Radiolocalization of Xenografted Human Lung Cancer with Monoclonal Antibody 8 in Nude Mice

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ABSTRACT

A monoclonal antibody (MAb) 8 (immunoglobulin G3 (IgG3)), directed against a M, 48,000 human lung cancer-associated antigen, was radiolabeled with either 125I or 131I, and its biodistribution was studied in nude mice bearing human lung cancer (TKB-2) over a 7-day period. 125I-labeled MAb 8 increased its binding to the tumor during the period, while the binding of 131I-labeled control IgG3 declined after initial uptake. At Day 7, percentages of injected dose of 125I-labeled MAb 8 bound to the tumor rose to 7.4%, which was a 4.4-fold increase from Day 1 and 16-fold binding of 125I-labeled control IgG3 at the same day. Tumor: blood ratios became 2.7:1 at Day 7, and tumor: liver, tumorspleen, and tumor: kidney ratios were more than 9:1. Normal organs showed no significant uptake of 125I-labeled MAb 8, compared with those of 125I-labeled control IgG3. A clear image of the xenografted tumor was obtained at Day 5, and it further improved at Day 7, when 60% of whole body radioactivity was localized to the tumor. Autoradiography of the mouse with tumor confirmed the excellent localization of 125I-labeled MAb 8 to the tumor, although the radioactivity of the tumor was not uniformly distributed.

INTRODUCTION

The use of specific antibodies for the detection and treatment of malignancy has been suggested. Early attempts at tumor imaging showed limited success (1-3). The major difficulty has been the production of antibodies that react only with neoplastic cells. The recent development of monoclonal antibodies against tumor-associated antigens gives promise to such an approach (4).

We developed a number of monoclonal antibodies against human lung cancer. One of these antibodies, MAbs 8, was produced using TKB-2, a morphological variant cell line of human small cell carcinoma of the lung, as the immunogen. It was IgG3k in isotype, and recognized an M, 48,000 glycoprotein. MAb 8 reacted with 87% of large cell carcinoma, 82% of both squamous cell carcinoma and adenocarcinoma, and 19% of small cell carcinoma of the lung by immunoperoxidase staining. The overall reactivity with lung cancer was 68%. It bound to the cytoplasm as well as the cell membrane of the cancer cells. Cross-reaction with normal tissues was limited to epithelium were slightly stained.

As preliminaries for clinical trials, a detailed assessment of the biodistribution and pharmacokinetics of the antibody is important. In this study, we radiiodinated MAb 8, and examined its localization in tumor-bearing nude mice.

MATERIALS AND METHODS

Monoclonal Antibodies. The monoclonal antibody, MAb 8, was produced by fusion between mouse myeloma cells (P3U653Ag8U1) and mouse spleen cells immunized with TKB-2 cells, a morphological variant line of human small cell carcinoma of the lung (5). Its isotype was IgG3k. As a control, another monoclonal antibody of the same isotype directed to an irrelevant antigen was used. The antibodies were purified from ascitic fluid of mice by saturated ammonium sulfate precipitation and DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography. The immunoglobulin concentration was determined by absorbance at A280.

Radiolabeling of Monoclonal Antibodies. Purified MAb 8 and the control IgG3 were radiolabeled with either 125I or 131I (Amersham Corporation, Arlington Heights, IL) using the IODO-GEN (Pearce Chemical Company, Rockford, IL) method. Briefly, 100 μg of the antibody were mixed with 1 to 2 mCi of the radioisotope in a tube coated with 100 μg of IODO-GEN for 10 min on ice. The reaction was stopped by adding 1 mM tyrosine, and free isotope was separated with a Sephadex G-25 column (Quick-Sep QS-2A; Isolab, Inc., Akron, OH). Specific activity of the radiolabeled antibodies ranged from 1 to 10 μCi/μg. Immunoreactivity of the radiiodinated antibody preparation was evaluated by enzyme-linked immunosorbent assay (5) using the nonlabeled antibody as a control.

In Vivo Localization. BALB/c female nude mice 4 to 5 weeks old were inoculated 1 × 10^7 s.c. in the flank with TKB-2 cells. The tumor grew to 1.0 to 2.0 cm in diameter by 4 to 6 weeks. The mice were administered i.p. 4 to 10 μCi of either 125I-labeled MAb 8 or 131I-labeled control IgG3. They were sacrificed successively over a 7-day period by exsanguination, and radioactivity of the tumor and every organ (cpm per mg) was counted. Percentage of injected dose per gram of tissue, corrected for physical decay, was calculated. Tumortissue ratio was determined from this data. The thyroid was blocked with 0.1% KI in drinking water, starting 5 days before injection.

