JB. Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. *J Clin Invest* 1983, 72, 1844–1850.
19. Miller RG, Jr. *Simultaneous Statistical Inference*. New York, McGraw-Hill, 1966.
20. Siegel S. *Nonparametric Statistics for the Behavioral Sciences*. New York, McGraw-Hill, 1956.
21. Snedecor GW, Cochran WG. *Statistical Methods*, Ames, Iowa, Iowa State University Press, 1967.
22. Carswell EA, Old LI, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* 1975, 72, 3666–3670.
23. Haranaka K, Satomi N, Sakurai A. Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. *Int J Cancer* 1984, **34**, 263–267.
24. Haranaka K, Satomi N. Cytotoxic activity of tumor necrosis factor (TNF) on the human cancer cells *in vitro*. *Jpn J Exp Med* 1981, **51**, 191–194.
25. Maruyama H, Kawamura H, Takemoto N, Komatsu Y, Aburada M, Hosoya E. Effect of kampo medicines on phagocytes. *Jpn J Inflammat* 1988, **8**, 65–66.
26. Ito H, Shimura K. Antitumor effects of Juzen-taiho-to (TJ-48) and other kampo medicines. In Hosoya E, Yamamura Y, eds. *Recent Advances in the Pharmacology of Kampo (Japanese Herbal) Medicine*. Tokyo, Excerpta Medica, 1988, 281–290.
27. Kawamura H, Takemoto N, Murayama H, Komatsu Y, Aburada M, Hosoya E. Effect of Juzen-taiho-to (TJ-48) on immune responses in mice. In Hosoya E, Yamamura Y, eds. *Recent Advances in the Pharmacology of Kampo (Japanese Herbal) Medicine*. Tokyo, Excerpta Medica, 1988, 291–296.

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Relation of Early Photofrin® Uptake to Photodynamically Induced Phototoxicity and Changes of Cell Volume in Different Cell Lines

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For efficacy of photodynamic therapy, selective uptake and retention of photoactive substances has been postulated. Therefore, measurements were performed to find out whether the photosensitiser Photofrin® is taken up differently in malignant and non-malignant cells *in vitro*. In addition, the sensitivity of malignant cells and non-malignant cells to photodynamic exposure was investigated, by quantifying viability and volume alterations of the cells. Bovine aortic endothelial cells, mouse fibroblasts and amelanotic hamster melanoma cells were suspended in a specially designed incubation chamber under controlled conditions (e.g. pH, pO_2, pCO_2 and temperature). After establishing constant baseline conditions, the cellular fluorescence intensity per cell volume, indicative of the uptake of Photofrin®, and cell volume were assessed by flow cytometry, and cell viability was quantified by the trypan blue exclusion test. Photodynamic exposure of cells was performed using an argon-pumped dye laser system via a 600 μm optical fibre at energy density of 4 Joules at the cell surface (40 mW/cm², 100 s). In comparison to endothelial and fibroblast cells, the melanoma cells exhibited no increased uptake of Photofrin®, and no enhanced sensitivity to photodynamic therapy (PDT). However, the fluorescence intensity/volume of endothelial cells was two to three times higher at each concentration of the photosensitiser. Following PDT, reduction in cell viability was dependent on the concentration of Photofrin®, and directly correlated with fluorescence intensity per cell volume. In addition, the cells of all three lines, treated by PDT, revealed dose-dependent changes in cell volume. Melanoma cells exhibited the most excessive increase. It is suggested that selective uptake of photosensitiser *in vitro* is not characteristic for tumour cells. The high uptake of Photofrin® by endothelial cells may indicate that the vascular endothelium is a major target for PDT, leading to cessation of tumour blood flow and subsequent destruction of tumour tissue. In addition, PDT-induced swelling of tumour cells might represent and effect synergistically impairing tumour perfusion, and thereby promoting tumour death.
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INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) is a promising treatment modality in the management of malignant tumours. Therapy using haematoporphyrin derivative (HPD) or Photofrin® is based on preferential labelling of neoplastic tissue by these drugs, producing transiently toxic oxygen species under laser irradiation [1–3]. The mechanisms of tumour destruction in response to PDT are the subject of intensive investigations. In some studies, malignant cells have been shown to take up greater amounts of porphyrin or to exhibit a higher sensitivity to PDT

compared to normal cells *in vitro* [4–6]. Other studies indicated that malignant and normal cells accumulate comparable amounts of porphyrin during incubation *in vitro* [4, 7–9].

Alternatively, there is increasing evidence of the importance of the vasculature in determining tumour destruction following PDT. At the macroscopic level, PDT results in destruction of tumour vessels but also vessels from normal tissue [10, 11]. Further effects, including stasis of blood flow, haemorrhage, vasoconstriction, platelet aggregation and platelet thrombus formation in the microvessels, have been implicated in the tumoricidal effect of PDT [11–14]. Some investigators supposed that damage of the endothelium is the primary cause of tumour response to PDT [15]. Gomer *et al.* [16] demonstrated *in vitro* that bovine aortic endothelial cells (BAEC) were more sensitive to photodynamic treatment than smooth muscle or fibroblast cells although they could not discriminate whether the effect was due to greater accumulation of the photosensitiser in endothelial cells as compared to control cells. In contrast to these results West *et al.* [17] have shown that BAEC took up two to three times more haematoporphyrin derivative as did adenocarcinoma cells. We have designed a study to determine whether differences either in uptake of Photofrin®, as evaluated by measuring cellular fluorescence, or in sensitivity to PDT can be demonstrated in BAEC as compared to amelanotic melanoma and fibroblast cells.

