Table III. *Change of serum tumor marker level by the vaccine therapy (phase I/II study).*

Evaluation[1]	Number of patients
Continuous decrease	0 (0%)
Transient decrease[2]	11 (73%)
No change	1 (7%)
Continuous increase	2 (13%)
Inability	1 (7%)

[1]A representative tumor marker was measured by ELISA every month after the vaccine therapy.
[2]Decrease lasted more than one month

Table IV. *Clinical outcome (phase I/II study).*

Response	Number of patients
CR	0 (0%)
PR	0 (0%)
SD	6 (40%)
Long SD[1]	8 (53%)
PD	1 (7%)

[1]SD continuing for more than 6 months.

Table V. *Immune response (phase I/II study).*

	Number of patients	
	DTH reaction (+)	DTH reaction (-)
ELISPOT assay (+)	9	0
ELISPOT assay (-)	0	4

The DTH reaction and ELISPOT assay were assessed at 3 months after the therapy.

reactions occurred. Low-grade fever, recovering within 24 h after the DC vaccine, was found in 8 patients. A transient increase of eosinophils was found on the first day after the third DC vaccine therapy in 1 patient, but recovered to the normal level within 3 days without treatment.

A Kaplan-Meier survival analysis indicated the presence of long survival patients who lived for more than 6 months. Based on the laboratory data common to these long survivors, the eligibility criteria for this tumor-pulsed DC vaccine therapy was determined as described in Materials and Methods. According to this eligibility criteria, 19 patients were divided into 6 suited patients (responders) and 13 unsuited patients (non-responders). The 6 responders showed a longer overall survival compared with the 13 non-responders ($p=0.0018$, Figure 1).

Phase I/II study. Using the above eligibility criteria, a phase I/II trial was again performed with cancer patients with multiple metastases. Fifteen patients, including 4 large intestinal cancer, 4 gastric cancer, 2 pancreatic cancer, 2 breast cancer, 1 lung cancer, 1 thymic cancer and 1 cancer of unknown origin were entered into this trial. Eleven patients had received prior second-line chemotherapy and 11 patients had received prior surgery. In addition, 3 patients had received prior radiotherapy. All of these patients were evaluated as PD at the time of entering the study (Table II). No particular adverse reactions, including autoimmune reactions, were found during this observation period (4-22 months). These 15 patients again showed a longer overall survival compared with the 13 non-responders who were treated in the above phase I study ($p<0.0001$, Figure 2). The 50% survival time of the 13 non-responders and the 15 patients was 3.0 months and 10.0 months, respectively. The serum levels of tumor markers were measurable in all the 15 patients and estimable in 14 of the 15 patients (Table III). In 11 patients (73%) there was a continued decrease for at least 1 month, while 2 patients (13%) and 1 patient (7%) showed a continuous increase and no significant change, respectively. Although neither CR nor PR was found, it is noteworthy that 14 patients showed SD and that 8 of the 14 SD patients maintained this SD for more than 6 months, *i.e.*, long SD (Table IV).

The patients' immune responses against tumor-pulsed DCs were evaluated by both the DTH skin reaction and IFN-γ ELISPOT assay before and after the DC vaccination. Both the DTH reaction and ELISPOT assay were assessed at 3 months after the tumor-pulsed DC vaccine therapy in 13 of the 15 patients (Table V). Nine of the 13 patients became positive for both the DTH reaction and ELISPOT assay, while the remaining 4 patients were negative for both. In 3 of the 4 negative patients, however, the DTH reaction became positive within 6 months after the therapy.

Most DCs induced from each patient were shown to develop high levels of MHC class II and costimulatory molecules CD80 and CD86, and showed the absence of CD14 (data not shown). However, the expression levels of these molecules were significantly low compared to the DCs induced from healthy volunteers' PBMCs, as previously reported (16).

Discussion

The initial purpose of this clinical trial was to evaluate the feasibility and toxicity of tumor-pulsed DC vaccine therapy against far-advanced cancer patients. Low-grade fever or eosinophilia were observed in only limited cases throughout the phase I trial and the phase I/II trial (Tables I and II). These adverse reactions did not require any particular treatment. As a result, the vaccine therapy did not need to be cancelled for adverse reactions. In the phase I/II trial, the maximum duration of treatment was in a patient who received 36 DC vaccinations in 22 months during the observation period. After the thirtieth vaccine therapy, edematous erythema without itching appeared at the injection site immediately after the intradermal injection of tumor-pulsed DCs for the DTH skin test. Erythema was accompanied with an increased serum IgE and was macroscopically similar to a type I allergic reaction. The erythema and IgE elevation recovered to normal within one hour and on the next day, respectively. Rheumatoid factor, anti-nuclear antibody and anti-thyroglobulin antibody in the sera were all negative throughout this trial period.

The second purpose of the study was to assess if tumor-pulsed DC vaccine therapy can induce some immune reactions against autologous tumor cells in patients. In order to evaluate the induction ability of tumor antigen-specific cytotoxic lymphocytes (CTLs) of this vaccine therapy, both the DHT skin test and IFN-γ ELISPOT assay were used as surrogate markers (17). In this study, the positive rate of the DTH reaction was significantly higher in responders than non-responders (data not shown). In this phase I/II study, both a positive DTH reaction and increased ELISPOT reaction were markedly induced by the vaccine therapy. Interestingly, the DTH reaction completely harmonized with the ELISPOT reaction (Table V). The high induction rate of positive DTH reaction and increased ELISPOT reaction indicates a potent CTL induction ability of this vaccine therapy.

The third purpose of the study was to find out the advantage of autologous tumor cells as an antigen source. As described above, necrotic tumor cells were used as the antigen source for induction of multiple CTLs against both known TAAs and unknown TAAs (11). For this purpose, the patient's HLA-A phenotype-binding synthetic peptides were first prepared as described in Materials and Methods. Next, PBMCs obtained from a patient in whom the ELISPOT reaction became positive were cultured together with known TAA peptide-pulsed DCs. If T cells which react to the TAA peptide exist in the PBMCs, they produce IFN-γ. For example, in this study, PBMCs from a patient treated by CEA, MAGE-1 and HER-2/neu-expressing tumor cells produced IFN-γ by co-culture not only with tumor-pulsed DCs, but also these peptides-pulsed DCs (data not shown). This data indicated that the tumor-pulsed DC vaccine therapy can elicit specific T cell responses against multiple TAAs.

Finally, we examined whether the tumor-pulsed DC vaccine therapy can prolong the survival time. Both the phase I and I/II trials showed the possibility that this therapy can prolong the survival time of far-advanced cancer patients (Figures 1 and 2). Before the efficacy for prognosis is evaluated, however, there are many problems that should be solved. For example, in the current clinical trials, the patient numbers were low and many types of carcinoma were targeted. Therefore, a phase II study is now under way to assess if this vaccine therapy can prolong the survival time.

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Purification, Characterization and Biological Significance of Tumor-derived Exosomes

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Abstract. *Exosomes are nanovesicles that are released into the extracellular environment during the fusion of multivesicular bodies with the plasma membrane. Exosomes released from dendritic cells, dexosomes, have several biological functions, for example as immunostimulants. Some tumor cells also secrete exosomes (Tu-exosomes). Although experimental data obtained with the use of dexosomes suggest a biological function of Tu-exosomes, this still remains poorly understood. To examine the function of Tu-exosomes, we established a method for collecting highly purified Tu-exosomes, using paramagnetic beads coated with antibodies against tumor-specific proteins such as HER2/neu. With these antibody-coated beads (Ab-beads), it was possible to collect HER2-expressing Tu-exosomes of high purity. Tu-exosomes were also collected from malignant ascites, which contain exosomes secreted from various types of cells such as tumor cells, lymphoid cells and mesothelial cells. The isolation of Tu-exosomes was confirmed by FACS analysis. With regard to their biological functions, Tu-exosomes cultured with a human breast cancer cell line bound to the cell surface and increased tumor cell proliferation. These data indicate that Tu-exosomes may have physiological functions.*

Abbreviations: FACS, fluorescence activated cell sorting; HER2, human epidermal growth factor receptor 2; MHC, major histocompatibility complex; Mo-DCs, monocyte-derived dendritic cells; Dexosomes, exosomes derived from DC; TCR, T cell receptor; CTLs, cytotoxic T lymphocytes; PBS, phosphate-buffered saline; D_2O, deuterium oxide.

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Key Words: Exosomes, HER2/neu, dendritic cells, Herceptin (Trastuzumab), breast cancer.

Exosomes are small membrane vesicles of endocytic origin that are secreted by most cells, including some types of tumor cells (1-7). Exosomes can be identified morphologically by electron microscopy; they have a characteristic saucer-like shape that is limited by a lipid bilayer, and they range from 30 to 100 nm in diameter (8). The presence of known cellular proteins in exosome preparations from various cellular sources has been analyzed mainly by Western blotting (9-12). The protein profiles of dexosomes have been analyzed in greatest detail (12). We reported that dexosomes prolong the survival of naïve T cells *via* an interaction between MHC class II molecules on dexosomes and TCR on naïve T cells (13). Recently, microvesicles, including exosomes derived from platelets, were found to play an important role in tumor metastasis and angiogenesis in lung cancer (14). In addition, it has been shown that some tumors also secrete exosomes-like microvesicles that contain many proteins such as MHC class I, heat-shock proteins and HER2/neu (15). These data suggest that tumor-secreted exosomes may play a role in tumor progression.

To analyze the biological function of tumor-secreted exosomes (Tu-exosomes), highly purified Tu-exosomes are required. The most common procedure for collecting exosomes from cell culture supernatants involves a series of centrifugation steps to remove dead cells and large debris, followed by a final high-speed ultracentrifugation step to pellet the exosomes (8). Recently, a good manufacturing process for harvesting relatively pure exosomes secreted by Mo-DCs was reported (10). However, it is difficult to obtain dexosomes of greater than 70% purity by this procedure. Interestingly, some tumor cells secrete exosomes expressing tumor-specific proteins such as HER2/neu (9). This unique characteristic of Tu-exosomes indicated that it might be possible to selectively isolate Tu-exosomes with beads coated with antibodies against tumor-specific proteins.

