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Enhanced immune responses induced by vaccine using Sendai virosomes as carrier

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Abstract

Sendai virosomes can deliver encapsulated contents into the cytoplasm directly in a virus fusion-dependent manner. In this paper, Sendai virosomes-formulated melanoma vaccine was constructed and its anti-tumor effects were investigated. The melanoma vaccine was prepared by encapsulating mixture antigen into the Sendai virosomes. The antigen, mixture proteins were extracted from B_{16} melanoma cells. The cytotoxic T lymphocyte (CTL) response level was evaluated by ^{51}Cr release method, and the change of $CD4^+$ and $CD8^+$ expression as well as the concentration of IgG in serum of immunized mice was measured. The results showed that Sendai virosomes-formulated melanoma vaccine can effectively elicit not only systemic immune response but also strong CTL response. Sendai virosomes can be used as an effective vector for use in anti-tumor vaccine therapy.

Keywords: Vaccine; Melanoma; Sendai virosomes

1. Introduction

One reason that vaccines against tumors have not been remarkable is due to a lack of induction of cytotoxic T lymphocyte (CTL) responses against anomalous cells. Exogenous antigens are usually taken up into cells by phagocytosis or endocytosis. After degradation by lysosomal enzymes, these exogenous antigens are presented in an MHCII-restricted manner. Such antigen presentation induces antigen-specific antibody production, but not CTL responses. In contrast, endogenous antigens (cytoplasmic antigens) are degraded by proteasomes in cytoplasm and presented with MHC class I molecules, eventually leading to the induction of CTL responses. Exogenous antigen can, however, enter the class I processing pathway if it is delivered into cytoplasm with a vector that is able to undergo fusion with the plasma membrane under normal physiological conditions (Sheikh et al., 2000; Kunisawa et al., 2001). Thus, a key event for the induction of antigen-specific CTL responses is the delivery of antigens into cytoplasm using a proper vector.

Sendai virosomes are vesicular particles reconstituted from Sendai viral envelopes. On the surface of the virus membrane, two major proteins are involved in cellular infection. Hemagglutinating and neuraminidase (HN) proteins are required to bind to a receptor (sialic acid) on the cell surface (Markwell and Paulson, 1980). In addition, Fusion (F) protein interacts with the lipid layer of the cell membrane to induce cellular fusion (Ishida and Homma, 1978; Hosaka, 1988). As a result of their fusogenic capacity, virosomes are able to deliver encapsulated matters into cytoplasm. These properties make virosomes potentially attractive for use as antigen carriers (Daemen et al., 2000). Therefore, it might be effective as a vehicle to deliver a CTL-inducible vaccine.

Melanoma represents one of the relatively immunogenic human solid tumors where the host's immune system may play an essential role in controlling the tumor growth. To overcome the lack of knowledge of specific tumor antigens, tumor cells are the best source of antigens to activate the immune system. Previous attempts of melanoma vaccines focused on various forms of tumor cell preparations, including whole tumor cells (either autologous or allogeneic), tumor cell lysates, or cell extracts (Schadendorf et al., 2000). However, the results have been relatively disappointing.

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Table 1
The change of tumor size before and after immunization

Protocol no.	Groups	Before immunization ($L \pm$ S.D. $\times$ $W \pm$ S.D. mm)	After immunization ($L \pm$ S.D. $\times$ $W \pm$ S.D. mm)
1	Control	$6.7 \pm 1.3 \times 5.4 \pm 0.89$	$14.4 \pm 3.3 \times 11.8 \pm 2.7$
	Low dosage	$6.2 \pm 1.6 \times 4.8 \pm 1.9$	$16.4 \pm 4.1 \times 10.6 \pm 3.5$
	Middle dosage	$5.7 \pm 1.4 \times 5.1 \pm 1.3$	$13.1 \pm 3.8 \times 11.4 \pm 3.1$
	High dosage	$6.3 \pm 1.8 \times 4.8 \pm 0.79$	$12.4 \pm 2.9 \times 10.7 \pm 3.6$
2	Control	$6.4 \pm 2.2 \times 5.2 \pm 1.1$	$12.1 \pm 2.2 \times 10.6 \pm 2.4$
	Low dosage	$6.3 \pm 1.7 \times 5.8 \pm 1.2$	$10.4 \pm 3.6 \times 9.2 \pm 2.9$
	Middle dosage	$6.9 \pm 2.0 \times 5.7 \pm 1.5$	$8.2 \pm 3.4 \times 7.8 \pm 2.3$
	High dosage	$7.0 \pm 2.4 \times 6.5 \pm 1.8$	$7.1 \pm 2.7 \times 6.9 \pm 1.9$

