Combined Oncolytic Virotherapy with Herpes Simplex Virus for Oral Squamous Cell Carcinoma

FUMI OGAWA, HIROO TAKAOKA, SOICHI IWAI, KEIKO AOTA and YOSHIAKI YURA

Department of Oral and Maxillofacial Surgery,
Osaka University Graduate School of Dentistry, Osaka University, Suita, Osaka, Japan

Abstract. *Background: The effect of dual infection with herpes simplex virus type 1 (HSV-1) mutants on human oral squamous cell carcinoma (SCC) cells was examined. Materials and Methods: Human oral SCC cells were infected with $\gamma_1$34.5 gene-deficient HSV-1 R849 and HSV-1 HF that has multiple mutations and induces cell fusion. Cell viability was measured by LDH release assay. Athymic mice were injected with oral SCC cells into the buccal region to induce subcutaneous tumors. Results: Oral SCC cells were infected with R849, followed by infection with R849 or HF. Virus production was elevated by both strains of HSV-1. Although the release of LDH from R849-infected cells was increased by secondary infection with R849 or HF, the effect of HF was more remarkable. When nude mouse tumors were treated with R849, HF, R849+R849, or R849+HF, treatment with R849+HF was the most effective. Conclusion: These results suggest that fusion-inducing virus HF enhances the oncolytic ability of $\gamma_1$34.5 gene-deficient HSV-1 and provides a rationale for using fusogenic viruses as enhancing agents*

Oral squamous cell carcinoma (SCC) is a common cancer in the head and neck region. The majority of patients with oral SCC fail locally or regionally, with only a small fraction failing distantly. After recurrence, tumors are usually resistant to chemotherapy and radiotherapy, and a wide resection of the surrounding tissue is frequently needed, leading to a severe impairment of orofacial structures and functions (1, 2). A novel therapy to preserve surrounding normal tissues is required.

Correspondence to: Dr. Yoshiaki Yura, Department of Oral and Maxillofacial Surgery, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: +81 668792941, Fax: +81 668792170, e-mail: yura@dent.osaka-u.ac.jp

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Use of replication-competent herpes simplex virus type 1 (HSV-1) mutants is an approach to treat cancer because the replication of viruses within cancer cells can result in their destruction (3-6). The HSV vectors used today are deficient in the main neurovirulence gene $\gamma_1$34.5, which severely restricts their ability to replicate in the adult central nervous system and to achieve latency (7, 8). It has been shown that activation of mitogen-activated protein kinase (MAPK) kinase (MEK) mediates the suppression of RNA-dependent protein kinase and the status of MEK predicts the ability of $\gamma_1$34.5 gene-deficient HSV-1 to replicate in and destroy tumor cells (9-11). However, deletion of the $\gamma_1$34.5 gene reduces replication efficiency. To restore the capability to destroy tumors, HSV-1 mutants with various inserts able to produce cytokines and chemokines including interleukin (IL)-12 and granulocyte macrophage-colony-stimulating factor (GM-CSF) have been developed (12, 13). Another strategy is to combine the use of $\gamma_1$34.5 gene-deficient HSV-1 with that of chemotherapeutic agents or radiotherapy (14-19). Indeed, chemotherapeutic drugs such as cisplatin and mitomycin C in combination with HSV vectors were found to be effective for the treatment of head and neck cancer and gastric cancer (14, 17).

A clone of HSV-1 HF, HF10, was reported to have a defective UL56, which may be responsible for the attenuation of this HSV-1, and to induce syncytia in epithelial cells (20). In phase I trials for lymph node metastasis of breast cancer and for head and neck cancer, no serious side-effect was observed (21, 22). HF spreads by cell-to-cell interaction and is expected to spread in solid tumors (23, 24). HF10 was also used as a helper agent to enhance the release of an adeno-associated virus in pancreatic cancer cells both *in vitro* and *in vivo* (25). Whether the oncolytic effect of $\gamma_1$34.5 gene-deficient HSV-1 can be modified by other strains of HSV-1 has not been investigated extensively (26). In the present study, we examined the effect of a secondary infection of HF, the parental strain of HF10, on the antitumor effect of $\gamma_1$34.5 gene-deficient HSV-1 R849.

