Short communication

Comparison of conventional time–intensity curves vs. maximum intensity over time for post-processing of dynamic contrast-enhanced ultrasound

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ABSTRACT

Our aim was to prospectively compare two post-processing techniques for dynamic contrast-enhanced ultrasound and to evaluate their impact for monitoring antiangiogenic therapy.

Thus, mice with epidermoid carcinoma xenografts were examined during administration of polybutylcyanoacrylate-microbubbles using a small animal ultrasound system (40 MHz). Cine loops were acquired and analyzed using time–intensity (TI) and maximum intensity over time (MIOT) curves. Influences of fast ($50 \mu l/2 s$) vs. slow ($50 \mu l/10 s$) injection of microbubbles on both types of curves were investigated. Sensitivities of both methods for assessing effects of antiangiogenic treatment (SU11248) were examined. Correlative histological analysis was performed for vessel-density. Mann–Whitney test was used for statistical analysis.

Microbubble injection rates significantly influenced upslope, time-to-peak and peak enhancement of conventional TI curves ($p < 0.05$) but had almost no impact on maximum enhancement of MIOT curves (representing relative blood volume). Additionally, maximum enhancement of MIOT curves captured antiangiogenic therapy effects more reliably and earlier (already after 1 day of therapy; $p < 0.05$) than peak enhancement of TI curves. Immunohistochemistry validated the significantly ($p < 0.01$) lower vessel densities in treated tumors and high correlation ($R^2 = 0.95$) between vessel-density and maximum enhancement of MIOT curves was observed.

In conclusion, MIOT is less susceptible to variations of the injection's speed. It enables to assess changes of the relative blood volume earlier and with lower standard deviations than conventional TI curves. It can easily be translated into clinical practice and thus may provide a promising tool for cancer therapy monitoring.

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1. Introduction

Selective inhibitors of tumor angiogenesis have emerged as a new class of drugs besides established anticancer therapies. However, clinical cancer trials on novel therapy concepts demonstrated that classic end point analyses based on morphologic criteria (e.g. tumor size regression) are insufficient for monitoring antiangiogenic treatments [1,2]. In this respect, surrogate markers of vascularisation, which can be determined by MRI [3,4], CT [5], PET and SPECT [6], might be valuable to describe the angiogenic activity of tumors and thus to improve treatment planning and monitoring. In addition, contrast-enhanced ultrasound has been used successfully as a cheap, fast, and easily applicable method for serial imaging of cancer patients [7]. It provides physiological estimates of blood flow and blood volume by the use of time–intensity (TI) curves in a region of interest [8,9]. Nevertheless, it is a limitation of ultrasound that an arterial input function is usually not available, which could be used to balance variations in the injection rate of microbubbles and in the cardiac output [10]. Thus, accuracy, comparability and reproducibility of the acquired data are limited.

This constraint may be solved by introduction of a technique named "microvessel imaging" [8], which is based on measuring the maximum enhancement in "maximum intensity over time (MIOT)" curves (maxMIOT). maxMIOT registers the actively perfused vessel surface by recording the intensity of all enhancing voxels during

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an entire measurement. Thus maxMIOT predominantly reflects the relative blood volume (rBV) and should be independent of the injection speed or the cardiac output.

In this respect, we conducted a study to evaluate the influence of the injection rate on maxMIOT in comparison to descriptive parameters of TI curves. Subsequently, we investigated the sensitivity of maxMIOT and conventional TI curves for capturing vessel regression in human epidermoid carcinoma xenografts during antiangiogenic therapy (SU11248, [11]).

2. Materials and methods

2.1. Animal model and treatment

Experiments were approved by the governmental review committee on animal care. Human epidermoid carcinoma xenografts were induced by subcutaneous injection of 6×10^6 A431 cells in the right hind limb of 29 female nude mice. After 14 days of tumor growth, animals were divided randomly into four groups. Seven animals were used to evaluate the influence of the injection rate on the MIOT technique in comparison to conventional TI curves. To investigate the sensitivity of both techniques to antiangiogenic therapy effects, additional animals were divided randomly in a "therapy group" ($n = 7$) and in a "control group" ($n = 5$). Control animals received no medication. In the therapy group, animals received a clinically approved antiangiogenic selective multi-targeted receptor tyrosine kinase inhibitor (SU11248, Sutent®, Pfizer, NY, USA) [11]. Daily i.p. injections of 60 mg/kg body weight were administered as described previously [12].

Ten animals were carried along for histological analysis.

