Fluence Rate as a Modulator of PDT Mechanisms

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Background and Objectives: Molecular oxygen in the tissue to be treated by photodynamic therapy (PDT) is critical for photodynamic cell killing. The fluence rate of PDT light delivery has been identified as an important modulator of tissue oxygenation and treatment outcome. This article provides supporting evidence for the role of fluence rate in PDT and discusses the underlying mechanisms.

Study Design/Materials and Methods: Intratumoral pO2 was measured polarographically in murine tumors before and during PDT light treatment using the Eppendorf pO2 Histograph. Tumor response as a function of fluence rate and fluence was also assessed in murine tumor models. Changes in vascular permeability as a function of fluence rate were determined in murine tumors by measuring tumor uptake of fluorescent beads (200 nm diameter).

Results: Severe oxygen depletion is shown to occur within seconds of illumination at a fluence rate of 75 mW/cm2 in radiation-induced fibrosarcoma (RIF) tumors photosensitized with AlPcS2. This effect was reversible and consistent with photochemical oxygen depletion, which has been shown by us and others to be fluence rate dependent. It is demonstrated that fluence rate affects the PDT tumor response in the Colon 26 tumor model, high fluence rate diminishing or even totally inhibiting tumor control, low fluence rate promoting tumor control. The influence of fluence rate is not restricted to cytotoxic effects, but can also be seen in sublethal conditions such as vascular permeability.

Conclusions: Fluence rate of PDT light delivery exerts far-reaching control upon treatment outcome through its oxygenation modulating properties and possibly other mechanisms yet to be identified. This has been shown to be true in the preclinical and clinical setting. Further development of in situ dosimetry will be necessary to take full advantage of these discoveries. Lasers Surg. Med. 38:489–493, 2006. © 2006 Wiley-Liss, Inc.

Key words: photodynamic therapy; oxygenation; inflammation

INTRODUCTION

The rate of energy delivery (irradiance) has long been recognized as a principle factor that determines the biological consequences of treatment modalities that require absorption of energy in tissue. For ionizing radiation, especially with X- or γ-rays, lowering the irradiation dose rate usually results in reduced effectiveness, a phenomenon largely explained by the repair of sublethal damage during prolonged irradiation [1]. In contrast and in spite of evidence for sublethal damage repair following photodynamic therapy (PDT) in vitro [2], a low rate of irradiance (incident fluence rate) in PDT generally leads to enhanced anti-tumor activity. Some of the evidence supporting this statement will be discussed in this article.

Except for very early papers by Gomer et al. [3] and Chen et al. [4] (neither one showed fluence rate influencing responses to PDT in normal murine skin or brain), fluence rate in PDT has not been at the center of attention until the past decade. This changed with the publication of studies by Tromberg et al. [5] and Gibson et al. [6] that showed the differing effects of various photoirradiation regimens on the tumor control in a rabbit and rat model, respectively, and a paper by Foster et al. [7] who outlined in theoretical terms a mechanism called photodynamic oxygen consumption. The latter was centered on the hypothesis that under high fluence rate illumination of a photosensitized tissue the consumption of molecular oxygen during the process of singlet oxygen generation can exceed the rate at which oxygen can be resupplied by diffusion from the vasculature. It was further hypothesized that this will lead to PDT self-limiting hypoxic tumor regions. A series of experiments measuring oxygen concentration in multi-cell tumor spheroids undergoing PDT supported this hypothesis [8].

Having been keenly interested in the relationship between tumor oxygenation and PDT outcome, our group

Abbreviations used: PDT, photodynamic therapy; HPPH, 2-[1-hexyloxoyethyl]-2-devinyl pyropheophorbide-a; IL, interleukin; MAbs, monoclonal antibodies; MIP, macrophage inflammatory protein.

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set out to explore the biological consequences of fluence rate in a number of preclinical tumor models and in the clinical situation. In the following the major findings of these studies will be presented and discussed.

