Effects of Laser Irradiation on Hematoporphyrin-treated Normal and Transformed Thyroid Cells in Culture

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ABSTRACT

Laser irradiation of tissues treated in vivo with the hematoporphyrin derivative (HPD) is known to result in a cytocidal effect, reportedly more pronounced in the tumor than in the surrounding normal tissues. In order to ascertain if this phenomenon had a clear cellular basis, it has been now reproduced in vitro in a model system consisting of normal and transformed cell lines. Epithelial rat thyroid cells were infected and transformed with a RNA oncogenic virus. Both the original (normal) and the viral-transformed (tumorigenic) cells were incubated with HPD and exposed to two types of laser irradiation: 631 nm, continuous wave; and 337.1 nm, pulsed. Under the conditions tested, the percentage survival of the transformed cells was found to be lower (up to ~3 times) than that of the normal cells. The cytocidal effect was greater using the pulsed than using the continuous-wave irradiation. The difference between normal and tumor cells was more evident at 30 µg than at 50 µg of HPD per ml. The HPD not followed by laser irradiation had no effect on the cell growth rate.

The finding of a significant difference in the sensitivity to photoactivated HPD between normal and tumor cells under strictly controlled and highly comparable conditions opens new possibilities to the study of the cellular and molecular mechanisms involved in the phototherapy of tumors. Furthermore, studies in vitro on the active components of the photosensitizer and on their selectivity towards the tumor cells, explained at a cellular level, will lead to better approaches to photochemotherapy in vivo.

INTRODUCTION

Photosensitizers have been known to have photodynamic activity in the presence of oxygen and light, which results in cytocidal effect both in vivo (7, 8, 19, 33) and on isolated cells (9, 14, 16, 20, 22, 35). Such effects represent also the basis for several attempts for the phototherapy of experimental and human tumors (6, 8, 10, 19). It has been shown that HPD, as well as other porphyrins, administered in vivo to the tumor-bearing animal or patient binds selectively to tumor tissue (4, 11, 13, 17–19, 21, 23–25, 27, 29, 31, 32, 34, 36–39) and, when photoactivated by red light as 631 nm, is able to cause a noticeable regression of the neoplastic growth as well as, in some cases, a reduction in the size of the tumor in both animals and patients. The usefulness of such a therapeutic approach seems to be related to a selective sensitivity of the neoplastic tissue to phototherapy as compared to the surrounding normal tissue. The cytocidal effect has been also demonstrated in vitro on isolated or cultured cells of tumor origin (5, 9, 16, 20, 35). The cellular and molecular bases of this selectivity, however, are still unknown. There is no proof of an intrinsic increased sensitivity of the cancer cells towards light-activated HPD. If the normal and tumor cells are equally sensitive to the photoactivated drug, perhaps a selective localization in the tumor tissue of drug administered in vivo must be considered. In fact, a preferential accumulation and/or retention of the drug within the neoplastic tissue has been reported (13). One possibility is that the tumor cells have higher affinity for the drug. Alternatively, factors related to the organization of the neoplastic tissue, such as its vascularization and/or its peculiar parenchep-m-stroma interactions, could be responsible for the higher levels of HPD in the tumor. Clearly, to evaluate the selectivity of HPD effect in the tumor tissues, a more stringent comparison between normal and tumor cells in vitro is needed.

In the present study, we have used an experimental system in which a normal cell strain, recently obtained from adult rat thyroid glands, has been transformed in vitro by a murine sarcoma virus. The normal and viral-transformed cell strains have been compared in their response to the laser irradiation after incubation with same amounts of HPD. The transformed cells were found to have a survival rate significantly lower than that of normal cells, thus demonstrating a difference between normal and tumor cells in their response to photosensitization with HPD.

MATERIALS AND METHODS

Cell Strains. Normal and virus-transformed cells used in this research were in vitro-cultured permanent lines of rat thyroid origin. The establishment in culture of these lines has been described previously (1), and culture conditions were not varied here unless when specified.

