を伴うことがある．実際，症例2は下唇の麻痺という臨床症状での来院であった．

治療法に関して，エナメル上皮腫は再発傾向の高さから，顎骨の辺縁切除のみではなく，区域切除を選択される場合もあるが，今回は，摘出・開窓術を選択した．この場合は，再摘出をせねばならない場合もあるので，十分な経過観察が必要である．顎骨中心性扁平上皮癌では術前のPET-CT検査にて頚部リンパ節転移が疑われたので，左側頚部郭清術を行い，顎骨の腫瘍と一塊に切除し，チタンプレートにて顎骨を再建した．現時点では両症例とも，経過良好である．

結　　語

歯原性腫瘍の発見にはパノラマX線撮影は極めて有効であり，顎骨の透過像の解析により腫瘍の範囲の特定，良性または悪性の判断の参考になり得る．さらに顎骨の透過像を認めた際は，CTやMRIなどの追加の画像検査で精査する必要があり，臨床症状として神経症状が現れている時やパノラマX線所見で境界不明瞭な顎骨透過像を認める際は，悪性腫瘍の可能性を考慮し，早急な治療が必要となる．

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モーニングセミナーから

周術期院内紹介患者の歯性感染症治療

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-緒言-

総合病院における歯科口腔外科では，歯科診療における地域医療の中核病院としての機能のみならず，病院内においては入院患者の歯科治療や口腔ケアという支援機能を担っている．近畿大学医学部附属病院・歯科口腔外科では平成 18 年に院内口腔ケアチームが設立され，医科各診療科との連携をはかり，各科の診療を円滑に進めるべく，支援機能を充実させている．

歯性感染症とは，齲歯や歯周病が原因で細菌性の炎症が周囲の組織まで波及してしまう疾患である．悪性腫瘍患者の化学療法中や周術期においては，感染に対する抵抗力が減弱し，口腔内の慢性炎症性病変が急性化し，感染の増悪が原因で各診療科における治療の完遂に支障をきたしている例も少なくない．今回，各診療科から依頼を受け，歯性感染症の治療を行い，各診療科における治療が完遂できた症例を提示し，院内の医科歯科の連携について考察したい．

1．歯科口腔外科院内、院外紹介患者比率および紹介科、診療内容

近畿大学医学部附属病院・歯科口腔外科では，平成 22 年度(平成 22 年 4 月 1 日より平成 23 年 3 月 31 日)に 2616 人の新規患者があった(新規カルテ作成患者)．その内訳は院外よりの紹介 1734 人(66.3%)，院内よりの紹介 823 人(31.5%)，教職員 59 人(2.3%)であった．院内紹介患者は外科よりの紹介 189 人(23.0%)，呼吸器内科よりの紹介 164 人(20.0%)，耳鼻咽喉科よりの紹介 148 人(18.0%)，腫瘍内科よりの紹介 82 人(10.0%)，神経内科よりの紹介 51 人(6.2%)，血液内科よりの紹介 33 人(4.3%)，脳外科よりの紹介 23 人(2.8%)その他 132 人(16.0%)であった(図1A)．

院内紹介患者 823 人の診療内容を検討したところ，口腔ケア 419 人(51.0%)，抜歯 121 人(14.7%)，補綴処置 78 人(9.5%)，保存処置 71 人(8.6%)，歯性感染症 26 人(3.2%)，その他 108 人(13.1%)であった(図1B)．

院内紹介の大多数は口腔ケア依頼，補綴処置および保存処置といった一般歯科治療の依頼であった．歯性感染症の治療は 823 人中 26 人(3.2%)であったが，これらの患者さんはこの歯性感染症を有しているがために，原疾患の手術，移植治療，化学療法などが行うことがで

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きない状態での当科受診依頼であった．以下に歯性感染症が原因にて原疾患の治療に支障をきたし，当科にて紹介され，歯性感染症の治療を行い原疾患の治療を行った症例を4例提示する．

-症例 1-

患者：69歳，男性．

主訴：開口障害．

現病歴：数年前より両側下顎智歯部の歯肉腫脹・疼痛を繰り返すも放置していた．2日前より症状が再燃し，歯肉腫脹・疼痛および開口障害を認めた．3日後，脳動脈瘤手術時に挿管困難が予想されるため，脳神経外科より紹介された．

既往歴：高血圧症，前交通動脈瘤．

現症：

全身所見：体格中等度．

局所所見：両側下顎智歯（半埋伏歯）部の歯肉腫脹を認め，圧痛が著明であった．開口量は1横指半であった．

パノラマX線写真：両側下顎智歯（図2A，黄色矢印）

血液検査所見：CRPは1.3mg/dL，白血球数は9900/μL，分画は好中球58.1%（図2A）

診断：智歯周囲炎．

治療・経過：抗生剤投与，歯周ポケット洗浄を行った．炎症の軽減とともに，1横指半であった開口域が，手術前日には3横指と大幅に改善した．開口障害を改善することにより，挿管可能となり，脳神経外科での手術による治療が可能となった．

