

Glycosyl-phosphatidylinositol-anchored interleukin-2 expressed on tumor-derived exosomes induces antitumor immune response in vitro

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ABSTRACT

Aims and background. Tumor-derived exosomes (TEXs) have been considered as a new kind of cancer vaccine, but the antitumor effects are not satisfactory. In order to improve the efficacy of TEXs, we investigated whether exosomes derived from glycosyl-phosphatidylinositol-anchored interleukin 2 (GPI-IL-2) gene-modified bladder cancer cells can increase the antitumor effects.

Methods and study design. We transfected melanoma antigen-1 (MAGE-1)-expressing T24 tumor cells with a plasmid encoding GPI-IL-2 and prepared the TEXs. Exosomes expressing GPI-IL-2 were characterized by electron microscope and Western blot analysis.

Results. IL-2 was present on the cell surface in the GPI-anchored form as demonstrated by fluorescent microscope and ELISA analyses. Exosomes expressing GPI-IL-2 naturally contained bioactive GPI-IL-2 and tumor-associated antigen MAGE-1. Moreover, exosomes expressing GPI-IL-2-pulsed dendritic cells could induce the proliferation of T cells and the antigen-specific cytotoxic T-lymphocyte immune response more efficiently.

Conclusions. GPI-IL-2 gene-modified tumor cells can make the TEXs contain GPI-IL-2, resulting in increased antitumor effects. Our study provided a feasible approach for exosome-based tumor immunotherapy. Free full text available at www.tumori-online.it

Introduction

Exosomes are vesicles of approximately 30-100 nm in diameter that are secreted by different cell types including tumor cells and antigen presenting cells¹⁻³. Exosomes are formed by reverse budding of the membrane of late endosomes or multivesicular bodies and are released to the extracellular space by fusion of multivesicular bodies with the plasma membrane^{4,5}. Tumor-derived exosomes (TEXs) have been reported to have potential therapeutic efficacy as cancer vaccines. TEXs are enriched in antigen presentation molecules (MHC class I and II molecules, HSPs, costimulatory molecules), cell targeting molecules (CD11b, ICAM, lactadherin), and tumor antigens (OVA, Her2/neu, MART-1, PSA and gp100)⁶⁻⁸. So TEXs are a source of antigens for antigen-presenting cells and participate in antigen presentation to T lymphocytes, leading to antitumor immune responses^{1,8}. The immunogenic potential and efficiency of TEXs remain to be further improved to obtain satisfactory curative effects⁶. Therefore, we investigated the efficiency of exosomes derived from glycosyl-phosphatidylinositol-anchored interleukin 2 (GPI-IL-2) gene-modified bladder cancer cells.

Key words: exosomes, GPI, IL-2, cytotoxic T lymphocytes.

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IL-2 is a pivotal mediator of tumor-protective immune responses as it promotes the survival, proliferation, and functional differentiation of several lymphocyte subsets including natural killer (NK) cells, T lymphocytes, and lymphokine-activated killer cells. Preclinical and clinical studies have shown that IL-2 alone or combined with other treatments can exert strong antitumor effects⁹⁻¹¹. However, the short half-life of IL-2 and the severe toxicity caused directly or indirectly by it have been an obstacle to the development of routine treatment protocols for clinical application. A means of prolonged and sustained delivery of IL-2 therefore needs to be found.

GPI anchoring has been established as a unique mode of protein binding to the plasma membrane via a common lipid structure¹². Unlike conventional polypeptide anchors, which have different transmembrane domains and connect to specific cytoplasmic extensions, GPI is a posttranslationally added lipid anchor. Studies have shown that GPI-anchored proteins such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-12, IL-4 and B7-1 were effective in stimulating immune responses when they were engineered onto the N-terminus of GPI and expressed on cell membranes¹³⁻¹⁵. Therefore, GPI anchoring may be a possible strategy to engineer immunogenic molecules onto the membrane of exosomes.

