Reduction of Vascular and Permeable Regions in Solid Tumors Detected by Macromolecular Contrast Magnetic Resonance Imaging after Treatment with Antiangiogenic Agent TNP-470

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ABSTRACT

Purpose: The availability of noninvasive techniques to detect the effects of antiangiogenic agents is critically important for optimizing treatment of cancer with these agents. Magnetic resonance imaging (MRI) is one such noninvasive technique that is routinely used clinically.

Experimental Design: In this study, we have evaluated the use of MRI of the intravascular contrast agent albumin-GdDTPA to detect the effects of the antiangiogenic agent TNP-470 on the vascular volume and permeability of the MatLyLu prostate cancer model.

Results: TNP-470-treated tumors demonstrated a significant decrease of vascular volume, as well as a significant reduction in vascular and permeable regions, compared with volume-matched control tumors. Although the fractional volume of permeable regions in the tumor decreased, the average value of tumor permeability did not decrease significantly. This was attributable to increase in permeability in some regions of the tumor. These regions were mostly associated with low vascular volume. ELISA assays of control and treated MatLyLu tumors also detected a significant increase of vascular endothelial growth factor in the TNP-470-treated tumors.

Conclusion: MRI detected significant changes in tumor vascular characteristics after treatment with TNP-470.

INTRODUCTION

Antiangiogenic therapy is justifiably an exciting area of cancer therapeutics (1–4). As antiangiogenic therapies evolve, the requirement of techniques that can noninvasively monitor the effects of agents on tumor vasculature is becoming increasingly apparent, because these treatments frequently do not cause tumor regression. Therefore, without functional evaluation of the vasculature, it can be difficult to detect the effects of antiangiogenic treatment. MRI (5) and MRS (6) are noninvasive techniques that may become mainstream methods of choice to detect and understand the effects of antiangiogenic agents in experimental tumors models, and in the clinical setting. Depending on the type of contrast agent used or the sequence used, MR can currently provide information on a wide array of vascular and metabolic parameters (5). Such functional information can be useful not only to detect the response of tumors to the treatment but also to understand how the treatment alters vascular or metabolic characteristics. The insights gained with this information can be used to further refine the treatment.

Studies describing the use of MRI to detect antiangiogenic treatment are relatively few and have primarily been performed using antibodies targeted to VEGF in preclinical tumor models of the breast, ovary, and brain (6–8). A significant reduction of microvascular permeability in these preclinical models was detected with MRI after treatment with anti-VEGF antibodies.

In the study described here, we have used MRI to quantify changes in vascular volume and permeability after treatment with the antiangiogenic agent TNP-470. TNP-470 is a fumagillin derivative that has been shown to block angiogenesis by the inhibition of endothelial cell proliferation (9–11). TNP-470 has been observed to inhibit tumor growth in several preclinical models (12–14) as well as in clinical studies (15, 16). One molecular target of TNP-470 was recently identified to be methionine aminopeptidase-2 (MetAP2), although the precise mechanism underlying its selective effect on the proliferation of endothelial cells is yet to be understood (17, 18). We observed a significant reduction in vascular and permeable regions of the tumor after treatment with TNP-470. However, values of permeability increased in some regions of the treated tumors. These changes in permeability led us to evaluate VEGF levels in the treated tumors compared with control tumors, suggestive of a “compensatory” type mechanism.
MATERIALS AND METHODS

