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Impact of Different Selectivity between Soluble and Membrane-bound Forms of Carcinoembryonic Antigen (CEA) on the Target-mediated Disposition of Anti-CEA Monoclonal Antibodies

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ABSTRACT

Carcinoembryonic antigen (CEA) is a tumor-specific antigen overexpressed in multiple cancers. CEA is expressed as a membrane protein, a part of which is cleaved from the cell membrane and secreted into blood. The soluble form of CEA (sCEA) has been shown to accelerate the clearance of anti-CEA antibody, which limits the antibody distribution in the tumor. To overcome this issue, we developed an anti-CEA monoclonal antibody, 15-1-32, which shows a strong affinity for membrane-bound CEA (mCEA) and relatively weak affinity for sCEA. In this study, we compared the effect of sCEA on the pharmacokinetics of 15-1-32 in mice with that of another anti-CEA monoclonal antibody, labetuzumab, showing less selectivity to mCEA than 15-1-32. As expected, the effect of sCEA on the serum concentration of 15-1-32 was much smaller than that of labetuzumab. The decrease in the area under the curve (AUC) of serum concentration was 22.5% for 15-1-32 when it was coadministered with sCEA, while that of labetuzumab was 79.9%. We also compared the pharmacokinetics of these two antibodies in CEA-positive tumor-bearing mice. The AUCs of 15-1-32 and labetuzumab were decreased in tumor-bearing mice compared with non-tumor-bearing mice to a similar extent (approximately 40% decrease). These results suggested that mCEA also contributes to the clearance of anti-CEA antibodies in CEA-positive tumor-bearing mice. Although the increased selectivity to mCEA minimized the effect of sCEA on the pharmacokinetics of 15-1-32, it may be insufficient to improve the pharmacokinetics in CEA-positive cancer patients.

SIGNIFICANCE STATEMENT

Because previous studies reported the rapid clearance of anti-CEA antibodies mediated by soluble CEA, we obtained a monoclonal antibody, 15-1-32, selective to membrane-bound CEA and evaluated the effects of CEA on its pharmacokinetics. Although the effect of soluble CEA on the serum concentration of 15-1-32 was very small, the clearance of 15-1-32 in CEA-positive tumor-bearing mice was still rapid, suggesting membrane-bound CEA also contributes to the clearance of anti-CEA antibodies. These results indicated that increasing selectivity to membrane-bound CEA is not enough to improve the pharmacokinetics of anti-CEA antibody.

Introduction

Carcinoembryonic antigen (CEA or CEACAM5) is a 180 kDa glycosylated protein that is highly expressed on the cellular surface of multiple cancers, especially in gastrointestinal cancers (Thompson et al., 1991; Jothy et al., 1993; Kodera et al., 1993; Hammarström, 1999). CEA is a glycophosphatidylinositol (GPI) anchor protein, which is anchored on the cellular membrane via glycophosphatidylinositol (Hefta et al., 1988). Like the other GPI anchor proteins, a part of the membrane-bound CEA (mCEA) is cleaved by GPI phospholipase D and is secreted into blood (Yamamoto et al., 2005; Naghibalhossaini and Ebadi, 2006). Higher levels of serum soluble CEA (sCEA) are observed in cancer patients, and thus, sCEA is widely used as a diagnostic marker for various cancers, especially for gastrointestinal cancers (Polat et al., 2014; Shimada et al., 2014). Owing to its high and selective expression in cancer cells, CEA has been also considered as an attractive drug target for cancer treatment.

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Monoclonal antibodies are widely used for cancer therapy (Scott et al., 2012). A monoclonal antibody can recognize cell-surface proteins on target cancer cells in an antigen-specific manner and kill cancer cells or inhibit their growth by modifying the protein function, antibody-dependent cellular cytotoxicity, and/or compliment-dependent cytotoxicity. Recently, antibody-drug conjugate (ADC) technology also has been developed, where antibodies tagged with cytotoxic agents deliver the cytotoxic agents to the target cancer cells. There are four approved ADCs; CD33-targeted gemtuzumab ozogamicin (Mylotag; Wyeth Pharmaceuticals), CD30-targeted brentuximab vedotin (Adcetris; Seattle Genetics, Inc.), Her2-targeted trastuzumab emtansine (Kadcyla; Genetech, Inc.), and CD22-targeted inotuzumab ozogamicin (Besponsa; Wyeth Pharmaceuticals, Inc.) (Abdollahpour-Alitappeh et al., 2019).

CEA was first found in human colorectal tumors in 1965 and has long been recognized as a cancer antigen (Gold and Freedman, 1965). Multiple monoclonal antibodies, including some ADCs, targeting CEA have been developed, and some of them have entered clinical trials (Sharkey et al., 2005; Oberst et al., 2014; Govindan et al., 2015; Sahlmann et al., 2017).

ABBREVIATIONS: ADC, antibody-drug conjugate; AUC, area under the curve; CEA, carcinoembryonic antigen; GPI, glycophosphatidylinositol; mCEA, membrane-bound CEA; QC, quality control; sCEA, soluble form of CEA.

