preoperative imaging diagnosis. These imaging data were reviewed with respect to tumor location, tumor size, the largest diameter of the main pancreatic duct (MPD), and the presence of mural nodule. We retrospectively compared the preoperative imaging diagnostic data with histopathological findings to obtain sensitivity, specificity, and accuracy.

During ERP, brushing for cytologic analysis was performed, and then pancreatic juice was immediately collected for 10 minutes with a catheter inserted into the pancreatic duct after an intravenous injection of 50 U per body of secretin (Eisai Co Ltd, Tokyo, Japan). A sample was immediately put on ice and divided into 2 sections: one for cytologic examination and the other for measurement of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) levels. The cytologic samples of collected pancreatic juice were immediately put on ice after adding heparin. After pancreatic juice was centrifuged, the cell pellet was smeared on glass slides, fixed in 95% ethanol, and stained with the Papanicolaou technique. For measurement of CEA and CA19-9 levels, the pancreatic juice was centrifuged and the supernatants were measured with CEA or CA19-9 immunometric chemiluminescent assay kit (Bayer Medical Co, Tokyo, Japan) according to the manufacturer's instructions. Serum levels of CEA and CA19-9 were also measured.

The resected pancreas specimens were fixed with 10% formaldehyde solution and serially cut into 5-mm intervals and embedded in paraffin. All tissue sections were stained with hematoxylin-eosin. Then all slides of the resected pancreas were reviewed by independent pathologists. Histologically, 14 patients had benign IPMTs (11 adenomas, including 2 MPD type, 5 branch duct type, and 4 combined type, and 3 dysplasias), and 13 patients had malignant IPMTs (5 adenocarcinomas, including 1 branch duct type and 4 combined type, and 8 invasive adenocarcinomas, including 2 MPD type and 6 combined type) according to the classification by Japan Pancreas Society.[18] We retrospectively reviewed clinicopathologic and preoperative imaging diagnostic data to identify indicative signs of malignant IPMTs.

The optimal cutoff levels for tumor size, mural nodule size, and CEA in pure pancreatic juice for differentiation between benign and malignant IPMTs were sought by constructing receiver operating characteristic (ROC) curves, which were generated by calculating the sensitivities and specificities of tumor size, mural nodule size, and CEA data at several predetermined cutoff points.[19] All values are expressed as mean±SD. The χ^2 test was performed to assess the difference in accuracy of imaging studies and to calculate differences in cases with and without mural nodule between benign and malignant IPMTs. All other statistical analyses were performed with the Mann-Whitney *U* test. Statistical significance was defined as $P<.05$.

RESULTS

CLINICAL CHARACTERISTICS

Clinical characteristics of the enrolled patients are given in **Table 1**. There were no significant differences in age, sex, and symptoms between the patients with benign and malignant IPMTs. Fourteen (52%) of 27 patients were asymptomatic. The reasons for performing imaging studies in asymptomatic patients were as follows: follow-up of other diseases, including diabetes mellitus, in 3 benign IPMT patients; annual medical examinations in 6 benign IPMT patients; other diseases in 4 malignant IPMT patients; and annual medical examinations in 1 malignant IPMT patient. The main symptom was pain in 11 (41%) of 27 patients. Only the malignant IPMT group experienced weight loss (15%). Twenty (74%) of 27 patients had no history of pancreatic disease. Seven (26%) of 27 patients had diabetes mellitus or chronic pancreatitis.

Table 1. Clinical Characteristics of 27 Patients With Intraductal Papillary Mucinous Tumors (IPMTs) of the Pancreas

Characteristic	Benign IPMT Group (n = 14)	Malignant IPMT Group (n = 13)
Age, mean ± SD, y	65 ± 3	72 ± 1
Sex ratio, M:F	1.3:1	1.7:1
Symptoms, No. (%)		
Asymptomatic	9 (64)	5 (38)
Abdominal pain	5 (36)	6 (46)
Weight loss	0	2 (15)
Pancreatic disease, No. (%)		
None	11 (79)	9 (69)
Diabetes mellitus	1 (7)	3 (23)
Chronic pancreatitis	2 (14)	1 (8)

PREOPERATIVE IMAGING FINDINGS

The number of IPMTs located in the total pancreas was 4 (31%) of 13 in the malignant IPMT group and none in the benign IPMT group. Tumors were located in the pancreatic head in 6 patients in both the benign IPMT group and the malignant IPMT group and in the pancreatic body (tail) in 8 patients in the benign IPMT group and 3 in the malignant IPMT group. The dilatation of MPD measured by ERP was 7.8±6.5 mm in the benign IPMT group and 10.7±6.5 mm in the malignant IPMT group. There were no significant differences in location and dilatation of MPD between the benign and malignant IPMT groups. However, the mean tumor size of 81±18 mm in the malignant IPMT group was significantly larger than that of 31±4 mm in the benign IPMT group ($P=.002$). The mural nodules were present in 6 patients with benign IPMTs and 12 patients with malignant IPMTs. There was a significant difference between the benign IPMT group and the malignant IPMT group related to the presence of mural nodule ($P=.007$). In addition, the mean mural nodule size of 9.8±4.4 mm in the malignant IPMT group was significantly larger than that of 3.3±5.7 mm in the benign IPMT group ($P=.002$). Sensitivity, specificity, and accuracy for differentiating benign from malignant IPMTs were 75%, 50%, and 62% by US; 92%, 85%, and 89% by CT; 91%, 64%, and 78% by endoscopic US; and 91%, 57%, and 73% by ERP, respectively. The accuracy rate by CT was higher than that of US ($P=.009$).

CYTOLOGIC ANALYSIS OF PURE PANCREATIC JUICE

Brushing for cytologic analysis was performed. A total of 20 mL of pure pancreatic juice was collected in 23 patients by intravenous injection of secretin. The cyto-

Table 2. Comparison of Cytologic Analyses of Pure Pancreatic Juice Between Benign and Malignant Intraductal Papillary Mucinous Tumor (IPMT) Groups

Class	Benign IPMT Group (n = 11)	Malignant IPMT Group (n = 12)
I	0	1
II	7	7
III	3	2
IV	1	1
V	0	1

logic specimen of pure pancreatic juice after brushing could be examined in 11 patients in the benign IPMT group and 12 patients in malignant IPMT group (**Table 2**). In the remaining 4 patients, deep cannulation for brushing for cytologic examination could not be performed or a sufficient amount of sample of pure pancreatic juice could not be collected for cytologic analysis. Two (17%) of 12 patients with malignant IPMTs were diagnosed as having a malignancy (class IV or V), and 10 (91%) of 11 patients with benign IPMTs were diagnosed as having benign disease (class I, II, or III). One patient diagnosed as having a cytologic malignancy in the benign IPMT group had histologic dysplasia and premalignant lesions.

TUMOR MARKERS IN PURE PANCREATIC JUICE AND SERUM

Both CEA and CA19-9 levels in pancreatic juice could be measured in 11 patients in the benign IPMT group and 9 patients in the malignant IPMT group. In the other 7 patients, a sufficient amount of supernatant could not be collected after the centrifugation of pancreatic juice. Serum CEA and CA19-9 levels could also be measured in all patients. Carcinoembryonic antigen levels in pancreatic juice in the malignant IPMT group (3051±7556 ng/mL) were significantly higher than those (41±80 ng/mL) in the benign IPMT group ($P=.003$). However, we found no significant differences between the benign and malignant IPMT groups in serum CEA levels (1.8±1.2 ng/mL vs 3.0±2.4 ng/mL), CA19-9 levels in pancreatic juice (412±416 ng/mL vs 9630±13511 ng/mL), and serum CA19-9 levels (18±35 ng/mL vs 262±777 ng/mL).

PREDICTIVE FACTORS FOR MALIGNANT IPMT DIAGNOSIS

This study clarified that predictive factors for differentiating benign IPMTs from malignant IPMTs were tumor size, mural nodule size, and CEA levels in pure pancreatic juice. The ROC curves for tumor size, mural nodule size, and CEA levels of pure pancreatic juice are presented in **Figure 1**. With regard to the tumor size and mural nodule size by preoperative imaging findings, diagnostic cutoff levels for differentiation between benign IPMTs from malignant IPMTs were 30 and 5 mm, respectively (**Figure 2**). The sensitivities of the cutoff line in tumor size and mural nodule were 92% and 85%, respectively, and the specificity of the cutoff line was 71% for both. Therefore, the accuracies were 81% and 81%, respectively. The cutoff level of CEA in pure pancreatic juice was at 110 ng/mL for differentiation between benign and malignant IPMTs (**Figure 3**). The sensitivity of the CEA cutoff line was 78%, the specificity was 91%, and the accuracy was 80%.

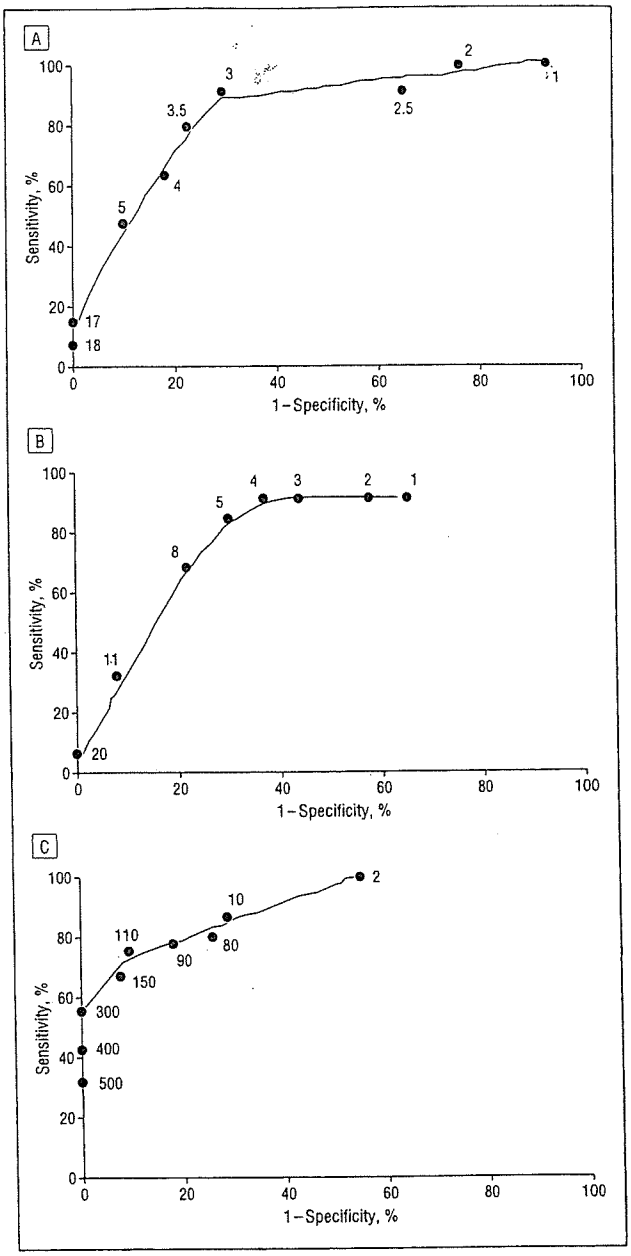

Figure 1. Receiver operating characteristic curve of tumor size (A), mural nodule size (B), and carcinoembryonic antigen level of pure pancreatic juice (C) for estimation of a cutoff level differentiating malignant from benign intraductal papillary mucinous tumors.

