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# An armed oncolytic herpes simplex virus expressing thrombospondin-1 has an enhanced *in vivo* antitumor effect against human gastric cancer

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**Advanced gastric cancer is a common disease, but the conventional treatments are unsatisfactory because of the high recurrence rate. One of the promising new therapies is oncolytic virotherapy, using oncolytic herpes simplex viruses (HSVs). Thrombospondin-1 (TSP-1) suppresses tumor progression *via* multiple mechanisms including antiangiogenesis. Our approach to enhance the effects of oncolytic HSVs is to generate an armed oncolytic HSV that combines the direct viral oncolysis with TSP-1-mediated function for gastric cancer treatment. Using the bacterial artificial chromosome (BAC) system, a 3rd generation oncolytic HSV (T-TSP-1) expressing human TSP-1 was constructed for human gastric cancer treatment. The enhanced efficacy of T-TSP-1 was determined in both human gastric cancer cell lines *in vitro* and subcutaneous tumor xenografts of human gastric cancer cells *in vivo*. In addition, we examined the apoptotic effect of T-TSP-1 *in vitro*, and the antiangiogenic effect of T-TSP-1 *in vivo* compared with a non-armed 3rd generation oncolytic HSV, T-01. No apparent apoptotic induction by T-TSP-1 was observed for human gastric cancer cell lines TMK-1 cells but for MKN1 cells *in vitro*. Arming the viruses with TSP-1 slightly inhibited their replication in some gastric cancer cell lines, but the viral cytotoxicity was not attenuated. In addition, T-TSP-1 exhibited enhanced therapeutic efficacy and inhibition of angiogenesis compared with T-01 *in vivo*. In this study, we established a novel armed oncolytic HSV, T-TSP-1, which enhanced the antitumor efficacy by providing a combination of direct viral oncolysis with antiangiogenesis. Arming oncolytic HSVs may be a useful therapeutic strategy for gastric cancer therapy.**

Gastric cancer currently ranks second in global cancer mortality.[1,2] Most patients are diagnosed at an advanced stage and curative surgical treatments are sometimes difficult due to the presence of peritoneal dissemination or extra-regional lymph node metastases. The long-term prognosis of curatively resected advanced gastric cancer remains unsatisfactory because of its high recurrence rate after surgery. The available chemotherapeutic reagents have only limited efficacy against these recurrent diseases. Therefore, new therapeutic strategies for advanced and recurrent gastric cancers are urgently needed.

**Key words:** oncolytic virus, herpes simplex virus, thrombospondin-1, gastric cancer, antiangiogenesis
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Replication-selective oncolytic herpes simplex viruses (HSVs) have emerged as a new platform for cancer therapy. Several oncolytic HSV mutants (1716, G207, NV1020 and OncoVex$^{GM\text{-}CSF}$) have already entered Phase I, II and III clinical trials for various solid tumors.[3–7] Despite the significant efficacy in preclinical models and safety in humans, however, the therapeutic benefits appear to be limited in cancer patients. It is therefore prudent to incorporate mechanisms in addition to direct oncolysis to enhance the tumor cell destruction. To this end, we have already shown that oncolytic HSVs with membrane fusion activity resulting from either genetically inserting a hyperfusogenic glycoprotein or random mutagenesis have an enhanced antitumor potency, while also exerting a synergistic effect on syncytial formation which facilitates the spread of the oncolytic virus in tumor tissue.[8–10] In addition, our collaborators have previously shown that HSV mutant G47Δ, in addition to enhanced viral replication, also possesses an immunoregulatory function, by which MHC Class I presentation was increased compared with its parent virus, G207, while maintaining the safety profile of G207.[11] This provides for the possibility of developing an enhanced cytotoxic lymphocyte response toward tumor cells and increased efficacy of the virus.

**What's new?**

**Oncolytic virotherapy using herpes simplex virus (HSV) engineered to destroy tumor cells represents a promising new anticancer strategy. In this study, to enhance the effects of oncolytic HSV, an "armed" virus expressing human thrombospondin-1 (TSP-1), an antiangiogenic protein, was developed. The armed virus, T-TSP-1, inhibited human gastric cancer cell growth both *in vitro* and *in vivo*. The enhanced viral antitumor efficacy observed suggests that T-TSP-1 may be a useful tool in the treatment of gastric cancer.**

Another problem with oncolytic virotherapy is the rapid innate immune responses that accompany viral infection, which induces the upregulation of angiogenic factors, such as vascular endothelial growth factor, and the downregulation of antiangiogenic factors, such as thrombospondin-1 (TSP-1) and thrombospondin-2 (TSP-2).[12,13] Moreover, Aghi *et al.* reported that TSP-1 reduction, accompanied with oncolytic virotherapy, induced increased angiogenesis of the residual tumor and resulted in the regrowth of tumors after oncolytic virotherapy.[12]

TSP-1 is a multifunctional 450 kDa homotrimetric glycoprotein and was originally described as a naturally occurring antiangiogenic factor and later as a potent tumor inhibitor.[14–16] The antitumor mechanisms of TSP-1 are reported to include antiangiogenesis *via* CD36,[17] induction of apoptosis,[18,19] latent transforming growth factor β (TGF-β) activation[20] and inhibition of matrix metalloproteinase 9 (MMP-9) activation.[21] TSP-1 mimetics and genes expressing them have been reported to have synergism when used with oncolytic HSV[12,22] and chemotherapeutic reagents, such as paclitaxel and cisplatin.[23] While TSP-1 is expected to have various effects that could be useful for cancer therapy, its use in infusion or injection treatments is limited because of its size and difficulty in large-scale production, and non-viral and replication-deficient viral vectors are thought to have limited success due to their poor distribution in the solid tumor mass and the tumor microenvironment.

To resolve these problems, we used replication-competent oncolytic HSVs as a vector to deliver TSP-1 to a tumor and its microenvironment, and hypothesized that, if oncolytic HSVs were combined with TSP-1, they would exert enhanced antitumor efficacy. Our viruses showed enhanced antitumor effects both *in vitro* and *in vivo via* direct antitumor and antiangiogenic mechanisms.