In this study we examined the possibility that exosomes can be modulated to carry GPI-IL-2 fusion proteins for efficient cancer immunotherapy. We characterized and evaluated the potential role of these GPI-IL-2-containing exosomes as a cell-free cancer vaccine. We found that exosomes engineered to contain GPI-IL-2 induced proliferative response and antitumor immune response of cytotoxic T lymphocytes (CTLs) more efficiently. These results suggest that exosomes from tumor cells genetically modified with cytokine adjuvants have the potential to be used as cancer vaccines.

Materials and methods

Reagents and cell lines

The human bladder cancer cell line T24 was purchased from the Institute of Cell Research, Shanghai, China. Amicon ultrafiltration centrifuge tubes (500 kDa) were purchased from Millipore, USA. Mouse anti-heat shock protein 70 (HSP70) polyclonal antibody, mouse anti-intercellular adhesion molecule-1 (ICAM-1) polyclonal antibody, and MAGE-1 polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc, USA. Recombinant human GM-CSF (rhGM-CSF) and recombinant human interleukin 4 (rhIL-4) were purchased from PeproTech, USA. Human IL-2 and IFN- γ ELISA were purchased from Bender MedSystems, USA. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Sigma, USA. AlamarBlue was purchased from BioSource International, Inc, USA.

Establishment of a stable cell line expressing GPI-IL-2

A fusion gene of a DNA oligo encoding GPI-anchor signal sequence of human placental alkaline phosphatase-1 attaching to the 3' end of human IL-2 cDNA was generated by annealing 2 synthesized cDNA oligos with Hind III at the 5' end and BamH I at the 3' end, and inserted into pEGFP-N1 plasmid, resulting in pEGFP-N1-GPI-IL-2 plasmid. pEGFP-N1-GPI-IL-2 and pEGFP-N1 were transfected into T24 tumor cells using LipofectamineTM2000 transfection reagents according to the manufacturer's protocol. After 48 hours of transfection, stable cell lines were selected in RPMI 1640 culture medium containing 400 g/mL geneticin (G418) for 3-4 weeks, and named T24/GPI-IL-2 and T24/p. To determine whether GPI-IL-2 was expressed in the transfectants, fixed cells were washed twice and incubated with rabbit anti-human IL-2 for 40 minutes at room temperature. After washing twice to remove unbound antibody, the TRITC-conjugated sheep anti-rabbit IgG antibody was added and incubated for 45 minutes in the dark. Fluorescence was determined by fluorescence microscopy. ELISA assays were used to measure the amount of GPI-anchored IL-2 expressed on the cell surface. This was performed as described previously¹⁶, and 1×10^7 T24/GPI-IL-2 cells, T24/p cells, and T24 cells were harvested. After 2 washings with PBS, each cell pellet was treated with 0.2 mL of GPI-specific lipase (PI-PLC) solution or PBS and incubated for 1 hour, then the supernatants were analyzed by human IL-2 ELISA kit.

Exosome purification

Cells were cultured for 48 hours in fresh medium with 10% exosome-free fetal calf serum obtained by overnight ultracentrifugation ($100,000 \times g$). Supernatants were collected and centrifuged successively at $300 \times g$ for 10 minutes, $1,000 \times g$ for 30 minutes, and $10,000 \times g$ for 30 minutes. After having been concentrated by ultrafiltration through a 500 kD MWCO hollow Millipore fiber membrane, the exosome-enriched fluid was purified by ultracentrifugation on a 30% sucrose D₂O cushion at $100,000 \times g$ for 2 hours. The collected sucrose layer was filtered through a 0.2- μ m filter after resuspension in a large volume of PBS for the final ultracentrifugation step. Exosomes prepared from T24/GPI-IL-2 cells, T24/p cells, and T24 cells were termed Ex/GPI-IL-2, Ex/p, and Ex, respectively. The protein concentrations of exosomes were measured by Bradford assay.

