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The photodynamic effect of a pulsed dye laser on human bladder carcinoma cells in vitro

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Summary. The photodynamic effect of a pulsed flashlamp pumped dye laser on cultured human bladder carcinoma cells was studied. MGH-U1 cells were incubated for 1 h in dihaematoporphyrin ether (DHE) and then exposed to green laser light (504 nm, 20 Hz) for varying laser power densities (50–100 mW/cm^2 and exposure times (2–15 s), representing incident pulse energy fluences of 2.5–5 mJ/cm^2 and energy densities of 0.1–1.5 J/cm^2. The cell survival was measured by clonogenic assay and controls exposed to either laser light alone or DHE in the dark showed no cytotoxicity. Sensitised cells were killed by energy densities of less than 1 J/cm^2 ($LD_{90} = 0.54$ J/cm^2). This demonstrates the probable effectiveness of a pulsed dye laser for photodynamic therapy provided that pulse fluence are below the saturation threshold of the photosensitiser (10 mJ/cm^2).

Key words: Photodynamic Therapy – Bladder cancer – Pulsed dye laser

Photodynamic Therapy (PDT) is an experimental technique which has the potential to treat malignant disease in many parts of the body, particularly superficial multifocal tumours. A photosensitising drug is administered which is taken up throughout the body. There appears to be some selective retention in neoplastic tissue over the next 24–72 h and then the tumour area is illuminated, usually by laser, with light of a specific wavelength corresponding to one of the absorption peaks of the photosensitiser. This leads to activation of the photosensitiser with production of a highly reactive form of tissue oxygen called singlet oxygen. Cell death results from oxidation of essential cellular components both in the tumour cells themselves and in vascular endothelial cells leading to disruption of its blood supply. One major application of PDT may be in the treatment of superficial bladder carcinoma, especially resistant carcinoma in situ (Tis), in which encouraging results have been reported by several authors [4, 5, 15, 16, 18]. PDT however can have serious side effects apart from the inevitable cutaneous photosensitivity which may last several weeks. There is usually severe bladder irritability with a significant incidence of a permanent reduction in bladder capacity, and even ureteric reflux leading to upper tract dilation [13]. Sometimes symptoms may be severe enough to require cystectomy [14]. This damage is most likely due to activation of photosensitiser in the muscle layers of the bladder which leads to fibrosis and subsequent contraction with reduction in compliance and functional capacity.

Most research and all published clinical work on bladder PDT have used porphyrin photosensitisers, either haematoporphyrin derivative (HpD) or the purified preparation dihaematoporphyrin ether (DHE). These are activated by red light (630 nm) from an argon ion laser pumping a rhodamine B dye laser.

Red light is generally used for PDT as it penetrates tissue well (up to 1 cm), but this is unnecessary for the treatment of Tis and probably causes the troublesome side effects mentioned above. One possible way of avoiding this might be by using green light which only penetrates 1–2 mm into tissue [22]. HpD also has a much stronger absorption peak for green light (around 507 nm) than for red light, and therefore a greater photodynamic effect would be expected. Recently the metal vapour lasers, particularly copper, have been developed for PDT and although they offer several advantages over the argon laser they also have no other urological application.

The flashlamp pumped dye laser is routinely used for fragmenting ureteric calculi [8]. This laser produces low frequency (less than 30 Hz) pulses of green light (504 nm) of short pulse length (1 μs) and very high peak powers in excess of 5 MW. A further use for this laser would be desirable and enable PDT to be undertaken without the expense of another laser.

Materials and methods

A human continuous bladder carcinoma cell line (MGH-U1) was maintained in monolayer culture in RPMI 1640 medium (Gibco Europe Ltd., Paisley, Scotland), supplemented with 5% heat-

