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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

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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

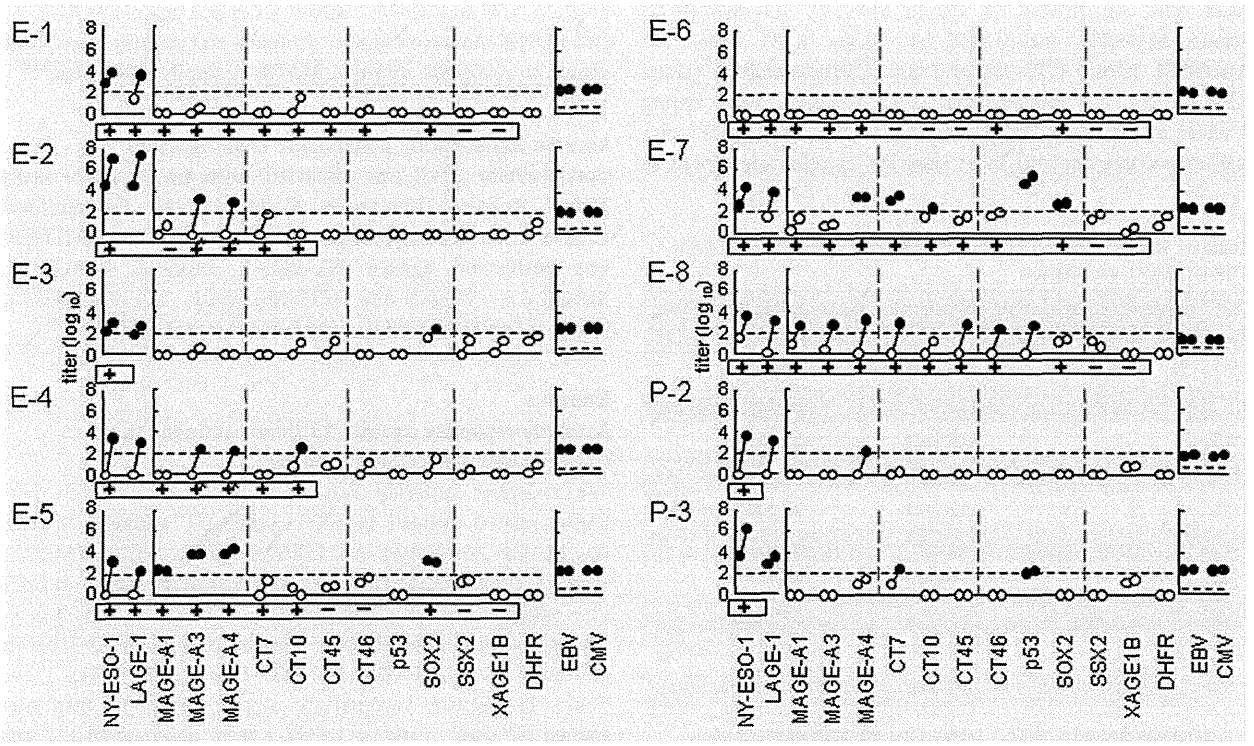

Figure 1. Reciprocal antibody titer against 13 tumor antigens in sera from patients vaccinated with CHP-NY-ESO-1 by ELISA. Serially diluted sera obtained before and after vaccination were assayed against N-His6-tagged recombinant proteins NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B and DHFR. The reciprocal titer was the maximal dilution showing significant reaction (open and closed circles). Closed circles indicate reciprocal titers exceeding 100 (positive reaction). In each assay, antibody positive and negative sera were included as controls. Positive (+) and negative (–) expression of tumor antigens indicated in boxes under each panel was analyzed by RT-PCR and/or IHC when sample was available (see Supporting Information Table). Titer of EBV and CMV antibody in sera were measured by EBV and CMV kits, respectively. Values exceeding 4.0 were positive by manufacturer's indication. +*; 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.

comparing seroreactivity among the various antigens tested.[31] In each assay, positive and negative control sera were included. A positive result was defined as reciprocal titers >100. For conventional ELISA, peroxidase-conjugated goat anti-human IgG or IgM (Jackson Immuno Research Laboratory, West Grove, PA) was added to the wells for second antibody. After washing, signals were developed with *o*-phenylene diamine dihydrochloride, and absorbance at 490 nm was read using an ELISA reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). Positivity was defined as sample optical density (OD) greater than three times that of the value for irrelevant control protein. Titers of Epstein–Barr virus (EBV) and Cytomegalovirus (CMV) antibody in sera were measured by EBV VCA kit (Denka Seiken, Tokyo, Japan) and CMV kit (Denka Seiken), respectively.

Western blot

Recombinant protein (20 ng) or cell lysate (20 μg) in sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS and 1 mM dithiothreitol) was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide BioRad Ready-Gels (Bio-Rad). After electrophoresis, the membrane (Hybond-P membrane, Amersham Pharmacia Biotech, Buckinghamshire, UK) was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:1,000 for recombinant protein or 1:200 for cell lysate for 1 hr at room temperature. After washing, alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratory) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Polyclonal rabbit anti-MAGE-A1 serum (Abcam, Cambridge, UK) and monoclonal anti-MAGE-A4 (clone 3D12; Abnova, Taipei, Taiwan), anti-p53 (clone PAb421; Enzo) and anti-His6-tag (clone OGHis; MBL, Nagoya, Japan) antibodies were used for positive controls at 1:1,000 dilution.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formalin-fixed paraffin-embedded specimens. Monoclonal antibodies

used were anti-MAGE-A1 (clone MA454), anti-MAGE-A3 (clone M3H67), anti-MAGE-A4 (clone 57B), anti-CT7/MAGEC1 (clone CT7-33) and anti-CT10/MAGEC2 (clone LX-CT10.5). For cancer-testis (CT) antigens, only strong nuclear and/or cytoplasmic staining as observed in testicular tissue (positive control) in at least 5% of cells was scored as positive. 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.[35,36]

Table 1. Heteroclitic antibody response and clinical response after CHP-NY-ESO-1 vaccination