MATERIALS AND METHODS

Cell culture

Amelanotic hamster melanoma cells (A-Mel-3), an established cell line derived from a spontaneous amelanotic melanoma in a Syrian golden hamster [18, 19], mouse fibroblasts (L929) and BAEC were used for the investigations. A-MEL-3 and L929 [20, 21] were grown in 175-cm^2 culture flasks (3028, Falcon, Oxnard, U.S.A.) using RPMI-1640 medium (Seromed, Berlin, Germany). The medium was supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, U.S.A.), 100 U/ml penicillin and 50 μg/ml streptomycin (Sigma Chemical Co., St Louis, U.S.A.). BAEC were isolated according to a modified method of Maruyame *et al.* [22]. Segments of bovine aorta were filled with Medium 199 (Sigma) containing 0.25% collagenase (CLS type 2, Worthington Biochemical, Freehold, U.S.A.) under sterile conditions. The aorta was placed in a plastic bag and incubated in a water bath at 37°C for 10 min. Thereafter, the effluent was collected into centrifuge tubes (2070, Falcon) containing 10 ml Medium 199 with 20% FCS to inactivate collagenase and the cells were sedimented at 1800 rpm for 10 min. The pellet was washed twice with 5 ml of Medium 199 with 20% FCS, and the cells were resuspended in 2 ml of fresh culture medium. The yield of this procedure was in the range of 0.5–3 × 10^6 cells/ml. The cell suspension was resuspended in 10 ml Medium 199 and divided between two 25-cm^2 plastic tissue culture flasks (3813, Falcon). All cell cultures were kept in an incubator (Heraeus, Hanau, Germany) at 37°C, in humidified room air with 5% CO_2. Cells were subcultivated three times a week. Only confluent monolayers obtained 2 days after subcultivation were used. For the experiments, cells were harvested with 0.05% trypsin–0.02% EDTA (Boehringer, Mannheim, Germany) in phosphate-buffered saline and subsequently resuspended in culture medium containing FCS for inactivation of trypsin. Washing twice in serum-free medium ensured removal of FCS. The final cell density was 0.5–1 × 10^6 cells/ml. Subsequently, the cell suspension was transferred into a specially designed incubation chamber [23,24] in which pH, pO_2 and temperature were controlled continuously by respective electrodes and a thermocouple, and maintained at pH 7.4, pO_2 100 mmHg, pCO_2 35 mmHg and temperature 37°C. A gas-permeable silicon rubber tube served as a membrane oxygenator to supply the medium with mixtures of O_2, CO_2 and N_2. The pH of the medium was controlled by adjustment of the CO_2 supply. Sedimentation of the cells was prevented by a magnetic stirrer. Suspended cells were collected from the incubation chamber via inserted stainless steel cannulas.

Measurements of cell fluorescence, volume and viability

Measurements of cell fluorescence and cell volume were performed by flow cytometry (Fluvo Metricell, HEKA-Electronic, Lambrecht/Pfalz, Germany) [25]. This system allows simultaneous measurements of fluorescence and volume of up to 5000 cells/s. Fluorescence of Photofrin® was excited with a high pressure mercury arc lamp (HBO-100, Osram, München, Germany) at wavelengths between 300 and 500 nm. Maximal excitation of Photofrin® was found for 388 nm. Excitation was induced through a 500-nm short-pass filter. Maximal emission of Photofrin® at 630 nm was measured by using a 580-nm long-pass filter to collect the emitted red fluorescence above 580 nm. Red fluorescence was calibrated by measurement of 5.72 μm solid latex polystyrol particles (Polysciences Inc., Warrington, U.S.A.). The results of the fluorescence measurements were given in relation to the solid fluorescent particles. The cell fluorescence intensity (CFI) was calculated in relative units from constant fluorescence quantum yields emitted by the excited latex beads. In addition, CFI was expressed per cell volume since large cells accumulate more Photofrin® per cell than do small cells [7]. Cell volume was measured according to the Coulter principle, employing a hydrodynamic focusing technique providing a volume resolution of ~1% [25] that was calibrated electrically. The measurements of fluorescence and volume of 40 000 cells lasted for less than 30 s. Cell viability was measured by the trypan blue exclusion test (Boehringer). One hundred cells were counted (three times) under the microscope and the percentage of blue cells per hundred determined.

