Modification of Photodynamic Therapy-induced Hypoxia by Fluosol-DA (20%) and Carbogen Breathing in Mice

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ABSTRACT

The administration of a perfluorochemical emulsion and carbogen (95% O₂, 5% CO₂) breathing before photodynamic therapy (PDT) was studied to determine how increased levels of tumor oxygenation may affect PDT-induced tumor destruction. C3H/HeJ mice bearing the RIF tumor were given injections of 5 to 10 mg/kg of dihematoporphyrin ethers 24 h prior to treatment. Animals were given injections of 12 ml/kg of Fluosol-DA (20%) followed by carbogen breathing or 12 ml/kg of saline and air breathing (controls) 1 h before tumors were exposed to 135 J/cm² of 630-nm light treatment. Changes in the hypoxic fraction of tumors, the time course for decreases in tumor cell clonogenicity, and tumor response were measured immediately and at various times after treatment. The administration of Fluosol-DA (20%) and carbogen breathing was found to delay the onset of PDT-induced hypoxia through the first hour posttreatment. Progressive tumor hypoxia was observed after 4 h posttreatment. The time period in which tumors remained well oxygenated coincided with observations of increased tumor cell survival. Decreases in tumor cell clonogenicity were observed only after tumor cells became hypoxic. These findings were consistent with the 24-h delay in complete tumor response in animals given Fluosol-DA (20%) and carbogen breathing before PDT. There were only minor variations in long-term tumor response and cure observed between the two groups tested.

A second series of experiments was done to assess any treatment advantage of the adjuvant use of Fluosol-DA (20%) and carbogen breathing with PDT at high tumor photosensitizer levels. At an injected dose of 50 mg/kg of dihematoporphyrin ethers, no such advantage was observed. The administration of Fluosol-DA (20%) and carbogen breathing did not reduce the extent of PDT-induced microvascular damage, maintain high levels of tumor oxygenation through light treatment, or modify the extent of tumor cell kill following treatment.

INTRODUCTION

PDT, using porphyrins, in particular DHE, is presently undergoing clinical evaluation for the treatment of a range of solid tumors (1). PDT is based on the systemic administration of tumor-localizing porphyrins and their subsequent excitation with visible light to produce tumor destruction. This destruction is primarily mediated by energy transfer from excited porphyrin molecules to molecular oxygen, thereby producing cytotoxic singlet oxygen (2). The presence of oxygen is therefore an absolute requirement for the photoinactivation of cells both in vivo and in vitro (3, 4). Anoxic conditions totally abolish PDT cell inactivation, and the half value for cellular photosensitivity lies at about 1% oxygen (4, 5).

Tumor treatment by PDT is accompanied by changes in tumor and normal tissue microvasculature. These include decreased blood flow, platelet aggregation, and hemorrhage, leading to vascular stasis and decreased tumor oxygenation (6–8). We have recently analyzed the development of tumor hypoxia in a rodent tumor model. It was shown that significant numbers of tumor cells are rendered hypoxic and thus presumably insensitive to further direct photodynamic damage, even after brief light exposure of the tumor and well before completion of treatment (5). Surprisingly, this potential self-limitation of PDT did not prevent tumor cure, indicating that ultimate tumor cell death in the tumor model used and under the given treatment conditions is apparently the result of vascular damage and its consequences, rather than direct photodynamic tumor cell inactivation. Based on these observations, it seemed of interest to determine what effects prolonged maintenance of tumor oxygenation during PDT might have on tumor response to this treatment modality.

Perfluorochemical emulsions have been studied as a means to increase oxygenation within tissues (9, 10). Their usefulness is based on the ability of fluorocarbons to dissolve large amounts of oxygen in the lung and release this oxygen in tissues. Also, due to the small size of the emulsion particles (one-tenth of the size of RBCs), they may travel into areas compromised by damaged vasculature (11). For these reasons, perfluorochemicals, in particular Fluosol-DA (20%), have been used clinically as blood replacements and to increase tissue oxygenation in cases of coronary ischemia (12, 13). Fluosol-DA (20%), combined with carbogen breathing, has been demonstrated to decrease hypoxic tumor cell fractions in rodent tumors as well as to sensitize these tumors to ionizing radiation (11, 14, 15) and is now being considered clinically as an adjuvant treatment to radiation therapy (16).

