Molecular profiling of angiogenesis with targeted ultrasound imaging: early assessment of antiangiogenic therapy effects

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Abstract

Molecular ultrasound is capable of elucidating the expression of angiogenic markers in vivo. However, the capability of the method for volumetric “multitarget quantification” and for the assessment of antiangiogenic therapy response has rather been investigated. Therefore, we generated cyanoacrylate microbubbles linked to vascular endothelial growth factor receptor 2 (VEGFR2) and αvβ3 integrin binding ligands and quantified their accumulation in squamous cell carcinoma xenografts (HaCaT-ras-A-5RT3) in mice with the quantitative volumetric ultrasound scanning technique, sensitive particle acoustic quantification. Specificity of VEGFR2 and αvβ3 integrin binding microbubbles was shown, and changes in marker expression during matrix metalloproteinase inhibitor treatment were investigated. In tumors, accumulation of targeted microbubbles was significantly higher compared with nonspecific ones and could be inhibited competitively by addition of the free ligand in excess. Also, multimarker imaging could successfully be done during the same imaging session. Molecular ultrasound further indicated a significant increase of VEGFR2 and αvβ3 integrin expression during tumor growth and a considerable decrease in both marker densities after matrix metalloproteinase inhibitor treatment. Histologic data suggested that the increasing VEGFR2 and αvβ3 integrin concentrations in tumors during growth are related to an up-regulation of its expression by the endothelial cells, whereas its decrease under therapy is more related to the decreasing relative vessel density. In conclusion, targeted ultrasound appears feasible for the longitudinal molecular profiling of tumor angiogenesis and for the sensitive assessment of therapy effects in vivo. [Mol Cancer Ther 2008;7(1):101–9]

Introduction

Onset and maintenance of tumor angiogenesis requires a complex molecular interplay among endothelial cells, stroma, and tumor cells (1, 2). This is negotiated through the regulation of interacting marker molecules on the cell membrane, such as vascular endothelial growth factor receptor 2 (VEGFR2) or Endoglin (1, 3) and by the release of proangiogenic and antiangiogenic factors (e.g., VEGF and thrombospondin; ref. 1). In this context, the ability to visualize noninvasively and quantify the regulation of different marker molecules longitudinally would be extremely valuable in preclinical research (4). Moreover, it would open new perspectives for the monitoring of tumor suppression (e.g., antiangiogenic therapies in patients; refs. 5, 6). In individualized therapy regimens, multimarker imaging would allow the recognition of changes in the molecular tumor profile during treatment that might indicate tumor resistance, thus giving indicators for the adaptation of the combination and dose of the therapeutics (7).

Different modalities for target-specific imaging have been applied successfully, but only few of them allow multimarker imaging, either simultaneously during the same examination setting or sequentially in a single examination: for instance, single-photon emission computed tomography can be applied with probes labeled with radiotracers of a different energy that the gamma camera can distinguish (8). Optical imaging with probes emitting photons at different wavelengths can also be used for multimarker imaging. However, quantification of marker concentrations in heterogeneous tissues remains difficult even when using tomographic methods (9). Furthermore, the sensitivity of optical imaging to probes decreases rapidly with increasing distance from the skin due to scattering and absorption. Ultrasound has proven to be highly sensitive for the identification of molecular structures when using targeted contrast agents (10–13). In this context, antibody-coated, gas-filled microbubbles with diameters of 1 to 5 μm were
used as specific contrast agents (11, 12) and have been applied to characterize arteriosclerotic disease (14), vascular thrombosis (15), neovascularation (16), lymph nodes (17) as well as cerebral encephalitis and other inflammations (18, 19).

Recently, nondestructive semiquantitative ultrasound measurements on representative tumor slices were applied to monitor the expression of several angiogenic targets during therapy (20). However, long intervals between the subsequent scans (1 h) were necessary to ensure clearance of targeted microbubbles from the marker molecule contrasting with new destructive scan techniques that would allow multitarget scans within short time intervals and for the absolute volumetric quantification of target densities within the entire tumor (21).

