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Tumour Destruction and Proliferation Kinetics Following Periodic, Low Power Light, Haematoporphyrin Oligomers Mediated Photodynamic Therapy in the Mouse Tongue

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Photodynamic therapy (PDT) is an experimental modality in the treatment of cancer. It involves photochemical reactions that require the interaction of a photosensitising drug, light and oxygen. The development of an efficient protocol based on assuring oxygen availability through modulation of the incident light power density and its mode of delivery was addressed in this study. An estimated energy dose of 180 J/cm^2 of 630 nm light from pulsed Nd:YAG dye laser was delivered 24 h after injection of 10 mg/kg haematoporphyrin oligomers in C3H/HeNCrj mice bearing the transplantable squamous cell carcinoma NR-S1, by either of these light regimens: (1) 5 mJ/cm^2/pulse for 30 min, 1 h dark interval, followed by another 30 min exposure to the same power (low power, periodic light regimen) or (2) 15 mJ/cm^2/pulse for 20 min (high power, continuous light regimen). Results showed a higher mean percentage area of tumour destruction with the low power, periodic light regimen at 54.34% in contrast to 12.44% of the high power, continuous light regimen 2 days after PDT. Furthermore, the mean bromodeoxyuridine labelling indices of the remaining viable-appearing cancer cells were 27.90 and 42.41, respectively, indicating a smaller tumour growth fraction with the former regimen. These results suggest that use of low power, periodically delivered light increases the antitumour efficacy of PDT.
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INTRODUCTION

THE PRINCIPLE utilised in photodynamic therapy (PDT) of cancer dates back to the beginning of this century [1, 2]. It involves the activation of a photosensitising drug retained in the tumour by a specific wavelength of light to produce a photochemical reaction in biological systems. The reaction consists of the excitation of the photosensitiser upon its absorption of light and the subsequent energy transfer from its triplet state to ground state, molecular oxygen ($^3O_2$) generating the product, singlet oxygen ($^1O_2$) [3]. This electrophilic product is highly reactive and causes irreversible oxidation of biomolecules in their electron-rich regions.

Porphyrin-mediated PDT is well investigated in this experimental treatment modality that utilises a wide variety of photosensitisers, partly because the potential of porphyrins as a diagnostic tool for tumour by emission of reddish fluorescence on light exposure was observed as early as 1924 [4]. Also, many years later, Dougherty *et al.* [5] reported the complete eradication of a transplanted mouse mammary tumour with haematoporphyrin derivative (HpD) and light from a xenon arc lamp.

Over 4000 patients have been treated with PDT worldwide [6]. The problems associated with clinical PDT, like those utilising the U.S. Food and Drug Administration-approved drug for clinical trials, Photofrin II, enriched with the "active ingredients" of HpD, dihaematoporphyrin ether, include poor tissue penetration of light, a low extinction coefficient, i.e. poor absorption of light at a certain tissue level, and skin photosensitivity [7]. The first two contribute significantly to less efficacious therapy. Presently, PDT can be indicated only for early stage, superficial cancers but there is also potential in utilising it as an adjunct to other treatment modalities.

Recognition of the need for sufficient quantities of light, drug, and $^3O_2$ to produce cytotoxic levels of $^1O_2$, for a successful PDT has led to numerous works which have employed different approaches but with a common goal of amplifying the therapeutic efficacy of PDT. The oxygen dependence of photosensitising action has been documented in solution [8], in culture [9] and *in vivo* [10]. The present study deals with assuring oxygen availability by modulating

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the light parameter. Related studies in the past include those aimed at improving tumour oxygenation by increasing arterial oxygenation through hyperbaric oxygen [11] and perfluorocarbon [12], or increasing blood flow by the use of vasoactive drugs like the calcium antagonist, verapamil [13]. The modification in light power density and delivery investigated in this work was motivated by the more encouraging results of delayed tumour growth from either low power light or periodic light delivery [14], and in an extended sense, the fractionation of X-ray dose in radiation therapy to exploit the increased radiosensitivity of well oxygenated tumours [15]. Furthermore, the PDT light protocols compared here (high power, continuously delivered light and low power, periodic light) have proven to be well tolerated by normal tongue tissues (unpublished observations).

