Localization of Human Renal Cell Carcinoma Xenografts with a Tumor-preferential Monoclonal Antibody

Rei-Kwen Chiou, Robert L. Vessella, Michael K. Elson, Ralph V. Clayman, J. Michael Gonzalez-Campoy, Michael J. Klicka, Rex B. Shafer, and Paul H. Lange

ABSTRACT

We previously described an immunoglobulin G1 monoclonal antibody (UMVA-RCC-A6H) that is highly reactive with human renal cell carcinoma (RCC) and has little cross-reactivity to other cell types both normal and malignant. In efforts detailed herein, radiolabeled A6H selectively localized to RCC xenografts and provided high resolution images of the xenografts. Also, A6H clearly discriminated between RCC xenografts and other human tumor xenografts. Consistent images of RCC xenografts (>60 mg) were obtained without background subtraction. The amount of radiolabeled A6H in the tumor usually ranged from five to twenty times that of the blood. Normal mouse tissues, abscesses, and other human tumor xenografts contained less radiolabel per mg than did blood. A control monoclonal antibody of the same isotype failed to exhibit any localization in xenografts or normal tissues. Approximately 40% of the radiolabeled A6H dose per g was localized in the RCC xenograft 2 days after injection, although at the time of imaging about 60% of the radiolabel remaining in the mouse was associated with the xenograft. These results demonstrate that a RCC restrictive monoclonal antibody does specifically localize to RCC xenografts and supports the hope that this approach may have clinical value for diagnosis, staging, or treatment.

INTRODUCTION

The detection of tumors by scintillation scanning has been attempted for many years with only limited success. Early efforts involved nonspecific tumor-seeking radiopharmaceuticals such as gallium and bleomycin-cobalt (1, 2) but lack of specificity limited their usefulness. Other investigators explored RIS (3), the combination of nuclear medicine techniques with tumor-"specific" xenogenic antibodies. Results did improve modestly in part because of advances in radiopharmacology and computer science but still success was insufficient for widespread clinical implementation. The polyclonal antibodies directed to membrane-associated antigens continued to lack high specificity and those which were specific for soluble tumor markers yielded results which were only partially successful.

In this paper we detail our efforts to utilize an IgG1 monoclonal antibody designated A6H (5) in RIS studies of human RCC xenografts. This MAB is highly reactive to an RCC membrane antigen which is also expressed on normal renal proximal tubules but no other normal tissues. It was generated from a tandem immunization protocol involving the sequential injection of 5 different fetal kidney homogenates. Radiolabeled A6H localized in the 4 RCC xenografts tested exhibited incorporation levels up to 60 times those of the blood and yielded clear scintigraphic images of xenografts as small as 60 mg without the need for background subtraction.

MATERIALS AND METHODS

Generation and Characterization of Monoclonal Antibody A6H.

Details on the generation and screening of UMVA-RCC-A6H have been reported (5). Briefly A6H was a product of a tandem immunization protocol (6). Barrier-reared BALB/c mice were injected with 5 different homogenates of fetal kidneys (16-22 wk, gestation) using an immunization schedule consisting of sequential i.p. injections over a period of 3 mo. Spleen cells were fused with P3X63-Ag8.653 (gift of Dr. Kearney, University of Alabama) (7) as described by Oi and Herzenburg (8). Antibody reactivity profiles were established using a cell-binding enzyme-linked immunosorbent assay and both fresh and paraffin-embedded tissue immunohistochemistry.

Purification of Monoclonal Antibodies. These procedures have also been described in detail (5). Briefly following clarification of ascites fluid, the immunoglobulin fraction was precipitated by the addition of ammonium sulfate, dialyzed, applied to a CM-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) chromatography column and the void eluate containing the immunoglobulin was collected. This fraction was then applied to a DEAE-Affi-Gel Blue (Bio-Rad Laboratories) column and the immunoglobulin subpopulations were eluted isocratically with buffer containing from 0.018-0.040 M NaCl. Appropriate fractions were pooled.
and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver stain analysis of the derived gel bands (9).

