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Relationship of Tumor Hypoxia and Response to Photodynamic Treatment in an Experimental Mouse Tumor[1]

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ABSTRACT

The relationship between tumor oxygenation and the effectiveness of photodynamic therapy (PDT) was studied *in vitro* and *in vivo* using the RIF mouse tumor model. The oxygen dependence of photodynamic inactivation of RIF cells, which had been exposed to 25 mg/kg porphyrin (dihematoporphyrin ether) *in vivo*, isolated and illuminated *in vitro*, was determined. No cell kill was achieved under anoxic conditions, full effect was reached at 5% O_2, and the half value of cell inactivation was found to be at 1% O_2. Tumor hypoxia was assessed after *in vivo* γ-irradiation of control and PDT-treated tumors by *in vitro* clonogenic assay of cell radiosensitivity. *In vitro* control experiments established that the radiosensitivity of PDT-surviving RIF cells was identical to that of untreated control cells. RIF tumors of treatment size (80–120 mg) contained no detectable hypoxic tumor cell fraction. PDT treatment consisting of i.p. injection of 10 mg/kg dihematoporphyrin ether 24 h prior to 45 J/cm² of 630 nm light, rendered approximately 9% of tumor cells severely hypoxic within 10 min of treatment time. An illumination period of 30 min (135 J/cm²) induced a hypoxic tumor cell fraction of 17%, which increased to 47% within 1 h posttreatment. Despite the prompt induction of tumor hypoxia during PDT light treatment, the tumors proved highly curable (81% cures) under the present treatment conditions (depilation of tumor area, 10 mg/kg dihematoporphyrin ether i.p., 135 J/cm²). Considering the reduced effectiveness of photodynamic cell kill at low oxygen concentrations, the rapid induction of tumor hypoxia by PDT itself, and the high tumor cure rate, it has to be concluded that in the RIF tumor hypoxic tumor cells are inactivated by a mechanism other than direct photodynamic cytotoxicity, and are thus not limiting to PDT tumor response.

INTRODUCTION

PDT,[3] a new experimental modality for the treatment of solid tumors, is now undergoing clinical evaluation (1). Its effect is based on the administration of tumor-localizing photosensitizers, in particular hematoporphyrin derivative and DHE, and their subsequent activation by visible light. Singlet oxygen ($^1O_2$) and other oxygen-derived species produced upon excitation of the photosensitizer appear to be the cytotoxic agents (2–4).

The usually rapid tumor response to PDT is characterized by initial damage to the tumor vasculature, which can occur after only brief periods of tumor illumination, and subsequent necrosis of tumor cells (5, 6). Vascular damage leads to decreased blood flow (7), blood stasis (6), hemorrhage (4, 6, 8), and decreases in tissue oxygen tension (9).

Analysis of clonogenicity of experimental animal tumors following PDT *in vivo* has revealed that tumor cells are not lethally damaged during the treatment (4). Inactivation seems to require additional factors provided by the posttreatment tumor environment, presumably related to the above described vascular changes.

The importance of the tissue oxygen supply and some possible implications of tissue hypoxia for PDT have recently been discussed by Freitas (10). The presence of oxygen, in addition to photosensitizer and light, seems necessary for photodynamic cytocidal effects to occur *in vitro* and *in vivo* (11–14). The reports concerning the extent of the PDT oxygen dependence, however, vary greatly. Lee See, *et al.* (11) observed a 50% reduction in cell photoinactivation at a pO_2 of about 50 mm Hg with a maximal effect at 90 mm Hg, while Moan and Sommer (12) found a 50% reduction in cell photosensitivity at a pO_2 of 7.5 mm Hg with full effect occurring at normal tissue oxygenation levels.

Another cancer treatment modality which is strongly oxygen dependent is ionizing radiation. Cellular radiosensitivity is reduced by 50% at a pO_2 of about 3 mm Hg. The limitations which may be imposed on the effectiveness of radiotherapy by hypoxic tumor regions have long been an area of intensive study and methods to quantitate hypoxic fractions of tumor cells have been devised (15, 16). One approach is based on the different radiosensitivity of tumor cell populations *in vivo* which are heterogeneous with respect to their status of oxygenation (17, 18). The dose-effect curves for mixed hypoxic and oxygenated cell populations are characterized by a biphasic nature with the two slope components reflecting the different sensitivities of hypoxic and oxygenated cells. This relationship permits determination of the fraction of hypoxic cells in tumors of the living animal by a comparison of the survival curves of aerated or partially aerated tumors and those rendered completely hypoxic by N_2 asphyxiation of the animal before irradiation (18). Considering the oxygen dependence of photodynamic effects, it is conceivable that tumor hypoxia might similarly limit the effectiveness of PDT tumor destruction. Hypoxic tumor regions may be present at time of treatment due to exhaustion of blood supply through rapid tumor growth, or they might be created during PDT light treatment through rapid shut-down of tumor circulation. The assessment of the degree of tumor hypoxia, as well as of its relationship to tumor response to PDT may be of immediate relevance to the clinical application of PDT for solid tumor treatment. In this study we have attempted to evaluate these parameters using the RIF mouse solid tumor model. First, we have defined the oxygen requirements for PDT effects on isolated RIF cells. Second, we have studied the development of hypoxia and quantitated hypoxic RIF tumor cell fractions after PDT *in vivo*, applying the above described radiobiological method. Finally, we have related the resulting observations to PDT tumor response.

