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VI. 研究成果の刊行
別刷

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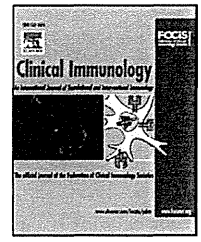

Anti-tumor effects of inactivated Sendai virus particles with an *IL-2* gene on angiosarcoma

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Abstract Cutaneous angiosarcoma is a life-threatening tumor that is resistant to conventional therapies. The therapeutic effects of Sendai virus particles (hemagglutinating virus of Japan envelope: HVJ-E) carrying *IL-2* gene (HVJ-E/IL-2) were examined in a mouse model of angiosarcoma. Intra-tumoral injection of HVJ-E/IL-2 effectively inhibited the growth of angiosarcoma cells (ISOS-1) inoculated in mice and improved tumor-free rates. HVJ-E/IL-2 stimulated local accumulation of CD8 (+) T cells and NK cells and reduced regulatory T cells in regional lymph nodes. Notably, the prevalence of myeloid-derived suppressor cells was lower in HVJ-E/IL-2-treated mice than in HVJ-E-treated mice. HVJ-E/IL-2 treatment promoted IFN-γ production from CD8 (+) T cells in response to tumor cells, more significantly than HVJ-E treatment. Greatly improved tumor-free rates were obtained when sunitinib, a tyrosine kinase inhibitor, was administered in combination with HVJ-E/IL-2. Immunogene therapy with HVJ-E/IL-2 with or without sunitinib could be a promising therapeutic option for cutaneous angiosarcoma.

1. Introduction

Cutaneous angiosarcoma is a life-threatening malignant tumor that commonly affects elderly patients, occurring mainly on the scalp and face. It develops as an erythematous or purple macule, forms nodules, and later ulcers. Local recurrence occurs frequently, despite radical surgical removal. Recent advances in chemotherapy, such as paclitaxel and docetaxel

Abbreviations: BMDC, Bone marrow-derived dendritic cells; DCs, Dendritic cells; HVJ-E, Hemagglutinating virus of Japan envelope; HVJ-E/IL-2, HVJ-E carrying *IL-2* gene; HVJ-E/pVAX1, HVJ-E carrying pVAX1 plasmid; ISOS-1/HVJ, ISOS-1 cells fused with HVJ-E; MDSC, Myeloid-derived suppressor cells; S.D, Standard deviations; TILs, Tumor infiltrating lymphocytes; Tregs, Regulatory T cells.

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[1–3], have improved clinical outcomes. Nevertheless, local recurrences are usually inevitable, and death frequently results from distant metastases to the lung, pleura, and brain, leading to a high mortality.

Inactivated, replication-defective, Sendai virus particles (hemagglutinating virus of Japan envelope: HVJ-E) are a safe and efficient tool for drug delivery [4]. Recent studies have demonstrated that HVJ-E can be used for anti-tumor immunotherapy. Intra-tumoral administration of HVJ-E exerted inhibitory effects on the growth of CT26 murine colon carcinoma cells inoculated in BALB/c mice [5]. These effects were, at least in part, mediated by the enhanced activation of tumor-specific CD8 (+) T cells and suppression of regulatory T cells (Tregs). In a subsequent study of mice with Renca renal cell carcinoma cells, HVJ-E promoted NK cell cytotoxicity through enhancing IFN-β production by dendritic cells (DCs) [6].

IL-2 has a stimulatory effect on activated T cells and NK cells, and thus administration of IL-2 has been recognized as a useful tool in immunotherapy for malignant tumors, such as malignant melanoma [7]. Additionally, local injection of IL-2 has been demonstrated to be an effective therapy for cutaneous angiosarcoma [8,9]. However, IL-2 immunotherapy has some clinical limitations because of its short half-life. In addition, a large amount of IL-2 cannot be administered due to systemic toxicity [10–12]. We hypothesized that local and persistent delivery of IL-2 by HVJ-E would overcome these problems and may be ideal for treating angiosarcoma as immunotherapy. The goals of this study were to verify the anti-tumor effects of HVJ-E carrying *IL-2* gene (HVJ-E/IL-2) in order to establish a beneficial therapeutic tool for cutaneous angiosarcoma, and to identify immunological changes provoked by HVJ-E/IL-2.

Sunitinib is a multi-targeted tyrosine kinase inhibitor that inhibits several growth factor receptors, including platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), C-kit (CD117), and fims-like tyrosine kinase receptor (flt3) [13]. This compound is now widely used to treat malignant tumors, such as metastatic renal cell carcinoma [14]. In this study, the therapeutic potential of sunitinib, a tyrosine kinase inhibitor, in combination with HVJ-E/IL-2 was also examined.

