Translational Relevance

Chemotherapy is one of the essential treatments in esophageal cancer. It is also important to predict the response to chemotherapy before treatment to avoid unnecessary treatment. In this study, we investigated whether we could predict the response to cisplatin-based chemotherapy for esophageal cancer by analyzing the miRNA expression using biopsy samples before treatment. Of the several miRNAs associated with resistance to cisplatin, let-7b and let-7c expression is potentially useful to predict the response to chemotherapy. We also found that let-7 modulates the chemosensitivity to cisplatin through the regulation of interleukin (IL)-6/STAT3 pathway in esophageal cancer. This result should help doctors and scientists dealing with chemotherapy for gastrointestinal cancers including esophageal cancer.

of upregulated miR-200c expression in chemoresistance in esophageal cancer and that this effect is mediated through activation of the Akt signaling pathway (14).

In the present study, we examined whether we could predict the response to chemotherapy before treatment in patients with esophageal cancer, by using endoscopic biopsies. The results showed that low expression of let-7 measured before treatment is associated with low sensitivity to cisplatin-based chemotherapy in esophageal cancer. The molecular mechanism of the involvement of let-7 expression in chemosensitivity was also investigated.

Materials and Methods

Patients, treatment, and samples

Biopsy samples were obtained under esophagoscopy from 98 patients with histopathologically confirmed primary thoracic esophageal squamous cell carcinoma who subsequently underwent surgical resection between 2000 and 2011 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan. Informed consent was obtained from each patient before participation in this study. These 98 patients were divided at random into 2 independent groups; 74 in the training set and the remaining 24 patients in the validation set. Biopsy samples of the patients were obtained before preoperative chemotherapy. The samples were confirmed to contain cancerous tissue. All patients received neoadjuvant chemotherapy, which consisted of 2 courses of 5-fluorouracil (5-FU), cisplatin, and Adriamycin, using the following protocol: Cisplatin was administered at 70 mg/m^2, Adriamycin was administered at 35 mg/m^2 intravenously on day 1, and 5-FU was administered continuously from days 1 to 7 at 700 mg/m^2/d. Two courses of chemotherapy were provided after an interval of 4 weeks (8). The median follow-up period was 22.4 months. Thirty (30.6%) patients died during the follow-up period. Patients were divided into 2 groups: the first 74 patients were categorized as the training set whereas the second group of 24 patients was categorized as the validation set (Supplementary Table S1).

Clinical and histopathological evaluation of response to chemotherapy

The clinical response to chemotherapy was evaluated according to the World Health Organization Response Criteria for Measurable Diseases (15). Complete response (CR) represented total regression of the tumor. Partial response (PR) represented more than 50% reduction in primary tumor size on computed tomography (CT). Progressive disease (PD) represented more than 25% increase in the primary tumor or appearance of new lesion. Stable disease (SD) represented cases that did not meet the criteria of PR or PD. For evaluation, both the CR and PR were grouped together into the responders whereas the SD and PD were grouped as non-responders. The clinical response was assessed retrospectively by 2 investigators (K. Sugimura and H. Miyata) in a blinded fashion. The histopathologic response was also categorized according to the criteria of the Japanese Society for Esophageal Diseases (16). The percentage of viable residual tumor cells within the entire cancerous tissue was defined as follows: grade III, no viable residual tumor cells; grade II, less than two-third residual tumor cells; grade I, more than two-third residual tumor cells; and grade 0, no significant response to chemotherapy. The histopathologic response was assessed retrospectively by 2 investigators (K. Sugimura and K. Tanaka) in an independent manner and any disagreements were resolved by consensus.

Cell culture

Human esophageal squamous cell lines, TE1/TE5/TE8/TE9/TE10/TE11/TE13, were obtained from the Riken Bioresource Center Cell Bank. All cells were cultured in RPMI-1640 media (Life Technologies), containing 10% FBS (Sigma-Aldrich Co.) and 1% penicillin/streptomycin (Life Technologies), in a humidified atmosphere under 5% CO_2 at 37°C.

Establishment of cisplatin-resistant cell lines

Cisplatin-resistant cell lines (TE8-R and TE10-R) were cultured through gradual increase in cisplatin concentration [*cis*-diamminedichloroplatinum (II), Wako], as described previously (14). The cultured cells were exposed cisplatin at an initial concentration of 2 μmol/L. Three days later, the cells were cultured in cisplatin-free medium until confluence. Next, cisplatin concentration was increased by 2- to 3-fold. This cycle was repeated until cisplatin concentration reached 35 μmol/L.

Isolation of RNA

Total RNA was isolated from cells or tissues using TRIzol reagent (Life Technologies) according to the protocol provided by the manufacturer. Briefly, 100 mg of tissue samples was homogenized with 1 mL of TRIzol reagent using a power homogenizer. After homogenization, the samples were mixed with 0.2 mL of chloroform. The samples were

shaken vigorously for 15 seconds and then centrifuged at 12,000 × *g* for 15 minutes at 4°C. The supernatant in the tube was mixed with 0.5 mL of 100% isopropanol and then incubated at room temperature for 10 minutes. After centrifugation at 12,000 × *g* for 10 minutes at 4°C, the supernatant was removed and washed with 1 mL of 75% ethanol. After centrifugation at 7,500 × *g* for 5 minutes at 4°C, the supernatant was removed and the pellet was dried for 5 minutes. The RNA pellet was resuspended in RNase-free water and adjusted into appropriate concentration.

Reverse transcription PCR and TaqMan miRNA assay

TaqMan miRNA Assay (Applied Biosystems) was used to measure miRNA levels. This assay detects only the mature form of the specific miRNAs. First, 10 ng of RNA was reverse transcribed and the resulting cDNA was amplified using the following specific TaqMan microRNA assays. Assay IDs were hsa-miR-135a ID 000460, hsa-miR-96 ID 000186, hsa-miR-141 ID 000463, hsa-miR-101 ID 2253, hsa-miR-146a ID 000468, hsa-miR-489 ID 0002358, hsa-miR-545 ID 0002267, hsa-miR-99a ID000435, hsa-let-7b ID 002619, hsa-miR-204 ID 000508, hsa-let-7c ID 000379, hsa-miR-202 ID 002363, hsa-miR-10a ID 000387, hsa-miR-136 ID 000592, hsa-miR-145 ID 002278, and RNU48 ID:001006. The PCRs were carried out in the 7500HT Sequence Detection System (Applied Biosystems), as recommended by the manufacturer. Amplification data were normalized to RNU48 expression. Quantification of relative expression was conducted using the $2^{-\Delta\Delta C_t}$ method (17).

Interleukin-6 quantitative reverse transcription PCR

For reverse transcriptase reaction, the Reverse Transcription System (Promega) was used according to the protocol provided by the manufacturer. Real-time quantitative reverse transcription PCR (qRT-PCR) was carried out using designed oligonucleotide primers and Light Cycler (Roche Diagnostics), and the amount of interleukin (IL)-6 mRNA expression was calculated. The expression of IL-6 was normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control. The designed PCR primers were as follows: IL-6: forward primer, 5′-CCTTCCAAAGATGGCTGAAA-3′, reverse primer, 5′-ATCTGAGGTGCCCATGCTAC-3′; GAPDH: forward primer, 5′-CAACTACATGGTTTACATGTTC-3′, reverse primer, 5′-AAATGAGCCCCAGCCTTC-3′.

miRNA microarray

The miRNA expression profiling was conducted with 1,000 ng of RNA extracted from 2 esophageal cell lines (TE8 and TE10) and the corresponding cisplatin-resistant cell lines (TE8-R and TE10-R) using the TaqMan Array Human MicroRNA Panel (version 1, Applied Biosystems). This qRT-PCR array contains the 365 target microRNAs as well as the endogenous controls. Normalization was conducted with RNU48. The expression of each miRNA in cisplatin-resistance cell line was compared with that in the control parent cell line, and the ratio of miRNA expression in cisplatin-resistance cell line to control cell line was calculated for all 365 miRNAs.

miRNA transfection

TE11 and TE13 cells were transfected with 30 nmol/L pre-miR miRNA precursor molecules of has-let-7c (#PM10436, Applied Biosystems) using SiPORT NeoFX (Ambion) in 6-well plates or 6-cm dishes according to the instructions supplied by the manufacturer. Pre-miR negative control (Applied Biosystems) was also used as a control.

MTT assay

Cell viability was determined by MTT (Sigma-Aldrich) assay. Let-7c or negative control miRNA–transfected cells were seeded into 96-well plates in culture medium. After 24 hours, the medium was changed with a medium containing the following concentration of cisplatin (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, or 400 μmol/L). After incubation for 6 hours, the medium was changed into normal medium. Seventy-two hours after culture, the cells were stained with 20 μL MTT (5 mg/mL) at 37°C for 4 hours and subsequently solubilized in 100 μL of 0.004N HCl-isopropanpol. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad Laboratories).

Apoptosis assay

Apoptosis was assessed by the flow cytometric detection of phosphatidyl serine externalization using Annexin V and propidium iodide staining (Bio Vision). TE13 cells, after transfection with pre-let-7c and pre-miR negative controls, were treated with 40 μmol/L cisplatin for 6 hours. The cells were harvested and processed for Annexin V staining using the procedure described by the supplier. Briefly, cells were trypsinized gently and resuspended with 500 μL of 1× binding buffer and then treated with 5 μL of Annexin V-FITC and 5 μL of phosphatidylinositol (PI). After incubation for 5 minutes on ice, each sample was analyzed immediately using the FACSCalibur flow cytometer (BD Bioscience).

ELISA assay

After 24-hours culture, the cells were exposed to 5 μmol/L CDDP (mentioned above) or medium only. The supernatants were collected (24, 48, or 72 hours) and centrifuged. IL-6 protein level was measured using ELISA kits (#D6050, R&D Systems) according to the protocol provided by the manufacturer.

Western blotting

Cells were washed with ice-cold PBS and harvested from the culture dish. The cells were lysed in RIPA buffer (25 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 500 KIE/mL aprotinin) containing phosphatase inhibitor. The extracts were centrifuged and the supernatant fractions were collected for Western blot analysis. The following antibodies were used

in this study: at 1:2,000 for anti-human p-STAT3 (Tyr705) antibody (#9145, Cell Signaling), 1:2,000 for anti-human STAT3 antibody (#9132, Cell Signaling), 1:2,000 for anti-human p-Akt antibody (#9271, Cell Signaling), 1:2,000 for anti-human Akt antibody (#4691, Cell Signaling), 1:2,000 for anti-human Erk antibody (#4370, Cell Signaling), 1:2,000 for anti-human Erk antibody (#4695, Cell Signaling), 1:10,000 for anti-human β-actin (#A2066, Sigma-Aldrich), and 1:2,000 for all secondary antibodies. Immune complexes were detected using the Detection Kit (GE HealthCare).

Statistical analysis

To validate the clinical significance of let-7c expression as a marker of chemosensitivity in patients with esophageal cancer, we used the cross-validation method. Data were expressed as mean ± SD. Clinicopathologic parameters were compared using the χ^2 test and continuous variables were compared using Student t test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. $P < 0.05$ denoted the presence of a statistically significant difference. Statistical analysis was conducted using the JMP Ver. 8.0 software.

Results

Altered expression of 15 miRNAs in cisplatin-resistant cells

PCR-based microarray analysis was conducted to compare the expression of miRNAs in cisplatin resistance cells and control cells using 2 pairs of cell lines; TE8/TE8-R and TE10/TE10-R. The miRNA microarray analysis in TE8/TE8-R and TE10/TE10-R cisplatin-resistant cells showed altered expression (by more than 1.7-fold) in 128 (35.0%) and 177 (48.5%) miRNAs among 365 miRNAs, respectively, compared with control cells. Among the miRNAs with altered expression in cisplatin-resistant cells, 15 miRNAs showed overlap in the 2 cell lines. Among these 15 miRNAs, miR135a, miR-96, miR-141, miR-101, miR-146a, miR-489, and miR-545 were upregulated, whereas miR-99a, let-7b, miR-204, let-7c, miR-202, miR-10a, miR-136, and miR-145 were downregulated in cisplatin-resistant cells, compared with control cells (Table 1). Accordingly, we selected these 15 miRNAs as candidates for the response to chemotherapy in esophageal cancer.