ID	Heteroclitic response No. of antigens	Weeks (the No.)	Clinical response
E-1	0	89 (31)	Regression
E-2	2	14 (7)	Partial regression
E-3	1	28 (12)	Stable
E-4	3	12 (6)	Progressive
E-5	2	22 (11)	Partial regression
E-6	0	4 (3)	N.E.
E-7	4	2 (2)	N.E.
E-8	7	54 (27)	Stable
P-2	1	28 (10)	PSA stabilization
P-3	2	29 (13)	PSA stabilization

Abbreviations: Weeks (the No.): weeks after the start of vaccination and the number of vaccinations given; N.E.: not evaluable.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed against NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2 and XAGE1B.[30,31]

Results

Antibody response against 13 tumor antigens in CHP-NY-ESO-1-vaccinated patients

We analyzed antibody responses against NY-ESO-1, NY-ESO-1-related antigen LAGE-1, other CT antigens MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SSX2 and XAGE1B, SOX2 and p53 in esophageal cancer patients E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8 and prostate cancer patients P-2 and P-3 before and after a cycle of CHP-NY-ESO-1 vaccination (Fig. 1 and Table 1). Before vaccination, strong antibody responses against NY-ESO-1 and/or LAGE-1 were observed in E-2 and P-3 and defined as baseline seropositive. Additionally,

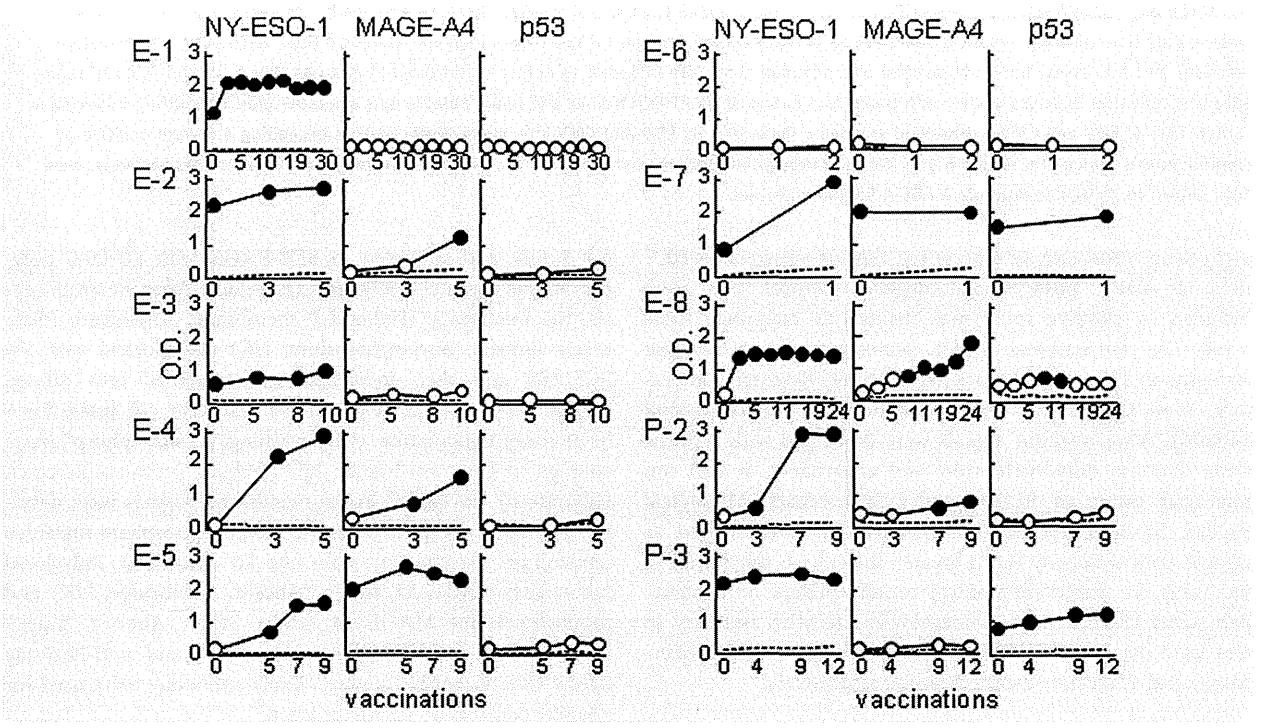

Figure 2. IgG antibody response against NY-ESO-1, MAGE-A4 and p53 in sera from patients before and after CHP-NY-ESO-1 vaccination by ELISA. Sera diluted at 1:100 were assayed against N-His6-tagged recombinant proteins NY-ESO-1, MAGE-A4 and Akt produced in *E. coli* and recombinant proteins p53 and CCDC-62 produced in *Baculovirus*. Akt and CCDC-62 were included as negative control (dotted line). Positive reaction (closed circles) represented the OD values exceeding three times the control OD value.

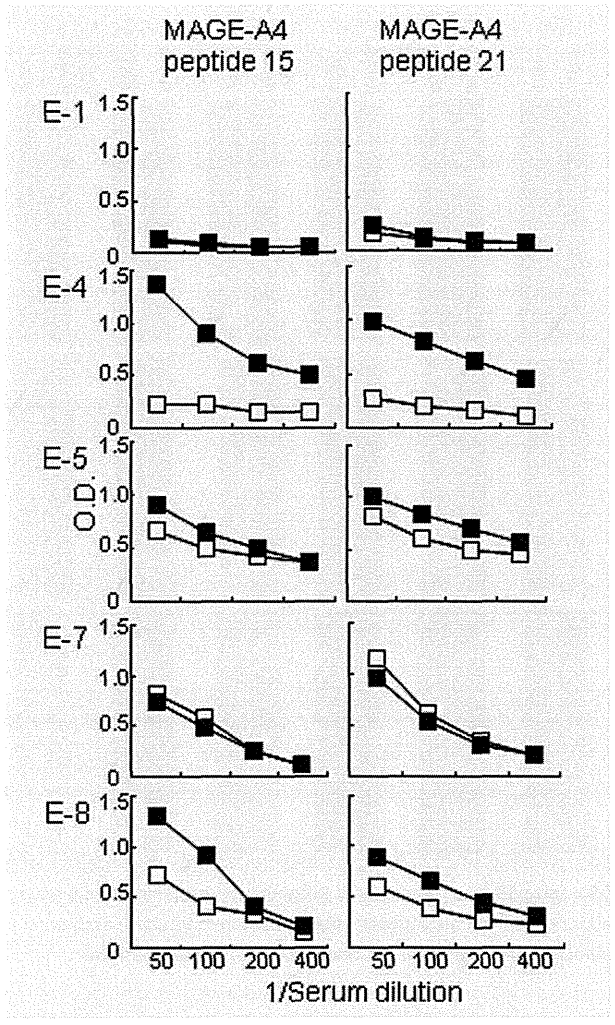

Figure 3. Serially diluted sera from patients before (open squares) and after (closed squares) CHP-NY-ESO-1 vaccination were assayed against MAGE-A4 peptide 15 and peptide 21 by IgG ELISA.

