Targeting and Therapy of Carcinoembryonic Antigen-expressing Tumors in Transgenic Mice with an Antibody-Interleukin 2 Fusion Protein

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ABSTRACT

The purpose of this study was to engineer a bivalent single-chain anticarcinoembryonic antigen (CEA) antibody and an interleukin 2 (IL-2) fusion protein derivative for selective tumor targeting of cytotoxins. The variable domains of a high affinity anti-CEA antibody, T84.66, were used to form a single-gene-encoded antibody [single-chain variable fragment joined to the crystallizable fragment, Fc (scFvFc)]. The fusion protein (scFvFc+IL-2) consisted of mouse IL-2-fused to the COOH-terminal end of the scFvFc. The engineered proteins were assembled as complete molecules and were similar to the intact anti-CEA monoclonal antibody (Mab) in antigen-binding properties. Based on IL-2 content of the fusion protein, its ability to support proliferation of CTLL-2 cells was identical with that of IL-2. Despite a molecular size similar to that of the intact Mab, the blood clearance of the fusion protein was markedly faster than that of the intact Mab or scFvFc. Incubation of radiolabeled scFvFc+IL-2 but not the intact or scFvFc antibodies in mouse serum was accompanied by the appearance of complexes, suggesting that the latter may contribute to the accelerated clearance of the fusion protein. Biodistribution and tumor targeting studies were carried out in CEA-transgenic mice bearing CEA-positive murine tumors as well as the antigen-negative parental tumor. The bivalent anti-CEA scFvFc had tumor localization properties similar to those of the intact Mab. Although fusion of IL-2 to the COOH-terminal end of the bivalent scFvFc altered its pharmacokinetic properties, the fusion antibody was able to target tumors specifically. Maximum uptake of the intact Mab, scFvFc, and scFvFc+IL-2 in CEA-positive tumors was 29.3 ± 5.0, 19.5 ± 2.1, and 6.6 ± 0.9% injected dose/g, respectively. Maximum tumor localization ratios (CEA-positive/CEA-negative tumor) were similar for all three antibody types (4.6–6.0), demonstrating the antigen specificity of the tumor targeting. Significant antigen-specific targeting to CEA-positive normal tissues of transgenic mice was not observed. Although the tumor-targeting properties of the fusion protein were low, the growth of CEA-expressing tumors was not antigen-relevant (P = 0.22) syngeneic tumor cells was inhibited after treatment of transgenic mice with the anti-CEA+IL-2 antibody. Therapy of CEA-expressing tumors was improved after i.v. administration of the fusion protein (P = 0.0001). These studies indicate that anti-CEA antibody-directed cytokine targeting may offer an effective treatment for CEA-expressing carcinomas. The availability of an immunocompetent CEA transgenic mouse model will also help to determine the immunotherapeutic properties of these fusion proteins.

INTRODUCTION

Although radiolabeled Mab3 may be considered one of the primary forms of immunomodulated tumor therapy (1), other methods may eventually prove more effective. One approach is to localize IL-2 to tumors by administering them joined to antibodies specific for tumor antigens (2–5). By altering the pharmacokinetics and biodistribution of IL-2, antibody-directed targeting of this cytokine could mitigate toxic side effects while enhancing IL-2 deposition at tumor sites. In animal studies, administration of IL-2 fusion proteins elicited and/or augmented antitumor responses presumably by increasing the local concentration of IL-2 (3, 6, 7). Alternatively, antibody-IL-2 fusion proteins, by their inherent tumor selectivity, can minimize normal tissue alterations of vascular permeability induced by IL-2 in efforts to augment tumor targeting of antibodies and other agents (8, 9). Studies of antibody-directed targeting of IL-2 have used conjugates prepared by chemical cross-linking (5, 10), but genetically engineered antibody-IL-2 fusion proteins have received more emphasis because of preparation ease (3, 4, 9, 11–13).

The expression of CEA in a majority of colon cancers (14) and in ~50% of breast (15) and lung (16) cancers has made it an attractive target for antibody-directed diagnosis and therapy. However, CEA is present in some normal tissues and is well expressed by the large bowel (17). In this study, we describe the generation of a genetically engineered bivalent single-gene-encoded antibody and its IL-2 fusion protein derivative. The tumor targeting properties of these antibodies were studied in an immunocompetent CEA transgenic mouse model (18). The antigen binding domains for the parent scFvFc antibody were derived from the high affinity anti-CEA murine antibody, T84.66 (19). Like humans, the transgenic line expresses CEA in the colon and thus provides a preclinical model more analogous to humans than the more commonly used animal hosts that lack normal tissue expression of the relevant antigen (20). It was found that the antigen-binding and blood clearance properties of the parent scFvFc antibody were relatively unaltered, and it retained its ability to target tumors specifically albeit at lower levels than the intact Mab. The colons from transgenic mice showed a marginal increase in accumulation of the scFvFc antibody compared with that appearing in non-transgenic colons. The antigen-binding and IL-2 activity properties of the fusion protein were preserved, whereas there was a marked decrease in tumor localization coincident with enhanced intravascular clearance. Despite the diminished tumor-targeting properties of the fusion protein, the growth of CEA-expressing but not antigen-relevant syngeneic tumor cells was inhibited after treatment with the anti-CEA+IL-2 antibody.

MATERIALS AND METHODS

DNA. The cDNAs encoding the light and heavy chains for the high affinity anti-CEA antibody T84.66, which has an IgG1 isotype, as well as the cDNA encoding the heavy chain for the anti-CEA antibody, T84.12, which has an IgG2a isotype, were used for fusion protein gene construction (19). The plasmid pEE12 was used as a mammalian expression vector to place cloned DNA under the control of a hCMV promoter (Lonza Biologicals, Slough, Berkshire). The plasmid pEE12 was used as a mammalian expression vector to place cloned DNA under the control of a hCMV promoter.

