Exosomes derived from Rab27a-overexpressing tumor cells elicit efficient induction of antitumor immunity

WENHAI LI[1*], DEGUANG MU[2*], FENG TIAN[1*], YUNSHENG HU[3], TAO JIANG[1], YONG HAN[1], JIAKUAN CHEN[1], GUOLIANG HAN[1] and XIAOFEI LI[1]

Departments of [1]Thoracic Surgery, [2]Respiratory Medicine and [3]Orthopaedic Surgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710038, P.R. China

Received July 2, 2013; Accepted October 10, 2013

DOI: 10.3892/mmr.2013.1738

Abstract. Lung cancer is the leading cause of mortality worldwide. However, there is a lack of effective therapeutic strategies. Currently, tumor immunotherapy based on exosomes, which are secreted by a variety of cell types including tumor cells, has drawn particular attention and are suggested to have the potential for exploitation in tumor therapy. Nevertheless, the therapeutic efficacy mediated via tumor cell-derived exosomes is not satisfactory. Rab27a, one of the Rab family of small GTPases, has been suggested to be important in exosome secretion. Thus, the purpose of the present study was to examine whether exosomes derived from Rab27a-overexpressing cells elicited more potent antitumor immunity. A Rab27a-overexpressing line was established via transfection of a Rab27a overexpression vector into the human non-small-cell lung cancer cell line, A549. Exosomes were isolated and the typical exosomal protein markers, CD9, CD63, heat shock protein (Hsp) 70 and Hsp90, were found to be enriched in the exosomes derived from Rab27a-overexpressing cells. Subsequently, these exosomes were demonstrated to be capable of upregulating major histocompatibility complex class II molecules as well as the co-stimulatory molecules CD80 and CD86 on dendritic cells (DCs), suggesting that more potent maturation of DCs was induced. Furthermore, DCs loaded with exosomes derived from Rab27-overexpressing cells significantly promoted $CD4^+$ T cell proliferation *in vitro*. In addition, *in vivo* immunization of exosomes derived from Rab27a-overexpressing cells inhibited tumor growth in a mouse model. It was also demonstrated that splenocytes from mice immunized with exosomes derived from Rab27-overexpressing cells expressed high levels of type I cytokines, including IL-2 and IFN-γ, which are important in the regulation of cell-mediated antitumor immunity. Collectively, it was demonstrated that exosomes derived from Rab27a-overexpressing cancer cells elicited more potent antitumor immune effects, which may provide novel insights for the development of efficient exosome-based cancer vaccines.

Correspondence to: Dr Wenhai Li or Dr Xiaofei Li, Department of Thoracic Surgery, Tangdu Hospital, The Fourth Military Medical University, 1 Xinsi Road, Xi'an, Shaanxi 710038, P.R. China
E-mail: li_wenhai@126.com
E-mail: xiaofeili_xian@163.com

*Contributed equally

Abbreviations: NSCLC, non-small-cell lung cancer; HSP, heat shock protein; DCs, dendritic cells; MHC, major histocompatibility complex

Key words: non-small-cell lung cancer, exosomes, Rab27a, tumor immunotherapy

Introduction

Lung cancer is the leading cause of mortality worldwide (1), with ~80% of lung cancers being non-small-cell lung cancers. Previously, it was demonstrated that lung cancer, not only has a high incidence of recurrence, but also poor overall survival (2). However, in spite of substantial progress made with traditional therapy, there remains a lack of effective therapeutic strategies. Therefore, novel potential therapeutic treatments should be developed.

Based on the concept of the tumor immune response (3-5), development of novel agents is crucial in order to re-engage the immune system to recognize and kill tumor cells. Exosomes secreted by immune or tumor cells have drawn particular attention and have been suggested to have the potential for exploitation in tumor immunotherapy (6,7). Exosomes are a type of nano-sized membrane vesicle with a diameter of 50-90 nm, first identified in the process of reticulocyte maturation, that discard unwanted membrane proteins such as the transferrin receptor (8-10). More recently, it has been demonstrated that exosomes are secreted by a wide range of cell types such as astrocytes, neurons and epithelial cells, in addition to tumor and immune cells [reviewed in (11)]. Exosomes contain various types of proteins that are inherited from their parental cells, such as tetraspanins and chaperones [Heat shock protein (Hsp) 70 and Hsp90] (12). Moreover, exosomes derived from different cell types exhibit selective enrichment of specific proteins. For example, exosomes secreted from antigen-presenting cells were enriched in major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules (13). In addition to proteins,

functional RNAs and lipids have also been observed in exosomes (14,15). The interaction between exosomes and target cells may result in the direct stimulation of target cells. Thus, exosomes are suggested to be important in cell-to-cell communication and are considered ideal agents to engage the immune response (16). Studies have demonstrated that exosomes derived from immune cells are able to directly activate $CD4^+$ or $CD8^+$ T cells (17,18). Exosomes derived from tumor cells containing tumor antigens are able to transfer tumor antigens to dendritic cells (DCs), eliciting a specific antitumor effect (6). Therefore, exosomes derived from tumor cells have been suggested as a novel type of cancer vaccine.