COMMENT

The early diagnosis of malignant IPMTs is important for improving prognosis. Invasive carcinoma derived from IPMTs has been reported to be the most important factor that influences survival in patients with IPMTs and

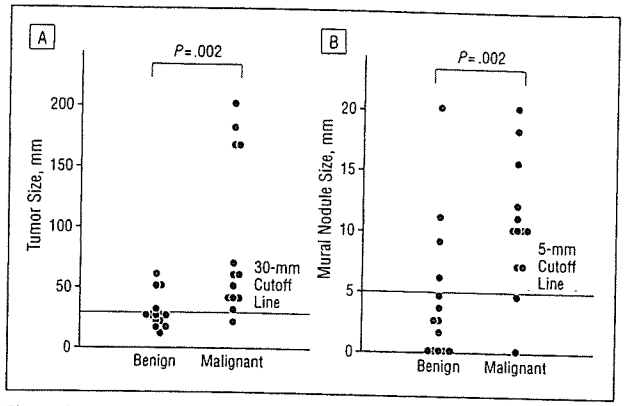

Figure 2. The distribution of tumor size (A) and mural nodule size (B) in the benign intraductal papillary mucinous tumor (IPMT) group (n=14) and the malignant IPMT group (n=13). The cutoff levels of tumor size and mural nodule size are 30 and 5 mm, respectively.

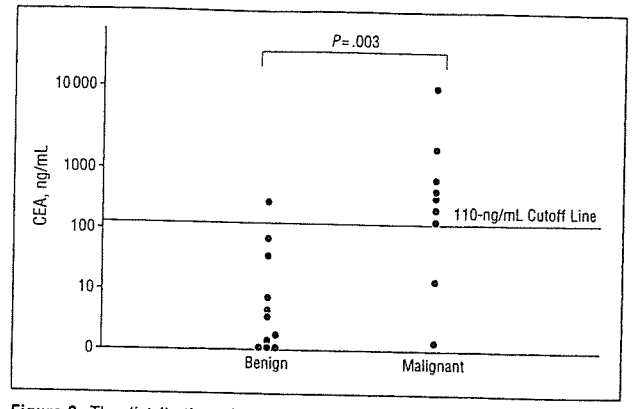

Figure 3. The distribution of carcinoembryonic antigen (CEA) level in pure pancreatic juice in the benign intraductal papillary mucinous tumor (IPMT) group (n=11) and the malignant IPMT group (n=9). The cutoff CEA level in pure pancreatic juice is 110 ng/mL.

predicts a low survival compared with noninvasive carcinoma.[5,20-23] In particular, prognosis in invasive carcinoma was reported to be influenced mainly by the extrapancreatic tumor extension or lymph node metastasis.[5,23] It is important to differentiate malignant from benign IPMTs before IPMTs invade the extrapancreatic tissue. However, differentiating malignant from benign IPMTs preoperatively using only a single diagnostic method is still difficult despite significant improvement in imaging techniques. Indeed, in this study, preoperative CT diagnosis had a higher accuracy rate than US; however, controversy still exists regarding which imaging modality is best for preoperative diagnosis.

In the present study, we simultaneously analyzed clinicopathological features, imaging findings, cytologic analysis results, and tumor markers (CEA and CA19-9) in pure pancreatic juice to evaluate the characteristics of invasive carcinoma derived from IPMTs. We clarified predictive factors for preoperative diagnosis of malignant IPMTs. Previous studies have shown that tumor size, the size of mural nodule, or the dilatation of MPD were important factors in diagnosing malignant IPMTs.[24-26] Although tumor size and mural nodule size are not uniform according to investigators, and no consensus concerning the early diagnosis of malignant IPMTs has been attained as yet,[9-15] we propose that tumors larger than 30 mm and mural nodules larger than 5 mm as determined by imaging findings indicate a strong possibility of malignancy. According to preoperative imaging diagnosis, one should select function-preserving procedures for benign IPMTs, and intraoperative frozen section of the cut end of the pancreas should provide the adequate pancreatic resection.

Although cytologic examination of pure pancreatic juice or detection of K-*ras* mutation has previously indicated the diagnosis of malignant IPMTs,[14,15,27-29] the accuracy was generally reported to be limited, and the diagnosis of malignant IPMTs is difficult using only a single method. In our study, the sensitivity of cytologic examination was low (17%) and indicated that it is difficult to differentiate malignant IPMTs only by cytologic examination of pure pancreatic juice. Some previous studies[30-32] reported that the CEA level in pancreatic juice in pancreatic cancer was significantly higher than that in benign pancreatic disease. However, the measurement of CEA and CA19-9 levels in pure pancreatic juice has not been performed for differentiation of malignant and benign IPMTs. We compare benign with malignant IPMTs for the first time, to our knowledge, by measurement of CEA and CA19-9 levels of pure pancreatic juice. The CEA levels in pancreatic juice in the malignant IPMT group were significantly higher than those in the benign IPMT group. In this study, we propose that the introduction of a cutoff value for CEA level (110 ng/mL) in pure pancreatic juice allowed most of the false-positive results for benign IPMTs to be ruled out. The measurement of CEA levels in pure pancreatic juice was strongly suggested to be a useful diagnostic method to differentiate malignant from benign IPMTs. In this study, we had incidents of high CEA levels in pure pancreatic juice in the benign IPMT group. The cases were histologically severe atypia with premalignant lesions, which indicates a high potential for CEA production.[2] On the other hand, with regard to the incidents of low CEA levels in the malignant IPMT group, there was a possibility that the collection of pure pancreatic juice was not appropriate for stenosis of the MPD by invasive carcinoma. It has been reported that the immunohistochemical staining of CEA is strongly positive in the cytoplasm of the tumor cells in the invasive IPMTs[22] and shows a relation to the cellular atypia grade of IPMTs.[2] Therefore, CEA may much more easily migrate to pancreatic juice than serum, and CEA levels in pure pancreatic juice may be much higher in malignant IPMTs than in benign IPMTs even if CEA levels in serum may be normal.

Future studies using immunohistochemical staining for CEA should be performed to clarify the correlation between pancreatic juice CEA and expression of CEA in IPMT cells. We must determine preoperative factors that are predictive for the accuracy of diagnosis of malignant IPMTs. In our study, tumor size larger than 30 mm, mural nodule size larger than 5 mm, and CEA levels higher than 110 ng/mL in pure pancreatic juice were identified as predictive factors for diagnosis of malignant IPMTs. Further studies in larger series of patients with IPMTs are necessary to confirm the data presented herein.

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Distribution of CD4(+)CD25^{high} Regulatory T-Cells in Tumor-Draining Lymph Nodes in Patients with Gastric Cancer

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Background. **Regulatory T-cells (T-regs) can inhibit the immune response mediated by T-cells. There is an increasing evidence that there is an increased proportion of T-regs in PBLs and tumor-infiltrating lymphocytes in several different human malignancies, although the mechanism remains unclear. In the present study, we evaluated the prevalence of CD4(+)CD25^{high} T-regs in tumor-draining lymph nodes in patients with gastric cancers.**

Materials and methods. **Regional lymph nodes in the stomach of the patients with gastric cancer (n = 44) were classified into N1 regional lymph nodes adjacent to the gastric tumor and N2 regional lymph nodes marginally distant from the tumor. The population of CD4(+)CD25^{high} T-cells as a percentage of total CD4(+) cells was evaluated by flow cytometric analysis with triple-color staining. Cytokine production (IL-10 and IFN-γ) was evaluated by intracellular cytokine staining and the antiproliferative function of CD4(+)CD25(+) cells positively selected by magnetic beads was measured by evaluating the proliferative activity of CD4(+)CD25(–) cells in response to anti-CD3 plus anti-CD28 in the presence of autologous CD4(+)CD25(+) cells.**

Results. **The percentage of CD4(+)CD25^{high} T-cells in N1 regional lymph nodes (3.1 ± 0.3%) was significantly higher than that of control mesenteric lymph nodes (1.2 ± 0.3%, $P < 0.01$). Furthermore, a more extended area (N2) of regional lymph nodes, as well as adjacent lymph nodes (N1) to the tumors, was involved in an increased prevalence of CD4(+)CD25^{high} T-cells according to the disease progression. The functional evaluations confirmed that CD4(+)CD25 ^{high} T-cells derived from the lymph nodes have an inhibitory activity corresponding to T-regs.**

Conclusions. **The populations of CD4(+)CD25^{high} T-cells in the regional lymph nodes in patients with gastric cancer were significantly higher in comparison to those in control lymph nodes. The increased prevalence of T-regs may be one of the explanations for impaired cell-mediated immunity in cancer-bearing hosts.**

Key Words: **regulatory T-cells; gastric cancer; lymph nodes; CD4(+)CD25(+) T-cells.**

INTRODUCTION

Regulatory T-cells (T-regs) are thought to be a functionally unique population of T-cells and function to maintain immune homeostasis [1–4]. T-regs can inhibit the immune response mediated by CD4(+) and CD8(+)T-cells, and it was reported that T-regs play an important role in preventing allograft rejection, graft-versus-host disease, and autoimmune disease [5, 6].

Patients and experimental models with cancer showed that T-regs down-regulated the activity of effector function against tumors, resulting in T-cell dysfunction in cancer-bearing hosts [7, 8]. Recently, an increased population of T-regs was reported in patients with gastric cancer [9–11], colorectal cancer, gall bladder cancer, pancreatic cancer [11, 12], ovarian cancer, [13] and lung cancer [14]. Moreover, we showed that the population of T-regs in tumor-infiltrating lymphocytes (TILs) of patients with advanced gastric cancer was significantly higher than that of TILs in patients with early gastric cancer [9]. In addition, gastric cancer patients with higher percentages of T-regs had a poorer prognosis than those with lower percentages [11].

Within the CD4(+) T-cells with suppressive func-

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tion, there were at least three different cell populations: CD25(+)CD4(+) naturally occurring T-regs, IL-10-producing Tr1 cells, and Th3 cells [15–22]. Furthermore, although CD25(+)CD4(+) naturally occurring T-regs were originally characterized by the coexpression of CD4 and CD25 in the mouse, it has recently been shown in humans that the CD4(+)CD25^{high} subset corresponded to naturally occurring T-reg populations with suppressive capacity, while CD4(+)CD25^{int} T cells contaminated effector or memory T-cells with no suppressive capacity [23]. In the present study, therefore, we focused on the prevalence of the CD4(+)CD25^{high} subset as T-regs in lymph nodes.

There is no clear evidence for the mechanisms of induction of T-regs in cancer-bearing hosts. There are several possibilities, including the specific expansion of T-regs induced by cancer-derived factors or physiological defense phenomena against continuous inflammation induced by cancer.

To further analyze the induction of T-regs, we evaluated the distribution of T-regs as characterized by CD4(+)CD25^{high} in tumor-draining lymph nodes with gastric cancer and clarified the difference of T-reg prevalence between the lymph nodes adjacent to tumors and those distant from tumors.

