Increased Immunogenicity of Tumor-Associated Antigen, Mucin 1, Engineered to Express α-Gal Epitopes: A Novel Approach to Immunotherapy in Pancreatic Cancer

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Abstract

Mucin 1 (MUC1), a bound mucin glycoprotein, is overexpressed and aberrantly glycosylated in >80% of human ductal pancreatic carcinoma. Evidence suggests that MUC1 can be used as a tumor marker and is a potential target for immunotherapy of pancreatic cancer. However, vaccination with MUC1 peptides fails to stimulate the immune response against cancer cells because immunity toward tumor-associated antigens (TAA), including MUC1, in cancer patients is relatively weak, and the presentation of these TAAs to the immune system is poor due to their low immunogenicity. We investigated whether vaccination with immunogenetically enhanced MUC1 (by expressing α-gal epitopes; Galα1-3Galβ1-4GlcNAc-R) can elicit effective antibody production for MUC1 itself as well as certain TAAs derived from pancreatic cancer cells and induced tumor-specific T-cell responses. We also used α1,3galactosyltransferase (α1,3GT) knockout mice that were preimmunized with pig kidney and transplanted with B16F10 melanoma cells transfected with MUC1 expression vector. Vaccination of these mice with α-gal MUC1 resulted in marked inhibition of tumor growth and significant improvement of overall survival time compared with mice vaccinated with MUC1 alone (P = 0.003). Furthermore, vaccination with pancreatic cancer cells expressing α-gal epitopes induced immune responses against not only differentiated cancer cells but also cancer stem cells. The results suggested that vaccination using cells engineered to express α-gal epitopes is a novel strategy for treatment of pancreatic cancer. Cancer Res 70(13): 5259–69. ©2010 AACR.

Introduction

Pancreatic cancer is the fourth most common cause of death from cancer in men and women, with 5-year survival for all stages of disease of <5% (1–3). Surgical resection remains the only potentially curative intervention but is contraindicated in most patients because the disease is diagnosed at an advanced stage. Potential treatments of pancreatic cancer include immunotherapy, gene therapy, and antiangiogenic agents.

Immunotherapy is an innovative approach that uses techniques such as vaccination to activate the patient’s immune system tumor-associated antigens (TAA) expressed in pancreatic cancer cells. Mucin 1 (MUC1) is one of the most considerable targets for vaccine immunotherapy (4, 5). It is a type I transmembrane protein composed of a heavily glycosylated extracellular domain containing a variable number of tandem repeats, a transmembrane domain, and a cytoplasmic domain (6–9). MUC1 is normally expressed on the apical surface of ductal and glandular epithelial cells. In benign cells of breast, prostate, ovarian, and pancreas, MUC1 is heavily glycosylated, limited to the apical side of the glands, and minimally expressed. Transformation of these cells into malignant cells is associated with the overexpression of MUC1, loss of MUC1 expression polarity, and dysregulation of O-glycans of MUC1 (6–8). Consequent to aberrant expression of a series of glycosyltransferases, the tumor-associated MUC1 carries a preponderance of shorter glycans (6–9). The ultimate result of these changes is the exposure of normally cryptic portions of the core protein structure, consisting of the tandem repeat array, which is the most immunogenic part of MUC1, toward the effector cells of the patient’s immune system; therefore, MUC1 is considered a potential target for vaccine therapies.

In fact, MUC1 vaccines were tested in several clinical trials (10–13). However, they have not been as successful as hoped, in part due to the immune evasion tactics applied by tumor
cells to escape immunorecognition (14, 15). A prerequisite for the induction of an effective antitumor immune response by MUC1 vaccine is the effective uptake of this molecule by professional antigen-presenting cells (APC). Thus, processing of the internalized MUC1 in the vaccine, and presentation of antigenic MUC1 peptides on APCs in association with MHC class I and class II molecules, could activate antitumor cytotoxic T cells and helper T cells. However, MUC1 proteins on pancreatic cancer cells do not express markers that contain modification of MUC1 to be recognized by APCs. To increase the immunogenicity of MUC1 to APCs, IgG bound to MUC1 could be a suitable strategy. In this study, we hypothesized that IgG-mediated targeting of MUC1 could enhance presentation to APC by exploiting the natural anti-Gal in humans.

