cells with a capacity for further maturation (24). Long *et al* demonstrated that SOX2 was implicated in the maintenance of pluripotency and esophageal epithelial development, and that it was preferentially expressed in ESCC (13). From these findings, we hypothesized that podoplanin and SOX2 expression were involved in tumor growth and invasion in ESCC after neoadjuvant CRT. We observed both podoplanin and SOX2 expression in the basal layer of adjacent normal mucosa after CRT. This finding suggests both podoplanin and SOX2 may play roles in the development of esophageal epithelium. Moreover, there was a significant correlation between podoplanin and SOX2 expression in immunohistochemical analysis (P=0.0404). Podoplanin expression was observed in the membranes of residual cancer cells at the outer edge of residual cancer nests, but not in the central region. Our finding was similar to previous reports of squamous cell carcinoma in head and neck, and the oral cavity. In addition, we found that residual cancer with high podoplanin expression showed a high immunoreactivity in stromal tissue. These results suggest that cancer cells and stroma might interact through podoplanin, and that podoplanin expression in stromal tissue surrounding cancer cells might be increased by CRT. Wicki *et al* reported that podoplanin expression in breast cancer cells was induced by epidermal growth factor, basic fibroblast growth factor and tumor necrosis factor α in *in vitro* study (14). On the other hand, SOX2 expression was detected in residual cancer cells after CRT, and was rarely detected in stromal cells. The specificity for residual ESCC may be diagnostically useful for both the accurate evaluation of a patient's response after CRT and the detection of micrometastases in lymph nodes. We observed that high expression of podoplanin was significantly correlated with lymph node metastasis, vascular invasion and poor prognosis, while, high expression of SOX2 was lymphatic, vascular invasion, poorly differentiated tumor and pathological incomplete resection. However, correlation of these expression levels with TRG multivariate analysis identified high PTLVD as independent risk factors for predicting poor prognosis. Several authors have reported that high peri- or intra-tumoral lymphatic vessel density is correlated with lymph node metastasis and poor prognosis in oral, head and neck squamous cell carcinoma and breast cancer (25-27). Our result was similar to previous studies, and suggested that PTLVD might be also associated with poor prognosis in patients with ESCC with neoadjuvant CRT. In conclusion, our results suggest that podoplanin and SOX2 expression levels may be useful prognostic markers for patients with ESCC after neoadjuvant CRT. However, data in this study should be interpreted with some caution. The major limitation is the small number of patients, and the retrospective nature of the study. We think that a larger study population and a long-term follow-up will allow us to validate our conclusions.

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SURGERY TODAY

Case Report

Multiple Solitary Leiomyomas in the Esophagus: Report of a Case

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Abstract
We herein report our findings for an asymptomatic 65-year-old man presenting with multiple solitary leiomyomas in the esophagus, who was undergoing follow-up for primary aldosteronism with high levels of serum progesterone. Esophageal endoscopy and computed tomography showed multiple submucosal tumors with calcification in the thoracic esophagus. A subtotal esophagectomy was performed because the possibility of malignancy could not be ruled out based on a needle biopsy taken of the specimen. The final resected specimen showed eight solitary, encapsulated nodules between the middle and lower thoracic esophagus. Histopathological examinations showed these nodules to have the typical histological findings of leiomyomas. In addition, staining with antibodies against the progesterone receptor revealed diffuse expression in the nuclei of the leiomyoma cells. Only four cases with more than eight solitary leiomyomas have been reported, including the current case. However, this is the first reported case where primary aldosteronism with elevated serum progesterone levels has been implicated in the pathogenesis of multiple solitary leiomyomas in the esophagus.

Key words Multiple solitary leiomyoma · Esophagus · Aldosteronism · Progesterone receptor

Introduction

Multiple solitary leiomyomas are characterized as well-defined, multiple encapsulated masses in the esophagus. They can be distinguished histopathologically from diffuse leiomyomatosis, which is characterized by diffuse hypertrophy of the whole esophageal muscular layer, rather than the presence of discrete tumor nodules.[1,2] Although Seremetis et al.[3] reported in a review of 838 leiomyoma cases that more than one tumor was occasionally found in the same patient (<2.4% of cases), more than three solitary lesions were not common.[3,4] The mechanism responsible for the development of multiple leiomyomas in the esophagus is unknown. However, many reports have suggested that progesterone can advance the growth of uterine leiomyomas.[5–8] We report a patient with eight solitary esophageal leiomyomas, who was undergoing follow-up for primary aldosteronism and elevated serum progesterone. It is possible that elevated progesterone levels and primary aldosteronism might thus have played a role in the growth of multiple solitary leiomyomas in this case.

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Case Report

A 65-year-old asymptomatic man, who was being followed up for primary aldosteronism with elevated serum progesterone, was admitted to our hospital for close investigation. On admission, his serum progesterone was elevated to 1300 pg/ml (normal range <700 pg/ml for males; Mitsubishi, Tokyo, Japan). In contrast, his serum level of estradiol was within the normal range for males (22 pg/ml; SRL, Tokyo, Japan). Multiple submucosal tumors in the esophagus were accidentally detected. A barium study showed multiple smooth filling defects below the bronchial bifurcation. Chest and abdominal computed tomography showed marked esophageal wall thickening and tumor-like lesions with calcification below the bronchial bifurcation (Fig. 1a). The wall thickness reached 24 mm in diameter, and the calcified lesion was adjacent to the left main bronchus. Distant metastasis was not found. Endoscopic examination and esophagography showed multiple protruding lesions covered with normal esophageal mucosa (Fig. 1b,c).

Endoscopic ultrasonography (EUS) showed more than four solitary hypoechoic lesions with a maximum diameter of 32 mm, accompanied by comet-tail signs due to calcification, arising from the muscularis propria. An EUS-guided fine-needle aspiration biopsy was performed, but it failed to produce a suitable specimen to make it possible to rule out the presence of any malignant lesions. Bronchoscopy showed no abnormalities. The patient underwent surgery because of the uncertain diagnosis and the remaining possibility of malignancy. Intraoperative findings revealed the presence of multiple nodules in the esophagus from the level of the azygos vein to the lower end of the intrathoracic esophagus. No invasion to the adjacent structures was found for any of the nodules. Because the masses occurred adjacent to each other, and because tumor growth was extensive, an esophagectomy with gastric pull-though was performed. Macroscopic examination revealed eight solitary, submucosal tumors with diameters ranging from 6 to 20 mm. These nodules were covered with thin, fibrous capsules (Fig. 2a,b). Microscopically, proliferating spindle cells were present in a tangled and crossed arrangement. These lesions were partially accompanied by calcification, and perinuclear vacuolation was found in some spindle cells. Mitotic figures and cytological atypia were not found (Fig. 3a). Immunohistochemical analysis was performed to allow differential diagnosis from other stromal tumors of the esophagus. Immunohistochemical staining using antibodies to α-smooth muscle actin (1:100; Dako, Glostrup, Denmark) produced positive results, but there was no staining with antibodies for c-kit (1:50; NovoCastro, Newcastle, UK) (Fig. 3b,c). To demonstrate a relationship between the multiple, solitary leiomyomas and hyperprogesteronism, staining with antibodies against the progesterone receptor (1:100; Dako) and estrogen receptor (1:100; Dako) was performed. The progesterone receptor was diffusely expressed in the nuclei of leiomyoma cells, but not in the smooth muscle in the adjacent normal mucosa and not in the nuclei of ordinary leiomyoma (Fig. 4a–c). In contrast, the estrogen receptor was not expressed in any of the leiomyoma cells (Fig. 4d). The postoperative histopathological diagnosis was of multiple solitary leiomyomas in the esophagus. Preoperative systemic evaluation with computed tomography and surgical findings did not indicate the presence of any other leiomyomas in other organs. The postoperative course was uneventful, and the patient was eventually discharged.

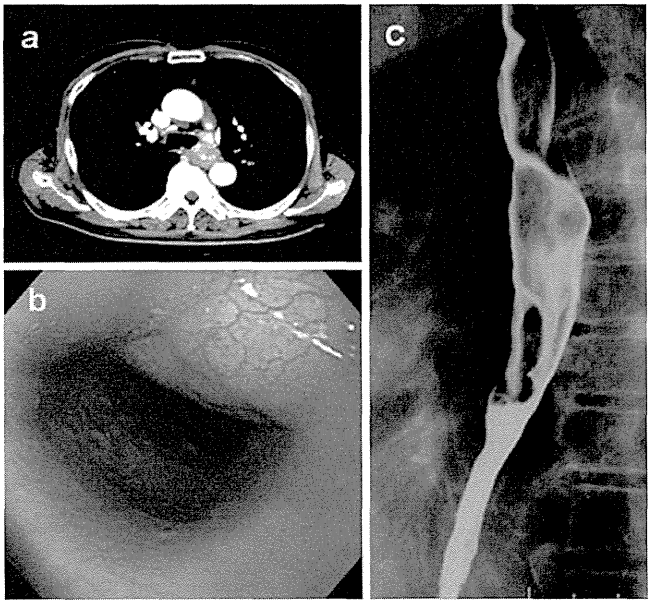

Fig. 1. **a** Chest and abdominal computed tomography showed marked esophageal wall thickening and tumor-like lesions with calcification below the bronchial bifurcation **b, c** Endoscopic examinations and esophagography showed multiple protruding lesions covered with a normal esophageal mucosa

Discussion

The incidence of esophageal leiomyomas is relatively low in comparison to esophageal carcinoma, and it accounts for only 0.4% of all esophageal neoplasms.[3,9] Leiomyomas are usually solitary, rounded, and well-demarcated masses, and multiple leiomyomas (more than three) are extremely rare.[3,4]

In a review of 838 cases, Seremetis et al.[3] emphasized that multiple solitary leiomyomas should not be con-