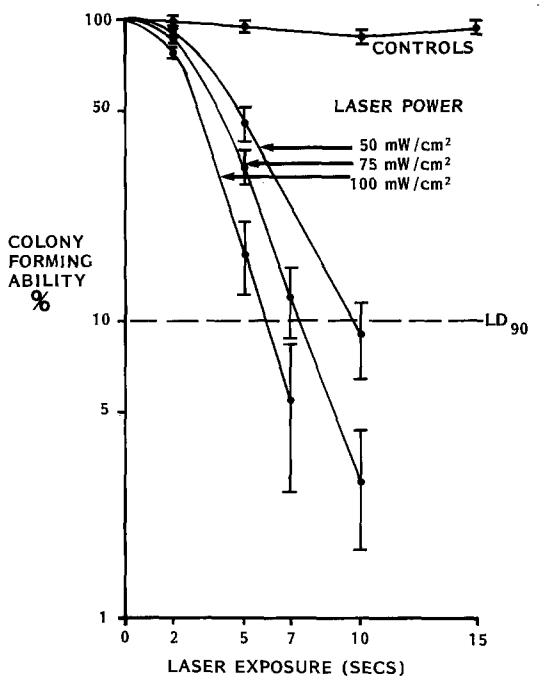

Fig. 1. Survival curves for MGH-U1 cells sensitised with DHE (20 μg/ml) and exposed to pulsed green light (514 nm, 20 Hz) from a flashlamp dye laser

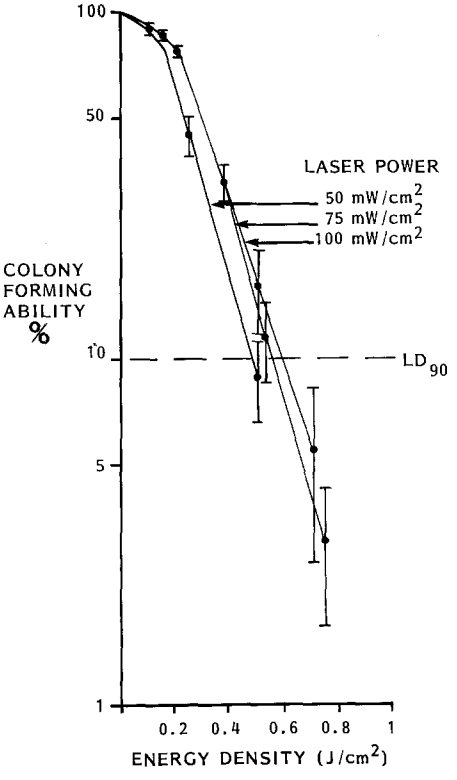

Fig. 2. Cell survival related to energy fluence at varying power densities

inactivated fetal bovine serum (Sera-Lab Ltd., Crawley Down, England) and 2 mM L-glutamine (Flow Laboratories Inc., Irvine, Scotland), at 36.5 °C in a humidified atmosphere of 5% CO_2 in air. The cells were routinely subcultured using an aqueous solution of 0.05% trypsin (1:250; Difco Laboratories, London) and 0.016% versene (EDTA disodium salt; BDH Chemicals, Poole, England). The cells were used over a restricted range of 10 passages to minimise any changes occurring as a result of long term culture. No antibiotics were used.

For photodynamic treatment exponentially growing cells were enzymatically detached, and a single-cell suspension was produced by repeated syringing through a 19 gauge needle. Viable cell numbers were estimated by trypan blue dye exclusion with the use of a haemocytometer. Approximately 150 cells were plated into 3.5 cm petri dishes containing 3 ml prewarmed and gassed medium, yielding about 100 viable colonies per dish. The cells were incubated for 48 h to allow the cells to attach and attain exponential growth.

The medium was then replaced with either fresh medium alone for the controls or with medium containing DHE (Photofrin II, Photomedica Inc., Ravitan, N.Y.) sterilised by passing through a millipore filter. All work was performed under very low level indirect lighting. Dishes were additionally protected from light, except during actual exposure, by covering with aluminium foil. After a 1 h incubation in DHE solution the dishes were washed with fresh medium to remove any unbound DHE and then placed in 1 ml phosphate-buffered saline (PBS) to prevent the cells from drying out during subsequent light exposure.

Light treatment was given as described below, following which the cells were washed again in fresh medium and incubated for a further 7 days. Colonies were fixed in methanol and stained with 10% Giemsa (Giemsa's stain, BDH chemicals) prior to counting. Colonies consisting of a minimum of 50 cells were scored with the use of a binocular dissecting microscope. The mean number of colonies of a least 3 dishes at each point was expressed as a percentage of the controls to calculate cell survival (colony forming ability). All experiments were repeated at least 3 times to plot cell survival curves.

A flashlamp pumped dye laser (Candela MDL-1P, Candela Corp., Natick, M.A., USA) was used, emitting green light (504 nm) transmitted along a 200 micron quartz fibre. This was positioned 12 cm above the culture dish, with the lid removed, thereby illuminating the whole dish evenly. Incident light energy was measured with a power meter at the level of the dish and the laser output adjusted to give an average power density of 50, 75 or 100 mW/cm² at 20 Hz. The pulse length was 1 μs representing a pulse energy density of 2.5–5 mJ/cm² and peak power density of 2.5–5 kW/cm². Measurements were repeated after each set of exposures to confirm that the power output was stable. 5 replicates were exposed at each parameter tested.