Anti-Gal is an IgG antibody (Ab) that is present in large amounts in normal subjects and patients with malignancies, comprising ~1% of serum-circulating IgG (16–18). Anti-Gal Ab specifically interacts with α-gal epitopes on cell surface glycolipids and glycoproteins, and it is produced throughout life due to antigenic stimulation by bacteria of the gastrointestinal flora (19). Once anti-Gal binds to α-gal epitopes on the cell, its Fc portion readily binds to FcyRIII on dendritic cells and macrophages. This interaction induces effective phagocytosis of the α-gal-opsonized cells by APC (20–22). The α-gal epitopes are absent in humans, but are abundantly synthesized on glycolipids and glycoproteins by the glycosyltransferase enzyme, αL3galactosyltransferase (αL3GT) within the Golgi apparatus of cells of nonprimate mammals, prosimians, and in New World monkeys (23). In contrast, humans, apes, and Old World monkeys lack α-gal epitopes; instead, they produce the natural anti-Gal Ab in very large amounts (24, 25). Anti-Gal Ab is an Ab that can be very active in vivo, as inferred from studies in xenotransplantation (26, 27). The in vivo binding of anti-Gal Ab to α-gal epitopes on transplanted pig heart or kidney is the main cause of hyperacute rejection of such grafts in humans and in Old World monkeys. Similarly, we hypothesized that MUC1 vaccine processed to express α-gal epitopes (MUC1 has five potential sites of N-glycans that are targets for αL3GT) can bind the natural anti-Gal IgG Ab in situ at the vaccination site. Such interaction would enhance recognition by APCs, resulting in more effective vaccination.

In the present study, we investigated the effects of vaccination with α-gal MUC1 on pancreatic cancer cells and examined its usefulness in the induction of tumor-specific T-cell responses, in vivo prevention of tumor growth, and prolongation of survival of α-gal MUC1 vaccine recipients. A rare population of cells with stem cell properties called cancer stem cells was identified recently. In 2007, Li and colleagues (28) reported that CD44+CD24+ epithelial-specific antigen (ESA)+ pancreatic cancer cells showed stem cell properties. It is necessary to develop an effective therapy for cancer stem cells to eradicate cancer cells; however, cancer stem cells are highly resistant to adjuvant therapy including chemotherapy. We hypothesized that biosynthesis of α-gal epitopes on the carbohydrate of cancer stem cell markers expressed on pancreatic cancer stem cells could effectively induce Ab production against these stem cells. We also investigated the immune response induced by vaccines expressing α-gal epitopes against both differentiated pancreatic cancer cells and pancreatic cancer stem cells.

Materials and Methods

Flow cytometry
To evaluate the expression of α-gal epitopes on the cell surface of α-gal PANC1 cells, flow cytometric analysis was performed. Parental PANC1 and αL3GT transfectants were incubated with 20 µg/mL biotinylated GS184 lection (Vector Laboratories), which specifically binds to α-gal epitopes or the monoclonal anti-Gal Ab M86 (29–35). Details of the procedure were previously described (26–30).

To examine the endogenous expression of MUC1 protein, cells were primarily stained with anti-MUC1 monoclonal Ab (mAb; clone VU4H5, Santa Cruz Biotechnology), and subsequently, cells were incubated with FITC-conjugated anti-mouse immunoglobulins as a secondary Ab (DAKO). Stained cells were washed, fixed, and then analyzed by a flow cytometer.

To evaluate the expression of CD44, CD24, and ESA, which are stem cell markers of pancreatic cancer cells on parental PANC1, parental PANC1 cells were stained with either FITC-conjugated antihuman CD44 mAb, phycoerythrin-conjugated antihuman CD44 mAb or FITC-conjugated antihuman ESA mAb, respectively, as previously described (BD Biosciences; ref. 36). To investigate Ab production against pancreatic cancer stem cells, isolated stem cells from PANC1 cells were stained with sera from vaccinated knockout (KO) mice. Cancer stem cells expressing both CD44 and CD24 were positively isolated by streptavidin-coated Dynalbeads M-280. The magnetic beads were incubated with either biotinylated antihuman CD24 mAb or antihuman CD44 mAb, respectively (Ancell Corp.). Subsequently, 10 × 10⁶ cells of parental PANC1 cells were incubated with anti-CD44 mAb-coated beads in 1% bovine serum albumin (BSA)/PBS for 2 hours at 4°C, and the CD44-positive cells were positively isolated by DynaMag (Invitrogen). The isolated cells were then incubated with anti-CD24 mAb-coated beads in a similar manner. Subsequently, CD44+ and CD24+ double-positive cells were isolated by DynaMag (Invitrogen). The isolated CD44+CD24+ pancreatic cancer cells were incubated with sera from KO mice that had been vaccinated with either parental PANC1 or α-gal PANC1 cells. Subsequently, these cells were stained with FITC-conjugated anti-mouse IgG1 (Bethyl Laboratories, Inc.) as a secondary Ab to detect Abs that interact with the pancreatic cancer stem cells. Moreover, PANC1 cells that did not bind with either anti-CD44 mAb- or anti-CD24 mAb-coated beads were combined and incubated with KO mice sera to assess reactivity with differentiated pancreatic cancer cells other than CD44+CD24+ cells.

