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Cell and Tissue Responses of a Murine Tumour to Phthalocyanine-mediated Photodynamic Therapy

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Mice bearing a subcutaneously growing tumour (Colo 26) were injected intravenously with the photosensitiser chloroaluminium sulphonated phthalocyanine (5 mg/kg) 24 h prior to irradiating the tumour with laser light (675 nm; 50mW, 100 J/tumour). Energy status of the tumour, as assessed by the loss of high energy phosphates in the ^{31}P-nuclear magnetic resonance spectra, was altered dramatically following treatment, such that the ATP fell to undetectable levels within 1 h of light irradiation. However, assessment of the clonogenic capacity of neoplastic cells isolated from dissociated tumours showed that these rapid changes in cellular metabolism were not reflected in similar rapid changes in cell viability. Reductions in clonogenic capacity, which fell to less than 0.1% of control values at 24 h postirradiation, closely mirrored those resulting from the cessation of vascular perfusion. Evaluation of tumour blood flow, using the technique of hydrogen washout, showed that the treatment protocol evoked a gradual and selective reduction in flow within the tumour resulting in complete vascular stasis by approximately 5 h after treatment. The results indicate that while chloroaluminium sulphonated phthalocyanine-mediated photodynamic therapy caused early metabolic damage in neoplastic cells, loss of viability paralleled the induction of complete inhibition of vascular flow in the tumour.
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INTRODUCTION

THE SYSTEMIC administration of tumour-localising photosensitisers which subsequently are activated by irradiation with light of the appropriate wavelength constitutes the technique of photodynamic therapy (PDT) [1,2].

We have been studying the characteristics of a new photosensitiser, chloroaluminium sulphonated phthalocyanine (AlSPc), which shows marked potential for use in PDT [3,4]. Although AlSPc plus light irradiation of experimental tumours produced dramatic changes in tumour mass and animal survival [3,4], the exact basis of this antitumour efficacy remains unknown. For the more commonly used photosensitiser, the haematoporphyrin derivative (HpD)/Photofrin II (PII), it has been suggested that PDT exerts much of its activity by inducing vascular damage rather than by exerting a direct effect against neoplastic cells [5]. Thus individual cancer cells isolated from PDT-responsive solid tumours showed limited sensitivity *in vitro* to photoirradiation [6]. Moreover, neoplastic cells isolated from tumours immediately after PDT exhibited little loss of viability, whereas those left *in situ* and only recovered later were considerably less viable, a response consistent with the possibility that PDT operates through an indirect effect [7].

Information regarding the mode of action of the metallophthalocyanine (MPc) photosensitisers is even more limited but there are suggestions that they too induce damage to the microvasculature [8] and produce alterations in tumour blood flow [9]. In the present study we have investigated the kinetics of neoplastic cell survival after AlSPc-based PDT by assaying for colony-forming efficiency (CFE) as a means of monitoring cell viability. Tumour cell energy status was assessed by ^{31}P-nuclear magnetic resonance (NMR) spectroscopy of high energy phosphate levels while tumour blood flow was determined by the technique of hydrogen washout.

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MATERIALS AND METHODS

Photosensitiser

AlSPc, which was obtained from Ciba–Geigy Dye Stuffs and Chemicals (Basel), is a complex mixture containing mono-, di-, tri- and tetra-sulphonated derivatives with, according to the supplier, an average of three sulphonated groups; this composition was confirmed by HPLC analysis [10]. AlSPc was dissolved with phosphate-buffered saline (PBS) (3–5 mg/ml) as stock solution.