In the present study, a new technique for collecting Tu-exosomes of high purity is described. Preliminary data concerning the effect of Tu-exosomes on tumor cell proliferation is also reported.

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Materials and Methods

Reagents. Herceptin (Trastuzumab), a humanized monoclonal antibody to HER2, was purchased from Roche Pharma AG (Reinach, Switzerland).

Tumor cell lines. The human breast adenocarcinoma cell lines BT-474 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA). BT-474 cells show high overexpression of HER2, whereas MDA-MB-231 cells show low overexpression of HER2, as described previously (16). These cells were maintained as monolayer cultures in complete medium composed of RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) and 10% v/v depleted-fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO, USA). FBS was predepleted of bovine exosomes by ultracentrifugation at 100,000 x g for 16 hours at 4°C.

Isolation and purification of exosomes. Exosomes were isolated as described previously but with minor modifications (13). Two hundred and fifty-ml volumes of culture supernatant were centrifuged at 300 x g for 10 minutes and then at 1,200 x g for 10 minutes to eliminate cells and debris. The cell-free supernatants were clarified through a 0.2-μm filter (Sartorius AG, Göttingen, Germany) to reduce the number of contaminating large vesicles shed from the plasma membrane. The supernatants were ultracentrifuged at 100,000 x g for 60 minutes at 4°C in a 70.1 Ti fixedangle rotor (Beckman Coulter Inc., Fullerton, CA, USA). The pellets were resuspended in 3.6 ml PBS. The exosomes were underlaid with 600 μl of a 30% sucrose/D_2O density cushion, followed by ultracentrifation at 100,000 x g and 4°C for 60 minutes. A 700-μl volume of the cushion layer was collected and pelleted at 100,000 x g for 60 minutes. The pellets were washed twice with PBS, resuspended in 250 μl PBS and stored at –80°C. Exosomal protein was measured by the Bradford assay with the Bio-Rad Protein Assay Reagent (Bio-Rad, Hemel Hemstead, UK). A similar process was used to isolate and purify exosomes from ascites of patients.

Herceptin beads. For further purification, the exosomes were isolated with Herceptin-coated paramagnetic beads. Briefly, Protein G-coated Dynabeads (Dynal Biotech, Oslo, Norway) were washed with PBS, and 10 μl of the beads was mixed with 100 μg Herceptin and incubated overnight at 4°C on a rotating plate. The Herceptin-coated beads (referred to as Ab-beads) were washed twice with PBS on a magnetic rack to eliminate unbound or excess Herceptin. Exosomes suspended in PBS were then mixed with the Ab-beads. The mixture was incubated overnight at 4°C on a rotating plate, and the beads were collected and washed twice with PBS on a magnetic rack to eliminate unbound or excess exosomes. Exosome-bead complexes were then used for FACS and electron microscopy analyses.

FACS analysis. The exosome-bead complexes were washed with PBS containing 3% bovine serum albumin (Sigma) and 0.1% NaN_3 (Sigma) to eliminate unbound or excess exosomes. The presence of HER2 protein on exosomes attached to the Ab-beads was examined by single-color immunofluorescence labelling with FITC-conjugated anti-HER2 monoclonal antibody (Becton Dickinson, San Diego, CA, USA) or FITC-conjugated isotype-matched monoclonal antibody (Becton Dickinson). After a 30-minute incubation at 4°C, labelled exosome-bead complexes were washed twice with PBS on a magnetic rack and the fluorescence intensity was measured with a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson).

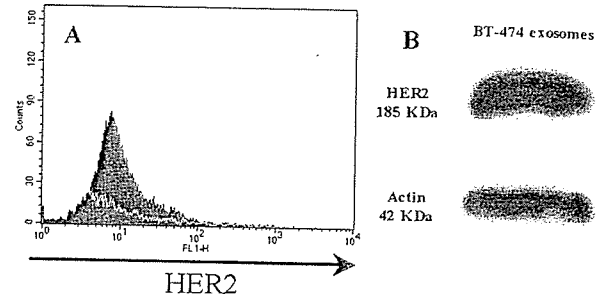


Figure 1. *FACS and Western blot analysis of HER2. A. HER2 protein in BT-474-derived exosomes. Filled histogram, Tu-exosomes with FITC-conjugated anti-HER2 antibody; open histogram, Tu-exosomes with FITC-conjugated isotype-matched antibody. B. Western blot analysis of proteins extracted from Tu-exosomes with anti-HER2 antibody and anti-actin antibody.*

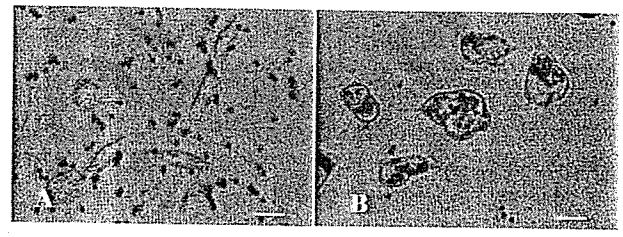


Figure 2. *Phase contrast images of Ab-beads cocultured with breast cancer cell lines. (A, MDA-MB-231 cells; B, BT-474 cells). Experiments were performed in triplicate with similar results. Bar=10 μm.*

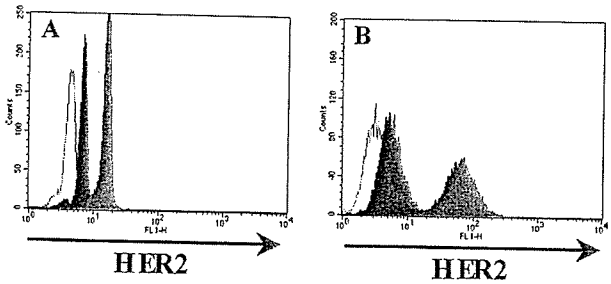


Figure 3. *FACS analysis of HER2 protein. A. Tu-exosomes, bound to Ab-beads, derived from BT-474 cells (gray) or MDA-MB-231 cells (black) with FITC-conjugated anti-HER2 antibody. Tu-exosomes, bound to Ab-beads, derived from BT-474 cells with FITC-conjugated isotype-matched antibody (open). B. BT-474 cells (gray) or MDA-MB-231 cells (black) with FITC-conjugated anti-HER2 antibody. BT-474 cells with FITC-conjugated isotype-matched antibody (open).*

Electron microscopy. The exosome-bead complexes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (CB) at pH 7.3 for 3 hours at 4°C and washed in 0.1 M CB. The complexes were resuspended and embedded in 4% agar, as described previously (17). The agar was

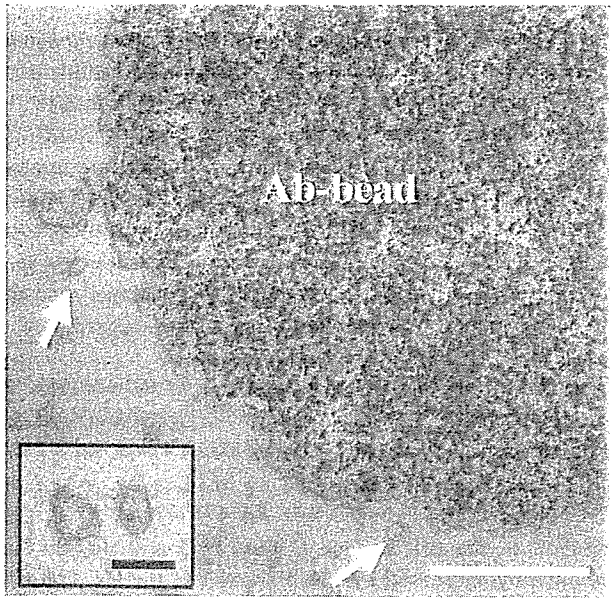


Figure 4. *Electron microscopic image of exosomes derived from BT-474 cells. Ultrathin sections of exosomes derived from BT-474 cells bound to Ab-beads were viewed with a transmission electron microscope. Small vesicles (arrows) are bound to the surface of an Ab-bead; bar=500 nm. Inset, two vesicles at higher magnification; bar=100 nm.*

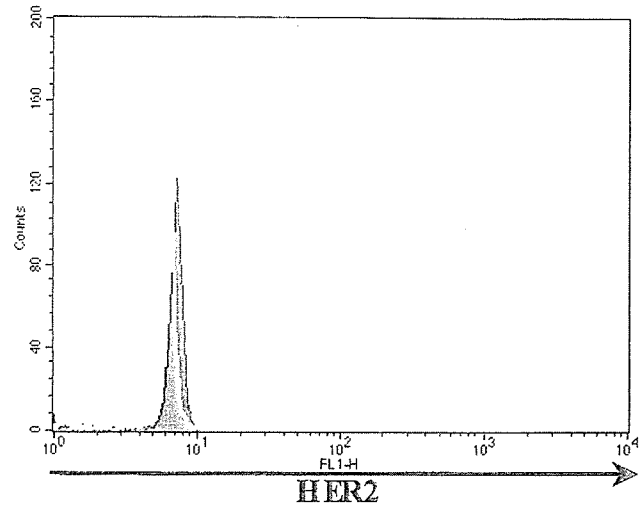
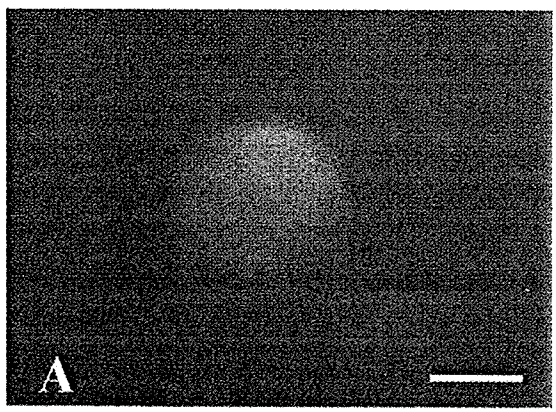