**FLUENCE RATE INFLUENCES PHOTOCHEMICAL OXYGEN CONSUMPTION IN PRECLINICAL TUMOR SYSTEMS IN VIVO**

In 1995 Henning et al. [9] developed computer software that allowed simulation of PDT oxygen consumption and singlet oxygen generation profiles in tissues. This program (PDT MODeM), which followed the calculations of Foster [7], was kindly provided to us by its creators and has been extremely useful in guiding our studies. Although it is based on simple photophysical and photochemical parameters, ignoring such complicating factors as photobleaching of the sensitizer [10], tumor-specific vascular physiology, etc., its predictions have been validated in several murine tumor models. Working with the radiation-induced fibrosarcoma (RIF) tumor in mice, which had been well characterized as to its vascular physiology [11], Sitnik et al. [12] defined the effects of fluence rate on photochemical oxygen consumption and its influence on the Photofrin-mediated PDT tumor response. Increasing oxygen consumption with increasing fluence rate from 0 to 150 mW/cm² could be predicted and proven by measurements of actual oxygen concentration with the Eppendorf pO₂ Histograph. Tumor response was inversely related to the levels of oxygen in the tumor during PDT. An extreme example of photochemical oxygen depletion is given in Figure 1 for another photosensitizer, the disulfonated phthalocyanine AlPcS₂ (for details see figure legend). This compound is particularly prone to induce photochemical oxygen consumption due to its high rate of light absorption (extinction coefficient $\epsilon_{672} = 170,000/M\cdot cm$). Panel A shows the simulated oxygen concentrations as a function of distance from a perfused blood vessel for controls (no light) and 75 mW/cm² illumination in a tumor photosensitized with AlPcS₂ (4 μmol/kg), predicting a severe drop in available oxygen levels within a distance of 20–60 μm. Since the inter-capillary distance in this tumor system is ~240 μm, one would expect the bulk of the tumor to become severely hypoxic during PDT treatment. This could be verified through Eppendorf pO₂ Histograph measurements, shown in Figure 1, Panels B and C. While Panel B shows the pooled data for tumors before illumination, indicating a wide range of oxygen levels from 0 to 40 mmHg, Panel C shows the pooled data for tumors after illumination.
Panel C shows that these same tumors were rendered essentially anoxic within the first minute of illumination at 75 mW/cm² (98% of measured values fell to ≤2 mmHg). Oxygen depletion was immediately and totally reversible upon termination of illumination after the first minute (data not shown). No PDT tumor response could be elicited under such conditions—as would be expected since the PDT response depends critically on the availability of molecular oxygen. These observations have since been refined and spatially resolved with the use of markers of tissue hypoxia [13,14].

**FLUENCE RATE INFLUENCES TUMOR AND NORMAL TISSUE RESPONSES IN PRECLINICAL MODELS**

Fluence rate-dependent photochemical oxygen depletion occurred with all photosensitizers and in all preclinical murine tumor models studied by our group, and this effect was accompanied by corresponding changes in PDT effectiveness. The impact of fluence rate on the tumor response is illustrated in Figure 2 for the Colon 26 (murine colon carcinoma) model and the photosensitizer 2-[1-hexyloxymethyl]-2-devinyl pyropheophorbide-a (HPPH, 0.4 μmol/kg). Kaplan–Meier analysis of tumor response for the fluence of 128 J/cm² shows increasing percentages of tumor cures as fluence rate decreases. From a complete tumor response surface, constructed by varying total fluence as well as fluence rate and assessing tumor control (cure at 90 days post treatment), it is apparent that for every stepwise increase in fluence rate, a similar increase in total fluence was required to achieve tumor cure (Fig. 3). The optimal fluence rate for these conditions in this tumor model was 14 mW/cm². Although only assessed for a low fluence of 28 J/cm², it is apparent that the fluence rate of 3.5 mW/cm² was less effective (0% cures) than a fluence rate of 7 mW/cm² (22% cures). This suggests the possibility that there exist lower limits of fluence rate beyond which mechanisms, such as repair of sublethal damage, override the advantages of low fluence rate. We have observed similar lower limits to fluence rate in a rat tumor model (data not shown). It is yet unclear what this implies for metronomic PDT [15,16], which involves the continuous slow delivery of photosensitizer and light, the latter delivered by implanted LED devices in the μW range of power. Tumor apoptosis has been observed with this treatment scheme but data on long-term tumor control are still outstanding. Fluence rate effects depend heavily on the amount of photosensitizer in the tumor as well as on the photophysical properties of the photosensitizer, thus translation from one experimental system to another may not be straightforward.

