Frequency of Myeloid Dendritic Cells Can Predict the Efficacy of Wilms' Tumor 1 Peptide Vaccination

SATOSHI OHNO[1,2,3*], FUMIHIDE TAKANO[4*], YASUYUKI OHTA[4], SATORU KYO[3], SUBARU MYOJO[3], SATOSHI DOHI[3], HARUO SUGIYAMA[5], TOMIHISA OHTA[4] and MASAKI INOUE[3]

[1]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan;*
[2]*Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan;*
[3]*Department of Obstetrics and Gynaecology, Kanazawa University, Graduate School of Medical Science, Ishikawa, Japan;*
[4]*Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy and Pharmaceutical Sciences, Kanazawa University, Ishikawa, Japan;*
[5]*Department of Functional Diagnostic Science, Osaka University, Graduate School of Medicine, Osaka, Japan*

Abstract. *Background: The object of this study was to investigate the clinical predictive capability of peripheral myeloid dendritic cells (DCs) in Wilms' tumor 1 (WT1) vaccine therapy for patients with gynaecological cancer. Patients and Methods: Six patients with WT1/human leukocyte antigen (HLA)-A*2402-positive gynaecological cancer were included in this study. The patients received intradermal injections of a modified 9-mer WT1 peptide every week for 12 weeks. Peripheral blood samples were obtained at 0, 4, 8 and 12 weeks after the initial vaccination. Circulating DCs were detected by flow cytometry. Results: The frequencies of $CD14^+CD16^+CD33^+CD85^+$ myeloid DCs were significantly higher in the therapeutically effective group than in therapeutically inert group ($p<0.05$). Conclusion: These results suggested that myeloid DCs, which should be associated with inducing cytotoxic T-cells, provided additional prognostic information in the use of cancer peptide vaccine.*

Recent advances in tumor immunology have resulted in the identification of a large number of tumor-associated antigens that could be used for cancer immunotherapy, since their epitopes associated with human leukocyte antigen (HLA) class I molecules were recognized by cytotoxic T lymphocytes. One such identified tumor-associated antigens is the product of the Wilms' tumor gene, *WT1* (1, 2).

WT1 was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor (3, 4). This gene encodes a zinc finger transcription factor and plays important roles in cell growth and differentiation (5, 6). Although the *WT1* gene was categorized at first as a tumor suppressor gene, it has recently been demonstrated that the wild-type *WT1* gene performed an oncogenic rather than a tumor-suppressor function in many kinds of malignancies (7). The *WT1* gene is highly expressed in various types of cancer, including gynaecological cancer (8, 9).

We have performed a phase I clinical trial to examine the safety of a WT1-based vaccine, as well as the clinical and immunological response of patients with a variety of cancer types, including leukemia, lung cancer and breast cancer (10). The WT1 peptide vaccine emulsified with Montanide ISA51 adjuvant and administered at a dosage of 0.3, 1.0, or 3.0 mg at 2-week intervals was safe for patients, other than for those with myelodysplastic syndromes. Furthermore, it has been confirmed that the potential toxicities of the weekly WT1 vaccination treatment schedule (3.0 mg per body) with the same adjuvant were also acceptable (11). In the past, clinical response to WT1 peptide-based immunotherapy in phase II trials with the weekly WT1 vaccinations has been reported for renal cell carcinoma (12), multiple myeloma (13), glioblastoma multiforme (14) and gynaecologic malignancy (15).

In clinical studies, the identification of predictive factor of treatment is extremely important for the improvement of clinical response. The most representative factor that predicts the outcome of cancer peptide vaccine therapy is the expansion and/or induction of tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs). Klebanoff *et al*. reported that not only the induction of effector CTLs but also maintenance of memory CTLs are required for ideal antitumor immune response in tumor-bearing patients (16).

*Both authors contributed equally to this work.

Correspondence to: Satoshi Ohno, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, 513, Wasedatsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan. Tel: +81 352721206, Fax: +81 352721208, e-mail: satoshi.ohno55@gmail.com

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Moreover, Fujiki *et al*. confirmed that occurrence of an antigen-specific helper T-cell (Th) response could predict good clinical response of CTL epitope vaccination (17). In animal models, Klages *et al*. showed that depletion of FOXP3 (+) regulatory T-cells (Tregs) had the potential to evoke efficient antitumor responses (18).

