Radioimmunoscintigraphy of colorectal cancer with technetium-99m-labeled murine anti-carcinoembryonic antigen monoclonal antibody in athymic nude mice

Naoyuki Watanabe,* Noboru Oriuchi,* Sumio Sugiyama,** Masahide Kuroki*** and Yuji Matsuoka***

*Department of Nuclear Medicine, School of Medicine, Gunma University.
** Department of Radiology, Takasaki National Hospital.
***First Department of Biochemistry, School of Medicine, Fukuoka University

Technetium-99m (Tc-99m) is an ideal radionuclide for clinical use. A murine monoclonal antibody (Mab) designated F33-104 binds to specific parts of carcinoembryonic antigen (CEA). In the present study, intact Mab F33-104 was labeled with Tc-99m, and the immunoreactivity and biodistribution of Tc-99m-labeled F33-104 were studied in athymic nude mice bearing human colorectal cancer xenografts. Mab F33-104, reduced under optimal conditions, was quickly and stably tagged with Tc-99m without loss of immunoreactivity. Higher tumor uptake of Tc-99m-labeled F33-104 was noted in the biodistribution, resulting in a higher localization index and specific-to-non-specific tumor ratio than those of radiiodinated F33-104. These results suggest the potential of Tc-99m-labeled Mab F33-104 for the radioimmunoimaging of colorectal cancer.

Key words: Tc-99m-labeled Mab F33-104, CEA, radioimmunoscintigraphy

INTRODUCTION

CARCINOEMBRYONIC ANTIGEN (CEA) is one of the most useful human tumor markers.1,2 Mabs to CEA have been labeled with radionuclides and studied for the diagnosis and therapy of colorectal cancer.3-7 It is generally accepted that Tc-99m is an ideal radionuclide for clinical use, due to its ready availability, low irradiation of the patients and low cost. Tc-99m-labeled antibodies have therefore been studied for radioimmunoscintigraphic studies of cancer, infection, thrombosis and myocardial infarction.3-7 However, there have been many problems such as the complexity of the processes, the long time required for preparation and in vivo unstableness in clinical use of Tc-99m-labeled Mabs.

Murine Mab designated F33-104 recognizes protein epitopes on domain A3-B3 of CEA, and is able to discriminate CEA in tumor tissues from normal fecal antigen-2, a soluble form CEA-counterpart in normal adult feces. F33-104 is currently used as the radiolabeled tracer in the radioimmunoassay (RIA) of serum CEA levels, resulting in the improvement of cancer specificity, sensitivity and accuracy.8-10 The present study employed a direct Tc-99m labeling method, where antibody could be quickly labeled with Tc-99m in the presence of a weak competing ligand hydroxyl methylene diphosphonate (HMDP) after the reduction of intrinsic disulphide bonds of Mab by the use of 2-mercaptopethanol.11

In the present paper, the in vitro and in vivo properties of Tc-99m-labeled Mab F33-104 are compared with those of radiiodination, and the potential of Tc-99m-labeled Mab for radioimmunoimaging is described.

MATERIALS AND METHODS

Monoclonal antibodies: The anti-CEA Mab F33-104 IgG was generated by conventional hybridoma methods with highly purified CEA preparations from liver metas-
tases of colon carcinomas as an immunizing antigen. The antibody was purified from ascitic fluid by affinity chromatography on protein A (Affi-Gel Protein A, Bio-Rad Laboratories, CA, USA) and was dialyzed against 0.05 M phosphate buffered saline (PBS), pH 7.5 for 24 hours. The binding affinity of F33-104 to CEA was calculated to be $1.8 \times 10^9$ M$^{-1}$ by Scatchard plot analysis. Mab BW431/26, which was used as positive control, recognized CEA. Tc-99m-labeled BW431/26 has been employed mainly in Europe for the diagnosis of patients with colorectal cancer. The Mab to human chorionic gonadotropin (hCG) was used as an irrelevant control Ab. The subtypes of both Mabs were IgG.$^5$

Cells and tumors: Female athymic nude mice (nu/nu) with a Balb/c background were obtained at approximately 4 weeks of age and were inoculated subcutaneously in the right rear flank with LS-180 human colorectal cancer cells (10$^6$ cells per animal) or PC-9 human lung cancer cells. LS-180 human colorectal cancer cells expressed CEA, whereas PC-9 cells, which were used as a negative control, did not express CEA. The sizes of LS-180 tumors were from 0.5 to 1.5 cm in diameter by 21 days after the inoculation and by 28 days for PC-9 tumors.