-症例 2-

患者：68歳，男性．

主訴：感染性心内膜炎にて入院中で，口腔内感染源の除去依頼を受けた．

現病歴：感染性心内膜炎にて循環器内科へ入院し，抗生剤投与にて消炎するも改善認めず，口腔内感染源スクリーニング目的にて当科紹介された．

既往歴：慢性心房細動，僧房弁閉鎖不全症．

現症：

全身所見：体格中等度．

局所所見：口腔内は右側下顎第一・第二大臼歯は歯周炎著明であり歯は動揺しており，歯周ポケットからの排膿認めた．

パノラマX線写真：右側大臼歯部にX線透過像を認めた（図3A，黄色矢印）．

血液検査所見：CRPは2.02mg/dL，白血球数は10000/μL，分画は好中球72%，血液培養にて，Staphyrococcus Aureusを検出した．

診断：全顎の慢性辺縁性歯周炎，右側下顎第一・第二大臼歯の急性歯槽膿瘍．

処置:抗生剤投与にて消炎し,右側下顎第一・第二大臼歯は抜歯し,消炎継続.歯周治療と保存不可能な歯の抜歯により,感染性心内膜炎の症状は経過し,退院となる(図 3B).

-症例 3-

患者:73 歳,男性.

主訴:左側頬部の腫脹.

現病歴:現在大腸癌再発につき,腫瘍内科にて化学療法中.3日程前から左側頬部腫脹認め,抗生剤処方するも改善認めず,精査加療目的にて当科初診.

現症:

口腔内所見:左側下顎犬歯部歯肉腫脹著明,発赤あり.波動を触知.

パノラマ X 線所見:左側下顎犬歯の根尖部に X 線透過像認めた(図 4A).

血液検査所見:CRP は 8.02mg/dL,白血球数は 9600/μL,好中球比率は 77%.

既往歴　大腸癌・腹膜播種・肝転移.

診断:左側下顎犬歯歯根嚢胞.

処置:抗生剤を投与し,切開排膿術を施行.消炎後に左側下顎犬歯抜歯および嚢胞摘出術施行した.消炎後,化学療法再開した(図 4B).

-症例 4-

患者:73 歳,女性.

主訴:下顎全体の疼痛および腫脹.

現病歴:20 年前に乳癌加療後,再発骨転移にて,3 年前よりゾレドロン酸投与.1ヵ月前より下顎骨疼痛および腫脹認め,当科初診.

現症:下顎骨が全顎的に腐骨化しており、排膿は著明。血液検査所見:CRP は 1.8mg/dL,白血球数は 13000/μL,好中球比率は 90%.

既往歴:乳癌・再発多発骨転移.

診断:ビスフォスフォネート系薬剤関連顎骨壊死.

処置:洗浄・抗生剤投与.平成 23 年 8 月 25 日,全身麻酔下にて下顎骨腐骨除去・掻爬術施行.現在化学療法再開し,経過良好である(図 5A,B,C).

-考察-

口腔ケアにより要介護者の肺炎の罹患を有意に減少させることができたとの Yoneyama ら報告以来[1],主として慢性期病院に行われていた口腔ケアが急性期医療にも応用されるようになってきた[2].近畿大学医学部附属病院においても,平成 18 年 5 月より歯科口腔外科のスタッフが各病棟を回診し,口腔ケアを担当している.現在までに,食道がん周術期における術後肺炎に対し口腔ケアの有効性を報告し[3],病棟での口腔ケアを行う対象疾患を少しづつ拡げ

つつある[4]. 平成 22 年度集計では, 歯科口腔外科総初診患者数の 31.5%が院内入院患者でその 51.0%が口腔ケア依頼となっており, 病院内で口腔ケアが浸透しつつあると考えられる.

今回は, 院内紹介の中で頻度は低いものの(院内入院患者数 823 人中 26 人で比率は 3.2%であった.), 歯性が原因の感染症が原疾患の手術, 移植治療, 化学療法などが行うことができない状態での当科受診依頼であった症例を検討してみた.

歯性感染症とは齲歯や歯周病などが原因で細菌性の炎症が周囲の組織まで波及してしまう疾患であり, 単に齲歯や歯周病を放置し, 感染を起こす場合もあるが, 感染に対する抵抗力が弱いときに慢性感染巣が増悪する場合もある. 感染に対する抵抗力が弱まる原因として, 周術期(手術, 化学療法、放射線治療), 白血病などの血液疾患, 糖尿病などの代謝疾患などのいわゆる宿主易感染状態が挙げられる. すなわち, 健康時に十分な歯科治療を行わずに放置し, 糖尿病などの全身的代謝疾患に罹患した場合や悪性腫瘍に罹患し, 易感染状態となり, 放置していた病巣が増悪したと考えられる. 症例1, 症例2, 症例3はこれに当てはまると考えられる. このようなケースは医科治療前に齲歯や歯周病の精査や歯科治療で確実に予防が可能であると思われる. 好中球減少が見込まれる医科治療では, 治療開始の 2 週間前までに歯科治療を済ませておくことを, NCI のガイドラインでは推奨している[5]. 本院においても, 歯性感染症が惹起してからの治療ではなく, 医科治療開始前の口腔ケアで対応できるよう, 医科へのアナウンスを徹底したいと考えている.