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3 The abbreviations used are: Mab, monoclonal antibody; IL-2, interleukin 2; BGP, biliary glycoprotein; EIA, enzyme-linked immunoassay; %ID, percent injected dose; l-MSX, l-methionine sulfoximine; scFv, single-chain variable fragment; scFvFc, scFv joined to the crystallizable fragment, Fc; Vκ, immunoglobulin light chain variable domain; Vκκ, immunoglobulin heavy chain variable domain; NHS, normal human serum.

United Kingdom (21). This plasmid also allows for colony selection based on glutamine synthetase activity. Mouse IL-2 cDNA (22) was obtained from the American Type Culture Collection (Manassas, VA). Oligodeoxynucleotides were synthesized by the City of Hope DNA synthesis facility with an Applied Biosystems 394 DNA RNA synthesizer.

**scFvFc Construction.** The light and heavy chain variable domains of T84.66 were joined by an 18-aminoc acid linker to form a scFv. This scFv sequence was then joined to a murine IgG2a constant region composed of hinge-CpG2C3 to form a scFvFc. An IL-2 fusion gene was created by joining the cDNA for murine IL-2 directly to the 3'-end of the scFvFc gene to form the scFvFc.IL-2 fusion gene.

For construction of the scFvFc, the T84.66 VLR was PCR amplified using the sense primer TCCCCGGGCGAGATGGACAGA and antisense primer TCCCCGGGCGAGATGGACAGAACA and were screened for the production of immunoglobulin by EIA. Selected clones were then subcloned and analyzed for expression of scFvFc.IL-2 fusion gene.

**Antigen Reactivity of Culture Supernatants and Purified Engineered Antibodies.** The antigen reactivity of culture supernatants and purified engineered antibodies was determined in a sandwich EIA. Goat antiserum against mouse IgG Fc antibody (250 ng/ml; Jackson ImmunoResearch, West Grove, PA) was coated overnight at 4°C in 0.1 M sodium bicarbonate, pH 9.6, onto polystyrene microtiter plates. Plates were then blocked for 1 h at room temperature with 5% BSA in coating buffer. After incubation at 37°C for 1 h with 10 μl of test sample or standard, plates were washed with PBS containing 0.05% Tween 20 and then exposed to 100 μl of alkaline phosphatase-labeled goat antiserum against mouse IgG Fc antibody (Jackson ImmunoResearch). After an additional 1-h incubation at 37°C, the plates were washed and then exposed to disodium 5-nitrophenyl phosphate (Sigma) in 1% (v/v) diethanolamine. The plates were read at 405 nm with a Bio-Rad Microplate reader. Standard curves were generated with purified hybridoma-derived Mab.

Antigen reactivity of culture supernatants and purified engineered antibodies was determined in competition EIA. Microtiter wells were coated with 250 ng/ml purified CEA coated in 0.1 M sodium bicarbonate, pH 9.6. After incubating with 0.5% BSA, 50 μl of standard sample were added to the wells, and the plates were incubated for 1 h at 37°C. Biotinylated hybridoma-derived T84.66 Mab (50 μl containing 50 ng) was then added, and incubation at 37°C for 1 h was continued. The T84.66 Mab was biotinylated with NHS-LC-biotin (Pierce, Rockford, IL) at 200 μl/m assay input ratio of biotin to antibody. The plates were washed, reacted with streptavidin-alkaline phosphatase conjugate (Jackson ImmunoResearch) for 1 h at 37°C and then exposed to disodium 5-nitrophenyl phosphate. The plates were read as described above.

**Measurement of Affinity Constants.** Antibody affinity (Kd = kassoc/kdiss) and rate constants were determined with a BIAcore 2000 (Pharmacia) biosensor. Data were analyzed with BIAevaluation software (version 3.1) using the biaalyte model under global conditions. NHS-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was used to attach the N-A3 two-domain CEA subunit (26) to F1 biosensor chips (Pharmacia). Fetusin was coupled to the sensor chip using NHS-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and was used for background subtraction. Antibody was applied to the chip at increasing concentrations (3–500 nM) with each concentration level followed by regeneration. A 20-min dissociation time was used to determine kDiss.

**Flow Cytometry.** Flow cytometry analysis was conducted with a FACSCalibur (Becton Dickinson, Mountain View, CA) as previously described (27).** IL-2 Assay.** The ability of the scFvFc.IL-2 fusion protein to support the proliferative activity of the IL-2-dependent cell line, CTLL-2 (American Type Culture Collection) was measured using either chromagrammetry using two 1-× 30-cm Superdex 200 columns in tandem (Pharmacia, Piscataway, NJ). Hybridoma-derived T84.66 was purified from ascites by affinity chromatography over a protein G immunoadsorbent (Pharmacia). The ascites was applied with 0.1 M phosphate buffer, pH 6.5; eluted with 0.1 M H3PO4, pH 2.2; and immediately neutralized with 1.0 M Tris, pH 8.0. The eluted intact Mab was dialyzed against PBS and concentrated. Purified antibody preparations were analyzed by SDS-PAGE. Protein content of purified antibody preparations was determined by amino acid analysis in the City of Hope Protein Chemistry facility.

**Enzyme Immunoassays.** The immunoglobulin content of culture supernatants was determined in a sandwich EIA. Goat antiserum against mouse IgG Fc antibody (250 ng/ml; Jackson ImmunoResearch, West Grove, PA) was coated overnight at 4°C in 0.1 M sodium bicarbonate, pH 9.6, onto polystyrene microtiter plates. Plates were then blocked for 1 h at room temperature with 0.5% BSA in coating buffer. After incubation at 37°C for 1 h with 100 μl of test sample or standard, plates were washed with PBS containing 0.05% Tween 20 and then exposed to 100 μl of alkaline phosphatase-labeled goat antiserum against mouse IgG Fc antibody (Jackson ImmunoResearch). After an additional 1-h incubation at 37°C, the plates were washed and then exposed to disodium 5-nitrophenyl phosphate (Sigma) in 1% (v/v) diethanolamine. The plates were read at 405 nm with a Bio-Rad Microplate reader. Standard curves were generated with purified hybridoma-derived Mab.

