Herpes simplex virus-induced, death receptor-dependent apoptosis and regression of transplanted human cancers

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(Received July 15, 2004/Revised October 6, 2004/Accepted October 12, 2004)

Inoculation of a live attenuated herpes simplex virus (HSV) vector, βH1, into human U87MG glioblastoma cells transplanted into athymic nude mice induced complete regression of tumors. The infected cells underwent histochemically confirmed apoptosis without lymphocyte infiltration after expressing CD30, CD30 ligand (CD30L), tumor necrosis factor (TNF)-α, TNF receptor 1 (TNF-R1), FAS, and FAS ligand (FAS-L) with activation of caspases 3 and 8. Induction of the transcripts of these receptors and ligands in inoculated tumors was confirmed by quantitative RT-PCR. To examine the specificity of apoptosis in the transplanted tumor, we inoculated βH1 into transplanted human lung, breast, gastric, and colon cancer tumors, and similar tumor regression with apoptosis was observed in all tumors. We analyzed the roles of expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in the tumors, and found that HSV-induced apoptosis was suppressed by the respective antibodies. These findings indicate that the CD30/CD30L, TNF-α/TNF-R1, and FAS/FAS-L interactions resulted in apoptosis and tumor regression in immunocompromised mice. In addition to the death receptor-dependent apoptosis induced by HSV, the expressed ligands and receptors might enhance the susceptibility of tumor cells to cell-mediated cytotoxicity and augment the activation of tumor-killing lymphocytes in immunocompetent models. (Cancer Sci 2004; 95: 990–998)

The use of replication-competent viral vectors, in particular herpes simplex virus (HSV) type 1 vectors, is a promising strategy for cancer therapy because the virus can replicate and spread *in situ*, exhibiting oncolytic activity through a direct cytopathic effect.[1] A number of oncolytic HSV vectors have been developed by introduction of mutations that disrupt genes associated with neurovirulence and/or viral DNA synthesis in order to restrict viral replication in transformed cells and to diminish the infectious disease.[2–11] Restriction of viral growth by introduction of a tissue-specific calponin promoter into the HSV genome resulted in efficient oncolysis of malignant soft tissue and bone tumors.[12] Evidence to date following administration of such vectors into the brain attests to their safety, an important observation in light of the neuropathogenicity of the virus. Phase 1 clinical trials of these vectors are either ongoing or completed, without any severe adverse events.[13–16] These variants have been demonstrated to be effective in the treatment of a wide variety of malignancies including brain, breast, colorectal, prostate, head and neck cancers, disseminated peritoneal, and soft tissue and bone tumors in animal models.[12, 17–22] On the basis of early studies using immunocompromised athymic or severe combined immunodeficiency mice, one mechanism of antitumor activity of oncolytic HSV is thought to be tumor cell destruction due to virus infection and intracellular replication.

Recent studies in immunocompetent syngeneic models have suggested that the host defense mechanisms provoked by HSV play important roles in oncolysis. Oncolytic HSV growing within a tumor elicits an immune response to HSV and tumor-specific antigens.[23] Colon cancer tumors were transplanted on the bilateral flanks of syngeneic immunocompetent mice, and HSV was inoculated into one of the established tumors. The tumors in the syngeneic immunocompetent mice showed greater and prompter regression than those in athymic animals. Further, the inoculation into a tumor on one side induced regression of the tumor on the other side. In addition, a $CD8^+$ T cell-mediated response is generated to a tumor-specific antigen on the cells. The recognition that conditionally replicating HSV vectors induce antitumor immune responses has led to engineering of HSV to carry genes for cytokines that potentiate the antitumor immune response.[11, 24–26]

We examined the antitumor effect of an attenuated HSV expressing β-galactosidase, βH1, using immunocompromised athymic models to analyze the direct effect of HSV on oncolysis in the absence of an antitumor immune response. We found that tumor regression due to HSV was mediated by apoptosis that occurred during HSV growth and subsequent expression of CD30, CD30 ligand (CD30L), tumor necrosis factor (TNF)-α, TNF-receptor 1 (TNF-R1), FAS, and FAS-L in transplanted human cancer cells. These observations suggest that expression of these ligands and receptors might enhance the susceptibility of tumor cells to NK cells or cytotoxic T lymphocytes, augment the activation of these tumor-killing lymphocytes, and thus potentiate the antitumor immune response and killing of tumor cells in immunocompetent models. Thus, unexpectedly, HSV induced expression of these ligands and receptors in tumor cells, leading to death receptor-dependent apoptosis and tumor regression. Apoptosis was not observed in βH1-infected U87MG glioblastoma cell cultures *in vitro*, indicating the specificity of apoptosis in the transplanted tumors. The elucidation of the mechanism of oncolytic activity of HSV by death receptor-mediated apoptosis may contribute to the development of more efficient oncolytic HSV vectors expressing ligand(s) or receptor(s) that activates apoptosis and cytotoxicity.

Materials and Methods

Cells and viruses. Human malignant glioma U87MG cells and human lung cancer A549 cells (RIKEN Cell Bank, Tsukuba, Ja-

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Abbreviations: HSV, herpes simplex virus; CD30L, CD30 ligand; TNF-α, tumor necrosis factor-α, TNF-R1, TNF receptor 1; FAS-L, FAS ligand; PBS, phosphate-buffered saline; PFU, plaque-forming units; RT-PCR, reverse transcriptase polymerase chain reaction; NK, natural killer; CTL, cytotoxic T lymphocytes.

pan) were propagated and used for transplantation. A live, attenuated HSV type 1 strain, βH1, expressing β-galactosidase and its parent, HF, and wild 7401H strains were prepared in Vero cells as described previously.[27–30]

Animals. Four-week-old male athymic BALB/c *nu/nu* mice (Japan SLC, Inc., Shizuoka, Japan) were housed in sterile cages. We conducted procedures involving animals and their care in the infection room of the Laboratory Animal Center, conforming to the animal experimentation guidelines of Toyama Medical and Pharmaceutical University.

