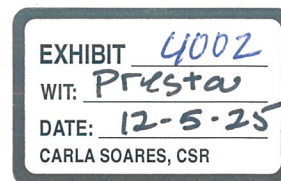


Chapter 9

The Design and Engineering of IgG-Like Bispecific Antibodies

Pei Jin and Zhenping Zhu



9.1 Introduction

Therapeutic monoclonal antibodies (mAb) have become an increasingly important asset for clinicians to fight cancer, inflammation, and infectious diseases (Chan and Carter 2010; Beck et al. 2010; Weiner et al. 2010). mAb as therapeutics exploit one of several mechanisms of action for their biologic effects: (1) Sequester antigens including growth factors and cytokines; (2) Act as receptor antagonists or agonists; (3) Act as activators of effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC); (4) Act as carriers for targeted delivery of cytotoxic agents, in the forms of immunoconjugates or immunotoxins to diseased tissues; and (5) Act as chemotherapy or radiotherapy adjuncts (Chan and Carter 2010; Beck et al. 2010; Weiner et al. 2010; Muller and Kontermann 2010). Despite recent success in commercial development of antibody therapeutics, current single-targeted mAb have demonstrated limited efficacy in clinic. This is because cells are evolved with multiple and redundant signaling pathways and are capable of escape from monotherapy. Although small molecule drugs can be developed to impact multiple signaling pathways, they often cause serious off-target toxicities. Therefore, therapeutics are needed that are capable of modifying multiple signaling pathways without augmented side effects. Solutions to circumvent limitations of current mAb therapies include antibody combination and the creation of dual-targeting bispecific antibodies (BsAb). In this chapter, we review the current status and future perspectives of IgG-like BsAb and their

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potential therapeutic applications. Advantages and limitations of the various approaches will be reviewed in the context of each BsAb format.

9.2 Antibody Combination Strategies and Drawbacks

Evidences are accumulating that the combination of mAb to impact multiple targets in disease pathways provides improved efficacy while limiting systemic toxicity. A clinical example is the combination of the anti-epidermal growth factor receptor (anti-EGFR, Erbitux[®]) and the anti-vascular endothelial growth factor (anti-VEGF, Avastin[®]) antibodies, both of which have been approved for colorectal cancer (CRC). A Phase II study among the irinotecan-refractory CRC patients receiving Erbitux[®], Avastin[®], and irinotecan showed a 37% response rate (Saltz et al. 2007) as compared to a 23% response among patients receiving Erbitux[®] and irinotecan only. Currently, multiple clinical trials of antibody combinations, e.g., the combination of anti-HER2 antibody Herceptin[®] and Avastin[®], and the combination of Erbitux[®] and Avastin[®] are being conducted in breast cancer and CRC patients, respectively.

Most available data supporting the antibody combination therapies are from pre-clinical studies, such as the combination of anti-EGFR and anti-vascular endothelial growth factor receptor-2 (anti-VEGFR2) (Ahmad et al. 2001; Jung et al. 2002; Tonra et al. 2009; Lamszus et al. 2005; Penar et al. 1997; Kunkel et al. 2001), the combination of anti-EGFR with anti-insulin-like growth factor receptor (IGFR) (Chakravarti et al. 2002; Krane et al. 1991; Kobari et al. 1998; Burtrum et al. 2003; Lu et al. 2005), and the combination of anti-EGFR with anti-HER2 (Ye et al. 1999), to treat a variety of xenograft tumors and other disease models. In each of the reported cases, the combination treatment achieved improved efficacy with additive or synergistic inhibitory activities. Antibody combination therapy has also been applied to target two distinct non-overlapping epitopes on the same molecule such as EGFR (Dechant et al. 2008; Pedersen et al. 2010) and HER2 (Nahta et al. 2004) to achieve synergistic therapeutic efficacy. However, therapeutic development of antibody combination encounters limitations, such as high cost, development delay, and regulatory hurdles. An alternative to antibody combination is the creation of dual-targeting BsAb capable of delivering biological activities of two mAb in the form of one molecule (see below).

If combination of mAb is therapeutically more effective than monotherapy, then the question is if more is better. Bulk of evidence in favor of oligoclonal antibodies (defined mixtures of mAb) comes from clinical indications such as neurotoxin poisoning in which escape from therapy can be instantly life threatening. In one case, the oligoclonal antibody cocktails were 200-fold more potent at neutralizing tetanus toxin in vivo than the monoclonal treatment (Volk et al. 1984). In another case, a cocktail of three antibodies was about 20,000-fold more potent against botulinum toxin than the monotherapy (Nowakowski et al. 2002). An alternative to defined mixtures of mAb is the use of polyclonal antibodies. Often regarded as primitive reagents best suited for molecular biology applications, polyclonal antibodies are regaining favor as potential therapeutic agents (Williams and Sharon 2002; Williams et al. 2002). While the concept of oligoclonal or polyclonal antibodies is appealing, therapeutic development of such mixtures might encounter huge regulatory challenges. Characterization of individual components in the

mixtures are prohibitively expensive (oligoclonal cocktails) or nearly impossible (polyclonal antibodies). An undefined or under-defined mixture of recombinant IgGs is both a safety and an efficacy concern. Also, batch consistency in the manufacturing process would be difficult to control from the regulatory perspective.