Studies were performed using the MatLyLu tumor model, which is an invasive and metastatic rat prostate cancer model (19). Prostate cancer cells were inoculated s.c. into the right flank of male severe combined immunodeficient mice, in a volume of 0.05 ml of HBSS (Sigma Ltd.) at a concentration of $10^6$ cells/0.05 ml. Cells in culture were maintained in RPMI 1640 (Life Technologies, Inc.) containing 300 mg/liter l-glutamine, supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μg/ml). In our initial protocol, mice were treated with 6.7 mg/kg s.c. for 7 days ($n = 10$; Ref. 12). However, this protocol was subsequently switched to 30 mg/kg ($n = 5$) given every 2nd day for a total dose of 90 mg/kg (20), because the rapid growth of MatLyLu tumors resulted in large control tumors at the end of the 7-day protocol. Both treatment protocols induced similar changes in tumor vasculature. Control animals were either injected with the vehicle using the 30 mg/kg protocol ($n = 3$) or were untreated ($n = 5$). There were no differences in vascular characteristics or growth rate between the vehicle-treated or the untreated tumors. Analyses of MRI data were performed using volume-matched treated tumors ($n = 9$) and control tumors ($n = 8$). Tumor volumes were calculated from caliper measurements using the equation, volume = $(\pi/6) \times a \times b \times c$, where $a$, $b$, and $c$ represent three orthogonal axes of the tumor, as well as from the $M_o$ map of MRI data. Mean matched tumor volumes ±1 SE used in the study were $442 ± 23$ mm$^3$ for the treated group and $430 ± 38$ mm$^3$ for the control group, using caliper measurements. Tumor volumes calculated from MRI data were $418 ± 18$ mm$^3$ for the treated group and $419 ± 42$ mm$^3$ for the control group. In the case of age-matched tumors, rapidly growing control tumors are significantly larger than slower-growing treated tumors at the end of treatment. As tumors grow, vascularization frequently decreases. Because antiangiogenic treatment also affects vascularization, differences in vascular characteristics between treated and control tumors may get masked for age-matched tumors. In this case, it is more valid to use volume-matched tumors.

Imaging studies were performed on a 4.7T GE Omega spectrometer as described previously (21). Briefly, mice were anesthetized with a mixture of ketamine (25 mg/kg; Aveco, Ltd., Fort Dodge, IA), acepromazine (2.5 mg/kg; Aveco), and 0.9% NaCl solution (1:1:2 by volume). The tail vein was catheterized before placing the animal in the magnet. Multislice relaxation rate ($T_1^{-1}$) maps were obtained by a saturation recovery method combined with fast $T_1$ Snapshot-FLASH imaging (flip angle of 10°, echo time of 2 ms). Images of 4–6 slices (slice thickness of 1 mm) acquired with an in-plane spatial resolution of 0.125 mm (128 × 128 matrix, 16-mm field of view, NS = 8) were obtained for three relaxation delays (100 ms, 500 ms, and 1 s) for each of the slices. An $M_o$ map with a recovery delay of 7s
was acquired once, at the beginning of the experiment. Images were obtained before i.v. administration of 0.2 ml of 60 mg/ml albumin-GdDTPA in saline (dose of 500 mg/kg) and repeated every 6 min, starting 3 min after the injection, up to 34 min. Relaxation maps were reconstructed from data sets for three different relaxation times and the $M_0$ dataset on a pixel-by-pixel basis. At the end of the imaging studies, the animal was sacrificed, 0.5 ml of blood was withdrawn from the inferior vena cava, and tumors were marked for referencing to the MRI images, excised, and fixed in 10% buffered formalin for sectioning and staining.

Vascular volume and PSP maps were generated from the ratio of $\Delta(1/T1)$ values in the images to that of blood. The slope of $\Delta(1/T1)$ ratios versus time in each pixel was used to compute PSP, and the intercept of the line at zero time was used to compute vascular volume. Thus, vascular volumes were corrected for permeability of the vessels. The fractional tumor volume with detectable vascular volume or PSP, values of vascular volume and PSP, as well as non-zero (detectable) values of vascular volume and PSP averaged over the entire tumor were determined. Three-dimensional volume data were processed with an operator-independent computer program that enabled selection, mapping, and display of the regions. Volume fractions of the regions were determined using the histogram analysis of the volume data. Values of vascular volume and PSP were computed for every voxel in the tumor. Thus, it was possible to determine the frequency of occurrence of values of vascular volume and PSP within the tumor, as well as to determine the region of the tumor occupied by specified values (21). The routine was written with Interactive Data Language (IDL, Research Systems, Boulder, CO) and is compatible with most operation systems.

