British Journal of Cancer (2003) 88, 760–766

www.bjcancer.com

Ischaemia-reperfusion injury in photodynamic therapy-treated mouse tumours

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Prompted by the observation of ischaemia development during the treatment of tumours by photodynamic therapy (PDT) that is typically followed by a restoration of tumour blood flow and by the indications of secondary superoxide generation after PDT, we aimed in this study to obtain evidence of the induction of ischaemia-reperfusion (I/R) injury in PDT-treated tumours. Using subcutaneous mouse FsaR fibrosarcoma model and Photofrin-based PDT treatment, we have examined the activity of xanthine oxidase (XO, a key enzyme in the I/R injury development) in tumours before and after the therapy. Compared to the levels in nontreated tumours, there was a five-fold increase in the activity of this enzyme in tumours excised immediately after PDT. This burst of elevated XO activity declined rapidly, returning to the pretreatment levels within the next 30 min. Visible reflectance spectroscopy confirmed the occurrence of a PDT-induced strong but temporary reduction in tumour oxygenation. The administration of XO inhibitor oxypurinol prevented this PDT-induced rise in XO activity. The oxypurinol treatment also decreased the extent of neutrophil accumulation in PDT-treated tumours and reduced the level of PDT-mediated cures. These results demonstrate the induction of I/R injury in PDT-treated tumours, and show that it can contribute to the therapy outcome. Since I/R injury is a well-recognised proinflammatory insult, we suggest that its induction in PDT-treated tumours promotes the development of inflammatory response that has become established as a key element of the antitumour effect of PDT.
British Journal of Cancer (2003) **88,** 760–766. doi:10.1038/sj.bjc.6600792 www.bjcancer.com

Keywords: photodynamic therapy; ischaemia-reperfusion injury; xanthine oxidase; visible reflectance spectroscopy; mouse fibrosarcoma

Recent insights into the mechanisms of photodynamic therapy (PDT)-mediated destruction of solid tumours have emphasised the important role played by the induced inflammatory response for the outcome of this therapy (Dougherty *et al*, 1998; Korbelik and Cecic, 2003; Sun *et al*, 2002). Evidence has accumulated documenting various inflammation-specific events following the treatment of tumours with PDT, including: (i) proinflammatory changes in vascular endothelium, (ii) complement activation and engagement of other plasma cascade systems (kinin-generation, coagulation, and fibrinolysis), (iii) release of inflammatory cytokines and chemokines, arachidonic acid metabolites and various other inflammatory mediators, (iv) activation of poly(ADP-ribose)polymerase and NFκB upregulation, and (v) invasion of inflammatory cells (Ryter and Gomer, 1993; Cecic and Korbelik, 2002; Korbelik and Cecic, 2003; Sun *et al*, 2002). The transcription factor NFκB is now a recognised key regulator of inflammatory response (Lentsch and Ward, 2000).

Ischaemia-reperfusion (I/R) injury is known as a potent instigator of inflammatory response responsible for severe tissue damage in a variety of common pathological conditions, including stroke, myocardial infarction, pulmonary and haemorrhagic shock, acute kidney and liver failure, and organ transplant rejection (Hernandez *et al*, 1987; Zimmerman and Granger, 1994; De Greef *et al*, 1998). Tissue ischaemia is associated with the conversion of xanthine dehydrogenase into oxidant-producing xanthine oxidase (XO), while concomitantly hypoxanthine accumulates because of the breakdown of ATP (Parks *et al*, 1988; Zimmerman and Granger, 1994). At the time of reperfusion, sudden reintroduction of oxygen enables XO to induce the formation of xanthine from hypoxanthine, which is accompanied with an intense release of reactive oxygen species, primarily superoxide anion (Parkins *et al*, 1998). The induced oxidative stress at the level of vascular endothelium promotes complement activation and elicits a series of inflammatory events culminating in a massive invasion of activated neutrophils and other inflammatory cells into the previously ischaemic area (Hernandez *et al*, 1987; De Greef *et al*, 1998; Kilgore *et al*, 1999).