## Material and Methods

### Cell lines and viruses

Vero (Africa green monkey kidney), AZ521, MKN1, MKN28, MKN45 and MKN74 (human gastric cancer cell lines) cells were originally obtained from the RIKEN BioResource Center (Tsukuba, Japan). All of the cell lines were authenticated according to the Cell Line Verification Test Recommendations of ATCC Technical Bulletin no.8 (2008) within 3 months. TMK-1 cells, a human gastric cancer cell line, were a gift from Dr. Eiichi Tahara (Hiroshima University, Hiroshima, Japan). The TMK-1, MKN1, MKN28, MKN45 and MKN74 cells were cultured in RPMI1640 containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY). AZ521 cells and Vero cells were cultured in dulbecco's modified eagle medium (DMEM) containing 10% FBS. T-01 is an HSV-1-based oncolytic virus, constructed by deleting the ICP6 gene, α47 gene and both copies of the γ34.5 gene. The details of its construction have been published previously.[11] Viral stocks were prepared by releasing the virus from infected Vero cells with heparin, followed by high-speed centrifugation, as described previously.[8]

### Cloning of thrombospondin-1 cDNA

Total RNA was extracted from normal human blood cells using an RNA Blood mini kit (Qiagen, Hilden, Germany), and reverse transcription PCR amplification with ReverTra Ace-α (Toyobo, Osaka, Japan). TSP-1 cDNA PCR amplification was performed with KOD plus (Toyobo). The oligonucleotide primer sequences used were follows: 5′-TA CAC ACA GGA TCC CTG CT-3′, sense, and 5′-TTA GGG ATC TCT ACA TTC GTA TTT CA-3′, antisense, for TSP-1 cDNA. The obtained human TSP-1 cDNA fragment was cloned into a cloning site of the pTA2 vector, named pTA2-TSP-1, using a TArget Clone Plus kit (Toyobo) according to the manufacturer's instructions. The sequence of obtained pTA2-TSP-1 was compared with the GenBank sequence of human TSP-1 (accession no. NM_003246) and confirmed. A 3.7-kb *EcoRV-SacII* fragment containing a human TSP-1 cDNA fragment was inserted into the *StuI-SacII* site of SV-01 to generate SV-TSP-1.

### Construction of the virus

Using a bacterial artificial chromosome (BAC) and Cre/loxP and FLPe/FRT recombinase systems, oncolytic HSVs were constructed. Mutagenesis of the T-BAC plasmid was done by a two-step replacement procedure as reported in a previous study.[24,25] The T-BAC plasmid (1.5 μg) and SV-TSP-1 (150 ng) were mixed and incubated with Cre recombinase (New England BioLabs, Ipswich, MA) and were electroporated into *E. coli* ElectroMaxDH10B (Invitrogen, Carlsbad, CA) using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). The bacteria were streaked onto LB plates containing Cm (15 μg/ml) and Kan (10 μg/ml) and incubated to select clones containing the mutant BAC plasmid. Recombinant T-BAC/SV-TSP-1 was digested with *Hind* III and electrophoresed on a 0.8% SeaKem GTG Agarose Gel (Takara Bio, Shiga, Japan) in TAE buffer at 2.5 cm/V for 18 hr with High MW DNA Markers

(Invitrogen). A total of 2 μg of T-BAC/SV-TSP-1 DNA and 0.5 μg of pOG44 (Invitrogen) were transfected into Vero cells with 10 μl Lipofectamine 2000 and 5 μl of Plus Reagent (Invitrogen). Virus was grown and selected as described.[24] The progeny viruses were further selected by limiting dilution, were cloned on Vero cells and were finally designated as T-TSP-1.

### *In vitro* immunocytochemical staining

Vero, TMK-1 and MKN74 cells were seeded in 6-well plates at $1 \times 10^6$ per well, then the cells were treated with PBS(−) and T-01 (Vero cells: multiplicity of infection (MOI) of 0.01, gastric cancer cells:MOI of 0.1) and T-TSP-1 (Vero cells:MOI of 0.01, gastric cancer cells:MOI of 0.1) after 24 hr of incubation and were incubated further at 37°C for 24 or 48 hr. Cells were fixed with 4% paraformaldehyde/PBS and washed in PBS(−) (pH 7.4), incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase, then washed in PBS(−) and incubated in protein block solution (Dako Cytomation, Glostrup, Denmark). They were incubated with an anti-human TSP-1 antibody [1:20] (R&D Systems, Minneapolis, MN). The samples were then rinsed with PBS(−), followed by incubation with Histofine Simple Stain MAX (MULTI) (Nichirei, Tokyo, Japan). Diaminobenzidine was used as a chromogen to detect the immunostaining as a brown product, and sections were counterstained with hematoxylin. Samples were observed using a Nikon ECLIPSE 80i (Nikon, Tokyo, Japan) microscope, and images were captured.

### Western blotting

TMK-1 gastric cancer cells were seeded in 10-cm dish at $2 \times 10^6$ cells per dish and incubated at 37°C. After a 24 hr incubation cells were infected PBS(−) and T-01 (MOI of 1.0) and T-TSP-1 (MOI of 1.0) and incubated further at 39.5°C for 20 hr and harvested. Proteins (30 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane (Bio-Rad) and blotted 2 hr with monoclonal mouse anti-TSP-1 antibody (diluted 1:500, R&D systems), or an hour with mouse anti-β-actin antibody (diluted 1:2000, Sigma). The membrane was then washed and blotted with an horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (diluted 1:4000, GE healthcare, Piscataway, NJ), washed, exposed to enhanced luminol-based chemiluminescent (ECL) Plus (GE healthcare) and developed.

### *In vitro* cytotoxicity of T-01 in gastric cancer cell lines

T-01 was used to treat gastric cancer cell lines *in vitro*. The cells were seeded on 24 well plates at $1 \times 10^4$ per well and incubated. Following a 24 hr incubation, the cells were infected with T-01 at an MOI of 0.1 and further incubated at 37°C. The number of surviving cells were measured daily using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer's instructions, and the survival was expressed as a percentage of the PBS(−) treated control cells.

### Comparison of virus yields and cytotoxicity of T-01 and T-TSP-1 *in vitro*

For virus yields studies, TMK-1 cells, which are moderately sensitive to T-01, MKN1 cells, which are only minimally sensitive to T-01, and Vero cells, were seeded on 12 well plates at $1 \times 10^5$ per well and incubated for 24 hr. Each well was infected with either T-01 or T-TSP-1 at an MOI of 0.1 (TMK-1 and MKN1 gastric cancer cells) or at an MOI of 0.01 (Vero cells) for 1 hr and further incubated at 37°C. After a 48-hr incubation, the cells scraped and lysed by three cycles of freezing and thawing. The progeny virus was titered on Vero cells by plaque assays. Each experiment was measured in triplicate. For cytotoxicity studies of T-01 and T-TSP-1, cells were seeded on 24-well plates at $1 \times 10^4$ per well and incubated for 24 hr. Each well was infected with either T-01 or T-TSP-1 at an MOI of 0.1 or 0.01, and further incubated at 37°C. The number of surviving cells was measured daily and was expressed as a percentage of the PBS(−)-treated control.

### *In vitro* apoptosis assay

To examine the apoptotic effect of TSP-1, we performed a TUNEL assay using TMK-1 and MKN1 gastric cancer cells infected with either T-01 or TSP-1. A total of $1 \times 10^6$ TMK-1 or MKN1 cells were plated on 6-well plates and were treated with T-01 (at an MOI of 0.1), T-TSP-1 (at an MOI of 0.1) or PBS(−) (control) after a 24-hr incubation. At 48 hr after treatment, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using an APO-BRDU kit (BD Pharmingen, San Jose, CA) according to manufacturer's instructions, and the cells were analyzed with a FACScaliber flow cytometer and the CellQuest software program (Becton Dickinson Immunocytometry System, Franklin Lakes, NJ).

