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In Vivo Microcartography & Subcellular Imaging of Tumor Angiogenesis: a Novel Platform for Translational Angiogenesis Research

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Abstract

Purpose—To eliminate the variable of tumor heterogeneity from a novel *in vivo* model of tumor angiogenesis.

Experimental Design—We developed a method to navigate tumor neovasculature in a living tissue microenvironment, enabling relocation of a cell- or microregion-of-interest, for serial *in vivo* imaging. Orthotopic melanoma was grown, in immunocompetent *Tie2GFP* mice. Intravital multiphoton fluorescence & confocal reflectance imaging was performed, on a custom microscope with motorized stage & coordinate navigation software. A point within a *Tie2GFP*+ microvessel was selected for relocation. Custom software predicted target coordinates based upon reference points (tissue-embedded polystyrene beads) & baseline target coordinates. Mice were removed from the stage to make previously-obtained target coordinates invalid in subsequent imaging.

Results—Coordinate predictions always relocated target points, *in vivo*, to within 10-200 μ m (within a single 40 $\times$ field-of-view). The model system provided a virtual living histology of tumor neovascularization & microenvironment, with subcellular spatial resolution & hemodynamic information.

Conclusions—The navigation procedure, termed *in vivo microcartography*, permits control of tissue heterogeneity, as a variable. *Tie2* may be the best reporter gene identified, to-date, for intravital microscopy of tumor angiogenesis. This novel model system should strengthen intravital microscopy in its historical role as a vital tool in oncology, angiogenesis research, and angiotherapeutic drug development.

Introduction

The Tumor Angiogenesis Subgroup of the Preclinical Models for Human Cancers Working Group, at the National Cancer Institute, has declared a need for “*in vivo* models of angiogenesis and the ability to monitor increases in angiogenesis *in vivo*”.[1] Dissecting the biological

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components of efficacy, in angiotropic therapy, has largely been the domain of preclinical research, relying upon histopathology, *in vitro* assays, & *in vivo* models.[2] Histological measures of microvessel density have been used, both clinically & preclinically, to assay tumor response to anti-angiogenic therapy, mainly using Chalkley counting [3] or the method of Weidner et al.[4,5]

Yet the internal micro-architecture of tumors is heterogeneous; angiogenesis-measurements from one biopsied, microscopic tumor sub-region may differ dramatically from those of another sub-region in the same tumor [5-7]. This has cast doubt on the reliability of tissue microvessel density measurements in angiogenesis research [8,9]. In particular, tissue heterogeneity, of tumor & tumor vasculature, poses a problem for assessing therapeutic response to novel angiotropic agents by serial tumor histopathology: i.e., are measured changes in angiogenesis, post-treatment, due to therapeutic-effects or merely tumor heterogeneity?

We have obviated the issue of tumor heterogeneity, by developing *in vivo* microcartography, a highly precise means for relocating a microscopic tissue region, even a particular tissue cell, *in vivo*, during serial intravital microscopy, with multiphoton fluorescence [10] & confocal reflectance [11] microscopy. We applied this technique to the study of angiogenesis, by imaging transgenic mice expressing green fluorescent protein (GFP) under the control of *Tie2*, an endothelial-specific promoter.[12-15]

Materials & Methods

Additional information regarding materials & methods is available, in Supplementary Methods.

Animal Model

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Sloan Kettering Institute for Cancer Research. *Tie2*/GFP mice (n=4) bore a dorsal skinfold chamber containing an ingrown wild-type B16 melanoma (kind gifts of Drs. Alan Houghton & David Schaefer). 90 μ m polystyrene latex microspheres were deposited in 4 locations upon the exposed subcutis, in the chamber. Tumors were observed, by transillumination & stereoscopy, until these were ~8mm, in diameter. Healthy, tumor-free *Tie2*/GFP DSFC mice (n=3) were studied, serially, for comparison.

Intravital Microscopy

A previously-described [11] custom-built microscope with motorized x-y stage acquired simultaneous near video-rate confocal reflectance & multiphoton fluorescence data. Mice were anesthetized & laid upon a heat-regulated tip-tilt adjustable platform under the heated objective lens of the microscope. The skin chamber was rendered immobile. Hemodynamic image sequences were acquired at ~ 20fps, with no frame-averaging per Z-slice. *Tie2*/GFP endothelium fluorescence Z-stack data was acquired at ~ 20fps, with 30 averages per frame. Pixel dimensions, using a 40 $\times$ 0.80NA objective, were 0.53 $\times$ 0.53 μ m. Selected slice thicknesses ranged from ~0.53-2.00 μ m. Sixteen serial imaging sessions (total), 2-30 hours apart, were performed on seven mice; between studies, mice were removed from the microscope. One mouse was imaged before and thrice after treatment with a single, high dose of external beam radiotherapy to the tumor (lead shielding protected the rest of the mouse.)