Figure 5. *FACS analysis of HER2 protein in exosomes isolated with Ab-beads from ascites of an ovarian cancer patient. Filled histogram, exosome-bead complexes with FITC-conjugated anti-HER2 antibody; open histogram, exosome-bead complexes with FITC-conjugated isotype-matched antibody.*



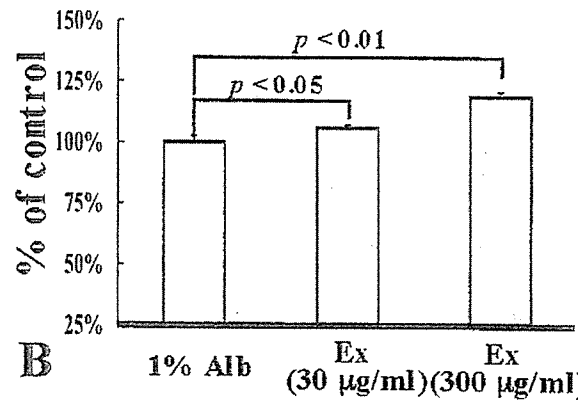


Figure 6. *Data from cell proliferation assay (MTT assay). A, Fluorescence microscopic image of exosomes derived from BT-474 cells cocultured with BT-474 cells. PKH26-labelled exosomes (Red) bound to PKH67-labelled BT-474 cell surface (Green); bar=10 μm. B, Exosomes derived from BT-474 cells increase tumor cell proliferation.*

cut into 1-mm^3 pieces, and the pieces were fixed in 1% osmium tetroxide in 0.1 M CB overnight and then washed in distilled water. The specimens were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were treated with uranyl acetate followed by lead citrate and were examined with an electron microscope (JEM-1200EX, JEOL, Tokyo, Japan).

Western blot analysis. Protein lysates of cells and exosomes (50 μg) were run on 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The blots were incubated with primary antibody to HER2 (rabbit polyclonal anti-HER2 IgG; Upstate Biotechnology Inc., Waltham, MA, USA) or actin (mouse anti-actin IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:100 at room temperature for 1 hour. The blots were incubated with secondary antibody (FITC-conjugated goat anti-rabbit or goat anti-mouse IgG; Santa Cruz Biotechnology) at a dilution of 1:200 for 1 hour at room temperature. Visualization was performed with a Molecular Imager FX System (Bio-Rad Laboratories).

3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromide assay. Cell proliferation was determined by the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazoliumbromide (MTT) assay (18). BT-474 cells (7.0x10^3

cells) were seeded onto 96-well plates and cocultured at 37°C with the indicated concentrations of exosomes in 1% Alb-RPMI. After incubation for 72 hours, cell proliferation was measured. The percent cell viability is expressed as the mean±SD for four independent wells.

Fluorescence microscopy. To examine exosomes cocultured with cancer cells by fluorescence microscopy, the exosomes were labelled at room temperature with the fluorescent membrane dyes PKH26 and PKH67 (Sigma), according to the manufacturer's instruction, and washed in PBS . PKH26-labelled exosomes (Red) and PKH67 labelled BT-474 cells (Green) were seeded onto 24-well plates and cocultured in 1% Alb-RPMI for 6 hours at 37°C, and random x400 fields were photographed with a fluorescence digital camera (VB7010, Keyence Corp., Osaka, Japan) coupled with a phase-contrast microscope (ECLIPSE TE300, Nikon, Tokyo, Japan).

Statistical analysis. The Student's *t*-test was used for statistical analyses. A *p*-value less than 0.05 was considered statistically significant.

Results

HER2 protein in Tu-exosomes. To isolate Tu-exosomes, high HER2-expressing BT-474 cells were used. The Tu-exosomes were collected from the culture supernatants by successive centrifugation steps, as described in Materials and Methods. HER2 protein in the Tu-exosomes was identified by FACS and Western blot analyses. Both the FACS (Figure 1A) and Western blot (Figure 1B) analyses confirmed the presence of HER2 protein in Tu-exosomes. FACS also revealed that Tu-exosomes contain HER2-negative components.

Isolation of HER2-containing Tu-exosomes. To selectively isolate HER2-containing Tu-exosomes, Herceptin-coated paramagnetic beads (Ab-beads) were used, as described in Materials and Methods. The Ab-beads were cocultured with low HER2-expressing MDA-MB-231 cells or high HER2-expressing BT-474 cells for 6 hours at 37°C. Phase contrast microscopy revealed that the number of Ab-beads bound to BT-474 cells (Figure 2B) was much greater than the number of Ab-beads bound to MDA-MB-231 cells (Figure 2A).

The Ab-beads were then mixed with Tu-exosomes overnight at 4 °C and collected with a magnetic rack. The bead-exosome complexes were incubated with FITC-conjugated anti-HER2 antibody or FITC-conjugated isotype-matched antibody. FACS analysis showed that almost 100% of the Ab-beads stained for HER2 and that the intensity of HER2 staining was narrow, suggesting that HER2-containing exosomes bound uniformly to Ab-beads (Figure 3A). HER2 protein in the cells themselves correlated well with the fluorescence intensity of the Tu-exosomes (Figure 3B).

Electron microscopic analysis confirmed that the exosomes bound to Ab-beads (Figure 4). At higher magnification (Figure 4, inset), bound entities showed the characteristic saucer-like morphology of exosomes ranging from 30 to 120 nm in diameter.

Isolation of HER2-containing Tu-exosomes from malignant ascites. To determine whether Ab-beads are useful as an experimental tool, we attempted to selectively isolate the HER2-containing Tu-exosomes from the ascitic fluid of a patient with advanced ovarian cancer. The exosome fraction was collected from ascitic fluids by several centrifugation steps. The presence of HER2-containing Tu-exosomes was confirmed by FACS analysis. The exosome fraction was then mixed with Ab-beads. FACS analysis indicated that the Ab-beads bound HER2-containing exosomes (Figure 5).

Effect of Tu-exosomes on the proliferation of BT-474 cells. To determine whether Tu-exosomes have biological functions, the effect of Tu-exosomes, derived from BT-474 cells, on BT-474 cell proliferation was examined by MTT assay. When the Tu-exosomes were cultured with BT-474 cells at 37°C, they attached to the cell surface (Figure 6A) and slightly, but significantly, increased the proliferation of the BT-474 cells (Figure 6B).

Discussion

One objective of this study was to develop a new procedure for collecting specific protein-containing exosomes of high purity. The successful isolation of exosome populations will enable the detailed analysis of the biological functions of exosomes and their possible use as therapeutic tools. In the present study, we used anti-HER2 antibody-coated paramagnetic beads (Ab-beads) to collect HER2-containing exosomes from crude exosome fractions collected by several centrifugation steps. The ability of these beads to specifically isolate HER2-containing exosomes was confirmed by FACS analysis. A humanized anti-HER2 monoclonal antibody (Herceptin) was used. Approximately 100% of the Ab-beads bound HER2-containing exosomes. Herceptin coupled to the beads may be functionally active, because the Ab-beads strongly inhibited the proliferation of high HER2-expressing BT-474 cells (data not shown).

Several types of tumors secrete exosomes (Tu-exosomes). It has been shown that high levels of exosomes accumulate in tumor ascites and pleural effusions of patients with various types of tumors such as breast or ovarian cancer (19). In addition, exosome-like vesicles have been collected from human serum (20). Because Tu-exosomes contain tumor antigens, such as melan-A/MART1 in melanoma tumor cells (9), they may act to transfer antigens from tumor cells to dendritic cells (DCs). It has been reported that Tu-exosomes are involved in the transfer of tumor antigens to antigen-presenting cells and in the stimulation of specific immune responses (15). Although these data indicate that Tu-exosomes obtained from malignant fluids are useful as antigen sources for immunotherapy, these fluid-derived exosomes include those secreted from various types of cells such as tumor cells, lymphoid cells, or mesothelial cells. To use exosomes as

potential antigen sources, Tu-exosomes should be selectively isolated. For this purpose we used Ab-beads to obtain, in a relatively selective manner, HER2-containing Tu-exosomes from ascitic fluid-derived exosomes of a patient with ovarian cancer. Thus, it may be possible to collect Tu-exosomes with beads coupled to antibodies to multiple tumor antigens.

Dexosomes, secreted from DCs, express both MHC class II molecules and costimulatory proteins such as CD80 and CD86; they can also stimulate naïve CD4+ T cells (21). Platelet-derived exosomes also have biological functions (13). Although the data obtained in the present study indicate that Tu-exosomes may also have biological functions, these functions remain unknown.

We determined whether Tu-exosomes can affect the proliferation of parental cells. Tu-exosomes derived from BT-474 cells stimulated the proliferation of BT-474 cells, suggesting a biological function. Membrane transfer has been reported *in vitro* in systems involving or not involving cell-cell contact. Furthermore, it has been suggested that exosomes bear combinations of ligands that can bind different cell-surface receptors simultaneously and that exosomes can fuse with target cells and exchange membrane proteins between the two cell types. Tu-exosomes bound to the surface of BT-474 cells; thus, there is a possibility that proteins in Tu-exosomes stimulated a proliferation-related signaling pathway in BT-474 cells. To examine the molecular mechanisms of Tu-exosome-mediated proliferation increases, we are investigating the expression of cell cycle-related proteins in BT-474 cells at both the mRNA and protein levels. In conclusion, Ab-beads may be useful as experimental and therapeutic tools in studies into the functional roles of exosomes.