症例4に関しては, 最近トピックスになっているビスフォスフォネート製剤 (以下 BP s) 使用症例である. BP s は病的骨折や脊髄圧迫などの骨関連事象 (skeletal-related events, 以下 SRE) の予防や治療と悪性腫瘍の骨転移に有効な薬剤である. しかしながら, BP s 投与患者に発症するビスフォスフォネート系薬剤関連顎骨壊死 (以下 BRONJ) という重篤な副作用の報告が頻出している[6, 7, 8]. 現時点では悪性腫瘍に対する注射用 BP s 症例に関しては, 原則投薬を継続し, 歯科口腔外科が局所の症状に対応するしか方法がなさそうである. そうであっても, 投与前に十分な齲歯や歯周病の精査や歯科治療が重要であることに変わりない.

-結語-

単なる齲歯や歯周病であっても, 宿主が易感染性であれば, 感染症は増悪する. 年齢を重ねるとともに歯周病や齲歯の本数は増え, 高齢になるほど何らかの歯科疾患を有している場合が多く, 特に全身状態の悪化が予測される場合, 医科治療中に悪影響を及ぼす可能性が高いことから, 医科と歯科が連携して, なるべく早い時期に口腔内の精査・加療を受けることが必要と考えられる. 当院では, 平成 18 年の院内口腔ケアチームが設立以来, 医科歯科の連携は密接になりつつある. これからも積極的に口腔ケア介入していき, 周術期患者のトラブルが少なくなっていくよう, 努力していきたいと考えている.

図表の説明

図1A:院内紹介患者の紹介元診療科　B:院内紹介患者の歯科治療内容

図2A:症例1のパノラマX線写真，両側智歯周辺にX線透過像を認めた(黄矢印)　B:消炎後3横指の開口域が確保された.

図3A:症例2のパノラマX線写真，全顎に歯槽骨の骨レベルの低下および右側第一，第2大臼歯周辺のX線透過像を認めた(黄矢印)　B:　当科にての消炎処置とCRP値の変化

図4:症例3のパノラマX線写真，左側下顎犬歯の根尖部に嚢胞を思わせるX線透過像を認めた(黄矢印)　B:　当科にての消炎処置とCRP値の変化.

図5A:症例4の初診時口腔内写真，下顎前歯部の骨が露出　B:Aののち下顎前歯部の歯が脱落し，骨の露出部が拡大した. C:腐骨掻爬後の口腔内写真，壊死を起こした骨を掻爬し，皮質骨のみを残した.

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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-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.