In an earlier work (Korbelik *et al*, 2000), we found indications that I/R injury may play a role in the response of tumours to PDT. A typical pattern of blood flow alterations in PDT-treated tumours consists of an initial marked drop that tends to recover after photodynamic light treatment, and such conditions are conducive to the induction of I/R injury. When examining the possibility of superoxide generation during the reperfusion episode, we found that the administration of superoxide dismutase (SOD) immediately after PDT resulted in a decrease in tumour cure rates (Korbelik *et al*, 2000). Since I/R injury, if indeed inflicted in PDT-treated tumours, would be of a considerable relevance for the development of inflammatory responses, microvascular dysfunction and tumour cures, our objective in the present study was to

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Received 29 May 2002; revised 29 November 2002; accepted 11 December 2002

obtain more conclusive evidence that would support the induction of this insult. We demonstrate that PDT results in a marked elevation in the activity of XO (a key enzyme that hallmarks the I/R process) in treated tumours, and show that XO inhibition attenuates the neutrophil infiltration into PDT-treated tumours and decreases tumour cure rates.

MATERIALS AND METHODS

Tumour model

Subcutaneous FsaR fibrosarcomas (Volpe *et al*, 1985) were inoculated by implanting 1×10^6 *in vitro* expanded tumour cells into the lower sacral region of syngeneic C3H/HeN mice. Tumours were used for experiments when reaching 6–8 mm in largest diameter with thickness around 2 mm. All animal procedures were conducted according to the approval issued by The Animal Ethics Committee of the University of British Columbia and meet the standards required by the UKCCR guidelines (Workman *et al*, 1998).

PDT protocol

Photofrin (porfimer sodium, Axcan Pharma Inc., Mont-Saint-Hilaire, Quebec, Canada) was administered intravenously at $10\,mg\,kg^{-1}$ at 24 h prior to the delivery of light generated by a high throughoutput fibre illuminator (Sciencetech Inc., London, Ontario, Canada) equipped with a 150 Q QTH lamp with integrated ellipsoidal reflector and 630 ± 10 nm interference filter. The light was delivered through an 8-mm core diameter liquid light guide model 77638 (Oriel Instruments, Stratford, CT, USA). The power density achieved for monodirectional superficial illumination of tumours and ~ 1 mm of surrounding normal tissue was around $110\,mW\,cm^{-2}$. During PDT light treatment, the mice were held unanaesthetised in restraining holders. For the evaluation of tumour cure or regrowth, the mice (eight per treatment group) were, after PDT, examined every second day for signs of tumour growth. Tumour cure was defined as no sign of recurrence at 90 days post-PDT. The XO inhibitor oxypurinol, purchased from Sigma Chemical Co. (St Louis, MO, USA), was dissolved in phosphate-buffered saline and administered intraperitoneally at $17\,mg/kg^{-1}$. The ethical guidelines were followed that meet the above-mentioned standards (Workman *et al*, 1998).

Measurement of XO activity

Amplex™ Red Xanthine/Xantine Oxidase Assay Kit (Molecular Probes, Eugene, OR, USA) was used for the measurement of XO activity in homogenates of the excised FsaR tumours. Briefly, this assay is based on the activity of hydrogen peroxide (formed by spontaneous degradation of superoxide, which is a major product in XO-mediated oxidation of hypoxanthine) that in the presence of horseradish peroxidase reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin fluorescence was measured in a fluorescence microplate reader using 530 and 590 nm wavelengths for excitation and detection, respectively. The results expressed in $mU\,h^{-1}\,mg^{-1}$ of tumour tissue were derived by preparing a XO standard curve. In obtaining the excised tumours, we followed the ethical guidelines that meet the above-mentioned standards (Workman *et al*, 1998).

Reflectance spectroscopy

The reflectance spectra were measured with a fibre optic spectrometer system developed in our laboratory (Zeng *et al*, 1995). A tungsten lamp is used for illumination through one branch of a bifurcated fibre bundle. Another branch of the fibre bundle collects and transmits the reflected light from the tissue to a spectrometer (Ocean Optics, FL, USA, model USB 2000) for spectral analysis. The fibre bundle has a holder to position itself at 45° to the skin surface to avoid the specular (mirror) reflection so that only the diffuse reflected light, which has gone into the tissue and sampled the tumour, was collected. The data acquisition of each spectrum is completed in less than 1 s and the measurements generate no significant PDT effect to the tissue. The spectral signals between 500 and 600 nm were used to assess the blood oxygenation status of the probed tissue volume. The ethical guidelines were followed that meet the above-mentioned standards (Workman *et al*, 1998).

