Quantitating Therapeutic Disruption of Tumor Blood Flow with Intravital Video Microscopy

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Abstract

Vascular-disrupting agents (VDA) kill tumor cells by selectively disrupting blood circulation in tumors. *In vivo* analysis of this intensely studied class of anticancer agents is invaluable for preclinical assessment of pharmacodynamic end points and effective therapeutic windows. In this review, we consider the role of intravital video microscopy in measuring tumor vascular response to VDAs, the potential of which lies in the opportunity to quantitate specific variables and to obtain real-time information on how VDAs affect tumor microcirculation. (Cancer Res 2006; 66(24): 11517-9)

Introduction

A functional vascular network is essential for the survival and continuing growth of solid tumors. Vascular targeting aims to exploit the differences between tumor and normal vasculature by irreversibly arresting tumor blood flow and producing tumor cell death by oxygen and nutrient starvation. The primary cell type in microvessels is the endothelial cell. Discovery of factors that specifically control endothelial cell proliferation has revealed several factors that stimulate or inhibit the proliferation of endothelial cells (1). These factors form the basis of the burgeoning field of tumor-selective vascular disruption.

Several classes of natural and synthetic agents target vascular endothelial cells, inducing vascular collapse and hemorrhagic necrosis in tumors. Vascular-disrupting agents (VDA) that naturally occur include cytokines and low molecular weight vasoactive substances, such as serotonin, histamine (2), and CM101, a bacterial polysaccharide exotoxin produced by group B streptococcus (3). Small-molecule VDAs, including flavonoids and tubulin-binding agents, several other agents, arsenic trioxide, fostriecin, and homoharringtonine (4) have been identified to have antivascular effects in tumors. This review focuses on the use of intravital video microscopy (IVVM) to assess small molecular VDA effects *in vivo*.

Why Tumor Vasculature?

Tumor vasculature is an attractive alternative to targeting tumor cells due to the following reasons: (*a*) easy accessibility of blood-borne agents to endothelial cells; (*b*) reliance of most tumor cells on vascular supply for survival; (*c*) endothelial cells are non-transformed, which means they are unlikely to develop acquired resistance; (*d*) selective expression of proteins on tumor endothelial cells; and (*e*) it is a prominent route for metastatic spread (5–7). VDAs exploit the differences between tumor and normal endothelial cells to induce selective vascular dysfunction (8). Blood flow disruption is influenced by functional changes in endothelial cells through increased vascular permeability, production of vasoactive agents, and involvement of inflammatory processes, leading to cessation of blood flow, vascular collapse, and hemorrhagic necrosis in tumors (2, 9).

In vivo Study

The use of *in vivo* models helps in evaluating the effects of VDAs in the actual tumor microenvironment. *In vivo* studies may be used to determine appropriate pharmacodynamic end points for efficacy testing of VDAs in early clinical trials (5). *In vivo* studies also provide invaluable information on therapeutic windows of VDAs (10, 11) and effects on systemic circulation (12). For instance, combretastatin A-4 prodrug (a tubulin-binding agent) induced potent and selective antivascular effects against tumor-associated endothelium, but significantly, the effects were at concentrations less than one tenth of the maximum tolerated dose showing a wide therapeutic window (11).

Intravital Video Microscopy

IVVM involves the use of a fluorescence microscope in a living animal for real-time observation, monitoring, recording, and quantitative analysis of specific variables and events. IVVM allows dynamic observation of cancer cell activity in the microcirculation of intact organs and tissue in live animals. A similar technique is used for direct visualization, monitoring, and analysis of VDAs on tumor microcirculation (9, 13, 14). In the work of Tozer et al. (9), visualization of vessels on tumor surfaces was possible; at the same time, IVVM enabled monitoring of arterioles and venules on VDA administration. Elucidation of the mechanism of action of various VDAs has been made possible through the use of IVVM (9, 14). Using an intravital video microscope, Hori et al. (14) showed that AC7700, a tubulin-binding agent, caused constriction of host arterioles, leading to narrowing of tumor vessels and hemolysis in tumor-draining vessels. The strength of IVVM lies in being able to quantify specific variables, such as vessel number, diameter, length, vascular density, permeability, and blood flow velocity. The fact that IVVM requires no prior assumptions to be made about the likely effects of a particular VDA is a major advantage over other methods in preclinical assessment (5). IVVM has its drawbacks: (*a*) motion artifacts as a result of respiratory and cardiac movements; (*b*) limited transillumination through thick tissues even when an inverted microscope is used (15); (*c*) time consuming and very technical; (*d*) invasive; and (*e*) necessitates anesthetics, which may have significant effects on variables assessed. Other methods, such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), have been used to assess VDA effects on tumor microcirculation.

