Abstract

Intrasplenic injection of the HT-29 LMM metastatic human colon cancer line reproducibly results in hepatic metastasis formation in congenitally athymic mice. HT-29-15, a murine monoclonal antibody (mAb) of the IgG1 class reactive with the HT-29 LMM line, and BL-3, an isotype-matched control antibody, were labeled with $^{125}$I. Labeled mAbs were injected i.v. in mice with hepatic metastases, and animals were sacrificed on days 3, 5, and 7. Specific mAb uptake by tumor was significantly greater than nonspecific mAb uptake, as evidenced by specific/nonspecific tumor/blood ratios (radiolocalization indices) of 3.47/1–25.6/1. Relative mAb uptake was greater by the hepatic tumors than by the splenic tumors from day 3 to day 7, although this was significant ($P < 0.05$) only on day 7 (5.12 ± 2.97 versus 1.79 ± 0.71). Tumor/uninvolved tissue ratios were also significantly greater ($P < 0.05$) for the hepatic metastases than for the splenic tumors on day 7 (12.23 ± 3.85 versus 6.63 ± 2.63). This murine hepatic metastasis model appears useful for evaluation of localization of mAbs to hepatic metastases from human colon carcinoma.

Introduction

Colorectal cancer is one of the most frequent malignant diseases and it is estimated that there are approximately 145,000 new cases and 60,000 deaths each year in this country (1). About 40% of these patients develop hepatic metastases in the course of their disease (2). The detection and therapy of hepatic metastases of colorectal cancer remain important goals of cancer treatment.

Since some initial success reported by Goldenberg et al. (3–6) for the clinical localization of primary and secondary colorectal cancer using polyclonal antibodies for carcinoembryonic antigen, the use of radiolabeled antibodies has shown promise as a clinically useful modality for the diagnosis and treatment of colorectal cancer (7). However, imaging sensitivity and specificity for hepatic metastases of colorectal cancer has been found to be lower than that for primary tumor because hepatic metastases have site-specific problems such as high normal hepatic vascular background. Additionally, there are few data on absolute tumor and normal tissue uptake of radiolabeled mAbs because there are considerable limitations in obtaining suitable tumor and normal tissue in humans.

The ability of radiolabeled mAbs to reach and bind the antigenic determinants accessible in a living tumor can be easily tested in the nude mouse model. Animal models with s.c. and visceral xenograft implants of HCC have already been developed and used widely for this purpose (9–12); a limitation of these is the low incidence of metastases. Recent studies from Fidler et al. (13–17) have shown that the implantation of established HCC cell lines with metastatic behavior (one of which was used in this study) into the spleen in nude mice reproducibly results in liver metastases.

This study was undertaken to determine the utility of the murine hepatic metastasis model developed by Fidler et al. for the localization of radiolabeled mAbs.

Materials and Methods

Mice. Ninety specific-pathogen-free athymic female BALB/c mice, 3 to 4 weeks of age, were cared for at the animal facility at this center. Mice were kept under sterile conditions in a laminar flow room in cages with filter bonnets and were fed sterilized mouse diet and sterilized water.

Tumor Cell Line. The human colon cancer cell line HT-29 LMM, a metastatic variant of the HT-29 cell line (18), was generously provided by Dr. J. J. Fidler (M. D. Anderson Hospital and Tumor Institute, Houston, TX). The cell line was grown under humidified conditions in the presence of 5% carbon dioxide in Dulbecco’s modified minimum essential medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY).

Establishment and Evaluation of Hepatic Metastases. Tumor cells grown in tissue culture were harvested by 5-min treatment with 0.05% trypsin and 0.02% EDTA and suspended in phosphate-buffered saline. The mice were anesthetized with i.p. pentobarbital (12 mg/g body weight), and a small left subcostal incision was made to expose the spleen. HT-29 LMM cells (2 x 10⁶) in 50 μl of phosphate-buffered saline were injected beneath the splenic capsule using a 30-gauge needle. The mice were sacrificed approximately 4 weeks after injection and tumor burden of hepatic metastases was evaluated by determining their number and weight. Hepatic metastases and splenic tumors were fixed in 10% buffered formalin and processed for histological examination.

Monoclonal Antibodies. HT-29-15, which was developed by Dr. J. Sakamoto in Dr. L. J. Old’s laboratory at this center (19), is a murine IgG1 monoclonal antibody reacting with a neuraminidase-sensitive cell surface antigenic determinant (M, 200,000) present on the HT-29 HCC cell line. It reacts with more than 60% of primary and metastatic colorectal cancers in immunohistopathology. A study in colorectal cancer patients with hepatic metastases demonstrated that radiolabeled HT-29-15 localized to tumor tissue (20).