Flow cytometry

Tumour neutrophil levels were assessed using a flow cytometry protocol described in detail elsewhere (Cecic *et al*, 2001). Briefly, the excised tumours were dissociated into single-cell suspensions and the cells were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies raised against specific murine membrane markers. Neutrophils were identified as cells stained positively for myeloid differentiation antigen GR1 (Ly-6G) and negatively for macrophage-specific antigen F4/80. Flow cytometry was performed with a Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL, USA) using standard techniques. In obtaining the excised tumours, we followed the ethical guidelines that meet the above-mentioned standards (Workman *et al*, 1998).

Statistical analysis

The unpaired Student's *t*-test was applied to test the difference between means for the data from XO measurement and flow cytometry. Log-rank test was used for the tumour response evaluation. The difference with $P < 0.05$ was considered statistically significant.

RESULTS

The activity of XO determined in the homogenates of nontreated FsaR tumuors was around $7\,mU\,h^{-1}\,mg^{-1}$ of tumour tissue (Figure 1). No significant change in this level was detected in the light-only and Photofrin-only treatment control groups. However, a five-fold increase in XO activity was found in the tumours exposed to Photofrin-based PDT and excised immediately after the termination of light delivery. The activity of this enzyme was still elevated, but at a lower level, in tumours excised at 15 min post-PDT, while it dropped to the control levels in tumours excised at 30 min post-PDT.

In additional experiments, we examined the effect of oxypurinol, a specific inhibitor of XO (Granger *et al*, 1986; Spector *et al*, 1986). Although the light dose was increased from a moderately curative $150\,J\,cm^{-2}$ (when used with $10\,mg\,kg^{-1}$ of Photofrin) to a highly curative $250\,J\,cm^{-2}$, there was no further increase in the XO activity in tumours excised immediately post-PDT (the average level was in fact somewhat lower) (Figure 2). A group of tumours was also excised at 10 min post-PDT and the results show that the XO activity markedly declined in this short time period. The administration of oxypurinol at 30 min before PDT light treatment completely prevented the PDT-induced burst in XO activity, while oxypurinol has not significantly affected the activity of this enzyme in nontreated tumours.

An obvious cause for the observed changes in XO activity would be a temporary decline in the oxygenation of PDT-treated tumours. To verify this, we monitored the oxygenation status in a group of six subcutaneous FsaR tumours starting before PDT and extending to 1 h after PDT using a noninvasive visible reflectance