Table 2
The change of $CD4^+$ and $CD8^+$ expression in serum before and after immunization[a]

Protocol no.	Groups	CD4 (%)		CD8 (%)	
		Before	After	Before	After
1	Control	39.5 ± 2.1	41.2 ± 1.6	34.7 ± 3.2	34.9 ± 2.8
	Low dosage	42.1 ± 2.4	44.1 ± 3.4	33.3 ± 2.5	33.6 ± 1.9
	Middle dosage	41.4 ± 3.1	48.7 ± 2.7**	34.9 ± 1.2	34.7 ± 2.7
	High dosage	40.7 ± 3.6	52.8 ± 1.8***	35.4 ± 2.2	35.6 ± 2.6
2	Control	41.3 ± 2.8	41.1 ± 1.7	34.1 ± 2.2	34.4 ± 2.5
	Low dosage	40.5 ± 1.3	45.4 ± 3.5*	34.6 ± 1.7	37.3 ± 2.9
	Middle dosage	42.4 ± 2.3	47.6 ± 1.7**	33.6 ± 2.1	40.7 ± 3.8**
	High dosage	39.8 ± 1.4	50.2 ± 2.2***	34.8 ± 1.5	42.6 ± 2.4***

[a] Results are expressed as means ± S.D. ($n = 5$).
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

In this study, the melanoma vaccine was prepared by encapsulating the antigen extracted from B_{16} melanoma cells into Sendai virosomes, and evaluated by measurement the systemic and cellular immunization responses of immunized mice.

2. Investigations and results

2.1. Change of tumor size before and after immunization

The tumor sizes in tumor-bearing mice were measured before and after immunization (Table 1). The data indicate that the tumor size bearing on mice immunized with Sendai virosomes-formulated vaccine did not increase on the whole than immunization before, especially at high dose. However, tumor size bearing on the mice immunized with proteins vaccine increased markedly.

2.2. Change of $CD4^+$ and $CD8^+$ expression before and after immunization

We measured the expression change both of $CD4^+$ and $CD8^+$ in serum before and after immunization (Table 2). The data indicate that the expression of $CD8^+$ increases remarkably after immunization with Sendai virosome-formulated vaccine. However, there is almost no change after immunization with free protein vaccine. The expression of $CD4^+$ enhances with the increasing of dosage no matter what kind of vaccine.

2.3. Comparison of CTL responses to the different vectors and dosage

To assess whether the vector of vaccine had an effect on the level of CTL response elicited, we immunized mice intramusclly (i.m.) with vaccine formulated in Sendai virosomes or free protein (Table 3). I.m. administration of free protein vaccine with three dosages induced low CTL levels. There were no difference between three dosage groups and control group (Fig. 1A). In contrast, corresponding dosage i.m. immunization with Sendai virosomes-formulated vaccine, induced significantly higher ($p < 0.001$) CTL responses. Furthermore, there were significant difference between three dosage groups and

Table 3
Experimental protocols used in this study

Protocol no.	Groups	Immunization route (i.m.)	Antigen (μg protein)
1	Control	Protein	–
	Low dosage	Protein	25
	Middle dosage	Protein	50
	High dosage	Protein	100
2	Control	Virosome vaccine	–
	Low dosage	Virosome vaccine	25
	Middle dosage	Virosome vaccine	50
	High dosage	Virosome vaccine	100

