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Affinity and Avidity in Antibody-Based Tumor Targeting

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Summation

Many factors contribute to successful tumor targeting by antibodies. Besides properties of the tumor tissue and general antibody pharmacology, a relationship exists between an antibody and its antigen that can shape penetration, catabolism, specificity, and efficacy. The affinity and avidity of the binding interactions play critical roles in these dynamics. In this work, we review the principles that guide models predicting tumor penetration and cellular internalization while providing a critical overview of studies aimed at experimentally determining the specific role of affinity and avidity in these processes. One should gain the perspective that binding affinity can, in part, dictate the localization of antibodies in tumors, leading to high concentrations in the perivascular space or low concentrations diffused throughout the tumor. These patterns can be simply due to the diminution of available dose by binding antigen and are complicated by internalization and degradation stemming from slow rates of dissociation. As opposed to the trend of simply increasing affinity to increase efficacy, novel strategies that increase avidity and broaden specificity have made significant progress in tumor targeting.

Key words: antibody, affinity, avidity, tumor targeting, internalization, scFv, specificity

Introduction

The focus on monoclonal antibodies (mAbs) as a distinct class of anticancer therapeutics has grown rapidly over the last 2 decades because of their innate ability to bind tumor-associated antigens. Areas such as antibody engineering, clinical trials, pharmacokinetics, mechanisms of action, antigen selection, and immunoconjugates have been extensively reported [1–8] and are not the focus of this article. Instead, the oft-overlooked field of tumor targeting, and, moreover, the importance of affinity and avidity are presented here. These two parameters of mAbs are indeed related and can be easily confused. In the following discussion, we remind the reader that affinity is the strength, which can be expressed in thermodynamic terms of the binding interaction between a single antigen and a single region of the mAb. Avidity, however, is the accumulated strength of multiple affinities summed up from multiple binding interactions and is commonly referred to as a functional affinity.

There are many factors that make effective tumor targeting with antibody-based molecules difficult. For example, one must overcome systemic clearance[3,9] capillary extravasation into the tumor,[10] and high interstitial pressure gradients[11,12] to even gain exposure to the intended target antigen. Affinity and avidity of mAbs becomes a critical issue in tumor targeting when one begins to consider interactions with tumor-associated antigens. Micropharmacological processes take place anywhere antigen recognition occurs. Not strictly limited to tumor cells, target antigen is commonly expressed on normal tissue, found in circulation, and shed into the tumor interstitial space. These nontarget pools of antigens can reduce treatment effectiveness, increase systemic clearance, and increase side-effects (especially for radioimmunoconjugates) by impairing mAb specificity for the tumor.[13] Therefore, this update aims to familiarize the reader with principles and examples of how affinity and avidity can affect tumor penetration, cellular internalization, and specificity.

The Binding-Site Barrier Model

After intravenous (I.V) delivery, it is commonly observed that mAbs have heterogenous, even perivascular, distribution within a tumor.[14] Presumably, poor penetration decreases efficacy and increases the risk of acquiring resistance because only small areas of the tumor are being exposed to cytotoxic concentrations of a drug while other regions may receive no drug at all. Heterogeneous mAb distribution within a tumor can arise in part from heterogeneous antigen expression, heterogeneous vasculature, and necrosis. With the exception of antigen expression, these typical characteristics of tumors

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can perturb the distribution and penetration of any molecule entering the tumor, and, therefore, properties of the mAb should be inconsequential. However, in a seminal study, parameters thought to influence distribution and penetration were analyzed in a model algorithm for full mAb and mAb fragments. By analyzing vasculature-wall penetration, molecular weight, valence, and antigen–mAb interactions, it was hypothesized that binding of the mAb to antigen can prevent its additional penetration.[15] Furthermore, the model predicted an inverse relationship between affinity and penetration (termed the binding-site barrier). Simply stated, as affinity increases, penetration decreases. In subsequent models, the system was refined by exploring the shape of the hypothetical tumor, dose of mAb, vascular permeability, antigen density, nonspecific binding, and lymphatic outflow.[16–19] The limited penetration into the tumor created by the binding-site barrier was concluded not to be of a mechanical nature, but successful binding of antigen, instead was concluded to decrease the concentration of free mAb such that an insignificant amount is available to diffuse further into the tumor. The critical factors predicted to affect the extent of the binding-site barrier are antigen density, mAb internalization and metabolism, and mAb binding affinity.[17]