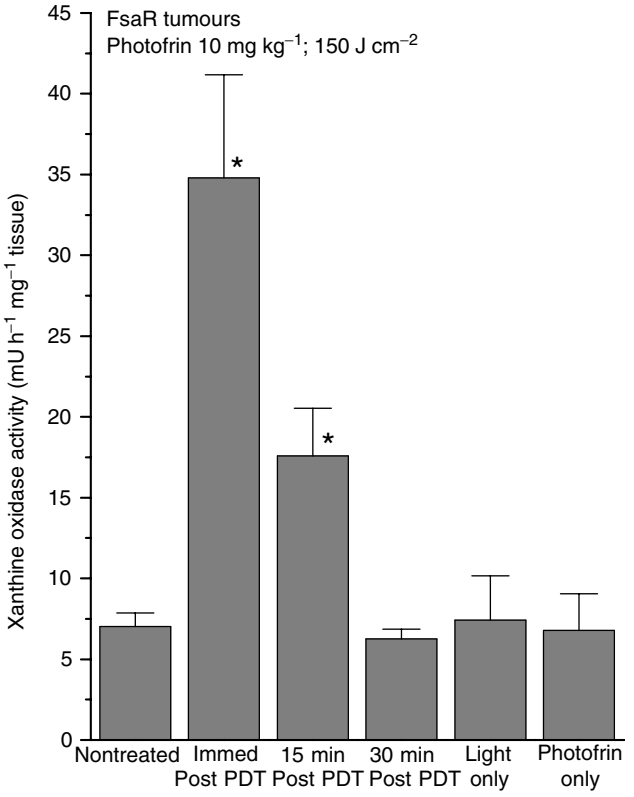

Figure 1 The effect of PDT on XO activity in FsaR tumours. Subcutaneous FsaR tumours growing in C3H/HeN mice were treated by PDT (Photofrin 10 mg kg^{-1} followed 24 h later by 150 J cm^{-2}). The tumours were excised either immediately post-PDT light treatment, or 15 or 30 min later. Their homogenates were used for the determination of XO activity as described in Materials and Methods. The samples were also prepared from nontreated tumours, tumours from mice not given Photofrin excised immediately after light treatment (light only), and those from mice given Photofrin 24 h earlier but not treated with light (Photofrin only). Bars represent s.d., $n = 4$; *depicts statistically significant difference from the level in nontreated tumours ($P < 0.01$).

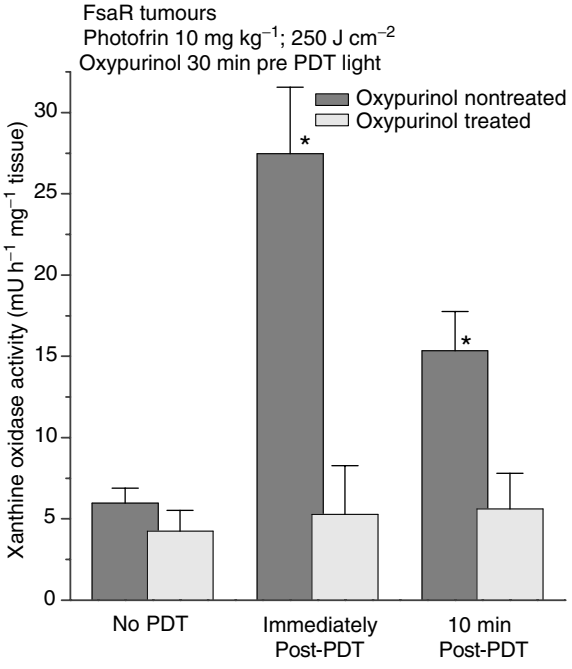

Figure 2 The effect of oxypurinol pretreatment on XO activity in PDT-treated FsaR tumours. Subcutaneous FsaR tumours were treated with PDT and the samples prepared for XO measurement as described in Figure 1, except that the time of excision was either immediately after PDT light treatment or 10 min later. Oxypurinol (17 mg kg^{-1}) was injected intraperitoneally 30 min before the onset of light treatment. Bars are s.d., $n = 4$; *depicts statistically significant difference from the level in nontreated tumours ($P < 0.01$).

measurement. Very similar results were obtained with all the tumours and a representative example is shown in Figure 3. The reflectance spectrum recorded with the tumour before PDT has the characteristic troughs (dips) at 542 and 577 nm (arrows) that result from the secondary absorption bands of oxyhaemoglobin (Figure 3A). The spectrum obtained with the same tumour immediately after PDT exhibits a striking absence of the 542 and 577 nm troughs, which shows that oxyhaemoglobin in the tumour tissue was replaced by deoxyhaemoglobin at that time. After 1 h, the reflectance spectrum of the tumour regained the characteristics seen in the pre-PDT treatment spectrum with the reappearance of 542 and 577 nm troughs. These changes in oxyhaemoglobin/deoxyhaemoglobin levels are more clearly presented by ratioing the post-PDT spectrum to pre-PDT spectrum (Figure 3B). The values around 542 and 577 nm are now depicted as prominent peaks emphasising the reduction in oxyhaemoglobin levels immediately after PDT compared to pre-PDT values. This drop in haemoglobin oxygen saturation obviously does not persist, since ratioing the reflectance spectrum taken at 1 h after PDT to the spectrum taken before PDT reveals no peaks around 542 and 577 nm; in fact, a small trough at 577 nm hints that oxygen levels might have even exceeded the pre-PDT values.