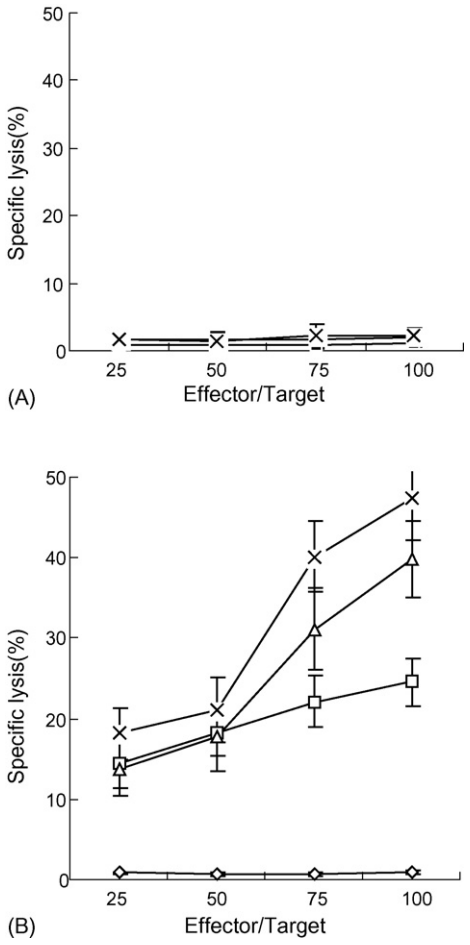

Fig. 1. The CTL response induced by the vaccines: (A) mice immunized with free protein vaccine and (B) mice immunized with Sendai virosomes-formulated vaccine ($n=5$). (◇) Control; (□) low dosage; (△) middle dosage; (×) high dosage.

control group ($p<0.001$). The percent specific lysis of target cells depends on the effector target ratio. The highest percent was obtained when E:T ratio was 100:1 (Fig. 1B).

2.4. Antibody responses to the different vectors and dosages

The results shown in Fig. 2 demonstrated that all animals immunized with vaccine developed high concentration of IgG in serum. Furthermore, with the increasing of dosage, the concentration of IgG in serum was rising. There were significant differences between different dosage groups and corresponding control groups ($p<0.01$) (Fig. 2). The reason is that free proteins in vaccine induce the production of melanoma-specific antibody.

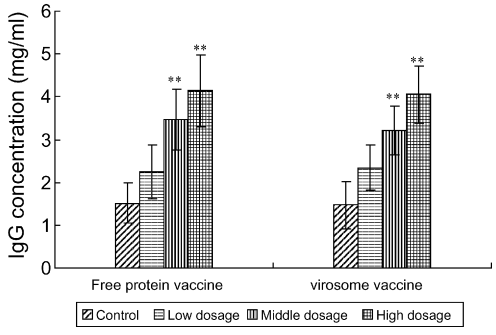

Fig. 2. The serum IgG concentration of immunized mice. Results are expressed as means ± S.D. ($n=5$). $^{**}p<0.01$ *vs*. control.

3. Discussion

Up to date, it was reported that vaccines based on the vector of Sendai virosomes only encapsulated one or two kinds of tool protein and were used to investigate the effect of antigen delivery. It was also reported that various forms of tumor cell preparations, including whole tumor cells, tumor cell lysates, or cell extracts were directly used to immunize people, but the results were not very good because they cannot induce sufficient CTL responses (Livingston et al., 1985). In this study, we integrated the above two methods and encapsulated mixture proteins extracted from B_{16} melanoma cells into Sendai virosomes to prepare the vaccine. The mixture antigens involve the tumor specific antigens, the tumor associated antigens of melanoma and some antigens that were not discovered. Therefore, it should be more effective than one or two kinds of protein antigens no matter directly immunization or encapsulating into Sendai virosomes. And reasonably, in evaluating antibodies induced by vaccine, total IgG, but not specific-IgG was measured.

The primary aim of this study was to determine whether the Sendai virosomes as vaccine vector could induce cellular and systemic immune responses to melanoma. On the basis of our results, it can be concluded that Sendai virosomes-formulated melanoma vaccine can elicit not only systemic immune response but also strong CTL responses. In other words, Sendai virosomes can act as an efficient and highly effective means of enhancing the CTL responses to B_{16} melanoma vaccine antigens, thus illustrating their use as vaccine delivery systems. It is known that CTL responses play a crucial role in protection against malignancy. However, the free protein antigen cannot induce any CTL response. In contrast, antigen-encapsulating Sendai virosomes can elicit strong CTL responses, suggesting that Sendai virosomes can deliver exogenous antigen into cytoplasm and present with the class I processing pathway.

