The tumor-associated glycoprotein (TAG)-72 is expressed in the majority of human adenocarcinomas but is rarely expressed in most normal tissues, which makes it a potential target for the diagnosis and therapy of a variety of human cancers. Here we describe the construction, affinity maturation, and biological characterization of an anti-TAG-72 humanized antibody with minimum potential immunogenicity. The humanized antibody was constructed by grafting only the specificity-determining residues (SDRs) within the complementarity-determining regions (CDRs) onto homologous human immunoglobulin germ line segments while retaining two mouse heavy chain framework residues that support the conformation of the CDRs. The resulting humanized antibody (AKA) showed only about 2-fold lower affinity compared with the original murine monoclonal antibody CC49 and 27-fold lower reactivity to patient serum compared with the humanized antibody HuCC49 that was constructed by CDR grafting. The affinity of AKA was improved by random mutagenesis of the heavy chain CDR3 (HCDR3). The highest affinity variant (3E8) showed 22-fold higher affinity compared with AKA and retained the original epitope specificity. Mutational analysis of the HCDR3 residues revealed that the replacement of Asn97 by isoleucine or valine was critical for the affinity maturation. The 3E8 labeled with 125I or 131I showed efficient tumor targeting or therapeutic effects, respectively, in athymic mice with human colon carcinoma xenografts, suggesting that 3E8 may be beneficial for the diagnosis and therapy of tumors expressing TAG-72.

Monoclonal antibodies (mAbs) are increasingly being used as therapeutic agents for cancer and other diseases. Murine mAbs have limited use as therapeutic agents because of a short half-life, an inability to trigger human effector functions, and the induction of a human anti-mouse antibody response (1, 2). To reduce the immunogenicity of murine antibodies in humans, chimeric antibodies with mouse variable regions and human constant regions were initially constructed (3). Although chimeric antibodies proved to be less immunogenic than murine mAbs, human anti-chimeric antibody responses have been observed (4). To further reduce the immunogenicity of the mouse variable regions, a humanized antibody has been constructed by grafting the complementarity-determining regions (CDRs) of a murine mAb onto the human framework regions (FRs) by a procedure commonly referred to as CDR grafting (5). Simple grafting CDRs, however, often decreased the affinity, because some FR residues directly contact the antigen or support the conformation of the CDR loops (6, 7). Therefore, humanized antibody is currently constructed primarily by CDR grafting, while retaining those rodent FR residues that influence antigen-binding activity (8). Since the FR residues often differ from one humanized antibody to another, the identification of key rodent FR residues is a crucial part of the humanization (9). Clinical studies have indicated that such humanized antibodies are, in general, less immunogenic than murine or chimeric antibodies and are tolerated by humans (10, 11). Unfortunately, the non-human CDRs of humanized antibodies can induce human anti-humanized antibody responses in patients (12).

Comprehensive analyses of the three-dimensional structures of the antibody-combining sites have revealed that only 20–33% of the CDR residues participate in antigen-binding (13–15). Most of the variable positions of CDRs are directly involved in the interaction with antigen (i.e. specificity-determining residues (SDRs)), whereas most of the conserved residues mainly serve to stabilize the structure of the combining site (15). The CDRs are observed primarily in the C-terminal part of light chain CDR1 (LCDR1), the first and sometimes also the middle positions in LCDR2, the middle portion of LCDR3, most of the heavy chain CDR1 (HCDR1), the N-terminal and middle parts of HCDR2, and most of HCDR3 except the terminal position. The CDRs form the center of the antibody-combining site. Therefore, one possible way to minimize the human anti-humanized antibody response is to graft only the SDRs of a murine antibody onto human FRs while maintaining the conformations of the CDRs of the murine antibody.

Tumor-associated glycoprotein (TAG)-72 is expressed by the majority of human adenocarcinomas in the colon, ovary, pancreas, breast, prostate, and lung but not in most normal tissues, except the endometrium in the secretory phase (16, 17). A murine mAb B72.3 (16) that specifically binds to TAG-72 is approved for in vivo imaging in patients...
with ovarian and colorectal cancers. A second generation antibody to B72.3, CC49 (18, 19), which has higher affinity for TAG-72 than B72.3 does, has shown efficient targeting to various carcinomas in clinical trials (20–22). The epitope for B72.3 or CC49 has been identified as sialyl-Tn (i.e. α-sialyl 2–6GalNAc), O-linked to Ser/Thr on mucin-type glycoproteins (23). However, CC49 elicits human anti-mouse antibody responses in patients (24). To overcome this problem, a humanized CC49 antibody (HuCC49) has been constructed by CDR grafting while retaining the buried FR residues that affect the structure of the antibody and those involved in V_{H}-V_{L} interaction or antigen binding (25). Subsequently, to reduce the immunogenicity of HuCC49, the mouse CDR and FR residues have been replaced individually with corresponding human residues, and the antigen-binding activity and potential immunogenicity of each variant have been analyzed (26–29).

