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Expert Review

Ligand-Targeted Delivery of Therapeutic siRNA

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Abstract. RNA interference (RNAi) is a post-transcriptional gene-silencing phenomenon that is triggered by double-stranded RNA (dsRNA). Since many diseases are associated with the inappropriate production of specific proteins, attempts are being made to exploit RNAi in a clinical settings. However, before RNAi can be exploited as therapeutically, several obstacles must be overcome. For example, small interfering RNA (siRNA) is unstable in the blood stream so any effects of injected siRNA are only transient. Accordingly, methods must be developed to prolong its activity. Furthermore, the efficient and safe delivery of siRNA into target tissues and cells is critical for successful therapy. Any useful delivery method should be designed to target siRNA to specific cells and to promote gene-silencing activity once the siRNA is inside the cells. Recent chemical modifications of siRNA have overcome problems associated with the instability of siRNA, and various ligands, including glycosylated molecules, peptides, proteins, antibodies and engineered antibody fragments, appear to be very useful or have considerable potential for the targeted delivery of siRNA. The use of such ligands improves the efficiency, specificity and, as a consequence, the safety of the corresponding delivery systems.

KEY WORDS: antibody; antibody engineering; ligand; RNA interference; targeted delivery.

INTRODUCTION

RNA interference (RNAi) is a natural phenomenon whereby a specific mRNA is targeted for degradation by complementary small interfering RNA (siRNA). Since many diseases are known to result from the inappropriate expression of specific genes, exploitation of RNAi appears likely to be a powerful method for the suppression of the expression of specific genes in mammalian cells for both functional analysis and potential therapeutic interactions (1). If RNAi is to be exploited for the treatment of human diseases, several issues must be addressed. These issues include the instability of siRNA and the requirement for efficient and safe delivery systems (2). Early studies in mice have involved high-pressure, large-volume injections into the tail vein (3–5). Lewis *et al.* reported successful gene silencing in the liver, kidney, spleen, lung and pancreas using such a system (4). However, it is unlikely that this method can be exploited to the treatment of humans. In this review, we shall focus on recent improvements in the chemical modifications of siRNA that increase its stability and recent developments in the targeted delivery of siRNA, or of plasmid DNA that encodes siRNA, that involve the use of ligands and, in particular, of antibodies and antibody fragments.

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STABILITY AND BIODISTRIBUTIONS OF siRNA

Corey and colleagues examined the stability and biodistribution of siRNA *in vitro* and *in vivo* (6–8). Because they are duplexes, siRNAs are much more stable than single-stranded RNAs although, in 50% murine serum, degradation of duplexes was observed after 24 h. Corey *et al.* also checked the biodistribution of siRNA after intravenous injection of ^{125}I-labeled siRNA into mice. The radiolabeled siRNA was detected in the kidney and liver at significant concentrations and lower concentrations in the lung, spleen, and heart. However, very little siRNA was detected in the brain. Because the molecular mass of siRNA is low, it is eliminated rapidly by renal clearance. Accordingly, levels of siRNA in all tissues decreases after 24 h. These observations indicate that, without increases in the bioavailability of siRNA, its silencing effects in living cells will remain limited.

CHEMICAL MODIFICATIONS OF siRNA

Various chemical modifications of siRNA have been reported (Fig. 1a). All of which were designed to increase the stability of siRNA. However, such chemical modifications must be designed such that they do not inhibit the activity. In other words, modifications should not interfere with the recognition of the siRNA by the RNA-induced silencing complex (RISC), which mediates the destruction of the target RNA in RNAi, and should also not interfere with subsequent reactions, including the degradation of the target mRNA. In general, the activity of modified siRNA depends on the position in the sequence that is modified and the structure of the modified nucleotide. One of the simplest and most promising modifications is the introduction of phosphoro-

