Interaction of Photodynamic Therapy and Hyperthermia: Tumor Response and Cell Survival Studies after Treatment of Mice in Vivo

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ABSTRACT

The interaction of photodynamic therapy (PDT) and hyperthermia was studied in the radiation-induced-fibrosarcoma experimental mouse tumor system by tumor regrowth experiments as well as in vivo to in vitro cloning assays. In vivo, PDT (Photofrin II, 10 mg/kg i.p.), followed 24 h later by light (135 J/cm², 630 nm) and/or heat (44°C, 30 min) caused severe vascular damage (congestion of tumor vessels and hemorrhage) and subsequent disappearance of palpable tumor mass. While heat-treated tumors always started to regrow within 2 days of treatment, regrowth if it occurred was delayed to 4–5 days after PDT and 6–7 days following combined treatments. Only PDT followed by heat cured a considerable number of animals (45%), while PDT alone and heat followed by PDT cured less than 10% of animals, and heat alone caused no tumor cures. The various treatments differed in their immediate as well as their delayed effects on tumor clonogenicity when observed over a 24-h period. Tumors treated with PDT showed no immediate changes in clonogenicity, but progressive delayed cell death occurred if tumors remained in situ. Heat alone led to an immediate reduction in the number of clonogenic tumor cells, followed by some additional cell death 6–7 days following combined treatments. Only PDT followed by heat caused markedly potentiated immediate reduction in clonogenicity in cell survival which may be the result of direct interaction of heat and PDT damage affecting the tumor cells. Some tumors rapidly progressed to total eradication, whereas others showed delayed survival values similar to those for tumor having received PDT only. In the reverse sequence, heat before PDT, the tumor cell survival kinetics resembled those following heat treatment alone. The comparative lack of effectiveness of this treatment regimen can be explained by the severe tumor hemorrhage caused by the initial heat treatment which reduces the transmission of light essential for the subsequent PDT treatment.

This study shows that despite pronounced similarities in the microscopic and macroscopic appearance shortly after treatment by PDT or hyperthermia, these two modalities lead to tumor destruction by different mechanisms. Furthermore the combination of these two modalities in the proper sequence leads to potentiated cytoidal effects on the tumor cells in vivo.

INTRODUCTION

The potential of hyperthermia to enhance other cancer treatment modalities, such as radiotherapy and chemotherapy, has been extensively investigated (1). We have recently reported another beneficial effect of hyperthermia, namely the potentiation of the effect of PDT (2). PDT combines the use of systemically administered photosensitizing porphyrins, which are preferentially accumulated in tumor tissue, and local application of light (3). This treatment frequently leads to selective, rapid tumor necrosis, and in many cases, in humans as well as in animal models, to complete tumor eradication. To increase the probability of complete tumor response, PDT has been studied in animals in combination with localized microwave hyperthermia, and a significant enhancement of tumor response has been observed which seems to be dose and sequence dependent (2). Various possibilities exist which would explain such an interaction. It could be occurring on the tumor cells directly since such interaction has been observed in vitro (4). Alternatively it could take place at the tumor vasculature; both modalities have been found to cause severe vascular damage under appropriate conditions (5, 6). Finally both types of interaction could occur simultaneously and/or influence each other.

The present study was undertaken to gain a better understanding of the mechanisms of the interaction of these two modalities as well as possibly shed some further light on the mechanisms of action of the individual modalities themselves.

MATERIALS AND METHODS

The RIF tumor system, carried in C3H/HeJ mice and derived from a radiation-induced fibrosarcoma (7), was used for all experiments. Its methods of propagation and handling have been described previously (8). This tumor, in our hands, has a 50% tumor take of 10 to 20 cells and a tumor volume-doubling time of 2.5 days. Tumors implanted on the right flank had a diameter of 6 to 8 mm and a thickness of 2 to 4 mm when used for treatment and/or explantation. The average tumor wet weight was 0.09 ± 0.061 (SE) g.

