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MUC1 Peptide Vaccination in Patients with Advanced Pancreas or Biliary Tract Cancer

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Abstract. *Background: To evaluate the immunogenicity of MUC1 peptide vaccine in advanced pancreatic and bile duct cancers, a phase I clinical trial was conducted. Materials and Methods: A 100-mer MUC1 peptide consisting of the extracellular tandem repeat domain and incomplete Freund's adjuvant were subcutaneously administered to 6 pancreatic and 3 bile duct cancer patients at weeks 1, 3 and 5 and doses ranging from 300 to 3000 μg. Circulating intracytoplasmic cytokine-positive CD4^{+} T cells and anti-MUC1 IgG antibodies were measured before and after vaccination. Results: There were no adverse events, except for mild reddening and swelling at the vaccination site. In 8 patients eligible for clinical evaluation, 7 had progressive disease and 1 stable disease with a tendency for increased circulating anti-MUC1 IgG antibody after vaccination. Conclusion: This phase I clinical trial revealed the safety of a vaccine containing 100-mer MUC1 peptides and incomplete Freund's adjuvant.*

MUC1 is a type I transmembrane glycoprotein with an extracellular domain composed of a polypeptide core containing multiple tandem repeats of a 20 amino acid sequence with numerous carbohydrate chains (1). The autoimmunogenicity of MUC1 was first shown by inducing HLA-unrestricted cytotoxic T lymphocytes (CTLs) against the tandem repeat region (2), which was confirmed by subsequent investigations (3-5). Thereafter, Domenech *et al.* demonstrated the presence of HLA-restricted CTLs against the tandem repeat sequence (6). The nanomer peptide STAPPAHGV, which corresponds to residues 9-17 of the 20 amino acid repeat sequence, was found to have significant binding affinity to several class I alleles, including HLA-A1, A2, A3 and A11, and to be able to elicit a MUC1-specific CTL response in an A11^{+} cancer patient. On the other hand, a humoral immune response to MUC1 was also revealed (7, 8) and circulating antibodies against the tandem repeat peptides were detected in various cancers (9, 10). These findings suggested the potential application of MUC1 in cancer immunotherapy and led to clinical trials of a MUC1 peptide vaccination (11-14).

The first study, by Goydos *et al.,* demonstrated the safety of a vaccine composed of a synthetic MUC1 peptide with 5 repeats of the 20 amino acid sequence and BCG (11). Karanikas *et al.* then reported the results of a clinical trial with the MUC1 peptide of 5 repeats fused with mannan in 25 patients with advanced breast, gastric or colorectal cancer (12). They detected large amounts of IgG_1 anti-MUC1 antibodies in 13 of the 25 patients, and could induce HLA-A2-restricted CTLs, but a significant CTL response was only seen in 2 out of 10 patients tested. Gilewski *et al.* reported the results of a vaccination with the MUC1 peptide consisting of 1.5 repeats conjugated with keyhole limpet hemocyanin (KLH) together with the immune adjuvant QS-21 in 9 breast cancer patients (13). High IgM and IgG antibody titers against the MUC1 peptide were detected; however, there was no evidence of T cell activation. Another of their studies, using a 106-amino-acid-long MUC1 peptide conjugated with KLH plus QS-21 in 6 breast cancer patients, again showed that the T cell response against the MUC1 peptide was minimal and inconsistent (14). These clinical data suggested that the tandem repeat peptide of MUC1 could be useful for inducing anti-MUC1 antibodies rather than CTLs.

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Recently, von Mensdorff-Pouilly *et al.* have reported that a positive test result for both IgG and IgM antibodies in pretreatment serum was associated with significant disease-specific survival in stage I and II breast cancer patients (15). We also revealed that circulating anti-MUC1 IgG antibody was a favorable prognostic factor for cancer of the pancreas (16). These results suggest that the antibodies might protect the host against cancer progression. In this study, we attempted a phase I clinical trial of a 100-mer MUC1 tandem repeat peptide with incomplete Freund's adjuvant in patients with advanced pancreatic or bile duct cancer.

Materials and Methods

Trial eligibility. Five patients with inoperable pancreatic cancer, 2 with recurrent disease of bile duct cancer, 1 with recurrent disease of pancreatic cancer and 1 with inoperable bile duct cancer were enrolled in this study. They were required to have computed tomography (CT) or magnetic resonance imaging (MRI) for evaluating clinical stage or recurrent disease. The eligibility criteria were as follows: age of 85 years or less, serum creatinine of less than 1.4 mg/dl, bilirubin of less than 1.5 mg/dl, platelet count of 100,000/μl or more, hemoglobin of 8.0 g/dl or more, and total WBC of 3000/μl or more. Hepatitis B surface antigen and Hepatitis C antibody were negative in all patients. The patients were untreated for at least 4 weeks before entry into the study, and had to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2 at the time of entry. Patients with evidence of other serious illness, immunosuppression, or autoimmune disease were excluded. Treatment of the enrolled patients was carried out at Yamaguchi University, Japan, from June 2000 through March 2004.