In this paper we describe the limited reduction of PDT-induced tumor hypoxia through the adjuvant use of Fluosol-DA (20%) and carbogen breathing, as well as the effects this treatment exerted on the response to PDT of an experimental mouse tumor model.

MATERIALS AND METHODS

Tumor System. The RIF tumor model was used in this study and was carried according to established protocols in female C3H/HeJ mice (17). Prior to intradermal inoculation of the RIF tumor, all hair was removed from the right axillary region of the mouse by shaving and depilation as described previously (18). Tumors were used for experimentation when they reached a surface diameter of 5 to 6 mm, a thickness of 2 to 3 mm, and weight of 80 to 120 mg. Tumors were free of evident necrosis.

Drugs. Photofrin II (Photomedica, Inc., Raritan, NJ) was the photosensitizer used in all experiments. It contained above 80% dihematoporphyrin ether as determined by high-performance liquid chromatography analysis and is subsequently referred to as DHE. Fluosol-DA (20%) was obtained from Alpha Therapeutics, Inc., Los Angeles, CA. It consisted of perfluorodecalin (14%, w/v) and perfluorotripropylamine (6%, w/v) in an emulsifying solution balanced with sodium and potassium salts to maintain osmolality.

Fluosol-DA (20%) and Carbogen Treatment. Fluosol-DA (20%) was equilibrated with carbogen by bubbling the gas mixture through the emulsion for 5 min. It was then injected i.e. into animals at a dose of 12 ml/kg of body weight. The animals were then placed in environmental chambers which were flushed with carbogen for 1 h before and during light treatment of the tumor. Animals given injections of saline

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PDT, photodynamic therapy; DHE, dihematoporphyrin ether; RIF, radiation-induced fibrosarcoma; i.e. intracardially.
and allowed to breath room air were used as controls in all studies. The administration of either Fluosol-DA (20%) or carbogen breathing alone to mice was used as additional controls for tumor response and hypoxic fraction studies.

In Vivo PDT. Tumor-bearing mice were given porphyrin doses of either 5, 10, or 50 mg/kg of DHE i.p. 24 h before light treatment. Localized, external light treatment was delivered using an argon dye laser system (Spectra Physics Corp., Mountain View, CA) at a power density of 75 milliwatts/cm² and a wavelength of 630 nm. Animals were restrained in plastic holders without anesthesia, and tumors were illuminated for 30 min for a light dose of 135 J/cm² through the transparent walls of the plexiglass environmental chambers. The power density of delivered light was adjusted to correct for any light attenuation by the walls of the chamber.

Tumor Clonogenicity Assay. Tumor cell survival following the varying treatment regimens was assessed as described previously (19). Briefly, tumors were excised, minced finely, and disaggregated using an enzyme mixture of Pronase, collagenase, and DNase. The resulting cell suspension was passed through a fine wire screen to remove any remaining clumps. Cells were counted, and appropriate cell numbers were plated in minimal Eagle’s α-medium with 1-glutamine and ribo- and deoxyribonucleosides, supplemented with 10% fetal bovine serum and antibiotics. All media and supplements were obtained from Grand Island Biological Co., Grand Island, NY. Cell colonies were counted 9 to 10 days later, and the number of clonogenic cells/g of tumor was calculated.

Evaluation of Hypoxic Tumor Fractions. The radiobiological determination of hypoxic tumor cell fractions following PDT in vivo has been described recently (5, 20). Briefly, at varying times after PDT with/without adjuvant treatment, animals were given graded doses of whole-body γ-irradiation of 10 to 26.7 Gy (1.08 Gy/min). Immediately after irradiation, tumors were excised, and tumor cell clonogenicity was measured as described. Cell survival following γ-irradiation in experimental and control groups was compared to cell survival in untreated tumors irradiated in N2-asphyxiated mice. Tumor hypoxic fractions were calculated by measuring the difference in cell survival between 100% hypoxic tumors (from N2-asphyxiated mice) and tumors from the other groups (21).