Experimental groups

A 30-min period preceded the measurements to ensure stable baseline values of cell size and cell viability prior to Photofrin® incubation. Preparations with unstable cell volume or impaired cell viability were discharged. The means of three measurements of cell volume and viability were taken as references. Following baseline measurements, Photofrin® was added to the cell suspension at concentrations of 1.5, 3, 4.5 or 15 μg/ml. It was received from Cyanamid Lederle (Wolfratshausen, Germany) at a concentration of 2.5 mg/ml in isotonic saline solution, and stored as 1-ml samples at −20°C. The cells were kept in the dark after exposure to Photofrin®. Fifty microlitre aliquots (5 × 10^4 cells) of the suspension were taken at intervals between 1 and 240 min after addition of Photofrin® for immediate measurement of: (a) CFI and cell volume prior to PDT; (b) cell viability by trypan

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blue exclusion test (Boehringer); (c) CFI and cell volume after PDT; (d) and cell viability after laser illumination. For PDT, 50 μl of the cell suspension were withdrawn from the chamber and introduced into an illumination chamber of 7 mm diameter and 1 mm depth. Laser illumination was performed at 630 nm with an argon-pumped dye laser (Aesculap Meditec, Heroldsberg, Germany) via a 600 micron optical fibre for a period of 100 s (40 mW/cm², 4 Joule). CFI, cell volume and cell viability were measured 5 min after PDT. A minimum of four individual cell samples were analysed for each experimental concentration.

Statistics

The results are expressed as median and standard error of the median. The data were analysed for statistical significance using the Kruskal–Wallis test for non-parametric one-way analysis of variance and multiple comparisons on ranks for unpaired samples [26]. Correlations were analysed with the Spearman test. Probability values smaller than 1% were considered significant.

RESULTS

Cell volume and cell density remained constant during the control period and 93.0 ± 1.2%, 90.0 ± 1.1% and 87.0 ± 0.6% of the A-Mel-3, L929 and BAEC populations, respectively, were viable. Without addition of Photofrin®, fluorescence was not detected in any of the cell types.

During incubation with Photofrin®, cell volume and cell viability remained constant. CFI increased in all cell lines in a dose-dependent manner as shown for BAEC (Fig. 1). Fifty per cent of the maximum CFI was obtained 5 min after addition of Photofrin®. After 60 min of incubation, maximum CFI was reached. No further increase could be measured during the entire course of the experiments. In comparison to A-Mel-3 and L929, the CFI values of BAEC were higher by a factor of 2–3 ($P<0.01$) during the whole observation period and at each concentration of Photofrin®. As indicated by the CFI values, differences in Photofrin® uptake between A-Mel-3 and L929 cell lines were not observed. However, all cell lines revealed a linear relationship between CFI and extracellular concentration of Photofrin® and regression analysis resulted in a statistically significant correlation for A-Mel-3 ($r=0.94$), L929 ($r=0.93$) and BAEC ($r=0.92$) (Fig. 2).

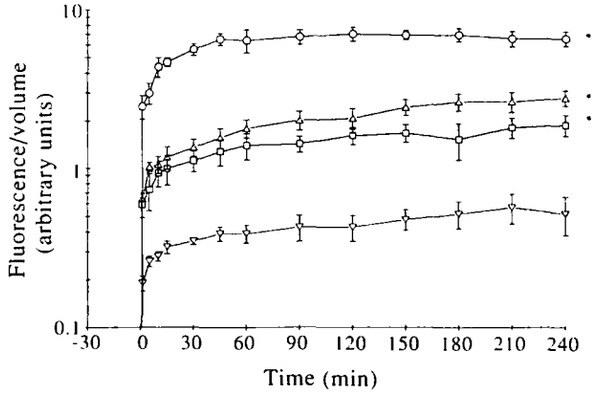

Fig. 1. Uptake of Photofrin® by BAEC as shown by CFI measurements. Cells were exposed to 1.5 (▽), 3.0 (□), 4.5 (△) or 15 (○) μg Photofrin®/ml in growth medium containing no fetal calf serum. 5 × 10⁴ cells were utilised per single volume measurement. Each point represents the median and the standard error of the median of four experiments (*$P<0.001$ versus ▽ at 1–240 min). The cellular fluorescence intensity was calibrated with 5.72 μm solid latex polystyrol particles to minimise fluctuations of the optical system and cell volume was calibrated electrically. In addition, CFI is expressed per cell volume. This allows comparison of large cells, accumulating more Photofrin® per cell, with small ones.

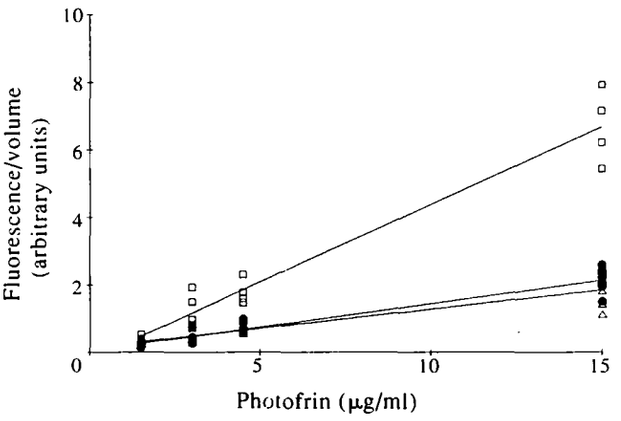

Fig. 2. CFI of Photofrin® after 60-min incubation as related to the concentration of Photofrin® incubated with BAEC (□), A-Mel-3 (●) and L929 (△). Correlation of Photofrin® medium concentration (1.5, 3.0, 4.5 or 15 μg Photofrin®/ml) and CFI had an r value of 0.92 (BAEC), 0.94 (A-Mel-3) and 0.93 (L929), with $P<0.001$. Each point represents four experiments.