Analysis of VEGF expression in control and in TNP-470-treated tumors was performed as follows. Tumors were removed and freeze-clamped in liquid nitrogen. The frozen tumor tissue was pulverized in liquid nitrogen and homogenized in a ratio of 1:10 in an extraction buffer containing 20 mM HEPES, 0.75 mM EDTA (pH 7.4), and protease inhibitors (Boehringer Mannheim, Indianapolis, IN). Homogenates were centrifuged at 14,000 rpm for 30 min, and the supernatants were used for VEGF analysis. Protein concentrations were estimated using the Bio-Rad DC assay (Bio-Rad, Melville, NY), which is based on the method of Lowry et al. (22). Fifty $\mu$g of tumor tissue homogenate (MatLyLu tumors) were used in the ELISA assay. Each sample was measured in duplicate. VEGF was measured by the Quantikine M (R&D Systems Inc., Minneapolis, MN) ELISA assay. This protocol provided consistent results with previous measurements (23).

Fig. 2. Triplanar views of three-dimensional reconstructed maps of (a) vascular volume (red), (b) PSP (green), (c) fused vascular volume and PSP maps, and (d) histological sections, stained with H&E, obtained from the untreated tumor shown in Fig. 1. A region of high permeability in the PSP map (b) and the corresponding area of necrosis in the histological map (d) are marked by arrows.
assay shows cross-reactivity with rat VEGF (70–80% cross-reactivity).

The experimental protocol was approved by the Institutional Animal Care and Use Committee. Statistical analyses were performed using an unpaired $t$ test. $P$-values of $<0.05$ were considered significant.

RESULTS

Multislice maps of (a) vascular volume and (b) PSP, together with (c) a histological section obtained from the central slice of a control tumor, are shown in Fig. 1. MatLyLu tumors were typically well vascularized and, as evident in Fig. 1, detectable levels of vascular volume and PSP were observed through most of the tumor. Triplanar views from three-dimensional reconstructed maps of the tumor in Fig. 1 of (a) vascular volume displayed in red, (b) PSP displayed in green, (c) fused vascular volume and PSP, and (d) histology are shown in Fig. 2. The three-dimensional volume data were visualized using a graphics-accelerated volume rendering technique supported on Silicon Graphics systems, (SGI Inc, Silicon Valley, CA). We used our dedicated in-house software Vortex, which uses the three-dimensional texture mapping hardware, to perform the volume rendering. Texture mapping provided interactive rendering speeds necessary to control transfer functions that map the voxel data to suitable color, intensity, and blending values. This facilitated effective visualization of the shape and distribution of the voxel data fairly easily and quickly. The salient features in these images were that regions of high permeability typically exhibited low vascular volume and that both vascular volume and PSP were detected throughout the tumor. We have previously observed in other tumor models that areas of high vascular volume and high PSP do not coincide spatially (21). Also apparent in this figure is a region of high permeability (Fig. 2b, arrow) that coarsely matched an area of cell death in the histology section (Fig. 2d, arrow).

Raw 1 s saturation recovery images obtained from a single slice from this tumor, at different time points, are shown in Fig. 3a. The corresponding relaxivity ($1/T_1$) maps derived for this slice (using 100-ms, 500 ms, 1 s and 7 s saturation recovery intervals) at different time points. Maps of (c) vascular volume and (d) PSP derived from the relaxivity maps for this slice. High magnification photomicrographs from (e) viable, high vascular volume and low permeability regions, and (f) dying, low vascular volume and high permeability regions, obtained from a 5-μm-thick H&E-stained section from this slice.
slice (using 100 ms, 500 ms, 1 s, and 7 s saturation recovery intervals) at different time points are shown in Fig. 3b. Maps of vascular volume and PSP derived from the relativity maps for this slice are shown in Fig. 3, c and d. High magnification photomicrographs from viable, high vascular volume and low PSP regions, and dying, low vascular volume and high PSP regions, obtained from a 5-μm thick H&E-stained section obtained from this slice are also shown in Fig. 3c and f respectively. The viable high vascular volume and low PSP regions show minimal extravasation of RBCs into tissue from the vessels. In contrast, vessels in the dying regions of the tumor, in which regions of low vascular volume and high PSP typically occur, almost always exhibit significant extravasation of RBCs into tissue, demonstrating that this vasculature is hyperpermeable. Within the 35-min period over which post-contrast-images were obtained, diffusion and convection of this large macromolecular agent within the tumor was virtually negligible. This is apparent from the 1 s saturation recovery images, as well as the relativity maps. The regions of high PSP were discrete, usually adjacent to low vascular volume regions, and did not increase spatially at the later time points. This, together with the histology data, provide further support that the parameters measured were representative of vascular volume and PSP, and that the occurrence of high PSP in regions with low vascular volume was not caused by diffusion or convection.

