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Microvascular Perfusion Changes following Transarterial Hepatic Tumor Embolization

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Abstract

Purpose—To quantify changes in tumor microvascular (< 1 mm) perfusion relative to commonly used angiographic endpoints.

Materials and Methods—Rabbit Vx2 liver tumors were embolized with 100–300- μ m LC Bead particles to endpoints of substasis or complete stasis (controls were not embolized). Microvascular perfusion was evaluated by delivering two different fluorophore-conjugated perfusion markers (ie, lectins) through the catheter before embolization and 5 min after reaching the desired angiographic endpoint. Tumor microvasculature was labeled with an anti-CD31 antibody and analyzed with fluorescence microscopy for perfusion marker overlap/mismatch. Data were analyzed by analysis of variance and post hoc test ($n = 3$ –5 per group; 18 total).

Results—Mean microvascular density was $70 \text{ vessels/mm}^2 \pm 17$ (standard error of the mean), and $81\% \pm 1$ of microvasculature (ie, CD31⁺ structures) was functionally perfused within viable Vx2 tumor regions. Embolization to the extent of substasis eliminated perfusion in $37\% \pm 9$ of perfused microvessels ($P > .05$ vs baseline), whereas embolization to the extent of angiographic stasis eliminated perfusion in $56\% \pm 8$ of perfused microvessels. Persistent microvascular perfusion following embolization was predominantly found in the tumor periphery, adjacent to normal tissue. Newly perfused microvasculature was evident following embolization to substasis but not when embolization was performed to complete angiographic stasis.

Conclusions—Nearly half of tumor microvasculature remained patent despite embolization to complete angiographic stasis. The observed preservation of tumor microvasculature perfusion with angiographic endpoints of substasis and stasis may have implications for tumor response to embolotherapy.

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An Appendix and Figures E1 and E2 are available online at www.jvir.org

Although transarterial embolization and chemoembolization have been performed for decades, there are relatively few quantitative data describing the effects of embolotherapy on the tumor microenvironment. Currently, angiography is used to visualize tumor-supplying arteries and monitor reduction in blood flow in these arteries to gauge residual tumor perfusion and treatment endpoints. However, only arteries > 1 mm in diameter can be sufficiently resolved with angiography, and smaller vessels remain difficult to evaluate despite magnification and other efforts to optimize imaging. This is a significant limitation, as tumor microvasculature, the small blood vessels (10–30 μm) responsible for nutrient delivery and oxygen exchange to individual tumor cells, are typically approximately 100 times smaller than arteries resolved with angiography. This highlights the importance of understanding changes in tumor microvascular perfusion in the optimization of embolotherapy. Although the relationship between angiographic embolization endpoints and global tumor perfusion has been quantified (1), the effect of embolization on the extent of tumor microvascular perfusion has not been well defined.

There are established tools and techniques used to assess microvascular density and perfusion within tumors in preclinical models and clinical pathology. For example, the CD31 antibody (which binds a glycoprotein expressed by endothelial cells) can provide an indicator of the microvascular density in tumors, and CD31 can be complemented by intravascular delivery of perfusion markers such as Hoechst (which binds nucleic acids) or lectins (which bind glycoproteins in the endothelial wall) to identify the fraction of functionally perfused microvasculature in histologic sections (2,3). Fluorescently conjugated lectins allow simultaneous polychromatic visualization of perfused vasculature (3). In addition, advanced microscopes with integrated mechanical slide scanning capability can now provide high-resolution (< 1 μm) histologic images over large tissue regions (> 1 cm) that permit regional/geographic microvasculature mapping of entire tumor cross sections. When perfusion markers are administered appropriately and analyzed with quantitative techniques, the influence of transarterial embolization on tumor microvascular perfusion may be determined.

The objective of the present study was to quantify changes in tumor microvascular (< 1 mm) perfusion relative to commonly used angiographic embolization endpoints in intrahepatic Vx2 tumors in a rabbit model. Transcatheter arterial delivery of fluorescently conjugated lectins and high-resolution microscopy were used to identify changes in microvascular perfusion caused by embolization to angiographic substasis and stasis.

MATERIALS AND METHODS

The materials and methods presented here are abbreviated. Additional details can be found in the Appendix (available online at www.jvir.org) (4).

Delivery of Embolic Material to Intrahepatic Vx2 Tumors

Femoral access and catheter placement in the proper hepatic artery was obtained with a 3-F sheath (Cook, Bloomington, Indiana) and 2.4-F microcatheter (Progreat, Terumo, Somerset, New Jersey). Routine fluoroscopy with a model 9600 or 9900 Elite Digital Mobile Super C-arm (GE Healthcare/OEC Medical Systems, Waukesha, Wisconsin) guided catheter

placement and confirmed catheter tip location. Intrahepatic Vx2 tumors were identified with angiography as shown in Figure 1.