Table 1. Fold change in the expression of 15 microRNAs in cisplatin-resistant cells compared with parental cells

microRNA	TE8R/TE8 fold change	TE10R/TE10 fold change
Upregulation		
miR-135a	6.08	12.07
miR-96	3.40	3.85
miR-141	2.41	25.37
miR-101	1.75	2.21
miR-146a	1.97	1,556.1
miR-489	1.78	5.30
miR-545	1.84	3.09
Downregulation		
miR-99a	0.49	0.12
let-7b	0.37	0.39
miR-204	0.35	0.29
let-7c	0.26	0.11
miR-202	0.02	0.01
miR-10a	0.57	0.06
miR-145	0.52	0.03
miR-136	0.54	0.002

Low expression of let-7c is associated with poor response to chemotherapy and poor prognosis

To determine whether the 15 miRNAs are implicated in the response to chemotherapy, we carried out qRT-PCR using pretreatment biopsy samples in 74 patients in training set group with esophageal cancer who underwent preoperative chemotherapy followed by surgery (Table 2). With regard to the clinical response in 74 patients of the training set, CR and PR was achieved in 3 and 30 patients, respectively, whereas SD and PD was observed in 35 and 6 patients, respectively. Thus, 33 (44.6%) patients were categorized as responder whereas the remaining 41 (55.4%) patients were categorized as nonresponders. Expression of the 15 miRNAs was confirmed in the biopsy samples. We also divided the 74 patients of the training set into 2 groups on the basis of the median value of the expression level of each miRNA; the high expression group ($n = 37$) and the low expression group ($n = 37$). Among 15 selected miRNAs, high expression levels of let-7b and let-7c correlated significantly with the clinical response to chemotherapy in esophageal cancer ($P = 0.019$, $P = 0.005$ respectively). However, the expression of the other microRNAs did not correlate with chemosensitivity. Next, we examined whether the expression of let-7b and let-7c is associated with the histopathologic response. With regard to the histopathologic response in 74 patients of the training set, complete tumor regression (grade III) and major tumor regression (grade II) was observed in 3 and 9 patients, respectively, whereas minor tumor regression (grade I) and almost no tumor regression (grade 0) was observed in 54 and 8 patients, respectively. Similar to the clinical response, high expression of let-7b and let-7c correlated significantly with better histopathologic response (Fig. 1A and B). Thus, the expression of let-7b and let-7c in pretreatment biopsy samples determined the response to chemotherapy in patients with esophageal cancer.

Next, we examined whether the expression of let-7b and let-7c is associated with the prognosis of patients who underwent preoperative chemotherapy followed by surgery for esophageal cancer. High expression of let-7c correlated significantly with longer survival in patients who received preoperative chemotherapy (Fig. 1D). High expression of

Table 2. Relationship between the expression of 15 microRNAs and clinical response

miRNA	Responders (n = 33) high/low	Nonresponders (n = 41) high/low	P
miR-135a	23/10	14/27	0.640
miR-96	19/14	18/23	0.350
miR-141	19/14	18/23	0.350
miR-101	19/14	18/23	0.350
miR-146a	20/13	17/24	0.160
miR-489	18/15	19/22	0.640
miR-545	19/14	18/23	0.350
miR-99a	15/18	22/19	0.640
let-7b	22/11	15/26	0.019
miR-204	15/18	22/19	0.640
let-7c	23/10	14/27	0.005
miR-202	16/17	21/20	1.000
miR-10a	21/12	16/25	0.061
miR-145	20/13	17/24	0.160
miR-136	16/17	21/20	1.000

NOTE: Data are number of patients.

let-7b also tended to correlate with longer survival, but this tendency did not reach statistical significance (Fig. 1C). We could not find significant relationship between let-7c expression and any clinicopathologic parameter in patients who received preoperative chemotherapy followed by surgery.

To validate the clinical significance of let-7c expression as a marker of chemosensitivity in patients with esophageal cancer, we examined the relationship between let-7c expression and chemosensitivity using biopsy samples of the second group of 24 patients in validation set group. The results confirmed that high expression of let-7c also correlated significantly with the clinical response in esophageal cancer.

Induction of let-7c expression restores chemosensitivity and increases apoptosis after genotoxic chemotherapy

In the next series of studies, we established the relationship between let-7c expression and chemosensitivity using esophageal squamous cell carcinoma cell lines. First, we determined let-7c expression in each esophageal cancer cell line and found relatively low expression of let-7c in TE11 and TE13 cells compared with other esophageal cancer cell lines (Supplementary Fig. S1a). To evaluate the biologic effect of let-7c, pre-let-7c was transfected into TE11 and TE13 cells, and let-7c expression was confirmed in the let-7c–transfected cells (Supplementary Fig. S1b). The MTT assay showed that let-7c–transfected cells were significantly more sensitive to cisplatin than control cells. Furthermore, the IC_{50} of let-7c–transfected

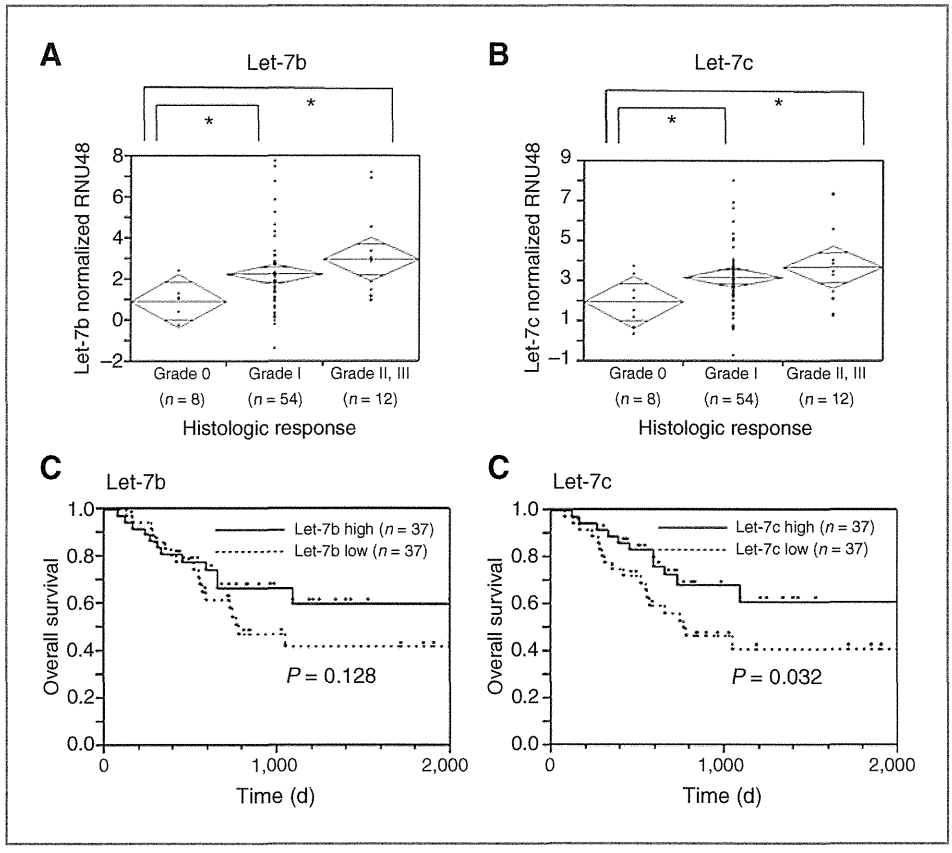

Figure 1. Association of let-7b and let-7c expression with histologic response and overall survival of patients treated with preoperative chemotherapy. A and B, the expression of let-7b and let-7c was higher in patients with histologic response of grade II–III/I than in those with grade 0 (let-7b: P = 0.014/0.02; let-7c: P = 0.032/0.025). C and D, overall survival curves of 74 patients with esophageal cancer according to let-7b and let-7c expression. High expression of let-7c correlated significantly with longer survival (P = 0.032). High expression of let-7b showed similar effect (P = 0.128).

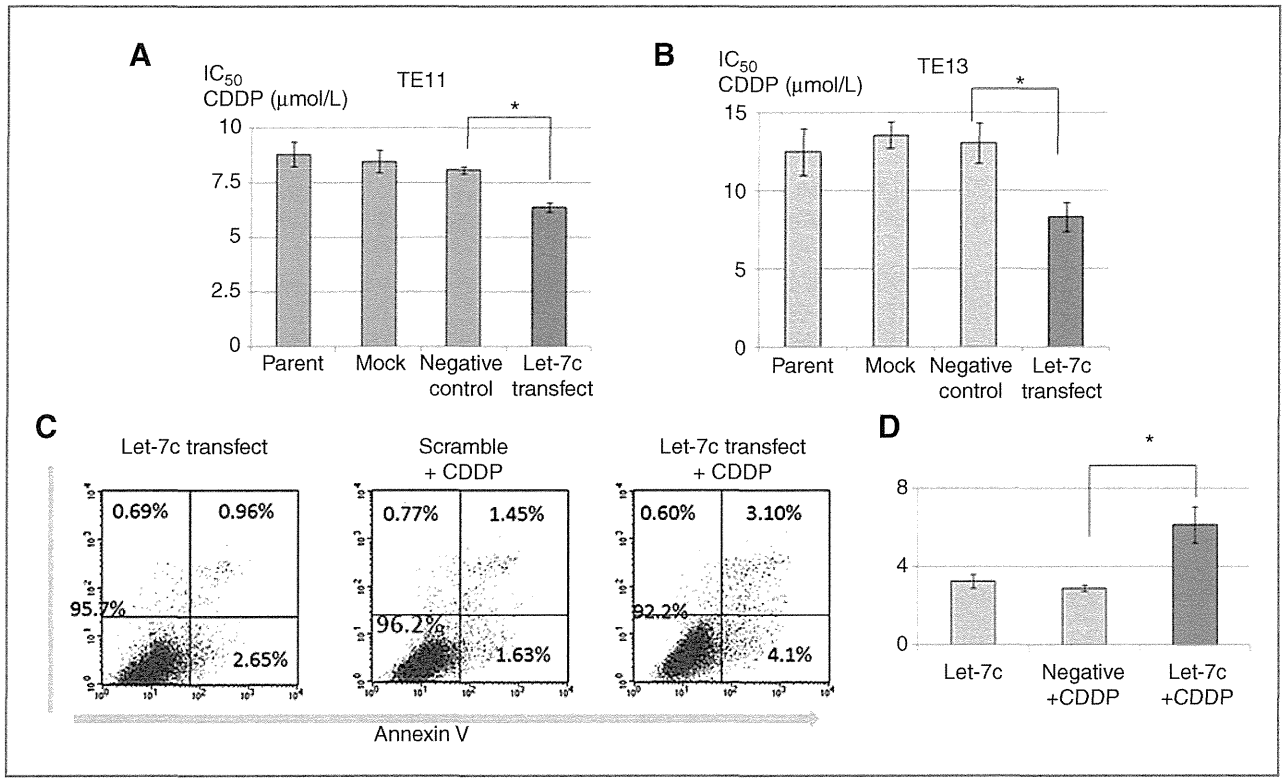

Figure 2. Overexpression of let-7c increases chemosensitivity in esophageal cells. A and B, the IC_{50} levels of cisplatin in TE11 and TE13 esophageal cells were significantly lower in let-7c transfected cells than in negative control transfected cells. Data are mean ± SD. *, $P < 0.01$. C, apoptotic cells were detected by flow cytometry using Annexin V and PI staining. Apoptotic cells were regarded as Annexin-V–positive cells. D, transfection of let-7c significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control. Data are mean ± SD of 3 experiments. *, $P < 0.01$.

cells was significantly smaller than that of the negative control (Fig. 2A and B).