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DCE-MRI is a noninvasive technique that provides derived variables relating to tissue perfusion, vascular permeability, and vascular surface area. Data obtained from DCE-MRI measure relative change in dynamic variables and are not quantitative (16). DCE-MRI has been used in both preclinical models and clinical trials to measure changes related to tumor blood flow, permeability, and perfusion on VDA administration (17, 18). Whereas DCE-MRI provides a noninvasive average and has extended usage in clinical trials, IVVM directly measures tumor vascular response to VDAs and provides real-time information within a research setting.

Quantification of Tumor Vascular Response to VDAs

A simplistic approach of IVVM by directly measuring the effects of VDAs on tumor blood flow and vascular function provides an easier assessment of these variables. For adequate analysis, images are recorded on a video/computer recording system, which enables "off-line" analysis. This has advantages in (*a*) off-line observation of pharmacodynamics of treatment, (*b*) direct measurements of specific variables (i.e., diameter), and (*c*) comparing posttreatment images with pretreatment images and (*d*) time is not wasted collecting and analyzing results during an experiment. Choosing a specific region of interest (ROI) within the tumor is paramount as it provides consistency with results. By injecting fluorescent dyes, such as FITC-dextran, vascular spaces within the tumor can be defined (13, 14, 19), as they provide intravascular fluorescence contrast hence precise delineation. However, it is a concern that exogenous markers may tone down vessel margins through extravasation. Tozer et al. (9) attempted to label plasma with TRITC-albumin to identify vessels under fluorescence conditions. However, results were inconclusive, as there was leakage from the vessels. As an alternative fluorescent labeling of donor red cells has enabled identification of that which is intravascular through blood cell movement. Measuring the width of a column of fluorescence red cells will give one the diameter of a vessel, although it will not be accurate. Using the width of fluorescence red cells, Tozer et al. (9) were able to detect changes in vessel diameter after VDA treatment. This technique worked for them, as they were more interested in vessel diameter changes than absolute values of vessel diameters.

Vessel numbers. Vessels are chosen for their length, uniformity of flow, and range of diameters within each tumor. Vessel identification and counting is done by image analysis software. However, manual identification and counting is also used. In the work of Tozer et al., automated identification and counting using in-house developed software in Lab Windows CVI 5.0 correctly identified 60% of the visible vessels with the remainder identified and counted manually. Drawbacks to this include the following: (*a*) human errors and (*b*) it leaves identification and counting of vessels at the discretion of the user. Using still images or video recordings, quantitative data on vessel numbers are obtained from images acquired before and after treatment (9). While analyzing vessel numbers, it is very important to define a single vessel, the end of which will have a branch, tethering point or will move out of the plane of focus. Assessment of nonfunctioning vessels after treatment involves counting the number of vessels within the area of interest that have disappeared from the field of view when compared with pretreatment images. It is also important to note vessels that contain stationary labeled red cells for a specified period, as they will not have any significant flow through them. Using these techniques, IVVM showed a decrease in the number of visible rat carcinosarcoma blood vessels at various doses of combretastatin A-4 phosphate (CA-4-P) that was significant at higher doses with no subsequent recovery in numbers after treatment (9).

Vessel diameter. Analysis of vessel diameter is obtained from selected vessels under high magnification (9, 14). Observing images under high magnification increases the risk of artifactual appearance of reduced vessel diameter, as slight movements can cause loss of focus. Measurement of a vessel diameter is more accurate with images in which the fluorescent dye defines the vascular space, as measuring the width of fluorescent red cell columns may underestimate the diameter. Vessel diameter analysis is vulnerable to operator errors, as fluorescent dyes that define vascular spaces may cause vessel wall obscurity. Sometimes it is impossible to measure the diameter of the vessels after drug administration, as tumor vessels may constrict as shown by Hori et al. (14), where significant vasoconstriction in tumor-feeding vessels occurred when AC7700 was given i.v. and the vessels disappeared from view.