LC Bead particles (100–300 μm ; Biocompatibles BTG UK, Farnham, United Kingdom) were mixed at a 1:20 ratio in saline solution/Isovue 300 contrast agent (1:1 ratio; Bracco Diagnostics Inc., Monroe Township, New Jersey) and delivered under fluoroscopic monitoring to the desired angiographic endpoints of substasis or stasis. Embolization to substasis was defined as delivery of a fixed bead volume (0.05 mL total bead volume) that resulted in occlusion of tumoral and peritumoral vessels while maintaining patency of the feeding hepatic artery. Embolization to stasis was defined by complete occlusion of tumoral and peritumoral vessels as well as the more proximal feeding hepatic artery branches, with apparent reflux on further injection. The total bead volume required to reach complete stasis ranged from 0.15 mL to 0.3 mL. Intermittent angiography was performed throughout the embolization to monitor antegrade flow and reflux, and angiographic endpoints were evaluated by one of two interventional radiologists (each with more than 6 y of clinical experience). Following embolization, the animals were euthanized, and tumor tissue was harvested, flash-frozen, and stored at -80°C before ex vivo analysis.

Microvascular Perfusion Assessment

Pre- and posttreatment microvascular perfusion was evaluated by administering different fluorophore-conjugated perfusion markers through the catheter before embolization to assess native tumor perfusion (lectin/fluorescein isothiocyanate [FITC]) and 5 minutes after reaching the desired angiographic endpoint to assess the embolization effect (lectin/Texas red). The perfusion markers were delivered at a rate of 1 mL/min (0.5 mg of lectin in 5 mL of heparinized saline solution; tomato lectins; Vector Laboratories, Burlingame, California). Animals treated with coadministration ($n = 3$) and sequential administration ($n = 5$) of perfusion markers served as control groups, and animals embolized to the extent of substasis ($n = 5$) and stasis ($n = 5$) served as treatment groups (18 subjects evaluated in total).

Euthanasia was performed 5 minutes after the final perfusion marker was delivered, and livers were harvested and flash-frozen. Frozen tissue was cryosectioned and then stained for cell nuclei (4',6-diamidino-2-phenylindole; Invitrogen, Grand Island, New York) and blood vessels (CD31; Abcam, Cambridge, Massachusetts; Cy5 conjugated with Zenon labeling; Invitrogen). Microvascular perfusion markers were delivered through the catheter from the same catheter tip location used for embolization. Epifluorescence imaging was conducted with a $10\times$ objective on a Scanscope FL system (Aperio, Vista, California) equipped with a monochrome CCD camera. Image processing software (ImageScope; Aperio) was used to export grayscale images as full-resolution TIFF files for each fluorescent channel.

Image Analysis

Image acquisition and display parameters were held constant to allow for quantitative comparison between groups. For each treatment group, the viable tumor was manually segmented from the surrounding liver parenchyma and necrotic tumor by using 4',6-diamidino-2-phenylindole nuclei stain. Quantitative image analysis was performed with custom MATLAB scripts (Math-Works, Natick, Massachusetts).

The tumor microvascular structures were identified by a CD31 endothelial marker. For every CD31⁺ vessel, the perfusion markers were analyzed for overlap or mismatch. Microvasculature was considered perfused before treatment if it stained positive for only lectin/FITC, newly perfused if it stained positive for only lectin/Texas red, and stably perfused (ie, perfused before and after embolization) if it stained positive for both lectin/FITC and lectin/Texas red. The custom MATLAB script was used to calculate baseline parameters such as microvascular density (in vessels per square millimeter) and percentage of microvasculature perfused before treatment (with a denominator of CD31⁺ structures). The influence of embolization was reported as the percent of microvessels that lost perfusion and the percentage of microvessels that gained perfusion (denominator was perfused microvessels at any time). A microvascular perfusion map was generated by MATLAB to document and illustrate the results as a color-coded image.

Statistics

All statistical analyses were performed by using Graph-Pad Prism 5.0 (GraphPad Software, San Diego, California). Each tumor was considered an independent sample, with two to four replicates per tumor. Groups were compared for differences by one-way analysis of variance and Tukey post hoc test ($n = 3-5$). All P values were two-sided, and a P value less than .05 indicated statistical significance. Values are reported as mean $\pm$ SEM unless otherwise indicated.