marginal antibody responses were observed in E-1, E-3 and E-7. In E-5, antibody against MAGE-A1, MAGE-A3, MAGE-A4 and SOX2 was observed. In E-7, antibody against MAGE-A4, CT7/MAGEC1, p53 and SOX2 was observed. In P-3, antibody against p53 was observed. After vaccination, in all patients except E-6, antibody response against NY-ESO-1 and LAGE-1 was increased or induced. In E-2, antibody responses against MAGE-A3 and MAGE-A4 were induced. In E-3, antibody response against SOX2 was induced. In E-4, antibody responses against MAGE-A3, MAGE-A4 and CT10/MAGEC2 were induced. In E-5, antibody responses against MAGE-A3 and MAGE-A4 were increased. In E-7, antibody responses against CT7/MAGEC1, p53 and SOX2 were increased and that against CT10/MAGEC2 was induced. In E-8, antibody responses against MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT45, CT46/HORMAD1 and p53 were induced. In P-2, antibody response against MAGE-A4 was induced. In P-3, antibody response against CT7/MAGEC1 was induced and that against p53 was increased. No antibody against DHFR included as a control was detected in any patient. Furthermore, no increase of antibody response was observed against EBV and CMV after CHP-NY-ESO-1 vaccination.

Expression of 13 tumor antigens in tumor specimens

Expression of NY-ESO-1 was detected by RT-PCR and IHC in tumors from all patients before vaccination. Expression of other tumor antigens except p53 was analyzed by RT-PCR in E-1, E-5, E-6, E-7 and E-8, and expression of MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1 and CT10/MAGEC2 was also analyzed by IHC in E-1, E-2, E-4, E-5, E-6, E-7 and E-8 (Fig. 1 and Supporting Information Table). Mutation of p53 was not determined in our study. Expression of corresponding antigen was confirmed with tumor specimens in patients who showed antibody against tumor antigens.

Antibody response against tumor antigens in CHP-NY-ESO-1-vaccinated patients: No involvement of antibody against His6-tag and the product of E. coli present in the vaccine

Antibody responses against selected tumor antigens were further confirmed in sera obtained at each time during multiple vaccinations. As shown in Figure 2, IgG antibody against MAGE-A4 was detected in sera from E-5 and E-7 before vaccination, and the response was increased or induced in E-2, E-4, E-5, E-8 and P-2 after vaccination. IgG antibody against p53 was detected in sera from E-7 and P-3 before vaccination, and the response was increased or induced in E-7, E-8 and P-3.

Induction of IgM antibody against MAGE-A4 was detected in sera from E-8 after vaccination (Supporting Information Fig. 1). IgM antibody against p53 was detected in sera from E-7 before vaccination. Increase or induction of IgM antibody against p53 was detected in E-7 and E-8 after vaccination. Interestingly, in E-8, transient IgM response against MAGE-A4 and p53 was followed by IgG response.

Recombinant NY-ESO-1 protein used for vaccination has His6-tag in the N-terminus and was produced in *E. coli* as the host cells. All antigens shown in Figure 1 also have His6-tag and were produced in *E. coli*. To exclude the possibility of detecting antibody against His6-tag and/or the product of *E. coli* in the assay that might be raised by vaccination, DHFR was tested as control. No antibody against DHFR was detected (see above). To further exclude the possibility, the antibody response against control antigens was examined by IgG ELISA using serum samples obtained in each time during multiple vaccinations. As shown in Figure 2, antibody against Akt protein with His6-tag and produced in *E. coli* was within a background level (<0.2 OD value). p53 used in the experiments shown in Figure 2 and Supporting Information Figure 1, but not in Figure 1, and CCDC-62 protein

