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ORIGINAL ARTICLE

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Pharmacokinetics and biodistribution of a light-chain-shuffled CC49 single-chain Fv antibody construct

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Abstract Murine monoclonal antibodies to tumor-associated glycoprotein 72 (anti-TAG-72 mAb B72.3 and CC49) are among the most extensively studied mAb for immunotherapy of adenocarcinomas. They have been used clinically to localize primary and metastatic tumor sites; however, murine mAb generally induce potent human anti-(mouse antibody) responses. The immunogenicity of murine mAb can be minimized by genetic humanization of these antibodies, where non-human regions are replaced by the corresponding human sequences or complementary determining regions are grafted into the human framework regions. We have developed a humanized CC49 single-chain antibody construct (hu/muCC49 scFv) by replacing the murine CC49 variable light chain with the human subgroup IV germline variable light chain (Hum4 V_L). The major advantages of scFv molecules are their excellent penetration into the tumor tissue, rapid clearance rate, and much lower exposure to normal organs, especially bone marrow, than occur with intact antibody. The biochemical properties of hu/muCC49 scFv were compared to those of the murine CC49 scFv (muCC49 scFv). The association constants (K_a) for hu/muCC49 and muCC49 constructs were 1.1×10^6 M^{-1} and 1.4×10^6 M^{-1} respectively. Pharmacokinetic studies in mice showed similar rapid blood and whole-body clearance with a half-life of 6 min for both scFv. The biodistribution studies demonstrated equivalent tumor targeting to human colon carcinoma xenografts for muCC49 and hu/muCC49 scFv. These results indicate that the human variable light-chain subgroup IV can be used for the development of humanized or human immunoglobulin molecules potentially useful in both diagnostic and therapeutic applications with TAG-72-positive tumors.

Key words Monoclonal antibodies · Single-chain antibodies · Antibody engineering · Recombinant antibody · Human colon cancer xenografts

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Introduction

The clinical utilization of murine monoclonal antibodies (mAb) is limited by the induction of a human anti-mouse antibody (HAMA) response [29, 37]. The HAMA response may elicit allergic reactions in patients [3], and it can alter the rate of antitumor antibody clearance from serum [11, 32]. Various attempts have been made to produce human-derived monoclonal antibodies using human hybridomas [23]. Unfortunately, the yields of human mAb from such hybridomas are low and transient compared to antibody production by mouse hybridomas. Another approach to reduce the immunogenicity of murine mAb is genetic humanization of these antibodies, in which non-human constant regions are replaced by corresponding human ones (chimerization) or/and complementary determining regions are grafted into human framework regions. However, these humanized antibodies retain various portions of the variable domains of light- and heavy-chain variable regions of non–human origin that can evoke an immunogenic reaction in patients [18]. Therefore, it is most desirable

to obtain a completely human antibody or antibody fragments capable of binding to a selected antigen.

mAb CC49 is a second-generation murine mAb B72.3 that reacts with tumor-associated glycoprotein (TAG-72). The TAG-72 antigen is expressed by the majority of human adenocarcinomas of the pancreas, colon, ovary, prostate, lung, and esophagus and is absent in most normal tissues [36]. Radiolabeled CC49 IgG has shown excellent tumor localization in several ongoing clinical trials; however, one major complication in its clinical use is the development of a HAMA response in patients [35]. Additional limitations for the successful clinical application of CC49 IgG have been its slow elimination from the circulation and relatively poor penetration into a tumor mass.

In an attempt to modify the pharmacology and biodistribution of the mAb, genetically engineered single-chain antibody fragments (scFv) have been developed. ScFv are comprised of the immunoglobulin heavy- and light-chain variable regions that are connected by a flexible peptide linker [6, 12]. In comparison to the corresponding IgG, $F(ab')_2$ or Fab′ fragments, scFv have shown very rapid blood clearance, excellent penetration into the tumor from the vasculature, reduced immunogenicity, and higher tumor:normal-tissue ratios (radiolocalization indexes: RI) in animal models [1, 8, 21, 26, 40, 41].

In this report, we describe the development of a new humanized CC49 scFv construct (hu/muCC49 scFv) where the murine CC49 variable light chain was entirely replaced by a homologous human light chain (the human subgroup IV germline variable light chain; Hum4 V_L). The properties of hu/muCC49 scFv were compared to those of murine CC49 scFv (muCC49 scFv) to determine if the replacement of the CC49 V_L by Hum4V_L would affect its antigen binding, pharmacokinetics, and biodistribution.

