A Fully Human Recombinant IgG-like Bispecific Antibody to Both the Epidermal Growth Factor Receptor and the Insulin-like Growth Factor Receptor for Enhanced Antitumor Activity*

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Both the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGFR) have been implicated in the tumorigenesis of a variety of cancers. Here we propose that simultaneous targeting of both receptors with a bispecific antibody would lead to enhanced antitumor activity. To this end, we produced a recombinant human IgG-like bispecific antibody, a Di-diabody, using the variable regions from two antagonistic antibodies: IMC-11F8 to EGFR and IMC-A12 to IGFR. The Di-diabody binds to both EGFR and IGFR and effectively blocked both EGFR- and IGFR-stimulated receptor activation and tumor cell proliferation. The Di-diabody also inherited the biological properties from both of its parent antibodies; it triggers rapid and significant IGFR internalization and degradation and mediates effective antibody-dependant cellular cytotoxicity in a variety of tumor cells. Finally, the Di-diabody strongly inhibited the growth of two different human tumor xenografts in vivo. Our results underscore the benefits of simultaneous targeting of two tumor targets with bispecific antibodies.

The lack of specificity of currently available chemotherapeutic and radiotherapeutic agents constitutes the major obstacle to the effective treatment of cancer. The common use of the combinatorial therapeutic regimens comprising several cytotoxic agents, e.g. various chemotherapeutics and radiations that hit cancer cells via different mechanisms, is often associated with severe toxicities to the patients. Because of their exclusive specificity and high affinity toward defined targets, monoclonal antibodies (mAb)1 are emerging as a promising new class of effective cancer therapeutics (1, 2). So far the United States Food and Drug Administration has approved eight antibody-based products, including unmodified (or naked) antibodies (Rituxan®, Herceptin®, Campath®, Erbitux®, and Avastin®), radiolabeled antibodies (Zevalin® and Bexxar®), and an antibody conjugate (Mylotarg®), for several oncology indications. Because of limited intrinsic cytotoxicity, unmodified mAb, when used alone, usually only yielded from marginal (e.g. Herceptin® and Avastin®) to 10–12% (e.g. Erbitux®) objective responses in patients with solid malignancies (3, 4). The therapeutic efficacy of these antibodies is significantly enhanced when combined with conventional chemotherapeutics and/or radiation, for example, Erbitux® plus a regimen of irinotecan, 5-fluorouracil, and leucovorin in third line refractory colorectal cancer patients yielded a 22.9% of objective response rate compared with that of 11% in patients treated with Erbitux® alone (5), and when combined with high dose radiation Erbitux® significantly prolonged the survival of patients with squamous cell carcinoma of head and neck (6). The dose-limiting toxicities of these combined therapies are usually associated with the cytotoxic components in the regimens. Because of their high specificity and low toxicity, it is generally believed that combination of the antitumor antibodies directed against different tumor-associated targets may yield enhanced therapeutic activity without adding significant toxicity. Clinical application of antibody combination is, however, greatly hindered by a number of factors, including limited availability of antibody products, high cost of each product, and the Food and Drug Administration-associated regulatory issues; it is most likely that each antibody has to be separately tested and approved before being tested and approved in combination. To this end, the development of bispecific or multi-specific antibodies that target two or more tumor-associated antigens simultaneously may offer a novel and promising solution.