Laser illumination of the cells incubated with Photofrin® reduced cell viability, which was dependent upon the CFI value (Fig. 3). In comparison to A-Mel-3 and L929, cell viability of BAEC was significantly reduced with less than 10% of BAEC surviving after drug doses of 4.5 or 15 μg/ml Photofrin®. Correlation of viability with CFI prior to laser irradiation revealed respective r values for BAEC, A-Mel-3 and L929 of 0.86, 0.91 and 0.89, with $P<0.001$ (Fig. 4). After laser illumination, CFI fell significantly for A-Mel-3 by 15.5 ± 5.6%, for L929 by 21.2 ± 7.9% and for BAEC by 10.4 ± 7.4% of the values prior to PDT. This phenomenon was neither photosensitiser dose- nor time-dependent. In addition, preincubation of A-Mel-3, L929 or BAEC with Photofrin® and subsequent illumination with laser light provoked significant changes of cell volume. At Photofrin® concentrations of 3.0 μg/ml, cell volume

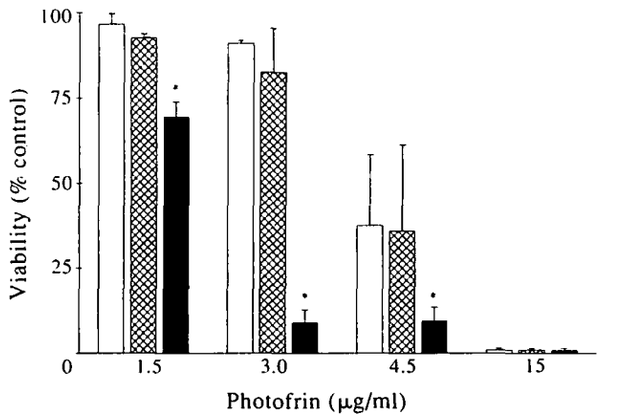

Fig. 3. Surviving fraction (5 min after PDT) of A-Mel-3 (□), L929 (▨) and BAEC (■). Cells were incubated with 1.5, 3.0, 4.5 or 15 μg Photofrin®/ml medium. Laser irradiation (40 mW/cm², 100 s) was performed after 60-min incubation with Photofrin®. Each symbol represents four experiments (*$P<0.01$ versus □ and ▨).

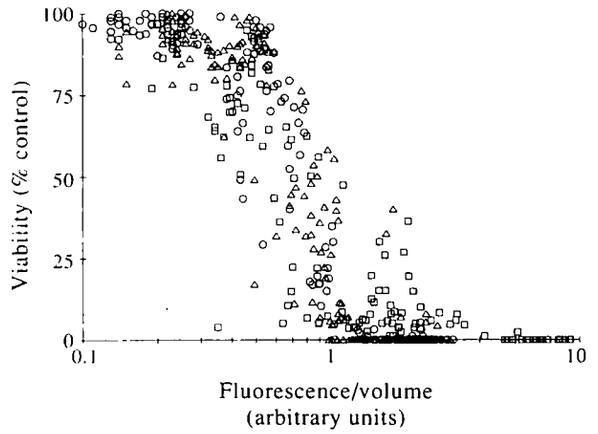

Fig. 4. Surviving fraction (5 min after PDT) of A-Mel-3 (○), L929 (△) and BAEC (□) as a function of CFI after Photofrin® incubation. Cells were incubated for 1–4 h with 1.5, 3.0, 4.5 or 15 μg Photofrin®/ml medium. Laser irradiation (40 mW/cm², 100 s) of cell samples was performed at intervals from 60 to 240 min. Correlation of the CFI prior to treatment and laser irradiation revealed respective r values for BAEC, A-Mel-3 and L929 of 0.86, 0.91 and 0.89, with $P<0.001$. For A-Mel-3, L929 and BAEC, four experiments were performed for each concentration.

of BAEC increased by 20.0 ± 5.5% 5 min after PDT. However, at 4.5 μg and 15 μg Photofrin®/ml, when all BAEC were killed, the increase in cell volume was either less pronounced, by 11.0 ± 8.1%, or cell volume was reduced to 94.3 ± 2.7%.

L929 and A-Mel-3 tended to swell more compared to BAEC when incubated at higher concentrations of Photofrin®. At 4.5 μg Photofrin®/ml, cell volume of L929 and A-Mel-3 increased after PDT by 21.8 ± 9.4% and 24.8 ± 5.7%, respectively (Fig. 5). However, when A-Mel-3 tumour cells were exposed to 15 μg Photofrin®/ml and subsequently irradiated, cell volume increased by 59.3 ± 11.6% of initial cell volume.

