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Evaluation of high frequency ultrasound methods and contrast agents for characterising tumor response to anti-angiogenic treatment

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ABSTRACT

Purpose: To compare non-enhanced and contrast-enhanced high-frequency 3D Doppler ultrasound with contrast-enhanced 2D and 3D B-mode imaging for assessing tumor vascularity during antiangiogenic treatment using soft-shell and hard-shell microbubbles.
Materials and methods: Antiangiogenic therapy effects (SU11248) on vascularity of subcutaneous epidermoid-carcinoma xenografts (A431) in female CD1 nude mice were investigated longitudinally using non-enhanced and contrast-enhanced 3D Doppler at 25 MHz. Additionally, contrast-enhanced 2D and 3D B-mode scans were performed by injecting hard-shell (poly-butyl-cyanoacrylate-based) and soft-shell (phospholipid-based) microbubbles. Suitability of both contrast agents for high frequency imaging and the sensitivity of the different ultrasound methods to assess early antiangiogenic therapy effects were investigated. Ultrasound data were validated by immunohistology.
Results: Hard-shell microbubbles induced higher signal intensity changes in tumors than soft-shell microbubbles in 2D B-mode measurements (424 ± 7 vs. 169 ± 8 A.U.; $p < 0.01$). In 3D measurements, signals of soft-shell microbubbles were hardly above the background (5.48 ± 4.57 vs. 3.86 ± 2.92 A.U.), while signals from hard-shell microbubbles were sufficiently high (30.5 ± 8.06 A.U). Using hard-shell microbubbles 2D and 3D B-mode imaging depicted a significant decrease in tumor vascularity during antiangiogenic therapy from day 1 on. Using soft-shell microbubbles significant therapy effects were observed at day 4 after therapy in 2D B-mode imaging but could not be detected in the 3D mode. With non-enhanced and contrast-enhanced Doppler imaging significant differences between treated and untreated tumors were found from day 2 on.
Conclusion: Hard-shell microbubble-enhanced 2D and 3D B-mode ultrasound achieved highest sensitivity for assessing therapy effects on tumor vascularisation and were superior to B-mode ultrasound with soft-shell microbubbles and to Doppler imaging.

1. Introduction

Tumor angiogenesis is a crucial precondition for progression of various types of cancer [1]. For interfering with tumor progression, many antiangiogenic drugs have been developed and new promising substances are in the pipeline. Prior to a clinical application, these novel substances need to be tested in animal models of human cancers in order to evaluate their potential value [2]. Until now, the potential of new antiangiogenic substances is mostly investigated by calliper measurements of the tumor size combined with invasive histological examinations. Alternatively, sophisticated and rather expensive techniques of non-invasive imaging have already been applied for testing drug efficiency [2].

In contrast, ultrasound has been established as a cheap and easy applicable method that allows longitudinal animal studies with high throughput in a time and cost effective manner [3–5]. Due to the higher spatial resolution necessary for visualizing millimeter sized rodent tumors, high-frequency ultrasound systems (>20 MHz) are used [4,5]. These dedicated small animal imaging systems make a wide variety of different measurements possible, ranging from non-enhanced Doppler to contrast-enhanced Doppler

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and dynamic contrast-enhanced B-mode imaging [6–10]. Despite the fact that these techniques have shown convincing results in assessing antiangiogenic therapy effects, a comparison and systematic evaluation has not yet been performed.

Ultrasound-based examinations of tissue vascularisation can be performed in 2D [9] and 3D [6–8]. Up to now, most studies were performed with 2D ultrasound. However, a major limitation of 2D scans is the reduced reproducibility of the data. In heterogeneous tumor tissues, the chosen 2D-slice might not be representative for the entire tumor. Even if the acquired slice is representative, it will be difficult to relocate the identical slice position when performing longitudinal studies. Hence, a 3D contrast-enhanced scanning technique might be more accurate in determining the vascular status of a tumor, and thus more sensitive in assessing antiangiogenic therapy effects.

Additionally, optimal contrast agents for preclinical imaging at high frequencies remain to be defined. Currently, only a few commercial contrast agents are specially designed for high frequency contrast-enhanced ultrasound imaging. One of the most frequently applied commercial contrast agents consists of phospholipid-stabilized soft-shell microbubbles with a mixed nitrogen and perfluorcarbon core (Vevo Mikromarker™) [10]. Besides soft-shell based contrast agents, microbubbles with a hard polymeric shell, composed of polybutylcyanoacrylate (PBCA) filled with room air have been introduced into experimental high-frequency imaging recently [11].