9.3 Engineering IgG-Like Bispecific Antibodies

BsAb are antibodies or antibody-like molecules specific for two distinctive epitopes or antigens. Initially, they were generated by fusing two antibody-producing hybridoma cell lines, each with a different specificity (Cotton and Milstein 1973). However, due to random pairing between the heavy chains (HC) and light chains (LC), the resulting hybrid hybridoma can produce only a minor population of heterodimeric BsAb among a pool of randomly formed active or inactive monomers and dimers. Purification of the BsAb out of such a pool is difficult and expensive. Chemical crosslinking can also be used to covalently link two mAb of different specificities to form BsAb (Nisonoff and Rivers 1961). However, chemical conjugation tends to inactivate the molecules. Further, the resulting antibody conjugates are heterogenous, and extensive purification is required to obtain limited amounts of BsAb.

Approaches to generating BsAb by recombinant technology have been more fruitful (Marvin and Zhu 2006; Coloma and Morrison 1997; Muller and Kontermann 2010). Recombinant BsAb are generated by protein design and genetic engineering. The resulting cDNA constructs are expressed in bacteria or mammalian cells. The expressed active BsAb are easily purified to homogeneity. While BsAb have been studied for over 2 decades, their development into clinically useful therapeutics has been hampered by limitations such as low expression, poor stability, heavy aggregation, and insufficient safety and efficacy profiles. However, recent progress in the field of antibody engineering and expression has resulted in new approaches to address these issues (Miller et al. 2009, 2010; Ewert et al. 2004; He et al. 2010; Michaelson et al. 2009). This has led to a renewed enthusiasm in engineering and therapeutic application of novel BsAb.

Recombinant BsAb can be broadly categorized into two classes: (1) the small bispecific formats derived from Fv or Fab that do not have an IgG Fc domain; and (2) the Fc-containing IgG-like bispecific formats. While small bispecific formats have the advantages of improved penetration into disease tissues or cells, they have significantly shorter half-life in vivo and thus require repeated dosing, or further genetic or chemical modification to extend their circulating half-life in vivo. This review focuses on the Fc-containing IgG-like bispecific formats and their potential therapeutic applications.

9.3.1 Heterodimeric IgG-Like Bispecific Antibody

Since one of the major barriers in the production of BsAb in a single cell system, e.g., hybrid hybridoma, is the undesired formation of HC homodimers, therefore

a first and obvious solution is to re-engineer the CH3 domain of the Fc, so as to favor the formation of HC heterodimers versus homodimers.

9.3.1.1 “Knobs-Into-Holes” BsAb IgG

An elegant experiment was designed by Carter and colleagues 15 years ago (Ridgway et al. 1996; Merchant et al. 1998) to facilitate the heterodimer pairing of two different IgG HC. In the experiment, single amino acid substitutions were introduced into the CH3 domains of the opposite Fc chain, so that the steric complementarity required for CH3/CH3 association obligates the CH3 domain with mutations to pair with a CH3 domain that has different accommodating mutations (Fig. 9.1b). Co-expression of the pair of HC mutants resulted in the formation of a mostly (92%) heterodimeric product, but with a stability that is significantly lower than that of the wild-type (knob:T366W + hole:Y407A mutant, $T_m = 65.4^\circ\text{C}$; wild-type, $T_m = 80.4^\circ\text{C}$). Further optimization using a phage display library resulted in a variant (knob: T366W; hole: T366S, L368A, Y407V), which had an improved T_m of 69.4°C (Atwell et al. 1997). These results demonstrate the utility of the knobs-into-holes approach. It is possible that other residue sets in the CH3 domain could also be optimized, to further improve stability and the heterodimer to homodimer ratio.

While this approach solves the HC homodimerization problem, it did not address the equally problematic mispairing between the LC and HC from two different antibodies (e.g., $\text{LC}_A\text{-HC}_B$ and $\text{LC}_B\text{-HC}_A$ mispairing, instead of the correct $\text{LC}_A\text{-HC}_A$ and $\text{LC}_B\text{-HC}_B$ pairing). This can be resolved by similarly redesigning the LC-HC interaction to incorporate knobs and holes at the VL-VH interface (Zhu et al. 1997). Based on inspection of the VL-VH interface of the anti-HER2 Fab fragment, 4D5 Fab (Eigenbrot et al. 1993), knob mutations, and the complementary hole mutations were designed for a diabody fragment containing the Fvs of 4D5 and an anti-CD3 antibody (Zhu et al. 1996). For one of the variants (termed “v5”; knob: VH-V37F, L45W; hole: VL-Y87A, F98M), 92% of the products were heterodimers, and near wild-type affinity for antigens was retained (Zhu et al. 1997). Although these mutations were used to promote heterodimerization in a bispecific diabody, the principle is applicable to a full length IgG format.

