Immunoscintigraphy of Human Mammary Carcinoma Xenografts Using Monoclonal Antibodies 12H12 and BM-2 Labeled with $^{99m}$Tc and Radioiodine

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ABSTRACT

For immunoscintigraphic localization of human breast cancer two monoclonal antibodies (mabs) 12H12 (immunoglobulin G1) and BM-2 (immunoglobulin G3) were developed. The mabs, directed against two different epitopes on the mucin glycoprotein TAG-12, showed reactivity with 96% of all primary mammary carcinomas. The antibodies were labeled with either $^{99m}$Tc or $^{131}$I. In addition, 12H12 was directly labeled with $^{99m}$Tc according to the method of Schwarz and Steinstrasser (A. Schwarz and A. Steinstrasser, J. Nucl. Med., 28: 721, 1987). Biodistribution was measured in female nude mice bearing the human mammary carcinoma SF-15. Both radiolabeled mabs showed similar biodistribution with fast tumor uptake (8.5% injected dose/g at 6 h postinjection), which increased to 10-11% injected dose/g at 24 h and subsequently remained constant up to 120 h. $^{99m}$Tc-Labelling of the mab 12H12 led to an enhanced tumor uptake of 10.5 and 14% Injected dose/g at 6 and 24 h postinjection, respectively, and to significantly accelerated blood clearance of radioactivity. Similar results were obtained with a second mammary tumor (AB-1), while an endometrial tumor (EK-3) showed a 3-fold lower accumulation of radioactivity and no difference in uptake of radiolabeled and $^{99m}$Tc-labeled 12H12. Scintigraphic imaging of tumor-bearing nude mice with the $^{99m}$Tc 12H12 at 24 h postinjection clearly demonstrated a diagnostic potential of the new mab for tumor localization and staging.

INTRODUCTION

Appropriate therapy for breast cancer depends upon reliable tumor detection and tumor staging. Since the presence of metastases in local lymph nodes is one of the major prognostic factors in the absence of distant metastases, large efforts have been made to better determine the extent of lymph node involvement by means of immunolymphoscintigraphy (1-5). However, the method seems to be limited by the specificity of the antibodies administered and the resolution of the equipment. Another important diagnostic problem is the detection of distant metastases. Radiolabeled monoclonal antibodies directed against tumor-associated antigens could answer specific questions in this field of clinical oncology. One well established indication for immunoscintigraphy, e.g., in colorectal carcinomas, is the localization of suspected tumor recurrences indicated by increasing tumor market levels (6).

A large number of antibodies against different epitopes of human neoplastic breast tissue have been investigated previously. Many of them were directed to carcinoembryonic antigen (1, 2, 7), TAG-72 (8, 9), cell surface antigens like the mabs2 B6.2 (10-12), anti-MME (13) or RCC1 (4) and various others (3, 14). But the majority of the mabs used for breast cancer imaging like HMFG1 and HMFG2 (5, 15), SM-3 (16), DF3 (17), Mc5 (18), and MA5 (19) have been shown to recognize epitopes on mucin molecules (20). However, a highly specific and sensitive monoclonal antibody for immunoscintigraphy and radioimmunotherapy has not yet been found (21). This study was undertaken to evaluate whether the newly developed anti-mucin mabs 12H12 and BM-2 could improve tumor/tissue ratios and thus facilitate immunoscintigraphic detection and staging of breast cancer. For this purpose radiiodinated mabs were characterized with respect to their biodistribution in nude mice bearing human tumor xenografts. Two breast carcinoma lines differing in antigen expression level and localization of antigen-expressing cells (luminal, basal), as well as an endometrial cancer cell line were used. Additionally a kit for $^{99m}$Tc-labeling of the mab 12H12 was used for biodistribution and imaging studies.

MATERIALS AND METHODS

mab

The mab 12H12 was raised against tumor cells from a human mammary carcinoma xenograft (22). mab 12H12 ($K_{d} = 8.7 \times 10^{-8}$ M$^{-1}$) is of IgG1 subtype and reacts with carbohydrate side chains on the tumor-associated antigen TAG-12. The corresponding antigen was purified from T47D cells and effusion fluids by 12H12 affinity chromatography and gelfiltration. The high-molecular-weight glycoprotein, termed TAG-12, was shown to be a member of the heterogeneous mucin family (23). Purified antigen was used to develop a second generation of mucin-specific antibodies. Deglycosylated TAG-12 was used as immunogen to select mab BM-2 (formerly called 2E11). The mab is of IgG3 subtype and reacts with high affinity ($K_{d} = 6.6 \times 10^{-10}$ M$^{-1}$) with multiple epitopes expressed on native and deglycosylated TAG-12 (24). mab BM-2 was found to react with synthetic peptides of the 20-amino acid tandem repeat area of the human Muc1 gene, its minimum epitope being the sequence A-P-D-T-R. Both mabs were produced in hollow-fiber bioreactors (Cellpharm AG, Wiesbaden, Germany) and purified to homogeneity by a combination of protein A-Sepharose 4 Fast Flow (Pharmacia, Freiburg, Germany) and fast protein liquid chromatography gel filtration by using Superose 6 (Pharmacia).