The therapeutic effect was established on the basis of the percentage area of destruction of the implanted tongue tumour. We were also interested in characterising the biological activity of the remaining viable-looking tumour cells through bromodeoxyuridine (BRDU) immunohistochemistry.

MATERIALS AND METHODS

Animals and tumours

Six- to eight-week-old, 22–25 g, male C3H/HeNCrj mice (Charles River, Osaka, Japan) were used in all the experiments. The institutional guide for the care and use of laboratory animals was strictly adhered to. Transplantable mouse squamous cell carcinoma, NR-S1 [16], was originally obtained from the National Institute of Radiological Sciences (Chiba, Japan) and was maintained by serial passage on the hind flanks of mice. Mouse tongues were inoculated with the tumour cells (1×10^6) which reached the size of at least 3 mm × 3 mm in about 5 days at which time the photosensitiser was administered. All tumours grew and no spontaneous involutions were noted.

Photosensitising drug

Haematoporphyrin oligomers (Hp oligomers) were synthesised and provided by Seikagaku Kogyo (Tokyo, Japan). They were received in isotonic frozen saline solution and thawed before use. The synthesis procedures were outlined, the chemical structure clarified and the advantages emphasised in previous articles [17–19].

PDT

The light source was a laser system consisting of a pulsed Nd:YAG laser and a pulsed dye (DCM) pump laser (Quanta-Ray® DCR-3 and PDL-2, Spectra-Physics, Mountain View, California, U.S.A.) with 630 nm wavelength emission of red light, 10 Hz frequency and 10 nsec pulse width. The wavelength was documented with a spectrometric multichannel analyser (SMA™ Systems, Tokyo Instruments, Tokyo, Japan). Photoirradiation of the tumour of anesthetised (Nembutal®, 40 mg/kg body weight) mice was performed 24 h after the intraperitoneal (i.p.) injection of Hp oligomers (10 mg/kg body weight). Each tumour received a total energy fluence of 180 J/cm^2. The light intensity was measured by a power meter (30A-P, Ophir Optics, Jerusalem, Israel). Experimental groups included mice ($n = 8$ per group) receiving: (1) 15 mJ/cm^2/pulse of light for 20 min (high power, continuous light regimen), the protocol usually utilised in most investigations, or (2) 5 mJ/cm^2/pulse for 30 min, 1 h dark interval, then another 30 min exposure to the same power (low power, periodic light regimen). The control groups included those receiving neither drug nor light, drug only and light only which was either high power, continuously delivered or low power, periodically delivered. As there were no significant morphological and quantitative differences between these controls, their results were pooled. The temperature of the tumour during photoirradiation, as measured by a thermal sensor (TM-54 H, Nippon Crescent, Chiba, Japan), did not rise above 36°C.

Assessment criteria

The therapeutic effect was assessed by the size of photonecrosis or destruction and the proliferative activity of the tumour.

All animals were killed by lethal ether inhalation 48 h after PDT. The tumour was cut at its centre, fixed with formalin and subsequently treated using routine histological techniques.

Tumour destruction was measured through computer-assisted image analysis (Image 1.44 program, Macintosh® IIci, Apple Computer, Japan). The area of destruction was measured and its percentage in relation to the total cross-sectional tumour area was computed.

Proliferative activity was assessed using BRDU immunohistochemistry. *In vivo* BRDU labelling was performed according to the modified method of Sano *et al.* [20]. Briefly, 1 h before killing, 40 mg/kg body weight of the thymidine analogue, 5-bromo-2′-deoxyuridine (Sigma, St. Louis, Missouri, U.S.A.) was injected i.p. Once tissues had been processed, BRDU was detected immunohistochemically by the use of anti-BRDU monoclonal antibody (B-44, Becton-Dickinson No. 7580, Mountain View, California, U.S.A.) and routine avidin–biotin peroxidase complex method (ABC kit, Vectastain Elite, Vector, Burlingame, California, U.S.A.). Diaminobenzidine–H_2O_2 demonstrated the reaction product. Counterstaining was by Mayer's haematoxylin. Sections of mice which were not administered with BRDU, and where the monoclonal antibody to BRDU was omitted and replaced by phosphate buffered saline, were used as controls. BRDU positive cells, which appeared dark brown (Fig. 1), were

