Cancer Chemother Pharmacol (2000) 46 (Suppl): S8–S12

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Recombinant anti-carcinoembryonic antigen antibodies for targeting cancer

Abstract Antibodies can be used to target cancer therapies to malignant tissue; the approach is attractive because conventional treatments such as chemo- and radiotherapy are dose limited due to toxicity in normal tissues. Effective targeting relies on appropriate pharmacokinetics of antibody-based therapeutics, ideally showing maximum uptake and retention in tumor and rapid clearance from normal tissue. We have studied the factors influencing these dynamics for antibodies against carcinoembryonic antigen (CEA). Protein engineering of anti-CEA antibodies, in vivo biodistribution models, and mathematical models have been employed to improve understanding of targeting parameters, define optimal characteristics for the antibody-based molecules employed, and develop new therapies for the clinic. Engineering antibodies to obtain the desired therapeutic characteristics is most readily achieved using recombinant antibody technology, and we have taken the approach of immunizing mice to provide high-affinity anti-CEA single-chain Fv antibodies (sFvs) from filamentous bacteriophage libraries. MFE-23, the most characterized of these sFvs, has been expressed in bacteria and purified in our laboratory for two clinical trials: a gamma camera imaging trial using ^{123}I-MFE-23 and a radioimmunoguided surgery trial using ^{125}I-MFE-23, where tumor deposits are detected by a hand-held probe during surgery. Both these trials showed that MFE-23 is safe and effective in localizing tumor deposits in patients with cancer. We are now developing fusion proteins that use the MFE-23 antibody to deliver a therapeutic moiety; MFE-23:: carboxypeptidase G2 (CPG2) targets the enzyme CPG2 for use in the antibody-directed enzyme prodrug therapy system and MFE::tumor necrosis factor alpha (TNFα) aims to reduce sequestration and increase tumor concentrations of systemically administered TNFα.

Key words Antibody targeting · Cancer · Antibody-directed enzyme prodrug therapy · Carcinoembryonic antigen · Single-chain Fv antibodies · Fusion protein

Work presented at the 15th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium, "New Immunological Approach to Cancer Treatment," 10–11 September 1999, Nagoya, Japan

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Introduction

Antibodies are highly specific recognition molecules which have great potential for targeting cancer therapies to malignant tissue when the appropriate delivery systems are optimized. We have investigated the parameters for developing such systems using antibodies directed against carcinoembryonic antigen (CEA), an oncofetal antigen which, with highly specific antibodies, is only detectable on tumors and the luminal surface of the gut. CEA is highly expressed in most gastrointestinal carcinomas and in a number of breast, lung, and ovarian carcinomas. Originally, chemically modified antibody fragments were made to facilitate these studies [17] but later work has focused on using recombinant molecules. Recombinant antibody fragments have many advantages over chemically modified fragments. There is no requirement for complex manipulation after production. They are readily defined at a molecular level and selected or genetically engineered to give the required antigen-binding characteristics. They can be produced in bacteria or yeast, which is comparably cheap and rapid and circumvents the problem of contamination with mammalian or viral DNA. This is particularly important for clinical use.

Single-chain Fv antibody fragments (sFvs) consist of the variable heavy and variable light chain regions tethered by a flexible linker. Since they are expressed as a

single protein they are ideal building blocks for fusion proteins with therapeutic as well as tumor-localizing properties. Another attractive feature of this approach is that recombinant Fabs and sFvs can be displayed in functional form on the surface of filamentous phage, and this technology may be used to generate and select for antigen binders with the desired characteristics [30].

The phage approach is much more efficient than the screening approach used in hybridoma technology; while hybridoma screening allows investigation of 10^2 to 10^3 clones, phage technology selects antibodies with the desired characteristics from 10^{10} or more clones. Pragmatically, the phage selection process is also advantageous as it favors stable antibodies that give high yields when expressed in bacteria. Phage technology was used to produce MFE-23, an sFv antibody with high affinity for CEA that localized to colorectal tumor xenografts in experimental in vivo models [11].

Clinical trials with MFE-23

Guidelines for the preparation of products derived from recombinant DNA technology for phase I trials were developed in the form of a Cancer Research Campaign Operational Manual [5]. These were subsequently applied to the production of the bacterial seed lot containing MFE-23 in a pUC 119 plasmid vector in *Escherichia coli* TG1 cells, and to the expression and purification of MFE-23 from that seed lot [18]. Purification of MFE-23 for the clinic was performed by immobilized metal-affinity chromatography and size exclusion [10].