Tumor cell vaccination αL3GT KO mice were immunized by pig kidney fragments, and the presence of anti-Gal in their serum was confirmed by ELISA as described above. Subsequently, the mice were vaccinated i.p. thrice at 1-week intervals with 1 × 10⁶
One week after vaccination, the mice were assessed for the immune response elicited by tumor cell vaccination as described below.

**Enzyme-linked immunospot analysis**

The enzyme-linked immunospot (ELISPOT) assay was used to measure the expansion of anti-MUC1–secreting B cells or MUC1-specific T cells, respectively (30, 37, 38). ELISPOT wells (MultiScreen 96-well Filtration Plate, Millipore) were coated with either MUC1-BSA (10 μg/mL) or BSA (5 μg/mL) for 20 hours at 4°C, then blocked overnight with RPMI containing 0.4% BSA and 50 μmol/L of 2-mercaptoethanol. Splenocytes prepared from mice that had been vaccinated with α-gal PANC1 cells or parental PANC1 cells were plated in the wells at a concentration of 1 × 10^6 cells per well, and the plates were incubated for 24 hours at 37°C. Subsequently, the wells were washed, incubated with horseradish peroxidase (HRP)–conjugated goat antimouse immunoglobulin (Bethyl Laboratories, Inc.), and the spots representing Abs secreted from individual plasma cells were identified with dianimobenzidine (DAB, Sigma-Aldrich). The number of anti-MUC1 producing plasma cells in each well was determined by subtracting the background number of spots in wells containing BSA as solid-phase antigen from the number of spots in the corresponding wells with MUC1-BSA.

To identify MUC1-specific T cells in the vaccinated KO mice, the secretion of IFN-γ from the activated T cells was measured by ELISPOT assay. ELISPOT plates were coated with anti-IFN-γ mAb (clone AN18, Mabtech). Splenocytes were incubated in the coated ELISPOT wells (1 × 10^5 cells/well) in the presence or absence of 100 μg/mL MUC1 peptide. Furthermore, to investigate whether the reactivity of MUC1-specific T cells was mediated through MHC class I, splenocytes were incubated in the wells in the presence of both 100 mg/mL MUC1 peptide and mouse antimouse H-2Db mAb as anti-MHC class I–blocking Ab (BD Biosciences) at a dilution of 1:50. After 24 hours of incubation at 37°C, the wells were washed and then stained with biotinylated anti–IFN-γ mAb (clone R4-6A2, Mabtech) for 1 hour at room temperature. Subsequently, the wells were washed, incubated with HRP-conjugated streptavidin (DAKO), and the spots representing activated T cells were developed with DAB. The numbers of developed spots in ELISPOT assays were counted by the ChemiDoc XRS image analyzer (Bio-Rad Laboratories) using Quantity one software (Bio-Rad).