In the present study, humanized antibody was constructed by grafting only the SDRs onto a human Ig germ line segment with CDRs of the same canonical structures as those of the murine antibody while retaining two key murine FR residues. The resulting humanized antibody AKA showed only about 2-fold lower affinity compared with CC49. Subsequently, antibody affinity was increased by random mutagenesis of HCDR3 residues followed by affinity selection. The resulting humanized antibody (3E8) showed 27-fold lower sera reactivity compared with HuCC49 and higher affinity compared with original CC49. The antibody showed specific tumor targeting and anti-tumor therapeutic effects in athymic mice bearing human adenocarcinoma xenografts expressing TAG-72.

**EXPERIMENTAL PROCEDURES**

Construction of Humanized Antibody—To select human FRs for SDR grafting, the V_{H} and V_{L} sequences of CC49 were compared with those of human Ig variable and joining region germ line segments. It was found that DP25-1_{H} and DK24-1_{L} were the most homologous to the VH and VL of CC49, respectively (Fig. 1). To construct the V_{H} of the humanized light chain (HzK), only Tyr^{94} in the LCDR3 of CC49 was grafted onto DP25-1_{H} and VK4 to construct the VL of the humanized antibody (Fig. 1A). The numbering follows Kabat et al. (30).

The V_{H} sequence of HzK was synthesized by recombinant PCR using a humanized V_{H} (pCLS2-neo) (31), which is also based on human DPK24-1_{L}, as a template and eight mutagenic PCR primers (primer 1, 5'-CCGCAGCAGCCCGCTCGTGATGAC(T/C)CAGTCTCC; primer 2, 5'-CTTATTGTGCTGTCTGATAA; primer 3, 5'-CGCCAGCACAATAAGAACTACTT; primer 4, ATATTGCTGACAGTAC; primer 5, 5'-ATAAAC; primer 6, 5'-CATATTCAAGATCTTGTACAG(A/T)AAATAGCCCGTGTC; primer 7, 5'-AACAGATCCTGGAATATGGCTTACCGGAGGCT; primer 8, 5'-GCTAGTAGACTGTTTGGTACAAGATTG); Primers 9 and 18 were contained EcoRI and SalI sites, respectively. The humanized V_{H} was fused to human C_{Y1} in pCHS2-neo (31) by recombinant PCR. The final PCR product was digested with EcoRI and SalI and then subcloned into pCDMV-dhfr to yield pdCMV-dhfr-akt. Another humanized heavy chain aka was constructed from akt by recombinant PCR using pdCMV-dhfr-akt as a template and mutagenic PCR primers; this was then subcloned into the EcoRI-Apal sites of pdCMV-dhfr-akt to construct pdCMV-dhfr-aka.

Expression and Purification of Humanized Antibody—The humanized antibody was transiently expressed in COS7 cells using Lipofectamine (Invitrogen), and the culture supernatant was subjected to ELISA to determine its antigen-binding activity and affinity. For the stable expression of humanized antibody, expression plasmid DNA was transfected into a dihydrofolate reductase-deficient Chinese hamster ovary (CHO) cell line, DG44. After selection in minimal essential medium α containing G418 in 96-well plates, the resistant cell clones were screened for the production of assembled antibody by an indirect ELISA and were subjected to stepwise methotrexate adaptation, as described previously (33).

For the production and purification of antibody, the CHO cell line that stably expressed the antibody was grown in serum-free medium (CHO-SFM II, Invitrogen), and the culture supernatant was subjected to affinity chromatography on a Protein G-Sepharose column (Amer sham Biosciences), as described previously (32). The integrity and purity of the purified antibody were determined by SDS-PAGE. For quantification of the purified antibody, an optical density of 1.43 at 280 nm was used for a protein concentration of 1 mg/ml (34).