BL-3 was kindly provided by Dr. J. Schlam (National Cancer Institute, NIH, Bethesda, MD). This mAb reacts with a human B-cell lymphoma and was used as an isotype-matched control. BL-3 does not react with HT-29 LMM cell line.

Radiolabeling. MAbs were labeled with sodium $^{125}$I-iodide (New England Nuclear, Boston, MA) by a modified chloramine-T method (21). The radiolabeled mAbs were purified by exclusion chromatography on a Sephadex G-25 column (Pharmacia, Piscataway, NJ). The percentage of labeling was between 60 and 80%. The fraction corresponding to the radiolabeled mAbs typically had more than 95% of radioactive iodine bound to protein, as determined by trichloroacetic acid precipitation. Immunoreactivity was tested by a modification of the method of Lindmo et al. (22) on each batch and was between 30 and 55% for the HT-29-15 mAb and less than 10% for the BL-3 mAb.

Biodistribution. Thirteen mice with hepatic metastases established approximately 4 weeks after injection were given i.v. injections of 30–50 μCi/2–4 μg of $^{125}$I-labeled HT-29-15. Ten mice were given injections of 15–25 μCi/2–4 μg $^{125}$I-labeled BL-3. At days 3, 5, and 7 after injection, animals were sacrificed (Fig. 1). Normal tissue (liver, spleen, heart, lung, kidney, large intestine, thyroid, muscle, and brain), blood, splenic tumor, and all hepatic metastases were removed and weighed, and their radioactivity was counted in a gamma well counter.

Abstract

Intrasplenic injection of the HT-29 LMM metastatic human colon cancer line reproducibly results in hepatic metastasis formation in congenitally athymic mice. HT-29-15, a murine monoclonal antibody (mAb) of the IgG1 class reactive with the HT-29 LMM line, and BL-3, an isotype-matched control antibody, were labeled with $^{125}$I. Labeled mAbs were injected i.v. in mice with hepatic metastases, and animals were sacrificed on days 3, 5, and 7. Specific mAb uptake by tumor was significantly greater than nonspecific mAb uptake, as evidenced by specific/nonspecific tumor/blood ratios (radiolocalization indices) of 3.47/1–25.6/1. Relative mAb uptake was greater by the hepatic tumors than by the splenic tumors from day 3 to day 7, although this was significant ($P < 0.05$) only on day 7 (5.12 ± 2.97 versus 1.79 ± 0.71). Tumor/uninvolved tissue ratios were also significantly greater ($P < 0.05$) for the hepatic metastases than for the splenic tumors on day 7 (12.23 ± 3.85 versus 6.63 ± 2.63). This murine hepatic metastasis model appears useful for evaluation of localization of mAbs to hepatic metastases from human colon carcinoma.

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This study was undertaken to determine the utility of the murine hepatic metastasis model developed by Fidler et al. for the localization of radiolabeled mAbs.
RADIOLOCALIZATION OF Mabs IN HEPATIC METASTASES

TIMua/Blood

HT-29-15

«on
EH
Li Sp Ht Lu Kl In Th Mu Br HM ST

•¿ DAY3 (n-3) ÏZà DAY9 (n-9) EU DAY 7 (n-9)

Fig. 1. Tissue/blood ratio for $^{125}$I-labeled HT-29-15 and BL-3 in mice with hepatic metastases. At days 3, 5, and 7 following i.v. injection, tissues were removed and radioactivity was counted. The tissue/blood ratio was calculated. $n$, number of animals/group. Li, liver; Sp, spleen; Ht, heart; Lu, lung; Kl, kidney; In, large intestine; Th, thyroid; Mu, muscle; Br, brain; HM, hepatic metastases; ST, splenic tumor.

The %ID/g and tissue/blood, hepatic metastases/liver, and splenic tumor/spleen ratios were calculated.

Results

Establishment and Evaluation of Hepatic Metastases. There was no operative mortality. Ninety-eight % (88 of 90) of mice developed hepatic metastases approximately 4 weeks after i.s. injection; 38% (34 of 90) developed less than 5 macroscopic metastases; while 30% (27 of 90) had 5–10 and the remaining 30% (27 of 90) had more than 10 metastatic foci in the liver. Average total weight of hepatic metastases was 0.29 g and maximum total weight was 1.0 g; average liver replacement by hepatic metastases (metastasis weight/total liver weight) was 21% (maximum liver replacement 71%).