Ten patients received ^{123}I-MFE-23 prior to single-photon emission computerized tomography imaging [6]. The median half-lives of the α and β phases of clearance from blood were 0.42 and 5.32 h, respectively. The antibody was stable in plasma and most of the antibody cleared from the circulation within 1 h. MFE-23 localized in all known tumor deposits and the ability of ^{123}I-MFE-23 imaging to be more sensitive than diagnostic technology of similar invasiveness was illustrated. The median peak tumor uptake of 2.4% of the injected radioactivity per kilogram occurring 1 h after injection was unexpectedly high in view of the rapid fractional blood clearance of MFE-23, but is consistent with good tumor penetration and efficient antigen binding of the sFv. This was achieved with median tumor to blood ratios of 1.54 by 4 h and 5.6 after 22 h. MFE-23 is the first sFv to be used in a clinical radioimmunoimaging trial. The antibody injection was well tolerated by all patients, and there was no evidence of anti-MFE-23 antibody formation in patients after 2 weeks. High renal uptake, thus limiting detection of small tumors in this region, was found to be one of the shortcomings in this trial.

A subsequent trial used ^{125}I-labeled MFE-23 for radioimmunoguided surgery (RIGS) of colorectal cancer. RIGS is based on the preoperative injection of a radiolabeled antibody and intraoperative use of a hand-held gamma-detecting probe. The technique has the advantage that the proximity of the probe exploits the inverse square law and therefore has the potential to detect small tumor deposits. Thirty five patients with primary or metastatic colorectal or pancreatic cancer received ^{125}I-labeled MFE-23 at 24, 48, 72, and 96 hours prior to surgery. Counts three standard deviations above normal tissue were regarded as positive, and results of probing were compared with histology for validation. Laboratory gamma counting acted as quality control and allowed calculation of the percentage of injected dose per kilogram of tissue, tumor to normal tissue ratio, and tumor to blood ratio. Preliminary results for MFE-23 showed an accuracy of 88% (79% true positive and 9% true negative) at the main tumor site [8].

Multivalent molecules

Effective targeted therapy relies on efficient antibody retention in tumor after clearance from normal tissue. This residence time is primarily influenced by the intrinsic affinity (monovalent) and functional affinity (multivalency or avidity) of the antibody for its antigen. We have not formally investigated the effect of intrinsic affinity on localization of our anti-CEA fragments but the importance of increased avidity has been demonstrated with chemically modified fragments. Compared to monovalent antibodies, the divalent Di-Fab′-maleimide (DFM) and trivalent Tri-Fab′-maleimide (TFM) delivered more radioactivity to tumor and had better tumor to blood ratios than IgG [9, 15]. In addition, recent work using a model based on data from biodistribution studies has confirmed that increasing avidity prolongs residence time in tumor and improves therapy [14]. Perhaps more importantly, avidity also influences the distribution within the tumor mass. A quantitative comparison of images of antibody distribution and tumor morphology has shown that tumor-specific antibodies preferentially localize in viable, sensitive tumor areas, whereas nonspecific antibodies are not retained in viable tumor and penetrate into the necrotic regions [13]. Further analysis has shown that by increasing avidity, the amount of antibody and its residence time are significantly improved in viable areas (Fig. 1). This can lead to a more than four-fold increase in antibody concentration in viable compared with necrotic areas and can signify a distinct therapeutic advantage over less avid antibodies. This knowledge has formed our thinking about the design of multivalent recombinant antibody-based molecules and led to the choice of divalent and trivalent molecules for therapy, as described below.

Antibody-directed enzyme prodrug therapy

Antibody-directed enzyme prodrug therapy (ADEPT) is a two-stage system for treatment of solid tumors. First,

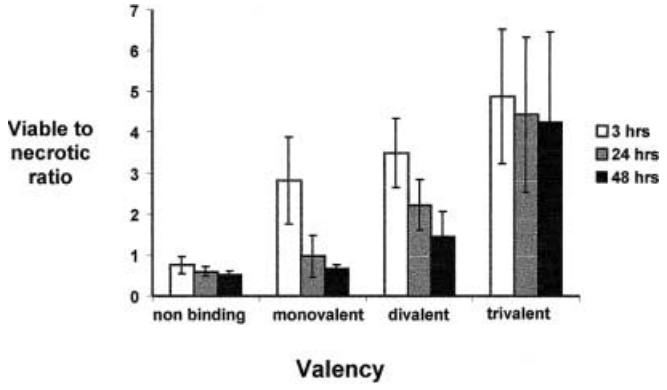

Fig. 1 Amount of antibody in viable parts of the tumor relative to necrotic areas. These regions were delineated using image registration which allows correlation of radiolabeled antibody distribution with tumor morphology [13]. The increase in avidity obtained with increasing valency leads to higher functional affinity and better retention in viable parts. The group with zero avidity contained pooled data from non-CEA-specific whole IgG antibodies. The monovalent group contained pooled data from MFE-23 and A5B7-Fab. The divalent group contained pooled data from A5B7-IgG, A5B7-F(ab′)$_2$, and DFM. The trivalent antibody was TFM