All patients were required to comprehend and sign an informed consent form approved by the Institutional Review Board of Yamaguchi University School of Medicine.

Vaccine preparation and administration. The MUC1 peptide, consisting of 100 amino acids (5 repeats) of the extracellular tandem repeat domain, was synthesized at the Peptide Synthesis Facility, University of Pittsburgh (Dr. O. J. Finn, Pittsburg, PA, USA), in accordance with the U.S. FDA Good Laboratory Practice Regulations and the Japanese GLP Standard. Montanide ISA-51 (incomplete Freund's adjuvant) was manufactured by Seppic, Inc. (Paris, France) and supplied in glass ampoules containing 3 ml of sterile adjuvant solution.

An appropriate amount of MUC1 peptide was diluted with sterile 0.9% NaCl solution and added in a 1:1 volume to Montanide ISA-51 and then mixed using a stopcock and two glass syringes for 5 min. The resulting emulsion was injected, using a glass syringe, subcutaneouly into the frontal thigh in a volume of 1 ml. Alternative thighs were used for a total of 3 injections, which were done 2 weeks apart. Skin tests were performed using 50 μg of the peptide in 0.9% NaCl solution injected intradermally in a volume of 100 μl using a 1-ml disposable syringe. The injection site was observed at 15 min and 48 h. For patients who requested the additional administration of MUC1 peptides, vaccination was repeated with monitoring for adverse events.

Evaluation of adverse events and clinical response. All adverse events were evaluated by the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 2.0 (17) at every vaccination. All known sites of disease were evaluated by CT scan before and after 3 vaccinations. Patients were assigned to a response category according to the response evaluation criteria for solid tumors, given in a revised version of the WHO criteria published in June 1999 in the WHO Handbook for reporting results of cancer treatment.

Intracellular cytokine assays. Peripheral blood samples were collected and the proportions of $CD4^+$ T cells producing intracellular cytokines were determined using flow cytometry, as reported previously (18). In brief, peripheral blood samples were collected by venapuncture into syringes containing sodium heparin anticoagulant. Phycoerythin-cyanine 5 (PC5)-conjugated anti-CD3 monoclonal antibody (mAb) and energy-coupled dye (ECD)-conjugated anti-CD4 mAb were purchased from Coulter Immunology (Hialeath, FL, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ mAb, phycoerythrin (PE)-conjugated anti-IL4 mAb and FITC/PE-conjugated control mAbs were purchased from Becton Dickinson (San Jose, CA, USA). PE-conjugated anti-interleukin (IL)-6 mAb and anti-IL-10 were purchased from R & D (Minneapolis, MN, USA) and PharMingen (San Diego, CA, USA), respectively. The proportions of CD3/CD4-positive lymphocytes producing IFN-γ, IL-4, IL-6 or IL-10 were measured using flow cytometry according to the instructions of the reagent manufacturer (Becton Dickinson). Briefly, 1 ml blood samples were treated immediately with 10 μg/ml of Brefeldin A (BFA) (Sigma Chemical, St. Louis, MO, USA) to block cytokine secretion, keeping the products within cells, and were kept at ambient temperature. Cell surfaces were stained with anti-CD3 and anti-CD4 mAbs. The red cells were lysed with 1 x FACS Lysing Solution (Becton Dickinson) for 10 min at room temperature. After washing with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and NaN_3, the cells were permeabilized with 0.5 ml of 1 x FACS Permeabilizing Solution (Becton Dickinson) for 10 min at room temperature. After two washes, the cells were incubated with optimal concentrations of anti-IFN-γ, anti-IL-4, anti-IL-6 or anti-IL-10 mAb. Samples were analyzed on an EPICS/XL flow cytometer (Coulter Electronics, Inc., Hialeath, FL, USA), and the data were analyzed using a System II software program (Coulter Electronics). The percentages of cytokine-producing $CD4^+$ T cells were calculated. Negative control reagents were used to verify the staining specificity of the experimental antibodies and to serve as a guide for setting markers to delineate positive and negative populations.

