厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

光感受性ROS産生蛍光タンパク質を発現する遺伝子改変アデノウイルス製剤を用いた新たな癌の光線力学療法システムの開発

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研究代表者　藤原　俊義

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【研究要旨】

テロメライシンは岡山大学で開発された国産の抗癌アデノウイルス製剤であり、テロメラーゼ構成成分であるhTERT遺伝子のプロモーターにより、癌細胞のみで選択的に増殖して腫瘍融解を引き起す。本研究では、テロメライシンに光感受性蛍光タンパク質をコードする遺伝子を搭載した改変アデノウイルス製剤を開発する。腫瘍選択的に光感受性蛍光タンパク質を発現させ、治療用デバイスにて励起光照射することで癌細胞のみで活性酸素（reactive oxygen species：ROS）を産生して細胞死を誘導する新たな癌の光線力学療法を確立する。

本研究の最終到達目標は、テロメラーゼ依存性増殖機能を持つアデノウイルス製剤テロメライシンをベクターとして癌細胞選択的に光感受性蛍光タンパク質*KillerRed*遺伝子を発現する次世代型武装化アデノウイルス製剤を作成し、新たな癌の光線力学療法としての臨床応用を行うことである。

本年度は、テロメライシンのゲノム改変を行ってE3領域にサイトメガロウイルス・プロモーターで駆動される*KillerRed*遺伝子を搭載し、またKilerRedタンパク質の励起光照射のための治療用デバイスを試作した。さらに、*KillerRed*遺伝子を恒常的に発現するヒト肺癌細胞株を樹立し、励起光照射による細胞死の分子機構について解析した。

A．研究目的

テロメライシン（Telomelysin、開発コード：OBP-301）は岡山大学で開発された国産の抗癌アデノウイルス製剤であり、癌細胞で選択的に増殖・複製する機能を有しており、自立的に癌細胞を破壊することで増殖したウイルスが拡散していく。したがって、テロメライシン自体が新しい作用機構に基づく新規の画期的な医薬品となるが、ゲノム構造が明らかなアデノウイルスを基本骨格としているため、テロメライシンはベクターとして応用可能であり、導入遺伝子の癌選択的かつ長期的発現が期待できる。

最近、GFPをはじめとする蛍光タンパク質は分子イメージングのマーカーとして注目されているが、マーカー機能では細胞毒性が低いものが選択される。研究代表者らも、GFP遺伝子を搭載したテロメスキャン（TelomeScan、OBP-401）を診断用医薬品として開発してきたが、GFP自体の細胞毒性は極めて低い（Kishimoto *et al.*, Nature Med., 12, 1213-1219, 2006）。その発想を転換し、本研究では診断と治療を融合した新しい研究分野「Theranostics」の医薬品として、光誘導細胞死を生じる新規蛍光赤色タンパク質*KillerRed*遺伝子を用いた光感受性武装化アデノウイルス製剤を開発する。標的癌細胞でウイルス増殖に伴う強い蛍光タンパク質発現により癌細胞の可視化を可能とし、また同時に励起光照射で産生されるROSによる強力な細胞傷害活性が発揮される。すなわち、遺伝子改変ウイルス製剤を基盤とする本技術は、分子イメージングと分子手術による全く新しい独創的な治療戦略と言える。

本研究では、テロメラーゼ依存性増殖機能を持つアデノウイルス製剤テロメライシンをベクターとして癌細胞選択的に光感受性蛍光タンパク質遺伝子を発現する次世代型武装化アデノウイルス製剤を作成し、新たな癌の光線力学療法としてその機能解析、小動物・大動物を用いた有効性･安全性の検討を終了することを目指す。

B．研究方法

1）*KillerRed*遺伝子発現テロメライシンの作成

KillerRedは*Anthomedusae*クラゲの発色タンパク質であるanm2CPを改変して開発された新しい赤色蛍光タンパク質であり、540～580nmの緑色光照射によって活性酸素（reactive oxygen species：ROS）を産生して細胞死を誘導することができる。テロメライシンはhTERTプロモーターでアデノウイルスの増殖に必要なE1A、E1B遺伝子を駆動する増殖カセットを搭載しており、今回、サイトメガロウイルス・プロモーターの下流に*KillerRed*遺伝子を組み込み、テロメライシン・ゲノムのE3領域に搭載した。*KillerRed*遺伝子発現テロメライシンは、癌細胞で選択的に増殖し、強力な*KillerRed*遺伝子発現を来

すものと期待される。

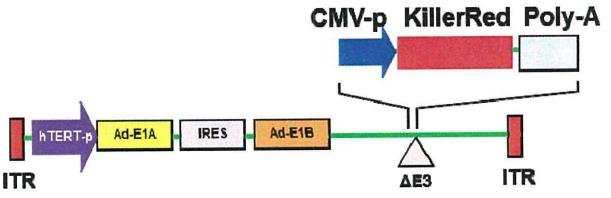

図1　*KillerRed*遺伝子発現テロメライシンの構造

2）KillerRed用励起光照射デバイスの試作

オリンパスと共同で、KillerRedタンパク質に蛍光発現させるための励起光照射用ビデオスコープを試作した。

3）*KillerRed*遺伝子発現ヒト癌細胞株の作成

励起光照射による*KillerRed*遺伝子発現細胞の殺細胞効果の分子機構の解析のために、ヒト非小細胞肺癌細胞株H1299に*KillerRed*遺伝子発現プラスミドをリポフェクション法で導入し、G418（ネオマイシンアナログ）投与で細胞選択を行った後に限界希釈法でstable cloneを樹立した。

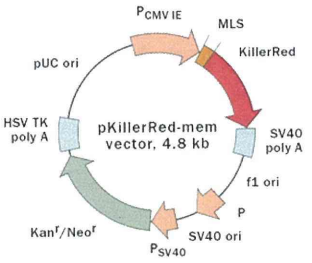

図2
*KillerRed*遺伝子発現プラスミドの構造

4）*KillerRed*遺伝子発現ヒト肺癌細胞のタイムラプス観察と分子機構の解析

*KillerRed*遺伝子発現H1299細胞に励起光照射し、蛍光顕微鏡下でタイムラプス観察を行うことで細胞死誘導を観察した。また、その分子機構を解析するために、活性化カスパーゼ3の免疫染色を行い、さらに活性酸素反応性蛍光試薬（DCF-DA）を用いた細胞内活性酸素（ROS）レベルの測定を行った。

（倫理面への配慮）

制限増殖型ウイルス製剤を用いる本研究は「大臣確認実験」となるため、「第二種使用等拡散防止措置確認申請書」を作成、学内の担当部署での検討の後に文部科学省に申請し、研究計画実施の承認を得ている。

C．研究結果

1）*KillerRed*遺伝子発現テロメライシンの作成

E3領域へ外来遺伝子を組み込むためのアデノウイルスDNA pAdHM20にテロメライシンの増殖カセットphTERTp-E1A-IRES-E1Bと*KillerRed*遺伝子発現カセットCMVp-KillerRed-polyAを挿入し、相同組み換えが生じないようにH1299ヒト肺癌細胞株を宿主細胞としてウイルス作成を行った。

2）KillerRed用励起光照射デバイスの試作

光源、スコープ、カメラアダプタ、カメラ、プロセッサ、モニターから成る鏡視下手術用ビデオスコープシステムである。励起波長570～590nmの励起光を発し、*KillerRed*遺伝子発現細胞にて蛍光波長600～655nmの近赤外蛍光を誘導することができる。

