(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 1×10^6 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 2×10^6 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 1×10^4 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 1×10^5 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 1×10^4 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 1×10^6 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 1×10^6 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, 1×10^7 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.

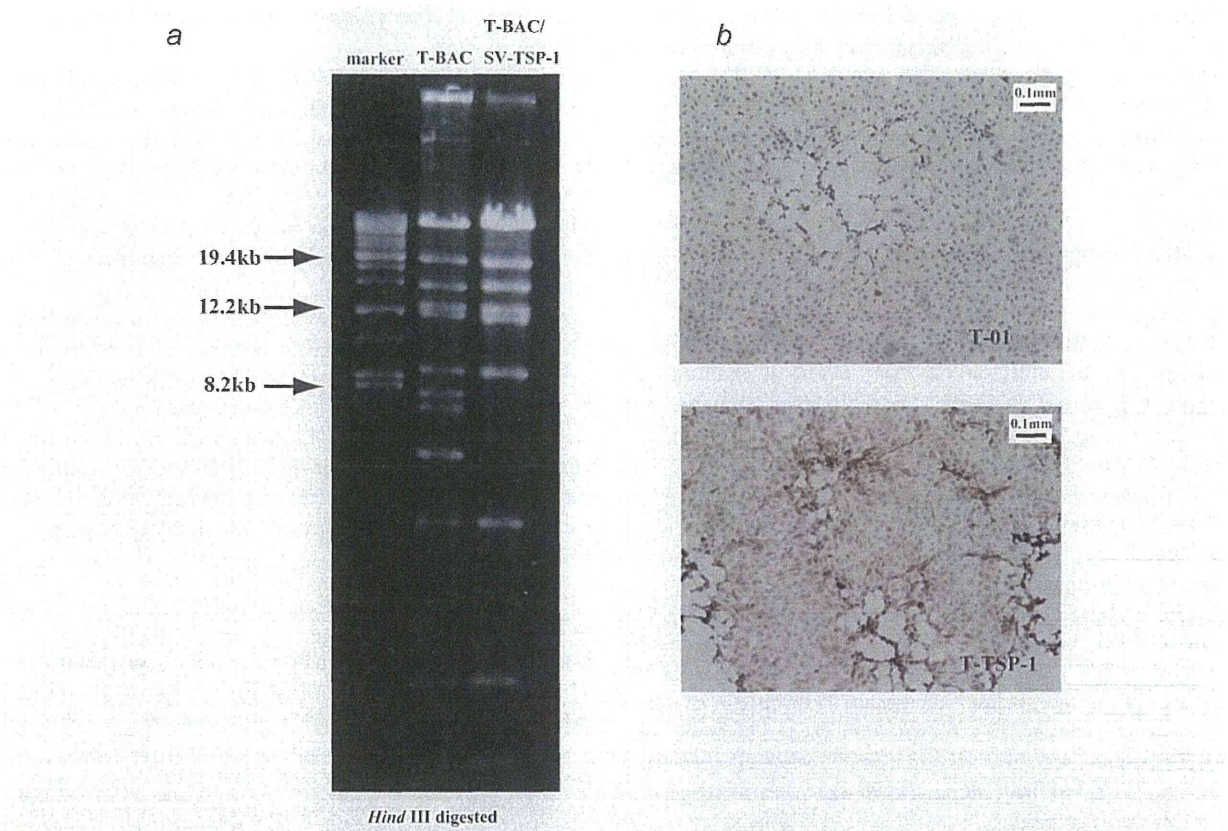

Figure 1. Verification of T-TSP-1 and TSP-1 expression in oncolytic HSV-1-infected Vero cells. (*a*) BAC plasmids were digested with *Hind* III. The digested BAC plasmids were electrophoresed, T-BAC (left) and Cre-recombinant BAC plasmid, T-BAC/SV-TSP-1 (right). (*b*) Vero cells infected with T-01 (MOI 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*).