Photosensitizer. Photofrin II (Photofrin Medical, Inc., Cheektowaga, NY) was used in all experiments and contains above 90% of dihematoporphyrin ether (9).

In vivo PDT. Tumor-bearing mice were given i.p. injections of photosensitizer (10 mg/kg). After 24 h, they were restrained without anesthesia in specially designed holders, and tumors were given external light treatment of 135 J/cm² (75 mW/cm², 630 nm, 30 min) using an argon dye laser system (Spectra Physics Corporation, Mountain View, CA) (8). Light was delivered through 400-µm quartz fibers (Quartz Silice, Paris, France), the tips of which were fitted with microlenses (focal length, 6 mm) for improved homogeneity of light distribution throughout the treatment field. A control group of animals was exposed to the same light treatment without prior administration of porphyrin.

Hyperthermia Treatment. Microwave radiation (2450 MHz) was delivered as described previously (2). Tumor temperature was maintained at 44 ± 0.2°C for 30 min. For control purposes, mice receiving heat treatment alone were also given injections of photosensitizer (10 mg/kg) 24 h prior to treatment. A control group of animals was treated at 44°C for 30 min and thereafter maintained at 39°C for an additional 30 min.

The abbreviations used are: PDT, photodynamic therapy; RIF, radiation-induced fibrosarcoma; PE, plating efficiency.
INTERACTION OF PHOTODYNAMIC THERAPY AND HYPERTHERMIA IN VIVO

Experimental Design. Four different treatment modes were evaluated: PDT only; hyperthermia only; PDT immediately followed by hyperthermia; hyperthermia immediately followed by PDT.

Temperature Measurement. Tumor temperatures were monitored in a select group of animals during PDT light treatment and in all animals during hyperthermic treatment as described before (8). Insertion of the microthermocouple caused no apparent effects other than minimal hemorrhage at the base of the tumors. For this reason, insertion of thermocouples for most PDT treatment groups was avoided so as not to interfere with light transmission. The temperature distribution throughout the tumor was examined in a group of tumors by insertion of thermocouples at various tumor depth. Temperatures were found to be within 0.2°C of the desired treatment temperature throughout the tumor tissue.

Assessment of Tumor Response. Animals were observed and tumor diameters were measured each day for 14 days following all treatments and once a week thereafter up to 91 days.

Light Transmission Measurements. Light transmission through shaved skin plus 3 mm thickness of tumor was assessed before and after standard treatment of heat as described previously (10).

Tumor Clonogenicity Assay. The techniques for preparation of tumor cell suspensions and colony formation assay have been described previously (8). The following parameters were determined: cell yield (number of recovered cells) per g tumor tissue; PE of recovered cells and clonogenic cells per g tumor tissue. In tumor samples treated with combined treatments 24 h prior to explant, cell yield had frequently fallen below countable levels and therefore PE also could not be evaluated. In these cases, the entire cell suspension obtained after tissue dispersion was distributed onto two to three tissue culture plates. All resulting tumor cell colonies were then counted and the number of clonogenic cells per g tissue was calculated.

Histology. Tumors were removed from animals before and immediately after varying times of PDT, hyperthermia, or combined treatments; fixed in buffered formalin; sectioned 5 µm thick, and stained with hematoxylin-eosin.

Data Analysis. The results of tumor clonogenicity were expressed as the geometric mean of the values from individual tumors. The significance of the differences between the various treatment groups was analyzed for selected experimental points utilizing the Mann-Whitney test.

