Original Article

Inactivated Sendai Virus Suppresses Murine Melanoma Growth by Inducing Host Immune Responses and Down-regulating β-catenin Expression

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Abstract

Objective　This paper aims to investigate the anti-tumor mechanism of inactivated Sendai virus (Hemagglutinating virus of Japan envelope, HVJ-E) for murine melanoma (B16F10).

Methods　The murine dendritic cells (DCs) were treated with HVJ-E, and then the cytokines secreted from DCs and costimulation-related molecules on DCs were measured. Meanwhile, the expression of β-catenin in HVJ-E treated murine melanoma cells was detected. In addition, HVJ-E was intratumorally injected into the melanoma on C57BL/6 mice, and the immune cells, CTL response and tumor volume were analyzed.

Results　HVJ-E injected into B16F10 melanoma obviously inhibited the growth of the tumor and prolonged the survival time of the tumor-bearing mice. Profiles of cytokines secreted by dendritic cells (DCs) after HVJ-E stimulation showed that the number of cytokines released was significantly higher than that elicited by PBS ($P<0.05$). The co-stimulation-related molecules on DCs were comparable to those stimulated by LPS. Immunohistochemical examinations demonstrated the repression of β-catenin in B16F10 melanoma cells after HVJ-E treatment. Meanwhile, real-time reverse transcription PCR revealed that HVJ-E induced a remarkable infiltration of CD11c positive cells, chemokine ligand 10 (CXCL10) molecules, interleukin-2 (IL-2) molecule, $CD4^+$ and $CD8^+$ T cells into HVJ-E injected tumors. Furthermore, the mRNA expression level of β-catenin in the HVJ-E injected tumors was also down-regulated. In addition, B16F10-specific CTLs were induced significantly after HVJ-E was injected into the tumor-bearing mice.

Conclusion　This is the first report to show the effective inhibition of melanoma tumors by HVJ-E alone and the mechanism through which it induces antitumor immune responses and regulates important signal pathways for melanoma invasion. Therefore, HVJ-E shows its prospect as a novel therapeutic for melanoma therapy.

Key words: HVJ-E; Dendritic cell; Melanoma; β-catenin

INTRODUCTION

Dendritic cells (DCs) are the most potent and professional APCs that determine either Th1 or Th2 polarization of naive T cells, and represent a promising tool for cancer immunotherapy[1-2]. The immature state of DCs is known to be appropriate for antigen processing, and it in turn must be matured to fully activate DCs, which expresses high levels of cell surface MHC-antigen complex and co-stimulatory molecules, for a sufficiently productive CTL response[3]. Enormous early clinical trials suggested the potential of DC-based immunotherapy, but the extensive follow-up studies concluded that the current strategy remained immature as a standard therapeutic for cancer treatment[4]. Current issues on DC-based cancer immunotherapy include lack of information regarding such aspects, as the most effective DC subtypes, the optimal conditions and activation stimuli to generate activated DCs showing optimal antitumor effect *in vivo*[5]. Since no one knows about the optimal performance and precise clinical indications of DC-based cancer immunotherapy at present, relevant preclinical assessment in detail is absolutely required to obtain more data about DC therapy in clinical settings.

The inactivated Sendai virus (Hemagglutinating virus of Japan envelope, HVJ-E) is a safe and efficient nonviral vector for drug delivery as it can incorporate DNA, RNA, proteins and drugs and deliver them into cells both *in vitro* and *in vivo*[6-9]. Moreover, HVJ-E has been applied to DC activation and used as an anticancer strategy[10-11]. Recently, colon carcinoma and renal carcinoma therapy using HVJ-E to induce host immune responses through DC maturation have been reported[10,12]. To our knowledge, the anti-melanoma effect of inactivated replication-defective viral particles that lack oncolytic and transgene encoding abilities has not been studied. Therefore, the present study aims to demonstrate that HVJ-E alone may suppress tumors growing in mice without exogenous gene expression not only by tumor-specific anti-melanoma immunity but also by the down-regulation of β-catenin expression, which will be important for melanoma development.