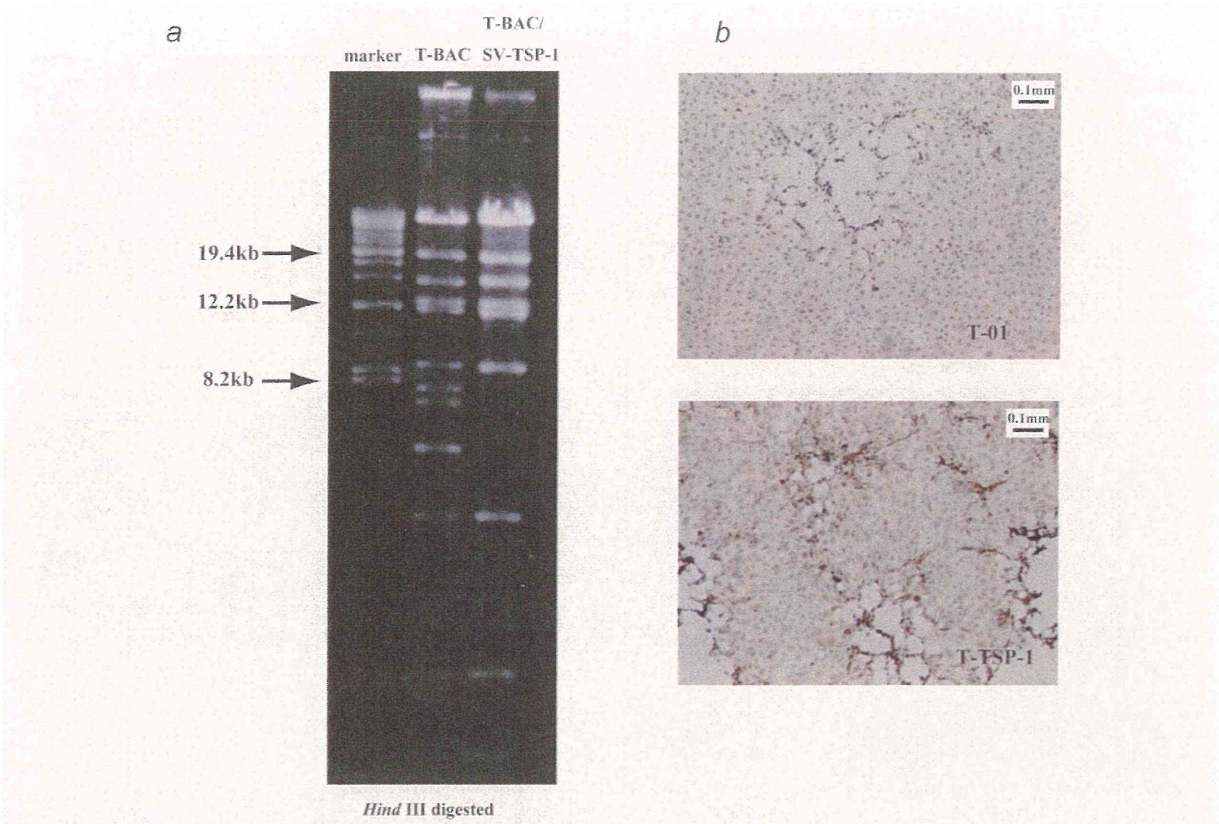

Figure 1. Verification of T-TSP-1 and TSP-1 expression in oncolytic HSV-1-infected Vero cells. (*a*) BAC plasmids were digested with *Hind* III. The digested BAC plasmids were electrophoresed, T-BAC (left) and Cre-recombinant BAC plasmid, T-BAC/SV-TSP-1 (right). (*b*) Vero cells infected with T-01 (MOI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

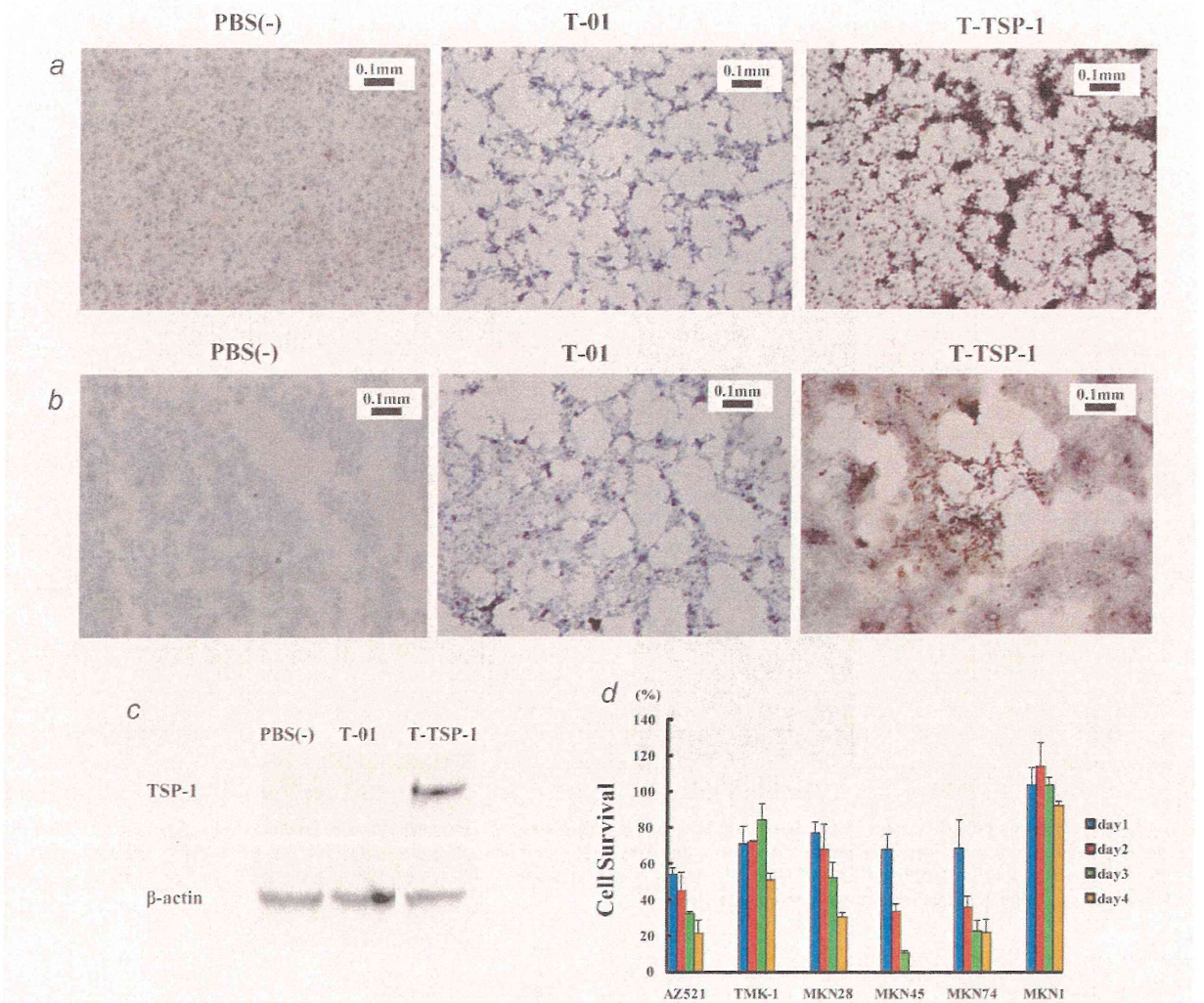

Figure 2. Immunocytochemical detection of TSP-1 and the cytotoxicity of T-01 in gastric cancer cell lines *in vitro*. Gastric cancer cells were infected with PBS(−), T-01 or T-TSP-1 and immunostained for human TSP-1 48 hr after infection. (*a*) TMK-1 cells after infection with PBS(−)(left), T-01 (middle)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