The surface glycoproteins CD4 and CD8 are expressed on functionally distinct subpopulations of mature T-lymphocytes in a mutually exclusive fashion. Expression of $CD4^+$ and $CD8^+$ correlates with the ability of T-lymphocytes to recognize antigenic peptides presented on class II and class I MHC molecules, respectively. Antigen presentation results ultimately in T-lymphocyte activation and proliferation. Several works reported that the encapsulated antigen can induce both the MHC-II and MHC-I responses. The tumor size bearing on mice immunized with Sendai virosome-formulated vaccine were smaller than control mice, indicating that the vaccine could inhibit the increment of tumor at least.

In addition, we should admit that fusion-mediated delivery of antigen to the cell cytosol does not imply the involvement of 'alternative' mechanisms for processing of exogenous protein antigens into the class I presentation pathway. Several

recent studies have provided evidence for the existence of such alternative processing routes in a limited subpopulation of APCs (Rock et al., 1993; Reis e Sousa and Germain, 1995; Jondal et al., 1996). However, antigen delivery by Sendai virosomes does not involve such special presentation mechanisms. Due to the membrane fusion activity of the HN, the Sendai virosomes deliver their contents directly to the cytosol and thus to the normal MHC class I presentation route.

It is well know that tumor-induced activation of immune suppress remains a major obstacle for successful anticancer therapy. However, the vaccine constructed in the present study could induce augmentation of immune response. In summary, we conclude that the Sendai virosomes could be used as a safe and promising vaccine vector for inducing systemic and cell-mediated immune responses. Furthermore, a number of novel vaccine antigens are being incorporated into Sendai virosomes in an attempt to develop a variety of new tumor vaccines.

4. Experimental

4.1. Materials

BCA-100 Protein Quantitation Kit was purchased from Shanghai Shenergy Biocolor BioScience & Technology Company (Shanghai, China). Triton X-100, was purchased from Sigma Chemical Co. (USA). Fetal bovine serum and RMPI-1640 were purchased from Gibco Co. (New Island, NY, USA). PE anti-mouse $CD4^+$ and PE-Cy5 anti-mouse $CD8^+$ monoclonal antibody were purchased from e-bioscience (CA, USA). CELLection™ Mouse CD8 Kit was purchased from Dynal Biotech ASA (Oslo, Norway). Mouse-IgG ELISA Kit and Bio-Beads® SM-2 Adsorbent were purchased from Roche (Mannheim, Germany) and Heraules (CA, USA). All other reagents and solvents were of analytical grade.

4.2. Animals

Six-week-old female C57BL/6 mice (Zhejiang university animal center, Hangzhou, China) were used in this study. Each group consisted of five mice. Tumor-bearing mice were prepared by inoculating s.c. a suspension (2×10^6 cells) of cultured B_{16} melanoma cells directly into the abdomens of mice. The mice were immunized when the tumor size was in the range from 6 to 8 mm in diameter.

4.3. Cells

B_{16} melanoma cells were obtained from Shanghai cell institute (China). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml of streptomycin and 100 unit/ml of penicillin, 2% $NaHCO_3$, final pH 7.4, at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was determined by Trypan blue exclusion and was routinely above 95%.

4.4. Virus

Sendai virus (Z strain) was kindly provided by Prof. Shinsaku Nakagana of Osaka University and grown for 72 h at 36 °C in the allantoic cavity of 10-day-old specific pathogen-free embryonated chicken eggs, purified by discontinuous sucrose density gradient centrifugation and stored at −70 °C in phosphate buffered saline (PBS). The hemagglutinin titer and hemolytic activity were determined as previously described (Amselem et al., 1985). Protein concentration of virus was determined by BCA-100 Protein Quantitation Kit according to the manufacture's instruction.