MATERIALS AND METHODS

Mice and Tumor Cell Lines

Male 6-to 8-week-old C57BL/6 mice were obtained from Yangzhou University (Yangzhou, China), and all animals were maintained in a temperature-controlled and pathogen-free room. Murine malignant melanoma B16F10 cells were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). The cell line was maintained in RPMI 1640 supplemented with 10% FCS (GIBCO, NY, USA), penicillin, and streptomycin under a humidified atmosphere containing 5% CO_2 at 37 °C.

Reagents and Antibodies

Recombinant murine IL-2, GM-CSF, and IL-4 were purchased from PeproTech (USA), and mouse IFN-β, IFN-γ, TNF-α, and IL-6 immunoassay kit were bought from R&D Systems Inc. (USA); Fluorescein isothiocyanate (FITC) antimouse CD11c, Phycoerythrin (PE) antimouse CD40, Allophycocyanin (APC) antimouse CD80, Phycoerythrin (PE)-Cy5 antimouse MHC II antibodies were obtained from ebioscience (San Diego, CA). Anti-β-catenin was acquired from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Preparation of HVJ-E

HVJ (Z strain) was preserved in our laboratory and prepared as described[13]. The inactivated HVJ that was unable to replicate is referred to as HVJ-E. Infectious particles were not produced by injecting HVJ-E either into fertilized eggs or in cultured Vero cells.

Preparation and Culture of Dendritic Cells

Murine dendritic cells derived from bone marrow were generated as described from C57BL/6 mice[14]. Dendritic cells (1×10^6) were seeded in 24-well plates in 1 mL of culture medium supplemented with 50 ng/mL of recombinant mouse GM-CSF and IL-4. The cultures were nourished every other day by gently aspirating the spent medium and adding fresh medium. Six days later, nonadherent and loosely adherent clusters of proliferating dendritic cells were used in subsequent experiments as immature dendritic cells.

Flow Cytometric Analysis for Costimulation-related Molecules on DCs

DCs were placed in fresh medium (1×10^6 cells/mL) and incubated with HVJ-E at an MOI of 150, or LPS (2 μg/L) or PBS (endotoxin free) for 48 h. Then the DCs were incubated with anti-mouse CD40, anti

mouse CD11c, anti-mouse MHC Class II, and anti-mouse CD80 respectively. The stained cells were then analyzed with the BD FACScalibur system and CELLQUEST software.

Cytokine Measurements

Immature murine dendritic cells (1×10^5) were cultured in 96-well plates and then HVJ-E was added to the culture medium for 48 h. Harvested supernatants were evaluated by ELISA. Murine TNF-a, IFN-β, IFN-γ, and IL-6 were measured according to the manufacturer's instructions. The geometric means and standard deviations for triplicate sets of samples were calculated. Supplied standards were used to generate standard curves.

Immunohistochemistry Examination of β-catenin in Melanoma Cells

To evaluate the β-catenin expression in B16F10 tumor cells after HVJ-E treatment, B16F10 cells were seeded in 60 mm plates at the density of 2×10^5 and incubated overnight; then, HVJ-E of 1500 MOI or endotoxin-free PBS were added into the dishes; 48 h later, the treated cells and controls were fixed by paraformaldehyde; finally the cells were washed and stained with anti-β-catenin antibodies that had been diluted at 1:100, followed by reacting with horseradish peroxidase labeled secondary antibody (Boshide, Wuhan, China) diluted at 1:200. The expression of β-catenin was visualized after color development with DAB.

Intratumoral Injection of HVJ-E and Measurements of Tumor Volume

B16 F10 cells (2×10^6) were injected into the intradermal space in the backs of C57BL/6 mice. After tumors inoculated into the mice reached 5 mm in diameter, virus particles of HVJ-E (1.5×10^{10}) dissolved in 100 μL of PBS or PBS (100 μL) were injected into tumors on Day 4, 8, and 12. Tumor volume was measured in a blinded manner with slide calipers by using the following formula: tumor volume (mm^3) = length × (width)^2/ 2.