**In vivo studies of tumor cell vaccine in an experimental animal model**

The efficacy of tumor vaccines expressing α-gal epitopes cannot be studied in a regular experimental animal model because normal wild-type mouse cells and the majority of mouse tumor cell lines, including mouse pancreatic cancer, express α-gal epitopes. Therefore, B16F10 melanoma cells (American Type Culture Collection) from C57BL/6 mice were used in the present study because they lack α-gal epitopes caused by inactivation of the α1,3GT gene (30). First, B16F10 cells were transfected with the full-length MUC1 gene (5, 39) using Lipofectamine, and a stable B16F10 transfectant expressing MUC1 protein was established (designated MUC1-B16F10). High anti-Gal KO mice were generated by immunization with pig kidney fragments then vaccinated with parental PANC1 or α-gal PANC1 cells after irradiation. One week later, all mice were challenged with s.c. injection with 0.5 × 10^6 live MUC1-B16F10 cells. Subsequently, mice were examined serially for both tumor growth and survival. The production of anti-MUC1 Abs and Abs toward other tumor antigens was examined by Western blot analysis, using α1,3GT KO mice sera. Subsequently, 3 μg of cellular proteins extracted from PANC1 were loaded to 6% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking with PBS containing 3% BSA, the blot was incubated for 20 hours at 4°C with α1,3GT KO mouse serum obtained from either nonimmunized, high anti-Gal, parental PANC1–vaccinated, or α-gal PANC1–vaccinated KO mice at 1:20 dilution, respectively. After washing, the blot was incubated with HRP-conjugated anti-mouse IgG (Bethyl Laboratories) as the secondary Ab and developed using the enhanced chemiluminescence detection system.

Details of the mice used in these experiments, cell lines, gene construct, methods used to establish stable pancreatic cancer cells expressing α-gal epitopes, Western blot analysis and immunoprecipitation, in vitro stimulation of lymphocytes, ELISA, and statistical analysis are presented as Supplementary Material in the electronic version of the journal.

**Results**

**Generation of stable PANC1 transfectant expressing α-gal epitopes**

No expression of α-gal epitopes was observed in parental PANC1 cells, whereas high expression levels of α-gal epitopes were observed on the cell surface of α1,3GT transfectants (Fig. 1A). Similar levels of MUC1 expression were observed in parental PANC1 and α-gal PANC1 cells on both flow cytometry and Western blot analyses (Fig. 1B). Immunoprecipitation with anti-Gal Ab showed a strong band at 150 kDa, which was consistent with MUC1 (Fig. 1B).

**Vaccination with α-gal PANC1 elicits the production of anti-Gal and anti-MUC1 Abs**

The anti-Gal Ab, as a natural Ab, was negative in nonimmunized α1,3GT KO mice. Repeated immunizations (four times) with pig kidney fragments resulted in the appearance of anti-Gal Ab, with the titer of anti-Gal IgG being similar to that observed in a large proportion of human sera (Fig. 1C and D; refs. 23, 24). As shown in Fig. 1C, vaccination with α-gal PANC1 resulted in 8-fold increase in production of anti-Gal IgG compared with high anti-Gal KO mouse generated by pig kidney immunization. In contrast, no significant increase in the titer of anti-Gal IgG was detected when anti-Gal KO mice were vaccinated with parental PANC1 cells (Fig. 1D). A similar difference in anti-MUC1 IgG response was observed in mice vaccinated with either α-gal PANC1 or parental PANC1 cells. Three vaccinations...
with $1 \times 10^6$ of α-gal PANC1 elicited a strong anti-MUC1 IgG response, whereas vaccination with parental PANC1 cells did not elicit such an Ab response (Fig. 2A and B). Approximately 8- to 16-fold increase in anti-MUC1 IgG production was elicited by α-gal PANC1 vaccination compared with parental PANC1 vaccination (Fig. 2A and B). Analysis of anti-MUC1 isotypes in sera from KO mice vaccinated with α-gal PANC1 cells showed large production of anti-MUC1 IgG, a small amount of anti-MUC1 IgM, but no IgA isotype (Supplementary Fig. S1A). The isotypes of immunoglobulin reactivities in the sera from parental PANC1–vaccinated KO mice also showed a similar pattern of anti-MUC1 isotypes to that of sera from α-gal PANC1–vaccinated mice (Supplementary Fig. S1B).

Analysis of IgG subclasses of anti-MUC1 IgG response in α-gal PANC1–vaccinated mice showed relatively large amounts of IgG1 (Fig. 2C), IgG2b, and IgG3 subclasses of anti-MUC1 IgG, but no IgG2a subclass of this Ab (Fig. 2C). On the other hand, similar analysis of IgG subclasses of this Ab response in parental PANC1–vaccinated mice showed small amounts of IgG1 production, but no IgG2a, IgG2b, and IgG3 subclasses (Fig. 2D).
Vaccination with α-gal PANC1 results in the expansion of systemic B- and T-cell response to MUC1

The marked increase in anti-MUC1 IgG titers in mice vaccinated with α-gal PANC1 was associated with expansion of the B-cell clone with anti-MUC1. As shown in Fig. 3A, the expansion of anti-MUC1–secreting B cells was detected in vitro by ELISPOT assay with MUC1-BSA as the solid-phase antigen. Splenocytes obtained from high anti-Gal KO mice without PANC1 vaccination displayed no dots in ELISPOT wells (data not shown). Parental PANC1–vaccinated KO mice had 319.5 ± 18.9 spots per 1 × 10⁶ splenocytes. However, α-gal PANC1–vaccinated KO mice displayed 553.6 ± 66.7 spots per 1 × 10⁶ splenocytes, and the proportion of anti-MUC1 B cells was significantly increased (P < 0.0001; Fig. 3A).