MATERIALS AND METHODS

Patients

Forty-four patients with gastric cancer were divided into two groups: those with early disease (n = 31) corresponding to stage I according to the TNM classification for gastric cancer (UICC) and those with advanced disease corresponding to stages II, III, and IV (n = 13). Regional lymph nodes in the stomach of patients with gastric cancer were classified into N1 regional lymph nodes adjacent to the gastric tumor and N2 regional lymph nodes marginally distant from the tumor according to the Japanese Classification of Gastric Carcinoma [24]. N1 and N2 regional lymph nodes and mesenteric lymph nodes used as controls which were not regional lymph nodes of the stomach were collected during surgery. None of the patients received radiotherapy, chemotherapy, or other medical interventions before the surgery. The characteristics of the study subjects are summarized in Table 1. This study was approved by the ethical committee of the University of Yamanashi, and written informed consent was obtained from all individuals.

Cell Preparations

The lymph nodes were homogenized by mechanical mincing and suspended as a single-cell suspension after being passed through a cell strainer (Becton–Dickinson Labware, Franklin Lakes, NJ).

For the separation of CD4(+)CD25(+) or CD4(+)CD25(-) cells, lymph node cells were separated with Macs CD4 Multisort kit and CD25 Microbeads (Miltenyi Biotec, Germany) using magnetic separation columns according to the manufacturer's guidelines. The enriched cells were >93% CD4(+)CD25(+) or CD4(+)CD25(-) cells as determined by flow cytometry.

TABLE 1

Patient Characteristics

	Gastric cancer (n = 44)	Gastric cancer with early disease[a] (n = 31)	Gastric cancer with advanced disease[a] (n = 13)
Gender (male: female)	32:12	24:7	8:5
Age (years)	65.7 ± 11.5	66.6 ± 8.3	63.6 ± 17.2
TNM stage[b]			
Ia	24	24	
Ib	7	7	
II	4		4
IIIa	3		3
IIIb	2		2
IV	4		4

[a] Gastric cancer with early disease corresponds to stage I and those with advanced disease corresponds to stages II, III, and IV.

[b] Stage according to the TNM classification for gastric cancer (UICC).

Flow Cytometric Analysis

Lymph node cells were stained for the molecules to determine their immunophenotype using anti-CD25-FITC, anti-CD4-PerCP, anti-CD3-APC, anti-CD152 (CTLA4)-PE, anti-CD45RO-PE, and anti-CCR2-PE (DAKO, Glostrup, Denmark) antibodies. Triple- or four-color flow cytometry was performed using FACSCalibur (Becton–Dickinson, San Jose, CA). Cells were analyzed using Cell Quest software.

To analyze the prevalence of T-regs, CD4(+)CD25^{high} cells after gating on CD3(+) were evaluated and expressed as a percentage of total CD4(+) cells.

Intracellular Cytokine Assay

Briefly, cells were incubated in RPMI 1640 (Sigma-Aldrich Cheme, Taufkirchen, Germany) with 5% FCS, 2 μl of IC block (Biosource, Camarillo, CA), and 2 μl of phorbol myristate acetate (PMA, Sigma-Aldrich C; final concentration of 25 ng/μl) for 4 h at 37°C. After staining with anti-CD25-FITC and anti-CD4-PerCP (DAKO) for 30 min on ice and followed by washing, cells were fixed in IC-Fix (Biosource) for 10 min on ice and washed with IC perm (Biosource) twice. Thereafter, cells were stained with IgG negative control (Biosource), rat antihuman IL-10-PE (Biosource), or rat antihuman IFN-γ-PE (Biosource) and washed twice.

Cell Proliferation Assay

Purified CD4(+)CD25(−) cells (1.25×10^4) from N1 regional lymph nodes in patients with advanced disease were incubated with the indicated ratio of autologous CD4(+)CD25(+) cells on anti-CD3 mAb (10 ng/ml, DAKO)-coated 96-well round-bottomed plates (Becton–Dickinson) in the presence of anti-CD28 mAb (10 μg/ml, PharMingen, San Diego, CA). Cell proliferation was measured by the incorporation of [^{3}H]thymidine (Moravek Biochemicals, Inc., California, CA, 1 μCi/well). The cells were harvested after 16 h and thymidine incorporation was expressed as counts per minute.

Immunohistochemistry

N1 regional lymph nodes from the all patients were collected during surgery and were immediately embedded in OCT compound (Sakura Finetek U.S.A, Torrance, CA) and frozen at −80°C. Sections

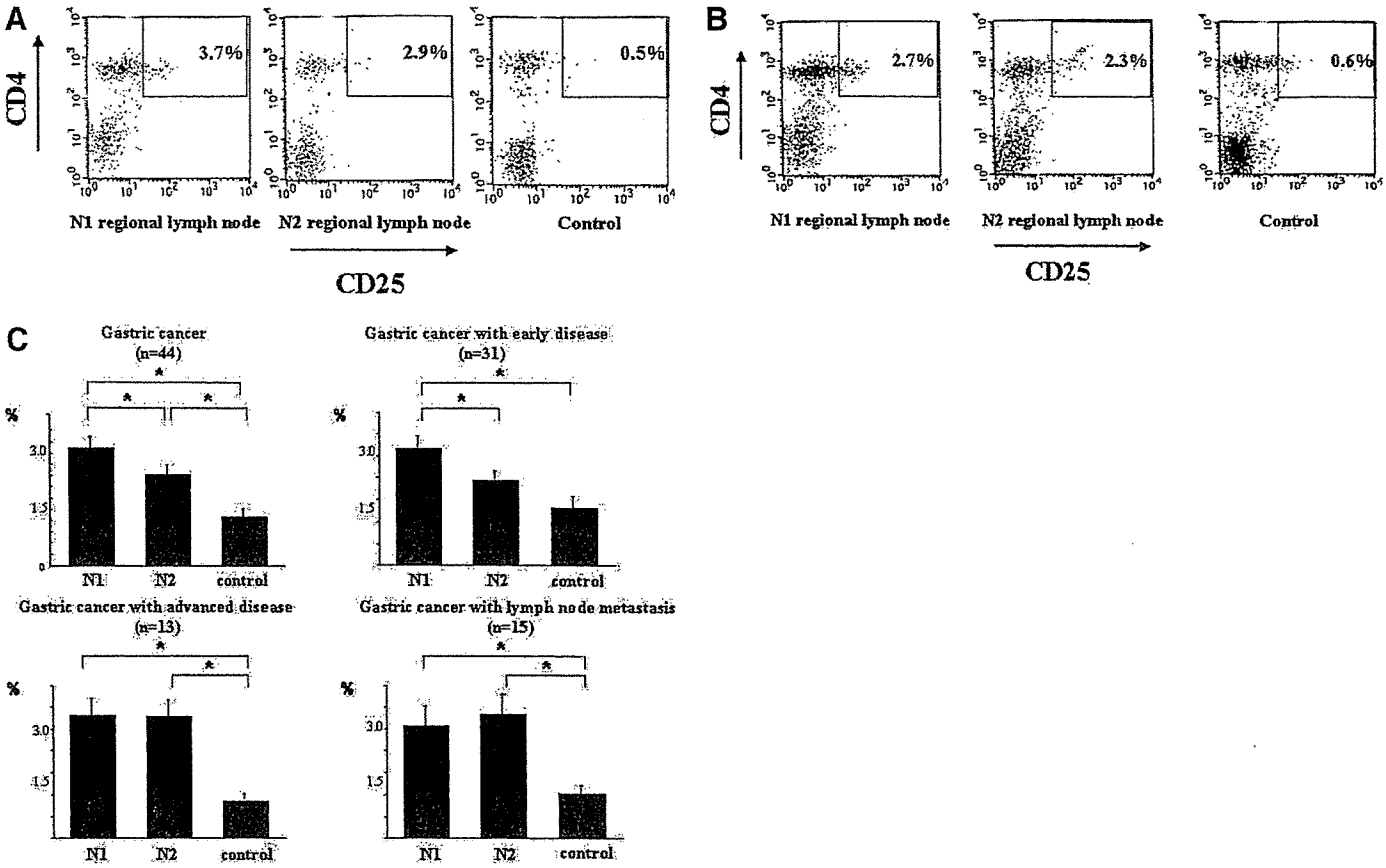

FIG. 1. Increased populations of CD4(+)CD25^{high} T-cells in regional lymph nodes with gastric cancer. The population of CD4(+)CD25^{high} T-cells as a percentage of total CD4(+) cells in lymph nodes was evaluated by flow cytometric analysis with triple-color staining. Regional lymph nodes in the stomach of patients with gastric cancer were classified into N1 regional lymph nodes adjacent to the gastric tumor and N2 regional lymph nodes marginally distant from the tumor. N1 and N2 regional lymph nodes and mesenteric lymph nodes as a control were collected during surgery. Representative flow cytometric data from gastric cancer patients with advanced disease (A), those from the patients with lymph node metastasis (B), and summarized data from all individuals (C) are shown. Rectangular gates in A and B indicate the CD4(+)CD25^{high} T-cell populations. $^*P < 0.05$.

5 μm thick were prepared and fixed in acetone for 10 min. Endogenous peroxidase activity was blocked by incubation for 10 min with Peroxidase Blocking Reagent (DAKO Envision System, Peroxidase; DAKO Corporation, Carpinteria, CA). Sections were preincubated with normal rabbit serum (1:10, DAKO, Glostrup, Denmark) for 10 min and incubated for 1 h with primary mAbs directed against CD1a (1:20, DAKO) or CD83 (1:20, DAKO). As a negative control, normal mouse serum was used without primary antibody. The sections were incubated with Envision-labeled polymer reagent (DAKO, Copenhagen, Denmark) according to the manufacturer's recommendations. This polymer reagent is a peroxidase-labeled polymer conjugated to goat antirabbit and goat antimouse immunoglobulins in Tris–HCl buffer containing carrier protein and an antimicrobial agent. Diaminobenzidine (DAKO) was then used as a chromogen, and the sections were counterstained with hematoxylin. Positive cells were counted by light microscopy and expressed as the average number of positive cells in five randomly selected areas at 400× magnification (Olympus, Tokyo, Japan).

Statistical Analysis

Differences between the values were determined using the Student's t test. The correlation between the prevalence of CD1a- and CD83-positive cells and CD4(+)CD25^{high} T-cells was analyzed by Spearman's rank correlation coefficient. Significance was determined when P values were <0.05.