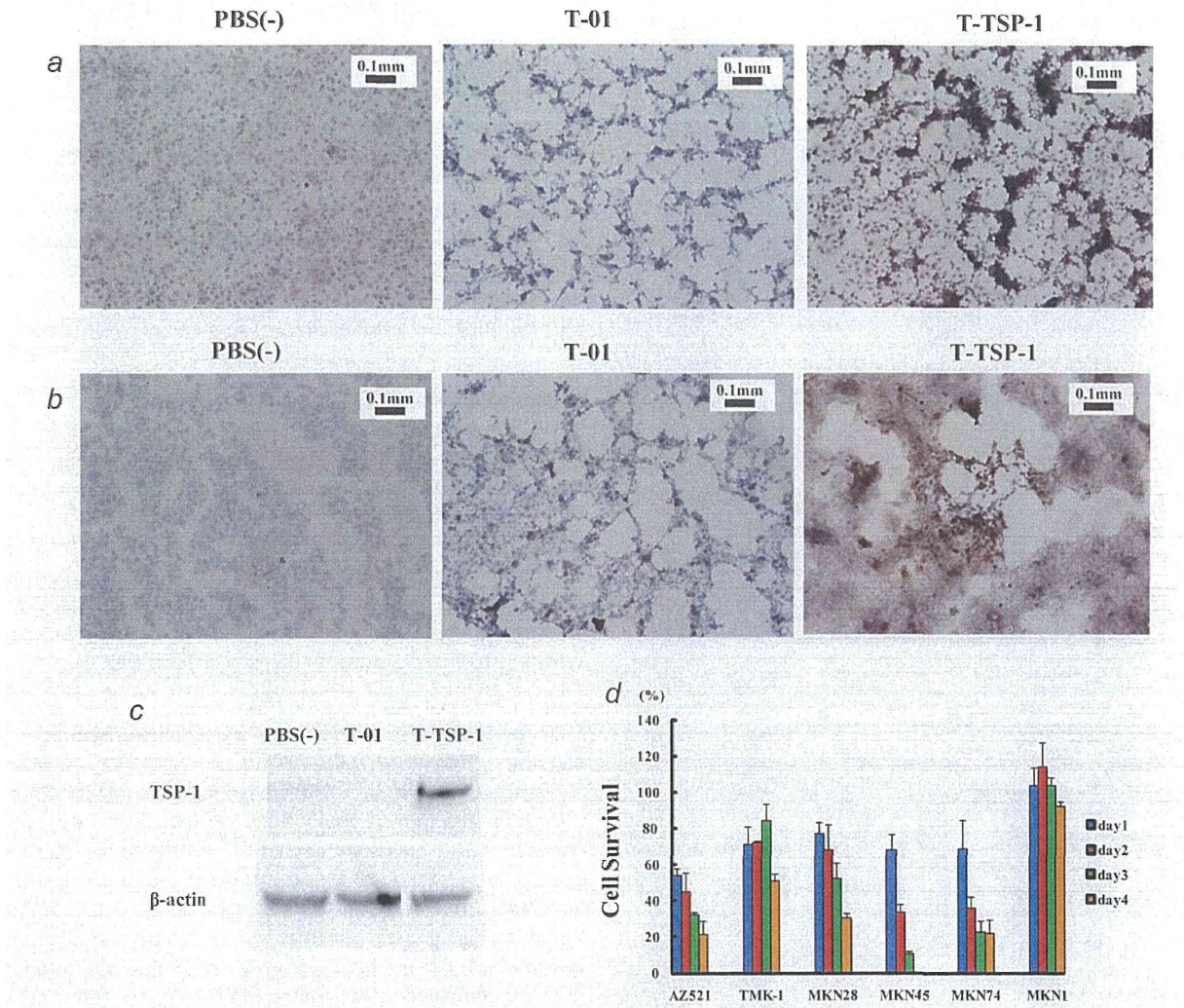

Figure 2. Immunocytochemical detection of TSP-1 and the cytotoxicity of T-01 in gastric cancer cell lines *in vitro*. Gastric cancer cells were infected with PBS(−), T-01 or T-TSP-1 and immunostained for human TSP-1 48 hr after infection. (*a*) TMK-1 cells after infection with PBS(−)(left), T-01 (middle) 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 1×10^4 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