Radiolabeling of Monoclonal Antibodies. Purified A6H or a control IgG1 specific for α-fetoprotein (UMVA-AFP-22) (10) was radiolabeled with either 125I or 131I using the chloramine-T procedure (11). The MAB (70-150 μg) was mixed with 1-2 mCi of radioiodine and 5 mg chloramine-T in 0.1 M phosphate buffer, pH 7.2, for 20-30 s after which tyramine was added to stop the reaction. The iodinated immunoglobulin was separated from excess reactants by gel filtration (Bio-Gel P-6DG; Bio-Rad Laboratories). Specific activities of the radiolabeled MAB ranged from 5-20 μCi/μg. Each radiolabeled A6H preparation was evaluated for immunological activity. An aliquot of the preparation (approximately 104 cpm) was added to replicate tubes containing 105 cells of an RCC cell line (7860), previously shown to be highly reactive with A6H (5). The tubes were incubated at room temperature with periodic mixing. After 1 h, the cells were pelleted by centrifugation and the amount of radiolabel bound to the cells was determined and expressed as the percentage of total radiolabeled MAB added to the tubes. This value, representing the iodinometrically reactive portion of the radiolabeled preparation, generally ranged from 50-70%, although on occasion low immunological activity (<30%) was noted. These preparations of low activity were not utilized in the studies presented herein.

Human Tumor Xenografts. Human RCC xenografts were established s.c. in nude mice from fresh surgical specimens and maintained in the colony by serial passage. The 4 RCC xenografts utilized in these studies were established by one of us (R. V. C.) and are designated TK-39, TK-82, TK-177C, and TK-177G. These xenografts closely resemble the histology of their original human tumors (12). TK-39 is a grade 3 clear cell RCC; TK-82 is a grade 3 clear cell and granular cell RCC. These 2 RCC xenografts have rapid growth rates in nude mice with doubling times of approximately 7-8 days. TK-177C and TK-177G were originally from the same patient who had 2 distinct histological patterns in the same tumor. The clear cell type was established as TK-177C and the granular cell type as TK-177G. These 2 xenografts have slower growth rates with doubling times of approximately 10-12 days and tend to stop growing after reaching a certain size (1.5-2 cm diameter) in nude mice. The fourth passage of TK-177G and TK-177C, the eighth passage of TK-39, and the seventh passage of TK-82 are used in this study.

Control human tumor xenografts incorporated in this study were VC-2, an endometrial carcinoma (gift of Dr. P. G. Satyaswaroop, Milton S. Hershey Medical Center, Philadelphia, PA) and 1411H, a human testicular carcinoma initially established as a cell line in our department (13).

In Vivo Localization and Radiocommunoscintigraphy. The majority of mice included in these studies bore 2 s.c. xenografts, (a) 1 RCC and the other a non-RCC, or (b) both RCC. For studies of radiolabel biodistribution which did not include scintigraphy, mice were given injections i.v. of 1-2 μCi of either 125I-radiolabeled A6H or AFP-22. Mice which were to be imaged received between 20-40 μCi of the 125I-radiolabeled MAB but always equivalent doses of A6H and AFP-22 per study. Immediately following scintigraphy, all of the mice in the study were sacrificed and samples of the xenograft type, 8 other tissue types, blood, and urine were collected. These were weighed, the amount of radiolabel in each was determined, and from these data (cpm/mg) the T:B ratios were calculated. These T:B ratios are referred to as the biodistribution index. Mice with skin abscesses were included in one series to determine the affects of acute inflammation on RIS. Initial RIS studies were performed at 5 h, and 1 and 2 days after injection of the radiolabeled MAB. Thereafter images were obtained 2 days after injection of radiolabel.

Scintigraphy was performed using a Siemens Pho Gamma V camera equipped with a pinhole collimator. These studies were done in a blinded manner; investigators performing the imaging were not aware of the xenograft types (RCC or a control tumor) or of which radiolabeled MAB was injected (A6H or AFP-22). Ten thousand-count images were normally acquired over a 3- to 5-min period and data were stored in a Modume D computer for further analysis and production of color imagings. Background subtraction techniques were not utilized.

RESULTS

Biodistribution of Radiolabeled MABs A6H and AFP-22. Biodistribution studies were performed on all of the mice bearing xenografts or skin abscesses and were designed to evaluate (a) the specificity of MAB tissue distribution using both RCC and non-RCC xenograft combinations, (b) the changes in T:B ratios with time after injection, (c) the influence of xenograft size on the T:B ratios, and (d) whether areas of acute tissue inflammation nonspecifically radiolabeled MABs accumulated. As detailed in "Materials and Methods," mice undergoing RIS received 20-40 μCi (2-4 μg) of 131I radiolabeled MAB, whereas those used solely for biodistribution studies received 1-2 μCi (0.1-0.2 μg) of radiolabel (125I). Since the T:B ratios were similar between the 125I and 131I studies, these data have been combined.

Biodistribution studies were done on 24 mice bearing either 1 RCC xenograft (N = 10), more than 1 RCC xenograft (N = 5), RCC and a non-RCC tumor xenograft (N = 4), non-RCC xenografts (N = 2), or skin abscesses (N = 3). After receiving either radiolabeled A6H or AFP-22, mice were sacrificed on day 2 or 3, and the T:B ratios for xenograft(s) and tissues were determined. RIS was performed on 11 of the mice prior to sacrifice (vide infra).