Materials and methods

Construction of hu/mu scFv

The hu/muCC49 scFv was constructed through a collaborative effort with the National Cancer Institute's Laboratory of the Tumor Immunology and Biology, and the Dow Chemical Company (Midland, Mich.). Briefly, CC49 V_H was obtained from variable regions of the murine monoclonal antibody CC49 [31]. For the V_L region, the Hum4 V_L germline gene coding sequence was used because of its homology with the murine CC49 light-chain sequence. The Hum4 V_L scFv segment was amplified by the polymerase chain reaction (PCR) using a 5′ primer with the *Nco*I site and the 3′ primer with the *Hind*III site from the pRL1001 vector containing the Hum4 V_L germline template (US patent 37777A). The final CC49 scFv cDNA (V_L-linker-V_H) was constructed by PCR by combining a *Nco*I-*Hind*III DNA fragment of the Hum4 V_L gene and the CC49 V_H region sequence (*Xho*I and *Nhe*I fragments) via a linker sequence [22]. The previously published 205C linker [24] was modified to accommodate *Hind*III and *Xho*I restriction sites by changing the terminal amino acids, resulting in the sequence LSADDAKKDAAKKDDAKKDDAKKDL, and used for scFv construction. The fragment was cloned into the pRW83 vector, which contains a chloramphenicol-resistance gene for recombinant selection, the *penP* gene with a *β*-lactamase *penP* promotor and terminator, and a pectate lyase *pelB* signal peptide [20].

Purification and characterization of CC49 scFv protein

The expression vectors containing the CC49 scFv sequences were transformed into *Escherichia coli* strain AG1 using Strategene competent cells (La Jolla, Calif.) by following the manufacturer's protocol. The bacteria were grown at 37 °C in Luria-Bertani medium containing 34 μg/ml chloramphenicol. The CC49 scFv proteins were prepared from the periplasmic fraction of a 1.0-l overnight culture, as previously described by Pavlinkova et al. [27]. The periplasmic fraction was dialyzed against 20 mM BIS-TRIS propane, pH 9.1, and applied to a Mono-Q HR 16/10 column (Pharmacia, Piscataway, N.J.). The murine CC49 scFv was eluted with 40 mM sodium chloride in 20 mM BIS-TRIS propane, pH 9.1, the hu/mu scFv was eluted with 75 mM sodium chloride in 20 mM BIS-TRIS propane, pH 9.1. ScFv were further purified by size-exclusion chromatography on a 1.6 × 70 cm Superdex 75 column (Pharmacia, Piscataway, N.J.). Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% polyacrylamide gel [16] and by enzyme-linked immunosorbent assay (ELISA) [25]. The protein concentration of purified fragments was determined by the method of Lowry et al. [17].

Competition ELISA

CC49 forms were analyzed at various stages in the purification process by a competition ELISA. Threefold serial dilutions of the sample (10 μl) in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) were added to plates coated with 50 ng/well bovine submaxillary gland mucin (BSM; Sigma, St. Louis, Mo.) and incubated with 8 ng/well biotinylated CC49 IgG for 2 h at room temperature [25]. After washing with PBS containing 0.05% Tween 20, the plates were incubated for 1.5 h with alkaline-phosphatase-conjugated streptavidin (AP-streptavidin; Jackson ImmunoResearch Lab., West Grove, Pa.) and developed with p-nitrophenyl phosphate substrate. The absorbance was determined by a Dynatech MR 5000 reader. The percentage binding was compared on a molar basis.

Isoelectric point (pI) analysis

The pI of folded scFv proteins was determined by isoelectric focusing (IEF). Samples of 5 μl (2–5 mg/ml) were applied to IsoGel agarose IEF plates (FMC Bioproducts, Rockland, Me.) pH 3–10 and isoelectric focusing was performed following the manufacturer's instructions. Immediately after focusing, the protein bands were fixed, washed, and silver-stained, using the Ag Stain KIT (Isolab Inc., Akron, Ohio). Standards with isoelectric points between 9.3 and 3.6 (Sigma, St. Louis, Mo.), and 9.6 and 4.45 (Bio-Rad, Hercules, Calif.) were used to calculate the pI of the samples.

The theoretical pI values of scFv were determined from the primary amino acid sequence, using a sequence analysis program (GCG Inc., Madison, Wis.).

Radiolabeling and determination of immunoreactivity

mAb CC49 scFv forms were labeled with $Na^{125}I$ or $Na^{131}I$ using Iodo-Gen (Pierce Chemical, Rockford, Ill.) [7]. The iodination produced antibody fragments with specific activities of approximately 3–9 mCi/mg. The immunoreactivity of the radiolabeled CC49 scFv was assessed in an antibody-capture assay with BSM, as the positive control and BSA as the negative control. The proteins were immobilized on a solid support as detailed earlier [26]. The radiolabeled samples (20 μl) were added to each tube containing 0.5 ml of beads in binding buffer (PBS containing 1% BSA, pH 7.2) and the samples were vortexed every 10 min to ensure com-

plete suspension. Following a 1-h incubation at room temperature, the unbound radiolabeled protein was removed by washing with PBS containing 1% BSA and 0.1% Tween 20. The beads were counted for 1 min in a gamma scintillation counter.

High-performance liquid chromatography (HPLC)

Samples (50–200 μg unlabeled or 0.2–0.4 μCi radiolabeled protein in 50 μl) were injected onto TSK G3000SW and TSK G2000SW (Toso Haas, Tokyo, Japan) size-exclusion columns connected in series, and eluted with 0.067 M phosphate and 0.1 M KCl buffer (pH 6.8) at a flow rate of 0.5 ml/min. Elution was monitored by absorption at 280 nm and by measuring the radioactivity of the eluted fractions in a gamma scintillation counter. For the serum stability test, radiolabeled scFv were mixed with normal mouse serum or 1% BSA and incubated at 37 °C for up to 4 days. Samples of 50 μl were taken at 0, 0.5, 1, 2, 4, 5, 24, 48, and 72 h and analyzed by HPLC size-exclusion chromatography.