Materials and Methods

Cells and virus. The human oral SCC cell line SAS was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and grown in an incubator at 37˚C in a humidified atmosphere with 5% CO_2. SAS cells were maintained in DMEM containing 5% FBS. HSV-1 mutant R849 (27) and HF (23, 24, 28) were grown in semi-confluent Vero cell monolayers. The infectivity of HSV-1 was determined by plaque formation on Vero cell monolayers covered with 0.3% methylcellulose. For purification of R849, infected cells were subjected to three cycles of freezing and thawing and then centrifuged at 3,000 ×g for 15 min at 4˚C. The supernatant was centrifuged at 9,000 ×g for 4 h at 4˚C, and the virus pellet was suspended in phosphate-buffered saline (PBS).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells grown in 96-well culture dishes were infected with HSV-1 at a multiplicity of infection (MOI) of 0.01, while controls were mock-infected. After incubation for different intervals, 10 ml of a 5 mg/ml MTT solution were added to each well with 100 ml of medium. Cells were allowed to incubate for 4 h at 37˚C and then 100 ml of 0.04 N HCl in isopropanol were added and mixed thoroughly to dissolve the dark blue crystals. After standing overnight at room temperature, the plates were read on a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) with a reference wavelength of 630 nm and a test wavelength of 570 nm. Background absorbance at 690 nm was subtracted from the 570 nm reading. Changes from room air controls were calculated.

LDH release assay was performed using LDH-Cytotoxic Test (Wako, Osaka, Japan) following the manufacturer's instructions. Cells were infected or mock-infected with HSV-1 and incubated for different intervals. After the experiment, the culture medium was harvested and cells were dissolved in 0.1% Triton®-X 100 to release intracellular LDH. LDH reagents were added to the medium. After incubation for 15 min at 37˚C, the plates were read on a Benchmark Plus microplate spectrophotometer at a wave length of 560 nm. The values for the medium were divided by those for the medium and cells and percentages were determined as the released LDH.

Reverse transcription-polymerase chain reaction (RT-PCR) and PCR analyses. Total RNA was extracted from mouse tissues (see below) using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was performed using a Takara RNA PCR kit (Takara, Tokyo, Japan). One microgram of RNA was reverse-transcribed using AMV reverse transcriptase, and cDNAs encoding the HSV-1 genes ICP0, ICP 27, LacZ and β-actin gene sequences were amplified by PCR using specific primers. The sequences of the primers used were as follows: ICP0 forward, 5'-GACAGCAAAAATCCCCTGAG-3'; reverse, 5'-ACGAGGGAAAA CAATAAGGG-3'; ICP 27 forward, 5'-GACGGCCTCCTCTACCA-3'; reverse, 5'-CTGGAATCGGACAGCAGCCGG-3'; LacZ forward, 5'-GCGTTACCCAACTTAATCG-3'; reverse, 5'-TGTGAGCGA GTAACAACC-3'. β-actin forward, 5'-GTGGGCCGCTCTAGGC ACCAA-3'; and reverse, 5'-CTCTTTGATGTCACGCACGA TTTC-3' (29). To confirm the integrity of each RNA sample, a PCR analysis of the β-actin gene was performed. The PCR amplification of cDNAs was carried out in volumes of 50 ml for 25 cycles at a denaturing temperature of 94˚C for 1 min, an annealing temperature of 55˚C for 2 min, and an extension temperature of 72˚C for 3 min using a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA). PCR products were subsequently size-fractionated on 1.5% agarose gels, stained with ethidium bromide and photographed under transmitted UV light.

Animal experiments. Experiments using SAS xenografts in nude mice were performed with the approval of the Institute of Laboratory Animals, Osaka University Graduate School of Dentistry. Athymic 5-week-old BALB/c (*nu/nu*) female mice were obtained from Clea Japan (Tokyo, Japan). Mice were subcutaneously injected with 1×10^6 SAS cells into the buccal region. Once the subcutaneous tumor reached approximately 5 mm in diameter, animals were divided into 5 groups of 6 animals each. Animals in groups 1 (R849), 2 (R849+R849) and 3 (R849+HF) were administered a single intratumoral (*i.t.*) injection of 1×10^6 plaque-forming units (PFU) of R849 suspended in 100 ml of PBS. Three days later, group 2 and 3 animals received an i.t. injection of 1×10^6 PFU of R849 and HF, respectively. Group 4 (HF) animals were given an i.t. injection of HF only. Group 5 animals received PBS instead of R849 and were used as a control. The experiment was started at the time HSV-1 was injected into the animals. Bidimential tumor measurements were performed with calipers and tumor volume was determined using the formula for a rotational ellipsoid (L × W2 × 0.5).

To detect mRNA of HSV-1 in mouse tissues, an experiment with acute HSV-1 infection was performed. Tumor-bearing mice were treated the same as group 1 or group 3 animals. Mice received an i.t. injection of 1×10^6 PFU of R849 and were maintained for 6 days. Alternatively, 3 days after the injection of R849, mice were given an i.t. injection of 1×10^6 PFU of HF and maintained for another 3 days. Six days after the inoculation of R849, mice were sacrificed and tumors were removed. Tumors were snap frozen for extraction of RNA and histopathological examination. For determination of the viral titer in tumor tissue, each frozen tumor was dissolved in 1 ml of PBS and homogenized. After centrifugation in a microtube, the supernatant was used for plaque assay in Vero cells (24).

