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Correlation of ultrasound contrast agent derived blood flow parameters with immunohistochemical angiogenesis markers in murine xenograft tumor models

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ABSTRACT

Purpose: In this study we used temporal analysis of ultrasound contrast agent (UCA) estimate blood flow dynamics and demonstrate their improved correlation to angiogenesis markers relative to previously reported, non-temporal fractional vascularity estimates.

Materials and methods: Breast tumor (NMU) or glioma (C6) cells were implanted in either the abdomen or thigh of 144 rats. After 6, 8 or 10 days, rats received a bolus UCA injection of Optison (GE Healthcare, Princeton, NJ; 0.4 ml/kg) during power Doppler imaging (PDI), harmonic imaging (HI), and microflow imaging (MFI) using an Aplio ultrasound scanner with 7.5 MHz linear array (Toshiba America Medical Systems, Tustin, CA). Time-intensity curves of contrast wash-in were constructed on a pixel-by-pixel basis and averaged to calculate maximum intensity, time to peak, perfusion, and time integrated intensity (TII). Tumors were then stained for four immunohistochemical markers (bFGF, CD31, COX-2, and VEGF). Correlations between temporal parameters and the angiogenesis markers were investigated for each imaging mode. Effects of tumor model and implant location on these correlations were also investigated.

Results: Significant correlation over the entire dataset was only observed between TII and VEGF for all three imaging modes ($R = -0.35, -0.54, -0.32$ for PDI, HI and MFI, respectively; $p < 0.0001$). Tumor type and location affected these correlations, with the strongest correlation of TII to VEGF found to be with implanted C6 cells ($R = -0.43, -0.54, -0.52$ for PDI, HI and MFI, respectively; $p < 0.0002$).

Conclusions: While UCA-derived temporal blood flow parameters were found to correlate strongly with VEGF expression, these correlations were also found to be influenced by both tumor type and implant location.

1. Introduction

Ultrasound contrast agents (UCA; for list of abbreviations see Appendix A) are gas filled microbubbles encapsulated by an outer shell (generally of polymer or lipid) for stability. These agents provide ultrasound contrast due to differences in the compressibility and impedance of the gas core relative to the surrounding medium [1]. These agents act as excellent blood pooling agents due to their size (1–6 μm), which makes them too large to extravasate out of the blood stream, but small enough to transverse the capillary bed [1,2]. The ability to track the perfusion of these agents in real time has also been shown to be useful for the quantification of blood flow dynamics in a variety of applications [3–5].

Contrast-enhanced ultrasound (CEUS) has been used extensively in the imaging of angiogenesis [6–13]. The ability to visualize the tortuous neovasculature within a tumor using CEUS allows for improved identification of cancer [8–10], while changes in this vascularity have also shown to be a useful indicator of treatment response [9,10]. This power of detection appears to increase when using dynamic-CEUS [8,13]. Due to the real time nature of ultrasound and the blood pooling properties of UCA, visualization of UCA perfusion provides an indicator of the blood flow kinetics within the area of interest. Parameters such as the time required from injection to contrast arrival, rate of UCA inflow, rate of UCA washout, and cumulative UCA signal over time (an indicator of

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net blood flow) have all been shown to be potentially useful indicators of lesion malignancy or response to treatment [8,13], and packages to quantify these parameters are now commercially available. Additionally, this analysis can be performed on a pixel-by pixel basis to create parametric maps. These maps illustrate differences in blood flow kinetics within a given region of interest (which are often heterogeneous in tumors), and can also be used for diagnosis [6].

Several ultrasound imaging modes have been explored for angiogenesis imaging. Contrast-enhanced Doppler is unable to image tumor microcirculation due to a resolution limit of approximately 100 μm [14] and the tendency of contrast to create large "blooming artifacts" [15] but the contrast signal has been shown to correlate best to vessels of 20–39 μm in diameter [16]. Pulse inversion harmonic imaging (HI) is now a well established contrast imaging mode which suppresses linear tissue harmonic signals while improving the detection of nonlinear harmonics generated by UCA; and this imaging mode has been shown to improve the visualization of tumor microcirculation [1,17]. Additionally, the use of destruction/replenishment pulse sequences has been shown to increase the ability to visualize microcirculation and perfusion [18–20]. Microflow imaging (MFI) is one such emerging contrast imaging mode based on UCA destructive pulses followed by lower intensity imaging to generate cumulative maximum intensity images of microvasculature [18–20].