2. Materials and methods

2.1. Antibodies

PE-Cy5 conjugated anti-mouse CD3e (CD3e-PE-Cy5, 145-2C11), FITC conjugated anti-mouse CD8 (CD8a-FITC, 53–6.7), CD4-FITC (GK1.5), CD25-PE (PC61.5), Foxp3-PE-Cy5 (FJK-16 s), Ly-6G (Gr-1)-FITC (RB6-8C5), CD11b-PE-Cy5 (M1/70), CD86-FITC (GL-1), CD31-FITC (390), CD34-FITC (RAM34) and rat IgG2a isotype control antibodies were purchased from eBioscience (San Diego, CA, USA). PE-conjugated anti-mouse ESAM (endothelial cell-selective adhesion molecule) (1G8/ESAM) was from BioLegend (San Diego, CA, USA). CD11c-PE (HL3), biotin-conjugated anti-mouse CD49b (CD49b-Biotin, DX5), and CD16/32 (2.4G2) antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). CD11c microbeads, CD8a (Ly-2) microbeads, and streptavidin-conjugated microbeads were purchased from Miltenyi Biotec GmbH (Bergisch, Gladbach, Germany). CD31 (P2B1) was from Abcam, Cambridge, UK, and Alexa FluorR488 goat anti-mouse IgG and Hoechst 33342 trihydrochloride trihydreate were obtained from Life Technologies, Carlsbad, CA, USA.

2.2. Cell line

ISOS-1 cells, a mouse angiosarcoma cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum [15]. These cells were positive for CD31, CD34, and ESAM as assessed by flowcytometric, Western blotting, and/or immunohistochemical analyses (Fig. 1). All culture media were supplemented with penicillin 50 U/mL and streptomycin 50 μg/mL (Life Technologies). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Mouse model of angiosarcoma

ISOS-1 cells (3×10^6 cells) [15] were inoculated into subdermal spaces of the dorsal skin of 7- to 9-week-old female BALB/c mice (Sankyo Labo Service Corporation, Tokyo, Japan) on day 0. Tumor size was measured with slide calipers, and the tumor volume was calculated according to the following formula:

Tumor volume (mm^3) = length × $(width)^2$ / 2.

Mice were maintained under specific pathogen-free conditions in our animal facility. The use of animals was in full compliance with the guideline of the Committee for Animal Experiments of Tokyo Medical and Dental University.

2.4. HVJ-E treatment

HVJ-E, an inactivated HVJ that is unable to replicate, was purchased from Ishiharasangyo Kaisha, Ltd., Osaka, Japan (GenomONE™-Neo®). Then, 1.5 assay units (AU) of HVJ-E (1 AU = 10^9–10^{10} particles)/50 μL saline was injected into a tumor on days 12, 16, and 20. Preliminary experiments demonstrated that inhibition of tumor growth was more effective when HVJ-E was administered on days 12, 16, and 20 than on days 4, 8, and 12 [5] (Data not shown). In some experiments, HVJ-E carrying plasmid DNA, such as pVAX1-mouse IL-2 (pVAX-IL-2), was prepared according to the manufacturer's instructions before the intra-tumoral injection. The pVAX1-IL-2 was constructed by cloning the mouse *IL-2* gene from pORF-mouse IL-2 (InvivoGen, San Diego, CA, USA) into pVAX1 at the EcoRI and XhoI sites, as described previously [16].

2.5. Cell proliferation assay

Cell proliferation was assessed by MTS assay with CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega Corporation Madison, WI, USA). ISOS-1 cells were seeded in 96-well microtiter plates (1×10^3 cells/well/100 μL). Then, 24 h later, they were treated with 10 μL of HVJ-E (multiplicity of infection (MOI): 6.25×10^5). Three days later, cell proliferation was assessed by measuring absorbance at 490 nm after adding 20 μL of CellTiter 96®AQ_{ueous} One Solution Reagent.