Acknowledgements

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Evaluation of a Dysfunctional and Short-lived Subset of Monocyte-derived Dendritic Cells from Cancer Patients

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Abstract. *Monocyte-derived dendritic cells (Mo-DCs) were generated from peripheral blood monocytes of 12 healthy volunteers (hMo-DCs) and 11 patients (pMo-DCs) with malignancies by culture for 7 days with granulocyte-macrophage colony-stimulating factor and interleukin-4. In this study, we focused on the cytogram pattern by FACS analysis. A gate (R1) was set up by which more than 95% of hMo-DCs were contained. Mo-DCs having lower side scatter than the R1 (R2) comprised 4.5% of hMo-DCs and 24.2% of pMo-DCs. Expressions of antigen presentation-related molecules and phagocytic ability in the R2 of pMo-DCs were lower than those in the R1 population. The R2, but not R1, in pMo-DCs decreased in number between days 7 and 14, and expression levels of antigen presentation-related molecules in the living pMo-DCs on day 14 increased. The 11 patients received dendritic cell vaccine therapy with autologous, tumor-pulsed mature Mo-DCs (day 7). The low R2 group (R2≤10%, 3 patients) had a significantly higher positive delayed-type hypersensitivity reaction against autologous tumor-pulsed Mo-DCs than that of the high R2 group (R2>10%, 8 patients) ($p<0.001$). These results indicate that the R2 of pMo-DCs may be a dysfunctional and short-lived subset.*

Dendritic cells (DCs), which are known as professional antigen-presenting cells (APCs), can induce both the generation and proliferation of specific cytotoxic T lymphocytes (1-6). DCs capture and process antigens, move to the T-dependent areas of secondary lymphoid organs and stimulate naive T cells. Only DCs are capable of inducing primary sensitization against specific antigens in naive T cells (1). The ability to present exogenous antigens to CD8+ T cells through "cross-presentation" is an important feature of DCs (7, 8). Recent advances in biotechnology have made it possible to generate DC-like APCs (Mo-DCs) from peripheral blood mononuclear cells (PBMCs) with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (9). Thus, Mo-DCs have become popular candidates for DC-based immunotherapy for patients with malignancy of various types (10-19). Unfortunately, the therapeutic efficacy of these DC vaccines has been quite limited. One possible reason is that the antigen-presenting capacity of Mo-DCs generated from cancer patients (pMo-DCs) is impaired (20-26). It has been proposed that DCs mediate both T cell immunity and T cell tolerance and that these opposite functions may be linked to the dynamic maturation of DCs (27). If pMo-DCs have impaired maturation ability, pMo-DCs administered to cancer patients may remain immature and sensitize T cells to cancer-related antigens. Although it has been shown that several tumor-secreted factors such as venous endothelial growth factor, IL-6, IL-10 and transforming growth factor β1 (TGF-β1) are able to inhibit the full maturation of functional DCs (28-30), little is known about the reasons why the antigen-presenting ability of pMo-DCs is impaired. To potentiate the efficacy of Mo-DC-based vaccine therapy, a greater understanding of antigen presentation-related functions of pMo-DCs is needed.

Our previous study showed that pMo-DCs of advanced gastrointestinal cancer patients are not only dysfunctional in antigen-presenting ability, but that they also have a relatively short lifespan (26). In this study, therefore, we focused on the identification of this dysfunctional and short-lived pMo-DC subset.

Materials and Methods

Patients. Eleven patients with stage IV carcinoma (3 pancreas, 3 rectum, 2 colon, 2 stomach and 1 gall bladder carcinoma), for whom no other standard therapy option was possible, were enrolled in the

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Table I. *Characteristics of study patients.*

No.	Age[a]	Gender	Primary site	% of R2	DTH	Survival time[b]
1	43	M	stomach	2.8	+	10
2	38	M	rectum	5.7	+	24
3	49	F	rectum	6.2	+	16
4	64	F	colon	14.6	+	5
5	65	F	pancreas	18.0	+	10
6	72	M	colon	23.4	-	6
7	45	F	rectum	23.4	-	15
8	65	M	stomach	31.7	-	2
9	49	M	gall bladder	41.6	-	7
10	55	M	pancreas	45.1	-	2
11	72	F	pancreas	54.0	-	4

Eleven patients with advanced cancer (stage IV), who were enrolled in the present study and were not involved in any previous chemotherapy, radiotherapy, or immunotherapy. Patients were divided into a low R2 group (R2≤10%, 3 patients) and a high R2 group (R2 > 10%, 8 patients).
[a]years
[b]months

phase I study; for ethical reasons, no control group was created. The research ethics committee of the Faculty of Medicine, Kyushu University, Japan, approved the study protocol. All patients gave written, informed consent at the time of enrollment. Patient profiles are shown in Table I. Staging was done in accordance with the American Joint Committee on Cancer criteria (31). Twelve healthy volunteers, whose sex and age were matched with those of the patients, were enrolled as control subjects. Informed consent was also obtained from the healthy volunteers.

Generation of Mo-DCs. PBMCs were isolated from heparinized peripheral blood from the patients and healthy volunteers by Ficoll Paque (Life Technologies, Gaithersburg, MD, USA) density gradient centrifugation. PBMCs were resuspended in GMP-grade RPMI 1640 (Hy-Media, Nipro, Tokyo, Japan) with 1% human albumin (RPMI medium), plated at a density of $2x10^6$ cells/ml and allowed to adhere in 24-well culture plates (Nalge Nunk International, Chiba, Japan). After 4-h incubation at 37°C, the non-adherent cells were removed, and the adherent cells were harvested and cultured in RPMI medium supplemented with GM-CSF (200 ng/ml; Genetech Co., China) and IL-4 (500 U/ml; Osteogenetics, Wuerzburg, Germany). On day 7, non-adherent cell fractions were collected as immature Mo-DCs and examined.

Tumor cells. Autologous tumor cells were collected from malignant effusions, CT-guided biopsy specimens, or probe laparotomy specimens. Tumor specimens were minced with scalpels and passed through metal meshes of decreasing pore size. Cells were cultured in serum-free enriched culture medium (EBM2; Sanko Junyaku, Tokyo, Japan) containing basic fibroblast-growth factor, epidermal growth factors and insulin. To avoid any decrease in tumor-associated antigens, no chemical digestion was done. This procedure yielded a tumor-enriched cell line for Mo-DC-vaccine therapy. A human gastric adenocarcinoma cell line, GCTM-1, was used for *in vitro* experiments.

Flow cytometry (FACS) analysis. To analyze the cytogram and the expression of antigen presentation-related molecules in Mo-DCs, the cells were incubated for 1 h with anti-CD80 or anti-HLA-ABC (BD Pharmingen, San Diego, CA, USA) conjugated to FITC or anti-CD11c or anti-HLA-DR conjugated to PE (BD Pharmingen). The isotype controls were IgG1 and IgG2a (BD Pharmingen). For staining, cells were washed two times with phosphate-buffered saline (PBS) and then incubated for 1 h at 4°C in (FACS buffer) containing 3% BSA (Sigma, St. Louis, MO, USA) and 0.1% NaN_3 (Sigma) in PBS as well as the appropriate concentration of labelled mAb. After a washing with FACS buffer, the fluorescence intensity of gated Mo-DC populations was measured with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data were analyzed by CELLQuest software (Becton Dickinson).

Cytogram pattern analysis of Mo-DCs. The cytogram pattern of hMo-DCs was more homogeneous than that of pMo-DCs. Mo-DCs were divided into two populations according to the cytogram pattern. A gate was set up in which more than 95% of hMo-DCs were contained; these hMo-DCs were designated as the R1 population in this study; hMo-DCs in the lower side scatter (SSC) gate were designated as the R2 population.

Capture of lysed GCTM-1 tumor cells by Mo-DCs. The membrane components of lysed GCTM-1 cells were labelled with the PKH 67 green fluorescent cell linker kit (Sigma), and Mo-DCs were labelled with PE-conjugated HLA-DR mAb (BD Pharmingen), according to the manufacturers' protocol. Fluorescence-labelled Mo-DCs and lysed tumor cells were co-cultured at an original cell ratio of 1:1 for 4 h at 37°C or 4°C, washed, and then applied to a FACS Calibur flow cytometer. The fluorescence intensity data were analyzed with CELLQuest software. Both PKH 67-positive and HLA-DR-positive cells (double-positive cells) in gated Mo-DCs populations were defined as lysed tumor cell-captured Mo-DCs.

Procedure for Mo-DC vaccine. Autologous tumor cells were resuspended in 2 ml of serum-free RPMI medium and lysed by 5 freeze and thaw cycles. Immature Mo-DCs were incubated with the lysed tumor cells overnight (Mo-DCs:tumor cells=5:1). Tumor-pulsed Mo-DCs were further cultured in the presence of 40% monocyte-conditioned medium for Mo-DC maturation, as previously described (32). Tumor-pulsed mature Mo-DCs ([1-10] $x10^6$ cells) suspended in 2 ml of 1% human albumin-containing physiological saline solution were injected subcutaneously in a left supraclavicular lesion every 2 weeks for as long as possible.

Delayed-type hypersensitivity (DTH) skin-test reaction. For testing the tumor-specific response, tumor-pulsed Mo-DCs (10^5 cells/ml) were administered intradermally before and after the treatment. A positive DTH skin-test reaction was defined as an induration greater than 5 mm after 48 h.

Statistical analysis. Fisher's exact probability test was used for statistical analyses. Calculations were carried out with StatView software (Abacus Concepts, Berkeley, CA, USA). All results with a p value of less than 0.05 were considered statistically significant.