Here, we report on the use of experimental cyanoacrylate microbubbles to target the VEGFR2 and $\alpha_v\beta_3$ integrin in squamous cell carcinoma xenografts in nude mice that were treated with the potent matrix metalloproteinase (MMP) inhibitor AG3340. Quantification of microbubble concentrations over the entire tumor volumes was done using sensitive particle acoustic quantification (SPAQ; ref. 21). Scan time for the quantification of two specific markers in the tumors was less than 20 min. Molecular ultrasound clearly showed that during tumor growth VEGFR2 and $\alpha_v\beta_3$ integrin are up-regulated and that, in line with our histologic findings, after therapy the density of both markers is decreased significantly compared with untreated controls.

**Materials and Methods**

**Synthesis and Characterization of Target-Specific Microbubbles**

Air-filled cyanoacrylate microbubbles were synthesized by adding monomeric butyl-2-cyanoacrylate (Sichel Werke) to an aqueous acidic solution (pH 2.5) containing 0.02% of the surfactant Triton X-100 (Sigma-Aldrich) under vigorous stirring for 1 h that resulted in a microbubble suspension. The suspension was then subjected to a flotation process to separate air-filled from non-air-filled microbubbles. Purified by repeated flotation, the air-filled microbubbles were treated under alkaline conditions that created carboxylic groups on the particle surface by partial hydrolysis of ester groups. Subsequently, streptavidin (Calbiotech) was linked to the bubble surface by means of a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma-Aldrich Chemie) reaction between the amine of the streptavidin and the carboxylic group of the bubble surface.

Size distribution of the microbubbles was determined with the particle counter Multisizer 3 (Beckman-Coulter). The system calibration ranged from 0.5 to 18 $\mu$m. The result was displayed as volume-weighted mean diameters and SD.

The loading degree of the microbubbles with streptavidin was determined by the addition of increasing concentrations of biotin-FITC (Sigma-Aldrich) to a constant amount of microbubbles ($4 \times 10^5$). For optical measurements of the fluorescence signal per bubble, a flow cytometry analyzer (Becton-Dickinson FACSCalibur) was used. Saturation of the signal intensity reflected the loading capacity of a single microbubble with biotin-FITC and consequently reflected the number of streptavidin molecules bound to the microbubble surface.

The in vitro stability of the target-specific microbubbles under static pressure was measured in a 1 mL syringe containing 0.5 mL solution. The pressure was metered with a fiber optic pressure sensor (Samba 3000, Samba Sensors AB) and was maintained for 60 s. The number of intact bubbles within a size range of 1.5 to 5 $\mu$m was then counted with a particle counter (Multisizer 3, Beckman-Coulter). The destructibility of the microbubbles under sonication was measured using an Acuson Sequoia 512 (Siemens Medical Solution) ultrasound imager with a linear array transducer (15L8w) and a CASY Cellcounter. For sonication, the solution was filled in a tube (without ultrasound absorption) and placed in a basin with deionized and degassed water. The solution was sonicated with a mechanical index ranging from 0.14 to 1.10.

Target specificity of streptavidin-coated microbubbles was generated immediately before their use by adding of either 5 $\mu$g biotinylated goat anti-mouse VEGFR2 antibodies (R&D Systems) or 5 $\mu$g biotinylated RGD peptides (Peptides International; both diluted in 50 $\mu$L PBS buffer) to 50 $\mu$L microbubble suspension ($2 \times 10^7$ microbubble/mL). Reagents were allowed to react for 5 min at 20°C, resulting in 100 $\mu$L injection volume containing $10^7$ target-specific microbubbles. Biotinylated mouse anti-human IgG control antibodies (Antibodies Online GmbH), and amino-terminal biotinylated RAD peptides (Biotin-GRADSP; Biomol GmbH) served as negative controls and were connected to the microbubbles as described above.