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Radioiodination of Antibodies. Proteins were radioiodinated to 5–10 μCi/μg using the IODO-GEN method (28). Briefly, 40 μg of protein were added to 40–80 μl of 0.1 M phosphate, pH 7.5, in a polypropylene tube coated with 20 μg of IODO-GEN (Pierce). Carrier-free 131I (500 μCi; ICN, Costa Mesa, CA) in the above buffer was allowed to react for 3 min at room temperature. Antibody-bound radioactivity was separated from free 131I by molecular sieve chromatography over a PD-10 column (Pharmacia) equilibrated in PBS containing 1% BSA. The purity of the each radioantibody was examined by molecular sieve chromatography using two Superdex 200 (1 × 30 cm; Pharmacia) columns connected in tandem. The immunoreactivity of the radioantibodies was evaluated with an immunoadsorbent containing CEA, whereas a BSA immunoadsorbent was used to determine nonspecific binding.

Pharmacokinetics and Biodistribution. Biodistribution and blood clearance studies were conducted in female and male CEA-transgenic mice with a C57BL/6 background (18). After i.v. injection, small volume (20 μl) blood samples from the tail vein were removed at various times (0.5, 1, 3, 6, 24, 48, 96 h). The %ID/g of blood for each time point was normalized to an initial sample removed immediately after injection of the radioantibody. Blood clearance curves were represented by biexponential functions using the ADAPT II software package (29). Two half-lives (t1/2α and t1/2β) and their associated amplitudes were estimated for each curve. For tumor localization, mice were implanted s.c. with MC-38 tumor cells in one flank, whereas MC-38.CEA cells were injected into the contralateral flank. Animals were injected with 1.0 × 106 MC-38.CEA tumors cells, followed in 2–3 days by injection with 0.5 × 106 parental MC-38 tumor cells. Differences in the in vivo growth rates of the parental MC-38 cells and the CEA-transfected clone necessitated injecting a different number of cells and delaying the time of injection of the former cells. Ten days after injection of MC-38.CEA cells, −2 μg (5–10 μCi of 131I) of radioantibody were administered by tail vein injection. Animals were anesthetized and exsanguinated by cardiac puncture at various times after injection (4, 8, 24, 48, 96 h). Tumor and reference normal tissues (liver, spleen, kidney, lung, stomach, colon, muscle) were removed and counted along with blood samples. Contents of the gastrointestinal tract were removed before counting. The radioactive content of tumor, tissues, and blood samples was expressed as %ID/g by dividing the measured count rate by the rate obtained counting. The radioactive content of tumor, tissues, and blood samples was expressed as %ID/g by dividing the measured count rate by the rate obtained counting. The radioactive content of tumor, tissues, and blood samples was expressed as %ID/g by dividing the measured count rate by the rate obtained counting.

Results were subjected to Student’s t test. In addition, a restricted/residual maximum likelihood-based repeated measure model (mixed model analysis) with various covariance structure was used. The latter procedure is a repeated measures analysis for correlated continuous outcome variables and is designed for longitudinal data analysis with multiple observable vectors for the same subject. SAS version 7.0 was used for all analyses.

RESULTS

Expression and Purification of scFvFc and scFvFc.Fc.II-2 Proteins. Transfection of NSO cells with the scFvFc gene using the pEE12-based glutamine synthetase expression vector system resulted in 73 wells with colonies. The supernatants from 71 (97%) of the latter colonies tested positive in assays detecting IgG or anti-CEA activity. Production of scFvFc protein by 13 clones with the highest activity ranged between 1.6 and 26.6 μg/l × 106 cells in 24 h (mean, 14.7 ± 5.8). One of the highest producing clones was recloned and used for further studies. From the transfection with the scFvFc.Fc.II-2 gene, 68 colonies were obtained, 55 (81%) of which were active in assays detecting IgG or anti-CEA activity. The expression of the scFvFc.Fc.II-2 protein by 11 clones with the highest activity ranged between 0.06 and 1.7 μg/l × 106 cells in 24 h (mean, 0.64 ± 0.60). The latter clones were placed in different concentrations of 1-MSX (10–50 μM) in an attempt to augment the expression of the scFvFc.Fc.II-2. Cell growth was obtained with all clones at either 10 or 20 μM 1-MSX concentrations, but none grew at higher drug levels. Of these, 6 clones showed a decrease in expression, 1 remained unchanged, whereas 4 others increased expression 2- to 4-fold. One scFvFc.Fc.II-2 clone that produced 2.3 μg/l × 106 cells in 24 h was selected for further study. Although only one transfection was conducted with each of the constructs, the lower expression of the scFvFc.Fc.II-2 gene suggests a decrease in its intracellular stability or secretion rate as compared with the parent scFvFc product.

