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Accelerated blood clearance of targeted ultrasound contrast reduced molecular imaging signal intensity: Secreted Frizzled Related Protein-2 signal remained significantly higher than signal from either Vascular Endothelial Growth Factor Receptor-2 or alpha$_V$beta$_3$ integrin.

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Multiple doses of polyethylene glycol (PEG) decorated pharmaceuticals cause accelerated blood clearance (ABC) due to the generation of antibodies reactive to the PEG moiety. Using molecular imaging to monitor response to therapy could be complicated by the ABC effect due to PEG chains in microbubble lipid shells. Our objective was to measure the half-life of targeted contrast flowing through non-tumor tissue during longitudinal imaging studies, and to determine which targeted agent returned the highest signal intensity within tumors. The molecular imaging signals from contrast agents targeted to three distinct molecular targets, Secreted Frizzled-Related Protein-2 (SFRP2), Vascular Endothelial Growth Factor Receptor-2 (VEGFR2), Alpha$_V$Beta$_3$ Integrin (avb3) were all significantly correlated to contrast half-life. The molecular imaging signal from SFRP2 remained significantly higher than the signal returned by ultrasound contrast targeted to either VEGFR2 or avb3 before and after restricting analyses to imaging exams with similar half-lives. We hypothesize that increasing immune clearance rates during our longitudinal studies limited the amount of targeted contrast able to perfuse tumor vasculature, and that this resulted in a global dose-dependent decrease in molecular imaging signals. Molecular imaging may underestimate biomarker levels as longitudinal studies progress and as contrast half-lives decrease, unless contrast dosing is normalized by the amount of contrast able to reach the tumor and surrounding tissue rather than by the injected dosage.

Keywords—ultrasound molecular imaging, accelerated blood clearance, contrast half-life

I. Introduction

Our laboratory recently showed that the circulating half-life of ultrasound microbubble contrast agents decorated with PEG chains decreased with repeated administration over the course of four weeks in rats. This phenomenon of accelerated blood clearance (ABC) was attributed to increasing immune clearance rates in response to the PEG moieties associated with microbubble contrast agents [1]. When circulating half-life decreased, the dose of contrast agent perfusing normal tissues or tumors also decreased as a function of concentration integrated over time. Our objective was to determine if targeted ultrasound contrast agents administered to immune-compromised nude mice would also demonstrate the ABC effect, and to determine which of our three targeted contrast agents (recognizing SFRP2, VEGFR2 or avb3 biomarkers) returned the greatest molecular imaging signal.

II. Materials and Methods

A. Angiosarcoma xenograft model

Female nude mice approximately six weeks of age were injected subcutaneously with 1x10^6 murine SVR cells (ATTC, Manassas, VA) by personnel from the University of North Carolina (UNC) Animal Studies Core. Animal care and monitoring was provided by the UNC Division of Comparative Medicine. All animal procedures were approved by the UNC Institutional Animal Care and Use Committee.

B. Ultrasound targeted contrast agent formulation

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SFRP2-targeted contrast used a recombinant Fab antibody [2] to direct binding to SFRP2. VEGFR2-targeted contrast used recombinant vascular endothelial growth factor (VEGF, SibTech, Brookfield, CT) to direct binding to VEGFR2 [3]. $Alpha_v beta_3$ integrin-targeted contrast used a synthetic cyclic arginine-glycine-aspartic acid peptide [4] (cRGD, Peptides International, Louisville, KY) to direct binding to $alpha_v beta_3$ integrin. SFRP2 Fab antibody was covalently attached to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000], (DSPE-PEG2000-maleimide, Avanti Polar Lipids, Alabaster, AL) via its free cysteine. Biotinylated VEGF or cRGD were bound to microbubbles containing DSPE-PEG2000-NeutrAvidin™ [2]. Targeted PEG-lipids were incorporated into a 1 mM lipid solution composed of 90 mole % 1,2-distearoyl-sn-glycero-3-phosphocholine, (DSPC, Avanti Polar Lipids) and 10 mole % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000], (DSPE-PEG2000, Avanti Polar Lipids) in PBS containing 15% (v/v) propylene glycol and 5% (v/v) glycerol. Targeted lipid solution was converted to a microbubble emulsion by shaking in the presence of perfluorobutane (C_4F_{10}) gas. Unincorporated reagents were removed from microbubbles by centrifugal separation (5 x 1 minute washes at 180g, 3 ml each in 5ml syringes). The concentration and size distribution of final preparations of targeted ultrasound contrast were measured using an Accusizer AD780 (Particle Sizing Systems, Port Richey, FL).