### *In vivo* subcutaneous tumor therapy

The 6-week-old female BALB/c nu/nu mice were purchased (CLEA Japan, Tokyo, Japan). Subcutaneous tumors were generated by injecting $1 \times 10^6$ TMK-1 cells in 50-μl medium into the right flank of the mice. When subcutaneous tumors reached ~6 mm in diameter, usually 5–7 days after implantation, animals were randomized into three groups, and 20 μl of PBS(−) containing 10% glycerol, $1 \times 10^7$ pfu T-01 or the same concentration of T-TSP-1 in 20 μl PBS(−) containing 10% glycerol were inoculated into the subcutaneous tumors (Day 0). Tumor growth was determined by measuring the tumors twice a week using calipers and calculating the tumor volume as: volume = 0.5 × (long axis) × (short axis)$^2$ and was expressed tumor growth ratio as previous reports.[26–28] Observations were continued until 4 weeks after virus inoculation. The mice were euthanized when the tumor reached >20mm. All animal studies were conducted under the guidelines approved by the Animal Care and Use Committee of Wakayama Medical University.

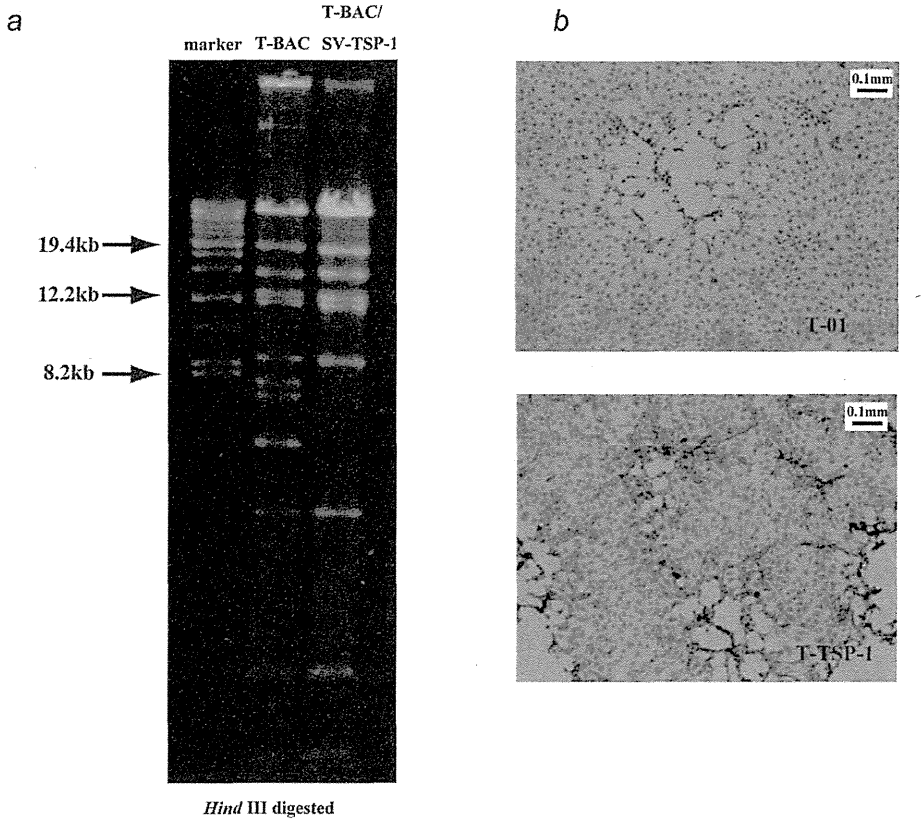


**Figure 1.** Verification of T-TSP-1 and TSP-1 expression in oncolytic HSV-1-infected Vero cells. (*a*) BAC plasmids were digested with *Hind* III. The digested BAC plasmids were electrophoresed, T-BAC (left) and Cre-recombinant BAC plasmid, T-BAC/SV-TSP-1 (right). (*b*) Vero cells infected with T-01 (MOI of 0.01) and T-TSP-1 (MOI of 0.01) were immunostained with an anti-TSP-1 antibody. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



### Histological examination

Animals were sacrificed on Day 7 after viral inoculation and tumor tissues were embedded in O.C.T. compound, were frozen in liquid nitrogen and stored at −80°C. Five micrometers thick sections were mounted on silanized slides (Dako Cytomation). Sections were used for HE, CD31 and TSP-1 staining. For immunohistochemical staining, samples were fixed, followed endogenous peroxidase blocking, protein blocking and were then rinsed. For CD31 staining, samples were incubated with a rat anti-CD31 antibody [1:200] (BD PharMingen), followed incubation with secondary antibody Histofine Simple Stain MAX(PO)(R) (Nichirei). For TSP-1 staining, the sections were incubated with an anti-human TSP-1 antibody [1:20] (R&D Systems Inc.), rinsed and then incubated with Histofine Simple Stain MAX(PO) (MULTI) (Nichirei). Diaminobenzidine was used as a chromogen to detect all immunostaining as a brown product, and sections were counterstained with hematoxylin. The microvessel densities (MVD) of tumors stained with an anti-CD31 antibody was measured for five individual areas with no overlap at 200-fold magnification (0.724 $mm^2$) for each section.

### Statistical analysis

The statistical analyses were performed using Student's *t*-test. A $p$ value $< 0.05$ was considered to be statistically significant. The StatView 5.0 software program (SAS institute Inc., Cary, NC) was used for all of the statistical analyses.

## Results

### Construction of an oncolytic herpes simplex viruses expressing thrombospondin-1

Using a BAC and Cre/loxP and FLPe/FRT recombinase systems, we generated an oncolytic HSV armed with human TSP-1, which we named T-TSP-1. This oncolytic HSV had deletions in both copies of the γ34.5 gene and in the ICP6 and α47 genes. The transgene, driven by a cytomegalovirus (CMV) promoter and with the *lacZ* gene as a marker, was inserted into the deleted ICP6 locus as previously reported.[24] The TSP-1 gene was inserted into the multicloning site of the shuttle vector SV01, and a TSP-1 expressing shuttle vector, named SV-TSP-1 was generated. Then, the recombinant BAC plasmid (T-BAC/SV-TSP-1) and T-BAC were digested with *Hind* III and electrophoresed to confirm the insertion of SV-TSP-1 (Fig. 1*a*).

