delayed-type hypersensitivity by the injection of dendritic cells pulsed with tumour lysate. The patient died of lung metastasis 3 months after initiation of dendritic-cell therapy.

There seems to be some difficulties regarding immunotherapy for patients with graft-versus-host disease, even with use of donor dendritic cells. A previous study[5] showed that steroid treatment inhibits dendritic-cell function in vitro. In our patient, immunosuppressive treatments could have suppressed antigen presentation or T-cell stimulation by dendritic cells; T-cell function also might have been suppressed. In conclusion, effective immunotherapy with dendritic cells for patients with graft-versus-host disease who have undergone allogeneic peripheral-blood stem-cell transplantation can be problematic.

Conflict of interest

We declare no conflicts of interest.

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ORIGINAL ARTICLE

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Effects of docetaxel on antigen presentation-related functions of human monocyte-derived dendritic cells

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Abstract *Purpose*: Docetaxel (TXT) is a unique chemotherapeutic agent that has been approved for treating various types of malignancies. TXT stabilizes microtubule assembly in cells and causes various dysfunctions of microtubule-dependent cellular events. Patients with advanced malignancies are beginning to receive TXT in combination with immunotherapy; however, the influence of TXT at clinically achievable serum concentrations (less than 10^{-6} *M*) on antigen presentation-related functions of human monocyte-derived dendritic cells (Mo-DCs) remains unclear. *Methods*: Immature Mo-DCs (imMo-DCs) were generated from peripheral blood monocytes with interleukin-4 and granulocyte-macrophage colony-stimulating factor in vitro. Mature Mo-DCs (mMo-DCs) were induced from imMo-DCs with tumor necrosis factor-α and prostaglandin E_2. *Results*: TXT at concentrations lower than 10^{-7} *M* did not significantly affect cellular viability, phagocytosis, or expression of antigen presentation-related molecules of Mo-DCs. In contrast, TXT at concentrations lower than 10^{-9} *M* significantly suppressed directional motility of imMo-DCs toward MIP-1α and of mMo-DCs toward MIP-3β. However, TXT had no effect on either CCR1 expression by imMo-DCs or CCR7 expression by mMo-DCs. No gross changes in the microtubule skeleton were evident by immunofluorescence microscopy after treatment with TXT at less than 10^{-8} *M*. However, reduced numbers of imMo-DCs with podosomes localized primarily in one cell region were observed. *Conclusions*: The present results indicate that different concentrations of TXT influence antigen presentation-related functions differently. In particular, TXT at relatively low therapeutic doses disrupts chemotactic motility of Mo-DCs.

Keywords Taxane · Non-directional migration · Directional migration · Microtubules · Immunotherapy

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Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and are capable of inducing primary sensitization against specific antigens in naive T cells [3]. Immature DCs (imDCs) exist in most tissues. They capture and process antigens. Following activation, they display these antigens in the form of MHC-peptide complexes at their surface [9]. Mature DCs (mDCs) enter lymphatic vessels, migrate to T-dependent areas of secondary lymphoid organs, and stimulate naive T cells [2]. Thus, the ability of DCs to migrate is crucial to the transmission of immunological events in peripheral tissues to secondary lymphoid organs.

It is now possible to generate in vitro DC-like APCs (Mo-DCs) from human peripheral blood mononuclear cells (PBMCs) with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) [29]. Immature Mo-DCs (imMo-DCs) and mature Mo-DCs (mMo-DCs) also migrate toward MIP-1α and MIP-3β, respectively [8, 22]. Mature Mo-DCs are able to enter lymphatic vessels and migrate to regional lymph nodes in animal models, and mMo-DCs might be involved in induction of tumor-specific cytotoxic T lymphocytes (CTLs) [11, 44]. Based on these experimental findings, a number of studies have shown that

subcutaneous injection of mMo-DCs loaded with tumor-associated antigens leads to antitumor immune responses in patients with various types of malignancies [4, 11, 13, 28]. In these DC-based vaccine therapies, the ability of injected mMo-DCs to migrate plays an essential role in CTL induction.

For a cell to invade, the front of the cell must protrude and attach to a substrate and then the rear part of the cell must be able to retract. These processes are directly driven by the actin cytoskeleton [35, 36]. Podosomes are unique actin-rich adhesion structures of monocyte-derived cells [7]. They are highly dynamic and actively engage in matrix remodeling and tissue invasion [25]. Microtubules also play a role in the locomotion of most, but not all, cell types, and they may be involved in the coordination of the direction of cell movement [15, 33]. Linder et al. [26] have shown that microtubules are essential for podosome formation in primary human macrophages. These findings indicate the significance of actin and microtubule cytoskeletons in cell motility. Thus, compounds that damage actin or microtubules may affect cell motility.

Docetaxel (TXT) is a new chemotherapeutic agent that has been approved for treatment of various types of malignancies [16, 18–20, 23, 30]. TXT is a semisynthetic taxane derived from the needles of the European yew (*Taxus baccata*). It binds to tubulin, leading to microtubule stabilization, mitotic arrest, and subsequent cell death [14, 17, 39]. TXT has been reported to affect the migratory capacity of certain cells such as endothelial cells [21], smooth muscle cells [1], some cancer cells [5, 37, 40], and neutrophils [32]. However, there is limited information concerning the influence of TXT on Mo-DC motility.

Cancer patients receiving chemotherapeutic agents, including TXT, sometimes also receive DC vaccine therapy. In addition, recent animal experiments suggest that chemotherapeutic agents administered in combination with Mo-DC-based vaccine therapy may be effective for treating cancer patients with multiple drug resistance [41]. Thus DC vaccine therapy is likely to become a more common component in regimens for treatment of cancer. Because TXT affects microtubule function, which is important for motility of DCs used in vaccine therapy, we investigated the effect of TXT on immunological functions of Mo-DCs, with particular emphasis on Mo-DC motility.

Materials and methods

Reagents and antibodies

TXT was purchased from Rhone-Poulenc-Rorer (Antony, France). Streptococcal preparation OK-432 was provided by Chugai Pharmaceutical Company (Tokyo, Japan). Human MIP-1α and MIP-3β were purchased from Diaclone Research (Besançon, France). The following monoclonal antibodies (mAb), conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE), were purchased from BD Biosciences Pharmingen (San Diego, Calif.): CD14, CD80, CD83, CD86, HLA-DR. Unlabeled mouse anti-human CCR1 and CCR7, FITC-conjugated gout anti-mouse immunoglobulins, and isotype controls, IgG1 and IgG2a, were also purchased from BD Biosciences Pharmingen.

Mo-DC preparation

Mo-DCs were generated from the PBMCs of healthy volunteers as previously described with minor modifications [43]. Briefly, PBMCs were suspended in RPMI 1640 medium (Sanko Pure Chemicals, Tokyo, Japan) with 10% fetal calf serum (FCS) (referred to as RPMI-FCS medium) for 4 h at 37°C, and the adherent cells were cultured in RPMI medium supplemented with 200 ng/ml GM-CSF (GeneTech, Beijing, China) and 500 U/ml IL-4 (Osteogenetics, Würzburg, Germany). On day 7, non-adherent cells were collected and further purified by negative selection with magnetic beads coated with mouse monoclonal anti-CD2, anti-CD3, and anti-CD19 antibodies (Dynabeads, Dynal Biotech, Oslo, Norway). This depletion procedure yielded over 90% $CD14^-$, $CD80^+$, and $HLA\text{-}DR^+$ imMo-DCs as assessed by fluorescence-activated cell sorting (FACS) with a FACS Calibur (Becton Dickinson, Franklin Lakes, N.J.). To induce maturation, imMo-DCs were cultured with RPMI-FCS medium supplemented with tumor necrosis factor-α (TNF-α, 200 U/ml; Dainippon Pharmaceutical, Osaka, Japan) and prostaglandin E_2 (PGE_2, 1 μg/ml; Sigma, St. Louis, Mo.) for two additional days [27]. These cells were used as mMo-DCs [24].