Histopathological examination and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) staining. Tumors were removed, placed in 10% buffered formalin for fixation and embedded in paraffin wax. Sections were stained with hematoxylin and eosin. For X-gal staining, R849-infected cells were placed in a fixative containing 0.2% glutaraldehyde and 2% formaldehyde in PBS for 1 h, and submerged in cold PBS. Cells were then left overnight in a substrate solution containing 1 mg/ml X-gal (Sigma, St. Louis, MO, USA), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride in PBS and washed with PBS.

Statistical analysis. LDH release and mean tumor volumes were compared using the unpaired *t*-test. $p<0.05$ indicated a significant difference among groups.

Results

Cytopathic effect of R849 and HF on oral SCC cells. SAS cells were infected with either R849 or HF at a MOI of 0.01 and examined for a cytopathic effect by phase-contrast

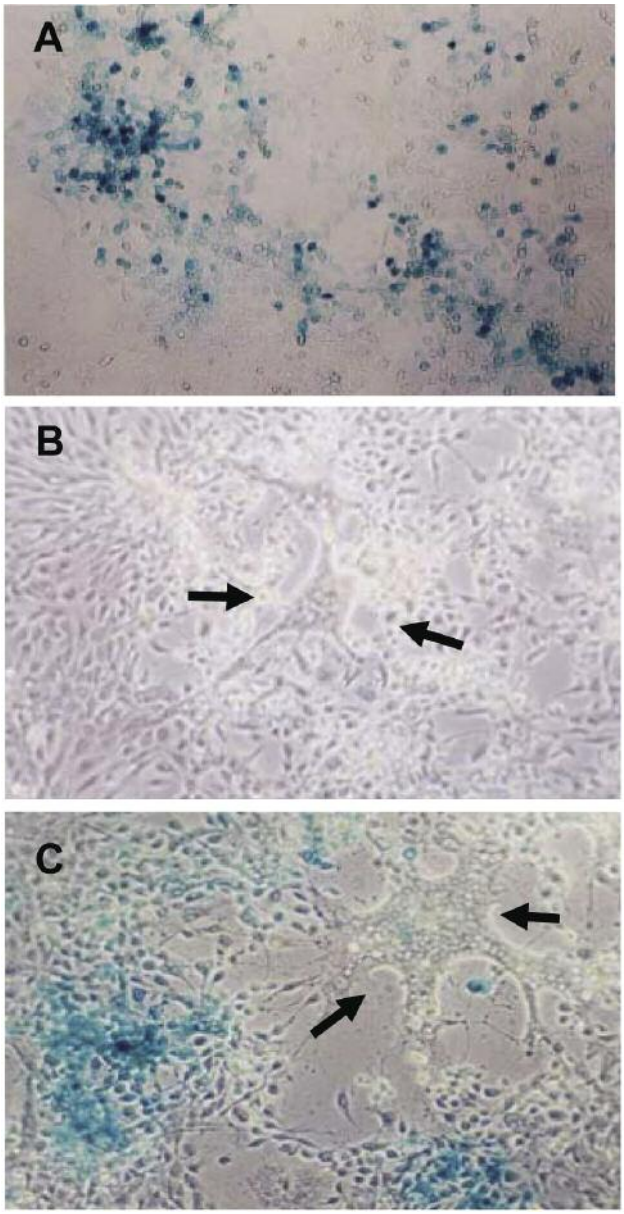

Figure 1. *Cytopathic effect of R849 and HF in human oral SCC cells. SAS cells were infected with either R849 or HF at an MOI of 0.01 and cultured at 37˚C for 24 h. A, Uninfected cells were left untreated. B, The morphology of SAS cells was observed with a phase-contrast microscope. R849-infected cells were fixed and subjected to X-gal staining. C, For dual infection, R849-infected SAS cells were super-infected with HF at 24 h after the initial infection, cultured for a further 24 h, and subjected to X-gal staining. Arrows indicate syncytial formation.*