**In vivo Studies**

All experiments were approved by the governmental review committee on animal care. Human squamous cell carcinomas (HaCaT-ras-A-5RT3; refs. 22, 23) were induced by s.c. injection of $2 \times 10^5$ cells in the left hind leg of nude mice. Experiments were initiated after 20 days of tumor growth. Animals (four groups: two therapy groups and two control groups; each $n = 5$) were anesthetized by continuous inhalation of sevofluorane (0.5-1.5 vol. % in oxygen), and unspecific microbubbles ($10^7$ microbubbles in 100 $\mu$L of 0.9% NaCl) were injected i.v. via a tail-vein catheter. Subsequently, 50 $\mu$L of 0.9% NaCl were injected to clear the catheter from microbubbles. Imaging was done 7 min after microbubble administration as suggested by prior studies in which timing variables had been optimized (18, 24). The initial ultrasound scan of the whole tumor volume was followed by three control scans. After 5 min, an additional scan was done to confirm that no intact microbubbles remained within the tumor. Then, one therapy group and one control group received RGD-conjugated microbubbles and the animals were scanned with the identical protocol. Subsequently, anti-VEGFR2 antibody-conjugated microbubbles were administered. An additional therapy group and control group served as negative controls.
and received anti-IgG antibody-conjugated microbubbles followed by RAD peptide-conjugated microbubbles. Administration of all control and specific microbubbles in one animal group could not be done because of the limited injection volume tolerated by nude mice. After imaging, animals received either the MMP inhibitor AG3340 (Prinomastat, 110 μL, 150 mg/kg i.p. twice a day for 7 days, n = 5) or a NaCl (control group, 110 μL, twice a day i.p. for 7 days, n = 5). After 7 days of therapy, animals were examined again as described above.

To further investigate the specificity, in vivo blocking experiments were done on six additional tumor-bearing nude mice. Three animals received VEGFR2-conjugated microbubbles and were scanned by SPAQ. After imaging, animals received 100 μg “unbiotinylated” goat anti-mouse VEGFR2 antibodies (R&D Systems) to block the target. Subsequently, VEGFR2-conjugated microbubbles were administered and their accumulation within the tumor was investigated. The same procedure was done with RDG-conjugated microbubbles and “unbiotinylated” RGD peptides (R&D Systems).

**SPAQ Imaging**

During ultrasound examination, the anesthetized nude mouse was set on a gel pad. The tumor implanted s.c. on the hind leg was positioned downward. The pad was placed on a navigable motor table (servo-motor Limes 50, motor controller DC 500, OWIS GmbH) that can be moved in micrometers increments over a fixed ultrasound transducer (Sector Scanner S12, Sonos 5500, Philips Medical Systems). Quantification of SAE signals was done with the SPAQ technology (21). The following settings were used: Doppler frequency, 5 MHz; mechanical index, 1.6; persistence, 0; priority, maximum; penetration depth, 3 cm; focus, 2.5 cm; frame rate, 25 Hz; table speed, 1.25 mm/s; incremental move, 50 μm.

For the postprocessing, a three-dimensional region of interest was drawn around the tumor. SAE signals within the region of interest were automatically quantified by video densitometry using the QuantiCon system (Echotech 3D Imaging Systems GmbH) and expressed as color pixel density (21). Tumor size was determined using the formula: \(\frac{a \times b^2 \times \pi}{6}\) expressed as mean size ± SD (mm³).

**Immunohistochemistry**

Immediately following final imaging, tumors were dissected for histologic phenotyping, covered with Tissue-Tek (Sakura), and then frozen in liquid nitrogen vapor. Tumor sections (6 μm thick) were cut with a Reichert-Jung Frigocut 2700 microtome (Leica) and were methanol/acetone fixed. Four additional tumor-bearing animals were sacrificed and their tumors were removed after 20 days of tumor growth to provide a baseline reference.

Double immunostaining was done using either a hamster anti-mouse CD61 antibody (BD Biosciences; 1:20 concentration) or a goat anti-mouse VEGFR2/FLK-1 antibody (R&D Systems; 1:10 concentration). Inner vessel walls were labeled using a rat anti-mouse CD31 antibody (BD Biosciences; 1:100 concentration). As secondary antibodies, Cy2-conjugated goat anti-hamster IgG (Jackson Immuno-Research; 1:100 concentration) and alternatively Alexa 488–conjugated donkey anti-goat IgG secondary antibody (Mobitec; 1:800 concentration) in combination with Cy3-conjugated donkey anti-rat IgG secondary antibody (Jackson ImmunoResearch; 1:800 concentration) were used. Cell nuclei were counterstained by 4,6-diamidino-2-phenylindole (Invitrogen; 1:100 concentration).

Tissue fluorescence sections were viewed in a Leica microscope (DMRE) with an adapted digital camera (F-view XS; Soft Imaging System GmbH). Quantitative analysis of marker density on histologic sections was done by calculating positive area fractions using the Analysis Software (Soft Imaging System GmbH). Three sections per tumor were analyzed for expression of VEGFR2 (n = 15 per control and therapy group) and CD61 (n = 15 per control and therapy group). CD31⁺ area fractions were analyzed of all tumor sections to determine vessel density.

