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Organ-specific pancreatic tumor growth properties and tumor immunity

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Abstract We established a model of orthotopic injection of a syngeneic pancreatic tumor cell line in C57BL/6 mice and evaluated the effects of organ site on induction of immunity to a tumor-specific antigen, MUC1. Mice were challenged with a syngeneic pancreatic adenocarcinoma cell line that expressed MUC1 (Panc02-MUC1) by orthotopic injection into the pancreas, or by subcutaneous injection. Tumor cells injected into the pancreas grew much faster than those injected subcutaneously. Mice challenged subcutaneously with Panc02-MUC1 rejected tumors or developed slowly growing tumors that were negative for MUC1 expression. In contrast, mice challenged orthotopically into the pancreas developed progressive tumors that were positive for MUC1 expression. Sera from mice that rejected Panc02-MUC1 (tumor-immune mice) showed no detectable IgG1 and IgM titers against the MUC1 tandem-repeat peptide, whereas mice with progressive tumor growth had significant titers of IgG1 and IgM specific for MUC1. This suggests that the humoral immune response was ineffective in mediating tumor rejection. The results show that the growth properties and immunological rejection of pancreatic tumors is affected by the organ site at which the tumor grows.

Key words Pancreatic tumor · Orthotopic injection · MUC1 · Tumor immunity · Syngeneic

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Introduction

The prognosis for patients with pancreatic cancer is poor. Virtually all human pancreatic adenocarcinomas are resistant to radiotherapy or currently available chemotherapy protocols. Although there has been some progress in early diagnosis of this disease by ultrasonic and radiographic techniques [7], surgical therapy for patients with all but the earliest stages of disease is palliative at best and often not indicated because of disseminated disease. Most patients die of complications from the primary tumor and metastatic lesions. One major problem in studying and developing therapies for pancreatic malignancies is that most patients are diagnosed with a large tumor burden at late stages of the disease. There is no effective therapy for pancreatic adenocarcinoma in early or late stages of the disease. Thus, there is interest in attempting novel therapies for treatment of pancreatic adenocarcinoma, and we are investigating the potential of immunotherapy for treating this tumor.

One candidate antigen for immunotherapy of pancreatic tumors is MUC1, a mucin-like glycoprotein that is secreted by and associated with the cell surface of pancreatic ductal epithelia. Human pancreatic tumors overexpress MUC1 and attach to it blood-group carbohydrate structures that are distinct from the structures attached by normal ductal cells [5]. MUC1 is being investigated by several laboratories for use in antibody-directed therapy and tumor vaccines.

All diagnostic and therapeutic strategies that involve pancreatic adenocarcinoma are complicated by the anatomical location of the primary tumor (pancreas) and metastases (usually liver, peritoneal lymph nodes, and other internal sites). Moreover, the ability of immunotherapeutic strategies to detect and destroy tumors at different organ sites is frequently not addressed. One method of evaluating organ-specific parameters of tumor growth and metastasis in animal models is the use of orthotopic injection into different organ sites.

Orthotopic tumor models have been reported for colorectal, lung, gastric, bladder and other cancers [8]. In the case of pancreatic cancer, tumor masses [2, 10, 11, 20, 24] and tumor cell suspensions [1, 19] have been implanted or injected orthotopically into the pancreases of nude mice. However, nude mice are not suitable for evaluating immune responses against tumors. In addition, these mice are also susceptible to viral and bacterial infections that may occur following surgical procedures.

The first goal of the studies described here was to develop an orthotopic murine model of pancreatic cancer in immunocompetent mice, mice that develop pancreatic tumors in the pancreas. Tumor growth properties were characterized at pancreatic and subcutaneous sites. The second goal was to investigate the development of immunity against MUC1 when mice were challenged with tumors subcutaneously and in the pancreas.

Materials and methods

Mice

C57BL/6 female mice 8–10 weeks old were purchased from Jackson Laboratories. The "Principles of laboratory animal care" published by NIH were followed in this study.

Tumor cell culture and transfection

The C57BL/6 syngeneic pancreatic tumor cell line, Panc02 [6], was obtained from Dr. J. Nelson, University of Texas, M. D. Anderson Cancer Center. This cell line was maintained in McCoy's 5A medium with 10% fetal bovine serum (FBS) and no antibiotics. Panc02 was transfected with a human MUC1 cDNA in the expression vector pHβAPr-1-neo [5] and cloned lines constitutively expressing MUC1 were selected and used in the studies reported here. Cloned cell lines expressing neomycin resistance were derived from transfections of the vector with no insert and used as negative controls. Transfections were performed with the Lipofectin reagent (Gibco, Gaithersburg, Md.) according to the manufacturer's specifications. After transfection, neomycin-resistant clones were selected with cloning cylinders. MUC1 expression was confirmed by Western blot analysis and immunocytochemistry using HMFG-2, a murine monoclonal antibody that recognizes human MUC1 tandem-repeat peptide.

Subcutaneous injection of tumor cell suspension

Tumor cells were harvested from in vitro culture by trypsinization and centrifugation in serum-containing media. Tumor cells were resuspended in McCoy's 5A medium with no additives. Subcutaneous injection was performed at a site on the back between the scapulae; 50 µl tumor suspension at a concentration of 2×10^6 cells/ml was injected to administer 1×10^5 cells.

The experimental endpoint (indicated as "death" in the survival curves) was defined as the time at which mice developed a tumor of 1 cm^3, at which time the animals were euthanized and examined. Statistical differences between survival for all groups of animals were calculated using the log-rank test.