However, no CEA-targeted therapeutics have been approved to date. There are at least two possible reasons for this. First, the biologic function of CEA in tumorigenesis is unclear. Like the other GPI anchor proteins, CEA has no intracellular domain required for signal transduction. Therefore, it is difficult to kill cancer cells or inhibit their growth by modifying the function of CEA. Second, sCEA limits the availability of anti-CEA antibodies to the tumor site. Beatty et al. (1990a) demonstrated that the blood concentrations of ^{111}In-labeled anti-CEA antibody (Indacea) were largely decreased by coadministration of sCEA compared with those without coadministration in mice. The blood clearance of Indacea also increased in CEA-expressing tumor-bearing mice compared with those of normal mice, suggesting the limited availability of the antibody to tumor.

Completely a human monoclonal antibody, 15-1-32 binds to the *N*-terminal domain of mCEA (Shinmi et al., 2017) and has a stronger affinity for mCEA than other anti-CEA antibodies but a poor affinity for sCEA. Shinmi et al. (2017) demonstrated that sCEA has a minor effect on the binding of 15-1-32 to MKN45, a CEA-positive gastric cancer cell line, whereas sCEA shows a greater effect on that of labetuzumab, another anti-CEA antibody with high binding affinity to sCEA. Labetuzumab was reported to bind to the A3B3 domain of CEA (Ma et al., 2004). When conjugated with monomethyl auristatin E, a cytotoxic agent, 15-1-32 showed an antigen-dependent cytotoxic effect on MKN45 that was not affected by the presence of high-level sCEA. Thus, it is expected that 15-1-32 could overcome the negative influence of sCEA, which reduced serum concentrations of antibody and its availability to the tumor site.

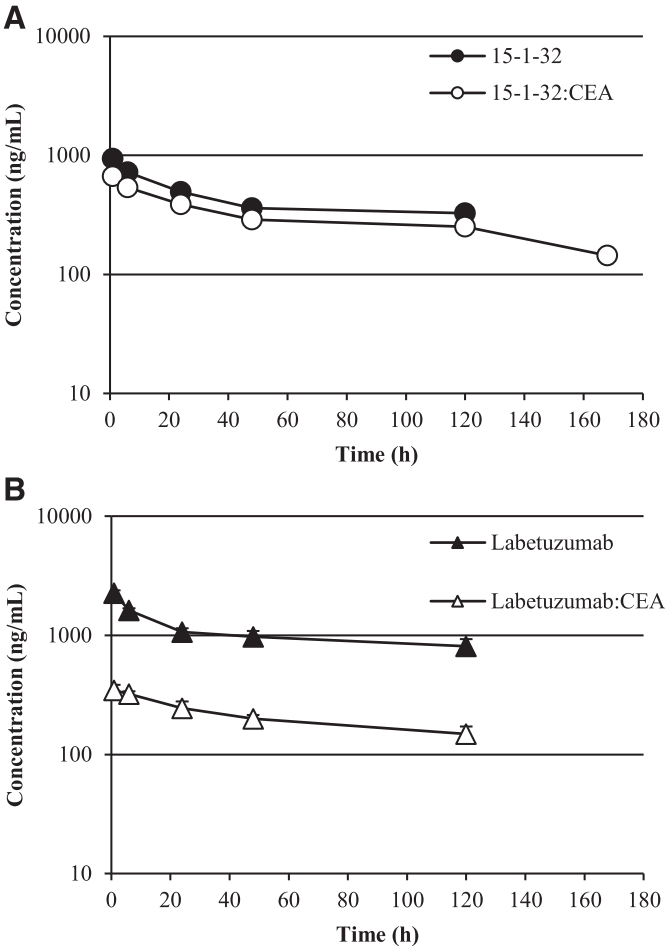

Fig. 1. Serum concentration–time profiles of 15-1-32 (A) and labetuzumab (B) after intravenous administration (0.1 mg/kg) with or without coadministration of soluble CEA (0.5 mg/kg) to male BALB/c mice. Each symbol represents mean ± S.D. (n = 4).

Our study investigated the effects of sCEA on the pharmacokinetics of two anti-CEA antibodies: 15-1-32 and labetuzumab. We also prepared CEA-expressing tumor-bearing mice in which serum sCEA levels are maintained over 5 ng/ml (the reference value of sCEA as a cancer diagnostic marker) (Perkins et al., 2003; Suzuki et al., 2017). We compared the pharmacokinetics of the two anti-CEA antibodies between tumor-bearing mice and normal (non-tumor-bearing) mice. Further, we discuss the biologic significance of sCEA selectivity based on our results.

Materials and Methods

Materials

As described previously elsewhere, 15-1-32 and labetuzumab were internally produced (Shinmi et al., 2017). Briefly, 15-1-32 or labetuzumab were stably expressed in DG44 cells, and the antibodies were purified from the culture supernatant using MabSelect SuRe Protein A resin (GE Healthcare, Piscataway, NJ). Trastuzumab was cloned into the mammalian expression vector and stably expressed in CHO-K1 cells. Trastuzumab was purified from the culture supernatant as described earlier. The purity of each antibody was examined by SDS-PAGE; it showed the presence of a single band of about 150 kDa, indicative of the desired purity (data not shown).