For production, the selected clones were grown in medium lacking glutamine and supplemented with nondialyzed fetal bovine serum. Static cultures were grown to extinction, at which time the concentration of scFvFc and scFvFc.II-2 was ~80 and 10 μg/ml, respectively. After an initial purification on an antiidiotype affinity column, the scFvFc antibody contained <10% material that was of a larger molecular size than that of dimeric scFvFc as determined by size exclusion chromatography. By contrast, ~50% of the scFvFc.II-2 chromatographed as high molecular weight material (data not shown). Dimeric scFvFc.II-2 was obtained after purification by size exclusion chromatography. However, after storage of the dimeric fusion protein (1.4 mg/ml) in PBS at 10°C or −20°C for 4–6 weeks, the high molecular weight material reappeared. The latter occurred at a faster rate with storage at 10°C. Additional aggregation of the scFvFc over that observed after its initial purification did not appear. Despite the aggregation properties of the scFvFc.II-2, SDS-PAGE analysis showed that both the scFvFc and scFvFc.II-2 were assembled as dimeric molecules (Fig. 1A). The molecular weights of the scFvFc (M_r 106,774) and scFvFc.II-2 (M_r 145,501) were similar to that predicted by sequence information. In addition, a second band was variably observed at M_r ~129,000 for the scFvFc.II-2 (Fig. 1A). The nature of this smaller band is not known but its size is similar to a molecule that is missing 1 mol of IL-2. Analysis of reduced samples of the purified scFvFc and scFvFc.II-2 produced single bands that migrated at M_r ~53,000 and 71,000, respectively, which closely agrees with that predicted by sequence information (Fig. 1B).

Biological Properties of the scFvFc and scFvFc.II-2. Antigen-binding activities of the hybridoma-derived Mab and the two engineered antibodies were analyzed in competition ELISA assay using biotinylated hybridoma-derived Mab. As depicted in Fig. 2A, both the scFvFc and scFvFc.II-2 were slightly less active than the parent intact T84.66 Mab does not cross-react with the BGP CEA-related antigen. The 50% inhibition concentration (x 10^-50 μM) for the T84.66 Mab, scFvFc, and scFvFc.II-2 was 3.6, 6.4, and 19.3, respectively. Irrelevant mouse IgG1 (MOPC-21) failed to compete with the biotinylated T84.66 Mab at 100 μg/ml (data not shown). Analysis by surface plasmon resonance revealed that the K_off of the scFvFc.II-2 was 4–5 times lower than that of the intact Mab as a result of a faster off rate (Table 1). The magnitude of the decrease in CEA binding by the scFvFc.II-2 was similar when determined by ELISA assay or surface plasmon resonance. Because the off rates for the scFvFc and scFvFc.II-2 were identical, the addition of IL-2 to the
scFvFc does not appear to be responsible for the change in binding properties of the fusion protein.

The binding specificity of the engineered antibodies was analyzed by flow cytometry using mouse and human cell lines that were transfected with CEA. Fig. 2B compares the binding of T84.66 Mab and the engineered antibodies with the CEA-expressing MC-38 syn-

genic colon carcinoma cell line. The scFvFc and the scFvFc.IL-2 reacted with a similar percentage (>90%) of MC-38.CEA cells as that obtained with the intact Mab. The level of staining of the antigen-negative cells with all antibodies was the same as that obtained when MC-38.CEA cells were reacted with the irrelevant MOPC-21 control immunoglobulin (data not shown). Similar results were obtained with the CEA-transfected MDA-MB-231 breast carcinoma cells (data not shown). The intensity of staining with the engineered antibodies was slightly lower than that of the T84.66 Mab (Fig. 2B). The latter difference was most likely due to a variation in reactivity of the fluorescein-labeled antimouse IgG Fc second antibody between the IgG1 isotype of the T84.66 Mab and the IgG2a of the engineered antibodies.

Purified scFvFc.IL-2 was also analyzed for IL-2 biological activity in a cell proliferation assay. The concentration of IL-2 in the purified fusion protein was determined as 2 mol eq of IL-2 per mol of fusion protein. As depicted in Fig. 3, the IL-2 activity of the scFvFc.IL-2 was equal to that of comparable amounts of human IL-2. Thus, the fusion protein contained 3.978 IU IL-2 activity/mg. The parent scFvFc molecule did not have an effect on cell proliferation (not shown).

Size, Immunoreactivity, Serum Stability, and Pharmacokinetics of Radiolabeled scFvFc and scFvFc.IL-2. The scFvFc, scFvFc.IL-2, and native Mab were radiolabeled with 131I for in vivo studies. Analysis of the radiolabeled preparations by molecular sieve chromatography showed that >90% of the activity for each antibody type routinely eluted as a single peak in the expected size range (Fig. 4A).

As shown, both the native IgG (M_r ~150,000) and the scFvFc.IL-2 (M_r ~145,000) eluted slightly earlier than the scFvFc antibody (M_r ~106,000). Immunoreactivity of the labeled antibody preparations was tested using a CEA-containing immunoadsorbent. Between 85 and 95% of the activity associated with each of the three labeled antibody types bound to the latter immunoadsorbent (data not shown). Binding to a control immunoadsorbent containing BSA varied between 5 and 10% of the activity for each antibody.

The stability of all three radioantibody types was examined after incubation at 37°C in the presence of nontransgenic mouse serum or buffer. Sterile serum or buffer containing BSA was spiked with a trace amount of radiolabeled antibody, the samples were incubated for various times and then analyzed by molecular sieve chromatography. For the intact antibody or scFvFc, 85–95% of the radioactivity eluted at the expected molecular size whether they were incubated in serum...
or buffer and at all time points to 96 h. Representative profiles of the latter antibodies are shown in Fig. 4A. When radiolabeled scFvFc-IL-2 was studied, a different pattern emerged after incubation in serum in which there was a gradual appearance of activity that chromatographed at a higher molecular size than that of the dimeric antibody. A small amount (4% compared with 0 time point) of high molecular size activity was already evident at 0.5 h, which increased to 49% by 96 h (Fig. 4B). The high molecular weight activity did not appear when the scFvFc-IL-2 was incubated in buffer. The high molecular weight activity appearing in the latter preparation after incubation in serum was broadly distributed between the void volume and the dimeric antibody molecule, although the void volume peak became more pronounced at the latter time points (Fig. 4C).