9.3.1.2 Common Light Chain “Knobs-Into-Holes” BsAb IgG

An alternative approach to solve the problem of LC-HC mispairing is to construct BsAb using two antibodies of different specificities that can share the same LC (Merchant et al. 1998) (Fig. 9.1c). In a proof-of-concept study, a large single chain Fv (scFv) phage library was used to screen for a variety of antigen specificities. The VL regions of the selected scFv were compared. Those with identical amino acid sequences, but paired with the VH regions of different antigen-binding specificities, were selected to create the BsAb. Functional IgG-like BsAb were produced at

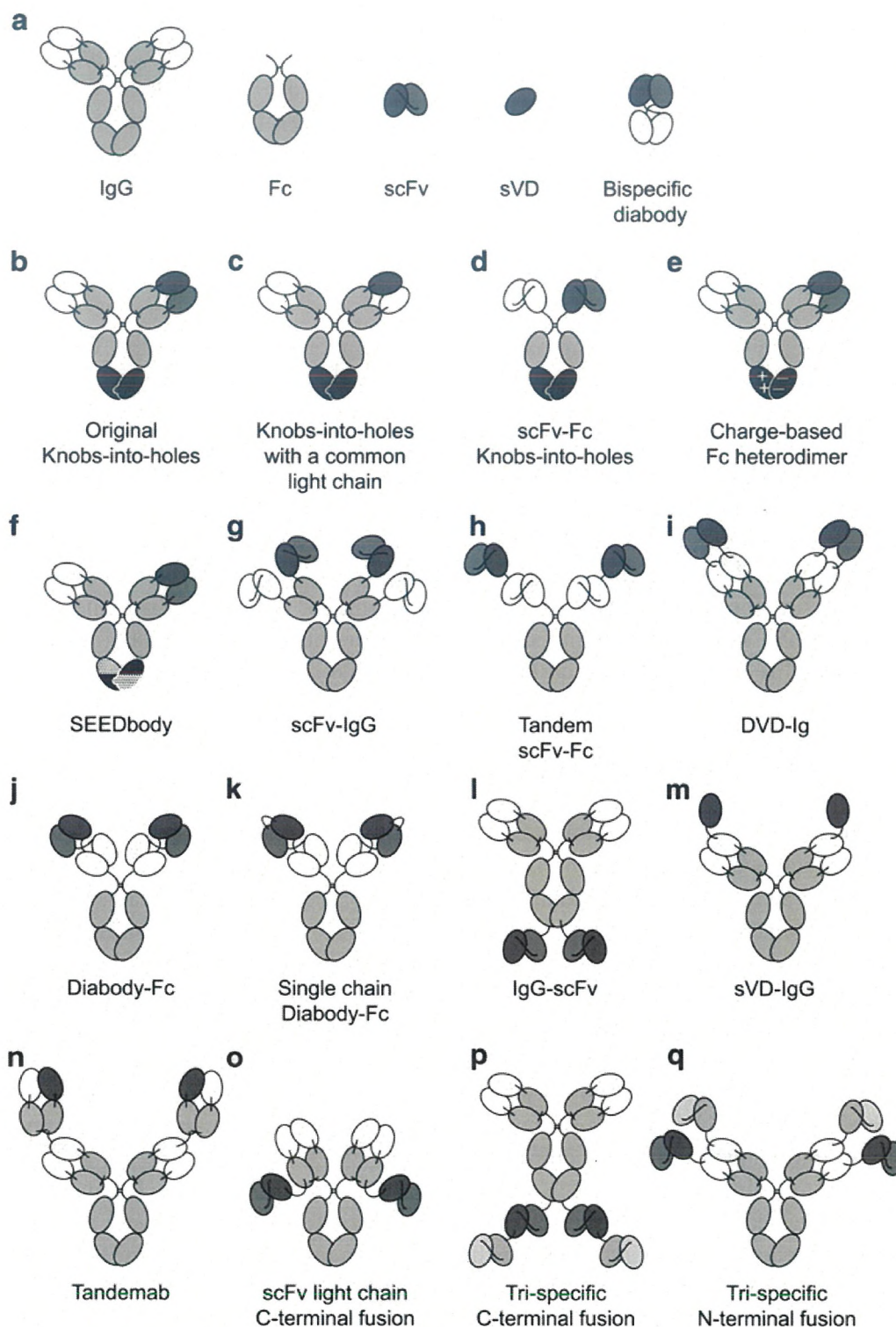


Fig. 9.1 Schematic drawings of the IgG-like bi- and tri-specific antibodies. (a), Components as building blocks to construct bi- and tri-specific antibodies; (b–q), Engineered IgG-like bi- and tri-specific antibodies that are discussed in this chapter

a high percentage (>95% of total IgGs produced), after a single LC was co-transfected into a host with two HC of different specificity that were engineered to incorporate the original knobs and holes mutations (knob: T366W, hole: T366S, L368A, Y407V), together with a heterodimer stabilizing disulfide bond (S354C, Y349'C). However, it is quite a challenge in practice to identify antibodies that can share common LC. This is particularly true for high affinity antibodies.