The priming of T cells, which specifically react with MUC1 peptide, was also determined by ELISPOT assay, by measuring IFN-γ secretion following the in vitro activation of T cells in the presence of MUC1 peptide. In parental PANC1–vaccinated KO mice, 211 ± 33.4 and 153.2 ± 15.2 spots per 1 × 10⁶ splenocytes were detected in the presence or absence of MUC1 peptide, respectively, and no significant increase in the number of spots was observed (Fig. 3B). In contrast, α-gal PANC1–vaccinated KO mice displayed 1,237.5 ± 283.1 spots per 1 × 10⁶ splenocytes in the presence of MUC1 peptides and 313.8 ± 48.9 spots in the absence of MUC1 peptides.

In the blocking assay, anti-MHC class I mAb completely blocked the elicited increase in the number of spots observed in α-gal PANC1–vaccinated KO mice. Splenocytes treated with anti-MHC class I mAb displayed 316 ± 70.4 spots, and this number of spots was comparable with that in the absence of MUC1 peptides (Fig. 3B). In parental PANC1–vaccinated KO mice, no significant blocking effects for anti-MHC class I mAb were observed (169.5 ± 15.2 spots/1 × 10⁶ splenocytes, Fig. 3B). Taken together, KO mice vaccinated with α-gal PANC1 displayed a significant expansion of both B- and T-cell responses to MUC1 (Fig. 3A and B). Furthermore, such elicited T-cell responses against MUC1 peptide were induced through MHC class I molecule.

Immune responses of α-gal PANC1–vaccinated α1,3GT KO mice are significantly stronger and more specific against PANC1 cells and MUC1 peptide

As shown in Fig. 3C and D, proliferation of lymphocytes significantly induced in the presence of parental PANC1, α-gal PANC1, or MUC1 peptide was likely to result from specific immune response to vaccination with either parental PANC1 or α-gal PANC1 because lymphocytes obtained from...
both nonimmunized α1,3GT KO mice and α1,3GT KO mice bearing high levels of anti-Gal Ab displayed almost no proliferative response when incubated with parental PANC1, α-gal PANC1, or MUC1 peptide. Furthermore, the elicited immune responses expressed by lymphocytes from α-gal PANC1–vaccinated α1,3GT KO mice were significantly stronger than those from parental PANC1–vaccinated α1,3GT KO mice (P < 0.001–0.01). Pig endothelial cells stimulated the proliferation of lymphocytes from both vaccinated KO mice and high anti-Gal KO mice.

Lymphocyte stimulation was also tested with irradiated cells from other species, including monkey COS7 cells, mouse fibroblast NIH3T3 cells, and human HEK293 cells. All types of stimulatory cells failed to induce lymphocyte proliferation. These data indicate that a significant proportion of the stimulatory effect was due to the specific recognition of peptide antigens present in PANC1 cells and MUC1 peptide but absent in mouse, monkey, or human cells. Interestingly, the proliferation induced by α-gal PANC1 cells was slightly lower than that induced by parental PANC1 cells. However, the proliferation rate observed in α-gal PANC1–vaccinated mice was significantly higher than in parental PANC1–vaccinated mice. In all the assays, the lymphocytes were viable and competent.
because they effectively proliferated as a result of nonspecific stimulation by concanavalin A.

In the blocking assay with anti-MHC class II mAb, specific lymphocyte proliferation elicited by vaccination with α-gal PANC1 was significantly reduced by ∼50% (Fig. 3C). Moreover, the specific immune response against MUC1 peptide expressed by lymphocytes from α-gal PANC1–vaccinated KO mice was also markedly suppressed by treatment with anti-MHC class II mAb (Fig. 3D). Taken together, these results indicate that the specific immune responses against PANC1 and MUC1 peptide are mainly induced through MHC class II molecule.

