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Melanoma growth effects on molecular clearance from tumors and biodistribution into systemic tissues versus draining lymph nodes☆

Nathan Andrew Rohner [a,b], Susan Napier Thomas [a,b,c,d,*]

[a] George W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia, United States
[b] Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, United States
[c] Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia, United States
[d] Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia, United States

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ABSTRACT

Factors produced within or administered directly into the tumor interstitium, such as cytokines, chemokines, proteases, exosomes, microvesicles, or therapeutic agents, play important and multifaceted roles in the regulation of malignant disease progression. Their bioavailability to mediate signaling in distributed tissues outside of the tumor microenvironment, however, has not been well described. We therefore sought to elucidate the relative extent to which factors from within the primary tumor disseminate to systemic tissues as well as how these distribution profiles are influenced by both hydrodynamic size and the remodeling tumor vasculature. To accomplish this goal, we intratumorally co-infused into the dermal lesions of B16F10 melanoma-bearing mice at prescribed times post tumor implantation a near infrared fluorescent tracer panel ranging from 5 to 500 nm in hydrodynamic diameter and compared the in vivo clearance and biodistribution profiles to that of naïve animals. Our results indicate that tumor growth reduces tumor-draining lymph node accumulation and alters the distribution of tumor-derived factors amongst systemic tissues. Despite these changes, previously developed principles of size-dependent lymph node drug targeting are conserved in melanomas, suggesting their applicability to sentinel lymph node-targeted drug delivery. Tumor progression was also found to result in a significant increase in the hydrodynamic size of factors originating from the tumor that accumulated within systemic tissues. This suggests that tumor vascular remodeling may redirect the organism-wide signaling activity of tumor-derived factors and may negatively contribute to disease progression by altering the bioavailability of molecules important to the regulation of pre-metastatic niche formation and the induction of anti-tumor immunity.

1. Introduction

Tumor-secreted soluble factors (TSF) such as cytokines, chemokines, proteases, and microparticles (including microvesicles and exosomes) mediate intercellular signaling at the cell- and tissue-levels to regulate cellular proliferation [1], angiogenesis [2] and lymphangiogenesis [3], recruitment of regulatory immune cells [4,5], as well as extracellular matrix remodeling [6] via their direct effects or nucleic and/or protein transfer. Accordingly, numerous TSF and their associated signaling pathways activated within the primary tumor have emerged as potential therapeutic targets given their implicated role in the promotion of malignant disease progression associated with poor prognosis including metastasis and anti-tumor immune suppression.

In addition to their function within the tumor microenvironment, TSF have reported activities in signaling to distant tissues, such as the lymph nodes, liver, lungs, kidneys, and spleen, which exacerbate disease progression. For example, intravenous (i.v.) infusion of melanoma-derived exosomes promotes metastasis by directing host progenitor cell differentiation toward a pro-metastatic phenotype [7]. Treatment with mammary tumor-conditioned media can also induce lung and lymph node remodeling to accelerate spontaneous metastasis [8]. Moreover, tumor antigen presentation in tumor-draining lymph nodes (TDLN) is implicated in directing anti-tumor immune suppression [3,9]. These data therefore suggest mechanisms of signaling to distributed tissues that direct the activity of TSF in facilitating disease progression that are not restricted to the primary tumor.

As such, mechanisms of TSF clearance from the tumor microenvironment and distribution to disseminated tissues likely play important roles in the regulation of TSF signaling locality and resulting function in cancer. This has been widely considered in the context of tumor lymphatic transport function, in particular for its role in facilitating cellular invasion and metastasis [10–12] and as a conduit for immune modulatory TSF to reshape the TDLN immune microenvironment [3,13]. The role of TSF clearance via the blood vasculature and resulting distribution in systemic tissues on the other hand has been largely overlooked.

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* Corresponding author at: George W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, 315 Ferst Drive NW, Atlanta, GA 30332, United States.
E-mail address: susan.thomas@gatech.edu (S.N. Thomas).

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However, treatment with melanoma-conditioned media redirects Lewis lung carcinoma metastasis from the lung to the kidney, spleen, intestine, and oviduct [14], tissues more typical of melanoma dissemination, suggesting a potential role for systemically circulating TSF in directing organism-wide responses to growing tumors. Interestingly, TDLN-targeted, but not non-targeted, toll-like receptor ligand adjuvant therapy has been shown to be efficacious in reducing tumor burden [9] by exploiting localized depots of endogenously produced tumor antigen within TDLN delivered via tumor-draining lymphatics. Thus, the relative contribution of blood versus lymphatic-mediated TSF clearance and resulting accumulation in associated tissues may not only influence TSF signaling activity and role in disease progression but also susceptibility to therapeutic interventions neutralizing or exploiting TSF function.

An added level of complexity in this problem is the influence of vascular remodeling that occurs within growing tumors. Hyper-angiogenic signaling during tumor formation and growth causes haphazard tissue remodeling and a tortuous, dilated, and leaky [15,16] tumor blood vascular network that is accompanied by lymphatic hyperplasia [17]. These characteristics are well recognized to manifest in the enhanced permeability and retention effect in which systemically circulating particulates at the nano- and microscales accumulate to a greater extent within malignant relative to healthy tissues [18]. Yet while this is often exploited for tumor-targeted drug delivery applications, its impact on the clearance and biodistribution of TSF and therapeutic agents administered directly to the tumor remains as-of-yet unknown.

