Initial Studies of Monoclonal Antibody PAM4 Targeting to Xenografted Orthotopic Pancreatic Cancer

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Abstract
To resemble the clinical presentation of pancreatic cancer in an animal model more closely, we developed an orthotopic xenograft of CaPan-1 human pancreatic cancer in athymic nude mice. Within 3 weeks after implantation into the body and head of the pancreas, animals had palpable tumors. By 8 weeks, metastases to the liver and spleen were observed, and at 10–14 weeks, ascites formation, with and without seeding of the diaphragm, and jaundice were evident. Thus, this tumor model exhibited many of the common features of human pancreatic cancer. Radiolabeled monoclonal antibody PAM4 showed specific localization of the primary orthotopic and metastatic tumors. On day 3, PAM4 accumulation within the primary tumor (0.5 g) was 11.3 ± 5.1% injected dose/g with a localization index of 11.3 ± 4.0. The estimated tumor:blood radiation dose ratio for PAM4 was 4:1, whereas a nonspecific antibody (Ag8) would provide only 40% of the blood dose to the tumor. Based on these observations, animals bearing 4-week-old orthotopic tumors (estimated volume, 0.25 cm3) were administered either 131I-labeled PAM4, 350 μCi, or nonspecific Ag8, 350 μCi, and compared with an untreated control group. Radiolabeled PAM4 provided a significant (P < 0.01) increase in survival time with less morbidity compared with the untreated control group, whereas nonspecific Ag8 was not significantly different from the control group. These studies provide a rationale for initiating a Phase I clinical study for detection and therapy of pancreatic cancer with PAM4.

Introduction
The development of new cancer therapeutics has, for the most part, relied on the use of human cancers carried as s.c. xenografts in athymic nude mice as a means of determining uptake and antitumor activity. However, the s.c. site for tumor growth lacks many of the hallmarks found in a clinical setting, including metastatic behavior and the development of signs and symptoms that may arise as a consequence of tumor growth within visceral organs. Recently, a number of laboratories have established that human cancers, when xenografted to the orthotopic site within athymic nude mice, provide tumor models that exhibit metastatic growth. Orthotopic tumor models have been reported for colorectal (1, 2), lung (3, 4), gastric (5), bladder (6), and other cancers. With respect to human pancreatic cancer, tumor cell suspensions (7, 8), as well as pieces (9–11), have been xenografted into the pancreases of immunosuppressed mice. In both situations, metastases, as well as ascites formation and jaundice, were shown to occur.

The objective of the current study was to use the orthotopic pancreatic tumor model for analysis of the biodistribution and therapeutic efficacy of a radiolabeled MAb, PAM4. This antibody was generated against a pancreatic cancer-derived mucin (12). By immunohistology, it was shown to react with pancreatic cancer specimens (85%) but not with normal pancreas. Biodistribution studies performed with s.c. xenografts derived from four different tumor cell lines covering the range of expected differentiation showed excellent uptake within tumor (range, 22%–48% ID/g), with no evidence of targeting to non-tumor tissues (13). We report now the feasibility of using radiolabeled PAM4 in a xenografted pancreatic orthotopic model to target in pancreatic and metastatic tumor, as well as to decrease the morbidity and mortality due to progressive tumor growth.

Materials and Methods
Tumor Transplantation. CaPan-1, a moderately well-differentiated human pancreatic cancer, was obtained from the American Type Culture Collection (Rockville, MD) and established in 5–6-week-old female nude/nude mice by s.c. injection of 105 cells. After tumors reached about 1 cm3 in size, they were minced in 0.9% sterile saline and passed through a 40-mesh screen to produce a 20% (w/v) suspension. Tumors were passaged serially in athymic nude mice as s.c. injections of 0.2 ml tumor suspension. For orthotopic growth of CaPan-1, a clean, single-cell suspension (20%) of a serially passaged s.c. tumor was prepared by mechanical disruption through a screen sieve and aspiration and expulsion through a 10-ml syringe fitted with sequentially smaller needles (to 26 g). Nude mice were anesthetized with sodium pentobarbital (70 mg/kg). Following anesthesia, a left lateral abdominal incision (1 cm) was made, and the spleen and attached pancreas were exteriorized with traction using forceps. Tumor cells, 50 μl 20% suspension, were injected into the pancreas. The pancreas and spleen were returned to the abdominal cavity, and the incision was closed in two layers using a 4–0 silk suture on the peritoneum and approximation of the skin using either 9-mm autoclips or 5–0 chromic gut sutures.