CTL Assay

A CytoTox 96® Non-Radioactive Cytotoxicity Assay-lactate dehydrogenase (LDH) release (Promega, Beijing, China) was used to measure cytotoxic activity according to the manufacturer's instructions. Briefly, to examine the tumor-specific CTL treated by HVJ-E, C57BL/6 mice were injected

intratumorally three times with 4-day interval by HVJ-E. On Day 7 after the last injection, splenocytes were obtained and further incubated in complete medium for 90 min. Nonadherent cells (3×10^6 cells/mL) were collected and re-stimulated in 24-well culture plates with mitomycin C (25 μg/mL)-treated B16F10 cells (10^6 cells/mL). These splenocytes, as effector cells, were cultured in the presence of 10 U/mL of IL-2 for 5 days at 37 °C. Then, effector cells (E) were incubated with B16F10 cells, target cells (T) in 96-well culture plates at different E:T ratios of 10:1, 20:1, 40:1, and 80:1. After 4 h incubation, the plate was centrifuged at 250×g for 4 min and the supernatants were harvested. Maximal release of LDH was performed by completely lysing target cells. Target cells without effector cells were used as negative controls (spontaneous release). The percentage of cytotoxicity was calculated as follows: [(experimental release -spontaneous release)/(maximum release - spontaneous release)] × 100.

Real-time Quantitative RT-PCR

Intradermal melanoma tumors were produced in C57BL/6 mice as described above. When tumors reached 5 mm in diameter, HVJ-E (1.5×10^{10} particles in a total volume of 100 μL) or PBS was injected into each tumor mass. After 24, 48, and 120 h, the tumors were removed and RNA was isolated with an RNeasy Mini Kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. A total of 1 μg of RNA was reverse transcribed into cDNA and amplified by real-time quantitative PCR by using an ABI PRISM 7500 Sequence Detection System. Primer pairs specific for murine CD11c, CD4, CD8b, CXCL10, IL-2, β-catenin, and β-actin were designed as shown in Table 1 and synthesized by Invitrogen (Shanghai, China). The concentration of target genes was determined by using the comparative CT method (threshold cycle number at the cross-point between amplification plot and threshold) and values were normalized to an internal β-actin control.

Statistical Analysis

All data were expressed as the mean±SD, and analyzed by one-way ANOVA with Fisher's adjustment, except for animal survival. Survival was plotted by using Kaplan-Meier curves and statistical relevance was determined via log-rank comparison. A probability value of P<0.05 was considered significant.

Table 1. The Primers Specific to Determined Genes

Gene Name	Primer (5'-3')	PCR Product (bp)	Accession No.
CD8b	GGCCTGACATGCAGCCTTAC CAGTCTTGAATGCGCTGTCTCT	237	NM_009858
CD4	TGCCTTCCACATCAGCTCTC CAGGACAGCGACTTCTGGAAC	189	NM_013488
CXCL10	CCGGATTCAGACATCTCTGCT CGCTGCAACTGCATCCATA	148	NM_021274
CD11c	TGATGCCACTGTCTGCCTTC TACTCAGACGGCCATGGTCTA	106	NM_021334
IL-2	CCGCAGAGGTCCAAGTTCAT CTCCTGAGCAGGATGGAGAAT	131	NM_008366
β-catenin	GCTGCACAGGTGACCACATT GAGTGCTGAAGGTGCTGTCTGT	237	NM_007614
β-actin	TTCATGGATGCCACAGGATT TGACGGCCAGGTCATCACTA	100	NM_007393

RESULTS

Activation of mBM-DCs Treated by HVJ-E

We evaluated the maturation and activation of C57BL/6-derived mBM-DCs induced by HVJ-E without any other stimulant in direct comparison with PBS or LPS (2 μg/mL). As shown in Figure 1, with repeated FACS analyses, DCs treated with HVJ-E resulted in the high level expression of the co-stimulatory molecules of CD40, CD80, MHC II, and CD11c on the DCs surface. The expression level of surface markers on DCs after HVJ-E stimulation was nearly comparable to those treated with LPS. Meanwhile, in comparison with those elicited by PBS, the expression level of all co-stimulatory molecules was significantly increased (*P*<0.05). These results demonstrated that HVJ-E could transform immature DCs to near fully activated and matured DCs without any other stimulant.