Results

Cytogram pattern of Mo-DCs on day 7. R1 and R2 populations of immature Mo-DCs on day 7 were identified

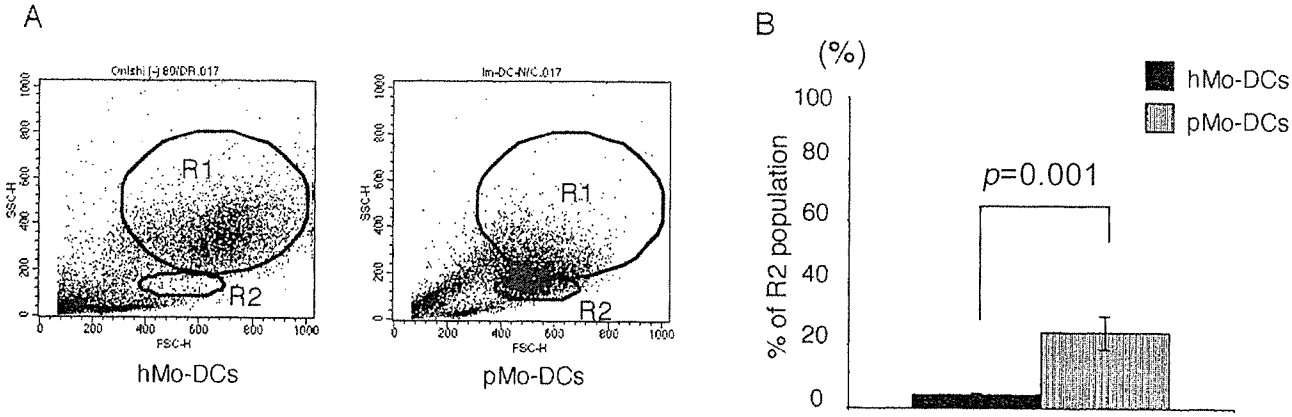

Figure 1. *(A) FACS analysis of representative cytogram patterns of hMo-DCs and pMo-DCs on day 7. The R1 gate, which contains more than 95% of hMo-DCs, is determined by forward and side scatter on day 7. R2 comprises Mo-DCs not in R1. (B) Percentages of the R2 population of hMo-DCs (filled column, n=12) and pMo-DCs (dotted column, n=11) on day 7. The results are presented as mean±SE (bars) values.*

by the cytogram pattern of FACS analysis, and hMo-DCs had a higher SSC pattern, whereas pMo-DCs had a lower SSC pattern (Figure 1A). The percentage of R2 in pMo-DCs (n=11, 24.2±5.2%) was significantly higher than that in hMo-DCs (n=12, 4.5±0.6%) (p=0.001, Figure 1B).

The expressions of antigen presentation-related molecules in R1 and R2 populations of Mo-DCs on day 7. The expressions of antigen presentation-related molecules in R1 and R2 populations were compared between hMo-DCs (n=6) and pMo-DCs (n=6) (Figure 2). The expressions of CD80, CD11c and HLA-ABC in the R2 population of hMo-DCs were significantly lower than those in the R1 population (p=0.031, 0.033 and 0.020, respectively). The expressions of CD80, CD11c, HLA-DR and HLA-ABC in the R2 population of pMo-DCs were also significantly lower than those in the R1 population (p=0.013, 0.033, 0.028 and 0.048, respectively). When the expressions of these molecules in the R1 populations of hMo-DCs and pMo-DCs were compared, only CD80 expression in the R1 population of pMo-DCs was lower than that in the hMo-DCs (p=0.050). Similarly, only CD80 expression in the R2 population of pMo-DCs was significantly lower than that in the R2 population of hMo-DCs (p=0.006).

Phagocytic ability of pMo-DCs in R1 and R2 populations on day 7. A dot plot pattern of a representative case is shown in Figure 3A. In this case, even though 47% of the R1 population of pMo-DCs captured the lysed GCTM-1 cells, only 7.7% of the R2 population of pMo-DCs captured the lysed GCTM-1 cells. Data for pMo-DCs generated from 5 patients are shown in Figure 3B. The percentage (10.0±2.2%) of Mo-DCs capturing the lysed GCTM-1 in the R2 population was significantly lower than that (39.0±7.7%) in the R1 population (p=0.023), suggesting that the phagocytic ability of the R2 population was lower than that of the R1 population. Capture of lysed GCTM-1 cells by pMo-DCs was not due to non-specific binding because the percentage of double-positive pMo-DCs at 4°C was less than 5%.

Cytogram pattern and expressions of antigen presentation-related molecules of Mo-DCs on day 14. The percentage of the R2 population in hMo-DCs and pMo-DCs on day 14 was measured. In hMo-DCs, no significant change in cell number was found between day 7 and day 14. In pMo-DCs, however, a significant decrease in cell number was found on day 14 compared to day 7, as found in our previous study (26). A representative cytogram is shown in Figure 4A. The cytogram pattern of pMo-DCs was very similar to that of hMo-DCs on day 14. No significant difference in the percentage of the R2 population was observed between pMo-DCs (n=8, 5.1±1.0%) and hMo-DCs (n=8, 4.9±1.1%) on day 14 (Figure 4B).

The expressions of antigen presentation-related molecules in the R1 population of hMo-DCs (n=6) and pMo-DCs (n=6) on day 14 are compared in Figure 5. No significant differences in the expressions of molecules examined were observed between hMo-DCs and pMo-DCs that survived until day 14.

Relationship between the DTH reaction after Mo-DC-vaccine therapy and the R2 population of pMo-DCs. Eleven patients received Mo-DC-vaccine therapy with autologous tumor-pulsed mature Mo-DCs. Patient profiles are provided in Table I. Autologous tumor-pulsed mature pMo-DCs on day 7 were injected subcutaneously every 2 weeks. Two months after therapy, the reaction of DTH was estimated using

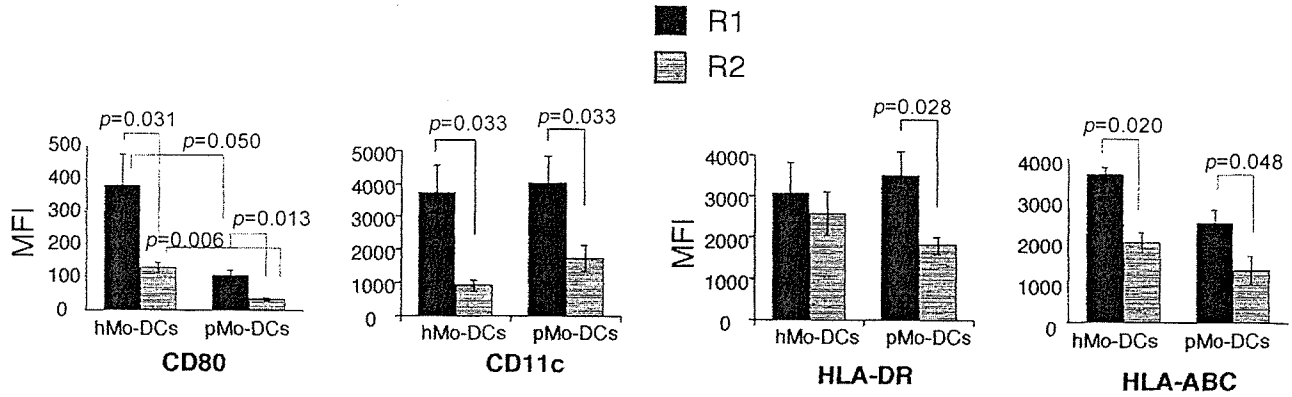

Figure 2. *The expressions of antigen presentation-related molecules in R1 (filled column) and R2 (dotted column) populations of hMo-DCs (n=6) and pMo-DCs (n=6) on day 7. The results are presented as mean±SE (bars) values.*

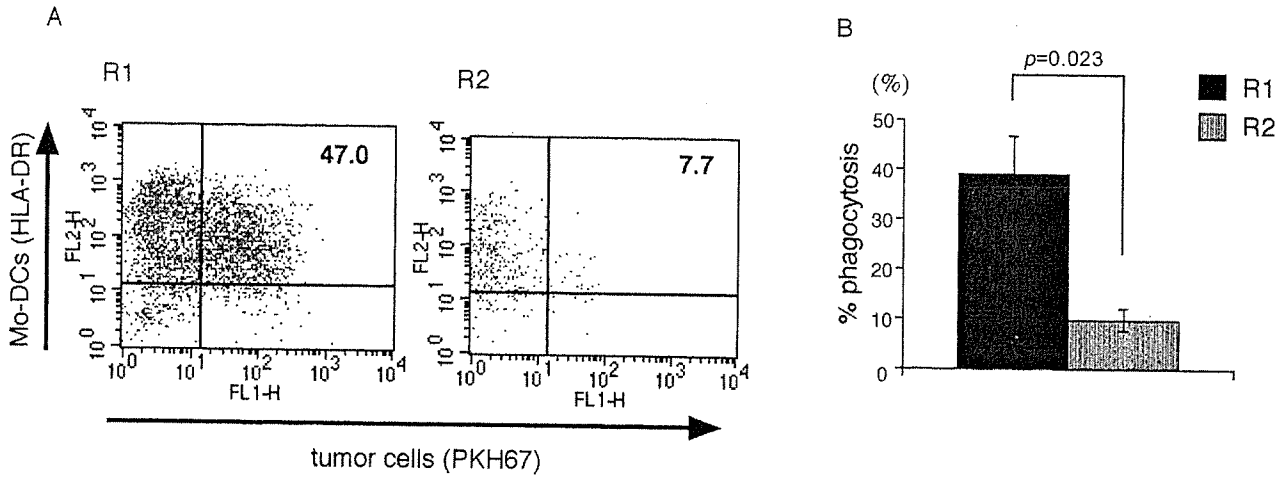

Figure 3. *Phagocytic ability in immature pMo-DCs on day 7. (A) The data are the log of fluorescence intensity of the R1 and R2 populations. The percentage of double-positive cells is shown in the upper right corners. (B) Percent phagocytosis of R1 populations (filled bars) and R2 populations (dotted bars) derived from 5 different pMo-DCs. Results are presented as mean±SE (bars) values.*

autologous tumor-pulsed Mo-DCs to assess the effectiveness of the immunotherapy. The patients were divided into a low R2 group (R2≤10%, 3 patients) and a high R2 group (R2 > 10%, 8 patients). The low R2 group (3/3) had a significantly stronger positive DTH reaction than that of the high R2 group (2/8) ($p<0.001$, Table I). Patients in the low R2 group had a significantly longer survival time than that of patients in the high R2 group (Figure 6).

