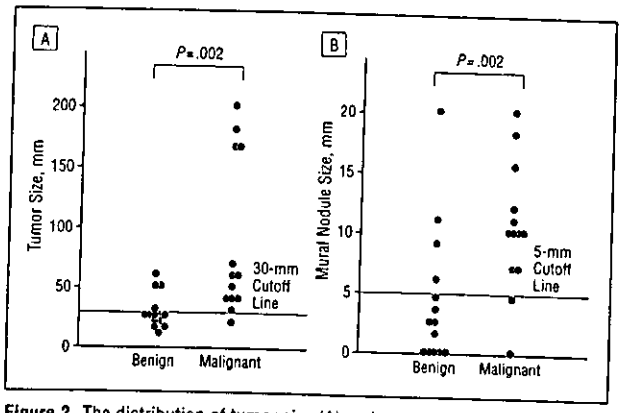

Figure 2. The distribution of tumor size (A) and mural nodule size (B) in the benign intraductal papillary mucinous tumor (IPMT) group ($n=14$) and the malignant IPMT group ($n=13$). The cutoff levels of tumor size and mural nodule size are 30 and 5 mm, respectively.

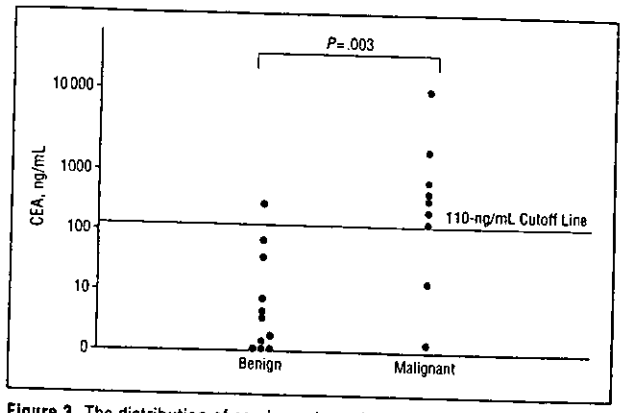

Figure 3. The distribution of carcinoembryonic antigen (CEA) level in pure pancreatic juice in the benign intraductal papillary mucinous tumor (IPMT) group ($n=11$) and the malignant IPMT group ($n=9$). The cutoff CEA level in pure pancreatic juice is 110 ng/mL.

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.

Accepted for publication August 25, 2003.

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Journal of Surgical Research 124, 151–157 (2005)
doi:10.1016/j.jss.2004.10.004

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

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Submitted for publication September 1, 2004

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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0022-4804/05 $30.00

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

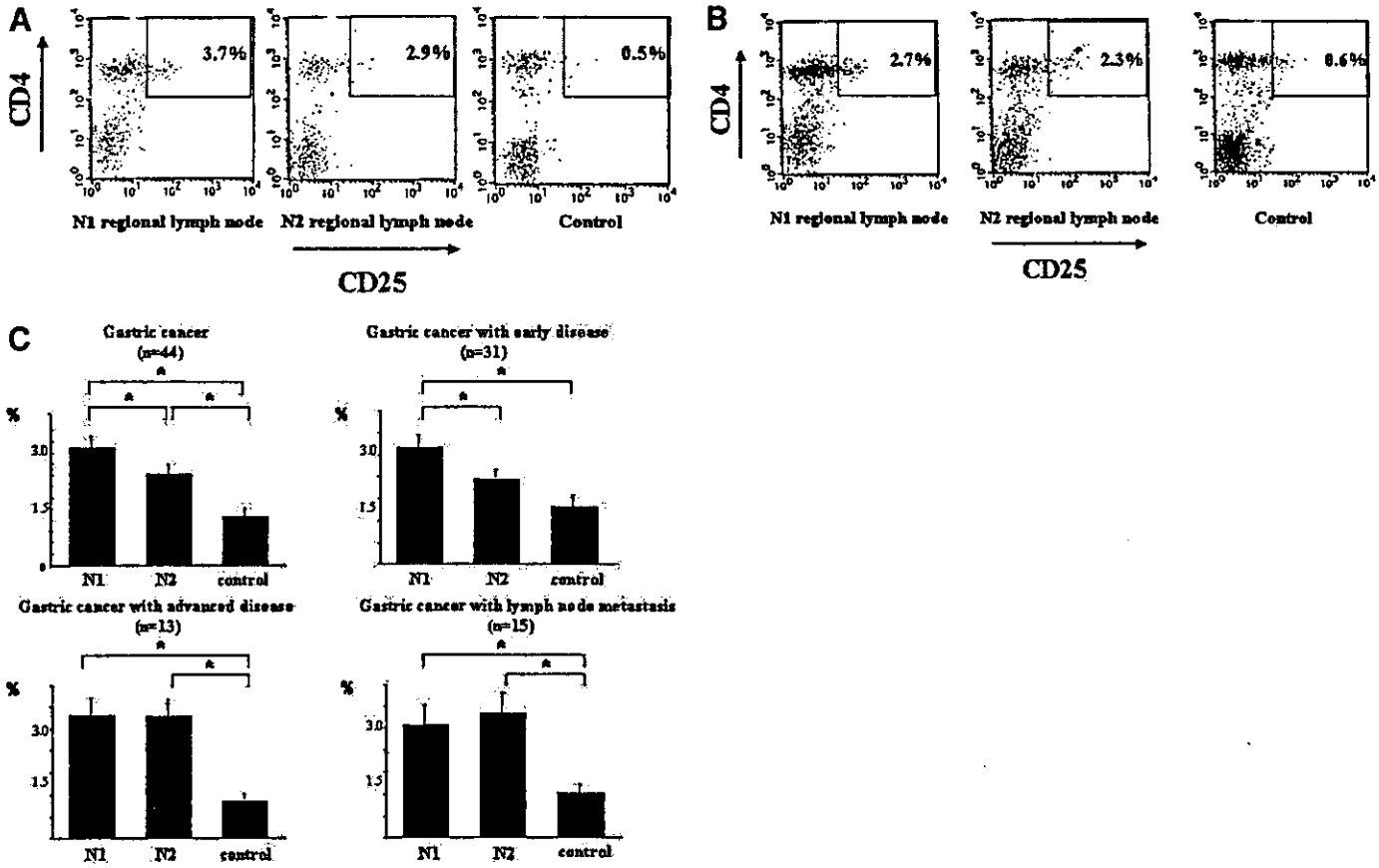

FIG. 1. Increased populations of CD4(+)CD25^{high} T-cells in regional lymph nodes with gastric cancer. The population of CD4(+)CD25^{high} T-cells as a percentage of total CD4(+) cells in lymph nodes was evaluated by flow cytometric analysis with triple-color staining. Regional lymph nodes in the stomach 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-

