Hematoporphyrin is a photodynamic substance which accumulates and is retained in tumor far more than in normal cells.\(^1\) Photodynamic effects on retinoblastoma cells have been investigated using hematoporphyrin derivative (HpD) and light.\(^2,3\) Clinically, photodynamic therapy for malignant tumors was first applied by Dougherty et al in 1978.\(^4\)

Since 1981, we have used photodynamic therapy for 14 patients with retinoblastoma. A HpD and argon laser were used. This therapy has proven to be effective for treating selected cases of retinoblastoma.\(^5\) The mechanism of this therapy seems to relate to the phototoxicity of singlet oxygen produced by HpD exposed to light.\(^6\) As cellular sites of the action of singlet oxygen and the relationship between singlet oxygen and cell death are poorly understood,\(^6\)–\(^18\) we investigated relationships between increases in intracellular lipid peroxide (LP) and cell death, under conditions of photoradiated HpD, with or without oxygen.

**Materials and Methods**

HpD was prepared following the procedure of Lipson et al.\(^19\) Retinoblastoma cell line Y79 cells were cultured in RPMI supplemented with 10% fetal calf serum. Y79 cells were incubated in culture medium containing 10 \(\mu\)g HpD per 10\(^6\) cells/ml for 4 hr, and then were irradiated 5 cm distant from a 15-W fluorescent bulb for 0–20 min in an incubator at 37°C. These cells were washed and homogenized ultrasonically in 1.15% KCl, and the LP levels were measured (Fig. 1). Anaerobic conditions were obtained by replacing the air in the experimental box with argon gas at 4°C. Cell viability was estimated by trypan blue staining.

For the morphologic study, the Y79 cells were fixed, dehydrated, embedded, sectioned, and observed under a transmission electron microscope.

### Results

First, the optimal HpD concentration and optimal incubation time for Y79 retinoblastoma cells taking up sufficient amounts of HpD were determined. Cell-associated HpD was measured fluorophotometrically, according to the method of Kessel and Cheng.\(^20\) When the Y79 cells were incubated with 0–200 \(\mu\)g HpD per 10\(^6\) cells/ml of culture medium for 4 hr, the uptake of HpD increased with increase in the HpD concentration in the culture medium. The uptake was adequate at 10 \(\mu\)g/10\(^6\) cells, and the incubation time was 4 hr unless otherwise indicated.

The formation of LP after light exposure was determined using various concentrations of HpD (Fig. 2). After 10 min of photoradiation, the intracellular LP level was increased as the concentration of HpD in the medium was increased. However, no increase of the LP level was observed in the absence of pho-
toradiation. In another control experiment, Y79 cells incubated with 100 or 200 µg HpD/10⁶ cells were photoradiated under anaerobic conditions. In this case, the formation of LP did not increase over the baseline value.

The LP formation of cells incubated with 10 µg HpD/10⁶ cells was measured at various times of photoradiation (Fig. 3). The LP level increased with the time of photoradiation. Photoradiation without HpD did not yield any increase in LP formation. Under anaerobic conditions, LP formation by cells incubated with HpD did not increase for up to 20 min of photoradiation. These results show that Y79 cells taking up HpD essentially need both light and molecular oxygen (O₂) for LP formation, and suggest that the active oxygens mediate the formation of LP. We then performed experiments using radical scavengers. Y79 cells taking up HpD were exposed to light in the existence of various radical scavengers (Fig. 4). Superoxide dismutase (SOD), the scavenger for su-
peroxide ($O_2^-$), and catalase, the scavenger for hydrogen peroxide ($H_2O_2$), did not inhibit the formation of LP. Mannitol, the scavenger for the hydroxyl radical (·OH), and 1,4-diazabicyclo[2,2,2]octane, the scavenger for the singlet oxygen, partially inhibited the production of LP by the cells. Thus, either singlet oxygen or ·OH is the mediator of LP formation.

The relationship between LP formation and cell death also was investigated (Fig. 5). At 5 min of photoradiation, LP formation occurred but cell death ratio was identical to the ratio in control experiment. When the cells were photoradiated for over 5 min, the cell death ratio increased abruptly and reached 83% at 20 min of photoradiation. The cell death ratio in the absence of oxygen, light, or HpD was significantly lower than that in the presence of all three elements (Table 1). These results suggest that the oxygen-radical-mediated reaction might lead to tumor cell death in cases of photodynamic therapy with HpD.