To determine whether the enhanced tumor response elicited by low fluence rate PDT is due to increased direct photodynamic tumor cell kill, Sitnik and Henderson [17] determined the number of clonogenic RIF tumor cells surviving Photofrin PDT at 30 and 150 mW/cm², respectively. Indeed, the low fluence rate treatment significantly decreased the number of clonogenic cells, while the high fluence rate treatment showed only insignificant reductions.

The greater effectiveness of low fluence rate in damaging tumor cells could also be demonstrated by assessing tumor cell apoptosis following HPPH PDT in Colon 26 tumors, either through TUNEL staining or through measuring caspase 3 activation [14]. TUNEL staining after high fluence rate PDT was characterized by heterogeneous distribution of TUNEL-positive cells, which were usually located close to blood microvessels, while apoptotic cells were distributed more evenly throughout the tumor parenchyma after low fluence rate treatment. This distribution pattern is consistent with the prediction of high fluence rate-mediated photochemical oxygen depletion at a distance from blood vessels, which would limit tumor cell damage to the proximity of blood vessels.

Since the effectiveness of tumor control always has to be seen in the perspective of treatment selectivity, the effect of fluence rate in response to Photofrin (5 mg/kg) PDT was determined in the normal mouse skin and foot [18,19].
Vascular perfusion was maintained under high fluence rate (150 mW/cm²) treatment (100 J/cm²), while the vasculature was shut down at fluence rates of 75 and 30 mW/cm², respectively. In the mouse foot response, equal doses of light produced more damage at low fluence rate than high fluence rate, mirroring the findings with regard to tumor response. However, when light doses were adjusted for equal tumor response, that is, adjusted downward for the higher effectiveness at low fluence rates, the foot response was diminished. These findings emphasize the need for caution in a clinical setting—the enhanced efficiency of PDT at low fluence rates may have to be balanced by lowering the total fluence to avoid excessive normal tissue damage.

Not only major endpoints such as tumor control or vascular occlusion can be controlled by fluence rate modification, but more subtle effects at the vascular and cellular level are also influenced. Such an effect is the induction of vascular permeability by PDT, which is shown in Figure 4 and in greater detail in a paper by Snyder et al. [20]. Measured as the tumor uptake of 200-nm fluorescent beads (for details see figure legend), which enter the tumor parenchyma through leaky vasculature, vessel permeability was minimal at high fluence rate, peaked at low to intermediate fluence rates, but diminished towards very low fluence rates. Again, this suggests a lower limit where further decreases of fluence rate do not add any further enhancement to PDT efficiency or where they actually might be detrimental.

**Fig. 4.** FluoSphere® (carboxylate-modified fluorescent microspheres, Molecular Probes, Eugene, OR; 200 nm diameter) uptake as a function of fluence rate in Colon 26 tumors, measured 24 hours after HPPH (0.4 µmol/kg) PDT (48 J/cm² at 665 nm). FluoSpheres® (100 mg/kg) were injected immediately after light treatment. Tumors were digested overnight in Solvable (Packard BioScience Co., Meriden, CT); fluorescence of the tumor lysate was measured by fluorometer (Fluoromax2, Jobin-Yvon, Inc., Edison, NJ). \( \lambda_{ex} = 580 \text{ nm}, \Delta\lambda_{em} = 593–700 \text{ nm} \); three to five tumors per point.