Fluorescent-labeled antibodies were prepared using Alexa Fluor 647 Protein Labeling Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Human soluble CEA was purchased from Origene Technologies (Schillerstraße, Germany). Mouse anti-human IgG antibody and monkey anti-human IgG antibody were internally produced and were biotinylated using Biotin Labeling Kit – SH (Dojindo Laboratories, Kumamoto, Japan) or ruthenylated using MSD GOLD Sulfo-TAG NHS Ester (Meso Scale Discovery, Rockville, MD) following the manufacturer's instructions.

The human gastric cancer cell line MKN45 was transfected with pMSCV-luc to establish a luciferase-expressing stable cell line, MKN45-Luc. The luciferase-expressing human gastric cancer cell line 44As3-Luc was prepared and kindly provided by Dr. Kazuyoshi Yanagihara (National Cancer Center Exploratory Oncology Research & Clinical Trial Center, Chiba, Japan). Both the cell lines were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator maintained with 5% CO_2 at 37°C.

Animals

Male BALB/cA Jcl (BALB/c) mice and BALB/cA Jcl-nu (nude) mice were purchased from Clair Japan (Tokyo, Japan). The animals were habituated for at least 7 days and were used between the ages of 7 and 11 weeks. All animal studies were performed in accordance with Standards for Proper Conduct of Animal Experiments at Kyowa Hakko Kirin Co., Ltd., under the approval of the company's Institutional Animal Care and Use Committee. Tokyo Research Park of Kyowa Hakko Kirin Co., Ltd., is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (15J0135).

Animal Studies

Effect of Coadministration of Soluble CEA. The dose solution of each antibody was prepared at a concentration of 0.02 mg/ml with or without 0.1 mg/ml of sCEA in saline (Otsuka Pharnaceutical, Tokyo, Japan) just before administration. We intravenously administered 15-1-32 and labetuzumab (5 ml/kg, bolus) to male BALB/c mice (8 weeks old, n = 4) at a dose of 0.1 mg/kg with or without coadministration of sCEA (0.5 mg/kg). Blood samples (approximately 30 μl) were serially collected from the tail vein of each mouse at 1, 6, 24, 48, and 120 hours. Blood samples at the last time point (168 hours) were collected from abdominal vein under isoflurane anesthesia.

The blood samples were placed at room temperature for coagulation and then centrifuged (4°C, 10,000g) for 5 minutes. The serum samples obtained were stored in frozen condition at −20°C until the measurement of serum concentrations of 15-1-32 and labetuzumab. In the following experiments, serum samples were prepared from blood and stored until use as described previously.

TABLE 1

Area under the curve (AUC) comparison for the serum concentrations of 15-1-32 and labetuzumab after intravenous administration (0.1 mg/kg) between with and without coadministration of soluble carcinoembryonic antigen (CEA) to male BALB/c mice

Each value represents mean ± S.D. (n = 4). $AUC_{0-120\ hours}$ percentage ratio (%) = $AUC_{0-120\ hours,with\ CEA}/AUC_{0-120\ hours,without\ CEA} \times 100$.

Antibody	$AUC_{0-120\ h}$		$AUC_{0-120\ h}$ Percentage Ratio (%)
	Without CEA	With CEA	
	ng·h/ml		
15-1-32	51,000 ± 2400	39,500 ± 1300	77.5 ± 2.6
Labetuzumab	125,000 ± 12,000	25,200 ± 1300	20.1 ± 1.0

Dose-Dependent Effect of Soluble CEA. The dose solution of each antibody was prepared at a concentration of 0.02 mg/ml with or without 0.01–0.04 mg/ml of sCEA in saline just before administration. We intravenously administered 15-1-32, labetuzumab, and trastuzumab (5 ml/kg, bolus) into male BALB/c mice (9 weeks old, $n = 4$) at a dose of 0.1 mg/kg with or without coadministration of soluble CEA (0.05–0.2 mg/kg). Blood samples were collected at 15 minutes after administration from the abdominal vein under isoflurane anesthesia.

Liver Distribution. The dose solution of antibody was prepared at a concentration of 0.02 mg/ml with or without 0.02 mg/ml of sCEA in saline just before administration. Alexa Fluor 647–labeled 15-1-32, labetuzumab, or trastuzumab was intravenously administered (5 ml/kg, bolus) into male BALB/c mice (9 weeks old, $n = 4$) at the dose of 0.1 mg/kg with or without coadministration of soluble CEA (0.1 mg/kg). Liver samples were collected at 15 minutes after administration under isoflurane anesthesia and flushed remaining blood by saline injection from the hepatic vein.

The fluorescent intensity in the liver samples was measured by IVIS Spectrum (PerkinElmer, Waltham, MA) with excitation at 640 nm and emission at 680 nm filters. Whole liver of each sample was traced as the region of interest, and the fluorescence intensity ([photons/s]/[$\mu W/cm^2$]) was calculated with the Living Image 4.3.1 software (PerkinElmer).

Preparation of Tumor-Bearing Mice. Five million MKN45-Luc or 1 million 44As3-Luc cells were intraperitoneally inoculated into nude mice at the ages of 8 and 7 weeks, respectively. The day of inoculation is defined as day 0, and subsequent days are named from day 1 until the last day of study. Luciferase activity was monitored as an index of tumor growth on appropriate days after inoculation. Luciferin (VIvoGlo Luciferin; Promega Corporation, Fitchburg, WI) was intraperitoneally administered at the dose of 150 mg/kg, and the whole body luminescence intensity (photons per second) was measured by IVIS Spectrum. Blood samples were collected from tail vein on the appropriate days.

