2 µl/ml HpD and exposed to light for 1 min swelled 117 ± 15 µm in 3 hr, compared to corneas perfused...
with 0.2 μl/ml HpD and exposed to light for 1 min which swelled 36 ± 4 μm (P < 0.05, (Fig. 1).

**Corneal Swelling as a Function of Duration of Light Exposure**

Control corneas perfused with 2 μl/ml HpD which were not exposed to light swelled either 43 ± 3 μm or 45 ± 3 μm after 3 hr (Fig. 1). Experimental corneas similarly perfused with 2 μl/ml HpD and exposed to light for 1 min swelled 117 ± 15 μm after 3 hr, which was significantly greater (P < 0.05) than the paired controls not exposed to light (Fig. 1). Exposure to light for 5 min resulted in a corneal swelling of 314 ± 16 μm after 3 hr which was significantly greater (P < 0.05) than the 117 ± 15 μm that resulted after exposure to light for 1 min. A similar direct dependence of the amount of corneal swelling as related to duration of light exposure was found in corneas perfused with 0.2 μl/ml HpD (Fig. 1).

Scanning electron microscopy of corneal endothelial cells following a 3-hr perfusion with 2 μl/ml HpD and a 5-min exposure to light showed swollen cells with loss of the endothelial mosaic pattern (Fig. 2A). In contrast, corneas perfused with 2 μl/ml HpD which were not exposed to light showed a normal corneal endothelial mosaic pattern (Fig. 2B). Transmission electron microscopy of corneal endothelial cells following a 3-hr perfusion with 2 μl/ml HpD and a 5-min exposure to light showed swelling of cytoplasm and cytoplasmic organelles, as well as disruption of the nuclear chromatin (Fig. 3A). Corneas perfused with 0.2 μl/ml HpD which were not exposed to light showed normal cytoplasmic organelles and nuclei (Fig. 3B).

Scanning electron microscopy of corneal endothelial cells following a 3-hr perfusion with 0.2 μl/ml HpD and a 5-min exposure to light showed a normal endothelial mosaic pattern (Fig. 4A) which was similar to corneas perfused with 0.2 μl/ml HpD and not exposed to light (Fig. 4B). Transmission electron microscopy of corneal endothelial cells following a 3-hr perfusion with 0.2 μl/ml HpD and a 5-min exposure to light showed swelling of the mitochondria and the appearance of cytoplasmic vacuoles but preservation of apical cell membrane, intracellular space, and apical junction (Fig. 5A). Corneas perfused with 0.2 μl/ml HpD which were not exposed to light showed normal cytoplasmic organelles and nuclei (Fig. 5B).

**Corneal Swelling as a Function of Time following Exposure to Light**

Corneas perfused with 0.2 μl/ml HpD and exposed to light for 5 min swelled 30 ± 2 μm after 1 hr. A second group of corneas similarly exposed to light for 5 min swelled 74 ± 4 μm after 2 hr. A third group of corneas similarly exposed to 5 min light swelled 71 ± 4 μm after 3 hr (Fig. 6).

**Modification of Corneal Swelling following HpD Photosensitization of Corneal Endothelium**

The addition of either 100 μg/ml or 500 μg/ml catalase to the perfusing medium reduced the corneal swelling that was induced by perfusion with 0.2 μl/ml HpD and 5 min exposure to light. Corneas perfused with a solution containing 100 μg/ml catalase + 0.2 μl/ml HpD swelled 44 ± 2 μm compared to 70 ± 2 μm (P < 0.05) for controls that were perfused with a similar solution that did not contain catalase (Fig. 7). Increasing the concentration of catalase to 500 μg/ml did not further reduce the corneal swelling and 1 μg/ml catalase was ineffective in reducing corneal swelling (Fig. 7). The addition of superoxide dismutase, D-mannitol, ascorbic acid, DMSO, EDTA (with and without FeCl₃), DETAPAC, L-histidine or sodium azide did not modify the corneal swelling induced by perfusion with 0.2 μl/ml HpD and exposure to light for 5 min (Fig. 7).