Experimental Analysis of Affinity in the Binding Site Barrier

Support for the binding-site barrier principle was demonstrated *in vivo* by comparing distribution of tumor-specific and nonspecific radiolabeled mAbs after I.V. administration.[17,20] Tumors were initiated in guinea pigs by implantation of cell lines in the peritoneum or by creating small lung metastases by I.V. injection of cell lines. Tumors ranged from 300 μm to 1 cm and had little discernable vasculature. In both studies, it was observed that the nonspecific mAb attained a low-level, ubiquitous distribution throughout the tumors, thereby demonstrating that mAbs can diffuse freely in the tumor, and no mechanical barrier inhibits penetration. Large amounts of the specific mAb were retained on the perimeter of antigen-rich regions when given at a low dose.[20] When given at very high dose, the distribution of specific mAb was very similar to the pattern of antigen expression, showing that dosing can be used to overcome the binding-site barrier. Similarly, in multiple subcutaneous tumor xenograft models, it was observed that a low affinity mAb was more homogenously distributed throughout the tumor than a mAb with a fivefold higher affinity.[21] While the range of affinities used in these studies was limited, or only compared a functional mAb to a nonspecific isotype matched control, the studies did demonstrate successfully that binding of antigen can limit penetration into a tumor.

Single-chain antibody fragments (scFv) are comprised of individual antigen-recognition sites from a mAb and have proven to be a very useful tool for investigating the role of affinity in tumor targeting and the binding-site barrier principle. Through phage display and sequential mutagenesis, a panel of antihuman Her2 (c-erbB-2) scFv were generated that target the same epitope with a wide range of binding affinities.[22,23] Following I.V. injections, the low-affinity scFv, G98A ($K_D = 3.2\times10^{-7}$ M), failed to accumulate more than the negative control scFv in small-cell tumors of SCID mice. After taking into account the high rate of scFv clearance by the kidneys, G98A also failed to achieve tumor levels higher than that in circulation after 24 hours in nephrectomized SCID mice bearing tumors. Higher affinity scFv of the same panel (C6.5, $K_D = 1.6\times10^{-8}$ M and ML3.9, $K_D = 1.0\times10^{-9}$ M) did show significant accumulation, illustrating that a minimum binding affinity is required for retention in the tumors.[24] Accumulation in the tumor ceased to increase with affinity and was nearly the same for scFv with K_D of 1.0×10^{-9} M (ML3.9), 1.2×10^{-10} M (H3B1) and 1.5×10^{-11} M (B1D2).[23,25] Immunohistochemical (IHC) and immunofluorescent (IF) analysis of well-vascularized tumors ($\sim$100 mg) showed the highest affinity scFv limited to tumor space adjacent to the blood vessel while the low-affinity scFv diffused uniformly throughout the tumor interior.[25] Taken together, these results show that binding affinity for the antigen has a strong role in the total concentration and penetration of scFv into the tumor in support of the binding-site barrier models.

Three of the anti-Her2 scFv above, G98A, C6.5, and H3B1, were successfully expressed as IgG variants. The monovalent immunoglobulin G (IgG) K_D remained nearly the same as the initial scFv, but the functional affinity (avidity) increased to 5.0×10^{-10} M, to 5.4×10^{-11} M, and 4.7×10^{-11} M, respectively.[26] The moderate-affinity IgG, C6.5, showed the highest accumulation in small-cell tumors 24 hours and 72 hours post I.V. injection in tumor-bearing SCID mice (Adams GP, et al., in preparation). Furthermore, IHC analysis revealed that C6.5 diffused further into the tumor than H3B1, which remained perivascular. When used in *in vitro* antibody-dependent cellular cytotoxicity (ADCC) assays with donor-derived peripheral blood monocytes (PBMC), potency proved to increase with monovalent affinity.[26] This relationship was consistent as antigen density increased from 2.8×10^4 to 1.3×10^6 receptors per cell as determined by quantitative flow cytometry. Therefore, given that penetration decreases with affinity, and ADCC increases with affinity, future therapeutic designs will benefit from *in vivo* studies monitoring antitumor efficacy as a function of affinity and antigen density.

