Magnetic Resonance Imaging Evaluation of Photodynamic Therapy-Induced Hemorrhagic Necrosis in the Murine M1 Tumor Model

BeAtrice G. Winsborrow1, Hiltrud Grondey1, Huguette Savoie1, Colin A. Fyfe1,2, and David Dolphin*1

1Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada and
2Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

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ABSTRACT

Proton magnetic resonance imaging (MRI) and histological methods were used to evaluate photodynamic therapy (PDT)-induced hemorrhagic necrosis in the murine M1 tumor within 72 h of treatment of male DBA/2 mice. The effects of three photosensitizing drugs were investigated: Photofrin (n = 4), Zn (II) phthalocyanine (n = 7) and benzoporphyrin derivative monoacid ring A (n = 11). As noted in previous studies of PDT using MRI, MRI makes possible serial, noninvasive, in vivo observation of tissue response to PDT. Our serial study of MRI and histological data confirms that tumors responded in the same way to PDT treatment using the three photosensitizing drugs: vascular damage followed by hemorrhagic necrosis. Most importantly and unlike previous MRI studies of PDT, we used a very high field magnet that enhanced the effect of magnetic susceptibility on image signal when blood is processed by the body after PDT-induced hemorrhagic necrosis. This last finding demonstrates the utility of high field magnets and the importance of localized, serial experiments in future magnetic resonance studies of PDT.

INTRODUCTION

Photodynamic therapy (PDT) is based on the cytotoxic effect of sensitizing drugs photoactivated in the presence of oxygen. Most of the clinical work on PDT has used either hematoporphyrin derivative (HPD) or Photofrin, both preparations contain a large number of porphyrin derivatives. New, pure sensitizing drugs are being developed with several design objectives in mind such as, enhanced photodynamic effect, reduction of drug retention time in the patient, and tissue specificity (1). The most generally accepted mechanism of PDT activity is the formation of singlet oxygen (1). The intrinsic properties of water in normal, necrotic and viable tumor tissue can be used in proton (2). Magnetic resonance (MR) experiments to identify discrete regions within the tissue anatomy. These properties, tissue water concentration (proton density) and 1H nuclear spin relaxation times (T1 and T2) in tissue, have been the focus of a number of recent PDT-related 1H MR studies (5-8) and MR studies using other nuclei (8-11). In this study, we use 1H MRI methods to evaluate PDT-induced hemorrhagic necrosis in the murine M1 tumor (12). The effects of three photosensitizing drugs are investigated: Photofrin, Zn (II) phthalocyanine (ZnPc) (13) and a chlorin-like benzoporphyrin derivative monoacid ring A (BPD-MA, Venteporfin)(14). Unlike previous MRI studies of PDT, we used a very high magnetic field strength. Consequently, our results show a significant effect of hemorrhagic necrosis on image signal. This effect should be an important factor in the design of future PDT-associated MRI and MRS studies.

MATERIALS AND METHODS

Animals and tumors. Male DBA/2 mice (20-25 g, Charles River Laboratories, St. Constant, Canada) were housed with alternating 12 h cycles of light and dark. They had free access to food and water. A cell suspension of the M1 tumor model, 3-methylcholanthrene-induced rhabdomyosarcoma (15), was injected intradermally 4.3 cm below the animal's neck, just to the right of the spine. When the tumors attained a size of about 3.5-7.0 mm in diameter, approximately 11 days postinoculation, they were treated by PDT. Photosensitizers and PDT. Treatment included the intravenous injection of a photosensitizing drug via the tail vein and then irradiation of the tumor with light. During the time following the injection of a photosensitizer or placebo, the animals were kept in the dark. The three photosensitizing drugs were: BPD-MA (Quadrac Logic Technologies, Vancouver, Canada), Photofrin II (QLT) and ZnPc (Novartus, Basch, Switzerland and QLT). The drug dose and the delay between injection and irradiation for each photosensitizer was 2 mg/kg by weight, 3 h for BPD-MA (n = 11); 2.5 mg/kg

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by weight, 24 h for Photofrin® (n = 4); and 0.125 mg/kg by weight, 48 h for ZnPc (n = 7). The mice were exposed to filtered red light (610–750 nm) from a 250 W quartz–tungsten-halogen lamp (Oriel, model 66184, Stratford, CT) fitted with a 4 cm diameter water filter (Oriel, model 61945) and a filter of appropriate wavelength for each drug. The illumination field was 7 mm in diameter from a fiber optic light source (6 mm diameter) held 1 cm immediately above the tumor. The fluence rate was 312 mW/cm² as measured by a Gentec power meter (Gentec, Plattsburgh, NY) and the duration of irradiation was 24 min for a net fluence of 450 J/cm².