Cell viability and detection of apoptosis

ImMo-DCs were seeded into 96-well plates and cocultured with the indicated concentrations of TXT at 37°C in RPMI 1640 supplemented with 1% human albumin (hereafter referred to as RPMI-Alb medium). Following incubation for 24 h, cell viability was determined by the 3-[4,5-dimethythiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) assay. Percent cell viability is expressed as the mean ± SD of three independent wells.

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the membrane-permeable dye Hoechst 33342 (Wako Chemicals, Osaka, Japan). Briefly, cells were plated in 96-well plates, treated with TXT for 24 h, stained with Hoechst 33342, and then observed by fluorescence microscopy. A total of 300 cells were counted in three randomly chosen fields at ×100 magnification. Cells with condensed or fragmented nuclei were considered to be apoptotic. The proportions of apoptotic cells are

expressed as mean ± SD percentages of three independent wells.

Immunofluorescence microscopy

ImMo-DCs and mMo-DCs were pretreated with the indicated concentrations of TXT at 37°C for 24 h. After incubation, cells were washed with RPMI to eliminate TXT. Washed cells were allowed to adhere to glass coverslips at 37°C overnight. Cells were then fixed with 100% methanol at −20°C for 5 min. After washing with PBS containing 0.1% Tween 20 (Nacalai Tesque, Kyoto, Japan), non-specific binding was blocked with 10% goat serum in PBS. Actin and microtubule cytoskeleton were visualized by immunofluorescence staining with mouse mAbs to actin (1:200; Sigma) and to α-tubulin (1:500; Sigma), respectively. Alexa 594-conjugated goat anti-mouse IgG antibody (Wako Chemicals) was used as a second antibody. Fluorescence signals were detected with a Radiance 2000 confocal laser-scanning microscope (Bio-Rad Laboratories, Hercules, Calif.). Images were processed with Laser Sharp 2000 software (Bio-Rad Laboratories).

Migration of Mo-DCs

Migration of Mo-DCs was determined by counting the number of cells that migrated through Transwell inserts with filter membranes of pore size 8 mm (BD Biosciences Pharmingen). The effect of TXT on non-directional Mo-DC migration was determined as follows. Mo-DCs treated with designated doses of TXT for 24 h were suspended at a concentration of 2×10^5 cells/ml in RPMI-Alb medium. Cell suspension (500 μl) was added to the upper compartment, and RPMI-Alb medium (400 μl) was added to the lower compartment. The cells were incubated at 37°C for 6 h. After incubation, the filter was fixed with 100% methanol and stained with Giemsa solution, and the cells on the upper surface were completely removed. Mo-DCs that had migrated from the upper side to the lower side of the filter were counted under a light microscope at a magnification of ×200. Non-directional migration is expressed as the mean ± SD migrating cell number of five microscopic fields.

The procedure for determining the effect of TXT on directional migration was the same as that for determining non-directional migration; however, to determine directional migration of imMo-DCs or mMo-DCs, MIP-1α (10 ng/ml) or MIP-3β (100 ng/ml), respectively, was added to the lower compartment.

Chemokine-induced invasiveness of Mo-DCs

Chemokine-induced invasiveness of Mo-DCs was measured by the invasion of cells through Matrigel-coated Transwell inserts [42]. Briefly, the upper surface of the filter (pore size 8.0 μm; BD Biosciences Pharmingen) was coated with basement membrane Matrigel (BD Biosciences Pharmingen) at a concentration of 250 μg/cm^2 and air-dried overnight at room temperature. The invasion assay was similar to the directional migration assay described above. MIP-1α (for imMo-DCs) or MIP-3β (for mMo-DCs) was added to the lower compartment. After 24 h, Mo-DCs that had migrated from the upper side to the lower side of the filter were counted under a light microscope at a magnification of ×200. Chemokine-induced invasion is expressed as the mean ± SD migrating cell number of five microscopic fields.

Evaluation of phagocytosis

Immature Mo-DCs (1×10^5/well) pretreated with designated doses of TXT for 24 h were suspended in RPMI 1640 medium with FITC-conjugated dextran (FITC-DX, Sigma) and incubated for 12 h. The percentage of imMo-DCs that captured FITC-DX was determined by examining 100 imMo-DCs under a fluorescence microscope. The percent phagocytosis is expressed as the mean ± SD of three wells.

Alternatively, imMo-DCs were labeled with PE-conjugated anti-HLA-DR mAb. The fluorescence-labeled imMo-DCs were cultured with FITC-DX for 12 h at 37°C or 4°C, washed, and applied to a FACS Calibur flow cytometer. The fluorescence intensity was analyzed with CellQuest (Becton-Dickinson). FITC-positive cells in gated HLA-DR-positive imMo-DC populations were defined as dextran-captured imMo-DCs.

Expression of antigen presentation-related antigens of Mo-DCs

For analysis of the effect of TXT on expression of maturation-related molecules of Mo-DCs, the following mouse anti-human mAbs, conjugated with either FITC or PE, were used: CD14, CD80, CD83, CD86, and HLA-DR. Unlabeled mouse anti-human mAbs CCR1 and CCR7 were visualized with FITC-conjugated anti-mouse immunoglobulins. Isotype controls, IgG1, and IgG2a were also included. Cells were stained at a concentration of 1×10^5 in 100 μl. Samples were incubated with the conjugated mAbs for 60 min at 4°C and then washed twice with PBS containing 3% bovine serum albumin (BSA; Sigma) and 0.1% NaN_3 (Sigma). The samples were analyzed with a FACS Calibur flow cytometer and CellQuest.

IL-12 secretion in Mo-DCs

Because our previous study had shown that OK-432 induces IL-12 secretion in imMo-DCs [24, 31], imMo-DCs

(1×10^5/ml) were incubated with the indicated doses of TXT for 24 h at 37°C, washed to eliminate TXT, and then incubated with OK-432 (0.02 KE/ml) for 24 h. Cell-free supernatants were collected by centrifugation and stored at −80°C. The concentration of IL-12 p40 and p70 in the supernatants was determined using an enzyme-linked immunosorbent assay (ELISA) kit specific for measuring IL-12 p40 and p70 (BioSource International, Camarillo, Calif.) according to the manufacturer's instructions. The detection limit for IL-12 p40 and p70 was 7.8 pg/ml and 1.56 pg/ml, respectively. Concentrations of IL-12 p40 are expressed as the mean ± SD of the data from three independent experiments.

Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) was carried out in U-bottomed 96-well plates with a total volume of 200 µl per well. Irradiated imMo-DCs (2×10^4 cells), which were treated with designated doses of TXT for 24 h at 37°C, were suspended in RPMI-Alb medium. Allogenic PBMCs (1×10^5) were used as responders. Wells were pulsed 3 days after the initial culture with 1 µCi (0.037 MBq) of [^{3}H]-thymidine (Amersham Pharmacia Biotech, Piscataway, N.J.). [^{3}H]-Thymidine incorporation was measured 24 h after the addition of [^{3}H]-thymidine with a liquid-scintillation counter (Beckman Coulter, Palo Alto, Calif.). [^{3}H]-Thymidine uptake is expressed as the mean ± SD counts per minute of three wells.

Statistical analysis

Statistical analysis was performed with the unpaired two-tailed Student's t test; $P < 0.05$ was considered significant.