C. Ultrasound molecular imaging

Prior to ultrasound imaging, animals were anesthetized with inhaled isoflurane/oxygen (approximately 3% isoflurane) and anesthesia was maintained during imaging procedures with 1-2% isoflurane/oxygen. Tumors were coupled with ultrasound gel to a layer of Saran Wrap that formed the bottom of a water-filled container. The dimensions of the water-filled container were sufficient to accommodate the translation of the linear array transducer required to acquire three-dimensional (3-D) ultrasound scans of the tumor and surrounding non-tumor tissue.

3-D ultrasound images of angiosarcoma tumors established in the flank of nude mice were captured with an Acuson Sequoia 512, a 15L8 linear array transducer, and motion stage. A 3-D B-mode scan was captured for anatomical reference at 15 Mhz. The B-mode scans were used for drawing regions of interest (ROI) in normal tissue and around tumors. Contrast-specific Cadence™ pulse sequence mode (CPS) operated non-destructively at 7 MHz with MI = 0.18, CPS gain = -7 dB, and dynamic range = 80dB. CPS scans were captured continuously for two minutes after bolus injection of contrast. Additional scans were collected every two minutes out to ten minutes followed by a scan measuring free flowing, unbound contrast.

D. Analysis of molecular imaging data

Average pixel intensity was calculated with custom scripts written in MATLAB (Natick, MA) for 3-D tumor volumes and for non-tumor, normal tissue ROIs. Targeted enhancement (TE) refers to the average pixel intensity within the tumor ROI, 10 minutes after bolus injection of targeted contrast agent. The ten minute timepoint was selected because the signal from free-flowing contrast in the vasculature was low at that time. Correcting the TE value by subtracting the signal from free-flowing, unbound targeted contrast (measured at 12 minutes post-injection) yielded the differential TE (*d*TE) which represented only the signal from contrast agent bound within the tumor ROI.

Time-intensity curves (TICs) were constructed using the average, log-compressed pixel intensity within the 3-D tumor and normal tissue ROIs. The tissue TIC was fit to a one-phase exponential decay curve in order to derive the half-life of contrast agent in circulation through non-tumor tissue. The tissue TIC was subtracted from the tumor TIC to derive the differential TIC (*d*TIC) which approximated the signal from contrast agent bound within the tumor vasculature. TE, *d*TE, TICs, *d*TICs and half-life were calculated for all imaging exams. In addition, the area under the curve (AUC) was also calculated for some curves.

Curve fitting and statistical analyses were performed in GraphPad Prism. Tukey's test was used to correct statistical tests for multiple comparisons. Differences were considered statistically significant if $p < 0.05$.

III. RESULTS

A. Angiosarcoma xenografts

Tumors became palpable between 7 – 14 days after implantation. Animals were humanely euthanized before tumors reached a diameter of 2 cm.

B. Targeted contrast agents

Centrifugal washing of targeted microbubble preparations increased the average diameter from approximately 1 micron to approximately 2 ± 1 micron. After washing, targeted microbubble contrast was stored at concentrations above 1×10^9 microbubbles/ml in PBS with 15% (v/v) propylene glycol and 5% (v/v) glycerol in syringes with headspace at 4ºC or on ice. Size distributions of microbubble preparations were stable for at least two weeks when processed and stored in this manner.