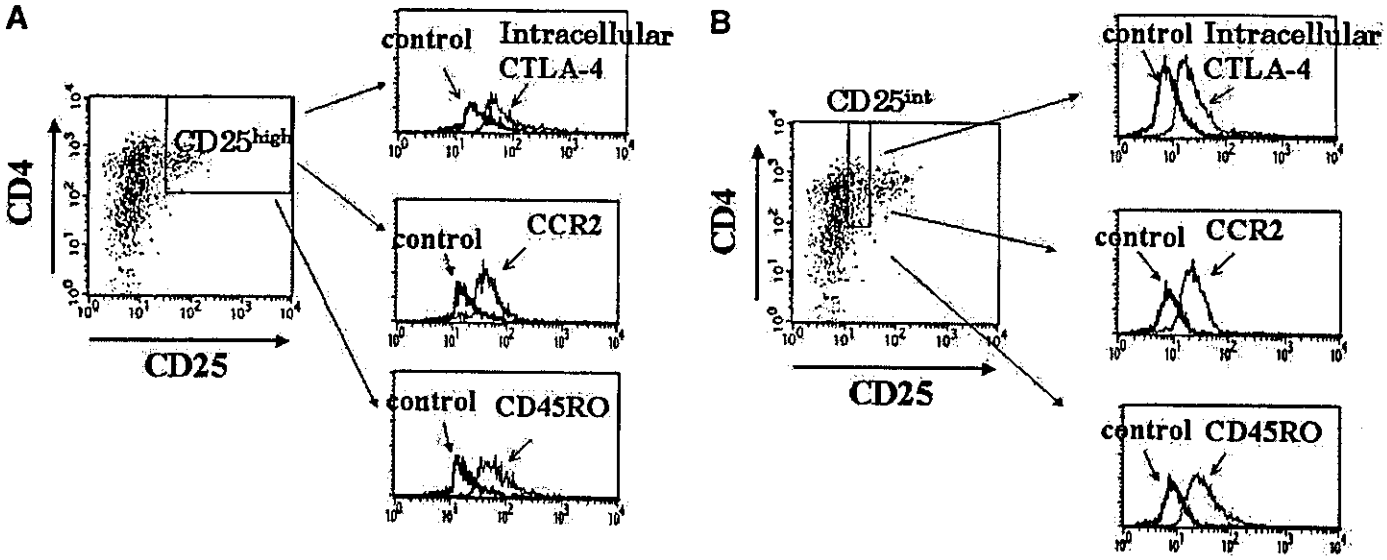

FIG. 2. The expression of CD45RO, CCR2, and intracellular CTLA-4(CD152) on CD4(+)CD25^{high} T-cells. Representative flow cytometric data from N1 (A, B) from advanced disease patients showed the expression of CD45RO, CCR2, and intracellular CTLA-4 after gating of CD4(+)CD25^{high} (A) or CD4(+)CD25^{int}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

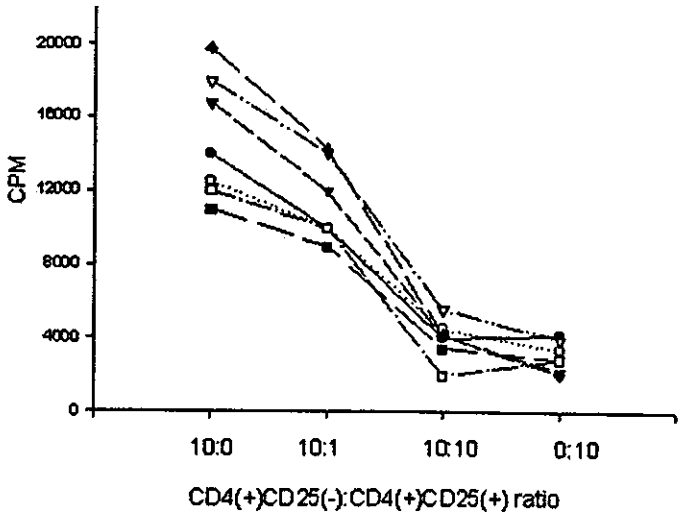

FIG. 3. Functional analysis of CD4(+)CD25(+) T-cells. CD4(+)CD25(+) and CD4(+)CD25(−) cells were purified from the N1 regional lymph nodes in gastric cancer patients with advanced disease (n = 7) using magnetic beads. The proliferation activity of CD4(+)CD25(−) cells in response to anti-CD3 plus anti-CD28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

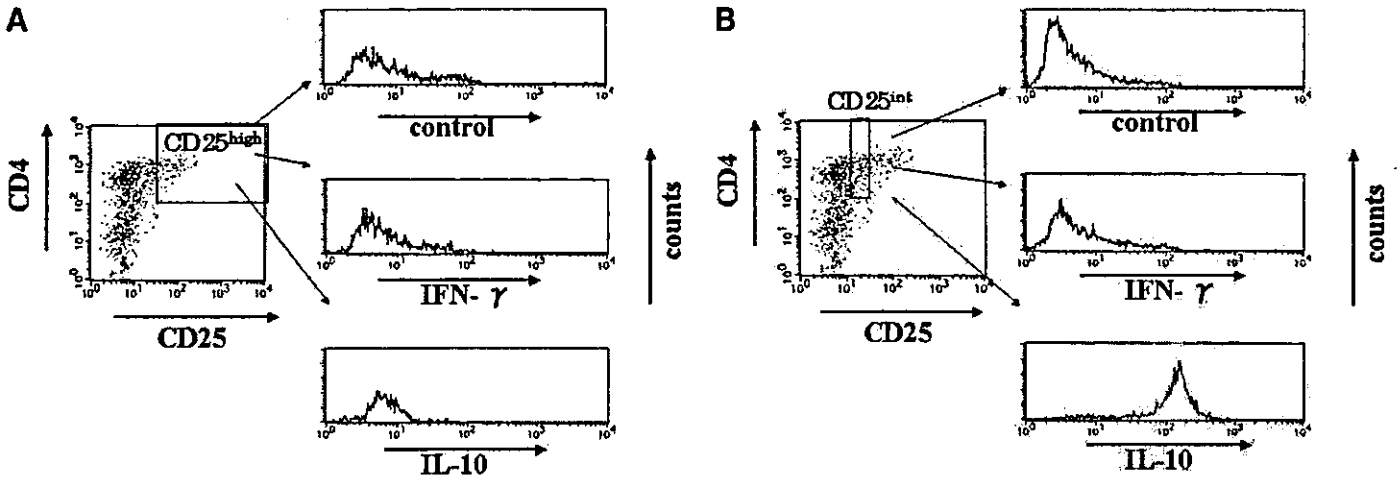

FIG. 4. Intracellular cytokine staining of CD4(+)CD25^{high} T-cells. Intracellular cytokine staining (IFN-γ and IL-10) was performed gated on CD4(+)CD25^{high} T-cells (A) or CD4(+)CD25^{int} T-cells (B) derived from N1 regional lymph nodes.

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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British Journal of Cancer (2005) 00, 000–000

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Full Paper

Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma

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The utilisation of antitumour T cells induced by cancer vaccination with HER-2 peptides or antibodies (Herceptin) against HER-2, as immunotherapy for oesophageal cancer, is a novel and attractive approach. It is important to clarify the frequencies of HER-2 expression and gene amplification in patients with oesophageal squamous cell carcinoma (SCC) and to evaluate the relationship between HER-2 status and HLA haplotype, since the candidates for HER-2 peptide-based vaccination are restricted to a certain HLA haplotype. We determined the frequency of HER-2 expression using the HercepTest™ for immunohistochemistry and HER-2 gene amplification by fluorescence *in situ* hybridisation (FISH) assay in oesophageal SCC ($n = 66$). HER-2-positive tumours (1+/2+/3+) analysed by a HercepTest were observed in 30.3% of all the patients and HER-2 gene amplification evaluated by FISH was observed in 11.0% of all the patients, in which all HercepTest (3+) tumours were found to have gene amplification and three of six moderately positive (2+) tumours showed gene amplification. Furthermore, HER-2-positive cells were present more diffusely and were larger within each tumour in the patients who were HercepTest 3+ than those who were HercepTest 1+. Moreover, the survival rate in HER-2-positive group was significantly worse than that in HER-2-negative group. Also, the survival rate in the patients with HER-2 gene amplification was significantly worse than that without HER-2 gene amplification. In addition, oesophageal SCC patients with both HLA-A24-positive and HER-2-positive tumours (1+/2+/3+) accounted for 26% of these cases, and both HLA-A2- and HER-2-positive tumours accounted for 18% of them.

British Journal of Cancer (2005) **0**, 000–000. doi:10.1038/sj.bjc.6602499 www.bjcancer.com

Keywords: HER-2; neu; FISH; immunohistochemistry; HLA; immunotherapy

While most patients with oesophageal cancer in Western countries have adenocarcinoma, most of those in Japan have squamous cell carcinoma (SCC). Despite various treatments such as surgical resection with extensive lymphadenectomy (Millet *et al*, 1982; Altorki and Skinner, 1990; Kato *et al*, 1991; Akiyama *et al*, 1994) and the surgery combined with chemotherapy (Hayashi *et al*, 2001) and/or radiotherapy (Le Prise *et al*, 1995; Adham *et al*, 2000; Ishikura *et al*, 2003), the prognosis for advanced patients with oesophageal SCC remains poor. The utilisation of antitumour T cells or antibodies against tumour antigens, as immunoadjuvant therapy for oesophageal SCC, is therefore an attractive approach.