Tumour cells and the in vitro *phototoxicity assay*

Colo 26 cells from a murine colorectal carcinoma [11], were grown in E4 medium supplemented with 10% heat inactivated fetal calf serum (FCS) for 1–3 days before being refed with medium containing 10 μmol/l AlSPc. 24 h later, tumour cells were detached by trypsinisation and replated at known cell numbers (10^2–10^6) in 60 mm Petri dishes containing 5 ml medium lacking the photosensitiser. The cells were then exposed to red light [12] for periods of time varying from 0 to 30 min. Following light exposure, cultures were incubated at 37°C for approximately 10 days, when they were fixed in methanol and stained with Giemsa. Colonies (>50 cells) were counted (3–6 Petri dishes/time point) and numbers were related to the numbers of colonies obtained from the control dishes (AlSPc-untreated cells following 30 min red light irradiation) which was expressed as 100% survival. The data presented are the mean of three independent experiments. The red light source used derived from fluorescent tubes filtered with a red gelatin filter

and grease proof paper as previously described [12]. Light intensity measured at cell level was 1.2 (S.D. 0.2) mW/cm^2 (Coherent Power Meter, model 212; Coherent, Cambridge, UK).

^{31}P-NMR spectroscopy of perfused cultured cells

Cultured Colo 26 cells treated with AlSPc as described above were cast in agarose threads using the method originally described by Foxall and Cohen [13]. The cells were suspended in low temperature gelling agarose (Sigma) by mixing 2 ml 1.8% agarose, made up in Hank's balanced salt solution (HBSS) and maintained at 37°C, with 1 ml of a cell suspension containing approximately 10^8 cells. The agarose–cell solution was then extruded through polyvinyl chloride (PVC) tubing with an internal diameter of 0.7 mm into a 20 mm NMR tube containing Krebs–Henseleit buffer at 4°C. Cell perfusion was achieved using an adapted brain slice perfusion rig [14]. Cells were kept in a viable state by the continuous perfusion (5–10 ml/min) of Krebs–Henseleit buffer containing 124 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $CaCl_2$, 26 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4 and 10 mmol/l glucose equilibrated with 95:5 O_2/CO_2 at 37°C. ^{31}P-NMR spectra were recorded using a ^{1}H/^{31}P double tuned probe. A pulse angle of 45° was applied every 0.5 s. Spectra were acquired in blocks of 800 scans, and transformed with 30 Hz line broadening. All NMR studies (including the *in vivo* experiments described below) were carried out on a Bruker AM-360 spectrometer with a 8.5T vertical magnet.

Colo 26 cells contained within the agarose threads were irradiated with red light for up to 30 min as described previously, before being perfused continously and ^{31}P-NMR spectra recorded as detailed for up to 12 h after irradiation. The shortest time between red light irradiation and commencing acquisition of the ^{31}P-NMR spectra was 10–15 min, the time taken to set up the NMR experiment.

Photodynamic therapy of subcutaneously growing Colo 26 tumours

The technique has been described previously [3]. Subcutaneously growing Colo 26 tumours were produced by injection of 10^6 cells/0.1 ml PBS into the right flank of syngeneic mice (female BALB/c mice 12–14 weeks old, obtained from the Imperial Cancer Research Fund Breeding Unit, Clare Hall). When the tumours reached 5–7 mm diameter (approximately 10 days after injection), mice received an intravenous injection of 5 mg/kg (0.1 ml) AlSPc in PBS via the lateral tail vein. PDT was performed 24 h later. The laser used was either an argon ion pumped tunable dye laser (Aurora–Cooper, Lasersonics, UK) or a copper vapour pumped dye laser (Oxford Lasers, UK). The laser dye was either 4-dicyanomethylene-2-methyl-6 (*p*-dimethylaminostyrl)-4-H-pyran in ethylene glycol and propylene carbonate or oxazine 720, respectively. Laser power was adjusted to 50 mW and the light, with emission at 675 nm, was delivered via a quartz fibre with a flat cut end which was inserted into the centre of the tumour (100 J/tumour).