Dendritic cells (DCs) are immune cells forming part of the mammalian immune system. Their main function is to process antigen material and present it on their surface to other cells (*e.g*. Th and CTLs) of the immune system. To date, however, the role of DCs, which should be associated with inducing CTL in cancer immunotherapy, remains unclear.

In the present study, we investigated the clinical predictive capability of peripheral myeloid DCs in WT1 vaccine therapy for patients with gynaecological cancer.

Patients and Methods

The WT1 peptide. The immunization consisted of an HLA-A*2402-restricted, modified 9-mer WT1 peptide (amino acids 235-243 CYTWNQMNL), in which Y was substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. This variant induces stronger cytotoxic activity than the natural peptide (19). The WT1 peptide [Good Manufacturing Practice (GMP) grade] was purchased from Multiple Peptide Systems (San Diego, CA, USA) as lyophilized peptides.

Trial protocol. The entry criteria were as follows: 16-79 years of age; expression of WT1 in the cancer cells determined by immunohistochemical analysis; HLA-A*2402-positivity; estimated survival of more than 3 months; performance status 0-1; no severe organ function impairment and the written informed consent of the patient. At least 4 weeks prior to immunotherapy, the patients were free from antitumor treatments such as surgery, chemotherapy and radiation. Patients with brain metastasis were excluded. The protocol was approved by the Institutional Review Board and the Ethical Committee at Kanazawa University.

Vaccination. The patients received intradermal injections of 3.0 mg of HLA-A*2402-restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant (SEPPIC S.A., Paris, France). The WT1 vaccinations were scheduled to be given weekly for 12 consecutive weeks.

Preparation of peripheral blood mononuclear cells (PBMCs). Peripheral blood samples from individual patients enrolled in the clinical trial were collected at 0, 4, 8 and 12 weeks. Collected blood in the vacutainer tube was transferred to a 50 ml conical tube (BD Falcon, Franklin Lakes, NJ, USA), diluted to a volume of 30 ml with HBSS (Gibco Invitrogen Corporation, Grand Island, NY, USA), and underlayed with 10 ml of Ficoll-Paque PLUS™ (GE Healthcare UK Ltd.). The 50 ml tubes were centrifuged at 400 × *g* for 30 min, after which the PBMCs were collected at the interface layer. PBMCs were collected by gently inverting the collection tube several times and drawing off the PBMCs containing plasma with a pipette. PBMCs from both sets of tubes were washed twice with HBSS and counted for recovery and viability using 0.4% Trypan Blue (Sigma, St. Louis, MO, USA).

Flow cytometric analysis. Flow cytometric analysis of stained DCs in PBMCs was performed on a flow cytometer (FACScalibar™; Becton Dickinson, San Diego, CA, USA). An acquisition gate was established based on a forward scatter and side scatter parameter that included only white blood cells, except for dead cells and debris as illustrated in Figure 1A.

Immunophenotyping of circulating DCs. To evaluate the phenotype of DCs in PBMCs isolated from the vaccinated patients, we used a panel of fluorescein isothiocyanate (FITC)- or phycoerytrin-conjugated monoclonal antibodies: mouse anti-human CD14/CD16 and mouse anti-CD33/CD85k, as well as FITC- or PE-conjugated isotype control antibodies (IgG2a and IgG1; Beckman Coulter, Hialeah, FL, USA). PBMCs (1×10^6 cells) were washed twice with ice-cold phosphate-buffered saline (PBS), and the resultant cells were counted and resuspended in PBS. Cells were stained directly with fluorochrome conjugated with specific antibodies or isotype control antibodies. After 30 min of incubation at 4˚C in the dark, the cells were washed and resuspended in the same buffer. The DC population in the PBMCs was analyzed using flow cytometry as described below.

Data were acquired using CellQuest software (Becton Dickinson). Between 10,000 and 20,000 events were acquired per sample. All data are indicated as quadrant analysis in the PBMC gate, and were representative, being derived from triplicate analyses.