We also examined the effect of let-7c transfection on apoptosis. For this purpose, we used flow cytometry to determine the percentages of Annexin-V–positive cells among let-7c–transfected cells and control cells treated with cisplatin. Transfection of let-7c significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control (2.9% vs. 6.1% at 24 hours, $P = 0.049$, Fig. 2C and D). Thus, induced expression of let-7c restored chemosensitivity and increased apoptosis after genotoxic chemotherapy in esophageal cancer cells.

Cisplatin activates IL-6/STAT3 prosurvival signaling pathway

What is the mechanism of let-7c–mediated chemosensitivity of esophageal cells? To answer this question, we hypothesized that let-7c expression regulated apoptosis in cisplatin-treated cells through downregulation of IL-6–mediated signaling pathway. This was based on Target scan and miRBase Targets database, which showed that IL-6 is potential target of let-7c, and also on previous finding of IL-6 as a putative let-7 target (18). In addition, a recent study has shown that IL-6 is released by genotoxic chemotherapy to protect cancer cell from cell death (19). First, we showed that cisplatin activated IL-6 mRNA in esophageal cancer cells (Fig. 3A). Next, we assayed IL-6 levels by ELISA. Cisplatin significantly increased the amount of IL-6 in the conditioned media (Fig. 3B). Furthermore, phosphorylated STAT3, which is downstream of IL-6, was induced by cisplatin in esophageal cancer cells (Fig. 3C and D). These results suggest that cisplatin activates the IL-6/STAT3 signaling pathway in an autocrine manner in esophageal cancer cells.

Next, we investigated whether activation of IL-6/STAT3 pathway protects cisplatin-exposed cancer cells from apoptosis. For this purpose, we examined cell viability and apoptosis in cisplatin-treated IL-6 knockdown cells and control cells. MTT assay showed that knockdown of IL-6 in esophageal cancer cells significantly reduced cell viability (Fig. 3E), and Annexin V assay showed that knockdown of IL-6 in esophageal cancer cells significantly increased the rate of apoptosis (Fig. 3F and G). These results indicate that cisplatin activates IL-6/STAT3 pathway in cancer cells, paradoxically providing protection of cancer cells against cell death.

Let-7 represses IL-6/STAT3 prosurvival pathway after genotoxic chemotherapy

We examined whether let-7 represses the activation of IL-6/STAT3 signaling pathway after cisplatin chemotherapy. Expression of IL-6 mRNA was significantly reduced after

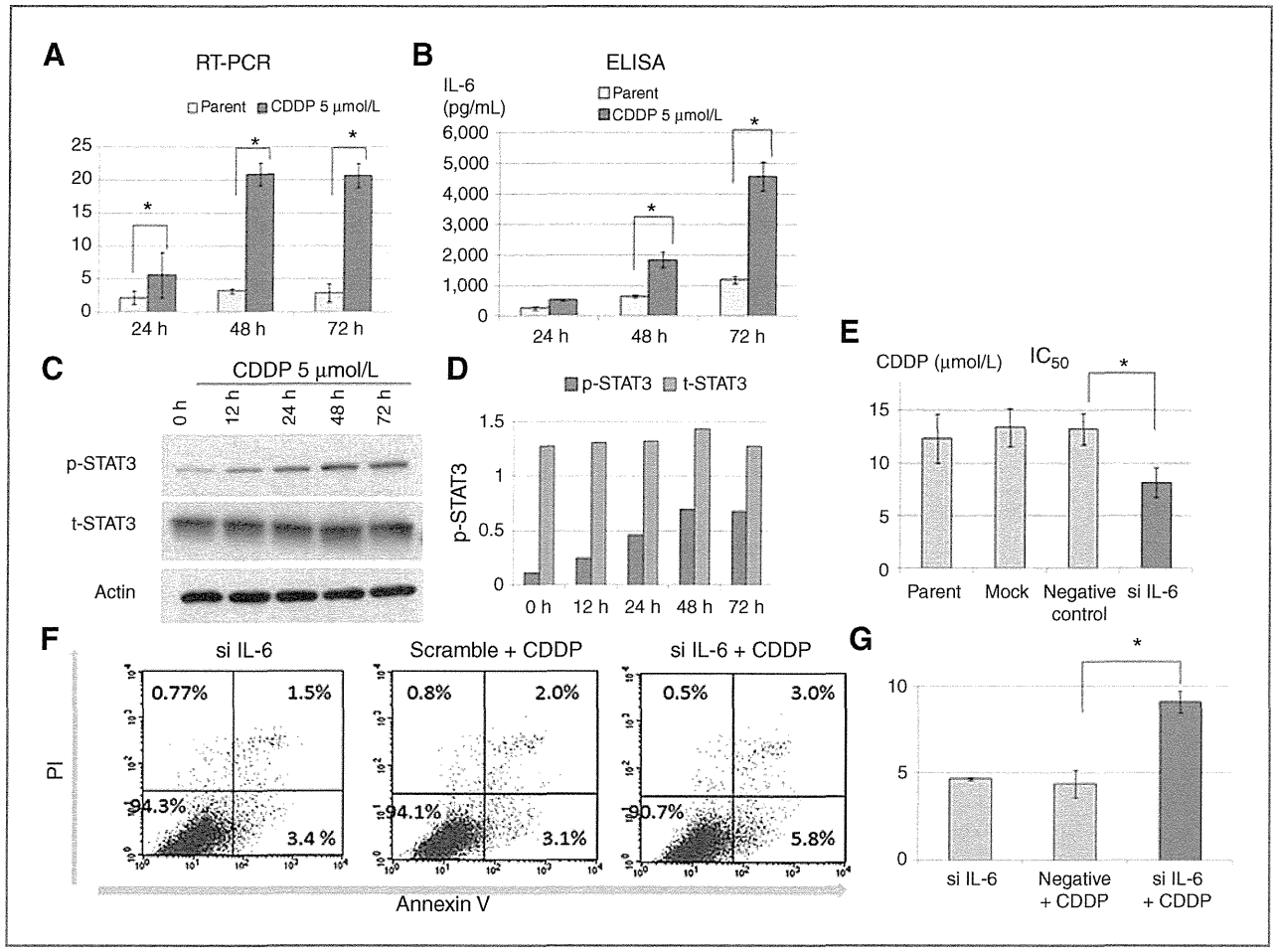

Figure 3. Cisplatin activates prosurvival IL-6/STAT3 signaling pathway. A, cisplatin significantly increased the expression of IL-6 mRNA in esophageal cancer cells at 24-, 48-, and 72-hour exposure. B, cisplatin significantly increased the expression of IL-6 protein in supernatants of conditioned medium at 24-, 48-, and 72-hour exposure. C, Western blot analysis of phosphorylated STAT3 and total STAT3 after cisplatin exposure. Exposure to cisplatin induced phosphorylated STAT3 in esophageal cancer cells. p-STAT3, phosphorylated STAT3; t-STAT3, total STAT3. D, semiquantitative analyses of expression of pSTAT3 and t-STAT3 in (C) by using densitometer. E, the IC_{50} level of cisplatin in siIL-6–transfected cells is significantly lower than in negative control transfected cells. F and G, transfection of siIL-6 significantly increased the proportion of apoptotic cells after cisplatin, compared with the negative control. Data in (A), (B), (E), and (G) are mean ± SD of 3 experiments. *, $P < 0.01$.

cisplatin treatment in let-7c transfected cells compared with control cells. The level of secreted IL-6 in the conditioned medium after cisplatin treatment was also significantly reduced in let-7c–transfected cells compared with control cells (Fig. 4A). Furthermore, phosphorylated STAT3 was significantly reduced in let-7c–transfected cells compared with control cells after cisplatin treatment, although the induced expression of let-7c had no apparent effect on the expression of Akt and extracellular signal–regulated kinase (Erk), which are downstream of IL-6 (Fig. 4B and C). Taken together, these results indicate that let-7 represses IL-6/STAT3 prosurvival pathway after genotoxic chemotherapy in esophageal cancer cells.

Finally, we examined the relationship between let-7c and IL-6 expression in clinical samples obtained from 40 patients with esophageal cancer. Let-7c expression of cancer tissue is significantly lower than that of noncancerous tissue (Fig. 4D). In contrast, IL-6 expression was significantly higher in cancer tissue than in noncancerous tissue (Fig. 4E). Moreover, IL-6 expression correlated inversely with let-7c expression in noncancerous tissue and esophageal cancer tissue (Fig. 4F).

Discussion

In multimodal therapy for esophageal cancer, chemotherapy is often combined with radiation and/or surgery. If prediction of the response to chemotherapy before surgery is possible, one can offer another treatment option for patients who show resistance to chemotherapy. In the present study, we investigated whether we could predict the response to cisplatin-based chemotherapy by analyzing the miRNA expression in esophageal cancer using biopsy samples before treatment. The results showed that low expression of let-7b and let7c is associated with low chemosensitivity in patients with esophageal cancer. The