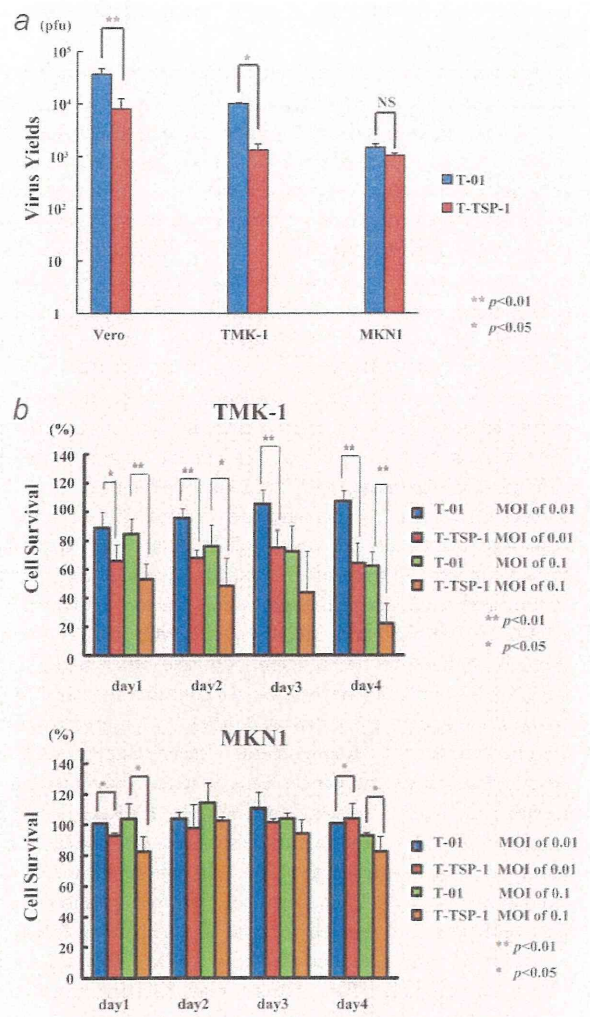

Figure 3. *In vitro* viral replication and cytotoxicity of HSVs against gastric cancer cell lines. (*a*) The *in vitro* virus yield was determined 48 hr after infection of Vero cells (1×10^5 per well) with T-01 or T-TSP-1 at an MOI of 0.01, and TMK-1 and MKN1 cells (1×10^5 per well) at an MOI of 0.1.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

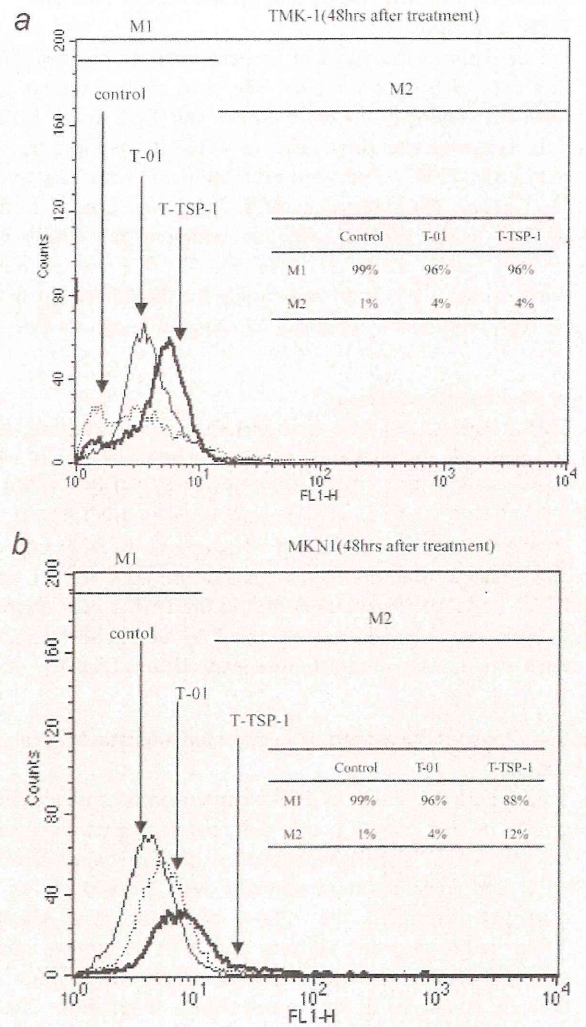

Figure 4. *In vitro* apoptosis assay of gastric cancer cells infected with oncolytic HSVs. (*a*) We performed an *in vitro* TUNEL assay using TMK-1 gastric cancer cells 48 hr after infection with PBS(−) (control), T-01 (MOI of 0.1) or T-TSP-1 (MOI of 0.1). (*b*) The results of the *in vitro* TUNEL assay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