Internalization and Catabolism

As discussed above, if a mAb is to diffuse into a tumor, there must be an appreciable concentration of free molecules. Binding antigen with slow dissociation rates (high affinity) can reduce the concentration of free mAb or scFv and limit penetration. The concentration of free mAb can be reduced further if the antigen is internalized before the mAb dissociates. After internalization, the mAb can be degraded in the endosome and lysosome, but the antigen can be recycled or replaced by newly synthesized protein. In this case, the tumor, especially a large tumor with dense antigen expression, can become a significant sink for free mAb, limiting homogenous distribution. This effect is especially pronounced when the mAb off rate is slower than the rate of antigen internalization.[27] Mathematical and *in vitro* models predict, and animal studies demonstrate that antigen density and internalization rates can perturb mAb penetration into tumors and make a significant impact on pharmacokinetics,[10,28–30] however, few researchers investigate a direct correlation between internalization and mAb binding affinity, valency, or avidity. Researchers that have investigated internalization with multiple mAbs or similar molecules are not in total agreement if properties of the targeting molecule have any effects on

internalization or are is totally dependent on the antigen of interest.[28,31–34]

In the earliest study reviewed here, Kyriakos et al. investigated internalization with seven ^{125}I-labeled mAbs and four antigen-expressing cell lines.[32] After a 2-hour incubation to bind cells, excess or unbound mAbs were removed, and the culture supernatants were assayed multiple times over 2–3 days using trichloroacetic acid (TCA) precipitation. For six of the seven mAbs tested, the investigators observed that, within the first few hours, 20% of the bound mAbs dissociated and remained intact. This fraction was suggested to have only bound in a monovalent fashion. Over the next 2 days, subsequent assays revealed that 80% of each bound mAb was slowly released after being degraded. It was concluded that these mAbs were bound divalently, endocytosed, and degraded in the endosomal and lysosomal compartments. Due to the high rate of degradation without dissociation, the authors concluded that, "bivalent binding of antibodies to cells is essentially irreversible, so that the concepts of equilibrium and affinity are not applicable."[32] Unfortunately, because the affinity constants of the mAbs used in this study was not determined, one cannot fairly come to a conclusion correlating affinity and internalization. It does, however, seem reasonable to *speculate* from this study that, if the mAb dissociation rate is much slower than the rate of antigen internalization, mAb association with cellular antigen can be viewed as irreversible given that degradation will take place before equilibrium can be established.

In a recent study, Schmidt et al. used two anti-CEA scFv monomers and their analogous disulfide-linked dimers to address the roles of affinity and valency directly in mAb internalization.[33] By adapting the method using fluorescent dye–conjugated scFv,[31] the researchers were able to avoid the issue of scFv dehalogenation, which may have contributed to degradation observed by Kyriakos et al. The anti-CEA scFv, whether in the form of monomers, disulfide stabilized monomers, or disulfide-stabilized dimers, all had essentially the same net uptake half-times ranging between 11 and 15 hours.[33] From this, one can approximate the internalization rate constant in their system to be on the order of 0.001 minutes^{-1} for CEA. The authors concluded that the affinity and avidity of the scFv examined had no impact on their internalization rates. It should be noted that the K_D for the two scFv monomers (sm3E and shMFE) were 26 pM and 160 pM. Upon dimerization, the functional affinity of cells increased to 9.6 pM and 85 pM, respectively.[33] Because these are relatively high affinity molecules where the added valency did not make a dramatic impact on avidity, it may be not be appropriate to apply these researchers' conclusions broadly, especially for lower-affinity scFv and mAbs. As opposed to more-readily internalized antigens, the slow rate of internalization suggests CEA recycling may be due to membrane turnover and not to active endocytosis.

In an effort to analyze the regulation of Her2 surface expression, Austin et al. developed imaging and kinetic trafficking assays using fluorescently labeled trastuzumab (Herceptin, Genentech, San Francisco, CA), pertuzumab (Omnitarg, Genentech, San Francisco, CA), and their respective scFv, 4D5s, and 2C4s.[31] After incubating fluorescent anti-Her2 mAb or scFv with human mammary epithelial (SKBr3) cells for 2 hours, unbound IgG was washed, and surface fluorescence quenched with antifluorescent IgG, allowing for quantification of internalized anti-Her2 molecules. This method led to the conclusion that trastuzumab does not affect the endocytosis of Her2. Roughly 16% of trastuzumab and pertuzumab IgG were internalized in their assays, but only about 9%–11% of 4D5 and 2C4 scFv were internalized.[31] While the some of the difference between the mAb and scFv internalization may be attributable to labeling efficiencies, these results suggest increased internalization with valency. The levels of 4D5 and Herceptin internalization shown here are consistent with those previously determined for ^{125}I-based assays.[34] However, it is difficult to conclude whether the scFv were internalized less than the IgG because divalent interactions increased likelihood of binding a recycling receptor or if the increased avidity of the IgG decreased dissociation.