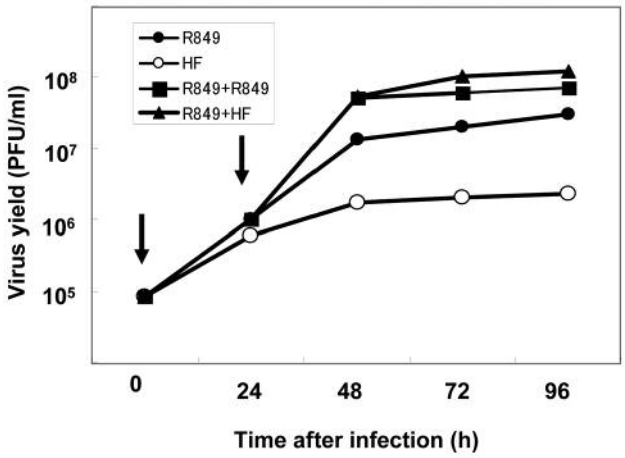

Figure 2. *Susceptibility of human oral SCC cells to R849 and HF in vitro. SAS cells were infected with either R849 or HF at a MOI of 0.01. To evaluate the effect of additional infections on virus yield, SAS cells infected with R849 at an MOI of 0.01 were cultured for 24 h. Thereafter, they were secondarily infected with either R849 or HF at an MOI of 0.01 and then virus yield was determined at various intervals. Data are means±SD of three determinations. Arrows indicate infection times.*

microscopy. Cells infected with R849 were also examined for the expression of the Lac Z gene by X-gal staining. A cytopathic effect showing cell rounding was observed in cultures infected with R849, whereas cell fusion appeared in the cells infected with HF (Figure 1A, B). For dual infection, SAS cells infected with R849 were super-infected with HF, cultured for 24 h and subjected to X-gal staining. There were X-gal-positive rounded cells and X-gal-negative syncytial formations. The incorporation of X-gal-positive cells into the syncytum was observed (Figure 1C).

Susceptibility of human oral SCC cells to R849 and HF in vitro. To determine the ability of oral SCC cells to support the replication of HSV-1 strains, SAS cells were infected with either R849 or HF at a MOI of 0.01 and assayed for virus production at 48, 72, and 96 h after infection. The virus yield of R849 was higher than that of HF during the experiment. At 96 h after infection, the difference between the yields of HF and R849 was 10-fold. To evaluate the effect of additional infection on the yield, SAS cells infected with R849 at an MOI of 0.01 were cultured for 24 h and then infected with either R849 or HF at the same MOI. By this secondary infection, the virus yield was elevated, irrespective of HSV-1 strain (Figure 2). As compared with cell cultures infected with R849 only, 96 h after the initial infection there was an approximate 1.8-fold and 2.6-fold increase in the viral yield in those secondarily infected with R849 and HF, respectively.

Suppressive effect of HSV-1 infection on the viability of oral SCC cells. The MTT assay measures the activity of mitochondria. When SAS cells infected with either R849 or HF at an MOI of 0.01 were cultured for 120 h, the viability decreased to 38% and 44% of the untreated control, respectively. Secondary infection with R849 or HF was