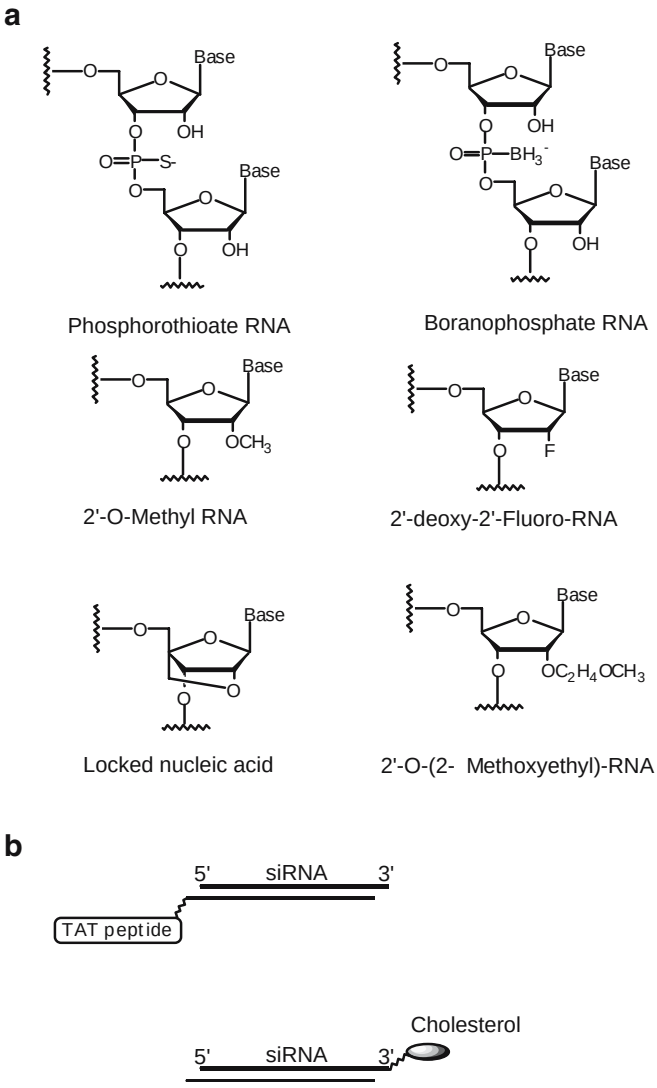

Fig. 1. (a) Six types of chemical modification of siRNA. (b) Two terminal modifications of siRNA.

thioate linkages (8–10). Phosphorothioate linkages enhance the resistance of siRNA to nucleases. The siRNAs that have phosphorothioated linkages at the 5′-end and/or 3′-end on the sense and antisense strand have close to wild-type activity in RNAi (10). However, oligonucleotides with extensive phosphorothioate linkages are often toxic *in vivo*. Another apparently useful modification is 2′-O-methylation (10–12). In view of the mechanism of hydrolysis of phosphodiester bonds in RNA, it is clear that protection at 2′ hydroxyl groups should be an effective method for stabilization of siRNA. Czauderna *et al.* reported that siRNA with 2′-methyl modifications at selected positions retained the activity to direct RNAi against specific targets (11). Other types of modification of 2′-hydroxyl groups including the preparation of 2′-fluoro, 2′-O-(2-methoxyethyl) and locked nucleic acid (LNA) nucleotides, have been also reported (8–13). The results of the cited studies indicate that RNAi does not require a 2′-hydroxyl group at all positions in the siRNA that induces gene silencing. Prakash *et al.* reported that modification of the sense strand has a smaller effect on activity than modification of the antisense strand (12). Hall *et al.* reported that some boranophosphate siRNAs, in which the non-bridging phosphodiester oxygen is replaced by an isoelectronic borane ($-BH_3$) (14) moiety, retain activity and, at low concentrations, they are more effective than the corresponding unmodified siRNA.

Terminal nucleotides on sense and/or antisense strand have also been modified (Fig. 1b). Such modifications significantly enhance resistance to degradation by exonucleases in serum and tissue homogenates. Again, the modifications must not to disturb the RNAi activity of the siRNA. When all four termini of an siRNA were blocked with amino-end modifications activity was dramatically reduced. However, siRNA with a normal 5′-end of the antisense strand but with all other ends modified had the same activity as unmodified siRNA (11). Terminal modifications of siRNA cannot only increase stability but can also incorporate additional functions. Chiu *et al.* incorporated protein transduction domains (PTDs) into siRNA for efficient delivery to cells (15). A Tat peptide, containing the cationic peptide, YGRKKRRQRRR, is rapidly internalized by cells and becomes concentrated in nuclei. Tat peptide conjugates have been used for the delivery of both DNA (16,17) and proteins (18,19). Chiu *et al.* linked Tat peptide to the 3′-terminus of the antisense strand of an siRNA [Fig. 1(B)] and they found that siRNA-Tat peptide conjugates were effectively delivered to cells and successfully silenced the expression of the target gene (15).

Soutschek *et al.* prepared chemically modified siRNA with partial phosphorothioate linkages, 2′-O-methyl sugars and a cholesterol moiety at the 3′end of the sense strand (20). Cholesterol improves pharmacokinetic properties *in vivo* by enhancing the binding of cholesterol- siRNA (chol-siRNA) to human serum albumin (HSA). Even 24 h after injection of their conjugate into mice, they detected significant levels of chol-siRNA in the liver, heart, kidney, adipose tissue and lung. Intravenous injection of the chol-siRNA resulted in the silencing of a gene for apolipoprotein B (apoB), an essential protein in cholesterol metabolism, and a consequent reduction in total levels of cholesterol. Although a relatively high dose of chol-siRNA (50 mg kg^{-1}) was required to achieve the desired effect, this report provided the first demonstration *in vivo* of a method that exploits RNAi and has potential therapeutic application.