9.3.1.3 scFv-Fc “Knobs-Into-Holes” BsAb

The LC-HC mispairing problem may also be circumvented by fusing the VL and VH in a scFv format (Xie et al. 2005). In this construct, an anti-HER2 scFv is fused to an Fc with the “knob” mutation T366Y, and an anti-CD16 scFv is fused to an Fc with the “hole” mutation Y407T (Fig. 9.1d). Efficient pairing of the two chains, via the knobs-into-holes mechanism, was demonstrated, and specificity for both antigens was confirmed by cell surface binding analysis. The scFv-Fc “knobs-into-holes” molecule is also able to crosslink the two target antigens, as shown by its ability to induce higher NK cell-mediated cytotoxicity than a monospecific anti-HER2 antibody alone. This format, which is smaller (120 kDa) than a normal IgG (150 kDa), might have slight gains in tumor penetration, while maintaining the longer half-life and effector functions provided by the intact Fc region.

9.3.1.4 Other Novel Fc-Heterodimer Formats

In addition to the knobs-into-holes technology, other approaches have been developed to achieve Fc heterodimerization. For example, heterodimeric IgG-like pairing can be made by charge polarity engineering of the opposite Fc chains within the CH3 domain (Fig. 9.1e) (Gunasekaran et al. 2010). It was demonstrated that mutations of a few charged residues along the interface of the opposite chains of the CH3 domains can promote Fc heterodimer formation, while effectively suppressing formation of homodimers (see Chap. 11 for a detailed description). If the knobs-into-holes technology is combined with the charge polarity engineering, a higher ratio of heterodimer formation may be achieved. However, a drawback of these methods is the inclusion of multiple mutations in the CH3 domains, which may pose an immunogenic risk in therapeutic settings.

In another approach, a SEED technology (the Strand-Exchange Engineered Domain) was developed, in which a pair of asymmetric fusion proteins were created based on structure modeling between the CH3 domains of the IgG and the IgA (Davis et al. 2010) (Fig. 9.1f). This approach resulted in the complementary AG and GA SEED CH3 domain-containing Fc chains, which preferentially form heterodimers with few or no homodimers when the opposite SEED chains were co-expressed in mammalian cells. Since the natural IgA Fc region does not bind FcRn and Protein A, the relevant surface residues of the AG SEED CH3 domain were mutated back to the IgG sequences to restore these binding activities.

The SEED CH3 domain-containing heterodimer scaffold can be genetically linked to one or more antigen-binding domains, resulting in the bi- or multi-specific antibodies. This novel scaffold was expressed at high levels, purified on recombinant Protein A resin, and had a long serum half-life comparable to that of the IgG1 controls.

9.3.2 *N-Terminal IgG-Like Fusion*

The fact that scFv can often retain the antigen binding affinity and specificity of their parent antibodies, coupled with their small size, makes them the ideal building blocks for construction of BsAb. The scFv can be covalently linked to the N-termini of IgGs resulting in the tetravalent BsAb.

9.3.2.1 scFv-IgG Fusion

In one such format, two scFv, each with a different specificity, were fused, respectively, to the N-termini of the constant light (CL) and the first constant heavy chain (CH1) domains of an IgG: (scFv_A)-CL and (scFv_B)-CH1-CH2-CH3. Co-expression of the two recombinant chains in a single host resulted in a tetravalent BsAb, Bs(scFv)₄-IgG (Zuo et al. 2000) (Fig. 9.1g). Only homogenous BsAb were generated due to the natural heterodimerization between the CL and the CH1 domains.

Two similar Bs(scFv)₄-IgG molecules were constructed using two scFv fragments specific for EGFR and IGFR, respectively (Lu et al. 2004b). The two constructs were in different fusion orientations: i.e., scFv_{anti-EGFR}-CL plus scFv_{anti-IGFR}-CH1-CH2-CH3, or scFv_{anti-IGFR}-CL plus scFv_{anti-EGFR}-CH1-CH2-CH3. Both constructs were expressed in mammalian cells and purified via one-step Protein-A chromatography. The BsAb prevented the binding of EGF and IGF to their respective receptors and blocked the ligand-induced receptor activation, whereas a parent mAb only inhibited receptor activation induced by an individual ligand, but not both. Further, both Bs(scFv)₄-IgGs inhibited proliferation of cultured tumor cell at levels similar to that of the combination of the two parental IgG antibodies. Both molecules demonstrated excellent stability when incubated in mouse serum in vitro at 37°C for up to 7 days. This Bs(scFv)₄-IgG format can be widely applied for construction of other BsAb.

In a more recent report (Dimasi et al. 2009), a scFv of different specificity was linked to the N terminus of either a full-length IgG LC (scFv-VL-CL), or a full-length IgG HC (scFv-VH-CH1-CH2-CH3), or both the LC and HC (scFv-VL-CL plus scFv-VH-CH1-CH2-CH3). Co-expression of the scFv-VL-CL fusion and the HC, or the scFv-VH-CH1-CH2-CH3 fusion with the LC, or both scFv-VL-CL and scFv-VH-CH1-CH2-CH3 fusions, led to the production of homogenous molecules that are either bispecific or trispecific (see Sect. 9.3.5 for more discussion).

