

Optimization of Radioimmunotherapy of Solid Tumors: Biological Impediments and Their Modulation

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Abstract In contrast to the overwhelming success of radiolabeled antibodies in treating hematologic malignancies, only modest success has been achieved in the radioimmunotherapy of solid tumors. One of the major limitations in successful application of radioimmunotherapy is the large molecular size of the intact immunoglobulin that results in prolonged serum half-life and poor tumor penetration and uptake. With the advent of antibody engineering, small molecular weight antibody fragments exhibiting improved pharmacokinetics and tumor penetration have been generated. However, their clinical application has been limited by suboptimal tumor uptake and short tumor residence time. There is a greater realization that optimization of the molecular size of the antibodies alone is not sufficient for clinical success of radioimmunotherapy. In addition to their size, radiolabeled antibodies encounter other impediments before reaching their target antigens expressed on the cell surface of solid tumors. Some of the barriers include poor blood flow in large tumors, permeability of vascular endothelium, elevated interstitial fluid pressure of tumor stroma, and heterogeneous antigen expression. Recent research has considerably improved our understanding and appreciation of these forces, and the new wave of optimization strategies involves the use of biological modifiers to modulate the impediments posed by solid tumors. In combination with radiolabeled antibodies, various agents are being used to improve the tumor blood flow, enhance vascular permeability, lower tumor interstitial fluid pressure by modulating stromal cells and extracellular matrix components, up-regulate the expression of target antigens, and improve the penetration and retention of the radiopharmaceuticals. This review outlines ongoing research efforts involving biological modifiers to optimize the uptake and efficacy of radiolabeled antibodies for the treatment of solid tumors.

Ever since their discovery, antibodies were envisioned as “magic bullets” that would deliver toxic agents, such as drugs, toxins, enzymes, and radioisotopes, specifically to the diseased site and leaving the nontarget normal tissues unaffected. With the success of radiolabeled antibodies in treating hematologic malignancies, the dream has been partially fulfilled (1). Recent approval of radiolabeled antibodies, such as Bexxar and Zevalin, by the Food and Drug Administration for the treatment of lymphoma has renewed enthusiasm in radioimmunotherapy, which involves using antibodies to target cytotoxic radionuclides to the tumor site (1–3). For solid tumors, radioimmunotherapy is an attractive approach as it is capable of targeting both known and occult metastatic sites. Monoclonal antibodies (mAb) against several tumor antigens, such as carcinoembryonic antigen (CEA), tumor-associated

glycoprotein 72 (TAG-72), and prostate-specific membrane antigen, are being used for imaging tumors in the clinics (4–7). Antibodies against these antigens and several others, such as MUC1 mucin, mesothelin, and CD56, are in clinical trials for treating various malignancies (3). However, the clinical success of radioimmunotherapy for solid tumors still seems to be a distant dream because only a very small amount of administered antibody (as low as 0.001–0.01%) localizes in the tumor and administration of higher amounts of radiolabeled mAbs causes myelotoxicity (8–10). To be clinically successful, radioimmunotherapy for solid tumors needs to be optimized so as to enhance the tumor uptake and retention of radiolabeled antibodies in the tumor and minimizing the exposure of nontarget tissues.

Limited success of the radioimmunotherapy of solid tumors can be attributed to several factors, including undesirable pharmacokinetics, poor tumor uptake, and high immunogenicity of radiolabeled mAbs. Owing to their large size (>150 kDa), intact antibody molecules have poor pharmacokinetics that lead to long circulation times, thereby causing radiotoxicity in nontarget tissues. Poor uptake of antibodies and other macromolecules by the tumor results from slow diffusion rates and long distances of diffusion in poorly vascularized tumors. The uptake of macromolecules, such as antibodies, in a tissue is driven primarily by convection resulting from the pressure gradients between interstitial fluid pressure (IFP) and microvascular pressure. However, the leaky nature of tumor vasculature, due to structural and functional abnormalities, and the

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absence of functional lymphatic system in tumor result in decreased microvascular pressure and increased IFP (11–13). This phenomenon leads to the lowering or complete elimination of pressure gradients, thus impairing the macromolecular transport in the tumor. Moreover, repeated administration of intact immunoglobulins leads to the generation of human anti-(immuno)globulin antibody (HAGA) responses (14). The HAGA responses limit the frequency of dose administration, thus ruling out any dose fractionation, which is an important aspect of most therapeutic regimens involving radioisotopes.

Most of the undesired properties of intact IgGs, which limit their use in radioimmunotherapy, result from their large size and can be eliminated by modifying their design. Antibodies can be modified to generate small molecular weight fragments without disturbing their specific antigen binding. Therefore, the first phase of optimization of radioimmunotherapy involved the utilization of smaller enzymatically derived antibody fragments $F(ab')_2$ and Fab' , which exhibited rapid and homogenous tumor localization and shorter serum half-lives. With the advent of genetic engineering, smaller antibody fragments, such as monovalent, divalent, and tetravalent single-chain Fvs (scFv), diabodies, and minibodies, have been developed (15–19). These new-generation antibody fragments, ranging from 30 to 120 kDa, when compared with intact mAbs and more conventional enzymatically derived fragments, offer several advantages as carriers for selective delivery of radioisotopes to tumors (summarized in Table 1). First, the rate of clearance of scFv from the blood pool and normal tissues is much more rapid than that seen with intact IgG, $F(ab')_2$, or Fab' fragments, offering the possibility of earlier imaging and, for therapy, the reduction of radiation-absorbed dose to normal tissues. Second, autoradiographic studies have shown that scFv molecules can penetrate into the tumor more efficiently than intact mAbs and larger fragments. This is important for radioimmunotherapeutic applications because of the potential for increasing the homogeneity of radiation dose deposition within tumors. The advantages of engineered antibody fragments in terms of pharmacokinetics and biodistribution have been discussed in detail in several review articles (15, 20–22). Several engineered antibody fragments are in the later stages of clinical development. However, despite exhibiting suitable pharmacokinetics, the absolute tumor uptake of engineered antibodies is much lower and tumor residence time is much shorter than that of intact mAbs.