In Vivo Microcartography

The authors developed a method to relocate a previously-visualized segment of a selected tissue microvessel within a living animal, for subjects returning to the microscopy unit, on multiple occasions, for serial imaging. Custom navigational software was developed in the LabVIEW

programming language (software available upon request) that utilized the motorized microscopy stage to establish initial X-Y coordinates of a microvessel segment-of-interest & selected groups of the previously-inserted latex polystyrene microspheres, with a 40× 0.8NA objective (a 270 × 270 μm field of view.) These microspheres served as navigation reference points, establishing the basis vectors of a coordinate system, and were found to be reliably-stable, in location. These spheres are also visible at lower magnifications (see supplementary figure 1); spheres in distinctive cluster-patterns could easily be relocated at lower magnification within the chamber at each imaging session, followed by moving to high magnification to establish the new (secondary) X-Y coordinates. Serial studies were separated by periods ranging from 2 hours to 3 days with removal of the mice from the microscope between imaging sessions. To relocate tissue microvessels-of-interest, a custom mathematical program calculated the affine-transformation between the initial & secondary sets of reference microsphere-coordinates, utilizing three sets of microsphere X-Y data (see supplementary figure 2). The accuracy of these calculations was confirmed by predicting the positions of two other reference microsphere clusters. The navigation software automatically moved the X-Y stage to position the field-of-view over the new coordinates. At 40×, relocation of the target microspheres was confirmed by recognition of the cluster-pattern (see supplementary figure 1) and the unique *Tie2*GFP+ vascular branching patterns, within the 270 × 270 μm field-of-view. It is known that other nonlinear distortions could be present as well; yet accounting for these was unnecessary, as the system performance was highly-precise, predicting new target coordinates to within a 270 × 270 μm field-of-view; i.e., microvessels-of-interest were always easily-relocated (see figure 3 & online videos.) The distance (μm) between the predicted and actual location of each target point was calculated from the coordinates obtained using the custom navigational software.

Image Processing

In addition to the aforementioned real-time image display & navigation microscopy software used during *in vivo* microcartography, datasets were processed for display (figures 1-3; supplementary figure 3; and supplementary videos 1-3) using Amira™ (3.0) software and ImageJ (v 1.37) freeware.[16] The focus of this experiment was validating a novel method for relocating a microvascular branch-of-interest *in vivo*, towards obviating the issue of tissue heterogeneity. For readers interested in image-based quantification of angiogenesis *per se*, the following excellent references are provided.[17]

External Beam Radiotherapy

The body of the mouse was protected by a custom lead shield, leaving only the dorsal skinfold chamber exposed. After baseline imaging, the skin chamber received 10 Gy of external irradiation at 1.15Gy per minute using an AGFA X-RAD 320 irradiator. This radiation dose was intended to produce tumor endothelial apoptosis, as described by Garcia-Barros et al, in a similar mouse model.[18]

Results

Animal Model & Intravital Microscopy

Multiphoton fluorescence microscopy visualized *Tie2*+ endothelial cells with subcellular spatial resolution & high vessel-to-background contrast (see figures 1 & 2). GFP concentrated in cell nuclei and was present, diffusely, in cytoplasm at a lesser concentration (see figure 1); the cellular distribution of *Tie2* protein differs [2] (GFP was not fused to Tie2 protein.)

In tumor-free mice, the expected orderly, hierarchical layout of tissue blood vessels was observed (see figure 2). In tumors, a seemingly-chaotic network of dilated blood microvessels was observed: the classic phenotypic finding of tumor angiogenesis. We observed vascular

morphology suggestive of sprouting angiogenic buds & intussusceptive vessels [19] (see supplemental figure 3).

In tumors, perivascular & stromal/interstitial *Tie2*⁺ cells were more abundant (see figure 3 and online video files). In tumor-free mice, *Tie2* expression was confined to blood vessel endothelium, with rare *Tie2*⁺ cell in perivascular & stromal/interstitial regions;

Circulating blood cells were clearly-visualized by confocal reflectance imaging, and microvascular blood flow velocity was quantifiable by imaging a single optical plane at 20.4 frames-per-second (fps) frame rate. Besides hemodynamic information, confocal reflectance data provided a 'virtual' histologic context for the neovasculature & cancer cells, visualizing, e.g., myocytes; amorphous connective tissue that could pose a barrier to cell movement or drug diffusion; [20] and adipocytes in the microenvironment.

In Vivo Microcartography

Relocating user-selected tissue cells & microspheres, in the dorsal skinfold chambers, at different time points, over a period of days, was rapidly-accomplished and semi-automated. Coordinate predictions relocated all microvascular endothelial cells-of-interest & microspheres-of-interest to within one 40× field-of-view (270 × 270 μm). Predicted & actual coordinates differed by 10-200μm.