Evaluation of clinical response. After the WT1 vaccine was administered 12 times, the antitumor effect of the treatment was assessed by determining the response of the target lesions on computed tomographic images. The tumor size was analyzed according to Response Evaluation Criteria in Solid Tumors (RECIST) (20), with results reported as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD).

The internationally approved RECIST guideline was originally developed for the evaluation of chemotherapy. However, peptide immunotherapy, especially if peptide is administered alone without adjuvant, may not lead to such drastic tumor regression as in chemotherapy. It is probable that some cancer patients treated with cancer vaccines can survive long-term without remarkable tumor regression (12-15). Their tumors could be stabilized or could regress following a temporary increase in size after vaccination since, in general, peptide-based immunotherapy does not act as quickly as chemotherapy due to the time needed to induce lymphoid activation. For this reason, it might be allowable to modify the RECIST guideline according to peptide-based immunotherapy. In this study, an assessment strategy in which the baseline of the sum of the longest diameters of the target lesions was shifted to 1 month after the initial WT1 vaccination was defined as 'modified RECIST'.

Statistical analysis. Differences between test groups were analyzed using Student's *t*-test. Calculations were performed using the statistical software package StatVeiw (Abacus Concepts, Berkeley, CA, USA).

Results

Patient characteristics. During the trial period, 6 patients were evaluated for frequencies of DCs at 0, 4, 8 and 12 weeks. The mean age of the 6 enrolled patients was 55.7 years (range 43-64 years). A summary of the patient's characteristics and response to WT1 immunotherapy is shown in Table I.