Recent advances in the field of radioimmunotherapy have attributed to the renewed enthusiasm for treatment of solid tumors. Delivery of radiopharmaceuticals in multiple steps (pretargeting), fractionated doses, or in combination with other therapeutic modalities, such as chemotherapy (combined modality radioimmunotherapy), has considerably improved the efficacy of radioimmunotherapy of solid tumors in clinics. Several review articles have adequately addressed the basic principles, experimental strategies, and clinical application of these approaches (3, 23–29). Despite these advances, the uptake, retention, and distribution of the radiolabeled antibodies in solid tumors are far from optimal due to significant biological impediments that are inherent in solid tumors. A better understanding and subsequent modulation of these impediments would further improve the efficacy of radioimmunotherapy.

It has recently been realized that the optimization of radioimmunotherapy of solid tumors cannot be achieved solely by engineering the design of mAbs, as large size of intact antibodies is not the only limiting factor. There are formidable barriers that radiolabeled antibodies encounter before reaching their target antigen on the tumor cells. After i.v. administration, the radiolabeled antibody has to cross the vascular endothelium and diffuse through the tumor stroma to reach the antigen-expressing tumor cells (Fig. 1). In addition, the blood supply and antigen expression are not uniform throughout the tumors. Recent studies have improved our understanding and appreciation of physical and physiologic barriers that play a pivotal role in the uptake and transport of radiolabeled antibodies in solid tumors. The next wave of optimization of radioimmunotherapy has focused on enhancing the tumor uptake, retention, and therapeutic efficacy of radiolabeled antibodies by using various biological modifiers to modulate the tumor microenvironment. There are several important determinants for the uptake of radiolabeled antibodies that can be manipulated. These include tumor blood flow, vascular permeability, structure and composition of tumor stroma, and tumor IFP (Fig. 1). This review discusses how these determinants can be modulated to enhance the uptake of radiolabeled antibodies for the optimization of radioimmunotherapy of solid tumors in preclinical studies.

Modulation of Tumor Vascular Flow

In contrast to normal tissues, the tumor blood vessels are abnormal, both structurally and functionally. They are tortuous,

Table 1. Characteristics of genetically engineered antibody constructs directed against tumor antigens

| Properties | Monovalent scFv | Divalent scFv | Tetravalent scFv | IgG | Diabody | Minibody |
|------------------------------|-----------------|---------------|------------------|--------|----------|----------|
| Molecular weight (kDa) | 30 | 60 | 120 | 150 | 55 | 80 |
| Serum half-life (min) | <10 | 80 | 170 | 330 | 173.4 | 288 |
| Peak tumor uptake (%ID/g) | 5 | 10 | 21 | >35 | 10.1 | 16.36 |
| Peak tumor uptake (time) | 30 min | 6 h | 6–8 h | 48 h | 4 h | 6 h |
| Tumor to blood ratio at 24 h | 13.2 | 22 | 34 | 2 | 9.5 | 44.5 |
| Antigen specificity | TAG-72 | TAG-72 | TAG-72 | TAG-72 | HER2/neu | CEA |
| Reference | (18) | (19) | (19) | (19) | (16) | (17) |

NOTE: In a scFv molecule, variable regions of heavy (V_L) and light (V_H) chain are joined by a flexible peptide linker. Two scFv molecules can be covalently linked via a peptide linker to form a divalent scFv, or they can associate noncovalently to form a diabody. Tetravalent scFv results from noncovalent association of two divalent scFvs, whereas minibody is a fusion of scFv with the CH3 for increased serum half-life. Abbreviation: %ID/g, percentage injected dose accumulated per gram of tissue.