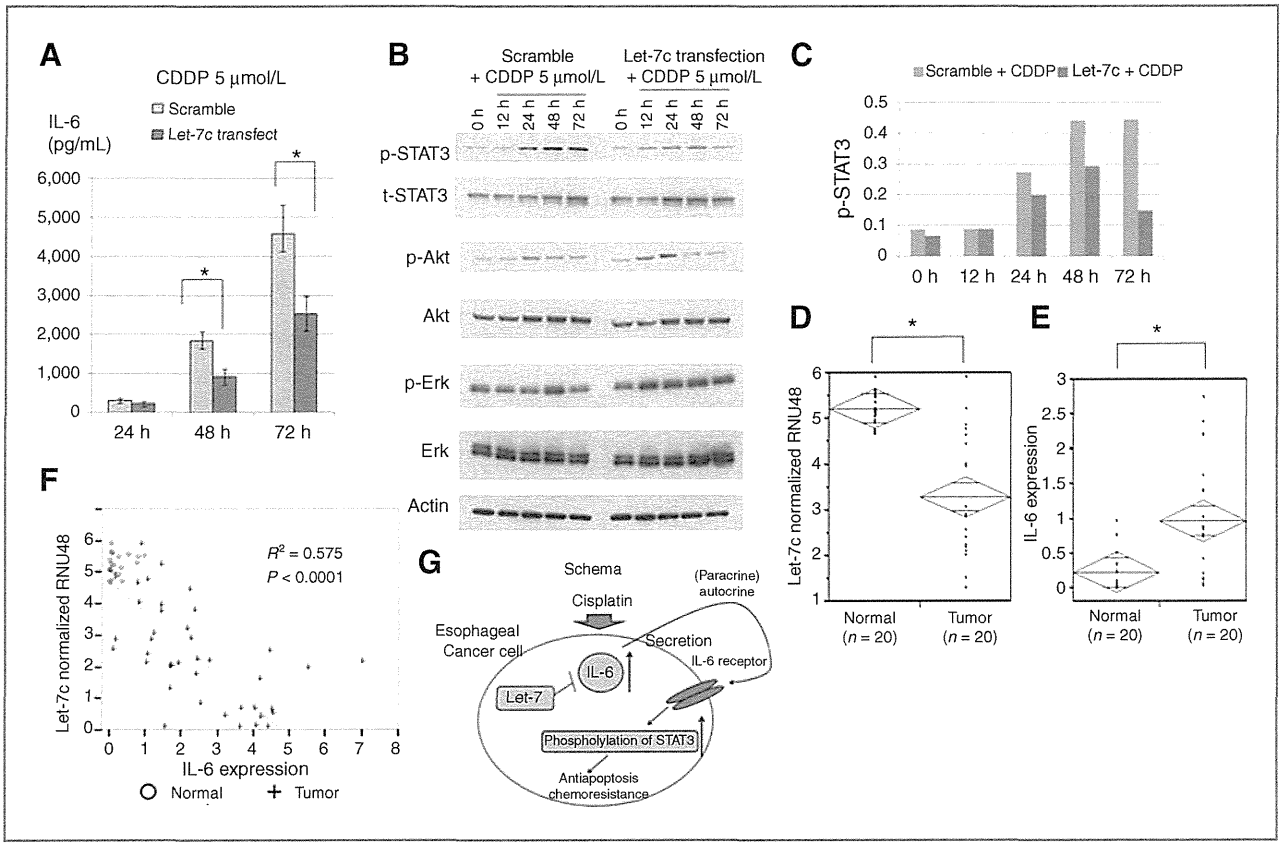

Figure 4. Overexpression of let-7c represses IL-6/STAT3 prosurvival pathway after cisplatin exposure. A, cisplatin significantly reduced IL-6 production in conditioned medium of let-7c–transfected cells compared with control cells at 48 and 72 hours. Data are mean ± SD of 3 experiments. *, $P < 0.01$. B, Western blot analysis of differential expression of proteins downstream of IL-6 after cisplatin exposure. Phosphorylated STAT3 was significantly reduced in let-7c–transfected cells compared with control cells. Representative data of 3 experiments with similar results. p-STAT3, phosphorylated STAT3; t-STAT3, total STAT3. p-Akt and p-Erk means phosphorylated Akt and Erk, respectively. C, semiquantitative analyses of expression of pSTAT3 in (B) by using densitometer. D, let-7c expression in esophageal cancer tissue is significantly lower than that of noncancerous tissue. E, IL-6 mRNA expression in cancer tissue is significantly higher than that of noncancerous tissue, determined by real-time RT-PCR. F, IL-6 mRNA expression correlated inversely with let-7c expression in noncancerous tissue ($n = 20$) and esophageal cancerous tissue ($n = 40$). G, schematic overview of relationship between let-7 and IL-6/STAT3 pathway in chemoresistance. IL-6 expression is upregulated after cisplatin exposure in esophageal cancer cells. In autocrine manner (although paracrine manner may also exist), increased expression of IL-6 upregulates phosphorylation of p-STAT3, resulting in antiapoptosis and chemoresistance. Let-7 restores sensitivity to cisplatin through repressing IL-6/p-STAT prosurvival pathway by inhibiting directly IL-6 expression.

results also showed that the effect of let-7 expression on chemosensitivity of esophageal cancer is mediated through let-7–induced repression of the IL-6/STAT3 pathway, which is prosurvival pathway activated through exposure to genotoxic agents such as cisplatin.

A few studies have reported the clinical use of miRNA expression for prediction of response to chemotherapy. Yang and colleagues (20) conducted miRNA microarray in 69 patients with epithelial ovarian cancer who had received cisplatin-based chemotherapy and reported significantly reduced let-7i expression in chemotherapy-resistant patients. They confirmed the clinical relevance of let-7i as a biomarker to predict chemotherapy response in a validation set of another 72 patients. However, the underlying mechanism of the involvement of let-7i expression in chemosensitivity of ovarian cancer was not clarified in their study. Another study by Nakajima and colleagues (21), which evaluated the expression of several miRNAs in 46 patients with recurrent or residual colon cancer, showed that upregulation of miR-181b and let-7g was significantly associated with poor response to 5-FU–based antimetabolite S-1. However, their finding of the correlation between high expression of let-7 and poor response to chemotherapy is different from our results.

The involvement of let-7 family in chemosensitivity has been examined in several *in vitro* studies. In pancreatic cancer cells, the expression of let-7b,c,d,e was significantly reduced in gemcitabine-resistant cancer cells, and upregulation of let-7 expression resulted in the reversal of epithelial–mesenchymal transition in gemcitabine-resistant cancer cells (22). In hepatocellular carcinoma cells, let-7 inhibited Bcl-xL expression, which is an antiapoptotic member of the Bcl-2 family and known to induce apoptosis in cooperation with anticancer drugs that target Mcl-1, antiapoptotic Bcl-2 protein (23). In oral cancer cells, let-7d negatively regulated EMT expression by targeting Twist and Snail and played an

important role in modulating the sensitivity to chemotherapy such as cisplatin and 5-FU (24). In the present study, let-7 expression modulated the chemosensitivity to genotoxic chemotherapy in esophageal cancer through the IL-6/STAT3 pathway.

IL-6 is an inflammatory cytokine known to be released from macrophages and T lymphocytes as well as from cancer cells (25). Previous studies indicated that IL-6 is associated with resistance to chemotherapy in a variety of malignancies. In ovarian cancer, Wang and colleagues (26) reported that autocrine production of IL-6 confers resistance to cisplatin and paclitaxel. Iliopoulos and colleagues (18) reported that IL-6 plays a pivotal role in chemoresistance by inducing the conversion of non–cancer stem cells to cancer stem cells in breast cancer cells. With regard to esophageal cancer, one recent study showed that intracellular IL-6 expression after cisplatin exposure is associated with reduced sensitivity to cisplatin treatment and that knockdown of IL-6 expression restored sensitivity to cisplatin treatment. In the present study, we showed that esophageal cancer cells release IL-6 after exposure to cisplatin and that IL-6 activated prosurvival JAK/STAT3 pathway in an autocrine manner, leading to cisplatin resistance. On the other hand, another recent report by Gilbert and Hemann (27) showed that IL-6 secreted from endothelial cells after treatment with doxorubicin created chemoresistant niche and is involved in increased resistance to DNA damaging agents in paracrine manner. Indeed, we showed in this study that let-7 repressed IL-6 activation in esophageal cancer cells in an autocrine manner during chemotherapy, but we think that let-7 can inhibit IL-6 production from the surrounding normal cells such as fibroblasts, endothelial cells, and macrophages. Further studies are needed to clarify whether let-7 represses paracrine IL-6 signal in the surrounding normal tissues in addition to its effect on autocrine IL-6 production from cancer cells.

In this study, transfection of let-7c resulted in a significant reduction in phosphorylated STAT3 in the cells, but it did not induce any significant change in the expression of Akt and Erk. Indeed, Akt and Erk are considered to be downstream of IL-6, similar to STAT3, and to be involved in antiapoptotic pathway (26), although their expression can be regulated by upstream signals other than IL-6. For example, Akt expression is reported to be regulated by phosphoinositide 3-kinase (PI3K), mTOR, and phosphate and tensin homolog (PTEN) deleted from chromosome 10 (28–31). Erk expression is also reported to be regulated by several receptors protein tyrosine kinases and the mitogen-activated protein kinase (MAPK) pathway (32–35). One possible explanation for the lack of significant effect of let-7c transfection on Akt and Erk could be that Akt and Erk pathways are regulated mainly by signals other than IL-6 whereas STAT3 is regulated by IL-6 expression in esophageal cancer cells.

There is increasing evidence that let-7 inhibits IL-6 signaling pathway directly by targeting IL-6. Iliopoulos and colleagues (18) showed that NF-κB, Lin28, let-7, and IL-6 form an inflammatory positive feedback loop. NF-κB induces Lin28 expression, leading to inhibition of let-7 and expression of the encoding IL-6. IL-6 can itself activate NF-κB, resulting in a positive feedback loop. Another recent report showed that downregulation of let-7 promotes the expression of IL-6 and IL-10 during *Salmonella* infection. Thus, the association between let-7 and IL-6 under an inflammatory environment has been described, but this is the first time to show that the association between let-7 and IL-6 plays an important role in the sensitivity to chemotherapy for cancer. This result suggests that treatment targeting this pathway is likely to enhance the response to anticancer chemotherapy.

The present study has certain limitations. First, the clinical results were based on retrospective analysis by using biopsy samples obtained from patients who underwent preoperative chemotherapy followed by surgery at only one institution. Second, the current results that let-7 modulates the chemosensitivity in esophageal cancer through the regulation of IL-6/STAT3 pathway may be adapted into cisplatin-based chemotherapy but not other chemotherapeutic regimens that do not include cisplatin, because cisplatin-resistant cell line used in this study did not show resistance to 5-FU nor Adriamycin (data not shown). However, cisplatin-based chemotherapy is the most widely used chemotherapeutic regimen for esophageal cancer, although other chemotherapeutic regimens are used occasionally, such as taxane-based chemotherapy for esophageal cancer which has low expression of let-7. Third, before one can apply the findings that let-7 expression can be used clinically to predict the response of esophageal cancer to chemotherapy, we need to validate this result in a prospective multicenter clinical trial.

In summary, we showed that evaluation of let-7 b and let-7c expression before treatment is potentially useful to predict the response to chemotherapy in patients with esophageal cancer. Moreover, the results also showed that the effect of let-7 expression on chemosensitivity is mediated though downregulation of IL-6/STAT3 pathway. Further studies are needed to explore the therapeutic potential of the let-7/IL-6/STAT3 pathway in genotoxic anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Sugimura, H. Miyata, K. Tanaka, R. Hamano, T. Takahashi, Y. Kurokawa, M. Yamasaki, K. Nakajima, S. Takiguchi, M. Mori, Y, Doki

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Received February 28, 2012; revised June 12, 2012; accepted July 10, 2012; published OnlineFirst July 30, 2012.

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Clinical Trial of the Intratumoral Administration of Labeled DC Combined With Systemic Chemotherapy for Esophageal Cancer

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Summary: Esophageal cancer is a highly aggressive disease, and improved modalities for its treatment are needed. We performed chemoimmunotherapy involving the intratumoral administration of ^{111}In-labeled dendritic cells (DC) in combination with preoperative chemotherapy in 5 esophageal cancer patients. Mature DC were generated and traced by scintigraphy after their administration. No adverse events that were directly related to the intratumoral DC administration were observed. Delayed-type hypersensitivity skin tests against keyhole limpet hemocyanin, which was added to the culture medium, detected a positive response in 3 patients, and keyhole limpet hemocyanin antibody production was observed in 4 patients, suggesting that intratumorally administered DC migrate to the lymph nodes, where they function as antigen-presenting cells. However, scintigraphic images obtained after the DC administration demonstrated that the DC remained at the esophageal tumor injection sites in all cases, and no DC accumulation was observed elsewhere. The accumulation of CD83$^+$ cells in the primary tumor was also observed in 2 out of 4 patients in an immunohistochemical analysis using surgically resected specimens. Although the induction of tumor-specific immune responses during chemoimmunotherapy was also analyzed in enzyme-linked immunosorbent assay against 28 tumor antigens, none of the antibodies against the antigens displayed enhanced titers. No changes of NY-ESO-1-specific cellular immune response was observed in a patient who displayed NY-ESO-1 antibody production before the DC administration. These results suggest that the intratumoral administration of ^{111}In-labeled mature DC during chemotherapy does not lead to detectable DC migration from the primary tumor to the draining lymph nodes, and therefore, might not achieve an optimal clinical response.

Key Words: intratumoral DC administration, esophageal cancer, DC migration, chemoimmunotherapy, scintigraphy, KLH, CD83$^+$ cells, antibody against 28 tumor antigens, NY-ESO-1 specific T-cell response, DTH

(*J Immunother* 2012;35:513–521)

Esophageal cancer is one of the most aggressive forms of cancer.[1] Despite recent technical advances in surgery, chemotherapy, and radiation therapy, the prognosis of esophageal cancer remains poor.[2,3] Thus, more effective modalities for treating the disease are needed.

One possible solution to this problem is immunotherapy. The tumor immune responses of esophageal cancer patients have been extensively analyzed. As a result, it has been found that reduced tumor tissue infiltration by lymphocytes[4] and dendritic cells (DC)[5,6] and the decreased expression of HLA class I[7] on tumor cells are correlated with a poor prognosis in esophageal cancer patients. Furthermore, several tumor antigens, example, NY-ESO-1, a cancer-testis antigen identified by the serological analysis of cancer antigens by recombinant cDNA expression cloning method using esophageal cancer specimens and autologous serum,[8,9] are frequently observed in esophageal cancer. Among the cellular components that exert antitumor immune responses, DC plays a central role in tumor tissue as professional antigen-presenting cells (APC).[6,10,11] DC captures the tumor antigens released from tumor cells; move to the draining lymph nodes; present the processed antigens to T cells; and induce or activate antigen-specific effector cells, example, T helper cells and cytotoxic T lymphocytes, resulting in the infiltration of these T cells into tumor tissue, where they can attack tumor cells. It has been shown that some chemotherapeutic drugs not only kill tumor cells directly but also induce tumor cell death indirectly by encouraging DC to engulf them by upregulating the expression of specific molecules, example, calreticulin and high mobility group box 1 protein, which are also known as "eat me" and "danger" signals, respectively,[10,11] on tumor cells.