In vivo *NMR measurements*

The tumour-bearing mice were anaesthetised with a mixture of halothane/oxygen delivered via a nose cone. Respiratory rate was monitored throughout the experiment using a technique described previously [15], and core temperature was maintained at 37°C by blowing warm air intermittently through the bore of the magnet. A radio frequency surface coil was adjusted to match the size of each tumour so that the sensitive volume of the coil did not exceed the tumour dimensions. The coil consisted of two concentrically wound coils, the outer one tuned to 360 MHz for adjusting field homogeneity, and the inner one tuned to 145 MHz and used to collect ^{31}P-NMR spectra. A pulse angle of approximately 45° at the centre of the coil, was applied every 0.5 s and spectra were averaged over 1000 scans and transformed with 35 Hz line broadening. Intracellular pH was determined from the chemical shift of the inorganic phosphate (P_i) resonance using a standard titration curve [16], with reference to the ^{1}H NMR signal from the water [17].

Assessment of tumour cell viability after in vivo *PDT by the CFE assay*

At designated times (1–24 h) after PDT, mice were killed humanely by cervical dislocation. Tumours were dissected free from surrounding normal tissues, weighed and minced with scissors under sterile conditions. This procedure could be carried out on the bench under room light since AlSPc is inert in such conditions [12]. Single cell suspensions were prepared as described elsewhere [4]; but briefly, minced tumour was disaggregated in 0.02% collagenase (Sigma Type I) and 0.01% DNase (Sigma Type I) with continuous stirring at 37°C for 1½–2 h. The cells were washed with HBSS, pelleted and then resuspended in E4 growth medium containing 10% FCS. Viable cells were counted using a haemocytometer (viability determined by trypan blue exclusion) and 100–2000 cells were plated on 60 mm petri dishes (3–6 dishes per time point). Colony numbers were assessed approximately 10 days later as detailed above and the total clonogenic cells per gram wet weight of tumour were calculated. Data points presented are the average of 5–7 tumours and represent the survival fraction relative to the control tumours (tumours obtained from mice which had received laser irradiation but no sensitiser injection) which was expressed as 100%. Additional experiments were performed to determine the effect of oxygen deprivation by killing the animals, leaving the tumour *in situ* and performing the cellular dissociations at various time after death.

Tumour blood flow measurement

Blood flow was measured in the tumour using a modification of the hydrogen flow technique used for measuring cerebral blood flow in the gerbil [18]. Three platinum electrodes (125 μm diameter) were inserted into the tumour to a depth of 2–3 mm. These electrodes had a polytetrafluoroethylene coating which was cut back to 1.5–2.0 mm to ensure that the recorded current was only measured from within the tumour and was not contaminated with flow from overlying skin. In the case of the treated animals a fourth electrode was inserted subcutaneously into the skin of the neck of the mice to record the skin flow for comparison. A silver chloride reference electrode was inserted subcutaneously in the lumbar region of the mouse. 5% hydrogen was administered by addition to the inspired anaesthetic gas mixture and, by polarising the electrodes to +400 mV, the current flow between the measuring electrodes and the reference electrode was monitored. Upon tissue saturation, the hydrogen gas supply was turned off and the washout of hydrogen was recorded by the electrode current. The flow data represent the mean flow averaged over the three electrodes in each tumour. Mean tumour blood flow was determined from the decay by assuming single exponential behaviour of the hydrogen washout.

Control experiments were performed to assess any effect on the tumour blood flow of changing from the horizontal to the vertical position, as required for the NMR experiment. A slight