Surface plasmon resonance

The BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden) was used to measure the binding kinetics of CC49 scFv. BSM was immobilized on a CM5 dextran sensor chip in 100 mM sodium acetate, pH 3.0, using the Amine Coupling Kit (Pharmacia Biosensor) as described by Pavlinkova et al. [26]. A similar surface was prepared using BSA as a control. Binding analyses were performed in 10 mM HEPES, pH 7.4/0.15 M NaCl/3 mM EDTA/0.005% surfactant P20 at a flow rate of 30 μl/min at 25 °C. The surface was regenerated with 6 M guanidine/0.2 M acetic acid, at a flow rate of 5 μl/min with no loss of activity. The association constant (K_a) and dissociation constant (K_d) were evaluated using the BIAevaluation 3.0.2 software supplied by the manufacturer where the experimental design correlated with the Langmuir 1:1 interaction model [13].

Biodistribution analysis

Female athymic mice (nu/nu), obtained from Charles River (Wilmington, Mass.) at 4–6 weeks of age, were injected subcutaneously on the back with 4×10^6 human colon carcinoma cells (LS-174T) [38]. Tumor-xenograft-bearing animals were used for the biodistribution study approximately 10 days after injection with 202 ± 29 mg tumor. The pharmacokinetic studies for the blood clearance were conducted by obtaining blood samples (5 μl) via tail bleeds at various times after injection of 10 μCi radioiodinated CC49 form (six tumor-bearing mice per group). The blood was collected from each animal 5-μl disposable micro-pipettes (Drummond Scientific Co., Broomall, Pa.) at each assay time point. For the whole-body retention studies, mice bearing the LS-174T xenograft (three/group) were injected via a tail vein with 1.5 μCi radiolabeled CC49 form and counted using a custom-built NaI crystal at various times after injection. Dual-label biodistribution studies were performed with mice injected, via the tail vein, with radiolabeled forms CC49 scFv (5 μCi ^{125}I-labeled hu/mu scFv and 2.5 μCi ^{131}I-labeled murine scFv in the first set of dual-label studies, and 2.5 μCi ^{131}I-labeled hu/mu scFv and 5 μCi ^{125}I-labeled murine scFv in the second set of dual-label studies). At specific times, mice (groups of six) were euthanized and dissected, and major organs were weighed and counted in a gamma scintillation counter. The percentage of the injected dose per gram of tissue (%ID/g) was calculated.

Biodistribution and pharmacokinetic data were statistically analyzed using Graphpad Prism software (GraphPad Software Inc., San Diego, Calif.). Two-way analyses of variance were used for comparison of changes in %ID/g over time among scFv constructs. When a significant interaction was detected, the differences between scFv constructs at each assay time were further compared by the *t*-test for unpaired data (significance assigned at the $P < 0.05$ level). The blood and whole-body clearance of the scFv was analyzed by fitting to a two-phase exponential decay curve.

Results

We have developed a new antibody construct containing human light and murine heavy variable chains bound to TAG-72 antigen. This hu/mu scFv construct was developed by combining DNA sequences for murine CC49 V_H with the human subgroup IVκ V_L gene. The hum4 V_L amino acid sequence is 84% homologous to that of the murine CC49 variable light chain (Fig. 1). Three amino acids are different in the complementary determining region CDR1 and one in CDR2 of the human light chain when compared to the mouse light-chain sequence. The sequence of human CDR3 is identical to the mouse sequence. The hu/muCC49 scFv gene (V_L-linker-V_H) was constructed by PCR, by combining the

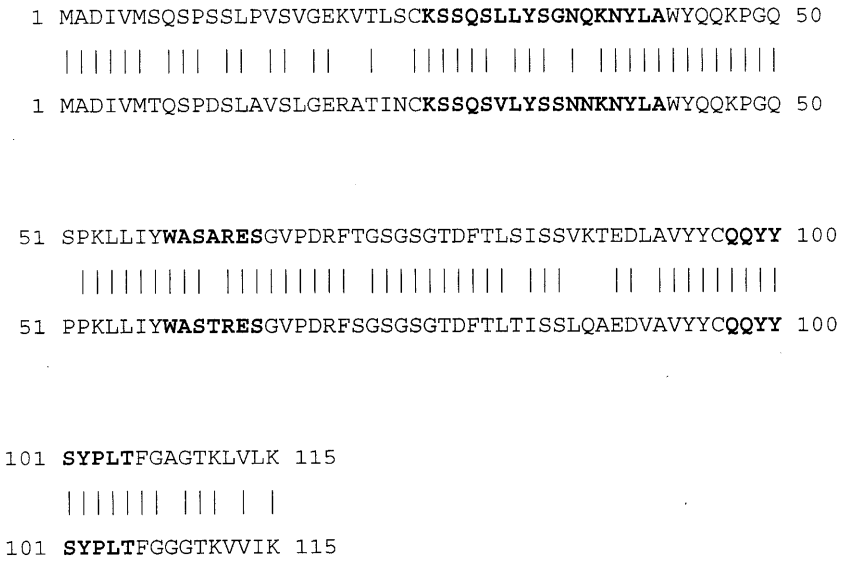

Fig. 1 Amino acid sequence of the human subgroup IV germline V_L (*lower lines*) aligned with the murine CC49 V_L (*upper lines*). Vertical marks indicate identity of the nucleotides. The complementary determining regions are in bold type