RESULTS

Tumor Response to PDT, Hyperthermia, and Combined Treatments. Both PDT and heat treatments were standardized so as to achieve optimal tumor response while sparing the surrounding normal tissues. They showed remarkable gross similarities with regard to the initial tumor response. Microscopic examination showed congestion of blood vessels and extensive hemorrhage, the latter especially at the base of the tumors, within 10 min of PDT-light treatment or 44°C heat treatment (Fig. 1). Hemorrhaging intensified as each treatment continued and was also pronounced in all combined treatments. Vascular structures were largely obliterated after complete PDT, hyperthermia treatment, and combined treatments (Fig. 2). Tumor cell damage was evident through nuclear pyknosis after hyperthermia and combined treatments (Fig. 2, b and c). Macrosopic tumor response to the various treatment regimens is shown in Chart 1. All treatments led to disappearance of palpable tumor within 24 h in the majority of animals which was due partly to tumor shrinkage and partly to edema surrounding the tumor. However, while tumor regrowth commenced immediately after heat treatments, regrowth was delayed to day 4 post-PDT treatment and to day 6 post-combined treatments, regardless of sequence. Only the combined treatment, PDT followed by heat, caused considerable long-term tumor control (45%), while heat alone did not lead to tumor control. PDT alone or heat followed by PDT occasionally cured tumors (less than 10%). Although tumor regrowth could occur as late as 28 days after treatment, none was seen after that time period in any of the experimental groups.

Tumor Temperature Changes during PDT Light Treatment. Tumor temperatures of previously untreated tumors monitored during PDT light treatment showed an increase of tumor temperature from a base level, ~36°C, of 3°C±0.3°C (SE) to 38–39°C within the first 5 min and thereafter remained constant. Tumors previously exposed to heat treatment (44°C/30 min) had cooled to 40–41°C by the time light treatment commenced and thereafter continued to cool to 38–39°C within 5 min. Temperatures remained at that level for the duration of the light treatment. The limited tumor temperature elevations caused by the laser light treatments had no effect on subsequent tumor growth. Likewise they had no effect on the response of tumors previously treated by heat (44°C/30 min).

Light Transmission. Light transmission in the C3H/HeJ mouse was low with only 37±6% of incident light transmitted through the skin and 18±4% transmitted through skin plus 3 mm of tumor thickness. However, when tumors had been heated (44°C/30 min) they had turned almost black by the end of treatment time due to heat-induced hemorrhage, and light transmission to the base of a 3-mm tumor was reduced below measurable levels.

Tumor Cell Survival Experiments. Tumors from animals which had received photosensitizer (10 mg/kg) 24 h prior to explant but were otherwise untreated were used for control values. The average number of recovered cells was 2.7±0.7×10⁶/g tissue, which represents 40 to 70% of cells present per g tumor tissue (11). More than 90% of recovered cells were viable as assessed by trypan blue exclusion. PE of recovered cells was 41±12%. The number of clonogenic cells per g of tumor was thus 1.06±0.3×10⁶. Taking tumor weight into account, the mean number of clonogenic cells per tumor was 8.8±5×10⁸.
The above values were identical to those of tumors taken from totally untreated animals. Laser light treatment (75 mW/cm², 135 J/cm²) to tumors not exposed to photosensitizer did not significantly alter these values. Above 90% of recovered cells were morphologically intact, as determined by trypan blue exclusion, in all subsequent experiments regardless of mode of tumor treatment, indicating that the most severely damaged cells were lost during the tumor dispersion procedure, and this loss is reflected in the values for cell yield. The changes in the above described parameters following the various tumor treatments applied in this study are shown in Chart 2.

PDT alone (Chart 2A), as in our previous studies (8), caused no significant differences in cell yield or PE compared to controls when tumors were excised immediately after treatment. If tumor excision was delayed after treatment, a steady, gradual decrease in PE and a more abrupt decrease in cell yield were observed, which by 24 h posttreatment resulted in a decrease in tumor clonogenicity to 0.1% of controls.