While CEUS has been shown to be useful for monitoring angiogenesis, results of correlating lesion vascularity with immunohistochemical markers have been mixed [21–23]. Previously, we compared UCA-derived fractional vascularity measurements with four immunohistochemical markers of angiogenesis in two murine xenograft tumor models using four ultrasound imaging modes (power Doppler, HI, MFI, and flash echo imaging). Results varied with imaging mode, marker, and tumor model, but showed correlations ranging from 0.00 to 0.41 [23]. While these results are encouraging, it is hypothesized that UCA wash-in derived blood flow parameters may more strongly correlate with immunohistochemical markers of angiogenesis than fractional vascularity measures from a single time point. In this manuscript we explore parametric analysis of this dataset to strengthen these correlations through the incorporation of temporal information.

2. Materials and methods

2.1. Ultrasound Imaging and pathology

All animal studies were performed under the guidance of a licensed veterinarian and all protocols were approved by Thomas Jefferson University's Institutional Animal Care and Use committee. Ultrasound imaging and pathology were originally obtained as part of a previous study comparing single time point based fractional vascularity measurements with immunohistochemical markers and has already been described in detail [23]. Briefly, 144 female 200–250 g, Sprague Dawly rats (Taconic Inc., Hudson, NY) were implanted with either C6 glioma cells or NMU mammary gland adenocarcinoma cells (ATTC, Manassas VA). Approximately 2E6 cells were injected subcutaneously into either the thigh or abdomen. Rats were then imaged and sacrificed at either 6, 8, or 10 days post implantation with 12 randomly assigned rats per group (3 time points × 2 implant location × 2 cell lines).

Prior to imaging, rats were anesthetized and a tail vein catheterized using a 24-gauge needle. Ultrasound imaging was performed using power Doppler imaging (PDI), pulse inversion harmonic imaging (HI), and microflow imaging (MFI) on an Aplio scanner with a 7.5 MHz linear array (Toshiba America Medical Systems, Tustin, CA). Ultrasound imaging was performed during bolus tail vein injection of the UCA Optison (GE Healthcare, Princeton, NJ; 0.4 ml/kg for each imaging mode). Contrast wash-in and washout were observed and cine loops stored for post processing. Injections were spaced out 3–5 min to avoid imaging of residual contrast.

Immediately after scanning, animals were sacrificed and the tumors excised. *Ex vivo* scanning was performed to identify the appropriate scan plane. Tumors were fixed in 10% formalin phosphate (Fisher Scientific, Houston, TX) for 12–24 h before being washed and fixed in paraffin. Tumor slices were then stained for Cyclooxygenase-2 (COX2; using a monoclonal antibody from Santa Cruz Biotechnology, Santa Cruz CA), platelet endothelial cell adhesion molecules (using a monoclonal CD31 antibody; Dako Corporation, Carpinteria, CA), basic fibroblast growth factor (bFGF; using a polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA), and vascular endothelial growth factor (VEGF; using a monoclonal antibody from Oncogene Research Products, San Diego, CA). Finally, stained tissue slices were mounted on glass slides and immunohistochemical markers quantified using a semi-automated histomorphology setup as previously described [23].

2.2. Derivation of blood flow parameters

Stored video data from PDI, HI, and MFI exams from each rat were loaded in Matlab (Version 2012a; The Mathworks Inc, Natick MA) for processing. All parametric analysis was performed while blinded to histological results. Because analysis was done on a pixel-by-pixel basis, five rats from the C6 group and four from the NMU were removed due to excessive motion in at least one contrast exam. Time intensity curves were then generated for each individual pixel within the imaging window from the time of contrast injection to peak enhancement. Briefly, this parametric algorithm calculates the signal intensity over time for each pixel to create a time intensity versus intensity curve. These curves typically show a stable baseline intensity level, followed by a sharp rise in intensity at contrast arrival, followed by a gradual decrease in signal intensity as the UCA washes out of the tumor vasculature. From this information parametric maps displaying a maximum intensity projection (MIP; identified as peak pixel intensity in arbitrary units), the time to peak (TTP; the time taken from injection to reach peak intensity in seconds), perfusion (PER; defined as MIP/TTP), and time integrated intensity (TII; defined as the area under the time intensity curve from contrast injection to peak enhancement) were calculated for each exam.

An unprocessed image from each video at baseline (prior to contrast arrival) was used to select as large a rectangular area as possible from within the tumor border. While a circular region of interest may have better encompassed the tumor area, it would also be more susceptible to sampling outside the tumor area at the occurrence of motion. Coordinates from this selection were then applied to the four parametric maps and the pixels within the border averaged to derive the four blood flow parameters for the entire tumor area. These four parameters from each imaging mode were then matched to the immunohistochemical findings quantified as part of our previously reported study [23].

2.3. Statistical analysis

All statistical analyses were performed in GraphPad Prism (Version 5.0, GraphPad Software, San Diego CA). Pearson coefficients (R; the standard measure of strength in correlation between two groups of variables) were computed to calculate the linear correlation of each temporal parameter to each immunohistochemical marker. Bonferroni correction of 16 multiple comparisons (from the 4 × 4 datasets, used to avoid errors of perceived significance