Radiation Vasculopathy

Microvessels selected for serial observation before, and multiply-after, a single high-dose of external radiotherapy (10Gy) demonstrated the *in vivo* course of radiation vasculopathy: confocal reflectance microscopy demonstrated microvessels undergoing thrombosis, with or without subsequent re-perfusion, and ultimately infarction (see figure 3; and online supplemental video files.) At each post-treatment time point, the number of tissue cells with high-reflectance increased. Concomitant with reflectance imaging, multiphoton fluorescence microscopy visualized, at each time-point, a progressive loss of endothelial *Tie2*/GFP signal in doomed vessels.

In contrast, in non-irradiated mice, endothelial *Tie2*/GFP signal did not weaken nor did cellular reflectance increase after repeated imaging. Green fluorescent protein that was exposed *in vitro* to 10 Gy, demonstrated no loss of fluorescence. Thus, neither photobleaching nor direct-effects of irradiation on GFP-fluorescence seem to account for the observed GFP-signal loss in endothelial cells post-irradiation.

Discussion

The biological milieu of a cancerous tumor is often significantly heterogeneous, internally, introducing uncertainty into assays that attempt to quantify tumor biomarker expression close to the cellular-level; tumor microvessel density, a standard angiogenesis biomarker, [3] in one microscopic tumor subregion may differ markedly in another subregion, in the same tumor. [5-7] Jacobs et al found microvessel density scores of samples taken contemporaneously from the same tumor could differ by 233%. [5] A means of eliminating the variable of tissue heterogeneity is evidently desirable.

The results presented in this article demonstrate that *in vivo* microcartography controls this variable, permitting researchers to ignore or explore tissue heterogeneity, in tumor microvasculature, by allowing serial monitoring of user-selected tumor microvascular regions rather than serial assays of random micro-regions. The model presented is a novel combination of microscopic coordinate navigation, intravital multiphoton & confocal microscopy, and imaging of endothelial gene expression in *Tie2GFP* mice. The subcellular resolution, afforded

by this system, enabled us to relocate individual microvascular segments & individual endothelial cells, within regions-of-interest. To our knowledge, this is the first report of directed-relocation of selected tissue cells, *in vivo*, during serial intravital microscopy. This *in vivo* model offers a platform for studying the vascular biology of the tumor, at the single-vessel level, during tumor growth or treatment, as the case of radiation vasculopathy illustrates (see figure 3 and online supplemental video files.)

Success in relocating microvascular regions-of-interest (ROIs) *in vivo* was accomplished using non-implanted inert microspheres. These proved to be accurate reference points for coordinate calculations; we recommend recording the positions of at least five microsphere clusters, away from the periphery of the chamber, to ensure positionally-stable reference coordinates for calculations made at subsequent time points. At each imaging session, the coordinates of reference microspheres & microvascular ROI center-points should be re-established for use in any subsequent session; gradual changes in the relative positions of the microspheres & microvascular ROIs may make the original coordinates less reliable, with time. Visualization of unique microvascular architecture confirmed successful relocation of microvascular ROIs; *in vivo* microcartography should be performed with sufficient frequency (i.e., temporal resolution) so that any changes in the microvascular architecture, between imaging sessions, appear gradual, allowing confident relocation of the ROI. Our results validate the feasibility, accuracy, and precision of *in vivo* microcartography for relocating tumor cells-of-interest, in the Tie2GFP+ mouse melanoma model tested. For other experimental models, its accuracy & precision may differ and the proper frequency of imaging will need to be determined, empirically, for each experiment. Tumor neovasculature of certain cancer-types or during particular anti-tumor treatments, for example, may undergo marked-changes so rapidly that cellular re-localization by *in vivo* microcartography may not be feasible.

Lastly, we suggest monitoring microvascular ROIs near the superficial center or ‘polar region’ of the tumor implant; we believe that any spatial-displacement of microvascular ROIs in this region, associated with tumor growth, would mostly occur in the Z-axis, relative to reference microspheres, rather than in the X-Y plane. With accurate X-Y coordinate predictions, it is relatively easy to find microvascular ROIs along the Z-axis.