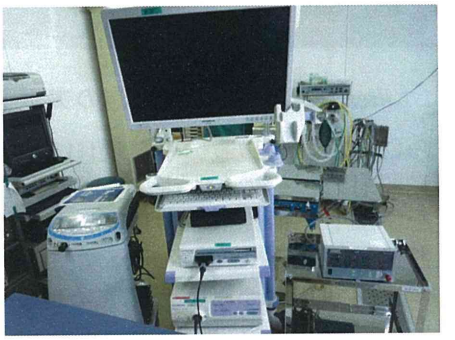

図3　KillerRedタンパク質励起用ビデオスコープ

3）*KillerRed*遺伝子発現ヒト肺癌細胞のタイムラプス観察と分子機構の解析

*KillerRed*遺伝子発現H1299ヒト肺癌細胞に蛍光顕微鏡下で励起光を照射しタイムラプス観察を行ったところ、5分以内に急速なPhotobleachingが生じて赤色蛍光は退色した。しかし、照射領域では1時間以内に強力な細胞障害活性が認められた。

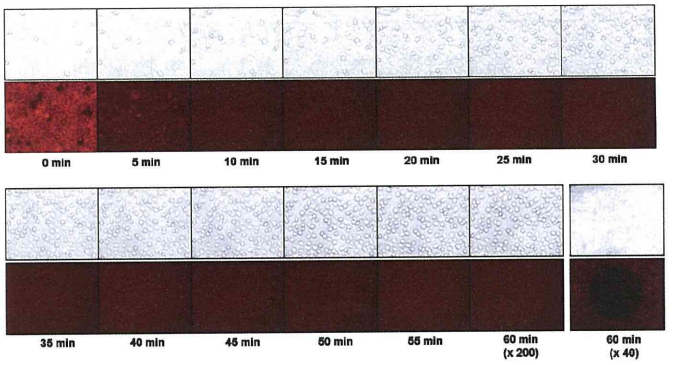

図4　励起光照射による細胞障害活性

また、照射部位の境界領域の細胞では活性化カスパーゼ3の免疫蛍光染色が陽性となり、励起光照射された*KillerRed*遺伝子発現細胞ではアポトーシスが誘導されていると考えられた。

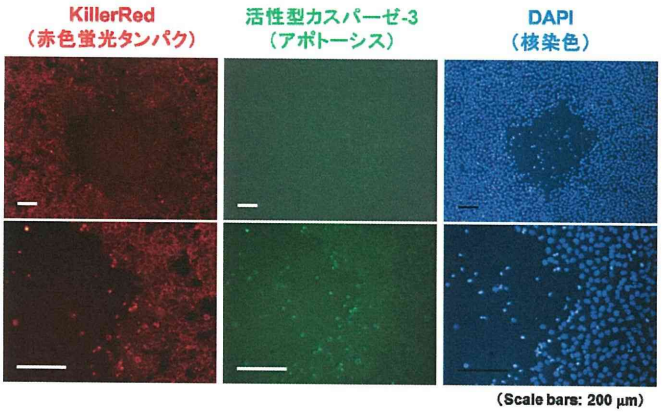

図5　*KillerRed*遺伝子発現細胞のアポトーシス誘導

さらに、*KillerRed*遺伝子発現H1299細胞に15分間励起光照射し、その1.5時間後に活性酸素反応蛍光試薬を添加して活性酸素（ROS）発現を調べたところ、無処置のH1299細胞やROS産生しない近赤外蛍光タンパク質Katushkaを発現するH1299に比較して、有意に高いROS産生が確認された。

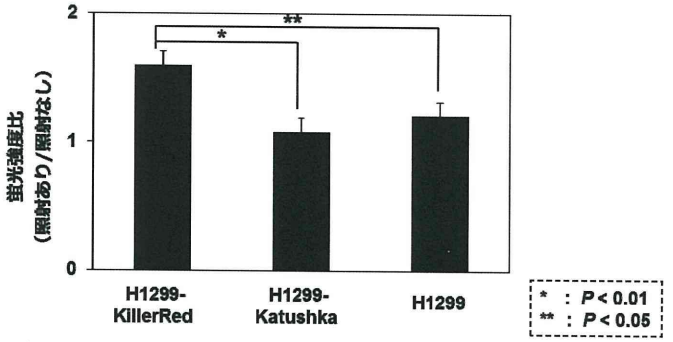

図6　励起光照射による活性酸素（ROS）産生

D．考察

光感受性物質と励起光照射を組み合わせて行う光線力学療法は、侵襲が少ない癌治療として一部の早期癌で臨床応用が試みられている。しかし、光感受性物質の標的とする生体内分子に対する選択性が小さいことや、光感受性物質の投与のために光線過敏症を引き起こすなどの問題点を残していた。本研究では、癌への選択性が強いテロメライシンをベクターとして*KillerRed*遺伝子を発現するため、癌集積性は極めて高い。生物製剤、特にウイルス製剤と光照射を組み合わせた光線力学療法は、未だ世界で報告がなく、臨床応用に向けた試みももちろん存在しない。一方、蛍光タンパク質自体の発見や開発は最近のトピックスであり、これらを結びつけることで現実的な治療開発につながる可能性が強い。

テロメライシンはテロメラーゼ活性を標的とする癌治療を目的とした生物製剤であり、生体内で自律性を持って増殖することによる従来の抗癌剤や分子標的薬剤にない抗腫瘍効果の増強が期待できる。本研究では、米国の臨床試験で安全性が確認されたこのテロメライシンをベクターとして全く新たな光線力学療法への応用を目指しており、その安全性と効果に関する理論的根拠が得られれば、創薬シーズとしては極めて魅力的なものになると思われる。中国では、すでに2種類の局所療法剤としてのウイルス製剤（Gendicine、H101）が中国FDAの承認を受けており、一般市場にて日常臨床に使用されている。しかし、遺伝子治療やペプチド医薬などに関しては中国以外の海外でも未だ実績がない分野だけに、新技術が製品の形で現実のものになれば、社会的インパクトは極めて大きく、抗癌剤市場はさらに高い伸びを示すと予測されている。

今後は、*KillerRed*遺伝子発現テロメライシンを大量製造・精製し、Good Laboratory Practice（GLP）レベルの光感受性武装化アデノウイルス製剤を調整し、マウスを用いて*in vivo*投与における安全性や体内動態、励起光照射による細胞障害活性が3次元構造の組織でも生じることを検証する。

光感受性武装化アデノウイルス製剤は低侵襲な局所制御療法であり、光線力学療法への応用で選択的な抗腫瘍活性を増強することで、集学的治療として癌患者の生活の質（QOL）の向上に貢献する。また、有効な癌治療戦略の一つとなることで、国民の健康増進や医療経済の節減にも役立つと期待される。

E．結論

緑色励起光により*KillerRed*遺伝子発現ヒト癌細胞を選択的に殺傷することができ、光感受性武装化アデノウイルス製剤を用いたKillerRedの光線力学療法への応用の可能性が示唆された。

F．研究発表

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Cancer Therapy: Preclinical

Clinical Cancer Research

Preclinical Evaluation of Telomerase-Specific Oncolytic Virotherapy for Human Bone and Soft Tissue Sarcomas

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Abstract

Purpose: Tumor-specific replication-selective oncolytic virotherapy is a promising antitumor therapy for induction of cell death in tumor cells but not of normal cells. We previously developed an oncolytic adenovirus, OBP-301, that kills human epithelial malignant cells in a telomerase-dependent manner. Recent evidence suggests that nonepithelial malignant cells, which have low telomerase activity, maintain telomere length through alternative lengthening of telomeres (ALT). However, it remains unclear whether OBP-301 is cytopathic for nonepithelial malignant cells. Here, we evaluated the antitumor effect of OBP-301 on human bone and soft tissue sarcoma cells.