Electron microscopy and Western blot analysis of exosomes

A 10- μ L drop of exosomes was pipetted onto a Formvar-coated copper grid and allowed to stand for 5 minutes at room temperature. The grid was stained for 1 minute with 2% uranyl acetate. Excess fluid was removed and the grid

was allowed to air dry for 10 minutes before viewing by transmission electron microscopy. Western blot was performed as described previously¹⁷. Briefly, exosomes (20 µg/lane) were separated on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. They were then stained with primary antibodies directed against MAGE-1, ICAM-1 and HSP70. The labeled proteins were visualized using horseradish peroxidase-conjugated secondary antibodies and an ECL kit. Human IL-2 ELISA kits were used to measure the amount of GPI-IL-2 expressed in the exosomes.

Culture of dendritic cells (DCs)

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by centrifugation on Ficoll-Hypaque. PBMCs were suspended in RPMI 1640 medium containing 10% human AB serum at 37 °C in 5% carbon dioxide on 6-well culture plates. After incubation for 2 hours, adherent cells were washed 3 times in PBS and cultured in RPMI 1640 medium containing 1000 IU/mL rhGM-CSF and 500 IU/mL rhIL-4. On day 7, loosely adherent cells were used for scanning electron microscope analysis and further studies.

Mixed lymphocyte reaction

On day 7, 1×10^5 DCs were collected and pulsed with 100 µL exosomes for 3 hours in 100 L of culture medium. DCs were then treated with mitomycin C (50 µg/mL) for 30 minutes, washed extensively with complete RPMI 1640, and used in the proliferation assay. Peripheral blood lymphocytes (PBLs) were added at a ratio of 3 lymphocytes to 1 DC in a final volume of 200 µL/well in round-bottomed 96-well plates for 3 days, then 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) was added to the plates and cultured for 4 hours. Absorbance was measured at 450 nm using a plate reader. Groups incubated without DCs served as controls, and each experiment was repeated 3 times.

The stimulation index (SI) was calculated as the OD value of experimental groups / the OD value of control groups.

Cytotoxicity assays

We stimulated the PBLs with exosome-pulsed and mitomycin-C treated DCs in 96-well plates once a week

for a total of 2 stimulations. Seven days after the second stimulation, lymphocytes were collected and washed as effector cells, while T24 cells served as target cells. The effector cells were incubated with the target cells at 3 different effector/target (E/T) ratios of 10:1; 25:1, and 50:1. At every E/T ratio, triplicate wells of effector and target cells alone were established in parallel. Twenty microliters of AlamarBlue was then added to each well and incubated for 24 hours. Following incubation, the fluorescence of the AlamarBlue was read on a plate reader with excitation at 530 and emission at 590 nm¹⁸. IFN-γ was measured in 24-hour supernatants by ELISA. The percentage of specific lysis was calculated using the following formula: % lysis = $100 [(AF \text{ of target alone}) + (AF \text{ of effector alone}) - (AF \text{ of mix})] / (AF \text{ of target alone})$, where AF represents the mean of the absolute fluorescence units.

Statistics

Results were expressed as mean ± SD. Statistical analysis was performed with 1-way ANOVA. Statistical significance was set at $P < 0.05$. All statistical analyses were performed with SPSS version 11.5.

Results

GPI-IL-2 expression

To determine whether GPI-IL-2 fusion proteins were expressed in the cells, T24/GPI-IL-2 cells, T24/p cells and T24 cells were analyzed by fluorescence microscopy before and after indirect immunofluorescence labeling. GPI-IL-2 was expressed in T24/GPI-IL-2 cells (Figure 1) but not in T24/p cells or T24 cells. Cells were treated with PI-PLC to remove the membrane-anchored IL-2 and the amount of IL-2 in the supernatants was assayed by ELISA. T24/GPI-IL-2 cells expressed significantly higher amounts of IL-2 on the cell surface than T24/p cells and T24 cells (Figure 2). This confirmed that IL-2 could be expressed on the plasma membrane of tumor cells through the GPI anchor.

Characterization of Ex/GPI-IL-2 isolated from GPI-IL-2-transfected tumor cells

Ex/GPI-IL-2, Ex/p, and Ex were isolated and purified by serial centrifugation and sucrose gradient ultracentrifugation.