DISCUSSION

Photodynamic therapy is now used in phase III clinical trials in various fields [27, 28], but the mechanism of how PDT inhibits tumour growth or kills the tumour remain unclear [29]. The selectivity of uptake and retention of HPD reported for malignant cells has not been convincingly reproduced *in vivo*. Moan *et al.* [9] examined cytofluorimetrically HPD uptake by mouse embryo fibroblast cells and found that uptake and retention of HPD did not correlate with the ability of these cells to produce tumours, although it was suggested that malignant cells incorporated marginally more HPD per unit volume than did normal cells [9]. Chang and Dougherty [4] also failed to show differences in uptake and clearance of HPD between normal and malignant cells *in vitro*. In some studies, malignant cells were found to incorporate higher amounts of porphyrins and to reveal increased sensitivity to PDT as compared to normal cells [5, 30–35]. Böhmer and Morstyn suggested that acidic pH, differences in serum concentrations of malignant tumour tissue, as well as the increased size of tumour cells may be important for selective uptake of HPD by malignant cells [7]. Other authors have demonstrated significant differences between tumour and normal tissue oxygenation after PDT [36, 37]. It was shown recently that the subcutaneous microcirculation of the hamster during PDT reveals haemorrhages and venous thrombosis within the tumour, and vasoconstriction of normal arterioles surrounding and supplying the tumour [12]. In addition, experiments were performed to study blood flow in transplantable rat bladder tumour and in rat jejunum after PDT, using radiolabelled microspheres [38, 39]. A comparison of the effect of PDT on blood flow in normal and tumour vessels using a rat skin flap model demonstrated a significant reduction of blood flow in both types of vessels, but no significant difference when exposed to identical doses of the photosensitiser [10].

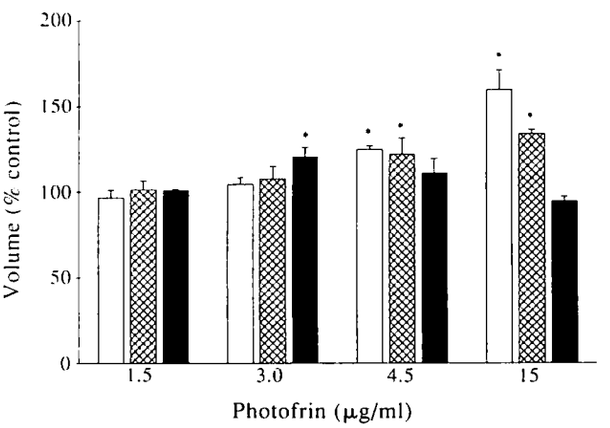

Fig. 5. Cell volume response of A-Mel-3 (□), L929 (▩) and BAEC (■) 5 min after PDT. Cells were incubated with 1.5, 3.0, 4.5 or 15 μg Photofrin®/ml medium. Laser irradiation (40 mW/cm², 100 s) was performed after 60-min incubation with Photofrin®. The increase in cell volume is given as a percentage of the control obtained prior to PDT. Each symbol represents four measurements. (*$P<0.01$ versus □, ▩ and ■ at 1.5 μg Photofrin®/ml).

We have used a recently established *in vitro* model to study uptake and phototoxicity of Photofrin® in cell lines with different phenotypes, i.e. endothelial versus fibroblast versus melanoma. A major advantage of this model is the close control of the extracellular environment as established in previous studies on volume regulation of glial cells [23, 24, 40, 41].

For assessing cell survival following PDT, viability was determined using trypan blue exclusion. A comparison of a dye exclusion, specifically the MTT test, and a clonogenic assay to assess cell survival after PDT showed that both gave similar shaped curves, but the dye exclusion method resulted in a slight overestimate of cell death [42]. Despite the lack of exact agreement between the assays, both show qualitatively the same results, and similar shaped curves were obtained with both assays, suggesting that short-term assays may be applicable for studies of PDT *in vitro* [43].

Our results of CFI measurements after incubation with Photofrin® showed no greater uptake of Photofrin® by A-Mel-3 tumour cells and no increased photosensitivity as compared to L929 and BAEC. In contrast, after standard incubation with Photofrin®, BAEC revealed 2–3-fold higher CFI. This might imply that the tumour selective effect of PDT *in vivo* is not due to greater uptake of the photosensitiser in tumour cells.

This finding is in agreement with results from *in vivo* studies indicating that the tumour microvasculature may be decisive for tumour destruction following PDT. Berenbaum *et al.* studied cerebral damage following PDT, and demonstrated selective initial damage to the microvascular endothelium [44]. Gomer *et al.* observed *in vitro* significant differences in cell photosensitivity when different cellular components of the vascular wall were examined by spectrofluorophotometry [16]. They specifically found that the endothelial cells were more sensitive to PDT in spite of the same cellular level compared to the other cell lines.

However, in the present study, the endothelial cells were more sensitive due to an increased uptake of sensitiser as indicated by cellular fluorescence intensity. This difference in cellular fluorescence may be explained by the different methods used, with Gomer *et al.* [16] measuring fluorescence per protein of the homogenised cells, while we determined fluorescence intensity per cell volume. In addition, Gomer *et al.* [16] compared cell lines originating from one species while we investigated cell lines taken from different species. Our results were supported by findings of West *et al.* [17], who compared bovine aortic endothelial cells and human colon adenocarcinoma cells, and found that, with both cell lines being in plateau phase, endothelial cells incorporated two to three times more hematoporphyrin derivative compared to WiDr (BAEC 204 ± 11ng/10^6 cells; WiDr 98 ± 5ng/10^6 cells), despite there being no difference in cell volume between the cells.