Then, we examined cytokines released from murine myeloid DCs induced by HVJ-E. The numbers of IFNs (IFN-β and IFN-γ) and representative proinflammatory cytokines (IL-6 and TNF-α) that were released into DCs culture medium at 48 h after addition of HVJ-E were measured by ELISA. As shown in Figure 2, the secretion of all the cytokines induced by HVJ-E was increased significantly, as compared with PBS (*P*<0.05). In addition, the up-regulation of IFN-β and IFN-γ treated with HVJ-E was more obvious than those treated with LPS (*P*<0.05). In contrast, higher expression levels of IL-6 and TNF-α

were seen in LPS-induced DCs.

Together with these results, HVJ-E induced spontaneous maturation and activation of mBM-DCs; however, their phenotypes were not equal to those found in the induction with LPS.
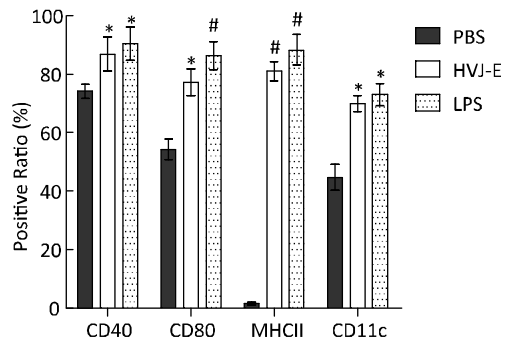

Figure 1. Effects of HVJ-E on surface markers on mBM-DCs. DCs were collected and treated by HVJ-E at MOI=150 or exposed to LPS (2 μg/mL) and PBS. 48 h later, DCs were subjected to the FACS analysis assessing the expression of surface markers. The experiment was carried out in triplicate, which produced similar results. *indicates significant difference *vs.* PBS control (*P*<0.05). #indicates significant difference *vs.* PBS control (*P*<0.01).

HVJ-E Down-regulated β-catenin Expression in Vitro

To explore whether HVJ-E had direct effects on β-catenin expression in B16F10 cells *in vitro*, we performed immunohistochemical analysis in tumor

cells that had been treated with HVJ-E. As shown in Figure 3, the β-catenin expression level in treated tumor cells was lower than that in the controls. This result indicated that HVJ-E could directly inhibit β-catenin expression in tumor cells, which might be used for cancer therapy. However, the specific mechanism is not really clear and further investigations are needed.
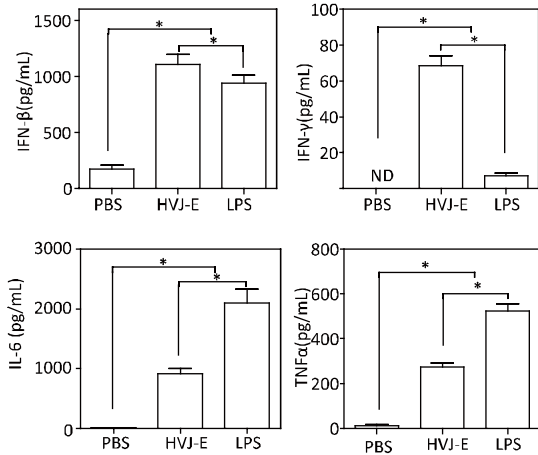