Two control groups were used in this study. In one, BPD-MA was injected intravenously and the tumor was not irradiated with light (n = 11). In the other, mice were irradiated with the same light dose used for the treated groups, but instead of photosensitizer they were injected intravenously with the drug delivery vehicle (liposomes for BPD-MA and ZnPc and saline for Photofrin®) prior to light treatment (n = 8). The liposomes were provided by QLT.

**MRI.** Proton images were acquired with a Bruker MSL-400 spectrometer equipped with a 9.4T vertical bore magnet and microimaging accessories. The clear space within the sleeve containing the field gradients was 2.5 cm. A mouse holder was constructed of plastic such that the tumor could be reproducibly positioned within the magnetic field with a 7.5 mm inner diameter (i.d.) single turn radio frequency (r.f.) surface coil (16) placed around the tumor. Both the holder and r.f. coil were built “in-house.” The r.f. circuit was tuned to 400.13 MHz proton frequency. In the holder, the mice were physically immobilized, without causing pain, minimizing the amount of anesthesia required. The anesthesia, 50 mg/kg ketamine (Ketalean®, MTC Pharmaceuticals, Cambridge, Canada) with 10 mg/kg midazolam (Versed®, Hoffmann-La Roche Ltd., Mississauga, Canada), was injected intraperitoneally. Serial images were obtained before and after injection of photosensitizer or placebo and at 1, 24, 48 and 72 h after light irradiation. B. field homogeneity within the tumor was optimized by shimming on its 1H O time domain signal. Coronal (plane of the r.f. coil, perpendicular to the incident light), transverse (perpendicular to the r.f. coil plane and spine length, parallel to the incident light) and sagittal (perpendicular to the r.f. coil plane and parallel to both the incident light and spine length) cross-sectional images were acquired with a spin echo pulse sequence (repetition time [TR] = 0.55 s, time to echo [TE] = 32 ms). The total acquisition time was 4.3 min for 128 gradient increments with four scans each, yielding images with a 256 × 256 matrix after zero filling and a 1.6 cm field of view to give an in-plane resolution of 126 × 126 μm. A 4 ms sinc r.f. excitation pulse was used in a 2.4 G/cm gradient to select a 1 mm thick slice. A 22 μs 180° “hard” pulse refocused the excited region within 4 mm above and below the plane of the r.f. coil. Each imaging session took 45–60 min.

**Histology.** In order to correlate the MRI images with tumor morphology, a proportion of the animals were sacrificed at each time point in the imaging series and their tumors underwent histological evaluation. The mice were euthanized by cervical dislocation after overanesthesia (halothane, MTC Pharmaceuticals, Cambridge, Canada). The spatial relationships between the tissue sections and the imaging planes were maintained by marking one ink spot on both sides of the tumor surface perpendicular to the spine. The tumor was then removed and fixed for histology without tissue shrinkage using Bouin’s solution. Then 5 μm thick sections were cut from the transverse plane of the tumor and then stained with hematoxylin and eosin (H&E).