The cause of enhanced porphyrin photosensitivity observed in endothelial cells might be the higher number of low density lipoprotein (LDL) receptors on endothelial as compared to other cells [45, 46]. The LDL carrier system is involved in delivery of porphyrin to tissue [47] and it has been suggested that higher LDL receptor activity is responsible for high drug uptake in BAEC [17]. There may be different mechanisms of transport of HPD into the different types of cell used in our study. Consistent with this assumption are results of Carrano *et al.*, who described different mechanisms of photosensitiser uptake into cells of different lines, both energy-dependent and independent [48]. The enhanced photosensitiser uptake and thereby higher phototoxicity for endothelial cells may play a role in the microvascular damages observed after PDT. *In vivo*, PDT produces, at a macroscopic level, destruction of tumour and normal tissue vasculature [10, 11]. A number of effects have been described, including stasis of blood flow, haemorrhage, vasoconstriction, platelet aggregation and platelet thrombus formation [11–14].

An interesting finding was the increase in cell volume after PDT, which was dose-dependent and also dependent upon CFI and concentration of extracellular Photofrin®. The cells of the three lines showed an initial cell swelling, although this only occurred at low concentrations of Photofrin®, i.e. 1.5 or 3.0 μg/ml with BAEC. Cell swelling after PDT appears to be a specific effect because photosensitiser or laser illumination did not influence cell volume. Cell swelling was most pronounced in A-Mel-3 tumour cells. The PDT-induced effects, leading to cell swelling and decreases in cell survival, are not clearly understood. Cell swelling might be attributed to the influx or formation of additional osmotic substances within the cells, such as lactic acid, originating from stress-induced glycolysis, or calcium following depolarisation of the plasma membrane [49]. Alternatively, influx or uptake of electrolytes into the cells after damage of the plasma membrane might equally contribute to cell swelling [49–51].

We suggest that swelling of tumour cells is of significant relevance to the *in vivo* situation, since experimental [52] and human tumours [53] exhibit interstitial hypertension after PDT. Moreover, it was recently observed that PDT induces an increase of both interstitial fluid pressure and volume of tumour [54]. In addition, interstitial hypertension is related to tumour blood flow as shown by Steen *et al.* [55]. Since tumour microvessels have a high permeability, and tumours lack functioning lymphatics, microvascular pressure is likely to alter interstitial fluid pressure [56]. Several factors are likely to increase the vascular resistance in tumour vessels after PDT, including vasoconstriction, microembolisation and partial occlusion of vessels by swollen endothelial and cancer cells. The latter finding might be a consequence of the preferential uptake of Photofrin® by endothelial cells and the swelling of tumour cells. We, therefore, suggest that damage of endothelial cells and swelling of tumour cells following PDT should be consequential, primary events, finally leading to cessation of blood flow and subsequent necrosis of tumours.