In past years, both laboratory and early clinical studies have demonstrated that bispecific antibodies (BsAb) may have significant potential applications in cancer therapy by targeting tumor cells with cytotoxic agents including effector cells, radioisotopes, drugs, and toxins (7–9). Here we explored a new concept of utilizing BsAb by constructing a novel IgG-like antibody molecule that targets two different (but relevant) tumor targets, i.e. growth factor receptors, thus blocking simultaneously two receptor activation and their downstream signaling pathways. In this “proof-of-concept” study, we chose the epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR) as our model targets. Both receptors have been implicated in the tumorigenesis of a variety of human cancers (10–15). Targeted inhibition of EGFR with mAb or small molecular kinase inhibitors, including Erbitux®...
and Iressa® (ZD1839), has shown great anticancer activity in a number of animal models as well as in various clinical studies (for reviews, see Refs. 16–20). Similarly, significant tumor inhibition has also been achieved in animal models with several EGFR targeting strategies including antisense oligonucleotides (21), dominate-negative receptor mutants (22), and neutralizing mAb (Refs. 23–25; for review, see Ref. 26). Using the variable domains of two neutralizing human antibodies as the “building blocks,” one directed against EGFR and the other against IGFR, we constructed and produced an IgG-like tetrameric BaAb, a so-called “Di-diabody.” The Di-diabody bound to both EGFR and IGFR, blocked the receptors from interacting with their respective ligands, and inhibited both EGFR- and IGF-stimulated activation of the receptors as well as the receptor-associated downstream signaling pathways. Further, the Di-diabody was able to trigger rapid and efficient IGFR internalization and degradation and mediate effective immune effector function such as antibody-dependent cellular cytotoxicity (ADCC). Finally, the Di-diabody strongly inhibited the growth of human tumor xenografts in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Proteins—**Human tumor cell lines, DiFi and HT29 (colorectal carcinoma), A431 (cervical carcinoma), MCF7 (breast carcinoma), and BxPC3 (pancreatic carcinoma) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (HyClone, Logan, UT) at 37°C in 5% CO2. Recombinant extracellular domain of EGFR and its ligand, EGF, and EGF were purchased from R & D Systems Inc. (Minneapolis, MN). Recombinant EGFR extracellular domain was produced at ImClone Systems Inc. (New York, NY). Identification from an antibody phage display library and production from mammalian cells of the anti-EGFR antibody, IMC-11F8, and the anti-IGFR antibody, IMC-A12, have been previously described (23, 27). IMC-C225, a clinical grade anti-EGFR antibody, and IMC-1121, a human anti-human vascular endothelial growth factor receptor 2 antibody (28), were produced at ImClone Systems Inc.

**Construction and Production of the Bispecific Di-diabody—**The variable light (VL) and the variable heavy (VH) domains of IMC-11F8 and IMC-A12 were cloned and assembled into two cross-over scFv fragments, A12VL-linker-11F8 VH and 11F8VL-linker-A12V VH, following a procedure previously described (29). A 5-amino acid sequence, Arg-Thr-Val-Ala-Ala, representing the first 5 residues from the N terminus of human κ light chain constant domain, was used as the linker (29). To construct the tetravalent bispecific Di-diabody, a gene encoding one of the “cross-over” scFv chains, 11F8VL-linker-A12V VH, was further fused on its C terminus by overlapping PCR to a gene encoding the Fc fragment (CH2-CH3 domains) of an IgG, via the hinge region, to form a fusion polypeptide, 11F8VL-linker-A12V VH, which was expressed in both E. coli and yeast cells. The fusion polypeptide chain was then solubilized into an expression vector along with its partner, the other cross-over scFv chain, A12VL-linker-11F8 VH (see Fig. 1 for details). The expression vector was transfected into NSO cells, followed by expression of the soluble Di-diabody in serum-free cell culture and purification of the antibody with protein A chromatography. The purity of the Di-diabody was assayed via SDS-PAGE analysis under both reducing and nonreducing conditions.

**Receptor Binding Assays—**Two different assays were carried out to examine specificity and efficacy of the Di-diabody. In the first assay, the cross-linking assay, the Di-diabody was tested for its capability in simultaneously binding two target antigens. Briefly, the Di-diabody or the monospecific antibodies (5 μM) were first incubated with a biotin-labeled IGFR (100 ng) in solution and then transferred to a microtiter plate coated with EGF (100 ng/well), followed by incubation with streptavidin-horseradish peroxidase (HRP) to measure the plate-bound biotin activity. In the second assay, the direct binding assay, various amounts of antibodies were added to triplicate wells of 96-well plates (Nunc, Roskilde, Denmark) precoated with human IGFR1 or EGFR extracellular domain (100 ng/well) and incubated at room temperature for 1 h, after which the plates were washed three times with PBS containing 0.1% Tween 20. The plates were then incubated at room temperature for 1 h with 100 μl of a rabbit anti-human IgG Fc-HRP conjugate (Jackson ImmunoResearch Laboratory Inc., West Grove, PA). The plates were washed, peroxidase substrate was added, and the absorbance at 450 nm was read following a previously described procedure (27).
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**RESULTS**