RESULTS

Increased Populations of CD4(+)CD25^{high} T-Cells in Regional Lymph Nodes with Gastric Cancer

Lymph node cells with gastric cancer were examined for the prevalence of CD4(+)CD25^{high} T-cells as T-regs. The population of CD4(+)CD25^{high} T-cells as a percentage of total CD4(+) cells was evaluated by flow cytometric analysis with triple-color staining. Regional lymph nodes in the stomach of patients with gastric cancer were classified into N1 regional lymph nodes adjacent to the gastric tumor and N2 regional lymph nodes marginally distant from the tumor. In representative flow cytometric data with gastric cancer with advanced disease, the prevalence of CD4(+)CD25^{high} T-cells in N1 lymph nodes was higher than that in control mesenteric lymph nodes (Fig. 1A). Also, in pa-

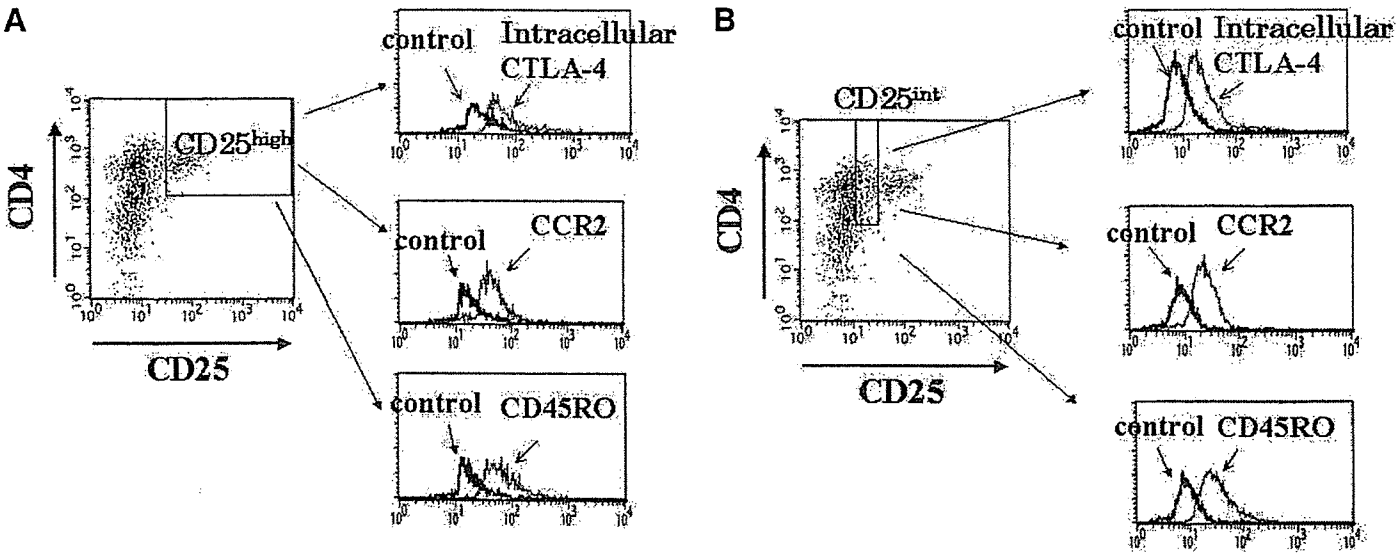

FIG. 2. The expression of CD45RO, CCR2, and intracellular CTLA-4(CD152) on CD4(+)CD25^{high} T-cells. Representative flow cytometric data from N1 (A, B) from advanced disease patients showed the expression of CD45RO, CCR2, and intracellular CTLA-4 after gating of CD4(+)CD25^{high} (A) or CD4(+)CD25^{int} (B).

tients with advanced gastric cancer with lymph node metastasis, the proportion of CD4(+)CD25^{high} T-cells in N1 lymph nodes increased in comparison to that in control mesenteric lymph nodes (Fig. 1B). Summarized data from all individuals indicated that the percentage of CD4(+)CD25^{high} T-cells in N1 regional lymph nodes (3.1 ± 0.3%) was significantly higher than that in control mesenteric lymph nodes(1.2 ± 0.3%, $P < 0.01$), as shown in Fig. 1C. In gastric cancer with early disease, the percentage of CD4(+)CD25^{high} T-cells in N1 lymph nodes (3.3 ± 0.3%) was significantly higher than that in N2 lymph nodes (2.4 ± 0.3%, $P < 0.05$). Moreover, in gastric cancer with advanced disease, the percentage of CD4(+)CD25^{high} T-cells in N2 lymph nodes (3.3 ± 0.2%), as well as N1 lymph nodes (3.3 ± 0.2%), was significantly higher than that in control mesenteric lymph nodes (0.9 ± 0.3%, $P < 0.02$). These observations indicated that tumor-draining lymph nodes with gastric cancer had an increased prevalence of CD4(+)CD25^{high} T-cells in comparison to control mesenteric lymph nodes. Furthermore, a more extended area (N2) of regional lymph nodes, as well as lymph nodes (N1) adjacent to the tumors, was involved in an increased prevalence of CD4(+)CD25^{high} T-cells according to the disease progression.

To characterize the CD4(+)CD25^{high} T-cells, we analyzed markers such as CD45RO, CCR2, and intracellular CTLA-4 (CD152). The expressions of CTLA-4, CD45RO, and CCR2 were analyzed in the gated CD4(+)CD25^{high} and CD4(+)CD25^{int} populations. Representative flow cytometric data from N1 lymph nodes showed that most CD4(+)CD25^{high} T-cells expressed CD45RO, CCR2, and intracellular CTLA-4 (Fig. 2A). In addition, most CD4(+)CD25^{int} T-cells derived from N1 lymph nodes also expressed CD45RO, CCR2, and intracellular CTLA-4 (Fig. 2B).

The CD4(+)CD25^{high} T-Cells Separated from Lymph Nodes Functionally Corresponded to T-Regs

To perform a functional analysis of CD4(+)CD25(+) T-cells, CD4(+)CD25(+) and CD4(+)CD25(−) cells were purified from N1 regional lymph nodes in gastric cancer patients with advanced disease ($n = 7$) with magnetic beads. The purity of CD4(+)CD25(+) was

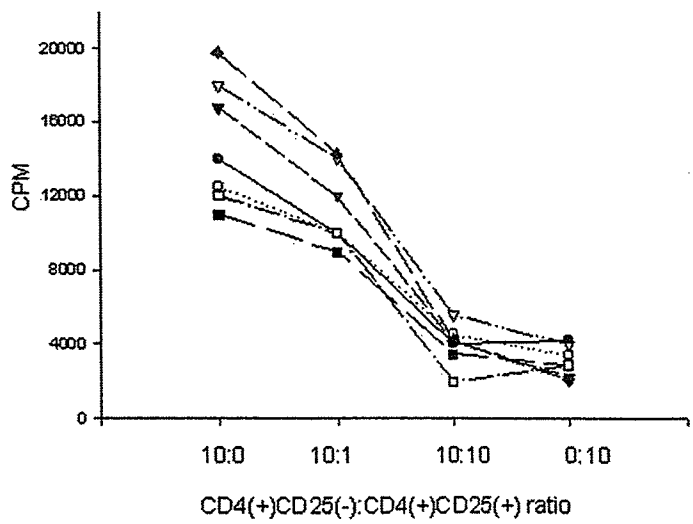

FIG. 3. Functional analysis of CD4(+)CD25(+) T-cells. CD4(+)CD25(+) and CD4(+)CD25(–) cells were purified from the N1 regional lymph nodes in gastric cancer patients with advanced disease ($n = 7$) using magnetic beads. The proliferation activity of CD4(+)CD25(–) cells in response to anti-CD3 plus anti-CD28 in the presence of autologous CD4(+)CD25(+) cells is indicated as a ratio.

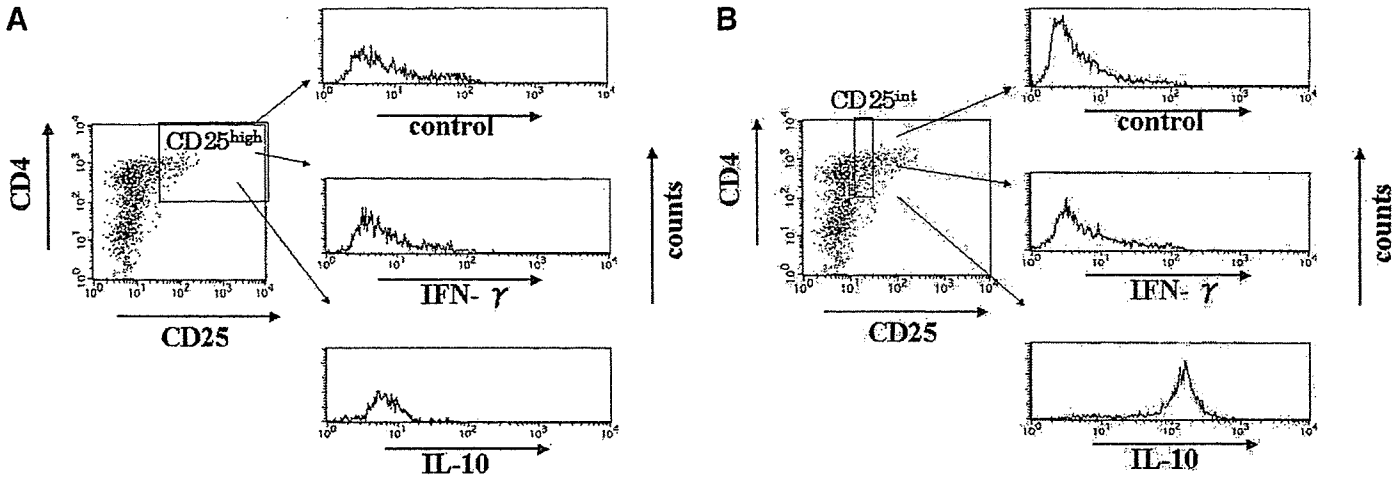

FIG. 4. Intracellular cytokine staining of CD4(+)CD25^{high} T-cells. Intracellular cytokine staining (IFN-γ and IL-10) was performed gated on CD4(+)CD25^{high} T-cells (A) or CD4(+)CD25^{int} T-cells (B) derived from N1 regional lymph nodes.

always more than 93%. Then we assessed the antiproliferative function of CD4(+)CD25(+) by evaluating the proliferative activity of CD4(+)CD25(–) cells in response to anti-CD3 plus anti-CD28 in the presence of autologous CD4(+)CD25(+) cells. The proliferation of CD4(+)CD25(–) cells was inhibited in the presence of CD4(+)CD25(+) cells in a dose-dependent manner (Fig. 3).

Furthermore, representative flow cytometric data with intracellular cytokine staining showed that CD4(+)-CD25^{high} T-cells derived from N1 lymph nodes produced small amounts of IL-10 (Fig. 4A). In contrast, CD4(+)-CD25^{int} T-cells derived from N1 lymph nodes produced large amounts of IL-10 (Fig. 4B). Thus, CD4(+)CD25^{high} T-cells separated from the lymph nodes functionally corresponded to T-regs.

Correlation of DC Maturation Status with the Prevalence of T-Regs in Regional Lymph Nodes

Since it was reported that repetitive stimulation with immature DCs induced the development of T-regs [25], we evaluated the prevalence of CD1a-positive cells as immature DCs and CD83-positive cells as mature DCs in N1 regional lymph nodes immunohistochemically and compared the prevalence of CD1a- or CD83-positive cells with a population of CD4(+)CD25^{high} T-cells in the same lymph node. As a result, there were no significant correlations between the prevalence of CD1a(+) cells and CD4(+)CD25^{high} T-cells ($r = -0.09$, $P = 0.62$) or between the prevalence of CD83(+) cells and CD4(+)CD25^{high} T-cells ($r = 0.003$, $P = 0.83$).