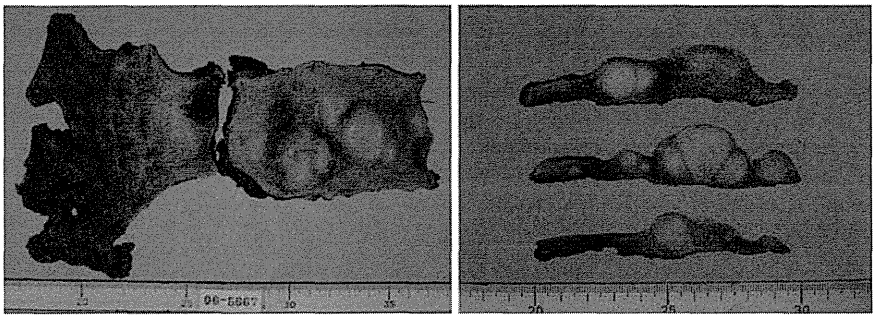

Fig. 2a, b. Macroscopically, eight solitary submucosal tumors, with diameters ranging from 6 to 20 mm, were revealed. These nodular lesions were covered with thin fibrous capsules

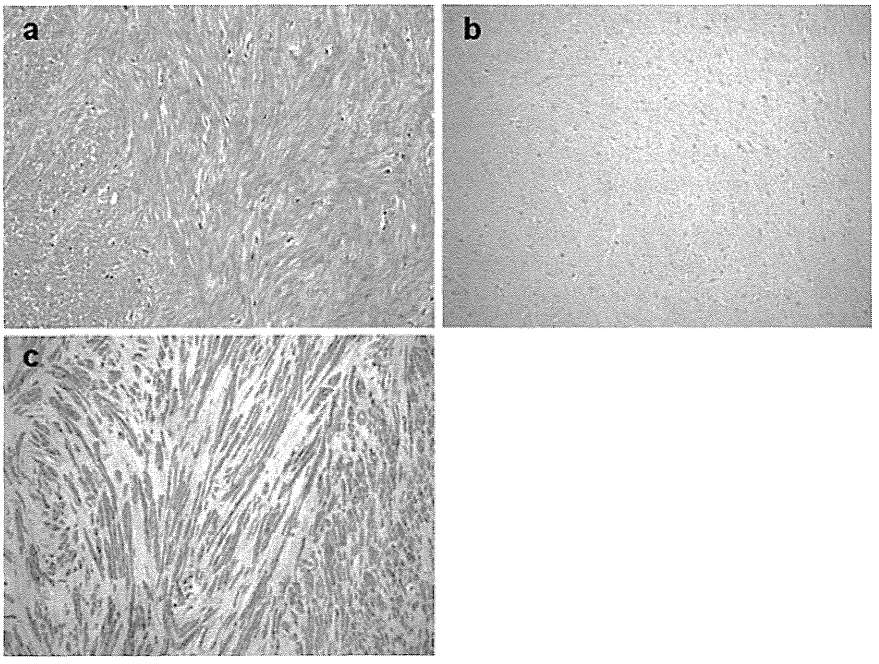

Fig. 3a–c. Immunohistochemistry. **a** Hematoxylin–eosin stain (×200). **b** Immunohistochemical staining for c-kit (×200). **c** Immunohistochemical staining for α-smooth muscle actin (×200)

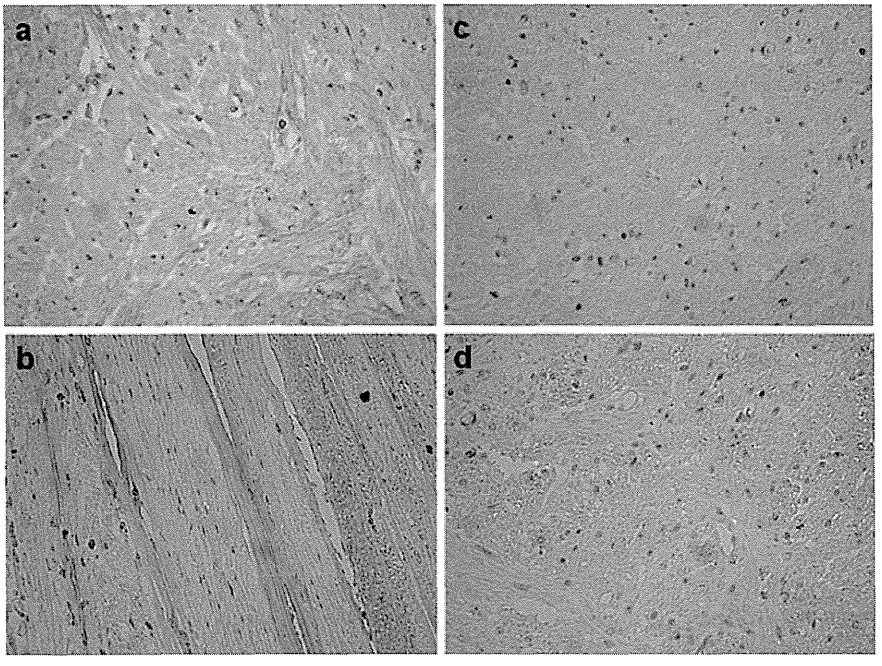

Fig. 4. Immunohistochemical staining for the progesterone receptor PgR in the tumor in the present case (**a**; ×400), in the esophageal wall (**b**; ×400), and in typical leiomyomas (**c**; ×400). **d** Immunohistochemical staining for the estrogen receptor in the tumor in the present case (×400)

fused with diffuse leiomyomatosis. Diffuse leiomyomatosis is a distinct entity histopathologically characterized by diffuse hypertrophy of the whole esophageal muscular layer, rather than by discrete tumor nodules.[1,2] Diffuse leiomyomatosis is not encapsulated and presents with considerable interstitial hypertrophy, and with lymphatic and plasma cell infiltration. It is most frequent in young women and is occasionally accompanied by Alport's syndrome, which is associated with larger deletions and rearrangements of the COL4A5/COL4A6 gene (Xq22.3).[10]

Multiple solitary leiomyomas appear as macroscopically well-demarcated, multiple solitary nodules with capsules. These nodules are covered with normal esophageal mucosa. Microscopically, the nodules have histological findings typical of leiomyomas, i.e., a low overall cellularity, and an increased growth from interlaced smooth muscle cells with hypovascularity.[11]

Table 1. Reports of multiple solitary leiomyomas with more than eight lesions in the esophagus

No.	First author, year[Ref.]	Age (years)	Sex	Evaluation	No. of lesions	Size (cm)	Surgery
1	Bradford, 1947[12]	23	Male	Barium swallow GIF	14	1–4	Removal of the tumor
2	Seremetis, 1974[3]	27	Male	Barium swallow GIF	15	0.4–6	Enucleation
3	Iwaya, 2006[11]	71	Male	Barium swallow CT GIF, EUS	>20 (with superficial cancer)	0.1–3	Esophagectomy
4	Present case, 2009	65	Male	CT GIF, EUS EUS-FNA	8	0.6–2	Esophagectomy

GIF, gastrointestinal fiber; CT, computed tomography; EUS, endoscopic ultrasonography; FNA, fine-needle aspiration

Although more than one tumor (generally two or three) is occasionally found in the same patient (<2.4% of cases), only four reported cases have presented with more than eight lesions (Table 1). Bradford et al.[12] reported 14 leiomyomas, averaging 1–4 cm, in the same patient. Seremetis et al.[3] reported on a patient with 15 independent esophageal leiomyomas, in whom enucleation of the lesions was successful. Iwaya et al.[11] encountered a patient with more than 20 leiomyomas, accompanied by esophageal carcinoma overlying the submucosal leiomyoma. However, the molecular mechanisms responsible for the development of multiple solitary leiomyomas still remain to be fully elucidated.

There have so far been no reports regarding the involvement of aldosteronism in the pathogenesis of leiomyomas, although some researchers have recently reported that progesterone and the progesterone receptor modulate myoma mitotic activity and play a critical role in the pathogenesis of uterine leiomyomas.[5–8] Kawaguchi et al.[13] found increased mitotic activity of leiomyomas during the secretory phase of the menstrual cycle, suggesting that leiomyoma growth is affected by progesterone levels. Furthermore, Tiltman et al.[14] reported the administration of medroxyprogesterone acetate to significantly increase the mitotic activity of leiomyomas, in comparison to an untreated control group. Treatment with the progesterone antagonist RU-486 (mifepristone) has been reported to induce the regression of leiomyomas, accompanied by a reduction in progesterone receptor immunoreactivity, thus suggesting a direct antiprogesterone effect.[15–17] Tori et al.[18] reported a case demonstrating multiple benign metastasizing leiomyomas, with the expression of both estrogen and progesterone receptors, which was successfully treated with a GnRH agonist. Although there has not been any reported association of leiomyoma growth with the effect of progesterone in any other organs except the uterus, these findings support the view that progesterone may play a vital role in promoting leiomyoma growth.

In our case, an elevation of serum progesterone with primary aldosteronism was observed at admission. The synthesis of steroid hormones is started from cholesterol in the adrenal cortex. Progesterone in particular is an intermediate in the formation of aldosterone from cholesterol. In addition, in males, progesterone is mainly formed from cholesterol in the adrenal cortex. It is therefore possible that the elevated serum progesterone levels in our patient were related to the excess production of aldosterone.

In addition to increased serum progesterone levels that accompanied the primary aldosteronism, immunohistochemical analysis demonstrated the expression of the progesterone receptor in the esophageal leiomyomas. These findings suggest that the elevated serum progesterone associated with aldosteronism could affect the growth of leiomyomas, resulting in the development of multiple solitary leiomyomas in the esophagus, as in our case. Furthermore, a progesterone antagonist and/or aromatase inhibitors might be useful for controlling the growth of leiomyomas in the esophagus in patients with hyperprogesteronism.

In conclusion, cases of multiple solitary leiomyomas in the esophagus with more than eight lesions are extremely rare, and only four cases, including the current case, have been reported. Furthermore, this case is the first one reported where primary aldosteronism with elevated serum progesterone levels might have contributed to the pathogenesis of the multiple solitary leiomyomas in the esophagus.