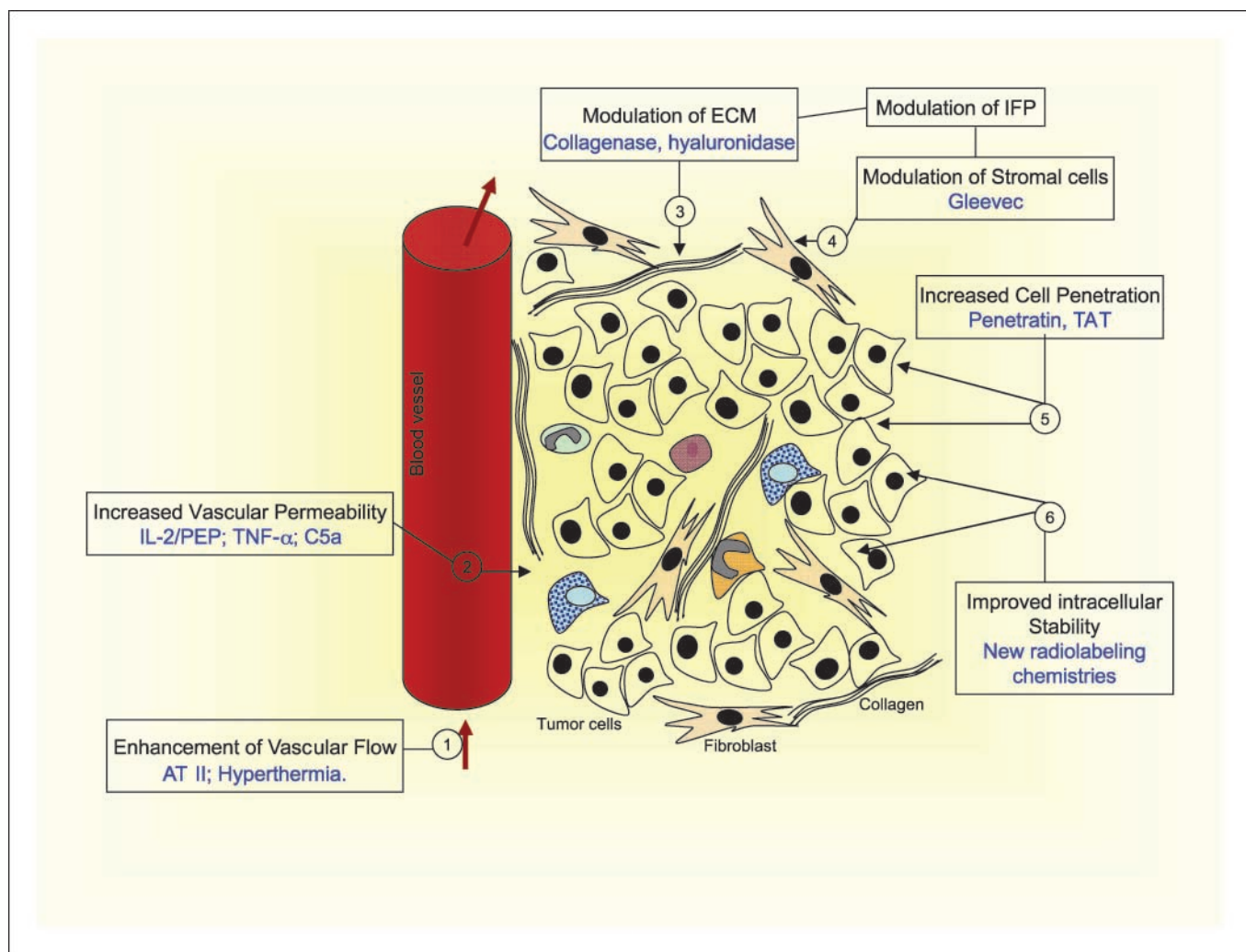


Fig. 1. Various impediments in the uptake and retention of radiolabeled antibodies in solid tumors and strategies to modulate these biological barriers. Following i.v. administration, radiolabeled antibodies reach tumor vasculature (1) and extravasate through the vascular endothelium (2) followed by diffusion through tumor interstitium (3 and 4). The radiolabeled antibodies recognize their target antigen on the surface of the tumor cells (5) and may remain surface bound or may be internalized (6). Adjacent boxes, various strategies that can improve the antibody uptake and/or retention at each of these steps; blue, examples of the modulators used.

poorly connected, and irregularly shaped with areas of dilation and constriction, thus resulting in turbulent and inefficient blood flow in the tumor (11, 30, 31). Additionally, the tumor blood vessels have discontinuous endothelial lining, abnormal pericytes and basement membrane, lack smooth muscles, and are hence hyperpermeable (31, 32). The leaky nature of the tumor vessels coupled with the absence of efficient lymphatic system results in elevated IFP in the tumors. The inefficiencies in vascular blood flow and elevated IFP result in poor uptake and heterogeneous distribution of macromolecules in the tumor tissue (12, 33, 34). Moreover, poor blood supply to the tumor results in regions of hypoxia, which contributes to radioresistance of tumor cells. Therefore, selective enhancement in tumor blood flow can not only lead to improved delivery of radiolabeled antibodies to tumors but also improve the sensitivity of tumor cells to radiation-mediated killing. Tumor blood flow can be improved by administration of vasoactive agents, such as angiotensin II (ATII), or by physical means, such as hyperthermia. Additionally, transient normalization of tumor vessels by antiangiogenic agents has been

recently shown to improve the delivery of antibodies in the tumors.

Angiotensin II. ATII is a promising vasoactive molecule, which has been shown to improve the uptake of radiolabeled antibodies in solid tumors. Systemic administration of ATII results in arteriolar constriction inducing widespread hypertension. On the other hand, the tumor blood vessels due to the lack of smooth muscles are unaffected. Consequently, there is an increased blood flow in the tumor accompanied by an enhanced fluid filtration across tumor vessels. Several studies have shown the utility of both continuous and periodic injection of ATII for improved uptake of antibodies, antibody fragments, antibody-drug conjugates, and immunotoxins with variable success. Continuous infusion of ATII using s.c. micro-osmotic pumps increased the uptake of ^{111}In -diethylenetriaminepentaacetic acid (DTPA)-labeled mAb A7 in LS180 colon carcinoma xenografts, although no change in the tumor to tissue ratios was observed (35). However, in a later study, tumor to normal tissue ratios improved when ATII was infused over a shorter time scale of 0.5 to 3 h (36). In combination