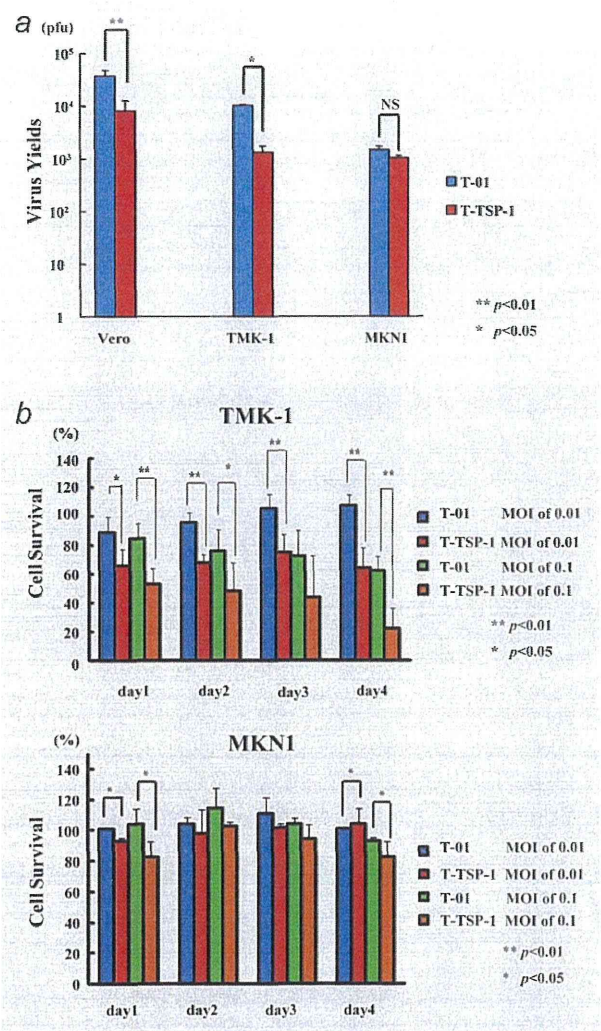

Figure 3. *In vitro* viral replication and cytotoxicity of HSVs against gastric cancer cell lines. (*a*) The *in vitro* virus yield was determined 48 hr after infection of Vero cells (1×10^5 per well) with T-01 or T-TSP-1 at an MOI of 0.01, and TMK-1 and MKN1 cells (1×10^5 per well) at an MOI of 0.1. (*b*) TMK-1 and MKN1 cells were seeded into 24-well plates at 1×10^4 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 1×10^6 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

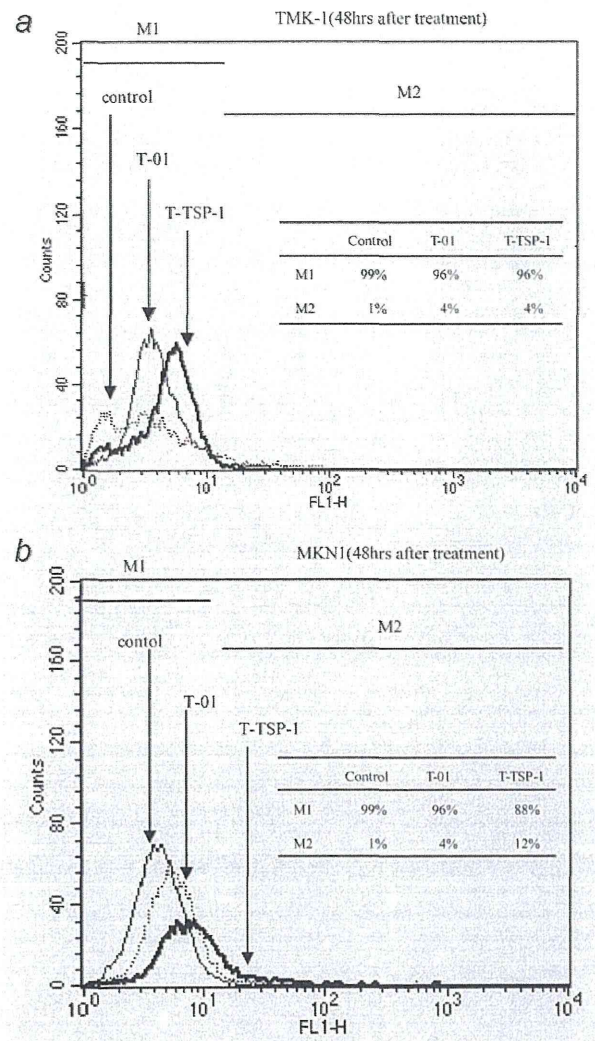

Figure 4. *In vitro* apoptosis assay of gastric cancer cells infected with oncolytic HSVs. (*a*) We performed an *in vitro* TUNEL assay using TMK-1 gastric cancer cells 48 hr after infection with PBS(−) (control), T-01 (MOI of 0.1) or T-TSP-1 (MOI of 0.1). (*b*) The results of the *in vitro* TUNEL assay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