The HER-2/neu (designated as HER-2) proto-oncogene located on chromosome 17(17q12–q21.32) (Popescu *et al*, 1989) encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity (Coussens *et al*, 1985). The HER-2 proto-oncogene is amplified and overexpressed in approximately 30% of human ovarian and breast tumours (Slamon *et al*, 1989), and in 8.2% of gastric cancers (Takehana *et al*, 2002). The humanised monoclonal antibody (mAb) Herceptin, which specifically targets HER-2, exhibits potent growth inhibitory activity against HER-2-over expressing tumours (Sliwkowski *et al*, 1999). Herceptin has boosted the interest of clinicians in immunotherapy based on this molecule as it represents the first mAb approved for therapeutic use with proved survival benefit in patients with HER-2-positive breast cancer with metastasis (Baselga *et al*, 1996; Slamon *et al*, 2001). Moreover, abundant examples from experimental models and clinical trials suggest that HER-2 can be immunogenic and generate antibodies, CTL- and helper T-cell-specific responses in individuals with HER-2-overexpressing tumours (Fisk *et al*, 1995; Kono *et al*, 1998). Based on the above reports, anti-HER-2 immune targeting could be utilised as an attractive approach to treat oesophageal cancer. Thus, it is important to clarify the frequency of HER-2 expression and gene amplification relating to the HLA haplotype in order to determine possible candidates for HER-2-based immunotherapy, since the candidates for HER-2 peptide-based vaccination are restricted to a certain HLA haplotype.

With respect to oesophageal SCC, the frequencies of HER-2 overexpression analysed by immunohistochemistry (IHC) ranged from 0 to 55.9% (Mori *et al*, 1987; Chang *et al*, 1992; Suo *et al*, 1992; Shiga *et al*, 1993; Suwanagool *et al*, 1993; Suo *et al*, 1995; Hardwick *et al*, 1997; Lam *et al*, 1998; Akamatsu *et al*, 2003). Furthermore, reports describing HER-2 gene amplification ranged from 0 to 25%, and these studies were performed by Northern blot, slot blot or RT–PCR analysis (Shiga *et al*, 1993; Ikeda *et al*, 1996; Tanaka *et al*, 1997; Friess *et al*, 1999). The discrepancy in the HER-2 frequencies among the reports may be related to the methodology, including the different mAbs used in IHC or inaccurate analysis for gene amplification. There has been no

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Received 26 October 2004; revised 26 January 2005; accepted 8 February 2005

Template: Ver 1.1.1

Journal: BJC ☐ Disk used Despatch Date: 7/3/2005
Article : npg_bjc_3390-04mp Pages: 1–8 Figs 1,2,3 col
Op: KGU Ed: SUJA

previous report describing HER-2 gene amplification in oesophageal SCC analysed by fluorescence *in situ* hybridisation (FISH) analysis.

In the present study, we determined the exact frequency of HER-2 abnormalities using the HercepTest™ for IHC and the PathVysion test for FISH in oesophageal SCC, and analysed patient's data for the survival rate. Both the HercepTest and the PathVysion FISH assay are approved by the US Food and Drug Administration (FDA) for determining the eligibility for Herceptin treatment in breast carcinoma. Furthermore, we have evaluated a possible candidate for anti-HER-2 immune targeting therapy for oesophageal SCC.

MATERIALS AND METHODS

Patients and samples

In all, 66 consecutive patients with primary oesophageal SCC who were histologically diagnosed and treated in the First Department of Surgery, University of Yamanashi Hospital, between 1998 and 1999, were enrolled in the present study and all the patients were followed up for 5 years. None of the patients had received any treatment before surgery (preoperative radiotherapy or chemotherapy) and all patients had undergone oesophagectomy with two-field ($n=39$) or three-field ($n=27$) lymph node dissection. The patients were classified using the tumour node metastasis (TNM) classification. The characteristics of the patients are shown in Table 1. The study was approved by the ethical committee of University of Yamanashi and written informed consent was obtained from all individuals.

Formalin-fixed, paraffin-embedded tissue blocks were used for IHC and FISH analysis.

Table 1 Clinical features of the patients ($n=66$)

Age (years old)	
Mean	65.3
Range	45–81
Gender	
Male	62
Female	4
Primary tumour[a]	
pTis	2
pT1a	8
pT1b	18
pT2	5
pT3	32
LNM	
Negative	29
Positive	37
SCC differentiation	
Well differentiated	15
Moderate differentiated	35
Poorly differentiated	14
Stage[a]	
0	9
I	5
II	25
III	19
IVa	6
IVb	1

LNM = lymph node metastasis; SCC = squamous cell carcinoma. [a]The grade of tumour and stages were defined according to the UICC (TMN) classification.

HLA class I typing

Heparinised peripheral blood was obtained from patients prior to the operation. Peripheral blood lymphocytes (PBLs) were purified by centrifugation on a Ficoll gradient (Pharmacia, Uppsala, Sweden). For class I typing, PBLs were subjected to a complement-dependent microcytotoxicity assay using antisera to HLA-A loci. Peripheral blood lymphocytes were typed for A loci 1, 2, 3, 9, 10, 11, 19, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 34, 36, 43, 66, 68, 69 and 74.

IHC analysis

Immunohistochemical staining was performed using the HercepTest™ (DaKoCytomation, Denmark) according to the manufacturer's recommendations. Archival, formalin-fixed, paraffin-embedded material was used to obtain 4-μm-thick sections from the main tumour and the regional lymph nodes. Briefly, deparaffinised and rehydrated tissue sections were incubated with the Epitope Retrieval Solution in a heat water bath for 40 min at 95–99°C. Then, the sections were cooled at room temperature for 20 min and washed with TRIS buffer for 5 min. Next, endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. The primary antibody was a rabbit polyclonal antibody to human HER-2, which recognises an intracytoplasmic part of HER-2, and the primary negative control antibody was an immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER-2. The sections were washed with a TRIS buffer for 5 min and incubated with the primary antibody or the primary negative control antibody at room temperature for 30 min. After rewashing with a TRIS buffer for 5 min × 2 times, the primary antibody was detected using the Visualisation Reagents, which were a dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins, for 30 min of incubation at room temperature. Subsequently, following rewashing with TRIS buffer for 5 min × 2 times, diaminobenzidine was added as a visualisation reagent for 10 min and the section was counterstained with haematoxylin. Control slides provided with the HercepTest™ kit, which contained three human breast cancer cell lines with staining intensity scores of 0, 1+ and 3+, were used in the present study. IHC analysis was performed by two observers (KM and KK) according to the staining intensity scores provided by the HercepTest™ kit. Each section was classified into four categories (0, 1+, 2+, 3+), in which tumour cells with complete absence of staining were scored as 0, those with incomplete membranous staining were classified as 1+, those with moderate, complete membranous staining were classified as 2+ and those with strong, complete membranous staining were classified as 3+ (Figure 1).

Furthermore, we evaluated the immunostaining pattern into two patterns, spot type or diffuse type (Figure 2), in which the diffuse type indicated tumour cells with membranous staining spread throughout the tumour tissue continuously. On the other hand, the spot type indicated tumour cells with membranous staining on one part or several parts separately in the section.

