Applicability of Carcinoembryonic Antigen-specific Monoclonal Antibodies to Radioimmunoguided Surgery for Human Colorectal Carcinoma1


ABSTRACT

Two carcinoembryonic antigen (CEA)-specific monoclonal antibodies (MAbs), PRIA3 and T84.66, were tested to determine whether they could accurately localize colorectal carcinoma and therefore be applicable in radioimmunoguided surgery (RIGS). Twenty-one tumors by three human colorectal carcinoma cell lines with various levels of CEA expression (KM-12c, C75, and Clone A) were successfully implanted in the intra-abdominal organs of 15 nude mice. The tumors were localized using a portable radioisotope detector (Neoprobe 1000) 48 h after injection of radiolabeled MAbs (10 mCi/mouse) when the precordial counts were <20 per 2 s. Histopathological identification of radiolabeled MAbs was also performed using immunohistochemistry and microautoradiography. Radioactivity counted on a portable radioisotope detector correlated well with that on a gamma counter. The distribution in the blood was significantly greater than in other organs (P < 0.001). Localization indices of the tumor in various organs was from 1.1 to 8.5 in the PRIA3-pretreated mice and 3.0 to 8.6 in the T84.66-pretreated mice. Silver grains and immune staining were distributed in the tumor cells of the PRIA3-pretreated mice, whereas they were in the necrotic debris as well as the tumor cells of the T84.66-pretreated mice. There were significantly more silver grains in the liver in the T84.66-pretreated mice than in the PRIA3-pretreated mice (P = 0.004). The sensitivity and specificity of tumor localization by RIGS were 71.4 and 91.4% in the PRIA3-pretreated mice, whereas they were 60 and 76% in the T84.66-pretreated mice. A study using specific anti-CEA MAbs suggested PRIA3 as an efficient immune probe for RIGS in colorectal carcinoma with a low rate of false-positive detection.

INTRODUCTION

Current chemotherapeutic agents offer <20% of survival benefits, and surgery still plays an important role in treating more than half of colorectal cancers (1). Although more than two-thirds of patients are candidates for curative surgery, locoregional recurrence occurs in as many as 40% after curative resection. Consequently, a diagnostic tool to enable accurate tumor localization is urgently needed to allow precise and complete removal. RIGS, originally designed by Martin et al. (2), allows intraoperative tumor detection by targeting the γ emission from tumor cells attached to radiolabeled antibodies. This depends, however, for its success on the availability of good and specific antibodies and of appropriate nuclides.

Various antibodies to tumor antigens have been developed and used for >15 years since the introduction of RIGS. The commonly used antibodies are TAG-72 and CEA. The former has been used more frequently than the latter in clinical practice. TAG-72 is a human pancarcinoma glycoprotein, and the two antibodies commonly used are B72.3 and CC49 (2). However, there are still several problems to be solved for their clinical application. One is the low expression of TAG-72, i.e., in <50% of colorectal carcinoma cells (3). The other is false-positive detection of tumor cells, even after histological identification by serial sectioning and specific immunohistochemistry (4).

Although serum CEA has intrinsic limitations to its use as a colorectal cancer marker, serial measurement has been used as a standard tool for use as a prognostic indicator or for detecting recurrence. CEA has been rather neglected in the field of RIGS, although a few studies using CEA-specific MAbs have revealed efficient localization of tumor of ~95% (5, 6). This study was primarily intended to verify whether specific anti-CEA MAAB could accurately localize colorectal carcinoma and therefore be applicable in RIGS. An animal model similar to human colorectal carcinoma was used.