However, the therapeutic efficacy of tumor cell-derived exosomes is unsatisfactory. Thus, additional improvement of exosomes to induce more potent antitumor effects is of particular importance. Rab27a, one of the Rab family of small GTPases, was suggested to be important in exosome secretion (19). Rab proteins are reported to control vesicular trafficking of different steps, such as budding, motility and docking, as well as fusion of various vesicular transport intermediates (20). Rab27a was also suggested to regulate azurophilic granule exocytosis of neutrophils and NADPH oxidase activity (21). Defects in Rab27a have been demonstrated to result in an immunodeficiency disorder, characterized by impaired cytotoxic T-lymphocytes and natural killer cell function (22,23). Knockdown of Rab27a in mice impaired the secretion of myeloperoxidase stimulated by lipopolysaccharides (LPSs) *in vivo* (24). Thus, whether exosomes from Rab27 expression-modified tumor cells have unique effects on antitumor immune activation remains unknown.

In the present study, investigation of the antitumor effect of exosomes derived from Rab27a-overexpressing tumor cells was conducted. A Rab27a-overexpressing cell line was initially established via transfection of a Rab27a overexpression vector in the human non-small-cell lung cancer line, A549. Exosomes were isolated and the typical exosomal protein markers CD9, CD63, Hsp70 and Hsp90 were identified as highly expressed compared with exosomes from control tumor cells. Subsequently, exosomes from Rab27a-overexpressing cells were demonstrated to be capable of inducing more potent maturation of MHC class II molecules and the co-stimulatory molecules CD80 and CD86, which were all highly upregulated in DCs. Furthermore, DCs loaded with exosomes derived from Rab27-overexpressing cells significantly promoted $CD4^+$ T-cell proliferation *in vitro*. In addition, *in vivo* immunization with exosomes derived from Rab27-overexpressing cells inhibited tumor growth in a tumor mouse model. It was also demonstrated that splenocytes from mice immunized with exosomes derived from Rab27-overexpressing cells expressed high levels of type I cytokines, including IL-2 and IFN-γ, which are important in the regulation of cell-mediated antitumor immunity. Collectively, it was demonstrated that exosomes derived from Rab27a-overexpressing cancer cells elicited more potent antitumor immune effects, which may provide novel insights for the development of efficient exosome-based cancer vaccines.

Materials and methods

Animals. BALB/c mice (specific pathogen-free grade, five weeks old, weighing 25-30 g) were obtained from the Medical Experimental Animal Center (Guangdong, China). The mice were raised under standard conditions of room temperature, dark-light cycle and humidity with free access to water. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University (Xi'an, China). All efforts were made to minimize suffering.

Cell culture. The human non-small-cell lung cancer cell line, A549 (SunBio Biomedical Technology Co., Ltd., Shanghai, China), was maintained in RPMI-1640 (Wuhan Boster Biological Technology, Ltd., Wuhan, China) supplemented with 10% fetal bovine serum. The cells were cultured at 37˚C in an incubator (Life Technologies, Baltimore, MD, USA) containing 5% CO_2.

Vector construction and transfection. Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Up to 5 μg of the total RNA was reverse-transcribed into cDNA using SMART MMLV reverse transcriptase (Clontech, Palo Alto, CA, USA). The cDNAs were used as templates for amplification of Rab27a using two designed primers (forward: 5'-aaatatgcggccgcatgtctgatggagattatgattacc-3' and reverse: 5'-ccgctcgagtcaacagccacatgccccttctctcc-3') according to the open reading frame of the Rab27a cDNA sequence (GenBank accession no. CR536496.1). The cloned Rab27a DNA fragments were subcloned into pCMV-Sport6 (Invitrogen, Carlsbad, CA, USA) using the *Not*I and *Xho*I restriction sites. The Rab27a DNA sequences in the recombinant vectors were confirmed by DNA sequencing (Sangon Biotech, Shanghai, China). For transfection, cells were seeded in a six-well culture plate ($2x10^5$ cells/well) and incubated at 37˚C with 5% CO_2 until the cells reached 80% confluence. Experimental procedures were performed according to the manufacturer's instructions. Briefly, plasmid DNA (1 μg) was diluted in 500 μl fresh medium with 5 μl lipofectamine (Invitrogen), mixed and incubated at room temperature for 15 min. Subsequently, the mixtures were applied to the cells at a final volume of 3 ml medium. The cells were cultured under the standard conditions for 24 h.