The normal strain (FRTL-5) was a subpopulation of fast-growing, high-serum (5%)-cultured, differentiated cells (2) recently derived from the slow-growing, low-serum (0.5%), differentiated strain FRTL (1). Thyroid biochemical markers such as thyroglobulin biosynthesis and iodide trapping, monitored on the original (FRTL) and high-serum cells (FRTL-5), are consistently present on both cell types. In addition, both cells have been monitored prior to the experiments and confirmed to be normal by all criteria tested, such as chromosome analysis, agar cloning (26), injection in syngeneic animals, and hormonal dependence.

The transformed strain (FRTL-5 KIKI) was produced by infecting in vitro the previous strain with a RNA tumor virus [Ki-MSV (Ki-MuLV)]. This strain has been shown to be transformed in vitro and tumorigenic
in vivo (12). Both strains were maintained in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum and a 6-hormone mixture consisting of insulin, 10 μg/ml; hydrocortisone, 10^{-6} M; transferrin, 5 μg/ml; glycyl-L-histidyl-L-lysine acetate, 10 ng/ml; somatostatin, 10 ng/ml; and thyroid-stimulating hormone, 10 milliunits/ml. Cells are grown in 5% CO₂/95% air, 37°C atmosphere and passaged bimonthly with a split ratio of 1/10 using the collagenase/trypsin described previously (1). Unless otherwise noted in the studies below, 1 x 10^5 cells are seeded in each well of a Costar 24-well plate and maintained in culture for 2 to 9 days before use in individual experiments.

HPD, HPD powder (13), kindly provided by T. J. Dougherty, Roswell Park Memorial Institute, Buffalo, N. Y., was dissolved as described in Ref. 10.

Laser Sources. Two laser sources have been utilized for irradiation. The first one was a continuous-wave dye laser (Rhodamine B) at 631 nm pumped by an argon laser. The second one was a nitrogen laser emitting pulses of 10-nsec duration at a wavelength of 337.1 nm, peak power up to 250 kilowatts (as measured by a calibrated TRG-105 C photodiode) and repetition rate of 30 Hz. The irradiance average power was measured by a calibrated thermopile and was adjusted to be equal for the 2 laser sources.

HPD incubation and Irradiation Experiments. Typically, 10^7 freshly trypsinized cells were suspended in 10 ml of complete culture medium containing HPD [none (controls), 30 μg/ml, or 50 μg/ml]. The incubations were carried out at 37°C in a CO₂ incubator for 2 hr with occasional mixing (every 10 to 15 min). Cell suspensions were then washed by centrifugation and kept in serum-free medium without HPD. When possible, procedures were carried out in the dark. Cell manipulations were carried out minimizing exposure to room light.

For laser irradiation experiments, samples of 200 μl (containing 2 x 10^6 live cells which survived to incubation with HPD) were uniformly irradiated, using suitable lenses, in a quartz cuvet with an optical path of 5 mm. Cell settling during irradiation was prevented by periodic (every 1 min) mixing of cell suspension.

In irradiation experiments (see Charts 4 and 5), the initial number of live cells was estimated close but not equal to 100%, because typically 10% of cell death occurred during the control incubations, without HPD. No significant change in cell survival was found for unirradiated samples during the time needed for the irradiation experiment. Each irradiation experiment was repeated 3 times. The experimental points on Charts 4 and 5 represent the average values. Variability between experiments was less than the experimental error in the cell counting (see “Evaluation of Cell Viability”) as performed with the hemocytometer (i.e., typically 5 to 10%).

Evaluation of Cell Viability. Cell counts, using a hemocytometer, were performed before and after HPD incubation and before and after laser irradiation. Cell viability was evaluated using trypan blue (1 g/100 ml) in isotonic solution (NaCl, 0.9 g/100 ml) (30).

The plating efficiency was determined by seeding duplicate dishes with a known number of cells. After 24 hr of incubation in complete growth medium, dishes were washed to eliminate cells in suspension, and attached (i.e., intact and viable) cells were trypsinized and counted. The plating efficiency represents the ratio of the number of attached cells over that of initially plated cells. The treatment of cells with HPD did not interfere with the use of trypan blue, inasmuch as consistent results were obtained when cell viability was measured independently by both trypan blue and plating efficiency methods on the same samples.