MATERIALS AND METHODS

Cell Lines, Monoclonal Antibodies, and CEA. Three colorectal carcinoma cell lines with different CEA expressions were used: KM-12c (University of Texas M. D. Anderson Cancer Center, Houston, TX), C75 (Imperial Cancer Research Fund), and Clone A (American Type Culture Collection). Their levels of CEA production were 2180, 118, and 2.7 ng/ml of medium, respectively. CEA expression on the cell membrane was also confirmed by indirect immunofluorescence. Two IgG1 murine anti-CEA MAbs used were: T84.66 (T84.66A3.1A1F2; American Type Culture Collection) and PRIA3 (Imperial Cancer Research Fund). They were proven as CEA-specific MAbs (7, 8). MOPC 31c (Sigma Chemical Co., St. Louis, MO), was used as an isotype-matched control. Human CEA was extracted from hepatic metastases of colorectal carcinomas by the perchloric acid method as described previously (9).

Radiolabeling of MAbs. The iodogen method was used for radiolabeling MAbs. Briefly, labeling was performed in a glass vial coated with 25 μg of iodine, dissolved in 1 ml of tetrahydrofuran. After vortexing in a ventilation hood, antibodies and 200 μCi of carrier-free Na125I (NEN Research Product, Boston, MA) were added. The reaction mixture was incubated for 30 min at room temperature. It was purified by running through a PD-10 column (Amer sham Pharmacia Biotech, Uppsala, Sweden) and sterilized by filtration (0.22 μm; Millipore, Bedford, MA). Radiochemical purity was verified by TLC (Merck, Darmstadt, Germany) eluted with 85% methanol, which left 125I-labeled antibodies remaining at the origin while free 125I was at the solvent front. A pyrogen test of final filtrates was carried out using a LAL kit (Limulus Amboceptor Lysate; Associates of Cape Cod, Inc., Woodhole, MA). The specific activity was determined using various amounts of radiolabeled iodine and MAbs.

Competitive Binding Assay. The immunoreactivity of radiolabeled anti-CEA MAbs was measured by a competitive binding assay in solid-phase. CEA was dried onto 96-well plates at a concentration of 1 μg/well in PBS for 24 h at 37°C. After blocking the free-binding sites with 4% BSA for 2 h at 37°C, 100-μl mixtures of labeled and unlabeled antibodies in various proportions (10 μg/ml; 11 dilutions from 0:100 to 100:1) were plated in triplicate for 2 h at 37°C. Nonreacting MAb was removed by washing three times with 0.5% BSA in PBS. The binding complex was stabilized with 1 N NaOH and collected on cotton-tipped swabs for gamma counting.

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3 The abbreviations used are: RIGS, radioimmunoguided surgery; CEA, carcinoembryonic antigen; MAb, monoclonal antibody; IHC, immunohistochemistry; ARG, microautoradiography.

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Tumorogenesis and Preparation for RIGS. Male athymic nude mice (athymic nude-nu; Asan Animal Laboratory, Seoul, Korea), 6–8 weeks of age, were raised in a laminar flow cabinet under specific pathogen-free conditions. Three cell lines cultured in log-phase were harvested and resuspended in PBS at 1 × 10^6 cells/ml. CEA (40 μg/0.2 ml/mouse) was injected i.p. in all mice 1 h before tumor cell injection. Tumor cells (1 × 10^7 cells each) were injected into the splenic subcapsule and cecal wall in 20 mice for each cell line. As visible or palpable abdominal masses appeared in ~25% of the mice at the eighth week after tumor cell injection, unlimited ingestion of potassium iodide dissolved in water (1 μl/ml H2O) was permitted for the purpose of saturation of the thyroid gland for 48 h prior to injection of radiolabeled MAbs. Then, 10 μCi/mouse (specific activity, 2 mCi/mg of MAb) of radiolabeled anti-CEA MAbs were injected i.p. into 48 mice (16 mice/cell line; 8 mice/each anti-CEA MAb). The same amount of 125I-labeled MOPC 31c was injected in 12 mice (4 mice/cell line) as a control. Twelve mice without tumor cell injection were also treated with radiolabeled MAbs and free 125I to assess the organ distribution.