Exosome isolation. Exosomes were isolated according to previously reported protocols (14) with minimal modification. Briefly, cell culture supernatants were collected at the indicated times by centrifugation (800 x g for 5 min and 12,000 x g for 20 min at 4˚C) and the collected supernatants were filtrated via a 0.22-μm diameter pore filter (Millipore, Billerica, MA, USA). Subsequently, ultracentrifugation at 110,000 x g for 3 h at 4˚C was performed. Thereafter, the ultracentrifuged pellets in the bottom of the tube were collected and resuspended in phosphate-buffered saline (PBS; sterile filtered), followed by ultracentrifugation at 110,000 x g for 2 h. Subsequently, the final pellets were collected and resuspended in sterile-filtered PBS and stored at -80˚C for further use. The total protein concentrations of the exosomes were quantified using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

Western blot analysis. A total of 20-30 μg of protein was fractionated by 12% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was treated by agitation and blocking at room temperature with 2% non-fat dry milk in Tris-buffered saline (TBS) for 1 h, followed by incubation with primary antibodies (Rab27a, CD9, CD63, HSP70 and HSP90 antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; CD80, CD86 and MHC class II antibodies from Abcam, Cambridge, UK) diluted (1: 10,000) in blocking buffer at 4˚C overnight and then washed three times with TBS and Tween (TBST; 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 10 min each time at room temperature. Thereafter, the membrane was incubated in the corresponding horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Wuhan Boster Biological Technology, Ltd.; diluted 1:5,000 in the blocking buffer) for 1 h. After being washed three times with TBST and once with TBS, each for 10 min, 1 ml 4-chloro-1-naphthol as a HRP substrate with 9 ml TBS and 6 μl H_2O_2 was used to visualize the target protein in the dark for 5-30 min.

DC generation and stimulation. Mouse bone marrow-derived DCs were obtained from a BALB/c mouse as described previously (25). The bone marrow cells were cultured in RPMI-1640 media with granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/ml) and IL-4 (10 ng/ml). On day 3, floating cells were discarded and fresh medium was added. On day 6, non-adherent and loosely adherent cells with the typical morphological features of DCs were collected for subsequent use. Immature DCs (~$5x10^5$) were resuspended in 1 ml complete culture medium supplemented with GM-CSF (10 ng/ml) and IL-4 (1 ng/ml), and incubated with exosomes at the indicated concentrations for 24 h. DCs stimulated with PBS or LPS (1 μg/ml) were used as controls. Cytokine levels in the culture supernatants were analyzed using the corresponding cytokine ELISA kit (Shanghai BlueGene Biotech Co., Ltd., Shanghai, China) according to standard procedures. For the protein expression analysis, cell lysates were detected by western blot analysis using the indicated antibodies.

$CD4^+$ T-cell proliferation by [^{3}H]-thymidine incorporation. $CD4^+$ T cells were collected from the spleens of BALB/c mice immunized with A549 cells (100 μg cell lysate per week) at day 6. The cells were purified with immunomagnetic beads (Miltenyi Biotech, Cologne, Germany) followed by culturing in complete RPMI-1640 media. Subsequently, DCs pulsed with exosomes were added ($CD4^+$ T cells/DCs: 10/1) and incubated for 56 h. Thereafter, 0.5 μCi [^{3}H]-thymidine per well was added, followed by another 16 h of continuous culture. Subsequently, [^{3}H]-thymidine uptake was detected by a MicroBeta counter (Beckmen Coulter, Krefeld, Germany).

Tumor inoculation and exosome challenge of mice. BALB/c mice were injected with exosomes (10 μg each time) or PBS (as the control) into the left groin subcutaneously on day -13, -11, -9 and -7. On day 0, a dose of $5x10^5$ A549 cells was injected into the right leg of each mouse close to the groin. The tumor volume, indicated by the length and width, was then determined each day post-tumor inoculation. To investigate the effect of exosomes on pre-established tumor models, BALB/c mice were injected with $3x10^5$ A549 cells on day 0. Exosomes or PBS was injected into the left groin on days +7, +9, +11 and +13. The tumor volume is presented as length x $width^2$ x $\pi/6$.

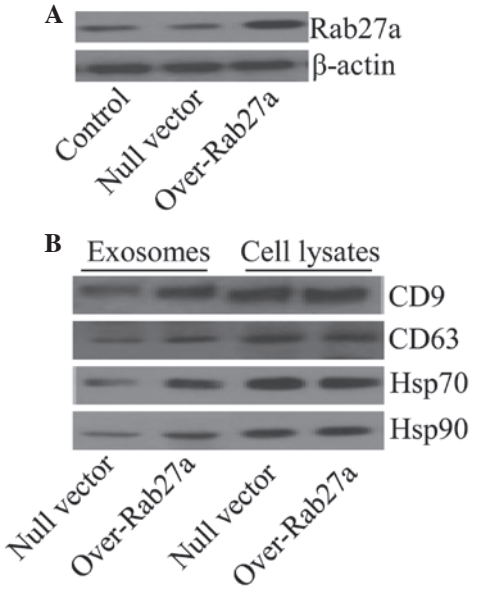

Figure 1. Effect of Rab27a overexpression on exosomes. (A) Western blot analysis was performed to detect Rab27a protein expression in control cells (without treatment), null vector-transfected cells and cells receiving the Rab27a overexpression vector. β-actin was used as an internal control. The results confirmed that Rab27a was highly expressed in the cells transfected with the Rab27a overexpression vector compared with the controls. (B) Typical exosome protein marker expression determined by western blot analysis in exosomes and in total cell lysates using the indicated antibodies. A total of 10 μg of protein from exosomes or from cell lysates transfected with either the null vector or the Rab27a overexpression vector were used for analysis. Three independent experiments were performed. Exosomes from Rab27a-overexpressing cells exhibited high levels of CD9, CD63, Hsp70 and Hsp90 compared with the controls. Hsp, heat shock protein.