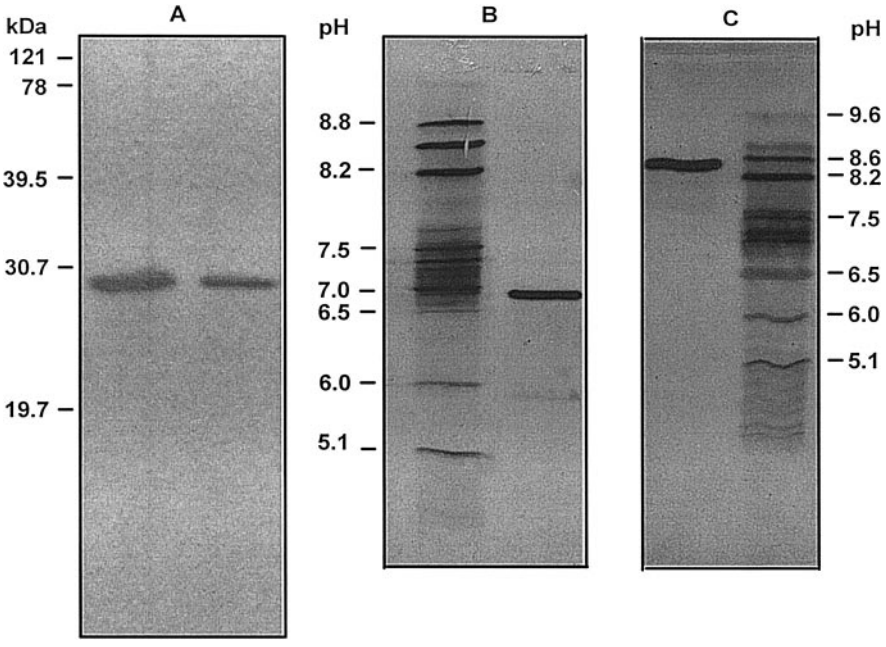

Fig. 2 A Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified CC49 scFv under non-reducing conditions. *Lane 1* hu/muCC49 scFv; *lane 2*, muCC49 scFv. Positions of the molecular mass marker proteins are indicated. **B, C** Isoelectric focusing (IEF) analysis of purified CC49 scFv forms on agarose IEF plates with a pH range of 3–10, revealed by silver staining. **B** *Lane 1* IEF standards (BioRad), *lane 2* hu/muCC49 scFv; **C** *Lane 1* muCC49 scFv, *lane 2* IEF standards (BioRad)

Hum4 V_L sequence and the murine CC49 V_H sequence via a linker sequence (205C). The recombinant construct containing hu/muCC49 scFv sequences was expressed in *Escherichia coli* as a soluble protein. The hCC49 recombinant protein was purified from the periplasm by ion-exchange and size-exclusion chromatography.

The properties of purified Hum $4V_L$/CC49 V_H and murine CC49 scFv were compared in SDS-PAGE, HPLC, IEF, and BIAcore analyses. The purified scFv analyzed by SDS-PAGE were 99% pure and migrated in accordance with their theoretical molecular mass of 28 kDa (Fig. 2A). Additional HPLC analysis of the CC49 scFv showed peaks corresponding to the expected M_r 28-kDa protein without any aggregates or breakdown products (data not shown). The computer-predicted isoelectric points from primary amino acid sequences of muCC49 and hu/muCC49 scFv were 7.8 and 6.5, respectively. The pI values of folded proteins observed in IEF were 8.3 for murine and 7.0 for hu/mu scFv (Fig. 2B, C). The immunoreactivity of the scFv forms was tested in a competition ELISA. Both species competed with biotinylated CC49 IgG for binding to BSM, which contains the epitopes recognized by mAb CC49, and were able to compete for 100% of the binding (Fig. 3). The kinetic and affinity constants of the CC49 antibody constructs for the BSM antigen were measured by surface plasmon resonance. This technique allows the measurement of real-time kinetic interactions to determine rates of association and dissociation, and the affinity constants. The hu/mu scFv showed a faster dissociation rate ($k_{off} = 4.01 \times 10^{-3}$) than murine scFv ($k_{off} = 1.83 \times 10^{-3}$). The derived association constants (K_a) for hCC49 and muCC49 constructs were 1.1×10^6 M and 1.4×10^6 M respectively (Fig. 4).