MAB Purification and Radiolabeling. MAbs PAM4 and nonspecific, isotype-matched P3X63Ag8 were purified from ascites by protein-A chromatography (14). Characterization of PAM4 has been reported (12). Radiolabeling of the antibodies with 125I or 131I was performed by the chloramine-T method (15). The specific activities of the radiolabeled antibodies were in the range of 7–12 μCi/μg. Routine quality assurance included size-exclusion high performance liquid chromatography (GF-250; DuPont, Wilmington, DE) to detect aggregates and unbound iodine, as well as TLC to detect unbound iodine (16). All radiolabeled antibody preparations were free of aggregates and contained less than 3% unbound iodine. Using antigen-specific affinity chromatography (13), radiolabeled PAM4 was found to be between 70 and 80% immunoreactive, whereas radiolabeled Ag8 showed less than 5% binding.

Biodistribution Studies. An initial growth curve of orthotopic tumor established that, at 5 weeks after tumor implantation, the tumor volume in the pancreas, as determined by caliper measurements in three dimensions and calculated by length × width × depth, was approximately 0.5 cm3. Thus, at 5 weeks after transplantation of CaPan-1, groups of animals (n = 5–8) were coadministered 25 μCi 131I-labeled PAM4 and 10 μCi 125I-labeled nonspecific Ag8. Animals were sacrificed on the days indicated, their tissues were removed, and the pancreas and attached pancreas were exteriorized with traction using forceps. Tumor cells, 50 μl 20% suspension, were injected into the pancreas. The pancreas and spleen were returned to the abdominal cavity, and the incision was closed in two layers using a 4–0 silk suture on the peritoneum and approximation of the skin using either 9-mm autoclips or 5–0 chromic gut sutures.

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2 To whom requests for reprints should be addressed, at The Garden State Cancer Center and The Center for Molecular Medicine and Immunology, 1 Bruce Street, Newark, NJ 07103.
3 The abbreviations used are: MAb, monoclonal antibody; ID, injected dose; MTD, maximum tolerated dose.
MAb-PAM4 TARGETING TO ORTHOTOPIC PANCREATIC CANCER

Histological observation. The tumor grew within the capsule of the pancreas but was not itself surrounded by a capsule (Fig. 2). An admixture of normal and tumor cells was evident at the periphery of the tumor. At 8 weeks, the normal pancreas was, in most instances, obliterated completely, and gross tumor metastases were observed within the spleens and livers of half of the animals. Metastatic tumors within the liver (range, one to three tumors/animal) were estimated to weigh 50–100 mg, whereas tumors within the spleen (range, one to three/animal) were smaller. At 10–14 weeks, most of the animals exhibited jaundice and ascites. The metastatic behavior of this tumor model seemed to be limited to the spleen and liver, with seeding of the diaphragm at the later stages. Gross and histological examination of the stomach, lungs, kidneys, and peritoneal lymph nodes did not reveal metastases to these sites.

Orthotopically grown pancreatic tumors grew quite large, in the range of a 10–20-fold increase over the normal weight of the pancreas at later stages of growth, without overt signs or symptoms of an unhealthy condition, as evidenced by animal body weight and gross motor control. Ascites formation was usually the first overt symptom of internal tumor growth. It should be noted also that by week 4, in addition to the orthotopic tumor, approximately half of the animals developed s.c. tumors at the surgical incision site, most probably as a consequence of leakage from the injection site. These tumors were usually equivalent in size to the orthotopic tumor. No correlation existed between animals bearing tumors at the incision site and animals having metastatic disease.