Transmission electron micrographs of Y79 cells exposed to light for 20 min without HpD infiltration revealed a normal appearance. The Y79 cells exposed to HpD for 4 hr but not to light showed a normal appearance (Fig. 6). There were numerous mitotic figures. The mitochondria in Y79 cells exposed to light for 5 min in the case of infiltration of 10 μg HpD/10^6 cells for 4 hr appeared contracted, and there was an increase in density of the matrix. Mitochondria in the Y79 cells exposed to light for 10 min with HpD infiltration were slightly swollen. The mitochondria in the cells exposed to light for 15 min with HpD infiltration were significantly swollen, and the cristae disrupted. These swollen mitochondria contained intramitochondrial bodies (Fig. 7). The mitochondria and endoplasmic reticulum in the cells exposed to light for 15 min with HpD infiltration were selectively destroyed, as were most of the cells (Fig. 8). The plasma membranes in the cells exposed to light for 20 min with HpD infiltration were disrupted, and the nuclei were pyknotic (Fig. 9). These morphologic studies revealed that the first changes occur in the mitochondria.

**Table 1.** Cell death ratio (%) in the absence or existence of oxygen, light, and hematoporphyrin derivative (HpD)

<table>
<thead>
<tr>
<th>Photoradiation time (min)</th>
<th>HpD + light</th>
<th>Light (−)</th>
<th>HpD (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>81 ± 8</td>
<td>26 ± 5</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>20</td>
<td>92 ± 7</td>
<td>27 ± 7</td>
<td>23 ± 9</td>
</tr>
</tbody>
</table>

Discussion

Porphyrin and hematoporphyrin are well-studied photosensitizers. Two types of mechanisms have been proposed for the photoinactivation of biomolecules. In the so-called type II reaction, singlet oxygen may mediate the photoinactivation. It remains to be determined whether singlet oxygen contributes to tumor cell death in cases of photodynamic therapy with HpD, and the requirement for oxygen is not well represented. We performed experiments using strict anaerobic conditions and obtained definite evidence for the requirement of active oxygen to kill retinoblastoma cells. In ophthalmic research, HpD retention in heterotransplanted tumor tissue has been noted, but we found no report on the mechanism of cell death in photodynamic therapy. We measured LP production to search for the effect of HpD on cells, because the method is simple and appropriate for elucidating the contribution of active oxygen. We used argon gas replacement for achieving the anaerobic condition. This condition is adequate to block the NADPH oxidase of neutrophils, to produce the superoxide anion. NADPH oxidase has an unusually high affinity for molecular oxygen. These experiments clearly showed that HpD, light, and oxygen are essential factors for cell damage to be induced by photodynamic therapy. We also used scavengers of...
active oxygen to delineate which molecular type contributes to the HpD mediated cell damage. SOD and catalase were without effect, but mannitol and 1,4-diazabicyclo[2,2,2]octane are effective for inhibiting lipid peroxidation. Therefore, in addition to singlet oxygen, •OH contributes to cell damage.

Fig. 6. Control study: Y79 retinoblastoma cells exposed to HpD but not exposed to light for 4 hr. The cells have a normal appearance. Note the mitosis. (×5200)

Cell-associated LP increased linearly with the time of photoradiation. The cell death ratio began to increase in a sigmoid fashion following the production of certain amounts of lipid peroxide. Therefore, it appears that a chain reaction of lipid peroxidation is required for cell death. Active-oxygen-mediated

Fig. 7. Y79 cells exposed to light for 15 min with HpD infiltration. The mitochondria are significantly swollen, and the cristae of the mitochondria are disrupted. The swollen mitochondria contain intramitochondrial bodies. (×7300)
mechanisms probably play a major role in cell lethality in cases of photodynamic therapy.

The site of photodamage with HpD remains controversial. One investigator has suggested that the cell membrane is damaged first\textsuperscript{10,24}; others have reported that the site is the mitochondrion, cell membrane,\textsuperscript{7,8,13,14,15} microsome,\textsuperscript{18} or chromosome.\textsuperscript{9} We noted abnormal findings in the mitochondria of the treated cells, and in the later stage, the endoplasmic reticulum was destroyed. The control cells were normal. It is apparent that the primary target of photo-induced damage is the mitochondrion.

Fig. 8. Y79 cells exposed to light for 15 min with HpD infiltration. The mitochondria and endoplasmic reticulum are selectively destroyed. (×7300)

Fig. 9. Y79 cells exposed to light for 20 min, with HpD infiltration. The mitochondria and endoplasmic reticulum are selectively destroyed, as are most of the cells. (×6500)
Key words: hematoporphyrin derivative, lipid peroxide, Y79 retinoblastoma cell, mitochondria, photodynamic therapy

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References