FISH analysis

FISH analysis was performed using the PathVysion® HER-2 DNA Probe Kit (VYSIS, Downers Grove, IL, USA). The HER-2/neu-SpectrumOrange probe is specific for the HER-2 gene locus (17q11.2–q12). The CEP 17 (chromosome enumeration probe)/SpectrumGreen probe is specific for the alpha-satellite DNA sequence (centromere region of chromosome 17). To determine the copy number for chromosome 17, we used CEP 17 as the control. FISH procedures were conducted according to the manufacturer's guidelines, except the removal of the protein from the section where we used our own protocol as described

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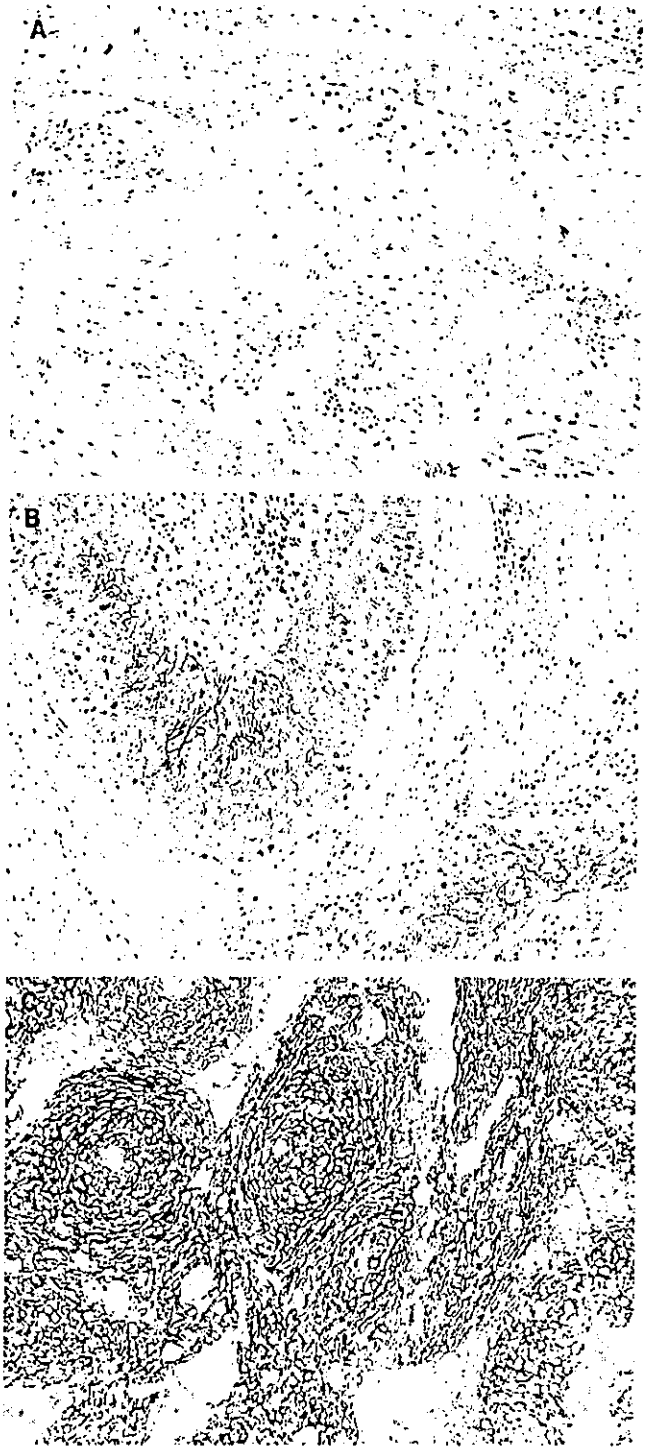

Figure 1 Representative immunostaining of HER-2-positive cells. (**A**) 1+ staining cases, (**B**) 2+ staining cases and (**C**) 3+ staining cases. Original magnification × 200.

previously (Takehana *et al*, 2002). Briefly, sections were deparaffinised, dehydrated and incubated in 20% sodium bisulphate/2 × standard saline citrate at 43°C for 20 min. Sections were washed with SCC and treated with proteinase K (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 25 min. Subsequently, denaturation, hybridisation and posthybridisation washing were performed according to the manufacturer's guidelines, and after hybridisation and posthybridisation washing, the sections were counterstained with DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride). FISH analysis was performed using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with Triple Bandpass Filter sets (Vysis). Signals were counted for at least 40 cancer nuclei per tumour. In accordance with earlier studies with FISH, a cell was considered to show amplification when a definite cluster or more than 10 orange signals of HER-2 was observed (Takehana *et al*, 2002). A positive control, which is breast tumour with previously identified HER-2 amplification and overexpression, was used as a positive control for HER-2 FISH.

Figure 2 Heterogeneity in the pattern of HER-2 immunostaining. The staining pattern was categorised as spot type and diffuse type. (**A**) Spot type and (**B**) diffuse type. Original magnification × 100.

Statistical analysis

The χ^2 test was applied to examine the differences in frequencies of the HLA-A haplotype and HER-2 expression in oesophageal SCC, the differences in HercepTest score and the rate of HER-2-positive cells in each tumour, and the differences in the lymph node metastasis of HER-2-positive patients and HER-2-negative patients. Actuarial overall survival rates were analysed by the Kaplan–Meier method and survival was measured in months

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from operation to death or last review. The log-rank test was applied to compare with the two groups. Univariate and multivariate survival analysis were calculated according to Cox's proportional-hazards model. All statistical analyses were performed using Statview 5.0 for Windows software and statistically significant difference was considered as *P*-values <0.05.

RESULTS

Frequencies of HER-2 expression and gene amplification

We studied 66 oesophageal SCC tumours and their regional lymph nodes. In IHC, each section was classified into four categories (negative, 1+, 2+, 3+) according to the staining intensity scores provided by the HercepTest kit. Positive immunostaining (1+/2+/3+) of HER-2 expression was found in 20 (30.3%) of the 66 patients with oesophageal SCC (Table 2). The clinicopathological data and their FISH analysis are summarised in Table 3. Three patients (4.5%) showed strong positive staining (3+) and 6 (9.1%) showed moderate positive staining (2+).

With respect to FISH, a cell was considered to show amplification when a definite cluster or more than 10 orange signals of HER-2 was observed in accordance with earlier studies with FISH (Takehana *et al*, 2002). In FISH analysis for IHC-positive oesophageal SCC ($n=20$), HER-2 gene amplification (cluster, Figure 3) was found in seven tumours (Table 3). In the three tumours, cancer nuclei showed more than three HER-2 signals accompanied with the same number of centromere 17 signals. They were judged as polysomy 17 (Table 3).

With respect to the comparison of FISH and IHC analysis, all the strong positive (3+) tumours were found to have gene amplification, as shown in Table 3. Among six moderate positive (2+) tumours, three showed gene amplification (cluster) and one showed polysomy. In 11 weak positive (1+) tumours, one showed gene amplification (cluster) and two showed polysomy.

Taken together, positive immunostaining (1+/2+/3+) for HER-2 expression was found in 30.3%. Moreover, moderate and strong positive patients (2+/3+) had a high frequency of gene amplification, while weak positive (1+) patients showed a low frequency of gene amplification.

Table 2 Frequencies of HER-2-positive patients detected by IHC in oesophageal SCC

	Patients (total = 66)
IHC scores	
3+	3 (4.5%)
2+	6 (9.1%)
1+	11 (16.7%)

IHC = immunohistochemistry; SCC = squamous cell carcinoma.

Immunostaining pattern and rate of HER-2-positive cells within each tumour

When the clinical application of anti-HER-2 immune targeting is considered, it is important to clarify the heterogeneity in the pattern of HER-2-positive tumour cells within each tumour. According to the DAKO HercepTest kit, the grading of the HercepTest depends on the intensity of membranous staining, indicating that the grading does not reflect how many cells are HER-2 positive. Thus, in the present study, we evaluated the immunostaining pattern and rate of HER-2-positive cells within the tumour.