DNA that encodes short-hairpin RNA (shRNA) also can be modified. However, in contrast to the modification of an oligonucleotide such as an siRNA, post-synthetic modification of long double-stranded DNA (linear or plasmid DNA) is both difficult and complicated. Some methods, such as diazocoupling (21) and photocoupling (22), allow the modification of DNA through covalent bonding, but the limited or nonexistent specificity of such reactions, with varying numbers of a particular modification introduced at a variety of sites in the target DNA, presents a serious problem. The undesirable excess in the extent of the chemical modification introduced by these methods results in the inhibition of transcription (23). However, our group has recently developed a straightforward method for the site-specific modification of long double-stranded DNA using a novel nucleoside analog (24). This novel nucleoside analog was efficiently introduced at the 3′-termini of dsDNA by

enzyme. In plasma, the hydrolysis of DNA is the result of exonucleolytic activity and occurs exclusively in the 3′ to 5′ direction (25). Thus, protection of DNA from such enzymatic degradation via modification of its 3′ termini should be advantageous in a clinical setting. Another type of small vector that encodes shRNA has also been developed. It is a dumbbell-shaped DNA molecule (26,27), whose stability toward exonucleolytic degradation results from the absence of free termini.

DELIVERY OF siRNA AND OF DNA THAT ENCODES siRNA

Soutschek *et al.* showed that RNAi can be exploited to silence endogeneous genes (20). However, the relatively high dose of siRNA required for gene silencing hampers the practical application of their technique. To reduce the amount of injected siRNA that is necessary for RNAi, the delivery system must be improved, with enhanced targeted delivery, efficient release from the endosomal compartment, and efficient release of negatively charged siRNA or DNA from the cationic vehicle. Moreover, in the case of the DNA-mediated delivery, efficient concentration in the nucleus is also essential (Fig. 2).

Strategies that target tumor cells appear to hold promise for the treatment of tumors because they focus the potential therapeutic benefits and might reduce the adverse side-effects associated with cancer therapy. In many cases, cancer cells have unique antigen(s) and/or receptor(s) on the cell surface that are not found on the normal cells. Targeted therapeutics require the construction of molecules that specifically recognize these macromolecules and ferry therapeutic agents to the target cells.

TARGETED DELIVERY BY GLYCOSYLATED AND FOLATE-MODIFIED MOLECULES

Numerous cell-targeting ligands, including glycosylated molecules, peptides, proteins and antibodies are available. For example, the asialoglycoprotein receptors are expressed on the surface of hepatocytes. The receptor mediates endocytosis and subsequent internalization of proteins that are conjugated to galactose-terminated oligosaccharide moieties (28). Several groups have exploited this system for the delivery of genes to hepatocytes, using polymeric carriers with ligands, such as galactose and lactose (29–31). Oishi *et al.* have constructed a lactosylated poly(ethylene glycol)-siRNA (lactosylated PEG-siRNA) conjugate with an acid-labile linkage (32). In this complex, siRNA is covalently linked to lactosylated PEG and, under the slightly acidic conditions of the intracellular endosomal compartment, the covalent linkage is disrupted and free siRNA is produced.

Unlike many other types of cell, the dendritic cells that form part of the mammalian immune system express mannose receptors and mannose receptor-related receptors. In efforts to develop DNA vaccine therapies, the introduction of antigen-encoding DNA is of particular interest (33). Macrophages also express mannose receptors. Gene delivery that targets macrophages would be very useful for the treatment of diseases such as Gaucher's diseases and AIDS, and mannose receptor-targeted delivery systems have developed by several groups (34,35).

The folate receptor is overexpressed in many human tumors, while its distribution in normal tissues is minimal (36). Attempts to exploit receptor as a tumor-specific receptor have, thus, been made. Attachment of folic acid, the ligand for the folate receptor, to various macromolecules allowed their binding to folate receptors and subsequent

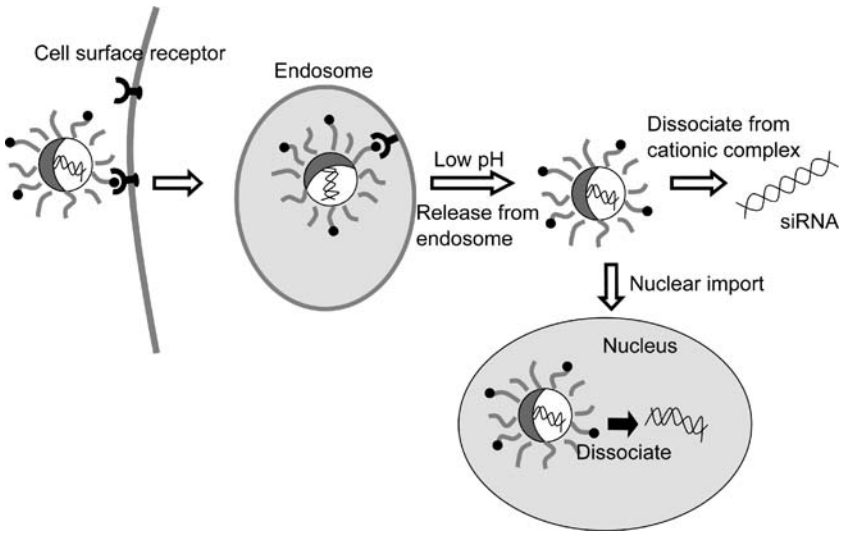

Fig. 2. The pathway for intracellular trafficking of non-viral gene vectors. After internalization by receptor-mediated endocytosis, the vector must be released into the cytosol to escape lysosomal degradation. Efficient dissociation of siRNA or DNA from the cationic compartment is also important. For the delivery of DNA, efficient localization within the nucleus is required.

internalization by endocytosis (37,38). Hwa Kim *et al.* reported the successful delivery of plasmid DNA that encoded siRNA by incorporating folate-modified poly(ethyleneimine) into the plasmid DNA (39).