Experimental Design: The cytopathic activity of OBP-301, coxsackie and adenovirus receptor (CAR) expression, and telomerase activity were examined in 10 bone (OST, U2OS, HOS, HuO9, MNNG/HOS, SaOS-2, NOS-2, NOS-10, NDCS-1, and OUMS-27) and in 4 soft tissue (CCS, NMS-2, SYO-1, and NMFH-1) sarcoma cell lines. OBP-301 antitumor effects were assessed using orthotopic tumor xenograft models. The fiber-modified OBP-301 (termed OBP-405) was used to confirm an antitumor effect on OBP-301–resistant sarcomas.

Results: OBP-301 was cytopathic for 12 sarcoma cell lines but not for the non–CAR-expressing OUMS-27 and NMFH-1 cells. Sensitivity to OBP-301 was dependent on CAR expression and not on telomerase activity. ALT-type sarcomas were also sensitive to OBP-301 because of upregulation of human telomerase reverse transcriptase (*hTERT*) mRNA following virus infection. Intratumoral injection of OBP-301 significantly suppressed the growth of OST and SYO-1 tumors. Furthermore, fiber-modified OBP-405 showed antitumor effects on OBP-301–resistant OUMS-27 and NMFH-1 cells.

Conclusions: A telomerase-specific oncolytic adenovirus is a promising antitumor reagent for the treatment of bone and soft tissue sarcomas. *Clin Cancer Res; 17(7); 1828–38. ©2011 AACR.*

Introduction

Bone and soft tissue sarcomas are annually diagnosed in 13,230 patients in the United States (1). They are the third most common cancer in children and account for 15.4% of all childhood malignancies. Treatment of patients with bone and soft tissue sarcomas requires a multidisciplinary approach that involves orthopedic oncologists, musculoskeletal radiologists and pathologists, radiation oncologists, medical and pediatric oncologists, and microvascular surgeons (2, 3). Despite major advances in the treatment of bone and soft tissue sarcomas, such as neoadjuvant and adjuvant multiagent chemotherapy and aggressive surgery, about one fourth of the patients show a poor response to conventional therapy, resulting in subsequent recurrence and leading to a poor prognosis (1). Therefore, the development of a novel therapeutic strategy is required to cure patients with bone and soft tissue sarcomas.

Recent advances in molecular biology have fostered remarkable insights into the molecular basis of neoplasia. More than 85% of all human cancers, but only a few normal somatic cells, show high telomerase activity (4–6). Telomerase activity has also been detected in 17% to 81% of bone and soft tissue sarcomas (7–10). Telomerase activation is considered to be a critical step in cancer development, and its activity is closely correlated with the expression of human telomerase reverse transcriptase

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Translational Relevance

Bone and soft tissue sarcomas frequently occur in young children and show aggressive progression, resistance to conventional chemotherapy, and poor prognosis, indicating a requirement for novel antitumor therapy to improve the clinical outcome. Telomerase-specific replication-selective oncolytic virotherapy is emerging as a promising antitumor therapy. We developed an oncolytic adenovirus, OBP-301, that efficiently kills human epithelial malignant cells in a telomerase-dependent manner. However, alternative lengthening of telomeres (ALT)-type nonepithelial malignant cells show low telomerase activity, suggesting lower effectiveness of OBP-301 in these cells. Here, we showed that OBP-301 has antitumor effects on both non–ALT-type and ALT-type sarcoma cells through upregulation of human telomerase reverse transcriptase mRNA. Furthermore, coxsackie and adenovirus receptor–negative sarcoma cells were efficiently killed by fiber-modified OBP-301 (termed OBP-405) through virus–integrin binding. Thus, a telomerase-specific oncolytic adenovirus would greatly improve the clinical outcome of young patients with advanced sarcomas.

(*hTERT*; ref. 11). Recently, telomerase-specific replication-selective oncolytic virotherapy has emerged as a promising antitumor therapy for induction of tumor-specific cell death. We previously developed an oncolytic adenovirus, OBP-301, in which the *hTERT* promoter drives the expression of the *E1A* and *E1B* genes linked to an internal ribosome entry site (IRES; ref. 12). We determined that OBP-301 efficiently induced the selective killing of a variety of human malignant epithelial cells, such as colorectal, prostate, and non–small cell lung cancers, but not of normal cells (12, 13). Furthermore, a phase I clinical trial of OBP-301, which was conducted in the United States on patients with advanced solid tumors, indicated that OBP-301 is well tolerated by patients (14).

There are 2 known telomere-maintenance mechanisms in human malignant tumors (15, 16): telomerase activation (4–6) and telomerase-independent alternative lengthening of telomeres (ALT; ref. 17–19). The ALT-type mechanism is more prevalent in tumors arising from nonepithelial tissues than in those of epithelial origin (20, 21). Therefore, ALT-type nonepithelial malignant cells frequently show low telomerase activity, suggesting that they have a low sensitivity to OBP-301, which kills cancer cells in a telomerase-dependent manner. However, it remains to be determined whether OBP-301 can exert an antitumor effect on human nonepithelial and on epithelial malignancies.

Adenovirus infection is mainly mediated by interaction of the virus with the coxsackie and adenovirus receptor (CAR) expressed on host cells (22). Therefore, while CAR-expressing tumor cells are the main targets for oncolytic adenoviruses, tumor cells that lack CAR can escape from being killed by oncolytic adenoviruses. It has been reported that CAR is frequently expressed in human cancers of various organs such as the brain (23), thyroid (24), esophagus (25), gastrointestinal tract (26), and ovary (27). Bone and soft tissue sarcomas also express CAR (28–30). However, some populations of tumor cells lack CAR expression, suggesting a requirement for the development of a novel antitumor therapy against CAR-negative tumor cells. We recently developed fiber-modified OBP-301 (termed OBP-405), which can bind to not only CAR but also integrin molecules ($\alpha v\beta 3$ and $\alpha v\beta 5$) and efficiently kill CAR-negative tumor cells (31).

In the present study, we first investigated the *in vitro* cytopathic efficacy of OBP-301 against 14 human bone and soft tissue sarcoma cells. Next, the relationship between the cytopathic activity of OBP-301, CAR expression, and telomerase activity in human sarcoma cells was assessed. The *in vivo* antitumor effect of OBP-301 was also confirmed using orthotopic animal models. Finally, the antitumor effect of OBP-405 against OBP-301–resistant sarcoma cells was evaluated *in vitro* and *in vivo*.

Materials and Methods

Cell lines

The human osteosarcoma (HuO9; ref. 32), chondrosarcoma (OUMS-27; ref. 33), and synovial sarcoma (SYO-1; ref. 34) cell lines were previously established in our laboratory. The human osteosarcoma cell lines OST, HOS, and SaOS-2 were kindly provided by Dr. Satoru Kyo (Kanazawa University, Ishikawa, Japan). The human clear cell sarcoma cell line CCS was maintained in our laboratory. These cells were propagated as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM). The human osteosarcoma cell line U2OS was obtained from the American Type Culture Collection (ATCC) and was grown in McCoy's 5a medium. The human osteosarcoma cell line MNNG/HOS was purchased from DS Pharma Biomedical and was maintained in Eagle's minimum essential medium containing 1% nonessential amino acids. The human osteosarcoma cell lines NOS-2 and NOS-10 (35), the human dedifferentiated chondrosarcoma cell line NDCS-1 (36), the human malignant peripheral nerve sheath cell line NMS-2 (37), and the human malignant fibrous histiocytoma cell line NMFH-1 (38) were kindly provided by Dr. Hiroyuki Kawashima (Niigata University, Niigata, Japan) and were grown in RPMI-1640 medium. The transformed embryonic kidney cell line 293 was obtained from the ATCC and maintained in DMEM. All media were supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant adenoviruses

The recombinant tumor-specific, replication-selective adenovirus OBP-301 (Telomelysin), in which the promoter

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element of the *hTERT* gene drives the expression of *E1A* and *E1B* genes linked with an IRES, was previously constructed and characterized (12, 13). OBP-405 is a telomerase-specific replication-competent adenovirus variant that was previously generated to express the RGD peptide in the fiber knob of OBP-301 (31). The *E1A*-deleted adenovirus vector dl312 and wild-type adenovirus serotype 5 (Ad5) were used as the control vectors. Recombinant viruses were purified by ultracentrifugation using cesium chloride step gradients, and their titers were determined by a plaque-forming assay by using 293 cells and they were stored at −80°C.

Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 20 hours before viral infection. All cell lines were infected with OBP-301 or OBP-405 at multiplicity of infections (MOI) of 0, 0.1, 1, 10, 50, or 100 plaque forming units (PFU)/cell. Cell viability was determined on days 1, 2, 3, and 5 after virus infection, using a Cell Proliferation kit II (Roche Molecular Biochemicals) that was based on an XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate, assay, according to the manufacturer's protocol. The ID_{50} value of OBP-301 for each cell line was calculated using cell viability data obtained on day 5 after virus infection.

Flow cytometric analysis

The cells (5×10^5) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Biotechnology), anti-human integrin αvβ3 (LM609; Chemicon International), or anti-human integrin αvβ5 (P1F6; Chemicon International) antibody for 30 minutes at 4°C. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories) and were analyzed using flow cytometry (FACS Array; Becton Dickinson). The mean fluorescence intensity (MFI) of CAR and integrin αvβ3 or αvβ5 for each cell line was determined by calculating the difference between the MFI in antibody-treated and nontreated cells from 3 independent experiments.

Quantitative real-time PCR analysis

U2OS cells, seeded on 6-well plates at a density of 5×10^5 cells/well 20 hours before viral infection, were infected with Ad5, OBP-301, or dl312 at an MOI of 10 or 100 PFUs/cell. Mock-infected cells were used as controls. Furthermore, to confirm the modulation of *hTERT* mRNA expression by OBP-301 infection, CAR-positive and *hTERT* mRNA-expressing human sarcoma cell lines were seeded on 6-well plates at a density of 5×10^4 cells/well 20 hours before viral infection and were infected with OBP-301 at an MOI of 100 PFUs/cell. Total RNA was extracted from the cells 2 days after virus infection by using the RNA-Bee reagent (Tel-Test Inc.). After synthesis of cDNA from 100 ng of total RNA, the levels of *hTERT* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression were determined using quantitative real-time PCR and a Step One Plus Real Time PCR System (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems). The relative levels of *hTERT* mRNA expression were calculated by using the $2^{-\Delta\Delta Ct}$ method (39) after normalization with reference to the expression of *GAPDH* mRNA.

To compare the *E1A* copy number between OBP-301- and Ad5-infected U2OS cells, U2OS cells, seeded on 6-well plates at a density of 5×10^5 cells/well 20 hours before viral infection, were infected with OBP-301 or Ad5 at an MOI of 10 PFUs/cell. Genomic DNA was extracted from serially diluted viral stocks, and tumor cells were infected with OBP-301 or Ad5 by using the QIAmp DNA Mini Kit (Qiagen). *E1A* copy number was also determined using TaqMan real-time PCR systems (Applied Biosystems).

In vivo OST and OUMS-27 xenograft tumor models

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. The OST and OUMS-27 cells (5×10^6 cells per site) were inoculated into the tibia or the flank of female athymic nude mice aged 6 to 7 weeks (Charles River Laboratories). Palpable tumors developed within 14 to 21 days and were permitted to grow to approximately 5 to 6 mm in diameter. At that stage, a 50 μL volume of solution containing OBP-301, OBP-405, dl312, or PBS was injected into the tumors. Tumor size was monitored by measuring tumor length and width by using calipers. The volumes of OUMS-27 tumors were calculated using the following formula: $(L \times W^2) \times 0.5$, where L is the length and W is the width of each tumor. The volumes of OST tumors were calculated using the formula: $(L + W) \times L \times W \times 0.2618$, as previously reported (40).

X-ray examination

The formation of osteolytic lesions was monitored using radiography (FUJIFILM IXFR film; FUJIFILM Co.) and an X-ray system (SOFTEX TYPE CMB; SOFTEX Co.).

Histopathologic analysis

Tumors were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections were stained with hematoxylin/eosin (H&E) and analyzed by light microscopy.

Statistical analysis

Data are expressed as means ± SD. Student's t test was used to compare differences between groups. Pearson's product–moment correlation coefficients were calculated using PASW statistics version 18 software (SPSS Inc.). Statistical significance was defined when the P value was less than 0.05.

Results

In vitro cytopathic efficacy of OBP-301 against human bone and soft tissue sarcoma cell lines

To evaluate the *in vitro* cytopathic effect of OBP-301 against nonepithelial malignant cells, 14 tumor cell lines

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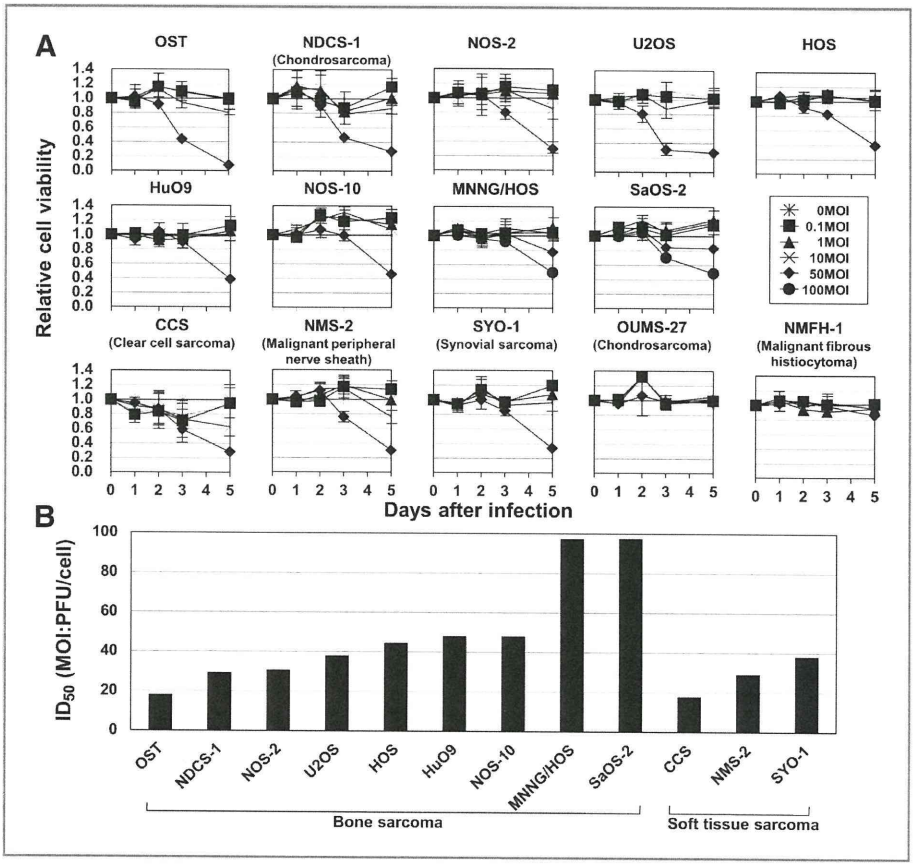