9.3.2.2 Tandem scFv-Fc Fusion

As an alternative to the N-terminal scFv-IgG fusion, tandem scFv [scFv(A)-scFv(B)] was fused to the Fc domain of an IgG to form a tetravalent BsAb (Fig. 9.1h) (Natsume et al. 2006). For example, a scFv specific for TAG-72 was fused to a second scFv specific for MUC1 and then to an Fc. The resulting scFv(A)-scFv(B)-Fc or tandem scFv-Fc was expressed efficiently in CHO cells, recognized both target antigens, and was able to mediate ADCC to tumor cells expressing either TAG-72 or MUC-1.

9.3.2.3 Dual-Variable-Domain Immunoglobulin

In this format, the VL and VH domains of a mAb specific for one antigen are covalently linked, via short linkers, to the N terminus of the respective VL and VH of a second mAb, which is specific for a different antigen, resulting in the dual-variable-domain immunoglobulin (DVD-Ig). Several different DVD-Ig constructs were produced and showed activities similar to that of the parental antibodies (Wu et al. 2007) (Fig. 9.1i). The DVD-Igs can be efficiently produced and exhibits good in vivo stability, suggesting that the approach may be widely applicable (see Chap. 12 for more details).

9.3.2.4 Diabody-Fc Fusions

Another method for homogenous production of an IgG-like BsAb is to replace the Fab fragment with a bispecific diabody. Diabodies are a derivative of the scFv fragment (Holliger et al. 1993). A scFv is composed of a VH and a VL domain, connected by a flexible linker of approximately 15 amino acids, e.g., [(Glycine)4-Serine] $\times$ 3, that permits self assembly into an antigen-binding competent form. If the linker is shortened to 5 amino acids, e.g., (Glycine)4-Serine, self assembly between VL and VH becomes impossible. This allows the two scFv to interact with each other to form a bivalent molecule of two interlinked polypeptides, the VL of one chain associating with the VH of the other. If VL and VH of different specificities comprise the diabody, i.e., VH_A-VL_B and VH_B-VL_A (the two so-called “cross-over” scFv), bispecific bivalent diabodies are formed with one binding site for each antigen. In addition to assembling into functional heterodimers, the cross-over scFv can also assemble into non-functional homodimers. Fortunately, purification of properly heterodimerized molecules can easily be achieved by a single round affinity chromatography. Further, as discussed earlier, the “knobs-into-holes” technique can be used to re-engineer the Fv interface to promote the correct heterodimeric VH-VL pairing (Zhu et al. 1997). Diabodies have shown to be useful for antigen cross-linking (Zhu et al. 1996; Holliger et al. 1993) and their small size is ideal for tumor penetration (Colcher et al. 1990). Diabodies can be fused to the Fc domain of an IgG to create tetravalent IgG-like BsAb, or so-called di-diabody (Lu et al. 2005) (Fig. 9.1j). A similar but smaller di-diabody construct can also be

created by using the CH3 domain for dimerization, i.e., VL_B-VH_A plus VL_A-VH_B-CH3 (Lu et al. 2003). As a precautionary measure to avoid potential immunogenicity in human therapy, the unstructured first five amino acids of the human IgG CL(Kappa) domain, instead of a “standard” (Glycine)₄-Serine linker, can be used as the linker between the variable domains to create the cross-over scFv (Zhu et al. 1996). A di-diabody that binds both EGFR and IGFR was constructed using the variable domains of an anti-EGFR and an anti-IGFR antibody as the building blocks. The di-diabody bound both EGFR and IGFR with affinity similar to that of their parent antibodies. It blocked binding of both EGF and IGF to their respective receptors and prevented the ligand-induced receptor activation. In addition, the di-diabody could efficiently induce ADCC activity against tumor cells that express EGFR and/or IGFR, indicating that the di-diabody possesses an intact and unhindered Fc domain. It also had an in vivo half-life that is equivalent to that of an intact human IgG. Lastly, the di-diabody effectively inhibited the growth of two different human tumor xenografts in nude mice. The di-diabody construct was transiently expressed in mammalian cells with excellent yield (>400 mg/L in non-optimized conditions), which could greatly facilitate the transition from “proof-of-concept” to therapeutic application. Unfortunately, the di-diabody construct has a tendency to form inactive molecules in vivo that lack the smaller cross-over scFv chain, a likely result of dissociation between the two cross-over chains. This shortcoming may be surmounted by introduction of the disulfide bonds (Zhu et al. 1997; Jung et al. 1994; FitzGerald et al. 1997) or packing improvement (Worn and Pluckthun 2001) to stabilize the VL-VH interfaces in the diabody.

9.3.2.5 Single Chain Diabody-Fc Fusion

In this approach, a single chain diabody (scDb) is first constructed by fusing both “cross-over” scFv of a bispecific diabody with a flexible linker (Kipriyanov et al. 1999; Alt et al. 1999; Kontermann and Muller 1999). The resulting fragment is fused to an Fc fragment (or just a CH3 domain) to create a tetravalent bispecific IgG-like molecule (Fig. 9.1k). In this format, one polypeptide with six domains is produced: VH_A-VL_B-VH_B-VL_A-CH2-CH3, which assembled into IgG-like dimers via the Fc domains. The scDb-Fc is bispecific and bivalent for both antigens, and has a full Fc. However, the stability of the scDb-Fc was not reported and the expression level was also low (~5 mg/L).