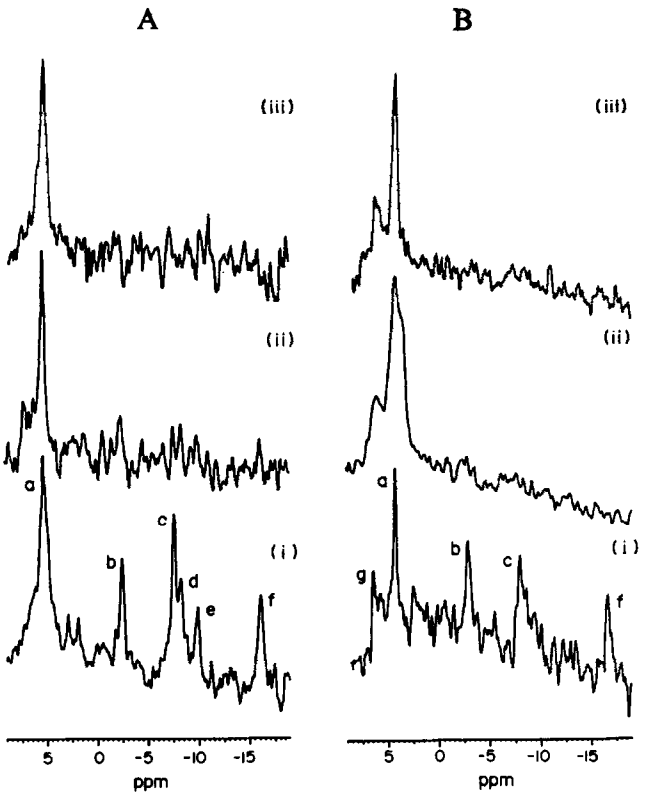

Fig. 1. ^{31}P-NMR spectra of (A) immobilised perfused cells previously treated with AlSPc for 24 h recorded (i) immediately prior to, (ii) 20 min following, and (iii) 12 h following 30 min exposure to red light. (B) *in vivo* Colo 26 tumours 24 h after injection of AlSPc (i) prior to (ii) 1 h following and (iii) 3 h following photoirradiation of the tumour. The peak assignments are (a) P_i; (b) contains contributions from the γ-phosphate of ATP and β-phosphate of ADP; (c) α-phosphate of both ATP and ADP; (d) NAD^+/NADH; (e) nucleoside diphosphosugars, principally UDP-glucose; (f) β-phosphate of ATP; (g) phosphomonoesters. Chemical shift scale is expressed relative to phosphocreatine at 0.0 parts per million (ppm).

transient decrease in tumour blood flow was observed which returned to normal in 2–3 min.

RESULTS

NMR studies on in vitro *perfused tumour cells and subcutaneously growing tumours*

Typical ^{31}P-NMR spectroscopy of intact perfused Colo 26 cells showed a rapid loss of the ATP signal within 20 min following PDT (Fig. 1A). Continued perfusion (up to 12 h) produced no return of the ATP signal.

^{31}P-NMR tumour spectra recorded *in vivo* before and after photoirradiation are illustrated in Fig. 1B. Because of the time taken to set up the NMR equipment, the earliest time point at which it proved possible to obtain measurements was 1 h following treatment. All tumours examined showed a dramatic change in the spectra following PDT. A substantial decrease in ATP was observed as early as 1 h postirradiation. The intracellular pH was estimated to be 6.97 (S.D. 0.17) (n=3) prior to treatment, and 6.73 (0.22) (n=4) following treatment (not significant).

Light irradiation alone was found to have no obvious effect on the tumour ATP levels.

Effect of AlSPc plus red light exposure on in vitro *cell survival*

The kinetics of cell death of Colo 26 cells following exposure to AlSPc and red light irradiation are presented in Fig. 2.

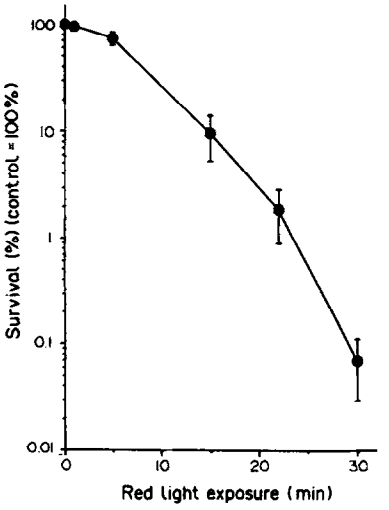

Fig. 2. Survival kinetics of Colo 26 cells *in vitro* after exposure to AlSPc for 24 h prior to irradiation by red light for the indicated time periods (0–30 min). The vertical bars represent S.E.