intravenously administered antibody delivers an enzyme selectively to a tumor deposit. When there is high tumor to plasma ratios of enzyme, a relatively nontoxic prodrug is administered systemically. The prodrug is catalyzed by the prelocalized enzyme at the tumor site to produce a potent cytotoxic agent. The active drug diffuses throughout the tumor mass, killing cells expressing tumor antigen and neighboring antigen-negative tumor cells.

Carboxypeptidase G2 (CPG2), a well-characterized, homodimeric, bacterial enzyme with no mammalian equivalent [21, 22, 26], has been exploited to cleave glutamic acid from a variety of prodrugs to release potent nitrogen mustards [28]. The F(ab′)$_2$ fragment of A5B7, an anti-CEA MAb conjugated to CPG2, has shown that CPG2 has great potential for ADEPT in model systems [23–25] and in the clinic [2–4]. However, a clearing antibody is needed to obtain high tumor to normal tissue ratios at early time points when the percentage of injected dose in tumor is at the highest. Furthermore, the system suffers from the complexity, expense, and difficulty of obtaining a reproducible product with chemical conjugation of the components [19].

It was proposed that genetic fusion protein of MFE-23 antibody and CPG2 would give a dimeric molecule with potentially superior targeting characteristics for ADEPT since recombinant fusion proteins can be reproducibly expressed and have the potential to be tailored to give optimal results and overcome hurdles such as immunogenicity and tumor penetration. MFE-23::CPG2 fusion protein was constructed, expressed in *E. coli*, and purified using CEA affinity chromatography [20]. The efficacy of MFE-23::CPG2 delivery to tumors in vivo was assessed by measuring CPG2 catalytic activity in excised tissue after intravenous injection of purified MFE-23::CPG2 into nude mice bearing CEA-positive LS174 T human colon adenocarcinoma xenografts [7]. Recombinant MFE-23::CPG2 cleared rapidly from the circulation, and catalytic activity in extracted tissues showed tumor to plasma ratios of 1.5:1 (6 h), 10:1 (24 h), 19:1 (48 h), and 12:1 (72 h). MFE-23::CPG2 catalytic activity was not retained in solid normal tissues, resulting in excellent tumor to tissue enzyme ratios 48 h after injection. These were 371:1 (tumor to liver), 450:1 (tumor to lung), 562:1 (tumor to kidney), 1,477:1 (tumor to colon), and 1,618:1 (tumor to spleen), as illustrated in Fig. 2. The favorable tumor:normal tissue ratios occurred at early time points when there was still 21% (24 h) and 9.5% (48 h) of the injected activity present per gram of tumor tissue (Fig. 2).

These results show that MFE-23::CPG2 delivers satisfactory quantities, with high tumor to normal tissue ratios, of CPG2 activity after a single injection with