On the basis of these findings, the adoptive transfer of autologous DC has been employed in several studies of cancer treatment. In particular, the direct administration of DC into tumors would probably be the most suitable way of capturing tumor antigens and inducing subsequent immune activation.[12,13] Although there have been several clinical studies of the administration of DC to tumors, the trafficking of DC from the tumor to the draining lymph nodes and the stimulation of antigen-specific immune responses have not been fully elucidated.

Received for publication December 20, 2011; accepted May 25, 2012.
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Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website, www.immunotherapy-journal.com.

In this study, we intratumorally administered ^{111}In-labeled DC during preoperative chemotherapy and traced the labeled cells by scintigraphy. Mature DC (mDC) generated by short-term culturing were used in this study.[14] We analyzed the DC in the tumor tissues and draining lymph nodes immunohistochemically, as well as the serological and cellular immune responses induced against tumor antigens.

MATERIALS AND METHODS

Patients

Patients were considered to be eligible for this study (UMIN 000000669, https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function = brows&action = brows&type = summary&recptno = R000000804&language = J) when they met the following inclusion criteria: they had resectable esophageal cancer and suspected metastasis in their draining lymph node(s), were younger than 80 years and without distant metastases, had received no prior treatment for esophageal cancer, had an Eastern Cooperative Oncology Group performance status of 0 or 1,[15] had pathologically confirmed esophageal cancer but no other severe disease, and had provided written informed consent. This study was approved by the Institutional Review Board of Osaka University.

Study Schedule

Safety and the detection of migrated DC by scintigraphy were the primary endpoints of this study. Two cycles of 4-week preoperative chemotherapy, which involved the administration of adriacin (30 mg/m^2) and cisplatin (70 mg/m^2) on day 1 and 5-fluorouracil (5-FU; 1000 mg) on days 1–7, followed by 3 weeks off, were scheduled before surgical treatment (Fig. 1). mDC generated by the short-term culturing of monocytes were administered to the primary esophageal tumor on day 3 using endoscopy. Adverse events were assessed according to the National Cancer Institute, Common Terminology Criteria for Adverse Events v3.0. The labeled DC were traced by scintigraphy (Symbia T6, Siemens, Tokyo, Japan), which was performed at 15 minutes, 24 hours, and 96 hours after the DC administration. The clinical response was evaluated according to the size of the tumor on computed tomography (CT) scans taken before and after chemotherapy.[16] Briefly, tumor status was assessed as the product of the longest diameter and the rectangular diameter, and the response rate was calculated using the following formula: (sum of the products obtained before therapy − sum of the products obtained after therapy)/(sum of the products obtained before therapy) × 100%, where the sum of the products includes the tumor diameter products of the primary tumor and the targeted lymph nodes. The clinical response was defined as "no change (NC)" when the response rate was between −25% and 50% and as "progressive disease" when the response rate was < −25% and/or new metastasis was observed. The blood samples used to analyze the patients' humoral and cellular immune responses were drawn during the leukapheresis and the surgical treatment. Surgically resected tissue was used for the immunohistochemical analysis. Tissues obtained from 5 esophageal cancer patients that were not subjected to DC administration were used as controls.

Generation of DC

Mononuclear cells were collected by leukapheresis using a COBE spectra blood separator (Gambro KK, Tokyo, Japan) and incubated in AIM-V Medium (Gibco, Invitrogen, Tokyo, Japan) for 2 hours at 37°C. Adherent cells were incubated in AIM-V Medium containing 75 μg/mL granulocyte macrophage-colony stimulating factor (GM-CSF; Primmune, Kobe, Japan) and 10 μg/mL interleukin (IL)-4 (Primmune) for 3 days at 37°C. For the last 24 hours, 5 μg/mL prostaglandin (PG; Ono Pharmaceutical Company, Tokyo, Japan), 500,000 IU/mL interferon α (IFN-α;

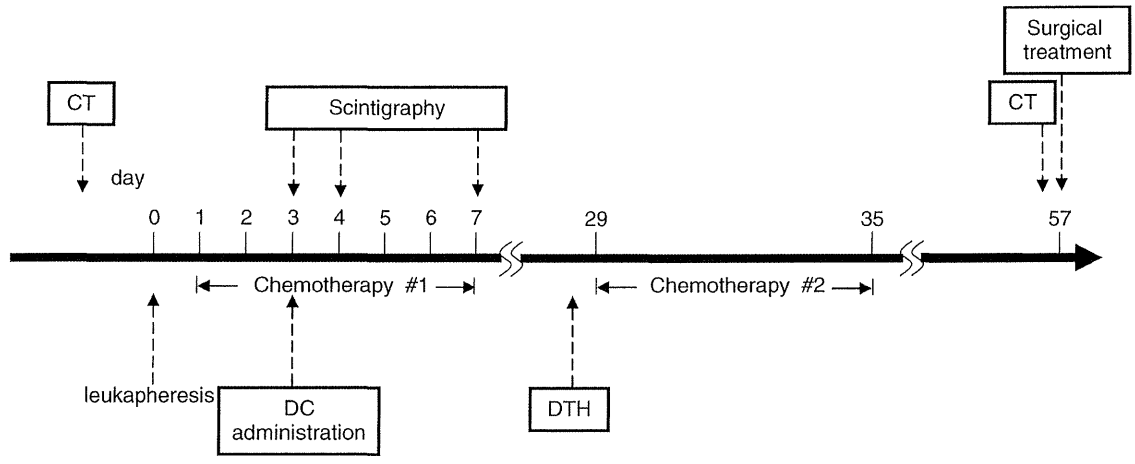

FIGURE 1. Schedule of our clinical trial of intratumoral dendritic cell (DC) administration. Two cycles of chemotherapy, which involved 4-week preoperative chemotherapy with adriacin and cisplatin on day 1 and 5-fluorouracil on days 1–7, followed by 3-week off, were scheduled before surgical treatment. Monocytes were collected by leukapheresis on day 0, and mature dendritic cell (mDC) were harvested after coculturing the adherent monocytes with granulocyte macrophage-colony stimulating factor, interluekin-4, prostaglandin, interferon-α, and OK-432 for 3 days. Then, the mDC were labeled with ^{111}In-oxine and administered to the patient's primary esophageal tumor on day 3 using endoscopy. Labeled DC were traced by scintigraphy at 15 minutes, 24 hours, and 96 hours after DC administration. Computed tomography (CT) scans were taken before and after chemotherapy. The delayed-type hypersensitivity (DTH) skin test was performed 2 weeks after the DC administration. All 5 patients completed this schedule. Finally, surgical treatment was performed in all patients except E-4, in whom a liver metastasis was detected on a CT scan taken after the second round of chemotherapy.

TABLE 1. Quality of DC Prepared From the Enrolled Patients

ID	Age/Sex	Stage	DC Administered			Clinical Response
			Viability (%)	Purity (%)	Number	
E-1	66/M	III	94.1	85.7	6.80×10^6	NC
E-2	66/M	III	98.4	97.9	6.50×10^7	NC
E-3	61/M	III	93.9	94.3	6.60×10^6	NC
E-4	51/M	III	99.1	92.1	1.40×10^7	PD
E-5	69/M	III	98.5	87.9	1.40×10^7	NC

DC indicates dendritic cells; NC, no change; PD, progressive disease.

Otsuka Pharmaceutical, Tokyo, Japan), 5 KE/mL Picibanil (OK-432; Chugai Pharmaceutical, Tokyo, Japan), and 200 μg/mL keyhole limpet hemocyanin (KLH; Calbiochem, Darmstadt, Germany) were added to the medium.[14] The cells were harvested and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Tokyo, Japan) using labeled CD11c, HLA-DR, CD14, CD40, CD80, CD83, and CD86 monoclonal antibody (mAb) (BD Biosciences).

Administration of Labeled DC

DC were incubated with ^{111}In-oxine (1 mCi) (Altana Pharma, Milan, Italy) for 15 minutes at room temperature. The cells were then washed, resuspended in a total volume of 1.2 mL of saline (Otsuka Pharmaceutical), and administered to the patient's primary esophageal tumor using endoscopy after checking the radioactivity of the labeled DC using a gamma counter.

Delayed-type Hypersensitivity (DTH)

The DTH skin test was performed 2 weeks after the DC administration.[17] Briefly, 20 ug of KLH were intradermally inoculated into each patient's forearm. The diameter of indurated tissue was measured after 48 hours, and a diameter of >2 mm was considered to indicate positivity.

Humoral Immune Response to KLH

The production of KLH antibody was measured by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates that had been coated with KLH (0.2 μg/mL) and left overnight were filled with serially diluted serum samples and incubated for 1 hour at room temperature. Anti-human total immunoglobulin G mAb (MBL, Nagoya, Japan) and 3,3′ 5,5-tetramethyl-benzide (Pierce, Rock Ford, IL) were added, and their optical density values at 450 mm were measured using a microtiter plate reader (Versa max, Molecular Devices, Japan). Ovalbumin was used as the control protein.

Humoral Immune Responses to Tumor Antigens

The production of antibodies to NY-ESO-1, LAGE-1, Melan-A, p53, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, SSX1, SSX2, SSX4, SOX2, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, CT47, XAGE1, GAGE2, Z1347, ZHP24, CT63, CT24, ERG, CT39, SAGE1, CT57/ACTL8, HERV-HGAG, and dihydrofolate reductase was measured by ELISA, and the reciprocal titers were calculated.[18,19] Serially diluted serum samples were added to 96-well plates that had been coated with 1 μg/mL recombinant protein, incubated overnight at 4°C, and blocked for 2 hours at room temperature. After overnight incubation, the plates were extensively washed with phosphate buffered saline containing 0.2% Tween 20. Antigen-bound serum immunoglobulin G was detected using an alkaline phosphatase-conjugated specific mAb (Southern Biotech, Birmingham, AL). After the addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a Synergy 2 fluorescence reader (BioTek, Winooski, VT). The reciprocal titer was calculated for each sample as the maximal dilution that displayed a significant reaction to a specific antigen. Specificity was determined by comparing seroreactivity among the various antigens tested. Positive and negative control sera were included in each assay. A positive result was defined as a reciprocal titer of >100.

Immunohistochemistry (IHC)

IHC was performed using formalin-fixed paraffin-embedded specimens. The anti-CD83 mAb was used for the staining (Beckman Coulter, Tokyo, Japan).

Cellular Immune Response to NY-ESO-1

$CD8^+$ or $CD4^+$ T cells were stimulated with NY-ESO-1 overlapping peptides (OLP) using autologous CD4-depleted and CD8-depleted peripheral blood leukocytes as APC in AIM-V Medium supplemented with IL-7 (40 ng/mL R&D Systems, Minneapolis) and IL-2 (20 IU/mL Takeda Pharmaceutical Company, Tokyo, Japan). After being cultured for 8 days, the cells were harvested and stimulated with APC that had been pulsed with NY-ESO-1 OLP for 24 hours, and their intracellular IFN-γ expression was analyzed using Cytofix/Cytoperm and the GolgiStop kit (BD

TABLE 2. Side Effects of Intratumoral Dendritic Cell Administration

ID	Related	Possibly Related	Unrelated
E-1	Stomatitis, alopecia, anemia, anorexia	Fever	Hyponatremia, hypoalbuminemia
E-2	Stomatitis, anemia	Fever	Hyponatremia, hypoalbuminemia
E-3	Stomatitis	Fever	Hyperkalemia, hyperglycemia, hyperkalemia
E-4	Malaise, anorexia, nausea	Fever	Diarrhea
E-5	Alopecia, anorexia, nausea	Fever	Hyponatremia

All toxicities were grade 1.