The basic principle of this navigation method – using markers of relatively-fixed position-markers as reference points for coordinate calculations – should be adaptable to other orthotopic tumor models studied by intravital microscopy. Intravital microscopy has already transformed the study of tumor angiogenesis, yielding laboratory findings with significant clinical impact. As notable examples, intravital microscopy provoked the first hypotheses that angiogenesis was stimulated by cancerous secretions;[21] provided the Chalkley method for quantifying tissue vessel density;[3,22] and, in conjunction with histopathological data, yielded the Jain concept of vascular ‘normalization’ as an effect of angioinhibitor therapy & a sign of its efficacy.[23]. The Jain concept is pursued in clinical trials of angioinhibitors; e.g., dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) detects changes in tumor perfusion and vascular permeability consistent with vascular normalization (reviewed by O'Connor et al.)[24] Recently, several researchers [25-28] have enhanced the study of tumor angiogenesis by combining intravital microscopy with molecular imaging, the *in vivo* visualization of biological molecules & biomolecular pathways.[29]

Direct visualization of tumor vascular transgene expression offers a key advantage over conventional intravital microscopy: the ability to detect the formation of angiogenic buddings, [27] a phenotypic hallmark of angiogenesis (by definition), which are not yet wholly canalized & perfused and, thus, poorly-accessible to endothelial stains or vascular contrast agents. Au et al obtained exquisite images of tissue microvasculature, *in vivo*, by confocal microscopy of transgenically-fluorescent vessels derived from human umbilical vein endothelial cells

implanted into immunodeficient mice.[30] We visualized of tissue microvasculature, in immunocompetent mice, with subcellular clarity & high target-to-background contrast, by employing confocal multiphoton fluorescence [10] microscopy with a more endothelial-specific transgene, *Tie2*, than has previously been used for imaging angiogenesis.

Tie2⁺ cells in the perivascular & stromal/interstitial regions of angiogenic beds were evident, more numerous in tumor tissues than in healthy dermis. De Palma et al previously-identified these cells as mesenchymal progenitor cells believed to be precursors of vascular pericytes and also as an infiltrating, *proangiogenic* subset of monocytes.[31] Activated neutrophils also express *Tie2* [32] and frequently infiltrate tumors.[33] Hence, this model may be useful for study of these tumor-infiltrating host cells.

The case of tumor radiation vasculopathy illustrates key features of our novel animal model that are well-suited for the study of angiogenesis & angio-therapeutic response. Controlling the variable of tumor heterogeneity, by *in vivo* microcartography, allows the study of vascular biology, *at the single-vessel level* & cellular level, *in vivo*. The fusion of multiphoton fluorescence & confocal reflectance imagery provides direct-visualization of microvascular architecture, with subcellular clarity, and microvascular hemodynamics. Imaging of tumor microvascular blood flow is likely indispensable, for any *in vivo* model used to evaluate novel angiotropic therapeutic agents, which may alter tumor hemodynamics, such as vascular disrupting agents.[34] Imaging of radiation vasculopathy was not the focus of this research article *per se*; yet the anecdotal data remains interesting. Endothelial *Tie2*GFP signal diminished, post-irradiation (but not in control mice), prior to tissue infarction, inviting speculation that loss of endothelial fluorescence and the increasing cellular/tissue reflectance, also-observed, are signs of radiotoxicity, in this model system. The vascular histopathology associated with the radiation dose used, in this case, was previously-detailed[18] and *Tie2* expression plays a key role in endothelial cell survival.[35]

Summary

This novel model system allows researchers to navigate a living tumor microenvironment, enabling the architecture & hemodynamics of tumor microvasculature in a *selected* microscopic region to be serially-monitored, at the cellular or single-vessel level, *in vivo*. We expect this model to be useful as a preclinical platform for studying tumor angiogenesis & development of anti-angiogenic treatments: the variable of tissue heterogeneity can be controlled, so that measured changes, in angiogenesis, post-treatment, can be more clearly attributed to therapeutic-efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tie2/GFP expression by endothelial cells as visualized by multiphoton fluorescence microscopy. The multiphoton image is formed from the maximum intensity projection of a 140 μm z-stack of 140 optical sections with 16 frame averages per section.

Figure 2.

The phenotype of pathological neovascularization compared to healthy vasculature. Upper Row: Tie2/GFP expression by tumor neovasculature (left) and normal microvessels of a tumor-free mouse (right); the scale bar measures 50 μm . These multiphoton images represent the maximum intensity projection of 138 μm z-stack with 61 optical sections (upper left) and 226 μm z-stack with 100 optical sections (upper right). Lower Row: Scanning electron microscopic imaging of polymer casts of tumor (left) and normal (right) microvasculatures (reprinted by permission from Macmillan Publishers Ltd: Nature Med (2003) 713-725, copyright 2003.) [36]

Figure 3.

In vivo microcartography relocates selected tumor microvessels for serial imaging of radiation vasculopathy, 1 hour before irradiation (*upper left*) and 3, 6, and 24 hours after external beam irradiation with 10 Gy (*upper right, lower left & lower right*, respectively). Each image represents a single optical plane of a $270 \times 270 \mu\text{m}$ field of view. Dynamic reflectance images (red) were combined with summed dynamic Tie2/GFP images (green). The dynamic reflectance images demonstrate tissue anatomy and hemodynamics, including radiation-induced thrombosis shown in the online supplemental video files.