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Antibody response to cancer/testis (CT) antigens: A prognostic marker in cancer patients

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Keywords: cancer/testis (CT) antigen, NY-ESO-1, XAGE1 (GAGED2a), overall survival, spontaneous antibody response

Immune responses to tumor antigens have been reported in cancer patients. However, the relevance of such spontaneous immune responses to the clinical course has not been studied extensively. We showed that the overall survival of patients with antibodies against NY-ESO-1 or XAGE1 (GAGED2a) antigen was prolonged in gastric or lung cancer patients, respectively.

Cancer patients respond to their own tumors immunologically, and antibody responses, or CD4 and CD8 T-cell responses, against tumor cells or tumor cell products have been recognized (**Fig. 1**). Using such antibodies or T cells as probes, many tumor antigens have been identified in various human tumors over the past 2 decades. Those include mutated, differentiation, over-expressed and cancer/testis (CT) antigens. More recently, T-cell responses to various mutated peptides have been detected by whole exome analysis. The findings clearly indicate the presence of tumor antigens and the occurrence of immune responses to them in cancer patients. However, the relevance of such spontaneous immune responses to the clinical course has not been studied extensively. The reason for this appears to be the difficulty of detecting anti-tumor immune responses in patients because of the generally weak antigenicity of the tumor antigens together with the lack of a reliable methodology. However, some CT antigens, including NY-ESO-1 and XAGE1 (GAGED2a), have been shown to be highly immunogenic. Besides CT antigens, the tumor suppressor gene product p53 is also known to be strongly immunogenic. A previous study on spontaneous NY-ESO-1 immune responses in cancer patients revealed that antibody responses and CD4 and CD8 T-cell responses occur concomitantly as an integrated immune response.[1] Because of its sensitivity and reproducibility as well as involving a simple assay procedure, an antibody response would be a useful immune biomarker to evaluate immune responsiveness in patients. Nevertheless, it should be noted that a split immune tolerance could take place depending on the antigens.[2] NY-ESO-1 antigen readily elicits a CD8 T-cell response, but p53 has been reported as a weaker elicitor, irrespective of a similar efficiency of antibody induction with these antigens.

By the detection of antibody responses against CT antigens, we have investigated the clinical relevance of such spontaneous immune responses in cancer patients. In gastric cancer patients, we previously showed that an NY-ESO-1 antibody response was present at 3–4% in stage I and II, and 20–25% in stage III and IV patients, suggesting a higher antibody response rate in more advanced stage patients.[3] Analysis of the overall survival in 310 gastric cancer patients with stages I to IV showed no difference between antibody-positive and -negative patients. However, in the 126 patients in stages III and IV, the overall survival of antibody positive patients was prolonged, although not significantly. It should be noted that NY-ESO-1 expression itself is tumorigenic, and patients with NY-ESO-1-antigen-positive tumors showed a shorter survival compared to patients with antigen-negative tumors in various cancer types.[4] In our recent prospective study on advanced stage (stages IIIB and IV) lung adenocarcinoma patients, patients with antibody responses to XAGE1 (GEGED2a) showed a significantly prolonged overall survival compared to patients with no antibody responses.[5] XAGE1 (GAGED2a) antigen expression was a worse predictor in patients with EGFR-mutated tumors. The XAGE1 (GAGED2a) antibody frequency in the advanced lung adenocarcinoma patients was similar (approximately 20%) to the frequency of NY-ESO-1 antibody responses in advanced gastric cancer patients. However, the patient cohort is more restricted at advanced stages in lung cancer compared to gastric cancer patients. Daudi, et al.[6] recently reported that ovarian cancer patients with antibody responses to any of the MAGE family antigens showed a shorter survival compared to patients without antibody responses. In their study, patients with all stages were included, and so the shorter survival of advanced cancer patients should be carefully considered, as they discussed. A stage-controlled study is necessary to elucidate the effect of the antibody response on the clinical course. A similar overall survival-shortening effect of the antibody response was observed in patients with antibody against p53.[7] Caution, as described in Daudi's report,

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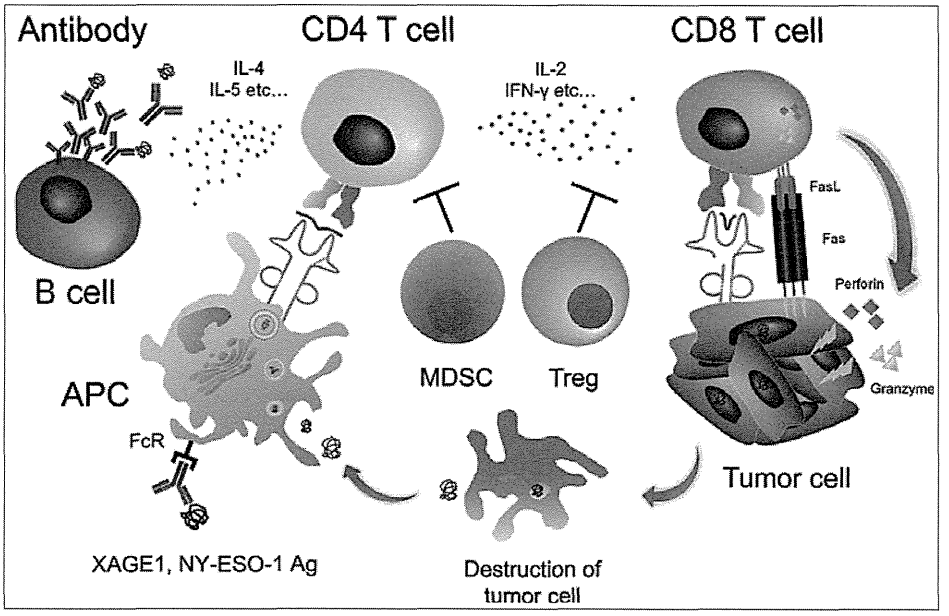

Figure 1. Spontaneous immune responses to tumors in cancer patients. The cancer/testis (CT) antigens NY-ESO-1 and XAGE1 (GAGED2a) are strongly immunogenic, and an integrated immune response, consisting of an antibody response and CD4 and CD8 T-cell responses is frequently elicited spontaneously. The antibody response is a useful biomarker of immune responses because of its sensitivity and reproducibility, as well as involving a simple assay procedure. CD4 T-cell responses to CT antigens would be enhanced by the antigen-presenting cells (APC) that efficiently internalize the antigen/antibody complex via the Fc receptor (FcR) and promote the antibody response via IL-4 and IL-5 cytokines, and the CD8 T-cell response via IL-2 and IFNγ. CD8 T cells lyse tumor cells via Fas-FasL, perforin, and/or granzyme. Tregs and MDSCs suppress CD4 and CD8 T cells.

should also be exercised when interpreting the results for p53.

Thus, in order to evaluate the clinical significance of the spontaneous antibody response in cancer patients, the clinical relevance of antigen expression should be carefully examined. The CT antigen expression itself generally worsens survival. Furthermore, it should be noted that the antibody response rate generally increases according to the stage. Therefore, the clinical benefit of the spontaneous antibody response should be evaluated in a patient cohort with restricted stages and antigen-positive tumors.

The presence of circulating NY-ESO-1-specific T-cells has been shown to be correlated with a favorable prognosis in melanoma patients.[8] Conversely, the presence of MDSCs has been shown to be correlated with a poor prognosis.[8] The clinical relevance of Foxp3-positive Treg infiltration is controversial, being correlated with poor survival in some cancers, but with better survival in others.[9] In the latter, the contribution of non-specific inflammation associated with Treg infiltration to the favorable prognosis has been suggested. Although such findings suggest an association of the immune phenotype with the clinical course in cancer patients, findings linking spontaneous immune responses to the clinical benefit are still limited. Our study, showing a link between NY-ESO-1 and XAGE1 (GAGED2a) antibody responses and prolonged overall survival, sheds light on the role of naturally occurring immune responses in cancer patients. Furthermore, we observed that genetic variants of immunoglobulin γ and κ chains influence the XAGE1 (GAGED2a) antibody response.[10] The present findings should be confirmed with different patient cohorts and extended to other antigens in other cancers to establish a firm basis for immunotherapy.

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Vaccination With NY-ESO-1 Overlapping Peptides Mixed With Picibanil OK-432 and Montanide ISA-51 in Patients With Cancers Expressing the NY-ESO-1 Antigen

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Summary: We conducted a clinical trial of an NY-ESO-1 cancer vaccine using 4 synthetic overlapping long peptides (OLP; peptides #1, 79–108; #2, 100–129; #3, 121–150; and #4, 142–173) that include a highly immunogenic region of the NY-ESO-1 molecule. Nine patients were immunized with 0.25 mg each of three 30-mer and a 32-mer long NY-ESO-1 OLP mixed with 0.2 KE Picibanil OK-432 and 1.25 mL Montanide ISA-51. The primary endpoints of this study were safety and NY-ESO-1 immune responses. Five to 18 injections of the NY-ESO-1 OLP vaccine were well tolerated. Vaccine-related adverse events observed were fever and injection site reaction (grade 1 and 2). Two patients showed stable disease after vaccination. An NY-ESO-1-specific humoral immune response was observed in all patients and an antibody against peptide #3 (121–150) was detected firstly and strongly after vaccination. NY-ESO-1 CD4 and CD8 T-cell responses were elicited in these patients and their epitopes were identified. Using a multifunctional cytokine assay, the number of single or double cytokine-producing cells was increased in NY-ESO-1-specific CD4 and CD8 T cells after vaccination. Multiple cytokine-producing cells were observed in PD-1 (–) and PD-1 (+) CD4 T cells. In conclusion, our study indicated that the NY-ESO-1 OLP vaccine mixed with Picibanil OK-432 and Montanide ISA-51 was well tolerated and elicited NY-ESO-1-specific humoral and CD4 and CD8 T-cell responses in immunized patients.

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The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000001857) on April 7, 2009 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

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The NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum.[1] NY-ESO-1 expression is observed in a wide range of human malignancies, but the expression is restricted to germ cells in the testis in normal adult tissues.[2] Therefore, NY-ESO-1 has emerged as a prototype of a class of cancer/testis (CT) antigens.