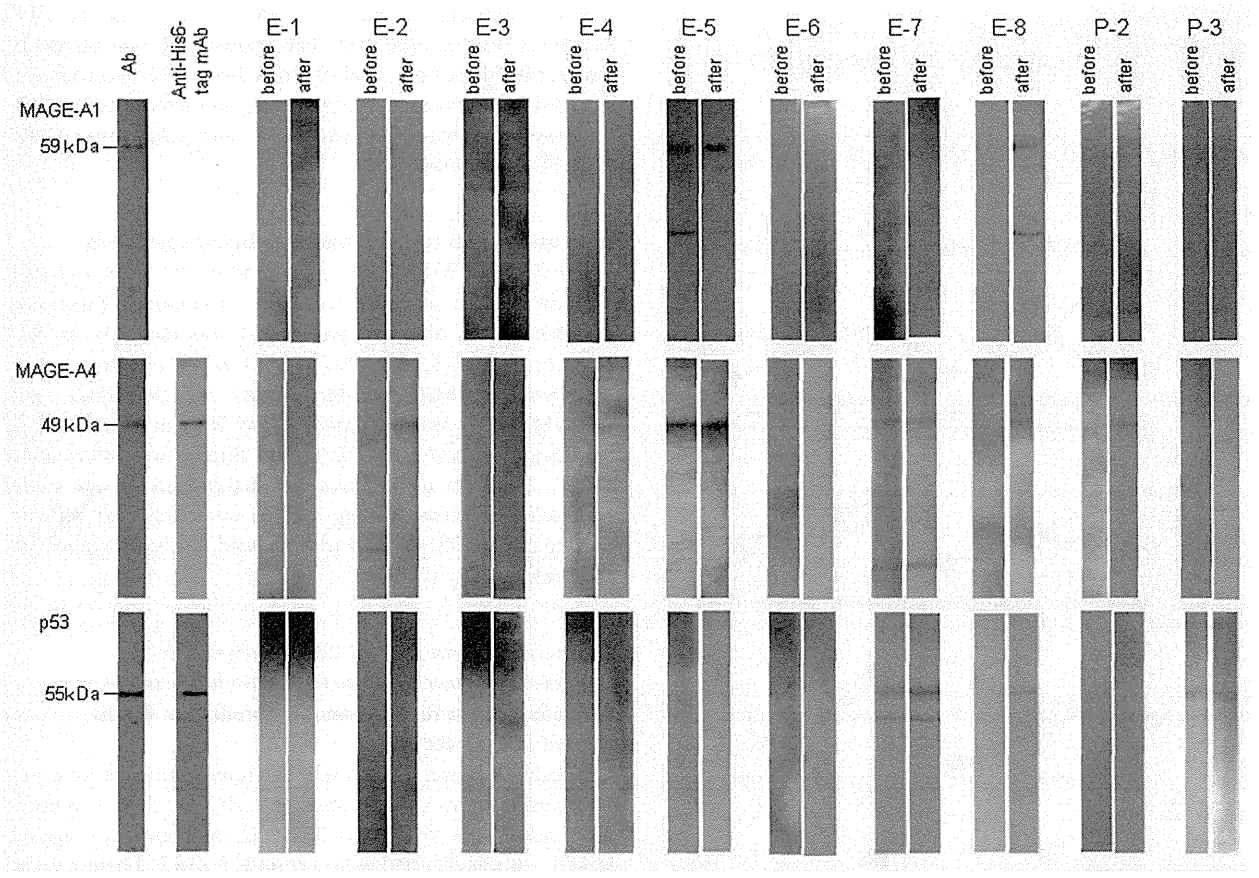

Figure 4. Western blot analysis. Reaction of sera against MAGE-A1, MAGE-A4 and p53 was investigated. Recombinant proteins (20 ng) were run by SDS-PAGE and transferred to a membrane by electrophoresis. Sera (1:1,000) from all patients obtained before and after vaccination were examined. Marker and control bands of each protein detected by monoclonal or polyclonal antibody (1:1,000) are also shown.

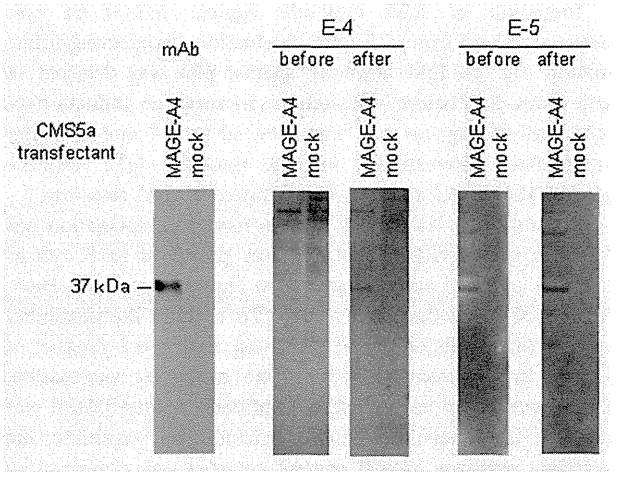

Figure 5. Western blot analysis of sera against MAGE-A4 in lysate of MAGE-A4-transfected CMS5a cells. Cell lysate (20 μg) was run by SDS-PAGE, transferred to a membrane by electrophoresis and sera (1:200) from E-4 and E-5 patients obtained before and after CHP-NY-ESO-1 vaccination were examined. Control band of the protein detected by monoclonal antibody (1:1,000) is shown.

share His6-tag and were produced by *Baculovirus*. Antibody against CCDC-62 was undetectable in sera from any patients.

Next, we synthesized MAGE-A4 OLPs and investigated antibody response by ELISA. Antibody response against MAGE-A4 peptides 15 and 21 was frequently observed in patients showing antibody response against MAGE-A4 protein (Supporting Information Fig. 2). Serially diluted sera from patients E-4, E-5, E-7 and E-8 obtained before and after CHP-NY-ESO-1 vaccination were examined against MAGE-A4 peptides 15 and 21 by IgG ELISA (Fig. 3). Increase or induction of antibody response was observed in E-4, E-5 and E-8, but not E-7 after vaccination. No antibody response was detected in E-1 included as negative control. These results were consistent with those by ELISA using recombinant MAGE-A4 protein in Figure 2.

Western blot analysis

The specificity of antibody against MAGE-A1, MAGE-A4 and p53 in sera from all patients vaccinated was further analyzed by Western blot (Fig. 4). Each antibody as positive control showed the representative band for MAGE-A1 protein at 59 kDa, for MAGE-A4 protein at 49 kDa and for p53 protein

at 55 kDa. Increase of reaction with the bands was observed with recombinant MAGE-A1 protein in sera from E-8, with recombinant MAGE-A4 protein in sera from E-2, E-4, E-5, E-8 and P-2 and with p53 in sera from E-7, E-8 and P-3 obtained after vaccination.

Specificity of the reaction was further confirmed using transfectants. As shown in Figure 5, sera from E-4 after vaccination and from E-5 before and after vaccination reacted to MAGE-A4 in lysate of MAGE-A4-transfected murine fibrosarcoma CMS5a cells. No reaction was observed with lysate of mock-transfected CMS5a cells.

Discussion

Efficient elicitation of host immune response is a prerequisite for successful immunotherapy using cancer vaccine, and immune monitoring of specific antibody, CD4 and CD8 T cell responses against tumor antigens after vaccination is crucial to evaluate the response. In our study, we investigated antibody response against 13 tumor antigens by ELISA using recombinant proteins to evaluate the immune response more precisely. Nine of ten patients analyzed except E-6 showed an increase or induction of antibody response against NY-ESO-1 and its related LAGE-1 antigen after CHP-NY-ESO-1 vaccination. Eight of these nine patients showed an increase or induction of antibody response to either of these antigens after vaccination. Previously, it was reported that sera from patients vaccinated with recombinant NY-ESO-1 protein and CpG in Montanide sometimes showed nonspecific production of antibody against other recombinant proteins used for control,[11,37] and some of these responses could be attributed to reactivity against bacterial components or His6-tag. To address this possibility, we performed specificity analysis of the antibody response using control recombinant proteins, synthetic peptides and by Western blot that showed heteroclitic responses were not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination.