Macroscopically, hepatic metastases were multiple irregular gray-white nodules of varying size evenly distributed in both liver lobes. Some nodules demonstrated scarring and retraction producing an umbilicated appearance. Microscopically, hepatic metastases revealed moderately differentiated adenocarcinoma of HCC, with no change from the primary splenic tumors evident on light microscopy. Hepatic metastases were demonstrated to form distinct nodules and to displace hepatocytes. However, areas of necrosis were rarely present in the center of the nodules. These pathological findings appeared similar to hepatic metastases in humans.

Biodistribution. Tissue/blood ratios of $^{125}$I-labeled HT-29-15 and control BL-3 in mice with hepatic metastases after i.v. injection are shown in Fig. 1. Relative mAb uptake was highest in hepatic metastases on day 7 and in splenic tumors on day 5. All other normal tissues had markedly lower ratios. Control mAb BL-3, in contrast, showed no evidence of specific uptake of radioactivity with no increased relative uptake in tumor tissue.

Tumor/blood ratios for each hepatic metastasis with $^{125}$I-labeled HT-29-15 ranged from 0.88 to 12.29 (Fig. 2), while uptake of $^{125}$I-labeled BL-3 by each hepatic metastasis was almost equal to that by normal tissue.

Biodistribution of $^{125}$I-labeled HT-29-15/BL-3 in hepatic metastases, splenic tumor, and blood, expressed as %ID/g, and tumor/blood, hepatic metastasis/liver, and splenic tumor/spleen ratios are given in Table 1. The hepatic metastasis/blood ratio of $^{125}$I-labeled HT-29-15 was significantly higher than

Table 1 Biodistribution of $^{125}$I-labeled HT-29-15 and BL-3

<table>
<thead>
<tr>
<th>Tumor/tissue ratio</th>
<th>mAb</th>
<th>Tissue</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
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<tr>
<td>Hepatic metastases</td>
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<td>Blood</td>
<td>1.55</td>
<td>4.84</td>
<td>5.12*</td>
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<td></td>
<td></td>
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<td>18.82</td>
<td>12.20*</td>
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<td>Blood</td>
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<td>0.26</td>
<td>0.20</td>
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<tr>
<td></td>
<td></td>
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<td>1.11</td>
<td>1.21</td>
<td>0.83</td>
</tr>
<tr>
<td>Splenic tumor</td>
<td>HT-29-15</td>
<td>Blood</td>
<td>1.18</td>
<td>2.26</td>
<td>1.79*</td>
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<tr>
<td></td>
<td></td>
<td>Spleen</td>
<td>4.71</td>
<td>8.97</td>
<td>6.63*</td>
</tr>
<tr>
<td></td>
<td>BL-3</td>
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<td>0.22</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
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<td>1.17</td>
<td>1.11</td>
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<tr>
<td>%ID/g</td>
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<td>3.08</td>
<td>7.73</td>
<td>2.81</td>
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<td>3.15</td>
<td>0.73</td>
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<td></td>
<td></td>
<td>5.95</td>
<td>5.13</td>
<td>3.56</td>
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</table>

* P < 0.05 (hepatic metastases/blood versus splenic tumor/blood with HT-29-15 on day 7).

Fig. 2. Relative uptake of $^{125}$I-labeled HT-29-15 and BL-3 by metastatic foci in the liver. O, ratio for hepatic metastasis/blood of $^{125}$I-labeled HT-29-15; ©, ratio for hepatic metastasis/blood of $^{125}$I-labeled BL-3.
splenic tumor/blood ratio only on day 7 ($P < 0.05$); the hepatic metastasis/liver ratio was also higher than the splenic tumor/spleen ratio only on day 7 ($P < 0.05$). %ID/g of $^{125}$I-labeled HT-29-15 was highest in hepatic metastases on day 5 and in splenic tumors on day 3. %ID/g of hepatic metastases was higher (not statistically significant) than that of splenic tumors from day 3 to day 7.

The specificity of reactivity of mAb HT-29-15 with HT-29 LMM is shown in Fig. 3. The uptake of HT-29-15 is normalized to that of BL-3 control antibody in calculating the localization index. From day 3 to day 5 there is a progressive increase for both hepatic metastases and splenic tumors in the localization index for tumor/blood and hepatic metastasis/liver or splenic tumor/spleen ratios. On day 3 the localization index was greater than 3.3 and by day 7 it ranged from 6.0 to 25.6.

**Discussion**

Several animal models using xenografted HCC cell lines for the study of radiolabeled mAbs have been described previously and are widely used. Mach et al. (9) developed s.c. xenografts of HCC in nude mice, and Hnatovich et al. (10) studied subcapsular renal implants of HCC in nude mice. Shah et al. (11) described the localization of HCC in the spleens of athymic mice, and Sharkey et al. (12) produced subcapsular liver xenograft implants of HCC in hamsters. Although radioimmunodetection of HCC xenografts in visceral organs has been attempted, no model utilizing hepatic metastases has been reported.