Transplantation of tumors and virus inoculation. Mice were injected with U87MG cells and A549 cells in the flank subcutaneously. Tumor fragments (3×3×3 mm) of MX-1 human breast cancer, KATO-III human gastric cancer, and RCA human colon cancer (kindly provided by Toyama Chemical Co., Toyama, Japan) were transplanted into the right flank subcutaneously. When the tumor volume reached more than 100 mm^3, the mice were randomly divided, and tumors were inoculated with 1×10^7 PFU of βH1 or phosphate-buffered saline (PBS: 10 m*M* (pH 7.4), 150 m*M* NaCl) in 50 µl. We measured the tumor diameter externally with calipers and calculated the tumor volume as $(l \times w^2)/2$, where l is the maximum length of the tumor nodule and w is the length perpendicular to l.

Histochemical analysis. Tumor nodules were removed on day 3, 7, 10, or 14 after HSV inoculation, fixed with 4% paraformaldehyde in PBS at 4°C overnight, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and cut into 4 µm sections.[29, 30] The sections were stained with standard hematoxylin and eosin, and immunostained with the following primary antibody: GK1.5 rat anti-mouse CD4 monoclonal antibody, 53-6.7 rat anti-mouse CD8a monoclonal antibody, PK136 mouse anti-mouse NK1.1 monoclonal antibody (PharMingen, San Diego, CA), Ki-1 mouse anti-human CD30 monoclonal antibody, MOMA-2 rat anti-mouse macrophage monoclonal antibody (Immunotech, Marseilles Cedex, France), IE8-G6 mouse anti-human TNF-α monoclonal antibody, H-5 mouse anti-human TNF-R1 monoclonal antibody, APO-1 mouse anti-human FAS monoclonal antibody, NOK-1 mouse anti-human FAS-L monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-HSV type 1 polyclonal antibody (Dako Laboratories, Glostrup, Denmark). Reacted antibody was detected with a DAKO LSAB2 Kit/HRP (Dako Laboratories). We performed immunohistochemistry for N-18 goat anti-human CD30L polyclonal antibody (Santa Cruz Biotechnology, Inc.) with a goat ABC Staining System (Santa Cruz Biotechnology, Inc.). We detected apoptotic cells by TUNEL staining using an Apop Tag In Situ Apoptosis Detection Kit (Intergen, NY).

Real-time quantitative RT-PCR. We extracted total RNA from tumors using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) and purified mRNA using an mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). A 5-µg aliquot of each RNA sample was reverse-transcribed into cDNA.

We performed real-time quantitative RT-PCR (*Taq*Man PCR) using a 7700 Sequence Detector according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA).[31, 32] Quantification and equalization of the amount of cDNA was achieved by amplifying GAPDH as an internal control (*Taq*Man GAPDH Control Reagents, Applied Biosystems). Primers and the probe for CD30L mRNA were CD30L-forward (5′-GCCACTATTATGGTGTTGGTCGTT-3′), CD30L-reverse (5′-TTCTGAGCAATTTCCTCCTTTGAC-3′), and CD30L-probe (5′-FAM-CGGACTCCATTCCCAACTCACCTGA-TAMRA-3′). Primers and the probe for CD30 mRNA were CD30-forward (5′-CCAGCAGAGCCCGAGTTG-3′), CD30-reverse (5′-TCTTCCACTGAGAGCATGACATC-3′), and CD30-probe (5′-FAM-ACCCCCCACTACCCCGAGCAGG-TAMRA-3′). Primers and the probe for TNF-R1 mRNA were TNFR1-forward (5′-CACCTGCCATGCAGGTTTCT-3′), TNF-R1-reverse (5′-CCTCAGTGCCCTTAACATTCTCA-3′), and TNF-R1-probe (5′-FAM-TCGTGCACTCCAGGCTTTTCTTA-CAGTT-TAMRA-3′). Primers and probes for TNF-α, FAS, and FAS-L (Applied Biosystems) were used to determine the expression ratio of each gene by the Comparative Cr Method (Applied Biosystems).

Western blot analysis. Tumors were homogenized and lysed, and their blots were probed with rabbit anti-human/mouse caspase 2 polyclonal antibody (MBL, Nagoya, Japan), rabbit anti-human/mouse caspase 3 polyclonal antibody (Genzyme Techne, Minneapolis, MN), rabbit anti-human caspase 8 and caspase 9 polyclonal antibodies (Santa Cruz Biotechnology, Inc). We visualized immunoblots with the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

Effect of antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L on apoptosis induced by inoculation of HSV. We mixed the following antibodies with HSV and inoculated tumors in order to examine the effect of each antibody on the induction of apoptosis: anti-human CD30 monoclonal antibody (200 µg), anti-human CD30L polyclonal antibody (70 µg), mouse anti-human TNF-α monoclonal antibody with neutralizing activity (D113-3, MBL: 35 µg), anti-human TNF-R1 monoclonal antibody (70 µg), mouse anti-human FAS monoclonal antibody with neutralizing activity on FAS-mediated apoptosis (MD-13-3, MBL: 35 µg), mouse anti-human FAS-L monoclonal antibody with neutralizing activity on FAS-mediated apoptosis (D041-3, MBL: 35 µg), or NCAM (H-94, Santa Cruz Biotechnology, Inc.: 70 µg) as a control, because a high percentage of cells is positive for NCAM expression in most gliomas.[33] They were concentrated or dialyzed using a Centricon (Millipore, Bedford, MA), if necessary. Tumor-bearing mice were inoculated with 1×10^7 PFU of βH1 combined with each antibody or PBS in a total volume of 100 µl. These tumors were fixed and stained with the TUNEL method.