Biosciences). Two-color analysis using fluorescein-conjugated IFN-γ mAb (eBioscience, San Diego) and CD8 mAb (Becton Dickinson) or CD4 mAb (BioLegend, Tokyo, Japan) was performed to determine the proportion of IFN-γ producing $CD8^+$ or $CD4^+$ T cells using a FACSCalibur flow cytometer (Becton Dickinson). SSX2 OLP were used as a negative control.

RESULTS

Quality of DC Prepared From Patients

Five esophageal cancer patients were studied (Table 1). Mononuclear cells were collected by leukapheresis and were used to prepare DC. More than 6×10^6 mDC were harvested from each patient after coculturing the adherent

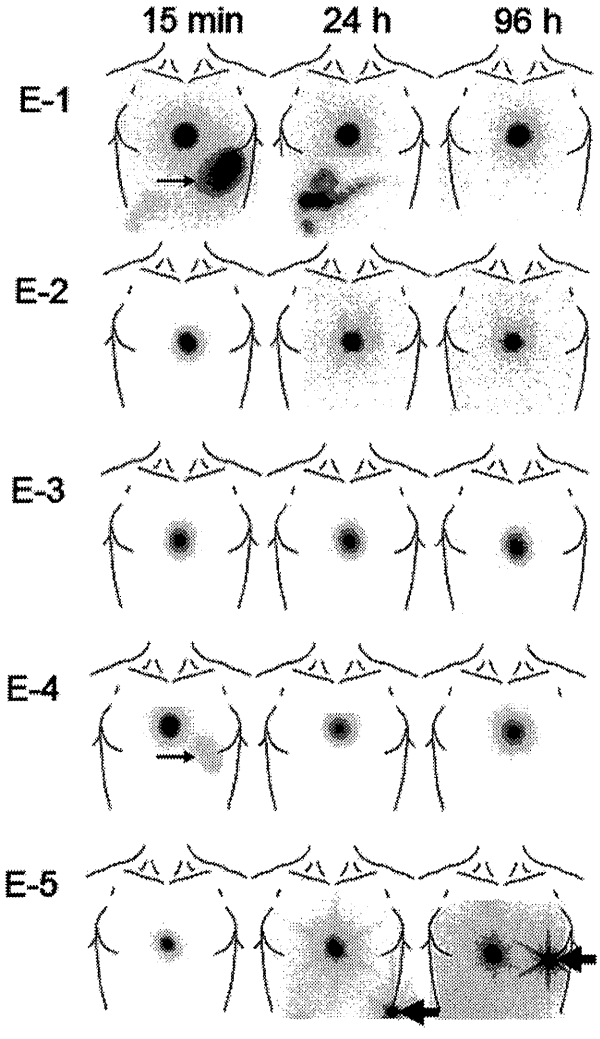

FIGURE 2. Scintigraphic images of ^{111}In-oxine-labeled dendritic cell (DC) in esophageal cancer patients after their intratumoral administration. Chest and abdominal scintigraphy images were taken at 15 minutes, 24 hours, and 96 hours after the intratumoral administration of labeled DC. The areas of accumulation observed in the abdomen of E-1 and E-4 at 15 minutes after the DC administration (arrows) were supposed to have been caused by the leakage of DC from the injection site. The arrow in E-5 indicates a hot reference used as a positive control.

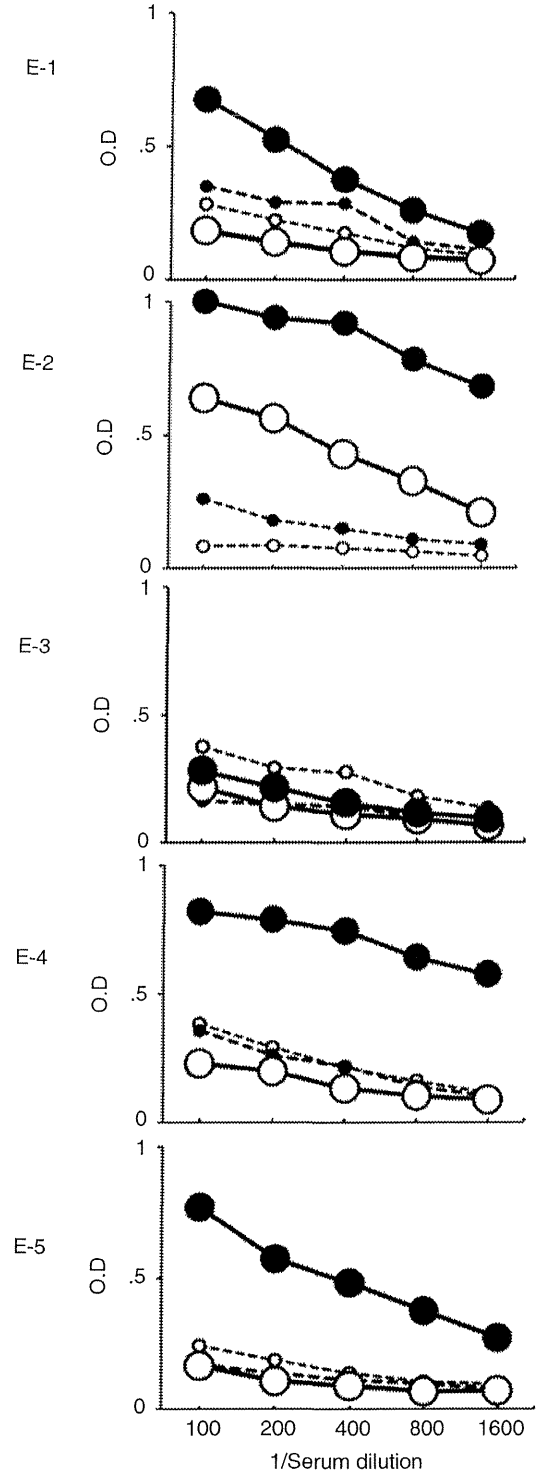

FIGURE 3. Serological analysis of the antikeyhole limpet hemocyanin (KLH)-specific immune response by enzyme-linked immunosorbent assay. Antibody titers in serum samples obtained before (open circles) and after (closed circles) the intratumoral DC administration were analyzed. Recombinant KLH of 0.2 μg/mL and serially diluted serum samples were applied as indicated, and the optical density values of the samples were measured. Ovalbumin was used as a control (dotted line).

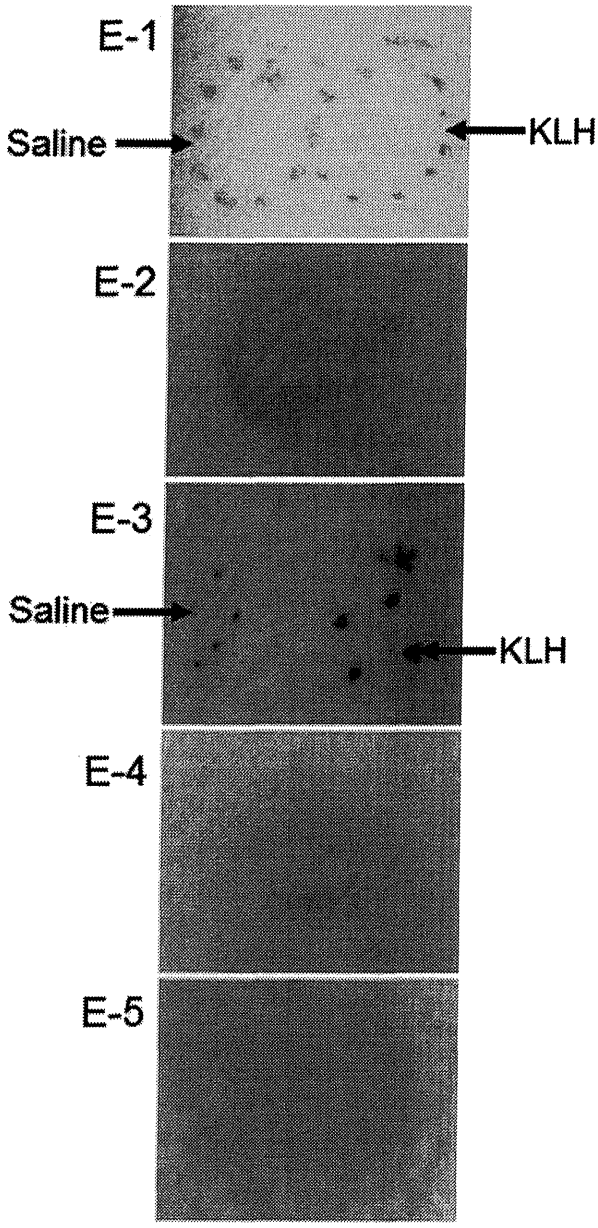

FIGURE 4. Delayed-type hypersensitivity (DTH) reaction against keyhole limpet hemocyanin (KLH). A total of 20 μg recombinant KLH protein were intradermally inoculated at 2 weeks after the dendritic cell administration, and the diameter of the indurated tissue was measured after 48 hours. A diameter of >2 mm was considered to indicate positivity. Positive DTH reactions were observed in patients E-2 (diameter; 22 mm), E-4 (52 mm), and E-5 (35 mm).

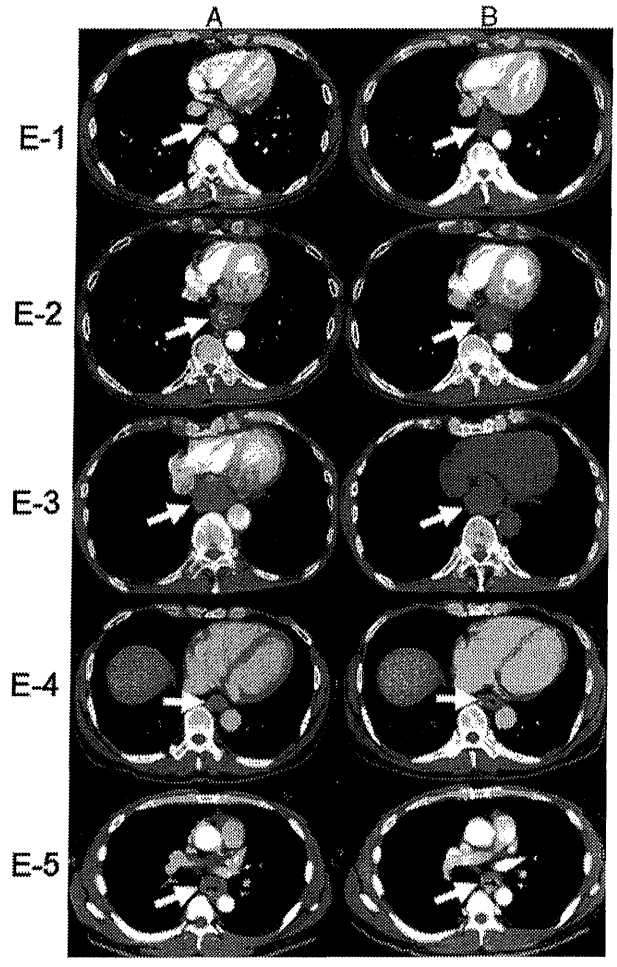

FIGURE 5. Computed tomography (CT) images of primary esophageal tumors obtained before and after the intratumoral dendritic cell (DC) administration. The primary esophageal tumor (arrow) was located in the lower intrathoracic esophagus region in patients E-1, E-2, E-3, and E-5 and in the middle region of the esophagus in patient E-4. CT images taken after the DC administration and after 2 cycles of chemotherapy (B) showed a marginal decrease in tumor status in E-2, E-3, E-4, and E-5 and a marginal increase in tumor status in E-1 compared with the images taken before the DC administration (A) (Supplementary Table, http://links.lww.com/JIT/A247).

monocytes with GM-CSF, IL-4, PG, IFN-α, and OK-432 for 3 days. The viability of the cells was >93% (Table 1), and the purity of the $CD11c^+$ $CD14^-$HLA-DR^+ cells was >85% (Supplementary Figure 1, http://links.lww.com/JIT/A244).