Statistical analysis. Assays were performed in triplicate and data are presented as the mean ± standard error of the mean. Differences between groups were analyzed by the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. Two asterisks (**) denote $P<0.01$ and one asterisk (*) denotes $P<0.05$.

Results

Overexpression of Rab27a increases the levels of selected exosomal proteins. To assess the effect of Rab27a on exosome secretion, a Rab27a overexpression vector was transfected into the non-small-cell lung cancer cell line A549. The expression levels of Rab27a in the cells were determined by western blot analysis and the results confirmed that Rab27a was highly expressed in the cells transfected with the Rab27a overexpression vector compared with the controls (Fig. 1A). Reportedly, Rab27a is responsible for exosome secretion (19); thus, in order to determine whether the overexpression of Rab27a affected the levels of particular molecules in exosomes, the typical exosomal protein markers CD9, CD63, Hsp70 and Hsp90 (26) were screened by western blot analysis. The

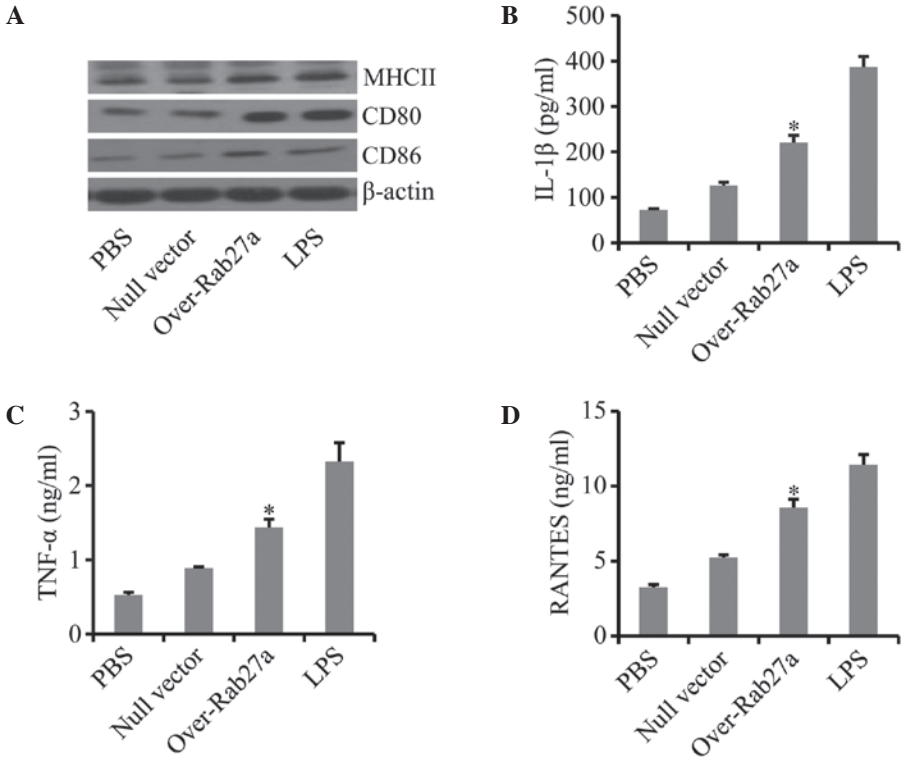

Figure 2. Effect of exosomes on DC maturation. Immature BMDCs were incubated with exosomes (10 μg/ml) derived from null vectors or Rab27a overexpression vector-transfected cells for 48 h. DCs stimulated with PBS or LPS (1 μg/ml) were used as the controls. (A) Western blot analysis was used to detect the DC activation markers MHCII, CD80 and CD86 using the indicated antibodies. DCs incubated with exosomes derived from Rab27a-overexpressing cells expressed higher levels of MHC class II, CD80 and CD86 compared with the controls The cytokines (B) IL-1β, (C) TNF-α and (D) RANTES in the supernatants were detected by ELISA. Results showed that increased levels of these cytokines were induced. Three independent experiments were performed. Data are expressed as the mean ± SEM of triplicates. *P<0.05 vs. cells receiving only PBS or a null vector was considered to indicate a statistically significant difference. MHC, major histocompatibility complex; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; DC, dendritic cell; BMDC, bone marrow DC.

results showed that exosomes from Rab27a-overexpressing cells exhibited high levels of CD9, CD63, Hsp70 and Hsp90 compared with exosomes from the control cells. In addition, the levels of all these proteins in the parental cells were not affected (Fig. 1B).