Antigen Expression

As previously shown, the high-molecular-mass mucin glycoprotein TAG-12 is expressed in more than 96% (n = 206) of primary tumors and in 99% (n = 107) of metastases of human breast cancer. However, the situation is different after transplantation to nude mice; only 1 of 7 tumor xenografts was still immunohistochemically TAG-12 positive after 15 passages in nude mice. The antigen TAG-12 can be found in cytoplasm, cell membranes, and secretory components. Western immunoblotting of tumor homogenates with 12H12 shows heterogeneous bands only in the high-molecular-weight range of 400,000, while BM-2 shows additional bands in the range of $M_{r}$ 220,000 and 180,000 (23). Immunocytochemical analysis of tumor cells in bone marrow, effusions, and peripheral blood of patients with breast cancer (n = 289) are characterized by a homogeneous TAG-12 expression (25). Both mabs do not react with human skin, liver, pancreas, spleen, small intestine, bone marrow, thymus, prostatic gland, lymphocytes, mesothelium, and other tissues. They show a slight reaction with apical secretory components of the normal breast gland and with fetal lung and kidney. The mabs BM-2 and 12H12 were used in a sandwich type enzyme immunoassay for TAG-12 analysis in sera of...
BREAST CANCER IMAGING WITH ANTI-MUCIN ANTIBODIES

patients with breast and other cancer types. In breast cancer elevated levels were found in 32.4% (n = 225) of patients with no clinical evidence of disease and in 67% (n = 196) of patients with metastatic disease (26).

Xenograft Tumors and Cell Lines

The mammary carcinoma xenograft SF-15 (generous gift from Dr. H. P. Fortmeyer, Frankfurt, Germany) was cut into three 3x3x1-mm slices and was implanted s.c. in the anterior lateral thoracic wall (27). Tumor xenografts that grew to a size of more than 1 cm within 8 weeks were removed aseptically and peripheral, nonneoplastic tissue was used for passaging. AR-1, a human breast carcinoma cell line recently established from a primary carcinoma in our laboratory, was transplanted from cell cultures. The cell line shows homogeneous mucin expression. EK-3, a primary human endometrial carcinoma cell line was cultured in our laboratory. It was additionally established as solid tumor xenograft on nude mice under the same conditions as reported for SF-15 tumor xenografts. In contrast to the commonly transplanted MX-1 and MCF-7 tumor cell lines, implantation of estrogen pellets was not necessary to enable growth of the three tumor xenografts.

T47D, a human breast carcinoma cell line (28) was used for the determination of the immunoreactive fraction of the mabs after radioiodination. All cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (GIBCO, Paisley, United Kingdom), 50 units/ml penicillin and 50 µg/ml streptomycin.

Immunohistochemical Examination of Tumor Xenografts

Paraffin-embedded tumor tissue was cut into 6-µm-thick sections. mabs 12H12 and BM-2 were derivatized with biotin N-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) and 0.5 µg mab in 500 µl PBS with 1% BSA (Boehringer Mannheim) was added to each tissue section and incubated for 2 h. After washing with PBS, 500 µl streptavidin-peroxidase (Dianova, Hamburg, Germany) 1/1000 in PBS with 1% BSA, were added to the tissue sections and incubated for 1 h at room temperature. Sections were then washed thoroughly with PBS and incubated with the substrate for 30 min. The substrates used were 3,3’-diaminobenzidine (Sigma, Deisenhofen, Germany), 50 mg dissolved in 45 ml PBS and 5 ml Tris buffer (pH 7.6) to which 50 µl H₂O₂ (30%) were added, and 3-amino-9-ethylcarbazole (Kem-En-Tec, Copenhagen, Denmark), 5 mg dissolved in 1 ml of 96% alcohol to which 9 ml distilled water and 10 µl of H₂O₂ (30%) were added.