**Construction and Production of the Di-diabody**—The variable regions of two previously characterized antagonistic antibodies, the anti-EGFR IMC-11F8 and the anti-IGFR IMC-A12, were used as the “building blocks” to construct the IgG-like tetravalent BsAb, the Di-diabody. A bispecific diabody was first constructed, followed by fusion of one of the diabody cross-over scFv chains to the Fc domain of an IgG (see Fig. 1, A and B, for details). Coexpression in mammalian cells of the Fc fusion (the top band) along with the other cross-over scFv resulted in an IgG-like tetrameric molecule. L, linker (Arg-Thr-Val-Ala-Ala) H, the hinge region of an IgG1. Note that the drawings are not to scale. C and D, SDS-PAGE analysis of the purified Di-diabody preparations. The purified Di-diabody was analyzed by gel electrophoresis under both nonreduced (C) and reduced conditions (D). Lane 1, IMC-11F8 IgG; lane 2, the Di-diabody. Also shown on the left side are the molecular mass standards.

**In Vivo Efficacy Studies**—Female athymic nu/nu mice, 6–8 weeks of age (Harlan Sprague-Dawley, Inc., Indianapolis, IN), were injected subcutaneously on the lateral dorsal surface with BxPC3 (2 × 10⁶/mouse) or HT-29 (5 × 10⁶/mouse) tumor cells. When tumors reached ~20 mm³, mice were randomized by tumor size and divided into treatment groups. The mice were treated by IMC-11F8 (or the equivalent IMC-C225), IMC-A12, IMC-11F8 (or IMC-C225) plus IMC-A12, the Di-diabody, or the control articles (saline or normal human IgG). Each antibody was administered by intraperitoneal injections at 40 mg/kg (or 80 mg/kg for the Di-diabody in the HT29 model) twice a week. Tumor growth was evaluated twice weekly with tumor volume calculated as: \( \text{volume} = \frac{1}{2} \times \text{length} \times \text{width}^2 \), where length = longest diameter and width = diameter perpendicular to length. Statistical analysis of tumor growth inhibition was performed using a Repeated Measures analysis of variance program (JMP 5.0.1).

The Di-diabody Blocks Signaling Pathways Stimulated by Both EGFR and IGF—The Di-diabody was examined on its efficacy in blocking EGFR- and IGF-stimulated receptor phosphorylation and downstream signal transduction. Although incubation of MCF-7 cells with individual growth factor, EGFR or IGF, results in significant levels of phosphorylation of the respective receptor, combination of EGFR and IGF yields activation of both EGFR and IGFR (Fig. 4, lanes 2–4). As expected, when the tumor cells were stimulated with both EGFR and IGF, treatment with either IMC-A12 or IMC-11F8 only inhibited phosphorylation of the individual receptor (Fig. 4, lanes 6 and 8).

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Fig. 2. Bispecific and dose-dependent binding of the Di-diabody to EGFR and IGFR. A, receptor cross-linking assay. Various antibody preparations were first incubated with a biotin-labeled IGFR in solution and then transferred to a microtiter plate coated with EGFR, followed by incubation with streptavidin-HRP to measure the plate-bound biotin activity. B and C, dose-dependent binding to immobilized EGFR and IGFR by the Di-diabody. Various amounts of antibodies were added to 96-well plates coated with human EGFR (B) or IGFR extracellular domain (C) and incubated at room temperature for 1 h, after which the plates were washed three times with PBS containing 0.1% Tween 20. The plates were then incubated at room temperature for 1 h with a rabbit anti-human IgG Fc-HRP conjugate. The plates were washed, peroxidase substrate was added, and OD$_{450}$ nm was read. The data shown are the representative of three similar experiments and are the means ± S.D. of triplicate samples.

Fig. 3. Anti-proliferative activity of the Di-diabody. DiFi cells in complete medium were seeded in 96-well plates and cultured overnight. Various amounts of the antibodies were added into the culture and incubated with the cells for 4 days, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added and incubated for additional 4 h. The plates were washed twice with PBS and incubated with HCl/isopropanol at room temperature for 10 min, followed by optical density reading at 570 nm. The data shown are the representative of three similar experiments and are the means ± S.D. of triplicate samples.

In contrast, the Di-diabody, like the mixture of both IMC-11F8 and IMC-12, significantly blocked activation of both receptors (Fig. 4, lanes 5 and 8). The control antibody, IMC-121, showed no effect on the phosphorylation of EGFR and IGFR (Fig. 4, lane 9).