Exosomes derived from Rab27a-overexpressing cells induced more potent maturation of DCs. Exosomes derived from cancer cells containing Hsps have been found to induce more potent immune activation (27). Thus, it was hypothesized that exosomes derived from Rab27-overexpressing cancer cells that harbored increased levels of Hsp70 and Hsp90 would induce more potent immune activation. The effect of the exosomes was analyzed on DCs, which are the professional antigen-presenting cells *in vivo*. The DCs were incubated with the exosomes for 48 h, and MHC class II molecules as well as the co-stimulatory molecules CD80 and CD86 in the DCs were examined by western blot analysis. The data showed that the DCs incubated with exosomes derived from Rab27a-overexpressing cells expressed higher levels of MHC class II, CD80 and CD86 compared with the controls (Fig. 2A), suggesting that exosomes derived from Rab27-overexpressing cells had a stronger effect on DC maturation. Furthermore, exosomes derived from Rab27-overexpressing cells also induced significantly higher levels of cytokines, including IL-1β (Fig. 2B), TNF-α (Fig. 2C) and RANTES, compared with the controls (Fig. 2D). These results indicated that exosomes derived from Rab27-overexpressing cells had a more potent immune-stimulatory activity.

DCs loaded with exosomes derived from Rab27a-overexpressing cells significantly promoted CD4$^+$ T-cell proliferation. To confirm that exosomes derived from Rab27a-overexpressing cells elicit more potent immune activation, the effect of DCs loaded with exosomes derived from Rab27a-overexpressing cells on the activation of CD4$^+$ T cells was determined. CD4$^+$ T cells were purified from the spleens of mice immunized with A549 cell lysates and co-incubated with DCs that were pre-pulsed with exosomes. Cell proliferation was evaluated by measuring the uptake of [^{3}H]-thymidine. The results showed that the proliferation of CD4$^+$ T cells was markedly enhanced by DCs loaded with exosomes derived from Rab27a-overexpressing cells (Fig. 3).

Exosomes derived from Rab27a-overexpressing cells induce an efficient antitumor effect. To examine whether exosomes derived from Rab27a-overexpressing cells induced efficient antitumor effects, mice were pre-immunized with exosomes and then challenged with A549 cells. The results showed that immunization with exosomes derived from Rab27a-overexpressing cells significantly inhibited tumor growth *in vivo* (Fig. 4A), compared with exosomes from cells transfected only with the null vector or treated with PBS (P<0.01). Furthermore, the effect on tumor growth

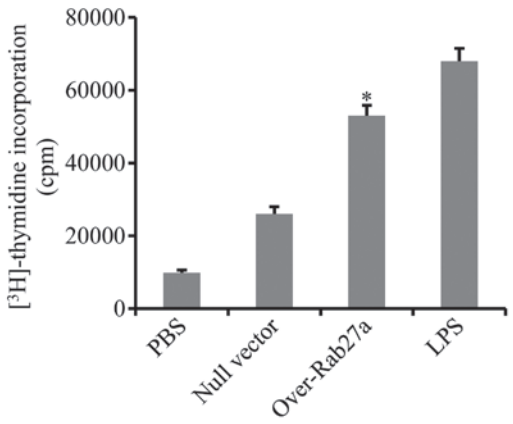

Figure 3. $CD4^+$ T-cell proliferation induced by exosomes derived from Rab27a-overexpressing cells. $CD4^+$ T cells were purified from the spleens of BALB/c mice immunized with A549 cell lysates at day seven. The cultured $CD4^+$ T cells were incubated with DCs pre-pulsed with exosomes (10 μg/ml) derived from cells transfected with either a null vector or the Rab27a overexpression vector. DCs stimulated with PBS or LPS (1 μg/ml) were used as the controls. The $CD4^+$ T cells and DCs were co-cultured for 56 h, then 0.5 μCi of [^{3}H]-thymidine per well was applied and the cells were continuously cultured for another 16 h. Cell proliferation was evaluated by measuring the uptake of [^{3}H]-thymidine (cpm). Each cell proliferation assay was performed in triplicate and repeated three times. *P<0.05 vs. cells receiving only PBS or the null vector was considered to indicate a statistically significant difference. Proliferation of $CD4^+$ T cells was markedly enhanced by DCs loaded with exosomes derived from Rab27a-overexpressing cells. PBS, phosphate-buffered saline; LPS, lipopolysaccharide; DC, dendritic cell; cpm, counts per minute.