Orthotopic injection of tumor cell suspension

Tumor cells were harvested as described above and resuspended in McCoy 5A media with no additives. Mice were anesthetized by intraperitoneal injection of a mixture containing ketamine and xylazine at an appropriate dose for the body weight of the mouse. Mice were set to a prone position and their abdomens were disinfected with 70% ethanol. An upper medial incision was carried out and the gastric lobe of the pancreas was exposed by traction with forceps. A 30-µl sample of tumor cell suspension was drawn into a 1-ml tuberculin syringe with a 27G needle and injected into the pancreas by penetrating the capsule of the pancreas. The pancreas was then returned to the correct position and the abdomen was closed in two layers using Chromic Catgut 5–0.

The experimental endpoint (indicated as "death" in the survival curves) was defined as the time at which mice developed a distended abdomen due to ascites or exhibited moribund behavior, at which time the animals were euthanized and examined. Tumor growth of 1 cm^3 or greater in these animals was confirmed by gross and microscopic post-mortem examination of the animals. Statistical differences between survival for all groups of animals were calculated using the log-rank test.

Immunohistochemistry

Tumor tissues from either subcutaneous or orthotopic sites were harvested from mice challenged with Panc02-MUC1 cells either subcutaneously or orthotopically, fixed in 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin and sectioned. The following reactions were performed at room temperature. Paraffin was removed by incubation in xylene for 1 min; xylene was subsequently removed by incubation in 100% ethanol for 10 s and 80% ethanol for 1 min. Endogenous peroxidase activity was blocked by incubating the sections with 0.3% H_2O_2 in 100% methanol for 30 min. After washing with PBS, sections were incubated with 10% normal goat serum for 30 min and then incubated with monoclonal antibody (mAb) HMFG-2 for 1 h. They were then washed three times with PBS and incubated with biotinylated goat anti- (mouse IgG) antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) for 1 h. Following three washes for 5 min with PBS, they were incubated with streptavidin-peroxidase solution for 45 min. After three washes in PBS, 0.5 mg/ml diaminobenzidine in PBS containing 0.03% hydrogen peroxide was added to develop color. After approximately 5 min the reaction was quenched by dilution into water. The tissue sections were counterstained lightly with hematoxylin.

Western blotting

Protein extracts were obtained by solubilizing the cells in Tri-Reagent according to the manufacturer's specifications (Sigma, St. Louis, Mo.). Extracts were separated by electrophoreses on 7.5% sodium dodecyl sulfate/polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, Mass.) electrophoretically. The membranes were prehybridized in blotto (5% dry milk in TRIS-buffered saline) overnight at 4 °C. HMFG-2 diluted in blotto was applied to the membrane for 1 h at room temperature. Following three washes (10 min each) with blotto, the membrane was incubated with horseradish-peroxidase-conjugated goat anti-(mouse IgG) antibody (Southern Biotechnology Associates, Birmingham, Ala.) at a 1:2000 dilution in blotto for 1 h. Enhanced chemiluminescence (ECL) reagents were applied following three washes with TRIS-buffered saline and the membrane was exposed to ECL-sensitive film for an appropriate time.

ELISA

Detection of MUC1-specific antibodies in the sera of mice was carried out by enzyme-linked immunosorbent assay (ELISA). A 40-residue peptide corresponding to two copies of the MUC1 tandem repeats (GVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAH) was synthesized and used for these purposes. Ninety-six-well microtiter plates were coated with 50 µl/well 10 µg/ml peptide and incubated at 37 °C for 2 h, before a blocking step with 200 µl/well blotto overnight at 4 °C. A 50-µl sample of diluted

serum (1:100 and 1:500) was added to each well in duplicate in the 96-well plates and incubated at 37 °C for 1 h. Following four washes with 200 µl 0.05% Tween-20 in PBS (washing buffer) per well, the plates were incubated with isotype-specific rabbit anti-(mouse immunoglobulin) antibodies (Zymed, San Francisco, Calif.) at a 1:250 dilution in PBS at 37 °C for 1 h. The plates were washed four times with 200 µl/well wash buffer and incubated with alkaline-phosphatase-conjugated goat anti-(rabbit immunoglobulin) antibody (Zymed, San Francisco, Calif.) at a 1:1000 dilution in PBS for 1 h at 37 °C. Following four washes with 200 µl/well wash buffer, to each well was added 50 µl 0.1% phosphatase substrate (Sigma, St. Louis, Mo.) in 10% diethanolamine pH 9.8, and the yellow color was developed for 5–10 min. This reaction was quenched while standards were still in the linear range by adding 50 µl/well 0.5 M NaOH. Color intensity was quantified as A_{405}.

Myeloma protein standards were used to generate isotype-specific antibody-binding curves. Ninety-six-well microtiter plates were coated with 50 µl/well purified IgG1, IgG2a, IgG2b, IgG3 or IgM murine myeloma proteins (Litton Bionetics, Kensington, Md.) at 11 serial two fold dilutions from 2 µg/ml to 1 ng/ml. The ELISA was carried out as described above, except that 50 µl wash buffer was used instead of diluted sera. Only the linear portions of standard curves were used to determine the concentration of peptide-specific antibody in the diluted sera.

Results

Transfection of the Panc02 murine pancreatic cancer cell line

Several clones that showed stable expression of MUC1 were derived following transfection and selection as described in Materials and methods. The Western blot in Fig. 1 shows MUC1 expression in three clones of Panc02 cells transfected with MUC1 (Panc02-MUC1), and lack of expression in one clone of Panc02 cells transfected with pHβApr-1-neo vector (Panc02-neo). Clone AA3 was used in the following experiments and is hereafter referred to as Panc02-MUC1.