We reported previously that those patients showed NY-ESO-1 specific antibody and CD4 and CD8 T cell responses during vaccination.[14,15] The findings suggest that increase or induction of antibody response against tumor antigens, *e.g.*, MAGE-A3 and MAGE-A4, as well as NY-ESO-1 after CHP-NY-ESO-1 vaccination may be caused by their release from tumor cells damaged by NY-ESO-1-specific immunity. Therefore, antibody response to multiple tumor antigens may suggest an intensity of the overall host immune response against the tumor, and detection of multiple heteroclitic serological responses using a panel of recombinant proteins would be a new tool of immunological monitoring for antitumor responses. A clear correlation between heteroclitic antibody responses and clinical outcomes could not be established in the limited number of patients analyzed in our study (Table 1). However, antibody response as well as CD4 and/or CD8 T cell responses to heteroclitic tumor antigens would be useful for evaluating overall immune response to tumor.

A number of studies have shown the relationship between heteroclitic immune response and clinical response. Germeau *et al.*[19] reported that the frequency of CTL precursor increased tenfold in some patients after vaccination using MAGE antigenic peptides, although they found no significant difference in the levels against immunizing antigens between the tumor-regressor and -progressor patients. They then analyzed CTL precursors against other tumor antigens than that utilized for vaccine and found that the immune responses elicited to those irrelevant antigens after vaccination might contribute to the whole immune response to a given tumor and was correlated to clinical responses. Similarly, Butterfield *et al.*[23,24] reported that peptide-specific T cell response was efficiently induced in most patients by immunization with MART-1/Melan-A peptide pulsed dendritic cells. However, cellular immune responses against not only MART-1/Melan-A but also gp100 and tyrosinase were detected only in a complete clinical responder. These findings suggest a relationship between heteroclitic CTL responses and clinical responses. Furthermore, Disis *et al.* reported induction of both cellular and humoral responses against other intramolecular determinants in patients immunized with HER-2/neu peptide vaccine, and of antibody response to p53 in patients immunized with HER-2/neu peptide vaccine.[17,22] They further studied the effect of HER-2/neu T-helper peptide-based vaccinated patients receiving trastuzumab therapy and observed prolonged immune responses against not only the vaccine antigen but also cryptic antigens.[38] Collectively, the presence of either humoral or cellular immune response to multiple tumor antigens appears to be indicative of the strength of overall response against the tumor and predictive of clinical response. In our study, we used a panel of 13 tumor antigens for the detection of the humoral response. Serological detection of responses to multiple tumor antigens that were shown to be highly immunogenic in cancer patients would be convenient and could be included in routine immune monitoring.

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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

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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.

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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;

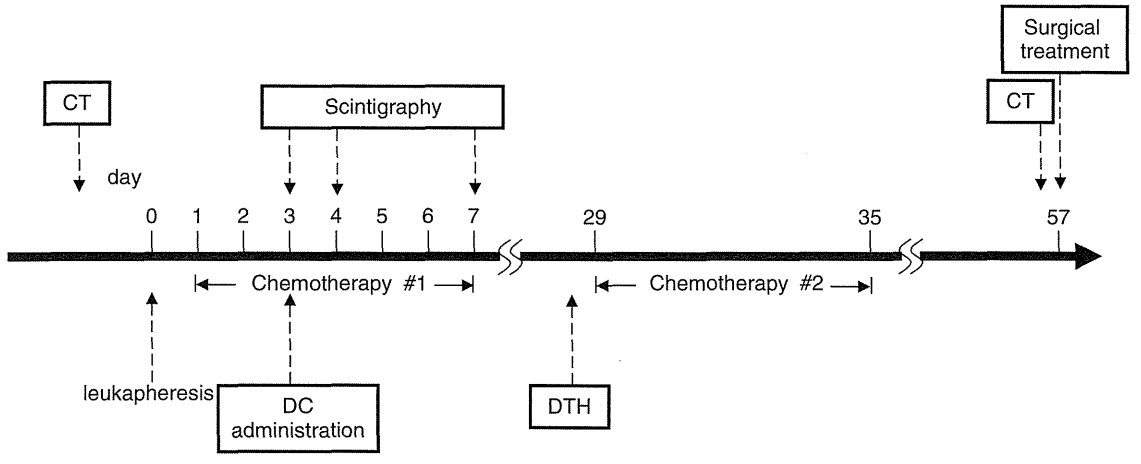

FIGURE 1. Schedule of our clinical trial of intratumoral dendritic cell (DC) administration. Two cycles of chemotherapy, which involved 4-week preoperative chemotherapy with adriacin and cisplatin on day 1 and 5-fluorouracil on days 1–7, followed by 3-week off, were scheduled before surgical treatment. 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