We recognised that there was heterogeneity in the pattern of HER-2 immunostaining and categorised the staining pattern into a spot type and diffuse type (Figure 2). Also, HER-2-positive cells were semiquantified by counting the average number in each tumour. As a result, all three strong positive (3+) cases showed the diffuse-type pattern (Table 3) and had HER-2-positive cells in more than 30% of tumours (Table 4), while most of the weak positive (1+) patients showed that HER-2-positive cells were less than 30% (Table 4) ($P=0.057$, χ^2 analysis). Thus, HER-2-positive

Table 3 Patients with HER-2-positive oesophageal SCC detected by IHC and their FISH analyses

Case number	Age (years)	Sex	Stage[a]	Histological[b] classification	IHC			FISH
					Score[c]	Pattern	Score[c] of LN	
1	73	M	III	Mod	3+	Diffuse	3+	Cluster
2	54	M	III	Por	3+	Diffuse	3+	Cluster
3	62	M	IVa	Mod	3+	Diffuse	3+	Cluster
4	62	M	IVa	Mod	2+	Spot	0	Cluster
5	59	M	III	Well	2+	Diffuse	0	Cluster
6	57	M	III	Mod	2+	Spot	2+	Polysomy
7	55	M	II	Por	2+	Diffuse	2+	No amplification
8	69	M	II	Well	2+	Spot	No	No amplification
9	76	M	I	Well	2+	Spot	No	Cluster
10	56	M	II	Mod	1+	Spot	0	Polysomy
11	74	M	III	Well	1+	Diffuse	0	Polysomy
12	60	M	III	Mod	1+	Spot	0	No amplification
13	60	M	III	Por	1+	Diffuse	0	No amplification
14	64	M	III	Mod	1+	Diffuse	0	No amplification
15	67	M	III	Mod	1+	Diffuse	0	No amplification
16	71	M	III	Mod	1+	Spot	0	No amplification
17	47	F	II	Por	1+	Diffuse	1+	No amplification
18	74	M	0	Por	1+	Spot	No	Cluster
19	70	M	II	Mod	1+	Diffuse	No	No amplification
20	80	M	II	Por	1+	Spot	No	No amplification

SCC = squamous cell carcinoma; IHC = immunohistochemistry; FISH = fluorescence *in situ* hybridisation; LN = lymph node; No = no lymph node metastasis. [a]Stages were defined according to the TNM classification. [b]Well = well-differentiated SCC; mod = moderately differentiated SCC; por = poorly differentiated SCC. [c]IHC score was defined by the staining intensity of tumour cells (0, 1+, 2+, 3+).

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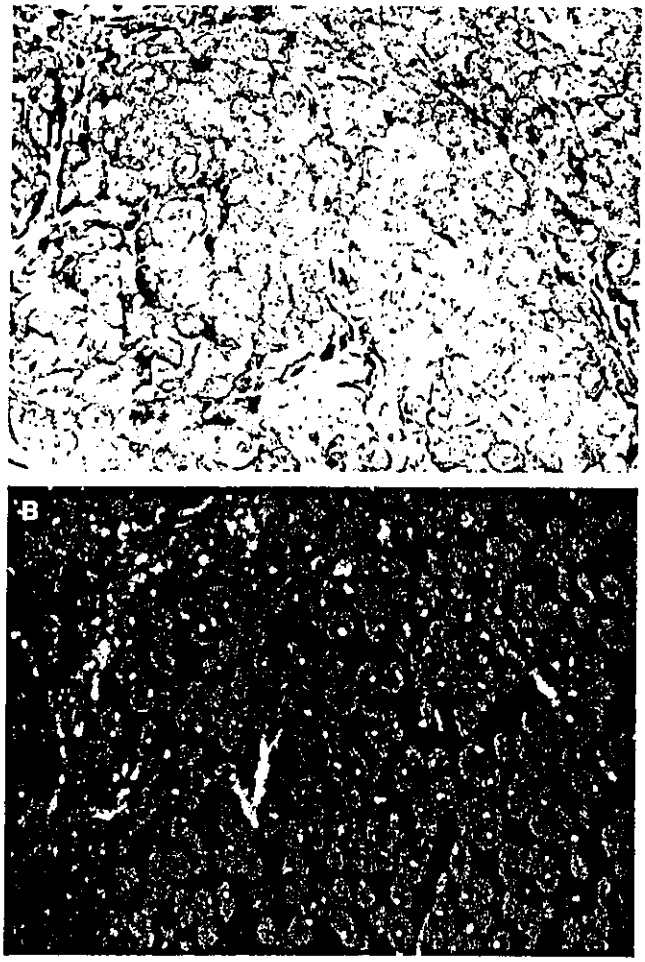

Figure 3 Representative FISH analysis of HER-2 gene amplification (cluster formation). The serial sections in the representative case were analysed by IHC (**A**) (3+ staining cases, × 400) and FISH analysis (× 400 in (**B**)).

Table 4 Correlation between IHC scores and the rate of HER-2-positive tumour cells in each tumour

	Rate of HER-2/neu-positive cells in each section		
	~30 (%)	31–60 (%)	61~ (%)
IHC scores			
3+ ($n=3$)	0	2	1
2+ ($n=6$)	5	0	1
1+ ($n=11$)	8	1	2
		$P=0.057$ by χ^2 test	

IHC = immunohistochemistry.

cells were present more diffusely within each tumour in the HercepTest 3+ patients than those who were HercepTest 1+.

Correlation of HER-2 expression in primary tumours and metastatic lymph nodes

Of all 66 cohorts, 37 (56.1%) had metastatic lymph nodes diagnosed by histopathological determination. While 15 (75.0%) in the 20 HER-2-positive patients had metastatic lymph nodes, 22 (47.8%) in the 46 HER-2-negative patients had metastatic lymph nodes (Table 3), indicating that there was a high frequency of lymph node metastasis in the HER-2-positive patients in comparison to HER-2-negative patients ($P<0.05$, χ^2 analysis). All the strongly positive (3+) patients in the primary tumours were found to have HER-2-positive tumours (3+) in the metastatic lymph nodes, while there was only one patient with a HER-2-positive tumour in the metastatic lymph node in the weak positive (1+) patients with lymph node metastasis ($n=8$).

Frequency of the HLA haplotype relating to HER-2 status

When anti-HER-2 immunotherapy such as cancer vaccination is considered for HER-2-positive patients, the candidates are restricted to a certain HLA haplotype (Kono *et al*, 2002a). It is important to clarify the HER-2 status relating to the HLA haplotype in patients with oesophageal SCC. In the patients tested for HLA haplotypes ($n=50$), the distribution of the HLA-A haplotype in patients with oesophageal SCC is shown in Table 5. The most frequent HLA-A haplotypes in oesophageal SCC are HLA-A24 (64%), HLA-A2 (52%) and HLA-A11 (28%). Also, oesophageal cancer patients with both HLA-A24-positive and HER-2-positive tumours accounted for 26% of these cases, and both HLA-A2-positive and HER-2-positive tumours accounted for 18% of them. There was no significant relation between the frequency of the HLA-A haplotype and the HER-2 status (Table 5).

Analysis of the survival of patients with oesophageal SCC

The survival rate in HER-2-positive (1+/2+/3+) group was significantly worse than that in HER-2-negative group (Figure 4A). Moreover, there was a significant difference in the survival rate between HER-2 (2+/3+) and HER-2-negative group (Figure 4B). The survival rate in the patients with HER-2 gene amplification was significantly worse than that without HER-2 gene amplification (Figure 5).

In univariate analysis, the factors such as HER-2 gene amplification, pT2 and pT3, lymph node metastasis, stages 3–4 were significant prognostic factors for survival (Table 6), although only pT2 factor reached to the significant level as independent risk factors for survival in multivariate analysis (Table 6).

Table 5 Frequencies of the HLA-A haplotype related to HER-2 expression in oesophageal SCC ($n=50$)

	HER-2 status detected by IHC	
	Positive ($n=18$)	Negative ($n=32$)
HLA-A2		
Positive ($n=26$)	9 (18%)[a]	17 (34%)
Negative ($n=24$)	9 (18%)	15(30%) NS[b]
HLA-A11		
Positive ($n=14$)	7 (14%)	7 (14%)
Negative ($n=36$)	11 (22%)	25(50%) NS
HLA-A24		
Positive ($n=32$)	13 (26%)	19 (38%)
Negative ($n=18$)	5 (10%)	13(26%) NS

SCC = squamous cell carcinoma; HLA = human leucocyte antigen. [a]Percentage indicates the number of patients out of all patients ($n=50$). [b]Not significant by χ^2 test.