ELISA—For an indirect ELISA, antibody samples were added to each well that had been coated with 1 µg of TAG-72-positive bovine submaxillary mucin (Type I-S, Sigma), as described previously (27), and were incubated at 4 °C overnight. After washing, 100 µl of goat anti-human IgG-horseradish peroxidase conjugate (1:1000 (v/v); Sigma) were added to each well and incubated at 37 °C for 2 h and were then added to each well that had been previously coated with 200 ng of the antigen. The bound antibody was detected by an indirect ELISA. The dissociation constant (K_{D}) of each antibody was calculated from a Scatchard plot (35).

To analyze the epitope specificity of 3E8, a competition binding assay between CC49 and 3E8 was performed as described previously (33). Briefly, CC49 or biotinylated 3E8 was incubated with bovine submaxillary mucin (250 ng/well; Sigma) in 96-well plates at 37 °C for 30 min in the presence of increasing concentrations of a competing antibody, 3E8.

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The bound CC49 or biotinylated 3E8 was detected by goat anti-mouse 
IgG (Fc-specific)-horseradish peroxidase conjugate (Pierce) or strepta-
vidin-horseradish peroxidase (Sigma), respectively. As a negative con-
trol of the competing antibody, humanized KR127, which specifically 
binds to the preS1 antigen of hepatitis B virus (36), was used.

**Serum Reactivity Assay**—The potential immunogenicity of AKA in 
humans was assessed by measuring the reactivity of the humanized 
antibody to the serum from patient EA, who received $^{177}$Lu-mCC49 in 
a phase I radioimmunotherapy trial (22). Since the serum from patient EA 
contained anti-murine variable region antibodies, it was used to com-
pare the reactivity of AKA with that of HuCC49. However, the serum 
also contained circulating TAG-72 antigen and anti-murine Fc antibodies, 
which may have interfered with the binding of the humanized anti-
tody to the anti-variable region antibodies in the serum. Therefore, 
prior to the serum reactivity assay, TAG-72 antigen and anti-murine Fc antibodies were removed from the serum by immunoadsorption to 
another TAG-72-specific murine mAb, CC92, that recognizes an 
etiotope of TAG-72 that is distinct from the one recognized by CC49 
(37). Briefly, CC92 was coupled to Reactigel (HW65F; Pierce). The 
patient serum was added to the CC92 gel and was incubated overnight 
at 4 °C with end-over-end rotation. The samples were centrifuged at 
1000 $\times$ g for 5 min, and the supernatants were saved and stored at 
$-20$ °C for the serum reactivity assay.

To test the serum reactivity of antibodies, a surface plasmon reso-
nance-based competition assay was carried out using a BIAcore X 
instrument (BIAcore), as described previously (38). Briefly, proteins 
were immobilized on the carboxymethylated dextran chip (CM5) by 
amine coupling. The dextran layer of the sensor chip was activated by 
injecting 35 $\mu$L of a mixture of N-ethyl-N’-(3-dimethylaminopropyl)car-
bodiimide hydrochloride and N-hydroxysuccinimide at a flow rate of 
5 $\mu$L/min. Proteins diluted in 10 mM sodium acetate buffer (pH 5.0) 
at a concentration of 100 $\mu$L/mg were injected until surfaces of 5000 reso-
nance units were obtained. The remaining reactive groups on the sur-
faces were blocked by injecting 35 $\mu$L of 1 M ethanolamine (pH 8.5). To 
determine the serum reactivity of HuCC49 and AKA, AKA or HuCC49 at 
different concentrations was incubated with the patient serum 
cleared of TAG-72 and anti-murine Fc antibodies at 25 °C, and then the 
mixture was applied to the HuCC49-immobilized CM5 chip in flow cell 
1 and a rabbit $\gamma$-globulin-immobilized chip in flow cell 2 as a reference 
to compete for binding to serum anti-variable region antibodies. After 
the binding was measured for 1000 s, the unbound samples were washed 
from the surface with running buffer using a flow rate of 100 $\mu$L/min, and 
the surfaces were regenerated with a 1-min injection of 10 mM glycine 
(pH 2.0). The percentage of binding at each antibody concentration was 
calculated as follows. Percentage of binding = (slope of the signal 
obtained with competitor (serum + antibody)/slope of the signal 
obtained without competitor (serum only)) $\times$ 100. The IC$_{50}$ for each 
antibody was calculated as the concentration required for 50% inhibi-
tion of the binding of the serum to immobilized HuCC49.