Figure 2. Representative cytokine secretion measured in dendritic cell supernatants by capturing ELISA 48 h after co-culture with HVJ-E, LPS, and PBS. The numbers of IFN-β, IFN-γ, IL-6, and TNF-α were increased after HVJ-E stimulation *vs.* PBS control (*P*<0.05), ND, not detectable.
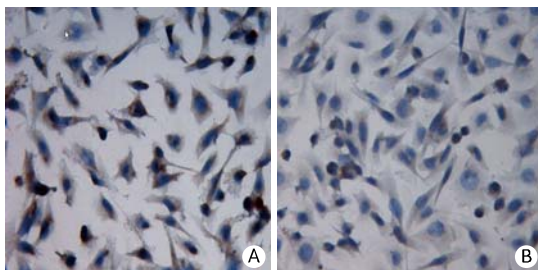

Figure 3. Immunohistochemical visualization of β-catenin in murine melanoma cells. The cells treated with PBS showed a strongly positive reaction to β-catenin in cytoplasm (A), while a weakly positive reaction to cytoplasm was observed in cells treated with HVJ-E. Original magnification (200×).

HVJ-E Therapy Induces Effective Inhibition of B16F10 in Vivo

We examined whether HVJ-E stimulated antitumor effects by injecting 1.5×10^{10} HVJ-E virus particles into melanoma growing in the backs of C57BL/6 mice. After three consecutive injections, the effective inhibition of melanoma growth of tumor-bearing mice and the prolonged survivals were observed, while the injection of endotoxin-free PBS had no effect against B16F10 melanoma tumors (Figure 4A and B).
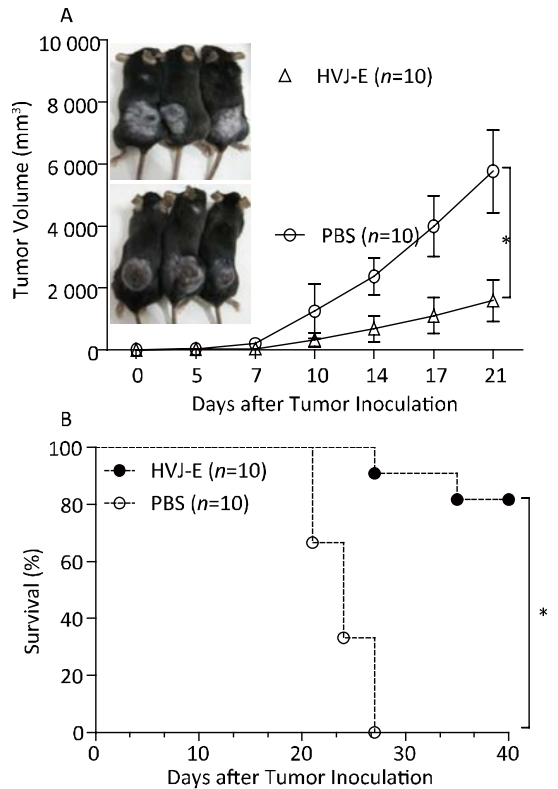

Figure 4. The inhibition of tumor growth by intratumoral injection of HVJ-E. B16F10 cells were inoculated into intradermal space in the backs of C57BL/6 mice. Replication defective particles (1.5×10^{10} each) of HVJ-E or endotoxin-free PBS were injected into tumors thrice (on Day 4, 8, and 12). The HVJ-E-treated tumors were effectively inhibited as compared with PBS-treated tumors. *P*<0.01 (A). Survival curve of the mice bearing B16F10 melanoma following three consecutive injections with HVJ-E or PBS, revealed a significant prolongation of survival in the HVJ-E-treated group. *P*<0.01 (B).

HVJ-E Promoted Immune Responses and Inhibited β-catenin Expression into Tumor

To determine how the host immune responses reacted to HVJ-E *in vivo*, the mRNA expression of CD11c, CD4, CD8, IL-2 and the interferon