Growth Curves. Cell population-doubling times and growth characteristics following incubation with HPD have been evaluated for both normal and transformed strains essentially as described previously (1, 12). After HPD incubation (see “HPD Incubation and Irradiation Experiments”), cells were usually plated in several dishes (6 cm in diameter) at densities between 3 and 20 x 10^4 cells/dish. Duplicate dishes were trypsinized and counted at different intervals, as indicated. The growth of transformed cells was evaluated in suspension cultures, these cells being anchorage independent for their growth.

RESULTS

Effect of HPD on Cell Growth Rate. In order to examine whether HPD had any effect by itself on the cell growth, i.e., independently from the laser photoactivation, both normal and virus-transformed rat thyroid cells were incubated with none (control), 30 μg, and 50 μg of HPD per ml. The growth curves of normal and transformed cells are reported in Charts 1 and 2, respectively.

The growth rates of normal and transformed cells after 2 hr incubation with HPD, at both concentrations tested, were found to be nearly identical to those of HPD-untreated control cells which followed their characteristic logarithmic growth up to confluency (6 days).

The actual number of HPD-treated cells, however, at any time between Days 1 and 6, was appreciably lower than that of the control cells. In order to explain this behavior, normal cells (FRTL-5) were counted at the end of the incubation period (2 hr) with or without HPD, as well as 22 hr later. The results obtained are reported in Chart 3. It is evident that in the absence of HPD (Chart 3A) no significant differences have been found in cell counts after 2 hr incubation. After 22 hr from plating, the cell number is still constant or only slightly increased, thus indicating that plating efficiency is not less than 90%. After 2 hr incubation...
in the presence of 30 μg (Chart 3B) or 50 μg (Chart 3C) of HPD per ml, viable cell counts are significantly lower than the initial counts, the decrease being proportional to the amounts of drug added. Even more important, in the case of the HPD-treated cells, the cell number decreased even further after 22 hr. These results demonstrate that HPD by itself affects the survival of both normal and transformed cells but has no effect on their growth rates.

**Cell Survival after HPD Treatment and Laser Irradiation.**

The survival curves of both normal (FRTL-5) and transformed (FRTL-5 KiKi) rat thyroid cells treated with HPD (30 μg/ml) and then irradiated with laser light are illustrated in Chart 4. It is evident that: (a) if the percentage of viable cells as measured by the trypan blue technique (see "Evaluation of Cell Viability") are plotted against the exposure intervals to the laser light (0 to 15 min), a straight line could be fitted on a semilog scale; (b) in each instance and for each exposure interval, the percentages of viable cells were found to be significantly higher for the normal cells than for the viral-transformed ones; (c) the photoactivated cytotoxic effect is more evident after UV (pulsed) excitation (Chart 4B) than after red (continuous-wave)-light irradiation (Chart 4A) delivered with the same average intensity of 100 milliwatts/sq cm. Similar results were obtained when both normal and transformed cells were preincubated with higher concentrations of HPD. The results with HPD (50 μg/ml) are reported in Chart 5. As expected, the cytotoxic effect was found to be considerably greater. The survival curves were linear for about 10 min of exposure to laser light, with the exception of the transformed cells irradiated with UV for which less than 10 min of exposure to CW laser caused a nearly total lack of cell survival. Again the pulsed UV excitation was more effective than the continuous red-light irradiation, and the transformed cells were more sensitive than the normal ones. When the irradiation experiments were repeated on HPD-untreated cells, no significant decrease in cell survival was found.

The sensitivities of both cell lines were directly compared by measuring the number of cells surviving after HPD treatment and laser irradiation. From the values reported in Table 1, it is evident that the percentage of cells killed by the photoactivation process is between 2 and 3 times larger for the transformed than for the normal cells, when the photosensitizer was used at the 30-μg/
the same cells with a RNA tumor virus such as the Kirsten murine
tumor cell has been made possible by infecting and transforming
control of the tumoral one; and the high variability between
genic mammalian cells.

