Dose Dependence of Intratumoral Perivascular Distribution of Monoclonal Antibodies

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ABSTRACT: Intravenously delivered antibodies have been previously found to distribute in a perivascular fashion in a variety of tumor types and despite targeting a range of different antigens. Properties of both the antibody and the targeted antigen, such as the administered dose, binding affinity, and antigen metabolic half-life, are predicted to influence the observed perivascular distribution. Here, the effect of antibody dose on the perivascular distribution is determined using an unbiased image analysis approach to quantify the microscopic distribution of the antibody around thousands of blood vessels per tumor. This method allows the quantitative determination of the localization of blood vessels, extravasated antibody, and tumor antigen following the administration of antibody doses covering two orders of magnitude in the dose range commonly utilized in preclinical studies. A mathematical model of antibody extravasation, diffusion, binding, and endocytosis in a Krogh cylinder geometry with parameters directly measured or taken from the literature is quantitatively consistent with the experimentally determined profiles. A previously reported scaling analysis is employed to extend these results to any tumor model in which the antigen density and turnover rate are known, allowing facile quantitative prediction of the minimum antibody dose required for complete tumor saturation. © 2011 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:860–867, 2012

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INTRODUCTION

Antibodies represent a significant and rapidly growing proportion of oncology therapeutics.[1] Although many have found success in a range of cancers, particularly hematologic malignancies, there remain substantial barriers to the effective use of antibodies to treat solid tumors. Solid tumors present a number of barriers to tumor targeting and penetration, including blood clearance, extravasation, diffusion through the interstitial space, binding to antigen, endocytosis, and degradation.[2,3] Many of these barriers are further exacerbated by the disordered physiology of solid tumors, which results in highly permeable and irregular vasculature and high interstitial fluid pressure.[4–6] For decades researchers have noted that the penetration into solid tumor tissue is often limited for drugs ranging in size and mechanism of action from chemotherapeutics to antibodies and nanoparticles.[3,4,7–11] Limited penetration has been linked to reduced therapeutic efficacy, even in cases in which bulk tumor uptake is high enough to exert an antitumor effect with a well-distributed therapeutic.[12] Recently it was shown that the US Food and Drug Administration-approved monoclonal antibodies cetuximab and trastuzumab penetrate poorly into tumors in animal xenograft models.[13,14]

Quantitative *in vitro* studies of antibody delivery to and distribution within tumor spheroids have yielded insights into the roles that antibody affinity and antigen internalization play in this process.[15,16] *In vivo*, for a range of antibodies, antigens, and cell lines,

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extravasation from tumor blood vessels has been shown to display a characteristic perivascular distribution in which the tumor cells within a few cell layers of the perfused vessels are often saturated with antibody, but more distal regions show little to no evidence of therapeutic targeting.[7,8,12–14] Common bulk measures of tumor uptake such as percent injected dose per gram fail to differentiate the heterogeneity of tumor targeting at the microscopic scale.

Here, we present an *in vivo* study of monoclonal antibody and antigen distribution around tumor blood vessels as a function of antibody dose covering two orders of magnitude. A computer-aided method of analyzing entire tumor cross sections in a quantitative and unbiased manner is utilized to generate data. These results are consistent with a Krogh cylinder model and scaling analysis, which predict the antibody dose necessary to saturate a tumor for a given antigen cell surface expression level and metabolic half-life. Although these modeling analyses are dramatic oversimplifications of the tumor microenvironment, they are nonetheless successful in quantitatively predicting the distribution of extravasated antibody averaged over the tumor cross section.

MATERIALS AND METHODS

Reagents

A low-picomolar humanized antibody to carcinoembryonic antigen (CEA), designated sm3e, has previously been engineered and characterized.[17] This antibody was secreted in transiently transfected human embryonic kidney 293 cells (Invitrogen, Carlsbad, California), purified by protein A resin (Millipore, Billerica, Massachusetts) and buffer exchanged into phosphate-buffered saline (PBS). The antibody was fluorescently labeled using the Alexa Fluor 488 protein labeling kit from Invitrogen. Labeling was conducted in a single batch of approximately 3 mg protein to yield a homogenously labeled reagent source for all experiments presented. Anti-CEA monoclonal antibody M85151a was purchased from Fitzgerald (Acton, Massachusetts), and goat anti-rat 546 secondary antibody was from Invitrogen. Antibody M85151a was labeled with the Alexa Fluor 647 protein labeling kit (Invitrogen) and has been previously determined to be noncompetitive with sm3e.[18]

Animal Model

Animal use and care was conducted in full compliance and under approval from the Committee on Animal Care of Massachusetts Institute of Technology. A CEA-positive human colorectal cancer cell line, LS174T, was used to induce xenograft formation in the flanks of 6–8 weeks old NCr nude mice (Taconic, Hudson, New York) by subcutaneous injection of 5×10^6 cancer cells. Tumors were allowed to establish and grow to a size of 5–10 mm, at which point antibody injections were conducted. Varying doses of fluorescently labeled sm3e, ranging from 5 to 500 μg, were supplemented as needed with immunoglobulin (Ig) G from human serum (Sigma–Aldrich, St. Louis, Missouri) to 500 μg total IgG and then injected retroorbitally into tumor-bearing nude mice. Mice were sacrificed 24 h after antibody administration and tumors were immediately excised and snap frozen in optimal cutting temperature medium (Sakura Finetek USA, Torrance, California) via isopentane over liquid nitrogen. Frozen blocks were stored at–80°C until sectioned by the Histology Core Facility of Koch Institute. Frozen blocks were sectioned approximately 1–2 mm into the tumor tissue at a thickness of 8 μm and stored at–80°C until stained and imaged.

Immunofluorescence Protocol

Frozen slides were first air dried for approximately 30 min and then the tissue samples were circled with a PAP pen (Invitrogen). Tissues were fixed for 15 min at room temperature in formalin and then washed three times with PBS. Blocking was performed with 5% goat serum (Invitrogen) in PBS for 1 h at room temperature. Primary antibody incubation was 5% goat serum in PBS + 1:100 rat anti-mouse CD31 (BD Pharmingen, San Diego, California) overnight at 4°C. Slides were then washed three times with PBS and incubated with PBS + 0.1% Tween 20 (Sigma–Aldrich) + 1:200 goat anti-rat 546 (Invitrogen) + 1:100 M85151a-647 anti-CEA antibody (Fitzgerald) for 1 h at room temperature. Slides were washed four times with PBS and then mounted in Vectashield + 4',6-diamidino-2-phenylindole (DAPI) medium (Vector Labs, Burlingame, California).

Fluorescence Imaging

Slides were imaged using a DeltaVision Spectris microscope (Applied Precision, Issaquah, Washington) equipped with a motorized stage and running Softworx software (Applied Precision). Emission and excitation filters were arranged to permit simultaneous four-color imaging of DAPI, Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 647. The paneling feature of Softworx (Applied Precision) was used to capture the entire tumor section at a resolution of 1.336 μm/pixel and to stitch together the fields into a single large mosaic image for subsequent analysis.

Modeling

Antibody extravasation was modeled using an extension of a previously described Krogh cylinder model of the tumor vasculature, as detailed in the Supplementary Material.[19] Criteria for tumor saturation were estimated using the Thiele modulus concept described previously and using parameters extracted from the