Radiolabeling of monoclonal antibodies
(1) Tc-99m-labeled monoclonal antibodies: Tc-99m labeling was performed by mixing a reduced Ab with Tc-99m-pertechnetate in the presence of a competing ligand HMDP (Clearborne, Nihon Mediphysics, Nishinomiya, Japan). Antibodies, 5 mg/ml of 0.05 M PBS, pH 7.5, were reduced by reaction with a molar excess of 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 100:1, 500:1, 1000:1 and 2000:1 (2-ME:Ab) at room temperature for 30 minutes. The reduced antibody was then purified on a PD-10 column (Pharmacia, Uppsala, Sweden) with 0.05 M PBS, pH 7.5 as a mobile phase. The antibody fractions were collected and divided into 0.5 mg aliquots. The antibody was frozen immediately at –80°C until use. For Tc-99m labeling, 50 $\mu$l of HMDP solution reconstituted with a 5 ml injection of 0.9% sodium chloride was added to the thawed antibody aliquot and mixed well for 10 seconds. Tc-99m-pertechnetate (740 MBq/ml) produced by generator (Ultra-TechnKow, Daiich Radioisotope Laboratories, Tokyo, Japan) was added to the antibody/HMDP mixture and incubated for 10 minutes. The specific activity of the resultant preparation was about 1,480 MBq/mg.

Labeling efficiency was assessed by cellulose acetate electrophoresis and gel-chromatography. Cellulose diacetate (Separax-5, Fuji Photo Film co, Tokyo), cut into $110 \times 10$ mm² strips was soaked in 0.06 M barbital buffer solution, pH 8.6 and then laid between the electrodes in a flat-bed electrophoresis tank. Tc-99m-labeled Mab, Tc-99m-labeled colloid and Tc-99m-pertechnetate control samples were applied with small pipettes and a current of 0.8 mA/strip was applied for 30 minutes at room temperature. After the strips were dried, the distribution of radioactivity was determined by images in a ZLC7500 gamma camera (Siemens, Ill, USA) equipped with a high resolution collimator for 10 minutes in a 512 by 512 pixel matrix with a digital computer (Scintipac 700, Shimadzu, Kyoto, Japan). Strips were also cut into fractions ($10 \times 10$ mm²) and the radioactivity was counted in a well-type gamma counter (Aloka, Japan). Gel-chromatography was performed on a PD-10 column. Approximately 0.1 $\mu$l of the sample was applied to the PD-10 column with 0.05 M PBS, pH 7.5 as a mobile phase. The fractions (0.5 ml) were collected into tubes, and counted in a well-type gamma counter.

(2) Radiiodinated antibodies: Mab was labeled with iodine-125 (I-125) by the chloramine-T method. In brief, I-125 sodium iodide (18.5 MBq/5 $\mu$l), Mab at 40 $\mu$g/180 $\mu$l in 0.3 M PBS, pH 7.5 and 10 $\mu$l of freshly prepared chloramine-T solution at 0.3 mg/ml in 0.3 M PBS, pH 7.5 were mixed, incubated for 5 minutes, and then separated from free I-125 by gel-chromatography on a PD-10 column. The specific activity of I-125-labeled Mab was approximately 440 MBq/mg.

Immunoreactivity of antibodies
(1) Immunoreactivity of reduced antibodies: The effect of reduction on the immunoreactivity of Ab was evaluated by competitive RIA with a commercial available CEA kit (Daiichi Radioisotope Laboratories), where I-125-labeled F33-104 is employed as an I-125-labeled tracer. I-125-labeled F33-104, standard CEA antigen (2.5 ng), and increasing amounts of reduced F33-104, non-reduced F33-104 or control Mab, and a bead covered with anti-CEA Mab were incubated at room temperature for 4 hours, washed 3 times and the radioactivity bound to beads was counted in a well-type gamma counter.