Figure 1. Cytopathic effect of OBP-301 on human bone and soft tissue sarcoma cell lines. A, cells were infected with OBP-301 at the indicated MOI, and cell survival was quantified over 5 days using the XTT assay. The cell viability of mock-treated group on each day was considered 1.0, and the relative cell viability was calculated. Data are means ± SD. The types of tumor except for osteosarcoma were shown in parentheses. B, the 50% inhibiting doses of OBP-301 on cell viability 5 days after infection were calculated and are expressed as ID_{50} values.

derived from human bone and soft tissue sarcomas were infected with various doses of OBP-301. The cell viability of each cell line was assessed over 5 days after infection by the XTT assay. OBP-301 infection induced cell death in a time-dependent manner in all sarcoma cell lines except for the OUMS-27 and NMFH-1 cell lines (Fig. 1A). Calculation of the ID_{50} values revealed that, of the 12 OBP-301–sensitive sarcoma cell lines, MNNG/HOS and SaOS-2 cells were relatively less sensitive than the other 10 sarcoma cell lines (Fig. 1B). Furthermore, to rule out the possibility that cytopathic effect of OBP-301 is due to nonspecific toxicity based on the high uptake of virus particles into tumor cells, we examined the cytopathic activity of replication-deficient dl312 in U2OS and HOS cells. dl312 did not show any cytopathic effect in U2OS and HOS cells, even when these cells were infected with dl312 at high dose (50 and 100 MOIs; Supplementary Fig. S1). These results indicate that OBP-301 is cytopathic for most human bone and soft tissue sarcoma cells line but that some sarcoma cell lines are resistant to OBP-301.

Expressions of the adenovirus receptor and *hTERT* mRNA on human bone and soft tissue sarcoma cell lines

Because adenovirus infection efficiency depends mainly on cellular CAR expression (22), we determined the expression level of CAR on the 14 sarcoma cell lines by flow cytometry. The 12 OBP-301–sensitive sarcoma cell lines showed CAR expression, determined as MFIs, at various levels, whereas the OBP-301–resistant OUMS-27 and NMFH-1 cells did not express CAR (Fig. 2A and Supplementary Fig. S2).

OBP-301 contains the *hTERT* gene promoter, which allows it to tumor specifically regulate the gene expression of *E1A* and *E1B* for viral replication. Thus, OBP-301 can efficiently replicate in human cancer cells with high telomerase activity but not in normal cells without telomerase activity (12). Recently, some populations of human sarcoma cells have been shown to possess low telomerase activity and to maintain telomere lengths through an ALT mechanism (17–19). Thus, it is probable that OBP-301 cannot efficiently replicate in, and kill, ALT-type human sarcoma cells because of their low telomerase activity. To assess whether the telomerase activity of human sarcoma cells affects the cytopathic activity of OBP-301, we analyzed *hTERT* mRNA expression levels in the 14 sarcoma cell lines by quantitative real-time reverse transcriptase PCR (RT-PCR) analysis. Thirteen of the sarcoma cell lines had detectable *hTERT* mRNA expression at variable levels, and only SaOS-2 cells did not express *hTERT* mRNA (Fig. 2B).

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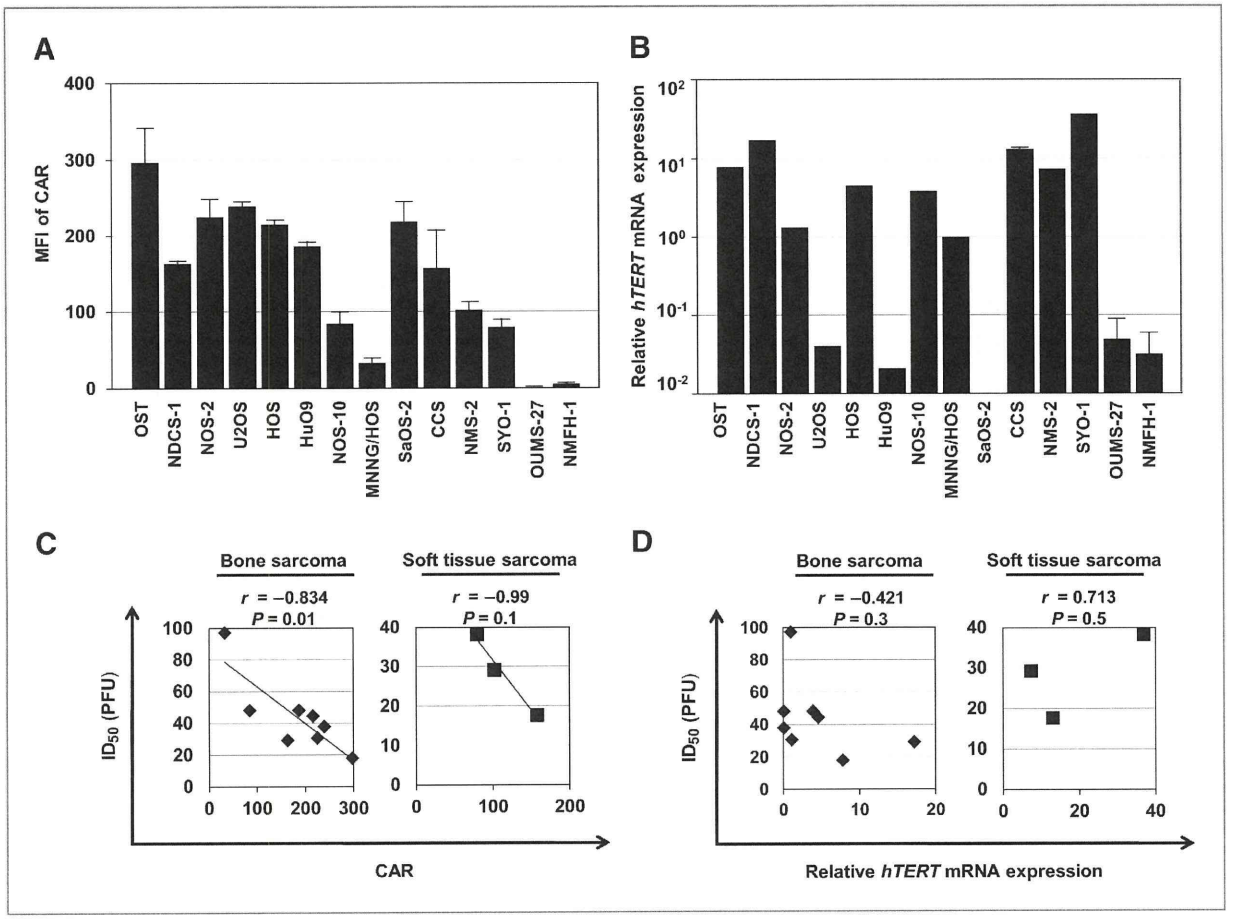

Figure 2. Relationship between the expression levels of CAR and *hTERT* mRNA and the cytopathic activity of OBP-301 against human bone and soft tissue sarcoma cell lines. A, the MFI of CAR expression on human bone and soft tissue sarcoma cells. The cells were incubated with a monoclonal anti-CAR (RmcB) antibody, followed by flow cytometric detection using an FITC-labeled secondary antibody. B, expression of *hTERT* mRNA in human bone and soft tissue sarcoma cells by quantitative real-time PCR. The relative levels of *hTERT* mRNA were calculated after normalization with reference to the expression of *GAPDH* mRNA. C, correlation between the MFI of CAR and the ID_{50} of OBP-301 on human bone and soft tissue sarcoma cells. D, correlation between *hTERT* mRNA expression and the ID_{50} of OBP-301 on human bone and soft tissue sarcoma cells. Statistical significance was determined as $P < 0.05$, after analysis of Pearson's correlation coefficient.