Toxicity of Intratumoral DC Administration

Table 2 shows the side effects observed during this clinical trial, which involved leukapheresis and 2 cycles of chemotherapy followed by intratumoral DC administration. All toxicities were grade 1. No serious adverse events that were directly related to the intratumoral DC administration, example, bleeding, ulceration at the injection site, or an unexpected immunologic response, were observed.

Tracing of Labeled DC

Figure 2 shows scintigraphic images obtained at 15 minutes, 24 hours, and 96 hours after the intratumoral administration of ^{111}In-oxine-labeled DC using an endoscope. The images taken at 15 minute after the DC administration demonstrated that the DC were localized at the primary esophageal cancer injection site in all cases. DC accumulation at another site; that is, the stomach, was observed in E-1 and E-4 at 15 minute after the DC administration (arrows in Fig. 2), and this was probably caused by the leakage of DC from the injection site because the CT

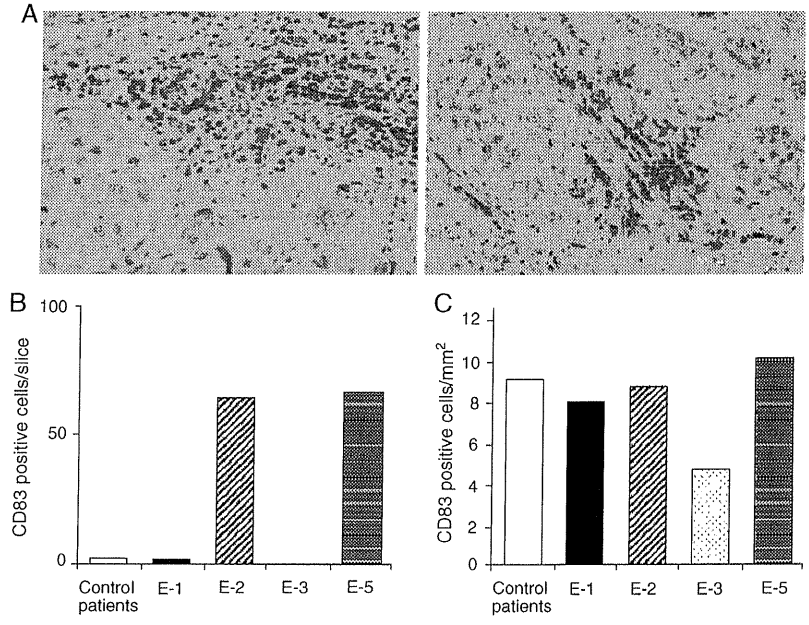

FIGURE 6. Immunohistochemical analysis of CD83$^+$ cells in surgically resected specimens obtained after intratumoral dendritic cell (DC) administration. Immunohistochemical analysis was performed with anti-CD83 monoclonal antibody using esophageal tumor and draining lymph node tissue from patients E-1, E-2, E-3, and E-5. E-4 did not undergo surgery after chemotherapy. Specimens from 5 esophageal cancer patients who did not undergo DC administration were used as controls. Abundant CD83$^+$ cell infiltration was observed in the tumors obtained from E-2 (A, left) and E-5 (A, right), whereas CD83$^+$ cells were rarely observed in the tumors from E-1, E-3, and the 5 control esophageal cancer patients. The mean number of CD83$^+$ cells in 10 slices obtained from a primary tumor tissue block taken from the injection site is shown in B. In the draining lymph nodes, the number of CD83$^+$ cells in a 1 × 1 mm^2 was counted, and the mean number of CD83$^+$ cells in 5 fields is shown in C.

images did not display any visceral tumors in the abdomen in these patients. The images obtained at 24 and 96 hours after the DC administration demonstrated that the DC remained at the injection site in all cases, although DC leakage was observed in the small intestine of E-1 at 24 hours. The arrows in E-5 indicate the hot reference used as a positive control.

Antibody Response Against KLH

The humoral immune response against KLH, which was added to the culture medium during the preparation of the mDC, was evaluated by ELISA using sera obtained before and after the DC administration. An increase in the titer of the antibody against KLH was observed in all patients, except E-3, after the intratumoral DC administration (Fig. 3). Positive DTH reactions were observed in 3 patients, E-2, E-4, and E-5 (Fig. 4).

Clinical Response

The statuses of the primary esophageal tumors and metastatic lymph nodes were evaluated in all patients using CT images obtained before and after chemotherapy (Figs. 1, 5, Table 1, Supplementary Table, http://links.lww.com/JIT/A247). In E-1, enlargement of the primary tumor and the shrinkage of a lymph node metastasis were observed. The response rate of the tumor was calculated to be 0.1%, and the clinical response was evaluated as NC. In E-2, the primary tumor and the 4 targeted metastatic lymph nodes had decreased in size. The response rate was calculated to be 9.6%, and the clinical response was evaluated as NC. In E-3, the primary tumor became smaller while the 2 targeted metastatic lymph nodes were enlarged. The response rate was −11%, and the clinical response was evaluated as NC. In E-4, the response rate was 2.8%, but liver metastasis was observed after DC administration. The clinical response was evaluated as progressive disease. In E-5, the primary tumor and the metastatic lymph node decreased in size. The response rate was calculated as 23.5%, and the clinical response was evaluated as NC.

CD83$^+$ Cells in the Primary Tumor Lesions and Lymph Nodes

CD83$^+$ cells were analyzed by IHC using surgically resected specimens from patients E-1, E-2, E-3, and E-5 (Fig. 6). CD83$^+$ cells were abundant in the primary tumor lesions obtained from E-2 and E-5 while they were rarely observed in the tumors from E-1, E-3, and the 5 untreated (control) esophageal cancer patients. In the lymph nodes, CD83$^+$ cells were observed in T-cell zones in both patients who had and had not been administered DC.

Serological Immune Responses to 28 Tumor Antigens

We analyzed the antibody responses to various tumor antigens using serum samples obtained from the patients before and after DC administration (Fig. 7). Antibody production was observed against MAGE-A3 and ERG in E-2; p53, CT7, and CT46 in E-3; NY-ESO-1, LAGE-1, and GAGE2 in E-4; and NY-ESO-1, LAGE-1, p53, MAGE-A1, and MAGE-A3 in E-5. None of the antibodies displayed markedly enhanced titers. Marginal increases were observed in the titers of the antibodies against p53, CT7, and CT46 in E-3 and that against p53 in E-5. Marginal decreases were observed in the titers of the antibodies against ERG in E-2,

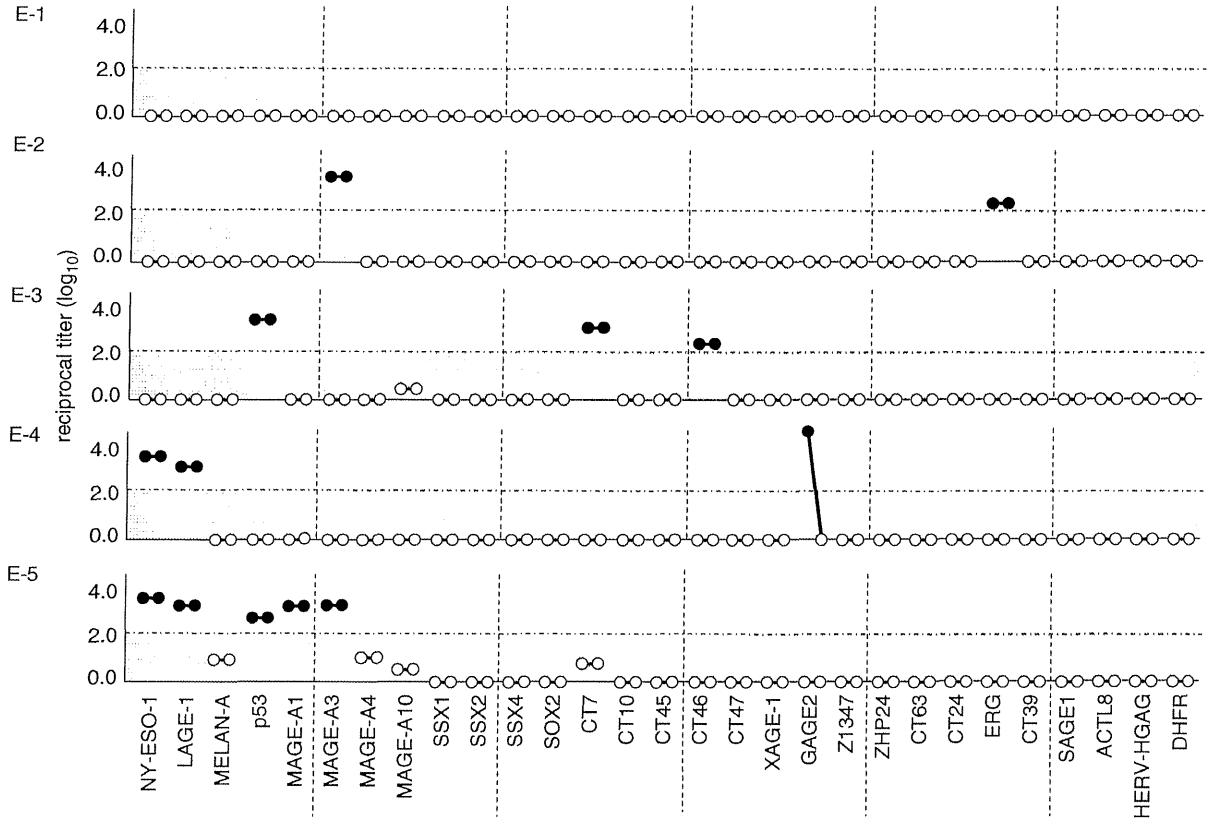

FIGURE 7. No induction of tumor-specific humoral immune responses against tumor antigens was observed in enzyme-linked immunosorbent assay. The reciprocal titers of antibodies against 28 tumor antigens in serum samples obtained before and after the intratumoral dendritic cell (DC) administration are indicated. Serially diluted serum samples obtained before the DC administration and after 2 cycles of chemotherapy were assayed against NY-ESO-1, LAGE-1, Melan-A, p53, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, SSX1, SSX2, SSX4, SOX2, CT7, CT10, CT45, CT46, CT47, XAGE1, GAGE2, Z1347, ZHP24, CT63, CT24, ERG, CT39, SAGE1, ACTL8, HERV-HGAG, and DHFR. The reciprocal titer was defined as the maximal dilution that showed a significant reaction. Closed circles indicate reciprocal titers that exceeded 100, which were considered to indicate a positive reaction.

and NY-ESO-1 and LAGE-1 in E-4, although the changes were not significant. The antibody against GAGE2, which was detected in E-4 before the DC administration, was not detected in this patient after the DC administration.

Cellular Immune Response Against NY-ESO-1

The NY-ESO-1-specific T-cell response was analyzed using PBMC obtained from E-4, who was NY-ESO-1 antibody-positive before the DC administration. IFN-γ-producing CD4 and CD8 T cells were analyzed after in-vitro stimulation with NY-ESO-1 OLP. No changes in their responses were observed after the DC administration (Supplementary Figure 2, http://links.lww.com/JIT/A246).

DISCUSSION

Immature DC (iDC) captures tumor antigens from tumor tissues. After they have captured tumor antigens, DC activation and maturation are induced by proinflammatory cytokines in the milieu. The resultant mDC then induce adaptive immunity, resulting in helper and effector T-cell differentiation and proliferation. Activated mDC in tumor tissue migrate through the afferent lymphatics and into the draining lymph nodes, where they present processed tumor antigens on MHC class I and II molecules to T cells. The migration of mDC has been shown to be influenced by cell surface CCR7 molecules through their interaction with transporter molecules, example, TREM-2, LTC4, LTD4, etc.[10,20,21] The expression of costimulatory molecules, example, CD80, CD86, and CD40, on DC, and the secretion of IL-12 from them are involved in the priming of naive T cells to the antigens present in the lymph nodes. In this study, we administered ^{111}In-labeled autologous mDC to primary esophageal tumors and traced their movement by scintigraphy. As the main objective of this study was to study the migration of labeled DC from the primary tumor site to the draining lymph nodes, mDC rather than iDC were used. The DCs were prepared from adherent cells by treating them with GM-CSF, IL-4, PG, IFN-α, and OK-432 for 3 days. These cells were fully functional mDC that strongly expressed CD40, CD80, CD83, CD86, HLA-DR, and CCR7 and displayed migratory activity towards CCL21.[14] We confirmed that CD80, CD83, CD86, CCR7, HLA-DR, and CD11c were expressed on the mDC before they were intratumorally administered. However, no migration of these cells to the draining lymph nodes was observed.