TARGETED DELIVERY BY PEPTIDES AND PROTEINS OTHER THAN ANTIBODIES

Another group of receptors that has received significant attention are the integrins. Integrins are heterodimeric cell-adhesion receptors that are composed alpha and beta subunits. The ligands of integrins include collagen and fibronectin (40). The arginine-glycine-aspartic acid (RGD) motif of fibronectin has often been used in attempts at the targeted delivery of drugs and genes because of its ability to bind to integrins that are expressed on the activated endothelial cells found in tumor vasculature. Schiffelers *et al.* attached a circularized RGD motif to the end of PEG-conjugated forms of branched polyethyleneimine and used the resultant nanoparticle to deliver siRNA *in vivo* (41).

Transferrin is a glycoprotein that delivers iron to cells (42). Tumor cells often overexpress transferrin receptors on their surface, and this protein has also been studied as a ligand for tumor-targeted delivery. Lieskovan *et al.* have constructed a multicomponent delivery system that included short polycationic cyclodextrins for condensing and protecting siRNA, adamantine-PEG for stabilization of the particle, and transferrin. This nonviral delivery system dramatically inhibited tumor growth in a murine model (43).

The non-antibody ligands described above are inexpensive and they are easy to prepare and handle. However, these natural ligands also bind to some non-target cells, and compete for binding with native molecules in body fluids. Accordingly, it would be better to use ligands to target receptors for which natural ligands do not have affinity. Recent developments suggest that antibodies and engineered antibody fragments have considerable potential in this regard, as discussed below.

THE NATURE OF ANTIBODIES

Antibodies, essential components of the human immune response, consist of a pair of identical light chains and a pair of identical heavy chains (Fig. 3). The amino-terminal domains are variable (*V*) regions that recognize antigens, and the other domains constitute the constant (*C*) regions. Antibodies have two antigen-binding sites and, thus, high affinity for their corresponding antigens. Mammen *et al.* suggested that a polyvalent interaction should be proportional to the *N*th power of the monovalent interaction, where *N* is the number of receptor-ligand interactions (44), such that $K_N^{poly} = (K^{mono})^{\alpha N}$ where α is the cooperativity factor). Thus, in the case of an antibody, if the cooperativity factor is 1, the bivalent interactions between two variable regions and two antigens will be proportional to monovalent interaction squared. In many cases, the enthalpically diminished binding and the entropic cost of polyvalent interactions results in cooperativity factors of less than one. However, because of their two antigen-binding sites, antibodies have high affinity for their target antigens.

Once an antibody recognizes antigen, it inactivates it by triggering an intracellular pathway or via activation of host immune mechanisms, such as complement- and antibody-dependent cellular cytotoxicity (45) (Fig. 4). In the latter case, the Fc domain is recognized by the high-affinity Fc receptors on monocytes and/or low-affinity Fc receptors expressed on natural killer (NK) cells and neutrophils. Carbohydrates at conserved positions in the constant regions of the antibody play important roles in these functions (46,47).

THE USE OF ANTIBODY FRAGMENTS FOR DELIVERY

There are more than 150 therapeutic monoclonal antibodies in clinical study at the time of writing. However, the application of antibody-mediated therapeutics has many problems, in particular, the high cost of preparation of intact antibodies. All antibodies on the market at the present time are produced by mammalian cells in culture. Preparation is expensive in terms of equipment and media, and there is limited opportunity for scale-up. Accordingly, many efforts to reduce the time and cost of manufacturing antibodies are being made (48). In addition to whole antibodies, some researchers are using antibody fragments, rather than intact antibodies, because fragments can be prepared in bacteria and mammalian cells are unnecessary (49,50) (Fig. 3). Antibody fragments such as $F(ab)_2$ and Fab, which can also be prepared by proteolytic digestion of intact antibodies, and smaller variants, such as Fv and single-chain variable (scFv) fragments can be produced in this way. With recent advances in antibody engineering, such antibody fragments can be prepared easily and resulted in a clinical setting. These fragments do not contain Fc domains and have several advantages over intact antibodies in clinical applications. First, the possibility is reduced that the antibody fragments will interact with non-target cells, when the Fc domain is absent, because Fc domains can bind to Fc receptors in normal tissues, in particular macrophages, and such binding results in the concentration of antibodies in the liver and spleen. Second, because fragments are smaller than entire antibodies, the potential for tissue penetration is enhanced. Third, fragments are cleared rapidly from the blood, and rapid clearance results in low background levels in diagnostic scans (51). Finally, fragments can be manufactured economically without a requirement for mammalian cells.

