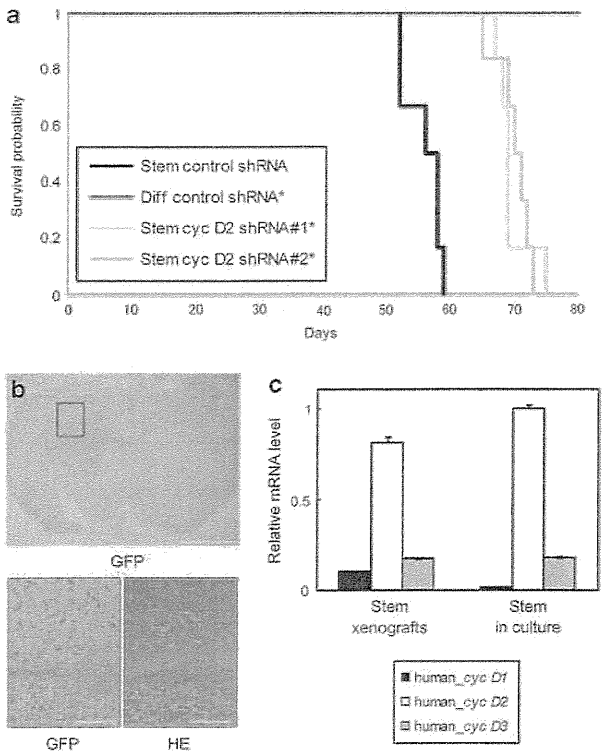

Figure 4. Critical role of cyclin D2 in the tumorigenicity of GSCs. (**a**) Undifferentiated (stem) or differentiated (diff) GB2 cells were infected with lentivirus harboring an shRNA targeting cyclin D2. After 1 week, cells were transplanted into the frontal lobe of immunocompromised mice. $^*P = 0.0006$ with comparison to stem control shRNA. (**b**) Histopathological analysis of representative tumor xenograft. Undifferentiated GB2 cells were infected with control lentivirus expressing GFP and transplanted into immunocompromised mice. GFP immunostaining (distribution of tumor cells) and hematoxylin and eosin (HE) staining are shown. Scale bars represent 2 mm (upper) and 200 μm (lower). (**c**) The mRNA levels of D-type cyclins in undifferentiated (stem) GB2 xenografts and undifferentiated (stem) GB2 cells in culture were evaluated by quantitative RT–PCR using human-specific primers. Error bars represent the s.d. ($n = 3$).

that block the expression of cyclin D2 could have a growth inhibitory effect on GSCs.

MATERIALS AND METHODS

Tumor specimens and primary tumor cultures

Following informed consent, tumor samples classified as primary glioblastoma were obtained from patients undergoing surgical treatment at the University of Tokyo Hospital as approved by the Institutional Review Board. Tumors were washed, and mechanically and enzymatically dissociated into single cells. Tumor cells were cultured in Neurobasal medium (Life Technologies, Carlsbad, CA, USA) containing B27 supplement minus vitamin A (Life Technologies), epidermal growth factor and basic fibroblast growth factor (20 ng/ml each; Wako Pure Chemical Industries, Osaka, Japan). For *in vitro* differentiation, tumor cells were cultured in Dulbecco's modified Eagle's medium/F-12 medium (Life Technologies) containing 10% fetal bovine serum. Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA).

Antibodies

Mouse monoclonal antibodies to cyclin B1, E2F1 and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies to cyclin D1, D2, D3 and RB were from BD Biosciences (Billerica, MA, USA). Mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Millipore (Bedford, MA, USA). Rabbit polyclonal antibodies to E2F2 and green fluorescence protein (GFP) were from Santa Cruz Biotechnology. Rabbit pAbs to phospho-RB S780, S795 and S807/811 were from Cell Signaling Technology (Danvers, MA, USA).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors). Lysates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was subjected to immunoblot analysis using horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (GE Healthcare, Pittsburgh, PA, USA) or sheep anti-mouse immunoglobulin G (GE Healthcare) as a secondary antibody. Visualization was performed using the Enhanced Chemiluminescence Plus Western Blotting Detection System (GE Healthcare) and LAS-4000EPUVmini Luminescent Image Analyzer (GE Healthcare).

Quantitative RT–PCR

Total RNA was extracted using NucleoSpin RNA Clean-up kit (Takara Bio Inc., Shiga, Japan) and reverse-transcribed into cDNA using ReverTra Ace qPCR RT Kit (Toyobo Life Science, Osaka, Japan). Real-time PCR was performed using LightCycler480 SYBR Green I Master and a LightCycler480 Instrument (Roche, Indianapolis, IN, USA). The results were normalized with the detected value for *GAPDH* or *ACTB*. Primers used in RT–PCR were as follows: *GAPDH* forward (5′-GCACCGTCAAGGCTGAGAAC-3′), *GAPDH* reverse (5′-TGGTGAAGACGCCAGTGGA-3′); *CD133* forward (5′-AGTGG CATCGTGCAAACCTG-3′), *CD133* reverse (5′-CTCCGAATCCATTCGACGA TAGTA-3′); *nestin* forward (5′-GAGGTGGCCACGTACAGG-3′), *nestin* reverse (5′-AAGCTGAGGGAAGTCTTGGA-3′); *cyclin D1* forward (5′-TGTCCTACT ACCGCCTCACA-3′), *cyclin D1* reverse (5′-CAGGGCTTCGATCTGCTC-3′); *cyclin D2* forward (5′-GGACATCCAACCCTACATGC-3′), *cyclin D2* reverse (5′-CGCACTTCTGTTCCTCACAG-3′); and *cyclin D3* forward (5′-GCTTAC TGGATGCTGGAGGTA-3′), *cyclin D3* reverse (5′-AAGACAGGTAGCGATCCAG GT-3′). Human-specific primers were as follows: *ACTB* forward (5′-CGTCACC AACTGGGACGACA-3′), *ACTB* reverse (5′-CTTCTCGCGGTTGGCCTTGG-3′); *cyclin D1* forward (5′-ACTACCGCCTCACACGCTTC-3′), *cyclin D1* reverse (5′-CTTGACTCCAGCAGGGCTTC-3′); *cyclin D2* forward (5′-ATCACCAACAC AGACGTGGA-3′), *cyclin D2* reverse (5′-TGCAGGCTATTGAGGAGCA-3′); and *cyclin D3* forward (5′-TACACCGACCACGCTGTCT-3′), *cyclin D3* reverse (5′-GAAGGCCAGGAAATCATGTG-3′). Mouse-specific primers were as follows: *ACTB* forward (5′-GGATGCAGAAGGAGATTACTGC-3′), *ACTB* reverse (5′-CCACCGATCCACACAGAGCA-3′).