Serum soluble CEA concentrations were measured using the CEA ELISA kit (Immunospec Corporation, Canoga Park, CA) following the manufacturer's instructions. Briefly, 1) Apply 50 μl of standards or samples to the anti-CEA antibody coated plate. 2) Dispense 100 μl of horseradish peroxidase–labeled anti-CEA antibody to each well and thoroughly mix. 3) Incubate at room temperature for 60 minutes. 5) Wash the plate 5 times with washing buffer. 6) Dispense 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate into each well and incubate for 20 minutes. 7) Measure optical density at 450 nm using ARVO-X3 (PerkinElmer).

Pharmacokinetic Study in Tumor-Bearing Mice. The dose solution of each antibody was prepared at concentration of 0.02 mg/ml in saline just before administration. In the following study, 5 ml/kg of the dose solution was intravenously administered (0.1 mg/kg) to tumor- and non-tumor-bearing mice. MKN45-Luc and 44As3-Luc cells were intraperitoneally inoculated into nude mice as described earlier (10 mice for each cell line).

For the MKN45-Luc model, based on serum sCEA concentrations on day 13, the mice were divided into two groups ($n = 4$ for each) to make the average serum sCEA concentration similar between the two groups. We intravenously administered 15-1-32 and labetuzumab (0.1 mg/kg) into the mice on day 17.

For the 44As3-Luc model, luciferase activity was measured on day 5, and the mice were divided into two groups ($n = 4$ for each) to make the average in vivo luminescence intensity of each group become similar. We intravenously administered 15-1-32 and labetuzumab (0.1 mg/kg) into the mice on day 6.

We also intravenously administered 15-1-32 and labetuzumab (0.1 mg/kg) to normal nude mice.

Blood samples (approximately 30 μl) were serially collected from the tail vein of each mouse at 0.5, 6, 24, 48, 72, and 96 hours. Blood samples at the last time point (168 hours) were collected from abdominal vein under isoflurane anesthesia.

Determination of Serum Concentrations of Anti-CEA Antibodies

The serum concentrations of 15-1-32 and labetuzumab were measured by electrochemiluminescent immunoassay by the following procedure. 1) Streptavidin-coated plates (Meso Scale Discovery) were treated with PBS containing 1% casein for 1 hour. 2) We incubated the plate with biotinylated anti-human IgG antibody for 1 hour. 3) Each antibody was diluted with blank serum to prepare calibration standards and quality control (QC) samples. 4) We applied standards, QCs, and test samples in duplicate and incubated for 2 hours. 5) We incubated with ruthenium-labeled anti-human IgG antibody. 6) We added Read buffer (Meso Scale Discovery) and measured the luminescence using a SECTOR Imager 2400 (Meso Scale Discovery). Between the individual steps, we washed the plates 3 times with PBS containing 1% Tween-20.

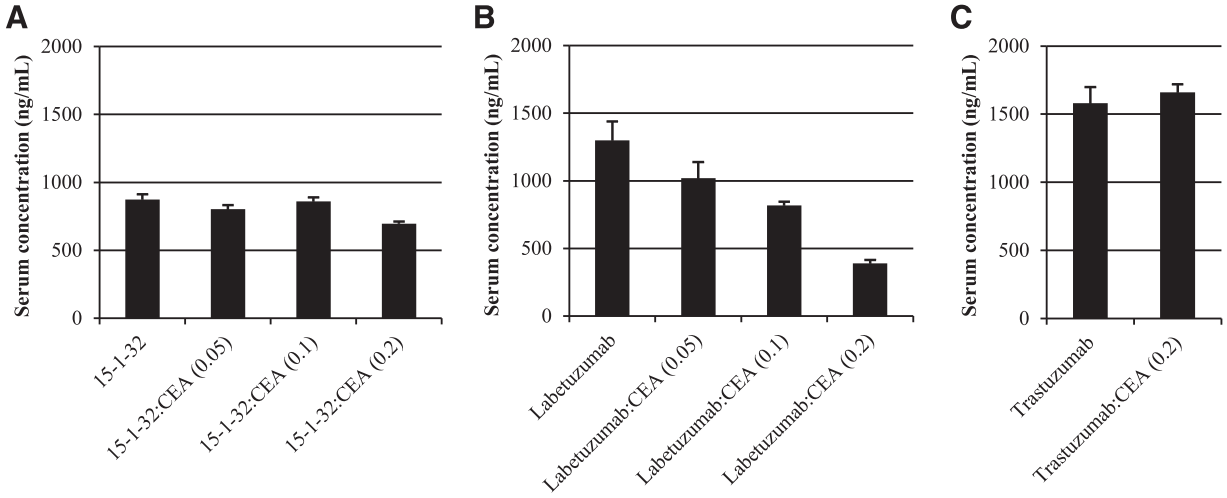

Fig. 2. Serum concentrations of 15-1-32 (A), labetuzumab (B), and trastuzumab (C) at 15 minutes after intravenous administration (0.1 mg/kg) with or without coadministration of soluble CEA (0.05–0.2 mg/kg) to male BALB/c mice. Values in parentheses represent the dose of soluble CEA. Each bar represents mean ± S.D. ($n = 4$).