**Results**

**Growth Characteristics of an Orthotopic Human Pancreatic Cancer Xenograft.** Orthotopic tumors first became palpable at 3 weeks after implantation. Development of intrapancreatic tumors, confirmed by gross and histological means, was observed in four of five animals at this time and in 100% of the animals at week 5. Growth was progressive, as determined by organ weight (Fig. 1) and histological observation. The tumor grew within the capsule of the pancreas but was not itself surrounded by a capsule (Fig. 2). An admixture of normal and tumor cells was evident at the periphery of the tumor. At 8 weeks, the normal pancreas was, in most instances, obliterated completely, and gross tumor metastases were observed within the spleens and livers of half of the animals. Metastatic tumors within the liver (range, one to three tumors/animal) were estimated to weigh 50–100 mg, whereas tumors within the spleen (range, one to three/animal) were smaller. At 10–14 weeks, most of the animals exhibited jaundice and ascites. The metastatic behavior of this tumor model seemed to be limited to the spleen and liver, with seeding of the diaphragm at the later stages. Gross and histological examination of the stomach, lungs, kidneys, and peritoneal lymph nodes did not reveal metastases to these sites.

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**Biodistribution of Radiolabeled PAM4 Antibody.** Tissue uptake of the radiolabeled antibody was determined on days 1, 3, 7, and 14 after injection. Accumulation of PAM4 within the orthotopic tumor was significantly higher than the accumulation of nonspecific Ag8 on both day 1 and day 3 (P < 0.001 and 0.01 by paired t test, respectively). However, no significant difference was observed at the later time points (Fig. 3). The blood level of 131I-PAM4 was significantly lower than the blood level of Ag8 at all time points examined (P < 0.01 at day 1; P < 0.001 at days 3, 7, and 14 by paired t test). The decrease of PAM4 from the blood (T1/2, 94 h) was five times faster than that for Ag8. Other nontumor tissues showed a similar rapid decrease of PAM4 over the 14-day period (T1/2, 82–97 h) in contrast to Ag8 (T1/2, 330–463 h). Thus, despite a continuing decrease in the percentage of PAM4 within the tumor, localization indices as shown

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**Fig. 1.** Orthotopic tumors, along with remaining normal pancreatic tissue, were excised and weighed at the time points indicated from groups of three to five animals.

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**Fig. 2.** Orthotopic CaPan-1 tumor xenograft, 5 weeks after implantation. An admixture of normal mouse pancreatic cells and CaPan-1 tumor cells is shown within the capsule of the pancreas. N, normal mouse acinar cells; T, tumor (hematoxylin and eosin stain; ×150).
in Table 1 demonstrated clearly the specific tumor targeting of PAM4. To demonstrate the tumor specificity of PAM4 further, a separate group of animals bearing 5-week-old orthotopic tumors was administered 50 μCi 131I-labeled PAM4. The animals were sacrificed on day 3, and microautoradiography was performed on 5-μm sections through the tumor. As shown in Fig. 4, a heterogeneous targeting of PAM4 was observed within the tumors. However, no targeting was observed within the surrounding normal pancreatic tissue. Based on the biodistribution data, the radiation doses to the tissues were estimated for PAM4 and Ag8 (Table 2). The dose that PAM4 would deliver to the tumor was about 4-fold higher than the dose to the blood, whereas Ag8 would deliver to the tumor only 40% of that delivered to the blood. The radiation dose given by PAM4 to each nontumor organ was lower than the dose delivered to the blood.

One animal within the day 3 group had a liver metastasis (78 mg) and a spleen metastasis (23 mg) that were each targeted by PAM4 with 32.3 and 23.9%ID/g, respectively, and localization indices of 93.1 and 130, respectively. Of further note, as indicated in Fig. 3, some of the animals had tumors growing as s.c. nodules at the incision sites (average tumor size at day 0, 0.2 cm³). Although the data are presented, they should be viewed with caution, because the number of animals bearing s.c. tumors was small (Table 1). Specific targeting at these tumor sites, as shown by the localization indices, was similar to that in the orthotopic tumors. Although it would seem that there was a decrease in the localization index at day 14 for the s.c. tumors, this value was not statistically different from the value calculated for the orthotopic tumors. Further studies with a larger number of s.c. tumors will be necessary to determine this point.