RNA interference

The stealth siRNA oligonucleotide sequences were 5′-CCACAGAUGUGAA-GUUCAUUUCCAA-3′ (cyclin D1), 5′-UGCUCCUCAAUAGCCUGCAGCAGUA-3′ (cyclin D2#1), 5′-UGACGGAUCCAAGUCGGAGGAUGAA-3′ (cyclin D2#2), 5′-AACUACCUGGAUCGCUACCUGUCUU-3′ (cyclin D3), 5′-GGGAGAUCA AGGUAACCCUGGUGUU-3′ (CDK4) and 5′-ACCGAGUAGUGCAUCGCGAU CUAAA-3′ (CDK6) (Life Technologies). Negative control stealth siRNA with medium GC content was purchased from Life Technologies. The silencer select siRNA oligonucleotide sequences were 5′-GGACCUUCGUAGCAUUG CATT-3′ (E2F1) and 5′-AGACAGUGAUUGCCGUCAATT-3′ (E2F2) (Life Technologies). Negative control silencer select siRNA was purchased from Life Technologies. Transfection of siRNA was performed using Lipofectamine RNAiMAX transfection reagent (Life Technologies). shRNAs targeting cyclin D2 were designed to harbor the same target sequences.

Flow cytometry

Cells were trypsinized, fixed in 70% ethanol and then stained with propidium iodide (Sigma, St Louis, MO, USA). Cells were passed through a FACSCalibur instrument (BD Biosciences) and the data were analyzed using the ModFit LT software (Verity Software House, Topsham, ME, USA).

Lentivirus production

Lentiviral vector CS-RfA-CG harboring an shRNA driven by the H1 promoter or CSII-CMV-RfA-IRES2-Venus harboring a cDNA driven by the CMV promoter was transfected with the packaging vectors pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev into 293FT cells using Lipofectamine 2000 Transfection Reagent (Life Technologies). All plasmids were kindly provided by H

Miyoshi (RIKEN BioResource Center, Ibaraki, Japan). Viral supernatant was purified by ultracentrifugation at 25 000 r.p.m. for 90 min (SW28 rotor, Beckman Coulter, Brea, CA, USA). Infection efficiency was monitored by GFP expression as it is driven by the CMV promoter.

Intracranial xenografts

At 1 week after lentivirus infection, 1×10^4 cells were injected stereotactically into the right frontal lobe of 5-week-old nude mice (BALB/cAJcl-*nu/nu*; CLEA Japn Inc., Tokyo, Japan), following administration of general anesthesia ($n = 6$). The injection coordinates were 2 mm to the right of the midline, 1 mm anterior to the coronal suture and 3 mm deep. Mice were monitored for 6 months. Survival of mice was evaluated by Kaplan–Meier analysis. *P*-value was calculated using a log-rank test. Tumors were histologically analyzed after hematoxylin and eosin staining. Tumor distribution was analyzed by GFP immunostaining. All animal experimental protocols were performed in accordance with the politics of the Animal Ethics Committee of the University of Tokyo.

Immunohistochemistry

Samples were fixed in 3.7% buffered formalin, dehydrated and embedded in paraffin. Sections (6 μm) were rehydrated, and endogenous peroxidases were blocked by incubation in 0.3% H_2O_2 for 30 min. The primary antibody was detected using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA). Slides were lightly counterstained with hematoxylin.

Microarray data

The expression profiles of undifferentiated GB1–3 and 5 cells were generated on the Affymetrix GeneChip HG-U133 Plus 2.0 microarray platform (Affymetrix, Santa Clara, CA, USA). The expression profiles of 15 GSCs were taken from the Gene Expression Omnibus database GSE7181 and GSE8049 (Lottaz *et al.*[9]).Data were analyzed using the software program GenePattern. The expression profiles of D-type cyclins in various histological grades of glioma were taken from GSE4290 (Sun *et al.*[17]). Data were analyzed using the software program R. *P*-value was calculated using a pairwise Wilcoxon's test. The expression profiles of D-type cyclins in patient glioblastomas, glioma cell lines and normal NSCs were taken from GSE4536 (Lee *et al.*[4]).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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 1×10^6 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.