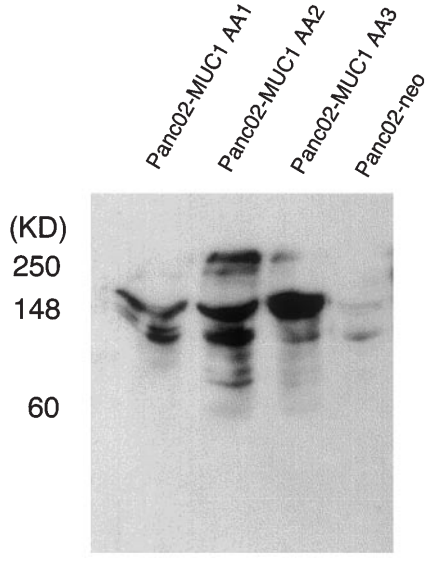

Fig. 1 Western blot analysis of three different clones of Panc02 cells transfected with MUC1. Panc02-MUC1 AA1, AA2 and AA3 represent separate clones of MUC1-transfected cells. Panc02-neo cells were control clonal cell lines of Panc02 transfected with the pHβApr-1-neo vector alone. Approximate molecular mass is shown to the *left*

Orthotopic tumor cell injection into the pancreas

Orthotopic injection of tumor cells into the pancreas was performed as described in Materials and methods. Anesthesia was deep enough to be effective for as long as 3 h. The incision, injection, and closure took less than 7 min/mouse. The maximum volume that could be injected without producing leakage from the injection site was 30 µl liquid, with no air. It was important to resuspend each tumor cell inoculum into the appropriate volume immediately prior to loading this into the syringe to achieve reproducibility in the injection technique. Mice recovered from anesthesia in good condition and exhibited normal behavior. No acute complications were observed.

Most mice developed ascites as the first clinical evidence of progressive tumor growth, and were sacrificed within 5 days of developing a distended abdomen. Necropsy showed moderate to large amounts of ascites (1–7 ml), a bleeding tumor 10–15 mm in diameter (equivalent to a 10- to 15-cm tumor in the pancreas of humans) and, in some cases, disseminated tumors in the abdominal cavity with sizes ranging from 1 mm to 10 mm.

Microscopically, tumors harvested from the orthotopic site were undifferentiated carcinoma with no well-formed ductal structures. Tumor growth at the primary site was usually accompanied by large amounts of angiogenesis and intratumor bleeding. Tumors harvested from subcutaneous sites were also relatively undifferentiated, and similar to tumors from the pancreatic site in morphology and degree of angiogenesis induced, but were not accompanied by bleeding.

Effect of injected cell number on tumor growth at the pancreatic site

Figure 2a, b shows survival curves of mice challenged in the pancreas with 1×10^6, 1×10^5 and 1×10^4 cells of Panc02-neo or Panc02-MUC1 respectively. Increasing the quantity of injected tumor cells resulted in a shortened time to onset of tumor growth and a decreased survival time. For all doses of cells used for tumor challenge, Panc02-neo grew progressively in all animals (100%). In contrast, a fraction (10%–50%, depending on dose) of the animals challenged with Panc02-MUC1 rejected the tumors.

Effect of injected cell number on tumor growth at the subcutaneous site

Figure 3a, b shows survival curves of mice injected subcutaneously with doses ranging from 1×10^4 to