Proinflammatory effects associated with XO activity are known to stimulate local neutrophil sequestration (Zimmerman and Granger, 1994), and it is also well established that PDT induces neutrophil accumulation in the treated tumours (Krosl *et al*, 1995; Gollnick *et al*, 1997; Sun *et al*, 2002). The oxypurinol treatment that inhibits XO activity produced a decrease in the levels of neutrophils found in PDT-treated tumours (Figure 4). Flow cytometry-based analysis of cell suspensions disaggregated from FsaR tumour tissue reveals that nontreated tumours contained only a minor neutrophil population (around 3%), which raised dramatically following PDT treatment. The time point of 12 h post-PDT depicted in Figure 4 is within the period of peak levels of the PDT-induced neutrophil invasion with the FsaR tumor model (Cecic, de Vit, Sluiter and Korbelik, unpublished results). While over 40% of cells in PDT-treated tumours at that time point were neutrophils, this level decreased significantly, although not dramatically, in the samples obtained from mice treated with oxypurinol.

The impact of oxypurinol treatment on PDT response of FsaR tumours is shown in Figure 5. The chosen PDT dose resulted in a rapid ablation of treated tumours, and only a minor fraction of these lesions recurred several weeks later. The oxypurinol administration performed before PDT showed no significant effect on the initial PDT response, but it increased the rate of tumour recurrence and this resulted in statistically significant decline in tumour cures. In contrast, the administration of oxypurinol at 1 h after PDT exhibited no significant effect on the tumour response to PDT.

DISCUSSION

Treatment of FsaR tumours with Photofrin-based PDT results in a burst of elevated XO activity peaking immediately after the termination of photodynamic light delivery that rapidly declines and fades away within 30 min post-PDT (Figures 1 and 2). This

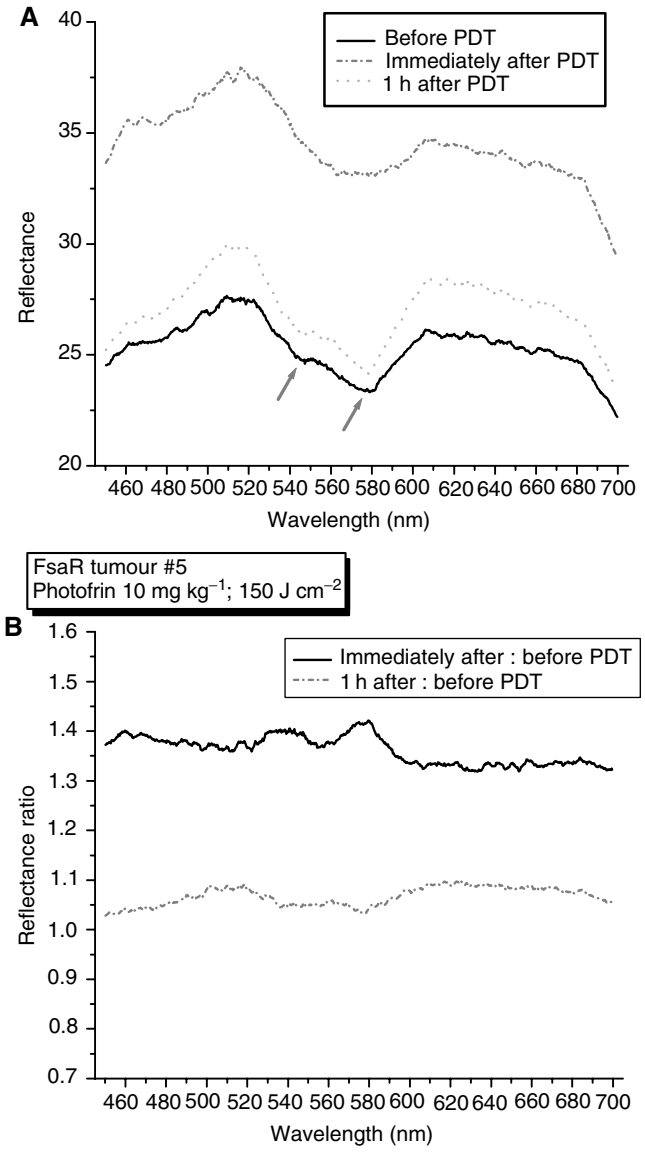

Figure 3 PDT-induced changes in the oxygenation of FsaR tumors monitored by reflectance spectroscopy. Subcutaneous FsaR tumours (six in total) were treated by Photofrin-based PDT as described for Figure 1. Visible light reflectance spectroscopy was performed with each tumour immediately before and immediately after PDT light delivery, as well as at 1 h after the termination of PDT light treatment. The results obtained with a representative tumour are shown as (**A**) reflectance spectra or (**B**) reflectance spectra ratios. The arrows identify characteristic troughs at 542 and 577 nm produced by oxyhaemoglobin.