**Affinity Maturation of AKA**—To improve the affinity of AKA, the 
HCDR3 of AKA Fab was randomly mutated, and the resulting Fab 
library was subjected to a modified colony lift assay (39). To begin, the 
Fab expression vector, pC3Q-dgIII, was constructed by deletion of the 
gene III from pComb3H (40) and by replacement of the first amino acid 
residue, glutamic acid, by glutamine. Next, the V$_{H}$ of aka and the V$_{L}$ of 
Hzk were subcloned into the Xhol-Apal and SacI-Xbal sites, respec-
tively, of pC3Q-dgIII to construct pC3Q-dgIII-aka. This plasmid DNA was 
used as a template for random mutagenesis of the HCDR3 by recombinant PCR. The first PCR was performed using the 5’ and 3’ 
primers, VH135 and HCDR3 BACK, respectively, or HCDR3 FOR-
WARD and LHS11, respectively. The HCDR3 FORWARD primer was 
mutagenic and introduced random mutations at the first five amino acid 
residues of the HCDR3 of AKA. The nucleotide sequences of the prim-
ers were as follows: VH135, 5’-AGTGGCACTGTCAAGTCTGG; 
HCDR3 BACK, 5’-TCTTGGACAGTAAGGACGGCCTGTC; 
HCDR3 FORWARD, 5’-CTCTATTCTGTGAAAGA/G(A/T)CN- 
(G)C/NNSNNSNNSNSTACTGGGGCCAAGGCACCTCG; LHS11, 
5’-CACCCGTTCCGGAAGT. The two first PCR products were then 
subjected to recombinant PCR. The final PCR product was 
digested with Xhol and Apal and was cloned into the Xhol-Apal sites of 
pC3Q-dgIII-aka to create a mutant Fab library.

After the transformation of *Escherichia coli* TG1 with the library, 10$^6$ 
cells were pipetted onto a nitrocellulose membrane placed on a 2× YT 
plate and were grown overnight at 37 °C (master membrane). A nitro-
cellulose membrane was coated with antigen by incubation in PBS con-
taining 10 $\mu$g/ml bovine submaxillary mucin at 37 °C for 6 h, rinsed 
twice with PBS, and blocked with 5% skim milk at 37 °C for 2 h. The 
blocked membrane was rinsed twice with PBS and soaked in 2× YT 
broth with 1 $\mu$m isopropyl-$\beta$-D-thiogalactopyranoside and 100 $\mu$L/ml 
ampicillin (capture membrane). This capture membrane was placed on 
a 2× YT plate containing 1 $\mu$m isopropyl-$\beta$-D-thiogalactopyranoside, 
and the master membrane was placed, cell-coated side up, on top of the 
capture membrane; the membranes were then incubated at room 
temperature overnight. The capture membrane was rinsed five times with 
PBS containing 0.05% Tween 20 (PBST), blocked with 5% skim milk at 
37 °C for 6 h, and incubated with goat anti-human IgG F(ab')$_2$-horse-
radish peroxidase conjugate (1:1000 (v/v); Sigma) at 37 °C for 1 h. After 
washing, the membrane was developed by enhanced chemilumines-
cence, and the spots generated on the film were used to identify the 
corresponding colonies on the original bacterial plate. Each positive 
colonies was grown in 2× YT and subjected to another round of selec-
tion as described above. Finally, 180 positive colonies were isolated, and 
the antigen-binding activity of soluble Fab was measured by an indirect 
ELISA. The Fabs with high affinity were selected, and the nucleotide 
sequences of the HCDR3s were determined.

**Conversion of Fab to Whole IgG**—The V$_H$ of selected Fab and Ig heavy 
chain leader sequences (32) were fused by recombinant PCR. The PCR 
products were digested with EcoRI and Apal and were subcloned into 
the EcoRI-Apal site of pcCMV-dhfr-aka. The resulting expression 
plasmid was transfected into COS7 cells, and the antibody secreted in 
the culture supernatant was analyzed by ELISA to determine the anti-
gen-binding affinity.