MATERIALS AND METHODS

Tumor System. The radiation-induced fibrosarcoma tumor, an experimental tumor model carried in C_3H/HeJ mice, was used for this study. It was maintained through *in vivo/in vitro* passages according to established procedures (19). Tumors were established on the right flank of animals by intradermal injection of 2–5 × 10^5 tumor cells harvested from exponentially growing cultures and suspended in Hanks' balanced salt solution. Prior to tumor cell injection, all hair was removed from the inoculation site by first shaving the skin and then applying a chemical depilatory (Nair; Whitehall Laboratories, New York, NY),

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[3] The abbreviations used are: PDT, photodynamic therapy; RIF, radiation induced fibrosarcoma; DHE, dihematoporphyrin ether.

followed by a warm water rinse. This treatment kept the tumor site completely hair free for 10 to 14 days. Depilation of the tumor area shortly before treatment was avoided since it was found to disturb the tumor surface by causing superficial hemorrhage. Tumors were used for experimentation 6 to 8 days after inoculation when they had attained a surface diameter of 5 to 7 mm, thickness of 2 to 3 mm, and weight of 80 to 120 mg. They were free from evident necrosis.

Photosensitizer. Photofrin II (Photomedica Inc., Raritan, NJ) was used in all experiments. This compound is a purified component of hematoporphyrin derivative (20).

***In Vivo* PDT.** Tumor-bearing animals were given i.p. injections of 10 mg/kg photosensitizer. Twenty-four h later they were restrained without anesthesia in specially designed holders, and tumors were given external light treatment. For light delivery, a 5-W argon laser (Spectra Physics model 164, Mountain View, CA) was used to power a dye laser (Spectra Physics model 375) containing Kiton red dye (Exciton Chemical Co., Dayton, OH). The output beam was split using a 50/50 beamsplitter (Oriel Optical Corp., Stamford, CT) and coupled to 400-μm quartz fiberoptic cables (Ensign-Bickford Optics Co., Avon, CT). Microlenses (focal length, 6 mm) were fitted to the ends of each fiberoptic to facilitate even light distribution through the treatment field. Wavelength was tuned to 630 nm by a birefringent filter, as measured by a monochromator (Bausch & Lomb Optical Co., Rochester, NY). Power density of delivered light was adjusted to 75 mW/cm^2 for a spot size of 1–2 cm diameter and measured by a radiometer (model 65A; Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Tumors were exposed to light for 10 min (45 J/cm^2) or 30 min (135 J/cm^2). This light treatment caused a maximum temperature rise in the tumor to 39.5°C, and by itself was without significant effect on tumor histology, clonogenicity, and response (5).

Evaluation of Hypoxic Tumor Fractions. Hypoxic fraction determinations were carried out according to Brown (21) by exploiting the difference in radiosensitivity of oxygenated and hypoxic cells. Briefly, mice bearing either untreated or PDT-treated tumors (unanesthetized, but restrained in plastic holders) were given whole body irradiation (1.08 Gy/min; 10–26 Gy) from a Cesium 137 γ source (Gammacell 40, Atomic Energy of Canada Ltd.). Immediately after γ-irradiation tumors were excised, finely minced, and single cell suspensions were prepared using 1 mg/ml neutral protease (Sigma, St. Louis, MO) in Hanks' balanced salt solution. The proliferative survival of these cells was then assessed by colony formation assay as described previously (5), using α-minimum essential medium supplemented with 10% fetal bovine serum and antibiotics (all from GIBCO, Grand Island, NY).