RESULTS

Perfusion Marker Delivery and Angiographic Embolization Endpoint

The substasis treatment arm showed a reduction in blood flow that was easily perceived with routine angiography. The main tumor-feeding hepatic artery still filled with contrast agent after substasis embolization, but there was substantially decreased or absent contrast agent delivery to the tumor/peritumor region (ie, pruning), with some contrast agent reflux into proximal gastric arterial branches after embolization. Figure 1a and Figure 1b show a representative example of angiographic feedback before and after embolization. Figure 1c and Figure 1d show a representative example of complete angiographic stasis with no opacification of the tumoral, peritumoral, or main tumor-feeding arteries. There was also substantially more contrast agent reflux into proximal gastric arterial branches after embolization at stasis.

Comparison of Angiography and Microvascular Perfusion Changes

Angiography provided identification of tumor vascular supply, guided selection of these vessels, and showed large vessel pruning and abolition of tumor blush during the procedure (Fig 1). Figure 2 illustrates the angiographic appearance of a tumor and the corresponding microvascular perfusion that was imaged with microscopy. The large vessels feeding the tumor and the surrounding parenchyma were easily visualized on digital subtraction angiography (Fig 2a). Within the same tumor, 5,016 microvessels were quantified on a histologic cross section (Fig 2b). The magnified regions (Figs 2c, 2d) illustrate the spatial inhomogeneity of perfused microvasculature in a tumor (ie, high microvascular density in some regions and low microvascular density in other regions). Tumor microvasculature also

has a diversity of geometry and size as has been previously reported (5), yet these images illustrate that the majority of the Vx2 tumor vasculature was smaller than the resolving limit of angiography systems. Within Vx2 tumor regions, $81\% \pm 1$ of the CD31⁺ vessels were perfused before treatment (across all treatment and control groups) and functional microvascular density was $70 \text{ vessels/mm}^2 \pm 17$. The coadministration and sequential administration of perfusion markers controls had perfusion marker overlap of $76\% \pm 2$ and $74\% \pm 3$, respectively ($P > .05$). Sequential administration of perfusion markers demonstrated $12\% \pm 2$ newly functional microvasculature and $15\% \pm 5$ of the microvasculature perfusion eliminated, indicating that there is some intermittent perfusion in Vx2 tumors.

Microvascular Perfusion Changes

Figure 3 illustrates representative microvascular perfusion in a Vx2 tumor that was embolized to an endpoint of substasis. The CD31⁺ microvessels are in regions of low cellular density. The majority of the microvasculature is shown in yellow in the perfusion map of the whole tumor cross section, indicating that many microvessels remained patent despite embolization to substasis with 0.05 mL of beads. There are also predominately green regions, indicating that the perfusion was eliminated, as well as regions that are red, indicating newly perfused microvasculature (ie, not perfused before embolization). The magnified perfusion map is at a scale at which individual microvessels can be better visualized. This magnification shows a region where there are variable effects of embolization: some microvessels remain functional, some become functional, and perfusion is eliminated in others. As shown in Figure 4, embolization to substasis stopped perfusion in $37\% \pm 9$ of the microvasculature ($P > .05$ vs coadministration and sequential administration controls) and resulted in $8\% \pm 2$ newly perfused microvasculature ($P > .05$ vs sequential administration control).

Figure 5 illustrates a representative Vx2 tumor that was embolized to the endpoint of stasis. The majority of the microvasculature is green and yellow in the perfusion map of the whole tumor cross section, indicating that some microvascular perfusion was eliminated and some remained patent despite embolization to angiographic stasis. In this example, there are regions in the tumor periphery (adjacent to normal liver) that are predominately yellow, suggesting that the perfusion was persistent in the tumor border more frequently than the tumor interior. The perfusion map zoom shows a region where there are variable effects of embolization on adjacent tumor microvessels. Embolization to angiographic stasis stopped microvascular perfusion in $56\% \pm 8$ of the microvasculature ($P < .05$ vs coadministration and sequential administration controls), yet, importantly, $44\% \pm 8$ remained patent. The level of microvessels that remained patent was not significantly different between stasis and substasis groups ($P > .05$). In contrast to substasis, newly perfused microvasculature was basically eliminated ($0.33\% \pm 0.07$) when embolization was performed to the endpoint of stasis ($P < .05$ vs substasis embolization and all controls).