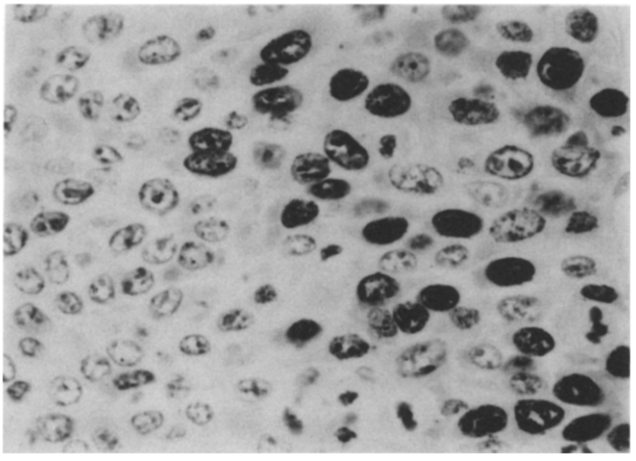

Fig. 1. BRDU-positive neoplastic cells. Labelled cells, very numerous on the right, appear with dark brown, homogeneously stained nuclei. (ABC (avidin–biotin complex) with haematoxylin stain, original magnification × 200.)

scored with the aid of a squared eyepiece graticule (Nikon, Tokyo, Japan) under high power magnification (×400) in about 2000 cells from nine areas of the tumour—three in the upper, three in the middle and three in the lower third, to make up the labelling index (LI). The examiner was blinded as to the identity of the slides and disregarded the obviously necrotic tumour cells and other cells (e.g. fibroblasts, erythrocytes).

Statistical analysis

The significance of the differences in percentage area of tumour destruction as well as the mean BRDU LI between groups were assessed using the Wilcoxon test. Results were considered significant at $P<0.05$.

RESULTS

Forty-eight hours after PDT, regions of necrotic, decomposed tumour cells as well as remaining viable tumour cells were observed in both regimens which differed only in the amount of these regions (see later). Their borders were usually marked with leukocytic infiltration. Necrotic cells were distinguished by the dense haematoxylinophilic masses formed by their chromatin (Fig. 2a). In other necrotic cells, a very granular appearance was observed. Cytoplasms were contracted and heavily stained. The cellular and nuclear membranes appeared disrupted. Lysis was apparent in the cytoplasm and nuclei of other necrotic tumour cells along with lysed erythrocytes and other necrotic tumour debris (Fig. 2b). On the other hand, the viable-appearing tumour cells were large, with intact cellular and nuclear membranes and were usually tightly bound in clusters (Fig. 2a, b). Blood vessels were intact; some exhibited blood stasis.

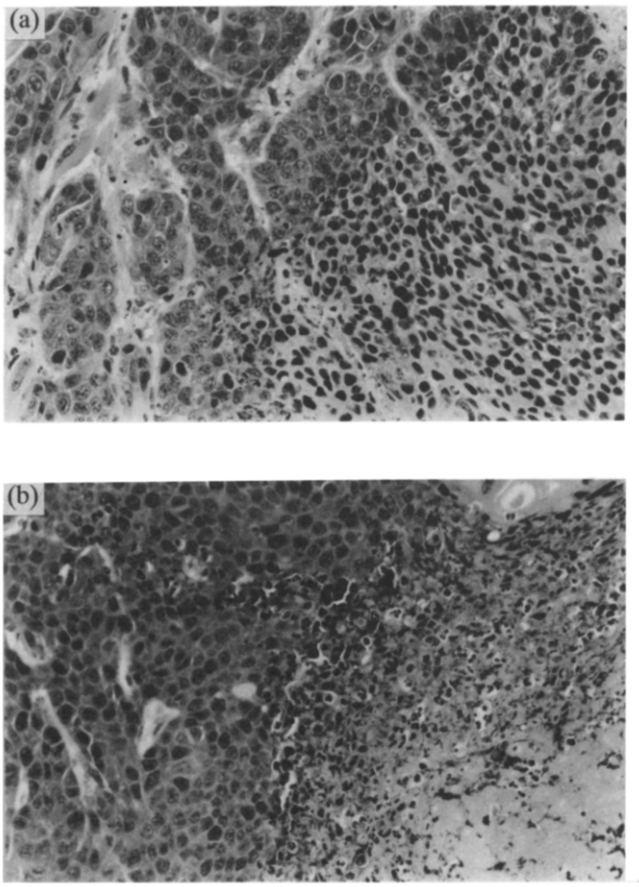

Fig. 2. Histopathological findings 48 h after PDT with low power, periodic light regimen. (a) Shrinking and dense haematoxylinophilic chromatin masses of the nuclei of necrotic cells (right) as well as viable-looking neoplastic cells (left) are shown. (b) An area of decomposing cells and tumour debris (right) can be appreciated. (Haematoxylin and eosin stain, original magnification × 100.)