DESIGN OF MULTIVALENT ANTIBODY FRAGMENTS

Variable domains of heavy and light chains can be joined together via a flexible polypeptide linker to yield so-called scFvs. Both scFvs and Fab fragments are monovalent and, consequently, they dissociate rapidly from their receptors (52). Therefore, dimeric and more complicated forms have been generated by chemical or genetic methods (52) (Fig. 3). Chemical cross-linking utilizes the thiol groups in cysteine that has been added carboxyl termini by genetic engineering. However, disulfide bonds are unstable in the reducing environment of living cells so another approach, utilizing bis-maleimide derivatives, has been developed (53). In this

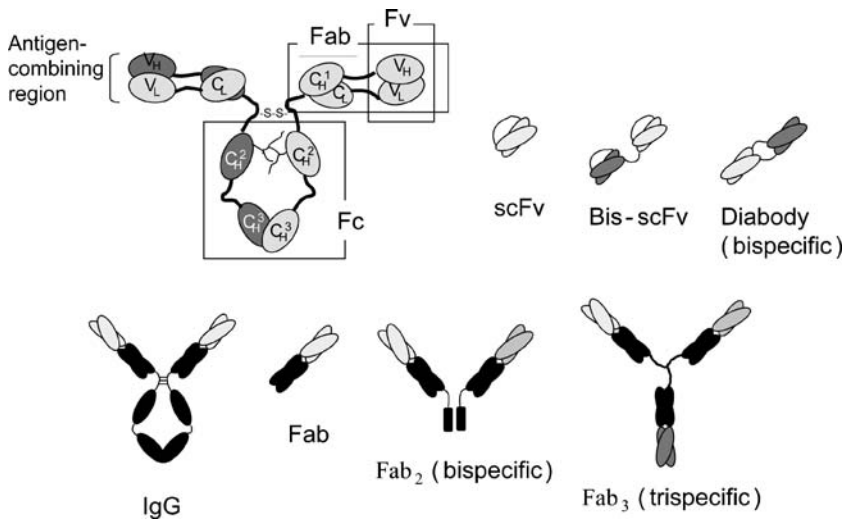

Fig. 3. Schematic representation of intact IgG and engineered antibody fragments. V_H Variable heavy chain, V_L variable light chain, C_H constant heavy chain, C_L, constant light chain. Fab and Fab_2 fragments can be produced by enzymatic cleavage of intact IgG. Engineered recombinant antibodies are shown as the scFv monomer, dimer (bis-scFv), and diabody, with linkers represented by a *black line* in each case. The Fab dimer (Fab_2) is shown as a conjugate with adhesive polypeptide or protein domains. A Fab trimer (Fab_3) is also shown as a chemical conjugate.

case, fragments are dimerized via a thioether bond and exhibit improved stability. Tri-maleimide derivatives allow construction of trimeric fragments (54).

In the most successful genetic approach to date, the scFv linker is limited to between zero and five amino acids (55). With reduced linker length, scFv dimers, known as diabodies are formed. Another approach to the construction of bivalent fragments involves dimerization of protein domains. Successful examples include helices that participate in self-assembly and use of the C_H3 domain of human IgG (56). Such bivalent fragments of intermediate molecular mass (50 to 80 kDa) exhibit increased stability in serum.

Bispecific antibodies (bisAbs) can bind two different epitopes, a property that can be exploited to increase both avidity and specificity (56,57) (Fig. 3). Successful application of bisAbs has been achieved in the recruitment of immune cells, such as cytotoxic T- and natural killer cells, for the treatment of cancer (58,59). Effective s dose are very low, and phase I and II clinical trials are underway. BisAbs can be prepared by the heterodimerization of two proteins. However, purification of the desired bisAbs, via removal of the unwanted pairs of heavy and light chains, has been a major problem in the large-scale production of bisAbs. Alternatively, bisAbs can be made by linking two scFvs in tandem (bisscFv) (57), via an additional linker oligopeptide. The sequence of the linker is critical for the successful construction of bisscFv.

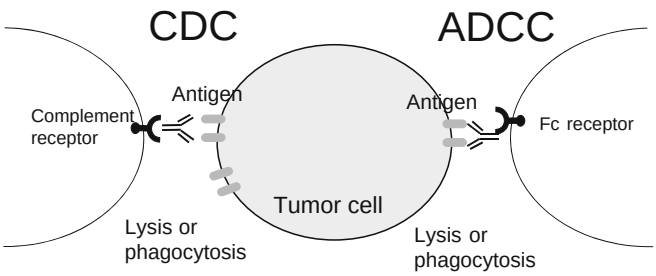

Fig. 4. Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Binding of antibodies to the antigens causes lysis of the target cell via direct contact with effector cells, which include macrophages and natural killer cells. In CDC, lysis is mediated by the complement cascade.