The effect of IMC-11F8, IMC-A12 and the Di-diabody on the two major downstream signal transduction molecules associated with both EGFR and IGFR, Akt and p44/p42 MAP kinases, were also studied in MCF-7 cells (Fig. 4). Stimulation with IGF results in significant phosphorylation of Akt (Fig. 4, lane 2), whereas EGF causes strong phosphorylation of p44/p42 MAPK (Fig. 4, lane 3). As expected, combination of IGF to EGF leads to activation of both Akt and p44/p42 MAPK (Fig. 4, lane 4). In the presence of both EGF and IGF, IMC-11F8 significantly inhibited the activation of MAPK but only moderately reduced the activation of Akt (Fig. 4, lane 7), whereas IMC-A12 strongly reduced Akt phosphorylation but was less effective in p44/p42 MAPK activation (Fig. 4, lane 6). Similar to the observation with the receptors, a combination of IMC-11F8 and IMC-A12, either as a mixture or as the Di-diabody, effectively blocked phosphorylation of both Akt and p44/p42 MAPK induced by EGF and IGF (Fig. 4, lanes 5 and 8).

The Di-diabody Triggers Efficient IGFR Internalization and Degradation—We previously demonstrated that IMC-A12 was capable of down-regulating tumor cell surface expression of IGFR by inducing rapid and efficient receptor internalization and degradation (23). Here we investigated whether the Di-diabody retained the receptor modulation activity of IMC-A12 on tumor cells. Consistent with our previous observation, IMC-A12 triggered significant IGFR internalization and degradation in MCF-7 cells after incubation at 37 °C for 4 h (Fig. 5, A and B). Incubation with the Di-diabody led to significant degradation of IGFR in both MCF-7 and BxPC3 cells (Fig. 5). This IGFR modulation effect of the Di-diabody is both dose-depend-
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**Fig. 4.** The Di-diabody inhibits both EGF- and IGF-stimulated activation of receptors and downstream signaling molecules, Akt and MAPK p44/p42. MCF-7 cells were first incubated with various antibodies at 37 °C for 30 min, followed by stimulation with EGF, IGF, or both at 37 °C for 20 min. Phosphorylation of EGFR, IGFR, as well as Akt and MAPK p44/p42 was analyzed following the procedure described under “Experimental Procedures.” Lane 1, no treatment; lane 2, IGF alone; lane 3, EGF alone; lane 4, IGF plus EGF; lanes 5–9, IGF plus EGF in the presence of the Di-diabody (lane 5), IMC-A12 (lane 6), IMC-11F8 (lane 7), IMC-A12 plus IMC-11F8 (lane 8), or IMC-1121 (a control antibody that does not bind to EGFR and IGFR) (lane 9). The results shown are the representative of three separate experiments. Similar results were also observed when BxPC3 and HT29 cells were used as the target cells.

**Fig. 5.** The Di-diabody triggers efficient IGFR internalization and degradation in tumor cells. Tumor cells were plated and incubated overnight in serum-free medium. IGF-1 (50 nM), EGF (50 nM), IGF plus EGF (I+E), or various antibodies was then added and incubated at 37 °C for up to 4 h. The cells were washed in ice-cold PBS, lysed, and electrophoresed using 4–12% Tris-glycine gels. The proteins were transferred to nitrocellulose membranes and detected by Western blotting using antibody C-20 (to IGFR) and IMC-11F8 (to EGFR), followed by an anti-rabbit (for C-20) or anti-human (for IMC-11F8) antibody-HRP conjugate. The signals were visualized with the ECL reagent. A, the Di-diabody induced IGFR degradation in a dose-dependent manner in MCF-7 cells. B, the Di-diabody (100 nM) induced IGFR degradation as efficiently as IMC-A12 and the combination of IMC-A12 and IMC-11F8 after 4 h of incubation at 37 °C. C, the Di-diabody (100 nM) induced IGFR degradation in BxPC3 cells in a time-dependent manner when incubated at 37 °C. DAb, the Di-diabody; A+F, IMC-A12 plus IMC-11F8; 11F8, the control antibody, IMC-1121. The data shown are representative of three similar experiments.

The Di-diabody Mediates Effective ADCC on Tumor Cells—In addition to blocking growth signals by interfering with growth factor/receptor interaction and down-regulating receptor surface expression, antitumor IgG antibodies can also cause direct tumor cell killing via mediating effective ADCC. Here we examined whether the Fc-containing Di-diabody is capable of mediating tumor cell killing in the presence of human effector cells. As shown in Fig. 6, IMC-11F8 showed good lysis activity to both A431 and BxPC3 cells but was ineffective to MCF-7 cells. On the other hand, IMC-A12 was only effective toward MCF-7 cells but failed to kill A431 and BxPC3 cells. The Di-diabody, similar to the combination of both IMC-11F8 and IMC-A12, was equally potent in mediating cell killing to all three tumor lines.