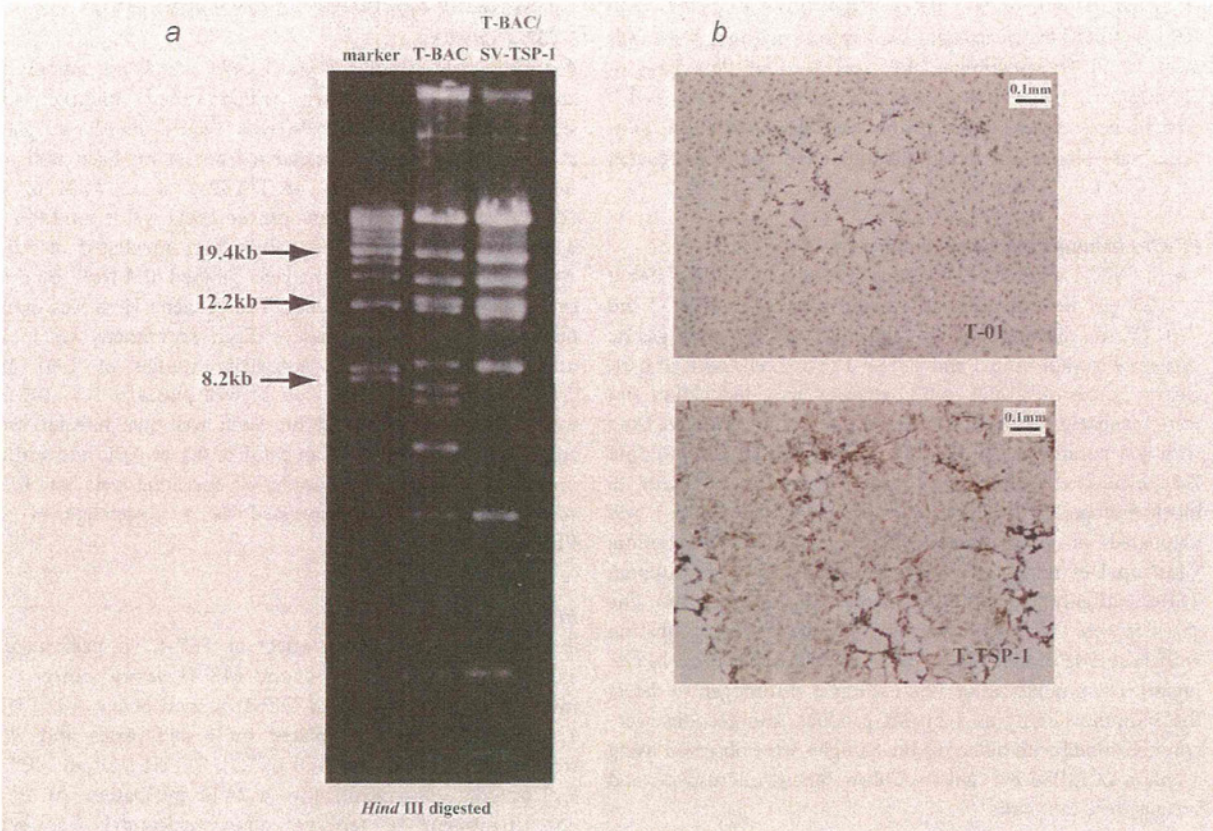

Figure 1. Verification of T-TSP-1 and TSP-1 expression in oncolytic HSV-1-infected Vero cells. (*a*) BAC plasmids were digested with *Hind* III. The digested BAC plasmids were electrophoresed, T-BAC (left) and Cre-recombinant BAC plasmid, T-BAC/SV-TSP-1 (right). (*b*) Vero cells infected with T-01 (MOI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

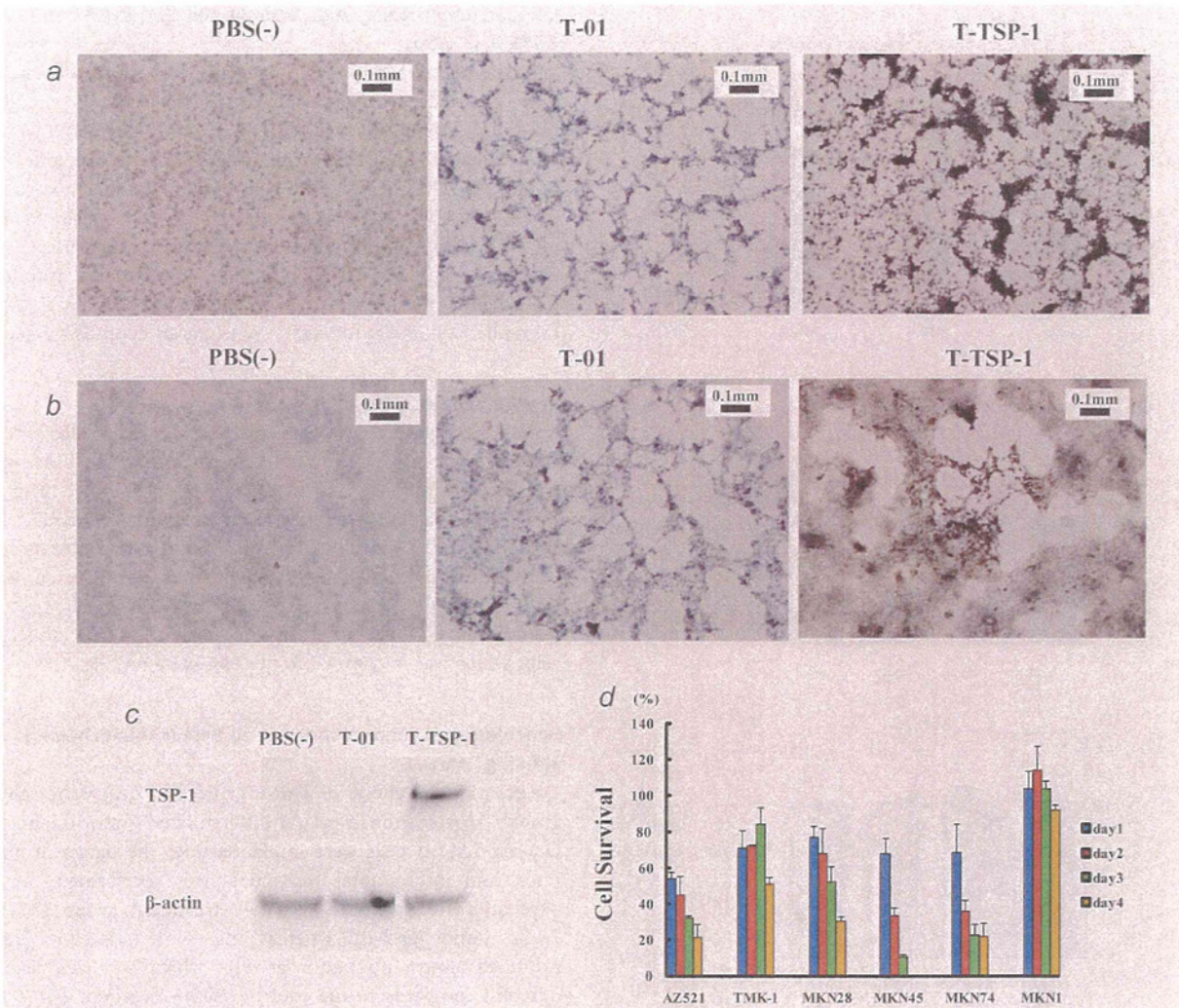

Figure 2. Immunocytochemical detection of TSP-1 and the cytotoxicity of T-01 in gastric cancer cell lines *in vitro*. Gastric cancer cells were infected with PBS(−), T-01 or T-TSP-1 and immunostained for human TSP-1 48 hr after infection. (*a*) TMK-1 cells after infection with PBS(−)(left), T-01 (middle)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

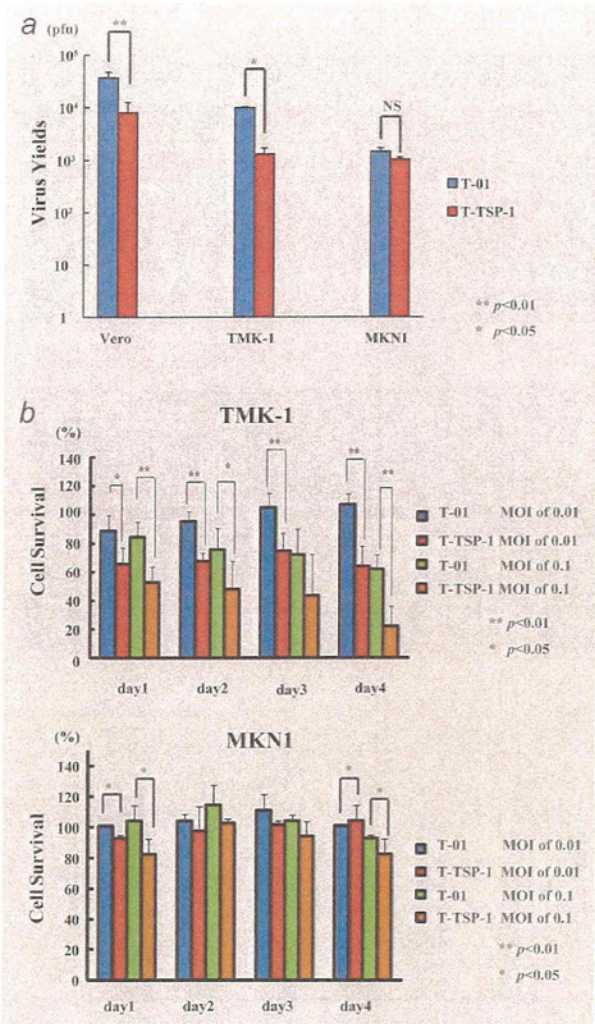

Figure 3. *In vitro* viral replication and cytotoxicity of HSVs against gastric cancer cell lines. (*a*) The *in vitro* virus yield was determined 48 hr after infection of Vero cells (1×10^5 per well) with T-01 or T-TSP-1 at an MOI of 0.01, and TMK-1 and MKN1 cells (1×10^5 per well) at an MOI of 0.1.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