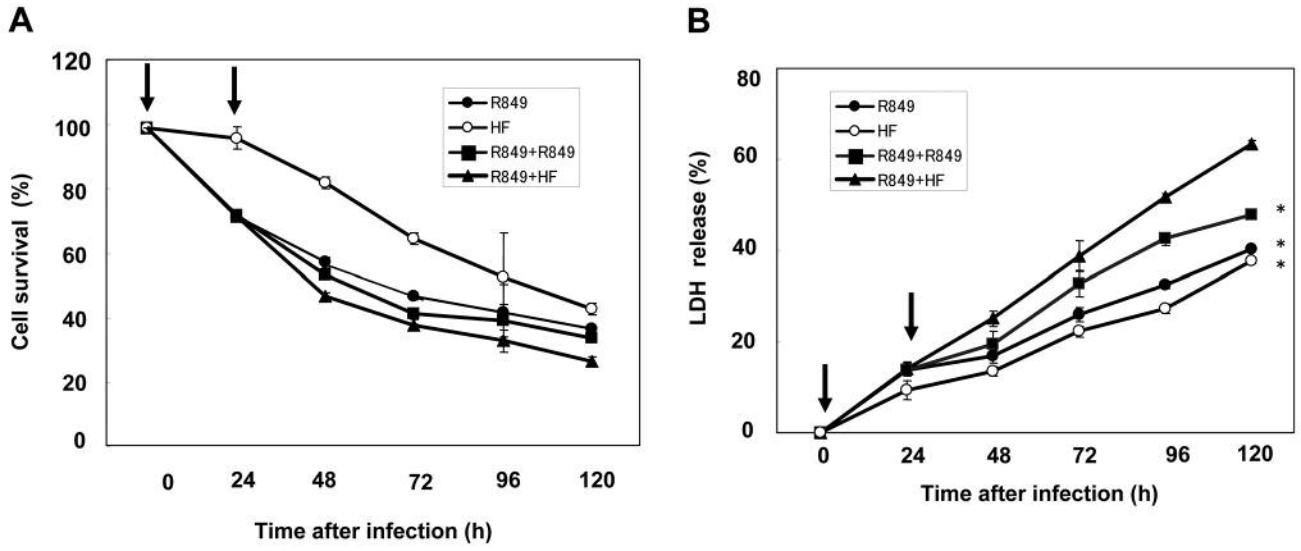

Figure 3. *Suppressive effect of dual infection on the viability of oral SCC cells. A, SAS cells were infected with either R849 or HF at an MOI of 0.01 and cultured at 37˚C. Cell viability was measured by MTT assay. R849-infected cells were also super-infected with R849 or HF at the same MOI 24 h after the initial R849 infection. At various intervals, cell viability was measured by MTT assay. B, Cells were infected with R849 or HF as described for the MTT assay. The LDH released into the culture medium from damaged cells due to disintegration of the cell membrane was harvested and the amounts were measured. Data are means±SD of four determinations. *p<0.05 vs. R849+HF. Arrows indicate infection times.*

performed 24 h after the R849 infection; cell viability at 120 h after the initial infection decreased to 35% and 28% of the control, respectively (Figure 3A).

LDH is released from damaged cells due to the disintegration of cell membranes, followed by degradation of the cells (30). At 120 h after infection with R849 or HF, a similar level of LDH release was observed; the proportions of free LDH were 40% and 38% , respectively. The amount of free LDH increased to 63% by secondary infection with HF (Figure 3B). The amount of free LDH in the cultures infected with R849 and HF was significantly ($p<0.05$) greater than that in those infected with R849 twice.

Virus yield in oral SCC xenografts in nude mice. To examine the replication of R849 *in vivo*, SAS xenografts in nude mice were given 1×10^6 PFU of R849 and virus production in the tumors was examined for 6 days. When the yield of virus in the tumors was measured by plaque assay, the viral titer reached a maximal level at 3 and 4 days after inoculation; the titer was 1.3×10^4 PFU/ml. Thereafter, the titer declined to 3.0×10^3 PFU/ml (Figure 4). When a secondary infection with HF was performed 3 days after the R849 infection, the yield was elevated up to 3.6×10^4 PFU/ml. The recovered virus after the injection of HF showed a syncytia type of cytopathic effect in Vero cells.

Histopathology and viral gene expression of R849-treated tumors. Oral SCC xenografts treated with R849 or a combination of R849 and HF were subjected to histopathologial examination 6 days after the initial injection of R849. No histological change caused by R849 was apparent, although central necrosis was observed (data not shown). In the tumors that received R849 initially and then HF, there was also central necrosis and multinucleated giant cells were observed as a specific feature adjacent to the necrotic portion (Figure 5A).

Total RNA was prepared from tumors and then subjected to RT-PCR analysis. The immediate early genes *ICP0* and *ICP27* were expressed in the tumors and the amounts of the transcripts were not altered, even if the tumors received a secondary inoculation of HF (Figure 5B). Three days after HF was injected, the expression of LacZ rather decreased, indicating that HF did not promote the replication of R849.

Suppressive effect of HSV-1 infection on the growth of oral SCC xenografts in nude mice. Group 1 (R849), 2 (R849+R849) and 3 (R849+HF) animals received injections of R849 into subcutaneous tumors. Three days later, group 2 and 3 animals were given either R849 or HF. Group 4 (HF) animals received HF only and group 5 (control) animals were given PBS as a control. In the control group, tumors grew rapidly and the tumor volume at 60 days was 4,500 mm^3 (Figure 6A), whereas the tumor growth of HSV-1 vector-treated (group 1-4) animals was suppressed and the tumor volumes were less than 1,500 mm^3. Although the injection of R849 or HF suppressed the growth of tumors, gradual tumor growth was observed in group 1, 2 and 4