COVALENT ATTACHMENT OF PEG TO ANTIBODY FRAGMENTS

Small antibody fragments, such as scFv and Fab, reach their target tumors rapidly and penetrate the tumors efficiently. However, the half-lives of antibody fragments are relatively short compared to that of intact IgG. Molecules of less than 30 kDa are easily removed from the blood by renal filtration, which reduces accumulation at target sites. One strategy to overcome this problem involves the covalent attachment of polyethylene glycol (PEG) to the antibody fragments (60) (Fig. 5). PEG is a non-immunogenic polymer that can shield proteins from recognition by the immune system and/or serum proteases with resultant increases in the half-lives of the proteins in the blood stream (61). Six so-called PEGylated proteins have been approved by the Food and Drug Administration (FDA) in the United States and several more are being tested in a clinical setting. In early studies, PEG was linked to various sites on antibodies via lysine residues (62). Although the half-lives of the PEGylated molecules were longer than those of the parent molecules, the nonspecific modifications resulted in loss of antigen-binding activity. Recently, Chapman *et al.* demonstrated that site-specific modification of antibody fragments at the termini of PEG diminishes the loss of activity of antibody fragments (60,62). They attached PEG via the cysteine residue that is located in the hinge region of the original intact IgG. They

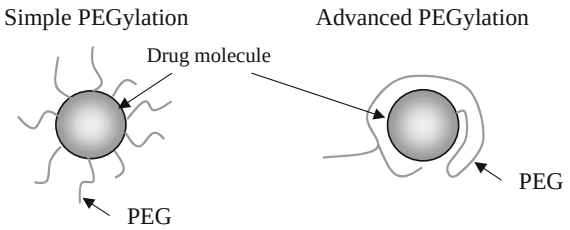

Fig. 5. Schematic representation of PEGylation. Two PEGylated drug molecules are shown. On the *left*, multiple small PEG molecules are attached at random sites via unstable linkages; on the *right* site-specific modification of the drug by a large, branched form of PEG, with a stable linkage, is shown.

also constructed PEGylated di-Fab, in which two Fabs are attached at one end of PEG (63). They prepared the Fab in *E. coli* with a yield of more than 1 g/l. The pharmacokinetic profile of the PEGylated Fab was better than that of the intact IgG *in vivo*, and the antigen-binding properties were fully retained. Several groups have failed to confirm these results (64,65), but this technique allows production of well-defined conjugates and should be useful for large-scale production.

ALTERNATIVES TO ANTIBODIES

The complicated structures of intact antibodies and their derivatives hamper efforts to exploit them in a therapeutic setting. For example, some antibodies tend to aggregate when they have been fused to additional proteins, such as toxins, for application as drugs. Thus, many attempts to construct simpler alternatives to antibodies have been reported, and many novel binding proteins that bind their target antigens with high specificity and affinity can now be easily generated by a variety of methods (66) (Fig. 6). The proteins are relatively small and their structures are simple, facilitating the construction of fusion proteins and conjugates. Moreover, preparation of such small proteins is convenient and economical.

The most successful method for creation of novel binding proteins is the phage-display method, in which randomized peptide libraries are displayed on the surface of phage molecules (67) (Fig. 6). This method has rapidly become a vital tool in studies aimed at identifying molecules that bind to specific targets. Antibody fragments, such as Fab and scFv, can also be displayed (68). Phage-display technology is straightforward but has several limitations. First, transformation limits library diversity (10^7–10^9). Second, because of the nature of phage itself, no harsh selection conditions and/or harsh washing conditions can be used to select for tight binders. Third, proteins that are toxic to host cells cannot be displayed.

Several technologies have been developed as alternatives to phage display. Ribosome display methods avoid the problems of phage display (69) (Fig. 6). In ribosome display, individual nascent proteins (phenotype) are coupled to their corresponding mRNAs (genotype) through the formation of stable protein-ribosome-mRNA (PRM) complexes. Because products of the polymerase chain reaction (PCR) are used directly as templates for transcription *in vitro*, this system allows huge potential diversity (10^{14}). In addition, toxic and unstable proteins that cannot be expressed in bacteria can be displayed. Recently, our laboratory reported the introduction of strong protein-RNA interactions that stabilized the PRM complex (70–73). Overall, advanced ribosome-display with strengthened association (ARISA) works best when it exploits stable interactions between an RNA-binding protein (mutant coat protein of bacteriophage MS2) and an RNA motif that is recognized by MS2 protein (72,73). Peptides with picomolar affinity constructs have been selected by ARISA. Another group has used puromycin for the covalent linkage between mRNA and protein (74), and this alternative strategy also stabilizes the PRM complex (Fig. 6).

INTERNALIZATION

When ligands are to be used for the delivery of drugs, in many cases, the binding of the ligands to the antigens must also allow receptor-mediated internalization. Important progress in this area has been made by Poul *et al.* (75), who developed a method for the recovery of internalized phage that display scFvs from living cells. Thus, even without prior knowledge of target antigens, internalizable scFvs can be generated (76).

TARGETED DELIVERY BY ANTIBODIES

To date, several researcher groups have used antibodies or antibody fragments for targeted delivery of genes. Marasco *et al.* have used selected fragment of HER2-specific monoclonal antibodies for the development of anti-HER2 immunoliposomes (77,78). Eaton *et al.* conjugated the CD3-specific antibody fragments to the end of a cationic derivative of PEG for the delivery of DNA (79). As noted by Eaton *et al.* the exposed cysteine residue at the hinge region of the original IgG is very useful for site-specific modification of antibody fragments with other macromolecules, such as PEG and liposomes.