The biodistribution of labeled DC has been investigated by several groups. Ridolfi et al[22] investigated the

migration of mDC and iDC labeled with ^{99m}Tc-HMPAO and ^{111}In-oxine to the draining lymph nodes after their intradermal or subcutaneous administration. Eight times more mDC (0.39%–3.14% of injected cells) than iDC (0.05%–0.42% of injected cells) migrated to the draining lymph nodes. Regarding the route taken by migrating DC, 3 times more intradermally administered mDC (~1%) than subcutaneously administered mDC (~0.4%) migrated to the draining lymph nodes. Similarly, Quillien et al[23] showed that 1%–2% of intradermally injected mDC labeled with ^{111}In-oxine migrated to the draining lymph nodes in melanoma patients while 80% remained in the afferent lymphatic vessels. The migration of DC from solid tumor tissues, but not skin, as was detected in esophageal cancer in this study, might be much rarer, thereby making any cells that do migrate hard to detect by scintigraphic imaging. In our study, a humoral immune response against KLH was induced in most of the patients after the administration of DC that had been pretreated with KLH, suggesting that some DC migrate to the draining lymph nodes after their intratumoral administration, where they act as APC.

We employed intratumoral DC administration combined with chemotherapy. Several chemotherapy regimens have been reported to enhance the immune response against tumor cells, which is known as immunochemotherapy. The treatment of tumors with anthracycline and oxaliplatin was shown to induce the expression of calreticulin, damage-associated molecular pattern molecules, and ATP and the release of high mobility group box 1 in tumors, resulting in the facilitation of their uptake and the processing of tumor antigens by DC followed by their migration to the draining lymph nodes, where T cells are stimulated.[10,11] 5-FU is selectively cytotoxic against myeloid-derived suppressor cells, which were identified as a population of immature myeloid cells with the ability to suppress T-cell activation,[24] and low-dose cyclophosphamide also selectively ablates $CD4^+$ $CD25^+$ regulatory T cells. Thus, both of these agents enhance the antitumor immune response.[25] In a mouse model, the cytotoxic effect of cisplatin on some tumor cells was diminished when immune-deficient RAG-1 mice were used to study tumor growth, suggesting that the immune system modulates the effectiveness of cisplatin-based chemotherapy.[26] Unfortunately, no antibody response against any of 28 tumor antigens was observed in the 5 patients in this study. Moreover, no increase in the NY-ESO-1-specific CD4 or CD8 T-cell response was observed in a patient who was NY-ESO-1 antibody-positive after chemotherapy. Although each of the chemotherapy reagents used in this study has been reported to elicit antitumor immune responses, our combined chemotherapy regimen induced myelosuppression and lymphopenia, which might have prevented the induction of antitumor immunity. After 1 cycle of chemotherapy, the number of lymphocytes in the peripheral blood had decreased by 81% in E-1, 49% in E-2, 57% in E-3, 57% in E-4, and 53% in E-5 compared with that observed before leukapheresis (data not shown).

The clinical responses of the 5 patients enrolled in this study to DC administration were unexpectedly poor compared with those reported for preoperative chemotherapy in esophageal cancer patients.[27] It is possible that the antigen capturing activity of mDC might be insufficient, although mDC were demonstrated to capture antigens efficiently in a previous study.[28] Alternatively, the leukapheresis might have decreased the number of lymphocytes, which might have resulted in a failure to activate the tumor immune response, even in the presence of fully functional mDC. These possibilities should be carefully examined in future. In our next study, we plan to intratumorally administer iDC and then provide a maturation stimulus in vivo, example, tumor necrosis factor,[29] IFN, IL-12,[12] OK-432, CpG,[30] or poly I:C, together with GM-CSF.

ACKNOWLEDGMENTS

The authors thank Ms Erika Ritter for excellent technical assistance, Mr Takashi Daimon for statistical analysis, and the members of Medical Center for Translational Research for invaluable suggestions.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

All authors have declared there are no financial conflicts of interest in regard to this work.

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Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination

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NY-ESO-1 is a prototypic cancer/testis antigen. In a recent phase I clinical trial, we vaccinated 13 patients bearing NY-ESO-1-expressing tumors with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1) and showed efficient induction of NY-ESO-1 antibody, and CD4 and CD8 T cell responses using peripheral blood from the patients. In our study, we analyzed heteroclitic serological responses in those patients after vaccination. Serological response against 11 tumor antigens including MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2, XAGE1B and p53 was examined by enzyme-linked immunosorbent assay (ELISA) using sera from ten vaccinated patients. Expression of tumor antigens was determined by reverse transcription-polymerase chain reaction or immunohistochemistry. Eight of nine patients who showed antibody responses against NY-ESO-1 also showed an antibody response against at least 1 of these 11 tumor antigens after vaccination. In one patient, seven tumor antigens were recognized. Specificity analysis of the antibody response by ELISA using control recombinant proteins and synthetic peptides and by Western blot showed that the response was not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination. Thus, heteroclitic serological responses appear to be indicative of the overall immune response against the tumor, and their analysis could be useful for immune monitoring in cancer vaccine.

NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum[1,2] and has been shown to be strongly immunogenic. Patients bearing NY-ESO-1-expressing tumors often show antibody and CD4 and CD8 T cell responses spontaneously.[3–5] Clinical trials using NY-ESO-1 peptide,[6–9] protein[10,11] and viral constructs[12] as cancer vaccine have been conducted. Those studies have demonstrated efficient induction of antibody, and CD4 and CD8 T cell responses, and have also shown in some sporadic cases efficacy of immunotherapy.[2,6–12]

In a recent phase I clinical trial, we vaccinated 13 patients including eight Stage IV esophageal cancer patients, four

Key words: NY-ESO-1, cancer vaccine, cancer testis antigen, heteroclitic antibody response

Abbreviations: CHP: cholesterol-bearing hydrophobized pullulan; CMV: Cytomegalovirus; CT antigen: cancer/testis antigen; CTL: cytotoxic T lymphocytes; DHFR: dihydrofolate reductase; EBV: Epstein–Barr virus; OD: optical density; OLPs: overlapping peptides; RT-PCR: reverse transcription-polymerase chain reaction; SEREX: serological expression cloning

Additional Supporting Information may be found in the online version of this article

The authors declare that there is no conflict of interest

Grant sponsors: Cancer Vaccine Collaborative of the Cancer Research Institute and the Ludwig Institute for Cancer Research, Ministry of Education, Culture, Sports, Science and Technology of Japan, New Energy and Industrial Technology Development Organization (NEDO), Japan

DOI: 10.1002/ijc.26074

History: Received 13 Sep 2010; Accepted 3 Mar 2011; Online 16 Mar 2011

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Stage D3 prostate cancer patients and a Stage IV malignant melanoma patient with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1).[13–16] We showed that the vaccine was well tolerated and had a potent capacity to induce NY-ESO-1 antibody and CD4 and CD8 T cell responses using peripheral blood from patients. Beneficial effects such as tumor regression or no tumor growth for a prolonged period were observed in six of seven disease-evaluable patients. However, all patients died eventually. Immunohistochemical analysis of the tumors that grew after vaccination suggested involvement of different mechanisms resulting in immune impairment.[15] NY-ESO-1-antigen loss was observed in a patient, disappearance of tumor-infiltrating CD4 and CD8 T cells was observed in two patients and an increase in the number of $CD68^+$ macrophages was observed in another patient. Further study will be necessary to elucidate cellular mechanisms including regulatory T cells causing immune suppression at the local tumor site.

It has been shown that cytotoxic T cell responses to tumor antigens other than the antigen used for immunization occur after vaccination.[17–26] This heteroclitic immune response following the initial response to a specific antigen was originally described in autoimmune disease and has been suggested to play a crucial role in clinical responses mediated by cancer vaccines.[27,28] Occurrence of CD8 T cell responses to unrelated tumor antigens was shown in studies of vaccination with MAGE-A1 and/or MAGE-A3,[19,20,25] HER-2/neu,[17,18,21,22] MART-1/Melan-A[23–25] and gp100.[26]

In our study, we analyzed the heteroclitic serological response against a panel of tumor antigens. Specificity analysis of antibody response was performed by enzyme-linked immunosorbent assay (ELISA) using control recombinant proteins and synthetic peptides, and by Western blot. The findings indicate frequent occurrence of heteroclitic serological responses in patients after CHP-NY-ESO-1 vaccination.

Material and Methods

Patients and sera

Eight advanced esophageal cancer patients (E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8) and two prostate cancer patients (P-2 and P-3) were enrolled in the clinical trial (protocol LUD 2002-005 of the Ludwig Institute for Cancer Research, New York, NY) (http://clinicaltrials.gov/ct2/show/NCT00106158?term=CHP+NY-ESO-1&rank=2).[13–16] Peripheral blood was drawn from patients with written informed consent with the permission of the ethics committees of Osaka and Okayama Universities. Sera from MAGE-A4 seropositive esophageal cancer patients (EC-1, EC-2 and EC-3) and five healthy donors were drawn with written informed consent with the permission of the ethics committees of Osaka University. Sera were stored in −80°C freezer until use.

Preparation of a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1)

Preparation of recombinant NY-ESO-1 protein for vaccine was described elsewhere.[29] A complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) and the schedule of vaccine were described previously.[14] Briefly, patients with advanced cancers expressing NY-ESO-1 were injected 2–31 times subcutaneously at biweekly intervals with 100 μg of NY-ESO-1 recombinant protein formulated with 2 mg of CHP.

Recombinant protein and overlapping peptides

N-His6-tagged recombinant proteins, NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B, dihydrofolate reductase (DHFR) and Akt,[30–32] were produced by using pQE30 vector (QIAGEN, Hilden, Germany) and expressed in M15 *E. coli* cells. N-His6-tagged p53 produced in a *Baculovirus* system was purchased (Enzo Life Sciences, New York, NY). N-His6-tagged CCDC-62 protein was produced in a *Baculovirus* system at Okayama University.[33]

A series of 22 25-mer MAGE-A4 overlapping peptides (OLPs) were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl chemistry on an ABIMED Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University. MAGE-A4 OLPs: 1 (1–25), 2 (15–39), 3 (29–53), 4 (43–67), 5 (57–81), 6 (71–95), 7 (85–109), 8 (99–123), 9 (113–137), 10 (127–151), 11 (141–165), 12 (155–179), 13 (169–193), 14 (183–207), 15 (197–221), 16 (221–235), 17 (225–249), 18 (239–263), 19 (253–277), 20 (267–291), 21 (281–305), 22 (295–317).

MAGE-A4 transfectants

CMS5a, a murine fibrosarcoma cell line from a strain of BALB/c origin, was stably transfected with pcDNA3.1 MAGE-A4 plasmid as described previously.[34] The plasmid was provided by Dr. A. Kuroda and Dr. M. Miyamoto (Hokkaido University, Sapporo, Japan) and purified using plasmid Mini kit.

ELISA

Serially diluted sera were added to 96-well plates coated overnight at 4°C with 1 μg/ml recombinant protein or 5 μg/ml peptide solution and blocked for 2 hr at room temperature. After overnight incubation, plates were extensively washed with PBS containing 0.2% Tween 20. For determining reciprocal titer by ELISA, shown in Figure 1, serum IgG bound to antigens was detected by alkaline phosphatase-conjugated specific monoclonal antibody (Southern Biotech, Birmingham, AL). After addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a Cytofluor Series 4000 fluorescence reader (PerSeptive Biosystems, Framingham, MA). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. Specificity was determined by