with enalapril, which is a kinase inhibitor, ATII administration resulted in further improvement of tumor uptake of ^{111}I -DTPA-A7, accompanied with a more uniform intratumoral distribution (37). Whereas ATII causes persistent distention of tumor vessels, thus improving the blood flow, kinase inhibition increases the concentration of bradykinin, which enhances the vascular permeability and hence antibody extravasation. The effect of this combination to improve the outcome of radioimmunotherapy was subsequently investigated in mice bearing colon cancer xenografts using ^{131}I -A7 (38). Following modulation of tumor blood flow by ATII and vascular permeability by enalapril, tumor quadrupling time was increased to 33.1 days from 24.3 days. The tumor-absorbed dose increased by 1.55-fold without affecting the doses to normal tissues and whole body clearance of radiolabeled antibody. Netti et al. (39) also studied the effect of periodic and continuous ATII administration on tumor uptake of anti-TAG-72 mAb CC49 in LS174T colon carcinoma xenografts. Both periodic and chronic infusion of ATII resulted in 40% enhancement of specific antibody (CC49) at 4 h after injection, whereas the tumor uptake of a nonspecific antibody was unaffected. It was proposed that periodic administration of angiotensin enhanced fluid filtration across tumor vessels, resulting in the extravasation of macromolecules across the vascular wall. The nonspecific antibodies, due to the lack of specific binding in the extravascular space, can cross the vessel wall in both directions and, thus, do not accumulate in the tumor interstitium. In contrast, transport of high-affinity antibodies across the vascular wall is unidirectional because the molecules that extravasate would remain trapped on tumor cell surface and extracellular matrix (ECM) due to specific binding to the cognate antigen (39).

The positive effect of ATII administration on tumor uptake of radioiodinated Fab fragment has also been shown in KT005 osteosarcoma (40). Three hours after injection, ~2-fold improvement was achieved in the tumor uptake of ^{125}I -labeled Fab fragments derived from mAb OST7, which recognizes alkaline phosphatase-related antigen in osteogenic sarcoma. This effect was only observed when the Fab fragments were radioiodinated using a metabolizable linker HML. ATII administration did not alter tumor uptake of the antibody fragments that were radioiodinated using chloramine-T. Additionally, ^{125}I -HML-labeled Fab exhibited lower kidney uptake in comparison with directly radioiodinated Fab. The genetically engineered fusion protein of divalent scFv of mAb CC49 with an intrinsic ATII sequence [sc(Fv)₂-ATII], without compromising the specific antigen-binding affinity of scFv or the biological activity of ATII, exhibited a more homogenous intratumoral distribution compared with the unmodified sc(Fv)₂ (41). However, the addition of ATII did not improve the absolute tumor uptake of the scFv.

Vascular normalization. Structural and functional abnormalities in the tumor vasculature are the primary cause of elevated IFP and inefficient and heterogeneous distribution of macromolecules in the tumor. There is growing evidence that antiangiogenic agents, such as antibodies directed against vascular endothelial growth factor (VEGF) or its receptor VEGF receptor 2, result in transient normalization of tumor vasculature, creating a so-called "normalization window" (31). The normalized vessels are less tortuous, have decreased vessel diameter, and reduced leakiness, with increased pericyte cover-

age and thinner basement membrane (31, 42). During the normalization window, there is a drop in the tumor IFP and improved delivery and penetration of chemotherapeutic agents (43). Recently, Nakahara et al. (44) studied the effect of VEGF inhibitor AG-01376 on the distribution of extravasated antibodies by confocal microscopy. There was an improved transport of antibodies from the surviving "normalized" blood vessels, and anti-E-cadherin antibodies were able to label the surface of the tumor cells. Antibodies were found to accumulate in the sleeves of basement membrane left behind by the regressing vessels, which were proposed to serve as the preferential routes of distribution in tumors (44). Studies have also shown that vascular normalization reduces tumor hypoxia, thereby sensitizing the tumor cells to radiation-mediated killing. Pretreatment with anti-VEGF receptor 2 antibody DC101 improved the efficiency of radiation therapy in the glioblastoma xenograft model (42). Considering the improved transport of antibodies and radiosensitization of tumors following vascular normalization, it will be interesting to evaluate the effect on antiangiogenic agents on the delivery and efficacy of radiolabeled antibodies in solid tumors.

Crossing Endothelium by Altering Vascular Permeability

To reach the target antigen expressed on tumor cells, radiolabeled mAbs and antibody fragments have to traverse through vascular endothelium, which is lined by endothelial cells. However, extravasation of macromolecules and cells across vascular endothelium is highly restricted (due to tight junctional contacts) and tightly regulated. Modulating the vascular permeability by using vasoactive agents is a promising approach to overcome the obstructive effects of vascular endothelium (Fig. 1). Several such agents have been used to improve tumor uptake of radiolabeled antibodies in both clinical and experimental studies. These include cytokines, such as interleukin (IL)-2 and tumor necrosis factor- α (TNF- α), VEGF, and activated complement 5a (C5a).

