Distinct molecular profiles characterize hypoxic breast tumor regions detected by combined MRSI, optical imaging, and imaging mass spectrometry

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Introduction

Hypoxia is one of the hallmarks of tumors, and triggers multiple signaling cascades that significantly impact upon biological outcomes including selection for resistance to apoptosis, resistance to radiation and chemotherapy, and increased tumor aggressiveness [1]. These hypoxic regions that arise from inadequate blood supply of tumors have a heterogeneous spatial distribution [1]. Understanding and characterizing the hypoxic response of tumors is therefore critically important and requires approaches that can evaluate the complexity as well as the spatial distribution of the response. To achieve this goal we have, for the first time, applied imaging mass spectrometry (IMS) [2, 3] to obtain comprehensive 3D data sets to discover and evaluate novel hypoxia-driven signaling pathways in human breast cancer xenograft models genetically engineered to express enhanced green fluorescent protein (EGFP) under hypoxic conditions (HRE-EGFP) [4, 5]. Cutting-edge IMS in combination with optical imaging of EGFP revealed the spatial distribution of a multitude of intact proteins and other biomolecules in hypoxic and well-oxygenated tumor regions. The incorporation of *in vivo* magnetic resonance spectroscopic imaging (MRSI) in this study allowed us to relate the molecular characterization obtained with IMS to functional metabolic changes obtained with MRSI in these tumors. Necrotic/hypoxic tumor regions displayed characteristic molecular-metabolic profiles that were very different from those of normoxic tumor regions and contained multiple to date unknown biomolecules.

Methods

Solid MDA-MB-231 HRE-EGFP breast tumor xenografts were obtained by orthotopically inoculating $2x10^6$ cells in the mammary fat pad of female severe combined immunodeficient (SCID) mice. We combined *in vivo* detection of metabolites using 3D MRSI with optical imaging of the hypoxia-reporter HRE-EGFP [4, 5] to detect hypoxic tumor regions as well as with IMS [2, 3] to detect to date unknown proteins and other biomolecules. The 3D MRSI data sets were acquired on a Bruker Biospec 9.4 T Small Animal Scanner using a standard 3D chemical shift imaging (CSI) spin echo sequence with VAPOR water suppression and the following parameters: echo time (TE) of 82 ms, repetition time (TR) of 1000 ms, field of view (FOV) of 8 mm x 8 mm x 8 mm, 8 x 8 x 8 voxels, number of scans (NS) of 4, block size (CB) 1024, sweep width (SW) of 7,000 Hz. Reference 3D CSI images of the unsuppressed water signal were acquired with TE=15 ms and NS=2, and all other parameters remaining the same. Data were visualized with in-house IDL-based software. Following MRSI, mice were sacrificed, each tumor was marked for spatial referencing, and sectioned to obtain freshly cut 2-mm thick slices. To visualize hypoxia, brightfield and EGFP fluorescence images of the same FOV were acquired of the fresh tumor slices with a 1x objective on Nikon inverted microscope. Tumor sections were cryo-sectioned, thaw-mounted on indium tin oxide (ITO)-coated glass slides, and covered with matrix and a thin gold layer. The matrix consisted of 2,5-Dihydroxybenzoic acid (DHB) 30mg/mL in 50% acetonitrile/0,1% trifluoroacetic acid. Matrix-assisted laser desorption ionization (MALDI) IMS was performed on a modified TRIFT-II instrument as described previously [2, 3] to detect small molecules as well as intact proteins up to 20,000 m/z. To image an entire breast tumor section, the sample stage was moved at 100 µm/s at a laser repetition rate of 10 Hz within a line-scan. Our in-house software Datacube Viewer was used to visualize any mass of interest from the generic data format.