Results

Effects of TXT on the viability of Mo-DCs

Achievable serum concentrations of TXT in the clinical setting are lower than 10^{-6} M and a concentration of 10^{-9} M is maintained for about 72 h [6]. When imMo-DCs were exposed to TXT at concentrations greater than 10^{-6} M for 24 h, viability decreased (Fig. 1a). The pattern of cellular death was apoptosis (Fig. 1b). Data are representative of six independent experiments with imMo-DCs generated from three different healthy donors.

Effects of TXT on cytoskeletal organization of imMo-DCs

Untreated imMo-DCs were oval in shape, indicating a polarized morphology (Fig. 2a). After treatment with

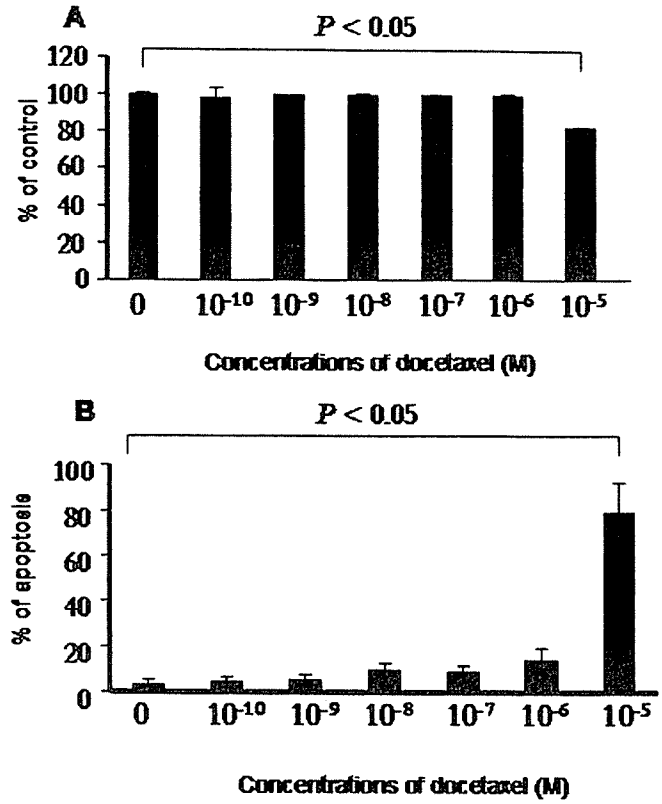

Fig. 1 Effect of TXT on viability of imMo-DCs. **a** The MTT assay was performed after 24 h of TXT treatment. Each experiment was performed in triplicate and the average optical density (OD) at 570 nm was calculated. The data presented are the means ± SD of three different wells. **b** Cells were stained with Hoechst 33342 and examined by fluorescence microscopy. A total of 300 cells were counted in three randomly chosen fields at ×100 magnification. Cells with condensed or fragmented nuclei were considered to be apoptotic. The data presented are percent apoptosis and are the means ± SD of three independent wells

TXT at concentrations greater than 10^{-8} M, imMo-DCs became rounded (Fig. 2d). The effect of TXT on cytoskeletal organization was examined by immunohistochemistry as described in "Materials and methods" (Fig. 2). In more than 90% of untreated imMo-DCs, multiple clear small actin foci resembling podosomes were found (Fig. 2b). Clusters of podosomes were localized primarily to one region in most untreated imMo-DCs, whereas in about 70% of imMo-DCs treated with TXT at 10^{-8} M, podosome clustering was mostly absent (Fig. 2e). In particular, almost all imMo-DCs treated with TXT at concentrations greater than 10^{-7} M failed to spread significantly on Matrigel-coated glass coverslips and lacked podosomes (Fig. 2h). No apparent change in microtubule cytoskeleton was found in cells treated with concentrations lower than 10^{-8} M TXT (Fig. 2f); however, an interwoven fabric of highly concentrated filaments or dense peripheral banding of filaments was found in almost all cells treated with greater than 10^{-7} M TXT (Fig. 2i).

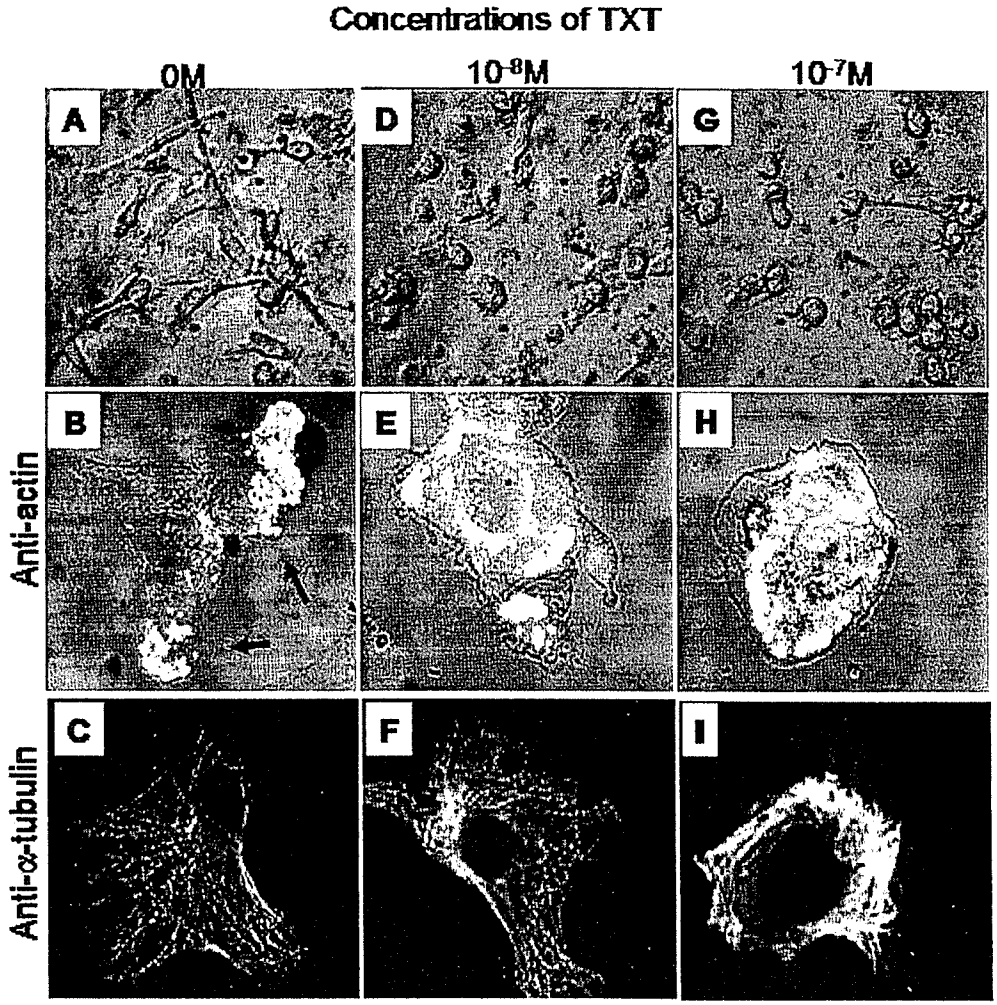

Fig. 2 Effect of TXT on cytoskeletal organization of imMo-DCs. ImMo-DCs were treated with the indicated doses of TXT for 24 h and observed under a phase contrast microscope (**a**, **d**, **g**; ×400) and confocal laser microscope (**b**, **c**, **e**, **f**, **h**, **i**; ×2000). Specimens were stained with either anti-actin mAb (**b**, **e**, **h**) or anti-α-tubulin mAb (**c**, **f**, **i**). *Arrows* indicate podosomes

Effects of TXT on the motility of Mo-DCs

A migration assay was performed with a modified Boyden chamber technique as described in "Materials and methods". TXT at 10^{-8} *M* significantly decreased non-directional motility of both imMo-DCs and mMo-DCs (Fig. 3a,b). TXT at 10^{-10} *M* significantly decreased MIP-1α-induced directional motility of imMo-DCs (Fig. 3c), and TXT at 10^{-9} *M* significantly decreased MIP-3β-induced directional motility of mMo-DCs (Fig. 3d). The data presented are representative of three independent experiments with Mo-DCs generated from three different healthy donors.