Results

Elimination of transplanted human malignant glioma by inoculation of βH1. We randomly grouped athymic BALB/c *nu/nu* mice harboring a subcutaneous U87MG tumor (100 mm^3), and inoculated 1×10^7 PFU of βH1 or PBS into the tumors. The tumors infected with βH1 shrank significantly and had disappeared by day 18, while no growth-suppressive effect was observed in tumors inoculated with PBS (control) ($P<0.0005$ by the unpaired t-test on day 18, Fig. 1, A and B). Expression of β-galactosidase was identified in βH1-inoculated tumors (data not shown).

We then evaluated the antitumor effect of βH1 on athymic BALB/c *nu/nu* mice bearing bilateral U87MG tumors. We inoculated 1×10^7 PFU of βH1 or PBS into the right-flank tumors (βH1- or PBS-inoculated group, respectively). Inoculation with βH1 resulted in significant regression of the tumors, but no reduction was observed in the non-inoculated tumors or PBS-inoculated controls ($P<0.0001$, versus PBS or non-inoculated tumors on day 18 by the unpaired t-test).

Infiltration of inflammatory cells into βH1-inoculated tumors was examined by staining with antibodies specific to mouse $CD4^+$ and $CD8^+$ lymphocytes, macrophages, and NK cells on days 3, 7, and 10. No inflammatory cell was apparent after βH1 inoculation (Fig. 1C). HSV-specific IgG antibody was not induced in mice inoculated with βH1 (data not shown).

βH1 induces apoptosis through activation of caspases 3 and 8. Hematoxylin and eosin staining of βH1-inoculated tumor sections revealed typical morphologic changes of apoptosis, i.e., fragmentation of the nucleus and condensation of nuclear chromatin, on days 3, 7, and 10. These apoptotic cells were stained

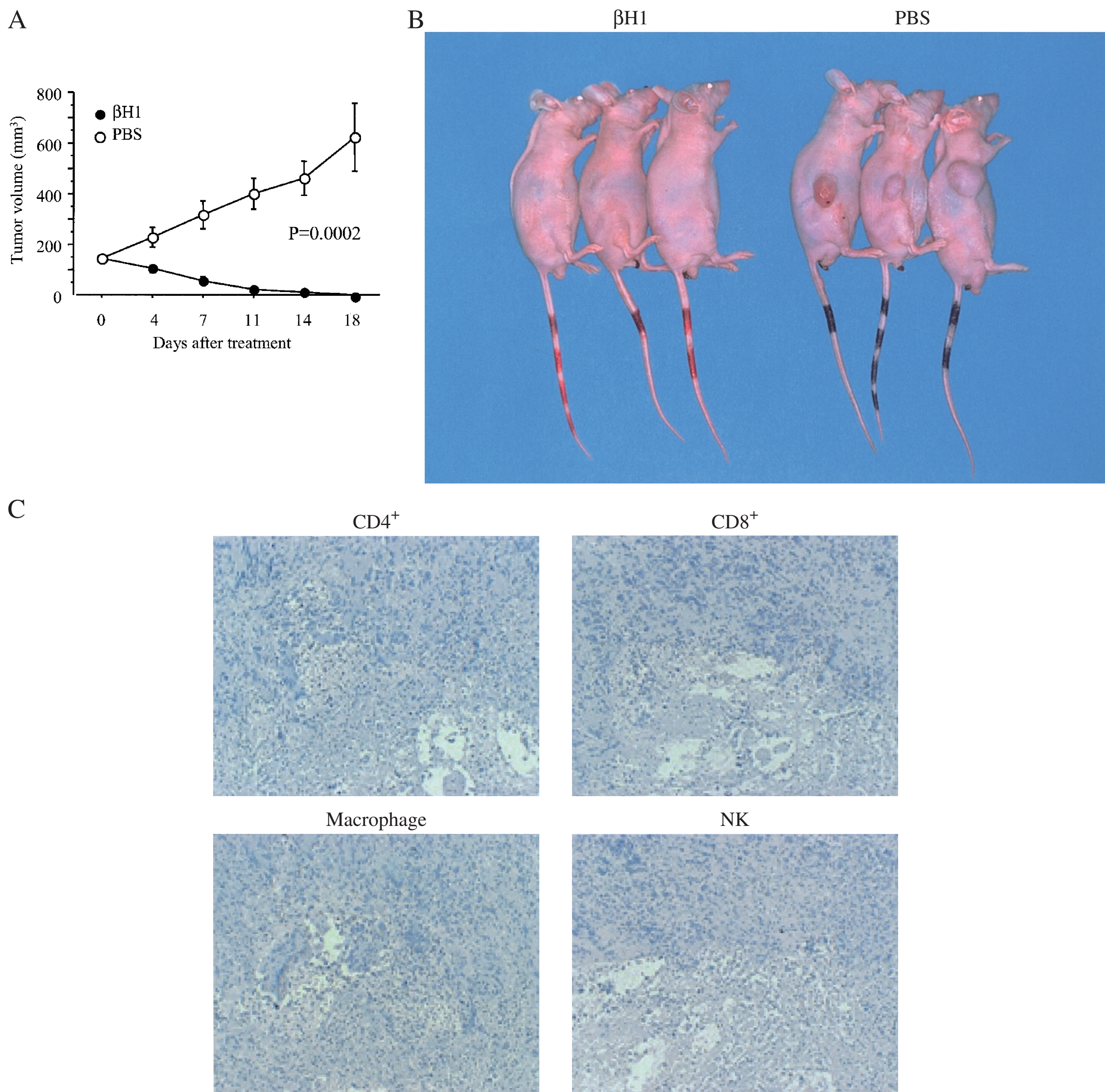


Fig. 1. Inhibition of human malignant glioma growth by βH1 infection in mice. A. Subcutaneous U87MG tumors in athymic BALB/c *nu*/*nu* mice were inoculated with 1×10^7 PFU of βH1 (●) or PBS (○). Bars represent means±SE. βH1-inoculated tumors had a significantly smaller tumor volume than uninoculated tumors ($P<0.0005$ by the unpaired *t*-test on day 18). B. All tumors inoculated with βH1 had disappeared, in contrast to uninoculated tumors, by day 18. C. Immunohistologic identification of inflammatory cell infiltrates in βH1-inoculated tumors on day 3. Magnification ×100. The sections were stained with antibodies to $CD4^+$ and $CD8^+$ lymphocytes, macrophages, and natural killer cells (brown-stained cells). No apparent inflammatory cell infiltrates were observed after βH1 inoculation.

positively by the *in situ* end labeling TUNEL method (Fig. 2A). These TUNEL-positive cells overlapped with HSV antigen-positive cells, as demonstrated by sequential sectioning (Fig. 2B).