The Di-diabody Inhibits the Growth of Human Tumor Xenografts in Nude Mice—We previously showed that both IMC-A12 and IMC-11F8, when used alone, were very efficacious agents in inhibiting the growth of human tumor xenografts in nude mice (23, 30). Here we compared the antitumor efficacy of the Di-diabody with its parent antibodies, alone and in combination. In these models, nude mice bearing established xenografts of ~200–300 mm³ were treated with the various antibodies twice a week by intraperitoneal injections. In the first model of BxPC3 pancreatic tumor xenografts (Fig. 7), both IMC-11F8 and IMC-A12 alone (at 40 mg/kg) yielded significant (77 and 58%, respectively; p < 0.05 compared with the PBS group, p > 0.05 between IMC-11F8 and IMC-A12) tumor growth inhibition at 6 weeks post-treatment initiation. The Di-diabody (at 40 mg/kg) demonstrated similar antitumor activity (52% tumor growth inhibition, p < 0.05 compared with the PBS group, p > 0.05 between IMC-11F8 and IMC-A12) tumor growth inhibition in 6 weeks post-treatment initiation. The Di-diabody (at 40 mg/kg) demonstrated similar tumor lysis activity (52% tumor growth inhibition, p < 0.05 compared with the PBS group, p > 0.05 among the Di-diabody, IMC-11F8 and IMC-A12) to the individual parent antibodies when given at the same dose (40 mg/kg). Combination of both IMC-11F8 and IMC-A12 (at 40 mg/kg of each antibody) resulted in the best antitumor activity (>90% tumor growth inhibition) among the groups (Fig. 7; p < 0.05 compared with the Di-diabody or the individual antibody group).

In the second xenograft model, a colorectal carcinoma cell line, HT29, was used. HT29 xenografts were less responsive to individual anti-EGFR and anti-IGFR antibody therapies (Fig. 7). Treatment with either IMC-A12 or IMC-C225, an anti-EGFR antibody that is functionally equivalent to IMC-11F8 regarding both in vitro and in vivo antitumor activity (31), resulted in tumor inhibition of 47 and 35% at day 40 post-
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**FIG. 6.** The Di-diabody mediates an effective ADCC activity in the presence of human peripheral mononuclear cells. Tumor cells were incubated in the wells of 96-well plates with IMC-11F8, IMC-A12, the Di-diabody, or a normal human IgG at 37 °C for 30 min, followed by the addition of the effector cells at an effector/tumor cell ratio of 100/1 and incubation at 37 °C for an additional 4 h. After centrifugation at 1500 rpm for 10 min, 100 μl of supernatant was transferred to 96-well flat bottom plates, followed by the addition of 100 μl/well lactate dehydrogenase assay reagent and a reading of the absorbance at 490 nm. The percentages of specific cell lysis were calculated as described under “Experimental Procedures.” The data shown are the representative of three similar experiments and are the means of triplicate determinations.

**DISCUSSION**

Compelling evidence suggest that both EGFR and IGFR play important roles in the growth and progression of a variety of human cancers; thus they may represent excellent targets for effective cancer intervention. In fact, consistent antitumor effects have been observed both experimentally and clinically with a number of strategies that antagonize either individual receptor activity, including the use of antagonistic antibodies and small molecular tyrosine kinase inhibitors (16–26). We hypothesize that a strategy that targets both EGFR and IGFR simultaneously, by using either a combination of two antagonistic antibodies or a BsAb, may yield greater antitumor activity than other approaches that address only a single receptor.

In our previous study we produced a BsAb, using IMC-11F8 (anti-EGFR) and IMC-A12 (anti-IGFR) as the building blocks, in a (scFv)2-IgG format (27, 32) and demonstrated that the BsAb was as potent as the combination of IMC-11F8 and IMC-A12 in neutralizing both EGFR- and IGF-stimulated receptor activation and downstream signal transduction (27). The BsAb was, however, difficult to produce in mammalian cells because of its low level of expression, thus preventing any further studies especially the in vivo animal testing. Here we engineered a novel IgG-like BsAb, the Di-diabody, that can be efficiently produced in mammalian cells and be purified by conventional protein A chromatography in a single step. This Di-diabody format should be readily applicable to the construction of other BsAb from antibodies recognizing any pairs of antigens.