**Endothelial Intracellular Glutathione**

The endothelial intracellular total glutathione content following a 3-hr perfusion with 0.2 μl/ml HpD and 5 min exposure to a 25-W incandescent light at 5 cm was 615 ± 42 ng glutathione/corneal endothelium compared to 487 ± 39 ng glutathione/corneal endothelium for paired corneas similarly treated but not exposed to light (mean ± SEM, P = 0.05, N = 6). The amount of oxidized glutathione was 13 ± 2% in corneas exposed to light compared to 19 ± 3% in corneas not exposed to light (mean ± SEM, P > 0.05, N = 6).

**Discussion**

This study has shown that rabbit corneal endothelial cells undergo anatomic and physiologic alteration fol-
Fig. 2. Scanning electron micrographs of corneal endothelial cells following a 3-hr perfusion with 2 μl/ml HpD and either 5 min light exposure (A) or no light exposure (B). A, The cells are swollen with loss of the endothelial mosaic pattern (×1260). B, Cells are flat with preservation of the endothelial mosaic pattern (×1200).
Fig. 3. Transmission electron micrographs of corneal endothelial cells following a 3 hr perfusion with 2 μM Hpd and either 5 min light exposure (A) or no light exposure (B). A, Cellular cytoplasm and cytoplasmic organelles are swollen and there is disruption of the nuclear chromatin. The intercellular space is intact (×30,000). B, Cytoplasmic organelles, nucleus and intercellular space is unremarkable (×21,500).
Fig. 4. Scanning electron micrographs of corneal endothelial cells following a 3 hr perfusion with 0.2 μl/ml HpD and either 5 min light exposure (A) or no light exposure (B). The endothelial mosaic pattern is preserved in A and B. (A, ×1200; B, ×1200).
Fig. 5. Transmission electron micrographs of corneal endothelial cells following a 3-hr perfusion with 0.2 μl/ml HpD and either 5 min light exposure (A) or no light exposure (B). A, The apical cell membrane, intercellular space and apical junction is intact. The mitochondria are swollen and there appear to be some large vacuoles in the cytoplasm (×27,720). B, The apical cell membrane, nuclei, intercellular space, and cytoplasmic organelles are normal (×27,000).
Following perfusion with hematoporphyrin derivative (HpD) and exposure to incandescent light, the alteration of the corneal endothelium results in swelling of the corneal stroma. The amount of damage increases with either increased concentrations of HpD or increased duration of light exposure. These results are consistent with previous work showing that the photototoxic effect of HpD plus light is proportional to the number of light quanta absorbed by HpD in each cell. The strongest porphyrin absorption band occurs at 400 nm; however, the 630-nm band is commonly used in phototherapy because of increased tissue transparency at this wavelength. The use of white light in this experiment, and upon which some previous data has been formulated, is of interest because of its flat spectrum in the visible range and the probability that much of the effect is probably attributable to the shorter wavelengths. The changes induced by 0.2 μl/ml (1.0 μg/ml) HpD are less dramatic than those caused by 2 μl/ml (10 μg/ml HpD), but the latter reflect almost total destruction of the endothelial barrier both functionally (Fig. 1) and anatomically (Figs. 2, 3). The lower concentration causes less swelling (Fig. 1) and less ultrastructural disturbance with intracellular changes occurring in the presence of an intact junctional complex (Figs. 4, 5). It is possible that the effect of dilute concentrations of HpD are restricted to alterations of pump activity. Comparisons of corneal swelling rates caused by hydrogen peroxide generated in other systems and the appearance of the cells indicate that adverse effects on pump activity may be initiated by low concentrations of hydrogen peroxide with higher concentrations inducing structural and barrier defects.