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**FLUENCE RATE CAN INFLUENCE THE ANTI-TUMOR HOST RESPONSE**

During the course of this intensive exploration of the role of fluence rate in PDT and parallel studies dealing with the role of anti-tumor mechanisms exerted by the host, it became apparent that fluence rate could also affect the character and extent of these mechanisms. Examination of Colon 26 murine tumors after either oxygen-depleting (high fluence rate) or oxygen-conserving (low fluence rate) HPPH PDT revealed that the heterogeneity of tumor oxygenation induced by high fluence rate also led to heterogeneity of the local inflammatory response [14]. Similar to the distribution of TUNEL-positive cells described above, tumor-infiltrating host cells, mainly neutrophils and macrophages, were restricted to the vicinity of blood vessels under high fluence rate PDT conditions, demarcating the extent of tissue injury to which they were called up to respond. Inflammatory host cells were widely distributed throughout the tumor under low fluence rate treatment condition, corresponding to the more far-reaching tumor injury. These differences were also reflected in the local levels of inflammatory cyto- and chemokines, such as interleukin IL-6, macrophage inflammatory protein (MIP)-1, and MIP-2. The possible implications of these effects on the adaptive anti-tumor immune response are discussed elsewhere in this issue.

**ARE FLUENCE RATE CONSIDERATIONS RELEVANT FOR CLINICAL PDT?**

This question has been explored in a study of nodular basal cell carcinomas undergoing Photofrin (1 mg/kg) PDT [21]. Based on measured values for Photofrin concentrations in these tumors, computer simulation of photochemical oxygen depletion predicted that the latter was likely to occur during 150 mW/cm² in tumors at the upper end of photosensitizer levels, but that it would not occur when the levels were low. Given the wide inter-tumoral variability of photosensitizer content, Eppendorf pO2 Histogram assessments of actual tumor oxygen pressure confirmed the predictions, that is, found oxygen depletion in some, but not all lesions during 150 mW/cm² irradiance, the conventionally used clinical fluence rate. Minimal oxygen depletion was observed at 30 mW/cm² irradiance. No assessment of the impact of fluence rate reduction on treatment outcome was possible in this study. Outcome was, however, one of the endpoints in a study by Ericson et al. [22], where ALA PDT was carried out in actinic keratosis patients with a range of fluence rates between 30 and 75 mW/cm². The authors concluded that low fluence rate illumination achieved favorable treatment responses.

While confirming that fluence rate can play a role in clinical PDT, these findings point towards numerous uncertainties that will not easily be dealt with without improved PDT dosimetry.

Photosensitizer concentration in the tumor, a major determinant of photochemical oxygen depletion (the higher the photosensitizer concentration, the higher the rate of singlet oxygen production during high fluence rate PDT)
needs to be known to predict outcome. It is clear from our publication [21] as well as a paper by Busch et al. [23], the latter finding 10-fold differences in Photofrin concentration in patients’ intra-abdominal carcinomatosis and sarcomatosis nodules that in many instances photochemical oxygen depletion would not be expected even with high irradiance. Fluence as well as fluence rate fall off with distance from the light source [24]. Thus, the optimal fluence rate close to the light source may not be optimal at distance and vice versa. To complicate matters further, Mitra and Foster [25] have reported that the status of tumor oxygenation greatly influences light penetration through the tumor in the wavelength range of ~580–800 nm. This is due to the oxygen-dependent light absorption of hemoglobin, fully oxygenated hemoglobin rendering the tumor more transparent and allowing greater tumor volumes to be treated.

Vascular tumor physiology is another unknown factor in the clinical situation. Tumors are known to have irregular and disturbed microvasculature [26], and preexisting hypoxia will exacerbate the oxygen depletion problem.

Finally, there is the factor of time to be considered. Although the enhanced efficiency of PDT at low fluence rates requires lower total fluences, the time it takes to deliver an effective treatment at low fluence rate is not entirely compensated by the lower light dose. In the clinical setting, the choice of fluence rate has been largely dictated by the need to keep patient treatment within acceptably brief limits. Metronomic PDT, where the light source is meant to be portable, may circumvent these treatment time limitations.

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