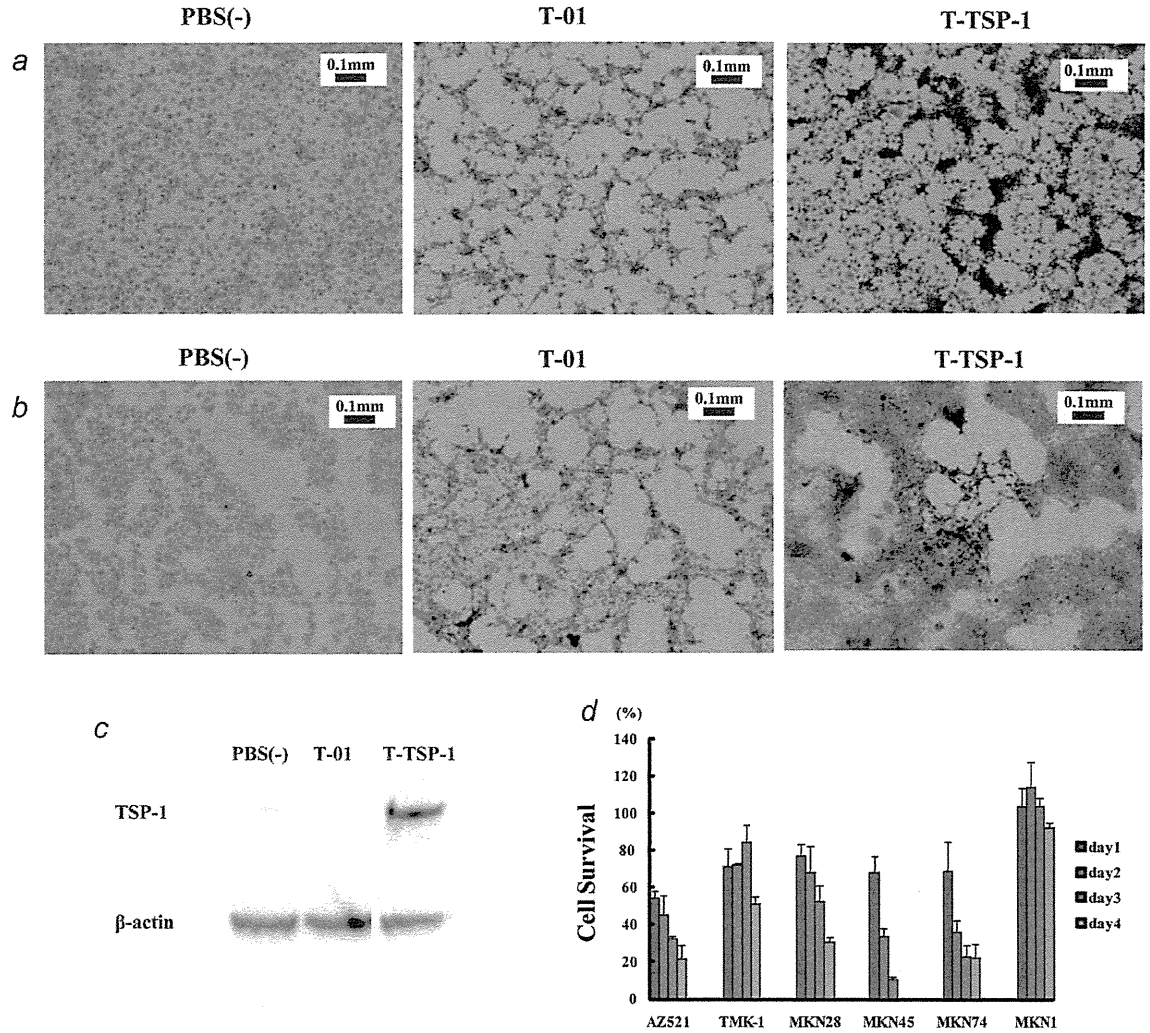


**Figure 2.** Immunocytochemical detection of TSP-1 and the cytotoxicity of T-01 in gastric cancer cell lines *in vitro*. Gastric cancer cells were infected with PBS(−), T-01 or T-TSP-1 and immunostained for human TSP-1 48 hr after infection. (*a*) TMK-1 cells after infection with PBS(−)(left), T-01 (middle) or T-TSP-1 (right). (*b*) MKN74 cells after infection with PBS(−)(left), T-01 (middle) or T-TSP-1 (right). (*c*) Expression of TSP-1 was confirmed by Western blotting. TMK-1 cells were infected with PBS(−) (left) or with T-01 (middle) or with T-TSP-1 (right). Note the presence of full-length TSP-1 in cells infected with T-TSP-1. (*d*) T-01 was administrated to gastric cancer cell lines *in vitro*. The cells were seeded on 24-well plates at $1 \times 10^4$ per well and were incubated for 24 hr. Following this incubation, the cells were infected with T-01 at an MOI of 0.1 and further incubated at 37°C. The number of surviving cells was measured daily and is expressed as a percentage of the PBS(−)-treated control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### *In vitro* immunocytochemical staining and Western blot analysis

To determine the activity of the virus expressing TSP-1 (T-TSP-1), Vero cells were treated with T-01 (MOI of 0.01), T-TSP-1 (MOI of 0.01) or PBS(−), and TMK-1 cells and MKN74 cells were treated with T-01 (MOI of 0.1), T-TSP-1 (MOI of 0.1) or PBS(−). Immunocytochemical staining with an anti-human TSP-1 antibody was performed 48 hr after treatment with PBS(−), T-01 or T-TSP-1. TSP-1 expression was detected in the Vero cells treated with T-TSP-1, but was not detected in Vero cells treated with T-01 (Fig. 1*b*). TSP-1 was expressed strongly in human gastric cancer cells infected with T-TSP-1, but was not expressed in gastric cancer cells treated with PBS(−) or T-01 (Figs. 2*a* and 2*b*). The expression of TSP-1 in T-TSP-1-infected Vero cells and human gastric cancer cells was confirmed. By Western blot analysis, moreover, expression of full-length TSP-1 in T-TSP-1 infected TMK-1 cells was confirmed, while TMK-1 cells infected by T-01 was not confirmed (Fig. 2*c*).

### *In vitro* cytotoxicity of T-01 in gastric cancer cell lines

At 96 hr after infection with T-01 at an MOI of 0.1, 79% of AZ521, 49% of TMK-1, 69% of MKN28, almost all MKN45