Experimental Protocols for Hypoxic Fraction Assessment. The following experimental conditions were used for the hypoxic fraction assay: (*a*) *in situ* γ-irradiation of tumors in either air-breathing or N_2-asphyxiated mice, immediate excision; (*b*) 30 min *in situ* PDT (135 J/cm^2), 5-min interval, *in situ* γ-irradiation in air-breathing mice, immediate excision; (*c*) 30 min *in situ* PDT (135 J/cm^2), 60-min interval, *in situ* γ-irradiation in air-breathing mice, immediate excision; (*d*) 10 min *in situ* PDT (45 J/cm^2), 5-min interval, *in situ* γ-irradiation in air-breathing mice, immediate excision; (*e*) 10 min *in situ* PDT (45 J/cm^2), 80-min interval, *in situ* γ-irradiation in air-breathing mice, immediate excision.

Assessment of PDT Oxygen Dependence *in Vitro*. RIF tumors cells were isolated from tumors by the above described enzyme procedure 24 h after i.p. administration of 25 mg/kg DHE to the animal. One ml aliquots of the tumor cell suspension in full medium containing about 1.5×10^6 tumor cells were transferred to small glass dishes (25 mm diameter) without lids. These were individually placed in specially designed glass gassing chambers (50 mm diameter, 30 mm high) which were fitted with gas in- and outflow ports. Graded mixtures of N_2 and O_2, both containing 5% CO_2 and saturated with water, were introduced into the chambers at a flow rate of 250 ml/min. Cells were exposed to gas mixtures for 1 h prior to and during light exposure. Throughout the preillumination gassing procedure all suspensions were kept in the dark and agitated to facilitate gas exchange with the medium by placing the chambers on a laboratory mixer (Thermolyne Corp., Dubuque, IA). Outflowing gas was analyzed using an oxygen analyzer (model E2, Beckman Instruments, Inc., Fullerton, CA). After gas equilibration, cells were exposed to graded doses of light (up to 50 J/cm^2, 630 nm, 75 mW/cm^2) from the dye laser described above. After illumination, known numbers of cells were transferred to 100-mm plastic culture dishes and incubated at 37°C for colony formation in an atmosphere of 5% CO_2 in air. Control cells in each experiment were handled identically except that they were shielded from light through the procedure.

Combined PDT and γ-Irradiation Treatment *in Vitro*. RIF cells, grown in culture to semiconfluency, were exposed to 25 μg/ml of DHE in complete growth medium for 24 h. The medium was then replaced with porphyrin-free medium for 4 h to remove loosely bound porphyrin from cells. Thereafter, cultures were divided into two groups: one was treated with graded doses of γ-irradiation alone, the other received a single dose of PDT light treatment (reducing cell survival to approximately 10%) followed immediately by graded doses of γ-irradiation. Light treatment of 1.0 J/cm^2 (4 mW/cm^2, 590–640 nm) was delivered using a GTE-Sylvania fluorescent light source (Salem, MA). Doses of γ-irradiation ranging from 0 to 10 Gy were delivered using the Cesium 137 γ source described before.

Following γ-irradiation cells were detached from plates using 0.25% trypsin, resuspended in fresh medium, replated at appropriate numbers, and incubated for 9 days for colony formation.

Assessment of Tumor Cure. Animals were observed daily following PDT *in vivo* and were considered cured if no tumor regrowth had occurred by 91 days posttreatment. The longest time to regrowth ever to be observed in this tumor system was 28 days. Control animals had to be sacrificed within 20 days posttumor implantation due to unacceptable tumor burden.

Statistical Analysis of Cell Survival Data. The surviving fraction of cells was calculated as the plating efficiency of treated cells (PDT and/or γ-irradiation) divided by the plating efficiency of appropriate control cells. Survival curves were established by linear least squares regression analysis of the surviving fractions. The values of D_0 (37% dose slope) and the corresponding 95% confidence intervals were calculated from the regression lines. Analysis of covariance was used to evaluate the statistical significance of differences between survival curves used for hypoxic fraction assessment. For biphasic survival curves only those data from the radiation-resistant portion of the curves were used for statistical analysis.

RESULTS

Oxygen Dependence of PDT Effects on RIF Cells

Relative cellular photosensitivity was calculated from the inverse values of the D_0 of survival curves established for each oxygenation level ranging from 0 to 20% O_2. No photoinactivation was achieved in the absence of measurable oxygen, while full effect was reached at approximately 5% O_2. Further increases in oxygen tension did not significantly increase photodynamic cell kill. Photosensitivity was reduced by half of its maximal effectiveness at oxygen levels of about 1% O_2.