Results

Initial experiments were designed to assess whether DHE caused any direct toxicity, and to determine a suitable concentration for use in subsequent laser experiments. MGH-U1 cells were incubated for 1 h in medium containing DHE at concentrations of 0–50 μg/ml. There was no direct toxicity throughout this range of DHE concentrations, though Chan et al. [7] found a toxicity of 20% at 25 μg/ml, and 60% at 100 μg/ml using HpD on fibroblast cultures. In clinical work DHE is generally administered at half the dose of HpD. When these experiments were repeated, and the cells exposed to strong white light for 30 mins, there was marked toxicity. Colony forming ability was reduced to 55% at a DHE concentration of 5 μg/ml, and there were no surviving colonies seen at concentrations above 15 μg/ml. A phototoxic concentration of 20 μg/ml DHE was therefore chosen for subsequent laser

exposures. Other workers have used varying concentrations of photosensitiser in their experiments; eg. Camps et al. [6] used 10 μg/ml HpD incubated for 2 h, Cowled et al. [9] and Gomer et al. [11] used 25 μg/ml HpD for 1 h, whilst Bellnier and Lin [3] used 50 μg/ml DHE for 12 h.

Figure 1 shows cell survival curves for varying laser exposures at power densities of 50, 75 and 100 mW/cm^2. 90% cell kill (LD_{90}) was achieved with exposures of 6 s at 100 mW/cm^2, 7.5 s at 75 mW/cm^2 or 10 s at 50 mW/cm^2. These LD_{90} values, expressed as the total light dose received by the cells, represent energy densities of 0.59, 0.55 and 0.48 J/cm^2 respectively. The energy density curve for each of the 3 laser powers studied is similar (Fig. 2), and the initial shoulder to the survival curves indicates that the cells are better able to withstand very short exposures which do not overwhelm cellular repair mechanisms. The cell kill achieved for very short exposures even at high powers though, is less efficient than that seen with longer exposures.

Discussion

This study demonstrates that the pulsed output at 504 nm from a flashlamp pumped dye laser kills sensitised human bladder carcinoma cells in vitro. Earlier studies showed that CW red light (630 nm) from an argon ion pumped dye laser kills tumour cells sensitised by HpD or DHE both in vitro and in vivo. The continuous wave laser therefore is already widely used for PDT.

Pulsed lasers differ from CW lasers in that they emit light of very short pulses with extremely high peak power. There are two main types of pulsed lasers; metal vapour lasers (usually copper) emitting nanosecond pulses at high frequency (above 10 kHz), and flashlamp pumped dye lasers which emit longer pulses (1–2 μs) at low frequency (5–20 Hz) with peak powers in the kilowatt range. Several workers have shown metal vapour lasers to have an equivalent effect in vitro to CW lasers [9]. Evidence for the efficacy of flashlamp pumped dye lasers in vitro was not available prior to this study and their action in vivo is less certain. Bellnier et al. [2] concluded that the toxicity they observed in transplantable transitional cell carcinoma in mice was the result of thermal damage alone. Although dish temperatures were not measured in our experiments the controls exclude a thermal effect. Barr et al. [1] did not demonstrate a PDT effect on rat colon with a similar laser to ours though they used a slower repetition rate (5 Hz) and a phthalocyanine photosensitiser.

It has been suggested that the very high pulse energy of the flashlamp pumped dye laser excites almost all the sensitiser molecules to the photoactive triplet state before completion of the pulse, with the result that most of the photons in the pulse are "wasted" as the sensitiser has become saturated due to depletion of the absorbing ground state molecules [1]. The cytotoxic singlet oxygen is generated from the interaction of oxygen with the triplet state of the sensitiser which has a lifetime of approximately 8 μs [21]; somewhat longer than the pulse length used here. In theory therefore, only 1 excitation cycle can be initiated each pulse, ie. in these experiments only 20/s. This limits the overall yield of singlet oxygen, below the threshold required for a PDT effect. These restrictions do not apply in our experiments unlike those of Bellnier et al. [2] and Barr et al. [1] since it can be shown that the pulse fluences employed here (2.5–5 mJ/cm^2) are below the saturation threshold (10 mJ/cm^2) for DHE at 504 nm. At this excitation wavelength the absorption cross section of DHE is 4×10^{-17} cm^2/molecule and from the saturation condition (absorption cross section $\times n_s = 1$), the saturation photon fluence (n_s) $= 2.5 \times 10^{16}$ photons/cm^2 or equivalently 10 mJ/cm^2. This figure is an underestimate since the analysis assumes a triplet quantum yield of unity. Therefore the pulse energies used in these experiments could be at least doubled before the photosensitiser became saturated. Barr et al. [1] used pulse energies of 25 mJ/cm^2 and a phthalocyanine photosensitiser (AlSPc) which is saturated at a pulse energy fluence of only 1 mJ/cm^2. Bellnier et al. [2] used very high pulse energies of 100–250 mJ/cm^2 per pulse from a similar laser to ours and a slow pulse repetition rate of 2–4 Hz causing thermal tumour damage and clearly also saturating the photosensitiser (HpD).