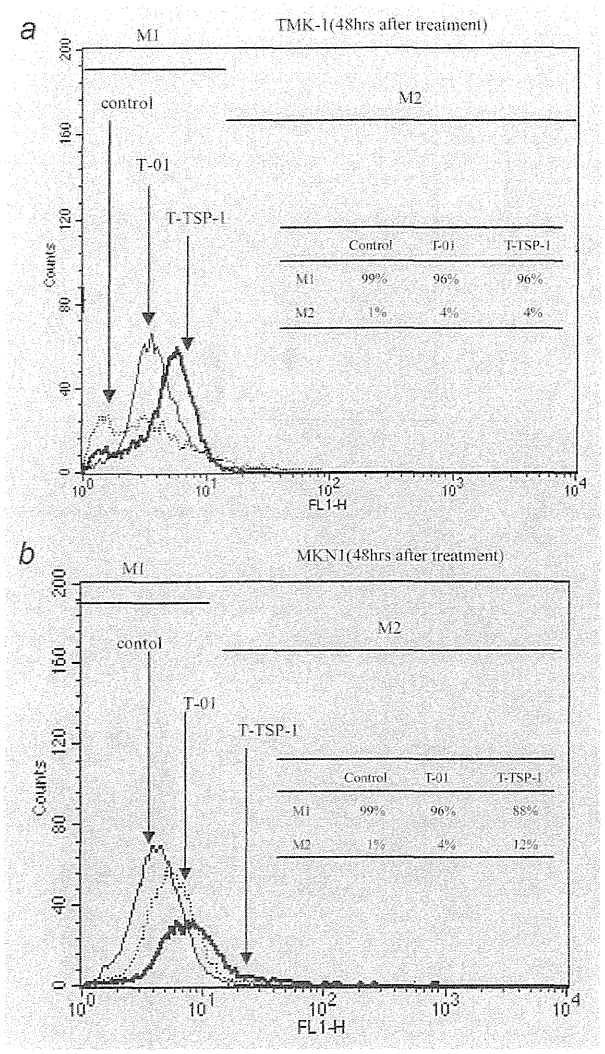

Figure 4. *In vitro* apoptosis assay of gastric cancer cells infected with oncolytic HSVs. (*a*) We performed an *in vitro* TUNEL assay using TMK-1 gastric cancer cells 48 hr after infection with PBS(−) (control), T-01 (MOI of 0.1) or T-TSP-1 (MOI of 0.1). (*b*) The results of the *in vitro* TUNEL assay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