Labeling of mabs

Radioiodination of mabs 12H12 and BM-2. Antibody (100 µg, 1 mg/ml 0.1 M phosphate buffer, pH 7.4, was placed in a glass tube coated with 5 µg of Iodo-Gen (Pierce, Rockford, IL). A solution of the mabs in 0.2 ml PBS with 10 µg human serum albumin was added as described by Schwarz and Steinsträsser (30), using a labeling kit (29) at room temperature. Unreacted ¹²⁵I ¹³¹I was separated by a centrifuged column procedure using Bio-Gel P30 (Bio-Rad, München, Germany). The iodination yield was 90%, resulting in a specific activity of 55 kBq/µg ¹²⁵I. Images were taken 24 h after injection. The mice were anesthetized with Halothane (Hoechst AG, Frankfurt/Main, Germany) and images were obtained in posterior view by using a large field-of-view gamma scintillation camera (Starcam 500 AT, General Electric, Frankfurt/Main) with a high-resolution germanium well-type detector coupled to a multichannel analyzer. The γ-rays at 27–35, 140, and 364 keV were used for determination of ¹²⁵I, ¹⁰⁹Tc, and ¹³¹I activity, respectively. Counting efficiencies amounted to 60% (¹²⁵I), 40% (¹⁰⁹Tc), and 18% (¹³¹I). The cross-talk of ¹³¹I with ¹²⁵I emission at 27–35 keV was determined to be 12% of the ¹³¹I counts at 364 keV.

Statistics

For statistical evaluation of differences in the distribution of radioactivity of mab 12H12 labeled with either ¹⁰⁹Tc or with ¹³¹I, Student’s t test (2-tailed) or the paired t test were used.

Radioimmunoeaging

For imaging studies, nude mice with the SF-15, AR-1, and EK-3 tumor xenografts were used. Each animal received 27 MBq (10 µg) of ¹⁰⁹Tc-labeled mab 12H12. Images were taken 24 h after injection. The mice were anesthetized with Halothane (Hoechst AG, Frankfurt/Main, Germany) and images were obtained in posterior view by using a large field-of-view gamma scintillation camera (Starcam 500 AT, General Electric, Frankfurt/Main) with a high-resolution low-energy collimator. Animals were imaged for 12 min, leading to a total of 7.5 × 10⁶ counts/animal. Subsequently, animals were killed and dissected, and biodistribution of radioactivity was measured as described above.

RESULTS

Immunoreactivity. The IF of the monoclonal antibodies 12H12 and BM-2 after radioiodination was determined by linear extrapolation to binding at the infinite antigen excess in a double-inverse plot according to the method of Lindmo et al. (31). The IF of mab 12H12 after labeling with ¹²⁵I was 55% and the IF of mab BM-2 after labeling with ¹³¹I was 87%. The IF after ¹⁰⁹Tc labeling of mab 12H12 was 45%. Unspecific binding amounted to 2–3%.

Immunohistochemistry. In immunohistochemical examination with the use of biotinylated mab 12H12, the SF-15 and AR-1 tumors showed an average staining of >80% of tumor cells. Wide areas showed homogeneous staining of all tumor cells beside areas with definite negative subpopulations, corresponding to a borderline type-I/II antigen pattern according to Matte et al. (32). Using the biotinylated mab BM-2, the AR-1 tumor showed strong, homogeneous staining of all cells (Fig. 1) corresponding to type I antigen pattern.
while the SF-15 tumor showed type 2 antigen pattern (Fig. 2). With both biotinylated mabs the endometrial carcinoma EK-3 showed staining of luminal edges of tumor cells forming glandular structures (Fig. 3) typical of type III antigen pattern.

**Biodistribution of mabs in SF-15-bearing Nude Mice.** The biodistribution of mabs 12H12 and BM-2 in nude mice carrying the mammary carcinoma xenograft SF-15 is shown in Table 1. Biodistribution of 125I-12H12 and 131I-BM-2 mabs were quite similar, whereas 99mTc accumulation following 99mTc-12H12 administration differed significantly from radiiodine accumulation in blood, kidneys, and tumor. A probability of error of <0.001 was calculated for the lower level of 99mTc in blood and the higher renal uptake (2-tailed t test for comparing the mean values of two groups). Differences in the tumor uptake of 99mTc-labeled mab 12H12 and 125I-labeled mab 12H12, respectively, appeared to be less pronounced, but these differences also proved to be of high significance ($P < 0.01$) because at all time points in each animal the accumulation of 99mTc exceeded that of 125I (paired t test). Based on the biodistribution in SF-15-bearing nude mice other mammary carcinoma xenografts were evaluated at 48 h p.i. where highest tumor:tissue ratios were expected.