A calibration curve was generated by log-log regression of the natural logarithmic transformed nominal concentration of the calibration standards (x-axis), and the natural logarithmic transformed mean values of the duplicate luminescence intensity (y-axis) using SOFTmaxPRO (Molecular Devices Japan, Tokyo, Japan). Each antibody concentration was calculated by substitution of the mean value of duplicate luminescence intensity to the regression equation of the calibration curve of each antibody on the same plate. The concentration range of standards was 30–10000 ng/ml. The concentrations of QC samples were 30, 100, 10,000 ng/ml. The coefficients of variation of luminescence intensity in duplicate measurement were less than 20%. The accuracies were within ±20%.

Pharmacokinetic Parameters

The serum concentrations of 15-1-32 or labetuzumab were plotted on a semi-log plot where the x-axis is time (linear) and the y-axis is serum concentration (log). The slope of initial linear phase was calculated, and the serum concentration at time 0 (C_0) was estimated by extrapolating the slope to time 0. The area under the curve (AUC) was determined by the trapezoidal rule with C_0.

Results

Effect of sCEA on Pharmacokinetics of Anti-CEA Antibody in Normal Mice. Effects of sCEA on pharmacokinetics of 15-1-32 and labetuzumab were investigated in normal mice by coadministration of sCEA with a molar ratio of 4 against each antibody, the same as in a previous report (Beatty et al., 1990a). Anti-CEA antibodies (0.1 mg/kg) were intravenously administered into male mice with or without coadministration of sCEA (0.5 mg/kg). The serum concentration–time curves of the anti-CEA antibodies are shown in Fig. 1. The serum concentrations of labetuzumab were largely decreased by coadministration of sCEA, while the influence on 15-1-32 serum concentrations was limited. The percentage ratios of the AUC of anti-CEA antibodies after administered with sCEA and alone were 77.5% ± 2.6% and 20.1% ± 1.0% for 15-1-32 and labetuzumab, respectively (Table 1).

The dose-dependent effects of sCEA were evaluated on serum concentrations of anti-CEA antibodies in mice, and herein trastuzumab (anti-Her2 antibody) was employed as a negative control. The dose of sCEA ranged from 0.05 to 0.2 mg/kg, while that of each antibody was fixed at 0.1 mg/kg. The sampling time point was set as 15 minutes because labetuzumab was cleared rapidly from serum within 1 hour after coadministration with sCEA. The molar ratios of sCEA versus each antibody were 0.4–2.0. Each antibody was intravenously administered with or without sCEA into mice, and the serum concentrations at 15 minutes after administration were measured. The serum concentrations of labetuzumab were clearly decreased in a dose-dependent manner of sCEA whereas the effects of sCEA were none or insignificant on the serum concentrations of 15-1-32 (Fig. 2, A and B). No effect of sCEA was observed on the serum concentrations of trastuzumab (Fig. 2C).

Effect of sCEA on Liver Distribution of Anti-CEA Antibodies in Normal Mice. We investigated the effects of sCEA on liver distribution of anti-CEA antibodies using fluorescent-labeled antibodies. Because clear effects of sCEA were observed on the serum concentrations of labetuzumab at a dose of 0.1 mg/kg for both labetuzumab and sCEA, the same dose was used. Trastuzumab was also used as a negative control in this study. Each antibody was intravenously administered with or without sCEA into mice. Then liver samples were collected at 15 minutes after administration, and the fluorescent intensity of liver was analyzed using the IVIS imaging system. The liver fluorescence intensities of labetuzumab were increased approximately 2-fold by coadministration of sCEA (Fig. 3). However, 15-1-32 showed only a slight increase and trastuzumab showed no increase in liver fluorescent by coadministration of sCEA (Fig. 3).