**Statistical Evaluation**

Data are presented as mean ± SD. Differences in microbubble concentrations and immunohistologic area fractions of CD31⁺, VEGFR2-positive, and α3β1 integrin-positive vessels between treated and untreated tumors were compared using the Mann-Whitney test (two-tailed). \(P < 0.05\) and \(P < 0.01\) were considered to show significant and highly significant differences, respectively. Statistical analysis was done with GraphPad Prism 4.03 (GraphPad Software).

**Results**

**Characterization of Target-Specific Microbubbles**

Air-filled microbubbles with a streptavidin coating were synthesized successfully by polymerization of butyl-2-cyanoacrylate and subsequent coupling of streptavidin to the bubble surface (Fig. 1A). The mean volume-weighted size of the microbubbles was 2.61 ± 0.81 μm (SD) as determined by the particle counter (Fig. 1B).

Coating of streptavidin on the microbubble shell was shown by flow cytometry. Addition of biotin-FITC with increasing concentrations to a constant amount of streptavidin-coated microbubbles (4 × 10⁶) resulted in an increased fluorescence signal per microbubble. Saturation of the signal intensity was achieved with 3 × 10¹⁰ biotin-FITC molecules, which is equivalent to a biotin-FITC molecule to microbubble ratio of 7,500:1, reflecting the high loading capacity of streptavidin-coated microbubbles for biotinylated antibodies or ligands.

Crucial variables for ultrasound imaging with SPAQ are \(a\) destructibility under high-power sonication and \(b\) stability of i.v. administered microbubbles within the circulatory system. Therefore, we investigated the behavior of microbubbles under static pressure and sonication. High-power sonication of the microbubbles in vitro revealed that at a mechanical index >0.25 decomposition of particles starts and that the majority of microbubbles are destroyed at a mechanical index greater than 0.6 (Fig. 1C).

For SPAQ imaging during the in vivo experiments, we chose a mechanical index of 1.6, ensuring the destruction
and consecutive detection of the majority of targeted microbubbles within the tumor.

The effect of static pressure on microbubble integrity is shown in Fig. 1D. The sigmoid curve indicates that static pressure of 80 mm Hg applied for 60 s leaves ~50% of the microbubbles intact.

Specificity of Targeted Contrast Agents

Using SPAQ, the concentrations of unconjugated, IgG antibody-conjugated, and RAD peptide-conjugated control microbubbles as well as VEGFR2-specific and \( \alpha_\text{v}\beta_3 \) integrin-specific microbubbles in tumors could be quantified in vivo during sequential measurements (Fig. 2A). In this context, the targeted microbubbles were destroyed by a high-powered Doppler ultrasound scan. The number of voxels containing Power Doppler signals was normalized to the total number of voxels of the tumor volume to reveal color pixel density values in percent.

In all tumors, the accumulation of specific microbubbles exceeded that of unspecific ones (Fig. 3). The highest mean color pixel density in tumors was found for VEGFR2-specific microbubbles (22.46 ± 8.36%) followed by \( \alpha_\text{v}\beta_3 \) integrin-specific ones (14.00 ± 7.36%). IgG control antibody-conjugated microbubbles (2.30 ± 0.75%), RAD control peptide-conjugated microbubbles (1.17 ± 0.92%), and unconjugated microbubbles (1.52 ± 0.95%) bound significantly less compared with the specific microbubbles (\( P < 0.05 \) for VEGFR2-specific and \( \alpha_\text{v}\beta_3 \) integrin-specific microbubbles versus IgG-conjugated control microbubbles; \( P < 0.01 \) for VEGFR2-specific and \( \alpha_\text{v}\beta_3 \) integrin-specific microbubbles versus RAD-conjugated and unconjugated control microbubbles; \( n = 5 \) tumors).

After administration of free ligands in excess (VEGFR2 antibodies or RGD peptides), binding of specific microbubbles was reduced by the factor 0.38 ± 0.19 (for VEGFR2-specific microbubbles; \( n = 3 \) tumors) and by the factor 0.68 ± 0.16 (for \( \alpha_\text{v}\beta_3 \) integrin-specific microbubbles; \( n = 3 \) tumors). Hence, competitive blocking of the target could be shown in vivo.