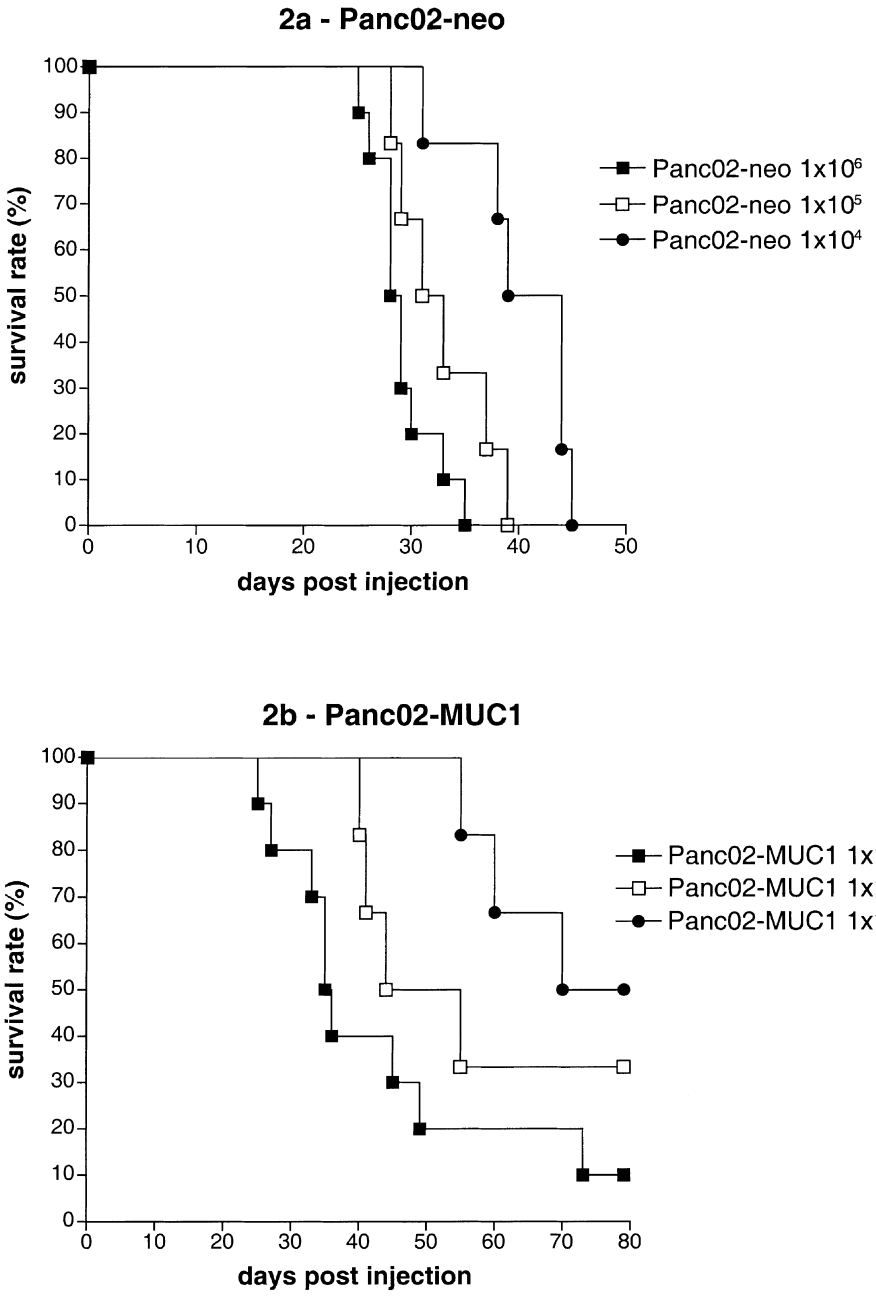

Fig. 2a, b Survival curves for mice challenged orthotopically with Panc02-neo (**a**) or Panc02-MUC1 (**b**). Mice were injected with Panc02-neo or Panc02-MUC1 at the indicated dose on day 0. Mice were killed when a distended abdomen became apparent

3×10^5 Panc02-neo cells, and from 1×10^6 to 3×10^7 Panc02-MUC1 cells respectively. Increasing the quantity of injected tumor cells resulted in a shortened time to onset of tumor growth and a decreased survival time. Panc02-neo grew progressively in most of the animals even at the relatively low doses of tumor cells used here (10^4–10^5). In contrast, a considerable proportion (up to 80%) of animals challenged with higher doses of Panc02-MUC1 (10^6–10^7) rejected the tumors (Fig. 3b). The percentage of the mice that did not develop tumors increased as the dose of injected tumor cells was decreased. No tumor growth was observed following injection of 3×10^5 or fewer cells of Panc02-MUC1 (data not shown).

Differences in growth properties of Panc02-MUC1 and Panc02-neo injected into the pancreas or at the subcutaneous site

Figure 4a, b shows survival curves of mice challenged with 1×10^6 cells of Panc02-neo or Panc02-MUC1 in the pancreas (Fig. 4a) or subcutaneously (Fig. 4b). As shown in Fig. 4a, challenge with Panc02-neo cells in the pancreas resulted in progressive tumor growth in 100% of the mice by day 35. Eighty percent of mice challenged with Panc02-MUC1 cells showed progressive tumor growth by day 50, which was significantly better than the group injected with Panc02-neo ($P = 0.005$). In the subcutaneous tumor challenge model (Fig. 4b), the