9.3.3 C-Terminal IgG-scFv Fusion

scFv can be fused to the C terminus of IgGs to create tetravalent BsAb with one antigen-binding specificity at the N-terminal and the other at the C-terminal scFv of the antibodies (Fig. 9.1l). In one such a BsAb, the specificity at the N terminus was for dextran and the specificity at the C-terminal scFv was for dansyl (Coloma and Morrison 1997). The C-terminal scFv in this BsAb showed a tenfold lower affinity

for dansyl than that of the parent IgG owing to a slower on-rate. This may result from steric hindrance of the scFv upon fusing to the IgG C-terminal end or from the conversion of the anti-dansyl IgG to a scFv. Regardless, the IgG-scFv construct is bispecific. Furthermore, it is capable of binding C1q, apparently through its intact Fc fragment, and triggering a partial complement cascade. This format has the potential to allow the creation of tetravalent molecules with effector function, but the full potential of the latter remains to be investigated.

A similar IgG-scFv antibody was constructed (Mabry et al. 2010) by fusing a scFv specific for IL-23 to the C terminus of a full-length anti-17A IgG. Before the construction of the bispecific molecule, a stability assessment was performed using a small group of phage library-derived scFv to select the most thermostable binder as the building blocks. The resulting anti-IL-17A/IL-23 BsAb showed an identical N-terminal affinity for IL-17A, and an eightfold lower C-terminal affinity of the scFv for IL-23, compared to their parental IgGs. The decrease in the C-terminal affinity of scFv for IL-23, however, did not affect its bioactivity.

While a BsAb may be functionally equivalent to the simple combination of their parental mAbs, in certain cases, BsAb can achieve significantly better efficacy. For example, an IgG specific for the TNF-Related Apoptosis Inducing Ligand Receptor-2 (TRAIL-R2) was linked at C terminus to a scFv specific for the Lymphotoxin-beta Receptor (LT β R). The BsAb was further engineered for improved thermal stability and reduced aggregation. The resulting anti-TRAIL-R2/LT β R BsAb demonstrated a much potent effect (IC₅₀ = 46 pM) in inhibiting the growth of tumor cells e.g., MDA-MB-231, Me180, and WiDr, while the individual parental mAb or the combination of them achieved only negligible or lower inhibitory activity (Michaelson et al. 2009). In a mouse MDA-MB-231-derived tumor xenograft model, the bispecific IgG-scFv demonstrated significant anti-tumor activity achieving up to 50% tumor inhibition, whereas the individual or combination of the mAb showed no efficacy. Interestingly, in the same xenograft model no tumor inhibition was observed when a differently formatted bispecific anti-LT β R/TRAIL-R2 antibody was administrated. In the latter format, the scFv specific for LT β R was linked to the N terminus of the anti-TRAIL-R2 IgG. The lack of efficacy in the xenograft model correlated well to the in vitro cell-based results. Both bispecific formats are capable of dual binding to the soluble TRAIL-R2 and LT β R. The difference in inhibitory activity between the IgG-scFv and the scFv-IgG formats in this case may be related to the proximity of the cell surface TRAIL-R2 and LT β R, which favor the IgG-scFv configuration for effective antibody-antigen interaction (Michaelson et al. 2009).

9.3.4 Other Novel IgG-Like BsAb Constructs

9.3.4.1 Single Domain Antibodies as Building Blocks

Some camel or shark antibodies are composed of only HCs (Muyldermans et al. 1994; Els et al. 2001). This observation has led to the development of human single

variable domain (sVD) antibody fragments, in which a VL or a VH alone comprises the binding unit (Ward et al. 1989; Riechmann and Muyldermans 1999; Jespers et al. 2004). These fragments can then be used to construct the IgG-like BsAb by fusing a sVD to the CL, and a second sVD of a different specificity to the CH1 of an IgG. Preferably, one sVD would be derived from a VL and the other from a VH to provide increased stability via a VL-VH interface, in addition to that of the CL-CH1 interface, i.e., VL_A-CL plus VH_B-CH1-CH2-CH3). sVD binders can also be directly fused to the N or C terminus of an IgG of a different specificity (Fig. 9.1m). In one such report, a single VH domain binder to the platelet-derived growth factor receptor- α (PDGFR- α) was isolated from a phage library, and fused to the N terminus of VL of an IgG antibody specific for PDGFR- β (Shen et al. 2006), or to either the N terminus of VH or the C terminus of the CH3 of an IgG antibody specific for mouse VEGFR-2 (Shen et al. 2007). All three BsAb constructs were expressed well in mammalian cells, and demonstrated the binding ability to both targets with affinity comparable with that of their parent antibodies. Further, the BsAb constructs were able to cross-link both target antigens in solution and also inhibited the function of both targets in cell-based assays.

In theory, sVD antibodies have the potential to satisfy the production criteria essential for drug development. In practice, however, while sVD binders are effective binding modules in isolation, their reconstitution into full IgG-like bispecific molecules often requires further engineering for improved product expression, stability, and retention of biological activity. This may be due to global instability resulting from the incompatible pairing of variable domains identified in isolation or local steric conflicts among the antigen-binding loops. In addition to VL and VH single domain scaffolds, other novel scaffold binders, such as fibronectin, ankyrin repeats, and lipocalin can be developed as building blocks for the construction of IgG-like BsAb molecules. For reviews, see Holt et al. (2003), Holliger and Hudson (2005), Saelens et al. (2008), and Wesolowski et al. (2009).

