Hypoxic Environments and the Extracellular Matrix: MRI and Second Harmonic Generation Microscopy Studies

S. M. Kakkad[1], M. Solaiyappan[1], K. Glunde[1], B. O'Rourke[2], A. Pathak[1], V. Raman[1], M-F. Penet[1], and Z. M. Bhujwalla[1]

[1]JHU ICMIC Program, The Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2]Institute of Molecular Cardiobiology, Johns Hopkins University School of Medicine, Baltimore, MD, United States

Introduction. To understand the relationship between hypoxic environments and macromolecular transport and drain in solid tumors, we characterized collagen fiber distribution in hypoxic and non-hypoxic tumor regions using second harmonic generation (SHG) microscopy of human breast or prostate cancer xenografts genetically engineered to fluoresce under hypoxia (1). Intrinsic SHG microscopy is proving to be valuable for understanding the remodeling of the collagen matrix in intact tissue. In tumors, the SHG signal primarily arises from fibrillar collagen (2). While quantitation of collagen structure is challenging, an advantage of this approach is that differences in fiber structure can be determined in 3D with micrometer resolution. It is possible that hindrance to the movement of macromolecules due to abnormal extracellular matrix (ECM) deposition in hypoxic areas may lead to poor drainage. On the other hand, hypoxia is known to upregulate expression of matrix metalloproteinases (3), making the ECM more porous under hypoxic conditions. Consequently, both functional and molecular characterization are necessary to provide insight into the dynamics governing interstitial fluid drain, ECM remodeling, and the mechanisms of clearance of macromolecules in tumors.

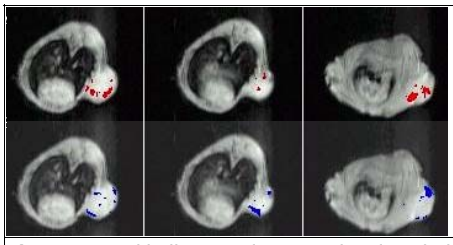

Figure 1: Multi-slice MR images showing draining (upper panel) and pooling (lower panel) of albumin-GdDTPA. Images from 1 mm slices were acquired from an MDA-MB-231 tumor of 160 mm^3, with an in-plane spatial resolution of 256 μm.

Methods and Results.

MR: MRI characterization of the macromolecular contrast agent albumin-GdDTPA was performed on a Bruker Avance 4.7T spectrometer, as previously described (4) to quantify draining and pooling voxels in tumors. As shown in Figure 1, distinct pooling and draining regions were typically identified in tumors, demonstrating the heterogeneity in the transport of macromolecules through the ECM of tumors.

Studies characterizing collagen fiber distribution in hypoxic and normoxic regions were performed on fresh tissue slices obtained from human cancer xenografts (MDA-MB-231 breast cancer or PC-3 prostate cancer), genetically engineered to express enhanced green fluorescent protein expression (EGFP) under the control of the hypoxia response element of VEGF (1).

Optical: Optical imaging was done using a Nikon E600FN upright fluorescence microscope with a Bio-Rad MRC-1024/2-P multi-photon imaging system attachment. Freshly excised tissue slices, approximately 1 mm thick, were obtained from tumors using a tissue slicer (Braintree Scientific). The fluorescence from EGFP expressing hypoxic cells was imaged by two-photon excitation at 880 nm. Collagen fibers from colocalized regions in the same slices were imaged using SHG microscopy at 880 nm. Fiber distributions in hypoxic and normoxic regions were visualized and quantified using a specialized 3D analysis tool that was developed to identify the collagen fiber distribution based on its tubular geometry, and quantify collagen fiber volume in hypoxic and normoxic regions of the tumor. Images from each channel were analyzed independently to avoid channel crosstalk and improve image quality. In this approach, we computed the 3D Euclidian distance maps and used the histogram profile to characterize the distribution of the collagen fibers. In addition, fiber volumes were computed for hypoxic and normoxic regions of interest (ROIs).