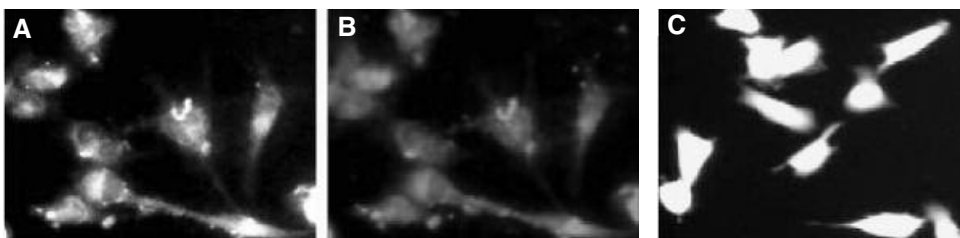


Figure 1 - Fluorescent microscopy of GPI-IL-2 expression on tumor cells (×200). A) Green fluorescence of pEGFP-N1-GPI-IL-2-transfected T24 tumor cells. B) Indirect immunofluorescence of T24/GPI-IL-2 after anti-IL-2 polyclonal antibody labeling. C) Green fluorescence in pEGFP-N1-transfected T24 tumor cells.

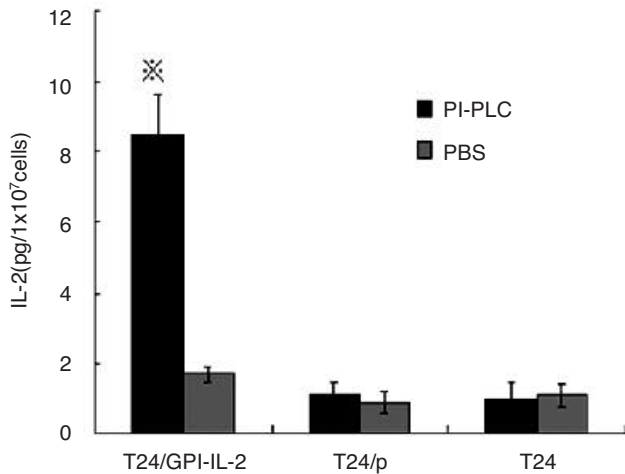


Figure 2 - GPI-IL-2 expression in T24 cells. Membrane-bound IL-2 was harvested from 1×10^7 T24/GPI-IL-2 cells, T24/p cells, and T24 cells by PI-PLC or PBS treatment, and measured with a human IL-2 ELISA kit. * $P < 0.01$

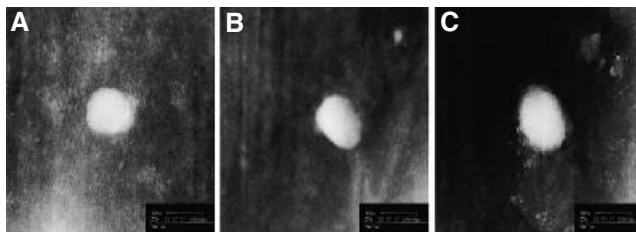


Figure 3 - Electron microscopy image of tumor-derived exosomes. Exosomes were isolated from T24 cells transfected with GPI-IL-2 gene or T24 cells without gene transfection, and then visualized by electron microscopy. They were spherical vesicles surrounded by a 2-layer lipid membrane and ranged in size between 30 and 80 nm. A) Exosomes derived from T24 cells. B) Exosomes derived from T24/p cells. C) Exosomes derived from T24/GPI-IL-2 cells. Bars = 60 nm ($\times 13, 500$).

trifugation. When visualized under an electron microscope, no significant differences in morphology and size were observed among the exosomes. They appeared as small vesicles of 30-80 nm diameter (Figure 3), which was consistent with the morphology of typical exosomes. ELISA was used to ascertain whether IL-2 could anchor onto exosomes. As shown in Figure 4A, 100 L of Ex/GPI-IL-2 contained 103.63 ± 5.23 pg IL-2, but IL-2 was not found in Ex/p and Ex. Western blotting confirmed that the exosomes contained HSP70, ICAM-1 and MAGE-1, but there was no obvious difference in the quantity of these proteins between Ex/GPI-IL-2, Ex/p, and Ex (Figure 4B). These results showed that GPI-IL-2 could be sorted into exosomes and GPI-IL-2 did not influence the protein components of exosomes.