(2) Immunoreactivity of Tc-99m-labeled antibodies: The immunoreactivity of radiolabeled Mab was determined by two methods, antigen binding assays and cell binding assays. In antigen binding assays, each labeled antibody (12 ng) in 100 $\mu$l of 0.05 M PBS, pH 7.5, increasing amounts of standard CEA antigen in 50 $\mu$l of 0.05 M PBS, pH 7.5 and a bead covered with anti-CEA Mab were incubated at room temperature for 4 hours. After washing 3 times, the radioactivity bound to beads was counted in a well-type gamma counter.

Cell binding assays were performed with LS-180 human colorectal cancer cells, which expressed CEA on their surfaces. Labeled antibodies (12 ng) were incubated with increased numbers of LS-180 cells in 200 $\mu$l of 0.05 M PBS, pH 7.5 at room temperature for 1 hour in microtubes. These microtubes were centrifuged at 10,000 g x 5 minutes. The supernatants were then removed by aspiration and the pellets were cut with a knife and then counted in a well-type gamma counter.
Stability of Tc-99m-labeled monoclonal antibodies

Tc-99m-labeled Mab was incubated with 0.05 M PBS, pH 7.5, human serum with normal CEA levels (1.9 ng/ml; normal range less than 2.5 ng/ml), or human serum with high serum CEA (128 ng/ml) at room temperature for 0, 1, 4 and 24 hours. The stability of Tc-99m-labeled F33-104 was assessed with both gel-chromatography and cellulose acetate electrophoresis as described above.

Biodistribution studies in tumor bearing athymic nude mice and normal mice

Mice were injected intravenously with a mixture of 12.5 μg (18.5 MBq) of Tc-99m-labeled F33-104 and 0.1 μg (44 kBq) of I-125-labeled F33-104 in 200 μl of 0.05 M PBS, pH 7.5. Groups of mice (n = 5) were killed with anesthetic at 3 hours and 18 hours after injection, and the tumors and all internal organs were removed, weighed and counted with a well-type gamma counter. A mixture of 12.5 μg of Tc-99m-labeled anti-hCG Mab and 0.1 μg of I-125-labeled anti-hCG Mab were used as control studies, and groups of mice (n = 5) were killed at 18 hours following the injection. Tc-99m activity was counted in a channel having windows set for 130–150 keV and I-125 activity for 15–85 keV. The samples were initially counted in the Tc-99m activity channel and were then counted in the I-125 activity channel after 7 days. No thyroid blocking agents were administered throughout these biodistribution studies. The results were expressed as % injection dose/gram. The localization index and specific-to-non-specific tumor ratio were described as follows: Localization index = LS-180 tumor-to-blood ratio of radiolabeled F33-104/LS-180 tumor-to-blood ratio of radiolabeled control Mab. Specific-to-non-specific tumor ratio = LS-180 tumor-to-blood ratio of radiolabeled F33-104/PC-9 tumor-to-blood ratio of radiolabeled F33-104.14-16

Imaging of athymic nude mice

Athymic nude mice bearing LS-180 human colorectal cancer xenografts were injected through a tail vein with 12.5 μg (18.5 MBq) of Tc-99m-labeled F33-104 for imaging studies. Images were obtained with a ZLC7500 gamma camera equipped with a high resolution collimator positioned at 0.5 cm above the dorsum of the mice, and collected on a 512 by 512 pixel matrix by means of a digital computer (Scintipac 700) for 5 minutes. A 10% energy window was centered over the 140 keV photopeak of Tc-99m.

Statistical comparisons were made by Student's paired t-test.