Radioimmunotherapy of Orthotopic Pancreatic Cancer. To determine whether radiolabeled PAM4 was able to control the growth of orthotopic transplants of CaPan-1 tumors, groups of 10 mice bearing 4-week-old orthotopic tumors (estimated weight, 0.25 g) were administered single doses of either 350 μCi 131I-labeled PAM4 or 350 μCi 131I-labeled Ag8. As shown in Fig. 5, 60% of the untreated animals died by week 10 after tumor implantation, and all of the animals were dead by week 15. In the group administered 350 μCi 131I-labeled, nonspecific Ag8, 60% were dead by week 7, and 100% were dead by week 14. When the Ag8-treated group was compared with the untreated control group by the log rank test, no significant survival advantage was observed. In fact, the data suggest that at early time points some deaths may have occurred due to radiation toxicity. In contrast, the group that was administered radiolabeled PAM4 showed significantly extended survival time with 70% survival at 16 weeks, the end of the study. The survival advantage of the PAM4-treated group over the untreated group was significant at the P = 0.001 level by the log rank test. At 16 weeks, the surviving animals were sacrificed to determine tumor size. Of seven surviving animals, two had tumors that were less than the estimated weight at the start of therapy, and the other five had tumors with a weight range of 1.0–2.8 g, with one or two small (<0.1 g) metastases evident in four of the seven animals.

Discussion

Previous studies from our laboratory demonstrated that PAM4, a MAb generated against pancreatic cancer-derived mucin (12), was able to target specifically to pancreatic tumors carried as s.c. xenografts in athymic nude mice (13). In addition, a single dose of 350 μCi 131I-radiolabeled PAM4 (the MTD), when administered to animals bearing large CaPan-1 tumors (1 g), achieved significant inhibition of tumor growth and extended survival time. 4 Recently, we, as well as others (1–11), have begun to consider whether growth of a tumor within the s.c. site provides an appropriate model for these

<table>
<thead>
<tr>
<th>Table 1 Biodistribution of MAb PAM4 in orthotopic and s.c. tumors</th>
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<tbody>
<tr>
<td><strong>Localization index</strong></td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Day 1</td>
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<tr>
<td>Day 3</td>
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<td>Day 14</td>
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<tr>
<td><strong>Tumor weight (g)</strong></td>
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<td>Day 1</td>
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<td>Day 3</td>
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<tr>
<td>Day 7</td>
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<tr>
<td>Day 14</td>
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</tbody>
</table>

Numbers in parentheses are the numbers of animals that have orthotopic and/or s.c. tumors.

4 D. V. Gold and R. Blumenthal, unpublished data.
therapy studies. Tumors grown within the s.c. site usually do not exhibit the characteristic patterns of tumor growth found in the patient (18, 19). Metastases of human pancreatic cancer xenografts from the s.c. site are rare. We have not observed spontaneous metastatic behavior from the s.c. site, nor did invasion of tissues by local extension occur in any of the pancreatic tumor lines used in our laboratory (CaPan-1, PANCl, AsPc-1, Hs766T, BxPc3, and RIP1). However, Kyriazis et al. (20), and Kajii et al. (21) each have reported metastatic growth of human xenografted pancreatic cancer lines, the former using the RIP1 cell line. The development of signs and symptoms associated with pancreatic tumor growth in its later stages, including jaundice and ascites, also have not been observed in the s.c. tumor model. In addition to these observations, the location of tumor growth within vital organs may influence the tumor:host toxicity ratio of therapeutic materials, particularly those that have an associated innocent bystander effect, such as radiolabeled materials, or those that may have a greater toxicity on faster growing metastases rather than the primary tumor. In this respect, Furukawa et al. (11) demonstrated that mitomycin C, although it had little effect on the growth of s.c. PANC4 tumors or primary orthotopic tumors, was effective in preventing metastatic growth from the primary tumor. For these reasons, we have examined the biodistribution and therapeutic efficacy of radiolabeled PAM4 in an orthotopic tumor model.