To ensure that the counted signals originated from stationary targeted microbubbles rather than from blood flow or unbound microbubbles within the blood pool, each scan was followed up by three control scans. Independent of the ligand bound to the microbubbles, a significantly (\( P = 0.007 \)) lower color pixel density was identified in the control scans (11.11 ± 5.93% during the first scan versus 1.69 ± 1.73%, 1.90 ± 1.53%, and 1.84 ± 1.56% during the three control scans; \( n = 6 \) tumors; Fig. 4). In this context, the reproducibly low concentration of microbubbles in the control scans indicates that all site-targeted microbubbles were destroyed during the first diagnostic scan using high-power sonication.

Monitoring of the Consequences of MMP Inhibition on the VEGFR2 and \( \alpha_\text{v}\beta_3 \) Integrin Expression in Tumors by Targeted Ultrasound

To investigate whether changes in the expression of VEGFR2 or \( \alpha_\text{v}\beta_3 \) integrin on tumor vessels can be assessed by molecular ultrasound, five tumor-bearing animals were investigated by SPAQ before and again 7 days after treatment with the MMP inhibitor AG3340. We did not choose a therapy against VEGFR2 or \( \alpha_\text{v}\beta_3 \) integrin to avoid competition of the diagnostic and therapeutic drug at the target. A group of five untreated tumor-bearing animals was carried along to investigate the marker expression during normal growth. Two additional groups of animals (one treated and one untreated) served as control groups and received unspecific control microbubbles.

Except for the measurement of one animal of the therapy group at the first time point, where accidental paravenous
injection of VEGFR2-specific microbubbles had taken place, all measurements were successful.

Before therapy, the mean size of tumors in the control and therapy group had not been significantly different (55.39 ± 52.88 and 36.39 ± 27.63 mm³, respectively; \( P = 0.54 \)). After 7 days, the mean tumor volume in the control group had increased by more than 200% to 133.05 ± 89.29 mm³. In the therapy group, a retarded tumor growth with a tumor volume increase by only ~80% to a mean volume of 62.37 ± 41.13 mm³ was found. This is in line with previously reported effects of AG3340 on tumor growth (25).

Before therapy started, no significant difference in the mean number of bound VEGFR2-specific and \( \alpha \nu \beta_3 \) integrin-specific microbubbles was found between tumors of the control and therapy group (Fig. 5). However, it is noteworthy that the interindividual variability of the microbubble accumulation was higher for \( \alpha \nu \beta_3 \) integrin-specific microbubbles than for VEGFR2-specific ones. Seven days after the first examination, significantly increased binding of VEGFR2-specific microbubbles (27.30 ± 8.26% before versus 47.97 ± 13.14% after 7 days; \( P < 0.05 \)) and \( \alpha \nu \beta_3 \) integrin-specific microbubbles (8.42 ± 2.55% before versus 14.63 ± 3.27% after 7 days; \( P < 0.01 \)) was observed in untreated tumors. In contrast, in treated tumors, accumulation of VEGFR2-specific microbubbles (27.76 ± 20.61% before versus 17.45 ± 9.74% after therapy; \( P = 0.55 \)) and \( \alpha \nu \beta_3 \) integrin-specific microbubbles (13.94 ± 7.42% before versus 9.72 ± 3.28% after therapy; \( P = 0.30 \)) was reduced and significantly lower than in the control group (VEGFR2, \( P < 0.01 \); \( \alpha \nu \beta_3 \) integrin, \( P < 0.05 \)). Accumulation of control microbubbles (unconjugated, IgG-conjugated, and RAD-conjugated microbubbles) in tumors was significantly lower than for both specific microbubbles and did not change significantly under therapy and over time.

Correlation of the Noninvasive Ultrasound Data with the Results from Immunohistochemistry

Immunohistochemistry of untreated tumors grown for 20 days (first examination time point) and 27 days (second examination time point) showed a decreasing vessel density over time (day 20, 3.08 ± 1.36%; day 27, 2.71 ± 1.29%). In contrast, during the 7 days, stained area fractions of VEGFR2 and \( \alpha \nu \beta_3 \) integrin increased significantly from 2.39 ± 1.07% to 4.25 ± 1.47% and from 2.39 ± 1.07% to 2.96 ± 0.76%, respectively.