RESULTS

In order to determine the optimal reduction conditions for F33-104, Mabs were reduced under various conditions (Fig. 1). The reaction with a molar ratio of 2-ME to Ab at above 1000 : 1 was required to obtain a high labeling efficiency of more than 95% (Fig. 1). Immunoreactivity of reduced F33-104 was determined by the competitive radiolimunoassays (Fig. 2). Reduced F33-104 obtained at 1000 : 1 and 2000 : 1 molar ratios and non-reduced F33-104 could equally compete with I-125-labeled F33-104, indicating no difference between the original and reduced F33-104 in immunoreactivity. In vitro and in vivo properties of Tc-99m-labeled F33-104 were examined in those
Fig. 3 Immunoreactivity of Tc-99m-labeled F33-104; Antigen binding assays (A) and Cell binding assays (B). Binding of radiolabeled Mab to antigens captured on beads was examined using a commercially available RIA kit. Cell binding assays were performed by incubating increasing numbers of LS-180 human colorectal cancer cells with radiolabeled Mabs. Tc-99m-labeled F33-104 (○), I-125-labeled F33-104 (●), Tc-99m-labeled anti-hCG Mab (□), and I-125 labeled anti-hCG Mab (■). Vertical bars represent Mean ± S.D. of 3 experiments.

at a molar ratio of 1000 : 1. There was little difference between Tc-99m- and I-125-labeled F33-104 as to binding to antigen captured on beads covered with anti-CEA antibodies* (Fig. 3A) and in the specific binding to LS-180 human colorectal cancer cells (Fig. 3B).

In cellulose acetate electrophoresis migration of Tc-99m-labeled F33-104 was identical to those of original and reduced F33-104 (Data not shown). There was also no release of Tc-99m-pertechnetate from Tc-99m-labeled Mab nor the formation of Tc-99m-colloid (Fig. 4). The stability of Tc-99m-labeled F33-104 was assessed by gel-filtration chromatography and cellulose acetate electrophoresis after incubation with human serum with or without high CEA values. There was no release of Tc-99m-pertechnetate from Tc-99m-labeled Mab even after 24 hours' incubation (Fig. 5).

Biodistribution studies of athymic nude mice bearing LS-180 human colorectal cancer xenografts showed significant differences between Tc-99m- and I-125-labeled F33-104 in the % injection dose/gram. Higher kidney uptake of Tc-99m-labeled F33-104 was observed at 3 hours after the injection (Fig. 6A). Higher tumor, liver, kidney and gut uptake of Tc-99m-labeled F33-104 was demonstrated at 18 hours, whereas higher stomach uptake of I-125-labeled F33-104 was seen at 18 hours (Fig. 6B). Scintigraphy of a nude mouse bearing a LS-180 human colorectal cancer xenograft demonstrated that a transplanted tumor was clearly visualized by Tc-99m-labeled F33-104, as confirmed by the biodistribution data (Fig. 7).
Fig. 6  Biodistributions of radiolabeled Mabs in athymic nude mice bearing LS-180 human colorectal cancer cells at 3 hours (A) and 18 hours (B) following intravenous injection. Tc-99m-labeled F33-104 (□), 1-125-labeled F33-104 (□). Results were expressed as % injection dose/gram. Vertical bars represent Mean ± S.D. *p < 0.05.

Table 1  Tumor/blood ratios of radiolabeled Mabs in athymic nude mice bearing specific (LS-180 human colorectal cancer) or non-specific (PC-9 human lung cancer) tumors at 18 hours postintravenous injection

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Labeled antibody</th>
<th>Tc-99m-labeled F33-104</th>
<th>1-125-labeled F33-104</th>
<th>Tc-99m-labeled control Mab</th>
<th>1-125-labeled control Mab</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS-180 human colorectal</td>
<td></td>
<td>2.10 ± 0.45</td>
<td>1.36 ± 0.20</td>
<td>0.51 ± 0.06</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-9 human lung cancer</td>
<td></td>
<td>0.41 ± 0.08</td>
<td>0.33 ± 0.13</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ± S.D.
Localization index of Tc-99m-labeled F33-104 was 4.12 and that of 1-125-labeled one was 3.16. Specific/non-specific tumor ratio of Tc-99m-labeled F33-104 was 5.12 and that of 1-125-labeled one was 4.12.
### Table 2  Kidney/blood ratios of radiolabeled Mabs in athymic nude mice bearing tumor xenografts and normal mice obtained 18 hours after the injection