In this study we demonstrated that simultaneous blockade of both EGFR and IGFR activation, either by a combination of an anti-EGFR and an anti-IGFR antibody or by a BsAb may lead to broader and enhanced antitumor activity. Tumor cells may gain their growth advantage and/or resistance to apoptosis by (over)expressing a number of growth factor receptors including EGFR and IGFR (10–15). Binding of ligands/growth factors leads to receptor activation and downstream signal transduction, resulting in cell proliferation, invasion, and increased resistance to apoptosis (10–15). Because of the redundancy of growth signaling pathways in tumor cells, inhibition of one receptor function (e.g. EGFR) could be effectively compensated by up-regulation of other growth factor receptor (e.g. IGFR)-mediated pathways. For example, a recent study has shown that malignant glioma cell lines expressing equivalent EGFR had significantly different sensitivity to EGFR inhibition depending on their capability of activating IGFR and its downstream signaling pathways (33). Other studies have also demonstrated that overexpression and/or activation of IGFR in tumor cells might contribute to their resistance to chemotherapeutic agents, radiations, and antibody therapy (34–37), and consequently, inhibition of IGFR signaling has resulted in increased sensitivity of tumor cells to these therapeutic agents (38, 39). Taken together, these observations strongly suggest that simultaneous blockade of both EGFR and IGFR may provide significant antitumor benefits over individual receptor-targeted therapies. Here we showed that the Di-diabody almost completely inhibited, as efficient as the combination of IMC-11F8 and IMC-A12, both EGFR and IGF-stimulated activation of EGFR and IGFR, as well as the downstream signaling molecules, including Akt and MAPK p44/p42 (Fig. 4). In contrast, treatment with IMC-A12 or IMC-11F8 alone only inhibited the activity of its respective receptor and receptor-associated signaling molecules (Fig. 4). When tested in vivo, the combination of IMC-11F8 and IMC-A12 yielded better antitumor activity than did each individual antibody in both BxPC3 and HT29 xenografted models, demonstrating the benefits of dual receptor targeting. Although less potent than IMC-11F8 in inhibiting EGFR-dependent tumor cell proliferation in vitro, the Di-diabody was equally efficacious to IMC-11F8 in vivo in BxPC3 xenografts and showed a trend of enhanced antitumor activity (similar to the combination of IMC-11F8 and IMC-A12) in the more antibody-resistant HT29 xenografts. The levels of receptor expression, the activation status of the receptor, and its downstream signaling pathways may be partly responsible for the different sensitivities of various tumor xenografts to antibody (alone and in combination) and Di-diabody therapies (40, 41). Taken together, these results indicate that the anti-EGFR × anti-IGFR Di-diabody may represent a novel and powerful approach to more effective cancer treatment with a broad antitumor spectrum and thus may be applicable to tumors with etiology based on EGFR, IGFR, or both.

In addition to direct blockade of growth factor/receptor interactions and inhibiting the activation of the subsequent signaling cascades, several other mechanisms of action may also play important roles in the antitumor activity of anti-receptor antibodies. Binding of anti-receptor antibodies to the tumor cell surface may trigger receptor internalization followed by degradation of the receptor in lysosome and/or proteasome compartments, leading to down-regulation of receptor expression, and ultimately, cell growth inhibition and/or apoptosis. In addition, antibodies may also recruit host effector mechanisms, such as ADCC and complement-mediated cytotoxicity, via its Fc region, to directly kill target tumor cells. Our Di-diabody inherited from its parent antibody, IMC-A12, the capability of inducing rapid and efficient IGFR down-regulation, and from both IMC-11F8 and IMC-A12, the efficacy in mediating ADCC activity toward tumor cells that express either EGFR or IGFR. It is
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like BsAb, these fragments are, however, incapable of promoting effector function such as ADCC. Here we described a novel approach to the efficient production of an IgG-like BsAb, the Di-diabody. There are several noteworthy characteristics associated with this BsAb molecule. Firstly, each Di-diabody is a tetravalent molecule comprising two binding sites to each of its targets. Bivalency in some instance may be required for certain antibodies to exert their therapeutic functions, e.g., cross-linking the receptors on target cell surface to stimulate activation, to induce apoptosis, or to promote receptor internalization (as in the IGFR case). In addition, antibody bivalency usually leads to higher binding avidity that is often desirable and may even be necessary for each arm of a BsAb destined for human therapy to demonstrate its activity (49). Secondly, the Di-diabody con-