As illustrated in Chart 1, the T:B ratios (i.e., biodistribution indexes) at day 2 or 3 postinjection of radiolabeled A6H ranged...
Enhanced accumulation of A6H in RCC xenografts

The accumulation of radiolabeled A6H within each RCC xenograft was compared to that with other nontarget tissues, blood, and urine and expressed as the selectivity index (cpm/mg):nontarget (cpm/mg). Quantization of radiolabel accretion occurred on day 2 or 3 post-I.v. injection of $^{125}$I- and $^{131}$I-labeled A6H (1-40 $\mu$Ci/dose; specific activities, approximately 10 $\mu$Ci/$\mu$g).

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<td>12</td>
<td>5-21</td>
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<td>5-19</td>
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Influence of Acute Inflammation on Biodistribution of MAB.

Mice with staphylococcal skin abscesses were involved in the final series of biodistribution studies. Two of the 13 specimens exhibited marginally elevated T:B ratios of 1.2 and 1.4. All of the other abscessed skin samples had T:B ratios of <1, although the actual values were higher than those usually found in normal tissues. These data demonstrate that the IgG1 MABs A6H and AFP-22 may accumulate slightly in areas of acute tissue inflammation but the levels rarely exceed the concentration of radiolabel in the blood.

Table 1

Enhanced accumulation of A6H in RCC xenografts

The accumulation of radiolabeled A6H within each RCC xenograft was compared to that with other nontarget tissues, blood, and urine and expressed as the selectivity index (cpm/mg):nontarget (cpm/mg). Quantization of radiolabel accretion occurred on day 2 or 3 post-I.v. injection of $^{125}$I- and $^{131}$I-labeled A6H (1-40 $\mu$Ci/dose; specific activities, approximately 10 $\mu$Ci/$\mu$g).

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CANCER RESEARCH VOL. 45 DECEMBER 1985

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frequently. We have used polyclonal antisera and reported the successful localization of primary tumor masses in 13 of 15 patients, all of which exceeded 4 cm² and were detected by other techniques (29, 30).

The number of laboratories actively engaged in the production of MABs to RCC-associated antigens is disproportionately few when contrasted to the potential clinical applications. Approximately 20,000 new patients/yr present with RCC, of which 60% have known or occult metastatic disease. There is no effective treatment for those patients with metastases, of which one-third are initially misstaged. Also there are no known tumor markers for RCC as there are for many of the other solid tumors making confirmatory diagnosis, staging, and follow-up more difficult. Classical pathological grading of RCC has not been of great benefit so far (31). If RCC-preferential MABs could be used clinically in RIS, it might contribute to the confirmation of suspected tumor masses, detection of occult disease prior to nephrectomy, and monitoring of recurrence during follow-up. Moreover immunopathological stratification of RCC might provide a more accurate method of pathological grading (32).

One of the first groups to utilize hybridoma technology in studies of RCC-associated antigens was Ueda et al. (33). They described several MABs which recognized antigenic determinants common to the RCC cell membrane with various degrees of restriction to normal cells or other tumors. All of these MABs recognized one or more organelle(s) of the normal kidney and their use in immunopathological studies have contributed to a better understanding of renal differentiation and RCC cellular origin (34). Scharf et al. (35) recently generated RCC-reactive MABs that were preferential for low grade tumors and did not react with normal renal structures (35). However, all of the MABs recognized antigenic determinants coexpressed by normal colon. The efforts of this laboratory in generating RCC-reactive MABs have also been described (36, 37) and the MAB A6H was one of these. It was shown to react strongly with RCC tissue sections, renal proximal tubules (classically considered to be the origin of RCC) (38) but no other normal tissues, and with 6 of 12 colon carcinoma sections. In the cell-binding enzyme-linked immunosorbent assay A6H reacted with some breast carcinoma cell lines, although this still needs to be confirmed immunohistochemically using fresh tissue specimens.

Compared to other radioimmunolocalization studies, A6H demonstrates unusually high and specific T:B ratios. Thus the accumulation of radiolabeled A6H within the 4 RCC xenografts ranged from 4 to 60 times that in the blood and from 13 to >200 times that in the liver, while none of the RCC tumors demonstrated nonspecific uptake of the control MAB AFP-22. These findings suggest that the high T:B ratios were not due to trapping of immunoglobulin (18). Furthermore, it is unlikely that accumulation of A6H within the RCC xenografts was due to an inflammatory effect since the amount of A6H in acutely inflamed skin abscesses was usually less than in the circulation, and histological evaluations of several RCC xenografts showed insignificant inflammation in the tumors.