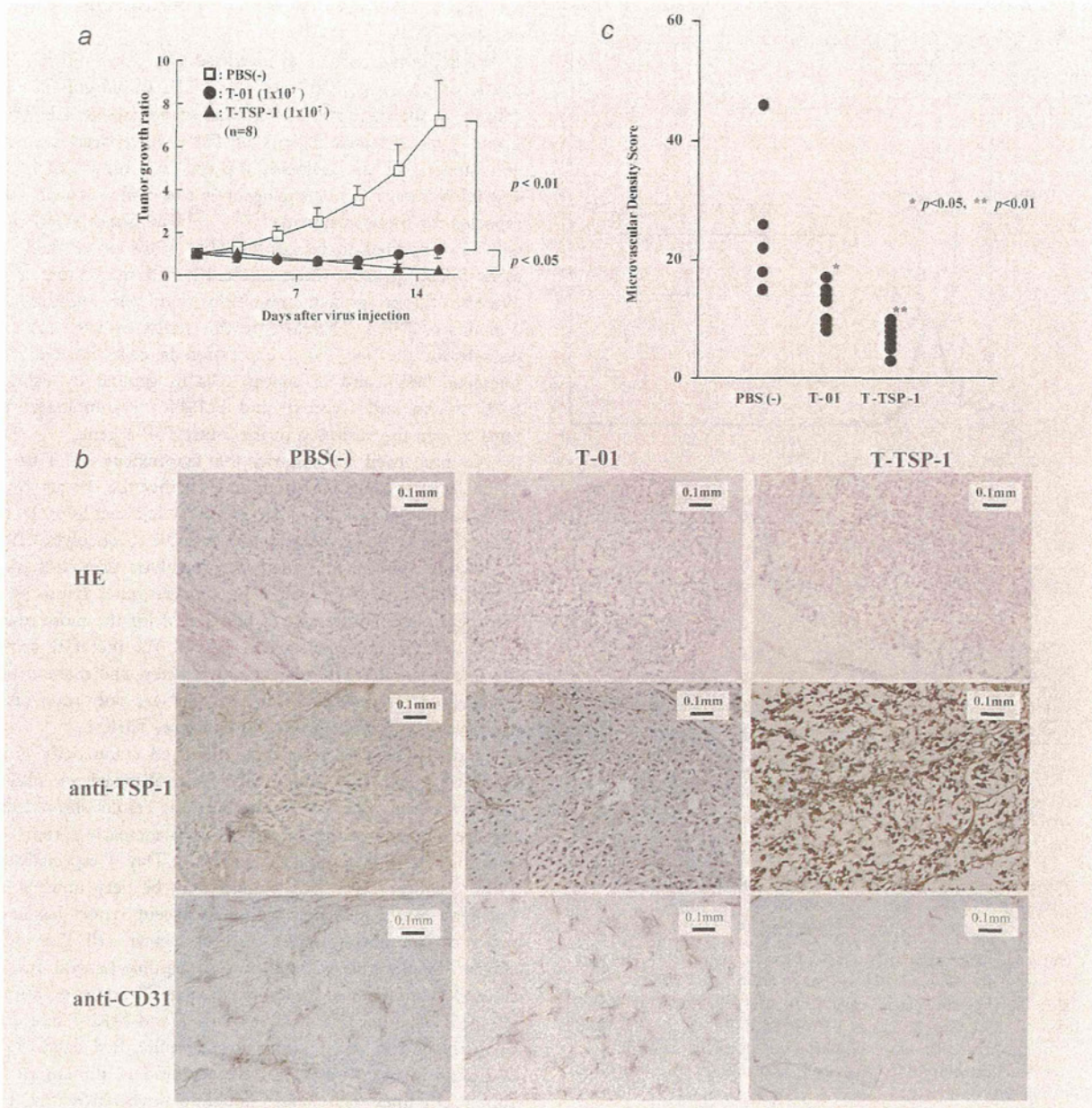

Figure 5. The efficacy of armed oncolytic HSV-1 vectors *in vivo*. (*a*) The antitumor effects of TSP-1-expressing oncolytic HSV-1s (T-TSP-1) and oncolytic HSV-1 not expressing any transgenes (T-01) was examined in BALB/c nu/nu mice bearing subcutaneous TMK-1 tumors. BALB/c nu/nu mice bearing subcutaneous 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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BJUI
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Leukopenia as a risk factor for osteonecrosis of the jaw in metastatic prostate cancer treated using zoledronic acid and docetaxel

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Study Type – Harm (case series)
Level of Evidence 4

What's known on the subject? and What does the study add?
The common clinical practice for advanced prostatic adenocarcinoma is combination therapy of zoledronic acid (ZA) and docetaxel (TAX). Little is known about the consequences of this combination therapy with regard to osteonecrosis of the jaw (ONJ).

This study shows that the combination therapy of ZA and TAX increases the risk of ONJ and that tooth extraction and leukopenia induced by TAX are the risk factors.

OBJECTIVE

- To determine whether docetaxel (TAX) can increase the risk of osteonecrosis of the jaw (ONJ) in patients with prostatic adenocarcinoma (PC) receiving zoledronic acid (ZA), a bisphosphonate (BP) used in the treatment of patients with cancer.

MATERIALS AND METHODS

- The medical records of 111 patients with PC who received ZA between September 2006 and March 2011 at our institutions were reviewed to assess the incidence and risk factors for ONJ.

RESULTS

- Nine patients (8.1%) developed ONJ during a median follow-up of 14.5 months.
- Using univariate analysis we found that TAX chemotherapy ($P = 0.037$, hazard ratio [HR] 6.611), tooth extraction during ZA therapy ($P < 0.001$, HR 11.254), and high prostate-specific antigen level ($P = 0.019$, HR 8.008) at the start of ZA were predictive factors.
- Using multivariate analysis we found that TAX chemotherapy ($P = 0.011$, HR 56.35), steroid use ($P = 0.044$, HR = 17.795), and tooth extraction ($P = 0.039$, HR 7.471) were independent predictors.
- Among those receiving TAX chemotherapy, multivariate analysis identified tooth extraction ($P = 0.009$) and nadir WBC counts <1000/μL during TAX chemotherapy ($P = 0.030$) as independent risk factors.

CONCLUSION

- Tooth extraction and nadir WBC counts <1000/μL were found to be risk factors for ONJ in metastatic prostate cancer treated with ZA and TAX combination therapy, showing that leukopenia is an important factor in the development of ONJ.

KEYWORDS

prostate cancer, zoledronic acid, osteonecrosis of the jaw, docetaxel, leukopenia, bisphosphonates

INTRODUCTION

Prostatic adenocarcinoma (PC) is the second leading cause of male cancer deaths in the USA [1,2]. PC is also one of the most common cancers in Japan, accounting for 10.6% of estimated new cancer cases and 5.1% of estimated cancer deaths in men [3]. Bone is the most common metastatic site, representing >80% of advanced PC cases [4]. In addition, complications from bone metastases, including pain, pathological fracture and abnormal serum calcium level, are a major cause of morbidity in PC.

Zoledronic acid (ZA) is a bisphosphonate (BP), which has a strong affinity for hydroxyapatite and concentrates within bone and shift bone turnover by inhibiting osteoclasts and promoting osteoblastic activity [4–6]. ZA has greater potency than pamidronate or other BPs used in several preclinical models of bone resorption [7], and has been shown to decrease and/or delay the onset of skeletal-related events, reducing tumour-induced hypercalcaemia and bone pain [4–6]. However, osteonecrosis of the jaw (ONJ), which has rarely been observed for other BPs [9], has been known to be a significant adverse event associated with use of ZA since the first report in 2003 [8–9].

The common clinical practice for advanced PC is combination therapy of ZA and docetaxel (TAX), an antineoplastic agent

[10,11], but little is known about the consequences of this combination therapy for ONJ. The aim of the present study was to determine whether co-treatment with TAX affects the risk of ZA-related ONJ in a retrospective study cohort.

PATIENTS AND METHODS

A retrospective chart review was conducted for men with PC who had bone metastasis and received ZA at the authors' hospitals between September 2006 and March 2011. The present study was approved by the institutions' Ethics Committee (No. 3124). ZA was administered at 4.0 mg every 21 days. TAX was given at 75 mg/m^2 by 1-h i.v. infusion every 21 days, along with a daily oral dose of 1.0–2.0 mg of dexamethasone [12]. Patients were followed at outpatient clinic every 3–4 weeks. We reviewed laboratory test data, including complete blood count, renal function, liver function and PSA levels. All patients with suspected ONJ were referred to a dentist for evaluation including panoramic x-rays. Biopsy of ONJ lesions was performed only when metastatic disease was suspected. ONJ diagnosis was made according to the following criteria stated in the American Association of Oral and Maxillofacial Surgeons (AAOMS) position paper: (i) current or previous treatment with a bisphosphonate; (ii) exposed, necrotic bone in the maxillofacial region that has persisted for more than 8 weeks; and (iii) no history of radiation therapy to the jaws [13]. Therapy for ONJ consisted of termination of ZA, patient education, antibacterial mouth rinse, antibiotic therapy, and/or surgical debridement [13]. ONJ-free survival was calculated from the first ZA administration. A log-rank test and Cox proportional hazards regression model were used for univariate and multivariate analyses of ONJ-free survival, respectively. All analyses were performed using the software, Stat Mate III ver. 3.07, ATMS Co., Ltd., Tokyo, Japan. A P value <0.05 was considered to indicate statistical significance.

RESULTS

A total of 111 patients with PC were included in the analysis. The median (range) patient age at the start of ZA treatment was 75 (56–91) years, and the median (range) initial serum level of PSA was 170.2 (0.48–9559) ng/mL. Radical prostatectomy (n = 20) and external radiotherapy (n = 1) had been performed as radical therapy. Of 94 men, 19 (20.2%, excluding 17 unknown) were smokers and 20 of 91 (22%, excluding 20 unknown) had diabetes. Dentures were used in 52 men (46.8%). Nine men had tooth extractions within 6 months. Extent of disease on bone scan was grade 1, grade 2, grade 3, grade 4, and unknown in 31, 36, 34, 4 and 6 men, respectively [14]. Owing to painful metastases, 37 (33.6%) needed analgesics.