Effects of PDT on Radiosensitivity of RIF Cells *in Vitro*. In order to use the radiobiological assay to monitor hypoxia induction by PDT, the lack of direct effects on cellular radiosensitivity of PDT preceding exposure to ionizing irradiation had to be established. *In vitro* experiments were therefore carried out, where a single PDT treatment, reducing cell survival to 10% of controls, was immediately followed by treatment with graded doses of γ-irradiation. The resulting cell survival curves (Fig. 1) showed nearly identical D_0 values (1.22 ± 0.03 and 1.15 ± 0.06 Gy) as well as D_q (quasi threshold dose) values (3.30 ± 0.56 and 3.48 ± 0.19 Gy), indicating that the lethal effects of γ-irradiation were unaffected by preceding PDT treatment.

Changes in Tumor Oxygenation following PDT Treatment *in Vivo*. The presence and size of hypoxic tumor cell fractions in RIF tumors was determined by evaluating the changes in radiosensitivity of tumor cells which were γ-irradiated *in situ* after

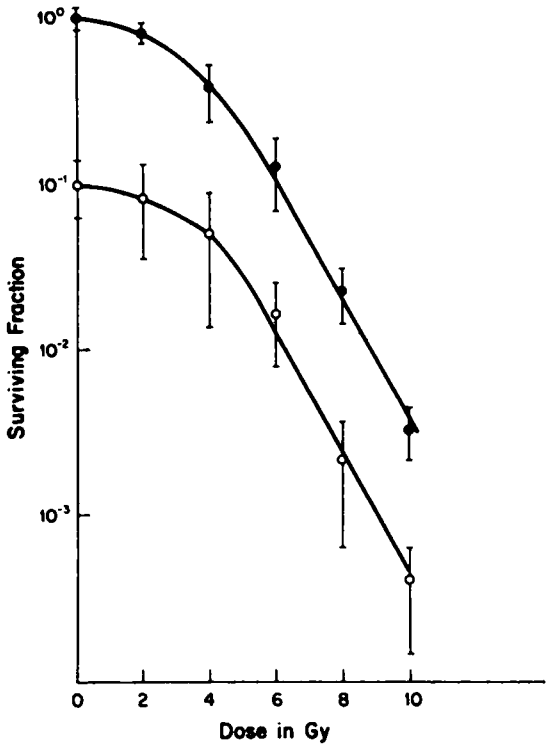

Fig. 1. Survival of RIF cells following exposure to graded doses of γ-irradiation *in vitro* with and without preceding *in vitro* PDT treatment. All cells were exposed to 25 μg/ml DHE for 24 h. γ-Irradiation alone (●); light treatment (1 J/cm²) immediately followed by γ-irradiation (○). *Points*, mean values from three experiments; *error bars*, ±2 SE.

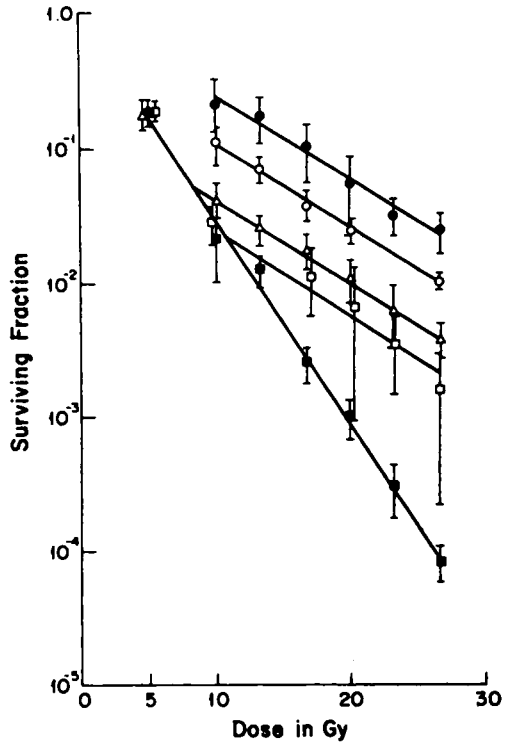

Fig. 2. Cell survival curves of the intradermal RIF tumor following γ-irradiation *in vivo* under different treatment conditions. All animals were injected with 10 mg/kg DHE 24 h prior to irradiation and were unanesthetized throughout the treatment procedures. Except for the hypoxic control group, all animals were breathing air throughout treatments. Aerated control tumors (■); hypoxic control tumors, N_2 asphyxiated before γ-irradiation (●); tumor given 10 min light (45 J/cm²) immediately followed by γ-irradiation (□); tumors given 30 min light (135 J/cm²) immediately followed by γ-irradiation (△); tumors given 30 min light (135 J/cm²) followed 60 min later by γ-irradiation (○). All tumors were excised for clonogenic assay immediately following γ-irradiation. *Points*, mean values of three individually analyzed tumors; *bars*, 2 SE.

varying tumor treatment conditions, then isolated and assayed for cell survival by clonogenic assay *in vitro*.