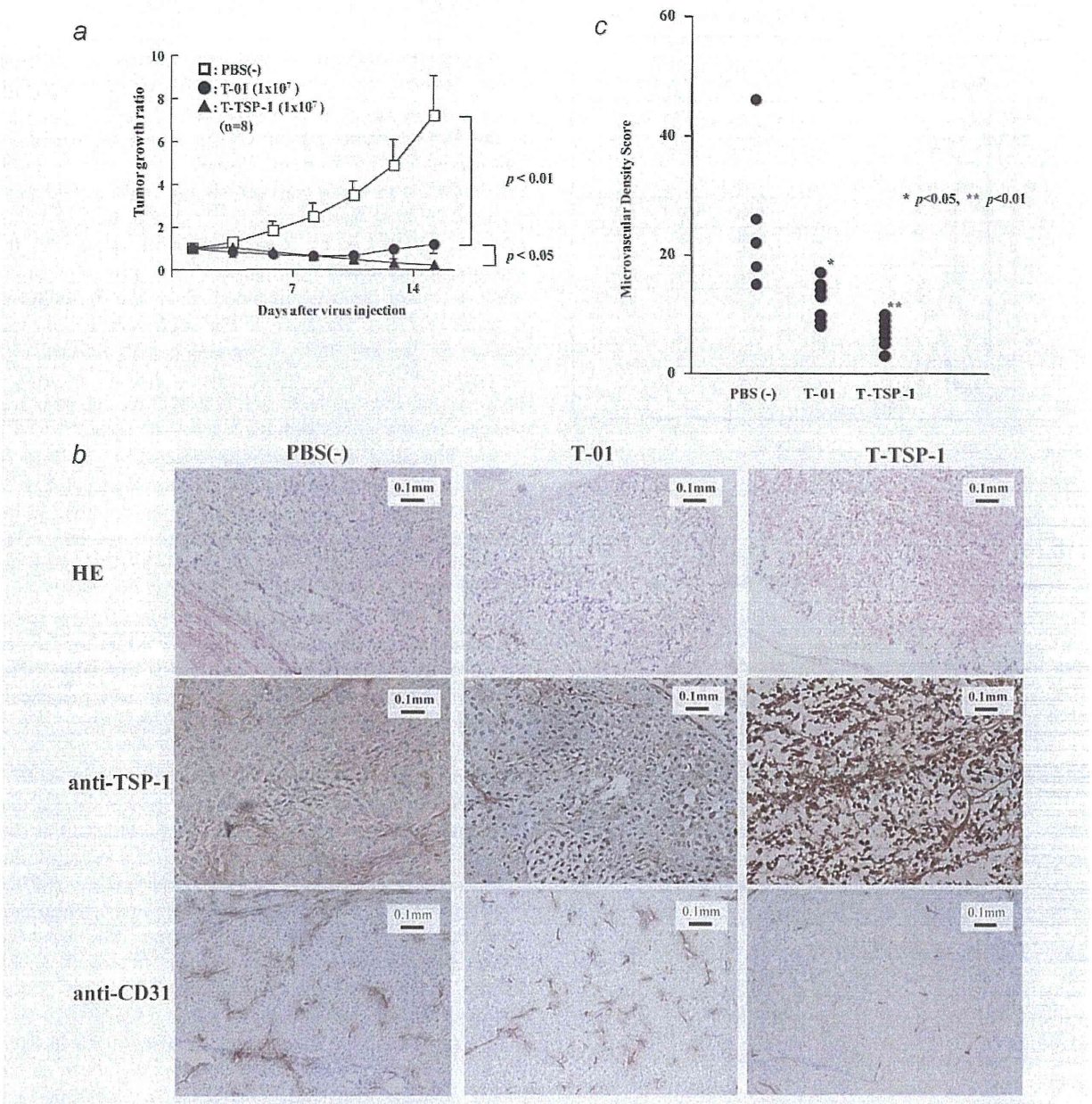

Figure 5. The efficacy of armed oncolytic HSV-1 vectors *in vivo*. (*a*) The antitumor effects of TSP-1-expressing oncolytic HSV-1s (T-TSP-1) and oncolytic HSV-1 not expressing any transgenes (T-01) was examined in BALB/c nu/nu mice bearing subcutaneous TMK-1 tumors. BALB/c nu/nu mice bearing subcutaneous TMK-1 tumors of ~6 mm in diameter were treated with intratumoral injection of PBS(−) or T-01 (1×10^7 pfu) or T-TSP-1 (1×10^7 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.