**Radioiodination of Humanized Antibody**—The IODO-BEAD method 
(Pierce) was used for radioiodinating the purified AKA and 3E8 with $^{125}$I 
(Amersham Biosciences) or $^{131}$I (Korea Atomic Energy Research Institute). 
The radiolabeled antibody was purified by gel filtration on a PD-10 column 
(Amersham Biosciences) and was sterilized by filtration (0.22 $\mu$m; Milli-
apore Corp.). The radioiodinating yield and radiochemical purity were 
determined with instant thin layer chromatography-silica gel (Gelman Scien-
tific) in the stationary phase and 70% methanol in the mobile phase.

**Biodistribution Study**—Female athymic mice (BALB/c-nu/nu; 5–6 
weeks old; 17–23 g) were obtained from Japan SLC, Inc. Tumors were 
grown after a subcutaneous injection of 5 × 10$^6$ human colon adeno-
carcinoma cell line (LS174T) in the left thigh. After 14 days, $^{125}$I-3E8 or 
$^{131}$I-aka (20 $\mu$L/740 kBq) were injected into the tail vein of the athymic 
mice bearing LS174T tumors. For each time point, a group of three mice 
was sacrificed to collect and weigh blood, tumors, and organs. Radioac-
tivity was measured in a $\gamma$-scintillation counter. The percentage of 
the injected dose/g of tissue was calculated.
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Radioimmunotherapy Studies—The radioimmunotherapeutic efficacy of $^{131}$I-3E8 was evaluated in the athymic mice bearing LS174T tumors. For the tumor growth inhibition study ($n = 8$), LS174T cells ($5 \times 10^6$) were subcutaneously injected into the back of athymic mice (5–6 weeks old; 17–23 g), and, after 14 days, saline or 7.4 MBq of $^{131}$I-labeled 3E8 (20 µg) was intravenously injected into each mouse every week during the first 6 weeks post-treatment. The body weight and tumor size were measured weekly for 42 days. The tumor volume on a given day was expressed relative to the initial volume on day 14. For individual tumors of the mice treated with saline or $^{131}$I-labeled 3E8, the tumor volume doubling time (Td) was calculated as the time required to reach a relative tumor volume of 2. The specific tumor growth delay was defined as (Td in mice treated with $^{131}$I-3E8 – Td in mice treated with saline)/Td in mice treated with saline.

For the survival study ($n = 7$), LS174T cells ($5 \times 10^6$) were injected intraperitoneally into the abdomen of the athymic mice; after 7 days, saline or 7.4 MBq of $^{131}$I-labeled 3E8 (20 µg) was injected intraperitoneally into each mouse every week during the first 6 weeks post-treatment. The survival of the mice was checked daily for 95 days, and their body weights were measured weekly for 76 days. Kaplan-Meier plots of percentage survival as a function of time were generated to assess the effect of treatment on the survival of mice in each group.

RESULTS

Design, Construction, and Affinity Determination of Humanized Antibody AKA—To design a humanized antibody, the amino acid sequences of the CC49 VL and VH were compared with those of human Ig germ line segments. The result showed that DP25-JH4 and DPK24-JK4 were most homologous to CC49 VH and VL, respectively (Fig. 1). The sequence comparison between CC49 VH and DP25-JH4 revealed that three positions in the HCDR1 and 11 positions in the HCDR2 were different, whereas 24 positions in the FRs were different. Their HCDR1 and HCDR2 domains have identical canonical structures, H1-1 and H2-3, respectively (7). The HCDR3 is not encoded by human germ line VH genes and has no canonical structure (7). To construct a humanized antibody (aka), the amino acid sequences of mouse, human, and humanized heavy chain (HzaK) are shown in Fig. 1A.

To design a humanized Vh, the amino acid sequence of CC49 Vh was compared with those of human DP25 and JH4. The sequence comparison between CC49 Vh and DP25-JH4 revealed that three positions in the HCDR1 and 11 positions in the HCDR2 were different, whereas 24 positions in the FRs were different. Their HCDR1 and HCDR2 domains have identical canonical structures, H1-1 and H2-3, respectively (7). The HCDR3 is not encoded by human germ line VH genes and has no canonical structure (7). To construct a humanized Vh, the SDRs (three residues in the HCDR1, seven in the HCDR2, and five in the HCDR3) were grafted onto DP25-JH4 while retaining two FR3 residues (Ala71 and Lys73) that were thought to influence the conformations of HCDR2 and HCDR3. The resulting humanized Vh was fused to human Cγ1 (hCy1) to construct the humanized heavy chain (aka). The amino acid sequences of mouse, human, and humanized Vh are shown in Fig. 1B.