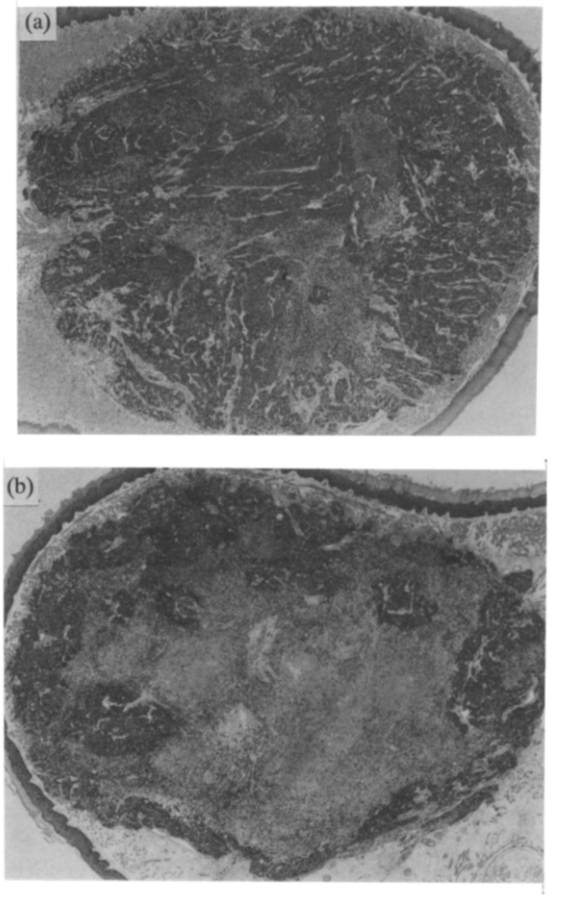

Fig. 3. Area of tumour destruction 48 h after PDT. The usual laboratory protocol of high power, continuous light (a) presents minimal necrosis whereas extensive tumour necrosis results from the low power, periodic light regimen (b). (Haematoxylin and eosin stain, original magnification × 10.)

In the high power, continuous light regimen (Fig. 3a), patchy regions of necrosis were observed. The low power, periodic light regimen (Fig. 3b) differed in its more extensive necrotic, decomposed regions which were most distinct in the centre and in that region immediately in the path of photoirradiation. Undamaged tumour cell regions were usually peripherally located.

Table 1 presents the levels of effectivness of the two PDT

Table 1. Percentage area of tumour destruction (48 h post-PDT with 10 mg/kg Hp oligomers and 180 J/cm² light)*

Pooled control $n=16$	High power (15 mJ/cm²/p) continuous (20 min) light regimen $n=8$	Low power (5 mJ/cm²/p) periodic (1 h interval between 2 30-min exposures) light regimen $n=8$
7.14±6.88	12.44±11.38†	54.34±15.95†

*Values are means ± S.D. †$P<0.01$.

Table 2. Intratumoral BRDU LI (48 h post-PDT with 10 mg/kg Hp oligomers and 180 J/cm² light)*

Pooled control $n=16$	High power (15 mJ/cm²/p) continuous (20 min) light regimen $n=8$	Low power (5 mJ/cm²/p) periodic (1 h interval between 2 30-min exposures) light regimen $n=8$
45.17±5.60	42.41±4.32†	27.90±3.81†

*Values are means±S.D. †$P<0.01$.

regimens as reflected by the percentage area of tumour necrosis (destruction) 48 h post-treatment. A much larger area of destruction was obtained from the low power, periodic light regimen. Its range of tumour necrosis was from 30.41 to 74.44% (range in the high power, continuous light regimen: 2.75–37.97%).

The BRDU LIs are summarised in Table 2. The range in the low power, periodic light regimen was 23.01–32.73. It was higher in the high power, continuous mode regimen (37.15–48.02) which did not vary much from that of the controls.