Localization Using Portable Radioisotope Detector. The precordial count was checked daily using a hand-held gamma detector tipped with a collimator (inner diameter, 11 mm) connected to a portable radioisotope detector (Neoprobe® 1000; Neoprobe Corp., Columbus, OH). When the count decreased to a level of <20 counts/s after 48 h, the mice were sacrificed by cervical separation. Because accurate probing is limited in the small organs of mice, assessment of the respective organs and tumors was made immediately after excision. Then, each organ was divided into two pieces, one for measurement in a gamma counter and the other for histopathological assessment. The distribution of radioactivity in each organ was corrected by the radioactivity in a gram of tissue measured by the portable radioisotope detector and gamma counter. The localization index of the tumor, i.e., the percentage of injected dose/g in the tumor divided by the percentage of injected dose/g in the organ, was calculated using the counts from the portable radioisotope detector. Positive localization was assessed when the counts in each organ and tumor were higher than that in the blood.

Indirect Immunohistochemical Staining and Microautoradiography. Five serial sections (5 μm in thickness) from the same tissues were prepared, one for H&E staining and two sections each for indirect IHC and ARG. Briefly, the technique for IHC was based on the labeled streptavidin-biotin method, using DAKO LSAB kit (DAKO Corp., Carpinteria, CA) and the standard protocol. Another two sections were deparaffinized and hydrated for ARG. The sections were coated with Ifford K5 (Ilford Imaging, Mobberley, United Kingdom) photographic emulsion for 5 s at 50°C in a dark room and then stored at −70°C for 1 week in dehydrated and light-tight boxes. The slides were then developed using Kodak photographic developer D-19 (Eastman Kodak Co., Rochester, NY) for 6 min and fixed using a photographic fixer. The IHC and ARG results were classified into four grades: grade 0, no staining; grade 1, staining of <25% of cells; grade 2, staining ≥25% to <50% of cells; and grade 3, staining ≥50% of cells.

Table 1 Mean grades of IHC and ARG in the tumor, liver, and spleen

<table>
<thead>
<tr>
<th></th>
<th>PR1A3</th>
<th>T84.66</th>
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<tbody>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
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<tr>
<td>IHC (n = 9)</td>
<td>1.8 ± 0.4</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>ARG (n = 4)</td>
<td>1.7 ± 0.4</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC (n = 9)</td>
<td>0.4 ± 0.2 (0.001)</td>
<td>0.5 ± 0.3 (0.003)</td>
</tr>
<tr>
<td>ARG (n = 9)</td>
<td>0.7 ± 0.2 (0.016)</td>
<td>2.0 ± 0.3 (0.033)</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
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<tr>
<td>IHC (n = 9)</td>
<td>0.9 ± 0.1 (0.019)</td>
<td>1.3 ± 0.7 (0.037)</td>
</tr>
<tr>
<td>ARG (n = 9)</td>
<td>0.8 ± 0.1 (0.03)</td>
<td>1.6 ± 0.4 (0.006)</td>
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*P, versus mean grade of tumor.

Statistical Analysis. Counts for distribution and localization indices in each organ or in different cell lines were analyzed using ANOVA or LSD test. Comparison of the counts between PR1A3 and T84.66 was assessed by an unpaired t test. The relation between counts from the portable radioisotope detector and gamma counter was assessed by using the significance of the product moment correlation test. The incidence of two or more groups was compared in a contingency table analysis using a χ² test. The significance level was set at 5% for each analysis, and all calculations were performed on an IBM-PC using Statistica (ver. 5.1; StatSoft, Inc., Tulsa, OK).

RESULTS

Tumorogenesis. Tumor cells were implanted onto the cecal wall and spleen similar to human colorectal carcinoma with liver metastasis. The KM-12c cell line was successfully implanted in six mice (30%), C75 in five mice (25%), and Clone A in four mice (25%). A total of 21 tumors was identified overall, 1.4 per tumor-bearing mouse. The cecum and adjacent mesentry (11 tumors) were the most frequent sites, the spleen (5 tumors) the next most frequent, and then the abdominal wall (3 tumors) and liver (2 tumors) in descending order of frequency. The numbers of tumor-bearing mice were four for PR1A3, eight for T84.66, and three for MOPC 31c.