The blood clearance properties of the three antibodies were examined in nontransgenic mice after i.v. injection. The blood clearance of the scFvFc-IL-2 was markedly faster than that of the T84.66 Mab with a $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 2.3 and 31.4 h, respectively, for the fusion protein compared with 5.0 and 101 h for the Mab for the same respective time points (Fig. 5A; Table 2). The faster clearance of the scFvFc-IL-2 is also reflected in the area under the curve, whereas the clearance of the scFvFc was intermediate between that of the latter and the intact Mab. Even with its more rapid clearance, ~40% of the initial amount of radiolabeled scFvFc-IL-2 injected remained in the circulation at 3 h postinjection. Similar blood clearance patterns were observed when antibodies were injected into CEA-transgenic mice (data not shown).

Biodistribution and Tumor Localization of the scFvFc and scFvFc-IL-2. The tumor targeting and biodistribution properties of the three antibody types were compared in CEA-transgenic mice. Mice were implanted s.c. on one flank with the CEA-transfected murine colon carcinoma cell line, MC-38.CEA, whereas the contralateral flank received the CEA-negative parental tumor. In this way, the specific tumor targeting properties of the native and engineered anti-CEA antibodies could be assessed. As depicted in Fig. 5B, the intact Mab gave the highest %ID/g of MC-38.CEA tumor wherein approximately a maximum 30% uptake was observed at 48 h post-antibody injection. The peak uptake of the scFvFc in MC-38.CEA tumors was lower and appeared earlier than that of the intact Mab (Fig. 5B). Approximately 20%ID/g of the radiolabeled scFvFc was present in the antigen-positive tumor 24 h after injection. The scFvFc-IL-2 demonstrated the lowest tumor uptake in that ~6%ID/g of this fusion protein was present in the MC-38.CEA tumor at 8 h post-antibody injection, the earliest time point examined with the latter antibody (Fig. 5B). There was not a significant difference in the peak level uptake of the intact or scFvFc, but the peak level of scFvFc-IL-2 was significantly lower than either the intact Mab ($P = 0.001$) or scFvFc ($P = 0.0002$). These experiments were repeated two to three times for each of the antibody preparations, and similar results were obtained.

All three antibody types showed specific accumulation of radioantibody in the CEA-positive tumor as shown by the generation of tumor localization ratios $>1$ (Fig. 5C). The tumor localization ratio for the intact antibody was slightly greater than 5 by 24 h postinjection and remained relatively constant thereafter. The scFvFc gave tumor localization ratios similar to that of the intact antibody until 96 h after injection, but by 144 h, this ratio had declined to <3 (Fig. 5C). Although the scFvFc-IL-2 had the lowest accumulation in
Fig. 5. Pharmacokinetics and tumor localization of the intact T84.66 and engineered antibodies. A, blood clearance of intact T84.66 Mab, scFvFc, and scFvFc.IL-2 in nontransgenic mice after i.v. injection. The percent injected dose was calculated from a sample removed shortly after injection as in the latter experiments. Each point represents five mice. B, localization of CEA-positive tumors growing in CEA-transgenic mice. Tumor weights for the different time points were 0.17 ± 0.02, 0.27 ± 0.03, and 0.33 ± 0.04 g for the intact Mab, scFvFc, and scFvFc.IL-2, respectively. Each point represents five mice. C, tumor localization ratios (CEA-positive tumor:CEA-negative tumor). Tumor weights for the CEA-positive tumors were as in B. Tumor weights for the CEA-negative tumors for the different time points were 0.36 ± 0.05, 0.28 ± 0.06, and 0.32 ± 0.04 g for the intact Mab, scFvFc, and scFvFc.IL-2, respectively. Each point represents five mice. Bars, SEM.

MC-38.CEA tumors of the three antibody types, specific uptake of scFvFc.IL-2 was evident (Fig. 5C). Tumor localization ratios for the latter antibody were similar to the other two antibody types until 48 h, after which there was a decline. At the time of peak uptake of antibody in the CEA-positive tumor, the level of intact (P = 0.01), scFvFc (P = 0.003), and scFvFc.IL-2 (P = 0.002) in the latter tumor was significantly higher than that in the contralateral CEA-negative tumor. Thus, although the accumulation of scFvFc.IL-2 in MC-38.CEA tumors was lower than that of the scFvFc or intact Mab, the scFvFc.IL-2 was still capable of antigen-specific targeting.

The MC-38.CEA tumor:nontumor ratios were compared for selected normal reference tissues (Fig. 6). Similar to the pattern observed when antibody uptake in the CEA-negative parental tumors was compared with that of MC-38.CEA tumors, the scFvFc and intact Mab produced similar tumor:nontumor ratios at earlier time points. After 48 h, ratios for the scFvFc declined whereas those for the intact Mab remained constant. For the scFvFc.IL-2, tumor:nontumor ratios for blood, colon, and muscle were similar to those observed for the scFvFc and intact Mab at early time points. However, the ratios for the remaining reference tissues (lung, liver, kidney, spleen, and stomach) were notably less than those obtained with the other two antibody types. The stomach of CEA-transgenic mice expresses CEA (18), but this does not appear to be the reason for the low tumor:stomach ratios because increased levels of fusion protein were also observed in the stomachs of nontransgenic mice (data not shown).

Non-tumor-bearing transgenic and nontransgenic mice were used to determine whether the scFvFc targeted to antigen-positive normal tissues of the former mice. The only CEA-positive tissue from transgenic mice that consistently showed an increase in accumulation of the antibody was the colon. All other tissues including the remaining CEA-positive tissues (stomach, cecum) from transgenic mice did not differ from the corresponding tissues obtained from nontransgenic mice in localization of the scFvFc (Fig. 7). Although the increase in localization to the colon was repeatedly observed at all time points, the increase was marginal (1.88 ± 0.41 and 1.05 ± 0.13%ID/g for transgenic and nontransgenic, respectively) and not significant. The blood levels shown in Fig. 7 are less than those derived from pharmacokinetic studies (Fig. 5A) because they were not normalized to a sample removed shortly after injection as in the latter experiments.