C. Ultrasound molecular imaging

Molecular imaging TICs and dTIC. The pixel intensity averaged over the tissue or tumor ROI volumes was plotted as molecular imaging TICs (Fig. A). Data from the tissue TICs fit a one-phase exponential decay when plotted from the maximum wash-in value out to ten minutes. The wash-out of signal from the tumor ROI was modelled better by a linear fit than by the exponential decay as we reported previously [2]. The accumulation of bound contrast agent was represented by the *d*TIC, obtained by subtracting the tissue TIC from the tumor TIC ('Delta' curve in Fig. A). The *d*TIC generally took the form of a rectangular hyperbola.

Molecular imaging TE and dTE. Correcting TE for the contribution of free-flowing contrast resulted in *d*TE values similar to the 10-minute value of the *d*TIC. This indicated that the free-flowing contrast signal determined at 12 minutes approximated the tissue TIC value at 10-minutes.

D. Half-life of targeted contrast agents affects molecular imaging signal.

The half-life of SFRP2-targeted contrast varied from approximately 6 minutes to less than 30 seconds (Fig. B). When

*d*TE values were binned by half-life and plotted against half-life, there was a significant correlation: as half-life decreased, *d*TE values also decreased. Within any animal, SFRP2 half-life generally decreased with repeated administration (data not shown). Molecular imaging signal decreased as a function of half-life (Fig. B) which was consistent with a decreased dose of contrast reaching the tumor ROI as contrast half-life decreased. This relationship between molecular imaging signal and contrast half-life was also observed for VEGFR2-targeted and avb3-targeted contrast agents (not shown).

E. Comparing molecular imaging signal intensities of ultrasound contrast targeted to different biomarkers.

Our SFRP2 molecular imaging exams had an average contrast half-life that was longer than the half-life of either VEGFR2 or avb3-targeted contrast agents. Exams for each of these contrast agents were binned and exams were excluded so that the average half-life for each of the sets of exams was approximately 1.7 minutes (Fig. C). SFRP2-targeted imaging returned a higher average pixel intensity than either VEGFR2 or avb3-targeted imaging when comparing exams of similar half-lives (Fig. C) or when comparing all available exams (data not shown).

F. Molecular imaging signal versus contrast 'dose' that reached the tumor.

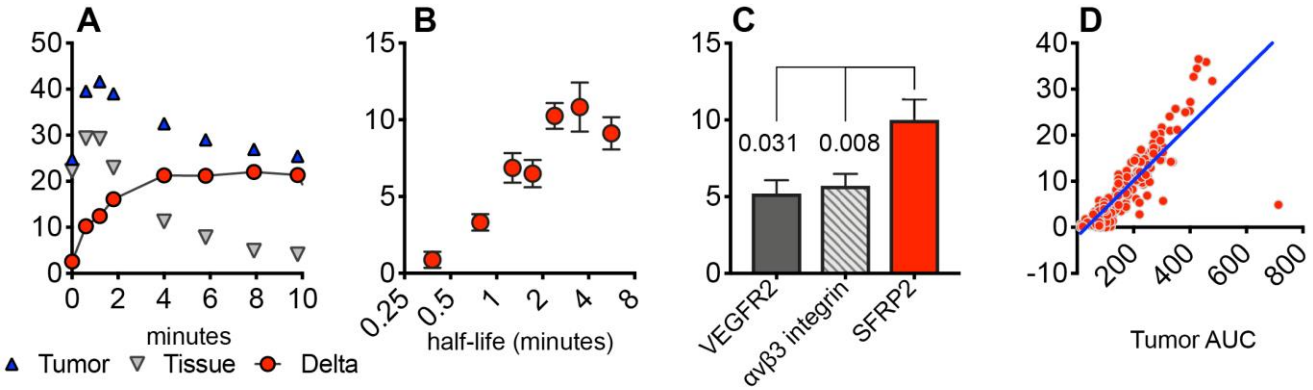
The molecular imaging signal for SFRP2 at 6 minutes (from the *d*TIC curve) was plotted against the AUC of the tumor TIC, and showed a significant positive correlation (Fig. D). When less contrast signal was found in the tumor ROI, the amount of bound signal was lower. This is consistent with the dose-responsiveness expected when targeted contrast has the opportunity to bind to its target. Given more targeted contrast, the final bound signal can be higher but in the absence of a high concentration of targeted contrast, the final bound signal remained low.