<table>
<thead>
<tr>
<th>Tumor</th>
<th>F33-104</th>
<th></th>
<th>Anti-hCG Mab</th>
<th></th>
<th>BW431/26</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tc-99m</td>
<td>I-125</td>
<td>Tc-99m</td>
<td>I-125</td>
<td>Tc-99m</td>
<td></td>
</tr>
<tr>
<td>LS-180 human colorectal cancer</td>
<td>1.42</td>
<td>0.32</td>
<td>1.37</td>
<td>0.30</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.49)</td>
<td>(0.03)</td>
<td>(0.37)</td>
<td>(0.03)</td>
<td>(0.17)</td>
<td></td>
</tr>
<tr>
<td>PC-9 human lung cancer</td>
<td>1.13</td>
<td>* 0.28</td>
<td>---</td>
<td>*</td>
<td>---</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>(0.12)</td>
<td>(0.02)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No tumor</td>
<td>0.71</td>
<td>0.33</td>
<td>0.82</td>
<td>0.28</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.09)</td>
<td>(0.09)</td>
<td>(0.03)</td>
<td>(0.09)</td>
<td></td>
</tr>
</tbody>
</table>

Values are Mean (S.D.) of 5 animals
*p<0.05.

athymic nude mice were carrying non-specific PC-9 cancer cells.

**DISCUSSION**

The present study shows that murine anti-CEA Mab, designated F33-104, was quickly and stably labeled with Tc-99m, without damaging antigen-binding activity, by the direct labeling method. Labeling of Mab with Tc-99m was achievable by only mixing reduced Mab and Tc-99m-pertechnetate produced by the generator. Labeling efficiency was so high that Tc-99m-labeled Mab could be injected without further purification, and was very convenient for clinical use. High labeling efficiency of F33-104 with Tc-99m was achieved by increasing the molar ratio of 2-ME to the antibody, probably due to the increased numbers of sulphhydril groups per antibody, cleaved with 2-ME by reducing the number of intrinsic disulphide bonds. The molar ratio of 2-ME to antibody of more than 1000:1 seemed to be optimal for Tc-99m-labeling of Mab F33-104. Furthermore, significantly higher tumor uptake was noted at 18 hours after the injection of Tc-99m-labeled F33-104 than for a radiiodinated version in the biodistribution of athymic nude mice bearing specific tumors. This resulted in a significantly higher localization index of 4.12 and specific-to-non-specific tumor uptake ratio of 5.12. Higher tumor activity of Tc-99m-labeled F33-104 than of I-125-labeled F33-104 may be due to the dehalogenation of radiiodine antibody in the circulation. Images of Tc-99m-labeled F33-104 showed predominant specific tumor uptake within 18 hours after injection, confirming the results of biodistribution studies.

Significant kidney uptake of Tc-99m-labeled F33-104 was observed at 3 hours and 18 hours after injection in athymic nude mice, especially in tumor-bearing athymic nude mice. Higher kidney uptake of Tc-99m-labeled Mabs was noted in athymic nude mice bearing both specific and non-specific tumors than that of corresponding iodinated Mab (Table 2). In addition, even Tc-99m-labeled control Mab showed higher kidney uptake in tumor-bearing athymic nude mice than in normal athymic nude mice.
High kidney uptake was already reported when F(ab')
fragments labeled with indium-111 (In-111) and Tc-99m
were administered. However, the reasons for high
kidney uptake when using intact Mab labeled with Tc-
99m are difficult to determine and have not been studied
extensively. Immune complex formation of Mab with the
circulating CEA seemed unlikely, since significant kid-
ney uptake of Tc-99m-labeled F33-104 in athymic nude
mice bearing CEA-negative tumors, and of Tc-99m-
labeled control Mab were also observed. Another possible
explanation is an in vivo breakdown of Tc-99m-labeled
Mab by unknown mechanisms at the tumor sites and/or
in the circulation which may contribute to significant kidney
uptake at a later time after injection.