Discussion

The results of our previous study indicated that Mo-DCs from patients with advanced cancer contain a dysfunctional and short-lived Mo-DC subset (26). In this work, we focused on the specification of the dysfunctional and short-lived Mo-DC subset to estimate the nature of pMo-DCs.

It is known that the cytogram pattern of FACS analysis differs between monocytes and Mo-DCs. When monocytes differentiate to Mo-DCs, the Mo-DC cytogram moves to the upper right. Based on these findings, to identify this short-lived Mo-DC subset, the cytogram pattern of Mo-DCs was analyzed by FACS. A gate (R1) was set up in which more than 95% of Mo-DCs generated from 12 healthy volunteers (hMo-DCs) were contained. The area having lower side scatter than R1 was named R2 and is similar to a gate for monocytes. On day 7, the percentage of the R2 population in pMo-DCs was significantly higher than that in hMo-DCs (Figure 1). In addition, pMo-DCs in R2 had significantly

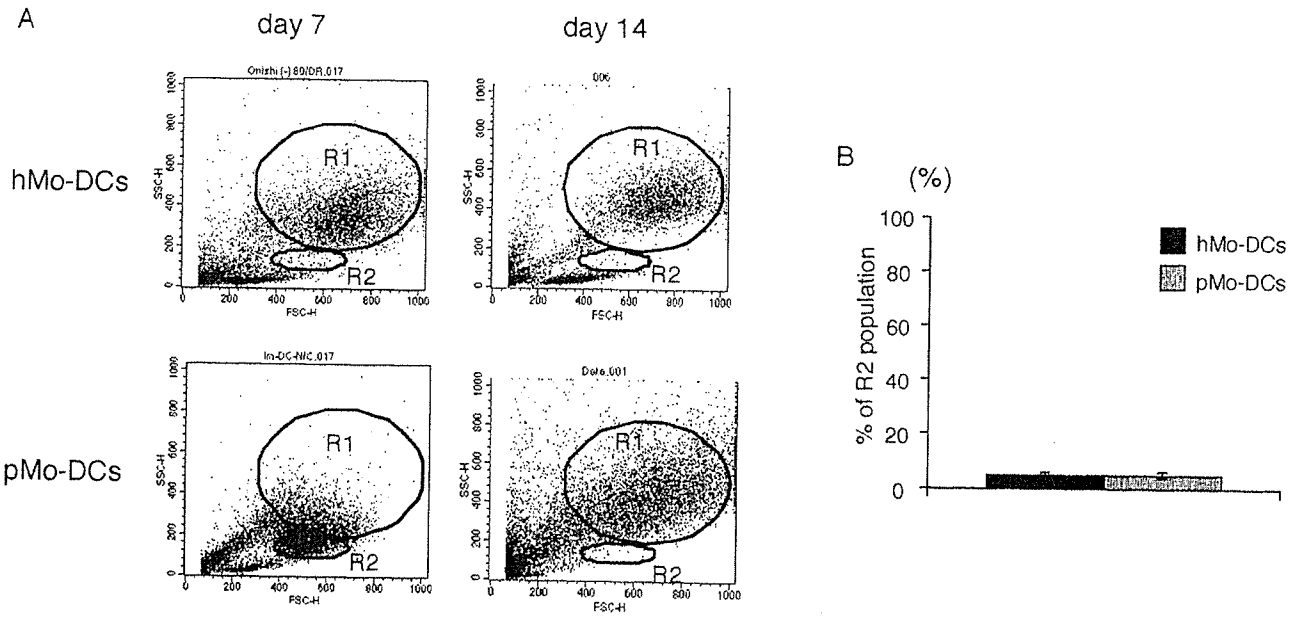

Figure 4. *(A) FACS analysis of representative cytogram patterns of hMo-DCs and pMo-DCs on days 7 and 14. (B) Percentages of R2 population of hMo-DCs (filled column, n=8) and pMo-DCs (dotted column, n=8) on day 14. The results are presented as mean±SE (bars) values.*

lower expressions of MHC and costimulatory molecules and a lower phagocytic ability than those in R1 (Figures 2 and 3). On day 14, however, the number of pMo-DCs decreased to three-quarters of that of day 7. The cytogram pattern of pMo-DCs also changed between day 7 and day 14 (Figure 4); the percentage of R1 in pMo-DCs increased. In hMo-DCs, however, neither cell number nor cytogram pattern changed between day 7 and day 14 (Figure 4). The cell number in R1 of pMo-DCs did not change significantly between day 7 and day 14, suggesting that mainly pMo-DCs in R2 were dying between day 7 and day 14. If so, R2 is a short-lived subset. Nevertheless, we cannot rule out completely the possibility that pMo-DCs in R2 changed to those in R1. This latter possibility is unlikely since it requires that Mo-DCs in R1 are short-lived.

Consistent with our previously reported findings (26), expressions of antigen presentation-related molecules of pMo-DCs were weak compared with those of hMo-DCs (Figure 2). Interestingly, most pMo-DCs in R2 disappeared between day 7 and day 14, and the difference in expressions of antigen presentation-related molecules between pMo-DCs and hMo-DCs also disappeared on day 14 (Figure 5). We conclude that the R2 population of pMo-DCs is dysfunctional and short-lived. Some investigators have shown that tumors impair dendritic cell differentiation from monocytes (33). In the present study, the R2 population had almost the same cytogram pattern as monocytes. We now speculate that those pMo-DCs which belong to R2 are insufficiently differentiated Mo-DCs. In fact, the mean fluorescence intensity of CD14 in R2 was higher than that in R1 (data not shown).

DC vaccine therapy with autologous tumor-pulsed Mo-DCs for patients with advanced malignancies is being evaluated in our laboratory. Based on our hypothesis that pMo-DCs that belong to R2 are insufficiently differentiated Mo-DCs, we analyzed the relationship between the percentage of the R2 population in pMo-DCs and the induction of tumor-specific immunological response. We used the DTH skin-test reaction against tumor-pulsed Mo-DCs as a tumor-specific response. As expected, a higher DTH-positive reaction after the therapy was induced in patients whose Mo-DCs contained a smaller R2 population (Table I). This suggests that Mo-DCs containing a smaller R2 population have a higher antigen presentation ability *in vivo*. This possibility is partly supported by the finding of a longer survival time in patients who received Mo-DCs containing a smaller R2 population (Figure 6). Our results indicate that we may be able to improve the efficacy of DC vaccine therapy by treating the R2 population in pMo-DCs. When anti-TGF-β1 antibody was added to the initial culture of monocytes, Mo-DCs in R2 significantly decreased, and both the expressions of MHC and co-stimulatory molecules as well as the phagocytic ability of pMo-DCs were improved (data not shown). It has been reported that the overexpression of TGF-β1 enhances cell invasion of fibrosarcoma, prostatic carcinoma and mammary adeno-carcinoma cells, with a consequent increase in the

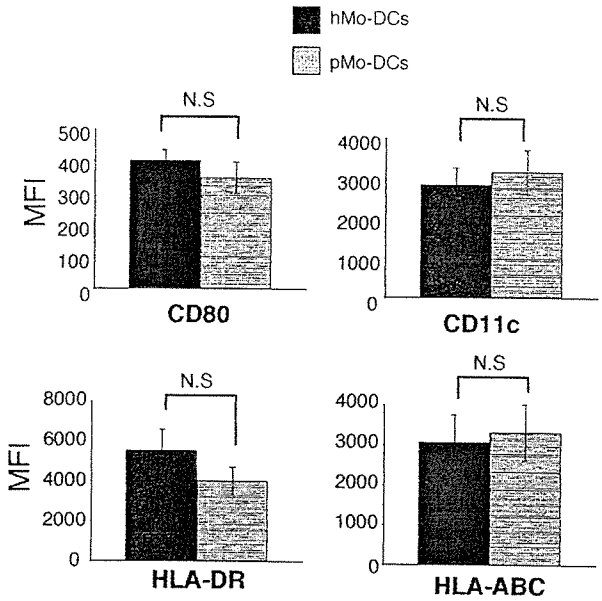

Figure 5. *Expressions of antigen presentation-related molecules in R1 populations of hMo-DCs (filled column, n=6) and pMo-DCs (dotted column, n=6) on day 14. The results are presented as mean±SE (bars) values.*

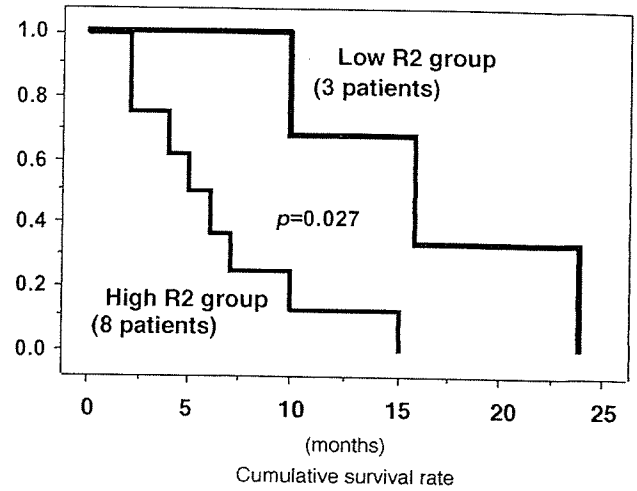

Figure 6. *Comparison of the cumulative survival rates of the low R2 group (R2≤10%) and high R2 group (R2>10%), in advanced cancer patients who underwent immunotherapy. The curve for the low R2 group is above the high R2 group at all time-points.*

metastatic potential of the tumor (34-36). In addition, it has been shown that tumor-derived TGF-β1 reduces the efficacy of DC vaccine (37, 38). Although these findings suggest that TGF-β1 may partly contribute to generation of the R2 population in Mo-DCs obtained from patients with malignancies, we have no definite recommendation for overcoming this problem.