Cells are able to grow indefinitely in culture yet retain all the
for the negative result. Actually, the experimental system of rat
inactivation. In the present study, a direct comparison with a
different cell types in their behavior towards the photodynamic
effect of HPD on tumor cells have faced 2 basic

- **Percentage of the initial number of cells killed per min of laser irradiation (continuous-wave or pulsed).** Values were calculated on the initial slopes of the survival curves.

- **Cells were incubated in medium containing HPD at 37° in a 5% CO₂ incubator for 2 hr with occasional mixing as for the irradiation experiments (see "Materials and Methods").** At the end of the incubation, cells were washed in fresh medium and then counted. Viable cells were determined with the dye exclusion test (0.1%
trypan blue).

ml concentration. At a drug concentration of 50 μg/ml, the
cytocidal effect was greater in absolute terms, but the "killing
ratio" of transformed to normal cell was not greater than 1.5 or
2. When HPD is present at this high level, the selectivity of the
action on the tumor cells is reduced or masked by the greater
cytocidal effect on the normal cells.

**DISCUSSION**

Several studies have demonstrated that either hematoporphyrin
or HPD has a cytocidal effect on isolated eukaryotic cells
in vitro. In fact, glioma cultured cells (16), ascitic TA-3 mouse
mammary carcinoma cells (9), Chinese hamster ovary cells (14),
and human NIH3T3 cells (5) have been shown to be inacti-
vated or killed in vitro by the photodynamic effect of hematoporphyrin
or HPD. However, all these cells are of tumor origin or
were established cell lines which have lost, while growing in
vitro, their normal characteristics (14), and no selective
cytocidal effects were demonstrated, prior to the present study, on mali-
nant versus normal eukaryotic cells. On the contrary, one normal
(C3H/10T1/2) and one chemically transformed mouse embryonic
fibroblastic cell line have been examined (28), and no difference in photo-
inactivation has been found although a slightly higher HPD
uptake was reported. Several differences in the experimental
conditions such as presence of serum during incubation with
HPD, different light sources (black-light fluorescent tubes at λ =
340 to 400 nm), and different evaluation of cell viability and
growth potential after photoinduction could account, however,
for the negative result. Actually, the experimental system of rat
thyroid cells used in this study is almost unique in that these
cells are able to grow indefinitely in culture yet retain all the
highly differentiated properties of the normal adult thyroid gland.
Using a such a system, we have now shown that the cytocidal
effect of HPD in vitro is evident also on normal and non-tumor-
ogenic mammalian cells.

All previous attempts to demonstrate a selectivity of the pho-
todynamic effect of HPD on tumor cells have faced 2 basic
difficulties: the lack of a suitable normal cell to be used as a
control of the tumoral one; and the high variability between
different cell types in their behavior towards the photodynamic
inactivation. In the present study, a direct comparison with a
tumor cell has been made possible by infecting and transforming
the same cells with a RNA tumor virus such as the Kirsten murine
sarcoma virus, strain Ki-MeV (Ki-MuLV). Again, the system now
used is unique in that direct comparison between well-charac-
terized differentiated normal and tumor cells in strictly controlled
identical conditions has been made possible.

The results obtained clearly demonstrate that normal and
transformed cells of the same origin have quite different sensitiv-
ities to the photoactivated HPD. The normal cells, when com-
pared to the otherwise similar but in vitro-transformed counter-
parts, are between 2 and 3 times more resistant to HPD photo-
sensitization (at the 30-μg/ml dose). Studies in vivo have also
shown an increased sensitivity of the tumor tissues to HPD
phototherapy. However, it was not clear thus far whether such a
difference could be related to an intrinsic property of the tumor
cell or, alternatively, to extracellular tissue factors such as
changes in vascularization, blood flow, cell-stroma interactions,
etc. Furthermore, our system will allow to test if a higher mortality
rate is related to higher uptake of the photosensitizing drug by
the transformed cells. Studies are in progress to elucidate this
point.