We examined activation of caspases 2, 3, 8, and 9 in βH1-inoculated tumors by western blot analysis and detected activated forms of caspases 3 and 8, but not 2 or 9 (Fig. 2C), suggesting that apoptosis was mediated by the death receptor-dependent pathway, but not by the mitochondrial pathway.

Expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in the apoptotic cells. To assess the apoptotic process in this tumor regression system, apoptotic areas were stained with antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L. Expression of all these molecules was confined to the apoptotic areas of tumors (Fig. 3A). We examined expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L transcripts in tumors from day 0 to 8 after the inoculation of βH1 by real-time quantitative RT-PCR. Increased transcripts of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L were detected in tumors by normalization using the amount of GAPDH transcripts as a control (Fig. 3B). The expression levels of receptors CD30, TNF-R1, and FAS on day 2 were elevated 9.2-, 2.1-, and 3.3-fold compared to those

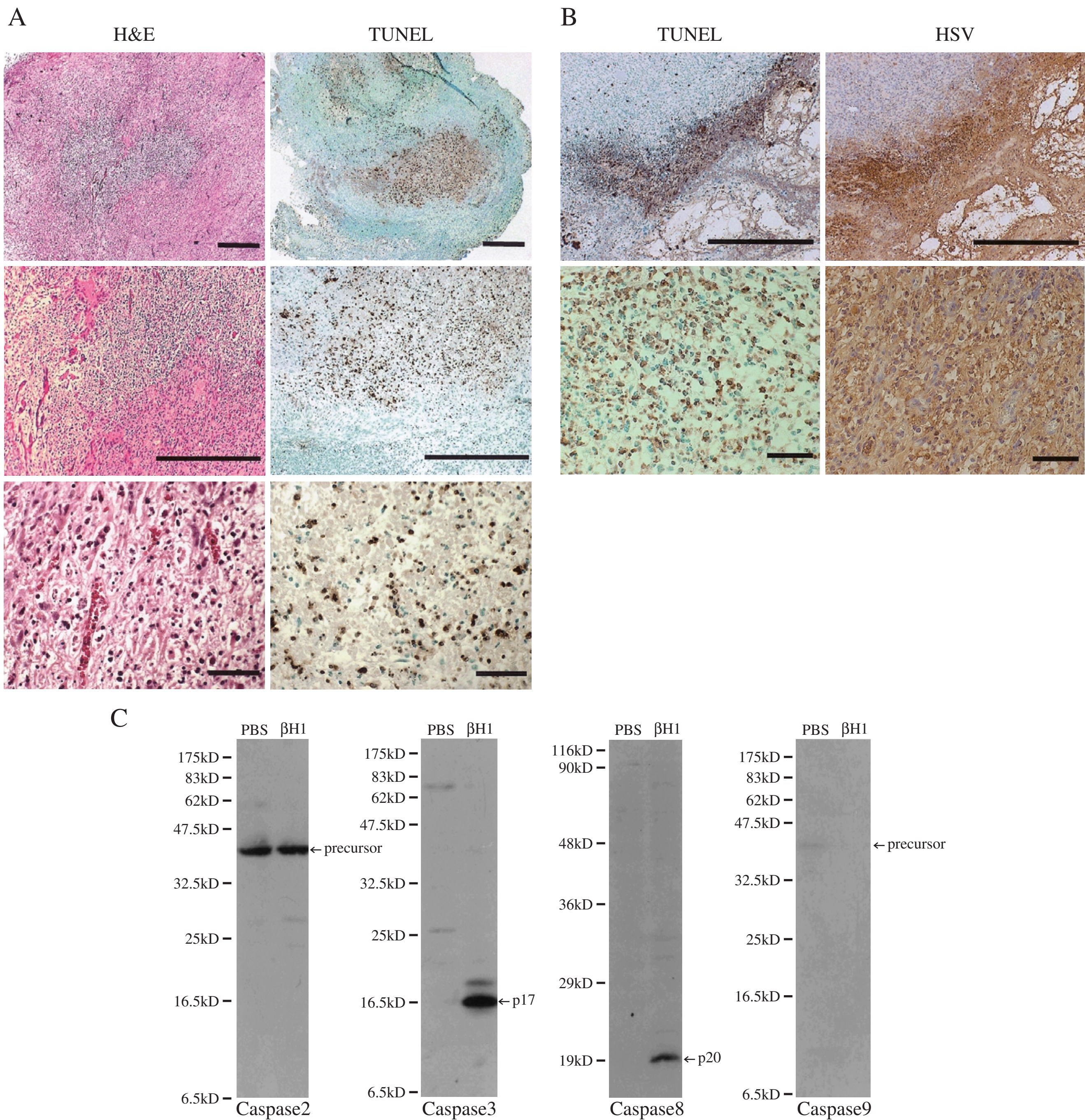


Fig. 2. Histochemistry of tumors inoculated with βH1 on day 3. A. Typical morphologic changes of apoptosis, i.e., fragmentation of the nucleus and condensation of nuclear chromatin, were obvious in hematoxylin and eosin staining, and those cells were stained brown by the TUNEL method. Magnification: upper panels ×40, middle panels ×100, lower panels ×400. Bar: 1 mm in upper panels, 1 mm in middle panels, 100 μm in lower panels. B. The distribution of TUNEL-positive cells and anti-HSV-positive cells were compared in sequential sections. Magnification: upper panels ×100, lower panels ×400. Bar: 1 mm in upper panels, 100 μm in lower panels. C. Detection of caspases 2, 3, 8, and 9 in βH1- and PBS-inoculated tumors by western blotting. Activated forms of caspases 3 (p17) and 8 (p20) were detected in βH1-inoculated tumors, while the precursors of caspases 2 and 9 without activated forms were detected in both tumors.