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Long-term results of radical prostatectomy with immediate adjuvant androgen deprivation therapy for pT3N0 prostate cancer

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Abstract

Background: Radical prostatectomy is used to treat patients with clinically localized prostate cancer, but there have been few reports of its use in locally advanced disease. We evaluated the long-term results of radical prostatectomy and immediate adjuvant androgen deprivation therapy in Japanese patients with pT3N0M0 prostate cancer.

Methods: We retrospectively reviewed 128 patients with pT3N0M0 prostate cancer who underwent radical prostatectomy at our institute from 2000 to 2006. All pT3N0 patients were treated with adjuvant androgen deprivation therapy shortly after radical prostatectomy. Immediate adjuvant androgen deprivation therapy was continued for at least 5 years. Twenty-three were excluded because of preoperative hormonal therapy, missing data, or others. Death from any cause, death from prostate cancer, clinical recurrence and hormone-refractory biochemical progression were analyzed by Kaplan-Meier graphs. Relative risks of progression were estimated using Cox proportional hazards models with 95% confidence intervals.

Results: The 10-year hormone-refractory biochemical progression-free survival rate was 88.3% and the cancer-specific survival rate was 96.3% after a median follow-up period of 8.2 years (range 25.6-155.6 months). Higher clinical stage ($p = 0.013$), higher Gleason score at biopsy ($p = 0.001$), seminal vesicle invasion ($p = 0.003$) and microlymphatic invasion ($p = 0.006$) were predictive factors for hormone-refractory biochemical progression by univariate analyses. Multivariate analyses identified Gleason score at biopsy ($p = 0.027$) and seminal vesicle invasion ($p = 0.030$) as independent prognostic factors for hormone-refractory biochemical progression. None of the patients with clinical T1 cancers ($n = 20$), negative surgical margin ($n = 12$), or negative perineural invasion ($n = 11$) experienced hormone-refractory biochemical progression.

Conclusions: Radical prostatectomy with immediate adjuvant androgen deprivation therapy may be a valid treatment option for patients with pT3N0M0 prostate cancer.

Keywords: Adjuvant androgen deprivation therapy, Pathological T3, Prognosis, Prognostic factor, Prostate cancer, Radical prostatectomy

Background

Tumor cell penetration of the prostatic capsule or invasion of the seminal vesicle is recognized as locally advanced prostate cancer of pathological T3N0. Patients with pT3N0 prostate cancers have the potential to suffer from disease relapse, and radical prostatectomy alone may fail to achieve a cure. The introduction of prostate specific antigen (PSA) assays means that more patients now undergo radical prostatectomy at earlier stages. However, pT3 disease still occurs in 25–58% of clinical T1 and T2 prostate cancer patients [1-4]. Although the management of patients with pT3 prostate cancer remains controversial, some reports recommend the use of adjuvant therapies in these patients [5-9]. Few studies have reported treatment outcomes of pT3 cancers, but some clinicopathologic factors, such as higher Gleason score, higher

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PSA level and seminal vesicle invasion are considered to be prognostic factors associated with poorer outcome [10-13]. However, to the best of our knowledge, there have been few reports of pT3N0 patients treated with adjuvant hormonal therapy, and more outcome data and accurate information are needed for these patients. We therefore analyzed clinical data from patients with pT3N0 prostate cancer to obtain detailed information and long-term outcome data. Importantly, a pathologic diagnosis of pT3N0M0 cancer is not necessarily accurate in patients who have undergone preoperative therapy, and this study therefore only included patients who had not received any preoperative treatment.