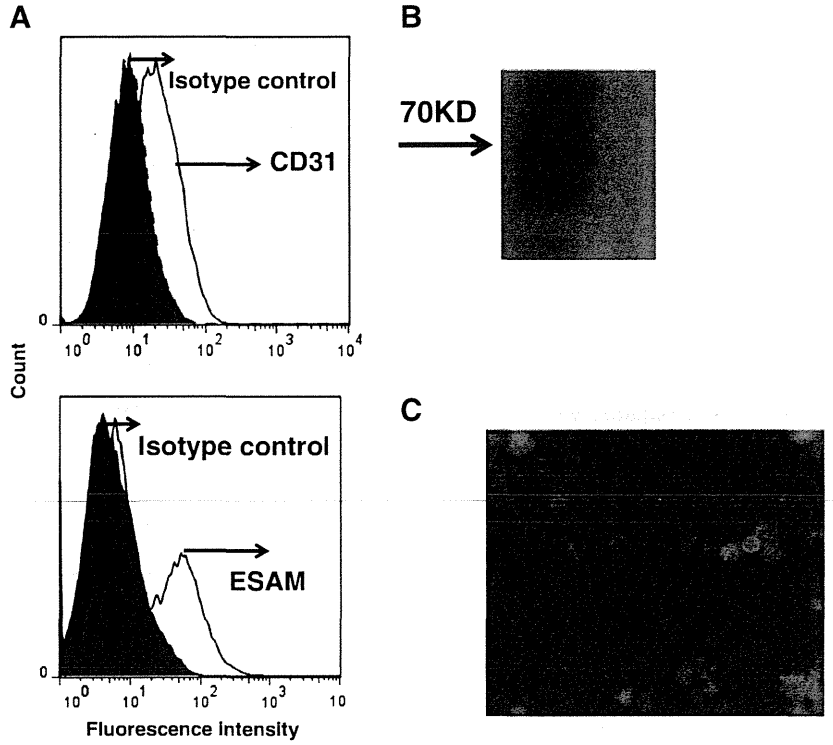

Figure 1 CD31 and ESAM expressions on ISOS-1 cell line. A: A flowcytometric analysis for CD31 and ESAM. B: 70 kDa of CD31 protein was detected by Western blotting analysis. C: Immunostaining of cultured ISOS-1 cells. Nuclei were counterstained with Hoechst 33342.

2.6. Flow cytometry

Single cell suspensions were prepared in PBS containing 5% FCS. Following the blocking of IgG receptors with anti-mouse CD16/32 mAb, cells were stained with antibodies against cell surface markers. For intracellular staining, cells were permeabilized with eBioscience Fixation/Permeabilization kit (eBioscience). Cells were then analyzed by FACSCalibur™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.7. Preparation of tumor-infiltrating lymphocytes (TILs)

Tumors were excised 2 days after the last HVJ-E injection. Tumors were mechanically sheared and incubated in dissociation solution (PBS (−) supplemented with 0.2 mg/mL DNase I (Roche Diagnostics GmbH, Mannheim, Germany) and 1 mg/mL collagenase D (Roche Diagnostics GmbH, Penzberg, Germany) for 1 to 2 h at 37 °C. Cell suspensions were passed through a 70-μm cell strainer and washed with PBS (−). The pellet was resuspended in 1 mL of RPMI-1640 and layered on top of discontinuous gradients of 68% and 44% Percoll solution [17] (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After centrifugation at 700 *g* at 25 °C for 20 min, lymphocytes at the interface were harvested.

2.8. Preparation of CD8 cells and NK cells

Spleens were removed 7 days after the last intra-tumoral injection of HVJ-E. Spleen cells (4.8×10^7) were cultured with mitomycin C (MMC) (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) -treated ISOS-1 cells (100 μg/mL, 37 °C, 30 min) at a ratio of 20:1 in a 250-mL flask in a volume of 100 mL. Six days later, the cells were harvested, and CD8 (+) cells were isolated by positive selection with immunomagnetic beads [5]. NK cells were directly isolated from spleens by the positive selection of CD49b cells with immunomagnetic beads 1 day after the last injection of HVJ-E treatment.

2.9. IFN-γ production from CD8 cells

CD8 (+) cells isolated as described above (1×10^5 cells) were stimulated with MMC-treated ISOS-1 cells (1×10^4 cells) in 96-well, round-bottomed, microtiter plates for 48 h. IFN-γ in the supernatants was measured with an immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA).

2.10. Cytotoxic assay

Anti-tumor cytolytic activity of effector cells was determined by the 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, target ISOS-1 cells labeled with CFSE (5-(6)-carboxyfluorescein diacetate succinimidyl ester) (1×10^5) were incubated with either CD8 cells or NK cells at various ratios (1:6.25, 1:12.5, and 1:25) for 4 h at 37 °C. They were then stained with 7-AAD (7-amino-actinomycin D) and analyzed by flow cytometry [18].

2.11. Preparation and stimulation of bone marrow-derived dendritic cells (BMDCs)

Bone marrow cells were cultured in 10 mL RPMI-1640 containing 10% FCS and GM-CSF (20 ng/mL, PeproTech, Rocky Hill, NJ, USA) in a 100-mm Petri dish. Three days later, 10 mL of medium containing GM-CSF (20 ng/mL) was added to the dish and cultured for another four days [19]. BMDCs were prepared by positive selection with CD11c-microbeads (Miltenyi Biotech) and stimulated with HVJ-E for 2 days.