Interleukin-2. IL-2 is a cytokine that mediates both immune and nonimmune responses. Due to its role in the activation and proliferation of lymphocytes and other immune cells, and the ability to recruit and activate lymphokine-activated killer cells, IL-2 has been used for the treatment of melanoma and renal cell carcinoma. However, systemic administration of IL-2 in high doses causes toxic side effect termed capillary leak syndrome, which leads to fluid accumulation in extravascular spaces culminating in multiorgan dysfunction. IL-2 induces enhanced vascular permeability due by the release of nitric oxide in the local environment, which in turn acts on endothelial cells, causing them to round up and develop microfenestrations that allow the fluid to leak into the surrounding tissue (45). This otherwise undesirable property of IL-2 has been harnessed to improve the uptake of radiolabeled antibodies. Pretreatment with free IL-2 resulted in improved tumor uptake of $^{99\text{m}}\text{Tc}$ -labeled anti-CEA mAb; although due to generalized vasopermeability, enhanced antibody uptake was observed in other tissues as well (46). This was due to the fact that, whereas the tracer antibody was targeted to the tumor by the virtue of antigen specificity, the delivery of IL-2 was not targeted. Subsequent studies were done by coupling IL-2 to tumor-specific antibodies for localized

enhancement of vascular permeability in the tumor. IL-2-conjugated anti-CEA mAb exhibited a ~4-fold increased uptake in tumor compared with unconjugated antibody administered alone or following pretreatment of free IL-2 without affecting the uptake in the nontarget tissues (47). Through a series of studies, Epstein et al. have established that the localization of IL-2 in the tumor need not be specific for inducing vascular permeability in the tumor (45, 48–50). IL-2 conjugated chemically or genetically to antibodies directed to tumor vasculature (anti-fibronectin mAb TV-1), tumor cell surface (anti-B-cell lymphoma mAb Lym-1 and anti-TAG-72 mAb B72.1), or necrotic regions [anti-DNA mAb tumor necrosis therapy (TNT)] induced approximately similar level of vasopermeability in several tumor types, including prostate, colon, lung, and lymphomas. In these studies, the tumor uptake of radiolabeled antibodies improved by 2- to 4-fold over controls following pretreatment with IL-2 conjugates (45, 48–50). The vasopermeability induced by IL-2-antibody conjugates was time and dose dependent and was abrogated by nitric oxide synthase inhibitor 1-NMA, thereby indicating the involvement of nitric oxide (45).

Recently, Epstein et al. (51) successfully identified the region of IL-2 that is responsible for inducing vasopermeability. A linear stretch of 37 amino acids (residues 22–58), subsequently termed permeability-enhancing peptide (PEP), was found to retain the entire vasopermeability activity of IL-2 but was devoid of its cytokine- and receptor-binding functions (51). The synthetic peptide exhibited maximal activity in dimeric form. Pretreatment with PEP-conjugated targeting mAbs Lym-1 and TNT, which recognize cell surface and intracellular antigens, respectively, resulted in 4-fold enhancement in the tumor uptake of ¹²⁵I-labeled tracer TNT mAb, which is similar to that obtained with intact IL-2 containing conjugates. In a step forward, recently, a fusion protein containing a PEP dimer with NHS76, a third-generation TNT mAb that recognizes nuclear debris present in necrotic regions of the tumors, was generated (52). Following 2 h of pretreatment with NHS76/PEP₂, the uptake of ¹²⁵I-labeled tracer mAb B72.3 increased by 4.4-fold in LS174T tumors. Additionally, the fusion protein was shown to improve the therapeutic efficacy of several antitumor drugs, including doxorubicin, Taxol, vinblastine, and taxotere, in human colon and murine lung carcinoma models (52).

Tumor necrosis factor- α . TNF- α is another cytokine that is known to enhance the tumor uptake of drugs and macromolecules by enhancing the vascular permeability by a yet unknown mechanism. TNF- α , when coadministered with ¹²⁵I-labeled antibody-carboxypeptidase G2 conjugate, resulted in the improved tumor uptake of antibody-enzyme conjugate in murine thymoma (53). Similarly, intratumoral injection of recombinant human TNF- α resulted in increased permeability of tumor blood vessels by 8- to 10-fold, thereby causing a 3-fold increase in the tumor uptake of i.v. administered ¹²⁵I-labeled anti-CEA mAb in colon carcinoma xenografts (54). However, in both instances, with improved tumor uptake of radiolabeled antibodies following TNF- α treatment, there was a concomitant increase in the accumulation of antibodies in normal tissues (53, 54). In another study, the effect of TNF- α administration on the tumor uptake of the specific and nonspecific IgG was investigated (55). A 2.6-fold increase in the tumor uptake of specific antibody was observed as early as 1 h, and at 3 days, the increase in the tumor accumulation was ~30% of the control

treatment. On the other hand, the nonspecific antibody exhibited transient increase in the tumor uptake in response to TNF- α ; however, this increase was not sustained for long. Again, treatment with TNF- α also resulted in increased uptake of antibodies in the nontarget tissues. Khawli et al. (56) conjugated TNF- α and several other vasoactive agents chemically to the TNT-1 antibody and studied its effect on the uptake of ¹²⁵I-labeled TNT-1 F(ab')₂ fragment in ME-180 human cervical carcinoma xenografts. TNF- α -antibody conjugate caused a ~3-fold increase in the tumor uptake of the antibody fragment and was most effective in improving the uptake compared with other vasoactive agents used in the study, which included IL-1, leukotriene B₄, histamine, bradykinin, and physalamin. Only IL-2-TNT-1 conjugate induced a greater increase in the tumor uptake of radiolabeled antibody fragment (56).