Therapy of MC-38.CEA Tumors. Therapy studies used either 30 μg of fusion protein administered i.p. every day for 2 weeks or 20 μg injected i.v. every other day for the same time period. These doses of fusion protein were equivalent to ~119,000 and 80,000 IU of IL-2 for the i.p. and i.v. injections, respectively. The total doses of IL-2 administered by the i.p. and i.v. routes were 1.67 × 10^6 and 0.56 × 10^6 IU, respectively. Preliminary toxicity experiments showed that these doses were also well tolerated. For the first experiment, mice were treated i.p. 1 day after s.c. implantation with either MC-38.CEA or MC-38.BGP tumor cells. Inhibition of MC-38.CEA tumor growth was observed in mice treated with the fusion protein as compared with mice receiving PBS (Fig. 8A; P = 0.026). At 24 days post-tumor implantation, the MC-38.CEA tumor size in mice treated with PBS was 1620 ± 204 mm^3 as compared with 825 ± 231 mm^3 after treatment with the fusion protein (P = 0.03). The scFvFc.IL-2 also caused inhibition of MC-38.BGP tumors as compared with treatment with PBS, but this inhibition was not significant (Fig. 8B; P = 0.22). However, the limited amount of growth inhibition of

Table 2. Kinetic parameters for blood clearance of scFvFc.IL-2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>A1 (%)</th>
<th>A2 (%)</th>
<th>t1/2α (h)</th>
<th>t1/2β (h)</th>
<th>AUC (% h)</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Mab</td>
<td>21.1 ± 8.1^d</td>
<td>52.7 ± 7.8</td>
<td>5.02 ± 4.4</td>
<td>101 ± 28.0</td>
<td>7886</td>
<td>0.864</td>
</tr>
<tr>
<td>scFvFc</td>
<td>41.3 ± 6.0</td>
<td>41.1 ± 5.7</td>
<td>3.45 ± 1.0</td>
<td>57.3 ± 10.0</td>
<td>3596</td>
<td>0.964</td>
</tr>
<tr>
<td>scFvFc.IL-2</td>
<td>87.3 ± 14.0</td>
<td>6.3 ± 20.2</td>
<td>2.3 ± 0.5</td>
<td>31.4 ± 101</td>
<td>580</td>
<td>0.981</td>
</tr>
</tbody>
</table>

^dEach blood curve (corrected for decay) was fitted using a biexponential model of the form. Blood (t) = A1 exp(-K1t) + A2 exp(-K2t).

^The A values give the relative magnitude of the two half-life components. R^2 is an indicator of goodness of fit.

^AUC: area under the curve.

^Mean ± SD.
MC-38.BGP tumors obtained with the fusion protein suggests that nonspecific effects appear to contribute only a small amount to the inhibition of MC-38.CEA tumor growth. In a second experiment, mice bearing MC-38.CEA tumors were treated in the same manner as the first with PBS, scFvFc, scFvFc.IL-2, or a mixture of scFvFc plus IL-2. Control mice received an equivalent µg or IU dose of scFvFc or IL-2, respectively. Only treatment with the scFvFc.IL-2 elicited significant \( (P = 0.001 \text{ versus scFvFc + IL-2 group}) \) inhibition of tumor growth that was similar to that observed with MC-38.CEA tumors in the first experiment (Fig. 8C). Although the scFvFc.IL-2 specifically inhibited MC-38.CEA tumor growth, all animals in the above two therapy experiments developed progressively growing tumors independent of treatment.

To determine whether the route of injection influenced therapy with the scFvFc.IL-2 antibody, tumor-bearing mice were also treated by i.v. injection. As with i.p. injection, MC-38.CEA \( (P = 0.001 \text{ versus PBS}) \) but not MC-38.BGP \( (P = 0.16) \) tumor growth was inhibited in mice receiving fusion protein i.v. (Fig. 9A). Compared with i.p. injection, MC-38.CEA tumor growth inhibition was slightly better after i.v. administration although this may have been due to a slower growth of both tumor types in the latter experiment. At 24 days post-tumor implantation, the MC-38.CEA tumor size in mice treated with PBS was 836 ± 92.7 mm³ as compared with 319 ± 123.5 mm³ after treatment with the fusion protein \( (P = 0.02) \). In a subsequent experiment, the fusion protein was administered i.v. for two additional weeks, and this treatment appeared to be more effective in suppressing tumor growth [Fig. 9B; \( P = 0.0001 \text{ versus PBS} \)]. Twenty-four days post-tumor implantation, the MC-38.CEA tumor size in mice treated with PBS was 874 ± 268.6 mm³ as compared with 209 ± 67.3 mm³ after treatment with the fusion protein \( (P = 0.01) \). As was the case with i.p. treatment, all mice injected i.v. with fusion protein developed progressively growing tumors. Although mice were not studied histopathologically, there did not appear to be any ill effects associated with administration of the fusion protein at these doses, particularly as a result of any potential targeting to antigen-positive normal tissues. However, higher doses of fusion protein were not well tolerated, but these mice were not studied further.

**DISCUSSION**

The scFvFc described in this study was generated to provide a molecule encoded as a single gene that retained antigen-binding as well as tumor-targeting properties similar to those of the native Mab. Upon transfection into mammalian cells, stable clones were derived that produced moderate levels of the scFvFc. Unlike scFv generated with other linkers bridging the Fv domains (31), the purified scFvFc had no tendency to aggregate, which may be due to the incorporation of the 218 linker as well as the bivalent structure of the molecule (23). However, the scFvFc blood clearance was approximately twice as fast as the intact Mab.