Cell isolation from control RIF tumors which had been exposed to porphyrin (10 mg/kg) in the animal but were otherwise untreated, resulted in a cell yield of $2.47 \pm 0.41 \times 10^8$ cells/g tumor. The plating efficiency of these cells in the colony formation assay was 49.9 ± 2.2%. Cell yields from tumors isolated after N_2 asphyxiation of the animal and after three of the four PDT treatment conditions (45 J/cm², immediate excision; 45 J/cm², excision 80 min later; 135 J/cm², immediate excision) were not significantly different from untreated controls. The cell yield from tumors exposed to 135 J/cm² and excised 1 h later was reduced by 50% as described previously (5). Plating efficiency under all the above conditions was not significantly different from untreated controls.

γ-Irradiation following any of the above conditions did not affect the yield of recoverable cells. Change in tumor cell survival as a function of dose of γ-irradiation to the tumor was thus solely expressed in changes of the plating efficiency of recovered cells. These changes, as observed under the varying experimental conditions, are summarized in Fig. 2.

To determine the *in vivo* radiosensitivity of aerated and hypoxic RIF tumor cells, tumors in air-breathing and N_2-asphyxiated animals were γ-irradiated. The survival curve generated from aerated tumors was characterized by a D_0 value of 2.84 Gy (±2 SE = 2.67–3.03 Gy), which was uniform over the range of radiation doses given. The survival curve from tumors rendered hypoxic via N_2-asphyxiation showed a D_0 value of 7.14 Gy (±2 SE = 5.97–8.87 Gy). The ratio of D_0 hypoxic tumors/D_0 aerated tumors was 2.51. The difference between these D_0 values was statistically significant ($P < 0.01$). The radiation survival curve for tumors which had received 45 J/cm² (10 min) light treatment immediately before γ-irradiation consisted of two components. The initial portion at radiation doses below 11 Gy showed cell survival to be identical to that of aerated control tumors, while the portion at higher radiation doses showed cell survival higher than for aerated control tumors and a curve slope identical to hypoxic tumors. A similar biphasic pattern was found when tumors were pretreated with 135 J/cm² (30 min) of light and irradiated immediately, except that the break in the survival curve occurred at a lower radiation level (~8 Gy). Tumor pretreatment as above plus a time interval of 60 min before irradiation caused even greater radiation survival, the curve slope again being parallel to that of hypoxic tumors. Since only radiation doses of 10 Gy and above were explored, an initial component for this curve was not documented.

Analysis of covariance showed that the terminal slopes of the survival curves generated from PDT-treated and hypoxic control tumors indeed were not significantly different, *i.e.*, they were parallel.

The biphasic nature of radiation survival curves from PDT-treated tumors in air-breathing animals indicates that PDT induced the formation of two cell populations with different radiosensitivities, one identical to that of aerated, the other like that of hypoxic RIF tumors. From the distance between the parallel terminal slopes for hypoxic controls and PDT-treated tumors, the fraction of hypoxic tumor cells under varying treatment conditions could be calculated (18). It was thus determined that within 10 min of tumor illumination (45 J/cm²), 9.3% (7.1–12.2%) of the tumor cell population had been rendered hypoxic. Tumors tested 80 min after this treatment showed the hypoxic tumor cell fraction had remained at the

same level (curve not shown). Within 30 min of tumor illumination, 17.0% (13.2–21.9%) of tumor cells had become hypoxic. This hypoxia progressed to a 46.5% (35.3–61.1%) hypoxic tumor cell fraction within 1 h post-PDT treatment.

Data concerning PDT treatment conditions and hypoxic fractions are compared to tumor cure data in Table 1. It is apparent that PDT doses which caused extensive, progressive tumor hypoxia were extremely effective in controlling the RIF tumor.