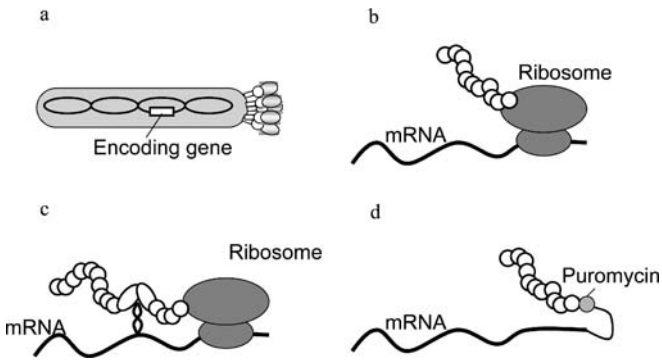

Fig. 6. Schematic representation of phage (a) and protein-ribosome-mRNA (PRM) complexes (b, c and d). a In the phage display system, randomized peptide libraries are displayed on the surface of phage molecules. b The PRM complex in the ribosome display system is a stable complex in which each individual nascent proteins is coupled to the corresponding mRNA. c The advanced ribosome-display with strengthened association (ARISA) system exploits stable interactions between an RNA-binding protein and an RNA motif. d Szostak *et al.* used puromycin to link mRNA and protein covalently.

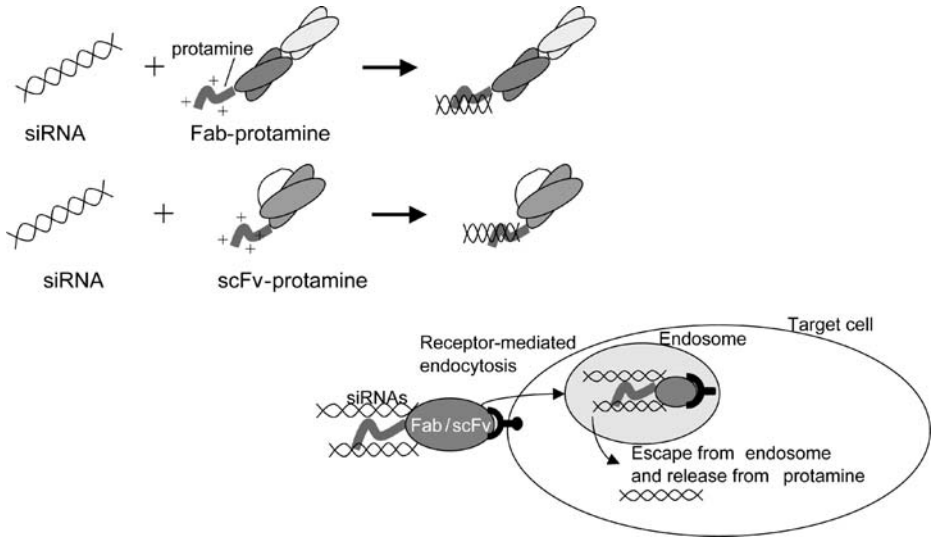

Fig. 7. Antibody-mediated delivery of siRNA. The antibody fragment targeted to a specific antigen receptor binds to the cell surface and then the complex,which includes siRNA, is internalized by endocytosis. After escape from the endosome and release from the cationic protamine, siRNA is incorporated into the RNA-induced silencing complex (RISC).

Recently, Zang *et al.* have reported a delivery system that crosses the blood–brain barrier (BBB) for treatment of brain cancers (80). The BBB is the specialized system of capillary endothelial cells that protects the brain from harmful materials in the blood-stream. The BBB limits transport of large molecules into the brain through both physical barriers (tight junctions) and metabolic barriers (enzymes). Accordingly, the BBB is often the rate-limiting factor in the permeation of therapeutic drugs into the brain (81). Zang *et al.* used a PEGylated immunoliposome (PIL) that had been modified with two monoclonal antibodies. One monoclonal antibody was targeted to the mouse transferrin receptor, which is found at BBB, and the second monoclonal antibody was targeted to the human insulin receptor, which is expressed on the plasma membrane of human brain cancer cells. The monoclonal antibody against transferrin allowed the PIL to enter the brain compartment. Once inside the compartment, the insulin receptor allowed entry into brain cancer cells.

Song *et al.* used an antibody for the targeted delivery of siRNA (82) (Fig. 7). They used the heavy chain of a monovalent Fab fragment targeted to the envelope glycoprotein (gp120) of human immunodeficiency virus (HIV) or an scFv fragment targeted to ErbB2 fused to protamine, which can bind siRNA. Gene expression was suppressed only in cells that expressed gp120 or ErbB2, respectively. Because of the strong cationic charge on protamine, we might expect that the release of siRNA would be limited. However, the cited study demonstrated the conclusive advantages of utilizing engineered antibodies for the targeted delivery of siRNA.