Numerous cancer vaccine strategies are under development.[3,4] For patients with hormone-resistant prostate cancer, a dendritic cell (DC) vaccine has recently been approved.[5] The therapy is based on loading autologous DCs ex vivo with a lysate of a cultured prostate cancer cell line transfected with the genes of acid phosphatase and GM-CSF, with subsequent administration to patients to induce specific T-cell responses. However, its clinical efficacy seems to be limited.[6,7] Cancer vaccines using recombinant proteins and peptides are thought to involve DCs in vivo and have advantages in that materials are easy to secure, there is little toxicity, and there are no complex regulatory matters when compared with cell therapy.[8] As cancer vaccines using short peptides showed only limited efficacy, cancer vaccines using synthetic long peptides have been introduced.[4,9–11] Synthetic peptides of 25–50 amino acids are internalized and processed by DCs efficiently, and presented the antigens on MHC class I and II for T cells.[12,13] Maturation of DCs is associated with upregulation of costimulatory molecules on their surfaces and is crucial for efficient induction of T-cell responses. Adjuvants such as TLR ligands induce DC maturation and strongly augment the immunogenicity of cancer vaccines.[3,14]

Detection of pathogen-associated molecular patterns by the pattern recognition receptors on DCs and activation of subsequent signaling induce specific CD4 and CD8 T-cell responses.[15,16] Thus, the stimulatory effect of innate immunity on adaptive immune responses is useful for cancer vaccines. TLRs, nucleotide-binding oligomerization domain–like receptors, the retinoic acid-inducible gene-I-like or RIG-like receptors, and the C-type lectin receptors are the 4 known families of pattern recognition receptors.[17] In this study, we immunized patients with advanced cancers expressing the NY-ESO-1 antigen with 30–32-mer NY-ESO-1 overlapping long peptides (OLP), and Picibanil

OK-432 and Montanide ISA-51 as immunomodulators. OK-432 is a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes*.[9,18,19] It was developed as a nonspecific immune stimulant, TLR2, 4, and/or 9 agonist. OK-432 induces various cytokines including tumor necrosis factor-α (TNF-α), interferon (IFN), interleukin (IL)-2, and IL-6.[20] Recently, it was shown that in vivo administration of OK-432 overcame regulatory T-cell suppression in mice.[21] The vaccine also induced efficient NY-ESO-1 immunity in patients.

MATERIALS AND METHODS

NY-ESO-1 OLP Vaccine

NY-ESO-1 OLPs [peptide #1: NY-ESO-1 79–108 (GARGPESRLLEFYLAMPFATPMEAELARRS), peptide #2: NY-ESO-1 100–129 (MEAELARRSLAQDAPPLP VPGVLLKEFTVS), peptide #3: NY-ESO-1 121–150 (VLLKEFTVSGNILTIRLTAADHRQLQLSIS), and peptide #4: NY-ESO-1 142–173 (HRQLQLSISSCLQQL SLLMWITQCFLPVFLAQ)] were synthesized by Multiple Peptide Systems (San Diego, CA). The vaccine, consisting of 1 mg of NY-ESO-1 OLP including 0.25 mg each of the 4 peptides, 0.2 KE OK-432 (Picibanil; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and 1.25 mL ISA-51(Montanide; Seppic, Paris, France), was emulsified under sterile conditions. Synthesis, production, formulation, and packaging of the investigational agent were in accordance with current Good Manufacturing Practices and met the applicable criteria for use in humans.

Study Design

A pilot, open-label, multi-institutional clinical trial of the NY-ESO-1 OLP vaccine was designed to evaluate the safety, immune response, and clinical response. Patients eligible for entry were those who had advanced cancers that were refractory to standard therapy and expressed NY-ESO-1 as assessed by immunohistochemistry (IHC), a performance status of 0, 1, or 2, were 20 years old or above, had a life expectancy of 4 months or more, and did not have impaired organ function. Patients ineligible were those who were positive for HIV antibodies, had multiple cancers, autoimmune disease, serious allergy history, or active brain metastasis, or received chemotherapy, systemic steroid, or immunosuppressive therapy in the last 4 weeks. Nine patients, including 7 patients with esophageal cancer, a patient with lung cancer, and a patient with malignant melanoma, were enrolled in a washout period after surgery, chemotherapy or radiation therapy. The vaccine was administered subcutaneously once every 2 (esophageal cancer patients) or 3 (lung cancer and malignant melanoma patients) weeks to achieve better performance status in esophageal cancer patients to complete a cycle of 6 vaccinations. Four weeks after the last administration, the safety, immune response, and clinical response were evaluated. Thereafter, the vaccine was administered additionally. The 9 patients received 5–18 immunizations. Clinical response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1)[22] and the immune-related response criteria (irRC).[23] Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) (http://ctep.cancer.gov/). The protocol was approved by the Ethics Committee of Osaka, Tokyo and Okayama Universities in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrollment in the study. The study was conducted in compliance with Good Clinical Practice. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (unique trial number: UMIN000001857) on April 7, 2009 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

Blood Samples

Peripheral blood was drawn from the patients at baseline, at each time point of immunization, and 4 weeks after the last immunization. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated by density gradient centrifugation using lymphoprep (AXIS-SHIELD Poc AS, Oslo, Norway). A CD8 T-cell–enriched population was obtained from PBMCs using CD8 microbeads with a large-scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). A CD4 T-cell–enriched population was then obtained from the residual cells using CD4 microbeads. The final residual cells were used as a CD4-depleted and CD8-depleted population. The 3 populations were stored in liquid N_2 until use. HLA typing of PBMCs was performed by sequence-specific oligonucleotide probing and sequence-specific priming of genomic DNA using standard procedures.

NY-ESO-1 18-Mer Series Peptides

The following series of 28 overlapping NY-ESO-1 18-mer peptides spanning the protein were synthesized: 1–18, 7–24, 13–30, 19–36, 25–42, 31–48, 37–54, 43–60, 49–66, 55–72, 61–78, 67–84, 73–90, 79–96, 85–102, 91–108, 97–114, 103–120, 109–126, 115–132, 121–138, 127–144, 133–150, 139–156, 145–162, 149–166, 153–170, and 156–173. A 30-mer peptide, 151–180, was also synthesized. These 29 peptides (NY-ESO-1 18-mer series peptides) were synthesized using standard solid-phase methods based on N-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on a Multiple Peptide Synthesizer (AMS422; ABIMED, Langenfeld, Germany) at Okayama University.

ELISA

Recombinant NY-ESO-1 protein was prepared as described previously.[24] Recombinant protein (1 μg/mL) or peptide (10 μg/mL) in a coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) was adsorbed onto 96-well Polysorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with PBS and blocked with 200 μL/well of 5% FCS/PBS for 1 hour at room temperature. After washing, 100 μL of serially diluted plasma was added to each well and incubated for 2 hours at room temperature. After extensive washing, horseradish peroxidase-conjugated goat anti-human pan-IgG, IgG_1, IgG_2, IgG_3, IgG_4, or IgM (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells, and the plates were incubated for 1 hour at room temperature. After washing and development, absorbance at 490 nm was read. Recombinant murine Akt protein[24] and ovalbumin (albumin from chicken egg white; Sigma, St. Louis, MO) were used as control proteins.

In Vitro Stimulation of CD4 and CD8 T Cells

Frozen cells were thawed and resuspended in AIM-V (Invitrogen, Carlsbad, CA) medium supplemented with 5% heat-inactivated pooled human serum (CM), and kept at room temperature for 2 hours. CD4-enriched and CD8-enriched populations (2×10^6) were cultured with irradiated

(30 Gy), autologous CD4-depleted and CD8-depleted PBMCs (2×10^6) in the presence of 29 NY-ESO-1 18-mer series peptides in 2 mL of CM supplemented with 10 U/mL rIL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/mL rIL-7 (Peprotech, London, UK) in a 24-well culture plate at 37°C in a 5% CO_2 atmosphere for 12 days. For the second stimulation, 1×10^6 instead of 2×10^6 responder cells were used in the culture described above. For nonspecific immune activation, T cells were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin with GolgiStop (BD Biosciences, Franklin Lakes, NJ) for 6 hours at 37°C.

IFN-γ Catch Assay

Responder CD4 or CD8 T cells (5×10^4) from the stimulation culture were cultured for 4 hours with autologous EBV-B cells (5×10^4) pulsed with mixed, or one of the 29 NY-ESO-1 18-mer series peptides. The cells were then treated with a bispecific CD45 and IFN-γ antibody (IFN-γ catch reagent; 2 μL) for 5 minutes on ice. The cells were diluted in AIM-V medium (3 mL) and placed on a slow rotating device (Miltenyi Biotec, Bergisch Gladbach, Germany) to allow IFN-γ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 1 hour, the cells were washed with cold buffer and treated with PE-conjugated anti-IFN-γ (detection reagent), and FITC-conjugated anti-CD4 or anti-CD8 mAb. After incubation for 10 minutes at 4°C, the cells were washed and analyzed by FACS Canto II (BD Biosciences).

IHC

IHC was performed as described previously.[9] E978[25] and EMR8-5 (Funakoshi, Tokyo, Japan) mAbs were used to analyze NY-ESO-1 and HLA class I expression, respectively. The reaction was evaluated as + + + (> 50% stained cells), + + (50%–25% stained cells), + (25%–5% stained cells), and − (< 5% stained cells).