Because CCR1 and CCR7 are receptors for MIP-1α and MIP-3β, respectively [8], we examined by FACS analysis the effect of TXT on expression of CCR1 on imMo-DCs and CCR7 on mMo-DCs. TXT at concentrations lower than 10^{-7} *M* did not significantly affect the expression CCR1 and CCR7 on Mo-DCs (data not shown).

Effect of TXT on chemokine-induced invasiveness of Mo-DCs

To invade a new territory, Mo-DCs must be able to penetrate the matrix, especially in response to chemokines. In this study, chemokine-induced invasive ability (chemo-invasive ability) was determined by a Matrigel invasion assay as described in "Materials and methods". TXT at 10^{-9} *M* significantly decreased the chemoinvasive ability of imMo-DCs or mMo-DCs toward MIP-1α or MIP-3β, respectively (Fig. 4a,b). The data presented are representative of three independent experiments with Mo-DCs generated from three different healthy donors.

Effect of TXT on phagocytic activity of Mo-DCs

Phagocytic ability of imMo-DCs was determined with FITC-DX by both fluorescence microscopy (data not

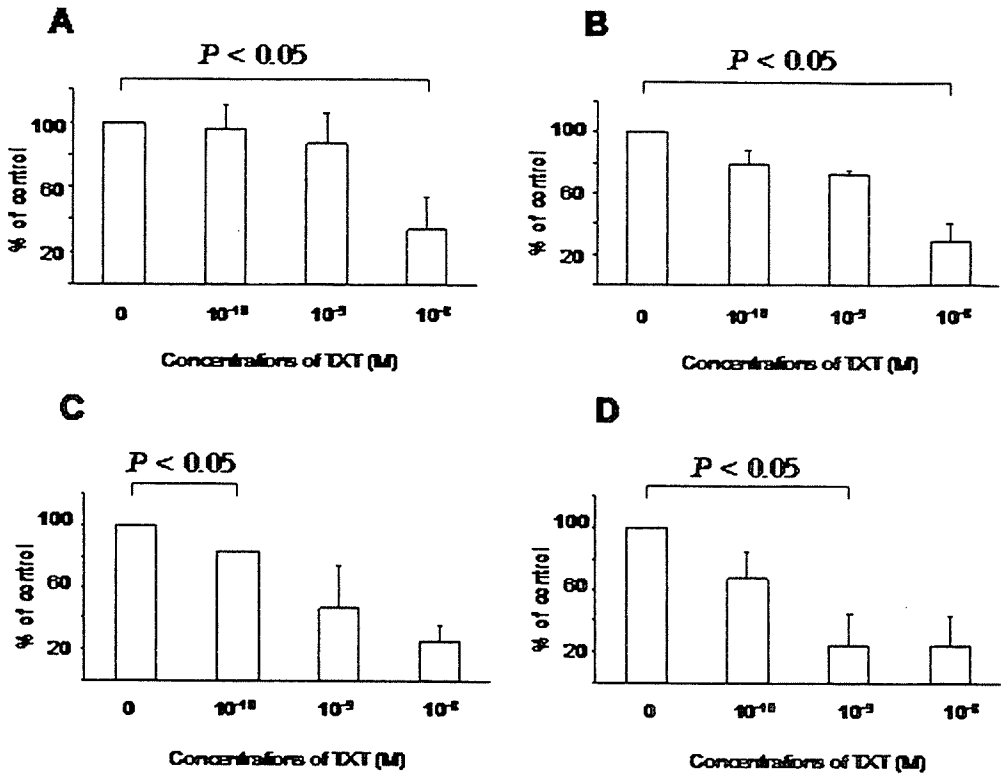

Fig. 3 Effect of TXT on the motility of Mo-DCs. The data presented are the ratios of TXT-treated migrating Mo-DCs to untreated Mo-DCs. **a** Non-directional migration of imMo-DCs; **b** non-directional migration of mMo-DCs; **c** directional migration of imMo-DCs against MIP-1α; **d** directional migration of mMo-DCs against MIP-3β

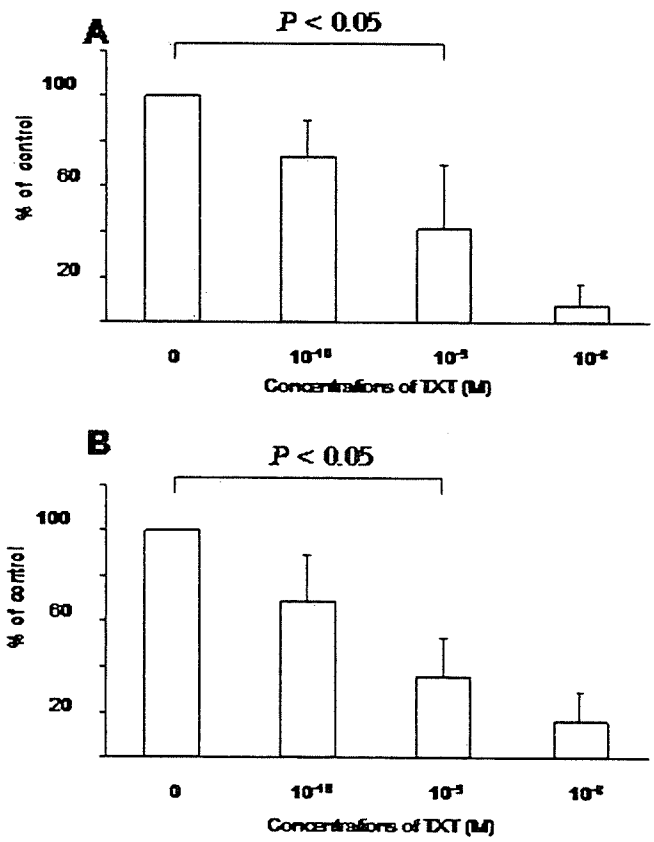

Fig. 4 Effect of TXT on chemoinvasive ability of Mo-DCs as determined by a Matrigel invasion assay. The data presented are the ratios of TXT-treated migrating Mo-DCs to untreated Mo-DCs. **a** Chemokine-induced invasion of imMo-DCs toward MIP-1α; **b** chemokine-induced invasion of mMo-DCs toward MIP-3β

shown) and FACS analysis (Fig. 5) as described in "Materials and methods". TXT at concentrations lower than 10^{-7} *M* did not affect phagocytic ability of imMo-DCs (Fig. 5). The data presented are representative of three independent experiments with Mo-DCs generated from three different healthy donors. When imMo-DCs were cultured with FITC-DX at 4°C (Fig. 5), their phagocytic ability was less than 5%, indicating capture of dextran by imMo-DCs rather than non-specific binding of dextran with imMo-DCs.