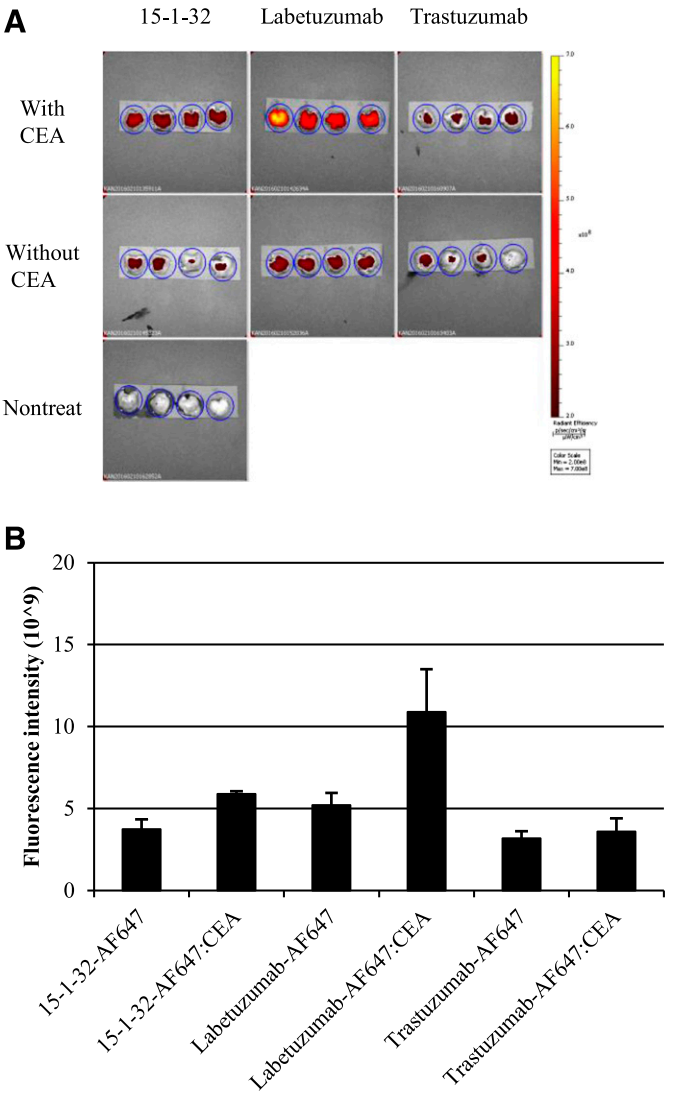

Fig. 3. Fluorescent image (A) and fluorescence intensity (B) in liver after intravenous administration of fluorescent-labeled antibodies (0.1 mg/kg) with or without coadministration of soluble CEA (0.1 mg/kg) to male BALB/c mice. In *B*, each bar represents mean ± S.D. (n = 4).

Pharmacokinetics of 15-1-32 and Labetuzumab in Tumor-Inoculated Mice. To investigate the effects of CEA-positive tumors on the pharmacokinetics of anti-CEA antibodies, tumor-bearing mice were prepared with CEA-positive cancer cell lines. MKN45 and 44As3 are human gastric cancer cell lines exhibiting a high expression of CEA. Luciferase-expressing MKN45 and 44As3 (MKN45-Luc and 44As3-Luc, respectively) cells were used to monitor the cancer cell growth in the peritoneal cavity of mice by in vivo imaging.

From our preliminary studies, higher serum levels of sCEA were obtained with these two cell lines by intraperitoneal inoculation rather than subcutaneous inoculation in nude mice (data not shown). Figure 4 shows the time courses of tumor-derived luminescent intensity after intraperitoneal inoculation of each cell line into mice. Compared with MKN45-Luc, 44As3-Luc showed higher luminescent intensity and continued to increase until day 15. The luminescent intensities from MKN45-Luc were almost constant until day 21.

The serum sCEA levels were monitored throughout the experimental period (Fig. 5). The serum sCEA levels increased rapidly after day 5 and became 50–60 ng/ml between days 10 and 13 in 44As3-Luc model.

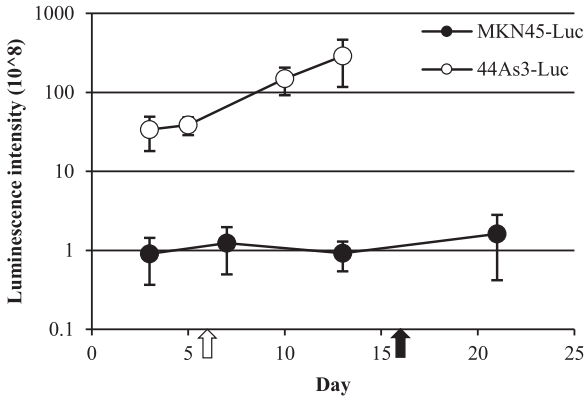

Fig. 4. Luminescence intensity–time profiles after intraperitoneal inoculation of MKN45-Luc and 44As3-Luc to nude mice. Each bar represents mean ± S.D. (n = 4). Each arrow represents the timing of the administration to MKN45-Luc (closed) or 44As3-Luc (open) intraperitoneally inoculated nude mice.

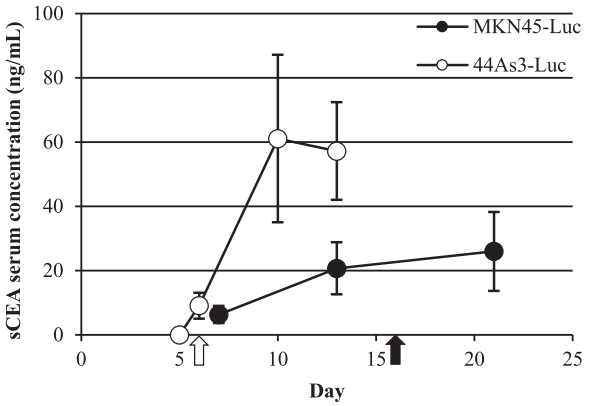

Fig. 5. Serum CEA concentration–time profiles in MKN45-Luc and 44As3-Luc intraperitoneally inoculated nude mice. The horizontal line represents days after inoculation. Each symbol represents mean ± S.D. (n = 6–8). Each arrow represents the timing of the administration to MKN45-Luc (closed) or 44As3-Luc (open) intraperitoneally inoculated nude mice.

