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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.

Keywords

carcinoembryonic antigen; tumor penetration; microdistribution; transport barriers; mathematical models; protein delivery; cancer; drug transport; macromolecular drug delivery; preclinical pharmacokinetics

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, 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 homogeneously 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 literature or measured directly in tumor sections. The key assumption underlying the Thiele modulus is that the extent of antibody distribution through the tumor volume is determined by the balance between endocytic consumption of bound antibody and extravasation/diffusion. The Thiele modulus represents the dimensionless ratio of these competing rates and is described mathematically as

$$\phi^2 = \frac{k_2 R^2 \left(\frac{[Ag]}{\varepsilon} \right)}{D \left(\frac{2PR_{cap}[Ab]_{plasma}}{D} + K_d \right)}$$

When $\phi^2 = 1$, these characteristic rates are equal and hence saturation is approximately achieved. k_e is the endocytic rate constant for antibody/antigen complexes. R is the average distance to the nearest blood vessel in the tumor. $[Ag]$ is the antigen concentration on a per-tumor-volume basis. ε is the fraction of tumor volume accessible to the antibody. D is the diffusivity of the antibody in tumor tissue. P is the vascular permeability coefficient. R_{cap} is

the average capillary radius. $[Ab]_{\text{plasma}}$ is the peak plasma concentration of antibody. K_D is the antibody/antigen equilibrium dissociation constant. Parameter values are given in Table 1.

RESULTS

Unbiased Image Analysis

Rather than introduce potential observer bias by manually selecting individual blood vessels for analysis, images were analyzed as entire tumor sections for features including intervessel distance, antibody penetration from blood vessels, and antigen distribution. The 546-nm (blood vessel) plane was isolated, thresholded to include vessels only, and converted to a binary image using ImageJ [National Institutes of Health (NIH), Bethesda, Maryland]. A Euclidean distance map was created from the vessel binary, and this map was used as a mask to measure the average intensity in the antibody (488) and antigen (647) planes as a function of distance from the nearest blood vessel by an automated MATLAB (Mathworks, Natick, Massachusetts) computer program (Fig. 1). Regions of the tumor revealed to be necrotic or stromal tissue by examination of hematoxylin and eosin staining or by the absence of antigen were excluded from the analysis.

Microdistribution Experimental Results

An anti-CEA IgG was dosed at levels ranging from 5 to 500 μg in mice with xenografted LS174T tumors. Mosaic images of full tumor cross sections that have been intensity scaled identically are shown in Figure 2. Corresponding imaging of antigen expression in these tumor sections confirms CEA expression throughout (Supplementary Data).

At low antibody doses, ranging from 5 to 50 μg , considerable perivascular binding and localization of extravasated antibody is observed, with a general trend of increasing penetration distance and perivascular antibody intensity with increased antibody dose (Figs. 2a–2c). In contrast, higher doses of 150 and 500 μg show near-complete penetration of the tumor, with no clear perivascular distribution of the antibody. When tumor sections are analyzed by computer to quantitatively determine the microdistribution trends, doses of 5 and 15 μg are seen to display perivascular distribution, which then decreases to near-background levels approximately 40–50 μm from the blood vessels on average. In contrast, the 50 μg dose also shows enhanced localization adjacent to blood vessels with decreasing signals with distance from vessels, beginning approximately 20 μm from the vessel, but this dose retains some degree of specific signal and never drops to background levels. The highest two-dose levels, which appear to saturate the tumor tissue, show no perivascular localization. Essentially, all tumor tissue is reached, with marginal additional binding detectable between the 150 and 500 μg doses.

Microdistribution Mathematical Modeling

To predict how antibody penetration will depend on parameters such as antigen density, endocytic half-life, and antibody affinity, a previously described Krogh cylinder-based model was extended (Supplementary Data).¹⁹ Parameter values were determined from the literature where appropriate and from direct measurement in the case of the Krogh cylinder radius (Supplementary Data). This model includes the effects of blood clearance, extravasation, antibody diffusion through the interstitial space, binding, unbinding, and endocytic clearance. Simulations of single-vessel extravasated antibody profiles over the experimental dose range show excellent agreement with the experimental results (Fig. 3). These experimental results represent the averages of the antibody microdistribution from thousands of blood vessels per tumor section analyzed. The model reproduces the

perivascular distribution seen at low doses as well as the complete penetration, which occurs at the highest doses, and the intermediate condition observed at the 50 μg dose level.