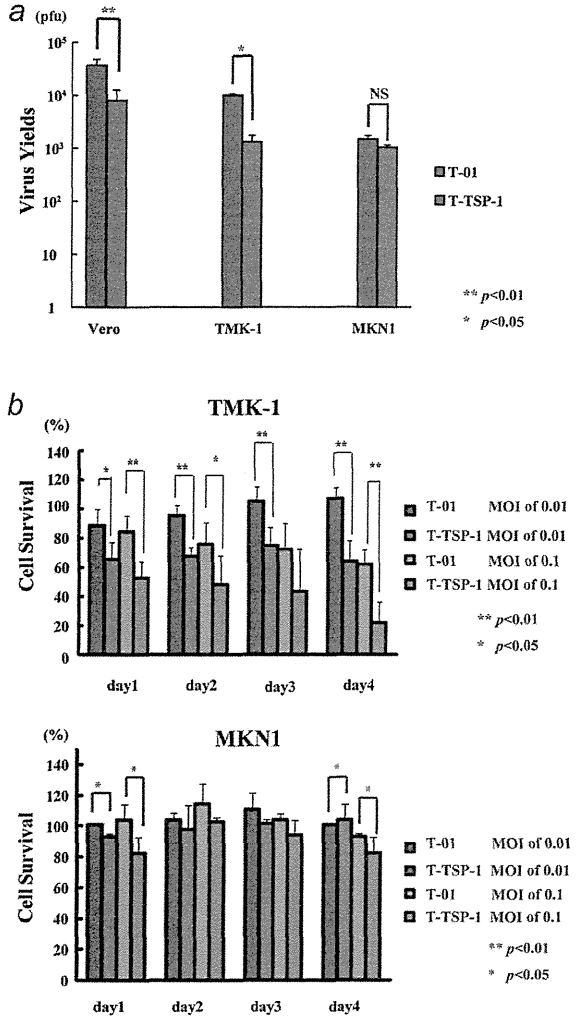


**Figure 3.** *In vitro* viral replication and cytotoxicity of HSVs against gastric cancer cell lines. (*a*) The *in vitro* virus yield was determined 48 hr after infection of Vero cells ($1 \times 10^5$ per well) with T-01 or T-TSP-1 at an MOI of 0.01, and TMK-1 and MKN1 cells ($1 \times 10^5$ per well) at an MOI of 0.1. (*b*) TMK-1 and MKN1 cells were seeded into 24-well plates at $1 \times 10^4$ per well. After a 24-hr incubation, the cells were treated with PBS(−)(control), T-01 (at an MOI of 0.01 or 0.1) or T-TSP-1 (at an MOI of 0.01 or 0.1). The number of surviving cells was quantified daily, considering control samples to be 100% viable. Bars: SE. $*p < 0.05$; $**p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and 78% of the MKN74 cells had been killed. On the other hand, only 8% of the MKN1 cells were killed by T-01 (Fig. 2*d*). The sensitivities to T-01 were different among the human gastric cancer cell lines. Therefore, we further examined the cytotoxicity and performed a virus replication assay of T-TSP-1 or T-01, in moderate and minimally sensitive gastric cancer cell lines, TMK-1 and MKN1.

### Comparison of virus yields and cytotoxicity of T-01 and T-TSP-1 *in vitro*

We determined the yields of progeny virus 48 hr after infection with each virus for 1 hr. The virus yields were not significantly different between T-TSP-1 and T-01 in the MKN1 cells. However, the virus yields of T-TSP-1 were significantly reduced in TMK-1 and Vero cells compared with those of T-01 (Fig. 3*a*). The cytotoxicity of T-TSP-1 was superior to that of T-01 in the TMK-1 cells, but neither of the viruses was effective against the MKN1 cells (Fig. 3*b*). We next examined the potential mechanism responsible for the differences in viral replication and cytotoxicity by using an apoptosis assay.

### *In vitro* apoptosis assay

TMK-1 and MKN1 cells were plated on 6-well plates at $1 \times 10^6$ per well, and after a 24-hr incubation, the cells were treated with PBS(−), T-01 (MOI of 0.1) or T-TSP-1 (MOI of 0.1). TUNEL assays were performed using an APO-BRDU kit. In MKN1 cells, the induction of apoptosis was observed in cells treated with T-TSP-1, but not in cells treated with PBS(−) or T-01 (Fig. 4). However, in the TMK-1 cells, apoptosis was not observed in either the T-01 or T-TSP-1-infected cells, which was in contrast to our expectations (Fig. 4).

### Subcutaneous tumor response and immunohistochemical staining

To examine the effects of TSP-1 expression on gastric cancer growth *in vivo*, human poorly differentiated gastric adenocarcinoma TMK-1 cells were implanted into the flanks of nude mice, and intratumoral treatments were performed. At 16 days after treatment, the PBS(−) treatment group showed 7-fold tumor growth, whereas the T-01 treatment group exhibited almost no tumor growth, which was significantly different compared to the control (PBS(−)) group ($p < 0.01$; Fig. 5*a*). Moreover, T-TSP-1 treatment group led to a significant tumor growth delay compared with T-01 treatment group ($p < 0.05$, compared with T-01; Fig. 5*a*).

Immunohistochemical staining of subcutaneous tumors treated with PBS(−), T-01 and T-TSP-1 was performed using an anti-TSP-1 antibody. No or slight TSP-1 staining was observed in tumor sections treated with PBS(−) or T-01, but strong TSP-1 staining was observed in samples from animals treated with T-TSP-1 (Fig. 5*b*). To determine whether the TSP-1-mediated inhibition of tumor growth in the different virus treatment groups reflected differences in angiogenesis, the MVD were determined. The MVD of subcutaneous tumors 7 days after treatment was determined by staining 5-μm thick frozen tumor sections with anti-CD31 antibodies, and the average densities of five independent fields were observed at a magnification of ×200. The MVD of T-01-treated tumors was significantly lower than that of PBS(−)-treated tumors ($p < 0.01$; Fig. 5*c*). In addition, that of T-TSP-1-treated tumors was significantly lower than that of T-01-treated tumors ($p < 0.05$; Fig. 5*c*). The decreased angiogenesis in tumors was thought to

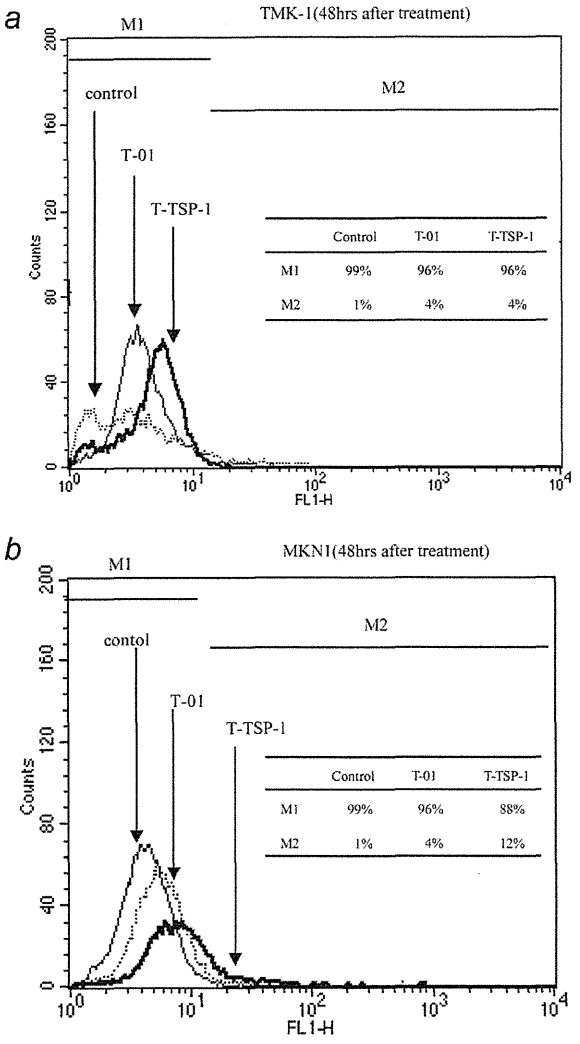


**Figure 4.** *In vitro* apoptosis assay of gastric cancer cells infected with oncolytic HSVs. (*a*) We performed an *in vitro* TUNEL assay using TMK-1 gastric cancer cells 48 hr after infection with PBS(−) (control), T-01 (MOI of 0.1) or T-TSP-1 (MOI of 0.1). (*b*) The results of the *in vitro* TUNEL assay of MKN-1 gastric cancer cells 48 hr after infection with PBS(−) (control), T-01 (MOI of 0.1) or T-TSP-1 (MOI of 0.1).

play an important role in the tumor growth inhibition induced by the virus.