on day 0, respectively, and those of ligands CD30L, TNF-α, and FAS-L were elevated 3.6-, 15.3-, and 53.2-fold, respectively. The relative expressions of TNF-R1 and FAS peaked on day 2, and the others peaked on day 4. The results of histochemistry and the time courses of increase of the transcripts demonstrated that expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L was associated with apoptosis induction in tumors.

Tumor regression and apoptosis induced by HSV strains. We performed immunohistologic analysis of tumors inoculated with HSV strains (Fig. 4). Tumor regression and typical morphologic changes of apoptosis were identified in tumors inoculated with an attenuated HF and wild 7401H strains, and these apoptotic cells were stained positively by the TUNEL method and with antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L, like those injected with βH1. This suggested that HSV

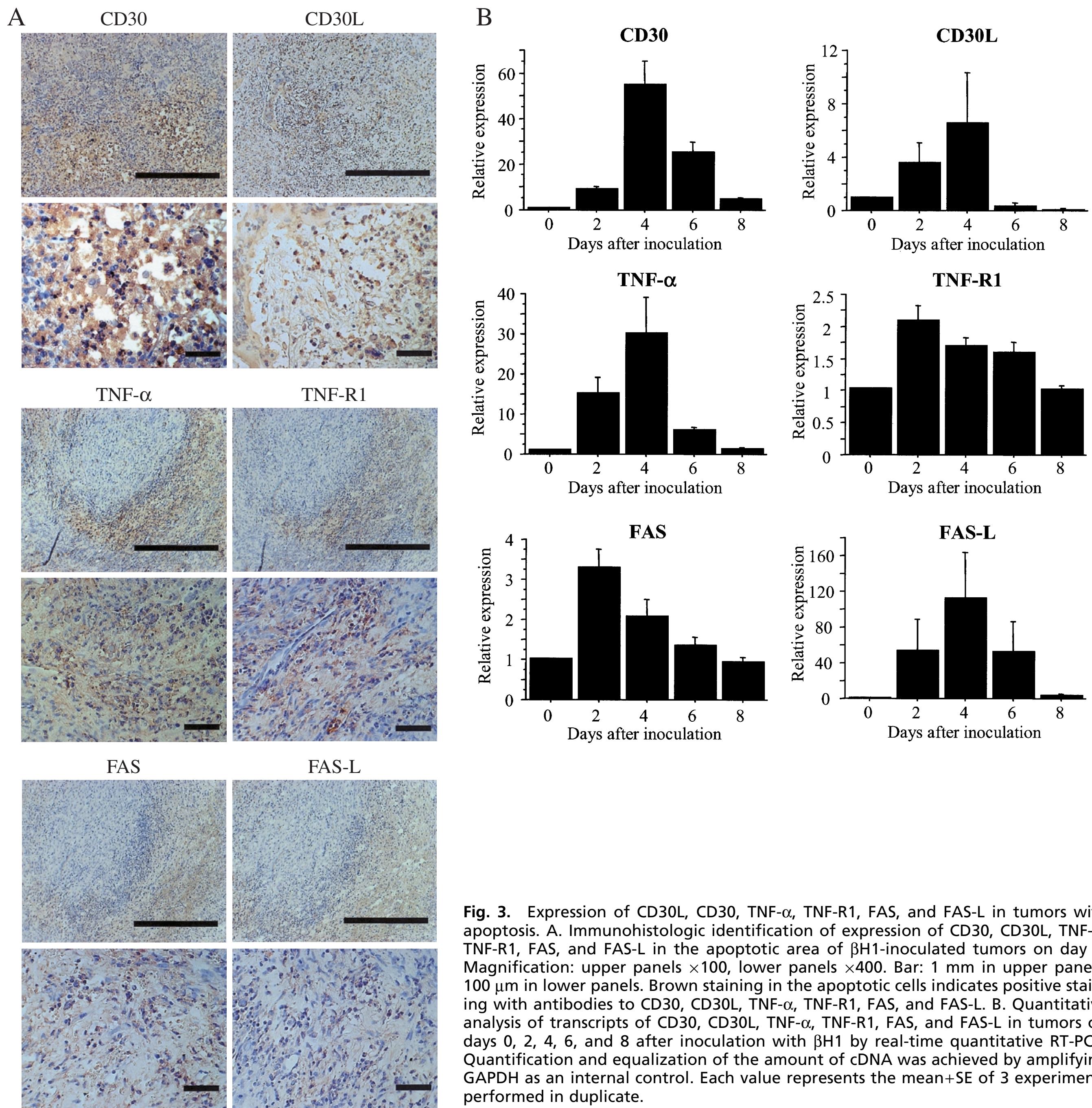


Fig. 3. Expression of CD30L, CD30, TNF-α, TNF-R1, FAS, and FAS-L in tumors with apoptosis. A. Immunohistologic identification of expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in the apoptotic area of βH1-inoculated tumors on day 3. Magnification: upper panels ×100, lower panels ×400. Bar: 1 mm in upper panels, 100 μm in lower panels. Brown staining in the apoptotic cells indicates positive staining with antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L. B. Quantitative analysis of transcripts of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in tumors on days 0, 2, 4, 6, and 8 after inoculation with βH1 by real-time quantitative RT-PCR. Quantification and equalization of the amount of cDNA was achieved by amplifying GAPDH as an internal control. Each value represents the mean+SE of 3 experiments performed in duplicate.

might be commonly oncolytic by inducing death receptor-dependent apoptosis in tumors.