Radiolabeling of MAbs. The affinity of PR1A3 and T84.66 was determined by the method of Beatty et al. (10) using an enzyme immunoassay. The affinity constant (Kd) for PR1A3 and T84.66 was 6.7 × 10^8 liter/mmol and 1.8 × 10^7 liter/mmol, respectively. Labeling yield was in the range of 80–85%. The radiochemical purity measured using TLC was 96.8% on 5 days after radiolabeling. There was a linear correlation of the reaction to solid-phase CEA between cold and labeled anti-CEA antibodies at a similar concentration of injection (Fig. 1). The binding ratios of labeled antibodies:cold antibodies, measured at the same concentration of cold and labeled antibodies, were 87% for PR1A3 and 68% for T84.66.

IHC and ARG. All tumors were histologically verified as poorly differentiated adenocarcinomas. They were grossly discernible, and microscopic metastasis in the liver, lung, spleen, kidney, and cecum was not identified on H&E staining. The mean grade of IHC in the tumor was <2 and that of ARG was 3 (Table 1). Silver grains and immune staining were distributed in the tumor cells of PR1A3-pretreated mice, and in the necrotic debris as well as the tumor cells of T84.66-pretreated mice (Fig. 2 and 3). Silver grains in the liver were distributed over the hepatocytes, Kupffer cells, and sinusoids, and those in the spleen were in the marginal zones surrounding the lymphoid follicles. The highest grade of IHC in the liver and spleen was grade 1, whereas that of ARG in the liver was grade 3. There were significantly more silver grains in the liver in PR1A3-pretreated mice (P = 0.004). Tumor showed significantly higher mean grades than the liver and spleen in both IHC and ARG (P = 0.001–0.037). The grade of IHC corresponded to that of ARG in the liver (P = 0.029), whereas it did not in the tumor and spleen. However, there was a significant correspondence in the grade of IHC of the tumor between PR1A3 and T84.66 (P = 0.033).

Distribution of Radiolabeled MAbs. The distribution of radiolabeled MAbs and free 125I in three mice, each without tumorogenesis,
was identified by measuring counts of the respective organs. The counts of free $^{125}$I were significantly lower than those of radiolabeled MAbs (free $^{125}$I/radiolabeled MAbs; range, 0.4–10.9%). The distribution in the blood showed significantly greater than in other organs ($P < 0.001$; Fig. 4). There was no remarkable difference between the MAbs except for MOPC 31c in the liver. In tumor-bearing mice, the distribution of T84.66 showed significantly greater counts than that of PR1A3 in the heart, spleen, lung, and cecum ($P = 0.023–0.05$). The distribution in the tumor of both anti-CEA MAbs was greater than that in the liver, spleen, lung, and cecum ($P = 0.01–0.009$). The radioactivity counted using a portable radioisotope detector correlated well with that using the gamma counter. The two sets of counts were significantly correlated, except the liver and cecum: tumor ($r = 0.899; P < 0.001$); heart ($r = 0.655; P = 0.006$); blood ($r = 0.539; P = 0.031$); spleen ($r = 0.655; P = 0.006$); kidney ($r = 0.794; P < 0.001$); and lung ($r = 0.696; P = 0.003$).