Morphology of cultivated DCs

After about 3 days, cellular aggregates appeared which attached to the layer of adherent cells (Figure 5A). Most cells had stretched out dendritic processes and were scattered the next day. On day 7 of culture, typical DCs were seen floating in the culture medium (Figure 5B). Scanning electron microscopy was carried out for further morphological observations. These typical DCs had a variety of branching forms: most pseudopods were long and uniform in width with blunt terminations, and smaller spinous processes were also evident (Figure 5C). This indicated that DCs constantly extended and retracted many fine cell processes.

Augmentation of activated T-cell proliferation by Ex/GPI-IL-2

In the mixed-lymphocyte reaction study, SI in the Ex/GPI-IL-2-pulsed DC group was 2.78 ± 0.15 . In the Ex/p-pulsed DC group, Ex-pulsed DC group, and DC

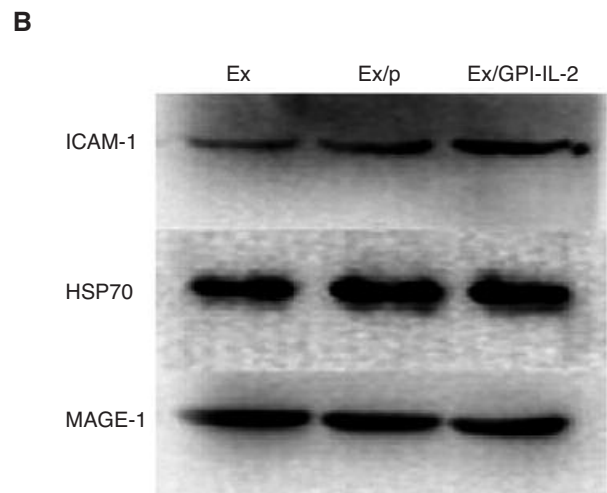
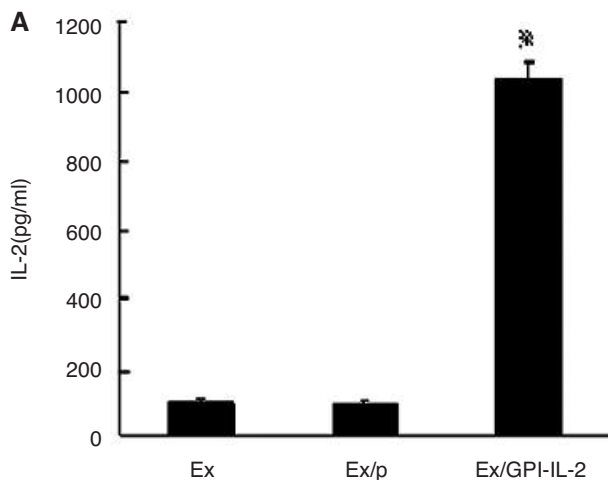


Figure 4 - Protein composition of exosomes analyzed by Western blot. A) The amount of GPI-anchored IL-2 expressed in the exosomes was measured by ELISA. * $P < 0.01$. B) Western blot assay of protein components of exosomes. Exosomal proteins were analyzed by Western blot using specific primary antibodies against ICAM-1, HSP70, and MAGE-1.