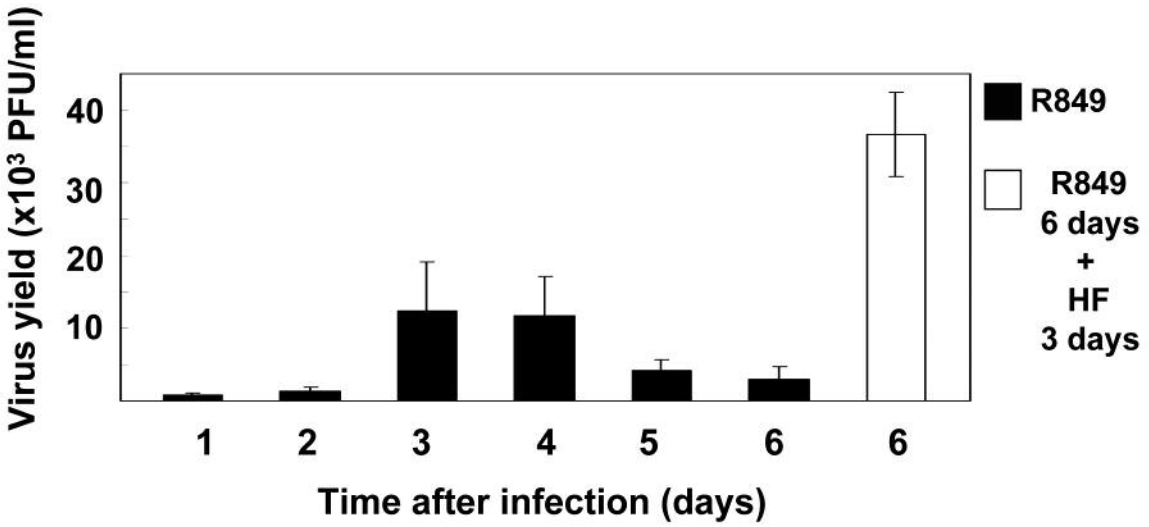

Figure 4. *Virus yield in oral SCC xenografts in nude mice. Oral SCC xenografts in nude mice were given 1×10⁶ PFU of R849 and virus production in the tumors was measured for 6 days after infection. In the case of secondary infection, tumors received 1×10⁶ PFU of HF 3 days after the R849 injection and were examined 3 days later. Data are means±SD of three determinations.*

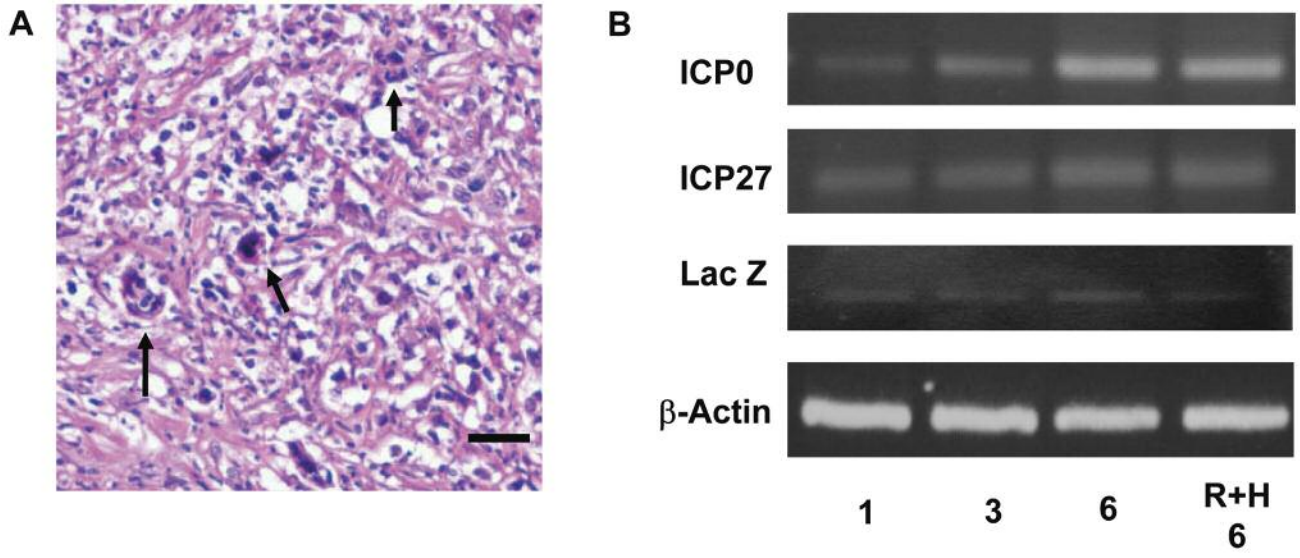

Figure 5. *Histopathological changes and virus gene expression in oral SCC xenografts in nude mice. A, Oral SCC xenografts in nude mice were treated with a combination of R849 and HF as described in Figure 3. Six days after the initial inoculation of R849, they were subjected to histopathologial examination. Arrows indicate multinucleated giant cells. Bar, 50 mm. B, Total RNA from tumor tissues injected with R849 or a combination of R849 and HF was subjected to RT-PCR analysis.*

animals. However, in group 3, there were animals that did not show further growth of tumors. When tumor volume was compared at day 60, a significant difference between group 3 (R849+HF) and group 1 (R849) ($p<0.001$), group 4 (HF) ($p<0.01$) and group 2 (R849+R849) ($p<0.01$) was found (Figure 6B). No symptoms of neurological abnormality or skin lesions at the injected sites were observed.