Assessment of Vascular Damage by Fluorescein Dye Exclusion. Immediately after varying PDT treatments and at 1 and 4 h posttreatment, 2 to 3 animals from each treatment group were ether anesthetized and given an i.c. injection of 2.0 mg of fluorescein dye (10 mg/ml of sodium fluorescein; Baker Chemical, Phillipsburg, NJ; diluted in Hanks’ balanced salt solution). Animals were sacrificed 1 to 2 min later, and a skin flap containing the tumor was lifted. The tumor and surrounding skin were illuminated with 365 nm of UV light (Blak-Ray long-wave UV lamp; Ultra-Violet Products, Inc., San Gabriel, CA) to assess dye exclusion in the tumor and surrounding tissues. Fluorescent patterns were photographed and recorded.

Assessment of Tumor Response and Cure. Following PDT treatments, tumor response was evaluated daily for 2 wk and at weekly intervals thereafter up to 91 days. Tumor control was defined as tumors being flat and necrotic, without any remaining palpable tumor mass. Animals were considered cured if no regrowth occurred within 91 days posttreatment.

Assessment of Effects of Fluosol-DA (20%) on PDT in Vitro. RIF cells grown in culture to semiconfluency were exposed to 25 μg/ml of DHE in minimal Eagle’s α-medium containing 10% fetal bovine serum for 24 h. The medium was then replaced with porphin-free medium for 4 h to remove loosely bound porphyrin from cells. The medium was then replaced with either 1 ml of Fluosol-DA (20%) or 1 ml of Hanks’ balanced salt solution. Cultures were exposed 1 h later to graded doses of 590- to 640-nm light (4 milliwatts/cm²) delivered using a GTE-Sylvania fluorescent light source (Salem, MA). Following the light treatment, cells were removed from dishes using 0.25% trypsin, replated in complete medium at appropriate numbers, and incubated 9 days for colony formation.

Measurement of Tumor Porphyrin Levels and Porphyrin Fluorescence Decay. Tumor-bearing animals were given injections i.p. of 30 mg/kg of DHE. Twenty-four h later they were given an i.c. injection of either 12 ml/kg of Fluosol-DA (20%) or saline. One h later, tumors were either excised for porphyrin extraction (22), or they were exposed in vivo to 135 J/cm² (75 milliwatts/cm², 630 nm). In the latter case, tumor fluorescence was monitored using a fluorescence photometer (22) at various times before, during, and after light treatment.

Tumor porphyrin levels were also determined in the RIF tumor by measuring the cellular uptake of DHE spiked with radiolaabeled [14C]-DHE (Oncologic Foundation of Buffalo, Buffalo, NY). Tumor-bearing animals were given injections of either 10 or 50 mg/kg of 14C-spiked DHE (0.22 mg of [14C]DHE/5.88 mg of cold DHE at an activity of 8.691 × 10² dpm/μg of DHE). The radiolabeled DHE has been shown to be identical in structure and biological activity to the DHE in Photofrin II.4 Tumors were excised and disaggregated, cells were counted as previously described, and the resulting tumor cell pellet and supernatants were collected. The tumor cell pellet was then resuspended in 2 ml of saline, and 12 ml of SintiVerse scintillation fluid (Fisher Scientific, Pittsburgh, PA) were added. Scintillation fluid was also added to a 2-ml sample of the collected supernatants. Samples were placed in a Beckman LS-1801 liquid scintillation counter (Beckman Instruments Co., Fullerton, CA), and 14C levels were counted. Sample dpm counts were calculated by the H# technique. Total DHE within samples were calculated by dividing the sample dpm counts by the specific activity of the [14C]DHE. Cellular DHE levels were calculated by dividing the total DHE within the sample by the number of cells in the sample.

Histological Evaluation. Tumors were excised at 4 h or 24 h after varying PDT treatments in vivo, fixed in buffered formalin (10%), sectioned at 5-μm thickness, and stained with hematoxylin-eosin.