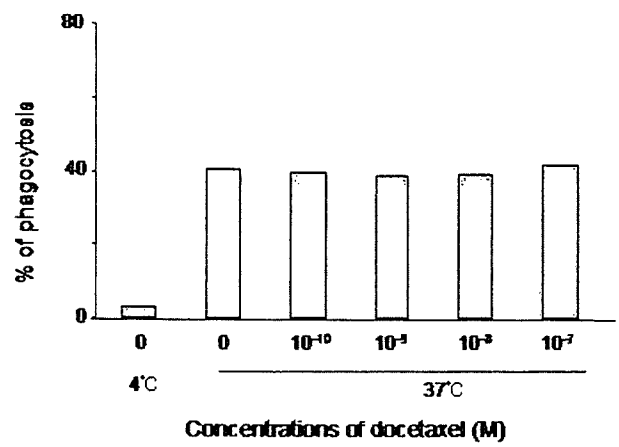

Fig. 5 Effect of TXT on phagocytosis by imMo-DCs. Cells were cocultured for 12 h with FITC-DX and subjected to FACS analysis. The data presented are representative of three different experiments

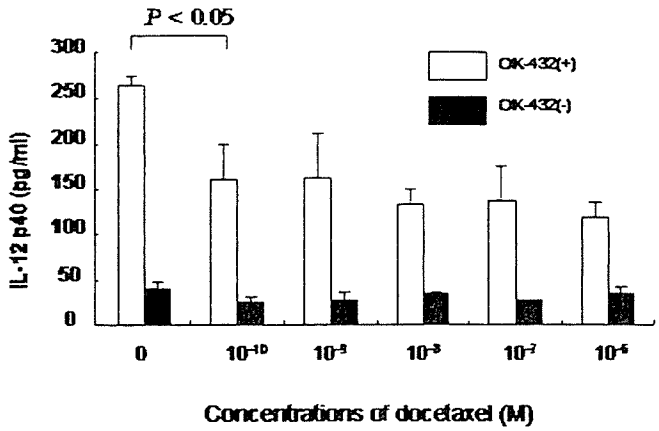

Fig. 6 Effect of TXT on IL-12 production by OK-432-stimulated Mo-DCs. ImMo-DCs treated with the indicated doses of TXT for 24 h were incubated with or without 0.02 KE/ml OK-432 at 37°C for 24 h. IL-12 p40 concentrations in the culture medium were determined by ELISA. The data presented are the means ± SD of three different wells

Effect of TXT on the expression of antigen presentation-related antigens of Mo-DC

TXT at 10^{-7} *M* did not affect the expression of antigen presentation-related antigens, including CD14, HLA-DR, CD80, and CD83, on either imMo-DCs or mMo-DCs (data not shown).

Effect of TXT on IL-12 production by Mo-DCs

When imMo-DCs capture antigens, such as streptococcal preparation OK-432, they secrete IL-12, which plays an important role in induction of CTLs [38]. Based on this finding, we examined the effect of TXT on IL-12 p40 production by imMo-DCs stimulated with OK-432. TXT at 10^{-10} *M* reduced IL-12 p40 production of imMo-DCs (Fig. 6) after coincubation with OK-432, but IL-12 p40 production from imMo-DCs without OK-432 stimulation was not affected by TXT. The data presented are representative of three independent experiments with Mo-DCs generated from three different healthy donors. OK-432 induced a considerable amount of IL-12 p70 in one of three cases. In this case, IL-12 p70 was also reduced at concentrations as low as 10^{-9} *M* (data not shown).

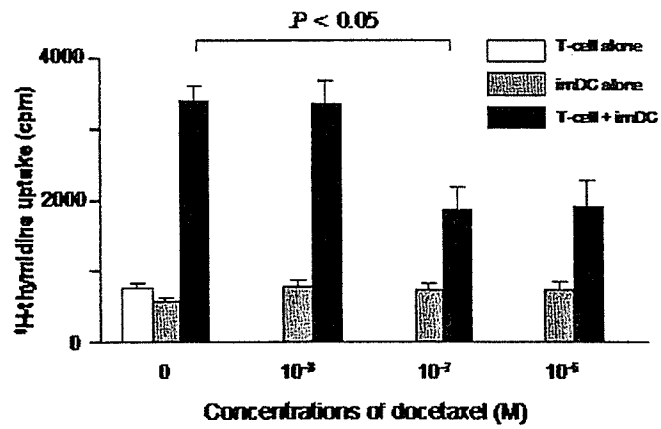

Fig. 7 Effect of TXT on allogenic T-cell stimulation by imMo-DCs. [^{3}H]-Thymidine incorporation into allogenic T cells cultured with TXT-treated imMo-DCs is shown. The data presented are the means ± SD of three different wells

Effect of TXT on imMo-DC-mediated allogenic T-cell response

To determine whether expression of antigen presentation-related molecules on imMo-DCs affects antigen-presenting potential, the ability of imMo-DCs to stimulate allogenic T-cell proliferation was determined by MLR as described in "Materials and methods". TXT at concentrations greater than 10^{-7} *M* significantly decreased [^{3}H]-thymidine uptake, suggesting that TXT decreases the ability of imMo-DCs to stimulate allogenic T-cell proliferation (Fig. 7). The data presented are representative of three independent experiments with allogenic PBMCs and Mo-DCs generated from three different healthy donors.

Discussion

In the present study, we showed that TXT at different concentrations produced different effects on antigen presentation-related functions of Mo-DCs. The effect of TXT on Mo-DC motility is particularly noteworthy.

ImDCs are capable of antigen capture [9]. However, DCs must mature and be adequately activated to become effective APCs. DCs that are insufficiently mature may induce immune tolerance rather than immune responsiveness [3]. It is generally accepted that antigen presentation-related functions of Mo-DCs are closely similar to those of DCs. Since TXT of 10^{-7} *M* did not affect Mo-DC viability, phagocytosis, or expression of antigen presentation-related surface molecules (Figs. 1 and 5), it is unlikely that patients receiving TXT in the clinic would have impaired imDC or imMo-DC function. Nevertheless, we have to recognize the possibility that TXT at high doses may weaken antigen-presenting ability, because TXT at 10^{-7} *M* suppressed allogenic T-cell proliferation by imMo-DCs (Fig. 7).

We wish to emphasize that TXT even at concentrations lower than 10^{-8} *M* impaired the motility of both imMo-DCs and mMo-DCs (Fig. 3). ImDCs respond to a large spectrum of chemokines through specific receptors. MIP-3α appears to be the most powerful chemokine guiding imDCs [8]. However, MIP-3α has no effect on imMo-DCs [3, 8]. In this

study, therefore, we chose MIP-1α as the chemokine for imMo-DCs [3]. Hotchkiss et al. [21] have reported that TXT at a very low concentration (10^{-12} *M*) reduces the migratory ability of endothelial cells. Substoichiometric binding of taxanes suppresses microtubule dynamics [10]. These findings imply that drug-mediated effects on microtubule plasticity/dynamics rather than on gross microtubule organization or expression are sufficient for inhibition of cell locomotion. Cell migration usually requires adhesion of cells to matrices such as integrins and fibronectin.

Podosomes are actin-rich adhesion structures found in monocyte-derived cells [12]. Although the roles of podosomes in cell spreading and migration and the mechanisms of their formation or dissolution are not yet clearly understood, a close relationship between microtubules and podosomes has been suggested [34]. For example, partial disassembly of microtubules leads to a more random pattern of podosome distribution [26]. Recently, it has been shown that microtubules are essential for podosome formation in human macrophages: freshly isolated monocytes undergoing adhesion fail to develop podosomes when treated with microtubule-depolymerizing drugs [26]. TXT at 10^{-8} *M* induced random podosome distribution in Mo-DCs and decreased their adhesive ability, even though TXT at this concentration only slightly impaired a radial microtubule array formation (Fig. 2). Moreover, our data suggest that TXT at concentrations as low as 10^{-9} *M* is able to alter microtubule dynamics and that a functional relationship exists between microtubules, podosomes, migration, and adhesion.

