Radioimmunotherapy of Human Colon Cancer Xenografts Using a Dimeric Single-Chain Fv Antibody Construct

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ABSTRACT

Progress in the use of monoclonal antibodies (MAbs) for the treatment of solid tumors is limited by a number of factors, including poor penetration of the labeled IgG molecule into the tumors, their inability to reach the tumor in sufficient quantities without significant normal tissue toxicity, and the development of a human antimouse antibody response to the injected MAb. One possible way to alter the pharmacology of antibodies is via the use of smaller molecular weight antibody fragments called single-chain Fvs (scFvs). A divalent construct of MAb CC49, CC49 (scFv)2, composed of two noncovalently associated scFvs, was generated and shown to bind a tumor-associated antigen (TAG-72) epitope with a similar binding affinity to that of the murine IgG. The therapeutic potential of this construct after labeling with 131I was examined in athymic mice bearing established s.c. human colon carcinoma (LS-174T) xenografts. Treatment groups (n = 10) received a single dose of 131I-labeled CC49 (scFv)2 (500–2000 μCi) or 131I-labeled CC49 IgG (250 and 500 μCi). The group of mice treated with the lowest dose of 131I-(scFv)2 (500 μCi) showed statistically significant prolonged survival, compared with controls (P = 0.036). Complete tumor regression was observed in 20% of mice given 1500 μCi of labeled (scFv)2 and 30 and 60% of mice treated with 250 and 500 μCi of labeled IgG, respectively. In conclusion, the CC49 (scFv)2 construct provides a promising delivery vehicle for therapeutic applications.

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INTRODUCTION

Radioimmunotherapy (RIT) is a promising therapeutic approach for the treatment of a wide variety of malignancies. The antitumor effect of RIT is based on the selective delivery of a sufficient radiation dose to tumors without significantly affecting normal tissues. Multiple clinical trials have shown the RIT treatment to be effective for some types of lymphomas and leukemias (1–5). However, despite the promising preclinical study results, only modest responses have been documented in patients with solid tumors (6–11). The efficacy of RIT is affected by numerous factors, such as vascular and tumor permeability, development of human antimouse antibodies, heterogeneity of antigenic expression, the intrinsic radiosensitivity of the targeted tumor, radiation emissions, properties of the radionuclide and chemical stability of the radioimmunoconjugate, and normal tissue toxicity that is secondary to circulating radiolabeled antibody (12).

The choice of antibody is another important factor in RIT. Several MAbs have been generated against tumor-associated antigens and have been used for radioimmunodetection of tumors. MAb CC49 IgG is one example of such an antibody. It reacts with a unique sialyl-Tn antigen expressed on a tumor-associated mucin, TAG-72. CC49 IgG exhibits high reactivity against tumor cells in most adenocarcinomas from colorectum, ovary, breast, stomach, and pancreas, but it shows little reactivity against normal tissues (13). The preclinical therapeutic studies using radiolabeled CC49 IgG showed reduced tumor growth in 80–100% of tumor-bearing animals (14). 131I-labeled MAb CC49 used in clinical trials demonstrated excellent tumor targeting. However, despite the promising preclinical results, patient trials with radiolabeled CC49 IgG failed to identify any clinically effective responses, even at the highest administered activities (300 μCi/m2; Ref. 9–11).

The difficulties in the clinical application of radiolabeled CC49 IgG and other radiolabeled MAbs are primarily caused by normal tissue toxicity, immunogenicity, and relatively poor penetration into tumor. The development of genetically engineered single-chain antibody fragments (scFvs) is one way to potentially overcome some of these limitations. The scFv molecule is comprised of the variable regions of the immunoglobulin heavy and light chains, which are covalently connected by a flexible peptide linker (15, 16). The scFvs showed very rapid blood clearance, excellent penetration into the tumor from the vasculature, reduced immunogenicity, and higher tumor:normal tissue ratios (RIs) than corresponding IgG, F(ab')2, or Fab' fragments in animal models (17–21). Larson et al. (22) showed that CC49 scFv is safe, that tissue equilibration and clearance are fast, and...
that same-day imaging of the primary and metastatic tumors is feasible in patients with colorectal carcinoma. However, due to their small size and monovalency, scFvs clear the body too rapidly to allow for sufficient tumor uptake and retention for therapeutic applications.

Recently, we developed a new CC49 scFv construct that forms high-affinity stable noncovalent dimers [(scFv)2]. The CC49 (scFv)2 showed a rapid blood clearance rate, excellent RIs, lack of uptake in normal tissues, and better tumor targeting properties than corresponding CC49 scFv or Fab’ (23). Here, we present the therapeutic potential of the 131I-labeled CC49 (scFv)2 construct in an experimental tumor system and compare it with that of the intact IgG molecule.