## Discussion

In this article, we described the impact of an oncolytic HSV armed with a therapeutic transgene, TSP-1. The expression of TSP-1 in cancer cells was previously reported to be repressed compared with that of normal cells.[29,30] A decreased expression of TSP-1 in cells infected with HSVs was also reported.[12,31] The administration of a TSP-1 mimetic reported enhanced the efficacy of chemotherapeutic reagents,[23] and it was also reported that the mimetic enhanced the activity of oncolytic HSVs.[12,22]

We hypothesized that an increased expression of TSP-1 in cancer cells infected with oncolytic HSVs would enhance the efficacy of the oncolytic HSVs. The whole protein and Type 1 and Type 3 repeat regions of TSP-1 have been used for anti-cancer and anti-leukemic therapy, and tumor apoptosis and inhibition of tumor angiogenesis and tumor growth were reported for these treatments.[19–21,32–34] The intact TSP-1 protein was reported to be considerably more active than the recombinant protein when injected[34] and may show more effective tumor growth inhibition than the recombinant domains of TSP-1. Therefore, in this study, we tried to compensate for the low TSP-1 expression in cells infected with oncolytic HSVs and in cancer cells in general by using a BAC system and Cre-loxP and FLP/FRT recombinase systems to arm the viruses with the intact TSP-1 gene.

We first tried to confirm the cytotoxicity of T-01 in human gastric cancer cell lines and whether the efficacy of T-01 was different in each of the gastric cancer cell lines. In the case of gastric cancer cells highly sensitive to oncolytic HSV-1 (AZ521, MKN45 and MKN74), oncolytic HSV-1 therapy alone is thought to be sufficient. On the other hand, other therapeutic modalities have to be selected for the more resistant gastric cancer cells, such as MKN1. We therefore armed the oncolytic HSV-1 to enhance its efficacy, and make it better adapted for gastric cancer cells that are only moderately sensitive to oncolytic HSV-1, for example, TMK-1.

In our *in vitro* experiments, enhanced cytotoxicity of an oncolytic HSV expressing TSP-1 was observed in TMK-1 cells compared with T-01 treatment. The results showed that the overall trend of the cell survival was increasing from Day 1 to Day 3, with a sudden decrease in Day 4 especially for TMK-1 cancer cells. This pattern may be very unusual for oncolytic HSVs mediated killing. A recent report has indicated that human gastroesophageal cancer cell lines with shorter doubling times were more susceptible to viral oncolysis and demonstrated faster cytotoxicity.[35] Some of human gastric cancer cell line such as TMK-1 and MKN1 had doubling times over 36 hr (Tsuji *et al.* unpublished data). Paradoxically, higher viral titers were achieved in human gastric cancer cell lines with longer doubling times, indicating that immediate cytotoxicity may be detrimental to ultimate viral replication. Therefore, we speculated that our phenomena *in vitro* have a close resemblance to the experimental data described previously.[35]

In terms of viral replication and apoptosis, the viral replication of T-TSP-1 was lower than that of T-01 in TMK-1 cells, but not in MKN1 cells. Moreover, the induction of apoptosis by T-TSP-1 was only observed in MKN1 cells but not in TMK-1 cells. Several studies have recently demonstrated that cancer cell apoptosis was induced by TSP-1.[19,33] Apoptosis is also a host cell defense mechanism that limits viral infection, and viral infection with HSV-1 often leads cells adjacent to HSV infected cells to apoptosis,[36] which can