1. Kessel D. Photosensitization with derivatives of hematoporphyrin. *Int J Radiat Biol* 1986, **49**, 901–907.
2. Kessel D, Chou T. Tumor localizing components of porphyrin preparation hematoporphyrin-derivative. *Cancer Res* 1983, **43**, 1994.
3. Weishaupt KR, Gomer CJ, Dougherty TJ. Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. *Cancer Res* 1976, **36**, 2326–2329.
4. Chang C, Dougherty TJ. Photoradiation therapy: kinetics and dynamics of porphyrin uptake and loss in normal and malignant cells in culture. *Radiat Res* 1978, **74**, 498–499.
5. Shulok JR, Klaunig JE, Selamn SH, Schafer PJ, Goldblatt PJ. Cellular effects of hematoporphyrin derivative photodynamic therapy on normal and neoplastic rat bladder cells. *Am J Pathol* 1986, **122**, 277–283.
6. Williams RD, Runge TC. Photodynamic therapy of human bladder cells *in vitro* correlated with cellular fluorescence levels of Photofrin II. *Photochem Photobiol* 1987, **46**, 733–737.
7. Böhmer RM, Morstyn G. Uptake of hematoporphyrin derivate by normal and malignant cells: effects of serum, pH, temperature and cell size. *Cancer Res* 1985, **45**, 5328–5334.
8. Henderson BW, Bellnier DA, Ziring B, Dougherty TJ. Aspects of the cellular uptake and retention of hematoporphyrin-deivative and their correlation with the biological reponse to PRT *in vivo*. *Adv Exp Med Biol* 1983, **160**, 129–138.
9. Moan J, Steen HB, Feren K, Christensen T. Uptake of hematoporphyrin derivate and sensitized photoinactivation of C3H cells with different oncogenic potential. *Cancer Lett* 1981, **14**, 291–296.
10. Reed MWR, Wieman TJ, Schuschke DA, Tseng MT, Miller FN. A comparison of the effects of photodynamic therapy on normal and tumor blood vessels in the rat microcirculation. *Radiat Res* 1989, **119**, 542–552.
11. Wieman TJ, Mang TS, Fingar VH, *et al.* Effect of photodynamic therapy on blood flow in normal and tumor vessels. *Surgery* 1988, **104**, 512–517.
12. Feyh J, Goetz AE, Heimann A, Konigsberger R, Kastenbauer E. Microcirculatory effects of photodynamic therapy with hematoporphyrin derivative. *Laryngorhinootologie* 1991, **70**, 99–101.
13. Pope AJ, Bown SG. The morphological and functional changes in rat bladder following photodynamic therapy with phthalocyanine photosensitization. *J Urol* 1991, **145**, 1064–1070.
14. Star WM, Marijnissen HPA, van der Berg-Blok AE, Versteeg JAC, Franken KAP, Reinhold HS. Destruction of rat mammary tumor and normal tissue microcirculation by hematoporphyrin-derivative photoradiation observed *in vivo* in sandwich observation chambers. *Cancer Res* 1986, **46**, 2532–2540.
15. Henderson BW, Waldow SM, Mang TS, Potter WR, Malone PB, Dougherty TJ. Tumor destructions and kinetics of tumor cell death in two experimental mouse tumors following photodynamic therapy. *Cancer Res* 1985, **45**, 572–576.
16. Gomer CJ. Rucker N, Murphree L. Differential cell photosensitivity following porphyrin photodynamic therapy. *Cancer Res* 1988, **48**, 4539–4552.
17. West CML, West DC, Kumar S, Moore JV. A comparison of the sensitivity to photodynamic treatment of endothelial cells and tumour cells in different proliferative states. *Int J Radiat Biol* 1990, **58**, 145–156.
18. Fortner JG, Gale Mahy A, Schrodt GR. Transplantable tumors of the Syrian (golden) hamster. *Cancer Res* 1961, **21**, 161–196.
19. Wolff HH, Balda RB, Birkmayer GD, Braun-Falco O. Zur Ultrastruktur des Hamster Melanoms A-Mel-3 von Fortner. *Arch Derm Forsch* 1971, **240**, 192–203.
20. Boegheim JPJ, Lagerberg JWM, Dubbelman TMAR, van Steveninck J. Damaging action of photodynamic treatment in combination with hyperthermia on transmembrane transport in murine L929 fibroblasts. *Biochim Biophys* 1989, **979**, 215–220.