Intracellular Staining (ICS) of Cytokines

CD4 or CD8 T cells were washed and treated with Cytofix and Perm Wash (BD Biosciences) according to the manufacturer instructions to stain them with IFN-γ-APC (BD Biosciences), IL-2-APC (BD Biosciences), IL-5-PE (BD Biosciences), IL-10-APC (BioLegend, San Diego, CA), IL-17 A-PerCP-Cy5.5 (eBioscience, San Diego, CA), and/or TNF-α-FITC (eBioscience) intracellularly. For Foxp3 staining, a human Foxp3 staining kit, Alexa 488 (BD Biosciences), was used. Cells were analyzed using a FACS Canto II (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

To amplify the *NY-ESO-1* cDNA segment, primers specific for *NY-ESO-1* were designed as described previously.[26] Primers for RT-PCR were: *NY-ESO-1 5′-AGT TCTACCTCGCCATGCCT-3′* (forward), *5′-TCCTCCT CCAGCGACAAACAA-3′* (reverse), *GAPDH 5′-ACCACA GTCCATGCCATCAC-3′* (forward), *5′-TCCACCACC CTGTTGCTGTA-3′* (reverse). The amplification program for *NY-ESO-1* was 1 minute at 94°C, 1 minute at 60°C, and 1.5 minutes at 72°C for 35 cycles after denaturing at 94°C for 1 minute. These cycles were followed by a 10-minute elongation step at 72°C. The amplification program for *GAPDH* was 1 minute at 94°C, 1 minute at 66°C, and 1.5 minutes at 72°C for 30 cycles after denaturing at 94°C for 1 minute. These cycles were followed by a 10-minute elongation step at 72°C. The PCR products were analyzed on a 1.3% agarose gel.

RESULTS

Patient Characteristics

Nine patients with advanced cancers expressing the NY-ESO-1 antigen were enrolled (Table 1). Seven were esophageal cancer patients. One was a malignant melanoma patient and 1 was a lung cancer patient. Eight patients completed a cycle of 6 vaccinations. One esophageal cancer patient, P-5, did not complete a cycle of vaccination due to disease progression and was given only 5 vaccinations, but was included in the analysis. Two patients, P-6 and P-7, were given additional vaccinations because of stable disease (SD) during treatment (Table 2).

Safety

Injection site reactions were observed in all patients (Table 1). The reaction was grade 1 in 7 patients and grade 2 with induration in 2 patients, but resolved without any treatment several months after vaccination. Fever (grade 1) was observed in 3 patients. No other adverse events either related or nonrelated to the vaccine were observed. The vaccine was well tolerated.

Monitoring of Humoral Immune Response

Serum antibodies against full-length NY-ESO-1 protein and also against 4 individual NY-ESO-1 OLP (peptides #1, 79–108; #2, 100–129; #3, 121–150; and #4, 142–173) used for the vaccine were investigated (Fig. 1). Three patients, P-2, P-3, and P-4, had antibodies against the NY-ESO-1 protein at the baseline (seropositive). Of those, P-2 and P-3 showed no antibodies against the 4 peptides, whereas P-4 showed antibodies against peptide #1 (79–108),

TABLE 1. Patient Characteristics

Patients	Age/Sex	Cancer	Vaccination	Vaccine-related Toxicity
P-1	69/M	Malignant melanoma	6	Injection site reaction (grade 1)
P-2	55/M	Lung cancer (adenocarcinoma)	6	Fever (grade 1), injection site reaction (grade 1)
P-3	66/M	Esophageal cancer (squamous cell carcinoma)	6	Injection site reaction (grade 1)
P-4	70/M	Esophageal cancer (squamous cell carcinoma)	6	Injection site reaction (grade 1)
P-5	58/M	Esophageal cancer (squamous cell carcinoma)	5	Injection site reaction (grade 1)
P-6	67/M	Esophageal cancer (squamous cell carcinoma)	18	Injection site reaction (grade 1)
P-7	74/M	Esophageal cancer (squamous cell carcinoma)	8	Injection site reaction (grade 1)
P-8	69/M	Esophageal cancer (squamous cell carcinoma)	6	Fever (grade 1), injection site reaction (grade 2)
P-9	70/F	Esophageal cancer (squamous cell carcinoma)	6	Fever (grade 1), injection site reaction (grade 2)

TABLE 2. Immune Responses and Tumor Responses After Vaccination With the NY-ESO-1 OLP Peptide

	IHC*		Antibody‡		CD4§		CD8§		Target Tumor		
Patients	MHC	NY-ESO-1	Pre	Post	Pre	Post	Pre	Post	Region	Total Diameter (mm)	Clinical Response‖ (Duration)¶
P-1	ND	+ †	−	+ + +	−	+	−	+	abLN + axLN	48	PD
P-2	+ +	+	−	+ + +	−	+ + +	−	+ +	meLN	24	PD
P-3	+ +	+	−	+ + +	−	+ + +	−	+ + +	abLN	32	PD
P-4	+ + +	+ + +	+	+ + +	ND	ND	ND	ND	liver	20	PD
P-5	+ + +	+ + +	−	+ + +	−	−	−	−	pleura	28	PD
P-6	+ + +	+ + +	−	+ + +	−	+	−	−	abLN + liver	33	SD (7 mo)
P-7	+ + +	+	−	+ + +	−	+ +	−	+	neLN	18	SD (4 mo)
P-8	+ +	+ + +	−	+ + +	−	+	−	+	pleura	30	PD
P-9	+ +	+ +	−	+ + +	ND	ND	ND	ND	pleura	25	PD

*IHC was performed using EMR8-5 mAb for MHC class I (MHC) and E957 mAb for NY-ESO-1. IHC-positive cells: + + + > 50%; 50% ≥ + + > 25%; 25% ≥ + > 5%; 5% ≥ −.
†NY-ESO-1 expression of P-1 was determined by RT-PCR.
‡Antibody response was determined by ELISA (see Materials and methods section) using OD values at 25× dilution for each OLP peptide and at 100× or 1600× dilution for NY-ESO-1 protein. Antibody response shown here represents OD for NY-ESO-1 OLP: + + + > 2; 2 ≥ + + > 0.5; 0.5 ≥ + > 0.1; 0.1 ≥ −.
§CD4 and CD8 T-cell responses were determined by an IFN-γ catch assay with the cells stimulated in vitro once (1°IVS). IFN-γ-positive cells: + + + > 10%; 10% ≥ + + > 5%; 5% ≥ + > 1%; 1% ≥ −. The response was confirmed by cells stimulated in vitro twice (2°IVS).
‖Clinical response was evaluated by RECIST and irRC.
¶SD duration was measured from the start of the immunization until the patients were defined as PD by RECIST (P-6) or removed due to disease progression (P-7).
Ab indicates abdomen; ax, axilla; LN, lymph node; me, mediastinum; ne, neck; ND, not done; PD, progressive disease; SD, stable disease.

but not against the others. After vaccination, antibodies against peptide #3 (121–150) were detected firstly and strongly in all patients. Antibodies against peptide #4 (142–173) were also detected in 7 of 9 patients. In contrast, the antibody responses against peptides #1 (79–108) and #2 (100–129) were relatively weak. The antibody responses against the NY-ESO-1 protein also increased in parallel, or with a delay, compared with antibody responses against the peptides in all patients. Notably, in patients P-6 and P-7 who showed SD, strong antibody responses against the peptides and the protein were observed. The dominant Ig subtypes were IgG_1 and IgG_3. An increased IgM response against peptide #3 after vaccination was observed in 6 patients.

Monitoring of CD4 and CD8 T-Cell Responses

CD4 and CD8 T cells purified from PBMCs using antibody-coated magnetic beads were cultured for 12 days with a mixture of 29 NY-ESO-1 18-mer series peptides spanning the entire NY-ESO-1 protein [stimulated in vitro once (1°IVS)] and assayed for IFN-γ production by an IFN-γ catch assay after stimulation with a mixture of 29 NY-ESO-1 18-mer series peptides for 4 hours. CD4 and CD8 T cells harvested from 1°IVS were again cultured in a similar way to 1°IVS, except using 1×10^5 instead of 1×10^6 responder cells [stimulated in vitro twice (2°IVS)] and assayed by an IFN-γ catch assay to confirm weak response. As shown in Figure 2, an increase in the CD4 T-cell response was observed in all 7 patients investigated in 1°IVS. In contrast, an increase in the CD8 T-cell response was observed in 5 of 7 patients in 1°IVS. A CD8 T-cell response was detected after 2°IVS in 2 patients (P-5 and P-6), who showed only a marginal response in 1°IVS. The Supplementary Figure (Supplemental Digital Content 1, http://links.lww.com/JIT/A313) shows the representative results of the IFN-γ catch assay for P-3 and P-7.

Next, the peptides recognized by CD4 and CD8 T cells were investigated by an IFN-γ catch assay using cells stimulated in 2°IVS and testing against individual 29 NY-ESO-1 18-mer series peptides. As shown in Figure 3, CD4 T cells dominantly recognized 18-mer peptides #15 and #21 and their adjacent peptides. A CD8 T-cell response was observed against various peptides, including 18-mer peptides #15 and #21, which were recognized relatively dominantly. In the case of CD8, a response against peptides (1–78) not included in the vaccine preparation was also observed. The Supplementary Table (Supplemental Digital Content 2, http://links.lww.com/JIT/A314) shows patient HLA.

$Foxp3^+$ CD4 cells were also examined by ICS. As shown in Figure 2, a decrease after vaccination was observed in 4 of 6 patients investigated. In contrast, a slight increase followed by a decrease was observed in 2 patients.

Clinical Observation

As shown in Table 2, patients P-6 and P-7 showed SD during vaccinations and were given additional 12 and 2 vaccinations, respectively. The other 7 patients showed PD during vaccinations. There was no discrepancy in evaluation between RECIST and irRC and no evidence of clinical benefit after immunizations. The results of immunomonitoring and clinical responses are summarized in Table 2. As previously described, in patients P-6 and P-7 who showed SD, strong antibody responses against the peptides and the protein were observed.