**Localization Indices of the Tumor.** The efficiency of the portable radioisotope detector to discriminate tumor from the normal tissues was assessed by the localization indices of the tumor (Table 2). Although the localization indices of the tumor appeared higher in T84.66 than in PR1A3, there was no remarkable difference except for the liver ($P = 0.049$). The localization indices of the tumor in three different cell lines did not show a difference except for the tumor localization to the lung between KM-12c and C75 ($P = 0.01$). A positive reading by a portable radioisotope detector is usually defined in two ways, either 2-s counts more than twice the counts of normal adjacent tissue or more than three times the SD above the background (11). However, these criteria could not be applied in this study,
because of limited size of the organs and negligible background (mean ± SD, 1.5 ± 1.1). Because the blood was distributed in all tissues, it was used as a standard for a cutoff value. Although localization indices were higher for T84.66 than PR1A3, the sensitivity and specificity were the reverse (Table 3). The accuracy of tumor localization was higher using PR1A3 than T84.66 (88.1% versus 70%). The accuracy using the heart count as a cutoff value was also higher for PR1A3 than T84.66 (73.8% versus 65%).

**DISCUSSION**

RIGS consists of three components, radioactive nuclide, immune based tumor-specific targeting agent, and intraoperative gamma detector (2). The radioactivity counted on a portable radioisotope detector correlated well with that from the gamma counter. The same result based tumor-specific targeting agent, and intraoperative gamma detection was higher using PR1A3 than T84.66 (88.1% versus 70%). The accuracy using the heart count as a cutoff value was also higher for PR1A3 than T84.66 (73.8% versus 65%).

A comparison of Fab’ and intact IgG revealed no substantial difference in maximal affinity except for plasma residence time (19). Robert et al. (20) recently attempted biparatopic MAbs directed against two different epitopes of the same molecule for the purpose of enhancing tumor uptake. They demonstrated a higher tumor uptake than that with the parental MAbs in certain combinations. MAbs to TAG-72 bind to adenocarcinomas of the colon, gastrointestinal tract, pancreas, ovary, breast, prostate, and lung (non-small cell; Ref. 21). The expression of TAG-72 detected by B72.3 seems to be about 40–82% (17). The detection rate of primary tumors was 60–75% for B72.3 and 86% for CC49 (13). Although tumor localization was good for the clinical application, RIGS using CC49 was reported to give as much as 34% false-positive detection of tumors (4). CEA expression in tumor cells is around 66–100% (22) and that appears to be greater than TAG-72 expression. CEA shows the highest specificity in colorectal carcinoma among several identified tumor markers, including TAG-72 and CA 19-9 (23, 24). Immunoscintiscan using the CEA-specific MAb, IMMU 4, showed accurate detection of colorectal carcinoma in 93% of cases without false-positive detection (25). Nevertheless, there are a few RIGS trials using anti-CEA MAbs. F(ab’)2 of F023C5 showed satisfactory sensitivity in tumors (81–96%) but low sensitivity in lymph nodes (58%; Ref. 5). Primary tumor detection was 92.6% with F(ab’)2 of F023C5, compared with 80.4% for B72.3 (26). Another anti-CEA MAb, A5B7, showed the highest localization by RIGS of as much as 97.8% for local lesions and 88.8% for recurrent lesions (17).

PR1A3 and T84.66 bind to the epitopes on the CEA-specific subdomains of A3 and B3, respectively, without cross-reactivity with other CEA gene family members (7, 8). The sensitivity of PR1A3 for CEA seemed high enough, because 59 of 60 colorectal carcinomas were bound by the MAb (27). PR1A3 is known to bind CEA close to the site of membrane attachment but absent in solution or loss of membrane attachment (8). The advantage of PR1A3 was also identified showing high positive (88–92%) and negative (93–100%) predictive value in the immunoscintiscan of recurrent colorectal cancers (28). There has been only one published clinical trial of RIGS using 99mTc-labeled PR1A3, and it presented a lower sensitivity (66%), probably because of the significant background activity of 99mTc (29).