The purified scFv proteins were radiolabeled to see the effect of radiolabeling on the immunoreactivity and

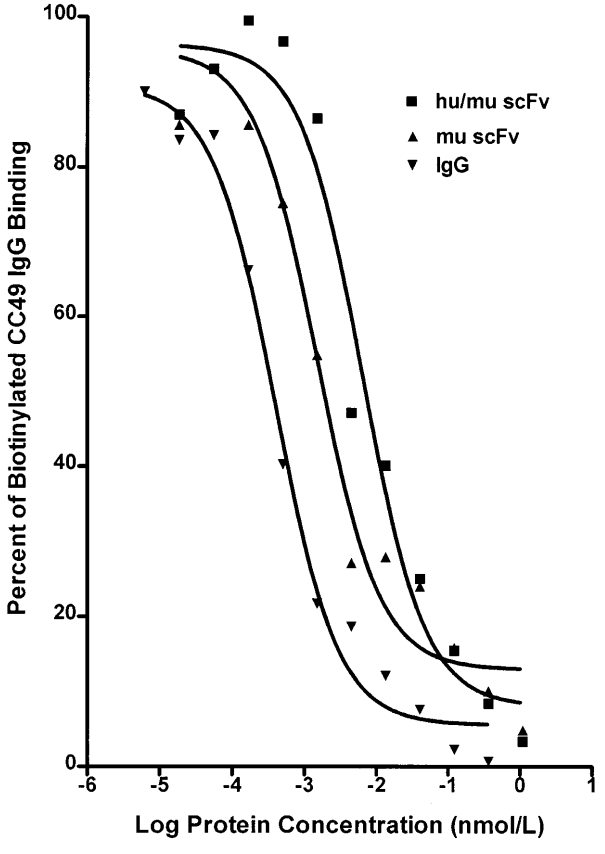

Fig. 3 Competition enzyme-linked immunosorbent assay using biotinylated CC49 IgG. Threefold serial dilutions of test samples were added to plates coated with 50 ng/well of bovine submaxillary gland mucin (BSM) to compete with biotinylated CC49 IgG. After washing and incubation with streptavidin-linked alkaline phosphatase, the plates were developed with *p*-nitrophenyl phosphate substrate. The percentage binding was compared on a molar basis

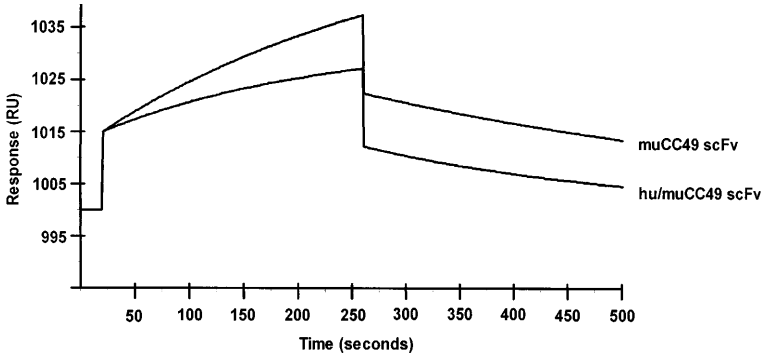

Fig. 4 Surface plasmon resonance analysis of the binding of CC49 scFv constructs. Sensogram demonstrating the binding to immobilized BSM on the biosensor chip

the stability of the CC49 scFv. The immunoreactivity of the labeled scFv proteins was determined by solid-phase radioimmunoassay using BSM and BSA attached to Reacti-Gel beads. The scFv showed similar binding to BSM (60%–66%). Radiolabeled scFv were analyzed by SDS–PAGE with and without addition of β-mercaptoethanol. A single major band representing approximately 98% of the incorporated radioactivity migrated with the molecular mass of the corresponding scFv (data not shown). The labeled scFv were also analyzed by HPLC size–exclusion chromatography (Fig. 5). The scFv were eluted as single peaks without any detectable aggregates and their elution profiles corresponded to their calculated molecular mass, indicating that they were not altered by iodination.

The radiolabeled scFv were further analyzed to determine the radiopurity and integrity after incubation in normal mouse serum. The radiolabeled scFv were mixed with mouse serum and incubated at 37 °C. Aliquots were taken at various times and were analyzed by HPLC size-exlusion chromatography. The radiolabeled scFv maintained their full integrity and stability throughout the testing period (24 h) (data not shown).

Pharmacokinetic studies of radiolabeled CC49 scFv showed a rapid, biphasic blood clearance. The blood clearance curves of the scFv were similar with $t_{1/2\alpha} = 2.7$ min and $t_{1/2\beta} = 315$ min (Fig. 6A). Whole-body clearance analysis also showed a rapid scFv clearance, suggesting that scFv was not being retained in the extravascular space or in any specific organ, but they were eliminated from the body through the kidneys (Fig. 6B).

The scFv were directly compared in two sets of dual-label studies (see Materials & methods). At various times after injection, blood, tumor, and normal organs were analyzed to determine the amount of each radionuclide retained per gram of tissue (Table 1). The hu/muCC49 scFv and muCC49 scFv demonstrated similar in vivo tumor targeting with 1.95 %ID/g and 1.68 %ID/g, respectively, 6 h after administration.