**RESULTS AND DISCUSSION**

**Histology and MRI of untreated tumors**

None of the tumors in the two control groups, treated by PDT without either sensitizer or light, were affected up to 72 h after treatment; the tumors continued to grow without the development of PDT-induced necrosis. The transverse tissue section in the left column of Fig. 1, row A was taken from a control tumor 48 h after injection of BPD-MA without light irradiation. The tumor cells were viable, except for a small pocket of solid necrotic cells with karyorrhectic (fragmented and shrunken) nuclei a few millimeters below the tumor surface. The tumor was well vascularized with healthy blood vessels, excluding the necrotic area that was devoid of blood vessels. It is suspected that this region underwent spontaneous necrosis due to the absence of any local blood supply. Below the tumor, a ruptured blood vessel with a small pool of old blood with a lot of lymphatic fluid can be observed. Such pools were noted in all of the control tumors and are likely caused by the pressure exerted on the underlying tissue by the enlarged, untreated, intradural tumor. In 17 of the 19 control tumors, a small amount of necrotic tissue was observed histologically in the region of the pooled blood. Presumably, the cells had necrosed spontaneously because the pooled blood was no longer oxygenated.

The corresponding, transverse, in vivo MRI image of the tumor region from which the above-mentioned histological section was taken is presented in the right column of Fig. 1, row A. The two small bright signal patches that appear above, to each side of the tumor are signals from the circular water reference sample placed above the r.f. coil (see the depiction in the central column of Fig. 1A). Because the reference sample is resting on the r.f. coil and the i.d. of the coil is 7.5 mm, the size of the tumor can be estimated in the image. The brighter signal at the sides of the tumor, nearest to the r.f. coil, is caused by a nonuniform B₁ field, which is a characteristic feature of surface coils. Under these conditions, qualitative rather than quantitative analysis of signal intensity is feasible. The top of this tumor protruded out of the r.f. coil such that the top 2 or 3 mm are not observed in the image. The small circle of dark signal near the top left of the tumor image corresponds to the solid necrotic region observed in the histological section. The signal void at the bottom of the image can be attributed to the pooled blood and lymphatic fluid observed at the base of the tumor (vide infra).

**Histology and MRI of treated tumors**

Within an hour of light treatment, the same observation was made for all three experimental groups (Photofrin®, BPD-MA, ZnPc). While histological examination revealed that the blood vessels were intact with damaged epithelial cells, particularly within a few millimeters of the tumor surface where the irradiating light was incident, the MRI images were the same as before irradiation. This suggests that the factors influencing the relative image signal intensity (water concentration and water proton signal relaxation) are not changed 1 h after irradiation under the conditions used. However 23 h later, histology showed that the blood vessels were no longer intact and hemorrhagic necrosis was observed in the tissue sections near the tumor surface. The MR images show a lack of signal in the regions of histologically detected necrosis (results not shown).

Forty eight hours after light treatment, larger areas of necrosis and significant blood vessel damage were detected histologically deeper into the tumor (Fig. 1B–D, respectively, Photofrin®, BPD-MA, ZnPc). With all three drugs, the pattern of necrosis is dependent upon tumor size at the time of light irradiation and not the choice of drug. Smaller tumors (<3.8 mm thick and <5.3 mm in diameter) were completely necrotic; the nuclei were karyorrhectic and the tissue...
Figure 1. Tissue section, descriptive representations and MRI images of murine MI tumors 48 h post-PDT treatment using no drug (row A), Photofrin® (row B), BPD-MA (row C) and ZnPc (row D). All representations are transverse cross sections of the tumors, parallel to the incident light, where the top of each figure corresponds to the tumor surface. The H&E-stained tissue sections (left column) were photographed with green-filtered light at 2.5× magnification. The letters in the descriptive representations (central column) summarize the general histological findings: tumor cells with karyorrhectic, K, or pyknotic, P, nuclei; viable tumor, V; scab, S; blood, B; old blood and lymphatic fluid, F. The dark, filled circles with open circles above represent, respectively, the r.f. coil and water reference sample used in the imaging experiments. The MRI images (256 × 256 matrix, four averages, 1 mm thick slice) in the right column were obtained using a spin echo imaging sequence (TR/TE = 0.55 s/32 ms) and have an in-plane resolution of 126 × 126 μm over a 1.6 cm field of view.
was essentially a dry crust. (Fig. 1C, left column). The transverse MRI image of this tumor (Fig. 1C, right column) has a signal void for the entire area of the tumor. Although the width of the incident light (7 mm) was greater than that of the larger, rounded tumors (≥3.8 mm thick and ≥5.3 mm in diameter, 10 out of 22), only the top central portion of the tumor surfaces closest to the light source received the full light dose. Accordingly, the larger tumors displayed a range of tissue damage (Fig. 1B, D, left column). Within the region coincident with the dark signal of the image (Fig. 1B, D right column), there was a mixture of necrotic tissue, with both karyorrhectic and pyknotic (dense and shrunken) nuclei, lysed cells, lymphatic fluid and blood, as shown schematically in Fig. 1B and D (central column). Cells close to the surface had karyorrhectic nuclei. Deeper into the tumor, the cells were necrotic with pyknotic nuclei. The tissue corresponding to the areas of bright signal at the sides of the tumor in the MRI images of Fig. 1B and D was viable. Below the larger treated tumors (≥4.5 mm thick, 5 out of 22), pooled blood and small regions of spontaneous necrosis were observed. As noted for the control tumors, this old blood was from vessel rupture under pressure exerted by the larger, intradermal tumor masses; the lack of oxygenated blood is considered the cause of the spontaneous necrosis (vide infra).