Before the start of ZA, all patients had received LHRH agonists in combination with anti-androgens (bicalutamide or flutamide), 62 (56%) had started 1.0–2.0 mg oral dexamethasone, 92 (83%) had become castration-resistant, and 56 (50.4%) had received chemotherapy with TAX (75 mg/m^2 every 3 weeks). The median (range) values for blood test results at starting ZA were: WBC, 6600 (2000–22 100)/μL; haemoglobin, 11.9 (4.3–15.2) g/dL; platelets, 21.7 (6.8–38.9) × 10^4/μL; alkaline phosphatase, 315 (77–6634) IU/L; C reactive protein, 0.33 (0.01–28.64) mg/dL; and PSA, 27.5 (0.02–2761) ng/mL. During ZA therapy, a WBC nadir <1000/μL occurred in eight men as an adverse event of TAX treatment.

During a median (range) follow-up of 14.5 (1.0–40.2) months, ONJ developed in nine (8.1%) cases. It occurred significantly more frequently in men treated with ZA and TAX combined chemotherapy (8/56, 14.3%) than men with ZA monotherapy (1/55, 1.8%, P = 0.040, chi-squared test with Yates' correction). Of the eight patients with ONJ receiving the combination treatment, three had a nadir WBC <1000/μL.

To assess the predictive factors for ONJ we performed the log-rank test using univariate analysis, which revealed the following predictive risk factors for ONJ: TAX chemotherapy (P = 0.037, hazard ratio [HR] 6.611), tooth extraction (P < 0.001, HR 11.254), and high PSA level at the start of ZA (P = 0.019, HR 8.008 [Table 1]). A Cox proportional hazards regression model identified TAX chemotherapy (P = 0.011, HR 56.346), steroid use (P = 0.044, HR = 17.795), and tooth extraction (P = 0.039, HR 7.471) as independent predictive factors (Table 1).

To evaluate risk factors for ONJ among patients with TAX chemotherapy, we carried out univariate and multivariate analyses which identified tooth extraction (P = 0.009) and nadir WBC counts <1000/μL during combination therapy (P = 0.030) as independent risk factors (Table 2).

DISCUSSION

In the present retrospective study we found nine (8.1%) cases of ONJ in 111 men receiving ZA therapy for metastatic PC. The incidence was in the range of that previously reported at 2.9–18.6% [15,16].

To date, several risk factors for ONJ have been suggested, including tooth extraction, use of dentures and diabetes [17,18]. Kyrgidis *et al.* [17], analysing 20 patients with breast cancer who had ONJ, found the risk of ONJ increased with tooth extraction during ZA treatment (P = 0.001, HR 16.4) and with use of dentures (P = 0.029, HR 4.9). Khamaisi *et al.* [18] reported a much higher incidence (58%) of diabetes in men with BP-related ONJ than men without (14%). Other possible risk factors, smoking and obesity, have not been substantiated [9]. Medications, such as steroids, thalidomide, and some chemotherapeutic agents, were also postulated to be risk factors, but the original AAOMS position paper concluded that there were no measurable associations with these potential risk factors [13].

A few studies have reported an increased risk of ONJ among patients exposed to chemotherapy agents. Jadu *et al.* [19] reported that cyclophosphamide and erythropoietin treatments were associated with an increased risk of ONJ. They also reported an increased risk with steroid use. By contrast, Wessel *et al.* [9] found no significant association between ONJ and use of cyclophosphamide, steroids, erythropoietin or thalidomide. As for TAX chemotherapy, Ortega *et al.* [20] detected a potential, but not significant, trend to increased risk of ONJ associated with TAX chemotherapy (HR 3.8, P = 0.24) in 52 men with PC, treated with ZA for bone metastasis. Similarly, in the present study, TAX chemotherapy was a significant predictive factor for ONJ in univariate and multivariate analysis. In addition, we identified tooth extraction and nadir WBC counts <1000/μL during TAX therapy as the

TABLE 1 Predictive factors for ONJ in all patients

Variable	*n*	Univariate analysis HR (95% CI)	*P*	Multivariate analysis HR (95% CI)	*P*
Age					
≤75 years	62	Reference	0.310		
≥76 years	49	1.393 (0.521–7.792)			
Karnofsky performance status					
≥80%	95	Reference	0.316		
≤70%	16	<0.001 (0.035–2.961)			
Diabetes mellitus					
No	71	Reference		Reference	
Yes	20	0.508 (0.111–3.003)	0.514	0.892 (0.054–14.695)	0.936
Smoking					
No	75	Reference	0.189		
Yes	19	<0.001 (0.048–1.826)			
Tooth extraction within 6 months of treatment					
No	103	Reference		Reference	
Yes	8	11.254 (21.189– >100.0)	<0.001*	7.471 (1.103–50.588)	0.039*
Denture use					
No	59	Reference		Reference	
Yes	52	2.651 (0.703–9.957)	0.150	1.008 (0.165–6.178)	0.993
Extent of disease					
Grades 0, 1 or 2	67	Reference			
Grades 3 or 4	38	0.873 (0.186–4.083)	0.862		
Visceral metastasis					
No	101	Reference			
Yes	10	<0.001 (0.013–9.250)	0.525		
Castration-resistant prostate cancer					
No	19	Reference			
Yes	92	>100.0 (0.569–20.761)	0.179		
PSA level					
≤27.5 ng/mL	56	Reference			
>27.5 ng/mL	55	8.008 (1.296–17.917)	0.019*	10.722 (0.637– >100.0)	0.100
Elevation of alkaline phosphatase >360 IU/L					
No	61	Reference			
Yes	50	0.308 (0.093–1.767)	0.229		
Anaemia (haemoglobin <13.8 g/dL)					
No	16	Reference			
Yes	95	2.227 (0.375– 24.272)	0.299		
Analgesic use					
No	74	Reference		Reference	
Yes	37	0.400 (0.104–2.304)	0.366	1.941 (0.213–17.656)	0.556
TAX therapy					
No	55	Reference		Reference	
Yes	56	6.611 (1.088–15.555)	0.037*	56.346 (2.550– >100.0)	0.011*
Steroid use					
No	49	Reference		Reference	
Yes	62	2.172 (0.515– 7.740)	0.317	17.795 (1.082– >100.0)	0.044*

**Significant ($P < 0.05$).*

significant risk factors. Thus, the present results indicate that TAX-induced leukopenia, rather than TAX use itself, is of more importance in ONJ development.

Anti-angiogenic properties of both ZA and TAX may additively or synergistically impair jaw vascularization and cause a predisposition to bone necrosis [20].