Discussion

$\gamma_1$34.5 Gene-deficient HSV-1 is generally used for oncolytic virotherapy with HSV-1 because of its lack of neurovirulence (7). The progeny viruses of $\gamma_1$34.5 gene-deficient HSV-1 R849 were expected to spread from the injected area to the surrounding tumor cells (27). The present study was

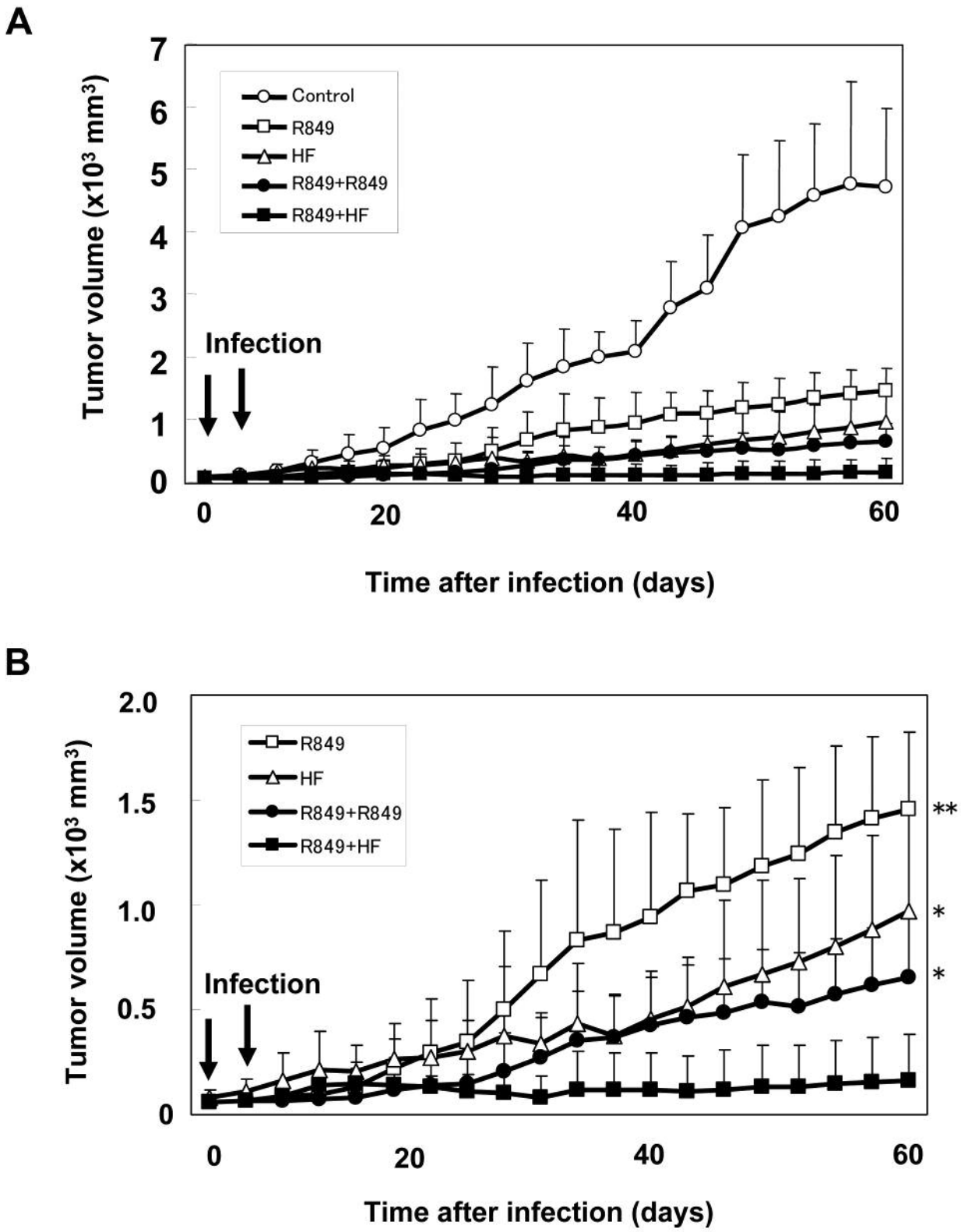

Figure 6. *Effect of R849 and HF on the growth of FI xenografts in nude mice. A, Group 1 (R849), 2 (R849+R849) and 3 (R849+HF) animals received injections of R849 into subcutaneous tumors. Three days later, group 2 and 3 animals were given either R849 or HF. Group 4 (HF) animals received HF only and group 5 (control) animals were given PBS and were used as a control. Tumor volume was measured during the experiment. B, The data from groups 1 to 4 are shown. (n=6) * p<0.01 vs. R849+HF, **p<0.001 vs. R849+HF.*