Plots of contrast media concentration versus time for representative voxels from within a tumor are shown in Fig. 4. These voxels are representative of high vascular volume and low PSP (Fig. 4a) or low vascular volume and high PSP regions (Fig. 4b). The linear fits have a correlation of 0.96 or higher. On the basis of the signal to noise levels of our images, we estimated the minimum detectable vascular volume to be 0.1 μl/g and the minimum detectable PSP to be 0.02 μl/g/min. The advantage of using an extrapolation to time zero to compute vascular volume corrected for PSP, was that the later time points yielded a higher signal to noise ratio, because of permeability increasing the change in contrast. Within the 34 min time period of measurement, over which time the concentration of albumin-GdDTPA in blood remains constant, back flow of contrast agent into the vasculature was unlikely.

Multislice maps of (a) vascular volume and (b) PSP, together with (c) a histological section from the central slice of a TNP-470-treated tumor are shown in Fig. 5. In contrast to the images in Fig. 1, there was a marked reduction of detectable vascular volume and PSP in the treated tumor. Histological sections obtained from this tumor exhibited extensive necrosis, as observed in Fig. 5c. Triplanar views from three-dimensional reconstructed maps of the tumor in Fig. 5 of (a) vascular volume displayed in red, (b) PSP displayed in green, (c) fused vascular volume and PSP, and (d) histology are shown in Fig. 6. Again, in contrast to Fig. 2, vascular volume and PSP were detected mainly around the periphery of the tumor. The spatial discordance between regions of high vascular volume and high permeability were also apparent in the treated tumor, especially in the peripheral region in which two distinct regions, one with high vascular volume and the other with high permeability, were detected adjacent to, but not overlapping with, each other.

The effect of TNP-470 on tumor growth is summarized in Fig. 7. Treatment of the tumors was initiated when tumor sizes were of the order of 100–150 mm$^3$. By the end of either dosing schedule, the treated tumors were usually ~400 mm$^3$, whereas the control tumors were ~700 mm$^3$. Although the treated tumors continued to grow over the course of the treatment, a small but significant growth inhibition was detected in the treated tumors compared with the control tumors ($P \leq 0.03$).

Changes in the percentage volume of the tumor with detectable vascular volume and PSP, as well as in the mean of non-zero (or detectable) values of vascular volume and PSP for control and treated tumors, are summarized in Fig. 8. A significant decrease in the fractional tumor volume with detectable vascular volume ($P < 0.0002$) and permeability ($P < 0.006$) was observed in the treated tumors (Fig. 8, a–b). A significant decrease in mean non-zero values of vascular volume ($P < 0.02$) but not permeability ($P = 0.5$) was also observed (Fig. 8, c–d).

This pattern of significant reduction of vascular volume, but not permeability, was also apparent for mean vascular volume and permeability even when zero values were included in the average. When zero values were included in the average, the mean (± SE) vascular volume decreased from 13.6 ± 1.4 μl/g in the control tumors to 7.48 ± 1.12 μl/g in the treated tumors ($P < 0.007$). The mean (± SE) PSP was 0.66 ± 0.15 μl/g-min in the control tumors and 0.4 ± 0.11 μl/g-min in the TNP-470 treated tumors.