*Fig. 4. Distribution of radiolabeled antibodies and iodine in three mice, each without tumorigenesis. Bars, SE.*

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*Fig. 4. Distribution of radiolabeled antibodies and iodine in three mice, each without tumorigenesis. Bars, SE.*

**Table 2** Localization indices (LI) of radiolabeled anti-CEA antibodies

<table>
<thead>
<tr>
<th></th>
<th>PR1A3 (n = 6)</th>
<th>T84.66 (n = 10)</th>
<th>PR1A3 and T84.66 (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.8 ± 0.4</td>
<td>8.6 ± 4.2</td>
<td>6.0 ± 2.7</td>
</tr>
<tr>
<td>Blood</td>
<td>1.2 ± 0.3</td>
<td>3.7 ± 1.3</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>Liver</td>
<td>1.6 ± 0.5</td>
<td>5.4 ± 1.4</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.8 ± 0.8</td>
<td>4.8 ± 1.1</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.1 ± 0.2</td>
<td>3.0 ± 0.7</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>Lung</td>
<td>8.5 ± 1.5</td>
<td>6.0 ± 1.5</td>
<td>6.5 ± 1.3</td>
</tr>
<tr>
<td>Cecum</td>
<td>3.1 ± 1.0</td>
<td>5.2 ± 1.2</td>
<td>4.5 ± 0.9</td>
</tr>
</tbody>
</table>

*a* LI, percentage of injected dose/g in tumor divided by percentage of injected dose/g in tissue.

*b* $P = 0.049$, between PR1A3 and T84.66.

**Table 3** Accuracy parameters of radiolabeled anti-CEA antibodies

<table>
<thead>
<tr>
<th></th>
<th>T84.66<em>a</em></th>
<th>PR1A3 and T84.66<em>a</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>5/7 (71.4)</td>
<td>6/10 (60) 11/17 (64.7)</td>
</tr>
<tr>
<td>Specificity</td>
<td>32/55 (91.4)</td>
<td>38/50 (76) 70/85 (82.4)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>5/8 (62.5)</td>
<td>6/18 (33.3) 11/26 (42.3)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>32/55 (91.4)</td>
<td>38/42 (90.5) 70/77 (90.9)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>37/42 (88.1)</td>
<td>42/60 (70) 79/102 (77.5)</td>
</tr>
</tbody>
</table>

*a* Numbers in parentheses, percentage.
tumor CEA. A minor reduction of ~20% of the binding affinity was demonstrated after radiolabeling MAb (19). Binding affinity was greater for PR1A3 as compared with T84.66 in the binding ratio of labeled antibodies:cold antibodies.

The main reason for lack of agreement between the grade of IHC and ARG was derived from the difference in CEA expression at the time of IHC, i.e., 48 h after radiolabeled MAb injection. Another might have been the formalin tissue fixation, resulting in the cross-linking of neighboring proteins (21). Other studies of combined IHC and ARG have also demonstrated a difference between shed and cellular CEA antigen (15). The distribution in the blood was significantly greater than in other organs and was quite similar to a previous study using the anti-CEA MAb, Col-1 (17). The profuse silver granules in the liver were in the hepatocytes, Kupffer cells, and sinusoids. Increased accretion in the liver and spleen was explained by the antigen-antibody complex, antigen-mediated, or Fc receptor-mediated interaction (21, 30). The kidney is also known as an organ of high accretion because of the trapped antibody aggregates (13), but that was also found in the PR1A3. The silver grains of PR1A3 were distributed mainly in the tumor cells, whereas those of T84.66 were distributed in necrotic tumor debris as well. These differences may partly explain the higher specificity in the PR1A3 than in the T84.66. The silver granules identified in the ischemic tumor necrosis might be trapped immune complexes, as shown in the study using the CX-1 cell line (15). Cote et al. (4) considered false-positive detection from breakdown products of the tumor including tumor antigen.

Although the sensitivity has been raised with various immune probes in RIGS, limited specificity has remained to be solved in the present situation without specific tumor markers. This study using specific anti-CEA MAbs suggested PR1A3 to be an efficient immune probe to detect colorectal carcinoma in RIGS. Further comparative trials using MAbs to TAG-72 or anti-CEA MAb fragments would be desirable to establish a clinical context.

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