intended to enhance the ability of R849 to destroy tumor cells by its combination with a fusogenic type of HSV-1 mutant HF. Virus yield is an important factor for successful oncolytic virotherapy. We found that the $\gamma_1$34.5 gene-deficient HSV-1 R849 had a cytopathic effect, causing the rounding of oral SCC cells, and expressed the LacZ gene, indicating that it replicated efficiently and produced a high titer of progeny virus in oral SCC cells. As expected, successive infection with R849 as a virus resulted in an increase of virus production. We also found that HF induced large syncytia in oral SCC cells, although the level of production of HF was 10-fold lower than that of R849. In the case of dual infection with R849 and HF, R849-infected cells and uninfected cells were fused to form large syncytia by the function of secondary virus HF. The amounts of progeny virus were also increased up to the level achieved by successive R849 infection.

Cytopathic effects are tightly correlated with the production of virus in cell-rounding viruses. R849-infected rounded cells would be degraded after production of progeny virus. Indeed, when mitochondrial activity was measured by MTT assay, R849 was found to decrease cell viability more efficiently than HF. However, a similar level of cytotoxicity was observed when oral SCC cells were infected with R849 or HF, assessed by the release of LDH. In R849-infected cells, subsequent HF infection reduced the viability more significantly than successive R849 infection. The LDH assay measures the damage to the plasma membrane (30). It can be stated that HF promotes the degradation of R849-infected cells as well as newly infected cells by increasing the permeability of the plasma membrane.

In the study of SCC xenografts in nude mice, the virus yields of R849 reached a peak at 3 days after injection and declined in 6 days. The experiment with a subsequent HF injection 3 days later indicated that HF induced multinucleated cells to form and caused necrotic changes in the central part of the tumors. In tumors, immediate early genes, *ICP0* and *ICP27*, were expressed but the expression was not altered by secondary infection of HF; the expression of LacZ rather decreased after HF injection. It was reported that the primary replication of one strain of HSV was generally unaffected by the simultaneous inoculation of another strain either at the same site or at a different site within the same dermatome (31). It is likely that additional HF infection does not resume the replication of the initially injected R849 in nude mouse tumors.

We examined the antitumor effect of R849 and HF on oral SCC xenografts in nude mice. In this case, mice received an intratumoral injection of R849 initially. Three days later, HF was injected into the tumors. The growth of R849-injected tumors was significantly suppressed, whereas that of control tumors increased continuously for 8 weeks. This is consistent with the results of our previous study, indicating a potent antitumor effect of R849 on human oral SCC (29). However, as described in other virus–tumor systems, a single injection of this $\gamma_1$34.5 gene-deficient mutant alone was insufficient to eradicate oral SCC in nude mice, and tumor growth occurred after an interval. Secondary HSV-1 infection, irrespective of strain, would exert a stronger antitumor effect in tumors. Notably, HF significantly enhanced the suppressive effect of R849 and, as a consequence, tumor remission was observed. Thus, we concluded that HF was more efficient against the growth of oral SCC xenografts than R849 as the secondary vector. HF may replicate in tumor cells devoid of R849 infection and fuse R849-infected cells together.

We used a $\gamma_1$34.5 gene-deficient virus that was incapable of replication in normal neuronal cells as an initial vector. Although it is possible to produce recombinant viruses by dual infection (31, 32), R849 cannot acquire the $\gamma_1$34.5 gene of HF inoculated 3 days later in the absence of replication of R849 (Figure 4). Indeed, we found neither general side-effects nor any neurological disorders in the mice. No viral gene was detected in the brains of the mice by PCR, indicating a lack of neurovirulent virus (data not shown). Thus, it is unlikely that a neurovirulent recombinant appeared in this experimental condition.

In conclusion, HF can enhance the oncolytic ability of $\gamma_1$34.5-deficient HSV-1, possibly by increasing the membrane permeability of tumor cells. Nakamori *et al*. (33) developed a class of oncolytic virus (Sinco-2D) which has fusogenic membrane glycoproteins of the gibbon ape leukemia virus and kills tumor cells by direct cytolysis, with syncytial formation induced by cell membrane fusion. A standard virus for oncolytic virotherapy, $\gamma_1$34.5 gene-deficient HSV-1, induced cell rounding and produced progeny viruses in human oral SCC cells (Figures 1 and 2). RNA viruses such as mumps virus and measles virus are known to induce syncytia in epithelial cells (34, 35). The results of the present study provide a rationale for using fusogenic viruses as enhancing agents of oncolytic virotherapy with $\gamma_1$34.5-deficient HSV-1.

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