**Table 2**

**Comparison of RCC xenograft weight with T:B ratio**

<table>
<thead>
<tr>
<th>RCC Xenograft</th>
<th>T:B mg</th>
<th>T:B mg</th>
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<th>T:B mg</th>
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</thead>
<tbody>
<tr>
<td>TK-39</td>
<td>60</td>
<td>24</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>TK-82</td>
<td>102</td>
<td>106</td>
<td>11</td>
<td>75</td>
</tr>
<tr>
<td>TK-177C</td>
<td>102</td>
<td>8</td>
<td>145</td>
<td>13</td>
</tr>
<tr>
<td>TK-177G</td>
<td>133</td>
<td>10</td>
<td>13</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>159</td>
<td>7</td>
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</tr>
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</table>

**DISCUSSION**

Numerous studies have been performed to test the hypothesis that antibodies can be used as specific carriers of radioisotopes to localize human cancer. These studies in both clinical and experimental settings have used either polyclonal or monoclonal antibodies, including antibodies against carcinoembryonic antigens, α-fetoprotein, human chorionic gonadotropin, prostatic acid phosphatase, and human tumor cell surface antigens. In xenograft studies utilizing human cancers, the T:B ratios usually have been less than 4 (14–18) and this ratio often has proven to be insufficient for useful RIS detection of tumors weighing less than 100 mg. In an effort to increase the T:B ratios, investigators have utilized a variety of methods. Some have used MAB fragments which are more rapidly cleared from the blood. Using such fragments of an anti-breast carcinoma MAB, Colcher et al. (19) reported T:B ratios up to 14. Also other radioisotopes have been used. For example, with 111In as a radiolabel rather than 131I or 125I Rainsbury and Westwood (20) achieved a T:B ratio of 18 using a MAB directed toward a milk fat globule. Finally tumor-directed MABs with especially good selectivity have been used for teratoma (T:B of 9) (21) and melanoma (T:B of 7) (22). These increased T:B ratios in mice were often accompanied by improved RIS.

Human RIS studies have focused predominantly on patients with colorectal cancer because of the availability of both polyclonal and monoclonal antibodies which recognize carcinoembryonic and other colorectal-associated antigens (23, 24). Also RIS with radiolabeled MABs was attempted in a number of other cancers including testicular, hepatoma, prostatic, melanoma, breast, and ovarian (25–28); however, relatively few patients were entered in these trials. Thus while enthusiasm for the potential of RIS remains high, an important issue in clinical studies is whether this diagnostic technique provides unique clinical information and for this the data are less clear. Certainly some examples do exist of RIS providing unique data but usually the findings have been confirmatory. For example, in the only reported RIS clinical studies involving RCC, the investigators used polyclonal antisera and reported the successful localization of primary tumor masses in 13 of 15 patients, all of which exceeded 4 cm² and were detected by other techniques (29, 30).

The number of laboratories actively engaged in the production of MABs to RCC-associated antigens is disproportionately few when contrasted to the potential clinical applications. Approximately 20,000 new patients/yr present with RCC, of which 60% have known or occult metastatic disease. There is no effective treatment for those patients with metastases, of which one-third are initially misstaged. Also there are no known tumor markers for RCC as there are for many of the other solid tumors making confirmatory diagnosis, staging, and follow-up more difficult. Classical pathological grading of RCC has not been of great benefit so far (31). If RCC-preferential MABs could be used clinically in RIS, it might contribute to the confirmation of suspected tumor masses, detection of occult disease prior to nephrectomy, and monitoring of recurrence during follow-up. Moreover immunopathological stratification of RCC might provide a more accurate method of pathological grading (32).

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In our RIS studies, clear tumor resolution was obtained consistently without using subtraction techniques even for the smallest RCC xenograft in the series, 0.4 cm diameter and 60 mg. Indeed this small xenograft accumulated 8.4% of the injected dose. Also in animals bearing both a RCC and non-RCC xenograft, RIS distinguished the 2 tumors and biodistribution analysis of the xenografts yielded a median tumor specificity index of 33. All these data would suggest that A6H localization is an antigen-mediated event which is highly preferential for RCC xenografts.