PHYSIOLOGICAL BARRIERS TO THE DELIVERY OF DRUGS BY ANTIBODIES

There have been many attempts to deliver drugs, including radionuclides, chemotherapeutic agents and toxins, using antibodies (83). The efficacy of delivery depends on the ability of each antibody to reach its target in adequate quantities. For therapy aimed at destroying all viable cells in a tumor, deep penetration, uniform distribution and high specificity are important. However, studies in animal models and in humans indicate that intact IgG is distributed heterogeneously in solid tumor (84) (Fig. 8). Various theoretical and experimental studies have revealed the factors that are responsible for this heterogeneity. The modeling analysis reported by Fujimori *et al.*, using parameters from experimental sources, led to the proposal of a 'binding site barrier' (85), whereby the high affinity of IgG decreases the uniformity of distribution of IgG. Adams *et al.* prepared a series of radioiodinated mutants of scFv with affinities from 10^{-7}–10^{-11} M and they observed a significant decrease in tumor retention even when affinity was increased from 10^{-10} to 10^{-11} M (86). Accordingly, an upper limit of

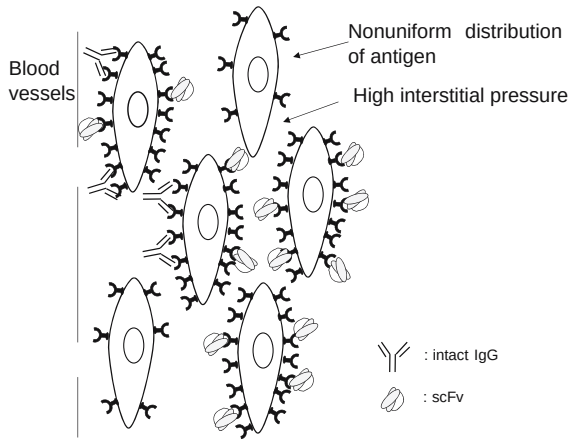

Fig. 8. A model showing the physiological barriers to the delivery of antibodies to solid tumors. The nonuniform distribution of antigens and high interstitial pressure cause nonuniform uptake of antibodies.

binding affinity appears to exist. Beyond this limit, further improvements in affinity have no beneficial effects.

Molecular mass is another important factor in the distribution of antibody-bound drugs in tumors. Yokota *et al.* observed greatly penetration by scFv than by Fab or intact IgG (84). Most intact IgG delivered to the tumor was concentrated immediately adjacent to vessels, while the scFv was more evenly distributed throughout the tumor. The high interstitial pressures within solid tumors also severely limits the diffusion of macromolecules from blood vessels to the center of the tumor (87). Other factors, such as nonuniform distribution of the antigen among tumor cells and a heterogeneous blood supply, are also responsible for the uneven distribution of intact antibodies in tumors (88).

In summary, because of its high affinity, the specificity of intact IgG against its target is high especially at sites immediately adjacent to blood vessels. However, percolation into the tumor is inefficient. Accordingly, for the treatment of a solid tumor, intact IgG is an inappropriate vehicle for drug delivery. As indicated by modeling analysis, a reduction in affinity (in the range of 10^7–10^8/M) should lead to better percolation without significant loss of specificity (85).

In the case of bivalent interactions such as those mediated by IgG, $F(ab)_2$ and diabodies, the dynamics are much more complex. The bivalent association occurs in two steps. In the first step, the antibody (or fragment etc.) combines reversibly with the antigen to form a monovalently bound antibody-antigen complex. Then, the second binding site can be exploited for reversible binding to a second antigenic site to yield a bivalently bound complex. The second-order rate constant is dependent not only on the affinity of the monovalent linkage but also on factors that includes the density and flexibility of the antigen, the distance between the two binding sites and the concentration of antibody etc. (89). Nielsen *et al.* showed that, at a high concentration of diabodies, for example, in regions adjacent to blood vessels, a large fraction of diabodies initially binds only one antigen. A diabody with a lower intrinsic equilibrium constant can participate rapidly in bivalent binding because diabodies that have bound antigens monovalently dissociate rapidly from and free up the antigen for bivalent binding by neighboring diabodies (90). Nielsen *et al.* also observed that a diabody with lower affinity allows significantly greater localization to the tumor than a diabody with higher affinity and a high-affinity scFV. This observation can be attributed, in part, to the longer retention in the blood of the larger diabody as compared to scFv. Moreover, this observation also indicates the existence of a threshold affinity for the improved delivery of bivalent molecules. Accordingly, for the two most important factors for the specific and uniform delivery of antibodies (and their fragments) to a solid tumor are stability in serum, which can be controlled by the size of the vehicle and/or PEGylation, and affinity, which can be controlled by genetic engineering and/or dimerization of monovalent fragments.

FUTURE PROSPECTS

Targeted delivery using of antibodies and antibody fragments may prove useful for the next generation of siRNA-delivery methods. However, many problems remain to be resolved, including low tumor-penetration by antibodies and the high cost of large-scale preparation. Moreover, there is considerable evidence for the heterogeneous nature of cancer cells (91). Despite these obstacles, antibody-mediated targeted delivery of siRNA to cancer cells appears to be an attractive strategy. Use of bivalent ligands for delivery, instead of the monovalent ligand used by Song *et al.* (82), and the display of bi-specific or multi-specific ligands on the surface of a complex might dramatically improve the efficiency of delivery, as indicated by Zang *et al.* (80). Many opportunities remain for the engineering of antibodies and selected peptides for the targeted delivery of siRNA.

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