α-gal PANC1 vaccines protect and prolong survival against tumor challenge using MUC1-B16F10 cells

As shown in Fig. 4A, α1,3GT KO mice immunized with pig kidney fragments were vaccinated with 1 × 10⁶ irradiated parental PANC1 or α-gal PANC1 cells. The efficiency of such vaccines was assessed by measuring the ability of irradiated α-gal PANC1 cell vaccines to induce a protective immune response against challenge with live MUC1-expressing B16F10 cells. Before the in vivo study, we established the same B16F10 clones that stably expressed MUC1. Although the parental B16F10 cells did not react with anti-MUC1 mAb (VU4H5), MUC1-transfected B16F10 cells showed strong reactivity with this mAb (Fig. 4B). The expression level of MUC1 gene in B16F10 transfectant was similar to that in parental PANC1 cells.

Representative pictures of mice treated with either α-gal PANC1 or parental PANC1 vaccines are shown in Fig. 4C. The tumor size in each mouse treated with α-gal PANC1 vaccines was much smaller than that of mice treated with parental PANC1 vaccines. Approximately 80% of parental PANC1–vaccinated mice died within 20 to 26 days postchallenge, whereas 75% to 80% of mice treated with α-gal PANC1 vaccine were still alive at 30 days after tumor implantation.
Tumor growth in mice vaccinated with \(\alpha\)-gal PANC1 or parental PANC1 was monitored (Fig. 5A). Tumors in mice vaccinated with parental PANC1 doubled their sizes every 3 to 7 days and reached the maximum size of \(\sim 980 \text{ mm}^2\) within 22 to 25 days. For tumors in mice treated with \(\alpha\)-gal PANC1, they continued to grow in nonvaccinated \(\alpha_1,3\)GT KO mice but displayed a much slower growth rate than most parental PANC1–treated KO mice. Tumors reached the size of 200 \text{ mm}^2\) within 20 to 36 days, whereas tumors in mice treated with parental PANC1 vaccines grew to a similar size within 12 to 14 days (Fig. 5A).

The beneficial effects of \(\alpha\)-gal PANC1 vaccines were further shown in the prolongation of survival after tumor challenge. As shown in Fig. 5B, the mean survival time of KO mice treated with \(\alpha\)-gal PANC1 was significantly prolonged (41.4 \pm 10.4 d) compared with that of parental PANC1 vaccine (21.1 \pm 10.5 d; \(P = 0.003\); Fig. 5B).

\(\alpha\)-gal PANC1 vaccines induce Ab production against MUC1 protein and other unknown tumor antigens

To understand the induced Ab response for other tumor antigens, the presence of immunostained PANC1 proteins was investigated by Western blots with mice serum before and after vaccination. As shown in Fig. 5C, sera from \(\alpha_1,3\)GT nonimmunized or pig kidney fragment–immunized KO mice did not contain any Ab that bound to MUC1 protein, and displayed only several nonspecific bands. Sera obtained from \(\alpha_1,3\)GT KO mice vaccinated with parental PANC1 also did not display obvious bands that were reflected by anti-MUC1 Ab. No detectable differences in these blot profiles were observed before and after parental PANC1 vaccine. In contrast, sera from \(\alpha\)-gal PANC1–vaccinated \(\alpha_1,3\)GT KO mice were positive for Abs that bound to not only MUC1 protein but also different immunogenic proteins in PANC1 cells. These findings indicate that the expression of \(\alpha\)-gal epitopes on TAAs in PANC1 cells can upregulate its immunogenicity and can induce the production of Abs against TAAs, including MUC1 and various tumor proteins.

Vaccination with \(\alpha\)-gal PANC1 can effectively elicit immune response against pancreatic cancer stem cells

To assess Ab production against parental PANC1 cells, cells were stained with sera from vaccinated KO mice. As shown in Fig. 6A, sera from \(\alpha\)-gal PANC1–vaccinated KO mice more strongly interacted with PANC1 cells than those from parental PANC1–vaccinated mice, as judged by the mean fluorescence intensity. Next, we confirmed that the subpopulation of pancreatic cancer stem cells expressed CD44, CD24, and ESA in parental PANC1 cells. Flow cytometric quantification (Fig. 6B) showed that 82.8% to 87.0% of parental PANC1 cells expressed CD44, 72.8% to 75.6% expressed CD24, and 91.4% to 93.8% expressed ESA. Examination of the expression of multiple surface markers showed 70.4% of PANC1 cells were CD44+CD24+ and 82.4% were CD44+ESA+. We isolated PANC1 cells with CD44+CD24+ phenotype by magnetic beads as described in Materials and Methods to investigate Ab production against both pancreatic cancer stem cells (i.e., binding PANC1 cells with beads).
and differentiated pancreatic cancer cells (i.e., nonbinding cells with beads). A strong Ab production against both pancreatic cancer stem cells and differentiated pancreatic cancer cells was effectively elicited by vaccination with α-gal PANC1 cells; however, vaccination with parental PANC1 cells did not induce any immune response against each subpopulation (Fig. 6C and D).