A similar distribution pattern was found at 48 h in AR-1-bearing mice (Table 2). However, difference in tumor accumulation of 99mTc-labeled mab 12H12 and 125I-labeled mab 12H12 was even higher than in SF-15-bearing mice. In contrast, no significant difference in tumor uptake was noted in EK-3 tumors (Table 3).

Tumor:tissue ratios of 99mTc-labeled mab 12H12 and radiiodinated mabs 12H12 and BM-2 at 48 h p.i. are shown in Table 4. 99mTc labeling of mab 12H12 resulted in significantly enhanced tumor:tissue ratios in SF-15 and AR-1-bearing mice compared to radiiodinated mab 12H12.

In EK-3 tumor-bearing mice, tumor uptake of 99mTc-labeled 12H12 was similar to that of 125I-labeled mab 12H12. Tumor:tissue ratios...
Table 1. Biodistribution of 125I-labeled mab 12H12, Tc-125I-labeled mab BM-2, and 131I-labeled mab BM-2 in SF-15 tumor-bearing nude mice.

<table>
<thead>
<tr>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor</strong></td>
<td><strong>Blood</strong></td>
<td><strong>Liver</strong></td>
<td><strong>Spleen</strong></td>
</tr>
<tr>
<td>125I-labeled mab 12H12</td>
<td>10.56 ± 2.1616</td>
<td>13.77 ± 2.61b</td>
<td>14.63 ± 4.0716</td>
</tr>
<tr>
<td>131I-labeled mab BM-2</td>
<td>15.83 ± 4.36a</td>
<td>12.21 ± 2.01a</td>
<td>6.38 ± 1.2616</td>
</tr>
</tbody>
</table>

Fig. 3. Immunohistochemical staining of endometrial carcinoma xenograft EK with mab BM-2-biotin/streptavidin-peroxidase and 3,3'-diaminobenzidine as substrate. Staining corresponds to type III antigen pattern with strong staining of luminal edges of tumor cells forming glandular structures. × 240.

**DISCUSSION**

In vivo distribution studies with monoclonal antibodies 12H12 and BM-2 in mice bearing different tumor xenografts were undertaken to evaluate whether the favorable in vitro characteristics of the mabs (high Kd, low cross-reactivity with other tissues, immunohistochemical staining of more than 96% of the primary mammary carcinoma) correlated with high tumor accumulation and significant tumor:matrix ratios in vivo. Inhibition or enhancement of Tc-125I and 131I-12H12 binding in the biodistribution studies using 1 µg of mab could be excluded, because preliminary experiments showed no differences in biodistribution up to 20 µg of the mab administered. Mabs 12H12 and BM-2 were shown to recognize different epitopes on the antigen TAG-12 and cross-competition was not observed in enzyme-linked immunosorbent assay and cell binding studies (data not shown).

Despite the 10-fold higher affinity of mab BM-2 to the antigen TAG-12 in vitro and the higher immunoreactive fraction compared to the mab 12H12, no increase in tumor accumulation was observed. This may be explained by the "binding site barrier" theory suggested by Weinstein et al. (33), stating that nonuniformity of the antibody

Table 2. Biodistribution of 125I-labeled mab 12H12, Tc-125I-labeled mab BM-2, and 131I-labeled mab BM-2 in AR-1 tumor-bearing nude mice, 48 h p.i.

<table>
<thead>
<tr>
<th>99mTc</th>
<th>125I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mab 12H12</strong></td>
<td><strong>Mab BM-2</strong></td>
</tr>
<tr>
<td><strong>Tumor</strong></td>
<td>20.65 ± 4.98bc</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>7.61 ± 0.75a</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>3.48 ± 0.18</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>2.95 ± 0.38</td>
</tr>
<tr>
<td><strong>Kidneys</strong></td>
<td>2.95 ± 0.38</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td><strong>Bone</strong></td>
<td>1.12 ± 0.1</td>
</tr>
<tr>
<td><strong>Lungs</strong></td>
<td>3.35 ± 0.46</td>
</tr>
</tbody>
</table>

*% ID/g tissue ± 1 SD, all data are means of 5 animals.

**a** 99mTc significantly different (P < 0.01) from 125I (paired t test).

**b** 99mTc significantly different (P < 0.01) from 131I (paired t test).

**c** 99mTc significantly different (P < 0.001) from 131I (t test, 2-tailed).
distribution will tend to be increased by a high affinity of binding or a high concentration of antigenic sites (34). An increase in binding affinity of the antibody from $10^9$ to $10^{10}$ was shown to have only a small effect on mab tumor uptake (35). Fujimori et al. (36) examined an increase in binding affinity of the antibody from $10^9$ to $10^{10}$ was shown to have only a small effect on mab tumor uptake (35).