Hence, although TSF and intratumorally administered therapeutic agents have been regarded for their role in local signaling within the tumor microenvironment, their bioavailability in distributed tissues and the impact of disease course on these biodistribution profiles has been overlooked. Unfortunately, detection and quantification of endogenously produced TSF are limited by significant dilutional effects as well as uncertainty in the spatial and temporal source of constitutive or inducible reporter systems. Furthermore, biodistribution analysis of specific exogenously supplied TSF is challenging due to degradation in vivo. To circumvent these limitations, we established a panel of near-infrared fluorescent tracers comprised of inert polymers that are resistant to hydrolysis and proteolytic degradation. Since TSF and therapeutic agents such as small molecule drugs and engineered drug delivery vehicles vary widely in hydrodynamic diameter and the rate and extent of blood versus lymphatic clearance from healthy tissues are acutely size-dependent [19,20], tracers over a physiological biomolecule size-matched range of 5–500 nm in hydrodynamic diameter were chosen. We examined in a temporal- and tissue-resolved manner the clearance and biodistribution of fluorescent tracers after infusion into the B16F10 melanomas of C57Bl6 mice at prescribed tumor growth phases or in the skin of naïve animals. Our findings support the hypothesis that lymphatic drainage significantly enriches levels of tumor-derived factors in draining lymph nodes (dLN), that melanoma progression attenuates lymphatic-mediated transport but not the predominant molecular size regime accumulating within TDLN, and that vascular remodeling within advanced melanomas increases the access of factors derived from the tumor interstitium to systemic tissues.

2. Materials and methods

2.1. Cell culture

B16F10 murine melanoma cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin/amphotericin *B*. Media, serum, and antibiotics were obtained from Life Technologies (Carlsbad, CA). Cell lines were routinely checked and confirmed to be negative for mycoplasma infection and rodent pathogens.

2.2. TSF analysis in tumor cell conditioned media

48 h B16F10 cell-conditioned serum-free medium, concentrated 20× by lyophilization after removing any possible cells by centrifugation at 300 ×g for 5 min, was fractionated in a size-resolved manner on a Sepharose CL-6B (GE Healthcare, Pittsburgh, PA) gravity chromatography column. The protein and deoxyribonucleic acid (DNA) content in fractionated media was analyzed using the bicinchoninic acid assay (Thermo Fisher Scientific Pierce, Waltham, MA) or by incubation with a 0.5× solution of GelRed (Biotium Inc., Hayward, CA) on an orbital shaker followed by absorbance or fluorescence measurements (BioTek Instruments Inc., Winooski, VT), respectively.

2.3. Near-infrared fluorescent tracers

500 and 50 nm fluorescent (580/610 nm and 660/680 nm excitation/emission, respectively) carboxylate-modified microspheres were purchased from Life Technologies. 500 kDa or 10 kDa amine-dextrans (Sigma-Aldrich, St. Louis, MO) were covalently labeled by incubation for 4 h in 0.1 M $NaHCO_3$ buffer at pH 8.4 on a shaker with Alexa Fluor 700 NHS-Ester or Alexa Fluor 610-X NHS-Ester dye (Life Technologies), respectively. Individual fluorescent dextran conjugates were purified from unreacted free dye by Sepharose CL-6B gravity column chromatography. Purified dextran-fluorophore conjugates were confirmed to be free of unconjugated dye by Sepharose CL-6B gravity column chromatography analysis. The size and zeta potentials of 500 and 50 nm fluorescent microspheres as well as 500 and 10 kDa dextran-AF700 conjugates suspended in Dulbecco's Phosphate Buffered Saline (D-PBS, Life Technologies) were confirmed using a Zetasizer Nano ZS (Malvern Instruments Ltd., WR14 1XZ, United Kingdom). All reagents were used and maintained under sterile conditions.

2.4. In vivo mouse melanoma model

C57Bl6 mice were purchased from Jackson Laboratories. All protocols were approved by the Institutional Animal Care and Use Committee. 0.5×10^6 B16F10 murine melanoma cells were intradermally implanted into the left dorsal skin of 6–8 weeks old mice on day 0. Tumors were monitored in anesthetized mice by caliper measurements of melanoma width, length, and depth and reported as an ellipsoidal volume. For intradermal injections, a depth-marked 27 gauge needle (Becton Dickinson, Franklin Lakes, NJ) was inserted perpendicularly into the center of the tumor of mice anesthetized with isofluorane and 10 μL of the fluorescent tracers (1.2×10^8 500 nm spheres, 7.9×10^{11} 50 nm spheres, 23.8 μg 500 kDa AF700-dextran, and 3 μg of 10 kDa AF610-dextran) were co-infused as a saline solution by syringe pump at a rate of ~300 nL per sec for a slow infusion rate that resulted in minimal alterations in interstitial pressure. The day post B16F10 implantation indicates the day on which tracer injections were made. Alternatively, naïve mice were injected in the same manner with the same fluorescent tracer panel solution in the dermal layer of the left dorsal skin. For i.v. injections, 3 μg of 10 kDa AF610-dextran in 100 μL saline was injected with a 29 gauge insulin syringe into the jugular vein of mice. The syringe was aspirated to withdraw a small amount of blood before and after injection to confirm that the entire volume was administered i.v.

2.5. Micro-computed tomographic imaging for vascular measurements

Animals were perfused with neutral buffered formalin for 10 min, a saline wash for five more minutes, and MicroFil (Flow Tech Inc., Carver, MA) catalyzed at a viscosity appropriate for small vessels (5 mL lead-based contrast agent: 2.5 mL diluent: 0.25 mL curing agent) by syringe with the application of constant pressure. Afterwards, perfused mice were carefully stored at 4 °C overnight to cure the contrast agent. The following day, skin or tumor samples were harvested and stored in D-