DISCUSSION

In cancer therapy, hypoxia, a characteristic of a tumour that exerts a significant influence on tumour biology and therapeutic response, should be dealt with efficiently. It may be a chronic form resulting from the inability of the capillaries to support the cellular metabolic oxygen requirements of the entire tumour volume [21] or an acute or transient type which is due to the temporary interruptions of blood flow within the tumour vasculature [22]. Foster and Gao [23] recently described PDT-induced hypoxia as the combined effects of large intercapillary distances in the tumour and oxygen consumption during PDT.

Mathematical calculations have predicted in the recent past that PDT is capable of consuming oxygen at a rate that is sufficiently high to take a part of the treated tumour into a state of very low oxygenation [24]. It was illustrated that such substantial consumption with respect to the metabolic $^3O_2$ consumption rate, would decrease the radial distance from a capillary at which oxygenation is sufficient to support $^1O_2$ production.

Overcoming this state of oxygen deficiency in order to attain enhanced antitumour efficacy might partially be achieved by: (1) using low power light density or (2) fractionating light delivery. High power light density (high fluence rate) appears to have an effect of more pronounced oxygen depletion [25] and thereby may contribute to diminished cell killing. Photobleaching (photodegradation) of the photosensitiser is also more likely to occur [26]. More severe vascular damage that hinders tumour reoxygenation is also a possibility. Arterioles and capillaries must remain viable during a significant fraction of photoirradiation. Fractionation may overcome oxygen depletion by allowing reoxygenation to occur [11]. Also worth citing is the concept in ionising radiation therapy, possibly translatable into the PDT setting, wherein oxygen, no longer utilised by irreversibly damaged, superficial layers of cells, is permited to diffuse to deeper sites during the time interval between exposures [15]. The finding that even a low incident light power of 50 mW/cm² (which is equivalent to 5 mJ/cm²/pulse in the pulsed laser) may still deplete oxygen levels at sites distant from the capillaries [23], was an area of concern when we designed our PDT protocol. Hence, we combined, to complement each other, the two modifications above into a single regimen. It was thought that this might eliminate a deterrent in augmenting the tumoricidal effect. In determining how fractionation should be modulated, we decided upon 1 h as a safe assumption of time interval which will allow reoxygenation. Of course, the factors of metabolic and PDT oxygen consumption rates and average intercapillary spacings can help to more precisely determine the duration of this time interval. Mathematical computations however, were not done in this study.

Our result of a higher percentage of tumour destruction with low power, periodically delivered light, in contrast to its high power, continuous counterpart lends qualitative support to the implications of the mathematical description mentioned earlier. The introduction of another variable in our regimen, i.e. the longer total treatment time, somehow worked to an enhanced effect.

Another matter addressed in this work is the proliferative status of the remaining histologically viable-looking tumour cells. DNA synthesis increases in malignant tumours [27]. Proliferative activities are vigorous. BRDU incorporates into the DNA of the S-phase cycling cell [28]; its LI is correlated with the growth fraction of the total tumour cell population and consequently represents a functional cytokinetic parameter.

BRDU immunohistochemistry, with its extreme sensitivity and ease and rapidity of sample processing, was utilised as a tool to assess the efficacy of the PDT regimens tried in this work. A high LI indicates a high growth fraction or a fast cell cycle progression [29]. While the 27.90 mean LI of the low power, periodic regimen is still relatively high, it definitely is significantly lower ($P<0.01$) than either the mean LI of the high power, continuous or control groups. No significant difference was found between the mean LIs of the high power, continuous light regimen and the pooled controls. The implication of the fate of those cells that form the discrepancy in LI between the experimental groups would be that they most probably have lost their reproductive integrity which is the ultimate goal of cancer therapy. Aside from the larger percentage of necrosis, this provides further evidence of an increased antitumour efficacy of PDT with low power light delivered periodically.

The same dose of Hp oligomers (10 mg/kg) and light (180 J/cm²) evoked different therapeutic responses in the NR-S1 tumour. The parameters responsible for this difference are the light power density and the mode of its delivery. Hence, dosimetry should not be based upon that of the photosensitiser and energy fluence alone but should also always consider the element of oxygen. We have presented a site-specific (tongue) work that utilises an experimental protocol that is not only safe for normal tissues but also enhances the tumoricidal effect through ample time provided for reoxygenation between exposures to low power light.

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