In a recent study, Pimm and Baldwin (39) discussed a number of extraneous factors that can influence T:B ratios. These included (a) prolonged periods between injection of the radionabeled MAB and analysis, (b) the use of MABs or MAB fragments with unusually fast clearance rates, and (c) the tendency for large xenografts (>800 mg) to have high T:B ratios. Consequently these authors postulate that T:B ratios may not be the best indicator of MAB localization and they suggested alternative parameters including the percentage of dose incorporated in the xenograft per unit weight and the percentage of whole body radioactivity in the tumor per unit weight.

We believe these extraneous factors effecting T:B ratios discussed by Pimm and Baldwin (39) have been satisfactorily addressed in our studies. For example, our T:B ratios were established on days 2 and 3 after injection which corresponds to the majority of studies in the literature. Also A6H had a blood half-life of 2.5 days, a rate which is similar to that of the authors and others but significantly slower than those MABs whose T:B ratios are hypothesized to be due to rapid blood clearance (39). In addition, the RCC xenografts ranged in size from 60–820 mg, with no discernible correlation between size and the T:B ratio.

Nonetheless our results have been analyzed using these other suggested parameters mentioned by Pimm and Baldwin (39). When the data is converted to percentage injected dose per gram, A6H continues to show exceptionally high levels of selective incorporation with a mean of 34% of injected dose per gram, A6H continues to show exceptionally high levels of selective incorporation with a mean of 34% of injected dose per gram on day 2 and 106% on day 3 in TK-177G. Also with A6H, over 0.1% of the total body radioactivity is present in the RCC per mg of tissue at day 2. In comparison, Pimm and Baldwin using an osteogenic sarcoma-preferential MAB, reported (a) T:B ratios ranging from 0.6–2.9, (b) 8.9% of the injected dose per gram on day 2, and (c) 0.03% of whole body radioactivity per mg in the xenograft. Parenthetically, their values are comparable to those obtained with other tumor preferential antibodies that have shown preferential biodistribution but lack the level of discrimination necessary for the detection of small tumors by RIS (40, 41). Thus A6H appears to show exceptional localization by these parameters as well as by T:B ratios.

One can only speculate why A6H seems to have better biodistribution and RIS properties than other reported tumor-preferential MABs because the factors that influence localization are many and often ill-defined (3, 4). One possibility is that our selection process of the appropriate MAB is especially favorable for RIS because of their vascularity or antigen expression. RCC is often highly vascularized and our xenografts maintain this property as judged by morphological and histological studies. It is also possible that the vascular permeability of the RCC xenografts may be conducive for RIS (44) and we are currently attempting to test this hypothesis. However, it does not seem probable that unusual xenograft vascularity could have concentrated immunoglobulin nonspecifically since AFP-22, the control MAB of the same isotype as A6H, did not accumulate in any of the xenografts. Finally it is possible that our xenografts have an unusually high concentration of antigenic determinants and preliminary studies on TK-177G suggest that this may also be a contributing factor. Yet even if RCC xenografts have properties which make them suitable for selective MAB localization, this suggests that a similar situation may be seen clinically.

Previous reports have taught that success of RIS in a nude mouse xenograft model does not guarantee its usefulness clinically. However, in these investigations, there is sufficient reason for enthusiasm. Additional facts which support this enthusiasm include (a) in preliminary studies of 2 of our other RCC reactive MABs, designated C5H and D5D (5), the T:B ratios have been comparable to those of A6H and provided discriminatory images (45), and (b) in other ongoing studies utilizing A6H, 6 additional RCC xenografts and 2 other non-RCC xenografts, the T:B ratios and scintigraphic images are closely paralleling those presented herein.

In summary, we have shown that a radionabeled MAB very selective for RCC can significantly and specifically accumulate within the tumor and can promote imaging of the xenograft with a high level of resolution. These preliminary efforts enhance the possibility that MABs with highly specific in vivo RCC localization might be useful in improving the diagnosis, staging, and treatment of this cancer.

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Fig. 1. Serial scintigraphy was performed at 5 h and 1 and 2 days following i.v. injection of 20 μCi of 131I-labeled A6H. The mouse bore 2 RCC xenografts, TK-177C and TK-177G. Immediately following the last scan, T:B ratios were determined and these are expressed along with the weight of each xenograft. Background subtraction techniques were not used. L, left; R, right.

Fig. 2. Mice bearing 2 xenografts, one RCC and one non-RCC (Scans A, B, and D) or 2 RCC (Scan C) received 20–40 μCi of either 131I-radiolabeled A6H (Scans A to C) or a similar dose of AFP-22 (specific activities, approximately 10 μCi/μg). Following scintigraphy, the mice were sacrificed and biodistribution of the radiolabel was assessed. The T:B ratio for each of the xenografts is indicated in the scans along with their weight. Background subtraction techniques were not used. CA, carcinoma; L, left; R, right.
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