Inhibition of βH1-induced apoptosis by antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L. We analyzed the effects of antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L on apoptosis of tumors by coinoculation of βH1 and each antibody to examine possible impairment of receptor-ligand interactions with the respective antibodies. TUNEL-positive apoptotic cells were quantitated by counting 3000 cells from each section (Fig. 5). Although the specificity and affinity of the antibodies might be different, each antibody treatment significantly inhibited apoptosis compared with the controls without antibody treatment by day 3 post-inoculation ($P<0.01$ by the unpaired Student's t-test). To confirm the role and specificity of these antibodies in the suppression of apoptosis, the effect of antibodies to CD30L and TNF-α on apoptosis by HSV was compared with that of antibody to NCAM under the same experimental conditions. Antibodies to CD30L and TNF-α suppressed apoptosis per βH1-infected area to 73.6 and 65.0% of the value in the case of treatment with antibody to NCAM, while treatment with PBS suppressed it to 98.4% of the value in the case of treatment with antibody to NCAM. Thus, antibody to the unrelated surface antigen on U87MG cells was not effective in preventing apoptosis, indicating the specificity of suppression of apoptosis by these antibodies. This indicated that interactions of the above receptors and ligands played an important role in the induction of apoptosis in the tumors.

Oncolytic efficacy of βH1 against transplanted human cancers. To confirm the oncolytic effect of βH1 on human cancers, we examined its effect against transplanted tumors of human lung

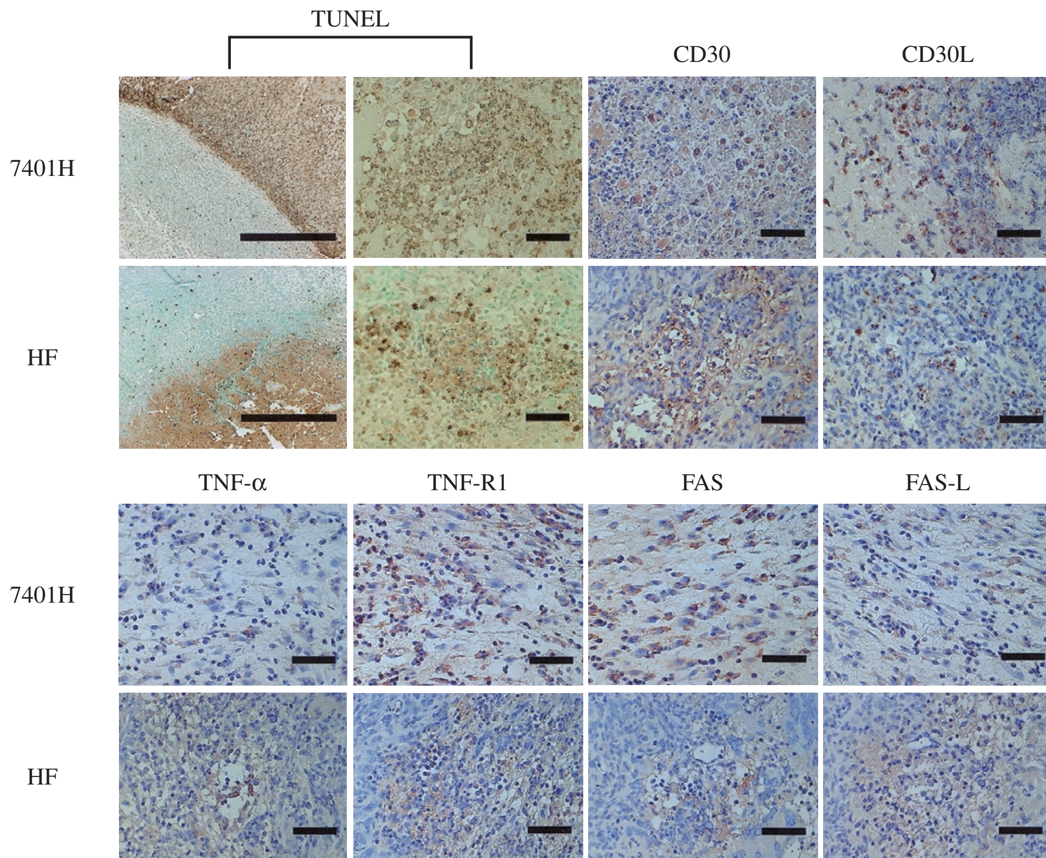


Fig. 4. Induction of apoptosis in tumors inoculated with attenuated HF and wild 7401H strains. Magnification: left panel of TUNEL ×100, right panel of TUNEL and others ×400. Bar: 1 mm in left panel of TUNEL, 100 µm in right panel of TUNEL and others. Immunohistologic identification of apoptosis by the TUNEL method and expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in tumors inoculated with HF or 7401H strain. Brown staining indicates TUNEL-positive and cells expressing CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L.

cancer A549 cell lines, human breast cancer MX-1, human gastric cancer KATO-III, and human colon cancer RCA. βH1 inoculation significantly inhibited tumor growth in the right flank in athymic mice (A549: $P<0.01$, MX-1: $P<0.05$, KATO-III: $P<0.05$, RCA: $P<0.01$, by the unpaired *t*-test at the end of the experiment, Fig. 6A), although the degree of inhibition of tumor growth seemed to vary. Apoptosis was revealed histologically by staining with hematoxylin and eosin and by the TUNEL method, and the apoptotic cells expressed CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in all transplanted human cancers (Fig. 6B).