A statistically significant difference in biodistribution of scFv species was demonstrated in the kidneys. Only at the early times after administration (0.5 and 1 h) was the hu/muCC49 scFv uptake by kidneys (81.9% ID/g and 29.9 %ID/g) significantly higher ($P < 0.05$) than that observed for muCC49 scFv (55.1 %ID/g and 17.6 %ID/g). The levels of the radiolabeled scFv in normal organs reflected their concentration in blood. For example, the hu/mu scFv showed statistically no difference in biodistribution in the liver or spleen when compared to murine scFv (Table 1).

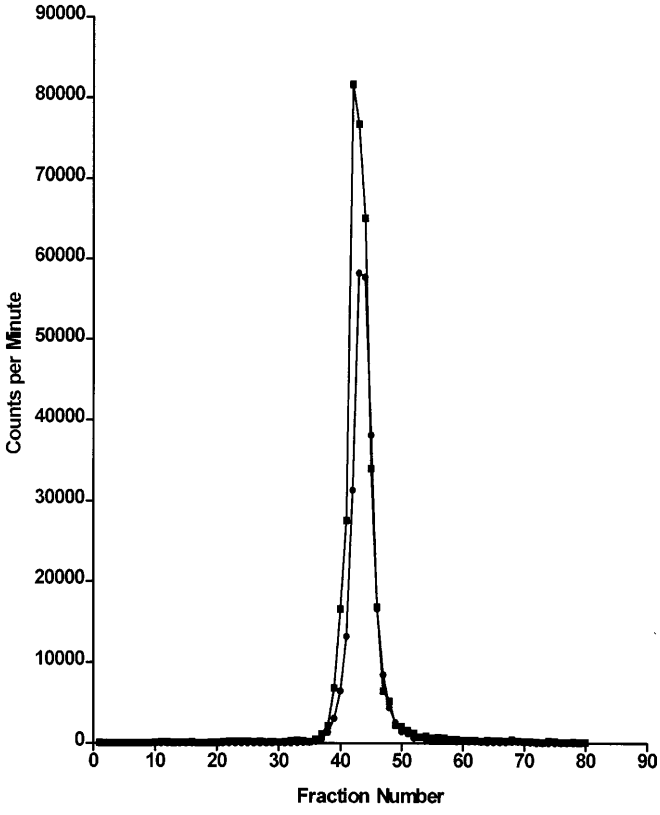

Fig. 5 HPLC size-exclusion profiles of the radiolabeled CC49 scFv. After radiolabeling, ^{125}I-scFv were analyzed on TSK G3000SW and TSK G2000SW size-exclusion columns connected in series. The CC49 murine scFv (●) and hu/muCC49 scFv (■) were eluted as a single peak of approximately 28 kDa

One important parameter is the radiolocalization index (RI: the ratio of the %ID/g in tumor to the %ID/g in normal tissue). RI values for well-vascularized organs were generally similar for both constructs with tumor:liver and tumor:spleen ratios of 11:1 and 15:1 for hu/muCC49 scFv and 11:1 and 19:1 for muCC49 scFv, respectively, at 24 h (Table 2).