DISCUSSION

The two major questions addressed in this study were (*a*) to what extent do the acute vascular effects induced by PDT *in vivo* affect the oxygen supply to the tumor cells and (*b*) does oxygen limitation, if it occurs, limit the effectiveness of PDT tumor treatment? The rationale for asking these questions lies in the observation that porphyrin-sensitized photodynamic effects are oxygen dependent as described previously by others (11–14). One aspect which needed further clarification due to discrepancies in the literature concerned the oxygen levels which would have to be considered limiting to PDT cell inactivation. According to the study by Lee See, *et al.* (11), full photoinactivation would not be reached under a pO_2 value of 90 mm Hg, more than twice the normal level of tissue oxygenation. Moan and Sommers' (12) data, on the other hand, indicate much lower oxygen requirements with full photosensitization at about 40 mm Hg. It is unclear whether these discrepancies stem from the different methods used to reduce O_2 concentrations or different porphyrin concentrations and endpoints used for assay. The experiments reported here followed more closely the procedure of Moan and Sommer (12), in particular the method of O_2 depletion. One major difference, however, was the route of porphyrin administration. While in the above mentioned studies cells in culture were exposed to various porphyrin concentrations, porphyrin uptake in this study took place *in vivo* with subsequent isolation and light treatment of tumor cells. The results reported here regarding PDT oxygen dependence are nearly identical to those reported by Moan and Sommer (12). Full photodynamic cell inactivation appears to be achieved at levels of normal tissue oxygenation with no increased effectiveness at higher O_2 concentrations. Lowering of O_2 levels below 5% (approximately 40 mm Hg), however, seems to be progressively limiting to cellular photoinactivation with a half-value of about 1% (approximately 7 mm Hg) O_2. Comparison to O_2 requirements for ionizing radiation reveals that the oxygen limitations for the two modalities are similar with a half-value for cellular radiosensitivity of approximately 3 mm Hg (15). We can, therefore, conclude that limited sensitivity towards cell inactivation by ionizing radiation due to insufficient oxygen supply would correlate with similarly impeded sensitivity towards photodynamic cell inactivation.

Having established the parameters of oxygen limitation for PDT in the experimental model used, it had to be determined whether hypoxia within these parameters occurred in these tumors. It was decided to evaluate tumor hypoxia by monitoring the radiosensitivity of tumor cells before and after PDT. That PDT and ionizing radiation act by independent mechanisms, *i.e.*, that PDT preceding γ-irradiation does not directly influence the radiosensitivity of PDT surviving cells, has been shown previously by Bellnier and Dougherty (22). This could be confirmed here for RIF cells. Both the radiation survival curve slopes and shoulders (Fig. 1) were nearly identical, whether or not cells had been exposed to a dose of PDT prior to graded doses of γ-irradiation. In order to assess PDT-induced tumor hypoxia, the radiosensitivity of aerated as well as hypoxic control tumors had to be assessed. As shown in Fig. 2, aerated control tumors of 80–120 mg weight did not show any evidence for the presence of hypoxic cells, but rather exhibited a uniform exponential slope which was 2.51 times steeper than that of hypoxic tumors. This value is typical for the oxygen enhancement ratio of radiation sensitivity (17, 18). In accordance with Brown (21), survival curves for larger aerated control tumors (300–400 mg) do show a break in the exponential slope (data not shown), which is taken to indicate the presence of hypoxic cells. Administration of PDT (45 and 135 J/cm^2 of light) immediately before γ-irradiation likewise resulted in biphasic survival curve slopes. The slope component at low radiation doses was identical to that of aerated controls, which can be taken as further evidence that prior PDT treatment did not change the radiosensitivity of the oxygenated tumor cell compartment. The high-dose slope component, on the other hand, was parallel to the hypoxic tumor slope, indicating that a portion of tumor cells had been shifted towards the radiosensitivity of hypoxic RIF cells. The extremely large scatter for the 45 J/cm^2 (10 min) light treatment seems to imply that, depending on the individual tumor in our experimental system, this PDT dose represents a near-threshold dose with some tumors not yet affected and others already rendered significantly hypoxic. Data resulting from the 135 J/cm^2 (30 min) light treatment were much more uniform and indicated that during light delivery a large portion of tumor cells (17% = 3.4×10^7 cells) had become insensitive to γ-irradiation due to hypoxia. Keeping the similarity between the oxygen limitation for ionizing radiation and PDT in mind, it seems clear that these cells would also be insensitive to any further PDT treatment.

The introduction of delay times between PDT completion and γ-irradiation showed that hypoxia, induced by extended PDT treatment (135 J/cm^2), was progressive with time while

Table 1 *Relationship of tumor hypoxia and tumor cure following PDT*

DHE Concentration (mg/kg)	630 nm Light power density (mW/cm^2)	Treatment time (min)	Total light dose (J/cm^2)	Time lapse treatment to γ-irradiation (min)	% of Hypoxic tumor cells	% of Tumor cure
10	75	30	135	5[a]	17.0 (13.2–21.9)[b]	
10	75	30	135	60	46.5 (35.3–61.1)	81 (N = 21)
10	75	10	45	5[a]	9.3 (7.1–12.2)	
10	75	10	45	80	7.08 (3.2–14.8)	0 (N = 19)

[a] Time interval necessary to transfer animals from PDT treatment to γ-irradiation.
[b] Values in parentheses, 95% confidence intervals.

hypoxia due to short PDT treatment did not progress.