Up to now, it remains unclear which method or contrast agent is most favorable for the assessment of antiangiogenic therapy effects in small animal studies. In the current study, we compared the accuracy of established non-enhanced and contrast-enhanced 3D Doppler techniques [8] to a novel contrast-enhanced 3D B-mode scanning protocol using subcutaneous human epidermoid carcinoma xenografts (A431) in nude mice. Furthermore, established dynamic contrast-enhanced 2D imaging [9] was included into the comparative analysis. In addition, we tested soft-shell (phospholipid-stabilized) and hard-shell (PBCA-stabilized) microbubbles for their suitability in high-frequency ultrasound. The imaging protocols as well as the microbubbles were tested with respect to their sensitivity and suitability in assessing antiangiogenic therapy effects using a clinically approved multi-tyrosine kinase inhibitor (SU11248). The results were validated by immunohistochemistry.

2. Materials and methods

2.1. Tumor model and animal handling

Experiments were approved by the governmental review committee on animal care. Human epidermoid carcinoma xenografts were induced by s.c. injection of 4×10^6 A431 cells (Cell Lines Service, Eppelheim, Germany) in the right hind limb of $n = 17$ female nude mice (body weight: 25 g) (Charles River, Sulzfeld, Germany) as described [8]. When the tumors reached a size of 5 mm in diameter, animals were divided randomly into different groups (described below).

All ultrasound measurements were performed using the VEVO770 Micro-Imaging System (VisualSonics, Toronto, Canada) equipped with the RMV-710B scanhead (B-mode frequency 25 MHz; Doppler frequency 25 MHz). Tumor bearing mice were anesthetized by inhalation of 1.5% isofluorane in air. A temporary tail vein catheter was placed for intravenous injection of microbubbles. The skin region covering the tumor was covered with ultrasound gel and the ultrasound transducer was fixed on a motor-driven manipulator above the animal. For 2D dynamic contrast-enhanced B-mode imaging, the scan head was kept stationary. For 3D imaging (B-mode and Doppler), the ultrasound probe moved perpendicular to the beam axis, thereby acquiring consecutive images (distance between consecutive images: 300 μm). Afterwards the acquired 2D images were merged into a 3D data set (Table 1.).

2.2. Contrast agents

- Soft-shell microbubbles (*SS-MB*; Vevo MicroMarker non-targeted contrast agent kit, Visualsonics Inc., Toronto, Canada) are phospholipid-shell microbubbles filled with a mixture of nitrogen and perfluorcarbon with a volume-weighted diameter range of 2.3–2.9 μm [10]. The freeze-dried microbubbles were reconstituted in 0.7 ml sterile sodium chloride at a concentration of 2×10^9 MB/ml. The composition of these microbubbles is comparable to SonoVue® (Bracco Imaging), an ultrasound contrast agent which is commonly used in clinical routine. Severe biological side effects in rodents are not to be expected.
- Hard-shell microbubbles *(HS-MB)* were freshly synthesized as described [11]. For in vivo use, the acidic MB suspension was diluted in phosphate buffer solution (PBS; pH: 7.2; $c = 2.1 \times 10^9$ MB/ml). Size distribution of the MBs was measured using a dedicated particle counter (Multisizer-3, Beckman-Coulter, Fullerton, USA). Their volume-weighted diameter range was 0.87–2.07 μm. HS-MB are comparable to SonaVist® (former Schering AG), which has been already applied in patients. They are rapidly cleared from the blood and are taken up by macrophages in the reticuloendothelial system of the liver and spleen [12,13]. Up to now, severe biological side effects have not been observed.

2.3. Part I: microbubble contrast properties at high frequencies

To investigate the signal intensity generated by SS-MB and HS-MB at high ultrasound frequencies, measurements were performed as follows:

- Firstly, 2D measurements were performed: untreated animals ($n = 5$) received randomly either 50 μl bolus injection of 1×10^8 HS-MB or SS-MB, followed by a flush of 20 μl saline solution. Simultaneously with the injection, 2D dynamic contrast-enhanced (DCE) imaging of the tumor was performed for 80 s (B-mode, frame rate 8 Hz, power setting 16%) and data were post-processed by creating time–intensity curves. 30 min after the first type of contrast agent was administered, the animal received the second type of contrast agent (either HS-MB or SS-MB) and the animal was investigated identically.
- 3D measurements were performed one day after the 2D measurements. First, a non-enhanced baseline scan was acquired. The intensity of each voxel of a baseline scan was subtracted from:

(i) a second non-enhanced scan (generating signals which might result from movement artefacts);
(ii) a first contrast-enhanced scan after injection of either SS-MB or HS-MB (during the phase of a stable blood level);
(iii) a second contrast-enhanced scan after injection of the second type of contrast agent (either SS-MB or HS-MB; after a delay of 30 min to ensure clearance of the contrast agents from the blood).

2.4. Part II: assessment of antiangiogenic therapy effects – imaging protocol and post processing

Treated and control animals ($n = 6$ per group; together $n = 12$ animals) were examined on days 0, 1, 2, and 4 of therapy (SU11248) and were investigated using the following protocols.