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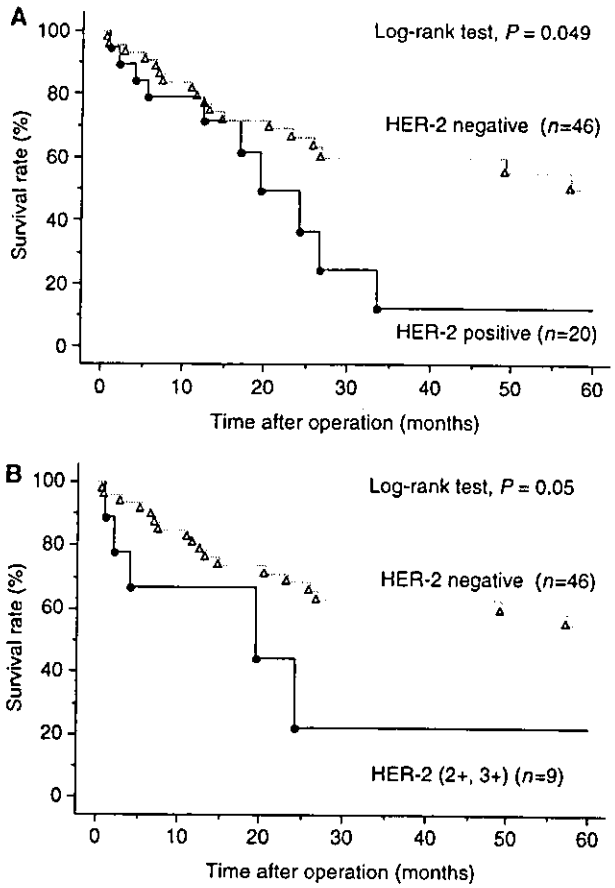

Figure 4 (**A**) Survival curves of HER-2-negative and HER-2-positive (1 + /2 + /3 +) groups. (**B**) Survival curves of HER-2-negative and HER-2 (2 + /3 +) groups.

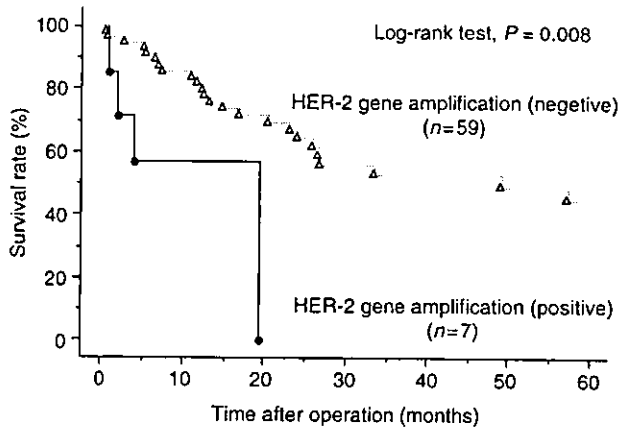

Figure 5 Survival curves of HER-2 gene amplification-negative and HER-2 gene amplification-positive groups.

DISCUSSION

The present study contains several important findings relevant to HER-2 status in oesophageal SCC. First, HER-2-positive tumours (1 + /2 + /3 +) analysed by the HercepTest were observed in 30.3% of all patients and HER-2 gene amplification evaluated by FISH was observed in 11.0% of all patients, of which all IHC (3 +) tumours were found to have gene amplification and three out of six tumours with moderate positive (2 +) tumours showed gene amplification. Second, HER-2-positive cells existed more diffusely and were larger within each tumour in HercepTest 3 + patients than those who were HercepTest 1 + . Thirdly, oesophageal SCC patients with both HLA-A24-and HER-2-positive tumours (1 + / 2 + /3 +) accounted for 26% of these cases, and both HLA-A2- and HER-2-positive tumours accounted for 18% of them.

Table 6 Significance of prognostic factors in univariate and multivariate survival analysis for patients with oesophageal SCC

	Univariate analysis			Multivariate analysis		
	Hazard ratio	**95% CI**	***P*-value**	**Hazard ratio**	**95% CI**	***P*-value**
FISH						
Negative	1.0	—	—	1.0	—	—
Positive	4.05	1.31–12.53	0.015	2.94	0.78–11.09	0.111
HER-2 expression						
Negative	1.00	—	—	1.0	—	—
Positive	2.06	0.93–4.57	0.074	0.92	0.35–2.41	0.861
Primary tumour[a]						
pTis-pT1b	1.0	—	—	1.0	—	—
pT2	5.85	1.56–21.91	0.009	5.00	1.06–23.52	0.042
pT3	5.28	1.91–14.55	0.001	3.01	0.76–11.95	0.117
LNM						
Negative	1.0	—	—	1.0	—	—
Positive	3.13	1.32–7.39	0.009	1.56	0.25–9.68	0.636
Stage[a]						
0–II	1.0	—	—	1.0	—	—
III–IV	5.21	2.33–11.64	<0.0001	2.09	0.30–14.53	0.458

SCC = squamous cell carcinoma; CI = confidence interval; FISH = fluorescence *in situ* hybridisation; LNM = lymph node metastasis. [a]The grade of tumour and stages were defined according to the UICC (TMN) classification.

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The frequencies of HER-2 overexpression in oesophageal SCC analysed by IHC ranged from 0 to 55.9% (Mori *et al*, 1987; Chang *et al*, 1992; Suo *et al*, 1992, 1995; Shiga *et al*, 1993; Suwanagool *et al*, 1993; Lam *et al*, 1998; Hardwick *et al*, 1997; Akamatsu *et al*, 2003). Furthermore, reports describing HER-2 gene amplification ranged from 0 to 25%, in which these studies were performed by Northern blot, slot blot or RT–PCR analysis (Shiga *et al*, 1993; Ikeda *et al*, 1996; Tanaka *et al*, 1997; Friess *et al*, 1999). This is the first report describing the HER-2 status in oesophageal SCC analysed by two FDA-approved tests, the HercepTest and FISH (PathVysion test). Moreover, there was no previous report describing HER-2 status evaluated by the HercepTest and FISH in relation to the survival rate in oesophageal SCC. As a result, HER-2-positive tumours analysed by the HercepTest were observed in 30.3% of all the patients and HER-2 gene amplification evaluated by FISH was observed in 11.0% of all the patients.

There is increasing evidence that there is a discrepancy in the detection of HER-2 status between the two FDA-approved test, the HercepTest and FISH (PathVysion test), in which the concordance rates ranged from 80 to 90% (Jacobs *et al*, 1999; Varshney *et al*, 2004). There have been several reports that cases with HER-2 overexpression without gene amplification mostly occurred in moderate positive cases (2+) (Perez *et al*, 2002; Varshney *et al*, 2004), in line with this study. Various explanations of this discrepancy have been proposed: transcriptional or post-translational activation (Slamon *et al*, 1989), artefactual high sensitivity of IHC (Varshney *et al*, 2004), the presence of chromosome 17 polysomy (Wang *et al*, 2002) or the low detection rate of FISH analysis (Jacobs *et al*, 1999). We found one case of polysomy in 2+ patients and two cases of polysomy in 1+ patients, suggesting that the presence of chromosome 17 polysomy might be one explanation for the discrepancy between the HercepTest and FISH in oesophageal SCC.

Interestingly, there was one case with HER-2 gene amplification in a HercepTest 1(+) tumour, indicating that the screening of HER-2 status by the HercepTest may underestimate HER-2 gene amplification. Since both the HercepTest and FISH assay have limitations in detecting HER-2 status, both methods should be applied when anti-HER-2 immune targeting, such as Herceptin or cancer vaccination, are considered in oesophageal SCC.

In this study, all the HercepTest 3+ patients had HER-2 gene amplification, and HER-2-positive cells in these cases were present diffusely and were larger within the tumours. Furthermore, the HER-2 expression in HercepTest 3+ patients was also preserved in the metastatic lymph nodes. The action of anti-HER-2-specific CTL correlated to the degree of HER-2 expression on the target tumour cells (Fisk *et al*, 1995; Kono *et al*, 1998). Also, the activity of ADCC induced by Herceptin correlated to the degree of HER-2 expression on the target tumour cells (Kono *et al*, 2002b). These results indicate that HercepTest 3+ patients in oesophageal SCC will be the best candidates for anti-HER-2 immune targeting. It has already been shown in breast cancer trials that there is a greater benefit from Herceptin therapy for 3+ patients compared to those who were 2+ (Cobleigh *et al*, 1999; Slamon *et al*, 2001).

The survival rate in patients with HER-2 expression or HER-2 gene amplification was significantly worse than that without HER-2 expression or HER-2 gene amplification. These results indicated that HER-2 status may be one of the prognostic factors to predict the clinical course of patients with oesophageal SCC, although the HER-2 status did not reach to the significant level as independent risk factors for survival in multivariate analysis in the present study. Since the sample size of the present study is limited, further studies with larger cohorts will be needed to draw valid conclusion.

