High-Frequency Doppler Ultrasound Monitors the Effects of Antivascular Therapy on Tumor Blood Flow

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Abstract

The effect of antivascular therapy on blood flow in superficial tumors was monitored using novel high frequency Doppler (HFD) ultrasound techniques. Human melanoma cells (MeWo) were injected orthotopically into the skin of athymic nude mice. Volumetric HFD imaging of established melanomas detected a significant reduction in blood flow 4 h after injection of the tumor vascular targeting agent ZD6126 followed by a recovery of flow by 24 h after injection. Measurements of tumor perfusion in situ by Hoechst 33342 staining correlated with the ultrasound results. This study demonstrates the feasibility of HFD as a noninvasive, quantitative tool for following longitudinally the effects of antivascular therapy on blood flow in superficial tumors.

Introduction

An issue of central importance in the development and evaluation of antiangiogenic and antivascular cancer therapies is the ability to quantitatively assess the effects on blood flow (1). Techniques that measure vascular function are particularly appealing for examining antivascular therapies because they provide an indication of the result on the direct therapeutic target: the tumor blood supply (2). At clinical frequencies (2–12 MHz) Doppler ultrasound is one method that is under evaluation for doing this in a noninvasive manner appropriate for serial monitoring (3, 4). High-frequency (>20 MHz) Doppler ultrasound is a newer method, capable of assessing blood flow in superficial tissue (<5–10 mm) with higher resolution (50–150 μm) and improved sensitivity to flow in small vessels (5, 6). The ability of a novel HFD combined pulsed-wave/flow imaging system to assess complex flow patterns in superficial mouse tumors was recently demonstrated (6, 7). In the present study, high-frequency three-dimensional flow imaging is evaluated as a technique to monitor longitudinally the effects of an antivascular agent on blood flow in experimental mouse tumors. This work is intended to be a proof-of-principle study to assess the feasibility of using HFD in studies involving superficial tumors.

The therapeutic agent used for our studies was ZD6126 (AstraZeneca, United Kingdom), a newly reported member of the tubulin-binding class of antivascular drugs (8). These drugs destabilize microtubules in the cytoskeleton of endothelial cells, thereby inducing neovascular endothelial cell rounding that can subsequently lead to vascular shutdown and necrosis in tumors. ZD6126 has been shown in preclinical studies to rapidly disrupt tumor blood vessels and subsequently produce growth delays in a range of tumor types (7) and is, therefore, a suitable model to induce blood flow changes in the present study. In addition to ultrasound monitoring, independent validation of the effects of this therapy on tumor blood flow was conducted using Hoechst perfusion staining (9).

Materials and Methods

Mice and Cell Lines. Human MeWo melanoma cells (10, 11) were used to establish tumors for therapy. We selected melanoma cells because superficial tumors derived from such cells growing in the skin would be considered orthotopic and would, therefore, be more physiologically relevant models of human disease. Tumors arising from this cell line have been examined previously with HFD ultrasound (6). MeWo cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. Tumors were established by subdermal (orthotopic) injection of 106 MeWo cells into athymic mice, as described by Cornil et al. (12). To reduce the effects of respiratory motion that can interfere with the detection of low blood velocities, the tumors were located in the upper hind leg region.

In Vivo Studies. The growth of tumors was monitored until an estimated volume of either 75 mm3 (“early stage”) or 150 mm3 (“later stage”) was reached. Volumes were determined using caliper measurements and the standard formula (π/6length2width). The mice then received a single tail vein injection of either ZD6126 (treated) or physiological saline (control). The treated mice received a therapeutic dose of 20 mg/kg ZD6126, a level well below the maximum tolerated dose. Ultrasound studies consisted of making serial measurements on the same tumor (either treated or control) at baseline, at 4 and 24 h postinjection. Perfusion staining studies were conducted on separate groups of mice at baseline, 4 and 24 h posttreatment. The acute nature of the perfusion staining experiments required that different groups of mice be used for each time point and that different mice be used than those for the serial Doppler measurements. After conducting either ultrasound or perfusion-staining experiments, mice were killed according to the Canadian Council on Animal Care guidelines.

Ultrasound Flow Imaging. Ultrasound flow imaging provides information about the spatial distribution of blood velocities and moving blood volume. The range of blood velocities that can be assessed is limited at the low end by the clutter filter that is used to remove the signal from tissue and at the high end by the ultrasound pulsing frequency (13). Because of attenuation effects, which result in a reduced penetration depth at higher frequencies (14), it was necessary to assess the larger tumors using a lower frequency than was used for the smaller tumors. Ultrasound flow imaging of the earlier-stage, smaller tumors was conducted at a center frequency of 38 MHz with system settings held constant (800-Hz pulse repetition frequency and 35-Hz wall filters) to provide sensitivity to velocities above 0.7 mm/s. The lateral and depth direction resolutions were 93 and 82 μm, respectively. Ultrasound flow imaging of later-stage tumors was conducted at a center frequency of 25 MHz with system settings held constant (800-Hz pulse repetition frequency and 25-Hz wall filters) to provide sensitivity to velocities above 0.75 mm/s. The lateral and depth direction resolutions were 113 and 122 μm, respectively.