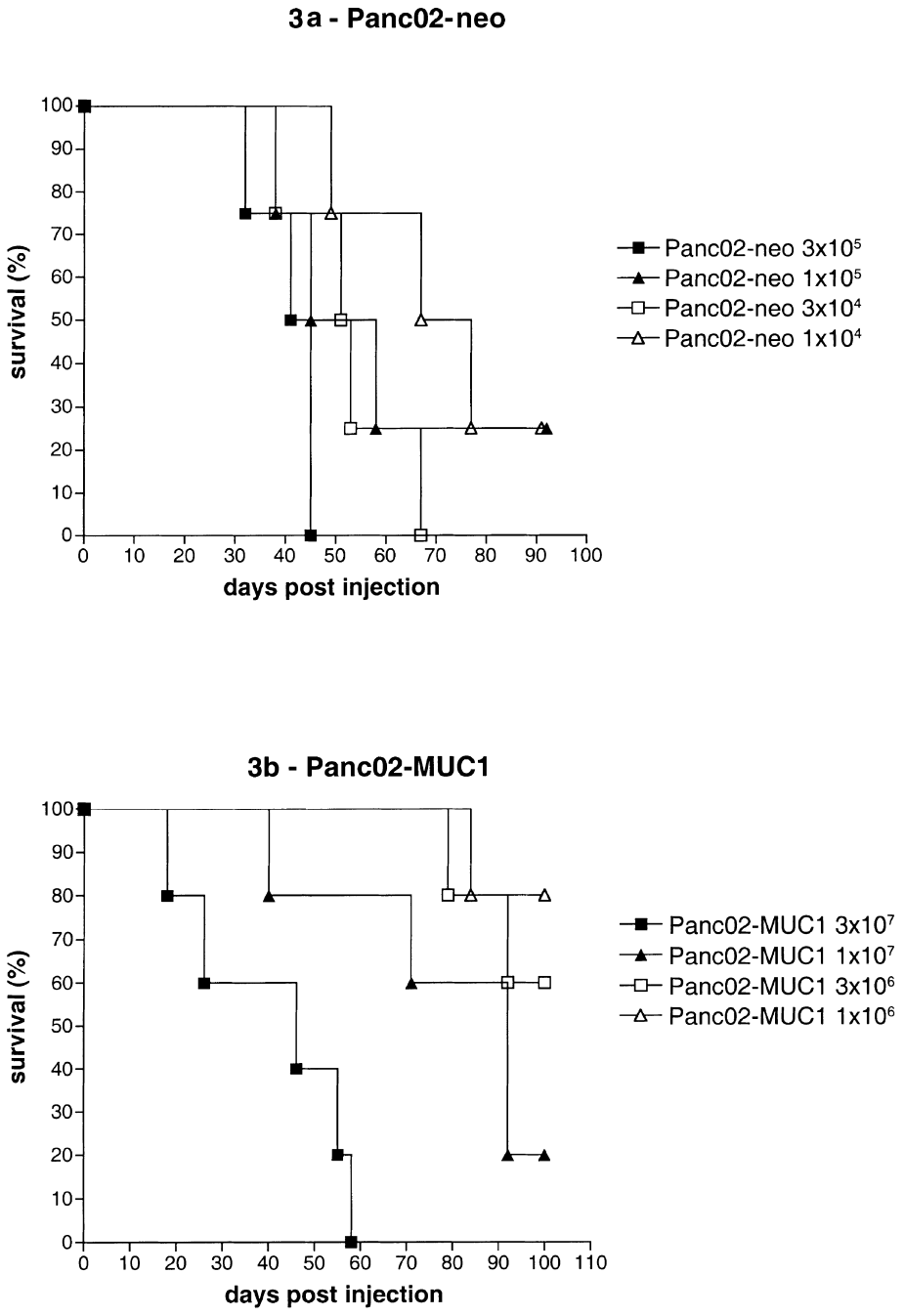

Fig. 3a, b Survival curves for mice challenged subcutaneously with Panc02-neo (**a**) or Panc02-MUC1 (**b**). Mice were injected with Panc02-neo or Panc02-MUC1 at the indicated dose on day 0. Mice were killed when tumor diameters reached 10 mm

survival of mice challenged subcutaneously with Panc02-MUC1 (50% at day 100) was significantly better ($P = 0.040$) than the survival of the group injected with Panc02-neo (20% at day 100).

Tumor growth rates are different following injection at subcutaneous and pancreatic sites

Mice were challenged orthotopically or subcutaneously with 1×10^6 cells of Panc02-neo or Panc02-MUC1. Mice challenged orthotopically in the pancreas with Panc02-neo were showing significant tumor-induced morbidity by 35 days after injection (Fig. 4a): all mice from this group developed tumors 10 mm in diameter or larger by day 35. In contrast, none of the mice challenged subcutaneously developed tumors larger than 10 mm in diameter (Fig. 4b) by day 35. Thus, Panc02-neo cells injected at the orthotopic site grew more rapidly than those injected subcutaneously, and resulted in a more aggressive pathogenesis of the disease, as shown by morbidity at early stages of tumor growth.

By day 45, 7 out of 10 mice challenged orthotopically with Panc02-MUC1 cells had tumors 10 mm in diameter or greater, as shown by findings at necropsy (Fig. 4a). In contrast, none of the mice challenged subcutaneously

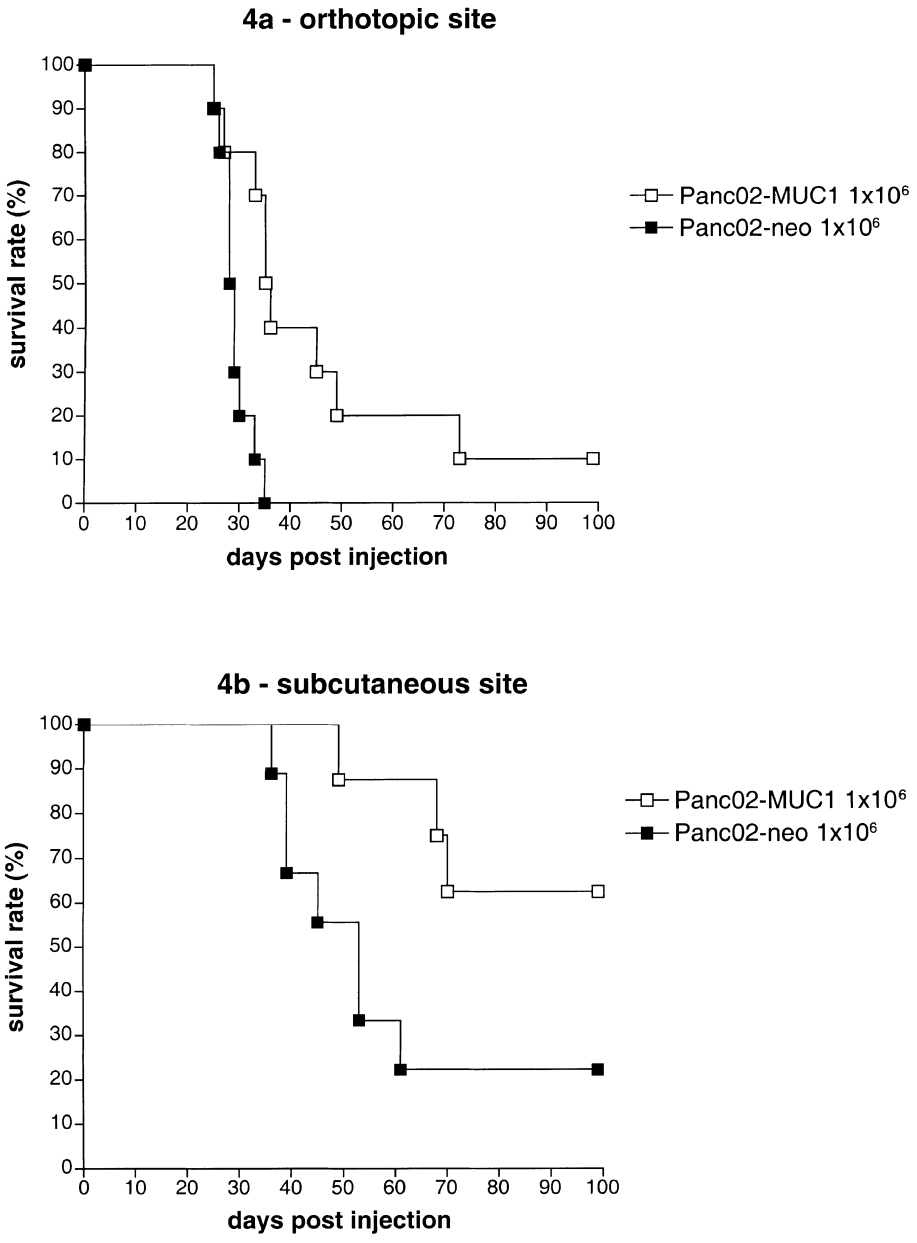

Fig. 4a, b Survival curves of mice challenged orthotopically with Panc02-MUC1 or Panc02-neo (**a**), or subcutaneously with Panc02-MUC1 or Panc02-neo (**b**) at a dose of 1×10^6 cells. *P* values were calculated by the log-rank test

developed tumors larger than 10 mm in diameter by day 45 (Fig. 4b). These organ-specific differences were also reflected in long-term survival: 60% of mice challenged in the pancreas with Panc02-MUC1 were free of tumor at 100 days, whereas only 10% of animals challenged in the pancreas with Panc02-MUC1 were free of tumor at 100 days.