tains a full IgG Fc region and possesses approximately the same molecular mass as an IgG (150 kDa). Compared with the other Di-diabody format we reported earlier in which the dia-

body was fused to the CH3 domain only (43–44), only limited success has been achieved in the past years in both engineering and production of full-length IgG-like BsAb (27, 32, 45–47, for review, see Ref 48). BsAb fragments are smaller than full-length IgGs, so they have bet-

ter solid tumor penetration rates, but their small size and lack of an intact Fc also results in their being cleared rapidly from circulation, leading to a short half-life. Further, BsAb fragments do not require glycosylation, so they can be produced in high yield in bacteria. Compared with the full-length IgG-

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body was fused to the CH3 domain only (i.e., no CH2 domain) of the Fc region (50), the full Fc-containing new format confers the Di-diabody not only a long serum half-life but also the capability of supporting secondary immune function, such as ADCC (Fig. 6). The Di-diabody had a clearance half-life in nude mice of ~7 days (as determined by an anti-human Fc enzyme-

linked immunosorbent assay), which is comparable with that of an IgG (for example, both IMC-11F8 and IMC-A12 demon-

strated a half-life in nude mice of ~5 days). The Fc region also allows the Di-diabody to be purified, like other IgG mAb prod-

cuts, directly via conventional protein A affinity chromatogra-

phy. Finally, the Di-diabody can be efficiently produced in mammalian cells at a high expression level. The Di-diabody is being routinely produced at 300–400 mg/liter from a stable NS0 cell line cultured under unoptimized conditions in a spinner bioreactor. In contrast, our previous version of the antiEGFR × anti-IGFR BsAb, the (scFv)4-IgG construct (27), expressed poorly in mammalian cells and presented significant challenges to the production of sufficient material for in vivo testing.

A number of issues associated with the Di-diabody construct remain, however, to be further addressed. First, in addition to the active tetravalent BsAb, there is evidence of production of nonactive scFv-Fc dimer that is devoid of the other cross-over scFv polypeptide. Careful control of the balance of production of both the scFv-Fc fusion and the partnering cross-over scFv, for example via genetic manipulation of the expression vector, to favor the formation of the tetravalent molecule may reduce the secretion of the nonactive component. Second, although the Di-diabody maintained its binding activity to both EGFR and IGFR when incubated in vitro in mouse serum at 37 °C for up to 7 days (not shown), the molecule appeared to be much less stable in vivo. For example, although both circulating IMC-
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11F8 and IMC-A12 retained >90% of their antigen binding activity 7 days after administration in mice, the Di-diabody appeared to lose its activity starting at 6 h post-injection; it retained >90, 30–40, and <10% of binding efficiency to both EGFR and IGFR at 6, 24, and 168 h post-administration, respectively. Based on the fact that there is no covalent linkage between the two polypeptides forming the bispecific diabody on each arm of the Di-diabody (Fig. IA), this loss of antigen binding activity in vivo is most likely a result of dissociation of the two polypeptides in the circulation followed by the rapid clearance of the smaller cross-over scFv fragment (~25 kDa) through the kidney, leading to the production and circulation of the nonfunctional half-molecule, the scFv-Fc dimer. This also explains the apparent discrepancy between the physical half-life (i.e. ~7 days) and the biological half-life (i.e. <24 h) of the Di-diabody in vivo, because the nonfunctional scFv-Fc was detected by the anti-human Fc enzyme-linked immunosorbent assay (used for the determination of the physical half-life) but not by the receptor-binding enzyme-linked immunosorbent assay (used for the determination of the biological half-life). Structural modifications in the Fv interfaces between each cognate VL and the VH domains within the diabody molecule, for example the installation of new disulfide bonds (51), may offer an efficient solution to enhancing the stability of the Di-diabody. It is plausible that a more stable Di-diabody should lead to an improved pharmacokinetics in vivo, hence enhanced antitumor activity.

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A Fully Human Recombinant IgG-like Bispecific Antibody to Both the Epidermal Growth Factor Receptor and the Insulin-like Growth Factor Receptor for Enhanced Antitumor Activity

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