Finally, the most recent study again utilized the G98A, C6.5, and H3B1 anti-Her2 IgGs discussed above. Following incubation of radioiodonated IgG, as described by Kyriakos,[32] with SKOV-3 cells for 24 hours, TCA precipitation revealed that the lowest affinity mAb, G98A, had the highest dissociation and least catabolism of 80% and 6.5%, respectively. In contrast, the highest-affinity mAb, H3B1, had the least dissociation and most catabolism of 14.4% and 50.1%, respectively (Adams GP et al., in preparation). These data show a clear trend of increased internalization with higher intrinsic (monovalent) affinity. As with any study using ^{125}I labeling, dehalogenation may have contributed to the percent of mAb catabolized.

The biggest variable in the above studies is the target antigen. While CEA internalizes slowly at a rate near 0.001 minutes^{-1},[33] Her2 monomer internalizes at rates approximately ten times faster than CEA and up to 100 times faster when bound to ligand-activated epidermal growth factor receptor (EGFR).[35] Rate of mAb and scFv internalization appears to be most dependent on the antigen's recycling,[28] and the rate of antigen endocytosis is unaffected by mAb binding.[31] Therefore, the likelihood of internalization must be considered in terms of relative dissociation and recycling rates. Practically speaking, one can maximize tumor penetration and minimize catabolism by selecting an antibody with a greater dissociation rate than the rate of antigen internalization. For rapidly recycling antigens, this can be problematic as a minimum affinity in necessary for specific retention of mAb in tumors.[24] In addition, mathematical and *in vitro* models suggest that mAb dosing can overwhelm internalization to achieve tumor penetration.[36]

Modifications in Avidity and Specificity

Using scFv for tumor targeting can be advantageous because they can be generated from phage-display libraries and have favorable tumor penetration rates. Due to their relatively small molecular weight, scFv are rapidly cleared by the kidneys. This limits the general therapeutic application of scFv monomers but aids in generating high tumor–blood ratios that are necessary for some imaging and diagnostic applications.[37] scFV are, however, ideally suited for the development of dimers, diabodies, IgG, or multivalent molecules to attenuate pharmacokinetics, to increase the avidity of low-affinity monomers, or to target multiple antigens. Detailed discussions on the engineering, properties, and variety of antibodylike molecules built from scFv can be found elsewhere[38–41] as the focus of this article is limited to tumor targeting. Molecular weight, flexibility, specificity,

and avidity are practically unlimited when creating a multivalent molecule due to the spectrum of domains, linkers, peptides, carbohydrates, and cargo available. Making generalizations of tumor-targeting abilities of antibody-like molecules engineered from monomers is therefore difficult and likely not to be all-inclusive. We highlight studies below that are thought to illustrate the utility of dimers, diabodies, tetramers, and bispecific diabodies in tumor targeting following their conversion from scFv monomers.

Diabodies are molecules resulting from the association of two scFv, held together by noncovalent intermolecular interactions of variable heavy and light (V_H and V_L) domains. Upon conversion of the C6.5 scFv (introduced earlier) to a diabody, the K_D decreased from 1.6×10^{-8} M to 4.0×10^{-10} M due to a slower rate of dissociation *in vitro* and consistent with longer cell-surface retention *in vivo*.[42] Due to the combination of increased molecular weight and avidity, the amount of C6.5 diabody in the blood and tumor was 6.5–7-fold more than the scFv in SCID mice bearing small-cell tumors.[42] When other scFv of the anti-Her2 series were converted to diabodies, similar increases in avidity were observed. Interestingly, the functional affinity of G98A, the low-affinity scFv, decreased from 361 n*M* to 5.6 n*M* upon conversion to a divalent molecule. In the same cell-based assay, the high-affinity scFv, ML3.9, only changed from 3.8 n*M* to 0.49 n*M* when gaining a second binding arm.[43] The G98A diabody had more than twice the accumulation in tumors after 24 hours than the ML39 diabody did. While these observations suggest that tumor accumulation of the ML39 diabody was limited due to a greater impact of the binding-site barrier and internalization than G98A, this study indicates that producing divalent molecules from scFv can be more efficient than affinity maturation for tumor targeting.[43]

Dimers of scFv ($scFv_2$) differ from diabodies in that the two monomers are typically tethered by peptides on the C-termini, and the interaction between V_H and V_L domains remains intramolecular. This scaffold was used to address directly whether decreasing first-pass renal clearance or increasing avidity is more important for dimer retention in tumors compared to scFv.[44] For comparison to the monomeric anti-Her2 scFv 741F8-1, a scFv homodimer was produced that is divalent for Her2. To test the same molecular weight, and therefore renal clearance rate, with only a monovalent binding with Her2, a heterodimer was made by pairing 741F8-1 with the antidigoxin scFv 26-10.[44] Homodimerization decreased the *in vitro* K_D of the scFv monomer from to 4.8×10^{-8} M to 9.2×10^{-9} M, while the heterodimer had no change.[44] After 24 hours, 741F8-1 scFv and the 741F8-1/26-10 heterodimer had the same retention in the tumor (1.25 and 1.13 %ID/g respectively), using a small-cell SCID xenograft. However, 3.57 %ID/g of 741F8-1 $scFv_2$ was retained in the tumor. These observations demonstrate that the increased valency and avidity, and not reduction in clearance from an increase in mass, is responsible for the increased tumor-targeting ability of $scFv_2$.[44]