Immunohistochemical analysis of the tumors

Expression of MUC1 was examined by immunohistochemical analysis of tumors that developed in mice challenged subcutaneously or orthotopically with Panc02-MUC1 (Table 1). The data reported in Table 1 were derived from tumors of representative animals in the experiments shown in Fig. 2b and Fig. 3b. These results were confirmed independently for representative animals from other groups reported in this paper (data not shown). All 12 tumors from the animals injected at the pancreatic site were uniformly positive or heterogeneously positive for expression of MUC1 protein. In contrast, all seven tumors from animals in this experiment injected at the subcutaneous site were negative for MUC1.

Metastatic potential of pancreatic tumors injected into the pancreas or at the subcutaneous site

Mice that died or were sacrificed because of tumor-induced morbidity from the orthotopic tumor injection were examined for macroscopic and microscopic metastasis in the liver, kidney and lung. No metastatic lesions were found among 28 mice that were examined. On

Table 1 Summary of immunohistochemistry results for tumor tissues harvested from dead or sacrificed mice. Mice that were challenged orthotopically ($n = 12$) or subcutaneously ($n = 7$) with Panc02-MUC1 and developed tumors in the experiment shown in Figs. 2b and 3b were evaluated for MUC1 expression by performing immunohistochemical analysis with mAb HMFG-2 on sections of tumors. $MUC1^-$ fewer than 10% of tumor cells stained positive, Heterogeneously $MUC1^+$ between 10% and 90% of the tumor cells stained positive, $MUC1^+$ more than 90% of the tumor cells stained positive

Tumor site	No. cells injected	Immunohistochemical results		
		$MUC1^-$	Heterogeneously $MUC1^+$	$MUC1^+$
Orthotopic	1×10^4	0	0	1
	1×10^5	0	0	4
	1×10^6	0	2	5
Total		0/12 (0%)	2/12 (17%)	10/12 (83%)
Subcutaneous	3×10^6	1	0	0
	1×10^7	2	0	0
	3×10^7	4	0	0
Total		7/7 (100%)	0/7 (0%)	0/7 (0%)

the other hand, from the subcutaneous tumor challenge model, one metastasis was found in the lung and one was found in the liver of 20 mice that were examined.

Humoral responses against MUC1 and their relationship to survival

Sera from 12 mice challenged orthotopically with 1×10^6 Panc02-MUC1 were collected on day 28 after tumor challenge, and examined for antibodies that bound a 40-residue peptide corresponding to two copies of the tandem repeat of MUC1. Some mice showed high concentrations of IgG1 and IgM, whereas others did not develop detectable antibody responses. Figure 5a, b shows the relationship between survival and concentration of serum antibodies specific for MUC1 (Fig. 5a: IgG1, Fig. 5b: IgM) for individual mice. Mice that showed progressive tumor growth and died early had relatively high concentrations of MUC1-specific antibody (above 20 μg/ml IgM or 5 μg/ml IgG). Mice that did not develop tumors (shown as "no tumor" cases in Fig. 5a, b) had no detectable or very low concentrations of antibodies specific for MUC1. IgG2a, IgG2b and IgG3 antibodies against MUC1 were not detected (data not shown).

Sera from five mice challenged subcutaneously with 1×10^6 Panc02-MUC1 cells were collected on day 28 and similarly evaluated for anti-MUC1 antibodies. One of these mice developed a tumor. This mouse had anti-MUC1 antibody concentrations of 180 μg/ml and 49 μg/ml sera of IgG1 and IgM respectively. The remaining mice (four animals) had not developed tumors by day 120, and showed no detectable IgG1 and IgM antibodies against MUC1.

Secondary tumor challenge by orthotopic injection

In the experiment presented in Fig. 4b, five out of eight mice challenged with 1×10^6 Panc02-MUC1 cells subcutaneously did not show progressive tumor growth. These mice were re-challenged with 1×10^5 cells of Panc02-MUC1 orthotopically into the pancreas. Five unmanipulated mice were also challenged with 1×10^5 cells of Panc02-MUC1 orthotopically into the pancreas. Mice in the former group (tumor-immune) developed no tumor and survived for more than 120 days. Mice in the latter group (unchallenged) developed progressively growing tumors by day 51.

Discussion

We describe here an orthotopic model of challenge with syngeneic pancreatic tumors in C57BL/6 mice. Orthotopic injection of a human tumor cell suspension into the pancreas has been previously reported [1, 19]; however, the previous studies were conducted in athymic mice, which do not permit investigation of immune responses against the tumors. The study reported here is the first that used syngeneic murine pancreatic tumor cells for orthotopic injection into the pancreas. We developed this orthotopic model and compared it to a subcutaneous model of tumor challenge to investigate the immunogenicity and therapeutic potential of tumor vaccine reagents that target the tumor-associated antigen MUC1 and facilitate responses that will kill pancreatic tumors growing at organ-specific locations. Ultimately, we will investigate organ-specific immunity and tolerance to MUC1 in a murine transgenic model [21].