The humanized heavy chain and light (HzK) chains were transiently expressed in COS cells, and the humanized antibody (AKA) secreted in the culture supernatant was assayed for antigen-binding affinity by a competition ELISA. The dissociation constant ($K_d$) of AKA was 1.05 × 10$^{-8}$ M (Fig. 2). To evaluate the importance of two mouse FR3 residues (Ala71 and Lys73) retained in AKA, either Ala71 or Lys73 was replaced with Arg71 (RTA) or Thr73 (ATA), respectively, of human DP25, or both residues were replaced with the human residues (RTA). The affinities of RTA, ATA, and RTA were 2.13 × 10$^{-8}$ M, 2.95 × 10$^{-8}$ M, and 5.83 × 10$^{-8}$ M, respectively (Fig. 2), indicating that both Ala71 and Lys73 are equally important in antigen-binding activity and thus need to be retained in this humanized antibody. The affinity of the humanized antibody (AKA-GQ) that consisted of the humanized heavy chain aka and the humanized light chain Hz-K-GQ was 0.75 × 10$^{-8}$ M (Fig. 2), which was slightly higher than that of AKA. This suggests that the two LCDR1 residues (Gly27 and Gln29) may not be SDRs. The affinity of the...
FIGURE 2. Affinity determination of humanized antibody AKA, AKA variants (RKA, ATA, RTA, and AKA/GQ), and chimeric constructs (ch/hK and ch/cK). ch/hK is a chimeric antibody that consists of chimeric CC49 heavy chain and humanized light chain HzK. ch/cK is a chimeric antibody that consists of chimeric CC49 heavy chain and light chains. Each of the constructs was transiently expressed in COS7 cells, and the culture supernatant was subjected to an indirect ELISA followed by a competition ELISA to determine the antigen-binding activity and affinity. The data are presented as a Scatchard plot. ψ, the fraction of bound antibody; i is the concentration of free TAG-72 at equilibrium. The slope of the straight line represents \( K_d \) (equal to \( 1/K_a \)) of each antibody.

TABLE 1

| Antibody | Relative affinity constant* \( (K_a) \) |
|----------|---------------------------------------|
| ch/cK    | 4.4 ± 0.1                             |
| ch/hK    | 9.7 ± 0.6                             |
| AKA      | 10.5 ± 1.2                            |
| RKA      | 21.3 ± 1.5                            |
| ATA      | 29.5 ± 0.4                            |
| RTA      | 58.3 ± 1.0                            |
| AKA/GQ   | 7.5 ± 0.2                             |

* \( K_a \) of each antibody was determined from Scatchard plots of three different experiments as described under “Experimental Procedures.”

Potential Immunogenicity of AKA—To assess its potential immunogenicity, AKA was produced from a stable CHO cell line and purified, as described under “Experimental Procedures.” Approximately 180 positive mouse residues was chosen for further study.

Affinity Maturation of AKA and Generation of the High Affinity Variant 3E8—To improve the affinity of AKA, AKA Fab was subcloned in the soluble Fab expression vector. The SDRs (amino acids 95–99) of the HCDR3 were then randomly mutated and subjected to panning, as described under “Experimental Procedures.” Approximately 180 positive Fab clones were selected and analyzed by ELISA for their antigen-binding activities. We were able to identify five different Fab clones (3E8, 3C4, NV, 3D5, and NI) with affinities higher than that of AKA Fab. For the accurate determination of affinity, the Fab format was converted into whole IgG and subjected to a competition ELISA. As shown in Fig. 4A, the affinities of the five variants (3E8, 3C4, NV, 3D5, and NI) were \( 0.65 \times 10^{-9}, 1.33 \times 10^{-9}, \) and...
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1.66 × 10⁻⁹, 2.27 × 10⁻⁹, and 3.38 × 10⁻⁹ M, respectively, which corresponded to 22-, 11-, 8-, 6-, and 4-fold increases in the affinity, respectively, compared with the AKA affinity. The HCDR3 sequences of the affinity-matured variants are shown in Table 2.