MATERIALS AND METHODS

Purification of MAb CC49 IgG. MAb CC49 IgG was developed by the immunization of mice with purified TAG-72, as described previously (24, 25). CC49 IgG was purified from ascitic fluid that was obtained from pristane-primed BALB/c mice using Protein G chromatography (Pharmacia Biotech, Piscataway, NJ). The protein concentration was determined by the method of Lowry et al. (26).

Expression and Purification of CC49 (scFv)2. Construction and expression of CC49/205C scFv (Vh-Linker-Vk) has been described previously by Pavlinkova et al. (27). The CC49 scFv protein was purified from the periplasmic fraction of a 1.0-liter Escherichia coli overnight culture by ion-exchange chromatography using a Mono-Q column (Pharmacia Biotech). Dimeric and monomeric scFv forms were separated by size-exclusion chromatography using a Superdex 75 column (Pharmacia Biotech).

Characterization of Purified CC49 Immunoglobulin Forms. CC49 (scFv)2 and IgG binding activity was determined by a competition ELISA (27) using microtiter plate wells coated with BSM (28). Purified proteins were analyzed by SDS-PAGE (29) and stained with Coomassie blue. The molecular weight of (scFv)2 was estimated by comparison to the relative mobility values of the gel filtration standard (Bio-Rad, Hercules, CA) using HPLC.

The binding kinetics of CC49 IgG and scFv dimer was measured by the BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden), as described previously (23). Briefly, BSM (positive control) or BSA (negative control; 200 μg/ml) was injected until a surface of 700 resonance units was realized. Binding analyses were performed in HBS buffer [10 mM HEPES (pH 7.4), 0.15 M NaCl, 3.4 μM EDTA, and 0.005% surfactant P20] at a flow rate of 30 μl/min at 25°C. The association constant (Kf) and dissociation constant (Kd) were evaluated using BIAevaluation Version 3.0 (Pharmacia Biosensor, Uppsala, Sweden), in which the experimental design correlated with the Langmuir 1:1 interaction model (30).

Radioiodination of CC49 Immunoglobulin Forms. The MAb CC49 IgG and the scFv form were labeled with Na125I or Na131I using Iodo-Gen (Pierce Chemical, Rockford, IL), as described by Colcher et al. (31). The iodination protocol yielded specific activities of ~3–9 mCi/mg. The purity of each radiolabeled preparation was analyzed by gel filtration HPLC on tandem TSK G3000SW and TSK G2000SW columns (Toso Haas, Montgomeryville, PA). The immunoreactivity was determined using BSM- or BSA-coated beads (Reacti-Gel HW-65F; Pierce). The coated beads (0.5 ml) were washed with 1% BSA-0.1% Tween 20 in PBS and resuspended in 0.5 ml of binding buffer (1% BSA in PBS). The radiolabeled samples were added to each tube and incubated for 1 h at RT, the unbound radiolabeled protein was removed by washing, and the pellet was counted in a gamma scintillation counter.

Biodistribution Studies. Tumors were grown in 4–6-week-old female athymic mice (nu/nu; Charles River, Wilmington, MA) after a s.c. injection of 4 × 106 human colon carcinoma cells (LS-174T; Ref. 32). Radiolabeled dimeric scFv and IgG (5 μCi of 125I-labeled MAb and 2.5 μCi of 131I-labeled MAb) were coinjected i.v. into tumor-bearing animals ~10 days after injection of the cells. At specific times, mice (groups of six) were euthanized and dissected, and the major organs were weighed and counted in a gamma scintillation counter. The percentage ID per gram of tissue (expressed as % ID/g) was calculated. Pharmacokinetic studies were conducted by obtaining blood samples (5 μl) via tail bleeds at various times after the i.v. injection of 10 μCi of the radiodinated CC49 forms. On average, six mice per group are presented. The whole-body retention of radiolabeled forms was also determined. Mice bearing the LS-174T xenograft (three mice per group) were injected via the tail vein with 1.5 μCi of radiolabeled CC49 forms and counted using a custom-built NaI crystal at various times after injection.

Biodistribution and pharmacokinetic data were analyzed by using Graphpad Prism, Version 2.01 (GraphPad Software Inc., San Diego, CA). To compare changes in % ID/g over time between IgG and (scFv)2, we performed statistical analyses using two-way ANOVAs. When a significant interaction was detected, the differences between IgG and (scFv)2 at each time point were further compared using the t test for unpaired data (significance assigned at the P < 0.05 level). The clearance of the (scFv)2 was very rapid, so single-phase exponential decay curve fit was used for pharmacokinetic analyses. The data for F(ab’)2, and IgG were analyzed using a two-phase exponential decay curve fit.