Cell survival kinetics after heat treatment followed a completely different pattern (Chart 2B). The number of recovered cells from tumors excised immediately after heating was reduced to 50% of controls. When tumors were left in situ after treatment, this cell yield decreased sharply during the 4 h following treatment, after which time it began to gradually increase. The mean PE of cells obtained immediately after heating was only 15% of controls, remained low for 4 h after treatment, and thereafter sharply rebounded to 50% of controls. As a consequence of these changes, in particular the low PE, the number of clonogenic cells per g tumor immediately after heating on the average was reduced to below 10% of controls. Tumor clonogenicity further decreased to below 1% of controls by 4 h but thereafter recovered in parallel with the recovery of PE. The variability of response to hyperthermia of individual tumors was extremely large, particularly in tumors removed immediately or shortly after heating with some tumors seeming particularly heat resistant. This variability could be attributed mainly to large differences in PE, which ranged from 20 to 0.5% in tumors removed immediately after heat treatment and from 44 to 2% in tumors removed 2 h later with the majority of values, however, lying close to or below 10%.

The combination of PDT followed immediately by heat (Chart 2C) led to a significant and uniform further reduction of all cell survival parameters in tumors which were removed promptly after treatment. Cell yield in these tumors, removed immediately after heat (30 min after PDT light), was reduced to 10% of controls and decreased steadily with time allowed in situ. PE was reduced to 1% of controls, ranging from 0.1 to 2%, but was higher in tumors left in situ for up to 4 h after treatment. By 12 h after combined treatments tumor responses could be clearly divided into two groups, those where no clonable tumor cells could be recovered at all and those where tumor clonogenicity could still be observed. By 24 h after combined treatments, tumor weight as well as cell yield in the latter group had further decreased so that in three of four tumors values for cell yield and PE could no longer be assessed. However, some proliferating tumor cells could still be recovered from those samples. The range of values obtained for the number of clonogenic cells/g from these tumors at 12 and 24 h post-combined treatment is not significantly different from that of tumors having received PDT only.

The reversal of treatment sequence, delivering heat before PDT (Chart 2D), resulted in tumor cell survival kinetics which resembled that of heat alone, although there was a tendency towards lower values. Statistical analysis of the data from the immediate explants proved that the differences between PDT, heat, and PDT plus heat were significant at P < 0.005. No significant difference was found between heat and heat plus PDT. Data from explants taken 24 h posttreatment showed statistically significant differences between PDT and heat, and heat and PDT plus heat, as well as PDT plus heat and heat plus PDT, all with P < 0.005.

Chart 3 shows the changes in the number of clonogenic cells...
The delayed progressive decrease in tumor cell survival following PDT is dependent on the tumors remaining in situ, thus implying the tumor environment as a major factor in the mechanism of PDT. We have shown previously (8) that tumor cell death after PDT follows a pattern very similar to that of tumor cells deprived of oxygen, and the vascular damage inflicted by PDT seemed to be a reasonable explanation for this observation. However, despite the histological evidence of severe vascular damage, the precise extent of functional vascular impairment in these tumors is unknown. Also the fact that all tumors recover after heat treatment despite vascular damage, which is at least as severe as that caused by PDT, implies that vascular damage alone does not cure RIF tumors.

The pattern of tumor cell survival at delayed explantation following heat shows progressive cell death for the first 4 h but recovery of tumor clonogenicity thereafter. These data are identical to those found by Kang et al. (6) for the SCK tumor following heat treatment at 43.5°C for 30 min, which implies that this might be the pattern of heat response of nonimmunogenic experimental mouse tumors, since both the RIF and the SCK tumors are nonimmunogenic. The strongly immunogenic EMT6 tumor shows a very different pattern of delayed heat response (12). Two possible explanations exist for the recovery of tumor clonogenicity after heat. It may be related to the recovery of vascular function which has been shown to occur in the SCK tumor commencing 5 h after heating and which coincides with recovery of clonogenicity (6). Alternatively it could be a reflection of repair of potentially lethal damage induced by the suboptimal tumor environment due to vascular damage following hyperthermia.

The large variability in heat response of individual tumors, i.e., the insensitivity to heat of some tumors, observed in our study is similar to that seen in EMT6 tumors by Marmor et al. (12) and may be reflected also in our experiments using combined treatments.