In-111-labeled Mabs to CEA have been successfully
used for the localization of colorectal cancer. The
antigenic nature of these antibodies has not been de-
scribed, whereas epitopes recognized by F33-104 have
been well characterized. In addition Tc-99m has many
advantages over In-111 in the imaging of cancer. Tc-99m-
labeled anti-CEA Mab F33-104 has the potential for
clinical application in diagnosing human colorectal can-
cer. We are investigating radiolabeled Mab with Re-186,
which has favorable nuclear properties for the treatment of
cancer.

ACKNOWLEDGMENTS

The authors wish to thank Dr. K. Endo for excellent advice and
help in this study. We thank R. Hirai and M. Goto for their help
in the preparation of this manuscript. This work was supported by
a Grant-in-Aid for Cancer Research from the Ministry of
Education, Japan.

REFERENCES

1. Gold P, Freedman SO. Demonstration of tumor specific
antigens in human colonic carcinomata by immunological

2. National Institutes of Health Consensus Development Con-
ference Statement. Carcinoembryonic antigen: its role as
a marker in the management of cancer. Cancer Res 41:

3. Begent RHJ. Recent advances in tumor imaging use of
radiolabeled anti tumor antibodies. Biochim Biophys Acta

4. Goldenberg DM. Current status of cancer imaging with

5. Hnatowich DJ. Recent developments in the radiolabeling
of antibodies with iodine, indium, and technetium. Semin

6. Goldenberg DM. Cancer imaging with CEA antibodies:
historical and current perspectives. Int J Bio Markers 7:

7. Shimura N, Kojima S, Kubodera A, Kubota K, Takahashi T,
Oyamada H. Radioimmunodetection of human colon can-
cer using Tc-99m-MDP-MoAb-A7 in mice. (letter) Int J

S, Nakazato H, et al. Epitope mapping of the nonspecific
cross-reacting antigen using various related recombinant
proteins expressed in chinese hamster ovary cells and eight
distinct monoclonal antibodies. Immunol Invest 21: 143–

S, Nakazato H, et al. Epitopes predominantly retained on
the carcinoembryonic antigen molecules in plasma of pa-
patients with malignant tumors but not on those in plasma of

Y, et al. Variations in radioimmunoscintigraphic detection
of tumor showed by five monoclonal antibodies to carcino-

11. Mather SJ, Ellison D. Reduction-mediated technetium-
99m labeling of monoclonal antibodies. J Nucl Med 31:

12. Baum RP, Hertel A, Lorenz M, Schwarz A, Encke A,
Hor G. Tc-99m-labeled anti-CEA monoclonal antibody for
tumour immunoscintigraphy: first clinical results. Nucl

13. Hunter WM, Greenwood FC. Preparation of iodine-131
labeled growth hormone of high specific activity. Nature

14. Moshakis V, McIllinney RA, Raghavan D, Neville AM.
Localization of human tumor xenografts after iv adminis-
tration of radiolabeled monoclonal antibodies. Br J Cancer

15. Beaumier PL, Krohn SA, Carrasquillo JA, Eary J, Hellstrom
with monoclonal Fab against p97. J Nucl Med 26: 1172–

16. Mach JP, Chatal JF, Lumbroso JD, Buchegger F, Forni M,
Ritschard J, et al. Tumor localization in patients by radio-
labeled monoclonal antibodies against colon carcinoma.

sarcoma xenografts in nude mice by a monoclonal antibody
labeled with radiodiode and indium-111. J Nucl Med 28:

18. Zimmer AM, Kazikiewicz JM, Rosen S, Spies SM.
Pharmacokinetics of Tc-99m(Sn) and I-131-labeled anti-
carcinoembryonic antigen monoclonal antibody fragments

19. Yokoyama K, Carrasquillo JA, Chang AE, Colcher D,
Roselli M, Sugarbaker P, et al. Differences in biodistribution
of indium-111 and iodine-131-labeled B7.2.3 monoclonal

S, Brechblie MW, et al. Comparative biodistribution of
yttrium and indium-labeled monoclonal B7.2.3 antibody in
athymic mice bearing human colon carcinoma xenografts.

of the 111 In-(Sn)-citrate method to label antibodies for
radioimmunotargeting studies. Int J Rad Appl Instrum B
Vol. 8, No. 1, 1994

Original Article 29