It is also noteworthy that TXT at 10^{-10} *M* reduced IL-12 p40 production from OK-432-stimulated imMo-DCs (Fig. 6). Although our previous data showed that phagocytic ability plays an important role in IL-12 production from OK-432-stimulated imMo-DCs, TXT at concentrations lower than 10^{-7} *M* did not affect phagocytosis of OK-432 by imMo-DCs (data not shown). We also note that TXT at 10^{-7} *M* did not affect IL-12 p40 production of imMo-DCs without OK-432 stimulation. These results underscore the complexity of OK-432-stimulated imMo-DC IL-12 production, which involves antigen capture, antigen processing, and finally IL-12 production.

The ability of TXT at very low concentrations to suppress the motility of DCs is important because only motile DCs are capable of inducing primary sensitization against specific antigens in naive T cells. When patients undergo chemotherapy with taxanes, we should pay more attention to their immunity. TXT-induced immunosuppression may increase the risk of infection in patients. Moreover, in the future, Mo-DCs are likely to be used in immunotherapy in combination with taxanes for treatment of cancer. The possibility that taxane treatment will decrease Mo-DC motility and consequently Mo-DC vaccine efficacy will have to be carefully considered.

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Combination Therapy with Tumor Cell-pulsed Dendritic Cells and Activated Lymphocytes for Patients with Disseminated Carcinomas

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Abstract. *This phase I study was performed to assess the safety and immune response of tumor cell-pulsed dendritic cell (DC) vaccine therapy against cancer patients with multiple metastases. DCs, generated from adherent cells of peripheral blood mononuclear cells (PBMCs) using interleukin-4 (IL-4) and granulocyte/monocyte colony-stimulating factor, were loaded with autologous necrotic whole tumor cells. Thereafter, the DCs were matured with culture supernatants of OK-432-stimulated PBMCs. Activated lymphocytes were also induced from non-adherent cells of PBMCs using OKT-3 and IL-2. Patients received a subcutaneous injection of DCs loaded with tumor cells every 2 weeks and received an intravenous injection of activated lymphocytes every 4 weeks. This combination therapy was named tumor-pulsed DC vaccine therapy. Tumor-pulsed DC vaccine therapy was continued as long as possible in 19 patients. No particular adverse reactions, except for low-grade fever, were found. The patients could be divided into two groups according to the survival time, i.e., 6 responders (long survival patients) and 13 non-responders (short survival patients). Based on the laboratory data of responders, eligibility criteria were determined. Using the eligibility criteria, a phase I/II study was recently performed with 15 patients. A delayed-type hypersensitivity reaction against tumor-pulsed DCs became positive in 13 of the 15 patients within 6 months after the therapy. This therapy was again safe, and no evidence of autoimmune disease was noted. The survival time of these 15 patients was significantly prolonged compared with that of the 13 non-responders of the phase I study ($p<0.0001$). This continuous tumor-pulsed DC vaccine therapy was well tolerated in patients with disseminated carcinomas.*

Although it has been shown that tumors possess antigenicity, which can induce specific immunity against tumors, immunotherapy targeting these antigenic molecules is successful only in limited cases (1). Recent advances in immunology have highlighted the importance of understanding the complex interactions between innate immunity and acquired immunity for the establishment of successful cancer immunotherapy. Among immune cells, the main players are dendritic cells (DCs) (2-4). Consequently, DC-based vaccine therapies with DCs loaded with various tumor-associated antigens (TAAs), such as tumor lysate (5), tumor-derived peptides (6), synthetic peptides (7), or tumor-derived RNA (8), are now under way. Synthetic peptides are very useful as antigen sources against known TAAs of target tumors. However, it is believed that the antigenicity of tumors is heterogeneous, and that some tumor cells do not contain the target TAAs (9, 10). Therefore, we used DCs loaded with whole tumor cells that contain both known and unknown TAAs (11). DC-based vaccine therapy is usually of 6-month duration (12, 13), however, in this clinical trial, the DC vaccine therapy was continued for as long as possible.

Materials and Methods

Patients. This phase I study included 19 inoperable cancer patients with multiple metastases, according to a protocol approved by the

Abbrevations: PBMCs, peripheral blood mononuclear cells; DCs, dendritic cells; TAAs, tumor-associated antigens; DHT, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; PR, partial response; SD, stable disease; PD, progressive disease.

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Key Words: Dendritic cells, activated lymphocytes, vaccine, autologous tumor cells.

Table I. *Patient characteristics and adverse events (phase I study).*

Patient no.	Age (yrs)/ Gender	Site of primary tumor	Site of metastases	Previous therapy	Adverse reaction
1	38/Male	Rectum	Lung, bone	Chemotherapy	Low-grade fever
2	49/Male	Bile-duct	Lung, Pl, LN	Chemotherapy	None
3	65/Female	Pancreas	Pt, Liver	Chemotherapy	None
4	46/Female	Stomach	Pt	Chemotherapy	Low-grade fever
5	72/Female	Pancreas	Liver, Pt, skin	Chemotherapy	None
6	72/Male	Large intestine	Liver, LN	None	Low-grade fever
7	56/Female	Gall bladder	Liver, Pt	Chemotherapy	Low-grade fever
8	49/Male	Gall bladder	Liver	None	None
9	73/Male	Gall bladder	Liver, Pt	Chemotherapy	Low-grade fever
10	41/Male	Stomach	Pt, LN	None	None
11	49/Female	Rectum	Pt, bone	Chemotherapy	Eosinophilia
12	49/Male	Stomach	Pt	None	Low-grade fever
13	45/Female	Rectum	Pt, bone	None	None
14	54/Female	Pancreas	LN	None	Low-grade fever
15	73/Male	Lung	Lung, skin, LN	Chemotherapy	None
16	66/Male	Stomach	Pt	None	Low-grade fever
17	68/Male	Lung	Lung, LN	Chemotherapy	None
18	54/Female	Ovary	Pl, Pt	Chemotherapy	None
19	65/Male	Rectum	Lung, Pt	None	None

Pl: pleural membrane, LN: lymph node, Pt: peritoneum.

Kyushu University Ethics Committee, Japan. Inclusion criteria were: histologically confirmed cancer, not amenable to cure by any standard therapy; performance status of 0, 1 or 2 on the ECOG scale; a minimum estimated life expectancy of 3 months; adequate hematological, hepatic and renal function; age>18 years; presence of obtainable tumor cells. The clinical details of the patients are summarized in Table I. Based on data of the phase I trial, a phase I/II trial was recently performed with 15 patients who satisfied at least 2 of the 4 following eligibility criteria: absolute lymphocyte count more than 1,000/μl, serum total protein level more than 6 g/dl, hemoglobin more than 10 g/dl, and a positive PPD skin test. The clinical details of the patients are summarized in Table II.

Study design. Each patient received a subcutaneous injection of 2-30x10^6 mature DCs loaded with necrotic tumor cells into the left supraclavicular area, every 2 or 3 weeks. Intravenous injection of 1-5x10^8 OKT3/IL-2-activated lymphocytes was combined with the above DC vaccine every 4 weeks. This combination therapy has been named tumor-pulsed DC vaccine therapy. In principle, this tumor-pulsed DC vaccine therapy was continued for as long as possible in the outpatient clinic.