Blood and cell velocity. Using IVVM, measurements on flow characteristics are obtained from selected vessels in the ROI. A chosen number of fluorescent red cells are monitored over a specified period. Velocity is calculated from the time it takes a specific fluorescent cell to travel between two points, a specified distance apart marked on vessel images (9, 20). The advantage of tracking individual cells gives a functional flow rate rather than the average flow rate within a vessel. Fluorescent markers not labeled in cells will show overall flow rate, which is not necessarily the same as the cell movement rate. Various measurements of velocity can be calculated both in preinjection and postinjection images. When considering a labeled cell technique, if no red cells are seen after drug administration, a minimum velocity is assumed, which is a small fraction of the average preinjection velocity. Tozer et al. (9) assumed a value of 0.85 μm/s when no red cells were observed after CA-4-P was administered. This was a small fraction of the mean pretreatment velocity and represented the maximum possible velocity under their conditions assuming equal spacing of fluorescently labeled cells. Derived variables of blood flow rates within specific vessels can be calculated from red cell velocity and diameter measurements assuming that red cells are moving with the bulk of plasma flow (9). CA-4-P effect on red cell velocity was extremely rapid, with the velocity falling throughout the time course of CA-4-P administration reaching <5% of its starting value within 60 minutes. Blood flow rates after CA-4-P treatment were almost identical to red cell velocity results, which reflected low average changes in venule diameter in all treated groups. They suggested that blood flow rates in treated groups were predominately determined by changes in local perfusion pressures and/or blood viscosity rather than by venular diameters. This was due to increased vascular permeability, production of vasoactive substances, and involvement of inflammatory processes. Using IVVM, the potential mechanisms of action of CA-4-P on tumor blood flow shutdown were thus explored (9).

Vascular function. Endothelial cell organization and confluence is an indirect measure of vascular cell function and can be determined using image analysis software to measure vascular cell density. Calculation of vascular density of tumor vessels by IVVM as a response to VDAs is calculated by dividing the total length of each vascular network by the tissue area within the ROI (13, 19). Density ratios are calculated by dividing the vascular density after injection at various time intervals by the vascular density before injection of a VDA. AC7700 treatment significantly reduced tumor

Table 1. A list of IVVM studies of agents with antivascular effects on tumor microcirculation (9, 13, 14, 19)

Agent and animal model	Hemodynamic variables	Tumor cell line	Outcome
CA-4-P in BD9 rats (9)	Blood flow, blood vessel diameter, blood vessel numbers, MABP	P22 rat carcinosarcoma	Visible vessel number decreased to 65% by 10 min, blood flow rate was 5% of starting value within minutes at 30 mg/kg, diminished blood flow within 60 min at 30 mg/kg
AC7700 in Donryu rats (13)	Blood vessel length, vascular density	AH-130 tumor cell (hepatoma)	Diminished tumor perfusion in 30 min, vascular density at 60 min, AC7700 26.3 ± 16.4%, control 88.5 ± 9.2%
AC7700 in Donryu rats (14)	Blood vessel diameter	Sato lung carcinoma	Change in diameter in 30 min, no blood flow, vessels disappeared from view, intravascular hemolysis
Endostatin (antiangiogenic agent with antivascular effects) in nude mice (19)	Blood vessel diameter, vascular perfusion, total and functional vascular density	C6 rat glioma cells	Reduced total vascular density by 67.6%, reduced blood vessel diameter by 37%, reduced vascular perfusion by 67%

Abbreviation: MABP, mean arterial blood pressure.

vascular density and hence reduced tumor perfusion as shown by Ohno et al. (13) using IVVM. However, tumor perfusion was a purely descriptive observation with no quantitative analysis. Although the link between vascular density and tumor perfusion has been made, a direct quantitative correlation is yet to be shown. A more direct assessment of vascular function is vessel permeability, which was assessed by comparing intravascular with extravascular fluorescence intensity of various individual tumor microvessels in peritumoral and intratumoral areas using IVVM (19). From these measurements, a permeability index was obtained, defined as the ratio of intravascular fluorescence to extravascular fluorescence. Using human endostatins (antiangiogenic agent with vascular-disrupting effects) on C6 rat glioma cells, the permeability index was assessed. No statistically important change in permeability index was seen, whereas significant reductions in total vascular density, perfusion, and vessel diameters were observed (19). So the indirect and direct indicators of vascular function, vascular density and vessel permeability, are independent of each other and cannot be investigated in isolation. Table 1 shows a summarized list of IVVM studies of agents with antivascular effects on tumor microcirculation.

Conclusion

VDAs have been shown to cause tumor vascular disruption *in vivo*. Using IVVM, different levels of quantification of vascular damage can be explored: (*a*) vessel diameter, (*b*) perfusion, and (*c*) endothelial cell function. However, there is a need to examine the relative importance of each of these levels and their interdependency while assessing effects of VDAs on tumor microcirculation. Use of IVVM to analyze VDA responses is still at its bare infancy.

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