ELISA assays. An enzyme immunoassay for detecting antibodies was performed, as described previously (16). Briefly, the MUC1 peptide was coated onto 96-well microtiter plates (ASAHI TECHNO GLASS Corporation, Japan) at 100 μg/ml in PBS (pH 7.4) at 4˚C for 12 h. The plates were washed with PBS, and non-specific binding sites were blocked with 3% HAS/PBS at 37˚C for 1 h. The plates were then incubated with patient sera diluted 1:40 in 1% HSA/PBS at 37˚C for 1 h. After washing with 0.05% Tween-20/PBS, they were incubated with the second antibody, a horseradish peroxidase-conjugated mouse anti-human IgG (DAKO Corporation, Carpinteria, CA, USA) diluted 1:5000 in 1% HSA/PBS, and washed with PBS. Substrate reaction using *O*-phenylenediamine dihydrochloride (DAKO) was determined at 492 nm in an autoreader (Labsystems, Helsinki, Finland). An anti-MUC1 mAb

Table I. *Patient characteristics and clinical response.*

Patient	Age/sex	Disease[a]	Prior therapy[b]	Dose of peptide (mg)	No. of vaccines received	Clinical response[c] (mos.)
1	77/M	PC	none	300	4	PD
2	66/M	BC	S	300	7	n.e.
3	58/F	BC	S, C, R	300	3	PD
4	65/M	PC	S	1000	3	PD
5	51/M	PC	R	1000	3	PD
6	57/M	PC	R	3000	3	PD
7	54/M	BC	none	3000	3	PD
8	49/M	PC	none	3000	3	SD (3)
9	56/M	PC	R	3000	3	PD

[a]PC, pancreas cancer; BC, biliary tract cancer.
[b]S, surgery; C, chemotherapy, R, radiotherapy.
[c]mos; months; PD, progressive disease; SD, stable disease; n.e., not evaluated.

E29 (DAKO) was used as a positive control. All of the serum samples were simultaneously measured in triplicate using one 96-well plate to compare each optical density (OD) value.

Results

Patient characteristics and clinical responses. Nine patients with advanced cancer of the pancreas or bile duct were enrolled in this phase 1 clinical study of a MUC1 peptide vaccination. The detailed characteristics of the patients are shown in Table I. The mean age of the patients was 59.2 years (range: 49-77 years). Six patients were in an inoperable state and 3 had recurrent diseases after surgery. The dose of MUC1 peptides ranged from 300 to 3000 μg; as no apparent toxicity was observed in patients 4 and 5 with a dose of 1000 μg, the highest dose (3000 μg) was started from patient 6.

It was difficult to draw any definitive results from this small-scale phase 1 study with regards to clinical responses and prognostic factor analysis. Nevertheless, the available results might be relevant from the point of view of developing a suitable peptide vaccine. In 9 patients who received MUC1 vaccinations, 8 were eligible for clinical evaluation. Of these, a stable disease (SD) in 1 patient (patient 8) and progressive diseases (PD) in 7 patients were diagnosed 2 weeks after the last vaccination (Table I). Patient 8 was diagnosed with SD by sequential CT scans and measurements of a tumor marker, CA19-9. The clinical response of patient 2 was unclear because recurrence was masked by bacterial cholangitis and subsequent liver abcesses during the observation period after vaccination. Patients 1 and 2 were vaccinated more than 3 times, to comply with their request.

Adverse events. All 9 patients were evaluated for adverse events according to the NCI-CTC (17). The vaccinations were generally well tolerated without hematological toxicity or symptoms of any autoimmune diseases. In all patients, mild reddening, swelling and itching at the vaccination site were observed, for which treatment was not required, and skin tests against MUC1 peptides were negative.

Immunological responses. Immunological responses could be evaluated in 7 out of 9 patients. Intracellular cytokine-positive $CD4^+$ T cell (%) and circulating anti-MUC1 antibody levels before and after vaccination are shown in Figure 1. IL-10 is a Th2 cytokine and IL-6 stimulates the proliferation of antibody-producing cells. In 5 out of 7 patients, both IL-10 and IL-6-producing $CD4^+$ T cell counts tended to decrease after vaccination (Figure 1a). Intracellular IFN-γ or IL-4-positive $CD4^+$ T cells were always under detectable levels (data not shown). The titer of circulating anti-MUC1 IgG antibodies also showed decrease or no change in 5 out of 7 patients. However, it tended to increase in the patient who showed SD for 3 months (Figure 1b).