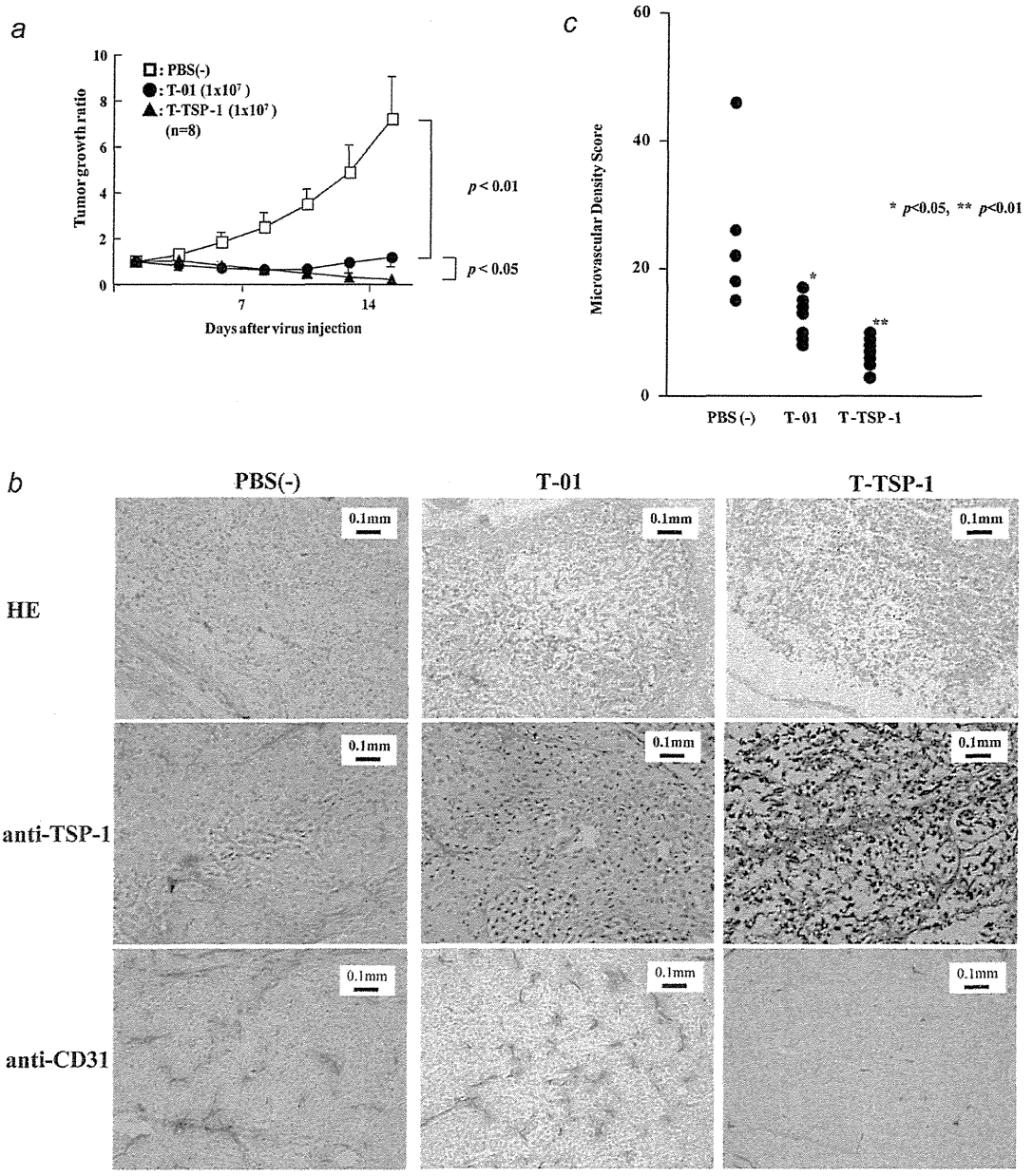


**Figure 5.** The efficacy of armed oncolytic HSV-1 vectors *in vivo*. (*a*) The antitumor effects of TSP-1-expressing oncolytic HSV-1s (T-TSP-1) and oncolytic HSV-1 not expressing any transgenes (T-01) was examined in BALB/c nu/nu mice bearing subcutaneous TMK-1 tumors. BALB/c nu/nu mice bearing subcutaneous TMK-1 tumors of ~6 mm in diameter were treated with intratumoral injection of PBS(−) or T-01 ($1 \times 10^7$ pfu) or T-TSP-1 ($1 \times 10^7$ pfu) on Day 0 ($n = 8$). The tumor growth ratio was determined by dividing tumor volume measured on the indicated week after virus injection by the tumor volume before treatment. (*b*) HE staining and immunohistochemical staining of subcutaneous tumors from mice treated with PBS(−) (left), T-01 (middle) and T-TSP-1 (right). (*c*) Subcutaneous TMK-1 tumors harvested at 7 days after treatment were stained with an anti-CD31 antibody and the MVD was evaluated for an average of five independent 200× fields. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

attenuate the viral oncolysis. Increased viral replication of γ34.5 deficient HSV-1 was observed when used with an anti-apoptotic agent.[36] For these reasons, we speculated that the apoptotic effect of TSP-1 derived from T-TSP-1 might reduce the viral replication. It has already been reported that HSV-1 infection block apoptosis of infected cells by viral protein.[37–39] In terms of the difference of apoptosis between TMK-1 and MKN1, we hypothesized that moderately sensitive gastric

cancer cell line TMK-1 to HSV-1 was blocked apoptosis by HSV-1 infection, and lower sensitive gastric cancer cell MKN1 exhibited more apoptosis.

In diffuse-type gastric cancers, TGF-β signaling was inhibited and tumor angiogenesis was induced by repressed TSP-1 expression, which led to accelerated tumor growth. The normalization of the TGF-β pathway by inducing TSP-1 was therefore considered to be a useful potential treatment for diffuse gastric cancer.[40] Strategies using TSP-1 are also thought to be useful in the treatment of advanced cancers with defects in the TGF-β signaling pathways, such as diffuse gastric cancer. With reduced virus yields in TMK-1 cells, T-TSP-1 could also achieve a significantly better cytotoxicity than T-01. It has been reported that TSP-1 and α3β1 integrin-binding peptide from TSP-1 induced inhibition of small cell lung carcinoma cells *in vitro*.[41] We speculated that the possible mechanism of enhanced cytotoxicity of T-TSP-1 compared with T-01 *in vitro* might be induced by signal transduction from TSP-1 to α3β1 integrin. To clarify our speculation, we need to study the mechanism by which T-TSP-1 increase the cytotoxic effect in adequate tumor model.

An improved *in vivo* therapeutic effect of T-TSP-1 was also observed compared to that of T-01 in TMK-1 cells. The main mechanism of the additional effect of T-TSP-1 *in vivo* was thought to be mainly antiangiogenesis and other effects of TSP-1, such as induction of apoptosis, activation of latent TGF-β signaling[20] and inhibition of MMP-9, which has been shown to increase the invasive potential of cells,[21] were thought to be comparably weak. Further important note is that a transgenic or orthotopic model would be much more informative in comparison with a subcutaneous tumor model. In this experiment, only immune-deficient mice were assessed, and therefore, the efficacy of the treatment in immune-competent models and patients may be different. To clarify the precise mechanism of T-TSP-1, in the future, we need to use the transgenic or orthotopic tumor models in immune-competent mice and examine an anti-tumor effect *via* viral oncolysis and mechanisms including immunological aspects.

According to a previous report, the repression of TSP-1 and upregulation of TXR1 induces resistance to taxanes, which are often used in gastric cancer chemotherapy, and TSP-1 is an effector of the apoptotic response to taxane chemotherapy.[42] Synergy between 2nd generation oncolytic HSVs (G207) and taxanes in thyroid cancer therapy was confirmed in a previous study.[43] An oncolytic virus, T-TSP-1, expressing TSP-1 may therefore enhance the sensitivity of gastric cancer cells to taxanes, and combination therapy using T-TSP-1 and a taxane may achieve more enhanced synergy. Further combination studies are needed to investigate this possibility.

Finally, to the best of our knowledge, this is the first report of oncolytic HSV-1 therapy using viruses armed with TSP-1 for human gastric cancer. We showed that an oncolytic virus armed with TSP-1 enhanced the efficacy of oncolytic HSV-1 for gastric cancer cells, and that the combination of TSP-1 and oncolytic HSV-1 inhibited human gastric cancer cell growth both *in vitro* and *in vivo*. These results demonstrate that arming with TSP-1 enhances the efficacy of HSV-1 and induces apoptosis in gastric cancer cells.