The association between nadir WBC count and ONJ in patients with cancer treated with BPs has not been reported. Leukopenia induced by chemotherapy might serve as an

TABLE 2 Risk factors for ONJ among patients receiving TAX and ZA treatment

Variable	*n*	Univariate analysis HR (95% CI)	*P*	Multivariate analysis HR (95% CI)	*P*
Age			0.247		
≤75 years	39	Reference			
≥76 years	17	2.208 (0.540–11.007)			
Karnofsky performance status			0.291		
≥80%	48	Reference			
≤70%	8	<0.001 (0.035– 2.722)			
Diabetes mellitus			0.805		0.298
No	45	Reference		Reference	
Yes	6	0.773 (0.111–5.524)		0.102 (0.001–7.561)	
Smoking			0.263		
No	44	Reference			
Yes	11	<0.001 (0.037–2.460)			
Tooth extraction within 6 months of treatment			<0.001*		0.009*
No	50	Reference		Reference	
Yes	6	10.747(10.940–>100.0)		15.974 (1.990–>100.0)	
Denture use			0.425		0.916
No	29	Reference		Reference	
Yes	27	1.753 (0.431–7.362)		0.889 (0.099–7.983)	
Extent of disease			0.311		
Grades 1 or 2	34	Reference			
Grades 3 or 4	20	0.365 (0.090–2.153)			
Visceral metastases			0.732		
No	53	Reference			
Yes	3	<0.001 (0.001–>100.0)			
PSA level			0.004*		0.165
≤27.5 ng/mL	24	Reference		Reference	
>27.5 ng/mL	32	10.976 (1.921–34.062)		25.737 (0.263–>100.0)	
Elevation of alkaline phosphatase 360 IU/L			0.322		
No	32	Reference			
Yes	24	0.376 (0.088–2.225)			
Anaemia (haemoglobin <13.8 g/dL)			0.192		
No	5	Reference			
Yes	51	>100.0 (0.529–23.894)			
Analgesic use			0.526		0.790
No	34	Reference		Reference	
Yes	22	0.533 (0.093–3.378)		0.706 (0.054–9.163)	
Steroid use			0.351		0.075
No	5	Reference		Reference	
Yes	51	0.389 (0.010–5.229)		35.710 (0.697–>100.0)	
No. of ZA administrations			0.815		
≤5	26	Reference			
>5	30	1.203 (0.248–5.878)			
Nadir WBC <1000/uL			0.083		0.030*
No	48	Reference		Reference	
Yes	8	3.230 (0.800–39.386)		12.450 (1.273–>100.0)	

**Significant ($P < 0.05$).*

aggravating factor for infection of the oral cavity such as mucositis. Although the pathogenesis of ONJ is not fully understood, colonization of the oral cavity and teeth by a complex microbial flora is considered an important initial step [21]. Once oral mucositis is induced, inflammation of the lesion might persist owing to microbes and other stresses, including mastication and dental procedures. Thus, it would be reasonable to postulate that oral infection resulting from

TAX-induced leukopenia could lead to ONJ aggravation.

Oral mucositis is associated with decreased granulocyte counts and occurs at rates as high as 20% in patients treated with TAX [10]. Resolution of mucositis is related to neutrophil recovery [22–26].. Indeed, Gabrilove *et al.* [27] observed the reduced severity of mucositis in patients who received granulocyte colony-stimulating factor (G-CSF) after standard dose chemotherapy. Earlier administration of G-CSF could decrease the risk of ONJ by recovering WBCs more rapidly.

In conclusion, we found three independent significant predictive factors for ONJ in PC cases with bone metastasis: tooth extraction, steroid use and TAX chemotherapy. In addition, among cases with ZA and TAX combination therapy, we found that tooth extraction and nadir WBC counts <1000/μL were independent significant risk factors, suggesting that leukopenia induced by TAX rather than TAX use itself might be essential for ONJ development. Obviously, limitations of the present study are its retrospective nature and its limited number of cases. Further studies are needed to show the pathogenetic and therapeutic implications of leukopenia in developing ONJ among patients with cancer receiving BP treatment.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **TAX**, docetaxel; **ONJ**, osteonecrosis of the jaw; **PC**, prostatic adenocarcinoma; **ZA**, zoledronic acid; **BP**, bisphosphonate; **HR**, hazard ratio; **WBC**, white blood cell; **AAOMS**, American Association of Oral and Maxillofacial Surgeons; **G-CSF**, granulocyte colony-stimulating factor.

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Association between lower urinary tract symptoms and sexual dysfunction assessed using the core lower urinary tract symptom score and International index of erectile function-5 questionnaires

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***Objective*: The aim of this study is to assess the association between lower urinary tract symptoms (LUTS) and erectile dysfunction (ED) using the Core Lower Urinary Tract Symptom Score (CLSS) and the International Index of Erectile Dysfunction (IIEF)-5 questionnaires. *Methods*: A total of 220 consecutive treatment-naive men completed the International Prostate Symptom Score (IPSS), CLSS, and IIEF-5 questionnaires. The clinical diagnoses were benign prostate hyperplasia (BPH, $n = 58$), prostatic cancer ($n = 51$), prostatitis ($n = 28$), overactive bladder wet (OAB wet, $n = 16$), other diagnoses ($n = 39$), and controls ($n = 28$). Simple statistics and predictability of low IIEF-5 score were examined. *Results*: The total IIEF-5 score significantly correlated with both the IPSS and CLSS questionnaires ($p = 0.0001$). Among IPSS and CLSS symptoms, factors other than daytime frequency and incomplete emptying showed significant correlation with the total IIEF-5 score. A multivariate regression model indicated nocturia and urethral pain as independent factors for low IIEF-5 scores. *Conclusion*: Urethral pain was identified as an independent factor for erectile dysfunction. The extraction of pain symptoms using CLSS questionnaire may be useful to evaluate LUTS in men with pelvic health problems.**

Keywords: LUTS, CLSS, IIEF-5, Erectile dysfunction, assessment

Introduction

Lower urinary tract symptoms (LUTS) and erectile dysfunction (ED) are common conditions in middle-aged and elder men that can strongly affect quality of life [1,2]. Recently, several community-based studies have been conducted to ascertain the associations between LUTS and ED [1–5]. Most of these studies suggest that the prevalence of ED correlates with the presence and severity of LUTS [1–5]. For example, a significant correlation between storage symptoms, especially nocturia, and ED has been reported [1]. However, the exact relationship between these two age-dependent conditions remains unclear.