DISCUSSION

The current report provides the first evidence of the distribution of CD4(+)CD25^{high} T-regs in tumor-draining lymph nodes in gastric cancer. We showed that tumor-draining lymph nodes with gastric cancer had an increased prevalence of CD4(+)CD25^{high} T-cells in comparison to nonregional mesenteric control lymph nodes. Furthermore, a more extended area (N2) of the regional lymph nodes, as well as lymph nodes (N1) adjacent to tumors, was involved in an increased prevalence of CD4(+)CD25^{high} T-cells according to disease progression.

There is accumulating evidence that there is an increased proportion of CD4(+)CD25(+) T cells in PBLs and TILs in several different human malignancies [9–11, 13–15]. We recently reported on increased populations of CD4(+)CD25(+) T-cells in PBL and TILs in patients with gastric cancer [9]. In the present study, we report that tumor-draining lymph nodes with gastric cancer had an increased prevalence of CD4(+)CD25^{high} T-cells in comparison to nonregional mesenteric control lymph nodes. Of note, a more extended area (N2) of the regional lymph nodes, as well as lymph nodes (N1) adjacent to tumors, was involved in an increased prevalence of CD4(+)CD25^{high} T-cells according to disease progression. These observations suggested that tumor-derived factors may induce and expand T-reg pools.

There is no clear evidence for the mechanisms of induction of T-regs in cancer-bearing hosts. There are several possibilities, including specific expansion of T-regs induced by cancer-derived factors or physiological defense phenomena against continuous inflammation induced by cancer. It was reported that there is a difference in sensitivity to clonal depletion against specific antigens or apoptosis induced by Fas–FasL interaction between CD4(+)CD25(+) T-cells and CD4(+)CD25(–) T-cells [26], in which CD4(+)CD25(+) T-regs are resistant to clonal depletion or apoptosis. This suggests that tumor-derived factors can induce apoptosis selectively in CD4(+)-CD25(–) T-cells. Alternatively, it was also shown that repetitive stimulation with immature DCs induced the

development of T-regs [25]. Since tumor-derived factors such as VEGF inhibit the functional maturation of immature DCs [27], it is possible that immature DCs inside a tumor microenvironment may induce the formation of T-regs. However, in the present study, we could not show a significant correlation between immature DCs and T-regs in tumor-draining lymph nodes with gastric cancer.

As a functional consequence of an increased proportion of T-regs in a cancer-bearing host, it was shown that T-regs can suppress immune responses of other CD4(+) and CD8(+) cells [14, 16]. Recently, it was also shown that CD4(+)CD25(+) T-regs suppress the proliferation, cytokine secretion, and cytotoxic activity of Vα24(+)NKT cells [22]. Thus, one of the explanations for impaired cell-mediated immunity in cancer-bearing hosts is the increased prevalence of T-regs.

Importantly, it has recently been shown that human CD4(+)CD25(+) T cells are not a homogenous population and could be split into suppressive and nonsuppressive fractions by sorting $CD25^{high}$ and $CD25^{int}$ cells [28]. Furthermore, it was proposed that only a subset of high levels of CD25 and CTLA-4 within CD4(+)CD25(+) T-cells was capable of inducing suppressive function and there was a difference in the cytokine production profile between $CD25^{high}$ and $CD25^{int}$ cells within CD4(+)-CD25(+) T cells [23, 29]. In the present study, we focused on CD4(+)$CD25^{high}$ T-regs and evaluated the prevalence of CD4(+)$CD25^{high}$ T-cells in the lymph nodes. These subsets showed strong expressions of intracellular CTLA-4 and CD45RO and small amounts of IL-10 production, as indicated in the previous report that CD4(+)$CD25^{high}$ T-cells may correspond to human naturally occurring T-regs [23]. These results indicated that the CD4(+)$CD25^{high}$ T-cells in the present study corresponded to T-regs functionally. Thus, further studies are desirable at a cloned T-cell level or molecular levels targeted to more specific markers or functional profiles.

Recently, immunotherapy for cancer, including cancer vaccination or adoptive transfer of T-cells, has been tested, but the results were limited in their effect on the regression of established tumors [30, 31]. The increased population of T-regs, especially in the tumor environment, is one of the problems to be resolved in the immunotherapy of cancer. It has been shown that the efficacy of therapeutic vaccination for cancer could be enhanced by removing T-regs [32]. A better understanding of the underlying mechanism of T-regs regulation or a strategy for controlling T-regs may lead to more effective immunotherapies against cancer.

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A Newly Identified MAGE-3-Derived, HLA-A24-Restricted Peptide Is Naturally Processed and Presented as a CTL Epitope on MAGE-3-Expressing Gastrointestinal Cancer Cells

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Key Words
MAGE-3 · HLA-A24 · Gastrointestinal tract cancer · Epitope

Abstract
Purpose: In order to broaden the possibility for anti-MAGE-3 immune targeting, it is important to identify HLA-A24-restricted epitopes derived from MAGE-3, since HLA-A24 is one of the most common alleles in Japanese and Asian people. In the present study, we defined a new MAGE-3 derived, HLA-A24-binding peptide presented as a CTL epitope on gastrointestinal cancer cells. ***Materials and Methods:*** A panel of MAGE-3-derived peptides (9mer and 10mer) with the HLA-A24-binding motif was selected, and identification of MAGE-3-derived, HLA-A24-restricted CTL epitopes was performed by a reverse immunology approach. To induce MAGE-3-peptide specific CTLs, PBMCs were repeatedly stimulated with monocyte-derived, mature DCs pulsed with the peptides. Subsequent peptide-induced T cells were tested for their specificities by ELISPOT, tetramer and cytotoxic assay. CTL clones were then obtained from the CTL line by limiting dilution. ***Results:*** The peptide-inducing CTLs revealed that MAGE-3(113)-peptide was reacted as a CTL epitope in a HLA-A24-restricted fashion, confirmed by ELISPOT and cytotoxic assays. In addition, the MAGE-3(113)-specific CTL clones, confirmed by tetramer assay, showed that the MAGE-3(113) epitope is naturally processed and presented as the CTL epitope on MAGE-3-expressing gastrointestinal cancer cells by evaluating the cold target inhibition assays. ***Conclusion:*** The newly identified MAGE-3(113)-peptide epitope is naturally processed and presented as the CTL epitope on MAGE-3-expressing gastrointestinal cancer cells, indicating that anti-MAGE-3 immune targeting with the MAGE-3(113) peptide is a promising approach for treatment.

Introduction

It is now well established that small peptide epitopes, when bound to MHC class I molecules on the surfaces of tumor cells, can be recognized as antigens (Ags) by cytotoxic T lymphocytes (CTLs). Tumor-specific CTLs, adoptively transferred or activated in vivo by tumor-associated CTL epitopes, have therapeutic activity and can induce regression of established tumors or micrometastases [1, 2]. The development of immunotherapeutic methods to treat cancer is critically dependent on the identification of tumor-associated Ags.

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Table 1. Sequence of MAGE-3-derived peptides with the HLA-A2402-binding motif

	Position[a] (mer)	Sequence
pep76	76 (9)	NYPLWSQSY
pep97	97 (9)	TFPDLESEF
pep113	113 (9)	VAELVHFLL
pep142	142 (9)	NWQYFFPVI
pep150	150 (9)	IFSKASSSL
pep175	175 (10)	LYIFATCLGL

[a] Residue number of the first position of the peptide in relation to the sequence of the entire MAGE-3 gene product.

As an alternative to the genetic and biochemical approaches for identifying tumor-associated CTL epitopes, a reverse immunology method has been developed [3–5]. In this method, MHC class I-binding epitopes are identified and their corresponding synthetic peptides are tested for their capacity to induce peptide- and tumor-specific CTLs derived from healthy individuals or cancer patients. This approach has recently been used for the definition of several new CTL epitopes in different melanoma Ags [3–5].

Cancer-testis antigens (CTA) such as MAGE, BAGE, GAGE and NY-ESO-1 are recognized as attractive targets due to their unique expressions in malignant tumors with the exception of male germ lines, which do not carry HLA molecules [6]. A number of antigenic peptides encoded by MAGE-3 or NY-ESO-1 in context with various HLA molecules have been identified [7–9]. Moreover, several examples from clinical trials suggest that MAGE-3-derived peptides can generate specific T cell responses in patients with melanomas [10] and gastrointestinal (GI) tract cancers [11], in which tumor regression was reported in some metastatic melanoma patients [10].

GI tract cancers are most common in Japan today. Despite the aggressive treatment modalities such as surgical resection with extensive lymphadenectomy and surgery combined with chemo-radiotherapy [12, 13], the control of GI tract cancer at the advanced stage remains difficult. Therefore, immunoadjuvant therapy such as the utilization of antitumor T cells or antibodies against tumor antigens is extremely appealing. It has been reported that MAGE-3 was expressed in 57% of esophageal carcinomas [14] and 38% of gastric carcinomas [15]. HLA-A24 is one of the most common alleles in Japanese people and is shared by more than 60% of the population [16]. Therefore, in order to broaden the possibility for anti-MAGE-3 immune targeting in GI tract cancer, it is important to identify HLA-A24-restricted peptide epitopes derived from MAGE-3. Furthermore, the expression of MAGE-3 is heterogeneous among patients and between individual tumor lesions [17], suggesting that immunotherapy targeting multiple antigenic epitopes is more desirable than a single epitope.

In the present study, we identified a new HLA-A24-restricted, MAGE-3-derived CTL epitope, which is naturally processed and presented as the CTL epitope on MAGE-3-expressing GI tract cancer cells.

Material and Methods

Cell Lines

MKN-7 (HLA-A26 gastric cancer), KATO III (HLA-A2402 gastric cancer), MRKnu-1 (HLA-A2402 breast cancer) and WiDr (HLA-A2402 colon cancer) were obtained from the IBL cell bank (Gunma, Japan). TE-4 (HLA-A0207 esophageal cancer) was a kind gift from Dr. Nishimura (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University, Japan). K562 was obtained from ATCC (Rockville, Md., USA). TISI cells were from a human B lymphoblastoid cell line expressing HLA-A24. These cell lines were kept in RPMI 1640 with 5% FCS, 50 U/ml penicillin and 2 m*M* *L*-glutamine.

Peptide Synthesis

MAGE-3-derived peptides were identified on the basis of the presence of an HLA-A2402-binding motif using a computer program which takes into account the effect of both primary and secondary anchor residues (table 1). Peptides were synthesized by standard solid phase methods and purified by HPLC. HIV peptide with HLA-A2402-binding capacity (ILKEPVHGV) was used as a negative control peptide.

Preparation of DCs

DCs were generated from PBMC in HLA-A24(+) healthy donors and gastric cancer patients. Briefly, PBMC were separated from peripheral blood by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and monocytes were enriched by adherence to a plastic tissue culture flask (Corning, N.Y., USA) for 90 min at 37°C. Adherent cells were cultured with 1,000 U/ml of granulocyte macrophage colony-stimulating factor (GM-CSF, Peprotech EC Ltd., London, UK) and 1,000 U/ml of IL-4 (Peprotech EC Ltd.) in X-VIVO (Life Technologies Inc., Gaithersburg, Md., USA). On day 5, DCs were matured with recombinant CD40 ligand (R&D System, Inc., Minneapolis, Minn., USA) and were used as mature DCs on day 7.