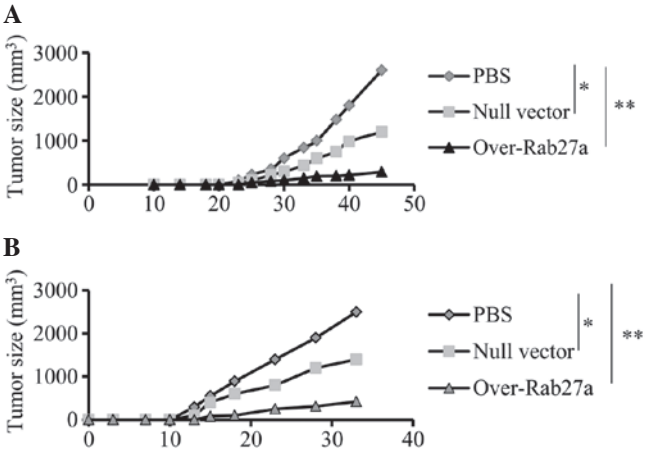

Figure 4. Effect of exosomes derived from Rab27a-overexpressing cells on tumor growth. (A) BALB/c mice (n=9 per group) were pre-injected with exosomes (10 μg) or PBS on day -13, -11, -9 and -7. On day 0, a dose of $5x10^5$ A549 cells was injected into the right leg of each mouse close to the groin. (B) BALB/c mice (n=9 per group) were injected with $3x10^5$ A549 cells on day zero. Exosomes or PBS was injected into the left groin on days +7, +9, +11 and +13. The tumor volume, indicated by length and width, was then determined each day post-tumor inoculation. *P<0.05 and **P<0.01 denote statistically significant differences. Exosomes derived from Rab27a-overexpressing cells induced stronger antitumor effects. PBS, phosphate-buffered saline.

in a pre-established tumor model was also examined and similar inhibitory effects on tumor growth were observed (Fig. 4B). The results indicated that exosomes derived from Rab27a-overexpressing cells induced stronger antitumor effects than normal exosomes.

Exosomes derived from Rab27a-overexpressing cells induced strong production of type I cytokines. To confirm whether exosomes derived from Rab27-overexpressing cells induced effective antitumor immunity, type I cytokines that are critical in cell-mediated antitumor immunity were screened (27). Splenocytes derived from exosome-immunized mice were re-stimulated with A549 cells *in vitro*. The levels of type I cytokines IL-2 and IFN-γ were then measured. The results showed that the concentrations of IL-2 (Fig. 5A) and IFN-γ (Fig. 5B) were higher in the splenocytes of mice immunized with exosomes derived from Rab27a-overexpressing cells than with exosomes derived from normal cells.

Discussion

Results of the present study have demonstrated that exosomes derived from Rab27a-overexpressing cancer cells induced more potent antitumor effects. The improved antitumor efficacy may be due to their enrichment in molecules that contribute to the induction of immune activation, such as Hsp70 or Hsp90 and tumor antigens. However, this theory requires further elucidation.

Exosomes derived from heat-shocked lymphoma cells have been demonstrated to contain increased amounts of molecules

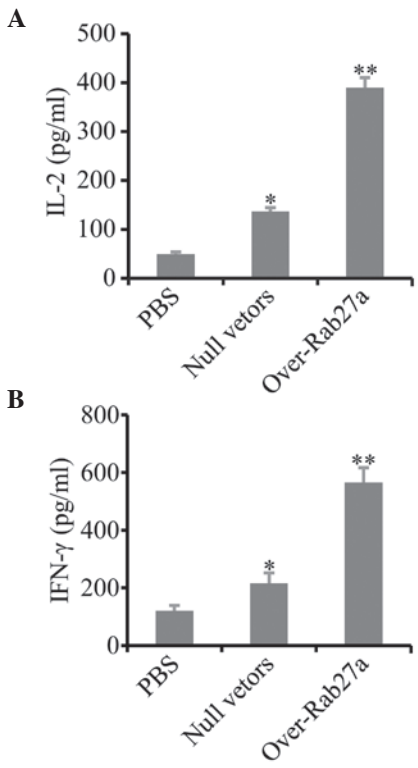

Figure 5. Effect of exosomes derived from Rab27a-overexpressing cells on the cytokine production of splenocytes. Splenocytes derived from mice one week after their final exosome immunization were incubated with inactivated A549 cells at a ratio of 10:1 for 48 h. The supernatants were then collected for detection of (A) IL-2 and (B) IFN-γ by the ELISA method. Treatment with PBS was used as the control. Three independent experiments were performed. Data are expressed as the mean ± SEM of triplicates. *P<0.05 and **P<0.01 indicate statistically significant differences. Concentrations of IL-2 (A) and IFN-γ (B) were higher in the splenocytes of immunized mice. PBS, phosphate-buffered saline.

involved in immunogenicity, including Hsp60, Hsp90, MHC class I and II, CD40 and CD86, and induce efficient antitumor T-cell immunity (27). In accordance, similar effects were obtained in the present study by modification of Rab27a expression in parental cancer cells. Recently, it has been reported that exosomes derived from anticancer drug-treated cancer cells elicit effective natural killer cell antitumor responses *in vitro* (28). Thus, it appears that improvement of exosome-mediated immune activation by modification of their parental cells is feasible.