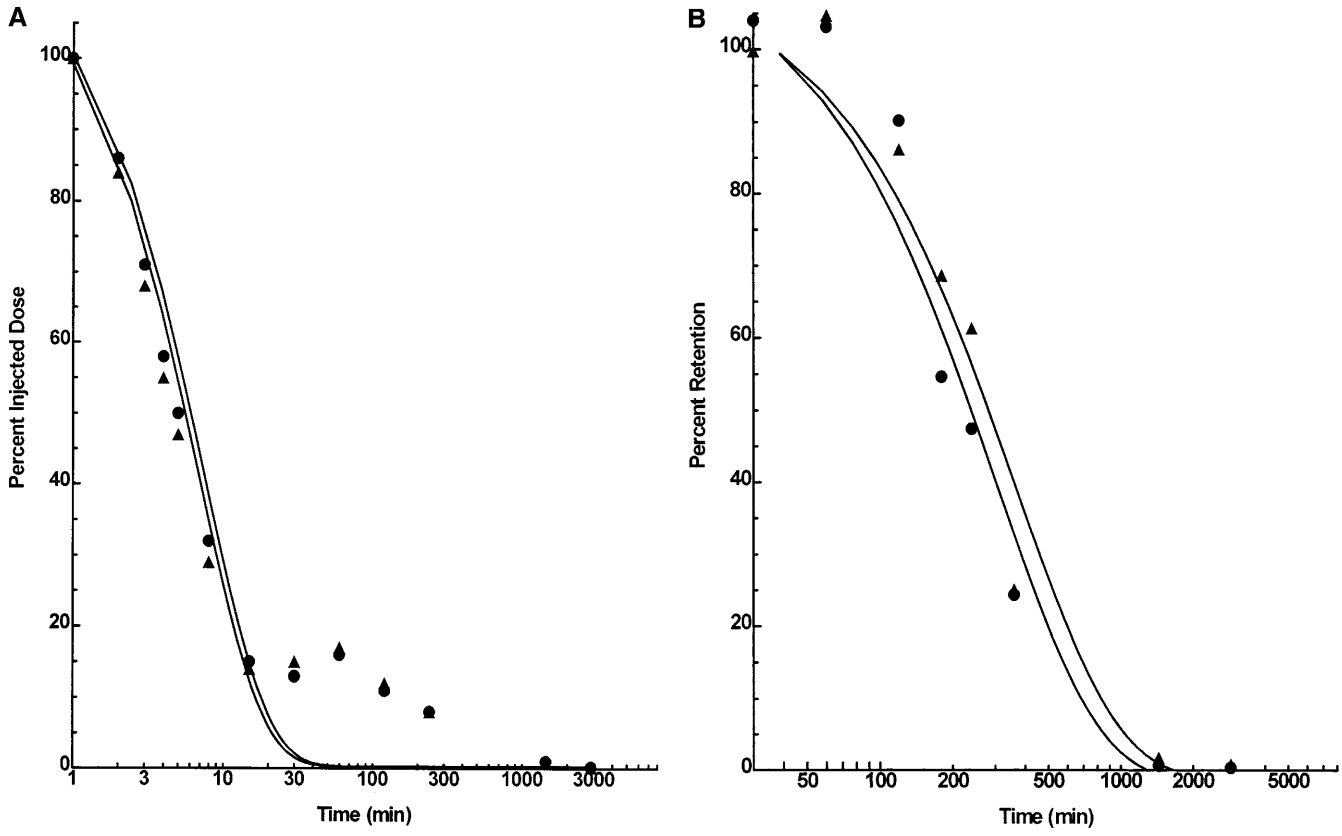

Fig. 6 A Blood clearance of CC49 scFv. Female athymic mice (six per group) bearing LS-174T colon carcinoma xenografts were injected with the radiolabeled muCC49 scFv (▲) and hu/muCC49 scFv (●), and blood samples were obtained at specific times. **B** Pharmacokinetics of whole-body retention of CC49 scFv. Murine scFv (▲) and hu/mu scFv (●) were injected into athymic mice (three per group) bearing LS-174T colon carcinoma xenografts. The radioactivity was measured in a custom-built NaI gamma scintillation counter at the indicated times

Discussion

CC49 antibody recognizes a tumor-associated antigen with characteristics of a mucin known as TAG-72. CC49 IgG has demonstrated efficient tumor localization in preclinical and clinical studies. For example, over 90% of tumor lesions were detected by using iodine-labeled CC49 mAb in patients with colorectal and breast carcinomas [10, 11]. Furthermore, a number of genetically modified forms of CC49 mAb have been developed with potential clinical advantages such as C_H2- and C_H1-domain-deleted chimeric CC49 [34] and single-chain Fv fragments [5, 21, 26]. Since these smaller Ig molecules, with a modified or missing constant region, have demonstrated more rapid blood and whole-body clearance rates and lower normal tissue toxicity, they may show reduced human anti-(mouse antibody) responses in patients. To minimize HAMA responses in patients further, CC49 mAb was humanized by grafting complementary determining regions of CC49 onto human frameworks of mAb LEN and 21/28′ CL [14]. These human mAb were most homologous to CC49 in the framework residues crucial to maintaining the combining-site structure. mAb LEN was used as the template for V_L, 36 of the 43 critical framework residues being

Table 1 Comparative biodistribution of CC49 scFv. Radiolabeled hu/muCC49 scFv and muCC49 scFv were coinjected into athymic mice (six per group) bearing LS-174T tumors. The mice were sacrificed at the indicated times and the percentage of the injected dose per gram of tissues (%ID/g) of the tumor and selected normal tissues was determined. The standard error of the mean (SEM) for the samples averaged approximately 10% of the value of the corresponding tissues. No significant interaction between ^{125}I-muCC49 and ^{131}I-muCC49 scFv, and between ^{125}I-hCC49 and ^{131}I-hu/muCC49 scFv was detected and the differences in the curves were not statistically significant, therefore the values from both experiments were averaged. Statistically significant differences in biodistribution between muCC49 and hu/muCC49 scFv were found in kidneys 0.5 h and 1 h after administration ($P < 0.05$)

Antibody construct	Tissue	Biodistribution (%ID/g) after:					
		0.5 h	1 h	4 h	6 h	24 h	48 h
hu/muCC49 scFv	Tumor	6.34	5.68	2.76	1.95	0.79	0.41
	Blood	6.06	5.93	2.00	1.38	0.07	0.05
	Liver	2.71	2.39	0.97	0.83	0.10	0.05
	Spleen	3.14	3.02	1.11	0.76	0.06	0.03
	Kidneys	81.93	29.91	2.09	1.29	0.11	0.07
muCC49 scFv	Tumor	5.17	4.61	2.29	1.68	1.06	0.79
	Blood	5.49	5.00	1.67	1.13	0.07	0.04
	Liver	2.28	1.95	0.93	0.73	0.16	0.06
	Spleen	2.67	2.50	0.94	0.64	0.08	0.04
	Kidneys	55.07	17.60	2.43	1.55	0.22	0.13