In conclusion, Mo-DCs in the R2 population are a dysfunctional and short-lived subset. The percentage of R2 population in Mo-DCs may be a useful index for evaluating the quality of Mo-DCs to be used in DC-based vaccine therapy.

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特集 癌に対する細胞療法の新しい展開

樹状細胞(DC)ワクチン療法の現況と将来

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要旨 われわれは，臨床レベルの癌はすでに免疫寛容の世界に存在していると考えているので，強力な抗腫瘍免疫を誘導するには生体に新たに免疫監視システムを作り上げなくてはならない。したがって，免疫監視システムで重要な役割を演じている樹状細胞（DC）の抗原提示能を向上させるための進行中の工夫に焦点を当てて紹介する。工夫の一つは，DCの活性化状態および生存時間を延長するための工夫である。二つ目は，腫瘍局所の免疫学的環境を免疫監視の世界に調整するための工夫である。そして最後に，現在進行中の人工免疫システムの開発について簡単に紹介する。

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Present and Future Outlook for Dendritic Cell (DC)-Based Vaccine Against Cancer

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Summary

We propose that tumors discovered in a clinical setting are already in the world of immune tolerance. In order to induce powerful antitumor immunity, therefore, we will have to develop a new immune surveillance system in the body. We focused on the ongoing noteworthy devices to improve antigen-presenting ability of dendritic cells (DCs) which play a key role in the world of immune surveillance. One approach is to develop strategies capable of prolonging both the activation state and life span of DCs. A second way is to adjust the immunological environment of tumor sites to the immune surveillance world. Finally, we briefly introduce the ongoing trials concerning artificial antigen-presenting systems.

Key words：Dendritic cells, Cancer, Vaccine therapy, Immunotherapy, Artificial antigen-presenting system
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はじめに

癌抗原の存在は十分予想されるものの，そのエビデンスを提示するには長い時間を必要とした。しかし，ここ10年の間に特異的な免疫反応を誘導可能な癌抗原が次々と報告された。また，これら癌抗原による特異的T細胞（CTL）誘導の機序が分子レベルで明らかとなり，CTL誘導には癌抗原を抗原ペプチドの形で対応するT細胞に提示（抗原提示）する，いわゆる抗原提示細胞が必要なことが理解されるようになった。そして，樹状細胞（DC）こそが生体においてナイーブなT細胞を活性化し，抗原特異的な免疫反応を誘導する抗原提示のプロフェッショナル細胞であることもわかってきた。DCはDC前駆細胞として骨髄から種々の組織へ遊走し，未熟な状態で組織中に存在している。未熟な状態では，旺盛な抗原補足能を有しておりスカベンジャー細胞として機能している。しかし，いったん成熟化すると抗原補足能は低下し，MHC分子やCD86などの補助分子や接着分子の発現が亢進するとともに抗原ペプチドをMHC分子に乗せ細胞表面へ提示し，スカベンジャー細胞から抗原提示細胞へと変身する。さらに，成熟化によりCCR7といったリンパ管への浸潤を可能にするケモカインレセプター発現も亢進し，成熟DCは二次リンパ組織へ移動し，そこで抗原特異的なナイーブT細胞を活性化する。すなわち，癌関連抗原で刺激したDCを利用すれば，癌細胞を特異的に攻撃するCTLを生体に誘導し得る可能性がでてきた。しかし，現時点では生体から十分な量のDCsを採取することは不可能である。そんな折，末梢血や臍帯血あるいは骨髄細胞から*ex vivo*においてDC様の抗原提示細胞を誘導する技術が開発された。これら成果が集合し，癌に対するDCワクチン療法が現実のものとなり，種々のDCワクチン療法のphase Iあるいはphase I/II studyがスタートした。理論的には非常に期待される治療法であるが，現時点での臨床効果は期待どおりとはいい難い。

この臨床効果が期待を下回っている一つの理由として，われわれは，少なくとも臨床レベルの進行癌は免疫監視の世界にいるというより免疫寛容の世界に存在していると考えている。つまり，進行臨床癌という状態と初期の発癌という状態における宿主免疫のかかわり方を区別して異なった次元で扱うべきだと思っている。しかし実際は，健康なヒトあるいは初期の発癌モデルを用いて得られた免疫反応の結果に基づいて，進行癌それも極めて進行した癌に対して免疫療法の理論が組み立てられているのが現状である。つまり，癌に対する免疫反応を誘導するには意識的に癌を免疫監視の世界へ引きだす努力が必要であろう。このような観点から，現在考えられているDCのCTL誘導能を高めるための工夫と腫瘍局所を免疫反応誘導に適した場に変える工夫および，臨床効果の客観的評価を可能にするであろう人工抗原提示細胞開発について解説する。

Ⅰ．樹状細胞（DC）の抗原提示能を高める工夫

1．DCによる抗原提示時間の延長

DCの抗原提示能を高めることはDCワクチンの臨床効果を高めるための基本的な条件である。現在行われている抗原提示能を高める工夫のうち，DCのT細胞への抗原提示時間を延長させる試みについて紹介する。

最近の研究成果は，*in vivo*でのT細胞免疫反応の誘導には活性化DCとT細胞との反応時間を長くすることの重要性を示唆している。つまり，領域リンパ節での抗原刺激DCの寿命はリンパ節到達後48時間程度だという報告があり，DCワクチンの問題点の一つは，誘導したDCの生存期間（life span）が短いためにリンパ臓器でのT細胞活性化が十分でないことである。DCの抗原提示能（抗原提示分子発現，補助分子発現，サイトカイン産生，クロスプレゼンテーション）や生存にDC表面に発現するCD40（TNFファミリーに対するレセプターの一つ）へのCD40Lの結合シグナルが重要であることはよく知られている。したがって，DCのCD40にシグナルを入れることがDCの抗原提示能を上げ，かつ寿命を延長する一つの方法である。問題は，CD40がB細胞，マクロファージ，血管内皮細胞といった多くの細胞で発現していることであり，CD40抗体やCD40Lの臨床応用時にこれら他の細胞に有害事象をもたらす可能性がある。これらの問題を克服するために，CD40の細胞外ドメインを欠き

CD40の細胞質ドメインに薬剤（AP20187）結合ドメインと細胞膜結合配列（myristoylation-targeting sequence, M）が結合したキメラ蛋白（ここではinducible CD40, iCD40）を恒常的に発現するDC（iCD40 DCs）を作製する試みがなされている[1]。このiCD40 DCにAP20187を投与すると細胞内に侵入したAP20187がCD40の薬剤結合ドメインに結合し，その結果CD40は三量体を形成し，恒常的にCD40シグナルが活性化した状態になる。CD40の細胞外ドメインを欠いているため，iCD40 DCの活性化はCD40L非依存性であり，活性化はAP20187によってコントロールできる。腫瘍抗原ペプチドをパルスしたiCD40 DCワクチンは，AP20187依存性に活性化されCTLを誘導しマウス移植腫瘍細胞の増殖を抑制する。ICD40 DCの代わりに通常のDCを用いた場合，抗腫瘍効果は非常に弱い。Hanksらは[1]この理由として，通常のDCではCD40の細胞外ドメインから入ってくる活性化シグナルをブロックするような逆のフィードバック機序が働いている可能性があるが，CD40の細胞外ドメインを欠いたiCD40 DCではフィードバック機序が働けないために活性化が強くかつ持続する可能性を想定している。また，iCD40 DCはAP20187依存性に生存期間も延長する。

前述のように，有効なCTLを誘導するにはDCによるT細胞への長時間の抗原提示が必要であるが，外から合成ペプチドをMHC class I分子に乗せたMHC/ペプチド複合体の作用は数時間程度である。この問題を解決するために，従来，外科手術時の吸収糸として臨床使用されてきたpoly（D,L-lactide-co-glycolide）microspheres（PLGA-MS）を抗原供給源として利用する方法が試みられている[2]。MHC class IおよびclassⅡに乗るペプチドや蛋白をPLGA-MS（数μm）に包埋し末梢血単球由来DC（Mo-DC）にパルスすると，Mo-DCに効率よく取り込まれMo-DC内部で抗原を約30日近くにわたって放出すると予想される。したがって，抗原包埋PLGA-MSは単独でワクチンソースとして使用可能であり，マウスの系では抗原特異的な抗体およびCTLが誘導されている。DCはPLGA-MS摂取によって成熟化は誘導されず，遊走能も影響を受けないとされている。また，ペプチド刺激Mo-DCのCTL刺激は4日であったが，PLGA-MS刺激Mo-DCsのCTL刺激期間は9日後にも確認されている。臨床使用に際しての問題は，PLGA-MSの最適な消毒法が未だ確立されていない点である。また，ワクチンに際してはDCの成熟化が必要であり，その方法としては抗原とCpG-ODNをともに包埋したPLGA-MSが有効だと思われる。

抗原提示時間を長くする方法にはDCの細胞寿命を延長させる方法もある。遺伝子銃により皮内投与されたDNAはDCに効率よく取り込まれるためにDNAワクチンに利用される。最近，種々の抗アポトーシス蛋白がDCの細胞寿命を延長可能なことがわかってきた。したがって，DCに腫瘍関連遺伝子と抗アポトーシス蛋白関連DNAを同時に導入することで腫瘍関連ペプチドを発現するDCの細胞寿命を延長し，より強力な抗腫瘍免疫を誘導する試みが行われ，動物モデルにおいて成功している。しかし，抗アポトーシス遺伝子の導入は発癌誘導の問題がある。そこでKimら[3]は，DCのアポトーシス関連蛋白をノックアウトすることでDCの細胞寿命を延長させることを思いつき，その方法としてアポトーシス関連因子（BakとBax）のsiRNAを遺伝子銃により皮下投与しDCに組み込んだ。本法により，腫瘍関連DNAとアポトーシス関連因子（BakとBax）のsiRNAを同時投与することによりDCの寿命は延長し，領域リンパ節内の腫瘍関連蛋白発現DCの数は有意に増加し，抗腫瘍効果は著明に増強した。