21. Boegheim JPJ, Lagerberg JWM, Tijissen K, Dubbelman TMAR, van Stevenick J. Preferential uptake of cytotoxic porphyrins from hematoporphyrin-derivative in murine L929 fibroblasts and Chinese hamster ovary K1 epithelial cells. *Biochim Biophys Acta* 1989, **1012**, 237–242.
22. Maruyama Y. The human endothelial cell in tissue culture. *Z Zelforsch Mikrosk Anat* 1963, **60**, 69–79.
23. Kempski O, Chaussy L, Gross U, Zimmer M, Baethman A. Volume regulation and metabolism of suspended C6 glioma cells. An *in vitro* model to study cytotoxic brain edema. *Brain Res* 1983, **27**, 217–228.
24. Kempski O, Zimmer M, Chaussy L, Baethmann A. *Volume and Metabolism of C6 Glioma Cells Suspended in Hypotonic Medium: an* in vitro *Model to Study Cytotoxic Brain Edema*. Go KG, Baethmann A, eds. New York, Plenum Press, 1984, 151–158.
25. Kachel V, Glossner E, Kordwig E, Ruhenstroth-Bauer G. Fluvometricell, a combined cell volume and cell fluorescence analyser. *J Histochem Cytochem* 1977, **25**, 804–812.
26. Theodorsson-Northeim E. Kruskal-Wallis test: BASIC computer program to perform nonparametric one-way analysis of variance and multiple comparisons on ranks of several independent samples. *CMPB* 1986, **23**, 57–62.
27. Feyh J, Goetz AE, Müller W, Königsberger R, Kastenbauer E. Photodynamic therapy in head and neck surgery. *J Photochem Photobiol* 1990, **7**, 353–358.
28. Jocham D, Baumgartner R, Stepp H, Unsöld E. Clinical experience with the integral photodynamic therapy of bladder carcinoma. *J Photochem Photobiol* 1990, **6**, 183–187.
29. Gomer CJ. Preclinical examination of first and second generation photosensitizers used in photodynamic therapy. *Photochem Photobiol* 1991, **54**, 1093–1107.
30. Andreoni A, Cubeddu R, De Silvestri S, *et al.* Effects of laser irradiation on hematoporphyrin treated normal and transformed thyroid cells in culture. *Cancer Res* 1983, **43**, 2076–2080.
31. Dougherty TJ. Porphyrin localisation and treatment of tumors. Doiron DR, Gomer CJ, eds. New York, Alan R. Liss, 1984, 301–334.
32. Mossman BT, Gray MJ, Silberman L, Lipson RL. Identification of neoplastic versus normal cells in human cervical cell culture. *J Obstet Gynecol* 1974, **43**, 635–639.
33. Patrice T, Praloran V, Le Bodic MF, Le Bodic L. Experimental aspects of *in vitro* and *in vivo* photochemotherapy. *Biochemie* 1986, **68**, 923–926.
34. Ryan US, Ryan JW, Whitaker C, Chiu A. Localising of angiotensin converting enzyme (kininase II). II. Immunocytochemistry and immunofluorescence. *Tissue Cell* 1976, **8**, 125–146.
35. Sprik SA, Sullivan MJ, Garey TE. *In vitro* photosensitization of human head and neck squamous cancer cells by dihematoporphyrin-ether. *Otolaryngology* 1988, **99**, 28–37.
36. Tromberg BJ, Orenstein A, Kimel S, *et al. In vivo* tumor oxygen tension measurements for the evaluation of the efficiensy of photodynamic therapy. *Photochem Photobiol* 1990, **52**, 375–385.
37. Tromberg BJ, Kimel S, Orenstein A, *et al.* Tumor oxygen tension during photodynamic therapy. *J Photochem Photobiol* 1990, **5**, 121–126.
38. Selman SH, Kreimer-Birnbaum M, Goldblatt PJ, Anderson TS, Keck RW, Britton SL. Jejunal blood flow after exposure to light in rats injected with hematoporphyrin-derivative. *Cancer Res* 1985, **45**, 6425–6427.
39. Selman SH, Kreimer-Birnbaum M, Klaunig M, Goldblatt PJ, Keck RW, Britton SL. Blood flow in transplantable bladder tumors treated with hematoporphyrin-derivate and light. *Cancer Res* 1984, **44**, 1924–1927.
40. Kempski O, Spatz M, Valet G, Baethmann A. Cell volume regulation of cerebrovascular endothelium *in vitro*. *J Cell Phys* 1985, **123**, 51–54.
41. Kempski O, von Rosen S, Weight H, Staub F, Peters J, Baethmann A. Glial ion transport and volume control. In Abbott NJ, ed. *Glial-Neuronal Interactions*, 633rd edition. New York, The New York Academy of Science, 1991, 306–317.
42. McHale AP, McHale ML, Blau W. The effects of hematoporphyrin derivative and human erythrocyte ghost encapsulated hematoporphyrin derivative on a mouse myeloma cell line. *Cancer Biochem Biophys* 1988, **10**, 157–164.
43. McHale AP, McHale ML, Blau W. Use of a tetrazolium based colourimetric assay in assessing photoradiation therapy *in vitro*. *Cancer Lett* 1988, **41**, 315–321.
44. Berenbaum MC, Hall GW, Hayes AD. Cerebral photosensitization by hematoporphyrin-derivative. Evidence for an endothelial site of action. *Br J Cancer* 1986, **33**, 81–89.
45. Gaffney J, West D, Arnold F, Satter A, Kumar S. Differences in the uptake of modified low density lipoproteins by tissue cultured endothelial cells. *J Cell Science* 1985, **79**, 317–325.
46. Netland PA, Zetter BR, Via DP, Voyta C. *In situ* labelling of vascular endothelium with fluorecent acetylated low density lipoprotein. *Histochem J* 1985, **17**, 1309–1320.
47. Barel A, Jori G, Perin A, Romandini P, Pagnan A, Buffant S. Role of high, low and very low lipoproteins in the transport and tumor delivery of hematoporphyrin *in vivo*. *Cancer Lett* 1986, **32**, 10–3-114.
48. Carrano CJ, Tsutsui M, McConnel S. Tumour localising agents: the transport of meso-tetra (p-sulfophenyl) porphine by Vero and HEp-2 cells *in vitro*. *Chemico-Biological Interactions* 1978, **21**, 233–248.
49. Specht KG, Rodgers MAJ. Plasma membrane depolarization and calcium influx during cell injury by photodynamic action. *Biochim Biophys Acta* 1991, **1070**, 61–68.
50. Lipson RL, Baldes EJ, Olsen AM. The use of a hematoporphyrin in tumor detection. *JNCI* 1961, **267**, 1–8.
51. Wijesekera TP, Dolphin D. Some preparations and properties of porphyrins. In Kessel D, ed. *Methods in Porphyrin Photosensitization*. New York, Plenum Publishing, 1985, 229–266.
52. Leunig M, Goetz AE, Dellian M, *et al.* Interstitial fluid pressure in solid tumors following hyperthermia: possible correlation with therapeutic response. *Cancer Res* 1992, **52**, 487–490.
53. Gutmann R, Leunig M, Feyh J, Goetz A, Messmer K, Kastenbauer E, Jain K, Interstitial hypertension in head and neck tumors of patients: correlation with tumor size. *Cancer Res* 1992, **52**, 1993–1995.
54. Lenning M, Goetz A, Gamarra F, Zcherer G, Messmer K, Jain RK. Photodynamic therapy–induced alterations in interstitial fluid pressure, volume and water content of amelanotic melanoma in the hamster. *Br J Cancer* 1994 (in press).
55. Steen RG. Edema and perfusion: characterization by quantitative 1H-MR imaging. *AJR* 1992, **158**, 259–264.
56. Zlotecki R, Boucher Y, Lee I, Jain K. Effekt of angiotensin II induced hypertension on tumor interstitial fliúid pressure and tumor blood flow. *Int J Radiat Oncol Biol Phys* 1992, **24**, 1123.

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