Subcutaneous sites of tumor challenge are usually selected for convenience of monitoring tumor growth properties; however, the pathogenesis of pancreatic cancer that accompanies organ-specific tumor growth usually does not occur at subcutaneous sites. Tumors grown at subcutaneous sites show greatly reduced incidences of metastasis, and often do not show properties of tumor growth (rate, architecture, or cellularity) that are similar to those seen in patients [8]. In contrast, orthotopic implantation of tumor cells in nude mice provides an improved model that includes rapid growth

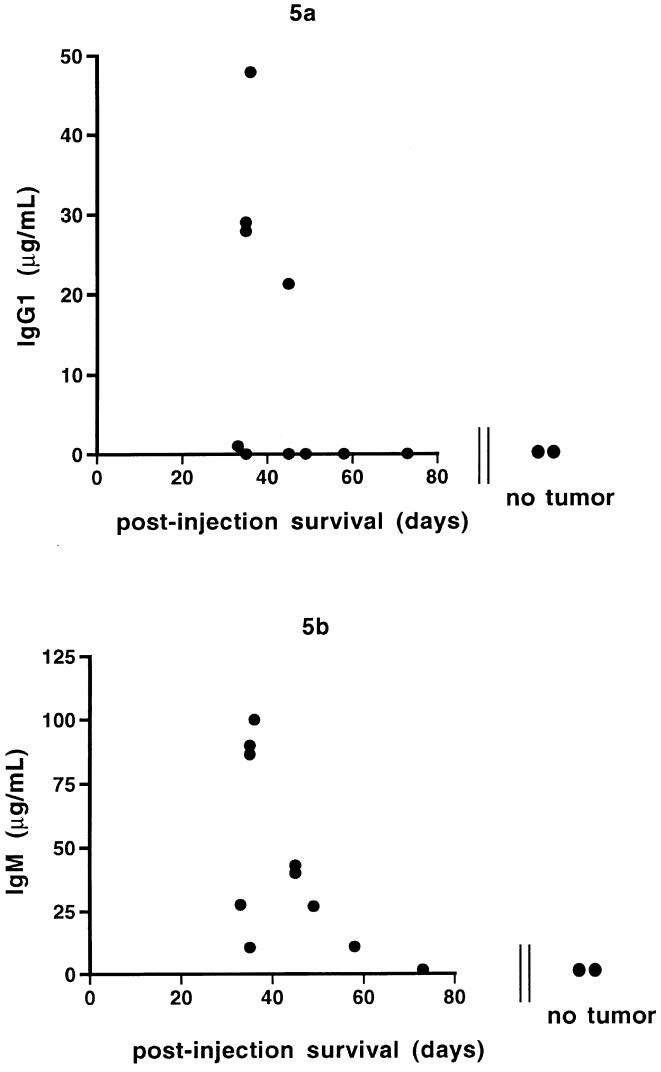

Fig. 5a, b The relationship between survival time and antibody titer (**a** IgG1, **b** IgM) in individual mice. Mice were challenged with Panc02-MUC1 and sera were collected 28 days after challenge. ● Antibody concentration for a single mouse. Antibody concentration data for mice that were challenged with Panc02-MUC1 but did not develop tumors are also included: these mice are designated *no tumor* in the figure

of local tumor and the development of spontaneous metastases [23].

Some investigators have performed orthotopic implantation of tumor masses instead of injecting defined numbers of tumor cells grown in vitro [2, 10, 11, 20, 24]. Subcutaneous tumors were resected from nude mice, dissected into small pieces, and implanted into designated organ sites. This method has several limitations, including difficulty in controlling the size and cellularity of the transplanted fragments and variability in the viability of the transplanted tumor mass. Direct injection of cultured tumor cells does not have these limitations. On the other hand, one disadvantage of directly injecting cultured tumor cells into the pancreas is the possibility that tumor cells may leak from the injection site or migrate along the route of injection. We examined the abdominal viscera of the mice challenged by direct injection with in vitro cultured tumor cells at several time points before they became moribund. In all cases the main portion of the tumor was localized to the pancreas and therefore we believe that leakage or migration is not a significant problem with this technique. Most primary tumors extended beyond the external capsule of the pancreas by direct invasion as a consequence of their inherent size. Disseminated tumors found in the peritoneal cavity were small in size, and were much smaller than tumors derived from peritoneal injections of tumor cells at similar times after injection.

There was spontaneous metastasis of Panc02-MUC1 and Panc02-neo to the liver and lung; however, the incidence of spontaneous metastasis was lower than is typically seen in humans. Given the hypothetical role of MUC1 in cell adhesion and anti-adhesion activities in tumor cells [5], it was possible that overexpression of MUC1 in the murine Panc02 tumor cell line would affect the incidence of spontaneous metastasis. We noted no differences in the incidences of spontaneous metastasis by Panc02-MUC1 or Panc02-neo following injection into the pancreas. These analyses of metastatic activity were not a principal subject of this investigation. Analysis of metastatic potential in this model is not well addressed by these studies, in part because the rapid rate of growth of the tumor at the pancreatic site led to relatively rapid deaths of the animals. Alterations in the incidence of metastasis may be observed when decreased numbers of tumor cells are initially injected to allow for slower progression of the primary tumor and longer survival times for the mice. Another possibility would be to transplant intact tumor fragments orthotopically into the pancreas, since this has been shown to enhance spontaneous metastasis in other model systems [2, 10, 11, 24].

Most studies use subcutaneous sites of tumor challenge to evaluate tumor immunity. The subcutaneous site is unlike many organ-specific sites: the subcutaneous site is well-populated by antigen-presenting cells and is frequented by other cell types that are responsible for producing immune responses. The relative abundance of antigen-presenting cells and other immune cells at other organ sites such as the pancreas is less well characterized. Hence, immunological responses against subcutaneous tumors may not accurately reflect the types of immune responses that can be produced in situ when tumors grow at organ-specific sites.