Other workers have demonstrated PDT responses on various cell lines with HpD or DHE. Camps et al. [6] looking at the PDT effect of CW red light (630 nm) on prostate cancer cells sensitised with HpD, found an energy density in excess of 20 J/cm^2 at 100 mW/cm^2 reduced cell viability to 10% (LD_{90}). Cowled et al. [9] using a higher power density, needed a similar energy density to achieve 50% cell kill with Raji cells, though a different assay method was used. Gomer et al. [11, 12] studying in vitro PDT with Chinese hamster ovary cells found, using red light, an LD_{90} of 0.25 J/cm^2 but when using relatively low level fluorescent illumination (0.35 mW/cm^2), lower energy densities were needed ($LD_{90} = 0.05$ J/cm^2). Bellnier and Lin [3], using a similar cell line to ours and CW red light found an LD_{90} of 0.15 J/cm^2. Even these 5 studies show a 100 fold difference in energy densities for the same PDT effect, a variation attributable in part to different assay methods and light sources. Our low frequency pulsed green light produces a PDT effect comparable to CW red light ($LD_{90} = 0.54$ J/cm^2.

Camps et al. [6] concluded that it was the length of light exposure needed to achieve cell kill which was important rather than the total light dose (energy density), so that cellular repair mechanisms for sublethal damage would be overcome. In contrast, Gomer et al. [11] found no significant dose-rate variation and felt that the cell killing was dependent on total light dose. The shouldered cell survival curves from our experiments indicate the ability to repair sublethal damage though, like them, we found that cell kill depended on the total light dose rather than the power density. Very short exposures which fall on the shoulder of the survival curve (e.g. 2 s), are unable to produce high cell kill even at very high power densities, whereas longer exposures of similar total energy are more effective (e.g. from Fig. 1 there is a 78% survival at 100 mW/cm^2 for 2 s (0.2 J/cm^2), compared with only a 60% survival at 50 mW/cm^2 for 4 s.

Care is needed when interpreting the results of in vitro PDT studies as they may not relate directly to clinical use.

Oxygen, which is essential for photodynamic action, is present at higher saturation in cell culture than in vivo, possibly enhancing the effect. In vivo studies have shown that a major part of the PDT effect results from damage to tumour blood vessels [10, 17, 20], whereas in vitro damage results from direct toxicity [19]. Nevertheless the mechanism of action is the same.

In conclusion, the flashlamp pumped dye laser emitting pulsed green light has a powerful PDT effect on human bladder carcinoma cells in vitro, sensitised with DHE. Cell survival curves indicate the ability to repair sublethal damage at low power densities, but with higher energy densities greater cell kill can be achieved ($LD_{90} = 0.54\ J/cm^2$). Comparison with other studies using CW red light is difficult owing to differences in cell lines and methodology, but it would seem that this laser is at least as effective for PDT as the CW argon laser. Other workers who have found a flashlamp pumped dye laser ineffective for PDT have used pulse fluences in excess of the saturation threshold of the photosensitiser which we estimate to be $10\ mJ/cm^2$ in our experiments. This is of interest to urologists as PDT may provide a second use for this type of laser. Further studies will be required to confirm these results in vivo but it is likely that the use of pulsed green light and porphyrin sensitisation will enable efficient PDT of carcinoma in situ of the bladder without the troublesome side effects which have been encountered with the use of CW red light.