VEGF levels obtained from homogenates of control and treated MatLyLu tumors are shown in Fig. 9. Treatment with TNP-470 resulted in a significant increase of VEGF levels.
Fig. 5 Multislice maps of (a) vascular volume, (b) PSP, and (c) a 5-μm-thick H&E-stained histological section of the central slice for a MatLyLu tumor (volume, 395 mm³) treated with the 90 mg/kg TNP-470 protocol.

Fig. 6 Triplanar views of three-dimensional reconstructed maps of (a) vascular volume (red), (b) PSP (green), (c) fused vascular volume and PSP maps, and (d) histological sections stained with H&E, obtained from the treated tumor shown in Fig. 3.
DISCUSSION

TNP-470 is a fumagillin derivative and its antiangiogenic effects are thought to be caused by the inhibition of endothelial cell proliferation (9, 23). The inhibition of endothelial methionyl aminopeptidase-2 has been proposed as one of its mechanisms of action, although recent data suggest that TNP-470 may also act directly on cancer cells (17, 18, 24). Whereas MRI has been used to follow changes in vascular volume and permeability after treatment with anti-VEGF antibodies (6–8), the effects of TNP-470 on tumor vasculature detected using MRI have not been previously reported. The changes detected in the MRI parameters are consistent with the known antiangiogenic effects of TNP-470. Similar to previous observations (25), TNP-470 treatment induced retardation of tumor growth in the MatLyLu model compared with control tumors.

In our study, two parameters that showed a significant reduction after treatment with TNP-470 were fractional tumor volume with detectable vascular volume, and fractional tumor volume with detectable PSP. These data indicate that there were large regions in the treated tumors in which vascular volume and PSP were virtually undetectable after treatment with TNP-470. Histologically these areas contained areas of necrosis. The necrosis induced was most likely a secondary effect of reduced vascular volume after treatment. Although a reliable noninvasive measure of tumor necrosis may be useful to detect antiangiogenic treatment, this would also depend on the tumor being studied and on the severity of the antiangiogenic effect. The outcome of antiangiogenic treatment may not always be an increase in necrosis, because the formation of necrosis will depend on other factors such as the rate of dead-cell clearance and the oxygen and substrate utilization rate of the cancer cells.

The significant reduction of average vascular volume, as well as average non-zero values of vascular volume, detected by MRI in treated tumors is in good agreement with data obtained by Kragh et al. (25). In their study, a significant reduction of vascularization was detected using near infrared spectroscopy as well as histological analyses, after treatment with TNP-470. Similarly, the reduction in permeable regions detected here by MRI is consistent with observations made by Lund et al. (26) of extensive accumulation of basement membrane material in vessels of tumors treated with TNP-470.

An additional observation made in this study was that some regions in the treated tumors exhibited higher values of permeability than did control tumors. As a result, the overall reduction of values of permeability was not significantly lower in the treated tumors. These regions of high permeability values were typically observed in regions with low vascular volume values.
Such regions are observed in most tumors, but the permeability values were higher in the TNP-470-treated tumors compared with the control tumors. VEGF levels in TNP-470-treated tumors were higher than in control tumors. These data suggest that reduced values, as well as regions, of vascular volume and the resultant hypoxia may have mediated a compensatory increase of VEGF and permeability after treatment with TNP-470. An increase of VEGF staining after treatment with hyperthermia and TNP-470 has been reported in a previous study (27). These authors also attributed the increase of VEGF levels after treatment to the induction of hypoxia.

Serial MR scans over the time course of the treatment would have provided further insight into the changes in tumor vasculature induced by TNP-470 treatment. This study, however, was performed to determine whether, at the end of a course of antiangiogenic treatment, MRI could detect changes in tumor vascular characteristics. The data obtained in this study demonstrate the complex nature of the changes in vascular characteristics induced by antiangiogenic agents. These data demonstrate the necessity of using techniques such as MRI, which provide both spatial as well as quantitative information of vascular characteristics, to understand the effects of antiangiogenic agents on tumor vasculature.

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