They increased gradually and became 20–30 ng/ml between days 13 and 21 in the MKN45-Luc model. The reference value for the serum sCEA to be used as a diagnostic marker for gastrointestinal cancers is 5 ng/ml (Compton et al., 2000). In the 44As3-Luc model, the serum sCEA level reached over 5 ng/ml on day 6. In the MKN45-Luc model, the serum sCEA level was comparable to 5 ng/ml on day 7 and became clearly above 5 ng/ml on day 13. Based on these results, pharmacokinetic studies of anti-CEA antibodies were begun on day 6 in the 44As3-Luc model and day 13 in the MKN45-Luc model.

The serum concentrations of anti-CEA antibodies in normal mice and 44As3-Luc and MKN45-Luc inoculated mice are shown in Fig. 6. Both labetuzumab and 15-1-32 showed decreased serum concentrations in 44As3-Luc and MKN45-Luc models compared with normal mice. In the MKN45-Luc model, the AUC decreased compared with normal mice between 0 and 96 hours, which were 36.6% and 33.9% for labetuzumab and 15-1-32, respectively (Table 2). In the 44As3 model, the serum concentrations of anti-CEA antibodies were detected until 24 hours, so the AUC values between 0 and 24 hours were compared with those of normal mice. The AUC decrease between 0 and 24 hours was 42.1% and 36.3% for labetuzumab and 15-1-32, respectively (Table 3). Unexpectedly, the decreases in AUCs were similar between the two anti-CEA antibodies in both the 44As3-Luc and MKN45-Luc models. It was suggested that mCEA majorly affects the pharmacokinetics of anti-CEA antibodies and subsequently the effect of sCEA is minor.

Discussion

Compared with other anti-CEA antibodies, 15-1-32, a unique anti-CEA antibody, shows a stronger binding affinity for mCEA but a weaker affinity for sCEA (Shinmi et al., 2017). We expected that 15-1-32 could avoid the influence of sCEA, which limits the availability of anti-CEA antibodies to tumors. We first compared the effects of sCEA on the pharmacokinetics of 15-1-32 and labetuzumab, the anti-CEA antibodies with less or no selectivity to sCEA.

By the coadministration of sCEA, the serum concentrations of labetuzumab largely decreased while the decrease was small for 15-1-32. In the dosing solution, the concentrations of each antibody and sCEA were 133 and 555 μM, respectively. Considering the binding affinity of 15-1-32 and labetuzumab to sCEA (66.3 and 16.5 nM, respectively) (Shinmi et al., 2017), both these antibodies have been thought to form complexes with sCEA in the dose solution. However, the dissociation constant of 15-1-32 for sCEA is approximately 10-fold greater than that of labetuzumab (104 and 8.38 $\times$ 10^{-4} s^{-1}, respectively). It is possible 15-1-32 dissociates from sCEA more rapidly in blood after injection into mice, which had a smaller impact of sCEA than labetuzumab.

Studies have found sCEA is mainly cleared up by Kupffer cells via receptor-mediated endocytosis (Toth et al., 1982; Gangopadhyay and Thomas, 1996). A previous report demonstrated that the liver uptake of Indacea, an ^{111}In-labeled anti-CEA antibody, was increased by coadministration of sCEA in mice (Beatty et al., 1990b). Similarly, with the reported results, increased liver uptake of labetuzumab was observed

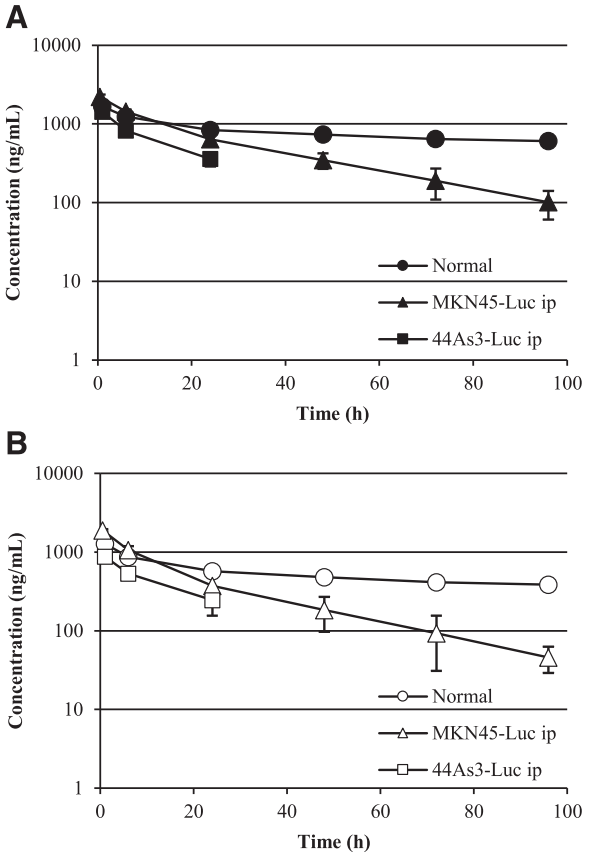

Fig. 6. Serum concentration–time profiles of 15-1-32 (A) and labetuzumab (B) after intravenous administration to normal nude mice and MKN45-Luc or 44As3-Luc intraperitoneally inoculated mice at the dose of 0.1 mg/kg. Each symbol represents mean ± S.D. (n = 4).