9.3.4.2 Two-in-One Antibodies

The first two-in-one antibody was developed by phage display of the anti-HER2 antibody Herceptin[®] with random mutations introduced into the CDRs of the VL domain. The phage library was screening for binding to both HER2 and VEGF (Bostrom et al. 2009). A variant with high affinity for both VEGF ($K_d = 3$ nM) and HER2 ($K_d = 0.2$ nM) was generated. The binding surface on the antibody for each antigen overlapped, with VEGF binding primarily mediated by the VL residues and HER2 binding by the VH residuals (see Chap. 13 for details).

9.3.4.3 Tandemabs

Another interesting bispecific format is the tandem Fab-Fc antibodies (tandemab; Fig. 9.1n). In such a format, a tetravalent IgG-like antibody was constructed (Zapata

et al. 1995; Miller et al. 2003), where two VH-CH1 units with distinct specificities were linked in tandem, which was then fused to the Fc. Each of the VH-CH1 units in this construct retained the binding affinity of the parent antibody when a common light chain (VL-CK) was co-expressed to produce the tetravalent tandemab. While tetravalent BsAb can be constructed into this format using Fab fragments of different specificities, the challenge remains to identify a common light chain that can be shared by both of the VH-CH1 units used, so that the high affinity and dual specificities of the tandemabs for the targets can be retained.

9.3.4.4 IgG Light Chain C-Terminal-scFv Fusion

In this format, a scFv is fused to the C terminus of the LC of an IgG via a [(Glycine) 4-Serine] $\times$ 2 linker resulting in a tetravalent BsAb (Orcutt et al. 2010) (Fig. 9.1o). The fusion of a scFv to the LC C terminus partially blocks formation of the disulfide bond between the LC and the HC, and thus requires engineering an additional disulfide bond between the IgG VL and VH domain to stabilize the LC-HC association. Two BsAb constructed in this format demonstrated their dual binding ability with the affinity of their parent IgGs and scFv. However, their expression levels was low (5–7 mg/L) in a transient 293 expression system.

9.3.5 Triple Specific IgG-Like Antibodies

Two trispecific IgG-like antibodies were recently constructed (Dimasi et al. 2009). In the first trispecific antibody (Ts1Ab), two scFv of different specificities were linked each other via a (Glycine)4-Serine linker. The tandem scFv was then fused to the CH3 domain of an IgG (Fig. 9.1p). In the second trispecific antibody (Ts2Ab), one scFv was linked via (Glycine)4-Serine linker to the N terminus of the VL domain of an IgG mAb, and the second scFv to the N terminus of the VH domain of the same IgG (Fig. 9.1q). The Ts1Ab and Ts2Ab were expressed at 120–150 mg/L, compared to 130–200 mg/L of the three parent mAb in a transient HEK293T expression system. Both Ts1Ab and Ts2Ab bound to their respective antigens simultaneously with reduced affinity (5- to 97-fold lower for Ts1Ab antigens and 2- to 4-fold lower for Ts2Ab antigens) as compared to that of their respective parent mAb. Further study showed that Ts2Ab bound to the FcRn and Fc γ receptors with the affinity of a classic IgG. Pharmacokinetic analysis in tumor-bearing nude mice confirmed an in vivo half-life that is comparable to that of their parent mAb. It is reasonable to expect that such a trispecific antibody, impacting multiple signal pathways, can provide additive or synergistic effect in therapy.

9.3.6 Novel Application of BsAb

9.3.6.1 Binding Avidity Enhancement

One of the valuable benefits of BsAb is the enhanced avidity for their antigens (Lu et al. 1999). High binding affinity (or avidity) is usually beneficial and, in some cases, required for the biological activity of a therapeutic antibody. In addition to the intrinsic high affinity for their antigens, normal IgG antibodies also exploit the avidity effect to increase their association with antigens because of their bivalent binding towards the targets. Thus, a BsAb directed against two separate (non-overlapping) epitopes on the same target molecule may most likely possess an increased avidity due to its bivalent paratopic (bi-paratopic) binding. Similarly, a tetravalent IgG-like BsAb, with bivalent binding for each antigen, are expected to have a very slow dissociation rate because two dissociation events must occur simultaneously for the BsAb to be free from the cell. In addition, bivalent binding to a target, particularly a cell-surface receptor, is under many circumstances a prerequisite for antibody function, e.g., cross-linking the receptors in order to stimulate activation, to induce apoptosis, or to promote receptor internalization.