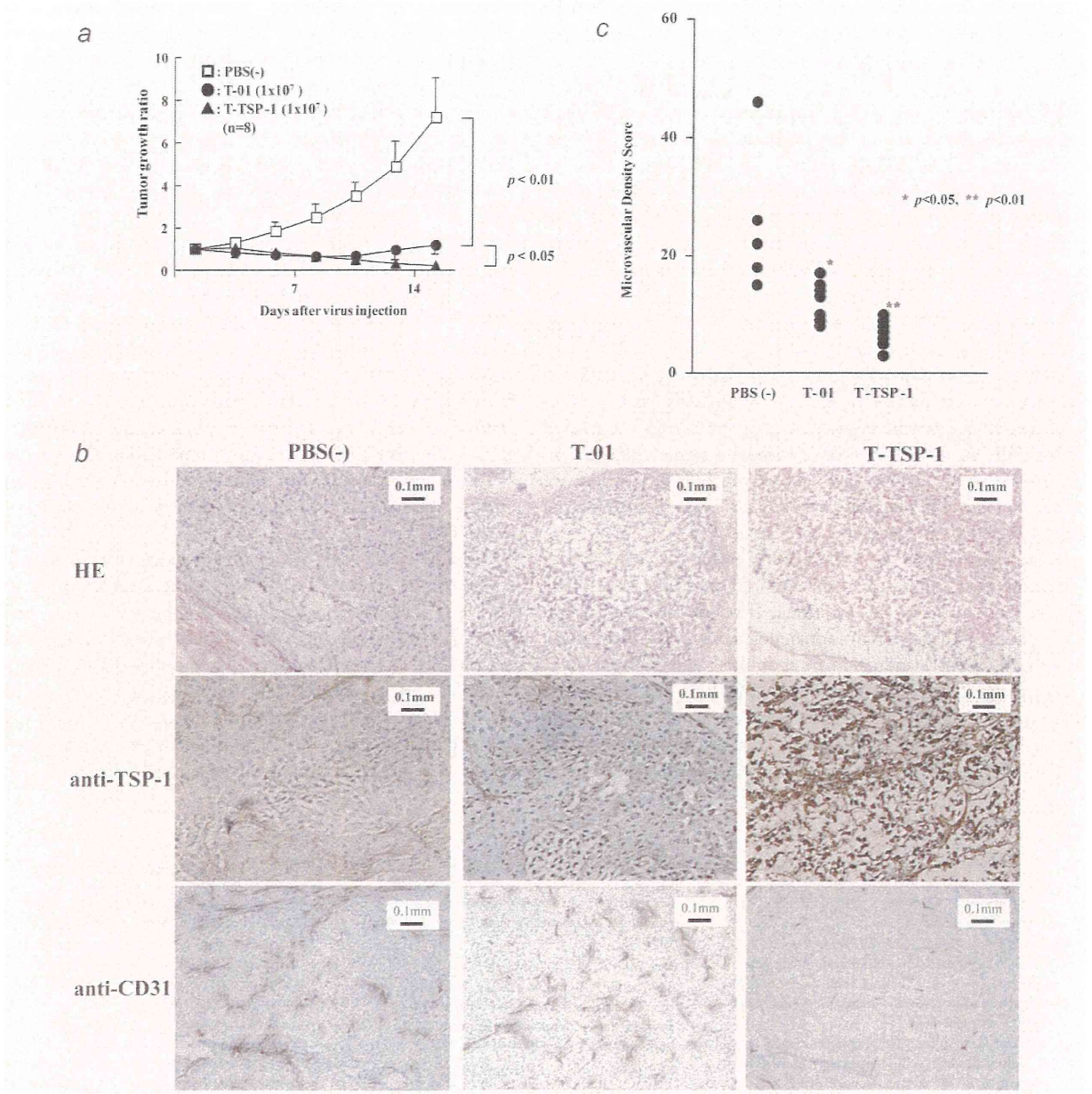

Figure 5. The efficacy of armed oncolytic HSV-1 vectors *in vivo*. (*a*) The antitumor effects of TSP-1-expressing oncolytic HSV-1s (T-TSP-1) and oncolytic HSV-1 not expressing any transgenes (T-01) was examined in BALB/c nu/nu mice bearing subcutaneous TMK-1 tumors. BALB/c nu/nu mice bearing subcutaneous 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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Surgical strategies for unresectable hepatoblastomas☆

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Abstract

Background: The aim of this study was to assess the surgical strategies for unresectable hepatoblastomas at the initial diagnosis based on the experience of two institutions.

Methods: The PRETEXT (Pretreatment evaluation of tumor extent) and POST-TEXT (Post treatment extent of disease) staging, surgical treatments, and clinical outcomes were retrospectively analyzed for 12 cases with PRETEXT III or IV and M(−) of 29 hepatoblastomas treated based on the JPLT-2 (The Japanese Study Group for Pediatric Liver Tumor-2) protocol at two institutions between 1998 and 2011.

Results: Two of the 9 cases with PRETEXT III status were downstaged to POST-TEXT II. One of the 3 cases with PRETEXT IV showed downstaging to POST-TEXT III. Four of the 7 cases with P2 or V3 (indicated for liver transplantation) in the PRETEXT staging system showed P2 or V3 in POST-TEXT staging after 2 cycles of CITA (JPLT-2 standard regimen), and one case showed P2 or V3 in POST-TEXT staging at the initial operation and underwent primary liver transplantation. The initial surgical treatments were 1 lobectomy, 2 segmentectomies, 6 trisegmentectomies, 2 mesohepatectomies, and 1 primary liver transplantation. Both patients who underwent mesohepatectomies had bile leakage, and 1 of 5 trisegmentectomies had an acute obstruction of the right hepatic vein. Two patients underwent rescue living donor liver transplantation. Both of these patients showed P2 or V3 positive findings in POST-TEXT staging after 2 cycles of CITA.