To examine whether 3E8 and CC49 bind to the same epitope, 3E8 was produced and purified from a stable CHO cell line, and a competition binding assay with CC49 was performed. As shown in Fig. 4B, the binding of CC49 to an immobilized TAG-72 was inhibited in a dose-dependent manner by increasing concentrations of 3E8 but not by an irrelevant antibody, anti-HBV humanized KR127 (36). This result indicates that 3E8 and CC49 may bind to the same epitope. The IC₅₀ (i.e. the concentration of the competitor antibody (3E8) required for 50% inhibition of the binding of CC49 to TAG-72) was ~5-fold less than that of biotinylated 3E8 to TAG-72, demonstrating that the affinity of 3E8 is higher than that of CC49.

A comparison of the HSDR3 sequences between the AKA and affinity-matured versions revealed that, despite large increases in their affinities, the sequence variations were limited (Table 2). In more detail, Ser⁹⁵ was maintained, Leu⁹⁶ was maintained or replaced by Trp⁹⁶, Asn⁹⁷ was replaced by Val⁹⁷ or Ile⁹⁷, Met⁹⁸ was maintained or replaced by Gln⁹⁸, and Ala⁹⁹ was replaced by Gly⁹⁹ or Gln⁹⁹. Notably, replacement of Asn⁹⁷ by Val⁹⁷ (NV) or Ile⁹⁷ (NI) resulted in an increase in the affinity by 8- or 4-fold, respectively, suggesting that Asn⁹⁷ in CC49 is suboptimal for TAG-72 binding. To verify this, Asn⁹⁷ of AKA was replaced by Ala⁹⁷ (N97A), and its antigen-binding activity was analyzed. Indeed, the activity of the N97A mutant was higher than that of AKA (Fig. 4C). To analyze the effect of the other amino acid changes on the affinity maturation of 3E8, Leu⁹⁶ or Ala⁹⁹ of AKA was replaced by Trp⁹⁶ (L96W) or Gln⁹⁹ (A99Q), respectively, and each mutant was analyzed for antigen-binding activity. As shown in Fig. 4C, the single mutation abrogated the activity of AKA, suggesting that the improvement of affinity from AKA to 3E8 may have resulted from the combined effect of the L96W, N97A, and A99Q mutations.

Biodistribution and Tumor Targeting Study—The tumor targeting abilities of 3E8 and AKA were studied in athymic mice bearing human colon adenocarcinoma xenografts. The 125I-AKA or 125I-3E8 was injected into the mouse model, and their biodistribution was examined at 4, 24, 48, and 72 h postinjection. The percentage of the injected dose/g of tissue of tumor and normal tissues are shown in Fig. 5. The AKA and 3E8 showed tumor localization, peaking at 24 h, with tumor uptake higher with 3E8 than with AKA at all time points examined. However, the 125I-AKA accumulated in the tumor was decreased in a time-dependent manner, whereas the 125I-3E8 in the tumor was almost maintained during the test period. Thus, tumor uptake of 125I-3E8 at 4, 24, 48, and 72 h postinjection became ~197, 167, 224, and 236%, respectively, of that of 125I-AKA. This is probably due to the increased affinity of 3E8 compared with AKA. The biodistribution study indicated that the 125I-3E8 showed ~2-fold higher tumor uptake than 125I-AKA.

Radioimmunotherapy with 131I-3E8—The radioimmunotherapeutic efficacy of 131I-3E8 was evaluated by the fractionated radioimmunotherapy protocol, because the use of dose-fractionation involving multiple injections of radiolabeled antibody is a strategy for producing more prolonged tumor growth inhibition, reducing hematological toxicity, and allowing higher total doses of radionuclide to be administered than with single-dose therapy (41, 42). To determine the therapeutic efficacy of 3E8, the 131I-labeled 3E8 (7.4 MBq) was injected once weekly for 6 weeks, and its anti-tumor effect in the athymic mice bearing human colon adenocarcinoma xenografts was evaluated by inhibition of tumor growth (Fig. 6A) or extension of the 50% survival rate (Fig. 6B). The