(IFN)-inducible chemokine CXCL10 were measured in B16F10 tumors of mice at 24, 48, and 120 h after HVJ-E injection. CD11c, CD4, CXCL10 and IL-2 expression was significantly increased in B16F10 tumors treated with HVJ-E compared with PBS treatment at all time points with a peak at 48 h. CD8 expression was also significantly increased in the HVJ-E treated group. Moreover, the expression level remained maximal even at 120 h after treatment (Figure 5 A-C). We also detected the mRNA expression level of β-catenin in B16F10 tumors of

mice at 24, 48, and 120 h after HVJ-E injection. The results indicated that the expression level of β-catenin was significantly decreased in B16F10 tumors treated with HVJ-E compared with PBS treatment at all the time points (Figure 5 A-C). We then performed CTL assays and measured LDH release to determine whether B16F10-specific cellular immunoreactions were induced. The results revealed that B16F10-specific CTL response was induced only in those mice in which melanoma was inhibited effectively (Figure 5 D).
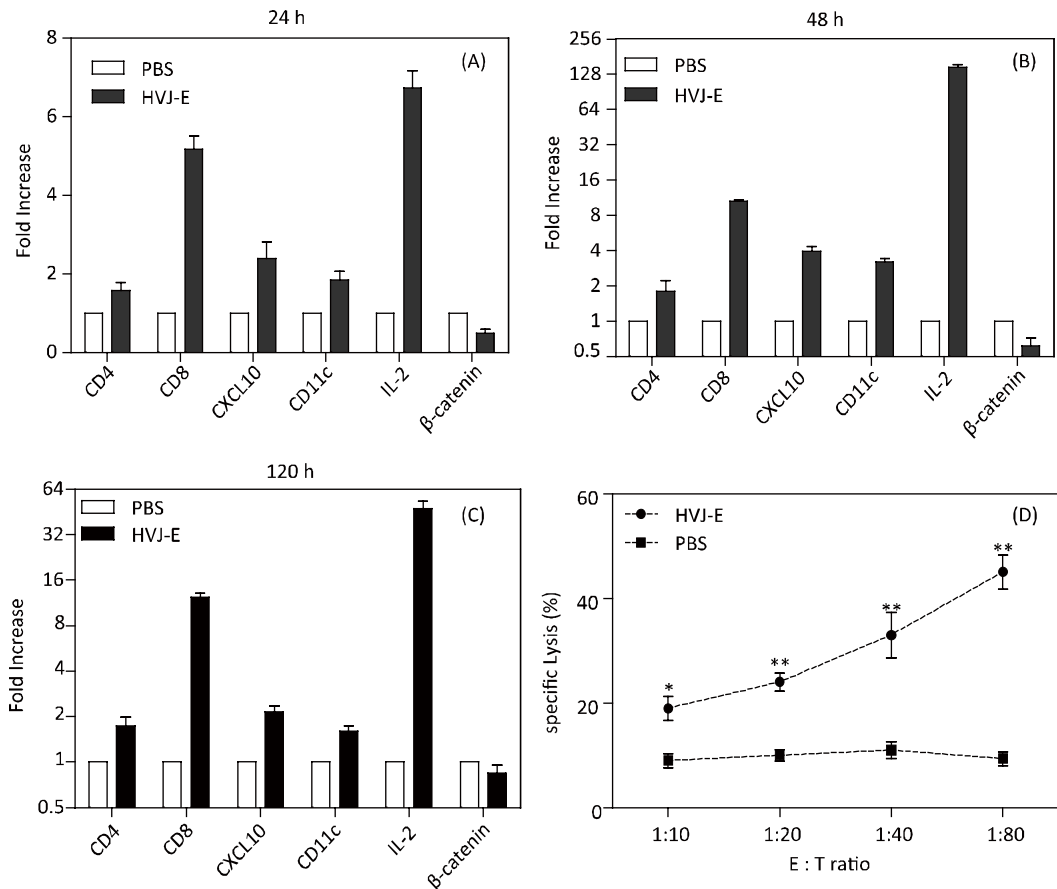