The presence of necrotic regions in the histological sections 48 h post treatment is reflected in the MRI images where the signal void corresponds well with the size and location of the necrotic tissue (Fig. 1B–D). This observation was also made 72 h after irradiation (result not shown), except there is less MRI signal within the tumor area and increased necrotic tissue than at 48 h after treatment. The evolving trend in signal loss in the images obtained over 72 h for all three experimental groups suggests that necrosis begins at the tumor surface and moves deeper into the tumor. This is in agreement with the histological observations.

These results are not at all like those reported in other MRI studies of PDT-treated murine tumor (5) and normal rat brain tissue (6). Where we observed signal loss in the MRI image in necrotic areas, the other studies found that necrotic tissue had more signal intensity than viable tissue. The $T_1$ and $T_2$ relaxation times of water in necrotic tissue were reported to be longer than in viable tumor tissue (5).

**Dependence of image signal on imaging parameters**

In order to find the cause of the signal loss that we observed and to relate it to the tissue condition, we investigated the dependence of the signal intensities upon the spin echo MRI experimental parameters ($TR$ and $TE$). In general, the spin echo signal intensity, $S$, is dependent upon the water concentration (proton density, $p$) and the water proton relaxation times ($T_1$ and $T_2$) (17):

$$S \propto p \rho \exp(-\frac{TE}{T_2}) + \mu G_2^2TE^3DD/12.$$ 

(1)

Accordingly, when $TE$ was decreased from 32 ms (Fig. 2A, as in Fig. 1B) to 7 ms (Fig. 2B), the contrast pattern changed for the tumor image obtained 48 h post-PDT treatment. In the areas where only necrotic tissue was present, without blood or lymphatic fluid, the relative signal is brighter with a 7 ms $TE$ than a 32 ms $TE$. This suggests that the signal contrast is weighted by $T_2$ relaxation. Interestingly, when $TR$ was increased from 0.55 s (Fig. 2B) to 1.55 s (Fig. 2C), no difference in the relative signal intensity of the low intensity region at the center of the tumor was observed (compare with the reference water signal in each image for scale). This indicates that the signal contrast in the necrotic region is not weighted by $T_1$ relaxation.

Our results show only that $T_1$ and not $T_2$ weighting affects the image contrast; this trend was also observed in a study of magnetic susceptibility effects (18). The source of the magnetic susceptibility change in parts of the tumor may be the particular biochemical form of iron as it is processed after blood vessel rupture (19). As part of the hemorrhaging process, iron is chelated to either short-term iron transport proteins (ferritin) or to long-term storage proteins (hemoglobin). These particles are superparamagnetic with very large magnetic susceptibilities that will establish different effective local magnetic fields (19). In this case, a more appropriate description of the MRI signal intensity, $S$, is (17)

$$S \propto \rho \exp(TE/T_2) + \gamma G^2TE^3/D/12.$$ 

(2)