DISCUSSION

Angiographic identification of tumor-supplying arteries and angiographic monitoring of residual flow to tumor tissue are key technical factors that guide transarterial embolization and chemoembolization and may affect treatment outcome. Efforts have been made to standardize angiographic endpoint reporting and perhaps improve reproducibility (eg, subjective angiographic chemoembolization endpoint [SACE] rating scales) (1,6). However, a poor understanding of the inherent limitations of angiographic information may have contributed to our slowly evolving understanding of the effects of chemoembolization on the tumor microenvironment. A more complete understanding of the biology and physiology of chemoembolization can inform ongoing efforts to optimize this promising locoregional therapy.

Current angiographic imaging systems visualize only vessels larger than approximately 1 mm in diameter and therefore do not provide sufficient resolution to monitor and document the changes in tumor microvascular perfusion that occur as a consequence of embolization. The present study assessed microvascular perfusion before and after embolization in Vx2 tumors. Eighty-one percent of the microvascular structures (ie, CD31⁺) were found to be perfused before any embolization. Sequential administration of perfusion markers in a control group demonstrated 12% newly functional microvasculature and elimination of perfusion in 15% of the microvasculature, indicating some intermittent perfusion in these tumors. These data represent a single snapshot in time and support the observation that microvascular perfusion in tumors is often incomplete and dynamic in nature (7,8). Intermittent tumor perfusion, which may result in perfusion-driven transient acute hypoxia, has also been previously reported with the use of other experimental tumor models (9–12).

A meaningful number of microvessels remained patent despite embolization of tumor-supplying arteries to routinely used angiographic treatment endpoints. Delivery of 100–300 μ m-diameter embolic microspheres to a substasis endpoint eliminated only approximately one third of tumor microvascular perfusion (37%). Although embolization to a complete stasis endpoint eliminated perfusion in a larger fraction of the microvasculature (56%), approximately 44% of the tumor microvasculature still remained perfused after embolization. This preservation of tumor microvascular perfusion at angiographic substasis and even stasis may provide at least a partial explanation for a lack of complete histologic response following embolotherapy: specifically, that persistent microvascular perfusion, despite embolization to the extent of angiographic stasis, still provides continued delivery of oxygen and nutrients to potentially hypoxic but viable tumor cells and tissue.

Following embolization to an endpoint of angiographic substasis, a small amount of newly perfused microvasculature (8%) was observed. This was not statistically different from the sequential-administration control (12%) and likely represents intermittent fluctuations in perfusion often found in solid tumors (7,8). However, following embolization to an endpoint of angiographic stasis, < 1% newly perfused microvasculature was observed—far less than the 8% observed following embolization to the extent of substasis ($P < .05$). These data suggest that embolization to the extent of complete angiographic stasis may prevent

perfusion of microvessels that become functional during normal perfusion fluctuations or after substasis embolization.

Tumor perfusion during embolization has been previously assessed by using transcatheter intraarterial perfusion (TRIP) magnetic resonance (MR) imaging (13–15) and computed tomography (CT) (16,17). For example, Jin et al (1) compared SACE and TRIP MR imaging perfusion reductions following chemoembolization and found a significant correlation between subjective angiographic feedback and the quantitative perfusion changes measured with MR imaging, highlighting the value of angiography during chemoembolization procedures. However, this study (1) also demonstrated that, even at angiographic SACE levels of 3 (ie, reduced antegrade arterial flow, eliminated tumor blush) and 4 (ie, eliminated antegrade arterial flow and eliminated tumor blush), there were still perfusion levels of 33.9% and 11.4%, respectively. These data, in combination with our microvessel perfusion results, suggest that traditional angiography is certainly valuable, but not well suited to monitor tumor microvasculature and quantify low levels of perfusion. However, we acknowledge that perfusion CT or TRIP MR imaging may be clinically useful to quantify blood flow and predict tumor response (1,6).

The present study has several limitations. First, the amount of perfusion and evolution of perfusion changes were not measured. The lectin stain indicates only the presence of residual perfusion (ie, binary result or yes/no) and does not report the amount of that perfusion or perfusion changes over time. However, the reduction in perfused microvessels was similar to the reduction observed with TRIP MR imaging, a technique that is capable of reporting the amount of flow (1,13). Second, collateral tumor blood supply that may ultimately impact response to chemoembolization was not investigated; only the arterially supplied microvessels that were perfused from the location of the catheter tip were evaluated in this study. Third, microvascular perfusion assessment with a lectin stain is dependent on the 5% overlap that was chosen based on the coadministration control that demonstrated a 76% overlap. The fact that it is incomplete overlap (not 100%) has been observed by others, including Debbage et al (3), who also observed some separation of the two perfusion markers in microvessels in their coadministration control groups. In addition, undersampling error and field selection bias could have been factors.