Generation of MAGE-3 Peptide Specific CTL Lines and CTL Clones

Mature DCs were pulsed with MAGE-3 peptides (20 μg/ml) in the presence of β_2-microglobulin (3 μg/ml) for 60 min at 37°C. Then, these peptide-loaded mature DCs were co-incubated with autologous PBMCs in a ratio of 1:10 in a 12-well plate in X-VIVO with 1% autologous serum and 100 IU/ml of IL-2 (Peprotech EC).

Subsequent cultured cells were re-stimulated with these peptide-loaded, irradiated (25Gy) mature DCs every 14 days. After 4 stimulations, cultured CTL lines were tested for reactivity with ELISPOT analysis and cytotoxic assay.

CTL clones were then obtained from the CTL lines by limiting dilution. Briefly, the CTLs were isolated in 96-well U-bottom plates in X-VIVO with irradiated allogeneic PBMC (5×10^4 cells/well) from two different donors in the presence of MAGE-3 peptide (20 μg/ml) and 100 IU/ml of IL-2. The CTL clones were expanded with irradiated allogeneic PBMC, MAGE-3 peptide, and 100 IU/ml of IL-2.

ELISPOT Analysis

MAGE-3 specific response was determined by IFN-γ enzyme-linked immunospot (ELISPOT) analysis. ELISPOT analysis was performed with the Mabtech assay system (Nacka, Sweden). After 96-well plates with nitrocellulose membranes (Millipor) were pre-coated with a primary anti-IFN-γ antibody (1D1K) for 24 h, the plates were pre-reacted with AIM-V containing 1% human serum albumin. Target cells ($2 \times 3\ 10^4$/well) and CTL ($2 \times 3\ 10^3$/well) were incubated in 200 μl of AIM-V for 24 h in triplicate. Thereafter, a biotinylated secondary anti-IFN-γ antibody (7-B6-1) was added for 2 h, and then the plates were incubated with streptavidin-alkaline phosphatase reagent and stained with NBT and BCIP (Gibco).

Cytotoxic Assay

A standard 4-hour ^{51}Cr release assay was performed. To assess the peptide-specificity of CTL, TISI cells were pulsed with MAGE-3 peptide for 16 h at 37°C. Thereafter, peptide-pulsed TISI cells were washed and subjected to cytotoxic assay as a target. After the target cells were labelled with 100 μCi ^{51}Cr for 60 min, target cells (5×10^3/well) and effector cells at various effector/target ratios were co-incubated in 200 μl of X-VIVO in a 96-well U-bottom plate in triplicate for 4 h at 37°C. Subsequently, cold target inhibition was done using nonradiolabeled TISI loaded with MAGE-3 peptide or with the irrelevant peptide (HIV peptide) used as a negative control at various hot/cold target ratios. Supernatants were harvested and radioactivity was determined using a gamma counter. The percentage of ^{51}Cr release was calculated according to the following formula: % lysis = 100× (experimental release-spontaneous release)/(maximum release-spontaneous release).

To perform the inhibition of cytotoxicity with anti-HLA class I mAb (W6/32), target cells were preincubated with mAb using a 1/10 dilution for 1 h at room temperature before the cytotoxic assay.

Tetramer Assay

To evaluate the specificity of the MAGE-3-reacted CTL clones, FITC-labeled anti-CD8 (MBL, Nagoya, Japan), PE-labeled HLA-A2402-MAGE-3(113)-tetramer (NH_2-OOH) or PE-labeled HLA-A2402-MAGE-3(195)-tetramer (NH_2-COOH)(MBL, Nagoya, Japan) was used for immunostaining, according to the manufacturer's recommendations.

RT-PCR for MAGE-3

Total RNA was extracted from tumor cell lines according to the standard protocol with an RNeasy Minikit (Qiagen K.K., Tokyo, Japan). One microgram of total RNA was added to the reaction mixture using the One-Step RT-PCR Kit (Qiagen), and was amplified in a GeneAmp PCR System 9700 (Applied Biosystems, Calif., USA) in a total volume of 50 μl, which contained 5 × one-step RT-PCR buffer 10 μl, dNTPMix 2.0 μl, one-step RT-PCR Enzyme Mix 2.0 μl and 0.6 μ*M* of each specific primer. Specific primers were designed as follows: *MAGE-3* primers, 5'>TGGAGGACCAGAGGCCCCC<3' (forward) and 5'>GGACGATTATCAGGAGGCCTGC<3' (reverse); β-actin primers, 5'>CTACAATGAGCTGCGTGTGC<3' (forward) and 5'>CGGTGAGGATCTTCATGAGG<3' (reverse).

RT reactions were carried out according to the manufacturer's recommendations with 1 cycle of 30 min at 52°C for reverse transcription and 15 min at 95°C for the initial PCR activation step. For *MAGE-3* PCR, the cycling conditions were as follows: 35 cycles of 1 min at 94°C for denaturation, 1 min at 73°C for annealing and 2 min at 72°C for elongation. The amplified product (725 bp for *MAGE-3* and 314 bp for β-actin) was electrophoresed on a 1.2% agarose gel (Ultra Pure, Gibco BRL, New York, N.Y., USA) and equilibrated in TAE (40 m*M* Tris-acetate, 2 m*M* EDTA). Ethidium bromide (0.5 μg/ml) was added to the agarose-TAE gels with TAE electrophoresis buffers to visualize the amplified DNA fragments and these were photographed using Polaroid film 667 under UV light.

Results

Generation of MAGE-3-Derived, HLA-A24-Binding Peptide-Specific CTL Lines

The sequences of MAGE-3 molecules for the probable HLA-A24-binding peptide were screened using a computer program (table 1). With these peptides, we generated 6 different peptide-inducing T cell lines from HLA-A24 (+) healthy donors (n = 7) using mature DCs pulsed with each peptide. Then, the T cell lines were each tested for their specificities against cognate peptides, which were used for each induction, with ELISPOT analysis. Representative data of the reactivities of peptide-inducing T cell lines is shown in figure 1. As a result, two peptide-inducing T cell lines (CTL113 and CTL142) out of 6 T cell lines significantly recognized TISI targets pulsed with each cognate peptide. These observations were confirmed in 6 of 7 different healthy donors.

MAGE-3(113) Peptide-Specific CTL Lines Can Specifically Recognize HLA-A24 Tumor Cell Lines Expressing MAGE-3

The expression of MAGE-3 mRNA on tumor cell lines was analyzed by RT-PCR (fig. 2). CTL113 and CTL142 were tested against MAGE-3-expressing tumor cell lines with ELISPOT analysis (fig. 3). Only CTL113 recognized HLA-A24-positive tumor cell lines expressing MAGE-3 (MRK-nu-1 and WiDr), but not HLA-A24-negative, MAGE-3-positive MKN-7 or TE-4 (fig. 3a), while CTL142 did not react with any tumors (fig. 3b).

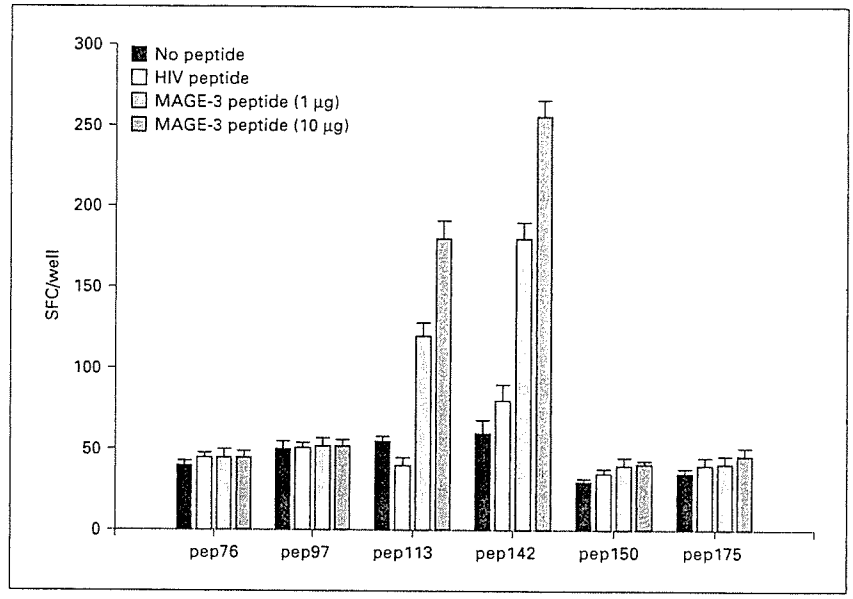

Fig. 1. Reactivities of MAGE-3-peptide-inducing T cell lines evaluated by ELISPOT assay. Peptide inducing CTL lines were generated from PBMCs in HLA-A24(+) healthy donors (n = 7) by repeated stimulation with matured DCs pulsed with the panel of MAGE-3 derived, HLA-A24-binding peptides. After four rounds of stimulation, the CTL lines were tested for their specificities against TISI pulsed with MAGE-3 peptides (1 or 10 μg/ml) or HLA-A24-binding HIV peptides (10 μg/ml) as a control by the ELISPOT assay. Representative data from 7 different donors is shown. SFC = Spot-forming cell.

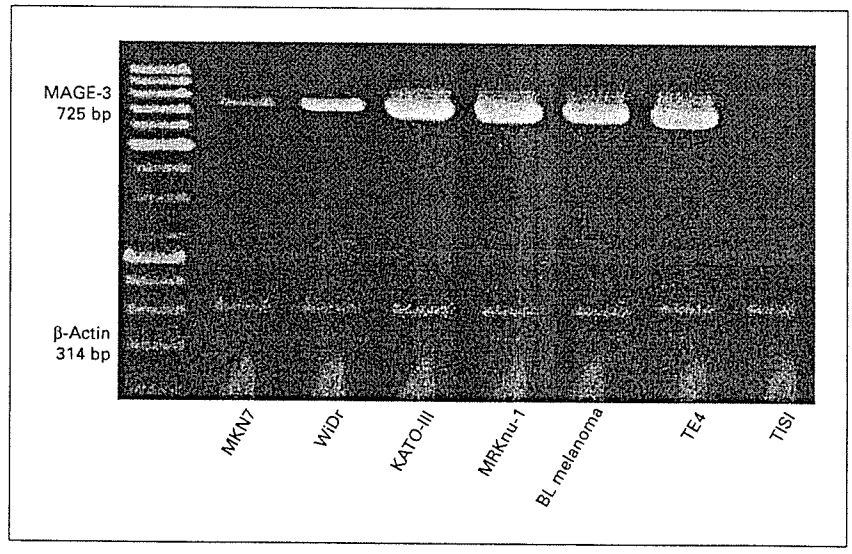

Fig. 2. The expression of MAGE-3 mRNA on tumor cell lines analyzed by RT-PCR. MAGE-3-specific bands by RT-PCR were confirmed for MKN7, WiDr, KATO-III, MRKnu-1 and TE4 cell lines. BL melanoma, a positive control for MAGE-3, and TISI, a negative control for MAGE-3, were included.