TABLE 2

Area under the curve (AUC) comparison for the serum concentrations of 15-1-32 and labetuzumab after intravenous administration (0.1 mg/kg) between normal and MKN45-Luc inoculated nude mice

Each value represents mean ± S.D. (n = 4). $AUC_{0-96\ hours}$ percentage ratio (%) = $AUC_{0-96\ h,\ MKN45\text{-}Luc}/AUC_{0-96\ hours,normal} \times 100$.

Antibody	$AUC_{0-96\ h}$		$AUC_{0-96\ h}$ Percentage Ratio (%)
	Normal	MKN45-Luc	
	ng·h/ml		
15-1-32	78,000 ± 5000	51,600 ± 6700	66.1 ± 8.7
Labetuzumab	52,500 ± 2000	33,300 ± 7800	63.4 ± 14.9

TABLE 3

Area under the curve (AUC) comparison for the serum concentrations of 15-1-32 and labetuzumab after intravenous administration (0.1 mg/kg) between normal and 44As3-Luc inoculated nude mice

Each value represents mean ± S.D. (n = 4). $AUC_{0-24\ hours}$ percentage ratio (%) = $AUC_{0-24\ hours,44As3\text{-}Luc}/AUC_{0-24\ hours,normal} \times 100$.

Antibody	$AUC_{0-24\ h}$		$AUC_{0-24\ h}$ Percentage Ratio (%)
	Normal	44As3-Luc	
	ng·h/ml		
15-1-32	27,600 ± 2100	17,600 ± 2200	63.7 ± 4.4
Labetuzumab	19,600 ± 1700	11,400 ± 1800	57.9 ± 9.0

with coadministration of sCEA while the increase in that of 15-1-32 was small.

In cancer patients, sCEA is constantly produced by the tumor, and a certain level of serum sCEA is maintained. To mimic this situation found in cancer patients, we prepared mouse models bearing CEA-positive tumors. By intraperitoneal inoculation of CEA-positive gastric cancer cell lines (44As3 and MKN45), more than 5 ng/ml of serum sCEA levels were achieved, which is the reference value of serum sCEA as a diagnostic marker for cancers (Perkins et al., 2003).

In the tumor-bearing mice, the serum concentrations of both 15-1-32 and labetuzumab were lower compared with those in normal mice. Different from the results of sCEA coadministration study, the decrease in serum concentrations was similar between 15-1-32 and labetuzumab: about a 40% decrease in both the 44As3 and MKN45 models. Because the impact of sCEA on the serum concentrations of 15-1-32 was small, it was suggested that 15-1-32 was mainly cleared by a pathway mediated by mCEA. Our previous report also demonstrated the internalization of 15-1-32 mediated by mCEA in MKN45, which led to intracellular degradation (Shinmi et al., 2017).

However, controversial results have been reported about the mCEA-mediated antibody internalization: no internalization was observed in some previous reports (Bryan et al., 2005; Boudousq et al., 2013). However, in those studies different antibodies and cell lines were used, and the incubation time was different. Internalization efficiency may differ depending on these experimental conditions. Considering the weaker binding affinity of labetuzumab to mCEA compared with 15-1-32, the clearance of labetuzumab mediated by mCEA may be smaller than that of 15-1-32. It has been reported that the liver and tumor uptake of ^{111}In labeled labetuzumab was decreased with the coadministration of unlabeled labetuzumab (Rijpkema et al., 2014). Therefore, it is possible that labetuzumab was cleared by both sCEA- and mCEA-mediated pathways, resulting in a similar decrease in tumor-bearing mice with 15-1-32.

In the sCEA coadministration study, the serum concentration–time curves of anti-CEA antibodies dropped from the initial time point with no change in half-life (data not shown). This may be because the antibody–sCEA complexes were cleared rapidly after injection and the remaining unbound anti-CEA antibodies were cleared with their original half-lives. On the other hand, in tumor-bearing mice the initial serum concentrations were not changed, but the half-lives of anti-CEA antibodies were increased. That may be because constant production of both sCEA and mCEA is the rate-limiting step for the clearance of anti-CEA antibodies. In cancer patients, sCEA is constantly produced by the tumor, so the pharmacokinetics of anti-CEA antibodies may be affected by CEA as observed in tumor-bearing mice.

In conclusion, it has been suggested that anti-CEA antibodies are eliminated by two different pathways mediated by sCEA and mCEA. Although the impact of sCEA on the pharmacokinetics of 15-1-32 was improved, becoming small with the reduction of the binding affinity to sCEA, 15-1-32 was still cleared rapidly by the mCEA-mediated pathway in tumor-bearing mice. Opposite to our expectations, increased selectivity to sCEA did not improve the pharmacokinetics in tumor-bearing mice. It only changed the contribution of sCEAs and mCEAs to the clearance of antibody.

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Authorship Contributions

Participated in research design: Iwano, Masuda, Enokizono.
Conducted experiments: Iwano, Shinmi.
Contributed new reagents or analytic tools: Murakami.
Performed data analysis: Iwano.
Wrote or contributed to the writing of the manuscript: Iwano, Enokizono.

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