Radiolocalization. The TKB-2 tumor-bearing mice were given i.v. 80 μCi of either 125I-labeled MAb 8 or 131I-labeled control IgG3. Scanning was performed using a gamma camera (Photogramma LFOV; Shimazu-Searle, Inc., Kyoto, Japan) equipped with a pinhole collimator of 8 mm in aperture. A 20% energy window was centered over 364 keV. Data were stored on a computer (Shimazu Scintipac 230; Shimazu, Inc., Kyoto, Japan). The region of interest with regard to the tumor was applied to the recorded image, and percentage of whole body radioactivity in the tumor was determined. Background subtraction was not utilized.

Autoradiography. The mice with TKB-2 tumor were given i.p. 4 μCi of 125I-labeled MAb 8. One mouse was sacrificed by dipping it into liquid nitrogen at Day 5. A sagittal whole-body section of 50 μm in thickness was cut at the left kidney plane. Autoradiography was performed using Fuji RX film (Fuji Photo Film Company, Ltd., Tokyo, Japan). In another mouse, the tumor was excised on Day 7, fixed with formalin, and embedded in paraffin. Both 15-μm sections kept in contact with Fuji RX film and 5-μm sections coated with Sakura autoradiographic emulsion NR-M2 (Konihiroku Photo Ind. Corporation, Ltd., Tokyo, Japan) were exposed for 2 months.

SDS-PAGE. A mouse bearing TKB-2 tumor was given an injection i.p. of 20 μCi of 125I-labeled MAb 8, and sacrificed at Day 7. The tumor was minced, homogenized in Ca^2+ and Mg^2+ free Dulbecco's phosphate-
Excised tumor from a mouse not given an injection of 125I-labeled MAb was performed. As a control, 125I-labeled MAb 8 was added to the sulfonylfluoride on ice, and centrifuged at 10,000 x g for 10 min at 4°C. 125I-labeled MAb 8, supernatant of the homogenized tumor, and buffered saline with 0.5% Nonidet P-40 and 1 mM phenylmethyl of 125I-labeled (O) and nonlabeled (•) MAb 8 starting with 4 jig/ml. Results are protein standard (Bio-Rad Laboratories, Richmond, CA) was used as a 8, and the sample was processed in parallel. Low-molecular-weight molecular weight marker.

RESULTS

Immunoreactivity of 125I-labeled MAb 8. Immunoreactivity of 125I-labeled MAb 8 was well maintained after iodination. Enzyme-linked immunosorbent assay demonstrated the similar binding of the labeled and non-labeled MAb 8 to TKB-2 target cells (Fig. 1).

**In Vivo Distribution.** The TKB-2 tumor-bearing mice were administered i.p. 4 to 10 μCi of either 125I-labeled MAb 8 or 125I-labeled control IgG3 and sacrificed over a 7-day period. The radioactivity (cpm per mg) of the tumor and various organs was counted and corrected for physical decay. The percentage of the injected dose per gram of tissue was calculated from this data; results are the mean ± SD of 3 to 5 mice.

![Fig. 1. Enzyme-linked immunosorbent assay of 125I labeled and nonlabeled MAb 8 to TKB-2 cells.](image)

![Fig. 2. Distribution of 125I-labeled antibodies in nude mice with TKB-2 tumor.](image)
Fig. 3. Scintigraphic image. $^{131}$I-labeled MAb 8, 80 $\mu$Ci, was injected i.v. into a nude mouse bearing TKB-2 tumor (a), and scanning was performed at Days 3 (b), 5 (c), and 7 (d). Data were stored on a computer, and counting time was adjusted to 100 s. Background subtraction was not utilized.

**Table 2** Distribution of $^{131}$I-labeled monoclonal antibodies in nude mice with TKB-2 tumor

Mice with TKB-2 tumor were given an i.v. injection of 80 $\mu$Ci of either $^{131}$I-labeled MAb 8 or $^{131}$I-labeled control IgG3. Gamma camera scanning was performed successively, and the region of interest in regard to the tumor was measured; results are the mean of 3 mice.

