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Jeffrey M. Tosk

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Abstract

SELENIUM-INDUCED ENHANCEMENT OF HEMATOPORPHYRIN DERIVATIVE PHOTOTOXICITY IN MURINE BLADDER TUMOR CELLS

by

Jeffrey M. Tosk

The phototoxicity of hematoporphyrin derivative (Hpd) to murine bladder tumor (MBT-2) cells was studied in vitro. It was observed that selenium in the form of sodium selenite enhanced Hpd-sensitized photodamage in MBT-2 cells under conditions where the selenite alone was non-toxic. Sodium selenite was found to enhance the fluorescence emission of Hpd and augment the Hpd-sensitized photooxidation of tryptophan. The data indicate that sodium selenite is able to disaggregate Hpd, thereby enhancing Hpd-sensitized phototoxicity.

LOMA LINDA UNIVERSITY

Graduate School

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PHOTOTOXICITY IN MURINE BLADDER TUMOR CELLS

by

Jeffrey M. Tosk

A Manuscript Submitted in Partial Fulfillment
of the Requirements for the Degree Master of Arts
in Biology

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Each person whose signature appears below certifies that this manuscript is adequate in scope and quality in lieu of a thesis for the degree of Master of Arts.

A handwritten signature in cursive script, reading "Benjamin H. S. Lau". The signature is written in dark ink on a white background.

_____, Chairman

Benjamin H. S. Lau, Professor of Microbiology and Biology

A handwritten signature in cursive script, reading "A. Graham Maxwell". The signature is written in dark ink on a white background.

A. Graham Maxwell, Professor of New Testament

A handwritten signature in cursive script, reading "Elwood S. McCluskey". The signature is written in dark ink on a white background.

Elwood S. McCluskey, Associate Professor of Biology and Physiology

A handwritten signature in cursive script, reading "Robert W. Teel". The signature is written in dark ink on a white background.

Robert W. Teel, Associate professor of Physiology and Pharmacology

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INTRODUCTION

Hematoporphyrin derivative (HpD) is a fluorescent dye mixture with a combination of tumor-localizing and photosensitizing properties (1-3). HpD has shown promise in the photodynamic therapy (PDT) of malignant tumors (4-6). Following parenteral administration of HpD to a tumor-bearing host, photoirradiation of the tumor site leads to extensive destruction of malignant cells with minimal injury to the adjacent normal tissues (7,8). Cytotoxicity is thought to result from photodamage induced by singlet oxygen (1O_2), generated via energy transfer from the excited triplet state of a porphyrin molecule to a ground state oxygen molecule (9-11). There is also evidence that hydroxyl radicals ($OH\cdot$) (12-14) and superoxide (O_2^-) (13,15) are involved. Porphyrin radical ($H_2P^\cdot$) formation has also been implicated as mediator of cytotoxicity from PDT (16,17).

Weishaupt, Gomer and Dougherty (9) identified singlet oxygen as an intermediate in HpD phototoxicity by the ability of 1,3-diphenylisobenzofuran, a specific singlet oxygen quencher, to inhibit HpD photoinactivation of tumor cells. Recently Keene et al. (11) provided direct evidence of its presence by spectroscopic detection during HpD photolysis. Forbes et al. (14) demonstrated that mannitol, a hydroxyl radical scavenger, partially blocked HpD phototoxicity. Although this suggests that hydroxyl radicals may be involved in PDT, the same report indicates that histidine and sodium azide, both singlet oxygen quenchers, are more effective than mannitol in protecting cells against HpD phototoxicity.

While in the course of investigating the effects of various inhibitors of reactive oxygen species on Hpd-sensitized photoinactivation of murine bladder tumor (MBT-2) cells, we decided to evaluate the effect of selenium in the form of sodium selenite. The protective effect of various selenium compounds against radiation-induced damage has been demonstrated previously (18,19), and was attributed to a free radical scavenging mechanism. In this communication we report that sodium selenite, contrary to our expectation, enhanced Hpd-sensitized phototoxicity toward MBT-2 cells. We provide experimental evidence to show that this effect may be due to a selenite-induced modification of the aggregation state of the Hpd.

MATERIALS AND METHODS

Cells. The murine bladder tumor cell line, designated MBT-2, was established from a chemically-induced murine transitional cell carcinoma of the bladder by Soloway (20). The cells were routinely grown and maintained in RPMI-1640 with 10% fetal bovine serum, 500 units/ml penicillin, 250 $\mu\text{g/ml}$ streptomycin and 2 g/l sodium bicarbonate. Cultures were maintained at 37° C in a humidified atmosphere of 5% CO₂.