2.12. Adoptive cell transfer

Naïve recipient BALB/c mice were irradiated with 6 Gy of total-body-irradiation 6 h prior to adaptive transfer. Then, 4×10^7 spleen cells/500 μL from donor mice that had received ISOS-1 inoculation and treatment with HVJ-E carrying *IL-2* gene (HVJ-E/IL-2) were transferred i.v. into irradiated recipient mice. One day later, they were inoculated with ISOS-1 cells.

2.13. Statistical analyses

For survival periods, statistical analysis was performed using the Kaplan–Meier method and the significance of differences in the survival distribution was evaluated by the log-rank test. Turkey's post hoc test or Student's non-paired *t*-test was performed for the other assays. $P < 0.05$ was considered significant.

3. Results

3.1. HVJ-E carrying *IL-2* gene (HVJ-E/IL-2) eradicated angiosarcoma and improved survival in mice

The in vivo effects of HVJ-E in the mouse angiosarcoma model were examined initially. BALB/c mice were inoculated with ISOS-1 cells on day 0 and treated with HVJ-E on days 12, 16, and 20. Intra-tumoral injection of HVJ-E effectively inhibited tumor growth of ISOS-1 cells in vivo (Fig. 2A).

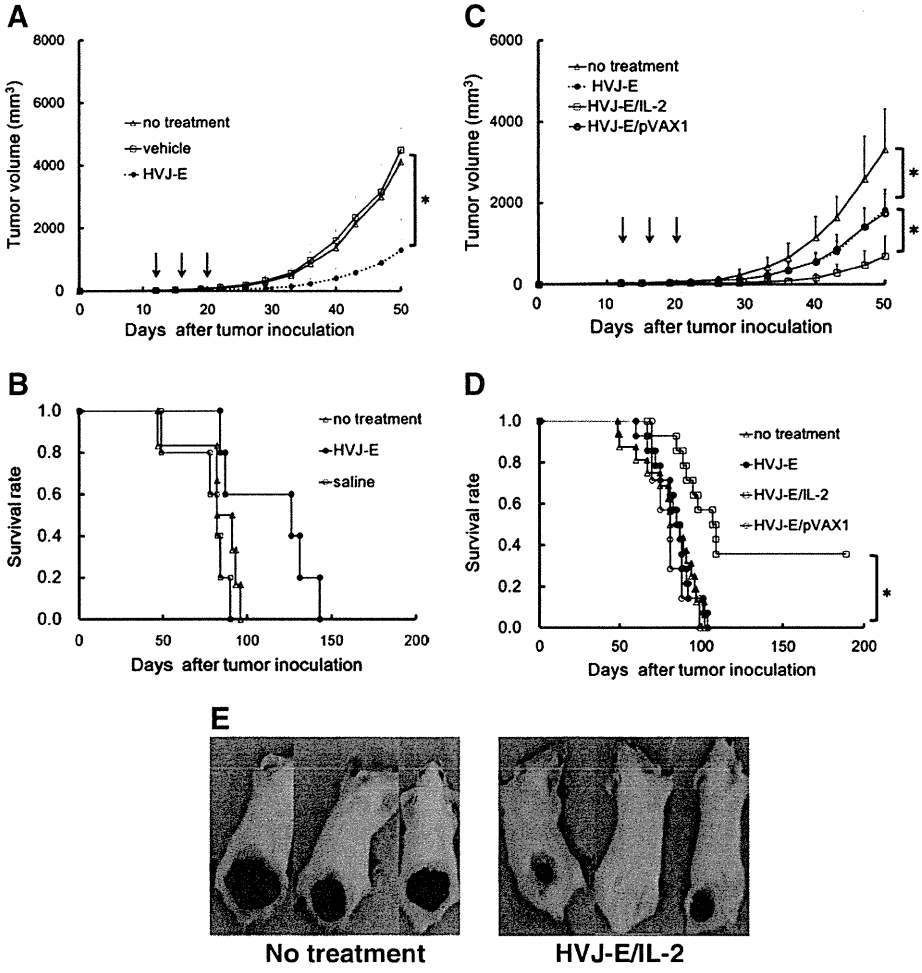

Figure 2 In vivo therapeutic effects of HVJ-E and HVJ-E/IL-2 on angiosarcoma. BALB/c mice were inoculated with angiosarcoma cell line (ISOS-1) on day 0. HVJ-E or HVJ-E/IL-2 was intra-tumorally injected on days 12, 16, and 20. Each group consisted of 8 16 mice. A and C: Time course curves of tumor volumes. HVJ-E/IL-2 is more potent than HVJ-E in suppressing tumor growth. B and D: Survival rates of treated mice. Unlike HVJ-E alone, HVJ-E/IL-2 significantly and consistently prolongs mouse survival. E: Macroscopic features of tumors. Data are presented as means ± S.D (standard deviations). *p < 0.05.