2.2. Synthesis of polymer-stabilized microbubbles

Microbubbles were synthesized as described previously [13]. Monomeric butyl-2-cyanoacrylate (Sichel-Werke, Hannover, Germany) was added to an aqueous acidic solution (pH: 2.0) containing 0.02% Triton X-100 (Sigma–Aldrich, Munich, Germany). Vigorous stirring for 1 h resulted in a microbubble suspension, which was then subjected to a flotation process to separate air-filled from non-air-filled microbubbles. For in vivo use, the acidic microbubbles suspension was diluted in phosphate buffer solution (PBS; pH: 7.2; concentration: 4.8×10^9 microbubbles/ml). Size distribution of the microbubbles was measured with a particle counter (Multisizer-3, Beckman-Coulter, Fullerton, USA). Their mean volume-weighted size was $2.5 \pm 0.76 \, \mu m$ (mean $\pm$ standard deviation).

2.3. Ultrasound protocol

Ultrasound measurements were performed using a small animal ultrasound system operating at 40 MHz (VEVO770, RMV704-transducer, VisualSonics, Toronto, Canada). Animals were anesthetized by inhalation of a mixture of isofluorane (1.5%), and O_2. A temporary tail vein catheter was placed for intravenous injection of microbubbles prior to each examination. The ultrasound transducer was fixed above the tumor, which was covered with ultrasound gel. Injection of microbubbles was performed manually. Dynamic contrast-enhanced imaging (8 frames per second) was performed during injection of microbubbles and cine loops of ~80 s length were stored for consecutive analysis.

2.4. Post-processing of cine loops

Analysis of cine loops was performed using the built-in software of the ultrasound system. A region of interest was drawn manually around the tumor. Each tumor-dataset was evaluated by two protocols:

(1) MIOT curves

MIOT describes a technique for mapping the trajectories of circulating microbubbles. The individual circulating microbubble causes an increase in the acoustic intensity of the corresponding pixel. A pixel-by-pixel analysis of the current frame with the previous frame is performed and the increase in acoustic intensity is registered. The highest amplitude of each pixel is preserved throughout the entire cine loop. The trajectories of the circulating microbubbles resemble the actively perfused vessel surface. The mean increase within a region of interest is displayed as a MIOT curve.

(2) Conventional TI curves

Each post-contrast image of the cine loop is compared with the pre-contrast baseline. The circulating microbubbles hereby cause a transient increase in the acoustic intensity of individual pixels. The mean increase within the region of interest is displayed as a TI curve.

2.5. Protocol of animal studies

Part (A) Influence of the injection rate – A group of untreated animals ($n = 7$) was used to investigate the influence of the injection rate on MIOT and on conventional TI curves. All animals received a rapid bolus injection of 50 μl microbubbles (injection time: 2 s). Cine loops were acquired and stored. A pause of 10 min was chosen to allow clearance of MBs from the circulation. Subsequently, animals received a slow injection of 50 μl microbubbles (injection time: 10 s) and data were acquired and stored as used after rapid injection. TI and MIOT curves were created. Time-to-peak, upslope and peak enhancement were determined from conventional TI curves and compared with the maximum enhancement (maxMIOT) of the MIOT curve. Values of the rapid injection (2 s) were set to 100% to calculate differences.

Part (B) Monitoring of treatment effects – Animals of the treatment ($n = 7$) and control ($n = 5$) groups were examined at days 0, 1, 2 and 4 of therapy. Imaging was performed after manual bolus injection of 50 μl microbubbles over 2 s. Peak enhancement was determined for conventional TI curves and maxMIOT for the MIOT curve (time-to-peak and upslope were not analysed for conventional TI curves due to their strong susceptibility to variations of the injection speed as reported in results of "part A"). Values of day 0 were set to 100%. Intra-individual changes were expressed as change in percent [%].

2.6. Quantitative immunohistochemistry

Six treated and four control animals from "*part B*" (*monitoring of treatment effects*) were sacrificed after the last imaging session on day 4. To investigate early time points (days 0, 1 and 2), ten additional animals, which had not undergone imaging, were used: Two untreated animals served as baseline at day 0. Two treated and two untreated mice were sacrificed at days 1 and 2 each. Tumors were resected, frozen in liquid nitrogen vapor, cut in 10 μm slices and fixed with methanol/acetone. Immunostaining of endothelial cells was performed using a rat-anti-mouse-CD31-antibody (BD Biosciences, San José, USA) in combination with a Cy3-conjugated donkey-anti-rat-antibody (Jackson Immuno-Research, West Grove, USA). Cell nuclei were counterstained by 4′,6-diamidino-2-phenylindole (Invitrogen, Karlsruhe, Germany). Six tissue slices from representative parts of the tumor were investigated entirely using a fluorescence microscope and digital images were captured. For quantitative analysis of fluorescence signals, area fractions with positive fluorescence were calculated. 24 representative area fractions were analyzed from each tumor.