The scFvFc showed excellent targeting to tumors and was similar to the intact Mab at the earliest time point in experiments with transgenic mice. In the latter model, animals had both an antigen-positive and -negative tumor that made it possible to demonstrate directly specificity of targeting. The ratio of antibody uptake between tumors growing in the same animal host that differ only in the expression of the targetable antigen (tumor localization ratio) is reminiscent of the paired-label technique introduced by Pressman et al. (32) for demonstrating specificity of antibody targeting. The ability to show specificity of tumor targeting in the transgenic model is a feature amendable for studies with engineered antibodies in which an identical irrelevant construct may not be easily fabricated or acquired. Thus, the scFvFc localized specifically to the MC-38.CEA tumors producing tumor localization ratios that were similar to those obtained with the intact Mab. As observed with the tumor localization ratio, the tumor-nontumor ratios were also very similar between the scFvFc and intact Mab for most of the reference tissues. Nonetheless, tumor uptake of the scFvFc was less than that of the intact Mab, and the latter was particularly evident after the peak of scFvFc uptake. The faster blood clearance of the scFvFc most likely accounted for the earlier peak accumulation of the scFvFc in tumors and its lower overall tumor targeting activity. The lower blood levels of the scFvFc at later time points coupled with its egress from the tumor contributed to the lower tumor localization and tumor-nontumor ratios observed at later time points as compared with the intact Mab. Others have also produced a single-gene-encoded antibody identical in structure with the scFvFc but its tumor-targeting properties were not reported (33). The demonstration that the scFvFc does have good tumor-targeting properties suggested that it may function well as a template for the creation of fusion proteins such as the anti-CEA scFvFc.IL-2.

Using the same expression system for both scFvFc and scFvF- c.IL-2, the secretion of the fusion protein was ~10% of that obtained with the scFvFc. Because all clones expressed the fusion protein in relatively low concentrations, it seems unlikely that the latter was due to insertion of the gene into unfavorable locations for transcription. Other factors such as message stability, assembly, or enhanced intracellular degradation may explain the lower secretion levels of the scFvFc.IL-2. Nonetheless, the scFvFc.IL-2 was expressed as fully assembled fusion protein containing 2 mol of IL-2 per antibody-IL-2 molecule. Like the scFvFc, the fusion protein was similar to intact Mab in its ability to bind CEA; in addition, IL-2 activity was fully preserved as has been reported by others (9, 34). Antibody activity was also found to be maintained after fusion of IL-2 at the COOH-terminal end of a chimeric Fd (4) or chimeric heavy chain (9, 35),
whereas Gillies et al. (34) observed either loss or enhancement of antigen binding with fusion proteins similar in structure to those reported in the latter studies. In addition, the scFvFc.IL-2 antibody retained its binding specificity for CEA-expressing cells.

The blood clearance of the scFvFc.IL-2 was considerably faster than that of both the intact Mab and the scFvFc. This phenomenon had been observed in studies of chimeric IgG fusion proteins with human IL-2 joined to the COOH-terminal end of the heavy chain (9, 12, 36). However, the clearance of the fusion protein reported here was up to 8 times slower than that reported for the latter fusion molecules. This difference may be due to our use of murine IL-2, to the nature of the engineered fusion protein, or to measurement methods. Because we used whole blood to evaluate blood clearance, it does not appear that binding of the fusion protein to circulating IL-2 receptor-bearing cells, and their coincident clearance explains the accelerated clearance of the fusion protein. Based on observations that changes in antigen-binding properties can occur in IL-2 fusion proteins, it was suggested that fusion of IL-2 alters antibody domain structure and thereby can alter both antigen reactivity and metabolism (36). This conformational change could mask, expose, and/or alter recognition sites on the C\(\text{H}2\) and/or C\(\text{H}3\) domains that are involved in immunoglobulin clearance (37). However, recent studies have shown that the apparent rapid clearance of the chimeric IgG fusion protein resulted from a cleavage within the cytokine portion of the fusion molecule (38). The heterologous immunoassay that was used to measure serum levels of fusion protein reflected disappearance of fusion protein as a result of both its clearance as well as a loss of IL-2 from the molecule. We also observed the formation of large molecular weight complexes on incubation of the fusion protein in mouse serum. Molecules smaller than the native fusion protein were not observed. As early as 4 h, >20% of the fusion protein appeared as complexes. This property was unique to the fusion protein because large molecular weight complexes did not appear when the parent scFvFc molecule was incubated in serum. Thus, the possible alteration in domain structure induced by fusion of IL-2 to the COOH terminus of the C\(\text{H}3\) domain may promote aggregation of the fusion protein in serum or binding to a factor in serum. The self-association properties of IL-2 may have contributed to fusion protein aggregation in serum (39). Possible candidates for a serum factor promoting aggregation are soluble IL-2 receptor that is present in low levels in the sera from normal mice (40), \(\alpha\)-macroglobulin (41), and/or anti-IL-2 antibody (42). Other binding factors may also be involved because we have observed similar aggregation on incubation in serum of an engineered antibody molecule with a scFv fused to the COOH-terminal end of the C\(\text{H}3\) domain (4). Nonetheless, although the clearance of the scFvFc.IL-2 was accelerated, its circulating half-life was considerably prolonged compared with that for recombinant IL-2 (43).