Preparation of DCs loaded with necrotic tumor cells. Peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis with a COBE spectrum apheresis system (GAMBRO BCT, Inc, CL, USA). PBMCs were suspended at a cell density of 4x10^6 cells/ml in GMP-grade RPMI 1640 (Hy-Media; Nipro, Tokyo, Japan) supplemented with 1% human albumin, and 500 μl of the cell suspension were cultured for 4 h in 24-well culture plate. After non-adherent cells had been removed, the adherent cells were further cultured in Hy-Media containing 1% human albumin, 100 ng/ml of recombinant human granulocyte/monocyte colony-stimulating factor (GM-CSF; North China Pharmaceutical group Corporation-Gene Tech, China) and 50 ng/ml of recombinant human interleukin-4 (IL-4; Osteogenetics, Wuerburg, Germany) for 7 days. After 7 days, the cells were harvested as immature DCs.

Table II. *Patient characteristics and clinical outcome (phase I/II study).*

Patient no.	Age (yrs)/ Gender	Site of primary tumor	Site of metastases	Prognosis (Months)
1	40/Male	Rectum	Lung, bone	22, alive
2	49/Female	Rectum	Lung, Pt	14, dead
3	65/Male	Large intestine	Lung, Pt	16, alive
4	43/Male	Stomach	Bone, LN	11, dead
5	65/Female	Pancreas	Liver, Pt	10, dead
6	50/Female	Unknown	Liver, Pt	6, dead
7	55/Female	Pancreas	Pt, LN	15, alive
8	75/Male	Lung	Lung, Pl	7, alive
9	65/Male	Large intestine	Adrenal, LN	6, alive
10	45/Female	Stomach	Lung, Pt	5, dead
11	50/Male	Thymus	Lung, Pt	10, dead
12	55/Female	Stomach	Pt	6, alive
13	54/Female	Breast	Lung, Pt	6, alive
14	53/Female	Stomach	Pt, LN	6, alive
15	49/Female	Breast	Lung, Pt	4, alive

Pl: pleural membrane, LN: lymph node, Pt: peritoneum.

Tumor specimens were obtained from the tumor mass or malignant effusions by surgical biopsy or parasenthesis, respectively. The tumor specimens were minced mechanically without chemical digestion. Thereafter, they were resuspended in 2

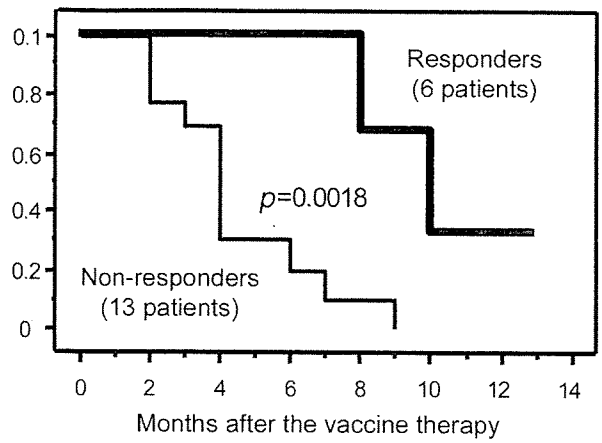

Figure 1. *Survival curves of the 19 patients enrolled in the phase I trial. According to the eligibility criteria described in Materials and Methods, 19 patients were divided into 6 suited patients (responders) and 13 unsuited patients (non-responders).*

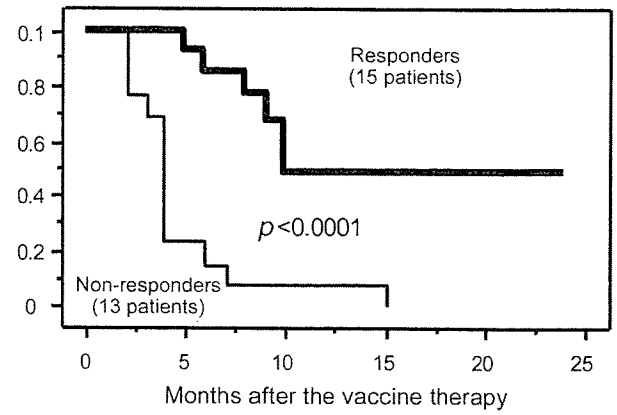

Figure 2. *Survival curves of the 15 patients who suited the eligibility criteria (phase I/II trial). The non-responders are the same as the non-responders of the phase I trial.*

ml of RPMI 1640 and lysed by 5 freeze and thaw cycles. The lysed cells (necrotic tumor cells) were used as a TAAs source.

Immature DCs were incubated with necrotic tumor cells overnight (DCs:tumor=5-10:1) and further cultured for 2 days in the medium containing 40% OK-432-induced PBMC culture supernatants to induce mature DCs (tumor cell-pulsed DCs). OK-432-induced PBMC culture supernatants were prepared by 1-day coculture of PBMCs (10^6/ml) of healthy volunteers and OK-432 (0.05 KE/ml) (14).

Preparation of activated lymphocytes. Non-adherent cells of patient's PBMCs were cultured for 2 weeks with Hy-medium containing 175 JRU/ml human recombinant IL-2 (Nipro) and immobilized monoclonal antibody to CD3 (10 μg/ml, OKT-3; Jansen-Kyowa, Tokyo, Japan).

Fluorescence-activating cell sorter (FACS) analysis. DCs ($1x10^5$) were suspended in 100 μl of diluted fluorescein-isothiocyanate or phycoerythrin-conjugated monoclonal antibodies (CD40, CD80, CD83, CD86, HLA-A, B and C, HLA-DR; Becton Dickinson, CA, USA) and assayed as described previously using a flow cytometer (FACS Caliber; Becton Dickinson) (14). The data were analyzed with CellQuest v3.2.1f1 (Becton Dickinson).

Measurement of serum tumor markers. Serum levels of tumor markers, including carcinoembryonic antigen (CEA), carbohydrate-antigen 19-9 (CA19-9), alpha fetoprotein (AFP) and DUPAN-2, were determined by enzymed-linked immunosorbent assay (ELISA).

Delayed-type hypersensitivity (DTH) reaction. One million tumor-pulsed DCs were injected intradermally into the forearm every 4 weeks. A positive DTH skin-test reaction was defined as >5 mm diameter erythema with induration 48 h after the DCs injection.

Enzyme-linked immunospot (ELISPOT) assay. Interferon-γ (IFN-γ)-producing PBMCs were assessed using an ELISPOT assay kit according to the manufacturer's protocol (Diaclone Research, Besancon, France) (15). Briefly, PBMCs ($5x10^4$ cells), together with tumor cell-pulsed or non-pulsed DCs (10^4 cells), were plated on nitrocellulose 96-well plates (Millipore, Bedford, MA, USA) coated with anti-IFN-γ antibody and incubated for 15 h at 37°C. After removal of the cells, bound IFN-γ could be detected *via* a secondary biotinylated antibody. Streptavidine alkaline phosphatase binds to biotin and is detected *via* the BCIP/NBT substrate. The spots were counted with a stereomicroscope.

Clinical outcome. A partial response (PR) was defined as a decrease in all measurable tumor tissue of over 50% for at least 4 weeks without any new sign of disease. Stable disease (SD) was defined as a decrease in measurable tumor tissue of less than 50% and an increase of less than 25%. In this study, SD continuing for more than 6 months was named long SD.

Statistical analysis. The data were analyzed with a SAS statistical software package. Categorical variables were compared using Fisher's exact test. *P*-values less than 0.05 were considered statistically significant. The estimated probability of survival was demonstrated using the Kaplan-Meier method. The Mantel Cox log-rank test was used to compare curves between responders and non-responders.

Results

Phase I study. Nineteen patients, including 5 large intestinal cancer, 4 gastric cancer, 4 biliary tract cancer, 3 pancreatic cancer, 2 lung cancer and 1 ovarian cancer, were entered into this phase I trial. All of these patients were evaluated as progressive disease (PD) at the time of entry to the study (Table I). The patients received the tumor-pulsed DC vaccine therapy for as long as possible. No patient had to leave the study for safety reasons during this trial period of 1 year, because no severe adverse

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.

