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Semi-automated rapid quantification of brain vessel density utilizing fluorescent microscopy

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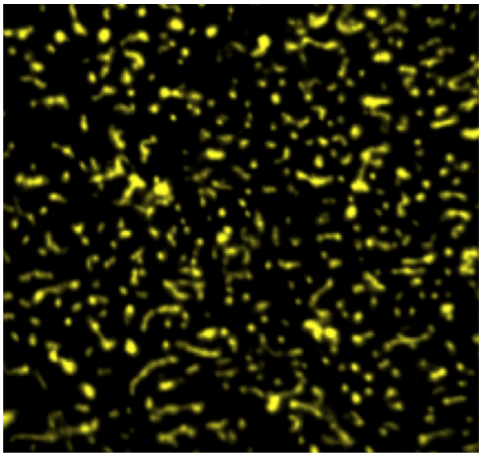
HIGHLIGHTS

- Semi-automated methodology utilizes fluorescence microscopy to calculate vascular density within brain and brain tumors.
- Novel methodology produces data comparable to previously hand counting pathology methods.
- Vascular density is decreased in experimental brain metastases compared to a preclinical glioma model.

GRAPHICAL ABSTRACT



Vascular density varies significantly between brain regions and between healthy and diseased tissue. However, current methods are arduous and time consuming. Herein we present a rapid simple method to quantify vascular density in brain. Normal brain vasculature is seen with indocyanine green fluorescence within the image.

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ABSTRACT

Background: Measurement of vascular density has significant value in characterizing healthy and diseased tissue, particularly in brain where vascular density varies among regions. Further, an understanding of brain vessel size helps distinguish between capillaries and larger vessels like arterioles and venules. Unfortunately, few cutting edge methodologies are available to laboratories to rapidly quantify vessel density.

Abbreviations: BBB, blood brain barrier; SD, Sprague-Dawley rats; CD-1, Charles River CD-1 Swiss mice; F344, Fisher 344 rats; VD, vessel/vascular density; kDa, kilodalton; ICG, indocyanine green; eGFP, enhanced green fluorescent protein; MW, molecular weight; TX Red, Texas Red; FITC, fluorescein isothiocyanate.

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New method: We developed a rapid microscopic method, which quantifies the numbers and diameters of blood vessels in brain. Utilizing this method we characterized vascular density of five brain regions in both mice and rats, in two tumor models, using three tracers.
Results: We observed the number of sections/mm^2 in various brain regions: genu of corpus callosum 161 ± 7, hippocampus 266 ± 18, superior colliculus 300 ± 24, frontal cortex 391 ± 55, and inferior colliculus 692 ± 18 ($n = 5$ animals). Regional brain data were not significantly different between species ($p > 0.05$) or when using different tracers (70 kDa and 2000 kDa Texas Red; $p > 0.05$). Vascular density decreased (62–79%) in preclinical brain metastases but increased (62%) a rat glioma model.
Comparison with existing methods: Our values were similar ($p > 0.05$) to published literature. We applied this method to brain-tumors and observed brain metastases of breast cancer to have a ~2.5-fold reduction ($p > 0.05$) in vessels/mm^2 compared to normal cortical regions. In contrast, vascular density in a glioma model was significantly higher (sections/mm^2 736 ± 84; $p < 0.05$).
Conclusions: In summary, we present a vascular density counting method that is rapid, sensitive, and uses fluorescence microscopy without antibodies.

1. Introduction

Vascular density varies from organ to organ, with some tissues such as lung containing 6-fold higher values than heart (Targan et al., 2003). The brain is a highly vascularized organ, although vessel density varies among regions i.e., thalamus contains 460 vessels/mm^2 which is double the number of blood vessels per unit of tissue compared to the superior colliculus (Klein et al., 1986; Wu et al., 2004). Alterations in vessel density are noted in pathological conditions like ischemia, aging, and cancer (Aronen et al., 1994; Eberhard et al., 2000; Farkas and Luiten, 2001; Mann et al., 1986). Clinically, low vascular density is used as a measure of a lack of developmental progress and as an indicator of cerebral palsy or mental retardation in infants (Miyawaki et al., 1998). Similarly a higher density of brain vasculature in stroke patients has been correlated with improved progress and survival due to the increased blood flow to the damaged area (Krupinski et al., 1993). In contrast, higher vessel densities have been associated with lower patient outcome in several types of cancer (Bevilacqua et al., 1995; Nico et al., 2008; Uzzan et al., 2004; Weidner et al., 1993).