To further confirm the reactivity of CTL113, it was tested against several targets in a cytotoxic assay. CTL113 lysed MAGE-3(+), HLA-A24(+) WiDr and the cytotoxicity was inhibited by treatment with anti-MHC class I mAb (fig. 4a). Furthermore, CTL113 lysed TISI pulsed with MAGE-3(113) peptide, but did not lyse TISI pulsed with HIV control peptide (fig. 4b). These results indicate that MAGE-3(113) peptide-inducing CTL recognized and lysed MAGE-3-expressing and HLA-A24(+) tumors, specifically.

CTL113 Clones Recognize HLA-A24 Tumor Cell Lines Expressing MAGE-3 and TISI Target Pulsed with MAGE-3(113) Peptides

To further analyze the specificity of the MAGE-3(113) epitope, CTL clones were generated by limiting dilution methods from the CTL113 line. Using the MAGE-3(113)-HLA-A24 tetramer, CTL113 clones were stained positive for the MAGE-3(113)-tetramer, but not for the MAGE-3(195)-tetramer (fig. 5), indicating that CTL113 clones were MAGE-3(113)-epitope specific. The ELISPOT as-

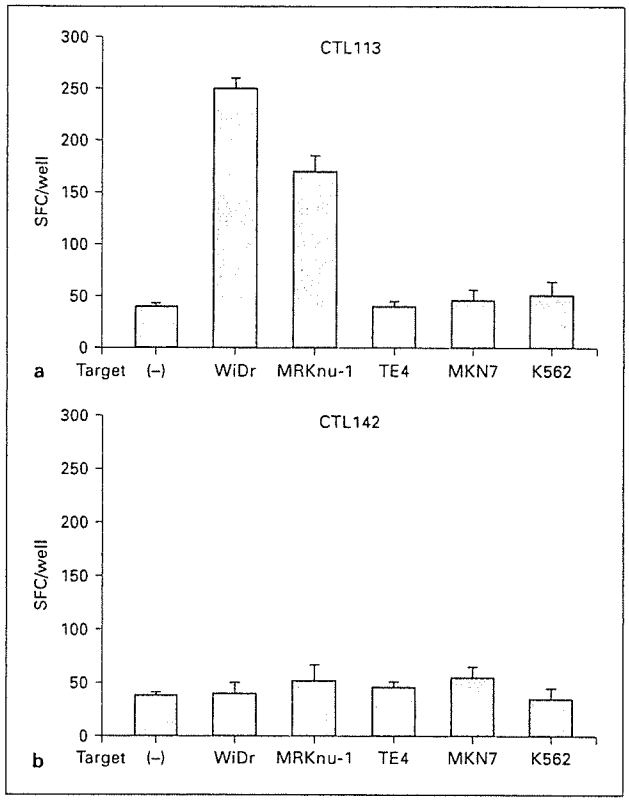

Fig. 3. Reactivities of CTL113 (**a**) and CTL142 (**b**) for MAGE-3-expressing tumor cells evaluated by ELISPOT assay. The reactivities of CTL113 and CTL142 were tested for HLA-A24-positive, MAGE-3-positive WiDr and MRKnu-1 cells or HLA-A24-negative MAGE-3-positive TE4 and MKN7 cells. CTL113 reacted with WiDr and MRKnu-1, but not with TE4 and MLN7, while CTL142 did not react with any MAGE-3-expressing tumor cells.

say indicated that CTL113 clones recognized TISI pulsed with MAGE-3(113) peptides and MAGE-3-expressing, HLA-A24(+) tumors (MRKnu-1 and WiDr), as shown in figure 6.

To further confirm the reactivity of the MAGE-3(113) peptide, various doses of MAGE-3(113) peptides were tested for their capacity to sensitize TISI by the CTL113 clones. As expected, the reactivity of the MAGE-3(113) peptide was dose-dependent (fig. 7).

Then, cold target inhibition assays were performed using non-radiolabeled TISI loaded with the MAGE-3(113) peptide or the irrelevant HLA-A24-binding HIV peptide at various hot /cold target ratios. A significant ((65.2%) at the 1:10 hot to cold ratio) inhibition of the killing for the WiDr mediated by CTL113 clones was observed when non-radiolabeled TISI loaded with the MAGE-3(113) peptide was added, but not TISI loaded with the HIV peptide (fig. 8). Thus, these data indicate that the newly identified MAGE-3(113) peptide is naturally processed and presented as a CTL epitope on MAGE-3 expressing tumors.

Generation of MAGE-3(113)-Specific CTLs from Patients with Gastric Cancers Expressing MAGE-3

To further confirm the reactivity of the MAGE-3(113) epitope in patients, MAGE-3(113)-specific CTLs were generated from HLA-A24(+) patients with gastric cancers expressing MAGE-3 (n = 4) by repeated stimulations with peptide-pulsing mature DCs, and their reactivities were evaluated by ELISPOT analysis. Representative data from two patients showed that MAGE-3(113)-peptide inducing CTLs recognized HLA-A24-positive tumor cell

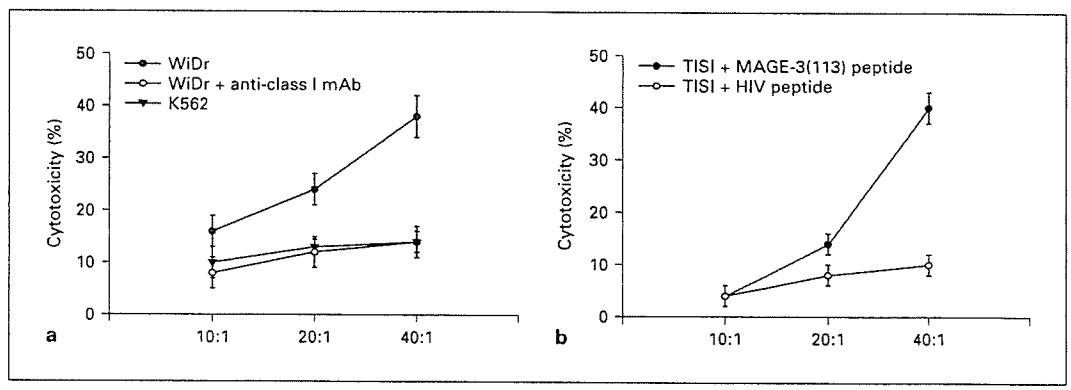

Fig. 4. Cytotoxicity of CTL113 analyzed by 4-hour ^{51}Cr release assay. CTL113 was tested for cytotoxicity against WiDr or K562 (**a**) and TISI pulsed with MAGE-3(113) peptides or HIV control peptides (**b**). The cytotoxicity against WiDr was inhibited by treatment with anti-class I mAbs (**a**).

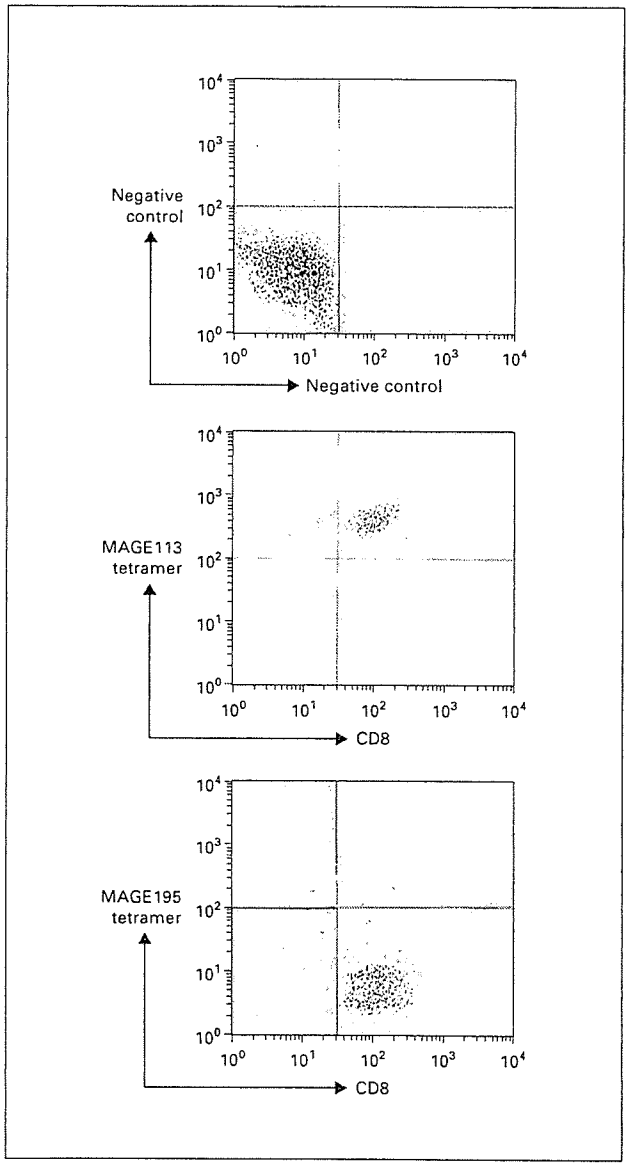

Fig. 5. Tetramer assay for CTL113 clones. CTL clones were generated by limiting dilution methods from the CTL113 line. Using the MAGE-3(113)-HLA-A24 tetramer, the CTL113 clones were stained positive for the MAGE-3(113) tetramer, but not for the MAGE-3(195) tetramer.

lines expressing MAGE-3 (MRK-nu-1 and WiDr), but not HLA-A24-negative, MAGE-3-positive MKN-7 or TE-4 (fig. 9). These results were confirmed in 4 of 4 patients, indicating that MAGE-3(113)-specific CTLs were able to be generated from the cancer patients.

Discussion

In the present study, we screened MAGE-3-derived peptides with the HLA-A24-binding motif using a reverse immunology approach, in order to identify HLA-A24-restricted CTL epitopes derived from MAGE-3. As a result, the newly identified MAGE-3(113) peptide was found to be naturally processed and presented as a CTL epitope on MAGE-3-expressing GI tract cancers.

MAGE-3 was expressed in 38% of gastric cancers [15], 57% of esophageal cancers [14] and 19% of colon cancers [18], in addition to a high proportion of melanomas [6]. These results indicate that anti-MAGE-3 immune targeting is a promising approach for the treatment of GI tract cancers. Until now, several MAGE-3-derived epitopes have been identified, including MAGE-3(195) as HLA-A24-restricted epitope [8], MAGE-3(271) as HLA-A0201-restricted epitope [19] or MAGE-3(168) as HLA-A1-restricted epitope [20]. Among them, it was shown that DCs pulsed with MAGE-3-derived, HLA-A2- or HLA-A24-restricted peptides can induce specific T cell responses in patients with gastric cancers [11]. HLA-A24 is one of the most common alleles in Japanese people and is shared by more than 60% of Japanese gastric cancer patients [16]. In contrast to HLA-A2, the HLA-A24 allele is much less heterogeneous and 99.3% of HLA-A24 is the HLA-A2402 subtype, which is the most common HLA allele in Asian and Japanese people [16]. Thus, it would be desirable to identify additional HLA-A24-restricted immunodominant epitopes derived from MAGE-3, in order to broaden tumor-specific immunotherapy based on MAGE-3.