Thiele Modulus Analysis

For the LS174T tumor and the CEA-targeting sm3e antibody used in this study, tumor saturation appears to occur at a dose between 50 and 150 μg . However, the dose required to saturate a tumor will be dependent on several antibody- and antigen-dependent factors, including antigen density and turnover rate as well as antibody affinity, permeability, diffusivity, and clearance rate. For the case of IgGs, previous analyses have strongly suggested that the factors limiting tumor penetration are not blood clearance related, but instead related to binding and endocytosis of antibody-antigen complexes.² A dimensionless number termed the Thiele modulus (ϕ^2) can be used to quantitatively describe the ratio of the rate of binding and endocytosis of an antibody to its rate of extravasation and diffusion into the tumor. This rate of extravasation and diffusion must be greater than the rate of binding and endocytosis (i.e., $\phi^2 < 1$) for the antibody to fully penetrate a given distance. Applying this dimensionless analysis using parameters obtained from the literature or directly measured from the experimental data (Supplementary Data) allows the determination of the saturation criteria for a tumor as a function of antigen density and turnover rate, parameters easily found in the literature or measured for a tumor model of interest (Fig. 4). Antibody affinity also can play a significant role, as very-high-affinity antibodies bind essentially irreversibly to tumor antigen and are eventually endocytosed and degraded. Moderate-affinity antibodies require lower doses for tumor penetration, as they are able to dissociate from bound antigen and diffuse farther into tumor tissue before being endocytosed and degraded (Fig. 4b).

DISCUSSION

We present here an unbiased image analysis of the microdistribution of antibody and antigen in xenograft tumors. The use of appropriately selected fluorophores allows independent imaging of extravasated antibodies, blood vessels, and antigen in entire xenograft tumor cross sections. The full cross-section, devoid of any nontumor or necrotic areas, can then be analyzed by the use of the MATLAB program (Mathworks), which measures and averages the pixel intensities in the antibody and antigen channels for each of the blood vessels in a tumor section. These data represent a snapshot of the average extravasated antibody distribution from all the vessels in an entire tumor section.

Using this image analysis method, we determined that for the particular antigen and antibody pair used here, complete tumor penetration occurs at a dose between 50 and 150 μg . Quantitatively, perivascular distribution is clearly observed in low doses (5 and 15 μg), less prominent at 50 μg , and absent at doses that fully penetrate the tumor. Interestingly, the normalized antibody-antigen intensity changes relatively little between the highest dose levels, suggesting that antigen saturation may have occurred and that increasing antibody dose beyond this level has little potential beneficial effect on targeting tumor cells.

A Krogh cylinder model of antibody distribution around a blood vessel was employed to predict these results. This model uses the parameters of the antibody and antigen that have been culled from the recent literature, or measured from the experimental results in the case of the Krogh cylinder radius. This parameter was estimated directly from intervessel distribution data measured in the tumor sections imaged (Supplementary Data). Using no-fit parameters, the model shows excellent agreement with the data. Despite the simplifications inherited in the use of the Krogh cylinder model, the model is able to quantitatively predict the antibody distribution profiles across the dose ranges examined. Low doses indicate an exclusively perivascular distribution, moderate (50 μg) doses show enhanced binding near

blood vessels but retain binding much further from the vessel, and high doses show uniform antigen saturation. The model predicts that the highest doses saturate all antigen binding sites; additional antibody accumulation at these high doses is due to the unbound antibody, which has extravasated into the tumor and is free to diffuse or bind free antigen. The experimental images may not capture this unbound antibody, as during the tumor excision, embedding, sectioning, and immunofluorescent staining processes there are abundant potential opportunities to wash away the free antibody.

These concepts can be generalized to different antibody–antigen combinations through the use of the Thiele modulus dimensionless quantity. This number, a ratio of the rate of binding and catabolism to the rate of extravasation and diffusion, succinctly captures the limiting rate processes for long serum half-life therapeutics like antibodies. It should be noted that molecules with substantially shorter half-lives may also be limited by their rapid blood clearance, although this is not the case for IgGs.² Setting the Thiele modulus equal to unity, at which point the rates are equal, allows the determination of the minimum criteria for full penetration of a tumor. These criteria will depend upon the easily measured quantities of antigen density and endocytosis rate, as well as the antibody properties. Typical antibody properties are used for the plots in Figure 4 and are listed in Table 1. The rate of antigen turnover can be seen to have a very strong effect on tumor penetration, suggesting that antigens that are endocytosed as a part of constitutive membrane turnover may be good targets with respect to beneficial intratumoral distribution.