Biodistribution of the radioiodinated mabs 12H12 and BM-2 at 120 h was within the range observed for several other radioiodinated antibodies against breast cancer antigens in athymic mice (11, 13, 17). Compared to another $^{131}$I-labeled anti-mucin mab Mc5 (18), radioiodinated mabs 12H12 and BM-2 showed 3- to 4-fold higher tumor uptake. $^{99m}$Tc-labeled mab 12H12 is characterized by fast tumor uptake and rapid blood clearance and therefore seems to be an alternative to the mabs so far used for radioimmunoimaging of breast cancer. The $^{99m}$Tc-labeled mab 12H12 is characterized by fast tumor uptake and rapid blood clearance and therefore seems to be an alternative to the mabs so far used for radioimmunoimaging of breast cancer.

In summary, we have established animal models for the preclinical testing of two novel monoclonal antibodies specific for human breast mucin. The $^{99m}$Tc-labeled mab 12H12 is characterized by fast tumor uptake and rapid blood clearance and therefore seems to be an alternative to the mabs so far used for radioimmunoimaging of breast cancer. Direct $^{99m}$Tc labeling of mab 12H12 was highly efficient and convenient (6).

These promising results have recently been confirmed by a clinical pilot study of 19 breast cancer patients, imaged with $^{99m}$Tc-labeled mab BM-7 (IgG1) labeled by this new method were obtained recently.$^3$  

$^{111}$In-Diethylenetriamine pentaacetic acid labeling of anti-breast carcinoma mabs may result in higher tumor:blood ratios after 3–4 days (42, 43) as compared to $^{99m}$Tc-labeled mab at 24 h p.i. However, $^{111}$In liver uptake in mice is 8–10%, which is three times that observed for the $^{99m}$Tc-labeled 12H12. Moreover, $^{111}$In liver uptake in patients is even higher than in mice (44). In breast cancer, the liver is one of the major targets of metastatic spread. Thus, $^{111}$In-diethylenetriamine pentaacetic acid labeling drastically reduces the sensitivity of immunoscintigraphic detection of lesions in the hepatic and upper abdominal region.

The immunohistochemical examination of the tumor xenografts showed a good correlation with the classification suggested by Mattes et al. (32), comparing the results obtained by immunohistochemistry and biodistribution studies. In both breast cancer xenografts, showing borderline type-I/II antigen pattern, a strong tumor accumulation was obtained, whereas the endometrial carcinoma EK-3 (type III) accumulated poorly. Immunohistochemistry of primary mammary tumors with mab 12H12, performed by Kaul et al. (23, 24) in 206 primary mammary carcinomas demonstrated that more than 75% showed type I or type II antigen pattern. In comparison, the study of Mattes et al. showed that the breast cancer specimen examined with puncarcinoma mab B72.3 showed type IV antigen pattern or were negative.

In summary, we have established animal models for the preclinical testing of two novel monoclonal antibodies specific for human breast mucin. The $^{99m}$Tc-labeled mab 12H12 is characterized by fast tumor uptake and rapid blood clearance and therefore seems to be an alternative to the mabs so far used for radioimmunoimaging of breast cancer. Direct $^{99m}$Tc labeling of mab 12H12 was highly efficient and convenient (6).

These promising results have recently been confirmed by a clinical pilot study of 19 breast cancer patients, imaged with $^{99m}$Tc-labeled

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Fig. 4. Scintigraphic images of SF-15 (a, b) and AR (c, d) tumor-bearing mice 24 h p.i. Each animal received 27 MBq (10 μg) of 99mTc-labeled mab 12H12 in 0.2 ml PBS. Data were taken with Starcam AT 500 (General Electric) without background subtraction. Animals were imaged for 20 min leading to 7.5 × 10^6 counts/animal. Tumors were located at the left anterior lateral thoracic wall (a, b), the right anterior lateral thoracic wall (c), and the upper right leg (d). —, tumor; —, liver; —*, kidneys; ▲, tail.

12H12 (45). Since the target antigen TAG-12 is also used as a tumor marker for breast cancer (26), it will be the goal of future studies to evaluate whether the 99mTc-labeled mab 12H12 could localize tumor recurrence indicated by increasing TAG-12 serum levels in breast cancer patients.

ACKNOWLEDGMENTS

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