Tumor Therapy Studies. In all RIT experiments, female athymic mice (nu/nu), obtained from Charles River (Wilmington, MA) at 4–6 weeks of age, were injected s.c. on the back with 4 × 106 human colon carcinoma cells (LS-174T; Ref. 32). Tumor growth was determined by caliper measurement of the long and short axis for each tumor and the tumor volume was calculated as follows:

\[ \text{Volume} = (\text{Length of short axis, in mm})^2 \times (\text{length of long axis, in mm})/2 \]

Eight days post-tumor implantation, the mice were distributed into groups (n = 10) to give similar tumor size distributions in all treatment groups. One group was injected with PBS (control group) or treated i.v. with a single dose of radiiodinated CC49 IgG or CC49 (scFv)2. Body weight and tumor size were monitored twice a week. Animals were observed until the animals died, the tumors exceeded 10% of...
the total body weight, the tumors began to ulcerate through the skin, or the animals lost 20% of their original weight, at which time the animals were removed from the group and killed. The survival fraction of each treatment group was evaluated according to the method of Kaplan and Meier. The survival curves were compared, and P values were generated using the log-rank test. GraphPad Prism, Version 2.01 (GraphPad Software Inc.) was used for these analyses.

**Table 1** Comparative biodistribution studies with CC49 IgG and (scFv)$_2$

<table>
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<th>4</th>
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Iodinated CC49 IgG and (scFv)$_2$ were injected into athymic mice (six per group) bearing LS-174T tumors. The mice were sacrificed at the indicated times, and values (in % ID/g) for each organ were determined. The values presented are the averages of multiple studies. The SEs for the samples were <20% ID/g of the values of the corresponding tissues. Tumor, blood, and kidney uptakes were analyzed using two-way ANOVA. The interaction was statistically significant for all analyzed data, with P < 0.001. The statistically significant differences in the blood and tumor uptake between IgG and (scFv)$_2$ were found for all time points (P < 0.001). The differences in the kidney uptakes were statistically significant for all time points (P < 0.05), except at 4 h postadministration. ND, not determined.

Fig. 1 HPLC size-exclusion profiles of the radiolabeled CC49 IgG and (scFv)$_2$. After radiolabeling, $^{125}$I-IgG and $^{125}$I-(scFv)$_2$ were analyzed using TSK G3000SW and TSK G2000SW size-exclusion columns connected in series. The IgG (●) and the (scFv)$_2$ (▲) were eluted as single peaks of $M_r$; 150,000 and $M_r$; 60,000, respectively.

Fig. 2 CC49 IgG, F(ab’)$_2$, and (scFv)$_2$ were injected into athymic mice (six per group) bearing LS-174T colon carcinoma xenografts, and blood samples were obtained at various times. IgG (▲), F(ab’)$_2$ (□), and (scFv)$_2$ (●).
RESULTS AND DISCUSSION

We have developed a new recombinant fragment-dimeric CC49 scFv \([\text{scFv}_2]\). We hypothesized that the characteristics of CC49 \([\text{scFv}_2]\) may satisﬁy the requirements for successful RIT. CC49 scFv noncovalent dimer combines a small size for fast elimination from circulation with a reduction of normal tissue toxicity and better penetration into tumor due to smaller size, compared with intact IgG.

Biochemical Characterization and Immunoreactivity of CC49 \([\text{scFv}_2]\). The CC49/205C \([\text{scFv}_2]\) was secreted as soluble, active protein using the pRW83 expression vector and puriﬁed by ion-exchange chromatography. SDS-PAGE analysis of the puriﬁed \([\text{scFv}_2]\) showed >90% purity. The molecular weight of the \([\text{scFv}_2]\) \([M_r \sim 60,000]\) was estimated using size-exclusion HPLC by comparison to the relative mobility values of the standards. The association constants were determined by surface plasmon resonance (BIACore) for CC49 IgG \([K_A = 1.14 \times 10^8]\) and \([\text{scFv}_2]\) \([K_A = 4.46 \times 10^5]\); Ref. 23).