The intravenous use of 2.5 mg HpD/kg body weight would lead to a maximal plasma concentration of about 60 μg/ml. The concentrations used here, 2 μl and 0.2 μl/ml HpD (10 μg/ml and 1.0 μg/ml), are considerably lower than the projected plasma level; thus, even if HpD was distributed in a maximum ratio of up to 1:40 (aqueous:plasma), the possibility exists that sufficient HpD could be present in the aqueous to cause an adverse effect.

The corneas showed evidence of swelling 1 hr following exposure to light in the presence of HpD, with swelling reaching a maximum level at 2 hr. There was no further increase in corneal thickness at 3 hr. The adverse effect was not blocked by superoxide dismutase, an agent that catalyzes the conversion of the superoxide anion to hydrogen peroxide. Further indirect evidence of the lack of effect of superoxide anion is demonstrated by the absence of protection by ascorbic acid, an agent that in an aerobic medium scavenges O2· but not H2O2. The adverse effect was in part blocked by the addition of 100 μM catalase to the perfusing solution. Since catalase catalyzes the divalent reduction of H2O2 to H2O, it is probable that the toxic effect of the HpD photosensitization process on corneal endothelial cells was in part secondary to hydrogen peroxide produced following perfusion with hematoporphyrin derivative (HpD) and exposure to incandescent light. The alteration of the corneal endothelium results in swelling of
during the dismutation of oxygen-free radical. It is improbable that hydroxyl-free radical, a species produced by interactions between hydrogen peroxide and superoxide anion, was at sufficient concentrations to participate in the toxic effect because the hydroxyl radical scavengers D-mannitol and DMSO did not offer protection. In addition, neither DETAPAC nor EDTA altered the HpD effect; these compounds could influence the reaction by complexing with trace amounts of iron present in the solution that some studies have indicated is necessary for the production of hydroxyl-free radical. The addition of EDTA-FeCl₂ chelate to the solution did not accelerate the corneal swelling rate, providing additional evidence against hydroxyl-free radical participation in this system.

The mechanism by which tumor cells are inactivated during photoradiation therapy is not well understood. It has been shown that oxygen is necessary for photocytotoxicity to occur and that the maximum effect occurs at a pO₂ of 90 mmHg. The pO₂ of the solution used in this experiment was 155 mmHg. It has been shown in a liposomal system that porphyrin photooxidation shifted from a type II (dye-oxygen) to a type I (dye-substrate) mechanism as the porphyrin concentration was increased. Some work has indicated that the cytotoxic effect of HpD plus photoradiation is mediated by singlet oxygen, while other work has demonstrated the production of superoxide anion. This study suggests that hydrogen peroxide, the dismutation product of superoxide anion, is in part responsible for the damage to corneal endothelial cells. The singlet oxygen scavengers ascorbic acid, L-histidine and sodium azide did not offer protection. However, because of characteristic difficulties and ambiguities in the establishment of singlet oxygen participation in a reaction, its precise role in this model cannot be specifically delineated. Intracellular total and percent oxidized glutathione were not altered by the HpD photoreaction. This is similar to the situation with rose bengal photosensitization of endothelial cells and indicates that the corneal swelling was not induced by failure of the glutathione redox system. This study has shown that corneal endothelial cells can be adversely effected by HpD in the presence of incandescent white light. Since the cornea is a transparent tissue, and the normal thickness cornea is known to transmit 80% of incident light at 400 nm and about 100% of light at 700 nm, the data suggests that further in-vivo studies should be performed to determine the possible susceptibility of endothelial cells in a clinical setting where patients are receiving photoradiation therapy for tumors. It is not known what concentration of HpD occurs in aqueous humor during photoradiation therapy and whether the aqueous humor components may diminish the effect. Until such information is available, the possible adverse effect of HpD photoradiation therapy should especially be kept in mind during treatment of ocular disease processes where intense levels of light pass through the cornea and may produce endothelial damage mediated by HpD in the aqueous humor.

**Key words:** hematoporphyrin derivative, photoradiation therapy, superoxide anion, hydrogen peroxide, cornea, endothelium, phototoxicity

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**References**