The signal depends on the magnetic field gradient ($G$) and the diffusion coefficient ($D$) of water through the tissue, in addition to water density ($p$), $TE$, $T_2$, and the proton gyromagnetic ratio ($\gamma$, an intrinsic nuclear property). The differences in susceptibilities at the boundary of the tissue containing superparamagnetic blood products and the surrounding diamagnetic tissue are the source of $G$. In these regions, if sufficient diffusion occurs during the imaging experiment, $T_2$ relaxation will be enhanced. This effect will cause signal loss at those boundaries on $T_2$-weighted images, without any signal loss on $T_1$-weighted images. This provides an explanation for the observation that the regions of low signal is the shortest $TE$ images (Fig. 2B, C) correspond directly to the tissue boundaries between viable or necrotic tissue and regions of pooled blood and lymphatic fluid that contain the blood breakdown products ferritin and hemoglobin (Fig. 2D). In addition, this also identifies the cause of the signal void below the tumors in the images in Fig. 1C because lymphatic fluid was observed histologically at the bottom of these tumors, including the untreated tumors.

The question at hand then is why has this effect not been observed in other MRI studies of PDT if hemorrhagic necrosis will occur with the tumor types and drugs used (2,4)? The magnetic susceptibility effect is proportional to the external magnetic field, $B_0$. The magnetic field used in the current study is at least twice as strong as that used by the other groups (5–8), thereby significantly increasing the influence of this effect on our results. To some extent, magnetic susceptibility did also affect the previous experiments. In one of these studies (6), reduced signal can be observed in the images within 24 h of PDT where higher signal is present at later times. This change in signal may be an indication of the change in the biochemical state of iron (19). In another study (5), an unexplained thin band of low signal can be observed between edematous and necrotic regions and between necrotic and viable regions. This may be due to blood breakdown products because the histological results indicate the presence of blood. In both studies, image intensities did not correlate well with the calculated signal intensities, based on the evaluation of $T_1$ and $T_2$ relaxation times for defined
tissue regions. Although the discrepancy was attributed to increased water content (5), magnetic susceptibilities may have played a more important role. Finally, in an MRI study of contrast agent uptake in mammary adenocarcinoma after PDT, the predicted effect of Gd-DTPA (gadolinium-diethylenetriamine pentaacetic acid) on tumor MRI signal was considered valid only at low concentrations (8). In the simulations, theoretical errors described as noise were included in the determination of relaxation results. However, above a limiting concentration of Gd-DTPA, the Gd-DTPA relationship to paramagnetic relaxation was too sensitive to these "errors" and consequently such data were not considered. It is our opinion that the limit may possibly be the result of magnetic susceptibility effects because it has been shown in an independent study (18) that at elevated Gd-DTPA concentrations, the paramagnetic Gd-DTPA becomes superparamagnetic and behaves as a negative contrast agent.

Because superparamagnetism may be present in discrete regions within the tumor, it would be advisable to perform localized MR experiments, rather than risk averaging the results over larger areas. This suggestion is supported by a number of in vivo MR studies of PDT effects using protons as well as other nuclei. The above-mentioned MRI study of Gd-DTPA is an excellent example of nonuniform tumor response to PDT (8). One 31P MRI study demonstrated the heterogeneity in regional tumor blood flow and regional effects of PDT within RIF-1 tumors (9). Moreover, a 31P MRS study has demonstrated heterogeneous tumor tissue effects of PDT by monitoring the high energy phosphorous metabolites within RIF-1 tumors (10).

Our serial MRI and histological study confirms that the photosensitizing drugs used here (Photofrin®, BPD-MA and ZnPc) have the same response to PDT treatment up to 72 h after light irradiation: vascular damage followed by hemorrhagic necrosis. Most importantly, unlike previous MRI studies of PDT, we used a very high field magnet that enabled us to show the effect of magnetic susceptibility on image signal when blood is processed by the body 24 h after PDT-induced hemorrhagic necrosis. Future work that could take advantage of the sensitivity to magnetic susceptibility effects at very high magnetic field strengths should include dynamic studies of T2* in gradient echo imaging experiments. Such experiments can yield regional information on diffusion of water and magnetic differences within the tumor. Both parameters should enable further understanding of tumor response to PDT treatment.

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