The puriﬁed CC49 \([\text{scFv}_2]\) and IgG were radiolabeled with Na\(^{125}\)I and analyzed for protein purity using SDS-PAGE and size-exclusion HPLC (Fig. 1). The immunoreactivity was evaluated using beads coated with BSM, which contains the epitope recognized by MAb CC49 on the human tumor-associated antigen (TAG-72), as a positive control or with BSA as a negative control. The \([\text{scFv}_2]\) showed 88—94% binding to BSM, compared with intact IgG (87—90%), with <5% nonspeciﬁc binding. Radiolabeled samples of the CC49 \([\text{scFv}_2]\) were stable for at least 35 days when they were stored at 4°C, as tested by HPLC and the binding assay. Radiolabeled \([\text{scFv}_2]\) was also analyzed to determine its stability in serum. Unfortunately, blood clearance of CC49 scFv dimer is fast, and its concentration in blood is not sufﬁcient to perform direct blood HPLC analysis. Therefore, we have designed conditions similar to in vivo conditions, where radiolabeled CC49 scFv dimer was incubated in murine serum at 37°C, and most importantly, the concentrations of radiolabeled CC49 \([\text{scFv}_2]\) were appropriate for analysis using HPLC for extended time period (4 days). The labeled samples mixed with murine serum or 1% BSA were taken at various times and analyzed by HPLC. The CC49 \([\text{scFv}_2]\) maintained its full integrity throughout the testing period (data not shown).

Pharmacokinetics and Tumor Targeting of the CC49 \([\text{scFv}_2]\). The blood clearance rate of radiolabeled CC49 \([\text{scFv}_2]\) was compared with the blood clearance of the intact IgG and the divalent fragment \([F(ab')_2]\) in pharmacokinetic studies. As seen in Fig. 2, the \([\text{scFv}_2]\) \([M_r \sim 60,000]\) blood clearance was faster than \([F(ab')_2]\) \([M_r \sim 110,000]\) or IgG \([M_r \sim 150,000]\) with 50% cleared from the blood pool in 50, 400, and 1200 min, respectively. Whole-body clearance analysis also displayed a rapid \([\text{scFv}_2]\) clearance, suggesting that CC49 noncovalent dimers were not being retained in the extravascular space or in any specific organ. The relative rates of clearance observed in the whole body experiments were similar to those observed with the blood clearance (data not shown).

The antigen-binding ability of the CC49 \([\text{scFv}_2]\) in vivo and its efﬁciency to target human colon carcinoma xenografts (LS-174T) were compared with CC49 IgG in biodistribution studies. At various times after injection, blood, tumor, and normal organs were analyzed to determine the amount of each radiolabeled IgG retained per gram of tissue. The \([\text{scFv}_2]\) % ID/g levels in tissues were generally lower than those of the IgG, due to a more rapid blood and whole-body clearance (Table 1). The differences in the relative rates of blood clearance in the biodistribution studies were statistically signiﬁcant \((P < 0.001)\). As expected, \([\text{scFv}_2]\) uptake in the kidneys was 5 times higher than that of IgG at 30 min \((P < 0.001)\) but 4 times lower that reported for the kidney uptake for similarly size Fab' fragments (23). However, the level of renal uptake detected with \([\text{scFv}_2]\) was similar to the levels found in the other major organs by 4 h postadministration.

A lower level of tumor uptake (10.87% ID/g) was observed with in vivo tumor targeting with \([\text{scFv}_2]\), as compared with IgG (23.12% ID/g) at 6 h postadministration \((P < 0.001)\). However, the CC49 dimeric scFv appears to remain intact in vivo, as evidenced by the relatively high levels of tumor uptake up to 120 h. The CC49 IgG showed noticeably higher nonspeciﬁc uptake in the liver, spleen, blood, and kidneys, compared with the \([\text{scFv}_2]\) construct after 24 h postadministration. Extremely high RIs \((RI = \text{ratio of the } % \text{ ID/g in tumor to the } % \text{ ID/g in normal tissue})\) were obtained with \([\text{scFv}_2]\) with a tumor:blood \((P < 0.001)\), tumor:lung \((P = 0.01)\), and tumor:spleen \((P = 0.02)\) ratios of 80.3, 25.5, and 31.2:1, respectively, compared with 3.4, 6.1, and 9.1:1 for IgG at 24 h.