Survival of Colo 26 cells was affected dramatically by AlSPc at 10 μm concentration such that approximately 90% of cells were killed by a 15 min irradiation. Exposure of the cells to light for a 30 min period resulted in a surviving fraction of less than 0.1%.

Survival of Colo 26 cells after in vivo *PDT*

From Fig. 3a it can be seen that the survival of Colo 26 cells, as determined in tissue culture by CFE, was not reduced substantially 1 h after *in vivo* PDT irradiation. Thereafter, there was a progressive reduction in tumour cell viability such that by 24 h post-treatment the surviving fraction of clonogenic cells had been reduced to approximately 0.1%. The trend of cell killing observed after PDT was comparable with those observed as a consequence of killing the animal and leaving the tumours *in situ* (Fig. 3b). However, leaving the tumours *in situ* for 24 h after PDT treatment did not result in complete loss of cell viability, as observed in the tumour after death. This may reflect residual tumour cell survival [3] and/or collateral blood supply to the periphery of the tumour.

Effects of phthalocyanine mediated PDT on tumour blood flow

Combined data on tumour blood flow derived from all the experiments conducted both before and after photoirradiation,

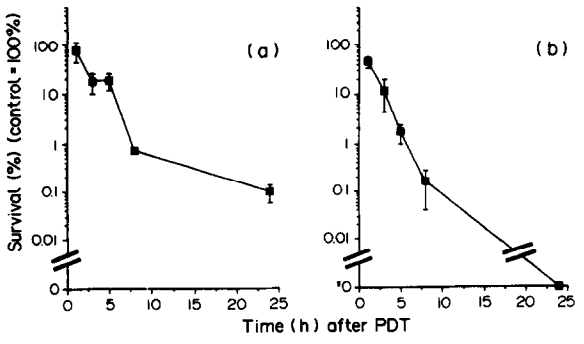

Fig. 3. *In vitro* tumour cell survival kinetics as a function of time remaining *in situ* following (a) PDT *in vivo* and (b) animal death. At the times indicated tumours were dissected out and dissociated. Cells were plated into replicate dishes and colony-forming efficiency assessed 10–12 days later. The vertical bars represent S.E. from a minimum of 5 animals (n=5–7).

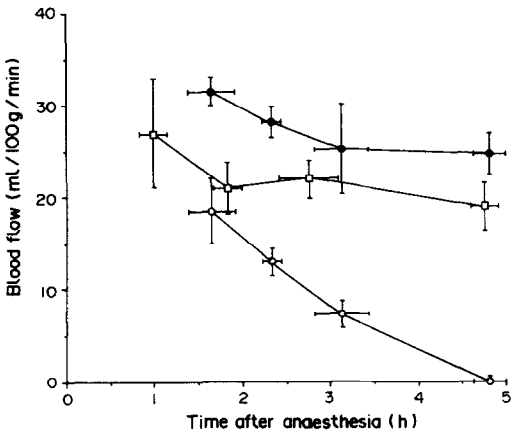

Fig. 4. The effect on tumour blood flow (○) following AlSPc mediated PDT (t=0). The skin flows (●) recorded from the neck of the same mice are included for comparison (n=5). Tumour blood flows (□) were also measured from both untreated mice and mice exposed to light alone (n=7). The data presented are obtained from a composite of individual experiments and the fact that measurements could not always be taken at precise times after PDT (i.e. at onset of anaesthesia) accounts for the horizontal error bars on the time axis.

are summarised in Fig. 4. It is clear that over the 5 h period following PDT there was a gradual, and eventually complete, cessation of tumour blood flow such that values had fallen to 50% of control by 2 h and to no detectable flow rate by 5 h. Measurements taken at 24 and 48 h post-PDT (data not shown) revealed no detectable re-establishment of blood flow. Included in the figure for comparison are the results obtained for skin blood flow taken from the same treated mice and for control animals (no treatment, n=5, and light alone, n=2), which show that the dramatic reduction in flow detected in the treated tumour is not a systemic phenomenon.