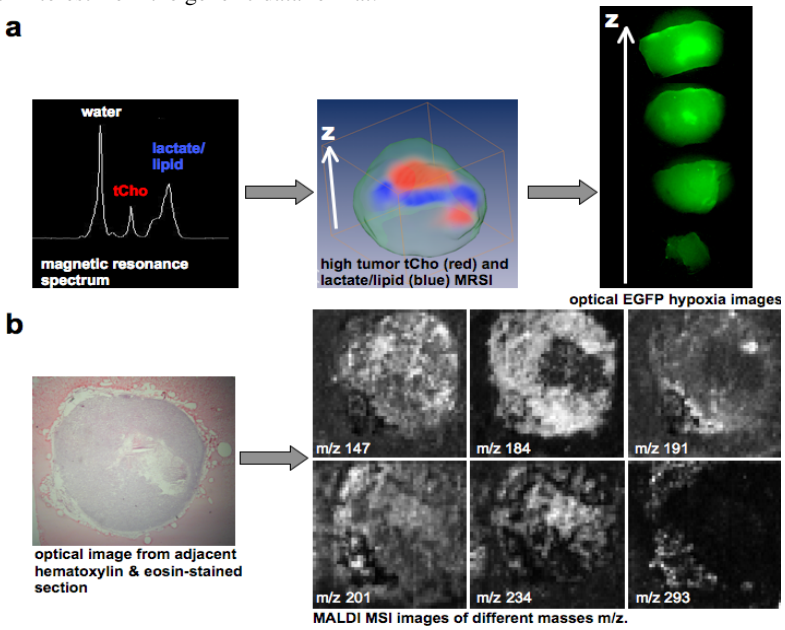

Figure 1: (a) 3D MRSI demonstrating the distribution of high total choline-containing compounds (tCho) displayed in red and high lactate/lipid displayed in blue corresponding to the ex vivo hypoxia maps of EGFP-fluorescing hypoxic tumor regions. tCho colocalizes with hypoxic regions. (b) MALDI MSI images of several m/z demonstrate the heterogeneous distribution of these small molecules in viable tumor regions detected in the corresponding hematoxylin & eosin-stained section.

Results

3D MRSI as well as IMS revealed high levels of total choline (Figure 1a) in hypoxic EGFP-fluorescing regions, and phosphocholine, which is m/z 184 (Figure 1b), in viable hypoxic tumor regions. Necrotic and hypoxic tumor regions showed a markedly different molecular and metabolic profile compared to other regions (Figure 1b). Other than m/z 184, which was unequivocally assigned to phosphocholine by using reference compounds, the spatially resolved mass spectrometric profiles displayed as single selected m/z images in Figure 1b that are high or low in necrotic and hypoxic regions remain to be assigned to specific biomolecular structures.

Discussion

IMS detected the spatial distribution of multiple different species of biomolecules, such as small molecules and proteins, which can be related to the corresponding MRSI and optical imaging data sets (Figure 1b). The elevation of total choline in hypoxic regions in this aggressive breast tumor model is in good agreement with our previous study showing elevated total choline levels in hypoxic regions in a prostate tumor model [5]. Confirming our multimodal imaging approach, which, for the first time, combined optical imaging, MRSI, and IMS, phosphocholine detected by m/z 184 in IMS images was elevated in hypoxic regions with high total choline. The obtained metabolic and molecular 3D distributions can in the future be used to differentiate distinct microenvironmental regions in breast tumors, and provide insights in the molecular processes in hypoxic regions. We are currently in the process of assigning the m/z's detected in the different tumor regions to specific biomolecular structures using proteomic-based approaches.

References : [1] Hockel and Vaupel, *J Natl Cancer Inst* 93, 266-76 (2001); [2] Luxembourg *et al*, *Anal Chem* 76, 5339-44 (2004); [3] Luxembourg *et al*, *Rapid Commun Mass Spectrom* 20, 3435-42 (2006); [4] Raman *et al*, *Cancer Res* 66, 9929-36 (2005); [5] Glunde *et al*, *Cancer Res* 68, 172-80 (2008). This work was supported by NIH R01 CA134695 and NIH P50 CA103175 (JHU ICMIC Program).