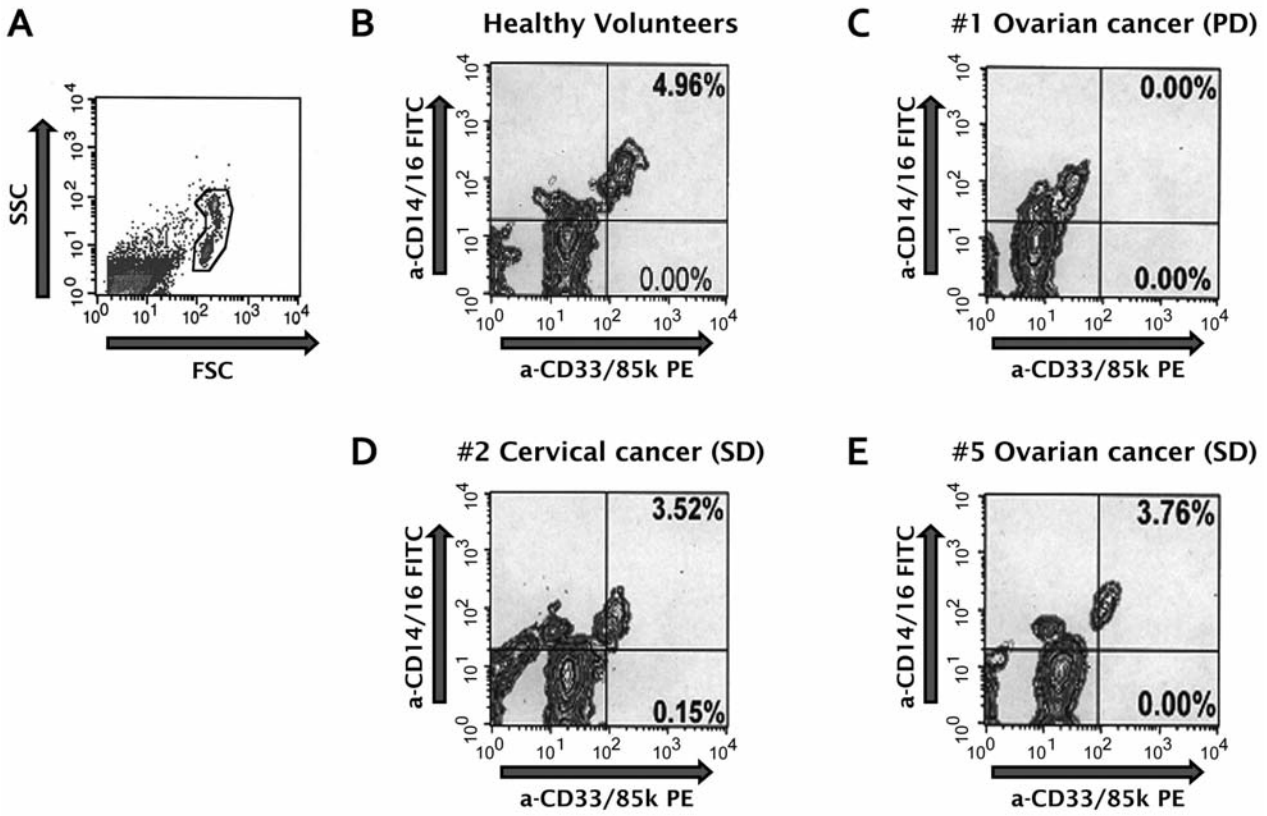

Figure 1. *Flow cytometric profiles of DCs in peripheral blood mononuclear cells (PBMCs). PBMCs collected from a healthy volunteer or cancer patients were stained with lineage-specific FITC- or PE-conjugated markers including anti-CD14-, anti-CD16-, anti-CD33- and anti-CD85 monoclonal antibodies. Gates to include viable PBMCs for analysis were set by forward and side scatter to delineate DCs. A: Dot-plot analysis of unlabeled PBMCs; B: quadrant analysis and population of lineage-specific markers for myeloid DC positive in PBMCs from a healthy volunteer; C: quadrant analysis and population of lineage-specific markers for myeloid DCs in PBMCs from a typical cancer patient treated with WT1 in the group with progressive disease; D and E: quadrant analysis and population of lineage-specific markers for mature DCs in PBMCs from two typical cancer patients-treated with WT1 in the group with stable disease.*

Analysis of DCs in cancer patients with WT1 vaccination. We evaluated the mature myeloid DCs ($CD14^+$-, $CD16^+$-, $CD33^+$- and $CD85^+$-positive cells) in PBMCs collected from healthy volunteers and the cancer patients with vaccination. As illustrated in Figure 1B, the population of myeloid DCs in PBMCs of healthy volunteers composed 4.96%. In contrast, the frequencies of myeloid DC in PBMCs from cancer patients divided into PD or SD groups were 0.0% (Figure 1C; in PD), 3.52% (Figure 1D; first case in SD) and 3.76% (Figure 1E; second case in SD), respectively.

Each population of peripheral myeloid DCs in the 6 cancer patients was compared according to the clinical response. The frequency of $CD14^+CD16^+CD33^+CD85^+$ PBMCs was significantly higher (p=0.0374) in the SD (3.206±0.543%) group than in PD group (2.026±1.443%) (Figure 2A). A significant difference (p=0.0027) between SD (3.182±0.520%) and PD (1.657±1.472%) groups was also observed using the 'modified RECIST' assessment (Figure 2B).

Table I. *Patient characteristics.*

No.	Age (years)	Gender	Diagnosis	RECIST	Modified RECIST
1	62	F	Ovarian cancer	PD	PD
2	57	F	Cervical cancer	SD	SD
3	43	F	Cervical cancer	PD	SD
4	55	F	Endometrial cancer	PD	PD
5	53	F	Ovarian cancer	SD	SD
6	64	F	Ovarian cancer	PD	PD

PD: Progressive disease; SD: stable disease.

Discussion

The present study demonstrated that the percentage of circulating myeloid DCs in patients with therapeutical effectiveness of cancer peptide vaccination were significantly