Figure 5. Immune cell infiltration into tumor beds and induction of tumor-specific CTLs *in vivo*. (A to C) mRNA expression quantified by real-time PCR at 24 h, 48 h, and 120 h in tumors growing in the backs of C57BL/6 after three consecutive intratumoral injections of 1.5×10^{10} HVJ-E or endotoxin-free PBS. CD4, CD11c, IL-2, and CXCL10 were significantly higher in HVJ-E treated tumors at all time points with the peak level at 48 h. CD8 expression obviously increased in HVJ-E injected tumors and was continuously elevated for 120 h. Expression levels were normalized by β-actin. Each value was calculated as fold increase relative to value of PBS-injected tumors. (D) Induction of tumor-specific cytotoxic T cells in tumor-bearing mice treated with HVJ-E by LDH release assay. C57BL/6 mice were intratumorally injected by HVJ-E three times at a 4-day interval. On day 7 after the last injection, spleen cells were isolated and restimulated with mitomycin C-treated B16F10 cells for 5 days. The restimulated effector cells were then assayed for cytolytic function with B16F10 cells as target cells. Results are given as means±S.D. [*]*P*<0.01 and [**]*P*<0.001 compared with PBS injected control groups.

DISCUSSION

HVJ-E without any other stimulant had antitumor effects on colon carcinoma and renal carcinoma through blocking regulatory T cells or by enhancing local CXCL10 expression and systemic NK cell activation[12,10]. In this paper, we demonstrated that the HVJ-E treated melanoma could be suppressed effectively not only by immune cells infiltration and IL-2 release, but also by down-regulation of β-catenin, which played critical roles in cell proliferation[15].

After direct intratumoral injection of HVJ-E, the B16 tumor growth was suppressed effectively and the survivals of the tumor-bearing mice was prolonged (Figure 4A and B). The recognition of pathogen-associated molecular patterns by antigen-presenting cells (APCs) such as dendritic cells through TLRs or RIG-I is important to induce not only the activation of innate immunity but also the development of acquired immunity[16-17]. Since we postulated that the activation of APCs by HVJ-E or HVJ-E fused tumor cells would be a pivotal step in eliciting innate immunity and tumor-specific adaptive immunity, we examined the DC maturation induced by HVJ-E. We found that DCs were matured by HVJ-E to a level comparable to that induced by LPS (Figure 1), a well-known robust mature DCs inducer, and a considerable amount of cytokine secretion was also observed (Figure 2). Moreover, DCs, effector T-cell and NK cell recruitment was also suggested from the remarkable increase of CD11c, CD4, CD8, IL-2, and CXCL10 mRNA expression in the established tumors injected with HVJ-E. As this is the first report, demonstrating the feasibility of using direct intratumoral injection of HVJ-E as melanoma immunotherapy, there are still some questions to be further addressed regarding this system. However, it is true that HVJ-E is a strong activator for DCs maturation, which may be useful for cancer therapy.

At present, although DC activation by HVJ-E or live HVJ *in vivo* and *in vitro* has been documented[11,18,5], the precise molecular and cellular mechanism of the anti-melanoma effects of HVJ-E injection on tumors is not well-understood. On the one hand, effector T-cell and NK cell recruitment and tumor-specific CTLs induction play important roles in melanoma inhibition (Figure 5). On the other hand, one of the findings of the current study also reveals that down-regulation of β-catenin expression (Figure 3, Figure 5 A-C) is an important inhibition

factor in the growth of many types of cancers, including melanoma, through regulation of PI3K-AKT, MAPK and Wnt/β-catenin pathways[19-21]. As a result, targeting this gene may therefore comprise a new potential therapy for treatment of melanoma. Suppression of B16 tumor growth has been demonstrated by intratumoral injection of siRNA targeting β-catenin[22], and down-regulating β-catenin signaling activity in various cancer cells has also been reported[23-25]. In view of these, the antitumor effects of HVJ-E by inducing DC activation or β-catenin down-regulation or by both are not yet clear.

All of these findings indicate that HVJ-E alone could induce strong anti-melanoma effects. Although dendritic cell maturation or cytokine release by HVJ-E and intratumoral injection of HVJ-E for tumor inhibition or eradication has also been reported[12,10], this paper is the first to show the potential contribution of HVJ-E to cancer suppression through its ability to down-regulate β-catenin signaling pathways which are important for melanoma growth.

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