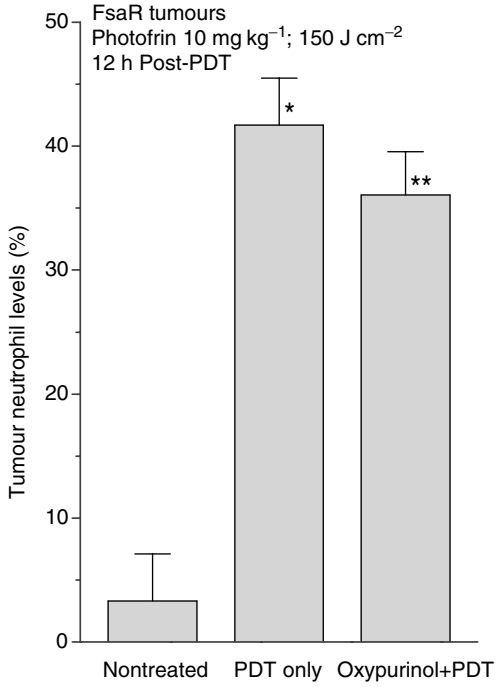

Figure 4 The effect of oxypurinol on the accumulation of neutrophils in PDT treated tumours. Subcutaneous FsaR tumours were PDT treated as described in Figure 1 and oxypurinol treatment was as described in Figure 2. Following their excision at 12 h post-PDT, the tumours were dissociated into cell suspensions, stained with antibodies for neutrophil identification, and analysed by flow cytometry. The results are presented as the percentage of neutrophils in total tumour cell populations. Bars are s.d., $n = 4$; *depicts statistically significant difference from the level in nontreated tumours ($P < 0.001$), **depicts statistically significant difference from the level in PDT-only group ($P < 0.05$).

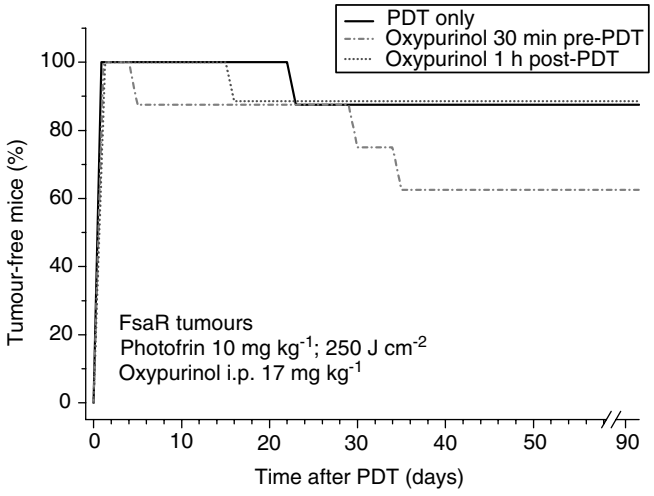

Figure 5 The effect of oxypurinol on the PDT response of FsaR tumours. Subcutaneous FsaR tumours were PDT treated as in Figure 2. Oxypurinol (17 mg kg^{-1} i.p.) was given to mice either 30 min before or 1 h after PDT light treatment. The mice were thereafter monitored for 90 days for signs of tumour growth. The difference in response between PDT-only group and PDT preceded by oxypurinol group is statistically significant ($P < 0.05$).