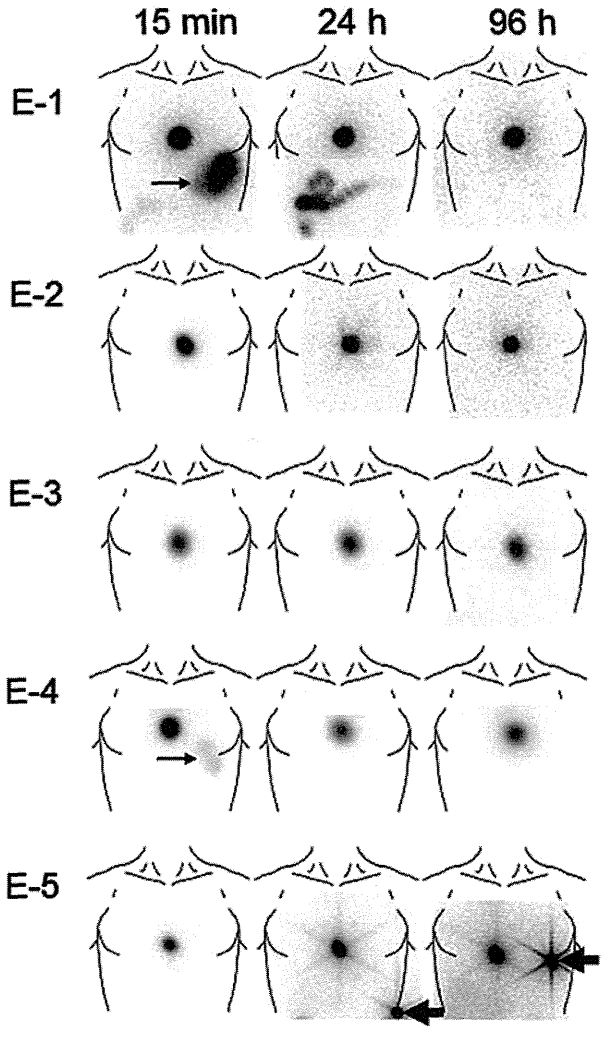

FIGURE 2. Scintigraphic images of ^{111}In-oxine-labeled dendritic cell (DC) in esophageal cancer patients after their intratumoral administration. Chest and abdominal scintigraphy images were taken at 15 minutes, 24 hours, and 96 hours after the intratumoral administration of labeled DC. The areas of 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.

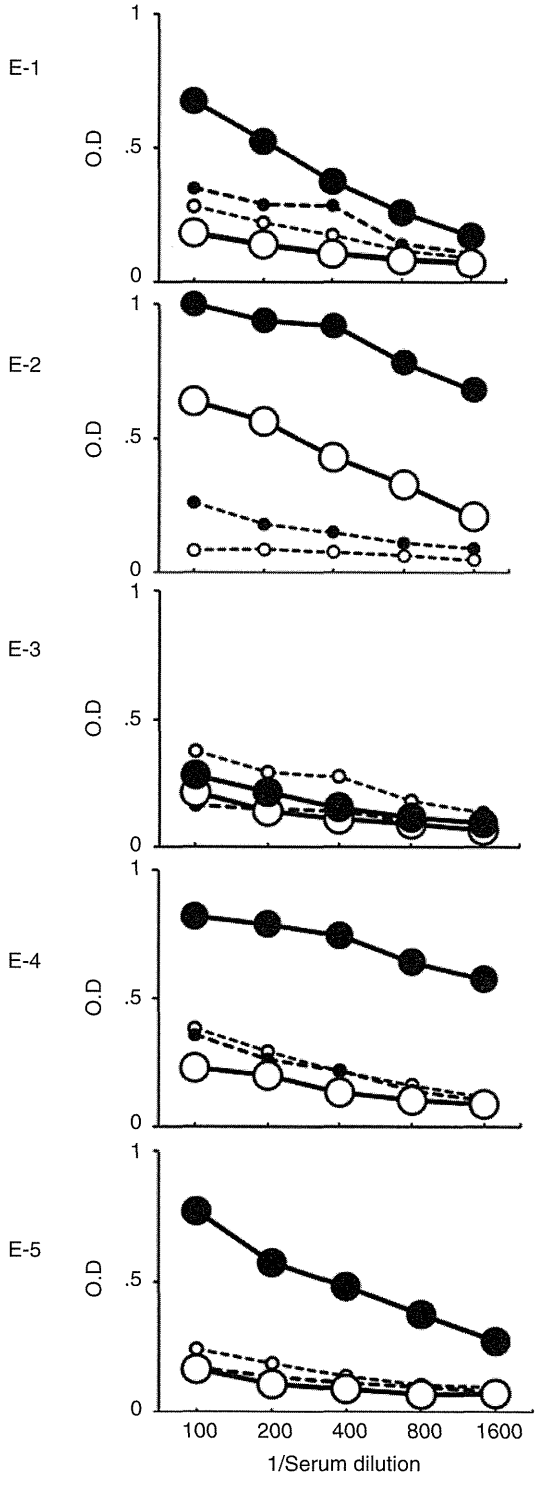

FIGURE 3. Serological analysis of the antikeyhole limpet hemocyanin (KLH)-specific immune response by enzyme-linked immunosorbent assay. Antibody titers in serum samples obtained before (open circles) and after (closed circles) the intratumoral DC administration were analyzed. Recombinant KLH of 0.2 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).