2．クロスプレゼンテーション

外来性抗原刺激DCによるCTL誘導能を高めるためには，外来抗原のクロスプレゼンテーション（外来抗原ペプチドをMHC class I分子に乗せて細胞表面に提示すること）効率を高め，さらにこのDCを長期間にわたりメモリーT細胞を誘導可能な抗原提示能の高い抗原提示のプロフェッショナル細胞に変換させることが重要である。すなわち次の2点，効率のよいクロスプレゼンテーション誘導のための抗原刺激と十分なDCの活性化である。外来抗原をクロスプレゼンテーションの系に乗せるには抗原をレセプター依存性

にphagocytosisの形で取り込ませる重要性が指摘されている。たとえば，抗原とラテックスビーズの結合物，菌体，アポトーシス細胞，腫瘍細胞，腫瘍細胞由来エクソゾームなどによるDC刺激がそれである。さらに，Fcレセプター依存性の抗原取り込みもクロスプレゼンテーション効率の高いことが報告されている。この抗原のレセプター依存性の取り込みが効率よいクロスプレゼンテーションを誘導する理由として，抗原が効率よくエンドソームへ取り込まれることが考えられる。すなわち，phago-endosomeがクロスプレゼンテーションにおいて重要な役割をしていると思われる[4]。また，DCの十分な活性化の手法としてはTLRファミリーへのシグナルが有効である。TLR4などのようにDCの膜上に発現しているTLRsはDCが十分に抗原を取り込む前にDCを成熟活性化してしまう可能性があるが，DCのエンドソームで発現しているTLR3やTLR9はDC活性化のよい標的となる。これらの点を考慮して，TLR9のリガンドである非メチル化CpG dinucleotidesと腫瘍蛋白抗原との結合物（Ag-CpG）をワクチンソースとして使用する方法が提唱されている[4]。その理論を繰り返すと，Ag-CpGはDC上のDNAレセプターを介してエンドソームに取り込まれる。エンドソーム形成時に小胞体が形質細胞と融合し，小胞体の一部が解放されTLR9やMHC class Ⅰ分子がエンドソーム内へ移行する。次いで，TAPやプロテオソームといったMHC class Ⅰ分子への抗原処理関連器がエンドソームの細胞質側へ集まってくる。これにより，エンドソーム内の抗原がプロセスされ，CD8エピトープがMHC class Ⅰに乗り，細胞膜へ移行する（クロスプレゼンテーション）。一方，エンドソーム内のCpG-ODNはTLR9に結合しToll/IL-1レセプターシグナル系を活性化させDCを成熟活性化する。すなわち，クロスプレゼンテーション能の高い活性化DCが誘導されることになる。

3. アロDC

末梢血中には，アロ抗原を認識するT細胞が1～10%くらい存在しているといわれている。これは外来抗原に反応するT細胞の割合よりも高い。この事実は，ワクチン源としてのアロ抗原の有用性を示唆している。最近，同種同系のDCと癌細胞との融合細胞よりも，同種異系のDCと癌細胞の融合細胞で免疫したほうが*in vitro*および*in vivo*の系においてCTL誘導能および抗腫瘍効果が強く，同種異系のDCと癌細胞の融合細胞のほうがワクチン源として有用である可能性が報告された[5]。その一つの理由として，通常行われているように同系DCと癌細胞の融合細胞により免疫したマウスの脾臓細胞と癌細胞を共培養するとTh1/Th2（IL-4，IL-10）サイトカインをともに誘導するが，アロDCと癌細胞の融合細胞による免疫マウス脾細胞ではTh1サイトカイン（IFN-γ）のみが誘導されており，このサイトカインプロファイルの差が関係していると予想している。また，このサイトカイン産生パターンの差は，「末梢血中には，アロ抗原を認識するT細胞の割合が外来抗原に反応するT細胞の割合よりも高い」ということが関係しているかもしれない。すなわち，アロDCによるアロ抗原認識$CD4^+$T細胞への多量の刺激がIFN-γ誘導を優位にするのかもしれない。

また，アロDCとIL-12遺伝子導入癌細胞の融合細胞はIFN-γ誘導作用がさらに高められ，抗腫瘍効果も高く，臨床設定での今後の治療モデルとなる可能性がある。

4. 遺伝子治療

現在，種々の遺伝子を組み込んだウイルスベクターを細胞に感染させ，任意の蛋白を発現させ治療する方法，いわゆる遺伝子治療が試みられている。この試みは，癌に対するDCワクチン療法においても始まっている。DCワクチンに遺伝子治療を応用する場合，特に次の点が重要である。抗原提示細胞以外の細胞に感染しないウイルスベクターの選択が必要となる。抗原提示能の不完全な細胞によって抗原が提示されれば，免疫学的トレランスを誘導する危険性がある。この点からは，DCに特異的に感染するウイルスベクターが理想的である。もう一つは，DCに取り込まれることによってTLRsシグナルなどを刺激しDCに成熟活性化を誘導する働きを有するウイルスベクターである。レンチウイルスベクターはDCに特異的に感染し，細胞毒性を示さず，ウイルス蛋白発現なしに安定的にDCに抗原蛋白を発現し，かつ

DCの成熟活性化を誘導可能であることが示されており，DCワクチンのためのウイルスベクターの最有力候補の一つであると思われる。事実，マウスモデルの系においてレンチウイルスベクターの直接投与による抗原特異的なCTLが，効率よく長期間にわたり誘導可能であったことが報告されている[6]。

Ⅱ．免疫の場の微小環境を改善する工夫

$CD4^+$T細胞中には，自己抗原に反応するT細胞の機能を押さえ込む作用をもつ$CD4^+CD25^+$ regulatory T cells（Tregs）と呼ばれるサブセットが存在する。Tregsは末梢血中のT細胞の5～10%を占めている。このTregsは癌患者末梢血や腫瘍組織中に増加しており，これがDCワクチン療法の効果も抑制していると考えられている。最近，卵巣癌患者の末梢血，腹水，リンパ節，腫瘍組織中のTregsの数をFACS解析で，Tregsの遊走を卵巣癌移植免疫不全マウスの尾静脈からTregsを注入する系で，またTregsの抗腫瘍効果抑制を卵巣癌移植免疫不全マウスを腫瘍抗原刺激DCによるワクチン療法の系で検討した興味ある報告がなされた[7]。その内容は，これまで報告されたようにTregsは主としてリンパ臓器に存在しているが，進行癌においてはTregsはリンパ節から腫瘍局所に移動し集積することを示唆する結果であった。また，腫瘍局所のTregsのリンパ節への移動はほとんどみられないことから，癌患者Tregsはリンパ節におけるナイーブT細胞の活性化を抑制するというよりは，リンパ節外での免疫反応を抑制することにより抗腫瘍免疫を抑制していると想定される。リンパ節と腫瘍局所のTregsは，リンパ節へのホーミング分子であるCCR7やCD62Lを同程度に発現しているにもかかわらず，腫瘍局所からリンパ節への移動がないことから，これらの分子が実際ホーミング分子として働いているかは未だ不明である。末梢血のTregsはCCR4とCCR8を発現しており，*in vitro*の系ではCCL1，CCL17やCCL22に向かって遊走することが知られている。また，腫瘍局所のTregsもCCR4を発現しており，CCL22抗体処理によってTregsの腫瘍局所への集積が抑制されたので，Tregsは卵巣癌細胞や腫瘍浸潤マクロファージの発現しているCCL22に向かって腫瘍局所に遊走してくることが想像されている。さらに，腫瘍局所のTregsは実際にCTL活性を抑制し，抗腫瘍活性を抑制することも示された。興味深いもう一つのデータは，臨床標本を用いた解析結果である。ヒト卵巣癌において，卵巣癌組織中のTregsの数は予後と逆相関を示し，卵巣癌局所のTregsによる免疫反応抑制が癌の進展に関与していることが示唆されている。すなわち，腫瘍局所のTregsの制御やTregsの腫瘍局所への遊走阻止が新たな治療戦略となる可能性がでてきた。

Tregsは，IL-2に対する高親和性レセプターIL-2R α鎖（CD25）を恒常的に発現するとともにT細胞の活性化を抑制するレセプターであるCTLA-4も恒常的に発現している。Tregsの基本的な特徴は，*ex vivo*の系ではTCR刺激に不応答（アネルギー）であり，反応性T細胞のIL-2産生抑制やIL-2感受性を抑制することで反応性T細胞を不応答にすると考えられる（免疫抑制機能）。多量の外来性IL-2添加はTregsに応答性を回復させ，T細胞に対する抑制機能をも減弱させる。また，成熟DCはTregsの抑制機能を抑制するとともにTregsの増殖を誘導することができる。これらの報告は，TregsのTCR刺激に対する不応答性と免疫抑制機能が連動していることを示唆している。しかし最近，Tregsの免疫抑制機能は成熟DCによって抑制することが可能で，このためにはTLRからのシグナルは必要でないこと，一方，TregsのTCR不応答性の克服にはDCのTLRを介した活性化が必要であることが報告された[8]。TLR活性化DCがTCR応答性を回復させる機序としてはDCの産生するIL-6とIL-1が共同してTregsのIL-2に対する反応性を高めることによると考えられている。つまり，TregsのTCR応答性（増殖能）にはTLRを介したDCの成熟化が必要だが，Tregsの免疫抑制反応を制御するには必要ではないということである。結論としては，Tregsの免疫抑制機能と不応答性は別々の機序によって制御されているらしい。一方で，Tregsの免疫抑制反応を消失させるにはTLRを介したDCの産生するサイトカインが必要だとする論文もある。この両論文の違い