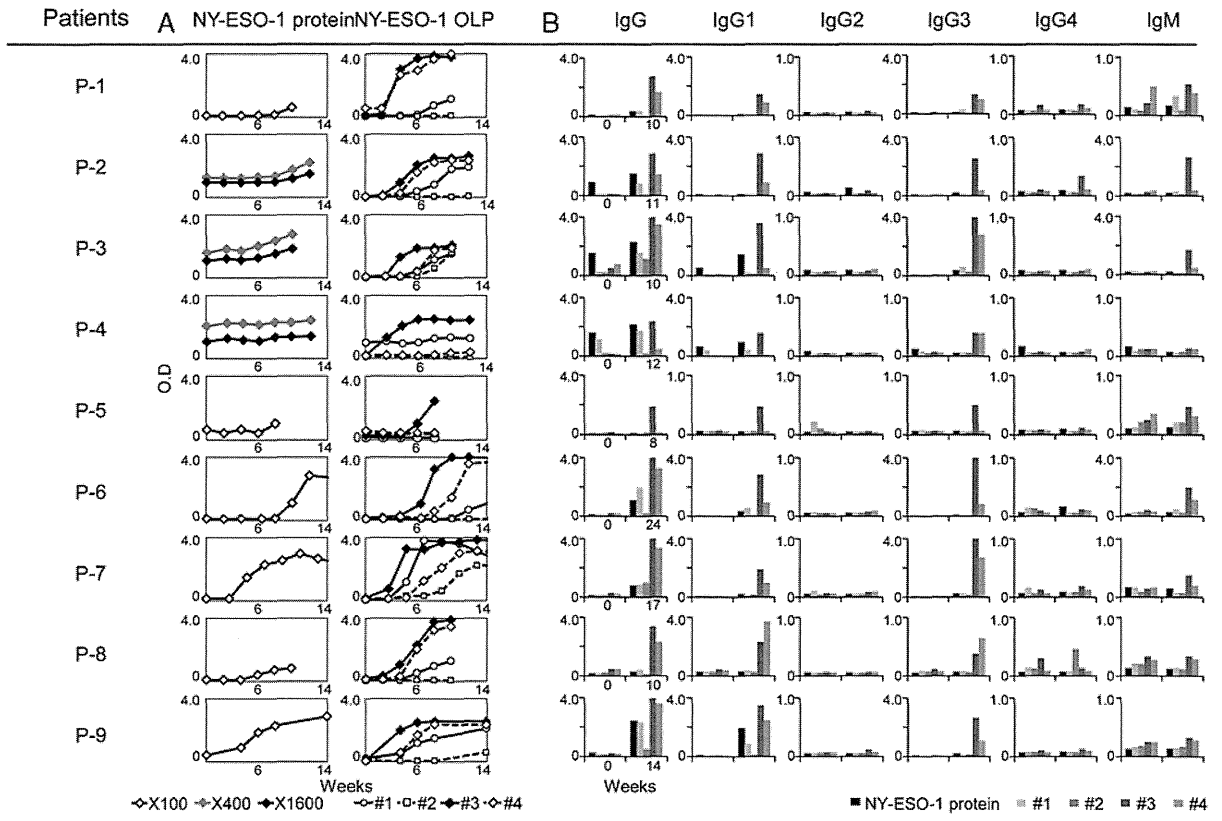

FIGURE 1. Immunomonitoring of humoral immune responses after NY-ESO-1 OLP vaccination. NY-ESO-1 antibody responses against recombinant NY-ESO-1 protein for each of the NY-ESO-1 OLP peptides were evaluated using plasma obtained before and after vaccination with NY-ESO-1 OLP from the 9 patients. A, Whole IgG antibody responses against recombinant NY-ESO-1 protein or each peptide included in the NY-ESO-1 OLP vaccine at a dilution of 1:100 (open diamond) or 1:1600 (closed diamond) for the protein and at a dilution of 1:25 for the peptides at the baseline (week 0) and at each vaccination. B, Whole IgG, IgG_1, IgG_2, IgG_3, IgG_4, and IgM antibody responses against the protein or each peptide at a dilution of 1:25 at the baseline and at the indicated week after vaccination.

Multiple Cytokine Production by CD4 and CD8 T Cells After Stimulation With NY-ESO-1 OLP

CD4 and CD8 T cells from patient P-3 and P-7 PBMCs purified by antibody-coated magnetic beads were stimulated in vitro with a mixture of 29 NY-ESO-1 18-mer series peptides for 12 days as previously shown and assayed for IL-2, TNF-α, and IFN-γ-producing cells by ICS using FACS. As shown in Figure 4, an increase in the number of single cytokine-producing cells was observed in CD4 and CD8 T cells after vaccination. A slight increase in double cytokine-producing cells was also observed. No triple cytokine-producing cells were observed. In addition, an increase in CD4 T cells producing IL-5 or IL-17, but not IL-10, was observed after vaccination, suggesting activation of Th2 and Th17, as well as Th1 (Fig. 4B).

Nonspecific Immune Activation by the Vaccine

In addition, nonspecific immune activation by the vaccine preparation was investigated using CD4 T cells from patient P-7 obtained before and after vaccination, and stimulated with PMA/ionomycin by ICS. As shown in Figures 4C and D, while no triple cytokine-producing cells were observed in either PD-1 (–) or PD-1 (+) CD4 T cells at day 0, these cells appeared even after a single vaccination (day 15). No further increase in these cells was observed in CD4 T cells obtained after the 10th vaccination (day 78). No significant change in PD-1 (–) or PD-1 (+) CD4 T-cell populations was observed during the vaccination period.

DISCUSSION

In this study, we showed that an NY-ESO-1 OLP vaccine with Picibanil OK-432 and Montanide ISA-51 was safe and induced NY-ESO-1 humoral and cellular immune responses in all patients. In our previous study on cancer vaccines with NY-ESO-1 protein[24–29] and the NY-ESO-1f peptide,[9] NY-ESO-1-specific humoral immune responses appeared to be useful as an immunological marker to predict the clinical responses of the patients vaccinated. This study showed that of the 4 peptides used, the antibody response against peptide #3 (121–150) was the most useful for monitoring than the responses against other peptides. The dominant epitope recognized by CD4 T cells (18-mer peptide #21, NY-ESO-1 121–138) in Figure 3 is included in the sequence of peptide #3. It is interesting to note that, the epitope peptide recognized by the antibody induced spontaneously in patients with NY-ESO-1-expressing tumors or in patients vaccinated with the NY-ESO-1 protein was NY-ESO-1 91–108, and was not included in peptide #3.[23] A mixture of four 30–32-mer long peptides used for the vaccine included a hydrophobic sequence located in a region approximately 121–170 amino acid, which is normally buried in the molecule[23] and therefore altered the immunological dominance of the antibody response to NY-ESO-1.

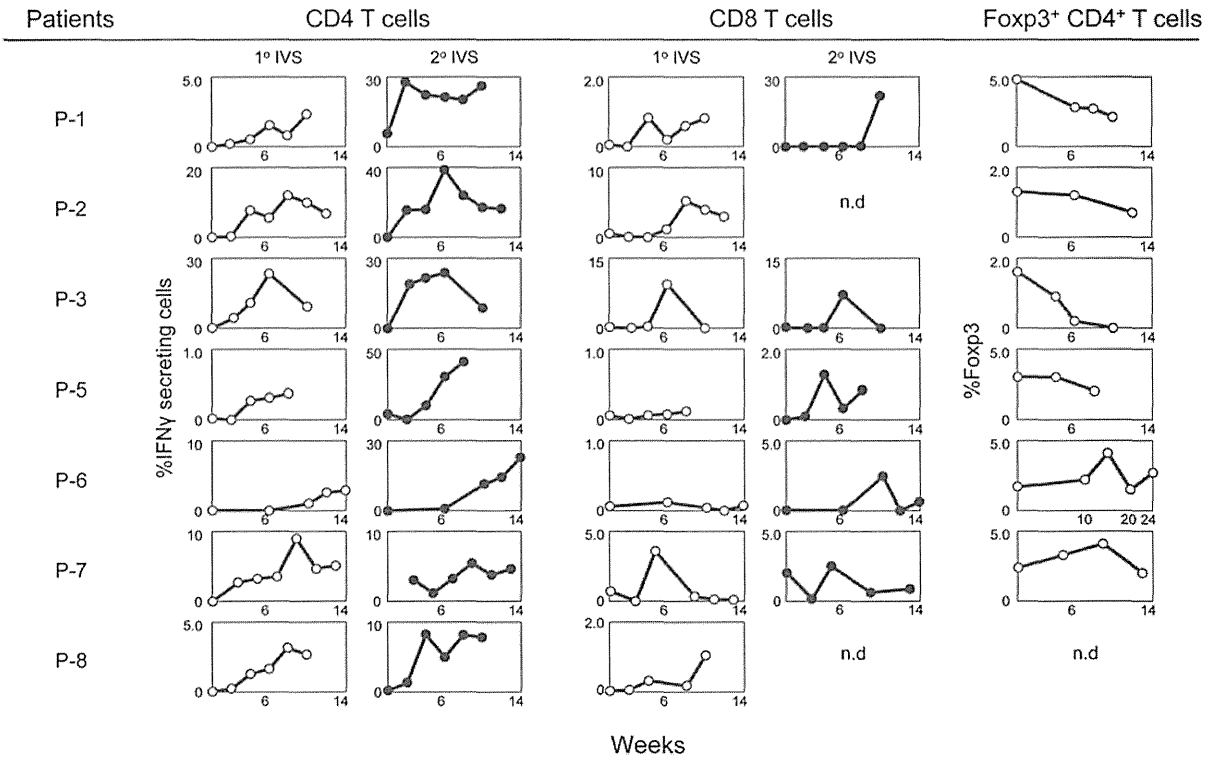

FIGURE 2. Immunomonitoring of CD4 and CD8 T-cell responses and Treg cells. CD4 and CD8 T cells were stimulated with 29 NY-ESO-1 18-mer series peptides once (1°IVS) or twice (2° IVS) and the net percentage of interferon (IFN)-γ-secreting cells was evaluated by an IFN-γ catch assay using bispecific CD45 and an IFN-γ antibody. Foxp3$^+$ CD4 Tregs were evaluated by ICS. The data are plotted with time. The analysis was conducted by FACS Canto II. ND indicates not done.