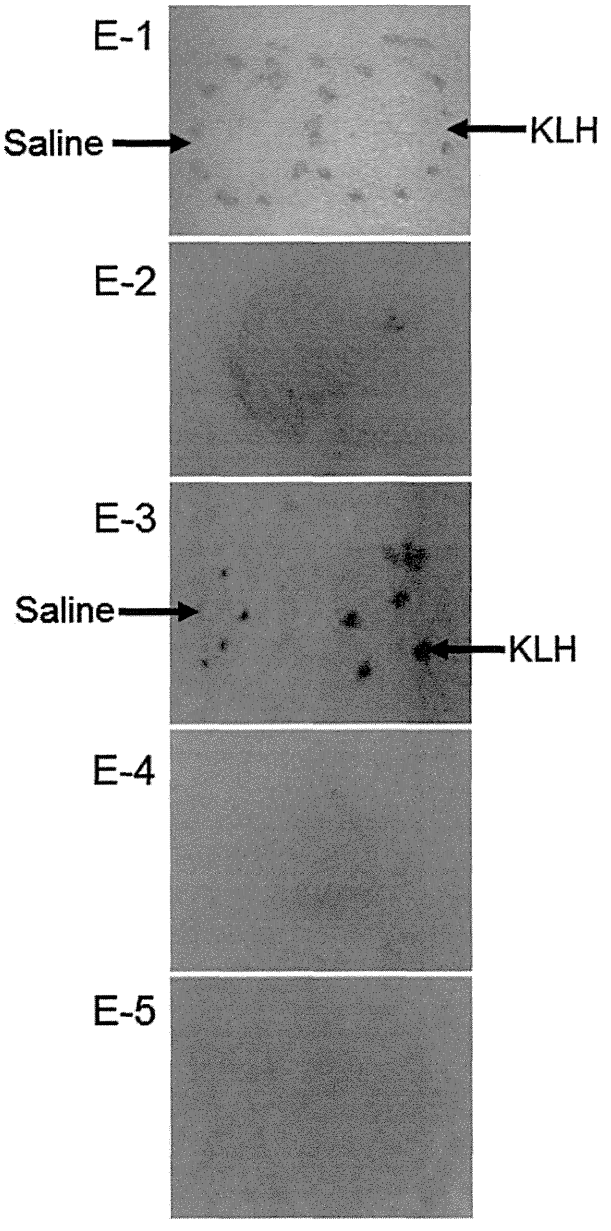

FIGURE 4. Delayed-type hypersensitivity (DTH) reaction against keyhole limpet hemocyanin (KLH). A total of 20 μg recombinant KLH protein were intradermally inoculated at 2 weeks after the dendritic cell administration, and the diameter of the indurated tissue was measured after 48 hours. A diameter of >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).

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.

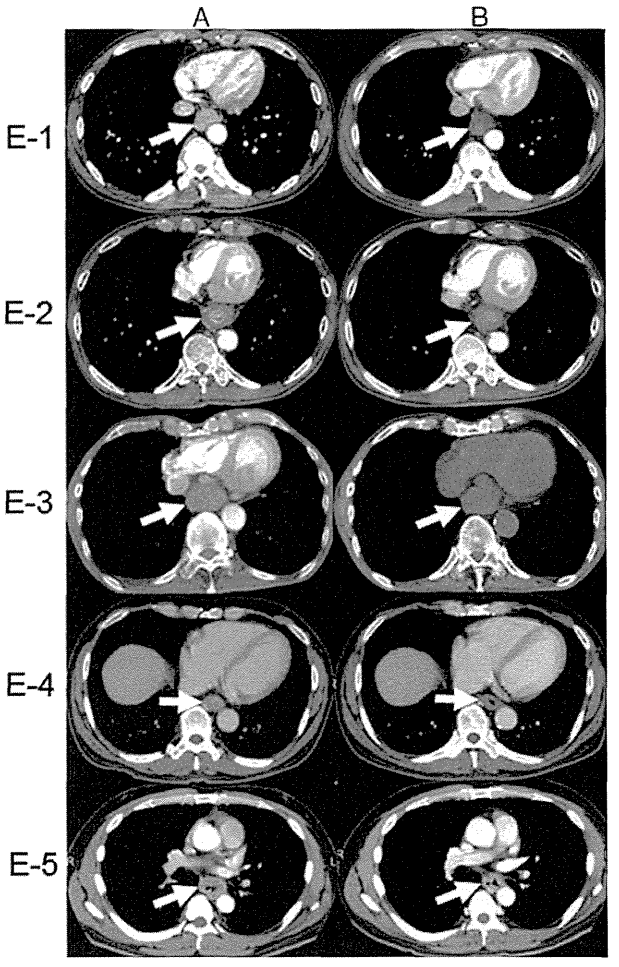

FIGURE 5. Computed tomography (CT) images of primary esophageal tumors obtained before and after the intratumoral dendritic cell (DC) administration. The primary esophageal tumor (arrow) was located in the lower intrathoracic esophagus region in patients E-1, E-2, E-3, and E-5 and in the middle region 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).

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

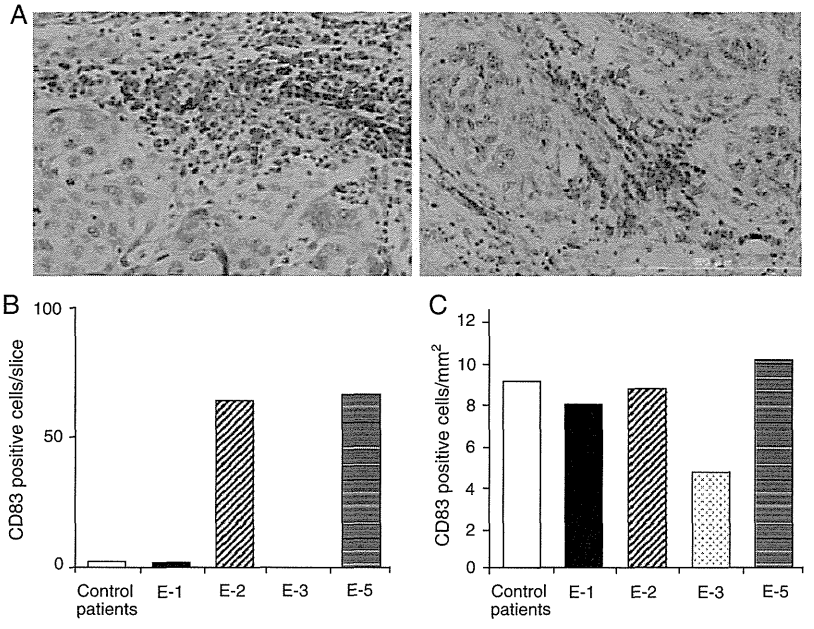

FIGURE 6. Immunohistochemical analysis of CD83$^+$ cells in surgically resected specimens obtained after intratumoral dendritic cell (DC) administration. Immunohistochemical analysis was performed with anti-CD83 monoclonal antibody using esophageal tumor and draining lymph node tissue from patients E-1, 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,