The studies reported here evaluated immune responses against MUC1, which has previously been shown to produce tumor-immune responses in C57BL/6 mice [21]. Human MUC1 is a candidate target for immunotherapy in the treatment of adenocarcinomas [3, 9, 13]. MUC1 is overexpressed in most human pancreatic adenocarcinomas [14]. MUC1 expressed by normal epithelial cells is heavily glycosylated, whereas it is underglycosylated in cancer cells, which reveal epitopes unique to the cancer cells [18]. Humoral [12, 17, 22] and cellular [4, 15, 16] immune responses to MUC1 have been observed in cancer patients.

Mice challenged subcutaneously with Panc02-neo developed tumors that grew rapidly and progressively and were not rejected by the endogenous immune response of the C57BL/6 mice. In contrast to a subcutaneous challenge, there was rapid and progressive tumor growth when either Panc02-MUC1 cells or Panc02-neo cells were injected orthotopically into the pancreas. There are at least three hypotheses that explain these findings: (1) the murine immune system recognizes MUC1 as a foreign antigen when presented at the subcutaneous or pancreatic sites; however, the immune response following the recognition is less when MUC1 is presented at the pancreatic site than at the subcutaneous site; (2) the biological environment of the pancreas supports a more rapid growth rate of Panc02-MUC1 cells than the subcutaneous site; (3) MUC1 expression is down-regulated in the subcutaneous environment. These are not mutually exclusive.

There are data in support of the first hypothesis, that the murine immune system recognizes MUC1 as a foreign antigen when it is presented at the subcutaneous site. Immunohistochemical analyses of tumors from mice challenged orthotopically were positive for MUC1, whereas the small tumors that arose at subcutaneous injection sites were negative for MUC1, suggesting that the MUC1-positive cells were eliminated at the subcutaneous site but not at the pancreatic site. Immunity to MUC1 in animals challenged at the subcutaneous site was also confirmed by re-challenging these animals with Panc02-MUC1 cells (Results). The immune response was specific for MUC1, since animals that rejected the Panc02-MUC1 cells were not protected against subsequent challenge with Panc02-neo cells (data not shown). Secondary tumor challenges by orthotopic injection into the pancreas were all rejected by these animals, strongly suggesting that they had developed an immune response that was capable of rejecting MUC1-bearing tumors at the pancreatic site. Further, this finding implies that immune responses to MUC1 produced at the subcutaneous sites can recognize and destroy tumors at the pancreatic site; however, we do not fully understand why tumors growing at the pancreatic site do not invoke an immune response that is sufficient to reject these tumors. It is possible that the pancreas is a poor organ site for establishing a primary immune response.

There is also support for the second hypothesis, that the biological environment of the pancreas supports a more rapid growth rate of Panc02 cells than the subcutaneous site. Panc02-neo cells injected into the pancreas grew at a faster rate than subcutaneously injected Panc02-neo cells. Thus, the biological environment in the pancreas is more favorable for growth by Panc02 cells than the subcutaneous site. It remains possible that rapid growth of pancreatic tumors at the pancreatic site resulted in a tumor that was too large to be rejected by the developing primary immune response. In effect the tumors growing in the pancreas may be "outrunning" the immune response by growing at a rate that is faster than the expansion of the primary immune response.

There are published and unpublished data that disprove the third hypothesis. In a separately published report [21] we showed that B16 melanoma cells transfected with MUC1 are rejected at the subcutaneous site in wild-type C57BL/6 mice but grow progressively in mice transgenic for MUC1 (MUC1.Tg) that are congenic with C57BL/6. The tumors that grew out of the MUC1.Tg mice expressed MUC1 [21]. Similar experiments have been conducted with Panc02 MUC1 cells (Morikane and Hollingsworth, manuscript in preparation) and the results showed that Panc02-MUC1 tumors grow progressively in MUC1.Tg mice and express MUC1. Taken together, these data disprove the hypothesis that the subcutaneous environment downregulates expression of MUC1 in these tumor cells.

Humoral immune responses were observed in mice challenged with Panc02-MUC1 subcutaneously (data not shown) and in the pancreas. The development of high titers of IgG1 and IgM did not correlate with tumor rejection and instead correlated with a poor prognosis. Mice that developed tumor immunity showed low titers of IgG1 and IgM specific for MUC1. This suggests that antibodies do not contribute significantly to immunity against Panc02-MUC1 in this system and may be an indicator of a poor prognosis. We are currently investigating the role of cellular immune response to MUC1.

It should also be noted that Panc02-neo cells, which are transfected with a control vector, express a neomycin-resistance gene that is apparently not sufficiently antigenic at either the subcutaneous site or the pancreatic site in this mouse strain to produce tumor immunity against Panc02-neo cells. We predicted that the product of the neomycin-resistance gene would be presented to antigen-presenting cells as a foreign protein or peptide and evoke an immune response against this molecule. However, Panc02-neo cells grew progressively at the subcutaneous and orthotopic site, and showed little evidence of rejection, on the basis of expression of the neomycin-resistance gene product. This observation suggests that MUC1 is highly immunogenic as compared to the product of the neomycin-resistance gene.

In summary, we established an orthotopic model of pancreatic cancer in C57BL/6 mice by injecting tumor cell suspensions into the pancreas. In this model we observed rapid and progressive growth of a syngeneic pancreatic cancer cell line in the pancreas compared with the tumor growth in the conventional subcutaneous injection model. We also observed differences in the immunogenicity of human MUC1 in this strain of mice with respect to the site of tumor challenge: subcutaneous tumors were rejected by an immune response, whereas tumors in the pancreas were not rejected. Nonetheless, immune responses produced by subcutaneous challenge resulted in rejection of tumors that were subsequently challenged at the pancreatic site. Antibody responses to MUC1 tandem-repeat peptides were not a factor in tumor rejection in this model.

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