Despite the rapid clearance of the scFvFc.IL-2 from the circulation, it specifically targeted antigen-positive tumors. Tumor localization ratios were similar to those obtained with either the scFvFc or the intact Mab until 48 h although the %ID/g of antigen-positive tumor of the fusion protein was significantly lower. Tumor:nontumor ratios for the fusion protein approximated those observed for the scFvFc and intact Mab in some reference tissues, whereas these values were lower with organs that have clearance functions such as liver and spleen. Becker et al. (6, 44) found that a chimeric anti-GD2-IL-2 fusion protein localized specifically to pulmonary or hepatic metastases of a human melanoma xenograft or murine melanoma. Targeting of the latter fusion protein to melanoma xenografts growing s.c. was also observed, although much higher tumor accretion (6- to 10-fold) and tumor:nontumor ratios were obtained than ours (44). Because the blood clearance of the fusion proteins appears similar between the...
higher doses of fusion protein. Whether targeting to antigen-positive tumors were not observed, whereas toxic effects precluded the use of IL-2 reported by others to have an anti-MC-38 effect (48). The ineffectiveness of antibody alone was not surprising because CEA-specific Mabs are poor mediators of growth of MC-38.CEA tumors. The ineffectiveness of antibody alone marginally affected by fusion protein treatment, whereas injection of CEA. The growth of antigen-negative parental tumors was only construct was sufficient to elicit growth inhibition of tumors expressing limiting factor.

increase in the level of fusion protein in normal tissues may be a more fusion protein is expected to reach the tumor, but a concomitant decrease in the level of fusion protein in normal tissues of transgenic mice parallels the distribution pattern of tumor vascular permeability to enhance the delivery of antibodies another potential use for the anti-CEA scFcFv.IL-2 is the modulation of tumor vascular permeability to enhance the delivery of antibodies and/or tumor location. Furthermore, the antitumor properties of the anti-CEA fusion protein may have been limited by its faster egress from the tumor possibly caused in part by its higher k<sub>off</sub>. Also, IL-2 fusion proteins demonstrating profound antitumor properties were constructed with antibodies that can mediate antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (CDC) activities that could potentiate the therapeutic effects of fusion proteins (3, 51, 52, 54). The present study examined the treatment of tumors growing s.c. It is possible that the anti-CEA fusion protein may show greater efficacy when studied in a more relevant metastatic tumor model.

Although immunotherapy with IL-2 has been most effective in patients with renal cell carcinoma and melanomas, responses have also been observed in patients with colorectal tumors treated with IL-2 in combination with lymphokine-activated killer cells (55). Cytotoxic T cell activity against CEA peptides has also been elicited in peripheral blood lymphocytes derived from normal individuals and cancer patients (56, 57), suggesting that it may be possible to induce CEA-directed T cell responses in vivo. Our studies have shown that an immunocompetent mouse model for CEA provides opportunities to define the therapeutic properties of anti-CEA fusion proteins such as the IL-2 fusion protein characterized in the present study. Furthermore, because anti-CEA antibodies target tumors in patients (58), another potential use for the anti-CEA scFcFv.IL-2 is the modulation of tumor vascular permeability to enhance the delivery of antibodies and other therapeutic reagents (5, 9). Finally, because CEA expression in normal tissues of transgenic mice parallels the distribution pattern in humans, the effect of fusion proteins on antigen-positive normal tissues can simultaneously be evaluated (18).

present and former study, differences in tumor uptake may be due to higher expression of antigen on melanoma cells, different murine systems, or different forms of the fusion proteins. Furthermore, the localization of a control fusion protein was not examined by Becker et al. (44) such that a high level of nonspecific tumor uptake may have contributed to the localization observed with the fusion protein. Other studies have shown that with antibody protein doses up to 2 mg in rodents the percentage of antibody reaching the tumor remains the same (45, 46). Thus, as protein doses are increased in therapy studies, more fusion protein is expected to reach the tumor, but a concomitant increase in the level of fusion protein in normal tissues may be a limiting factor.

The level of tumor localization obtained with the scFcFv.II-2 construct was sufficient to elicit growth inhibition of tumors expressing CEA. The growth of antigen-negative parental tumors was only marginally affected by fusion protein treatment, whereas injection of antibody alone or a mixture of antibody plus IL-2 did not alter the growth of MC-38.CEA tumors. The ineffectiveness of antibody alone was not surprising because CEA-specific Mabs are poor mediators of antibody-dependent cellular cytotoxicity (47). The daily dose of IL-2 delivered as a fusion protein was also less than that of recombinant IL-2 reported by others to have an anti-MC-38 effect (48). The therapeutic effects of the anti-CEA scFcFv.II-2 were improved by injecting i.v. and extending the duration of treatment. However, tumor cures were not observed, whereas toxic effects precluded the use of higher doses of fusion protein. Whether targeting to antigen-positive normal tissues contributed to toxicity at these higher doses of fusion protein is currently being examined. Using recombinated immunodeficient mouse models, it was found that anti-GD2-IL-2 fusion protein doses as low as 1 μg and containing 3000 IU of IL-2 cured mice of hepatic metastases of neuroblastoma xenografts (3, 49). Similar antitumor effects were also observed when slightly higher doses of fusion protein were used to treat melanoma or prostatic xenografts (44, 50) or syngeneic colon carcinomas or B-cell lymphomas (51–53). The blood clearance of the fusion protein used to treat murine B-cell lymphomas (51) was similar to that of the anti-CEA scFvFc.II-2, suggesting that a shortened intravascular residence time does not explain the lower efficacy of the latter fusion protein. Factors that may account for the differences in antitumor effects between the present study and those of others are tumor type, antigen type, antigen expression, and/or tumor location. Furthermore, the antitumor properties of the anti-CEA fusion protein may have been limited by its faster egress from the tumor possibly caused in part by its higher k<sub>off</sub>. Also, IL-2 fusion proteins demonstrating profound antitumor properties were constructed with antibodies that can mediate antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (CDC) activities that could potentiate the therapeutic effects of fusion proteins (3, 51, 52, 54). The present study examined the treatment of tumors growing s.c. It is possible that the anti-CEA fusion protein may show greater efficacy when studied in a more relevant metastatic tumor model.

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Targeting and Therapy of Carcinoembryonic Antigen-expressing Tumors in Transgenic Mice with an Antibody-Interleukin 2 Fusion Protein

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