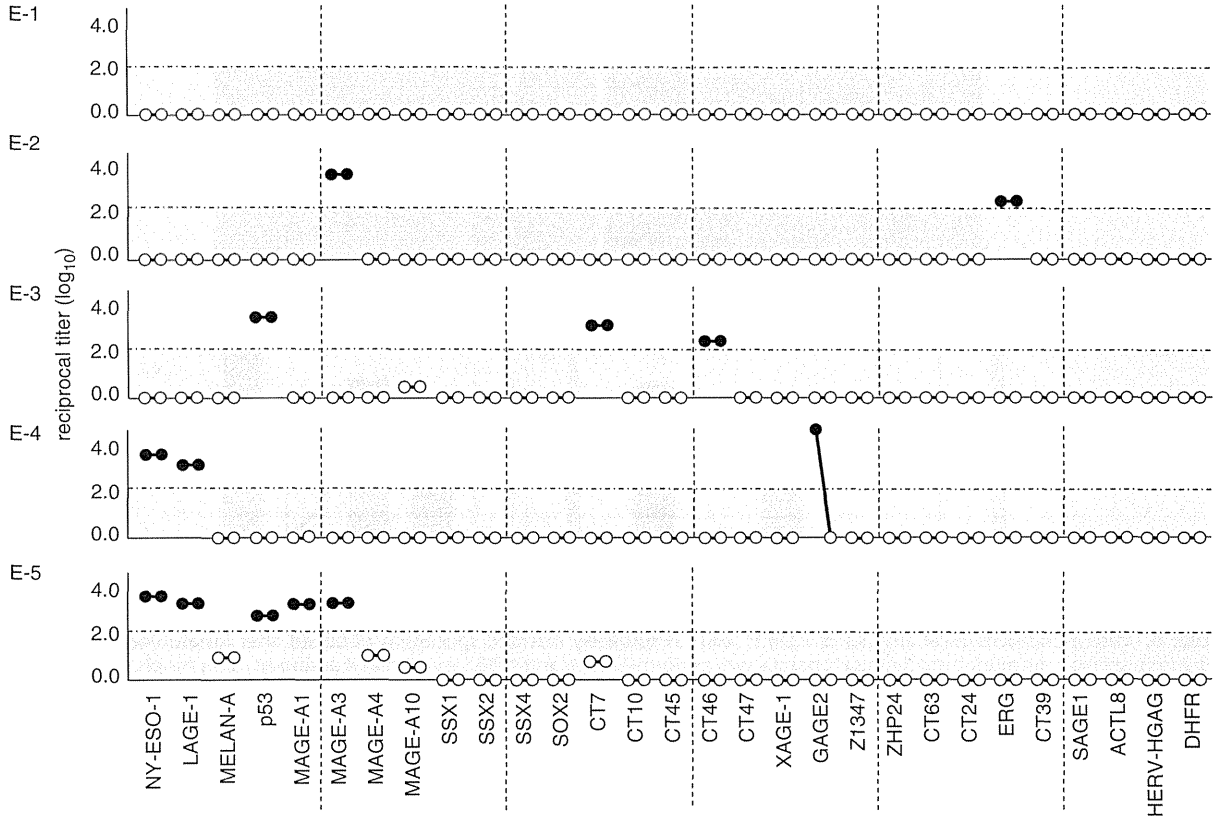

FIGURE 7. No induction of tumor-specific humoral immune responses against tumor antigens was observed in enzyme-linked immunosorbent assay. The reciprocal titers of antibodies against 28 tumor antigens in serum samples obtained before and after the intratumoral dendritic cell (DC) administration are 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.

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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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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

Let-7 Expression Is a Significant Determinant of Response to Chemotherapy through the Regulation of IL-6/STAT3 Pathway in Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: Cisplatin-based chemotherapy is widely used for esophageal cancer, sometimes in combination with surgery/radiotherapy, but poor response to chemotherapy is not uncommon. The aim of this study was to examine whether miRNA expression is useful to predict the response to chemotherapy in patients with esophageal cancer.

Experimental Design: Using pretreatment biopsy samples from 98 patients with esophageal cancer who received preoperative chemotherapy, we measured the expression level of several miRNAs whose expression was altered in cisplatin-resistant esophageal cancer cell lines compared with those parent cell lines and examined the relationship between the miRNA expression and response to chemotherapy. *In vitro* assays were conducted to clarify the mechanism of miRNA-induced changes in chemosensitivity.

Results: The expression levels of 15 miRNAs were altered in cisplatin-resistant cells. Of these, low expression of let-7b and let-7c in before-treatment biopsies from 74 patients of the training set correlated significantly with poor response to chemotherapy, both clinically and histopathologically. Low expression of let-7c also correlated with poor prognosis ($P = 0.032$). The relationship between let-7b and let-7c expression and response to chemotherapy was confirmed in the other 24 patients of the validation set. In *in vitro* assay, transfection of let-7c restored sensitivity to cisplatin and increased rate of apoptosis after exposure to cisplatin. Let-7c directly repressed cisplatin-activated interleukin (IL)-6/STAT3 prosurvival pathway.

Conclusions: Let-7 expression in esophageal cancer can be potentially used to predict the response to cisplatin-based chemotherapy. Let-7 modulates the chemosensitivity to cisplatin through the regulation of IL-6/STAT3 pathway in esophageal cancer. *Clin Cancer Res; 18(18); 5144–53. ©2012 AACR.*

Introduction

Despite recent advances in surgical techniques and perioperative management, the prognosis of patients who undergo surgery alone for esophageal cancer remains poor (1). Neoadjuvant chemotherapy or chemoradiotherapy followed by surgery has emerged as a promising strategy for advanced esophageal cancer and in fact, good responders to such preoperative therapy show better survival (2, 3). However, the reported response rate to cisplatin-based chemotherapy, which is widely used for esophageal cancer, is only modest, ranging from 25% to 48% (4–7) and nonresponders likely receive no survival benefit (8). The ability to predict the response to chemotherapy before treatment should limit the application of chemotherapy to selected patients who are likely to show some benefits, and allow tailoring such therapy to the individual patient with esophageal cancer.

miRNAs are noncoding RNAs of approximately 22 nucleotides in size and act by repressing the translation of target mRNA by binding to the 3′-untranslated region of those mRNAs (9). miRNAs exist stably in various tissues and play pivotal roles in differentiation and development (10). In addition, aberrant expression of miRNAs is reported in various types of cancers. In esophageal cancer, miR-21 and miR-93 are reported to be upregulated, whereas miR-375, miR-27b, miR-203, miR-205, and let-7c are downregulated (11, 12). Recent studies also showed the involvement of several miRNAs in resistance to anticancer treatment including chemotherapy and radiotherapy. Giovannetti and colleagues (13) reported that overexpression of miR-21was associated with poor outcome in gemcitabine-treated patients with pancreatic cancer. In our previous study using residual tumor after chemotherapy, we showed the involvement

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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