The combined treatments, PDT immediately followed by heat, led to markedly augmented cell kill in both early and delayed tumor responses. Various explanations offer themselves for the immediate drop of tumor clonogenicity of 2 orders of magnitude over that seen after heat alone and a drop of 3 orders of magnitude over clonogenicity after PDT alone. PDT with the associated disruption in tumor circulation and concomitant changes in tumor physiology (13) might lead to enhanced thermal sensitivity of tumor cells. Several authors have shown that disruption of tumor blood circulation by either clamping of the tumor or killing of the animal prior to heat application does indeed lead to enhanced heat sensitivity of tumor cells (14, 15). The argument against this explanation comes from our own studies, which have shown that the potentiating effect of combined PDT plus heat treatments decreases if time is allowed between PDT and heat (2). If potentiation were due to changes in tumor environment caused by preceding PDT, this effect should increase with time between treatments rather than decrease, unless quick recovery of vascular function occurs after PDT. However, evidence speaks against the latter (13). If the above explanation is ruled out, a direct interaction of the cytocidal effects of PDT and hyperthermia on the cellular level seems likely, much as is seen in in vitro experiments combining the two modalities (4). This, however, implies that PDT by itself does cause some direct, sublethal tumor cell damage even if none...
seems to be expressed in tumor cells recovered immediately after PDT treatment. The delayed response of the combined treatment clearly falls into two categories, those tumors which rapidly progress to total eradication and those where an observable number of clonogenic cells remains which presumably are capable of eventually repopulating the tumor. The 12- and 24-h survival values for these tumors are very similar to those of tumors treated with PDT alone and may represent tumors which are relatively insensitive to heat or capable of quickly recovering from heat damage. However, other differences among individual tumors which might affect the PDT treatment, such as tumor depth or differences in vascular supply, cannot be excluded from consideration.

The results from experiments with the reverse sequence of combined treatments, heat immediately followed by PDT, are not surprising. As shown in our light transmission measurements, hemorrhage caused by the heat treatment reduces light penetration to the base of the tumor to below measurable levels. It must also be assumed that this degree of vascular damage reduces tumor oxygenation. Both light and oxygen are essential to the mechanism of PDT. Furthermore the cellular interaction of the two modalities applied in this sequence can be expected to be less pronounced based on in vitro data (4).

Finally, if the cell survival kinetics obtained from the various treatment regimens are based on total tumor weight and compared, they reflect extremely well the overall gross tumor response and cure data, thus validating the results obtained by in vivo and in vitro determinations and confirming that studies like this can indeed provide considerable insight into therapeutic mechanisms. A comparison of animal studies like this to the human situation, however, remains problematic. Higher PDT doses than used clinically are required for the tumor model used here which, among other factors, is due to its high clonogenic potential (50% tumor take, 10 cells). Clinical studies combining PDT and heat have yet to be undertaken, but indications are that considerable benefit may be expected for the treatment of solid tumors using these combined modalities.

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REFERENCES

Fig. 1. a, untreated RIF tumor with peripheral vasculature (arrows); b, RIF tumor treated with PDT, dihematoporphyrin ether (10 mg/kg), 45 J/cm² (75 mW/cm², 10 min, 630 nm) showing congested, distended blood vessels (arrows) and beginning general hemorrhage; c, RIF tumor treated with hyperthermia (44°C, 10 min) showing congested blood vessels (arrows) and beginning general hemorrhage. Tumors were removed immediately after treatment. H & E, × 240.
Fig. 2. a, RIF tumor treated with PDT, dihematoporphyrin ether (10 mg/kg), 135 J/cm² (75 mW/cm², 30 min, 630 nm); b, RIF tumor treated with hyperthermia (44°C, 30 min); c, RIF tumor treated with PDT as in a followed by hyperthermia as in b. Tumors were removed immediately after treatment. H & E, × 240.
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