Notably, in the present study it was demonstrated that overexpression of Rab27a increased the levels of certain molecules in secreted exosomes without affecting the total amounts in the parental cancer cells. Silencing Rab27a has been demonstrated to block exosome secretion and downregulate protein levels of exosomal markers, including HLA-DR, CD63, Tsg101 and Hsc70, in human HeLa cells (19). However, the size and morphology of the exosomes was unaffected. Rab proteins are reported to control vesicular trafficking at different steps, such as budding, motility and docking, as well as the fusion of various vesicular transport intermediates (20). Rab27a has also been suggested to regulate azurophilic granule exocytosis by neutrophils and NADPH oxidase activity (21). Defects in Rab27a resulted in an immunodeficiency disorder exhibiting impaired cytotoxic T-lymphocytes and natural killer cell function (22,23). Knockdown of Rab27a in mice was shown to impair the secretion of myeloperoxidase stimulated by LPS *in vivo* (24). Rab27a has previously been demonstrated to be involved in the regulation of the secretion of secretory granules and lysosome-associated organelles in a variety of cell types (29-31). It is of note that knockdown of Rab27a blocks exosome secretion in mammary carcinoma cells, which results in the inhibition of tumor growth as Rab27a-dependent exosomes are able to modify the tumor microenvironment for the promotion of tumor growth (32). Silencing of Rab27a inhibited melanoma exosome secretion as well as angiogenic growth factors, resulting in the inhibition of tumor growth and metastasis (33). Considering these findings, it may be hypothesized that exosomes secreted in a Rab27a-dependent manner are more tumorigenic. Overexpression of Rab27 in cancer cells may render secreted exosomes greater immunogenicity, and thus elicit more potent antitumor immunity.

The results of the present study showed that exosomes derived from Rab27a-overexpressing cells induced a stronger immune response and, compared with the controls, it markedly enhanced maturation of DCs. Levels of MHC molecules and the co-stimulatory molecules CD80 and CD86 were found to be highly increased. The levels of cytokines, including IL-1β, TNF-α and RANTES, were also highly induced. Hsps have been shown to directly stimulate the maturation of DCs (34). In the present study, high levels of Hsp70 and Hsp90 were observed in the exosomes derived from Rab27a-overexpressing cells, which may be partly responsible for DC maturation. Moreover, DCs pulsed with exosomes were shown to be capable of boosting $CD4^+$ T cell proliferation, suggesting that they are able to induce strong T cell responses. Thus, it was hypothesized that exosomes derived from Rab27a-overexpressing cells may induce efficient antitumor immune effects *in vivo*. As expected, it was observed that immunization with exosomes from Rab27a-overexpressing cells significantly inhibited tumor growth *in vivo* in a mouse model. It was also determined that type I cytokines were highly induced, which are important in tumor suppression via activation of macrophages and CTL (35).

Exosomes have been proposed to be an ideal source of tumor antigens and have potential roles in tumor immunotherapy (36). As expected, cell-free tumor vaccines based on exosomes have been exploited in a number of experimental animals and cancer patients. It has been reported that use of DEX stimulates a specific T-cell response and promotes natural killer lytic-activity in non-small cell lung carcinoma-bearing patients (37). A method for ovarian cancer treatment via combination of tumor-associated ascites-derived exosomes and a Toll-like receptor 3 agonist has also been described and evaluated (7). Ascites-derived exosomes in combination with GM-CSF for immunotherapy of colorectal cancer was shown to induce a beneficial tumor-specific antitumor cytotoxic T-lymphocyte (CTL) response with well-tolerated side effects (38). Therefore, efforts have been made to improve the efficacy of exosome-based tumor vaccines. Exosomes derived from DCs pulsed with lymphocytic leukemia cell antigen in combination with cyclophosphamide and a sodium salt of polyinosinic-polycytidylic acid markedly induced spleen cell proliferation and cytotoxic effects *in vitro* and suppressed tumor growth *in vivo* (39). Notably, a study showed that intradermal immunization with exosomes induced more effective CTL than subcutaneous administration (40). It is possible that the efficiency of exosomes in antitumor immunity is not only associated with the processing protocol but also the source cell type, as a study has demonstrated that exosomes derived from ovalbumin (OVA)-pulsed DCs elicited more efficient antitumor immunity than exosomes derived from tumor cells expressing OVA (41).

In conclusion, results of the present study have demonstrated that exosomes derived from Rab27a-overexpressing cancer cells elicit effective antitumor immunity, providing novel insights for the development of efficient exosome-based cancer vaccines. However, more studies are required to confirm these findings.

References

1. Jemal A, Siegel R, Ward E, *et al*: Cancer statistics, 2007. CA Cancer J Clin 57: 43-66, 2007.
2. Schiller JH, Harrington D, Belani CP, *et al*: Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 346: 92-98, 2002.
3. Thomas L: On immunosurveillance in human cancer. Yale J Biol Med 55: 329-333, 1982.
4. Kaplan DH, Shankaran V, Dighe AS, *et al*: Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci USA 95: 7556-7561, 1998.
5. Shankaran V, Ikeda H, Bruce AT, *et al*: IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410: 1107-1111, 2001.
6. Wolfers J, Lozier A, Raposo G, *et al*: Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med 7: 297-303, 2001.
7. Navabi H, Croston D, Hobot J, *et al*: Preparation of human ovarian cancer ascites-derived exosomes for a clinical trial. Blood Cells Mol Dis 35: 149-152, 2005.
8. Pan BT and Johnstone RM: Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell 33: 967-978, 1983.