At each time point, volumetric imaging was performed by acquiring a series of image planes, spaced at 50 or 80 μm for the 38- and 25-MHz experiments, respectively, covering the entire tumor region (Fig. 1A). Scan times were on
Fig. 1. A, a series of adjacent planes are acquired, and the resulting volumetric flow data within the tumor is analyzed. Representative power Doppler images from a region of a treated 150 mm³ MeWo tumor at baseline (B), 4 h (C), and 24 h (D) posttreatment with (20 μg/kg) ZD6126. A pronounced reduction in blood flow is evident at the 4-h point, followed by a recovery at the 24-h point. Images are taken from near, but not precisely the same region. Scale is 7 mm × 7 mm; images are cross-sections parallel to the short axis of the tumor.

Ultrasound Image Processing. Region-of-interest selection was performed using custom written Matlab (Mathworks Inc., Natick, MA) software to include the entire tumor volume. Ultrasound flow imaging data consists of spatial maps of either blood velocity or Doppler power (P). In this study we consider only the Doppler power data. Using these data, a measure of the total IP (IP) flow was calculated within a tumor volume at each time point as follows:

\[ IP = \sum_{tumor} P(i, j, k) \]

where i, j, and k are the volumetric pixel indices, and the power P is in linear rather than in logarithmic form. The IP is related to the volume of moving blood within the tumors (13, 14) and, as such, it reflects the functional vascular volume. For each tumor, this measure was normalized with respect to its baseline value to infer relative changes in blood flow as a function of time.

To reduce the effects of tissue motion, the mice were anesthetized using 1–1.5% isoflurane during the flow imaging experiments. Heart rates and body temperature were monitored using a THM100 mouse pad (Indus Instruments, TX). Heart rates were not noticeably altered by the therapy. Three treated and 3 control mice were imaged for both the early- and later-stage tumors, making a total of 12 mice used in the Doppler component of the study.

Drug Preparation. ZD6126 was dissolved at a concentration of 10% (w/v) in a solution of PBS and 0.5% sodium carbonate.

Hoechst Perfusion Staining. Perfused vasculature in the MeWo tumors was identified by i.v. injection of 200 μl (10 mg/kg) of Hoechst 33342 dye (Sigma, St. Louis, MO) into the tail vein (11). Twenty min after injection with Hoechst, mice were euthanized by cervical dislocation. Tumors were quickly removed and embedded in Tissue-Tek OCT Compound (Miles Inc., Elkhart, IN), followed by rapid freezing over dry ice for subsequent sectioning. For each tumor sample, 6-μm cryosections were cut at four different levels and viewed under UV epifluorescence using an Axioskop 2 transmitted-light microscope (Zeiss, Munich, Germany). Using this method, perfused blood vessels could be visualized by the surrounding halo of fluorescent, Hoechst 33342-labeled cells. Digital images were captured using the Axiosvision 3.0 image analysis system at x10. Prior to analysis, a threshold for the vascular structures was determined for the stain used, based on the background level of brightness. Image analysis was performed with Northern Eclipse Version 6.0 software to measure the percentage image area occupied by perfused vasculature. Although this analysis does not directly quantify the number of perfused vessels, it provides an estimate of the relative degree of perfused tumor vasculature. For each tumor, an average percentage image area was calculated, a minimum of four random fields were analyzed per section, and a minimum of four sections per tumor were examined. A total of four mice were imaged at each time point for the later-stage tumors.

Statistics. Statistical analysis of data were conducted using NCSS software for Windows (Kaysville, UT). Mann-Whitney tests were used and Ps of < 0.05 were considered to be significant.

Results

High-Frequency Ultrasound Monitors Tumor Vasculature Changes. Representative examples of 25-MHz ultrasound power images of a plane through a later-stage tumor are shown in Fig. 1, B–D, at baseline, as well as at 4 and 24 h postinjection. A large reduction in perfused vessels was observed 4 h after the ZD6126 injection, relative to the baseline and...
24-h points. A similar pattern was observed for the 38-MHz data on early-stage tumors. This was consistent with the qualitative high-frequency pulsed-wave Doppler assessment, with which only minimal flow could be detected at the 4-h point in treated tumors. The mean IP for the control and treated tumors at different times after the ZD6126 injection are shown in Fig. 2, A and B, for the early- and later-stage tumors, respectively. In both cases, a statistically significant drop ($P < 0.05$) occurs at the 4-h point followed by a recovery at the 24-h point.