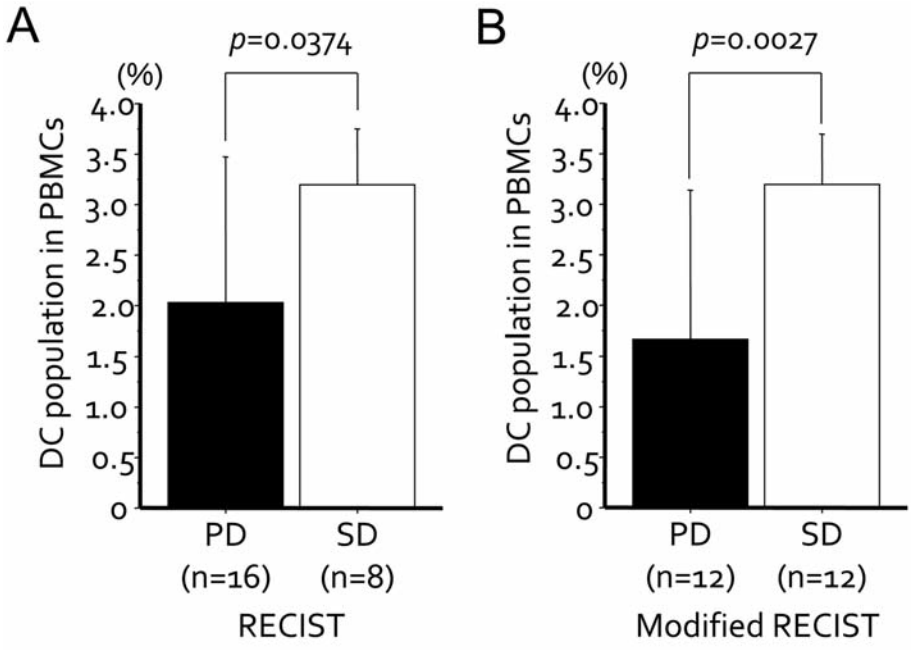

Figure 2. *Validation for circulating myeloid DCs in peripheral blood. Among the 6 gynaecological cancer patients with WT1 peptide vaccination, those in the progressive disease (PD) group (black histograms) showed a depletion of the myeloid DC subset, which was statistically significant compared with DC frequencies in those with stable disease (SD) (open histograms).*

higher than in those with therapeutical inertness. Recent studies point to a numerical decrease and sometimes even functional impairment of circulating DC subsets in various pathologies. In hematopoietic cancer patients, DC counts may be significantly reduced in lymphoid or myeloid leukemia (21, 22). A similar observation was made for certain solid cancers (23). Furthermore, numbers of circulating DCs are reduced in patients with metastatic cancer as compared to those with localized cancer (24). These findings suggest that DC deficiency may play a role in inducing cancer-related immunosuppression.

Moreover, chemotherapeutic techniques have a range of side-effects that depend on the type of medication used. The most common medications mainly affect the fast-dividing cells of the body, such as blood cells. Virtually all chemotherapeutic regimens can cause depression of the immune system, often by inactivating the bone marrow and leading to a decrease of white blood cells, red blood cells and platelets. In very severe myelosuppression, which occurs in some regimens, almost all the bone marrow stem cells (cells that produce white and red blood cells) are destroyed. Bone marrow has recently been shown to be an important site for T-cell priming and reactivation, generation of T-cell memory and recruitment of large amounts of circulating memory T-cells and antigen-loaded DCs (25-29). Therefore, myelosuppression associated with chemotherapy may block CTL activation in cancer patients.

In patients with advanced cancer, the basal metabolic rate declines and cachexia occurs. The pathophysiological pathway of cachexia is thought to be secondary to stimulation by enhanced levels of pro-inflammatory cytokines. Elevation of tumor necrosis factor-α and other plasma cytokines has been demonstrated in many conditions associated with cachexia (30). Cachexia is often associated with breakdowns in the host immune system and may result in reduced therapeutic response of peptide vaccine.

In tumor immunosurveillance, it is generally thought that $CD8^+$ CTLs are the main effector cells because they can effectively expand and kill malignant cells. Therefore, the most common approaches to combat tumors have centered on the induction of TAA-specific CTLs. In this study, the activity of WT1 peptide alone was examined and adjuvant that would activate DCs with subsequent induction of CTLs was not included. To enhance the therapeutic efficacy of cancer peptide vaccination, the use of a more suitable adjuvant, such as bacillus Calmette-Guerin cell-wall skeleton (31), granulocyte-macrophage colony-stimulating factor (32, 33), CpG (34), interferon-α (35) and interleukin-2 (36) should be allowed.

In conclusion, the demonstration of a diminished percentage of DCs in peripheral blood might represent a new interesting biological marker predicting a poor prognosis in patients treated with WT1 peptide vaccination. The reduced DC numbers may contribute to reduced therapeutic response and thus restoration of DCs may be a goal for cancer

peptide-based immunotherapy. The present study gives us an indication of enhancement of clinical response in WT1 protein-targeted immunotherapy.

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