9. Harding C, Heuser J and Stahl P: Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 97: 329-339, 1983.
10. Pan BT, Teng K, Wu C, Adam M and Johnstone RM: Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol 101: 942-948, 1985.
11. Yang C and Robbins PD: The roles of tumor-derived exosomes in cancer pathogenesis. Clin Dev Immunol 2011: 842849, 2011.
12. Stoorvogel W, Kleijmeer MJ, Geuze HJ and Raposo G: The biogenesis and functions of exosomes. Traffic 3: 321-330, 2002.
13. Mignot G, Roux S, Thery C, Ségura E and Zitvogel L: Prospects for exosomes in immunotherapy of cancer. J Cell Mol Med 10: 376-388, 2006.
14. Valadi H, Ekström K, Bossios A, *et al*: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9: 654-659, 2007.
15. Subra C, Laulagnier K, Perret B and Record M: Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. Biochimie 89: 205-212, 2007.
16. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A and Ratajczak MZ: Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20: 1487-1495, 2006.
17. Zitvogel L, Regnault A, Lozier A, *et al*: Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med 4: 594-600, 1998.
18. Raposo G, Nijman HW, Stoorvogel W, *et al*: B lymphocytes secrete antigen-presenting vesicles. J Exp Med 183: 1161-1172, 1996.
19. Ostrowski M, Carmo NB, Krumeich S, *et al*: Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 12: 19-30; sup pp 11-13, 2010.
20. Zerial M and McBride H: Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2: 107-117, 2001.
21. Johnson JL, Brzezinska AA, Tolmachova T, *et al*: Rab27a and Rab27b regulate neutrophil azurophilic granule exocytosis and NADPH oxidase activity by independent mechanisms. Traffic 11: 533-547, 2010.
22. Griscelli C, Durandy A, Guy-Grand D, *et al*: A syndrome associating partial albinism and immunodeficiency. Am J Med 65: 691-702, 1978.
23. Klein C, Philippe N, Le Deist F, *et al*: Partial albinism with immunodeficiency (Griscelli syndrome). J Pediatr 125: 886-895, 1994.
24. Munafó DB, Johnson JL, Ellis BA, *et al*: Rab27a is a key component of the secretory machinery of azurophilic granules in granulocytes. Biochem J 402: 229-239, 2007.
25. Guo J, Wang B, Zhang M, *et al*: Macrophage-derived chemokine gene transfer results in tumor regression in murine lung carcinoma model through efficient induction of antitumor immunity. Gene Ther 9: 793-803, 2002.
26. Chaput N, Taïeb J, Schartz NE, *et al*: Exosome-based immunotherapy. Cancer Immunol Immunother 53: 234-239, 2004.
27. Chen W, Wang J, Shao C, *et al*: Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. Eur J Immunol 36: 1598-1607, 2006.
28. Lv LH, Wan YL, Lin Y, *et al*: Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. J Biol Chem 287: 15874-15885, 2012.
29. Desnos C, Schonn JS, Huet S, *et al*: Rab27A and its effector MyRIP link secretory granules to F-actin and control their motion towards release sites. J Cell Biol 163: 559-570, 2003.
30. Stinchcombe JC, Barral DC, Mules EH, *et al*: Rab27a is required for regulated secretion in cytotoxic T lymphocytes. J Cell Biol 152: 825-834, 2001.
31. Barral DC, Ramalho JS, Anders R, *et al*: Functional redundancy of Rab27 proteins and the pathogenesis of Griscelli syndrome. J Clin Invest 110: 247-257, 2002.
32. Bobrie A, Krumeich S, Reyal F, *et al*: Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. Cancer Res 72: 4920-4930, 2012.
33. Peinado H, Alečković M, Lavotshkin S, *et al*: Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 18: 883-891, 2012.
34. Cho BK, Palliser D, Guillen E, *et al*: A proposed mechanism for the induction of cytotoxic T lymphocyte production by heat shock fusion proteins. Immunity 12: 263-272, 2000.
35. Fowler DW, Copier J, Wilson N, Dalgleish AG and Bodman-Smith MD: Mycobacteria activate γδ T-cell anti-tumour responses via cytokines from type 1 myeloid dendritic cells: a mechanism of action for cancer immunotherapy. Cancer Immunol Immunother 61: 535-547, 2012.
36. Tan A, De La Peña H and Seifalian AM: The application of exosomes as a nanoscale cancer vaccine. Int J Nanomedicine 5: 889-900, 2010.
37. Morse MA, Garst J, Osada T, *et al*: A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. J Transl Med 3: 9, 2005.
38. Dai S, Wei D, Wu Z, *et al*: Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. Mol Ther 16: 782-790, 2008.
39. Guo F, Chang CK, Fan HH, *et al*: Anti-tumour effects of exosomes in combination with cyclophosphamide and polyinosinic-polycytidylic acid. J Int Med Res 36: 1342-1353, 2008.
40. Hao S, Ye Z, Yang J, Bai O and Xiang J: Intradermal vaccination of dendritic cell-derived exosomes is superior to a subcutaneous one in the induction of antitumor immunity. Cancer Biother Radiopharm 21: 146-154, 2006.
41. Hao S, Bai O, Yuan J, Qureshi M and Xiang J: Dendritic cell-derived exosomes stimulate stronger CD8+ CTL responses and antitumor immunity than tumor cell-derived exosomes. Cell Mol Immunol 3: 205-211, 2006.