9.3.6.2 Epitope Cross-linking for Acquired Antagonistic Activity

In addition to avidity enhancement, a BsAb directed against two non-overlapping epitopes on the same antigen molecule may also acquire novel properties that are not associated with either of the parent antibodies. For example, a bispecific diabody that binds two distinct epitopes on VEGFR-2 cross-linked the epitopes and effectively blocks the binding of VEGF to the receptor, whereas the parent scFv from which the bispecific diabody was derived did not, on their own or in combination, block VEGF binding (Lu et al. 1999). Cross-linking two separate epitopes on the same receptor molecule may introduce new steric hindrance for ligand binding, and/or induce conformation changes in the receptor, preventing it from binding ligand. It will be interesting to see if this principle is applicable to other molecules for which ligand-blocking antibodies are difficult to identify.

9.3.6.3 Fine-Tuning Antibody Specificity Towards Tumor Cells

An emerging and intriguing concept is the use of BsAb to further fine-tune the specificity of anti-tumor antibodies to target cells. In this context, a BsAb is constructed from two antibodies specific for two different tumor antigens, each with low to moderate binding affinity. Only simultaneous bivalent binding (or cross-linking) of the two target antigens on the same tumor cell surface by the BsAb would result in strong association that is required for initiating biological processes, such as down-regulation of signaling pathways, internalization of BsAb-conjugated

toxins, ADCC, and CMC. In contrast, monovalent binding of the BsAb to cells that only express one of the target antigens would only result in weak association, which may not be sufficient for induction of any meaningful cellular activity, thus would preserve the cells from the side effects of antibody mediated activities. This is significant since most of the targets currently being used as tumor cell identifiers are, in fact, not truly “tumor-specific,” but rather “tumor-associated,” i.e., they are also expressed in certain normal tissues/cells, albeit at lower density than in tumor cells. By identifying and constructing BsAb to pairs of targets simultaneously expressed on a given type of tumor, one could expect the enhanced antibody specificity towards the targeted cells, while sparing normal cells of unwanted side effects. This concept was elegantly demonstrated in a recent report using an anti-HER2 $\times$ anti-HER3 BsAb (Robinson et al. 2008). In this report, the authors constructed an anti-ErbB3 $\times$ anti-ErbB2 bispecific scFv dimer, and demonstrated that the BsAb selectively targeted tumor cells in vitro and in vivo that co-expressed the two target antigens over tumor cells that express only one target antigen or normal cells that express low levels of both antigens. Further, the bispecific scFv also exhibited significantly greater in vivo targeting of ErbB2/ErbB3 double positive tumors than the derivative molecules that contain one functional arm only targeting either ErbB2 or ErbB3, but not both. Together, these results clearly suggest that BsAb selected to co-target critical functional pairs of tumor-associated antigens could enhance the targeting specificity of antibody-based cancer therapeutics.

9.3.7 *Stability Optimization of Bispecific Antibodies*

scFv and its derivatives are commonly used as the building blocks for construction of BsAb. However, in the absence of the stabilizing CH1 and CL domains and the disulfide bond that covalently joins these two constant domains, scFv are usually less stable than the Fab fragment of an IgG. The insufficient stability of scFv in the BsAb constructs often leads to their poor expression, multimeric aggregation, and short serum half-life, limiting their manufacturability and therapeutic applications. Attempts have been made to improve the stability of scFv molecules, for example, by interface engineering to improve packing (Worn and Pluckthun 2001), by introducing new disulfide bonds between the VL-VH (Zhu et al. 1997; Jung et al. 1994; FitzGerald et al. 1997; Michaelson et al. 2009), by CDR-grafting to the proven stable frameworks, and/or further structure-based framework engineering (Ewert et al. 2004; Michaelson et al. 2009; Jordan et al. 2009). Further, it has been shown that the orientation of the VH and VL could also affect the expression and antigen-binding activity of the final scFv constructs (Lu et al. 2004a). A new technology was recently reported to improve the stability of IgG-like BsAb using a combination of the sequence-based analyses of residue co-variations in IgG variable domains, as well as other structure-, and knowledge-based methods (Miller et al. 2010). Thermally stable scFv were identified using this technology that not

only retained full antigen-binding affinity, but also were much less prone to aggregation. For example, a mutant with the substitutions of S16E in the VH and S46L in the VL were identified that increased the thermal stability as much as 26°C, and decrease the soluble aggregation from ~40% of the parent scFv to ~7%. These results demonstrate that by applying appropriate strategies, scFv-based BsAb can be developed with satisfying stability and manufacturability.

9.4 Conclusions

BsAb equip two disease-targeting/modifying capabilities within one molecule, and thus may be able to circumvent certain limitations encountered by current mAb therapeutics, such as insufficient efficacy, and challenges associated with antibody combinations. BsAb development may open up new target space for the development of mAb therapeutics. For example, there are individual targets (pathways) that may not provide sufficient therapeutic benefit when being modified alone. However, significant therapeutic benefits of these targets may be achieved, due to additive and/or synergy effects, if they are combined with other targets/pathways. These targets thus represent excellent candidates for the development of dual-targeting BsAb therapeutics. Recent progress in the field of antibody engineering has led to novel approaches to optimizing the formatting, the expression, the stability, and the manufacturability of IgG-like BsAb. Many issues, however, remain to be carefully addressed, e.g., manufacturing and downstream process development (purification and formulation), analytical and quality control, preclinical toxicology and pharmacology studies, regulatory and clinical development pathways, etc., before we see that BsAb become bonafide mainstream therapeutics.

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