**Discussion**

Development of immunotherapy for pancreatic cancer is necessary to improve the poor prognosis associated with this disease. In this study, we showed the in vitro and in vivo effectiveness of immunotherapy through vaccination, with a resultant increase in immunogenicity of α-gal MUC1, and showed that repeated vaccination with α-gal PANC1 elicited the production of anti-MUC1 Ab as well as the generation of an effective cytotoxic T lymphocyte (CTL) response against the MUC1 molecule. Although MUC1 protein is a potential target for the immunotherapy of pancreatic cancer, vaccination against a single antigen is disadvantageous because it is not clear which antigens, including MUC1, have the potential to induce effective antitumor immune responses. In addition, immunity against various antigens is expected to be effective in heterogeneous cell populations of tumors.

Therefore, the use of unfractionated TAAs in the form of tumor cell lysates or whole cancer cells circumvented these disadvantages as described above. In this study, we used vaccination with whole cancer cells, and the immunogenicity of well-characterized as well as uncharacterized multiple TAAs contained in cancer cells was upregulated by α-gal epitopes; and thus, these TAAs would be effectively internalized by APC, which phagocytose-vaccinating tumor cells opsonized by anti-Gal. Multiple TAAs can be presented to T cells by both MHC class I and class II pathways, ultimately leading to polyclonal expansion of both B and T cells. In this study, whereas the sera obtained from parental PANC1–vaccinated KO mice failed to produce both anti-MUC1 Ab and anti–unknown TAA Abs, those from α-gal PANC1–vaccinated KO mice contained several Abs that bound to not only MUC1 protein but also different unknown TAAs. Future studies are required to identify and characterize these unknown molecules as novel TAAs for immunotherapy of pancreatic cancer. For the development of an effective immunotherapy of pancreatic cancer, we proposed that tumor cell lysate is a suitable source of tumor antigens because it contains several known as well as unknown antigens that could elicit an antitumor immune response.

Recently, the existence of cancer stem cells was confirmed in acute myelogenous leukemia and was subsequently verified also in breast cancer and brain tumors (39, 40). Putative pancreatic cancer stem cells express CD44, CD24, and ESA, which are glycoprotein marker molecules that can be used to identify cancer stem cells (28–48). In the present study, sera obtained from α-gal PANC1–vaccinated KO mice produced anti–CD44 CD24 PANC1 Ab, but those from parental PANC1–vaccinated KO mouse were negative for anti–CD44 CD24 PANC1 Ab. These results suggest that the buildup of α-gal epitopes on carbohydrates of cancer stem cell markers, thus...
allowing these molecules to be internalized by APC, is a novel strategy for treatment of cancer stem cells, including pancreatic cancer stem cells.

Tumor lysate is a suitable material for vaccination because it contains not only differentiated cancer cells but also cancer stem cell population. Because the number of cancer stem cells is small, it is difficult to induce immune response against cancer stem cells by standard vaccination with tumor cell lysates. In contrast, vaccination with tumor lysate enzymatically engineered to express α-gal epitopes might result in elicitation of immune response toward these cancer stem cells because putative pancreatic cancer stem cells express glycoprotein marker molecules that can identify cancer stem cells (28, 40, 46). Accordingly, the α-gal epitopes could be synthesized on the carbohydrates of both TAAs and stem cell markers, allowing these molecules to be internalized by APC.

Overall, the anti-Gal–mediated in situ targeting of immunizing tumor cells to APC might provide the immune system with the opportunity to be activated effectively by several TAAs, including MUC1 in tumor cells. Furthermore, novel approaches using vaccines of α-gal epitope–expressing pancreatic cancer cells are anticipated to induce immune response against not only differentiated cancer cells but also cancer stem cells, and may lead to cure pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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