Conclusions: POST-TEXT staging and P and V factors should be evaluated after 2 cycles of CITA for unresectable hepatoblastomas detected at the initial diagnosis. The patients should be referred to the transplantation center if the POST-TEXT IV, P2, or V3 is positive at that time. Liver resection by

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trisegmentectomy is recommended in view of the incidence of surgical complications. Careful treatment, such as back-up transplantation, should thus be considered for liver resection in the cases with POST-TEXT IV, P2, or V3 status after initial 2 cycles of CITA.

Hepatoblastoma is the most common childhood liver tumor. Multicenter trials have made it clear that pre- and/or postoperative chemotherapy is necessary to improve the outcome of the treatment of hepatoblastoma [1,2]. The Japanese Study Group for Pediatric Liver Tumor (JPLT) established JPLT-1, the first nationwide protocol for liver tumors in childhood, to improve the outcome in children with liver tumors in Japan [3].

Furthermore, a refined version of the protocol, designated JPLT-2, was opened to participating institutions in 1998. The PRETEXT (Pretreatment evaluation of tumor extent) staging system was adopted from SIOPEL (International Society of Pediatric Oncology Liver Tumor Study Group) as an internationally approved staging system for tumor localization. All patients other than non-metastatic PRETEXT I patients are subjected to preoperative chemotherapy. A second-line regimen including ifosfamide is applied for tumors showing chemoresistance. A protocol including high-dose chemotherapy with autologous stem cell transplantation is used for patients with metastatic disease. The JPLT2 protocol resulted in an improvement in the 5-year overall survival rate (OS) in non-metastatic cases to 100% for PRETEXT I, 87.1% for PRETEXT II, 89.7% for PRETEXT III, and 71.2%, for PRETEXT IV. The 5-year OS in metastatic cases was 43.9%. The outcome in unresectable and metastatic tumors remained unsatisfactory [4].

Liver transplantation is the only option for those children with disease contained to the liver that is deemed unresectable after a predetermined course of chemotherapy. Long-term survival of 80% to 90% has been reported in children with unresectable hepatoblastoma who undergo primary transplantation [5,6]. The excellent results of primary liver transplantation for unresectable hepatoblastoma, in contrast to the poor outcomes reported in children requiring rescue transplant for recurrence after a primary resection, have led some surgeons to advocate for the expanded use of primary transplantation [7].

There are questions associated with the surgical strategies for PRETEXT III or IV hepatoblastomas such as the following: Extensive liver resection or liver transplantation? Which patients truly need total hepatectomy with liver transplantation? When should this be decided? The indications for liver transplantation of hepatoblastomas depend on POST-TEXT (Post treatment extent of disease). The consensus of indication is POST-TEXT IV or P2 (Involvement of the main portal vein) or V3 (Involvement of all three hepatic veins and/or the IVC) in POSTTEXT [8]. P2 means the involvement of the main portal vein. V3 means the involvement of all three hepatic veins and/or the IVC [9].

The aim of this study was to assess the surgical strategies for unresectable hepatoblastomas at the initial diagnosis. The PRETEXT and POST-TEXT staging, surgical treatments, and clinical outcomes were retrospectively analyzed for PRETEXT III or IV and M (−) hepatoblastomas treated based on the JPLT2 protocol at two institutions between 1998 and 2011.

1. Patients and methods

All 29 patients treated for hepatoblastoma at the two institutions (Kyushu University Hospital and Kyoto Prefectural University of Medicine Hospital) from 1998 through 2011 were retrospectively reviewed. This study was performed according to the Ethical Guidelines for Clinical Research published by the Ministry of Health, Labor, and Welfare of Japan on July 30, 2003 (revised 2008) and complies with the Helsinki Declaration of 1964 (revised 2008).

The tumors were evaluated using state-of-the-art imaging, including computed tomography; clinical staging was based on PRETEXT (pretreatment extent of tumor) as applied in SIOPEL [9,10]. M (metastatic), E (extrahepatic invasion), P (main portal invasion), V (invasion of all three hepatic veins or the vena cava), and R (rupture at diagnosis) were added as annotations to PRETEXT staging.

All 29 patients with hepatoblastomas were treated based on the JPLT2 protocol in principle [4]. Briefly, PRETEXT I tumors were primarily resected, and PRETEXT II–IV cases were treated with preoperative chemotherapy. At least two courses of a combination of 80 mg/m^2 cisplatin on day 1, followed by 30 mg/m^2 pirarubicin on days 2 and 3, which was designated CITA, were repeated preoperatively. CITA was allowed to be substituted with transarterial chemoembolization using 30 mg/m^2 pirarubicin and 200 mg/m^2 carboplatin (CATA-L) at the discretion of the physician. CITA was repeated until surgical resection became feasible. A combination of 3 g/m^2 ifosfamide on days 1 and 2, 400 mg/m^2 carboplatin on day 3, 30 mg/m^2 pirarubicin on days 4 and 5, and 100 mg/m^2 etoposide on days 1–5 (ITEC) was given until the tumor became resectable if CITA (CATA-L) failed to induce PR (as defined below). Postoperative chemotherapy was given to all cases. PRETEXT I and II tumors were treated with four courses of half-dose CITA (low-CITA). PRETEXT III, IV, and metastatic cases were treated with two courses of CITA. Patients who required salvage with ITEC preoperatively were treated with two courses of ITEC.