We showed that MAGE-3(113) peptide-inducing CTLs recognized and lysed MAGE-3-expressing GI tract cancer in an HLA-A24-restricted fashion. Furthermore, the cold target inhibition assay using the MAGE-3(113)-specific CTL clones supported the view that the newly identified MAGE-3(113) peptide is presented as a CTL epitope on MAGE-3 expressing GI cancers. MAGE-3(113) peptide-specific CTLs could be generated from 4 of 4 cancer patients with MAGE-3 expressing tumors in the present study, suggesting that MAGE-3(113) epitope is not tolerated in a cancer-bearing host.

In general, cancer vaccination trials are reported to have a limited clinical response, and there are several problems to be resolved including the surrogate endpoints, trafficking of sufficient numbers of effector cells into the tumor or the presence of regulatory T cells [21]. With regard to MAGE antigens, it remains unclear why most vaccinated patients with MAGE epitopes, including those who displayed tumor regression, had either unde-

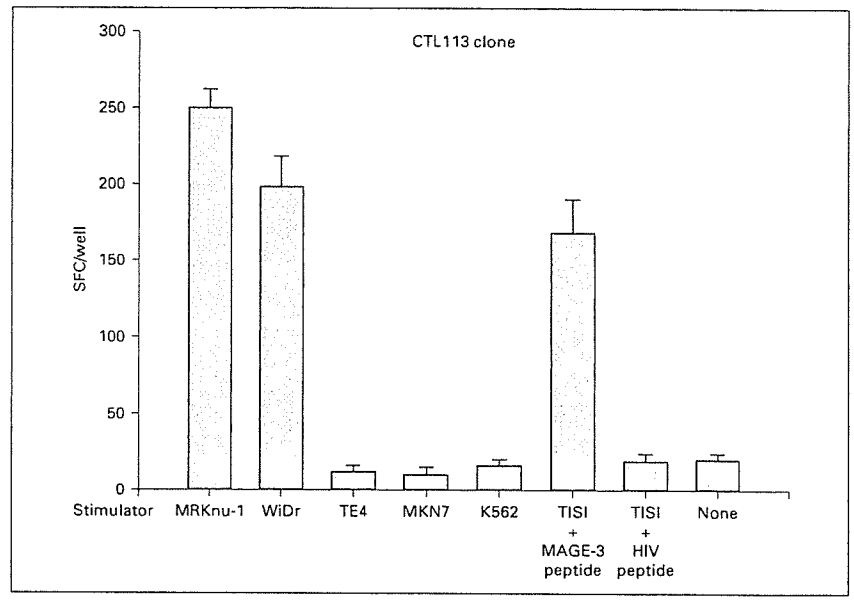

Fig. 6. Reactivities of CTL113 clones for MAGE-3-expressing tumor cells evaluated by ELISPOT assay. The reactivities of CTL113 clones were tested for HLA-A24-positive, MAGE-3-positive WiDr and MRKnu-1 cells or HLA-A24-negative MAGE-3-positive TE4 and MKN7 cells.

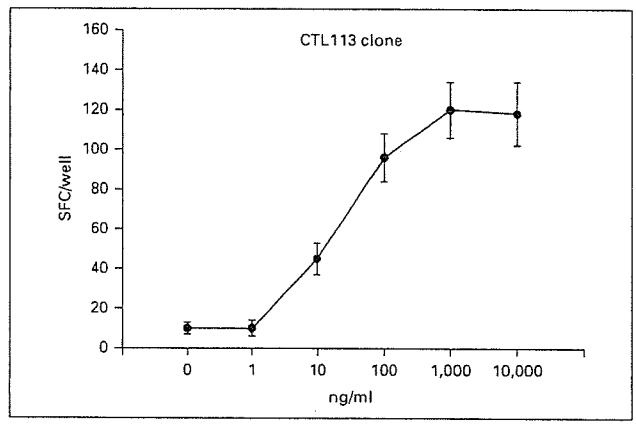

Fig. 7. Dose dependency of MAGE-3(113) epitope. Various doses of MAGE-3(113) peptides were tested for their capacities to sensitize TISI by the CTL113 clones, analyzed by the ELISPOT assay.

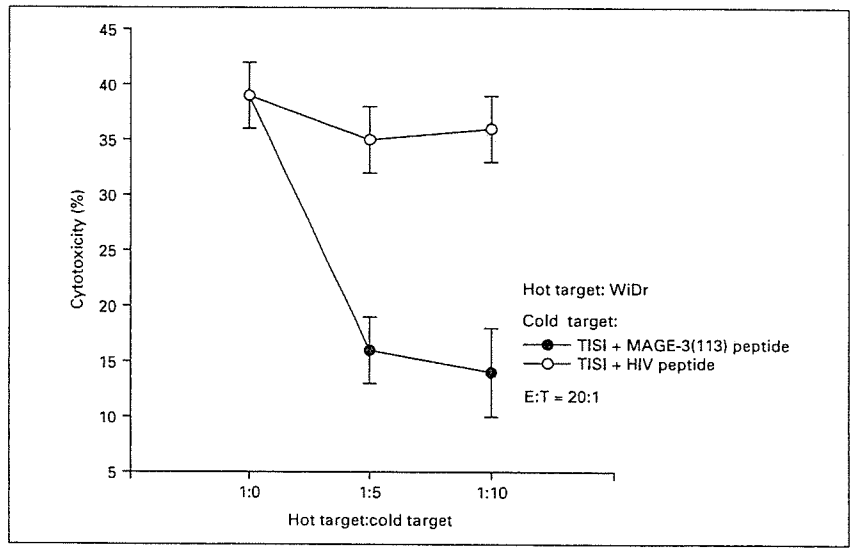

Fig. 8. Cold inhibition assay using CTL113 clones. Cold target inhibition assays were performed using nonradiolabeled TISI loaded with MAGE-3(113) peptides or the irrelevant HLA-A24-binding HIV peptides at various hot/cold target ratios. A significant ((65.2%) at the 1:10 hot to cold ratio) inhibition of the killing for the WiDr mediated by the CTL113 clones was observed when nonradiolabeled TISI loaded with the MAGE-3(113) peptide was added, but not TISI loaded with the HIV peptide.

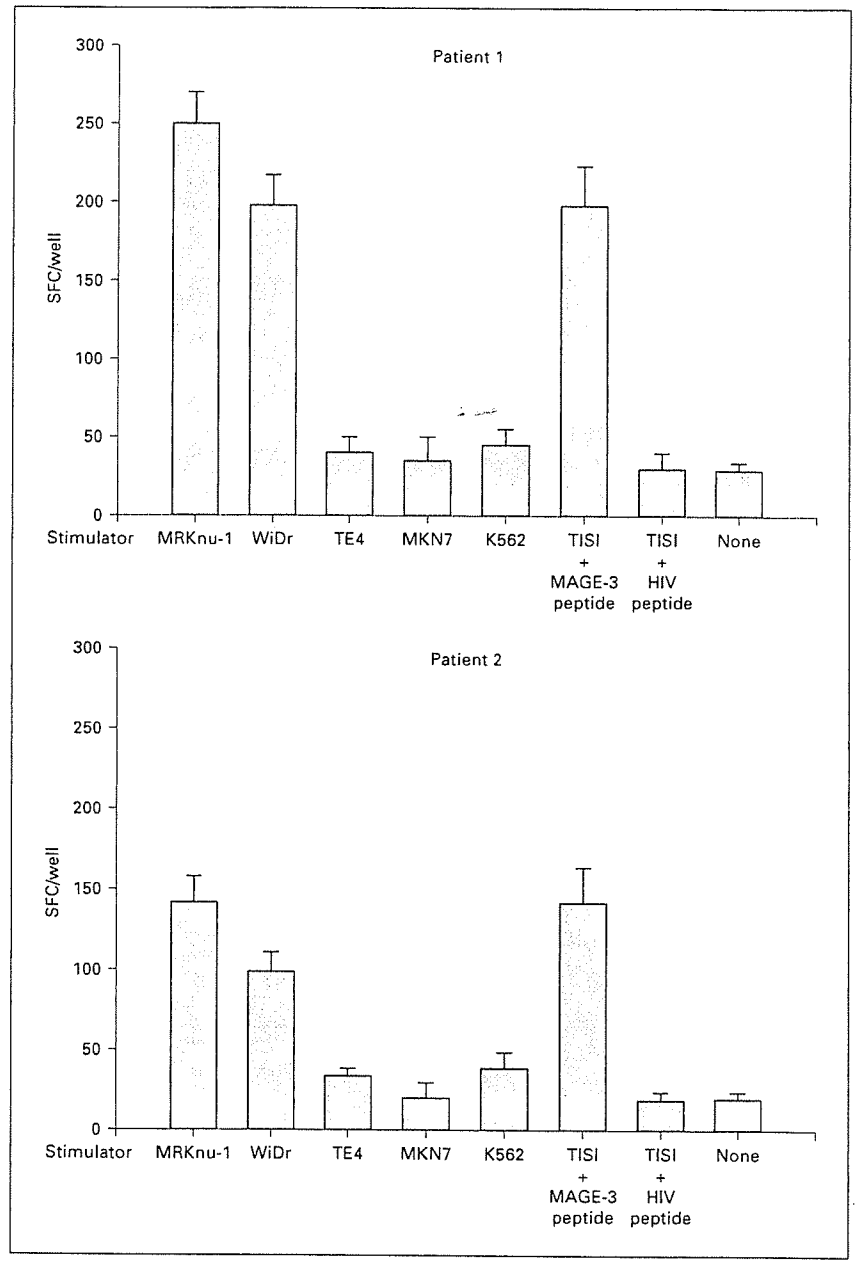

Fig. 9. Reactivities of MAGE-3 peptide-inducing T cell lines generated from cancer patients. Peptide-inducing CTL lines were generated from PBMCs in HLA-A24(+) patients with gastric cancer (n = 4) by repeated stimulation with matured DCs pulsed with the MAGE-3(113) peptide. After four rounds of stimulation, the CTL lines were tested for their specificities against TISI pulsed with MAGE-3 peptide or HLA-A24-positive, MAGE-3-positive WiDr and MRKnu-1 cells or HLA-A24-negative, MAGE-3-positive TE4 and MKN7 cells by the ELISPOT assay. Representative data from 4 different patients are shown.

tectable or very low frequencies of antivaccine CTLs in the peripheral blood [22]. However, it has recently been shown that vaccinated melanoma patients with MAGE had already mounted a strong spontaneous T cell response against several types of tumor antigens before vaccination and had antitumor CTLs at much higher frequencies than those of the antivaccine CTLs after vaccination, suggesting that the anti-vaccine CTLs are not direct effectors for killing the tumors, but that their interaction with the tumor generates conditions enabling the stimulation of large numbers of antitumor CTLs that proceed to destroy the tumor cells [10, 23]. These observations suggested that anti-MAGE targeting by vaccination could stimulate naïve T cells to form new antitumor clo-

notypes against several tumor antigens other than the vaccine antigen.

In the present study, we found that the newly identified MAGE-3(113) epitope is naturally processed and presented as a CTL epitope on MAGE-3 expressing GI tract cancers. Thus, anti-MAGE-immune targeting such as MAGE-3(113) peptide-based vaccination or the adoptive transfer of MAGE-3(113) peptide-inducing CTLs may be a promising approach for the treatment of GI tract cancers as well as melanomas.

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