<table>
<thead>
<tr>
<th>Days after injection</th>
<th>$^{131}$I-labeled MAb (n = 3)</th>
<th>$^{131}$I-labeled control IgG3 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total in mouse$^a$</td>
<td>% total in tumor$^b$</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>17.1</td>
</tr>
<tr>
<td>3</td>
<td>68.4</td>
<td>22.4</td>
</tr>
<tr>
<td>5</td>
<td>20.0</td>
<td>50.2</td>
</tr>
<tr>
<td>7</td>
<td>12.6</td>
<td>59.8</td>
</tr>
</tbody>
</table>

$^a$ Percentage of total radioactivity of Day 1 that remained in the mouse.
$^b$ Percentage of total radioactivity of the mouse found in the tumor.

Tumor:blood ratio became 2.7:1 at Day 7. Tumor:heart, tumor:spleen, and tumor:kidney ratios increased to more than 9:1, while those with $^{125}$I-labeled control IgG3 remained less than 2:1.

**Imaging of Tumor-bearing Mice.** The mice bearing the TKB-2 tumor were given injections i.v. of 80 $\mu$Ci of either $^{131}$I-labeled MAb 8 or $^{131}$I-labeled control IgG3, and studied by gamma camera scanning (Fig. 3). Up to Day 3, the whole body of the mice was imaged and the tumor border was difficult to define. The tumor image became increasingly clear as background subsided and was distinct at Day 5. At Day 7, most of the radioactivity was seen at the site of the tumor. The background activity cleared with a $t_1/2$ of approximately 8.7 days. As for $^{131}$I-labeled control IgG3, tumor image was not apparent throughout the study. Percentages of whole body radioactivity in the tumor (Table 2) increased from 17.0% at Day 1 to 59.8% at Day 7, while those of control IgG3 remained at less than 20%.

**Autoradiography of Mice.** The mouse xenografted with TKB-2 tumor was sacrificed 5 days after $^{125}$I-labeled MAb 8 injection, and autoradiography of the whole body section was performed (Fig. 4). Although radioactivity was observed throughout the body including liver, kidney, and lung, the tumor showed the strongest uptake of $^{125}$I-labeled MAb 8. Distribution of radioisotope at the tumor was not uniform, some areas showing strong binding and others remaining unbound. Except for the tumor, the blood vessels around the eye and those at the neck contained moderate radioactivity. Slight activity at the skin was due to contamination with urine and feces. As demonstrated in Fig. 5, the central necrotic area of the tumor showed no radio-
activity, but the surrounding viable area took up $^{125}$I-labeled MAb 8.

SDS-PAGE Analysis. SDS-PAGE analysis (Fig. 6) of the radioiodinated MAb 8 showed very strong radioactivity by the heavy chain compared with that of the light chain. In the tumor homogenate, most of its radioactivity was present at F(ab')$_2$ and the heavy chain, and only a trace amount at IgG. In the control tumor, on the other hand, most of the radioactivity was present at IgG. Both of the mouse sera showed $^{125}$I-labeled IgG.

DISCUSSION

Monoclonal antibodies conjugated to radioisotopes have been studied for localization of malignancy by a number of investigators. In experimental animals, antibodies directed to carcinomaembryonic antigen (6–10), melanoma (11, 12), colon carcinoma (13–15), breast carcinoma (16), hepatocarcinoma (17), osteogenic sarcoma (18, 19), renal cell carcinoma (20), germ cell tumor (21), and milk fat globule (22) have been used. In clinical studies, melanoma (23–25), colon carcinoma (26, 27), and breast carcinoma (28, 29) have been their targets. Most of these antibodies were highly specific to tumor-associated antigens, but some antibodies detected tumors in spite of the presence of cross-reacting normal tissues (30, 31). Not only intact IgG, F(ab')$_2$ and Fab fragments were also used successfully (7, 8, 13, 16, 23, 25–27). $^{131}$I, $^{125}$I, $^{121}$I (28), and $^{111}$In (9, 10, 17, 22, 24, 29) were used for labeling in most cases.

The present study demonstrated that radioiodinated MAb 8 had accumulated at the tumor xenografted in nude mice and had given a distinct image of the tumor by scanning. The specificity of this localization to the tumor was supported by the absence of any significant accumulation of $^{125}$I-labeled control IgG of the same isotype. The increasing tumor:tissue ratios over the period studied and the predominant radioactivity at the tumor by autoradiography also confirmed the specificity of radioiodinated MAb 8 for the tumor.