In the model described here, i.e., injection of the HT-29 LMM HCC cell line reproducibly resulted in hepatic metastases in 90 mice after 4 weeks with no operative mortality. This method results in discrete metastatic foci in the liver. The injection of cancer cells into the spleen represents an advantageous route of injection for development of liver metastases and is physiologically comparable to the metastatic process in HCC.

The reproducibility of hepatic metastases formation after i.s. injection of a primary HCC line has been reported in the past to be low (23). As Fidler et al. (13-17) have reported, the ability of HCC cells to produce liver metastases is not due to simple trapping in the liver; the formation of hepatic metastases from HCC is a selective process. Choosing appropriate metastatic cells and host conditions (i.e., young healthy mice) is essential for high reproducibility of hepatic metastases in nude mice. The HT-29 LMM cell line has been developed by Dr. Fidler expressly for the purpose of reproducibly generating hepatic metastases following i.s. injection. The present study confirms the high frequency of hepatic metastases (98%) obtained using this cell line.

Our study demonstrates specific radiolabeled mAb (HT-29-15) localization to both splenic tumors and hepatic metastases with simultaneous injection of an isotype-matched control mAb (BL-3). Tumor/blood ratio for hepatic metastases progressively increased from day 3 through day 7, perhaps reflecting rapid clearance of radiolabeled mAb from blood. Each hepatic metastasis demonstrated considerably different uptake of specific labeled mAb even in the same mouse. There was no correlation between tumor weight or size and mAb uptake (data not shown). One of the advantages of this model is the ability to assess the entire tumor burden which is not usually possible in humans.

The difference in specific uptake between hepatic metastases and splenic tumors may reflect the different vascularity of both uninvolved tissue and tumor, the selection of a metastatic subpopulation which expresses different characteristics (i.e., antigen level), or difference in the rate of dehalogenation and/or modulation of mAbs. It is conceivable that the mAb is catabolized more rapidly in the spleen than in the liver. This may account for the drop in mAb concentration in the spleen relative to that in the liver. However, it is interesting to note that although the hepatic metastasis/liver ratio increases from day 5 to day 7 while the splenic tumor/spleen ratio drops, the %ID/g of the hepatic metastases drops much more dramatically over the same period. Further studies including autoradiography are ongoing to address this issue.

In the human study of 23 colorectal cancer patients using $^{125}$I-labeled HT-29-15, hepatic metastases were imaged on days 5 and 7, and mAb localization was confirmed by biopsy (20). Analysis of tissue radioactivity showed that hepatic metastasis/liver ratio increased from day 1 to day 7 and ranged from 0.95 to 6.6 in patients with antigen-positive tumors; the hepatic metastasis/blood ratio also correlated with the time interval for biopsy and ranged from 0.2 to 3.01. Our study also showed a significant increase in relative hepatic metastasis uptake in this animal study, although this was confounded by the drop in %ID/g mentioned earlier.

There was considerable variation in mAb uptake by different hepatic metastases of the same patient. The %ID/g of hepatic metastases ranged from 0.0007 to 0.0106%. However, biopsy data in the human study did not reflect entire metastatic lesion(s). In our murine model, we confirmed the variation in the specific uptake by different metastases in the same mouse. The %ID/g was between 1.23 and 18.46%; this is consistent with the high documented %ID/g obtained in nude mouse xenografts. Hepatic metastases in the human are of varying size, usually being large and with considerable necrosis centrally; in the study referred to, logistic considerations precluded procurement of entire tumors on the same days post-mAb infusion; control mAb was also not simultaneously infused. These variables can be very well controlled in an animal model such as ours; we have shown, similar to the results from the study of Cohn et al. (20), that relative mAb uptake is time dependent, and we have validated this result in an animal model comparable to the human condition, taking into account radiolabeled mAb uptake in the entirety of the hepatic metastases in any given animal. Ongoing studies will address the issue of detection of very small hepatic metastases and consequently the effect of tumor burden. Additionally, we will begin utilizing this model to study questions such as extraction efficiency of radiolabeled mAbs by hepatic metastases following regional (i.e., intrahepatic artery/intraportal vein) injection.

We conclude that this murine hepatic metastasis model is a
good model for evaluation of the localization of mAbs to hepatic metastases. Ongoing studies in this hepatic metastases model will aid in the development of clinical imaging and treatment of patients with colorectal hepatic metastases.

Acknowledgments

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Radiolocalization of Monoclonal Antibodies in Hepatic Metastases from Human Colon Cancer in Congenitally Athymic Mice

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