IV. DISCUSSION

Collecting multiple molecular imaging scans over the course of ten minutes allowed us to construct TICs for both tumor and non-tumor ROIs. The non-tumor TIC was essential for determining the half-life of our targeted contrast agents as they perfused non-tumor tissue. Our study showed that targeted contrast agents were subject to the ABC effect in nude mice, this extended our previous report of the ABC effect for non-targeted ultrasound contrast administered to rats [1].

As the clearance rate for contrast increased, the amount of contrast able to reach our tumor and its surrounding tissue decreased. The half-life of contrast decreased over time as our longitudinal study progressed. As a consequence, the dose of contrast at the beginning of our longitudinal study was no longer sufficient to fully perfuse the region of interest near the end of our longitudinal studies. As the dose of contrast that was able to reach the tumor decreased, the molecular imaging signal also decreased. Although this drop in signal would be expected since receptor-ligand binding is supposed to be dose-responsive, saturable and able to be displaced by an excess of either ligand or receptor [5], [6] we did not anticipate that there would be a difference between the dose of contrast agent administered in the bolus injection, and the dose of contrast agent that was able to perfuse our region of interest.

Since it is standard practice to hold the administered dose of contrast agent constant during a longitudinal study to eliminate 'dose' as a variable, it is clear that a different practice will be needed if we are to compare imaging exams between animals or within the same animal when the contrast agent half-life has changed significantly. We propose that dosing be normalized by monitoring the contrast signal during the wash-in phase of a bolus injection or by dosing by infusion until a 'standard' level of contrast signal is achieved in the field of view. Either method would be assessing the amount of contrast that is able to perfuse the region of interest and would use that parameter to maintain a consistent dosing at the tissue level.



Y-axis represents average pixel intensity from molecular imaging. **[A]** The difference between the time-intensity curves (TIC) for tumor and tissue ROIs represented magnitude of targeted contrast signal retained by the tumor. Contrast half-life was determined by fitting the tissue TIC to one-phase exponential decay. **[B]** Average pixel intensity from SFRP2 molecular imaging was significantly correlated with contrast half-life (231 exams across 60 animals, p=0.0238, Spearman r = 0.8571). **[C]** SFRP2 molecular imaging signal was significantly greater than signal from either VEGFR2 or αvβ3 (exams binned so that T½ = ~1.65 min, p values were as listed in figure). **[D]** SFRP2 average video intensity from molecular imaging TIC at 6 minutes was signifcantly correlated to the integrated SFRP2 signal over the tumor ROI (p<0.0001, Spearman r = 0.8998).

In practice, we have restricted analyses of imaging exams to those obtained late in a longitudinal study where we have adjusted the administered dose of contrast to be the same between different contrast agents, and to be of sufficient dosage to fully perfuse the region of interest. This method allowed us to adhere to the practice of using the same injected dose of contrast to 'allow' comparison between imaging exams but at the expense of not using data collected earlier in the longitudinal study when half-lives were decreasing rapidly.

A sufficient number of imaging exams were taken over a wide range of contrast half-lives in this study so that we could restrict our analyses to imaging exams with similar half-lives. This allowed us to remove the contrast half-life as a variable and demonstrated that our SFRP2-targeted contrast agent returned a significantly higher molecular imaging signal than either VEGFR2-targeted or avb3-targeted contrast agents, and that this was independent of contrast half-life. This approach would have been more difficult to complete with a smaller number of imaging exams.

Further studies with additional tumor models are required to determine whether the higher signal returned by SFRP2-targeted ultrasound contrast was a general phenomenon or whether it indicated that SFRP2 was expressed at significantly higher levels in angiosarcoma compared to the other biomarkers.

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