Our results indicated that when CaPan-1 tumors are xenografted within the pancreases of immunosuppressed mice, the tumors exhibit features more characteristic of the clinical setting. Tumors grew quite

### Table 2. Dosimetry of \(^{131}I\)-labeled PAM4 and Ag8

<table>
<thead>
<tr>
<th>Tumor</th>
<th>PAM4 (cGy)</th>
<th>Ag8 (cGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthotopic</td>
<td>2346(^a)</td>
<td>1007</td>
</tr>
<tr>
<td>s.c.</td>
<td>2615</td>
<td>1067</td>
</tr>
<tr>
<td>Liver</td>
<td>319</td>
<td>799</td>
</tr>
<tr>
<td>Spleen</td>
<td>495</td>
<td>901</td>
</tr>
<tr>
<td>Kidney</td>
<td>156</td>
<td>603</td>
</tr>
<tr>
<td>Lungs</td>
<td>254</td>
<td>1,017</td>
</tr>
<tr>
<td>Blood</td>
<td>579</td>
<td>2,349</td>
</tr>
</tbody>
</table>

\(^a\)The estimated dose is for a single administration of the MTD, 350 \(\mu\)Ci \(^{131}I\)-labeled PAM4 or \(^{131}I\)-Ag8.

Fig. 4. Microautoradiography of orthotopic CaPan-1 tumors (5 weeks old) after administration of 50 \(\mu\)Ci \(^{131}I\)-labeled PAM4. At day 3 after injection, specific uptake of radiolabeled antibody was observed within the tumors, whereas no radiolabel was noted in the surrounding normal pancreatic tissue. A, high concentrations of antibody targeting appear in the central area of the tumor. No necrotic regions were evident (X15). B, PAM4 targeting was directed in a mostly diffuse manner within the tumor. A region of necrosis (n), devoid of radiolabeled antibody, was observed (counterstained with hematoxylin and eosin; X25).
The PAM4-treated group exhibited a significantly increased survival compared with either bulky, with metastases to the liver and spleen developing at week 8, with the early onset of s.c. tumors found at the incision site. Many of the most common findings associated with human pancreatic cancer, including the surrounding normal pancreas. The results of PAM4 targeting were similar to those of Marincola et al. (8), wherein pancreatic tumor cell suspensions grew in 70% of the nude mice, with 65% growth in the liver. However, in this study, to promote liver growth, the cell suspension was injected into the portal vein. Although in our system, we cannot rule out completely inadvertent injection into the circulation, the observed liver and spleen metastases occurred typically at a later time point, compared, for example, with the early onset of s.c. tumors found at the incision site. In addition, metastatic seeding of the diaphragm was observed only at very late stages of tumor growth.

A high concentration of PAM4 was achieved within the orthotopic as well as metastatic tumors. Targeting was specific to the tumor, without significant localization of the antibody in nontumor tissue, including the surrounding normal pancreas. The results of PAM4 targeting seemed to be similar in both orthotopic and s.c. tumors at least through day 7, with the results at day 14 unclear due to the small number of animals bearing s.c. tumors. Over the 14-day study period, the potential radiation dose to the orthotopic tumor, normalized to the residence time within the blood. Hence, it may prove possible to administer multiple therapeutic doses of radiolabeled PAM4 for greater efficacy.

A number of recent clinical studies support the notion of providing radiation as an adjunctive therapy to primary surgical tumor debulking. Intraoperative radiation therapy, approximately 20 Gy total, either alone (22) or with external beam radiation therapy (23), with curative tumor resection resulted in significantly longer survival times compared with those after curative resection alone. Patients treated with curative resection alone, who did not survive, had greater numbers of hepatic, lymph node, and peritoneal metastases at autopsy than did nonsurvivors treated with curative resection and radiation. Coia et al. (24) evaluated preoperative external beam radiation of 1.8 Gy/day, to a total of 30.4 Gy, in conjunction with chemotherapy (5-fluorouracil and mitomycin-C). Prior to treatment, 32% of patients were judged to have resectable disease, whereas after treatment, 55% were considered resectable. The investigators concluded that the chemoradiation treatment enhanced resectability. Survival for those undergoing resection was 43%, high compared with other reports of patients undergoing curative resection alone. These investigations suggest that further experimental studies of 131I-PAM4, including therapy with repeated doses and in combination with chemotherapy, are warranted.

References


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