While the anti-Her2 scFv above benefited from increased avidity, polymerization is not always constructive. In studying the effects of valency and molecular weight on tumor targeting, the anti-Her2 scFv 4D5 was assembled into dimers and tetramers with self-associating disulfide linked peptides.[45] Despite the added valency, only modest increases in avidity and tumor retention were noted. While the reason for these unexpected observations is not obvious from the available data, this result highlights the possibility that geometry and flexibility of multivalent molecules are important factors in antigen recognition and tumor uptake.

By combining scFv that target different antigens or different epitopes, one can make antibodies that are bispecific. Many bispecific reagents have been made,[46,47] and their common goal is to bind and kill tumor cells more selectively over other populations by requiring both antigens to be present. Careful consideration of possible combinations of tumor-associated antigens to target may ultimately increase the efficacy over targeting each antigen alone. Robinson et. al. hypothesized that targeting the ErbB2/ErbB3 receptor tyrosine kinase (RTK) heterodimer would increase selectivity and induce a therapeutic effect, given that signaling through the receptors is causally linked to a variety of cancers.[48] The ALM $scFv_2$ was made by linking the A5 anti-ErbB3 scFv to the ML3.9 anti-ErbB2 scFv with a 20 amino acid peptide. ALM selectively binds cells that are double positive for ErbB2/ErbB3 over cells that only express one of the RTK, demonstrating that both arms of the $scFv_2$ engage their antigens and aid in cellular retention. Selectivity of ALM was demonstrated further when it was tested in tumor xenografts where mice bore tumors from cells expressing one or both of the antigens. In xenografts from ErbB3-positive cells, ALM did not accumulate significantly in the tumors. ALM tumor targeting increased when tested in xenografts from ErbB2-positive cells. In two different-double positive xenografts, however, ALM accumulated to high levels compared to other tissues. The synergy between the two binding arms of ALM is key to its tumor-targeting ability, as inactivation of either significantly impairs its association with the double-positive xenograft. Administration of ALM $scFv_2$ to double-positive cell lines produces antitumor cell activity. Treatment of cells with parental A5 and ML3.9 scFv either alone or in combination demonstrates that the A5 scFv harbors ALM's cytotoxic property while ML3.9 increases avidity.[48] ALM is a rare example of how two scFv can be combined as a dimer to increase tumor uptake, selectivity, and efficacy above and beyond the two monomers alone. The synergistic relationship in targeting the functional ErbB2/ErbB3 RTK heterodimer is undoubtedly more important than simply increasing molecular weight or avidity of a $scFv_2$.

Conclusions

From the review of the literature primarily limited to mAb affinity and avidity, it is clear that appropriate tumor targeting is not a trivial task to engineer into a molecule. It has been well-documented that affinity can limit penetration into tumors, especially large or poorly vascularized tumors. However, one must decide if homogenous distribution is necessary for their application. For example, for imaging and diagnosis, it is adequate to use a high-affinity mAb as resulting heterogeneous distribution will achieve the goal of tumor detection. However, specificity and clearance, both affected by avidity and valency, will be crucial to systemic clearance in the appropriate imaging timeframe as well as limiting side-effects that radiation may have on other tissues.

As discussed, antigen selection will be a critical factor for internalization and catabolism of mAbs. The relative rates of antigen recycling and dissociation are important in mAb

penetration into tumors. Therefore, in applications dependent on targeting every cell of a tumor, the mAb needs to dissociate before it is internalized and degraded. In the case of ADCC, a slow internalizing antigen would be the best target. However, if one is trying to deliver a cytotoxic agent to the cytoplasm of cells in a limited region of a tumor, such as the vasculature, a mAb with slow dissociation targeting a rapidly recycling antigen would be appropriate. These are just simple examples of the interplay of affinity, avidity, and efficacy in tumor targeting. Strategies derived from constructing mAbs and mAb-like molecules from single targeting domains will continue to provide methods to create effective cancer diagnostics and therapies.

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