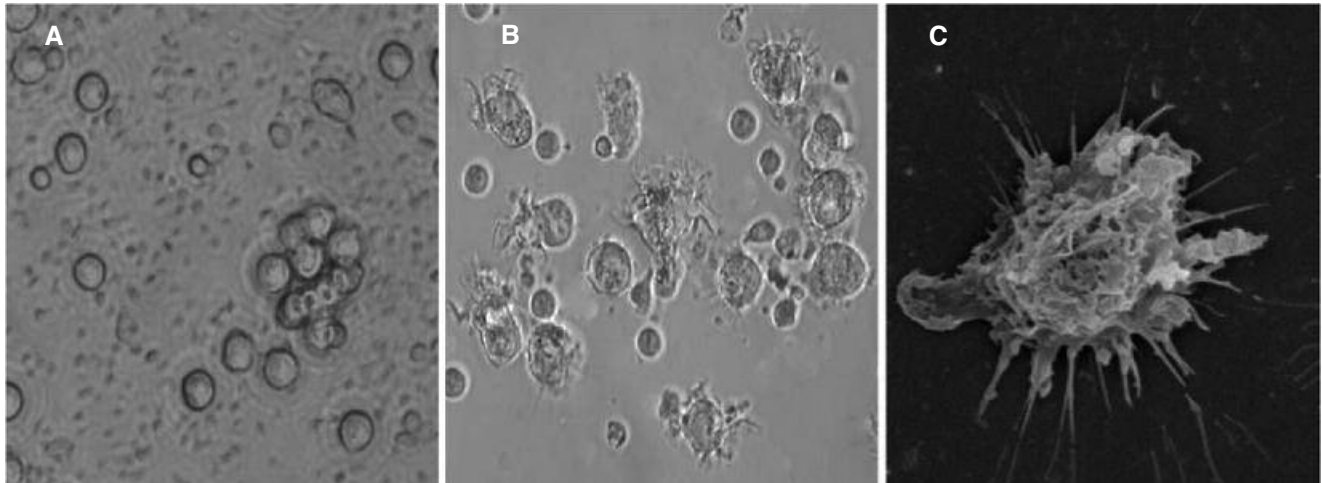


Figure 5 - Morphology of cultivated DCs. Adherent cells from PBMCs were cultured in RPMI 1640 medium containing 1000 IU/mL rhGM-CSF and 500 IU/mL rhIL-4. A) Cell aggregates appeared and attached to the layer of adherent cells (day 3, $\times 400$). B) Typical dendritic processes of DCs (day 5, $\times 400$). C) Scanning electron microscopy of DC (day 7, $\times 3000$).

group, it was 1.94 ± 0.12 , 1.92 ± 0.12 , and 1.50 ± 0.06 , respectively. The data showed statistical differences in the 4 groups ($P < 0.05$), but there was no statistical difference between the Ex/p-pulsed DC group and the Ex-pulsed DC group (Figure 6). Our results revealed that Ex/GPI-IL-2-pulsed DCs markedly induced proliferation of T cells compared with DCs of the other groups, and also indicated that the IL-2 in Ex/GPI-IL-2 was bioactive.

Ex/GPI-IL-2 increased the induction of the cytotoxic effect and release of IFN- γ by activated T cells

As observed above, Ex/GPI-IL-2 derived from MAGE-1-positive T24 cells contained MAGE-1. Cytotoxicity ex-

periments were performed that tested the efficiency of Ex/GPI-IL-2-pulsed DCs in the induction of MAGE-1-specific CTLs. The activity of MAGE-1-specific CTLs was valued by the lysis of T24 cells. IFN- γ produced by Ex/GPI-IL-2-induced CTLs was measured by ELISA. As shown in Figure 7, four groups of lymphocytes stimulated with exosome-pulsed DCs were able to lyse T24 cells, and the CTL activity was proportional to E/T in each group. The CTL activity of the Ex/GPI-IL-2-pulsed DC group was significantly higher than that of other groups, with CTL activity in the same ratio ($P < 0.01$). Correspondingly, CTL induced by Ex/GPI-IL-2-pulsed DCs produced more IFN- γ than other groups (Figure 8, $P < 0.01$). These results indicated that Ex/GPI-IL-2 could induce a CTL response more efficiently; meanwhile, MAGE-1 may be one of the tumor-shared rejection antigens in T24-cell-derived exosomes.