The immunoreactivity of $^{125}$I-labeled MAb 8, which had been labeled by the IODO-GEN method, was well maintained. SDS-PAGE analysis showed an intensive radioactivity by heavy chain and an only slight incorporation of $^{125}$I by light chain, even taking into account the difference in molecular weight between the two. Probably the light chain of MAb 8 contains a few exposed tyrosine residues in its chain. Although dehalogenation is one of the serious problems in studying radioiodinated antibodies in vivo (32, 33), radioiodinated MAb 8 was stable enough to give a clear image of the tumor in this study.

Several parameters have been used to indicate the localization of radiolabeled antibodies. Tumor:blood ratios increased progressively after $^{125}$I-labeled MAb 8 injection and reached 2.7:1 at Day 7, while those of the control IgG remained at less than 0.6:1 during the period. This increase of tumor:blood ratios was in part due to the clearance of the radiolabeled antibody from the blood but was also due to the continuous accumulation of $^{125}$I-labeled MAb 8 by the tumor for as long as 7 days. Tumor:blood ratio may be influenced by the amount of antibody injected and the size of tumor, and %ID/g is another parameter (19). %ID/g of the tumor continued to increase over the period, indicating that the accumulation of $^{125}$I-labeled MAb 8 by the tumor still lasted even after 7 days. A third parameter, percentage of whole body radioactivity in the tumor, also continued to rise during the period. This continuous accumulation of antibody in the tumor was different from other reports (9, 13, 15, 19) in which %ID/g of the tumor had reached maximum 2 to 3 days after antibody injection and had decreased slowly thereafter. This long accumulation and retention of MAb 8 in the tumor is particularly desirable for a therapeutic application, since the prolonged contact of drug- or isotope-conjugated antibody with tumor cells enables effective killing of them.

Interestingly, SDS-PAGE analysis of the tumor homogenate showed that most of the injected radioactivity was present at the tumor in the form of $^{125}$I-labeled F(ab')$_2$ and heavy chain, and $^{125}$I-labeled intact IgG was present in only a trace amount.
There are two possibilities to explain the degradation of administered $^{125}$I-labeled MAB 8 in the tumor. One possibility is that the injected MAB 8 was decomposed after homogenization with endogenous proteolytic enzymes released from broken cells. Another possibility is that MAB 8 was degraded in situ in the tumor before excision. Although the former possibility cannot be excluded, the latter is more likely: (a) the control sample, in which $^{125}$I-labeled MAB 8 was added after excision, showed only slight degradation; (b) MAB 8 reacted strongly with the cytoplasmic antigen and degraded in the cytoplasm. The slow and continuous accumulation of $^{125}$I-labeled MAB 8 may be acceptable to think that $^{125}$I-labeled MAB 8 was internalized into the cytoplasm of tumor cells in situ.

The heterogeneous nature of malignant tumor is one of the difficult problems to be resolved for the detection and treatment of malignancy with monoclonal antibodies. As revealed by autoradiography of the whole body section of a mouse, radioactivity at the tumor was not uniform. This inhomogeneity was largely due to the presence of necrosis in the tumor. Most of the viable regions reacted with $^{125}$I-labeled MAB 8, as shown by microautoradiography. However, this may not be the case in clinical situations, since immunoperoxidase staining with MAB 8 showed heterogeneous distribution of the antigen within a lung cancer (5). Two suggested resolutions for this difficulty are the administration of a group of monoclonal antibodies with different binding repertoires (4) and the coupling with an appropriate radioisotope that can kill several cell diameters in the hope that one cell, at least, in a tumor cluster expresses the antigen (15). Further studies are necessary to settle the problem.

In summary, radioiodinated MAB 8 localized well to the xenografted lung cancer with MAB 8 showed no significant binding to normal organs. The slight cross-reaction, which had been demonstrated with the human lung by immunoperoxidase staining (5), was not significant in this study. The discrepancy may originate in the difference of species or method of study and indicates that great care must be taken in applying to clinical cases the results of a study using mice (34–36).

In summary, radioiodinated MAB 8 localized well to the xenografted nude mice and gave an excellent image of the tumor. The continuous accumulation by the tumor of radioiodinated MAB 8 is desirable for therapeutic application. However, great care must be taken in interpreting these data for clinical trials.

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