Assessment of long-term tumor response showed that PDT consisting of 10 mg/kg of porphyrin and 135 J/cm^2 to the tumor was curative in 81% of animals. This value represents a large increase from the previously reported less than 10% cures for these treatment conditions in the RIF tumor system (23). This increase can be directly related to the different preparation of the tumor area prior to treatment. Thorough depilation of the skin within the light field doubled light penetration and thereby dramatically improved cure rates. This large improvement becomes understandable when one considers that the TD_{50} (number of tumor cells required to produce tumors in 50% of animals) for the RIF tumor in 10 cells (Ref. 19; own experience), and thus tumor cure depends on a reduction of tumor cells to 10 or less. The mechanism of this very efficient means of tumor control can now be more thoroughly analyzed. It appears that roughly 10% (approximately 2×10^7) of the tumor cells were severely hypoxic (less than 3 mm Hg) PDT survivors by the time 45 J/cm^2 had been delivered to the tumor surface, and thus would have been insensitive to any further direct photodynamic damage. Yet, delivery of additional 90 J/cm^2 led to over 80% long-term tumor control. It has to be concluded that these severely hypoxic cells were eventually killed by secondary mechanisms, *i.e.*, probably the very damage to the vasculature and its consequences which caused the hypoxia. The observation that PDT conditions which cause progressive tumor hypoxia are highly curative while those which cause only limited, nonprogressive hypoxia are ineffective, hints at oxygen and/or nutritional deprivation as being the overriding mechanism of cell kill in this tumor model. This assumption requires caution, however, since the light doses used to produce these conditions also are very different. Studies are in progress in this laboratory to determine the kinetics of tumor inactivation by PDT if oxygen deprivation is delayed or prevented. The results obtained in this study may also offer further explanation for the reported potentiation of PDT effects by misonidazole (24), a radiosensitizing compound which was found to be ineffective in sensitizing hypoxic cells to PDT damage *in vitro* (13) but which is also known for its direct cytotoxicity to hypoxic cells (25). A progressive shift of tumor cells into the hypoxic compartment, as reported here, would make them increasingly accessible to the toxicity of misonidazole, thus increasing tumor cell kill by misonidazole.

In summary, we have determined the oxygen dependence of photodynamic effects on RIF tumor cells to be similar to that of the effects of ionizing radiation. It was further shown that PDT at light fluences commonly used for tumor treatment produces a significant and progressive shift of oxygenated to severely hypoxic tumor cells within short time periods, probably rendering these cells progressively insensitive to further direct photodynamic effects. This induction of hypoxia is not limiting to tumor cure of this experimental model. While these observations shed some light on the mechanism of destruction of the RIF tumor, and probably s.c. animal tumors in general, their importance for the human situation is unproven. Although many human tumors respond to PDT by exhibiting gross vascular damage effects, no data are presently available describing the precise nature or extent of this damage. There is some indication from data concerning development of vascular stasis following hyperthermic treatment, that damage to the tumor vasculature, observed in rodents, may not be seen in human tumors (26). Continued work is necessary to put the here described observations into the proper perspective.