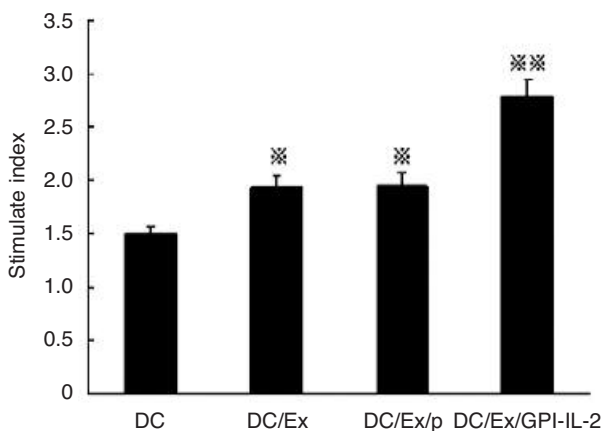


Figure 6 - Capacity of Ex/GPI-IL-2-pulsed DCs to stimulate the proliferation of autologous T cells in MLR assays. $\ast\ast P < 0.01$ compared with the other groups, $\ast P < 0.05$ compared with the control group. T-cell proliferation was determined on day 3 by addition of WST-1 to triplicate wells for 4 hours. T-cell proliferation was expressed as mean counts per minute obtained for triplicate wells.

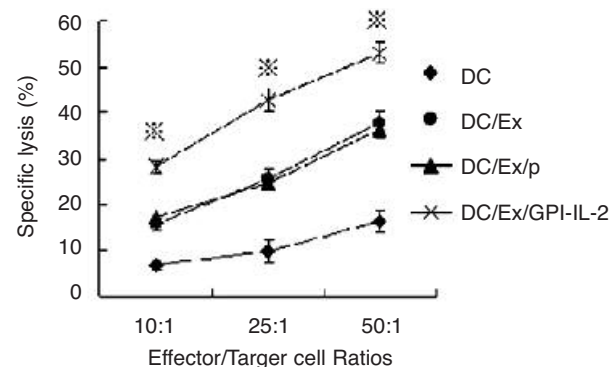


Figure 7 - Increased induction of specific CTL activity by stimulation of Ex/GPI-IL-2. Cytotoxic activity of T cells stimulated by different exosomes target to T24 cells. $\ast P < 0.01$ compared with the other groups.

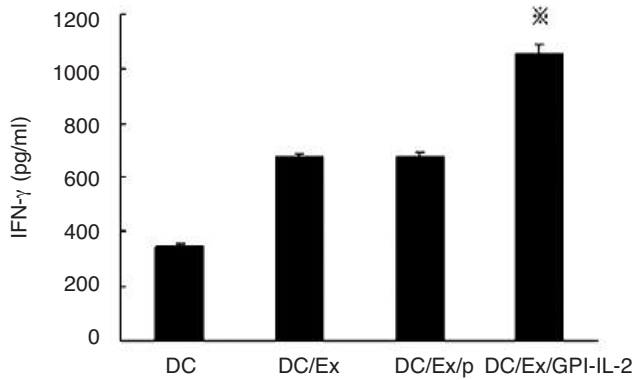


Figure 8 - IFN- γ release levels in the cytotoxicity effect. IFN- γ in supernatants was measured by ELISA. * $P < 0.01$ compared with the other groups.

Discussion

Studies have shown that exosomes are among the most promising cell-free tumor vaccines. Of notable interest, phase I trials of exosomes derived from DCs showed encouraging results in antitumor therapy^{19,20}. TEXs resemble exosomes of DC origin in their biophysical and biochemical properties. The most significant cell type-specific differences are the expression of tumor-associated antigens and/or tumor-specific antigens in TEXs^{21,22}. It was reported that none of the previous cancer immunization strategies achieved effective immunization across tumor types and MHC haplotypes except for exosomes¹. This indicates that cross-protection among different tumors might be possible. In this study, HSP70, ICAM-1 and MAGE-1 were detected in exosomes. Expression of HSP70 molecules in TEXs is a cofactor for receptor-mediated uptake, and is capable of stimulating DC maturation and T-cell immune responses^{17,23}. MAGE-1 is a member of the MAGE family of tumor-specific antigens expressed in various tumors including bladder cancer, melanoma, head and neck squamous cell carcinoma, lung carcinoma, and breast carcinoma^{24,25}. The MAGE gene family-encoded products can be recognized by autologous CTLs. Studies have shown that MAGE antigens are ideal targets for immunotherapy^{26,27}. TEXs were found to be effective in therapy studies on irrelevant allogeneic tumors¹; therefore, TEXs may be a novel source of tumor rejection antigens that could be useful for the characterization of immunorelevant tumor antigens and for cancer immunotherapy.