We next investigated the relationship between CAR and *hTERT* mRNA expressions and the cytopathic activity of OBP-301 among the 11 CAR-positive sarcoma cell lines with *hTERT* gene expression. CAR expression levels significantly ($r = -0.834$; $P = 0.01$) correlated with the cytopathic activity of OBP-301 against 8 of the bone sarcoma cell lines (Fig. 2C). CAR expression in 3 of the soft tissue sarcoma cell lines also correlated ($r = -0.99$) with the cytopathic effect of OBP-301, but the differences did not reach significance ($P = 0.1$) because of the low number of cell lines assayed. In contrast, there was no significant correlation between *hTERT* mRNA expression and the cytopathic activity of OBP-301 (Fig. 2D). These results indicate that the cytopathic activity of OBP-301, at least in part, depends on CAR expression.

Furthermore, SaOS-2 and U2OS cells have already been shown to be ALT-type sarcoma cell lines with low telomerase activity (9, 17). Among these ALT-type sarcoma cells, U2OS cells showed a sensitivity to OBP-301 that was similar to that of non–ALT-type sarcoma cells such as HOS and NOS-10 (Fig. 1B). These results indicate that ALT-type human sarcoma cells are sensitive to OBP-301 and that a low telomerase activity does not detract from the cytopathic activity of OBP-301.

Enhanced virus replication and cytopathic activity of OBP-301 through *hTERT* mRNA upregulation in ALT-type sarcoma cell lines

The high sensitivity of ALT-type sarcoma cells to OBP-301 prompted us to hypothesize that OBP-301 may activate the *hTERT* gene promoter, thereby enhancing the viral replication rate and subsequently inducing cytopathic activity in ALT-type sarcoma cells. Furthermore, it has been previously shown that the adenoviral E1A

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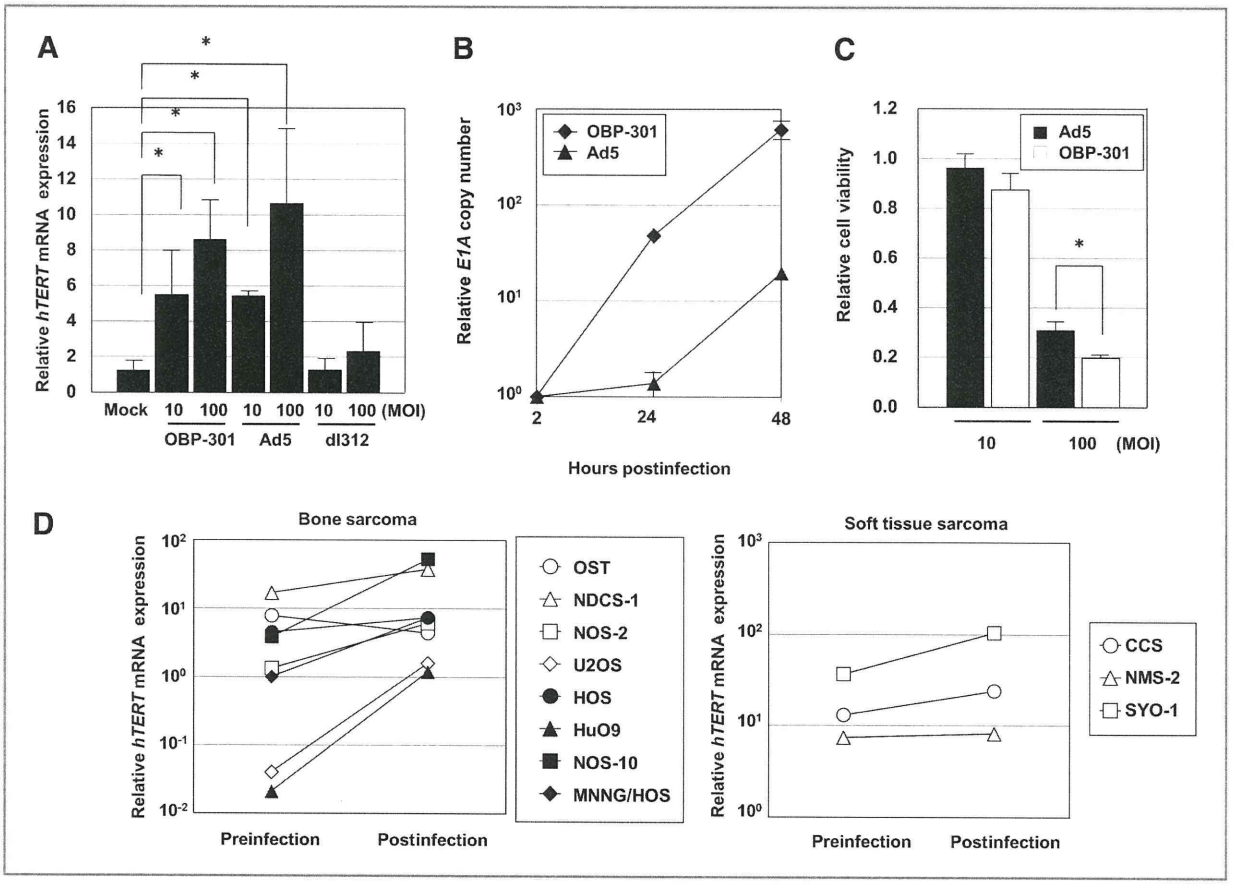

Figure 3. Upregulation of *hTERT* gene expression in ALT-type human sarcoma cell lines enhances the replication and the cytopathic effect of OBP-301. A, expression of *hTERT* mRNA in U2OS cells that were mock-infected or were infected with OBP-301, Ad5, or dl312. The cells were infected with OBP-301, Ad5, or dl312 at the indicated MOIs for 48 hour, and *hTERT* mRNA expression was analyzed using quantitative real-time RT-PCR. The value of *hTERT* mRNA expression in the mock-infected cells was set at 1, and relative mRNA levels were plotted. B, quantitative measurement of viral DNA replication in U2OS cells infected with OBP-301 or Ad5. The cells were infected with OBP-301 or Ad5 at an MOI of 10 PFUs/cell, and *E1A* copy number was analyzed over the following 2 days by quantitative real-time PCR. The value of the *E1A* copy number at 2 hours after infection was set at 1, and relative copy numbers were plotted. C, comparison of the cytopathic effect of OBP-301 and Ad5 in U2OS cells. The cells were infected with OBP-301 or Ad5 at the indicated MOIs, and cell survival was quantified 5 days after infection by using an XTT assay. D, expression of *hTERT* mRNA after infection of human bone (left) and soft tissue (right) sarcoma cell lines with OBP-301 at an MOI of 100 PFUs/cell. Statistical significance (*) was determined as $P < 0.05$ (Student's t test).

protein can activate the promoter activity of the *hTERT* gene (41, 42). Therefore, to determine whether OBP-301 infection activates *hTERT* mRNA expression, we examined the expression level of *hTERT* mRNA in ALT-type U2OS cells after infection with OBP-301 at MOIs of 10 and 100 PFUs/cell (Fig. 3A). Compared with mock-infected U2OS cells, OBP-301–infected U2OS cells showed a 6- to 8-fold increase in *hTERT* mRNA expression in a dose-dependent manner. Ad5 infection also increased *hTERT* mRNA expression in U2OS cells, whereas there was no increase in U2OS cells infected with *E1A*-deleted dl312. These results suggest that OBP-301 is cytopathic for ALT-type sarcoma cells through E1A-mediated activation of the *hTERT* gene promoter.