We used an IFN-γ catch assay to detect antigen-specific cellular responses using PBMCs in immunomonitoring. The assay can potentially give rise to false-positive reactions due to neighboring cells picking up IFN-γ by the bystander effect. However, it could be avoided by carefully performing the assay using a larger volume in a limited

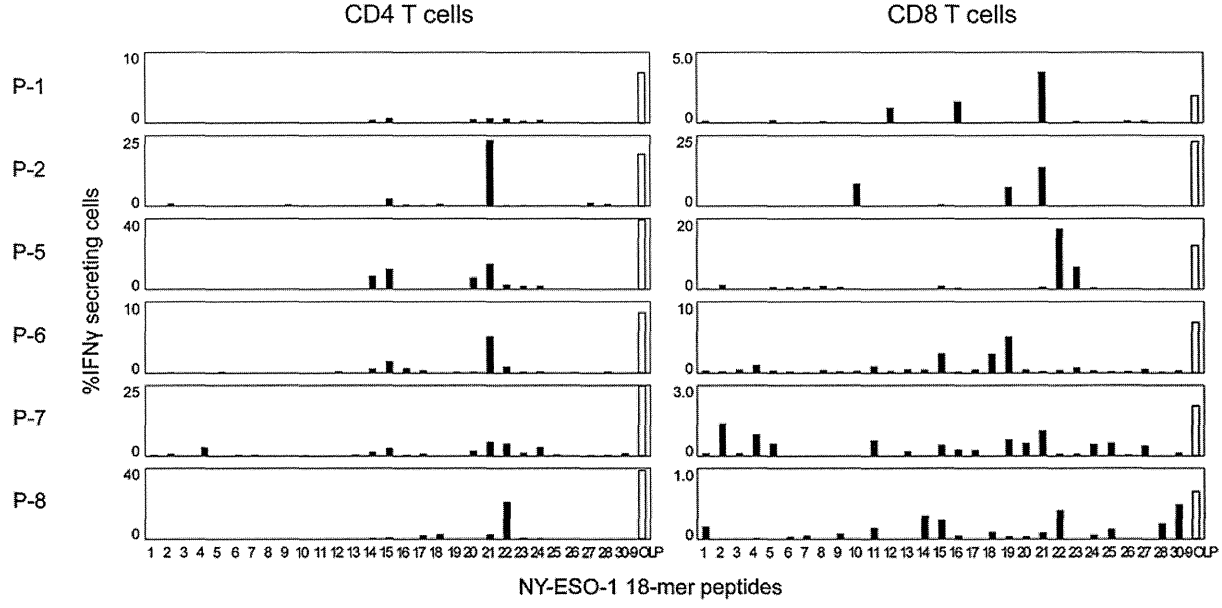

FIGURE 3. Epitope peptides recognized by CD4 and CD8 T cells. Using CD4 and CD8 T cells stimulated twice for 26 days with irradiated (30 Gy) autologous CD4-depleted and CD8-depleted PBMCs in the presence of 29 NY-ESO-1 18-mer series peptides, the epitope peptides recognized were determined by an interferon (IFN)-γ catch assay. In the assay, the cells were stimulated with autologous EBV-B cells pulsed with each 29 NY-ESO-1 18-mer peptide individually. The analysis was conducted by FACS Canto II.

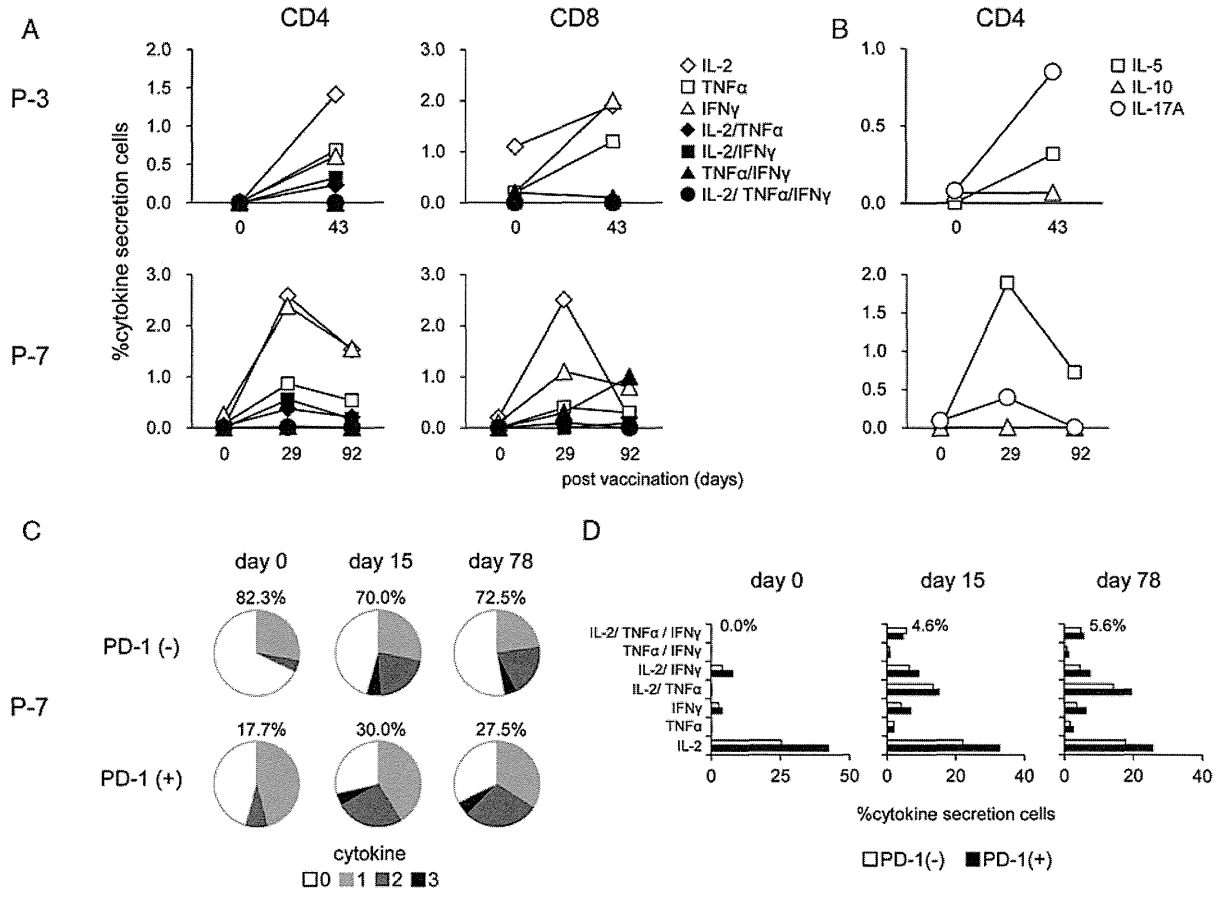

FIGURE 4. Multiple cytokine production by CD4 and CD8 T cells and nonspecific immune activation after NY-ESO-1 OLP vaccination. Multiple cytokine production by CD4 and CD8 T cells stimulated with a mixture of 29 NY-ESO-1 18-mer series peptides (A, B) or PMA/ionomycin (C, D) was analyzed. The cells from patients P-3 (A, B) and P-7 (A, B and C, D) obtained before and after vaccination were assayed for IL-2, TNF-α, and interferon (IFN)-γ-producing cells (A, C, D) or IL-5, IL-10, and IL-17A-producing cells (B) by intracellular staining. Frequency of single, double, and triple cytokine-producing cells in CD4 and CD8 T cells (A, B) or in PD-1 (–) or (+) CD4 T cells (C, D) before and after vaccination are depicted.

concentration of IFN-γ. The assay was highly reproducible and sensitive compared with intracytoplasmic staining or an ELISPOT assay. In the ELISPOT assay, it should be noted that the response resulting from a minor CD4 T-cell population contaminated in a purified CD8 population can sometimes make the interpretation of results difficult, especially after stimulation with longer peptides. Induction of CD4 T-cell responses was observed in all patients vaccinated and their increase during the vaccination period was consistent with the results in our previous study on a cancer vaccine with NY-ESO-1 protein and NY-ESO-1f peptide. For induction of NY-ESO-1-specific CD4 T cells, overlapping peptides appeared to be much more efficient than protein.[10] In contrast, induction of CD8 T-cell responses was similarly observed in all patients vaccinated with NY-ESO-1 OLP. However, the responses were relatively weak and fluctuated a lot during vaccination compared with the CD4 T-cell response. In our previous study with NY-ESO-1 protein, even a patient with a tumor that almost completely disappeared showed only a marginal CD8 T-cell response in PBMCs.[25,29] We observed some Foxp3$^+$ CD4 T cells infiltrating tumor tissue from this patient. In this study, we detected Foxp3$^+$ CD4 T cells in PBMCs from all patients analyzed during vaccination with NY-ESO-1 OLP. No increase in regulatory T cells was observed during vaccination in PBMCs. It is possible that those cells suppress CD8 T-cell responses in tumor microenvironments. Future studies combining cancer vaccines and inhibition of regulatory T-cell function will be intriguing.

TLRs are expressed either on the cell surface (TLR1, 2, 4, 5, 6, and 10) or on the membrane of intracellular organelles such as endosomes (TLR3, 7, 8, and 9). The bacterial CpG motif is the ligand for TLR9. Viral single-stranded RNAs are ligands for TLR7 and 8, and double-stranded viral RNAs such as PolyI:C are ligands of TLR3. Molecular patterns of extracellular microbes are recognized by the cell surface-expressed TLR1, 2, 4, 5, 6, and 10.[30] OK-432 is a ligand for TLR2, 4, and/or 9.[9] Triggering of TLR signaling leads to the activation of nuclear factor κB, activating protein-1, and/or IRF3, which results in secretion of type 1 IFNs and/or proinflammatory cytokines such as IL-1β, IL-12, and TNF-α.[17] We used OK-432 as an immunemodulator for the NY-ESO-1f peptide, CHP-NY-ESO-1 and CHP-HER2 cancer vaccine and observed efficient induction of tumor antigen-specific immune responses.[9,18,31] Sabbatini et al[32] used an NY-ESO-1 OLP vaccine with or without poly-ICLC in ovarian cancer patients. They observed that an efficient antibody response against NY-ESO-1 OLP was

induced with its use in combination with poly-ICLC and Montanide, but not without poly-ICLC. In this regard, OK-432 could be a feasible immune-modulator for a cancer vaccine with tumor antigens.

