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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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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).

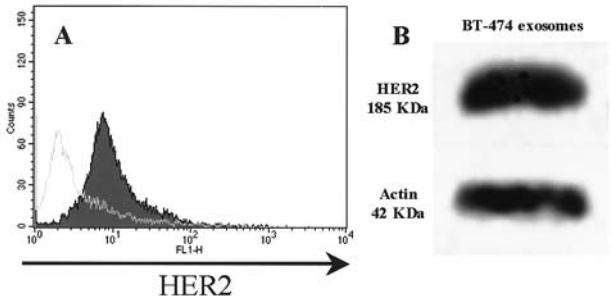


Figure 1. *FACS and Western blot analysis of HER2. A. HER2 protein in BT-474-derived exosomes. Filled histogram, Tu-exosomes with FITC-conjugated anti-HER2 antibody; open histogram, Tu-exosomes with FITC-conjugated isotype-matched antibody. B. Western blot analysis of proteins extracted from Tu-exosomes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

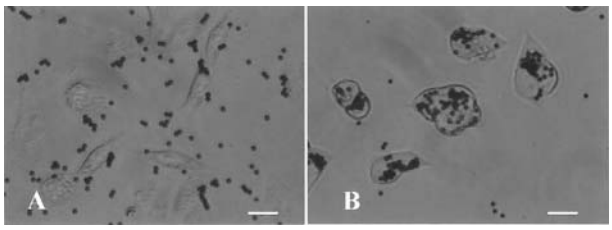


Figure 2. *Phase contrast images of Ab-beads cocultured with breast cancer cell lines. (A, MDA-MB-231 cells; B, BT-474 cells). Experiments were performed in triplicate with similar results. Bar=10 μm.*

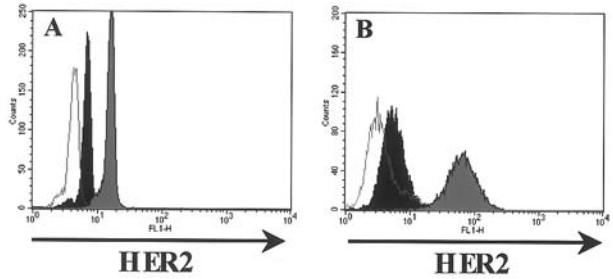


Figure 3. *FACS analysis of HER2 protein. A. Tu-exosomes, bound to Ab-beads, derived from BT-474 cells (gray) or MDA-MB-231 cells (black) with FITC-conjugated anti-HER2 antibody. Tu-exosomes, bound to Ab-beads, derived from BT-474 cells with FITC-conjugated isotype-matched antibody (open). B. BT-474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

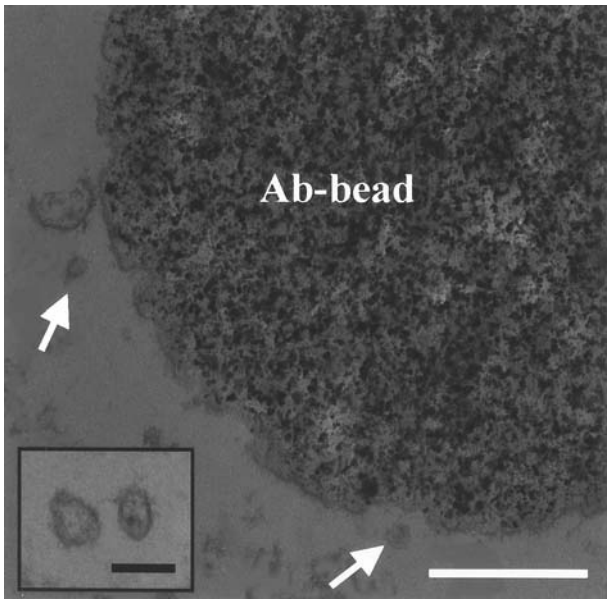


Figure 4. *Electron microscopic image of exosomes derived from BT-474 cells. Ultrathin sections of exosomes derived from BT-474 cells bound to Ab-beads were viewed with a transmission electron microscope. Small vesicles (arrows) are bound to the surface of an Ab-bead; bar=500 nm. Inset, two vesicles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

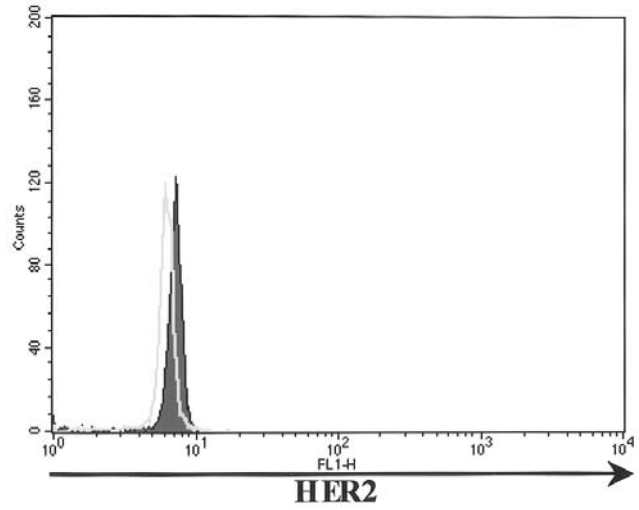
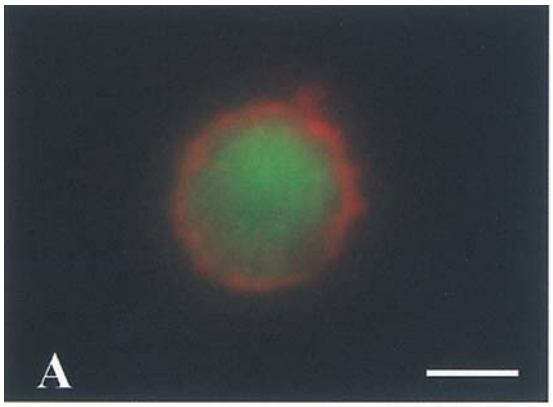


Figure 5. *FACS analysis of HER2 protein in exosomes isolated with Ab-beads from ascites of an ovarian cancer patient. Filled histogram, exosome-bead complexes with FITC-conjugated anti-HER2 antibody; open histogram, exosome-bead complexes with FITC-conjugated isotype-matched antibody.*



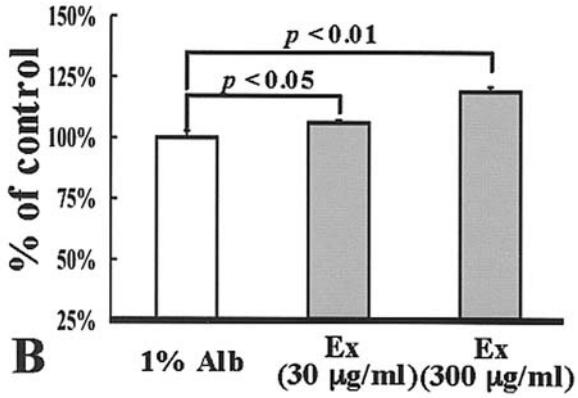


Figure 6. *Data from cell proliferation assay (MTT assay). A, Fluorescence microscopic image of exosomes derived from BT-474 cells cocultured with BT-474 cells. PKH26-labelled exosomes (Red) bound to PKH67-labelled BT-474 cell surface (Green); bar=10 µm. B, Exosomes derived from BT-474 cells increase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

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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