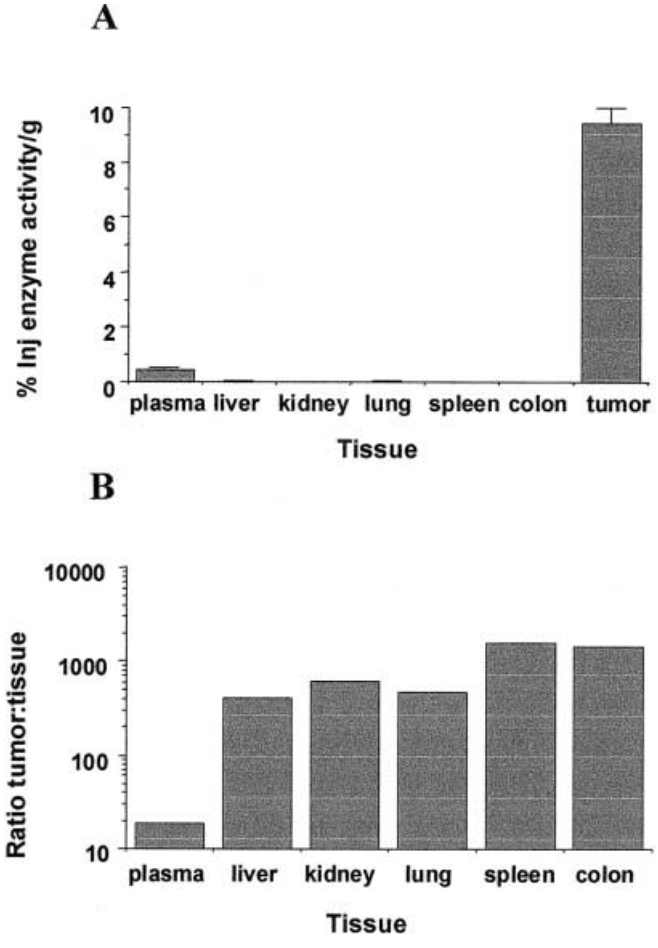

Fig. 2 Biodistribution of MFE-23::CPG2 in nude mice bearing LS147 T xenografts. Four mice received 25 U (176 mg) of MFE-23::CPG2 by intravenous injection. Animals were killed and CPG2 catalytic activity assessed 48 h later. Enzyme activity in plasma was assessed by spectrophotometry [21]. Liver, kidney, lung, colon, muscle, and tumor were homogenized and enzyme activity measured by high-performance liquid chromatography [16]. **A** Percentage injected activity per gram of tissue. **B** Tumor to tissue ratios of activity determined from results shown in **A**

no secondary clearance system in mice bearing human colorectal tumor xenografts. The high tumor concentrations and selective tumor retention of active enzyme establish that this recombinant fusion protein has potential to give improved clinical efficiency for ADEPT.

Reduced immunogenicity

Immunogenicity has been a major problem in ADEPT using CPG2 chemically conjugated to A5B7-F(ab′)$_2$. Treatment has so far been limited to two to three cycles within two weeks under cover of an immunosuppressive agent (cyclosporin) [1]. As part of our program to identify and modify immunogenic sites on CPG2, a phage library of sFv antibodies to CPG2 has been constructed from immunized mice. Different sFvs reacting with the enzyme have been identified. One of these, CM79, inhibits a substantial part of the CPG2 binding of sera of patients with antibodies to the enzyme after receiving ADEPT. Surface-enhanced laser desorption/ionization affinity mass spectrometry (SELDI) was used to determine the amino acid sequences of the CPG2 immunogenic sites. Two peptides were found to form the epitope for CM79. Another sFv, CM12, also bound to a third peptide. We were able to localize these peptides on the X-ray crystal-derived structure of CPG2 and found that they were adjacent on the surface of the enzyme [27]. The immunogenicity of the MFE-23 moiety of MFE-23::CPG2 is still unknown. However, human antibodies have not been detected after injection of the sFv MFE-23 in the clinical trials described above, and this may be predictive of its behavior as part of MFE-23::CPG2.

Selective delivery of tumor necrosis factor alpha

A potentially even more avid fusion protein can be obtained when the therapeutic arm is tumor necrosis factor alpha (TNFα), a molecule that is trivalent in active form. To test this, the fusion protein MFE::TNFα has been expressed in bacterial inclusion bodies and successfully isolated, refolded, and purified with a final yield of 20 mg/L. MFE::TNFα monomer was shown to have an apparent molecular weight of 48 kD by Western blotting under reducing conditions, and the native molecule showed a first protein liquid chromatography elution profile consistent with trimer formation (144 kD). The molecule retained CEA-binding activity as assessed by enzyme-linked immunosorbent assay and was biologically active in cellular cytotoxicity assays for TNFα. Furthermore, radiolabeled MFE::TNFα localized to colorectal xenografts in nude mice [12]. Experiments are underway to ascertain whether all three MFE-23 moieties in MFE::TNFα are sterically able to bind CEA at a given time.

Gene therapy

sFv targeting of genes to tumor-associated antigens is potentially a powerful way to achieve safe and efficient therapeutic effects in cancer gene therapy. MFE-23, expressed in fusion with the envelope protein of a murine retrovirus (MFE-env), was able to target specific retroviral infection and deliver therapeutic genes to human, CEA-expressing, cancer cells in vitro [16]. This approach is still a long way from the clinic but illustrates the future potential for sFv-directed gene therapy.

Acknowledgements The authors would like to thank the Cancer Research Campaign and the Ronald Raven Chair in Oncology Trust, which supported the work.

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