Discussion

We selected the most attenuated HF strain for mice from our HSV library and constructed βH1 expressing β-galactosidase activity.[28] Intramuscular inoculation of βH1 induces β-galactosidase activity in the bilateral anterior horn motor neurons of the spinal cord for prolonged periods without loss of function or significant pathological changes.[29, 30] Inoculation of the parent HF caused hemiparesis in some rats and inoculation of wild 7401H caused death in most rats. Thus, attenuated βH1 strain may be suitable for therapeutic use.

Suppression of tumor growth and induction of apoptosis were observed in U87MG tumors inoculated with βH1, its parent HF or wild 7401H. βH1 showed the most effective oncolytic activity among them (data not shown), and its inoculation was not lethal in the athymic mice because of its attenuation. Therefore, βH1 was used in most of the analyses of oncolytic activity, and tumor regression was induced by apoptosis mediated by CD30/CD30L, TNF-α/TNF-R1, and FAS/FAS-L interactions.[34–36]

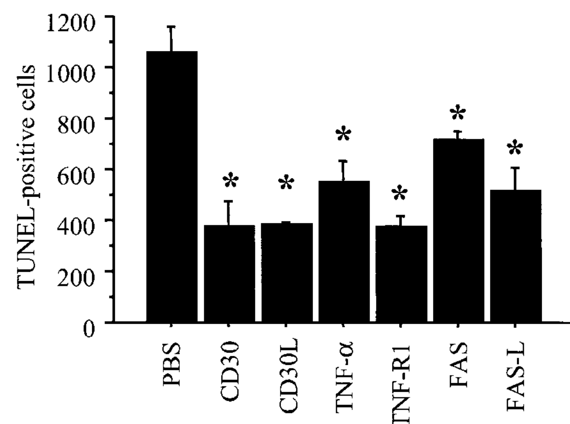
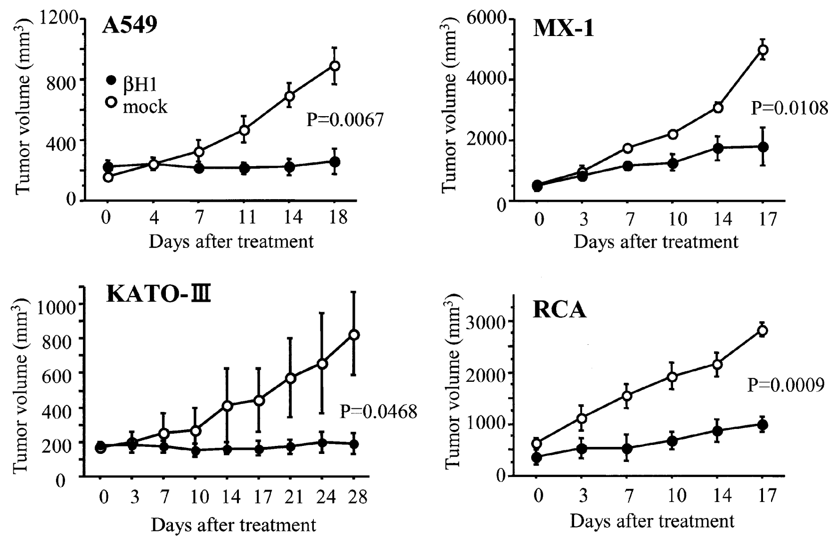


Fig. 5. Inhibitory effect of antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L on the induction of apoptosis in tumors coinoculated with βH1 on day 3. Tumors were inoculated with 1×10^7 PFU of βH1 together with PBS or antibodies to CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L. The effect of the respective antibody on the induction of apoptosis was compared by counting the number of TUNEL-positive apoptotic cells per 3000 cells from three areas in tumors inoculated with βH1 and each antibody. Results were expressed as the means+SE. * $P<0.01$ versus PBS by the unpaired Student's *t*-test.