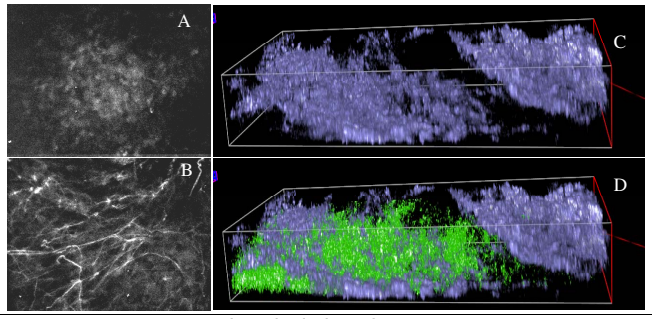

Figure 2: 3D reconstructed optical data from an MDA-MB-231 tumor. 34 slices of the section with 5 micrometer z step were acquired. (A) A 2D collapsed image of the volume in the two photon channel (excitation at 880nm) showing EGFP expression. (B) Collagen fibers from (A) ROI detected in the SHG channel. (C) 3D visualization of the collagen fibers overlaid with (D) EGFP expressing hypoxic cells.

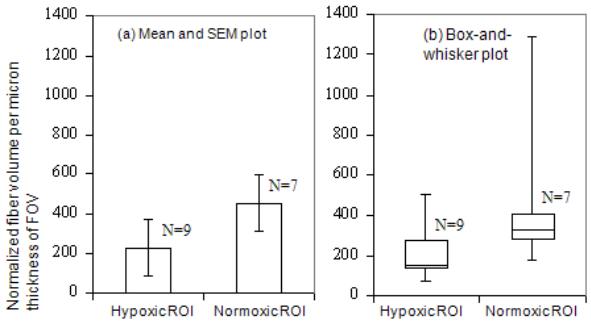

Figure 3: Comparison of normalized fiber volumes per micron thickness of FOV in normoxic (n=7) and hypoxic (n=9) ROIs. (a) depicts the difference by mean ±SEM plot and (b) depicts the difference by box and whisker plot. The fiber volumes are represented by voxel counts.

As shown in Figure 2 and summarized in Figure 3, collagen fiber volumes were different between hypoxic and non-hypoxic tumor ROIs. As evident in Figure 2, hypoxic EGFP expressing regions typically had fewer collagen fibers, as reflected from the lower mean values of fiber volume present in hypoxic regions shown in Figure 3. When we analyzed the distribution of the fibers in both hypoxic and normoxic regions based on the mutual distances between all the voxels that make up the fibers, a statistically significant difference was observed in the cumulative histogram plot of the fiber voxel distribution in ks (Kolmogorov–Smirnov) test with *p value* <<0.01.

Discussion. Our data provides evidence that the ECM is significantly altered in hypoxic tumor regions. The analyses revealed that the amount of collagen fiber in hypoxic ROIs was lower than that of non-hypoxic ROIs (Figure 3), and the fiber distribution was significantly different. These data suggest that the ECM underwent structural changes in hypoxic regions. Several matrix metalloproteinases (MMPs) that degrade the ECM are upregulated under hypoxia (3). The observation of fewer fiber voxels in hypoxic regions is consistent with this MMP upregulation under hypoxia, and with the possibility that hypoxic environments may facilitate the movement of macromolecules as well as dissemination of cancer cells in the metastatic cascade. To further quantify the effect of hypoxia on macromolecular transport, we are currently relating macromolecular transport characteristics to the distribution of hypoxia within the same tumor.

References. 1. Raman V, et al., Cancer Res, 12: 9929-36, 2006; 2. Brown, E., et al., Nat Med, *9:* 796-800, 2003; 3. Ackerstaff et al., Neoplasia, 9: 1138–1151, 2007; 4. Pathak AP, et al., Cancer Res.66:5151-8, 2006.

Acknowledgement. This work was supported by NIH P50 CA103175 and NIH U54 CA091409 (in collaboration with Dr. Paul Wang at Howard University).