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).

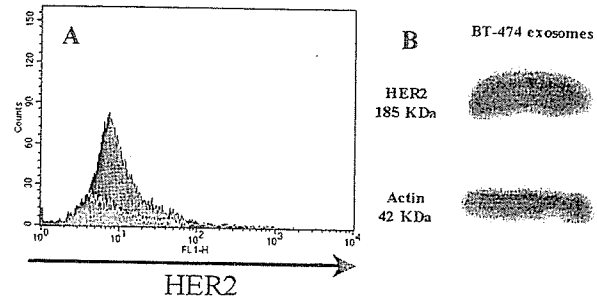

Figure 1. *FACS and Western blot analysis of HER2. A. HER2 protein in BT-474-derived exosomes. Filled histogram, Tu-exosomes with FITC-conjugated anti-HER2 antibody; open histogram, Tu-exosomes with FITC-conjugated isotype-matched antibody. B. Western blot analysis of proteins extracted from Tu-exosomes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

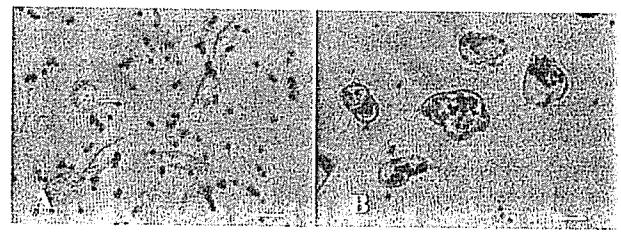

Figure 2. *Phase contrast images of Ab-beads cocultured with breast cancer cell lines. (A, MDA-MB-231 cells; B, BT-474 cells). Experiments were performed in triplicate with similar results. Bar=10 μm.*

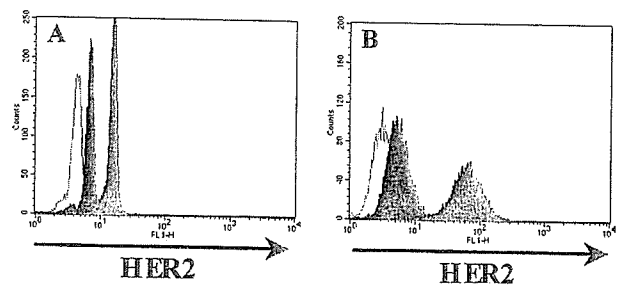

Figure 3. *FACS analysis of HER2 protein. A. Tu-exosomes, bound to Ab-beads, derived from BT-474 cells (gray) or MDA-MB-231 cells (black) with FITC-conjugated anti-HER2 antibody. Tu-exosomes, bound to Ab-beads, derived from BT-474 cells with FITC-conjugated isotype-matched antibody (open). B. BT-474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

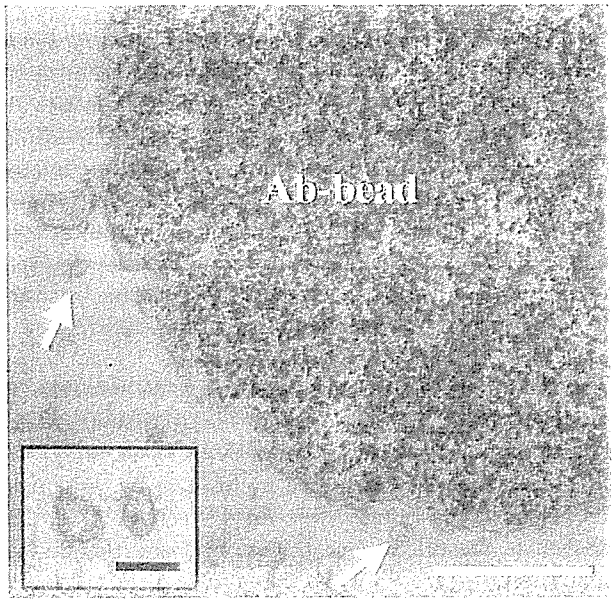

Figure 4. *Electron microscopic image of exosomes derived from BT-474 cells. Ultrathin sections of exosomes derived from BT-474 cells bound to Ab-beads were viewed with a transmission electron microscope. Small vesicles (arrows) are bound to the surface of an Ab-bead; bar=500 nm. Inset, two vesicles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

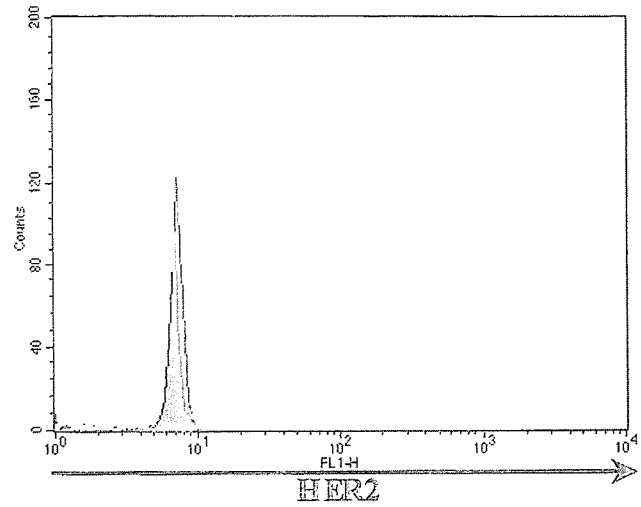

Figure 5. *FACS analysis of HER2 protein in exosomes isolated with Ab-beads from ascites of an ovarian cancer patient. Filled histogram, exosome-bead complexes with FITC-conjugated anti-HER2 antibody; open histogram, exosome-bead complexes with FITC-conjugated isotype-matched antibody.*

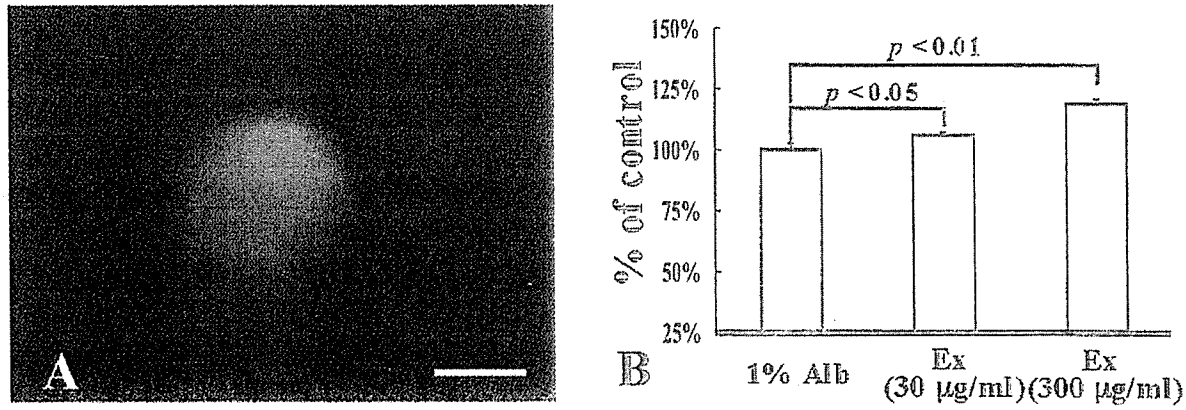

Figure 6. *Data from cell proliferation assay (MTT assay). A, Fluorescence microscopic image of exosomes derived from BT-474 cells cocultured with BT-474 cells. PKH26-labelled exosomes (Red) bound to PKH67-labelled BT-474 cell surface (Green); bar=10 μm. B, Exosomes derived from BT-474 cells increase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