Preparation of Hpd. Hematoporphyrin dihydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO) and Hpd was prepared by the method of Lipson, Baldes and Olsen (1). 50 mg of the resulting dried acetate were dissolved in 2.5 ml of 0.1 N NaOH, stirred in the dark for 1 h at room temperature and brought to pH 7.4 by the dropwise addition of 0.1 N HCl. This solution was adjusted to a final volume of 10 ml with normal saline, sterilized by Millipore filtration (0.22 μm pore size) and stored in the dark at -20° C. All experiments were performed using dilutions from this stock solution.

Drug treatment. MBT-2 cells were grown to near-confluence, at which time the medium was replaced with fresh medium containing 12.5 $\mu\text{g/ml}$ Hpd. The medium of control cultures was replaced with fresh medium containing no Hpd. All procedures involving Hpd were performed in the dark. Following a 12 h incubation, the Hpd medium and the control medium were discarded and the monolayers of MBT-2 cells were carefully washed and replaced with fresh medium. Following an additional 4 h incubation, the monolayers were washed once more and harvested by trypsinization. The cells were washed twice and finally resuspended in

fresh medium to yield a final concentration of 10^6 cells/ml.

The stock solution of sodium selenite was prepared in serum-free medium at a concentration of 1 mg/ml. It was added to Hpd-treated or untreated cells to give final concentrations of 5, 10, or 20 $\mu\text{g/ml}$. In some experiments the treated cells were photoirradiated immediately following the addition of selenite. In other experiments the cells were reincubated for an additional 2 h following addition of selenite. Cell survival was determined by trypan blue exclusion. The results were expressed as the means $\pm$ SE of triplicate cultures.

Photoirradiation. A quartz-halogen lamp (MK-2, Nikon, Inc., Garden City, NY), with infrared-filtered output was employed as the light source. The cells were irradiated in triplicate in 2 ml suspensions at a distance of 30 cm from the light source in clear plastic tubes (Falcon 2057). The mean fluence rate was 20 mW/cm^2 as determined by radiometry. Cell survival following photoirradiation was also determined by trypan blue exclusion.

Fluorescence spectroscopy. Fluorescence spectra were recorded on a Perkin Elmer LS-3 fluorescence spectrophotometer. Mixtures of Hpd (0.1 mM or 60 $\mu\text{g/ml}$) and various quantities of sodium selenite (0-0.07 mM or 0-12 $\mu\text{g/ml}$) were examined. A quartz cuvette with a 1 cm optical path was used. Excitation was at 400 nm, emission at 627 nm.

Photodegradation of tryptophan. Phosphate buffered saline (PBS, pH 7.2) containing 0.1 mM or 20 $\mu\text{g/ml}$ tryptophan (Trp) and 0.1 mM Hpd was photoirradiated in duplicate under the same conditions as the cells. The concentration of sodium selenite used was 20 $\mu\text{g/ml}$. During photoirradiation the concentration of undegraded Trp was determined by

diluting 20 μ l samples from the irradiated solution in 2 ml PBS and measuring the fluorescence of Trp ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 350$ nm). Under such conditions none of the fluorescence could be attributed to Hpd.

RESULTS

When administered to Hpd-treated MBT-2 cells just prior to photoirradiation, sodium selenite enhanced phototoxicity (Fig. 1); this effect was dose dependent. Under some conditions sodium selenite was cytotoxic toward MBT-2 cells. Fig. 2 shows that the cytotoxic effect required at least 2 h of exposure at selenite concentrations of 5-20 $\mu\text{g/ml}$. A 20 min exposure to sodium selenite was non-toxic.

Controls comprising cells which were photoirradiated without Hpd sensitization exhibited no phototoxicity regardless of the presence or absence of selenite. Similarly, no cytotoxicity was observed in Hpd-treated cells which were kept in the dark. Such cultures retained over 90% viability (data not shown).

When Hpd-treated cells were incubated in medium containing selenite for 2 h prior to photoirradiation, an additive effect of selenite toxicity and Hpd-sensitized phototoxicity was observed. The toxicity was directly related to photoirradiation time (Fig. 3).