We next compared viral replication rates after infection of ALT-type U2OS cells with OBP-301 or Ad5. As expected, the viral replication rate of OBP-301 was significantly higher than that of Ad5 (Fig. 3B). Furthermore, the cytopathic activity of OBP-301 was significantly higher than that of Ad5 against the ALT-type U2OS cells (Fig. 3C). Finally, to determine whether OBP-301 activates *hTERT* mRNA expression in both ALT-type and non–ALT-type human sarcoma cell lines, we infected 11 CAR-positive human sarcoma cells with OBP-301 at 100 MOI. Ten of the 11 CAR-positive human sarcoma cell lines showed an increase in the expression level of *hTERT* mRNA after OBP-301 infection that ranged from a 1.1- to 50.0-fold increase (Fig. 3D and Supplementary Table S1). In addition, the expression level of *hTERT* mRNA was also upregulated when OST cells were infected with 5 or 50 MOI of OBP-301 (Supplementary Fig. S3). These results suggest that OBP-301 is cytopathic for both ALT-type and non–ALT-type human sarcoma cells through activation of the *hTERT* gene promoter.

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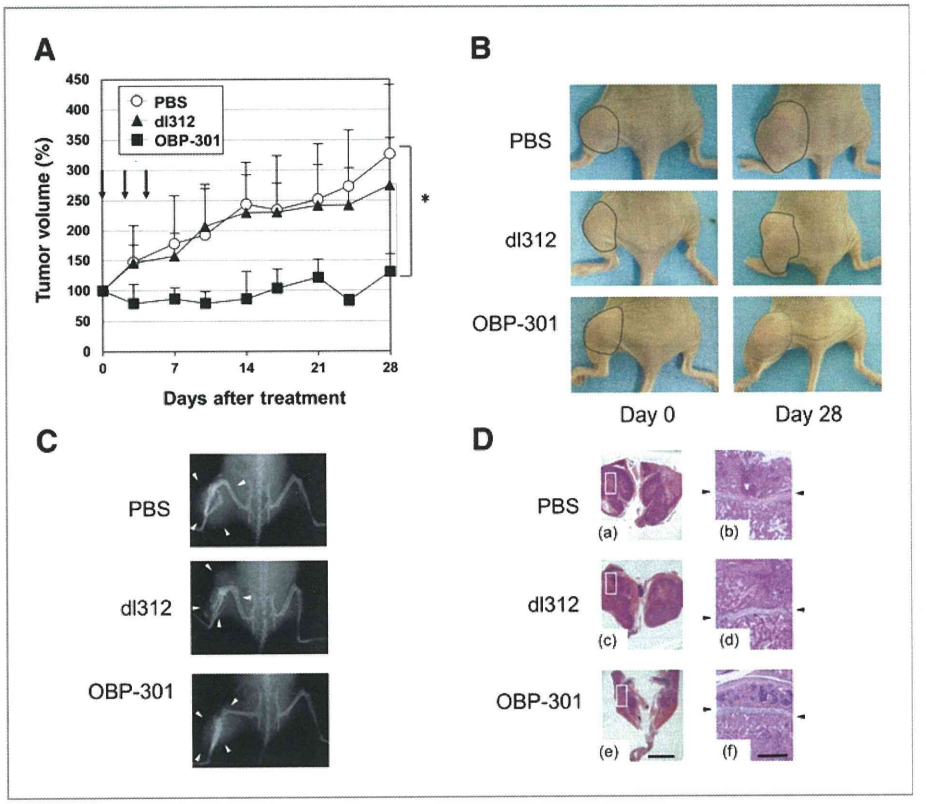

Figure 4. Antitumor effect of OBP-301 in an orthotopic OST bone sarcoma xenograft model. A, athymic nude mice were inoculated intratibially with OST cells (5×10^6 cells/site). Fourteen days after inoculation (designated as day 0), OBP-301 (■) or OBP-405 (▲) was injected into the tumor, with 1×10^8 PFUs on days 0, 2, and 4. PBS (○) was used as a control. Four mice were used for each group. Tumor growth was expressed as mean tumor volume ± SD. Statistical significance (*) was determined as $P < 0.05$ (Student's t test). B, macroscopic appearance of OST tumors in nude mice on days 0 and 28 after treatment with PBS, dl312, or OBP-301. Tumor masses are outlined by a dotted line. C, X-ray photographs of mice bearing OST tumors. The white arrowheads indicate the space occupied by the tumor mass. D, histologic analysis of the OST tumors. Tumor sections were obtained 28 days after inoculation of tumor cells. Paraffin-embedded sections of OST tumors were stained with H&E. The black arrowheads indicate growth plate cartilages. a, c and e, are low-magnification images and b, d and f are high-magnification images of the area outlined by a white square. Left scale bar, 5 mm. Right scale bar, 500 μm.

Antitumor effect of OBP-301 against 2 orthotopic tumor xenograft models

To evaluate the *in vivo* antitumor effect of OBP-301 against human bone and soft tissue sarcomas, we used 2 types of orthotopic tumor xenograft models: the OST bone sarcoma xenograft and the SYO-1 subcutaneous soft tissue sarcoma xenograft. We first identified a dose of OBP-301 that was suitable for induction of an antitumor effect in the subcutaneous OST bone sarcoma xenograft model (determined as $>10^7$ PFUs; Supplementary Fig. S4). We next assessed the antitumor effect of OBP-301 on the orthotopic OST bone sarcoma xenograft model. OBP-301 was injected into the tumor once a day for 3 days, with 10^8 PFUs per day (10). Replication-deficient adenovirus dl312 or PBS was also injected into control groups. Tumor growth was significantly suppressed by OBP-301 injection compared with injection of dl312 or PBS (Fig. 4A). Macroscopic analysis of the tumors indicated that OBP-301–treated tumors were consistently smaller than dl312- or PBS-treated tumors on day 28 after treatment (Fig 4B). We further determined whether OBP-301–treated tumors were less destructive to surrounding normal tissues than control tumors, using X-ray and histologic analyses (Fig. 4C and D). X-ray examination revealed that OBP-301–treated tumors resulted in less bone destruction than dl312- or PBS-treated tumors. Histologic findings were consistent with the X-ray results, showing that some tumor tissue had penetrated over the growth plate cartilage in dl312- and PBS-treated tumors but not in OBP-301–treated tumors.

With future clinical application in mind, we sought to establish a suitable protocol for repeated intratumoral injection of OBP-301 by using an orthotopic SYO-1 soft tissue sarcoma xenograft model. Doses of OBP-301 that were suitable for induction of an antitumor effect on SYO-1 tumors ($>10^8$ PFUs) were determined in a manner similar to that of OST bone sarcoma cells (data not shown). OBP-301 was injected 3 times into the tumor, with 10^9 PFUs and intervals of 1 day, 2 days, or 1 week between injections (Supplementary Fig. S5). A total of 3 OBP-301 injections, with intervals of 2 days or 1 week between injections, induced a significant