When considering the cancer vaccination with HER-2-derived peptide epitopes, there is an HLA restriction. It has been shown that HLA-A2- or HLA-A24-restricted peptide epitopes were identified for immunodominant CTL epitopes derived from HER-2 (Fisk *et al*, 1995; Kono *et al*, 1998, 2002b). In the present study, oesophageal SCC patients with both HLA-A24- and HER-2-positive tumours (1+/2+/3+) accounted for 26% of these cases, and both HLA-A2- and HER-2-positive tumours accounted for 18% of them. These populations are considered for cancer vaccination with the HER-2 peptide in oesophageal SCC. In fact, we and others reported that clinical vaccination trials in gastric or breast cancer patients using DCs pulsed with HER-2 peptides confirmed the fact that vaccination with HER-2 peptides is immunogenic, and that HER-2 could be a good target for immunotherapy (Disis *et al*, 1999; Kono *et al*, 2002c). Furthermore, we have recently shown that Herceptin enhances MHC class I-restricted antigen presentation in HER-2-overexpressing tumours, resulting in a higher susceptibility of HER-2-overexpressing tumours to lysis by HER-2-specific CTL (Kono *et al*, 2004). These results suggested that the combination of Herceptin and anti-HER-2-specific CTLs may result in a synergic antitumour effect in oesophageal SCC. Anti-HER-2 immune targeting such as Herceptin or cancer vaccination with HER-2 peptides is novel and attractive approach for oesophageal SCC and the candidates for HER-2-based immunotherapy were limited, but significant populations of oesophageal SCC.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology in Japan and from the Japanese Foundation for Multidisciplinary Treatment of Cancer.

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NPG.BJC.3390-04MP

Freeze-Thawing Procedures Have No Influence on the Phenotypic and Functional Development of Dendritic Cells Generated from Peripheral Blood CD14$^+$ Monocytes

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Abstract: Little is known about the potential influence of cryopreservation on the biologic activities of dendritic cells (DCs). In this study, we examined the effects of freeze-thawing on the phenotypic and functional development of human DCs obtained from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood CD14$^+$ cells. CD14$^+$ cells were cultured, immediately or after freeze-thawing, with granulocyte-macrophage CSF and interleukin-4 for 9 days, and then with added tumor necrosis factor-α for another 3 days. For both fresh and freeze-thawed monocytes, immature DCs harvested on day 6 and mature DCs harvested on day 9 of culture were examined under the same conditions. Cells were compared with regard to their 1) capacities for antigen endocytosis and chemotactic migration (immature DCs), and 2) allogeneic mixed lymphocyte reaction and antigen-specific cytotoxic T lymphocyte responses (mature DCs). Freeze-thawing did not affect the viability or subsequent maturation of DCs at any stage of development. Furthermore, essentially no difference was observed in phenotype or function between cells generated from fresh or cryopreserved/thawed cells. Although this study design was limited with the use of fetal bovine serum, the observation still suggests that freeze-thawing does not affect viability, phenotype, subsequent maturation, or functions of DCs at any stage of maturation.

Key Words: dendritic cells, cryopreservation

(*J Immunother* 2004;27:27–35)

Received for publication December 11, 2002; accepted May 21, 2003.

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Supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare.

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Dendritic cells (DCs) are potent antigen-presenting cells that have been characterized as a natural adjuvant[1–3] and have been examined in several studies for their possible clinical application in immunotherapy against cancer.[4–7] Immature DCs can capture and process exogenous antigens,[8] while in response to maturation stimuli, they start to express enhanced costimulatory molecules and migrate into secondary lymphoid organs to select and stimulate antigen-specific T cells.[2,9] However, only a few studies have examined the influence of cryopreservation/thawing on the subsequent expansion of culture-generated DCs, the expression of surface molecules, and the functional capacities of immature and mature DCs.

In the present study, isolated CD14$^+$ cells, rather than plastic adherent cells that have been used in most other studies, were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 9 days and then with added tumor necrosis factor-α (TNF-α) for 3 additional days according to the method described in other studies.[10] To investigate the influence of preceding cryopreservation and thawing on the subsequent development of DCs, we examined the phenotypic and functional development of DCs, including endocytic, chemotactic migratory, and antigen-presenting capacities at different maturation steps, ie, monocytes, immature DCs, and mature DCs. Our results suggest that freeze-thawing does not affect the induction of functional mature DCs.

MATERIALS AND METHODS

Culture Medium and Cytokines

The following culture media were used to generate DC: RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Catalog no. 30071, lot no.AHK9081, KitHycone, Logan, UT) and 1000 units/mL penicillin, 100 μg/mL streptomycin (GIBCO-BRL). Recombinant human GM-CSF was provided by Kirin Brewery-Company (Tokyo, Japan). Recombinant human IL-4, TNF-α, and IL-2 were purchased from R & D Systems (Minneapolis, MN).

Procedure for Obtaining Monocyte-Derived DCs from Mobilized Peripheral Blood $CD14^+$ Cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were collected by leukapheresis after mobilization with granulocyte-CSF (G-CSF) as previously described.[11] Small aliquots of cells (about 2×10^8 cells) remaining in the tubing system were used for this experiment after informed consent was obtained. The experimental program was approved by the IRB of National Cancer Center Hospital in Tokyo. Cells were washed twice with phosphate-buffered saline and $CD14^+$ cells were then positively selected with MicroBeads (Miltenyi Biotec, Bergish Glandbach, Germany) according to the manufacturer's instructions. Bead-bound cells were analyzed immediately after selection by flow cytometry and found to be composed of >90% $CD14^+$ cells. Isolated $CD14^+$ cells (approximately 1×10^6 cells/mL) were cultured in 25-cm^2 flasks (Corning Inc., Corning, NY) in culture medium supplemented with GM-CSF (50 ng/mL) and IL-4 (50 ng/mL) for 6 days to allow them to differentiate into immature DCs. Thereafter, immature DCs were further cultured with GM-CSF (50 ng/mL), IL-4 (50 ng/mL), and TNF-α (50 ng/mL) for 3 days to differentiate into mature DCs. Half of culture medium was replaced with the same volume of the fresh medium containing the cytokines every 3 days.

Freeze-Thawing Procedures

Freezing was performed as follows: the cells were resuspended in a freezing medium containing 10% DMSO and 90% fetal bovine serum at a cell concentration of 5×10^6 cells/mL per tube (CryoTubes™, catalog no. 375353, Nunc A/S, Denmark) and slowly frozen to −80°C overnight using a Cryo 1°C NALGEN Freezing Container (Nalge Nunc International, Rochester, NY) filled up with isopropyl alcohol. The frozen cells were then transferred to a liquid nitrogen tank for storage for 4 weeks. Thawing was performed as follows: the frozen cells in a freezing tube were thawed in a 37°C water bath and then washed with culture medium containing 10% acid citrate dextrose solution (Sigma-Aldrich, Steinheim, Germany) by centrifugation at 250*g* force for 10 minutes at room temperature. The thawed cells were counted using trypan blue dye and cultured overnight at 37°C in 5% CO_2 with an appropriate combination of cytokines for subsequent assay. To evaluate the effect of freezing/thawing procedure, the recovery rate in each step was calculated as follows:

Alive cell number per tube after thawing /Alive cell number per tube before freezing × 100 (%)

Definition of Cell Samples

Each stage in the maturation of DCs and points of cryopreservation/thawing are illustrated in Figure 1. Cryopreservation was performed at each maturation stage to obtain the following cell types for experiments: 1) 2 types of monocytes [fresh (fr/mono) and thawed monocytes (th/mono)], 2) 3 types of immature DCs harvested on day 6 of culture [freshly cultured immature DCs (fr/iDC), thawed immature DCs (th/iDC), and immature DCs generated from the culture of th/mono (iDCs from th/mono)], and 3) 4 types of mature DCs [freshly cultured mature DCs (fr/mDC), thawed mature DCs (th/mDC), mature DCs generated from th/iDCs (mDCs from th/iDC), and mature DCs generated from th/mono (mDCs from th/mono)]. At each maturation stage, the number of viable cells, cell surface markers, and function of cells were examined. The number of viable cells was counted with trypan blue dye under the microscope and the results represent the average of 4 calculations.