DISCUSSION

It has been noted that alterations in the blood flow [9] and obvious damage to the tumour vasculature [8] occur early after MPc mediated PDT. These findings are similar to those observed after HpD/PII mediated PDT [8,19–23], and suggest that tumour responses to PDT are independent of the photosensitiser used. Many of the earlier studies however consisted of static observations taken at selected time points in what is, clearly, a dynamic process. In the work reported here we have monitored temporal changes in the blood flow using a technique which does not require sacrifice of the animals and have attempted to correlate these measurements with the long-term survival capacity of isolated tumour cells as determined by their CFE.

Following AlSPc-PDT, the ^{31}P-NMR spectra of the subcutaneously growing Colo 26 tumours show a rapid loss of the high energy phosphates (Fig. 1B). ATP fell to undetectable levels within 1 h following treatment indicating an early metabolic response to PDT. These findings are similar to those obtained after HpD/PII mediated PDT [23–26] and to those observed in the perfused cells treated *in vitro* (Fig. 1A), suggesting that the loss of ATP is a direct effect.

In earlier investigations we have found that the AlSPc-PDT protocol we have used gives substantial reductions in tumour burden and may extend survival times but frequently there is subsequent regrowth of viable cells from the peripheral zone of the tumour [3]. Such regrowth could account for why, in comparable experiments using HpD/PII mediated PDT, other researchers have found a gradual return of ATP levels to nearly control values 24 h after tumour irradiation [24,26].

Despite the rapid and dramatic changes in metabolism of cells treated both *in vitro* (Fig. 1A) and *in vivo* (Fig. 1B) and the immediate decline in cell viability observed after irradiation of the target cells in tissue culture (Fig. 2), it is not clear that this treatment is actually killing cells *in vivo* via a direct effect. Thus the CFE assay shown in Fig. 3 detected no substantial decline in tumour cell clonogenicity until some considerable time after PDT. One possible interpretation that has previously been placed on the kinetics of tumour cell killing *in vivo* is that it is a reflection of an indirect mode of cell killing [7]. Certainly the curve obtained showing the kinetics of tumour cell death (Fig. 3a) is very similar to those obtained after killing the animal (Fig. 3b) where the mechanism of cell death is a consequence of cessation of vascular perfusion.

Damage of tumour vasculature induced by PDT may result in oxygen and nutrient deprivation which could contribute to the early cessation of ATP synthesis and the development of acidosis within the tumour [24]. However, data from other experiments examining the relationship between tumour blood flow (as measured with the hydrogen washout technique) and ^{31}P-NMR spectroscopy show that the levels of ATP only become undetectable at flows of 5 ml/100 g/min or less [27]. The hydrogen washout data presented here (Fig. 4) show a reduction in tumour blood flow from approximately 27 ml/100 g/min to 18 ml/100 g/min by 1 h after PDT. This decline is slower and less dramatic than that found after HpD/PII-PDT [19,20,23] or aluminium tetra-sulphonated phthalocyanine mediated PDT [9] and although probably not sufficient to explain the loss of ATP, it does correlate with the decline in cell viability. The differences between sensitisers may reflect differences in the mode of action of the various photosensitisers or it may be that the combined dose of light and sensitiser used in the present study did not generate thermal effects (C. J. Tralau, Rayne Institute, London).

The results presented in this paper show that while AlSPc-PDT can evoke rapid responses in the cells both *in vitro* and *in vivo*, this direct effect does not appear to account for the loss of cell viability *in vivo*. The absence of ATP does not indicate immediate cell death, suggesting the action of an additional event. The correlation between the observed decline in tumour blood flow and loss of CFE suggests that it is the damage to the tumour vasculature which may ultimately be responsible for tumour cell death and eventual necrosis.

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