Methods

We retrospectively reviewed 128 patients with pT3N0M0 out of a total of 431 patients with prostate cancer who underwent radical prostatectomy at our hospital from January 2000 to December 2006. These patients were selected because immediate adjuvant hormonal therapy was applied in patients with pT3N0M0 prostate cancer during this period. Twenty-three patients were excluded from the analysis; four because of incomplete data, 12 because they had received preoperative hormonal therapy and seven because they had received bicalutamide only or estramustine as hormonal therapy. Data from the remaining 105 patients were analyzed. Bilateral obturator lymph nodes were dissected in all patients at radical prostatectomy. Immediate adjuvant androgen deprivation therapy was started within 12 weeks of radical prostatectomy. Undetectable PSA levels or PSA nadir were not required to be confirmed following radical prostatectomy. Clinical diagnosis was defined according to the 2009 TNM guidelines based on a digital rectal examination (DRE), transrectal ultrasonography, biopsy results, computed tomography scans and/or magnetic resonance imaging, and bone scintigraphy. All specimens were reviewed by a single pathologist. After radical prostatectomy, patients were followed-up at 1-month intervals for the first 3 months following surgery, then at 3-month intervals for 5 years, and finally at 3–6-month intervals thereafter. Follow-up examinations included measurement of PSA levels, and a DRE, computed tomography scan, magnetic resonance imaging or bone scintigraphy in the event of suspected disease recurrence. Immediate adjuvant therapy included surgical orchiectomy, administration of luteinizing hormone-releasing hormone (LHRH) analogs, and combined androgen blockade consisting of orchiectomy or an LHRH analog together with anti-androgens. Immediate adjuvant androgen deprivation therapy was continued for at least 5 years after radical prostatectomy. Salvage additive or altered hormonal therapies were initiated when the PSA level rose rapidly by >0.4 ng/ml, or when it rose consistently by >0.2 ng/ml for more than three consecutive visits. Salvage additive or altered hormonal therapy included: 1) combined androgen blockade consisting of orchiectomy or LHRH analog with bicalutamide; 2) suspension of bicalutamide to confirm the effects of anti-androgen withdrawal; 3) estramustine; and 4) dexamethasone.

Hormone-refractory biochemical progression was defined as a PSA level >0.2 ng/ml despite the above hormonal therapies. Clinical recurrence was defined as recognizable disease relapse on imaging examination. Clinical recurrence was treated by salvage radiation therapy. This study was approved by the Ethics Committee, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo.

The study end points were death from any cause, death from prostate cancer, clinical recurrence, and hormone-refractory biochemical progression. These end points were analyzed by plotting Kaplan-Meier graphs and comparing them according to each clinicopathologic factor using log-rank tests. Relative risks for hormone-refractory biochemical progression according to each clinicopathologic factor were estimated using the Cox proportional hazards models with 95% confidence intervals. All statistical analyses were performed using JMP version 9 (SAS Institute, Cary, NC, USA) and differences were considered statistically significant at $p < 0.05$. The following clinicopathologic factors were evaluated: age at radical prostatectomy, preoperative PSA level, preoperative T stage (clinical stage), Gleason score of the biopsy specimen, seminal vesicle invasion (representing stage pT3b), surgical margin of operation specimen, microlymphatic invasion, microvascular invasion, perineural invasion, and Gleason score.

Results

The clinical and pathological data for all 105 patients are shown in Table 1. The median age at surgery was 67.0 years and the median preoperative PSA level was 15.1 ng/ml (range 3.5–160.7, with lower and upper quartile values of 8.18 and 24.9). The median number of lymph nodes removed was 7.0 (range 2–19). A total of 43% of patients were underestimated preoperatively as having stage T1 (n = 20; 19.0%) or T2 (n = 25; 23.8%) tumors. Regarding the Gleason score at biopsy, 38 (36.2%) patients had a score of ≥8. Seminal vesicle invasion (pT3b) was detected in 42 patients (40.0%). Microlymphatic and microvascular invasions were detected in 33 (31.4%) and 51 (48.6%) patients, respectively. Immediate adjuvant androgen deprivation therapy consisted of androgen suppression with orchiectomy (n = 17), LHRH analog (n = 64), combined androgen blockade with orchiectomy or LHRH analog and bicalutamide or other anti-androgens (n = 24). Three patients changed their hormonal therapies during follow-up because of adverse events.

The median follow-up period was 98.7 months (range 25.6–155.6). During the follow-up period, eleven patients