Complement 5a. Kurizaki et al. (57) recently used C5aAP, a conformationally constrained agonist of anaphylatoxin C5a, to improve the outcome of radioimmunotherapy of colon carcinoma xenografts. C5a is generated as a cleavage product of complement C5 protein during the activation of serum complement pathway. It is capable of enhancing the vascular permeability by recruiting and activating neutrophils, which in turn act on adheren junctions of vascular endothelial cells (58, 59). In LS174T colon carcinoma xenograft mouse model, C5aAP increased the tumor uptake of ¹²⁵I-labeled mAb B72.3 in a dose-dependent manner and was most effective when administered in fractionated doses 3 to 6 h before the radiolabeled mAb (57). Although the tumor uptake of radioiodinated mAb increased by 50% by 72 h, the tumor to normal tissue ratios were unchanged even at the highest dose of C5aAP administered because the increase in vascular permeability was not restricted to the tumor only. Nevertheless, the usefulness of C5aAP was evident in the therapy studies involving ¹³¹I-labeled B72.3, where C5aAP resulted in an increase in the tumor quadrupling time from 14.2 to 26 days.

Vascular endothelial growth factor. VEGF is another important molecule that can modulate the vascular permeability to enhance macromolecular uptake. Both VEGF and anti-VEGF antibodies are being regarded as futuristic modalities for treating cancer. The basis for this attraction is that VEGF receptors are differentially expressed in most of the cancers and the interaction between VEGF and its receptor is the driving force for the development of tumor vasculature. VEGF itself is a potential targeting agent and has been used for delivering toxins and thus inhibiting tumor growth in preclinical studies (60–63). To exploit the reputation of VEGF as vascular permeability factor, Halin et al. (64) generated fusion proteins containing VEGF and a scFv L19 that exhibits a high affinity for ED-B domain of fibronectin (a tumor vasculature-specific marker). However, the scFv-VEGF fusion proteins exhibited tumor accumulation similar to unconjugated scFv. Other studies involving VEGF inhibitors and anti-VEGF antibodies have shown usefulness in augmenting tumor uptake of drugs and antibodies. However, the underlying mechanism involves normalization of tumor vasculature and is discussed in detail in the previous section.

Overcoming Stromal Barriers

Following extravasation from the blood vessels, antibodies, like other therapeutic agents, have to diffuse through tumor

stromal compartment to reach the target tumor cells (Fig. 1). Transport of macromolecules in the interstitial compartment is facilitated by interstitial convection, which is governed by IFP gradients and hydraulic conductivity (12, 34). In addition to the leaky vasculature and defective lymphatics, tumor stroma is also a major factor contributing to elevated IFP in tumors. Hydraulic conductivity, on the other hand, is more dependent on the interstitial space, composition, and structure of stromal components (65). Tumor stroma comprises stromal cells, which include fibroblasts, dendritic cells, macrophages, lymphocytes, and endothelial cells, and ECM, which consists of a protein network (mainly collagen) embedded in a hydrophilic gel containing glycosaminoglycans and proteoglycans. In addition to IFP, hydraulic conductivity in the interstitium inversely correlates with the collagen and glycosaminoglycan content in the ECM (33, 65–67). Hyaluronan content directly correlates with the diffusion coefficient, whereas collagen content has an inverse correlation (68–70). In addition, collagen and glycosaminoglycan content also affect the physical characteristics, such as rigidity and IFP. Transport across the tumor interstitium can thus be improved by modulating the stromal compartment of the tumor.

Platelet-derived growth factor inhibitors. Generally, strategies that improve vascular blood flow and vascular permeability tend to lower the IFP and increase microvascular pressure, thereby increasing the transvascular pressure gradient. Tumor IFP can also be modulated by targeting the tumor stroma as it also contributes to high IFP in the tumors. Platelet-derived growth factor (PDGF) is one of the key regulators of IFP in normal connective tissues and acts by triggering signaling pathways via PDGF receptors (PDGFR), which are expressed on the stromal cells. Inhibition of signaling in tumor stroma has been shown to decrease the tumor IFP and improve the tumor uptake of chemotherapeutic agents (71). The utility of the PDGFR- β inhibitor STI571 (also known as Gleevec, imatinib mesylate) in improving uptake of radiolabeled mAb and efficacy of radioimmunotherapy was recently shown (72). STI571 treatment increased the tumor uptake of ^{125}I -labeled B72.3 ~2.4 times in the LS174T colon carcinoma xenograft model. The improved tumor uptake of radiolabeled antibody in response to STI571 translated into increased therapeutic efficacy of ^{131}I -B72.3, as the tumor doubling time increased from 19 to ~40 days.

Collagenase. Collagen is an important component of ECM, and several studies have suggested that modulating collagen can improve the access and uptake of antibodies in the tumor. Netti et al. (70) showed that mechanical stiffness of the tumor tissue is dependent on the collagen content and correlates with the poor penetration of the macromolecules. Further, collagenase treatment before the administration of nonbinding IgG increased its diffusion rate in the tumor interstitium by ~2-fold (70). In a similar study using osteosarcoma xenografts, it was shown that collagenase treatment decreased both IFP and microvascular pressure with differing kinetics such that it increased transcapillary gradient transiently. This was associated with ~2-fold increase in the uptake of TP-3, a mAb specific for osteosarcoma (73). In comparison with a nonspecific mAb, TP-3 also exhibited increased penetration and diffused farther away from blood vessels in response to collagenase treatment (73). A recent report studied the effect of collagenase treatment on the penetration of antibodies administered via i.p. route in