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References

1. Barr H, Boulos PB, MacRobert AJ, Traulau CJ (1989) Comparison of lasers for photodynamic therapy with a phthalocyanine photosensitiser. Lasers Med Sci 4:7
2. Bellnier DA, Lin C-W, Parrish JA, Mock PC (1984) Hematoporphyrin derivative and pulsed laser photoradiation. In: Progress in clinical and biological research, vol 170. Doiron DR, Gomer CJ (eds) Porphyrin localization and treatment of tumors. Liss, New York, p 533
3. Bellnier DA, Lin C-W (1985) Photosensitisation and split-dose recovery in cultured human urinary bladder carcinoma cell containing non-exchangeable hematoporhyrin derivative. Cancer Res 45:2507
4. Benson RC (1986) Integral photoradiation therapy of multifocal bladder tumors. Eur Urol 12 [Suppl 1]:47
5. Benson RC (1988) Treatment of bladder cancer with hematoporphyrin derivatives and laser light. Urology 31 [Suppl]:13
6. Camps JL, Powers SK, Beckman WC, Brown JT, Weissman RM (1985) Photodynamic therapy of prostate cancer: an in vitro study. J Urol 134:1222
7. Chan WS, Svensen R, Phillips R, Hart IR (1986) Cell uptake, distribution and response to aluminium chlorosulphonated phthalocyanine, a potential anti-tumour photosensitizer. Br J Cancer 53:255
8. Coptcoat MC, Ison KT, Watson G, Wickham JEA (1987) Lasertripsy for ureteral stones; 100 clinical cases. J Endourol 1:119
9. Cowled PA, Grace JR, Forbes IJ (1984) A comparison of the efficacy of pulsed and continuous wave red laser light in induction of phototoxicity by haematoporphyrin derivative. Photochem Photobiol 39:115
10. Fingar VH, Mang TS, Henderson BW (1988) Modification of photodynamic therapy-induced hypoxia by Fluosol-DA (20%) and carbogen breathing in mice. Cancer Res 48:3350
11. Gomer CJ, Rucker N, Razum NJ, Murphree AL (1985) In vitro and in vivo light dose rate effects related to haematoporhyrin derivative photodynamic therapy. Cancer Res 46:3348
12. Gomer CJ, Rucker N, Ferrario A, Murphree AL (1986) Expression of potentially lethal damage in chinese hamster cells exposed to haematoporphyrin derivative photodynamic therapy. Cancer Res 46:3348
13. Harty JI, Amin M, Wieman TJ, Tseng MT, Ackerman D, Broghamer W (1989) Complications of whole bladder dihematoporphyrin ether photodynamic therapy. J Urol 141:1341
14. Nseyo UO, Dougherty TJ, Boyle DG, Potter WR, Wolf R, Huben R, Pontes JE (1985) Whole bladder photodynamic therapy for transitional cell carcinoma of the bladder. Urology 26:274
15. Nseyo UO, Dougherty TJ, Sullivan L (1987) Photodynamic therapy in the management of resistant lower urinary tract carcinoma. Cancer 60:3113
16. Prout GR, Lin C-W, Benson R, Nseyo UO, Daly JJ, Griffin PP, Kinsey J, Tien M, Lao Y, Mian Y, Che X, Ren F, Qiao S (1987) Photodynamic therapy with hematoporphyrin derivative in the treatment of superficial transitional-cell carcinoma of the bladder. N Engl J Med 317:1251
17. Selman SH, Milligan AJ, Kreimer-Birnbaum M, Keck RW, Goldblatt PJ, Britton SL (1985) Hematoporphyrin derivative photochemotherapy of experimental bladder tumors. J Urol 133:330
18. Shumaker BP, Hetzel FW (1987) Clinical laser photodynamic therapy in the treatment of bladder carcinoma. Photochem Photobiol 46:899
19. Shulok JR, Klaunig JE, Selman SH, Schafer PJ, Goldblatt PJ (1986) Cellular effects of hematoporphyrin derivative photodynamic therapy on normal and neoplastic rat bladder cells. Am J Pathol 122:277
20. Star WM, Marijnissen HP, van-den Berg-Blok AE, Versteeg JA, Franken KA, Reinhold SH (1986) Destruction of rat mammary tumor and normal tissue microcirculation by hematoporphyrin derivative photoradiation observed in vivo in sandwich observation chambers. Cancer Res 46:2532
21. Truscott TG, McLean AJ, Phillips AM, Foulds WS (1988) Detection of haematoporphyrin derivative and haematoporphyrin excited states in cell environments. Cancer Lett 41:31
22. Van-Gemert JC, Berenbaum MC, Gijsbers GHM (1985) Wavelength and light dose dependence in tumour phototherapy with haematoporphyrin derivative. Br. J Cancer 52:43

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