Flow Cytometric Analysis

Cell surface markers were examined at each maturation stage using the following monoclonal antibodies: fluorescent isothiocyanate (FITC)-conjugated anti-CD14 and HLA-DR antibodies, and phycoerythrin (PE)-conjugated anti-CD1a, CD80, and CD86 antibodies, which were purchased from Becton Dickinson (San Jose, CA), and PE-labeled anti-CD83 antibody, which was purchased from Coulter-Immunotech (Miami, FL). Flow cytometry was performed using a FACSCalibur (Becton Dickinson). Expression rates of the surface markers were evaluated with the fluorochrome-conjugated isotype IgG intensity as a control. The results represent the percentage of positive cells calculated from 10^4 total events.

Endocytosis Assay with FITC-Dextran

FITC-Dextran (MW 40000; Sigma, St Louis, MO) was added to the cell suspensions at a final concentration of 1 mg/mL, and the cells were cultured for 45 minutes at either 37°C or 4°C as a control. After incubation, cells were washed twice with cold phosphate-buffered saline and analyzed by flow cytometry.

Transmigration Assay

To evaluate the chemotactic effects of DCs on stimulation, a previously described, the double-chamber system was used with a minor modification.[12,13] Briefly, polycarbonate membranes with 8-μm pore size filters (Chemotaxicell™; Kurabo, Osaka, Japan) were placed on 24-well culture plates to separate the upper and lower chambers. IL-16 (TECHNE, Minneapolis, MN) solution was diluted to concentrations from 0.0001 to 10 nmol/L with 500 μL of culture medium and placed in the lower chambers. Immature and mature DCs were then added to the upper chambers at 1×10^5 cells /100 μL. After incubation for 4 hours at 37°C, the cells migrated to the lower chambers and those larger than 12 μm were counted using a Coulter counter.

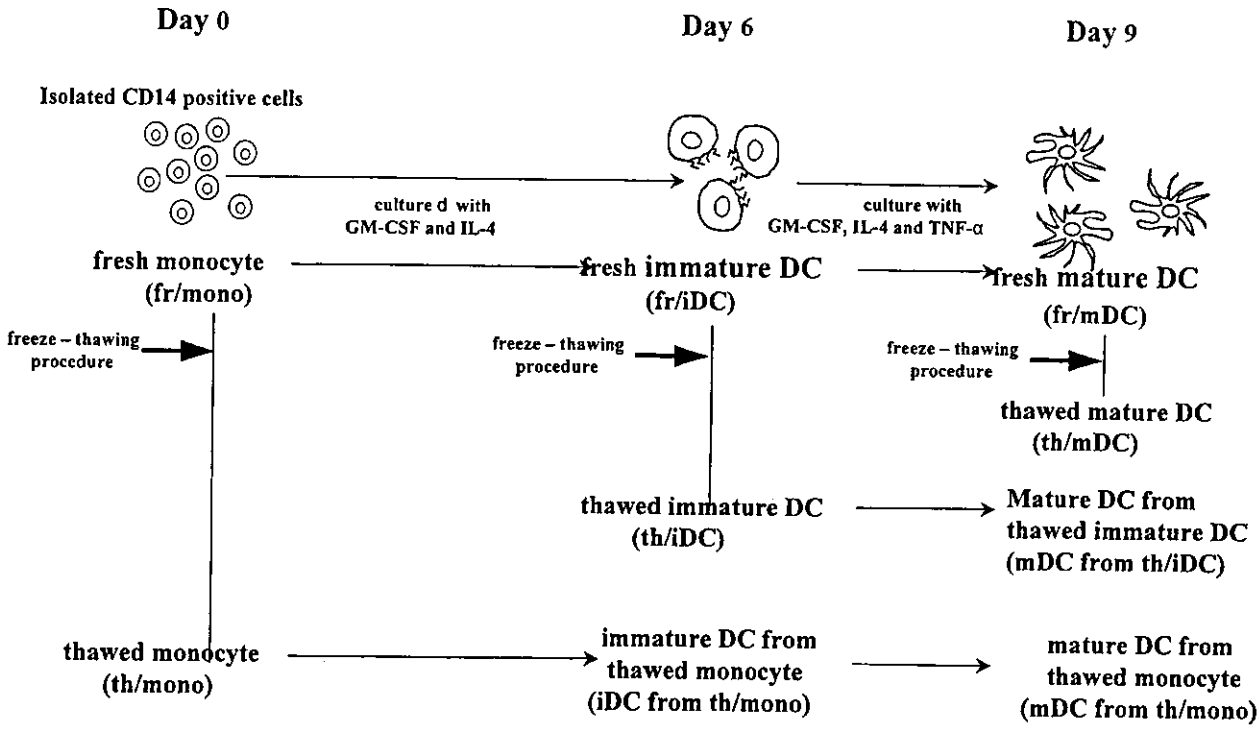

FIGURE 1. Definition of cell maturation stage and scheme of the experimental framework. $CD14^+$ cells were isolated from G-CSF-mobilized PBMCs using MACS, resuspended at 1×10^6 cells/mL, and cultured in the presence of GM-CSF (50 ng/mL) and IL-4 (50 ng/mL) for 6 days to generate immature DCs. TNF-α (50 ng/mL) was then added to the culture to induce mature DCs after an additional 3 days. Freshly isolated and cultured cells were resuspended in freezing medium at 5×10^6 cells/mL, cryopreserved for 4 weeks in liquid nitrogen, and then rapidly thawed for experimental use. Using this experimental culture system, 3 types of immature DCs and 4 types of mature DCs were generated.

Allogeneic Mixed Lymphocyte Reaction (allo-MLR)

Allo-MLR was performed according to a protocol previously described.[14] Briefly, irradiated (30 Gy) mature DCs were washed 3 times with RPMI 1640 medium, including 10% fetal bovine serum and plated into 96-well round-bottomed microplates at various cell concentrations. Allogeneic $CD3^+$ T lymphocytes as a responder cell were isolated from PBMCs by human T cell enrichment columns (R&D, Minneapolis, MN). Responder cells were added at 5×10^4 cells/well in a final total volume of 200 μL, and plates were incubated for 5 days. The cocultured cells were pulsed with 1 μCi [^{3}H]methylthymidine (5 Ci/mmol; Amersham Life Science, Buckinghamshire, UK) per well for 16 hours and then harvested and analyzed by a liquid scintillation counter.

Induction of Cytotoxic T Lymphocytes (CTL)

Mature DCs (1×10^5) prepared from $HLA\text{-}A24^+$ donors were loaded with 10 μM Epstein-Barr virus (EBV)-derived peptide (TYGPVFMCL: purity >95%) (Genetics, Huntsville, AL),[15] which is capable of binding to HLA-A2402 for 2 hours. In 24-well plates, autologous purified $CD8^+$ T lymphocytes, which were isolated from PBMCs by magnetic cell sorting using CD8 Microbeads (Miltenyi Biotec) as a source of effector cells, were cocultured at a ratio of 2:1 with mature DCs in 2 mL of CTL medium (RPMI 1640) and AIM-V serum-free medium (1:1), supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 1% penicillin-streptomycin, 1% nonessential amino acid (GIBCO-Invitrogen, Aukland, New Zealand), and 100 IU/mL IL-2 (Shionogi Pharmaceutical, Osaka, Japan) for 10 days.

Half of the medium was changed every 3 days. BEC-2 (HLA-A2402) and Bamb-2 (HLA-A1/A26) generated by EBV-transformed B-lymphoblastoid cell lines from EBV^+ healthy donor (kindly provided by Dr. K. Itoh, Kurume University, Kurume, Japan)[16] as target cells were also loaded at a concentration of 1×10^6 cells/mL with 10 μM of EBV peptide for 2 hours. The effector cells were cocultured with target cells in a total volume of 200 μL at effector to target (E:T) ratios of 2:1, 5:1, and 10:1, respectively, in 96-well round-bottom microplates. After overnight incubation, the concentration of interferon (IFN)-γ in the supernatant was