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REFERENCES

1. Dougherty, T. J. Photosensitization of malignant tumors. Sem. Surg. Oncol., *2:* 24–37, 1986.
2. Weishaupt, K. R., Gomer, C. J., and Dougherty, T. J. Identification of singlet oxygen as the cytotoxic agent in photoactivation of a murine tumor. Cancer Res., *36:* 2326–2329, 1976.
3. Moan, J., Petterson, E. O., and Christensen, T. The mechanism of photodynamic inactivation of human cells *in vitro* in the presence of haematoporphyrin. Brit. J. Cancer, *39:* 398–407, 1979.
4. Henderson, B. W., and Miller, A. C. Effects of scavengers of reactive oxygen and radical species on cell survival following photodynamic treatment *in vitro*: comparison to ionizing radiation. Radiat. Res., *108:* 196–205, 1986.
5. Henderson, B. W., Waldow, S. M., Mang, T. S., and Dougherty, T. S. Tumor destruction and kinetics of tumor cell death in two experimental mouse tumors following photodynamic therapy. Cancer Res., *45:* 572–576, 1985.
6. Star, W. M., Marijnissen, P. A., van den Berg-Blok, A. E., Versteeg, J. A. C., Franken, K. A. P., and Reinhold, H. S. Destruction of rat mammary tumor and normal tissue microcirculation by hematoporphyrin derivative photoradiation observed *in vivo* in Sandwich observation chambers. Cancer Res., *46:* 2532–2540, 1986.
7. Selman, S. H., Kreimer-Birnbaum, M., Klaunig, J. E., Goldblatt, P. J., Keck, R. W., and Britton, S. L. Blood flow in transplantable bladder tumors treated with hematoporphyrin derivative and light. Cancer Res., *44:* 1924–1927, 1984.
8. Bugelski, P. J., Porter, C. W., and Dougherty, T. J. Audoradiographic distribution of hematoporphyrin in normal and tumor tissue of the mouse. Cancer Res., *41:* 4606–4612, 1981.
9. Bicher, H. I., Hetzel, F. W., Vaupel, P., and Sandhu, T. S. Microcirculation modifications by localized microwave hyperthermia and hematoporphyrin phototherapy. Bibl. Anat., *20:* 628–632, 1981.
10. Freitas, I. Role of hypoxia in photodynamic therapy of tumors. Tumori, *71:* 251–259, 1985.
11. Lee See, K., Forbes, I. J., and Betts, W. H. Oxygen dependency of photocytotoxicity with haematoporphyrin derivative. Photochem. Photobiol., *39:* 631–634, 1984.
12. Moan, J., and Sommer, S. Oxygen dependence of the photosensitizing effect of hematoporphyrin derivative in NHIK 3025 cells. Cancer Res., *45:* 1608–1610, 1985.
13. Mitchell, J. B., McPherson, S., DeGraff, W., Gamson, J., Zabell, A., and Russo, A. Oxygen dependence of hematoporphyrin derivative-induced photoinactivation of Chinese hamster cells. Cancer Res., *45:* 2008–2011, 1985.
14. Gomer, C. J., and Razum, N. J. Acute skin response in albino mice following porphyrin photosensitization under oxic and anoxic conditions. Photochem. Photobiol., *40:* 435–439, 1984.
15. Churchill-Davidson, J. The oxygen effect in radiotherapy. Historical review. *In:* J. M. Vaeth (ed.), Frontiers of Radiation Therapy and Oncology, pp. 1–15. Basel: S. Karger, 1968.
16. Denekamp, J. Does physiological hypoxia matter in cancer therapy? *In:* Steel, Adams, and Peckham (eds.), The Biological Basis of Radiotherapy, pp. 139–155. New York: Elsevier Science, 1983.
17. Powers, W. E., and Tolmach, L. J. A multicomponent X-ray survival curve for mouse lymphosarcoma cells irradiated *in vivo*. Nature (Lond.) *197:* 710–711, 1963.
18. Van Putten, L. M., and Kallman, R. F. Oxygenation status of a transplantable tumor during fractionated radiation therapy. J. Nat. Cancer Inst., *40:* 441–451, 1968.
19. Twentyman, P. R., Brown, J. M., Gray, J. W., Franko, A. J., Scoles, M. A., and Kallman, R. F. A new mouse tumor model system (RIF-1) for comparison of end-point studies. J. Nat. Cancer Inst., *64:* 595–604, 1980.
20. Dougherty, T. J., Potter, W. R., and Weishaupt, K. R. The structure of the active component of hematoporphyrin derivative. Prog. Clin. Biol. Res., *170:* 301–314, 1984.
21. Brown, J. M., Twentyman, P. R., and Zamvil, S. S. Response of the RIF-1 tumor *in vitro* and in C3H/Km mice to X-radiation (cell survival, regrowth delay, and tumor control), chemotherapeutic agents, and activated macrophages. J. Nat. Cancer Inst., *61:* 605–611, 1980.
22. Bellnier, D. A., and Dougherty, T. J. Haematoporphyrin derivative photosensitization and γ-radiation damage interaction in Chinese hamster ovary fibroblasts. Int. J. Radiat. Biol., *50:* 659–664, 1986.
23. Henderson, B. W., Waldow, S. M., Potter, W. R., and Dougherty, T. J. Interaction of photodynamic therapy and hyperthermia: tumor response and cell survival studies after treatment in mice *in vivo*. Cancer Res., *45:* 6071–6077, 1985.
24. Gonzales, S., Arnfield, M. R., Meeker, B. E., Tulip, J., Lakey, W. H., Chapman, J. D., and McPhee, M. S. Treatment of Dunning R3327-AT rat prostate tumors with photodynamic therapy in combination with misonidazole. Cancer Res., *46:* 2858–2862, 1986.
25. Hall, E. J., Astor, M., Geard, C., and Biaglow, J. Cytotoxicity of Ro-07-0582; enhancement by hyperthermia and protection by cysteamine. Br. J. Cancer, *35:* 809–815, 1977.
26. Reinhold, H. S., and Endrich, B. Tumour microcirculation as a target for hyperthermia. Int. J. Hyperthermia, *2:* 111–137, 1986.