metastatic ovarian carcinoma model (74). Collagenase treatment lowered tumor IFP and resulted in enhanced penetration of antibodies in the ovarian tumors implanted in the abdominal wall. The concentration of both specific and nonspecific antibodies at the tumor surface was ~2-fold higher, whereas the mean distance of penetration increased ~4-fold following collagenase treatment (74). Given the role of collagenase in promoting metastasis, its use to improve the uptake of radiolabeled antibodies may not be feasible in a clinical setting. Relaxin, a peptide hormone primarily produced in the ovary and placenta during pregnancy, can alternatively be used to modulate collagen in tumor interstitium. Relaxin is a pleiotropic hormone that is involved in collagen remodeling by its action on fibroblasts, where it inhibits collagen synthesis and secretion and up-regulates matrix metalloproteinases (75). In fact, due to its antifibrotic properties, relaxin is used clinically in the treatment of scleroderma (76). Brown et al. (77) showed that relaxin administered via osmotic pumps increased the collagen turnover in the tumor and improved the diffusion coefficient of IgG in HSTS26T xenografts.

Hyaluronidase. Hyaluronan is another important component of the ECM, which can be modulated to improve antibody uptake. Intratumoral injection of hyaluronidase transiently decreased the IFP by 60% to 80% in both s.c. and orthotopic osteosarcoma xenografts (78). Periodic injection of hyaluronidase resulted in a 70% increase in the tumor uptake of ^{125}I -labeled TP-3 (antibody specific for osteosarcoma-associated antigen; ref. 79). However, the uptake of nonspecific mAb (UPC-10) in tumor and liver was decreased by 20% by hyaluronidase, thus indicating that the treatment resulted in increased specificity of the mAb for tumor (79). In contrast to these findings, hyaluronidase treatment had no effect on the transport and penetration of i.p. administered IgG in ovarian cancer xenografts grown on abdominal walls (74).

Improved Cell Surface Binding and Cell Penetration

The ultimate target for any anticancer therapy is the tumor cell. In radioimmunotherapy, the radiolabeled antibody has to bind to the antigen expressed on the tumor cell or in the ECM in the close proximity to the tumor cells. This is more relevant when the therapeutic radionuclide emits α -particles, which have very short path lengths, and the decay has to be in close proximity to the cell nucleus for maximal tumor cell death. Internalization of radiolabeled antibody can have a positive effect on the efficacy of radioimmunotherapy. If the uptake of the antibody within the tumor is a function of equilibrium between the concentration of molecules in circulating blood and the stroma/ECM, then, by internalization of the radioimmunoconjugates reaching the ECM, the equilibrium will be shifted toward the ECM, thus resulting in increased uptake of the radiolabeled antibodies. The molecules that are localized in the ECM and on the cell surface will be cleared from the blood later as their concentration falls in the blood. In such a situation, all the molecules that have been internalized will be resistant to being cleared by washout and, therefore, will have prolonged residence time in the tumor.

A novel class of biological modifiers called cell-penetrating peptides (CPP) has recently attracted attention as a means to improve uptake and penetration of therapeutic molecules. Most

of the CPPs are derived from various naturally occurring proteins and are capable of entering cells in a receptor- and energy-independent manner. Few studies have investigated the possibility of using CPPs for improved tumor uptake, penetration, and retention of antibody fragments. Conjugation of the TAT peptide to the Fab fragments of mAbs NRLU-10, a pancreatic carcinoma mAb, and NRML-05, an anti-melanoma mAb, improved their cell surface retention and internalization *in vitro* (80). However, there was some loss of specificity as a result of TAT conjugation. Niesner et al. (81) studied the tumor uptake and biodistribution of TAT-conjugated scFv L19, which exhibits specificity for ED-B domain of fibronectin. *In vitro*, conjugation with TAT peptide facilitated internalization of fluorescently labeled scFv in tumor cells. However, when analyzed *in vivo*, TAT conjugation abolished the tumor-targeting properties of scFv L19 (81). The uptake of ^{125}I -labeled L19 increased in liver and spleen, whereas the tumor uptake decreased drastically as a result of TAT conjugation. Recently, we studied the effect of CPP coadministration on the biodistribution of divalent scFv fragments of anti-TAG-72 mAb CC49 (82). Coinjection with penetratin and TAT resulted in improved tumor retention of ^{125}I -labeled sc(Fv) $_2$ without altering the biodistribution in nontarget tissues and thus improved tumor to normal tissue ratios. At 24 h, penetratin administration resulted in 80% retention of the peak dose in comparison with 48% retention with TAT, whereas only 29% of the peak accumulated dose was retained when the scFv was administered alone (82). Additionally, CPP administration resulted in improved penetration and homogenous distribution of radiolabeled scFv in the tumor. It has thus been established that the specific antigen-binding (and therefore tumor targeting) activity of antibody fragments can be combined with the nonspecific cell-penetrating activity of CPPs (82). However, caution should be exercised when using CPPs *in vivo* because the advantage of CPPs in biodistribution ceases at higher CPP to antibody ratios and a generalized increase in antibody uptake is observed even in the nontarget tissues (82).