4.5. Preparation of antigens

The antigens are mixture proteins extracted from cultured B_{16} melanoma cells. The cells (1×10^7) were harvested by centrifugation at $180 \times g$ for 10 min and washed twice with PBS. Pelleted cells were resuspended in 5ml of hypotonic buffer (10 mM Tris–HCl; 1 mM $MgCl_2$; 1 mM KCl; 0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.3) and subjected to 5–10 freeze and thaw cycles with −20 °C refrigerator and lukewarm water until the cells were completely lysed. Hereafter, cell lysates were centrifuged at $3000 \times g$ for 5 min to remove cell debris. Saturated ammonium sulfate solution was diluted with supernatant to the concentration of 30%, 50%, 70%, respectively. The pellet was collected respectively by centrifugation at $10{,}000 \times g$ for 20 min and resuspended in 1 ml PBS. The protein concentration was 509 μg/ml determined by BCA-100 Protein Quantitation Kit as previously reported (Qiang et al., 2004).

4.6. Preparation of virosomes and vaccine

Sendai virosomes were prepared essentially as described (Vainstein et al., 1984). Briefly, 10 mg of intact, pelleted Sendai virions was solubilized with 20 μl of Triton X-100 in a final volume of 400 μl of PBS (pH 7.4). The detergent was removed from the clear supernatant obtained after centrifugation by the direct addition of SM-2 Bio-beads. The vaccine was prepared by adding mixture protein after centrifugation and then remove the detergent. The morphology and size measurements of the Sendai virus and virosomes were performed by JEM-120DEX (JEOL, Japan) and Coulter N 4nd submicron analyser (Malvern, UK). Most virions were roughly spherical and their diameter was 50–200 nm.

4.7. Mice immunization

Two different immunization protocols were used in these studies (Table 3). Each group consisted of five mice. The mice were immunized on day 1, 7, 14, 21, 28, and on day 35, the blood was collected from orbit venous plexus.

4.8. Flow cytometry

Seven days after the last immunization, the heparinized whole-blood samples of mice collected from orbit venous plexus

were single stained for surface CD4, and CD8 using the following murine IgG monoclonal antibodies directly coupled to fluorochromes: phycoerythrin (PE) anti-mouse CD4 and PE-Cy5 anti-mouse CD8. Flow cytometry was performed on a FACS analyzer equipped with a FACSlite argon laser (Becton-Dickinson) and calibrated using standard techniques (Haczku et al., 1996).

4.9. Cytotoxic assays

Seven days after the last immunization, mice were sacrificed by cervical dislocation. The spleens were removed aseptically. The splenic tissue was macerated, and the supernatant separated by simple decantation. The erythrocytes were removed by treatment with Tris-buffered ammonium chloride (0.14 M; pH 7.2) for 3 min on ice. The cells were washed three times with PBS. The cell concentration was adjusted to 2×10^7 cells per ml in complete RPMI-1640. $CD8^+$ cells were isolated from the spleen cells using CELLection^{TM} Mouse CD8 Kit according to the manufacturer's instructions. The target cells were labeled for 1 h with 3.7 MBq ^{51}Cr per 10^6 cells in 100 μl medium. Subsequently, 100 μl of different concentrations labeled B_{16} melanoma cells and the same volume of effector cells (1×10^6) in complete RPMI-1640 were added to each of the wells of 96-well microplates and incubated at 37 °C in 5% CO_2 for 4 h. After centrifuged at $1500 \times g$ for 5 min 100 μl of the supernatant of each sample cells were harvested, ^{51}Cr levels in the supernatants were determined using a gamma counter. The determinations were carried out in triplicate and standard deviations were calculated. Specific lysis was calculated as follows:

$$\text{Specific lysis \%} = 100 \times \frac{[\text{Experimental release (cpm)} - \text{spontaneous release (cpm)}]}{[\text{Maximum release (cpm)} - \text{spontaneous release (cpm)}]}$$

Spontaneous release was determined from wells to which 100 μl of complete medium were added instead of effector cells. Maximum releasable activity was measured after treating the target cells with 1% Triton X-100 (Arkema et al., 2000; Al-Ahdal et al., 1985).

4.10. Measurement of IgG antibody levels by enzyme immunoassay

Seven days after the last immunization, the mouse-IgG in serum was determined by mouse-IgG ELISA kit according to the manufacturer's instructions.

4.11. Statistics

Student's *t*-test was used to compare mean values of different groups with the expression percentage of $CD4^+$ and $CD8^+$, IgG antibody concentration and specific lysis of CTLs. Statistical significance was designated as $p < 0.05$.

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