In treated tumors, immunohistochemistry of excised tumors confirmed the molecular ultrasound findings by

Figure 2. SPAQ-based molecular imaging. A, a nude mouse (tumor implanted s.c. on the right hind leg) is placed on an ultrasound pad. B, the ultrasound transducer is fixed below the navigable table that can be moved in micrometer increments. The frame rate of the ultrasound Doppler system (25 Hz) is synchronized with the movement of the table (1.25 mm/s), resulting in an accrual move of 50 \( \mu \)m. C, during the ultrasound scan (mechanical index = 1.6), the targeted microbubbles disintegrate and emit detectable signals (yellow dots; red, region of interest). The two-dimensional ultrasound images are reconstructed to a three-dimensional dataset that is analyzed quantitatively by an automatic video densitometry system as to the color pixel density within the region of interest.
showing significantly lower stained area fractions for VEGFR2 and αvβ3 integrin in the therapy group compared with the control group (VEGFR2, 1.97 ± 0.54% therapy group versus 4.25 ± 1.47% control group; P < 0.05; αvβ3 integrin, 1.44 ± 0.40% therapy group versus 2.96 ± 0.76% control group; P < 0.01). Likewise, CD31 staining indicated a lower vessel density in treated tumors (1.17 ± 0.49% therapy versus 2.71 ± 1.29% control; P < 0.05; Fig. 6).

Normalization of the stained area fractions of VEGFR2 and αvβ3 integrin to that of CD31 (vessel density) indicated that the absolute reduction of both markers in the treated group is related primarily to the reduced total vessel number. In contrast, the increasing ratios of VEGFR2/CD31 and αvβ3 integrin/CD31 during the observation time (days 20-27) point to the up-regulation of the expression of these proteins by the endothelial cells in the tumors of both groups: (a) ratio VEGFR2/CD31, additional baseline: 0.77, control group: 1.56, and therapy group: 1.68 and ratio αvβ3 integrin/CD31, additional baseline: 0.44, control group: 1.09, and therapy group: 1.23.

Discussion

Molecular imaging offers new perspectives to investigate the expression of marker molecules on tumor, vascular, and stroma cells during carcinogenesis and tumor growth (1, 4, 26), crucial for the development of novel therapeutic strategies (e.g., growth factor receptor blockade; refs. 4, 5, 7, 26). Molecular imaging may even be capable to indicate early therapy response of tumors (e.g., down-regulation of growth factor receptors) occurring before the change of tumor volume. Moreover, tumor resistance may be recognized earlier, allowing quick adaptation of therapy regimens. However, due to the large number of involved marker molecules interacting during these processes, reliable multi-marker imaging of the tissue will be required. This means the data obtained by such imaging methods has to be quantitative and representative for the tissue. This can be achieved by a volumetric scan of the entire tumor.

In this study, we show that volumetric molecular ultrasound is suited for multitarget imaging in the course of one animal experiment and that the method is highly sensitive for the assessment of changes of molecular marker profiles during tumor growth and antiangiogenic therapy. First, we found an up-regulation of VEGFR2 and αvβ3 integrin during growth of untreated tumors. This observation is in line with findings of Ellegala et al. in U87MG tumors who also observed an up-regulation of αvβ3 integrin during follow-up with targeted ultrasound (16).
Most likely the observed up-regulation is triggered by the increasing hypoxia that occurs during tumor growth, leading to strong stimulation of angiogenesis (27). In contrast, tumors treated with the MMP inhibitor AG3340 showed a lower density of both markers after 7 days. Our noninvasive ultrasound imaging protocol did not allow differentiating whether the decreased accumulation of targeted microbubbles is related to a down-regulation of the marker at the vessel surface or a decrease in relative vessel number. This shows the need for both surrogate marker imaging of vascular function (including the assessment of relative blood volume, vessel size, perfusion, and vessel permeability; refs. 26, 28) and molecular imaging for quantification of specific marker molecules (11). In additional studies, this differentiation could be achieved noninvasively by supplementing the ultrasound imaging protocol with a contrast-enhanced first-pass scan to assess the relative blood volume (29). In this study, the histologic results indicate an up-regulation of VEGFR2 and \( \alpha_v \beta_3 \) integrin expression in endothelial cells during growth of tumors, whereas the vessel density decreases. In contrast, comparing treated and untreated tumors 27 days after inoculation, concordantly lower densities of vessels, VEGFR2, and \( \alpha_v \beta_3 \) integrins are found after treatment. Thus, it can be assumed that the reduced density of both markers in the treated tumors is related primarily to the decrease in absolute vessel density and not to a down-regulation of the markers by the endothelial cells. However, preceding the decrease in vessel density, MMP inhibition could also cause a down-regulation of VEGFR2 and \( \alpha_v \beta_3 \) integrin expression on the endothelial cells [e.g., by inhibition of VEGF release from the matrix (30, 31) and ensuing depletion of the positive feedback on VEGFR2 expression]. This might need to be elucidated in further longitudinal in vivo studies with more closely spaced examination intervals, a process for which molecular ultrasound is highly suited.