Despite its apparent advantages, internalization of radiolabeled antibodies can result in the rapid loss of radioactivity following degradation of the antibodies in the tumor cells. Therefore, before internalization can be exploited as a means of improved uptake and retention of, conjugation chemistries to enhance the intracellular stability of the radiopharmaceuticals need to be developed. Zalutsky et al. (83) radioiodinated anti-HER2/neu mAbs using *N*-succinimidyl-5-iodo-3-pyridinecarboxylate (SPIC) or tyramine cellobiose (TCB). The intracellular radioiodine activity was 2.3 to 3.0 times higher in ovarian cancer cells *in vitro* with SPIC and TCB labeling than with iodogen-labeled antibodies. However, this did not translate into improved tumor accumulation in animal experiments (83). In a later study, an internalizing epidermal growth factor receptor variant III (EGFRvIII) mAb L8A4 was radioiodinated via positively charged *D*-amino acid peptide (*D*-KRYRR; ref. 84). In comparison with directly radioiodinated L8A4, radioiodinated *D*-KRYRR-L8A4 has four to five times higher accumulation in the EGFRvIII-overexpressing U8 Δ EGFR cells *in vitro*. *In vivo*, the radiolabeled *D*-KRYRR-L8A4 exhibited 5-fold higher uptake in tumor than that obtained with directly radiolabeled mAb (84). However, there was higher uptake of *D*-KRYRR-L8A4 in kidneys than that observed in directly radioiodinated antibody. To further improve the intracellular retention, the

D-amino acid prosthetic group was recently modified to have negative charge (85). A new radiolabeled prosthetic group, N^ϵ -(3[*I]iodobenzoyl)-Lys 5 - N^α -maleimido-Gly 1 -GEEEEK ([*I]IB-Mal-*D*-GEEEEK), was synthesized and conjugated to internalizing L8A4. The antibody radioiodinated with [*I]IB-Mal-*D*-GEEEEK exhibited ~15-fold increase in the internalized radioactivity in tumor cells *in vitro* than directly labeled antibody (85). Improvement in the tumor uptake *in vivo* with the [*I]IB-Mal-*D*-GEEEEK-L8L4 was similar to that observed with *D*-KRYRR-L8A4, and like *D*-KRYRR-L8A4, [*I]IB-Mal-*D*-GEEEEK-L8L4 too exhibited higher renal uptake. But the suitability of IB-Mal-*D*-GEEEEK to radiolabeled α -emitter halogen astatine-211 can be useful in the development of intracellularly stable, α -emitter-conjugated internalizing antibodies.

Hyperthermia

Several studies have shown the usefulness of hyperthermia for improving the efficacy of radioimmunotherapy. Hyperthermia not only results in enhanced tumor uptake and homogenous distribution of radiolabeled antibodies but also acts as a radiosensitizer and exhibits antitumor effects by killing hypoxic and S-phase cells (86). Enhanced tumor uptake of radiolabeled antibodies and improved efficacy of radioimmunotherapy using hyperthermia have been shown in several tumor types using various antigen-antibody combinations. Cope et al. (87) reported increased tumor uptake of ^{131}I -labeled F(ab') $_2$ fragment of Mel-14 (an antibody reactive with melanoma and gliomas) in glioma xenografts in response to tumor-localized hyperthermia. In colon carcinoma xenografts, hyperthermia improved the effectiveness of ^{131}I -labeled anti-CEA mAb without affecting the tumor uptake of the antibodies (88). Hyperthermia was also found to improve the absolute amount and rate of tumor uptake of chimeric antitenascin antibody in glioma xenografts (89). Localized hyperthermia resulted in increased antigen expression leading to improved tumor uptake of ^{111}In -NRLU-10 in colon adenocarcinoma xenografts (90). In small cell lung cancer-bearing animals, hyperthermia improved the uptake of ^{111}In -labeled NE150 (a neural cell adhesion molecule-binding mAb; ref. 91). This was associated with enhanced vascular permeability and faster blood clearance of radiolabeled antibody (91). In melanoma xenografts, hyperthermia at 43°C resulted in decreased tumor IFP associated with the damage to the tumor (92). In contrast, it was shown that the hyperthermia treatment, which enhances the uptake of radiolabeled antibody (41.8°C, 4 h), does not alter the tumor IFP in the glioma xenograft model (93). It was further shown that hyperthermia decreased the tumor catabolism and increased the urinary excretion of the radiolabeled antibody (94). Although the combination of hyperthermia with ^{131}I -anti-CEA has been evaluated in a phase I/II clinical trial, the temperature and duration of hyperthermia is still being optimized in preclinical studies (95–97).

Conclusion and Perspectives

Radioimmunotherapy has come a long way since the discovery of mAbs, and the advances in the molecular biology technologies have resulted in the generation of engineered antibodies, including humanized antibodies, chimeric antibodies,

and scFvs. Few of them have been approved and are being successfully used in clinics to treat various malignancies. Various optimization strategies described in this article have been tried with antibodies directed against different targets and in different tumor models with varying degree of success. Most of these studies have been limited to animal models and need further optimization before these approaches can be tested in clinics. ATII has a very short serum half-life and its continuous i.v. infusion (as described for animal studies) is difficult in the clinics. IL-2 suffers from lack of target specificity but might be useful as a fusion protein. The identification of PEP from IL-2 is an encouraging development from clinical perspective, and the PEP-antibody fusion proteins should be evaluated more

extensively. Collagenase and hyaluronidase lack specificity and cannot distinguish their targets in normal and cancer tissues and would lead to harmful side effects if administered in patients. Similarly, a right balance between the cell-penetrating activity and specific antigen binding has to be found for clinical exploitation of CPPs in radioimmunotherapy. Likewise, temperature and duration of hyperthermia need to be optimized in clinics. Nevertheless, these studies have helped in appreciating the contribution of various biological impediments that a radiolabeled antibody encounters on its way to the target cells and also raised hopes of finding new ways to overcome these barriers and improve the uptake and retention of radiolabeled antibodies in solid tumors.

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