RESULTS

The effects of PDT alone at an injected porphyrin dose of 5 mg/kg of DHE and PDT plus adjuvant treatment with Fluosol-DA (20%) and carbogen breathing on tumor response are shown in Fig. 1. Striking differences in the short-term effects were observed between these treatment groups. Animals treated with PDT alone showed tumor darkening due to hemorrhage within 1 to 4 h after treatment. Within 24 h complete response with disappearance of tumor bulk was observed, followed over the next 3 days by some normal tissue necrosis within the treatment field. These same effects were also observed in animals treated with PDT plus Fluosol-DA (20%)-carbogen, but the onset of changes was markedly delayed. Only slight tumor changes were observed at 4 h posttreatment, and most of the tumor bulk was still present at 24 h. Only after 48 h was complete response observed. Twenty-six % of animals were cured by PDT alone (n = 62), while only 13.3% were cured by the combined treat-

Tumor cell survival immediately following PDT treatment was therefore measured at an injected dose of 50 mg/kg of DHE in animals given Fluosol-DA (20%) and carbogen breathing, and for controls. Tumors from controls showed 43.7 ± 11.1% cell survival compared to 43.8 ± 4.2% tumor cell survival from animals given Fluosol-DA (20%) and carbogen breathing prior to treatment. Thus, although tumor cell survival immediately after treatment was reduced at the higher injected drug dose, the administration of Fluosol-DA (20%) and carbogen breathing did not modify tumor cell survival.

Observation of long-term tumor response after these high-dose treatments was impossible due to overwhelming normal tissue damage and subsequent mortality of the animals.

In order to understand the mechanisms responsible for the above described effects, the influence of Fluosol-DA (20%) and carbogen breathing on PDT-induced tumor hypoxia was assessed. These data are shown in Fig. 3 and summarized in Table 1 for the low drug dose conditions. In these tumors, which contain no detectable preexisting hypoxic cell population at treatment size (5), PDT-induced hypoxia was markedly delayed by administration of Fluosol-DA (20%)-carbogen. Approxi-
approximately 10-fold differences in hypoxic tumor cell fractions were measured in tumors from mice given Fluosol-DA (20%) and carbogen breathing when compared to controls at two different porphyrin doses (5 and 10 mg/kg of DHE) when tumors were assayed immediately or 1 h after light treatment. Hypoxia, however, was progressive in all treatment groups, and values for the adjuvant group at 4 h approached those of PDT-only controls at 1 h post-light exposure. In studies using porphyrin doses of 5 mg/kg of DHE, there were no significant differences between the hypoxic fraction of tumors assayed immediately after treatment from mice treated with PDT alone (13 ± 7%), PDT in combination with carbogen breathing alone (13 ± 6%), or PDT in combination with administration of Fluosol-DA (20%) alone (12 ± 4%).

A second series of experiments was done to assess if the administration of Fluosol-DA (20%) and carbogen breathing could maintain high levels of tumor oxygenation in animals given porphyrin doses of 50 mg/kg of DHE. In these studies, the Fluosol-DA (20%)-carbogen treatment was ineffective in maintaining tumor oxygenation, and significant levels of tumor hypoxia (28 ± 3%) were found immediately after the PDT treatment.

To assess whether the observed delay in the development of hypoxia at low porphyrin doses might be related to an effect of the Fluosol-DA (20%)-carbogen treatment on PDT-induced vascular damage, the extent of microvascular occlusion was visualized by a fluorescein dye exclusion technique. In PDT-treated animals given 5 or 10 mg/kg of DHE, complete exclusion of fluorescein dye from the tumor microvasculature was observed by the end of the 30-min light exposure and was maintained through 24 h posttreatment. In animals also given Fluosol-DA (20%) and carbogen breathing, fluorescein dye was found within tumor microvasculature immediately and 1 h after the light treatment, indicating that significantly less tumor microvasculature damage had occurred. However, by 4 h posttreatment, there was complete dye exclusion to the tumor microvasculature, indicating that vascular disruption had developed. This fluorescein exclusion was maintained for at least 24 h posttreatment. However, at 50-mg/kg porphyrin doses, there was complete dye exclusion from tumors exposed to Fluosol-DA (20%) and carbogen, as well as for controls immediately after light treatment, indicating rapid vascular damage in either treatment condition.

To determine whether the adjuvant treatment effects at low porphyrin dose could be traced to differences in photosensitizer distribution or cell inactivation due to Fluosol-DA (20%), various control experiments were carried out. In vitro cell survival experiments showed that the presence of Fluosol-DA (20%) did not alter cell inactivation by PDT. RIF cell survival to PDT in the presence of Fluosol-DA (20%) was identical to controls yielding survival curves with a 37% dose slope ($D_{0}$) of 0.15 J/cm$^2$ and a quasithreshold dose ($D_{T}$) of 0.85 J/cm$^2$. In both groups, 1.15 J/cm$^2$ of light at a porphyrin dose of 25 µg/ml of DHE produced 90% cell kill.