DCs are the most highly efficient specialized antigen-presenting cells, and DC-based tumor vaccines are regarded as having huge potential in cancer immunotherapy. Vaccines of DCs pulsed with tumor peptides, lysates or RNA, or loaded with apoptotic/necrotic tumor cells, or engineered to express certain cytokines or chemokines could induce significant antitumor CTL responses and antitumor immunity. Although exosomes

are a source of functional MHC-I-peptide complexes, they require DCs for efficient priming of MHC-restricted T-cell activation^{1,28}. One study found that exosomes can be internalized and processed efficiently by immature DCs in only 2 hours²⁹. Ligands on the exosome and DC surface may mediate the targeting of exosomes to DCs. Once internalized by DCs, exosomes were sorted into recycling endosomes and late endosomes/lysosomes. In this way, MAGE-1 and other immunorelevant tumor antigens in exosomes were processed and presented to T cells by DCs.

IL-2 remains the most effective among the large number of immunotherapeutic strategies. It has strong antitumor effects in lung, renal, prostate, and bladder cancer¹¹ when combined with other therapeutic modalities. The antitumor effect of IL-2 due to it induces the proliferation of CD4⁺ and CD8⁺ T cells by upregulating and maintains the expression of the IL-2 receptor (IL-2R) - subunit. In this study, IL-2 was successfully anchored onto the exosomes via GPI anchoring. GPI-anchored proteins are a form of protein expression of exosomes on the membrane³⁰; GPI anchored proteins can be released from the cells and reinsert onto the plasma membrane in the presence of G418³¹. This may explain why GPI-anchored IL-2 could be detected in exosomes. The experiments carried out by others³² and ourselves collectively proved that proteins of interest can be incorporated into exosomes from gene-modified tumor cells, and this may offer a new approach for the design of more effective exosome-based tumor vaccines in the future.

We suggest the following mechanisms to explain why Ex/GPI-IL-2 exhibit much more potent antitumor effects than the other exosome groups. First, Ex/GPI-IL-2 were a source of tumor rejection antigens and immunorelevant proteins. Stimulation with tumor rejection antigens can enhance the ability of T cells to respond to IL-2 by triggering the rapid upregulation of IL-2R³³; in addition, HSP70 and ICAM-1 can significantly enhance the immunogenicity of Ex/GPI-IL-2. Second, DCs express functional IL-2R³⁴, so it is possible that IL-2 in exosomes contributes to the maturation process and makes DCs highly efficient in antigen presentation³⁵. Third, DCs that captured Ex/GPI-IL-2 might release IL-2 persistently in an unknown manner, the released IL-2 then acts on the IL-2R of DCs, T cells and NK cells, and Ex/GPI-IL-2 could therefore have a longer half-life and trigger a stronger antitumor effect. As a result, the prolonged and sustained delivery of IL-2 might contribute to the much more potent antitumor effect of Ex/GPI-IL-2.

In conclusion, TEXs are a novel source of tumor rejection antigens that could be useful for cancer immunotherapy. We have demonstrated that exosomes derived from gene-modified tumor cells contain IL-2 and that Ex/GPI-IL-2 can promote PBL proliferation. More importantly, Ex/GPI-IL-2-pulsed DCs can induce

a more significant tumor-antigen-specific CTL response *in vitro*. This study provides a promising strategy to prepare exosomes containing immunogenic molecules for effective cancer immunotherapy.

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