Despite the identified limitations, these data emphasize microvascular perfusion changes that result during transcatheter embolization. These changes cannot be appreciated with routine angiography and may help educate and motivate future research into embolic material development and tumor response to embolotherapy. Polyvinyl alcohol microspheres 100–300 μm in size were chosen for this study because they are commonly used in clinical practice. However, the type and size distribution of embolic materials may influence microvascular perfusion (eg, smaller microspheres, which lead to more distal vascular distribution [18], may yield different microvascular perfusion observations). Persistent perfusion or recanalization despite embolization with particles, glue, and coils has been documented and is contrary to what we hope these devices achieve (19). Embolic particles with prothrombotic coatings have been developed and evaluated in vivo (20,21), which may hold promise for a more complete occlusion when required.

The impact of microvascular perfusion on ischemia, hypoxia, tumor biology, and therapeutic effects requires additional studies that may provide a better understanding of the impact of residual microvascular perfusion on tumor necrosis and local disease progression/recurrence despite good technical results following embolization and chemoembolization. Perfusion is essential to provide convective transport and cellular oxygenation, which could improve drug exposure and effectiveness during chemoembolization (22) and may be considered desirable depending on the therapeutic intent.

In conclusion, a complex and dynamic response of microvasculature perfusion was observed following transarterial embolization in the rabbit Vx2 liver tumor model. These findings suggest that angiography is not capable of detecting residual tumor microvascular perfusion. Embolization to substasis and complete stasis angiographic endpoints permitted persistent microvascular perfusion (44% with stasis and 55% with substasis) that was generally localized to the tumor periphery. One important difference was that embolization to the endpoint of complete stasis eliminated newly perfused microvasculature, and this may have important effects on tumor microenvironment. Dynamic changes in tumor microvascular perfusion following transarterial embolization/chemoembolization may impact effectiveness, and an improved understanding of these changes could help to improve the therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FITC	fluorescein isothiocyanate
SACE	subjective angiographic chemoembolization endpoint
TRIP	transcatheter intraarterial perfusion

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Figure 1.

Angiographic appearance of substasis (**a, b**) and stasis (**c, d**) embolization endpoints. Preembolization Vx2 intrahepatic tumor blush is clearly identified (asterisk) with digital subtraction angiography from the proper hepatic artery (**a, c**). Postembolization digital subtraction angiography of the tumors embolized to angiographic substasis and stasis demonstrates flow reduction (**b, d**). Arrowheads estimate the distal extent of contrast agent delivery following embolization at each endpoint.

Figure 2.

Angiographic and histologic tumor perfusion imaging. **(a)** The angiographic appearance of a Vx2 intrahepatic tumor shows perfused blood vessels (> 1 mm) and a tumor blush (blue circle). **(b)** A histologic cross section through the same tumor. The perfused microvasculature in the tumor is identified with staining of a CD31 antibody and a perfusion marker (lectin) delivered intraarterially from the catheter shown in **a**. The blue box indicates the region that is magnified in **c**. **(c)** A magnified region of the whole tumor cross section shows the complex network of microvasculature. The blue box indicates the region that is

magnified in **d. (d)** A small region of the tumor illustrates the size of the microvasculature and the resolution of this histologic technique ($< 1 \mu\text{m}$).

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Figure 3.

Microvascular perfusion analysis with embolization to substasis (0.05 mL of beads delivered). Representative perfusion maps generated from MATLAB processed data (CD31⁺ microvessels and lectin perfusion markers; Fig E1 shows raw data [available online at www.jvir.org]) from a Vx2 tumor illustrate localization of the microvasculature. The perfusion map legend indicates the color that represents the perfusion status of the microvasculature after substasis embolization. The box in the perfusion map of the entire tumor cross section indicates the region of magnification for the perfusion map zoom.

Figure 4.

Microvascular perfusion analysis. Groups were compared for differences in microvascular perfusion by analysis of variance and Tukey post hoc test ($n = 3-5$). Data are presented as mean $\pm$ standard error of the mean. Analysis of variance P values were .5620 (a), .0031 (b), and .0027 (c). * $P < .05$, Tukey pairwise comparisons.

Figure 5.

Microvascular perfusion analysis with embolization to angiographic stasis. Representative perfusion maps generated from MATLAB processed data (CD31⁺ microvessels and lectin perfusion markers; Fig E2 shows raw data [available online at www.jvir.org]) from a Vx2 tumor illustrate localization of the microvasculature. The perfusion map legend indicates the color that represents the perfusion status of the microvasculature after substasis embolization. The box in the perfusion map of the entire tumor cross section indicates the region of magnification for the perfusion map zoom.