## References


1. Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006;12:354–62.
2. Hohenberger P, Gretschel S. Gastric cancer. *Lancet* 2003;362:305–15.
3. Rampling R, Cruickshank G, Papanastassiou V, et al. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther* 2000;7:859–66.
4. Markert JM, Medlock MD, Rabkin SD, et al. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* 2000; 7:867–74.
5. Kemeny N, Brown K, Covey A, et al. Phase I, open-label, dose-escalating study of a genetically engineered herpes simplex virus, NV1020, in subjects with metastatic colorectal carcinoma to the liver. *Hum Gene Ther* 2006;17:1214–24.
6. Hu JC, Coffin RS, Davis CJ, et al. A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin Cancer Res* 2006;12:6737–47.
7. Kaufman HL, Kim DW, DeRaffele G, et al. Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding GM-CSF in patients with stage IIIc and IV melanoma. *Ann Surg Oncol* 2010;17:718–30.
8. Nakamori M, Fu X, Meng F, et al. Effective therapy of metastatic ovarian cancer with an oncolytic herpes simplex virus incorporating two membrane fusion mechanisms. *Clin Cancer Res* 2003;9:2727–33.
9. Nakamori M, Fu X, Rousseau R, et al. Destruction of nonimmunogenic mammary tumor cells by a fusogenic oncolytic herpes simplex virus induces potent antitumor immunity. *Mol Ther* 2004;9:658–65.
10. Nakamori M, Fu X, Pettaway CA, et al. Potent antitumor activity after systemic delivery of a doubly fusogenic oncolytic herpes simplex virus against metastatic prostate cancer. *Prostate* 2004; 60:53–60.
11. Todo T, Martuza RL, Rabkin SD, et al. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. *Proc Natl Acad Sci USA* 2001;98:6396–401.
12. Aghi M, Rabkin SD, Martuza RL. Angiogenic response caused by oncolytic herpes simplex virus-induced reduced thrombospondin expression can be prevented by specific viral mutations or by administering a thrombospondin-derived peptide. *Cancer Res* 2007;67:440–4.
13. Kurozumi K, Hardcastle J, Thakur R, et al. Oncolytic HSV-1 infection of tumors induces angiogenesis and upregulates CYR61. *Mol Ther* 2008;16:1382–91.
14. Weinstat-Saslow DL, Zabrenetzky VS, VanHoutte K, et al. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res* 1994;54:6504–11.
15. Sheibani N, Frazier WA. Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis. *Proc Natl Acad Sci USA* 1995;92: 6788–92.
16. Volpert OV, Dameron KM, Bouck N. Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene* 1997;14:1495–502.
17. Dawson DW, Pearce SF, Zhong R, et al. CD36 mediates the *In vitro* inhibitory effects of thrombospondin-1 on endothelial cells. *J Cell Biol* 1997;138:707–17.
18. Manna PP, Frazier WA. CD47 mediates killing of breast tumor cells *via* Gi-dependent inhibition of protein kinase A. *Cancer Res* 2004;64:1026–36.
19. Saumet A, Slimane MB, Lanotte M, et al. Type 3 repeat/C-terminal domain of thrombospondin-1 triggers caspase-independent cell death through CD47/alphavbeta3 in promyelocytic leukemia NB4 cells. *Blood* 2005;106:658–67.

20. Yee KO, Streit M, Hawighorst T, et al. Expression of the type-1 repeats of thrombospondin-1 inhibits tumor growth through activation of transforming growth factor-β. *Am J Pathol* 2004;165:541–52.
21. Rodriguez-Manzaneque JC, Lane TF, Ortega MA, et al. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc Natl Acad Sci USA* 2001;98:12485–90.
22. Hardcastle J, Kurozumi K, Dmitrieva N, et al. Enhanced antitumor efficacy of vasculostatin (Vstat120) expressing oncolytic HSV-1. *Mol Ther* 2009;18:285–94.
23. Campbell NE, Greenaway J, Henkin J, et al. The thrombospondin-1 mimetic ABT-510 increases the uptake and effectiveness of cisplatin and paclitaxel in a mouse model of epithelial ovarian cancer. *Neoplasia* 2010;12:275–83.
24. Fukuhara H, Ino Y, Kuroda T, et al. Triple gene-deleted oncolytic herpes simplex virus vector double-armed with interleukin 18 and soluble B7-1 constructed by bacterial artificial chromosome-mediated system. *Cancer Res* 2005;65:10663–8.
25. Liu TC, Zhang T, Fukuhara H, et al. Dominant-negative fibroblast growth factor receptor expression enhances antitumoral potency of oncolytic herpes simplex virus in neural tumors. *Clin Cancer Res* 2006;12:6791–9.
26. Fu X, Tao L, Jin A, et al. Expression of a fusogenic membrane glycoprotein by an oncolytic herpes simplex virus potentiates the viral antitumor effect. *Mol Ther* 2003;7:748–54.
27. Fu X, Tao L, Rivera A, et al. Virotherapy induces massive infiltration of neutrophils in a subset of tumors defined by a strong endogenous interferon response activity. *Cancer Gene Ther* 2011;18:785–94.
28. Fu X, Nakamori M, Tao L, et al. Antitumor effects of two newly constructed oncolytic herpes simplex viruses against renal cell carcinoma. *Int J Oncol* 2007;30:1561–7.
29. Tenan M, Fulci G, Albertoni M, et al. Thrombospondin-1 is downregulated by anoxia and suppresses tumorigenicity of human glioblastoma cells. *J Exp Med* 2000;191:1789–98.
30. Jo WS, Mizukami Y, Duerr EM, et al. Wnt signaling can repress thrombospondin-1 expression in colonic tumorigenesis. *Cancer Biol Ther* 2005;4:1361–6.
31. Choudhary A, Hiscott P, Hart CA, et al. Suppression of thrombospondin 1 and 2 production by herpes simplex virus 1 infection in cultured keratocytes. *Mol Vis* 2005;11:163–8.
32. Streit M, Velasco P, Brown LF, et al. Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am J Pathol* 1999;155:441–52.
33. Greenaway J, Henkin J, Lawler J, et al. ABT-510 induces tumor cell apoptosis and inhibits ovarian tumor growth in an orthotopic, syngeneic model of epithelial ovarian cancer. *Mol Cancer Ther* 2009;8:64–74.
34. Miao WM, Seng WL, Duquette M, et al. Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor-beta-dependent and -independent mechanisms. *Cancer Res* 2001;61:7830–9.
35. Wong J, Kelly K, Mittra A, et al. A third-generation herpesvirus is effective against gastroesophageal cancer. *J Surg Res* 2010;163:214–20.
36. Wood LW, Shillitoe EJ. Effect of a caspase inhibitor, zVADfmk, on the inhibition of breast cancer cells by herpes simplex virus type 1. *Cancer Gene Ther* 2011;18:685–94.
37. Leopardi R, Roizman B. The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. *Proc Natl Acad Sci USA* 1996;93:9583–7.
38. Benetti L, Roizman B. Herpes simplex virus protein kinase US3 activates and functionally overlaps protein kinase A to block apoptosis. *Proc Natl Acad Sci USA* 2004;101:9411–16.
39. Ahmed M, Lock M, Miller CG, et al. Regions of the herpes simplex virus type 1 latency-associated transcript that protect cells from apoptosis *in vitro* and protect neuronal cells *in vivo*. *J Virol* 2002;76:717–29.
40. Komuro A, Yashiro M, Iwata C, et al. Diffuse-type gastric carcinoma: progression, angiogenesis, and transforming growth factor beta signaling. *J Natl Cancer Inst* 2009;101:592–604.
41. Guo N, Templeton NS, Al-Barazi H, et al. Thrombospondin-1 promotes alpha3beta1 integrin-mediated adhesion and neurite-like outgrowth and inhibits proliferation of small cell lung carcinoma cells. *Cancer Res* 2000;60:457–66.
42. Lih CJ, Wei W, Cohen SN. Txr1: a transcriptional regulator of thrombospondin-1 that modulates cellular sensitivity to taxanes. *Genes Dev* 2006;20:2082–95.
43. Lin SF, Gao SP, Price DL, et al. Synergy of a herpes oncolytic virus and paclitaxel for anaplastic thyroid cancer. *Clin Cancer Res* 2008;14:1519–28.