Porphyrin extraction from tumors before light treatment was done to determine if an in vivo administration of Fluosol-DA (20%) would influence the uptake or retention of porphyrins in tumors. These experiments were carried out at injected DHE doses of 30 mg/kg due to the limited sensitivity of the extraction method. No difference was found in the amount of extractable porphyrin from mice given Fluosol-DA (20%) and carbogen breathing (15.8 ± 3.5 µg of porphyrin/g of tumor) compared to controls (15.1 ± 1.0 µg of porphyrin/g of tumor). Upon porphyrin extraction following light treatment, photosensitizer concentrations in tumors were found to be uninfluenced by in vivo administration of Fluosol-DA (20%) [6.5 ± 4.0 µg/g of porphyrin from animals given Fluosol-DA (20%) and carbogen versus 6.9 ± 2.2 µg/g of porphyrin from controls]. Finally, tumor fluorescence decay during treatment was monitored as a probe for in vivo porphyrin activation and was also found unchanged by the administration of Fluosol-DA (20%) (Fig. 4).

DISCUSSION

This study addresses two questions. (a) Can the administration of Fluosol-DA (20%) and carbogen breathing be used to maintain high levels of tumor oxygenation throughout PDT treatment? (b) What are the implications of increased tumor oxygenation on the effects of PDT in vivo?

We have been able to demonstrate that Fluosol-DA (20%) and carbogen breathing, administered as an adjuvant to PDT, produced a highly significant, temporary reduction in PDT-induced tumor hypoxia at DHE doses of 10 mg/kg and below. This reduction was paralleled by a delay and decreased severity of the microvascular occlusion which usually accompanies PDT treatment. The vascular disturbances observed in the control studies have also been confirmed for the SMT-F mouse tumor model by Doppler velocimetry (24). The limited protection of the vasculature, in addition to the superior oxygen-carrying capacity of Fluosol-DA (20%), was apparently responsible for the maintenance of tumor oxygenation. The mechanism of vascular protection is unclear. Control experiments showed that the presence of Fluosol-DA (20%) neither interfered with PDT cell inactivation in vitro, nor with photosensitizer uptake and activation in the tumor. However, the situation within the vascular space may well be different from that assessed in...
overall tumor measurements, and it must be further elucidated. It has to be emphasized that vascular obstruction was only delayed and not prevented.

Concomitant with the delay in vascular changes and development of hypoxia was a delay in tumor response, which was evident upon macroscopic and histological evaluation as well as analysis of tumor cell survival kinetics. In view of our previous contention, that tumor destruction by PDT in this tumor model under the given conditions (i.e., low porphyrin content of tumor cells) was primarily caused by vascular damage (19), these results were to be expected. They raise, however, the prospect of affecting the mechanism of tumor destruction by PDT in situations where direct photodynamic damage of tumor cells might be possible due to high porphyrin content. Injected doses of 50 mg/kg of DHE or more would be required in the RIF tumor model to produce significant levels of direct cell kill using the light doses in this study (23). Under these conditions, PDT appears to be self-limiting, since the rapid vascular shutdown and ensuing hypoxia prevent the delivery of an effective treatment. Oxygen limitations of PDT occur before sufficient light has been delivered to realize the potential for direct photodynamic tumor cell inactivation (5, 25). The maintenance of tumor oxygenation through the adjuvant use of Fluosol-DA (20%) and carbogen breathing might therefore have offered a treatment advantage at high tumor photosensitizer levels. However, no such advantage was gained by the use of Fluosol-DA (20%) and carbogen breathing at 50-mg/kg DHE injected doses in this tumor model. It is apparent that the vascular damage produced by PDT at high porphyrin doses was too much for the administration of Fluosol-DA (20%) and carbogen breathing to overcome.

Finally, the implications of these studies for human PDT treatment are not yet clear, since neither the kinetics of vascular damage nor the tumor cell porphyrin content in human tumors is known. Once this information becomes known, however, the present study might become helpful in designing new treatment approaches.

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