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High-resolution, serial intravital microscopic imaging of nanoparticle delivery and targeting in a small animal tumor model

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Summary Nanoparticles are under active investigation for the detection and treatment of cancer. Yet our understanding of nanoparticle delivery to tumors is limited by our ability to observe the uptake process on its own scale in living subjects. We chose to study single-walled carbon nanotubes (SWNTs) because they exhibit among the highest levels of tumor uptake across the wide variety of available nanoparticles. We target them using RGD (arginine-glycine-aspartic acid) peptide which directs them to integrins overexpressed on tumor vasculature and on the surface of some tumor cells (e.g., U87MG as used here). We employ intravital microscopy (IVM) to quantitatively examine the spatiotemporal framework of targeted SWNT uptake in a murine tumor model. IVM provided a dynamic microscale window into nanoparticle circulation, binding to tumor blood vessels, extravasation, binding to tumor cells, and tumor retention. RGD-SWNTs bound to tumor vasculature significantly more than controls ($P < 0.0001$). RGD-SWNTs extravasated similarly compared to control RAD-SWNTs, but post-extravasation we observed as RGD-SWNTs eventually bound to individual tumor cells significantly more than RAD-SWNTs ($P < 0.0001$) over time. RGD-SWNTs and RAD-SWNTs displayed similar signal in tumor for a week, but over time their curves significantly diverged ($P < 0.001$) showing increasing RGD-SWNTs relative to untargeted SWNTs. We uncovered the complex spatiotemporal interplay between

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these competing uptake mechanisms. Specific uptake was delimited to early (1—6 h) and late (1—4 weeks) time-points, while non-specific uptake dominated from 6 h to 1 week. Our analysis revealed critical, quantitative insights into the dynamic, multifaceted mechanisms implicated in ligand-targeted SWNT accumulation in tumor using real-time microscopic observation.

Introduction

Nanoparticles (nps) have the potential to revolutionize cancer diagnosis and therapy. Yet the mechanisms of np delivery to the disease sites remain poorly understood. When targeted nps are injected for diagnostic imaging or therapy, typically one only observes a macroscopic effect/picture (via imaging, e.g., PET, SPECT, bioluminescence, Raman, photoacoustics [1—3], or palpation) over time that is assumed to be due to nanoparticles in the tumor. However, due to the lack of spatial and temporal resolution, the underlying microscale interactions of nps that lead to bulk signal at that site are inaccessible and thus not understood. Even if histology or microscopy in living animals is performed to understand np interactions, it is generally at only a single time-point [4,5], or it is performed in cell culture [6] which while valuable does not recapitulate the complexity of the living mammal. We employ intravital microscopy (IVM), which employs lasers and photodetectors to microscopically image in living subjects [7], to directly and dynamically (repeatedly over weeks) image nanoparticle interactions at many time-points for the first time. This study design enabled us to uncover previously misunderstood, critical features of nanoparticle targeting to cancer in living subjects, such as the role of targeting ligands in tumor specificity.

There remains great debate about how, and whether, targeting ligands actually work in living subjects. Recent literature indicates targeting only increases np specificity of interaction with tumor cells, yet does not affect overall uptake [8—11]. However, many other groups have demonstrated differential levels of uptake between targeted and untargeted nps [12,13]. This critical question thus remains unresolved. We hypothesized that dynamic IVM could provide unique insights into np behavior in tumors in vivo. Our principal objective was to directly visualize and elucidate the entire spatiotemporal framework of microscale interactions between targeted nanoparticles and the tumor from injection through vascular binding, extravasation, tumor cell binding, and clearance. Herein we employ targeted single-walled carbon nanotubes (SWNTs) and frequently observe/quantify their behavior from injection until ~4 weeks post-injection to uncover the fundamental principles underlying np targeting. This enabled us to discover an underlying framework for np targeting which (1) helps resolve the dichotomy in the literature, (2) confirms the importance of temporal effects in targeting, and (3) provides a general structure to help others predict and understand how new nps may behave over space and time. Deep comprehension of the dynamic mechanisms of np delivery has the potential to lead to broad advancement and innovation in nanomedicine.

The enhanced permeation and retention (EPR) effect reflects the tendency in tumors of circulating macromolecules/particles to leak out of blood vessels (permeation/extravasation) and remain there (retention) [14]. EPR is the major mechanism accounting for np distribution into tumor interstitium, but is not well-understood for nps. For instance, in successful siRNA-nanoparticle work in living subjects by Moore and co-workers, their nanoparticles accumulate ''...in tumors, presumably resulting from enhanced permeability and retention'' [15]. This is characteristic of current studies, in which it is unknown (though commonly hypothesized) how nps arrive at/remain in tumors. Furthermore, the retention of nanoparticles is of critical interest to the pharmaceuticals field [16]. These questions of delivery/retention have remained difficult to answer since no dynamic microscopic studies of nps in live subjects have previously been done. Therefore, just as IVM was used to study the hematopoietic stem cell niche [17], we apply IVM to examine the dynamic niche of the tumor-targeted np. This leads to insights that could have far-reaching effects on guiding the engineering and chemistry of injectable nps' physical structure and ligands, on the imaging of nps as contrast agents or activation as therapeutic agents, and details the interstitial and cellular behavior of nps. Our dynamic, high-resolution approach could thus guide diagnostic tumor imaging and therapeutic treatment time-points and delivery of various nanomedicines.

Materials and methods

Nanoparticle conjugates

We prepared red-dye labeled, peptide-conjugated SWNT bioconjugates as previously reported, with slight modifications [18]. After sonicating raw Hipco SWNTs in an aqueous solution of DSPE-PEG_{5000}-Amine (NOF Corp) for 1 h, they were centrifuged at $24{,}000 \times g$ for 6 h to obtain short, PEGylated SWNTs in supernatant (~500 PEG chains per SWNT). SWNTs were filtered through 100 kDa filters (Millipore) to remove excess coating polymer. SWNTs were then conjugated to both RGD (or RAD) and Cy5.5. To perform the conjugation, Cy5.5-NHS (Invitrogen) and sulfo-SMCC (sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate) (Pierce) were mixed at a 1:5 molar ratio (0.2 mM:1 mM) and incubated with the SWNT solution at pH 7.4 for 2 h (on average the final solution contained ~8 Cy5.5 molecules per SWNT). Upon removal of excess reagents, the SWNT solution was split equally and reacted overnight with 0.2 mM of thiolated RGD (cyclo-(Arg-Gly-Asp-D-Phe-Lys)) or RAD (cyclo-(Arg-Ala-Asp-D-Phe-Lys)) in the presence of 10 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma—Aldrich) at pH 7.4, yielding SWNT-PEG-Cy5.5-RGD and SWNT-PEG-Cy5.5-RAD with both Cy5.5 and RGD/RAD conjugated onto the surface of