Table 2 Comparative biodistribution studies of hu/muCC49 and muCC49 scFv 24 h after administration (radiolocalization index, RI). Radiolabeled hu/muCC49 scFv and muCC49 scFv were co-injected into athymic mice (six per group) bearing LS-174T tumors. The mice were sacrificed 24 h after administration and the RI (%ID/g of the tumor divided by %ID/g of normal tissues) for each organ was determined. The SEM for the samples averaged less than 10% of the %ID/g of the corresponding tissues

Tissue	RI	
	hu/muCC49 scFv	muCC49 scFv
Blood	11.2	15.1
Liver	7.9	6.6
Spleen	13.1	13.3
Kidneys	7.2	4.8
Lungs	9.6	14.4

identical, and 21/28' CL was used for V_H, 28 of the 40 critical framework residues being identical.

The above applications have produced antibody forms in which some portion of variable domains is coding for non-human variable region-sequences. Such non-human regions may still evoke an anti-V-region HAMA response in patients [9, 15, 30, 32]. Therefore, it is most beneficial to acquire a human variable region capable of binding to a selected antigen.

A new CC49 single-chain fragment was developed, comprised of the human subgroup IV variable κ light chain and the variable region of the murine CC49 heavy chain covalently connected by a flexible peptide linker. The amino acid sequence of the human subgroup IV V_L is 84% homologous to the CC49 murine V_L chain sequence (Fig. 1). Frequently, the CDR3 of the light chain, and the CDR1 and CDR3 of the heavy chain are the most important regions for the maintenance of antibody immunoreactivity. Therefore, it was important, that the amino acid sequence of the human light chain replacement was identical to that of the mouse CC49 variable light chain in CDR3. The genetically engineered hu/muCC49 scFv formed a stable three-dimensional structure with high affinity to TAG-72 antigen and an association constant of 1.1×10^6 M^{-1}, as demonstrated by the BIAcore analysis. The binding of radiolabeled scFv to antigen was determined by radioimmunoassay using BSM-conjugated beads. The scFv demonstrated lower binding to BSM (60%–66%) than to bivalent scFv, $F(ab')_2$ or IgG (more than 90%), as published earlier [26]. This decrease in binding affinity is due to a decrease in the functional affinity (avidity) of these monovalent constructs. The scFv have a single antigen-binding arm from the parent immunoglobulin, therefore, they are incapable of simultaneous binding to two or more target antigens.

Since hu/muCC49 scFv showed a faster dissociation rate than muCC49 scFv, by surface plasmon resonance analysis, it was essential to analyze the ability of the hu/mu construct to localize human tumor xenografts in vivo. The hu/mu scFv demonstrated equivalent tumor-targeting properties in comparative dual-label studies with muCC49 scFv.

Statistically significant differences in the biodistribution between murine and hu/mu scFv were observed only with the kidneys at early assay times point after administration (0.5 h and 1 h; $P < 0.05$). The kidneys are the major site for catabolism of molecules smaller than 60 kDa [19] including light-chain dimers [39], Fab fragments [2], and scFv [42]. The renal accretion of small proteins is due to their glomerular filtration, metabolic degradation followed by tubular reabsorption and/or secretion of the substance [4, 19, 28, 33]. Consequently, 30%–60% of the dose administered can be found in the kidneys [19] and may cause renal radiotoxicity. The size of a molecule is the major factor determining its susceptibility to filtration across basement membranes [2]. Other factors affecting filtration are the conformation, electrical charge, and binding characteristics of the molecule [19]. An apparent difference between hCC49 and muCC49 that may influence differential kidney uptake is their pI. The isoelectric focusing analysis determined the pI of folded hCC49 to be lower (pI = 7.0) than that of muCC49 scFv (pI = 8.3). Another factor affecting the kidney uptake is the protein conformation. The conformation of hu/muCC49 scFv could be different from that of muCC49 scFv, with the exposure of positively charged amino acid(s) that could bind to negatively charged glomerular basement membranes of the kidneys and increased the tubular reabsoption of hu/muCC49 scFv.

The fast clearance rate of scFv reduces the radiation–absorbed dose to normal tissues in radioimmunotherapy applications and, in clinical diagnostics, permits earlier imaging times. Since the blood clearance is an important parameter for clinical applications, pharmacokinetic studies in mice were performed and demonstrated similar rapid clearance rates for hu/mu scFv and muCC49 scFv with a rapid equilibration phase and subsequent slower elimination from circulation (Fig. 5).

In summary, the stable hu/muCC49 scFv was produced and characterized in vitro and in vivo. In biodistribution studies, the hu/muCC49 scFv molecule exhibited excellent tumor-targeting properties and rapid clearance. The human subgroup IV light chain can be used to combine with any V_H capable of forming a three-dimensional structure with the ability to bind to TAG-72 to produce humanized or human antibody constructs. The V_L gene can also be used to isolate a gene encoding human V_H having the ability to bind TAG-72 from a combinatorial library. The hu/muCC49 scFv may provide a useful reagent for diagnostic and therapeutic applications.

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