Targeting of angiogenic markers in vivo was possible by conjugation of either biotinylated anti-VEGFR2-antibodies or biotinylated RGD peptides to the streptavidin-coated microbubble shell. Whereas most groups are using soft shell microbubbles (e.g., phospholipid) coated with biotin or sometimes polyethylene glycol (11, 12), experimental microbubbles used in this study have a hard polymeric shell, coated with streptavidin. The suitability of either type of microbubbles strongly depends on the applied imaging protocol: in nondestructive imaging, microbubbles serve as contrast enhancers by backscattering the ultrasound waves. As the pressure wave passes through, the bubble contracts and expands, creating an ultrasound echo the imaging device picks up (12). Microbubbles with a soft, flexible shell are superbly suited as such contrast enhancers. Their disadvantage is the substantial loss of gas volume, as the core gas may escape rapidly (11, 12). Consequently, the microbubbles shrink and change their acoustic characteristics. To achieve enhanced stability, gases with very low blood solubility are used (e.g., perfluorocarbon) that may enhance the lifespan of the microbubbles to a certain degree. A thicker and more robust polymer-stabilized shell, as used in our experimental microbubbles, prevents gas diffusion more effectively while also providing higher microbubble stability at the cost of a reduced backscatter signal (12). However, the lower backscatter signal is less relevant for destructive ultrasound techniques where the disintegrating microbubble emits a strong nonlinear signal that is being determined as stimulated acoustic emission. This signal is strong enough to detect even single microbubbles within tissues (21) and therefore enables imaging with superior sensitivity.
Quantitative analysis of the contrast agent concentration in the target is still not sufficiently realized for most molecular imaging modalities with the exception of those in nuclear medicine (8). This also holds true for most previous targeted ultrasound approaches. Ultrasound methods using the signal intensity enhancement caused by site targeted soft shell microbubbles (10–16, 20, 32) only provide semiquantitative data about the contrast agent concentration. We applied the destructive ultrasound method SPAQ (21) to obliterate the stationary microbubbles and to quantify the detected signals. The fact that even single microbubbles can be detected and that no microbubbles can be counted twice is an important precondition for quantitative multimarker imaging in narrow time intervals.

For successful quantification of targeted contrast agents via SPAQ analysis, it is essential to confirm that (a) no relevant unspecific accumulation of microbubbles occurs and (b) no relevant numbers of microbubbles circulate within the blood when doing the ultrasound scan. In this study, we were able to show the specificity of targeted microbubbles, which accumulated significantly within tumor vessels compared with unspecific ones. In a previous study about the pharmacokinetic behavior of cyanoacrylate microbubbles, we were further able to show that 1.5 min after injection the blood concentrations of microbubbles in mice already decreased to less than 2% of the maximum peak enhancement (24). Commensurate with these findings, this study also found no relevant replenishment of microbubbles in control scans following the diagnostic scan done 7 min after microbubble injection.

Moreover, SPAQ includes a volumetric scan of the entire organ and therefore generates reproducible three-dimensional quantitative data. Other methods have been applied with a fixed transducer at one particular region of the object, generating a representative slice (11) but no data of an entire organ (e.g., tumor). However, even if the examined ultrasound slice is representative of the entire relevant tissue, repeated imaging at the identical position is hardly feasible. Consequently, the reliability of repeated measurements for therapy monitoring is negatively affected by this shortcoming of conventional methods. Here, volume scanning offers improved comparability between consecutive examinations (33, 34).

In conclusion, molecular ultrasound is a promising quantitative technology for imaging of multiple angiogenic markers. It provides a high sensitivity for contrast agents, excellent biocompatibility, and significant potential for clinical translation. It is very well suited for the identification of angiogenic markers as well as the assessment of antiangiogenic therapy effects. Thus, using molecular ultrasound offers multiple options to improve biomedical research in the future.

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