Two out of 9 vaccinated patients showed SD in the clinical response. Although strong induction of the NY-ESO-1 antibody against both the peptides used for the vaccine and the NY-ESO-1 protein was observed in these patients, there is no convincing evidence as to whether the strong antibody response is related to the clinical response.

Recently, it was shown that antibodies against immune checkpoint molecules had a significant antitumor effect, and a combination of different antibodies augmented this effect. With the proviso of control of immunosuppression in the tumor microenvironment, the use of immunogenic vaccines will be relevant. Thus, the use of both reagents controlling immunosuppression and immunogenic vaccines will be important in the future. We are planning combination therapies of immune checkpoint modulators with NY-ESO-1 vaccine.

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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

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Production of NY-ESO-1 peptide/DRB1*08:03 tetramers and ex vivo detection of CD4 T-cell responses in vaccinated cancer patients

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ABSTRACT

We established CD4 T-cell clones, Mz-1B7, and Ue-21, which recognized the NY-ESO-1 121–138 peptide from peripheral blood mononuclear cells (PBMCs) of an esophageal cancer patient, E-2, immunized with an NY-ESO-1 protein and determined the NY-ESO-1 minimal epitopes. Minimal peptides recognized by Mz-1B7 and Ue-21 were NY-ESO-1 125–134 and 124–134, respectively, both in restriction to DRB1*08:03. Using a longer peptide, 122–135, and five other related peptides, including either of the minimal epitopes recognized by the CD4 T-cell clones, we investigated the free peptide/DR recognition on autologous EBV-B cells as APC and peptide/DR tetramer binding. The results showed a discrepancy between them. The tetramers with several peptides recognized by either Mz-1B7 or the Ue-21 CD4 T-cell clone did not bind to the respective clone. On the other hand, unexpected binding of the tetramer with the peptide not recognized by CD4 T-cells was observed. The clone Mz-1B7 did not recognize the free peptide 122–135 on APC, but the peptide 122–135/DRB1*08:03 tetramer bound to the TCR on those cells. The failure of tetramer production and the unexpected tetramer binding could be due to a subtly modified structure of the peptide/DR tetramer from the structure of the free peptide/DR molecule. We also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from patients after NY-ESO-1 vaccination in immunomonitoring.

1. Introduction

To analyze T-cell immunomonitoring after vaccination, peptide/MHC tetramers have become widely used [1]. Peptide/MHC tetramers identified and visualized antigen specific T-cells. MHC class I tetramers were originally developed by Altman and Davis [2], and used for various antigens including those of viral or tumor origin [3,4]. However, MHC class II tetramers have been used in only a few studies because of the difficulty in preparation [5]. The soluble form of MHC class II molecules is necessary to produce tetramers. However, production of such molecules using extracellular domains of MHC class II α and β chains is generally difficult because of a lack of assembly or aggregation [6]. These findings indicate the necessity of transmembrane regions for the proper assembly of the molecules. Kalandadze et al. [7] found that replacement of the hydrophobic transmembrane regions by the Fos and Jun leucine zipper dimerization motifs resulted in the assembly and secretion of DRαβ heterodimers in yeast. Novak et al. [8] developed MHC class II tetramers using DR molecules incorporating leucin zipper motifs to stabilize the DRα and β heterodimer. The procedure has been widely used, but successful production of MHC class II tetramers is still limited [9–13].

We recently analyzed CD4 T-cell responses against NY-ESO-1 in PBMCs from patients who were vaccinated with a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1) in our clinical trial and determined three novel NY-ESO-1 CD4 T-cell epitopes: NY-ESO-1 87–100 bound to DRB1*09:01, NY-ESO-1 95–107 bound to DQB1*04:01, and NY-ESO-1 124–134 bound to DRB1*08:03 [14]. CD4 T-cells that

Abbreviations: APC, antigen-presenting cell; CHP-NY-ESO-1, complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 whole protein; Fmoc, *N*-(9-fluorenyl)-methoxycarbonyl; HD, healthy donor; MFI, mean fluorescence intensity; OLP, overlapping peptide; PBMC, peripheral blood mononuclear cell.

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recognized these epitope peptides also recognized EBV-B cells or DC that were treated with recombinant NY-ESO-1 protein or an NY-ESO-1-expressing tumor cell lysate, suggesting that the epitope peptides are naturally processed. These CD4 T-cells had a cytokine profile with Th1 characteristics.

In this study, we showed that tetramers with several peptides recognized by the CD4 T-cell clones did not bind to the same clones. On the other hand, unexpected binding of the tetramer with a peptide not recognized by CD4 T-cells was observed. The failure of tetramer production and the unexpected tetramer binding could be due to a subtly modified structure of the peptide/DR tetramer from the structure of the free peptide/DR molecule. We also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from patients after NY-ESO-1 vaccination in immunomonitoring.

2. Materials and methods

2.1. Patients and blood samples

Peripheral blood samples were drawn from esophageal cancer patients E-1 and E-2, and a prostate cancer patient P-3, who were vaccinated with CHP-NY-ESO-1, and a lung cancer patient TK-OLP-01, who was vaccinated with NY-ESO-1 OLP in our clinical trials [15,16] after obtaining written informed consent. PBMCs were isolated by density gradient centrifugation using Histopaque 1077 (Sigma–Aldrich, St. Louis, MO). CD4 T-cells and $CD19^+$ cells were purified from PBMCs using CD4 and CD19 microbeads, respectively, using a large scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). The cells were stored in liquid N_2 until use. HLA typing was done using PBMCs with a sequence-specific oligo-nucleotide probe and sequence-specific priming of genomic DNA using standard procedures. Patient E-2 was found to possess homozygous alleles.

2.2. Peptides

Peptides were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on a Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University (Okayama, Japan).

2.3. Cell lines

E-2 bulk CD4 T-cells were stimulated in vitro twice as described previously [14]. Clones were then established by limiting dilution. EBV-B cells were generated from $CD19^+$ peripheral blood B cells using the culture supernatant from EBV-producing B95-8 cells.

2.4. Generation of HLA-DRB1*08:03 tetramers

HLA-DR tetramers were prepared as described previously [5]. The cDNA coding for the extracellular domains of the HLA-DRα chain was inserted by fusion PCR in a basic leucine zipper and His tag. The HLA-DRβ chain was fused with an acidic leucine zipper and the BirA substrate peptide for BirA enzyme-dependent biotinylation. The HLA-DRα and HLA-DRβ chimeric cDNA were cloned into the pcDNA3.1 vector, respectively. The expression vectors containing the HLA-DRα and HLA-DRβ chains were co-transfected into CHO cells.

2.5. ELISA

Supernatants (100 μl) from cultures of CD4 T-cells (5×10^3) stimulated for 18 h with autologous EBV-B cells (5×10^3) pre-pulsed for 30 min with peptide in a 96-well round bottomed culture plate, or with solid-phase peptide/HLA-DRB1*08:03 tetramers in a 96-well flat bottomed culture plate, were collected and the amounts of IFNγ were estimated by sandwich ELISA [14]. TNFα, IL-4, IL-10 and IL-17A in the culture supernatants were estimated by DuoSet Sandwich ELISAs (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.6. Flow cytometry

FITC-conjugated anti-human TCRαβ mAb (BD), PerCP Cy5.5-conjugated anti-human CD3 mAb and APC-conjugated anti-human CD4 mAb (eBioscience, San Diego, CA) were used for T-cell surface staining. The stained cells were detected by FACS Canto II (BD). Flow cytometry results were analyzed with FlowJo (Tree Star, Ashland, OR).

2.7. Tetramer staining

CD4 T-cells were incubated with tetramers for 1 h at 37 °C in a 5% CO_2 atmosphere. FITC-conjugated anti-human CD4 mAb (Miltenyi Biotec) was added at the end of tetramer staining and incubated for an additional 20 min at 4 °C.

2.8. IFNγ capture assay

The method has been described previously [14].

2.9. TCR Vβ and CDR3 sequence analysis

For TCR Vβ analysis, the IOTest Beta Mark kit (Beckman Coulter, Brea, CA) was used. The CDR3 sequence was determined by PCR as described previously [17].

3. Results

3.1. Determination of NY-ESO-1 minimal epitopes recognized by CD4 T-cell clones Mz-1B7 and Ue-21 established from PBMCs of an esophageal cancer patient E-2 immunized with CHP-NY-ESO-1

We established CD4 T-cell clones from PBMCs of an esophageal cancer patient E-2 immunized with CHP-NY-ESO-1 which recognized the 18-mer NY-ESO-1 121–138 peptide. The CD4 T-cell clones Mz-1B7 and Ue-21 produced IFNγ, TNFα, but not IL-4, IL-10 or IL-17A (Supplementary Fig. 1), indicating that they have Th1 characteristics. We determined restriction molecules by antibody blocking and minimal epitopes using various N- and C-termini truncated peptides. Assays were done by ELISA examining IFNγ in the culture supernatant from responding T-cells using autologous EBV-B cells as antigen-presenting cells (APC). As shown in Fig. 1A, recognition of the 18-mer NY-ESO-1 121–138 by CD4 T-cell clones Mz-1B7 and Ue-21 was inhibited by addition of anti-HLA-DR mAb, but not anti-HLA-DQ mAb. Since patient E-2 possessed homozygous haplotypes (DRB1*08:03, DQA1*01:03, DQB1*06:01, DPB1*05:01) according to genetic analysis (see Section 2), the two clones Mz-1B7 and Ue-21 recognized the NY-ESO-1 peptide 121–138 in restriction to DRB1*08:03.

We then investigated recognition of various N- and C-termini truncated peptides and found that a core peptide region recognized by either clone Mz-1B7 or clone Ue-21 was made up of amino acids 125–134 (Fig. 1B). Further analysis revealed that a minimal peptide recognized by clone Mz-1B7 was peptide 125–134 (10-mer) and that recognized by clone Ue-21 was peptide 124–134 (11-mer) (Fig. 1C). Thus, clones Mz-1B7 and Ue-21 recognized