In these studies, the International Prostate Symptom Score (IPSS), a specific questionnaire for benign prostate hyperplasia (BPH), was used as the assessment tool for LUTS. Actually, the IPSS would be the questionnaires most frequently used to assess LUTS in patients [6]. This questionnaire contains 7 items relating to storage, voiding, and post-micturition symptoms but none regarding incontinence or pain symptoms [6]. LUTS in males are diverse in nature and may reflect underlying diseases/dysfunctions of the lower urinary tract, prostate, nervous system, and other organ systems. Men with BPH sometimes complain of symptoms such as urgency, urgency incontinence, or lower abdominal pain, and men with prostatitis frequently suffer from urogenital pain [7]; none of these symptoms is addressed by the IPSS questionnaire.

Recently, we have developed the Core Lower Urinary Tract Symptom Score (CLSS) questionnaire for the core or important symptoms in various pathological conditions [8]. The CLSS questionnaire addresses 10 important symptoms (daytime frequency, nocturia, urgency, urgency incontinence, stress incontinence, slow stream, straining, incomplete emptying, bladder pain, and urethral pain) selected from 25 symptoms defined by the International Continence Society standardization committee. Notably, the CLSS questionnaire, unlike the IPSS questionnaire, includes questions on incontinence and pain. Recently, we also demonstrated the clinical significance of the CLSS for the assessment of LUTS in men with various diseases [9]. In that study, pain

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and urgency incontinence had a large impact on the Quality Of Life Index. In the present study, we attempted to clarify the association between LUTS and ED using the IPSS, CLSS, and International Index of Erectile Function (IIEF-5) questionnaires in order.

Patients and methods

The study was approved by our institutional ethics committee. A total of 220 treatment-naive men who visited our hospital, a tertiary referral institution, for urological conditions between April 2010 and July 2011 were consecutively enrolled (Table I). The subjects, aged from 37 to 85 years (mean, 65.3 years), were divided into disease groups by routine urological examination: BPH ($n=58$), prostatic cancer ($n=51$), prostatitis ($n=28$), over active bladder (OAB) wet ($n=16$), and others (i.e. urolithiasis, bladder tumor) ($n=39$). Controls ($n=28$) were subjectively free of LUTS. Prostate biopsy was performed to exclude cancer when the prostate-specific antigen level was >4 ng/ml. In such cases, questionnaire data prior to the biopsy were used for analysis.

Questionnaire

The subjects were asked to respond to three self-administered questionnaires, the IPSS, CLSS, and IIEF-5 at the first medical examination. The IPSS questionnaire comprises 7 questions on LUTS (incomplete emptying, frequency, intermittency, urgency, weak stream, straining and nocturia). As previously mentioned, the CLSS questionnaire addresses 10 symptoms [8]. Voiding frequency was scored as follows: 0 (<7 times); 1 (8–9); 2 (10–14); 3 (15 or over) for the day time, and 0 (0 times); 1 (1); 2 (2–3); 3 (4 or over) for the night-time. Other symptoms were scored according to the frequency of episode (0–3 [8]). These symptoms were chosen from among 25 LUTS as the most influential as reported by 1000 symptomatic individuals [7]. The CLSS questionnaire further inquires about the single symptom (the single core symptom or chief complaint) that patients consider to have the most significant impact on daily life. Erectile function was evaluated accord to the IIEF-5 [10].

Statistical analysis

Correlation between the IIEF-5 score, the total or individual score of CLSS or IPSS, and age was assessed using the Spearman correlation coefficient. Wilcoxon signed-rank test was used to assess the relationship between IIEF-5 score and comorbid conditions (hypertension, hyperlipidemia, diabetes mellitus, angina pectoris, cerebrovascular disease). A multivariate regression model was used to identify symptoms predicting a low IIEF-5 score. We used JMP software (SAS Institute, Cary, NC), and regarded p values <0.05 as statistically significant.

Results

Demographic data for the subjects are shown in Table I. Their mean age was 65.3 ± 10.4 years. Mean CLSS, IPSS, and IIEF-5 score were 8.1 ± 5.1, 11.6 ± 8.5, and 8.5 ± 6.2, respectively. Of the patients in this study, 97% reported some degree of ED (IIEF-5 score < 22).

As shown in Figure 1, a differential pattern was observed in symptomatic men. For example, men with OAB wet experienced nocturia, urgency, and urgency incontinence more frequently than the other groups of patients, while men with BPH experienced straining and slow stream most frequently. About 40% of men with prostatitis complained of urethral and/or bladder pain, while among men with BPH, 25.9% complained of urethral pain and 19.0% complained of bladder pain to some extent.

As to comorbid conditions, 17 and 8 patients experienced angina pectoris and cerebrovascular disease, respectively; 74 had hypertension, 31 had hyperlipidemia, and 34 had diabetes mellitus. Among comorbid conditions and age, only diabetes mellitus and age showed significant correlation with total IIEF-5 scores ($p=0.011$ and <0.0001, respectively). Consistent with previous reports, the total IPSS score inversely

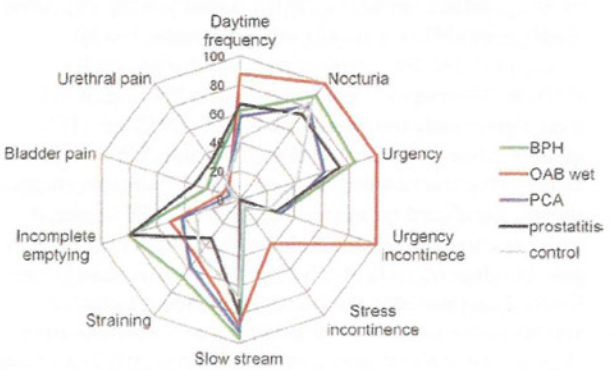

Figure 1. Positive rates of LUTS in men. Men with prostatitis or BPH sometimes complain of urgency incontinence, urethral and/or bladder pain, which are not addressed by the IPSS questionnaire.

Table I. Demographic data for the subjects.

	No.	mean age (year)	mean CLSS	mean IPSS	mean IIEF-5	mean PSA (ng/ml)	mean Prostate vol. (cm^3)
Total	220	65.3 (37–85)	8.1 (0–20)	11.6 (0–35)	8.5 (0–23)	7.3	35.0
Control	28	58.3	5.3	6.0	10.2	1.6	23.4
Overactive bladder (OAB wet)	16	67.8	12.7	17.1	5.0	1.2	30.1
Benign prostatic hyperplasia	58	67.9	9.3	15.0	9.0	7.1	43.5
Chronic prostatitis	28	64.7	9.5	13.3	7.2	8.5	43.6
Prostatic Cancer	51	67.4	6.5	8.3	9.0	13.5	29.8
Others	39	63.1	7.7	11.0	8.2	1.6	22.1

CLSS; Core lower urinary tract symptom score, IPSS; International prostate symptom score, IIEF; International index erectile function, PSA; Prostate-specific antigen, Prostate vol.; Prostate volume.