phenomenon correlates with the blood reperfusion in PDT-treated tumours occurring after an ischaemic period induced during the light treatment (Korbelik *et al*, 2000; van Geel *et al*, 1994). A combination of several factors is probably responsible for the generation of ischaemia in PDT-targeted tissues. One is the depletion of oxygen through its consumption by the photodynamic process (Foster *et al*, 1991; Bush *et al*, 2000). Also contributing is the reduced blood flow consequent to the vasoconstriction caused by inflammatory mediators like thromboxane whose release is known to be induced by PDT (Fingar *et al*, 1990, 1992), and blood flow stasis resulting from microvascular damage or obstruction by adhering neutrophils and platelets (Star *et al*, 1986; Henderson and Fingar, 1989; Fingar *et al*, 1992). As soon as its consumption drops with the termination of light delivery, oxygen will diffuse from the surrounding tissues into the ischaemic region. The reoxygenation will be supported by the vasodilating effect of mediators such as histamine and prostaglandins (Kamide *et al*, 1984; Henderson and

Donovan, 1989; Henderson and Dougherty, 1992), and nitric oxide (NO) produced by activated neutrophils (Cecic and Korbelik, 2002).

The occurrence of the above-discussed changes in tumour oxygenation is supported by the evidence obtained using visible reflectance measurement with FsaR tumours (Figure 3). Diffuse reflectance spectroscopy probes the scattering and absorption properties of tissue. Haemoglobin, one of the main chromophores in well-perfused tissues in the visible range, is characterised by the strong absorption bands of its oxygenated form (oxyhaemoglobin) at 542 and 577 nm (Zonios *et al*, 2001). These bands are lost when oxyhaemoglobin is converted into deoxyhaemoglobin under reduced oxygen concentrations, which enables the development of visible reflectance technique for noninvasive *in vivo* monitoring of changes in tissue oxygenation. In the spectral range 500–600 nm, the tissue penetration depth with this technique is up to 1 mm (Hillenkamp, 1979). This makes it adequate for monitoring the oxygenation of subcutaneous lesions such as FsaR tumours, particularly in mouse models since the skin overlying the tumours ($<200\,\mu$m) is thinner than human skin. The reflectance spectra that we obtained with a series of FsaR tumours before and after PDT were remarkably consistent and highly reproducible. At this point, presented are qualitative characteristics of dramatic PDT-induced changes in tumour oxygenation. They demonstrate that the oxygenation of FsaR tumours is markedly reduced at the end of PDT light delivery, but is restored to pretreatment or even higher values 1 h later (when these tumours were showing signs of a strong oedema). We are currently developing a ratio technique for processing data from pre- and post-PDT treatment spectra (based on a modification of the model described by Zonios *et al*, 2001) for deriving quantitative values for changes in tumour oxygenation. We have also carried out a preliminary examination of oxygen tension in FsaR tumours with Eppendorf pO_2 histograph. These measurements indicate that nontreated FsaR tumours are moderately oxygenated (average 39.5 mmHg) and that there is a greater than two-fold increase in these levels at 1 h post-PDT treatment as used in the reflectance spectrum protocol (M Korbelik, AI Minchinton, J Sun, unpublished results). Obviously, more investigation is warranted on the kinetics of oxygenation and blood perfusion changes in PDT-treated tumours as this is directly linked with the mechanism of antitumour effect of this modality, but such a task goes well beyond the scope of the present paper.

The reperfusion following an ischaemic episode is a well-recognised indication for the development of a classical physiological insult, the I/R injury (Zimmerman and Granger, 1994; Kilgore *et al*, 1999). This event is hallmarked by the burst in XO activity such as demonstrated in this report. Therefore, it can be concluded that the I/R injury was induced in PDT-treated FsaR tumours. The occurrence of this insult can obviously have important implications for the response of PDT-treated tumours.

Intense generation of superoxide mediated by XO is largely responsible for the damage inflicted by I/R injury (Parkins *et al*, 1998). The endothelium of the vasculature in PDT-treated tumours could sustain a heavy damage from the released superoxide and such event will have a powerful proinflammatory impact. The I/R injury is known to be associated with the activation of complement (a potent instigator of the inflammatory process whose engagement was recently demonstrated in PDT-treated tumours (Cecic and Korbelik, 2002)), proadhesive changes in the endothelium, release of various inflammatory mediators, PARP activation, and influx of activated neutrophils (Szabo and Dawson, 1998; Kilgore *et al*, 1999). Oxypurinol, which inhibits the PDT-induced XO activation (Figure 2), also attenuates the accumulation of neutrophils in PDT-treated tumours (Figure 4). In an earlier report, we have shown that the oxypurinol treatment reduces, as well, the extent of systemic neutrophilia that develops in mice bearing PDT-treated tumours (Cecic and Korbelik, 2002). These observations suggest that the impact from I/R injury contributes to the induction of neutrophil invasion into the tumours treated by PDT.