TABLE 2

HCDR3 sequences and relative affinity constants of AKA variants

| Antibody | Position in HCDR3 | Kᵦ \( \times 10⁻⁹ \) |
|----------|-------------------|-----------------|
| AKA | Ser Leu Asn Met Ala Tyr | 10.5 ± 1.2 |
| 3E8 | Ser Trp Ile Met Gln Tyr | 0.65 ± 0.02 |
| 3C4 | Ser Leu Val Gln Gly Tyr | 3.33 ± 0.11 |
| 3D5 | Ser Leu Ile Gln Gly Tyr | 2.27 ± 0.18 |
| NV | Ser Leu Val Met Ala Tyr | 1.66 ± 0.12 |
| NI | Ser Leu Ile Met Ala Tyr | 3.38 ± 0.25 |

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JOURNAL OF BIOLOGICAL CHEMISTRY

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The result from the tumor growth inhibition study showed that the Td in the \(^{131}I\)-3E8-treated mice (13.2 days) was significantly longer than that in the control mice injected with saline (4.9 days; Fig. 6A). The tumorspecific growth delay was calculated to be 1.7. The body weights of control mice increased to 119% of the initial body weight over time, saline-treated mice maintained their initial body weight, and the \(^{131}I\)-3E8-treated mice showed a reduction of up to 13% of the initial body weight (data not shown).

In the survival study, the 50% survival rate increased from 52.5 days in the control mice to 90.5 days in the \(^{131}I\)-3E8-treated mice (Fig. 6B), indicating that the 50% survival rate of the \(^{131}I\)-3E8-treated mice was 1.7 times that of the control mice. The body weight of the \(^{131}I\)-3E8-treated mice showed a reduction of up to 10% of the initial body weight (data not shown).

DISCUSSION

Murine mAbs induce human anti-mouse antibody responses in humans. To circumvent these problems, murine mAbs have been humanized mostly by CDR grafting. However, the non-human CDRs of humanized antibody can induce human anti-humanized antibody responses in patients (12). Alternative humanization strategies, such as veneering (43) and deimmunization (44), have been developed, but the veneered or deimmunized antibodies contain a higher number of mouse residues than do the CDR-grafted antibodies, and their effectiveness remains unproven in clinical studies. In this study, we constructed a humanized CC49 antibody by grafting only the SDRs within the CDRs of CC49 onto homologous human Ig germ line segments whose CDRs have the same canonical structures as those of CC49 while retaining only two mouse FR3 residues in the heavy chain that support the conformation of CDRs (Fig. 1). The resulting humanized antibody AKA showed only about 2-fold lower affinity compared with CC49 (Fig. 2) and 27-fold lower serum reactivity compared with the humanized antibody HuCC49 that was constructed by CDR grafting (Fig. 3). The results indicate that SDR grafting is an advanced strategy of antibody humanization.

The affinity of AKA was increased by random mutagenesis of the HCDR3 domain followed by affinity selection. The highest affinity variant, 3E8, showed ~22-fold higher affinity (Fig. 4A) and 2-fold better tumor targeting ability (Fig. 5) compared with AKA. In addition, it showed higher affinity compared with parental CC49 while maintaining the epitope specificity of CC49 (Fig. 4B). The sequence comparison and mutational analysis of the HCDR3 between AKA and affinity-matured antibodies revealed that Asn\(^{97}\) of CC49 is suboptimal for TAG-72 binding and that N97V or N97I is critical for affinity maturation (Fig. 4C, Table 2). The variant 3E8 has the least number of mouse residues and the highest affinity among the humanized versions of CC49 that have been constructed to date (26–29).

The 3E8 labeled with \(^{131}I\) showed an anti-tumor therapeutic effect in athymic mice bearing human colon adenocarcinoma xenografts (Fig. 6). The \(^{131}I\)-3E8-treated mice showed 1.7-fold increases of tumor-specific growth delay and 50% survival rate compared with the control mice treated with saline, and the \(^{131}I\)-3E8-treated mice showed a 10–13% reduction of body weight. Since bone marrow toxicity is evaluated by loss of body weight (45), the results suggest that the fractionated radioimmunotherapy protocol resulted in prolonged tumor growth inhibition and low hematological toxicity in the \(^{131}I\)-3E8-treated mice. To our knowledge, this is the first study to demonstrate the therapeutic efficacy of an anti-TAG-72 humanized antibody conjugated with radioisotope against human colon adenocarcinoma in an in vivo tumor model. The advantages of 3E8, such as high affinity, efficient tumor targeting, and therapeutic efficacy, will make it a useful reagent in the diagnosis and therapy of colon adenocarcinoma expressing TAG-72.
High Affinity Anti-TAG-72 Humanized Antibody

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