As expected, however, the normal cells, although to a lesser
degree, are also sensitive to the photoinduction by the HPD
plus laser irradiation. The mechanism of such an effect seems
to be related to the formation of singlet oxygen (22, 35). This
highly reactive intermediate is produced by energy transfer from
the triplet state of the HPD molecule after one photon absorption
under continuous-wave laser irradiation. It has been recently
observed, however, that, under high-intensity pulsed UV irradiation,
photoionization of HPD can occur following the absorption
of 2 photons (3). Thus, while some of the molecules are able to
form singlet oxygen from their triplet state, the photoionized
molecules may also interact as radicals within the cell. Actually,
cell damage at a higher extent is expected, due to the larger
absorption cross-section of HPD at 337.1 nm as compared to
that at 631 nm. However, from Table 1, it can be seen that, on
increasing the drug dose from 30 to 50 μg/ml, the selectivity
(i.e., the ratio of transformed to normal cells) of HPD activated
at 631 nm decreases from 2.17 to 1.42. This indicates the
occurrence of a saturation of the cytotoxicity induced by the
photodynamic action. This saturation effect would be expected
to be more pronounced for near-UV irradiation. On the contrary,
at a given HPD concentration, the selectivity is larger (e.g., 3.03
versus 2.17 for 30 μg/ml), thus indicating that an additional
cytocidal mechanism occurs. This is in agreement with the
observation (9) that, for a given light dose, pulsed irradiation
is more effective than continuous-wave irradiation at the same
wavelength. The target mechanism of the cell damage, however,
is not yet clarified. Intracellular effects on DNA have been claimed
by some (15), while alterations of the cell surface have been
advocated by other investigations (20). The molecular
mechanisms of the damage are also unknown (22).

HPD by itself, i.e., without light irradiation, is not completely
devoid of toxic effects. In our experimental system, at the drug
concentrations used, the growth rates of both normal and viral-
transformed cells are not affected by the previous 2-hr incubation
with HPD (Charts 1 and 2). A certain fraction of the cells,
however, is inactivated by the incubation, the effect being pro-
portional to the amount of HPD added (Chart 3). These results
are at variance with those of other authors (9, 13) which have
reported no significant toxicity of HPD by itself.

Following irradiation, however, cell survival decreases rapidly
and very significantly when the exposure time to the laser beam

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**Table 1: Photosensitivity of normal and transformed rat thyroid cell lines**

<table>
<thead>
<tr>
<th>Continuous-wave irradiation</th>
<th>Pulsed irradiation</th>
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<tr>
<td>30 μg/ml</td>
<td>50 μg/ml</td>
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<tr>
<td>30 μg/ml</td>
<td>50 μg/ml</td>
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<tr>
<td>Normal cells (FRTL-5)</td>
<td>1.38</td>
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<tr>
<td>Transformed cells (FRTL-5)</td>
<td>2.99</td>
</tr>
<tr>
<td>Normal cells (FRTL-5)</td>
<td>2.17</td>
</tr>
<tr>
<td>Transformed cells (FRTL-5)</td>
<td>3.03</td>
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is increased. This phenomenon, together with the higher sensitivity of the transformed cells to the photoinactivation, represents the basis of the phototherapy of tumors. Experimental or animal tumors are sensitive to the photodynamic action of several phototoxic drugs (7), and the first reports on preclinical trials of tumor phototherapy in humans are also available (10, 19). We hope that our experimental system will provide an useful tool for the optimization and the preliminary screening in vitro of the treatment protocols to be used in vivo. For instance, we have provided evidence that pulsed UV excitation seems to be more effective than the continuous-wave red-light irradiation at the same average intensity. Furthermore, the better ratio between transformed and normal cells is obtained at lower (i.e., 30 μg/ml) HPD concentration; at higher drug levels, the cytotoxic effect on normal cells becomes too important, and the therapeutic ratio is significantly decreased.

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