The PRETEXT and POST-TEXT staging, surgical treatments, and clinical outcomes were retrospectively analyzed for 12 cases with PRETEXT III or IV and M (−) of 29 hepatoblastomas.

2. Results

Table 1 shows a summary of the 12 cases with PRETEXT III or IV and M (−) hepatoblastomas treated based on the JPLT-2 regimen. Ten cases were PRETEXT III, and 2 cases were PRETEXT IV. Three cases underwent liver transplantation. One case had a primary liver transplantation due to POST-TEXT IV at surgery, while the other 2 cases underwent rescue liver transplantations. Two of the 10 cases with PRETEXT III showed a downstaging to POST-TEXT II at the initial operation. Five cases underwent trisegmentectomy, and 1 case underwent mesohepatectomy. One of 2 cases with PRETEXT IV showed downstaging to POST-TEXT III at the initial operation. One case with POST-TEXT III underwent mesohepatectomy, and another case with POST-TEXT IV underwent primary liver transplantation. A summary of the 12 cases with PRETEXT III or IV showed that 3 cases (25%) showed downstaging in POST-TEXT at the time of the operation. The initial surgical treatments were 1 lobectomy, 2 segmentectomies, 6 trisegmentectomies, 2 mesohepatectomies, and 1 primary liver transplantation.

The P and V factors in PRETEXT staging were retrospectively evaluated, POST-TEXT staging after 2 cycles of CITA, and POST-TEXT staging at surgery, respectively. Two of 5 cases with P2 or V3 in PRETEXT III showed P2 or V3 in POST-TEXT staging after 2 cycles of CITA, and no cases showed P2 or V3 in POST-TEXT staging at initial operation. Both two cases with P2 or V3 in PRETEXT IV showed also P2 or V3 in POST-TEXT staging after 2 cycles of CITA, and one case showed neither P2 nor V3, and another case showed P2 or V3 in POST-TEXT staging at the initial operation, and then underwent primary liver transplantation.

Two of the cases in the current series underwent rescue liver transplantation. One patient (Case No. 3) who underwent rescue liver transplantation was a 3 month old female with PRETEXT IV, and P2 and V0. The POST-TEXT staging after 2 cycles of CITA was III, and P2 and V0. POST-TEXT staging at the initial operation was III, and P1 and V0 after an additional 5 cycles of CITA. The patient underwent mesohepatectomy. The medical insurance would not cover transplantation for hepatoblastoma in Japan at that time. Multiple recurrence occurred in the posterior area 2 months after mesohepatectomy, and then a right lobectomy was performed. Multiple recurrence of the residual liver and liver dysfunction occurred 5 months after right lobectomy, and then living donor related liver transplantation was performed. However, the patient died due to graft failure 4 days after the operation. The other patient who underwent rescue liver transplantation was a 10 month-old male with a

Table 1 Summary of characteristics for 12 hepatoblastomas with PRETEXT III or IV and M (−).

	Patient age at diagnosis	PRETEXT	P	V	POSTTEXT after 2 cycles of CITA	P	V	neoadjuvant chemotherapy	POSTTEXT at surgery	P	V	operation	adjuvant chemotherapy	outcome
1	1 Y	III	0	1	II	0	0	CITA 4 cycles	II	0	0	right lobectomy	ITEC 3 cycles	Alive, NED
2	11 M	III	0	0	III	0	0	CITA 3 cycles	III	0	0	segmentectomy (S4+S5)	CITA 2 cycles	Alive, NED
3	3 M	IV	2	0	III	2	0	CITA 7 cycles	III	1	0	mesohepatectomy → right lobectomy → transplantation	ITEC → CPT11 4 cycles	transplantation related death
4	10 M	III	2	2	III	1	2	CITA 4 cycles	III	1	2	trisegmentectomy (left)	CITA 2 cycles	Alive, NED
5	1 Y	III	2	0	III	2	0	CITA 4 cycles	III	1	0	trisegmentectomy (left)	CITA 2 cycles	Alive, NED
6	7 Y	III	2	2	III	0	0	CITA 5 cycles	III	0	0	mesohepatectomy	CITA 2 cycles	Alive, NED
7	3 Y	III	2	2	III	1	1	CITA 4 cycles	III	1	1	trisegmentectomy (right)	CITA 2 cycles	Alive, NED
8	10 M	III	2	3	III	1	3	CITA 4 cycles	III	1	2	trisegmentectomy (left) → transplantation	(−)	Alive, NED
9	1 Y	IV	1	3	IV	1	3	CITA 4 cycles + ITEC 2 cycles	IV	1	3	transplantation	CPT-11 2 cycles	Alive, NED
10	1 Y	III	1	2	III	1	2	CITA 5 cycles	II	1	2	trisegmentectomy (right)	CITA 1 cycle	Alive, NED
11	1Y	III	0	0	III	0	0	CITA (4)	III	0	0	S2+3 segmentectomy + partial resection	CITA 4 cycles	Alive, NED
12	9 M	III	0	0	III	0	0	CITA (3)	III	0	0	trisegmentectomy (left)	CITA 2 cycles	Alive, NED