Currently, several methods are used to calculate vessel density including the Chalkley method, the Weidner approach, and capillary perfusion of fluorescein isothiocyanate (FITC)-labeled globulin or dextran. Each of these techniques involves manual counting of vessels, which has the limitations of being highly time consuming and difficult to reproduce (Chalkley, 1943; Gobel et al., 1991; Weidner et al., 1991). Human error can be a significant confounding variable in the identification of individual vessels. For example, when the thickness of the tissue sample is so thin that only a small portion of a vessel might be visible, variable human judgement can lead to errors in counting (Nico et al., 2008; Simpson et al., 1996). A review of 43 studies which evaluated vascular density and patient prognosis in breast cancer revealed that only three studies utlized a completely automated methodology to assess vessel density. Of the remaining 40 studies, four used a combined manual and automated system (Uzzan et al., 2004).

To address the limitations of time and human error, we have developed a semi-automated rapid methodology to quickly and accurately assess vascular density of brain tissue. Briefly, the method consists of injecting a large molecular weight fluorescent marker into the peripheral vasculature, allowing the dye to circulate in the blood for approximately 60–120 s followed by immediate sacrifice, removal of the brain from the skull within 45 s, and immediate freezing in isopentane. Similar to previously published work, in our initial studies, we used the large vascular-impermeant fluorescent dextrans, Texas Red 2000 kDa and Texas Red 70 kDa, to count the normal brain vascular density of both mice and rats. In subsequent studies we used indocyanine green (ICG), which upon entry to circulation becomes bound to albumin. Albumin is rapidly trapped in the vasculature, and has been used previously as a vascular marker (Habazettl et al., 2010). In studies using Texas Red, we observed that vascular density values in rat and mouse brain showed no significant difference from previously published results using manual counting methods. To expand the application of this method to pathological conditions, we incorporated an experimental brain metastases of breast cancer and an implanted glioma model. Comparison of the metastatic and glioma models showed striking differences in vascular density. Brain metastases generally had a significantly lower number of capillaries per unit of tissue, compared to normal brain, whereas the highly aggressive RG-2 glioma model was 5–6 fold higher in density. In summary, we demonstrate that this methodology has the ability to efficiently and accurately calculate vascular density in normal and pathological brain tissue. Further, this method is easily transferable to numerous labs, which may increase vascular density counting as a variable in neurological studies.

2. Methods

2.1. Chemicals

Lysine fixable dextrans Texas Red 2000 kDa MW and Texas Red 70 kDa MW were purchased from Molecular Probes (Invitrogen, Eugene, OR). Indocyanine green (ICG) and all other analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Human metastatic breast cancer cells transfected with enhanced green fluorescent protein (eGFP) over-expressing Her2 (MDA-MB-231-BR-Her2) and rat glioma (RG-2) cells were cultured in DMEM supplemented with 10% FBS (MDA required Zeocin 300 μg per 500 mL media, Invitrogen). All cells were used in passages 1–10 and maintained at 37 °C with 5% CO_2. For preparation of cells for injection, cells were grown to 70% confluency, trypsinized, and rinsed twice in 4 °C PBS to remove all traces of serum. Cells were resuspended in serum free 4 °C DMEM and placed on ice. All cell lines were kindly provided by the laboratory of Dr. Patricia Steeg at the National Cancer Institute.

2.3. Animals

Male F344 rats (220–330 g; $n = 12$), male CD-1 mice (25–30 g, $n = 12$), and female NuNu mice (~25 g; $n = 12$) were obtained from