Hoechst Perfusion Staining Results Correspond to Ultrasound Results. Perfused vessels were visualized in tumor cryosections by the surrounding Hoechst-labeled cells (9). Representative examples of baseline and 4-h and 24-h posttreatment perfusion staining are shown in Fig. 3 A–C. A large drop in the perfused area at the 4-h point was evident, whereas perfusion at 24 h resembled that of the control tumors. The quantitative analysis of the results is shown in Fig. 4. The data indicate a significant drop in the perfused area at 4 h ($P < 0.05$) and no significant difference at the 24-h point. These results indicate that at the dose and treatment schedule that was used (i.e., a single injection), ZD6126 induces a short-term reduction in tumor perfusion in the MeWo tumor xenografts.

Discussion

This study was conducted to evaluate the ability of HFD to monitor changes in tumor blood flow associated with antivascular therapy. If HFD is found to be sufficiently sensitive to detect therapeutic modifications of tumor blood flow, its noninvasive nature makes it well suited as a tool for quantitatively assessing blood flow in superficial tumors in a serial manner.

As noted earlier, conventional-frequency Doppler ultrasound is also under investigation as a method for monitoring and for antivascular and antiangiogenic therapy in superficial tumors and has shown
encouraging results (3). Two factors are improved by the use of high-frequency ultrasound over lower frequencies: spatial resolution and sensitivity to slow flow in small vessels (6). Spatial resolution scales linearly with frequency and goes from about 0.5 mm at 5 MHz to 50 μm at 50 MHz (14). The larger sample volume of conventional-frequency ultrasound will tend to encompass a number of microvessels, which may have a range of velocities and orientations. A velocity estimate in this situation will, therefore, represent an average velocity for the ensemble of vessels present. Improved spatial resolution permits an assessment of the spatial distribution and blood velocity of smaller vessels and may be useful in assessing differences in spatial patterns of flow that result from treatment. As with conventional frequency ultrasound, the potential exists for such measures to be complicated by heterogeneous attenuation patterns. The heightened sensitivity to small vessels at high frequencies is itself the result of two primary factors. First, the echo strength from blood increases with frequency (14), eventually becoming comparable with that from many tissue types. Second, Doppler shifts increase linearly with frequency, making it easier to separate blood and tissue signals (6). The precise detection limit depends on a number of parameters (e.g., tissue motion and tissue ultrasonic properties), but at conventional frequencies it is likely limited to vessels with diameters above 100 μm and velocities above ~1 cm/s (7). At 50 MHz, vessels as small as 15–20 μm in diameter with velocities on the order of mm/s have been detected and imaged (6). The improved sensitivity to slow flow in smaller vessels may be significant in applying HFD to monitoring antivascular or antiangiogenic therapy. In particular, sensitivity to smaller vessels may be advantages in providing insight into flow conditions at a level of circulation that is closer to that associated with nutrient and waste exchange and drug delivery.

The IP measurements made in this study are related to the total volume of blood flowing within the tumor with a velocity above the clutter filter value (16). A comparison of these data for the treated and control groups, therefore, indicate that the volume of blood flowing within the tumor was significantly reduced at the 4-h point (P < 0.05) and subsequently recovered by the 24-h point. The IP results followed a similar pattern to that of the perfusion staining data, which demonstrated an initial decrease in the density of perfused microvessels followed by a recovery. A rapid and extensive reduction in perfused vessels is characteristic of what has been observed previously with ZD6126 as well as other tubulin-binding agents (17, 18). The possibility of a recovery in flow after an initial drop has also been demonstrated in this study, suggest that high-frequency ultrasound flow imaging has the potential to be a viable tool for noninvasively monitoring antivascular and/or antiangiogenic effects (20, 21) using HFD.

A number of other techniques, such as magnetic resonance imaging, are also under evaluation for noninvasively assessing the therapeutic modulation of blood flow in tumors (2). It is likely that no single imaging technique will satisfy the requirements for experimental or clinical therapeutic blood flow monitoring. The combination of resolution, penetration depth, and cost, along with the sensitivity demonstrated in this study, suggest that high-frequency ultrasound flow imaging has the potential to be a viable tool for noninvasively imaging blood flow in superficial tumors, particularly in preclinical experimental therapeutic studies. In this regard, it would, therefore, be of considerable interest to study agents such as conventional chemotherapeutic drugs and antioncogenic signal transduction inhibitors for their potential ability to cause antivascular and/or antiangiogenic effects (20, 21) using HFD.

Acknowledgments

We thank AstraZeneca for providing the ZD6126 used in this study. We are also grateful to Mingyu Zhang for his assistance with drug preparation and Emmanuel Cherin for his assistance with transducer characterization.

References


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