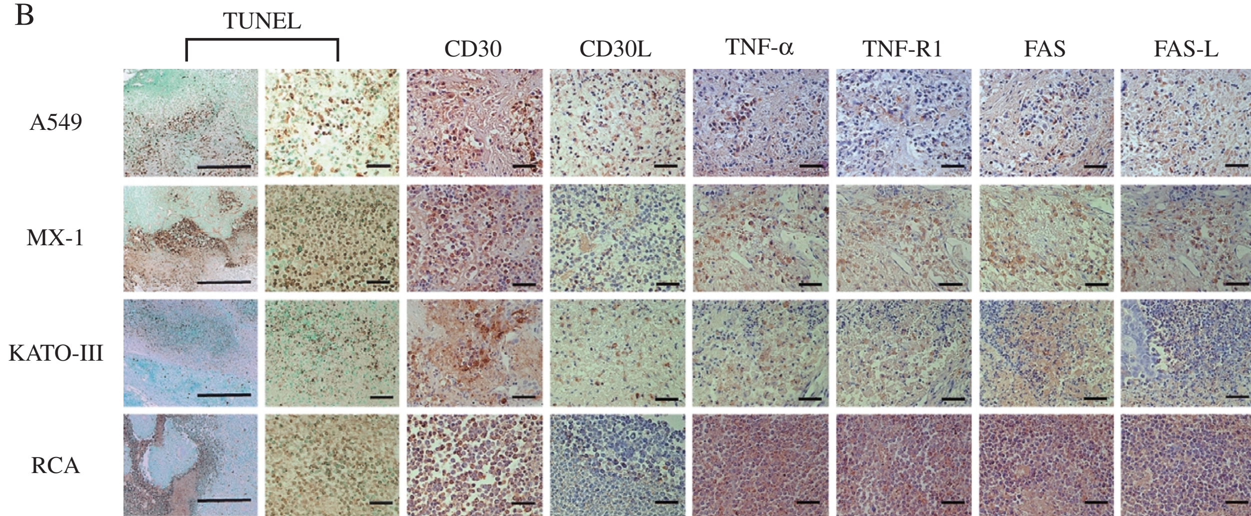


Fig. 6. Oncolytic efficacy of βH1 against transplanted human cancers. A. A549 human lung cancer cells, MX-1 human breast cancer cells, KATO-III human gastric cancer cells, or RCA human colon cancer cells in the right flank of athymic BALB/c *nu/nu* mice were inoculated with βH1, and the tumor size was monitored. Bars represent means±SE. The tumor volume was significantly reduced in tumors inoculated with βH1 compared with control tumors (unpaired *t*-test at the end of experiment). B. Immunohistologic identification of expression of CD30L, CD30, TNF-α, TNF-R1, FAS, and FAS-L in TUNEL-positive apoptotic area. Magnification: left panel of TUNEL ×100, right panel of TUNEL and the others ×400. Bar: 1 mm in left panel of TUNEL, 100 μm in right panel of TUNEL and others. Cells expressing these antigens are stained brown in transplanted human cancers.

We analyzed the direct oncolytic effect of HSV on tumors in the absence of antitumor immune response by using an immunocompromised mouse model. U87MG cells were transplanted into the bilateral flanks, and tumor regression was observed only in βH1-inoculated tumors. We observed no anti-HSV antibody production or apparent inflammatory cell infiltrates, $CD4^+$ and $CD8^+$ lymphocytes, macrophages, or NK cells, in the tumors. These results indicated that this system is suitable for analyzing the direct oncolytic activity of HSV.

The distribution of HSV-infected cells was consistent with that of TUNEL-positive apoptotic cells, and we detected activated forms of caspase 3 and 8, but not 2 or 9 (Fig. 2C), indicating that apoptosis was mediated through the death receptor-dependent pathway by HSV infection. We confirmed increased expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L in the apoptotic areas of tumors by histochemical analysis, and their increased expression was further confirmed by real-time quantitative RT-PCR. Although the quantity and expression of ligands and receptors were clearly increased, the importance of this in the apoptotic process remained unclear. Therefore, we examined the inhibitory effect of antibodies against CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L on apoptosis by coinoculation of the respective antibody and βH1 into tumors. Each antibody significantly decreased the number of apoptotic cells (Fig. 5). This clearly indicated that the interactions of CD30/CD30L, TNF-α/TNF-R1, and FAS/FAS-L played an important role in the apoptotic process in tumors inoculated with HSV. [34–36]

Surprisingly, antibodies to CD30 and CD30L similarly suppressed the induction of HSV-induced apoptosis. CD30 was expressed most strongly among the three receptors, CD30, TNF-R1, and FAS. CD30 is a TNF-R superfamily member, and is expressed on activated lymphocytes, as well as on neoplastic cells of Hodgkin's disease and anaplastic large cell lymphoma.[37, 38] CD30 does not contain death domain within the cytoplasmic tail, but CD30 signaling exerts a variety of effects, ranging from apoptosis to cellular proliferation.[35, 39–41] Inhibition of apoptosis by antibodies to CD30 and CD30L indicated

that the interaction of CD30/CD30L contributed to induction of apoptosis in HSV-infected cells in tumors. CD30/CD30L, TNF-α/TNF-R1, and FAS/FAS-L interaction might have induced activation of caspases 3 and 8, leading to apoptosis in tumors inoculated with HSV.[34–36, 40]

βH1 induced apoptosis in all transplanted tumors of human lung cancer cells, human breast cancer cells, human gastric cancer cells, or human colon cancer cells, with expression of CD30, CD30L, TNF-α, TNF-R1, FAS, and FAS-L, and tumor regression. However, the degree of tumor regression varied among the transplanted tumors used, and this might reflect different susceptibility to HSV infection. In spite of the apparent difference in the antitumor efficacy among the cell types, oncolysis of various transplanted tumors in immunocompromised athymic mice by HSV was commonly directed by apoptosis mediated by these receptor-ligand interactions. Apoptosis was not observed in tissue cultures *in vitro*, or in normal neuronal cells in the central nervous system,[28–30, 42] indicating the specificity of HSV-induced apoptosis in transplanted tumors.

We have clarified that tumor regression due to HSV is induced by the interaction of CD30/CD30L, TNF-α/TNF-R1, and FAS/FAS-L and the subsequent apoptosis is mediated by the death receptor-dependent pathway in the immunocompromised athymic mouse system. However, immunological factors have been shown to be important in the oncolytic activity of HSV in immunocompetent animal model systems.[10, 24–26] In addition to the host immunity to HSV-infected cells,[43] these receptors and ligands are important inducers and targets of oncolytic immune responses. FAS in particular is the major target for cytotoxic T lymphocytes, and its expression might enhance the susceptibility of tumor cells to cytotoxic T lymphocytes, NK cells, and lymphokine-activated NK cells.[44–46] TNF-α activates NK cells and controls the cytotoxicity of lymphokine-activated NK and NK cells *in vitro* and *in vivo*.[47] Thus, the expression of these ligands and receptors may augment the activation of these tumor-killing lymphocytes. Thus, in addition to death receptor-dependent apoptosis, the expression of these ligands and receptors might enhance the susceptibility of tumor cells to cell-mediated cytotoxicity and augment the activation of tumor-killing lymphocytes, thereby potentiating the antitumor immune response and killing of tumor cells in immunocompetent models. A more efficient oncolytic HSV may be engineered by incorporating an apoptosis and cytotoxicity-activating ligand or receptor for further improvement of cancer treatment with engineered HSV vectors.

We thank Y. Yoshida and F. Hiramoto for technical assistance, Katherine Ono for editorial assistance, and Toyama Chemical Company for supplying cancer cell lines. This study was partly supported in part by Grants-in-Aid (No. 10189101, 12210009, and 13558094) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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