Therapeutic Efficacy of the Radiolabeled CC49 \([\text{scFv}_2]\). We have compared the therapeutic potential of radiiodinated CC49 \([\text{scFv}_2]\) and IgG in two sets of RIT studies with athymic mice bearing established s.c. LS-174T tumors. Radiolabeled
MAb administered dose titration and the size of the established s.c. LS-174T tumors were the variables in these studies. In the first study, mice with large s.c. LS-174T tumors received a single injection of 500, 750, or 1000 mCi of 131I-CC49 (scFv)2. In the second study, animals with smaller established s.c. tumors received a single injection of 1000, 1500, or 2000 mCi of 131I-CC49 (scFv)2 by i.v. injection. Each study also involved groups of mice receiving radiolabeled MAb CC49 IgG at doses of 250, 500 mCi for comparison as well as a control group. Survival analysis (Fig. 3) showed significant tumor growth inhibition for all treated animal groups, as compared with controls. Even the lowest (scFv)2 administered dose of 500 mCi resulted in a statistically significant prolonged survival of treated mice when compared with untreated mice (P = 0.036), with median survival times of 27 and 18 days, respectively. Groups of mice treated with 750 and 1000 mCi of 131I-CC49 (scFv)2 demonstrated significant prolonged survival, compared with the control group (P < 0.001). However, complete tumor regression was observed only with higher levels of (scFv)2 (1500 mCi; 2 of 10) and with 250 mCi (3 of 10) and 500 mCi (6 of 10) of CC49 IgG (Fig. 3). An administered dose of 1500 mCi of 131I-labeled (scFv)2 was the maximum tolerated single dose.

As expected, the initial size of tumors was another important factor influencing the efficacy of RIT. In the second study using athymic mice with smaller s.c. LS-174T tumors, the median survival was higher for treated groups when compared with the first study. Both study groups, the group with larger tumors (initial volume = 305 ± 64 mm3) and the group with smaller tumors (initial volume = 176 ± 31 mm3) after treatment with 131I-labeled CC49 IgG or CC49 (scFv)2, and no therapy (Control). Data points, means for 10 animals in each group; bars, SE. The IgG administered dose was 250 or 500 mCi, and the administered dose of radiolabeled (scFv)2 was 500, 750, 1000, or 1500 mCi.

![Growth kinetics of LS-174T tumors (A), initial volume = 305 ± 64 mm³; and small tumors (B), initial volume = 176 ± 31 mm³) after treatment with 131I-labeled CC49 IgG or CC49 (scFv)2, and no therapy (Control). Data points, means for 10 animals in each group; bars, SE. The IgG administered dose was 250 or 500 mCi, and the administered dose of radiolabeled (scFv)2 was 500, 750, 1000, or 1500 mCi.](cancerres.aacrjournals.org)

Fig. 4 Growth kinetics of LS-174T tumors [large tumors (A), initial volume = 305 ± 64 mm³; and small tumors (B), initial volume = 176 ± 31 mm³] after treatment with 131I-labeled CC49 IgG or CC49 (scFv)2, and no therapy (Control). Data points, means for 10 animals in each group; bars, SE. The IgG administered dose was 250 or 500 mCi, and the administered dose of radiolabeled (scFv)2 was 500, 750, 1000, or 1500 mCi.
μCi of (scFv)₂, respectively, and 62 days for mice treated with 250 μCi of IgG. The mean volume quadrupling time of the untreated control tumors was <8 days for the group with small tumors. The best growth inhibition was observed in mice treated with 500 μCi of IgG, in which the tumor volume never quadrupled.

The selection of radionuclide is a critical factor to consider for RIT. The kidney was the dose-limiting organ in a preclinical therapy study with radiometal (⁹⁰Zr)-labeled Fab fragments (33). However, radiation nephrotoxicity was not a problem in the therapeutic applications of radiolabeled antibody fragments, but the dose-limiting organ was the red marrow in a study in which Behr et al. (34) reported excellent therapeutic antitumor efficacy using ¹³¹I-labeled anti-CEA Fab. In this study, the Fab fragments were more efficient at controlling human colon cancer xenograft growth than the respective IgG. Treatment-related toxicity of ¹³¹I radiolabeled CC49 (scFv)₂ and IgG was monitored by weight loss (Fig. 6). One of 10 animals died from a single injection in the group injected with 1500 μCi of ¹³¹I-CC49 (scFv)₂ at 41 days posttreatment and in 500 μCi of ¹³¹I-CC49 IgG group at 44 days posttreatment. Treatment with 2000 μCi of ¹³¹I-CC49 (scFv)₂ yielded 100% lethality at 26–59 days.

In conclusion, we showed that the radiolabeled CC49 (scFv)₂ construct is a promising candidate for therapeutic applications. The single administration of 1500 μCi of ¹³¹I-CC49 (scFv)₂ resulted in complete tumor regression in ~20% of treated mice and significant improvement in survival times. This was the maximal tolerated dose for a single-treatment regimen. Future studies for RIT with the CC49 scFv dimer should include dose fractionation of the administered radiolabeled construct. Several investigators have reported that dose fractionation can be less toxic and more efficacious than a single dose (35–37). The biological efficacy of time-dose fractionation depends on the total dose that can be administered, the dose rate, the biological half-life of the antibody, the physical half-life of the radionuclide, and the fractionation schedule (38).

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