The marked ability of sodium selenite to enhance the fluorescence emission of Hpd is shown in Fig. 4. The fluorescence enhancement depended upon the concentration of sodium selenite up to 0.07 mM (12 $\mu\text{g/ml}$). Fig. 5 shows the effect of selenite on the Hpd-sensitized photooxidation of Trp. Over the 8 min time course shown, only irradiated tubes containing Trp with Hpd and selenite exhibited a detectable loss of Trp. Hpd alone was observed to sensitize the photooxidation of Trp. However, this effect required at least 30 min of

irradiation to become evident (data not shown). Trp fluorescence in unirradiated controls remained constant throughout the time course studied.

Figure 1. Dose-dependent selenite enhancement of Hpd-sensitized phototoxicity in MBT-2 cells. ■—■ 2 min, ◆—◆ 4 min and ▲—▲ 10 min photoirradiation times. N = 3. Absence of SE bars indicates SE < the dimensions of the symbol.

Figure 2. Dose and time-dependence of sodium selenite cytotoxicity in MBT-2 cells. $\square$ — $\square$ 20 min, $\blacklozenge$ — $\blacklozenge$ 2 h, $\blacktriangle$ — $\blacktriangle$ 4 h and $\blacksquare$ — $\blacksquare$ 24 h incubation. N = 3.

Figure 3. Effect of 2 h preincubation in 20 $\mu\text{g/ml}$ sodium selenite on Hpd-sensitized phototoxicity in MBT-2 cells. $\blacklozenge\text{---}\blacklozenge$ Control without selenite and $\blacktriangle\text{---}\blacktriangle$ 2 h preincubation. N = 3.

Figure 4. Sodium selenite concentration-dependent enhancement of Hpd fluorescence emission. Aqueous solutions of 0.1 mM Hpd were buffered by PBS pH 7.4. Excitation was at 400 nm, emission at 627 nm. Fluorescence enhancement due to added selenite is expressed as relative emission of the Hpd-selenite solution/relative emission of Hpd solution alone (F/F_0). $N = 3$. SE never exceeded symbol size.

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Figure 5. Effect of combined Hpd and sodium selenite on the photodegradation of Trp. ■—■ Trp alone, ▲—▲ Trp + Hpd, ◆—◆ Trp + selenite and □—□ Trp + Hpd and selenite. (see text for experimental details). N = 2.

DISCUSSION

This investigation shows that the phototoxicity of Hpd toward MBT-2 cells was enhanced by the addition of sodium selenite to a cell suspension just prior to photoirradiation (Fig. 1). This was a synergistic effect, not attributable to the direct cytotoxicity of selenite, as the enhancement depends on the selenite concentration within a range of time exposure too short (2-10 min) for the selenite to exert a toxic effect of its own. This is important to consider, as sodium selenite has been shown to possess considerable antineoplastic activity in several experimental tumor systems (21-23).

We also demonstrated the cytotoxic activity of sodium selenite (Fig. 2). Significant toxicity was observed, however, only after two or more hours of exposure. When the Hpd-treated cells were incubated with selenite for two hours prior to photoirradiation, the net toxicity following irradiation may be attributed to the additive effects of selenite and Hpd. Under these conditions, the selenite cytotoxicity appeared to mask the aforementioned synergistic enhancement of Hpd photosensitization (Fig. 3).

While the mechanism of the observed enhancement of Hpd-sensitized phototoxicity by selenite remains obscure, a partial explanation may be derived from the observation that sodium selenite was able to enhance the intensity of fluorescence emission of Hpd (Fig. 4). We attribute this effect to a selenite-induced alteration of the aggregation state of Hpd. Ito and Ito (24) studied ethanol-induced disaggregation of hematoporphyrin (HP) administered to HP-treated alcohol-tolerant yeast,

and observed enhanced HP-sensitized phototoxicity under conditions where the added ethanol enhanced HP fluorescence intensity. They attributed both the augmented phototoxicity and the fluorescence enhancement to ethanol-induced disaggregation of HP. On the other hand, Smith (25) reported that solutions of HP containing aggregates have lower fluorescence and triplet state yields than solutions containing primarily the monomer. Margalit, Shaklai and Cohen (26) demonstrated fluorescence quenching of porphyrin solutions by salts which promote an increase in aggregation, while Kessel and Rossi (27) reported that protoporphyrin-sensitized oxidation of Trp was impaired under conditions which promote porphyrin aggregation beyond dimer formation. Our demonstration of the ability of selenite to augment the Hpd-sensitized oxidation of Trp (Fig. 5) is consistent with the hypothesis that selenite affects the disaggregation of Hpd. Additional studies are necessary to elucidate the mechanism of selenite-mediated disaggregation. The possible clinical application of these findings is under investigation.

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