Evidence that superoxide formation is associated with PDT treatment was provided in several earlier studies. In addition to our finding that intravenous SOD administration immediately after photodynamic light treatment decreases the cure rate of PDT-treated mouse FsaR and EMT6 tumours (Korbelik *et al*, 2000), Athar *et al* (1989) have shown that the effect of PDT on mouse skin can be augmented by an SOD inhibitor and diminished by an SOD mimic. Using ESR spectroscopy, the superoxide production was also documented in PDT-treated tissues (Athar *et al*, 1988) and in photoirradiated aqueous photosensitiser liposomal preparations (Hajdur *et al*, 1997a, b).

Superoxide and its dismutation product hydrogen peroxide have been described as stimulators of transcriptional activation of stress proteins (Demple *et al*, 1999). On the other hand, PDT-generated tumour ischaemia can elicit a specific stress response known to activate hypoxia-inducible factor-1 (HIF-1) that can be responsible for the induction of VEGF expression (Ferrario *et al*, 2000), and activation of early response genes including cyclooxygenase-2 and inducible nitric oxide synthase (Hierholzer *et al*, 2001). Thus, in addition to the primary stress response triggered by singlet oxygen generated directly in photodynamic reactions (Dougherty *et al*, 1998), several forms of secondary stress may be inflicted in PDT-treated tumours: oxidative stress mediated by superoxide, oxidative and nitrosative stress mediated by NO (Korbelik and Cecic, 2003), and hypoxic stress. The extent of PDT-induced injury and its contribution to the antitumour effect of this modality is likely to differ depending on the type of treated tumour and photosensitiser class used for PDT. It may also be influenced by the duration of PDT light treatment and the dose rate, although we have not observed significant differences following the treatment with 150 and 250 J cm^{-2}, both delivered at around 110 mW cm^{-2} (Figures 1 and 2). The rate of xanthine dehydrogenase conversion into XO during ischaemia differs in various tissues. In the ileum, nearly complete conversion occurs within 10 s, whereas in the heart XO levels double after 8 min of nonperfusion (McCord, 1985). Similar differences can be expected to exist among different types of tumours. Therefore, the extent of PDT-induced I/R injury, which with FsaR tumours is obviously contributing to the therapy outcome (Figure 5), is likely to vary in different types of lesions. With respect to the photosensitisers used for PDT, in addition to the XO activation following Photofrin-based PDT described in this study, we have evidence of a similar effect produced by benzoporphyrin derivative-based PDT (Korbelik and Cecic, 2003).

An important element that can influence the extent of I/R injury in PDT-treated tumours is the extent of endogenous NO production, which varies among solid human and animal tumours (Parkins *et al*, 1995; Tozer and Everett, 1997). NO reacts rapidly with superoxide forming peroxynitrite anion (Blough and Zafiriou, 1985; McCall *et al*, 1989) and this can result in superoxide detoxification (Wink *et al*, 2001). This element may contribute to the observed tendency of an increased resistance to PDT of tumours characterised by elevated intrinsic NO production (Korbelik *et al*, 2000). Low NO-producing tumours were shown to be more profoundly affected by transient clamping of blood vessels feeding subcutaneous tumours that results in I/R injury associated with substantial tumour cytotoxicity (Parkins *et al*, 1985, 1988).

In conclusion, this work demonstrates that PDT can induce I/R injury and the extent of this insult may be sufficiently pronounced to have an important impact on the therapy outcome. The infliction of I/R injury is a classical proinflammatory event, and we suggest that it participates in the induction of inflammatory response that has a major role in the antitumour effect of PDT (Korbelik and Cecic, 2003).

ACKNOWLEDGEMENTS

This study was supported by the Canadian Institutes of Health Research (Grant MT-12165). The photosensitiser used in this study was provided by Axcan Pharma Inc. The help of Ivana Cecic in reflectance measurement and Denise McDougal in flow cytometry is gratefully acknowledged.

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