Discussion

This phase I clinical trial revealed the safety of a vaccine containing 100-mer MUC1 peptides and incomplete Freund's adjuvant in advanced pancreas and bile duct cancer patients. The only adverse event observed was mild reddening and swelling at the vaccination site. A skin test against the MUC1 peptide before vaccination was negative in all patients. Although 1 pancreatic cancer patient showed SD with a modest increase of circulating anti-MUC1 IgG titer after vaccination, 7 other evaluable patients were PD, and the circulating cytokine-producing $CD4^+$ T cell and anti-MUC1 IgG levels tended to decrease in most patients. It seems that these results reflect a rapid progression of

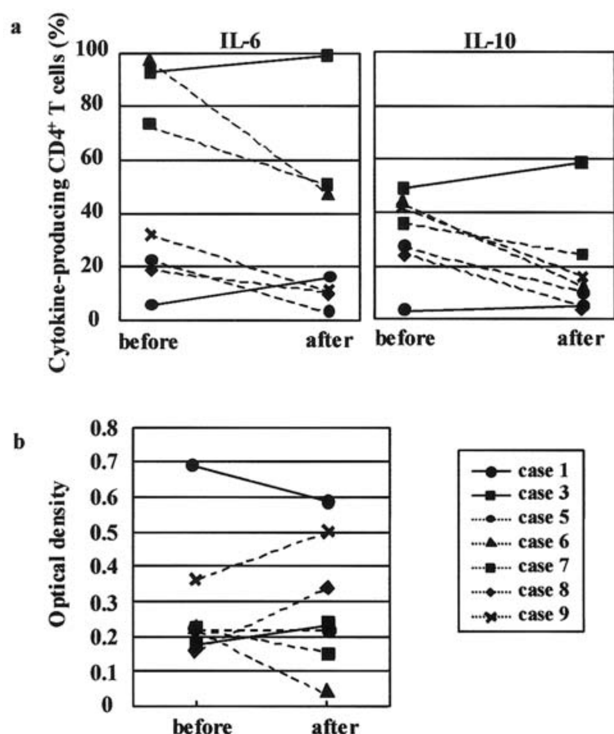

Figure 1. *Intracytoplasmic cytokine and anti-MUC1 IgG antibody assays before and after vaccination. (a) The effect of vaccination on the percentage of IL-6- or IL-10-producing CD4$^+$ T cells in peripheral blood. (b) The effect of vaccination on circulating anti-MUC1 IgG antibody levels. All samples were measured simultaneously in each assay. Results are shown as the mean from triplicate wells.*

advanced pancreatic or bile duct cancer and the presence of a profound immunosuppressive status in those patients.

Pancreatic and biliary tract cancers are two of the worst cancers with regards to 5-year survival rates (19, 20). In pancreatic cancer, several mechanisms for escaping immune surveillance have been shown, including the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, local hindrance of tumor infiltrating lymphocytes (TILs) and loss of the signal transducing CD3ζ chain of TILs (21). On the other hand, we have revealed that MUC1 is involved in the metastatic ability of pancreatic cancer cells (22) and is a poor prognostic factor for cancer of the pancreas (23). Recently, Monti *et al.* demonstrated that MUC1 mucins derived from pancreatic cancer cells suppress the maturation of dendritic cells, resulting in low immunostimulatory functions and the IL-10^{high}IL-12^{low} cytokine secretion phenotype of dendritic cells (24), suggesting that MUC1 *per se* could be a potent immunosuppressive factor. In this context, the findings of Hiltbold *et al.* should be noted. They showed that the efficiency of MUC1 processing by dendritic cells and the resulting strength of CTL activity were inversely correlated with the degree of MUC1 glycocylation (25), and that soluble MUC1 is not transported to late endosomes or MHC class II compartments for processing and binding to class II MHC (26). These suggest that the reduction of tumor burden, which leads to decreased immunosuppressive factors including MUC1, could be essential to cancer therapy with a peptide vaccine.

Ramanathan *et al.* recently reported the results of a phase I study of a MUC1 vaccine in patients with resected (n=15) or locally advanced (n=1) pancreatic cancer without prior chemotherapy or radiotherapy (27). Their MUC1 peptide was the same one as used in our study. Escalating doses of the peptide (100, 300, 1000 and 3000 μg) were admixed with SB-A2 and administered intramuscularly every 3 weeks for 3 doses. Two of 15 resected patients are alive and disease-free at follow-up of 32 and 61 months. Both patients were at stage T3N1M0 at surgical operation. Immunological parameters including delayed-type hypersensitivity, circulating CD8$^+$ T cell's number, the serum level of anti-MUC1 antibody and the cytokine (IFN-γ or IL-4) production of peripheral blood T cells were improved after vaccination in some patients. They observed an almost total suppression of the T cell's ability to make either IFN-γ or IL-4 in every patient before vaccination, which corresponds to our present results, but the production of cytokines increased significantly after vaccination in 5 patients. These findings suggested the importance of reduced tumor burden for peptide vaccine therapy in pancreatic cancer. A phase I study of MUC1 peptide vaccination for resected pancreatic cancer is now being prepared in our departments.

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