Antibody affinity may also play a significant role in tumor penetration; the criteria for penetration of a 10-pM binder are markedly different from those of a 1-nM binder. This phenomenon occurs as a consequence of the different time scales of unbinding for high versus moderate-affinity binders. High-affinity binders rarely dissociate over the lifespan of a bound antigen, whereas moderate-affinity binders are more likely to bind multiple antigens, diffusing between each one. This may allow moderate-affinity binders to penetrate farther into the tumor than very-high-affinity binders, albeit at the cost of fewer antibodies per targeted cell.

Quantitative analysis and modeling of perivascular antibody distribution can be a useful window into elucidating the factors that ultimately determine whether a given therapeutic is able to fully penetrate the tumor tissue. Experiments using a well-characterized antibody–antigen model system *in vivo* provide validation for the mathematical framework and show that tumor penetration should be possible at antibody doses near the high end of those commonly used in preclinical studies. However, penetration is much easier to achieve for slowly internalizing antigens such as those turned over constitutively with the cell membrane. Actively endocytosed antigens require much larger doses for complete penetration; this is an important consideration for antibody–drug conjugates, which are typically targeted to rapidly internalized antigens. The pharmacodynamic benefits of efficient antibody–drug conjugate internalization are obtained at micropharmacokinetic cost of impeded tumor penetration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Depiction of an example of immunofluorescence image. Objects pseudocolored red indicate blood vessels, and green pseudocolored represents extravasated antibody. Antibody was administered to the tumor-bearing mouse 24 h before euthanasia at a dose of 50 μ g. Not shown for clarity are DAPI and antigen, both of which are also imaged with blood vessels and antibody. Scale bar, 1 mm. (b) Blood vessels have been thresholded and a Euclidean distance map applied to the thresholded vessels. Antigen-negative and necrotic regions of the tumor section can be manually removed from the analysis of this image. Pixel intensities are directly proportional to the distance from the nearest blood vessel. (c) Output of the automated MATLAB analysis of the antibody distribution from blood vessels. Antibody

intensity values at large distances from the blood vessels are indistinguishable from those measured in tumors, which did not receive the antibody. Not shown for clarity is the corresponding antigen distribution.

Figure 2.

Selected images of tumor sections from xenografts in mice administered between 5 and 500 μg of anti-CEA antibody sm3e labeled with Alexa Fluor 488 24 h before sacrifice and tumor harvest. Blue pseudocolor represents DAPI; green pseudocolor represents sm3e; red pseudocolor represents blood vessels; not shown for clarity is antigen, which was also imaged in each section. Doses were 5, 15, 50, 150, and 500 μg in panels a–e, respectively. Note that low doses at 50 μg or less show clear perivascular distribution, whereas higher doses of 150 and 500 μg appear to show complete saturation of the tumor. All scale bars are 1 mm.

Figure 3.

(a) Experimentally observed perivascular antibody–antigen distribution ratio as a function of dose and (b) Krogh cylinder-based predicted perivascular distribution of extravasated antibody. The lower limit for signal in panel a was taken to be three standard deviations over the background signal. Equations and parameters used to generate panel b are detailed in the Supplementary Data.

Figure 4.

Plots of $\phi^2 = 1$ trendlines as a function of bolus dose IgG injected and the number of cell-surface antigens per tumor cell, allowing for the estimation of minimum doses required for tumor saturation. Data are plotted for a wide range of physiologically relevant antigen turnover rates. The shaded band represents a typical range of constitutive turnover half lives for membrane proteins. Parameters used in model calculations are given in Table 1. (a) 10 pM bivalent affinity and (b) 1 nM bivalent affinity.

Table 1

Model Parameters for Thiele Modulus Calculations

Model Parameter Values			
Parameter	Definition	Value	Citation
ε	Void fraction	0.2391	20
Mouse blood volume (to compute $[Ab]_{\text{plasma}}$)	Approximately 10% of mouse weight	2 mL	Assumed
D	Diffusivity	$2.54 \times 10^{-7} \text{ cm}^2/\text{s}$	20
P	Vascular permeability	$3.87 \times 10^{-7} \text{ cm/s}$	20
R_{Krogh}	Krogh cylinder radius	60 μm	Measured
R_{cap}	Capillary radius	8 μm	21
Tumor cell density	Tumor cells per unit volume	$3 \times 10^8 \text{ cells/mL}$	22
K_d	Apparent affinity	10 pM	Measured
CEA/LS174T cell	Antigens per cancer cell	$4 \times 10^5 \text{ Ag/cell}$	18

Antigen concentration was determined from tumor cell density and antigen per cell data.

Plasma concentration used was peak plasma concentration, calculated assuming that all injected dose is available.