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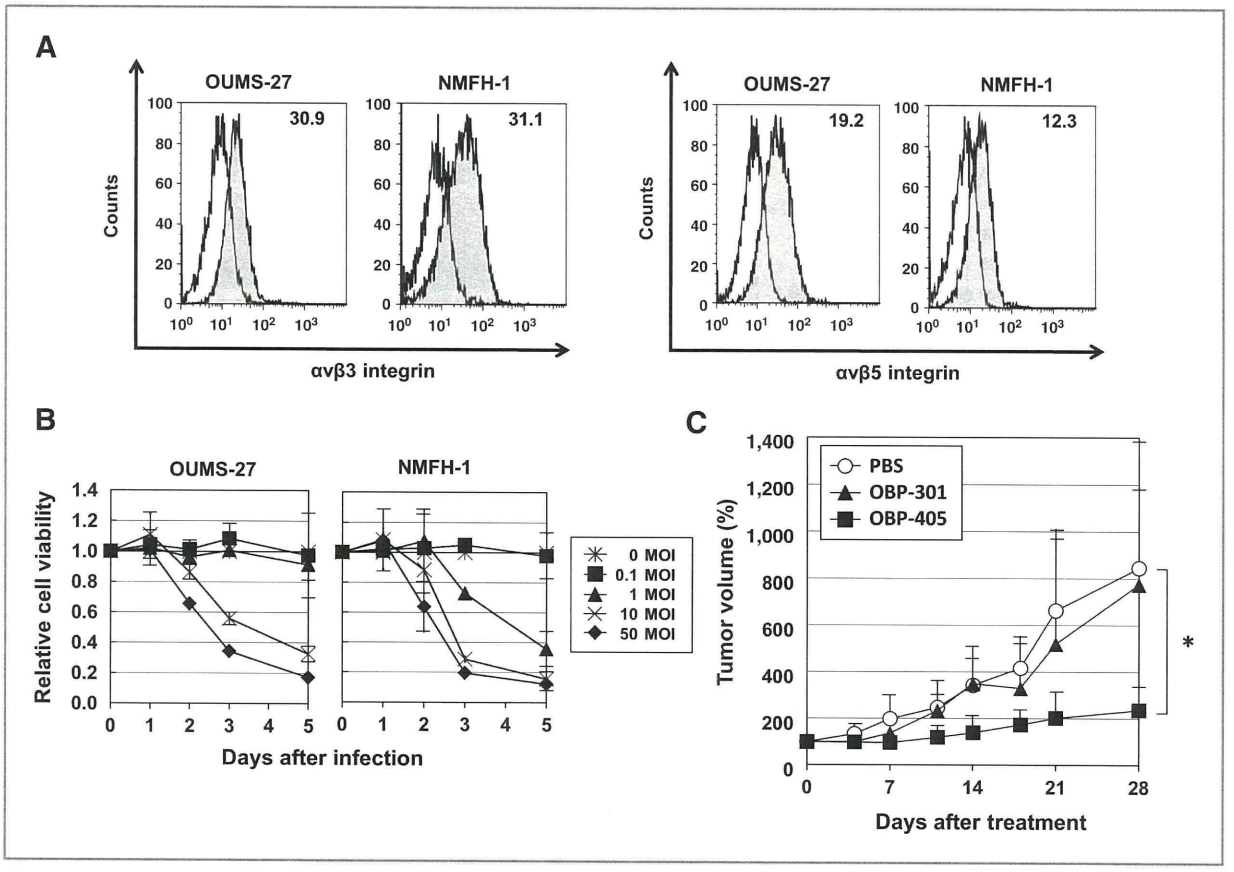

Figure 5. *In vitro* and *in vivo* antitumor effects of OBP-405 on OBP-301–resistant human sarcoma cell lines. A, expression of the integrins αvβ3 (left) and αvβ5 (right) on OUMS-27 and NMFH-1 cells. The cells were incubated with a monoclonal anti-αvβ3 integrin (LM609) or an anti-αvβ5 integrin (P1F6), followed by flow cytometric detection using an FITC-labeled secondary antibody. The gray histogram represents integrin antibody staining. The number at the top right-hand corner of each graph is the MFI. B, cytopathic effect of OBP-405 on OUMS-27 and NMFH-1 cells. The cells were infected with OBP-405 at the indicated MOI values, and cell survival over 5 days was quantified using an XTT assay. C, antitumor effect of OBP-405 in a subcutaneous OUMS-27 xenograft tumor model. Athymic nude mice were inoculated subcutaneously with OUMS-27 cells (5×10^6 cells/site). Fourteen days after inoculation (designated as day 0), OBP-301 (▲) or OBP-405 (■) was injected into the tumor, with 1×10^8 PFUs on days 0, 2, and 4. PBS (○) was used as a control. Ten mice were used for each group. Tumor growth was expressed as the mean tumor volume ± SD. Statistical significance (*) was determined as $P < 0.05$ (Student's *t* test).

suppression of tumor growth, whereas intervals of 1 day between injections were not effective. These results suggest that an interval of more than 2 days between injections is necessary to efficiently suppress tumor growth by repeated injections of OBP-301.

Antitumor effect of OBP-405 on OBP-301–resistant sarcoma cell lines

OUMS-27 and NMFH-1 cells are resistant to OBP-301 because they lack CAR expression (Fig. 1A and Supplementary Fig. S2). We previously developed a fiber-modified OBP-301, termed OBP-405, which can enter not only CAR-positive cancer cells but also CAR-negative cancer cells through binding to the cell surface integrins αvβ3 and αvβ5 (31). We therefore sought to evaluate the antitumor effect of OBP-405 on the OBP-301–resistant OUMS-27 and NMFH-1 cells. We first examined the expression levels of the integrins αvβ3 and αvβ5 on the surface of these cells by flow cytometry (Fig. 5A). OUMS-27 and NMFH-1 cells expressed both integrin molecules. We next examined the effect of OBP-405 on OUMS-27 and NMFH-1 cell viability by using the XTT assay (Fig. 5B). OBP-405 efficiently suppressed cell viability of both of these cell lines in a dose- and time-dependent manner. We further assessed whether OBP-405 has an *in vivo* antitumor effect by assaying the effect of 3 intratumoral injections of OBP-301 or OBP-405, with 10^8 PFUs or of control PBS, into subcutaneous OUMS-27 tumor xenografts. As shown in Figure 5C, administration of OBP-405 resulted in significant suppression of tumor growth compared with OBP-301- or PBS-treated tumors 28 days after treatment. These results suggest that fiber-modified OBP-405 is a potential antitumor reagent that is effective against CAR-negative human sarcoma cells.

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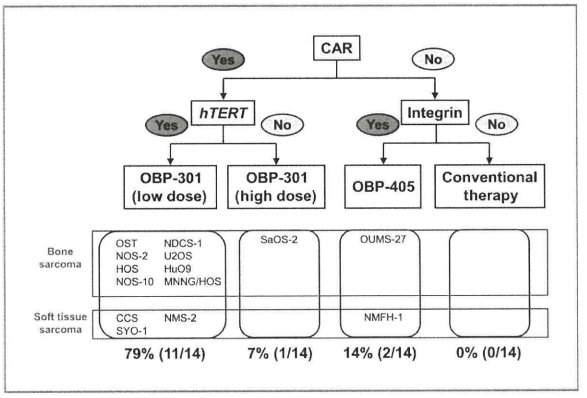

Figure 6. Outline of a therapeutic strategy for the use of telomerase-specific replication-selective oncolytic adenoviruses for human bone and soft tissue sarcoma cells. Assessment of CAR expression in tumor cells could serve as an indicator for OBP-301 or OBP-405 treatment. Of the 14 human sarcoma cell lines shown, the 12 CAR-expressing sarcoma cells (86%) should be treated with OBP-301 and the 2 sarcoma cells (14%) that lack CAR expression should be treated with OBP-405. The tumor expression level of *hTERT* mRNA would be useful in deciding the dose of OBP-301 to be used for treatment. The expression level of integrins on the tumor surface should be confirmed prior to OBP-405 treatment.

Discussion

Telomerase-specific replication-selective oncolytic adenoviruses are emerging as promising antitumor reagents for induction of tumor-specific cell death. We previously reported that OBP-301 has a strong antitumor effect on a variety of human epithelial malignant cells that have high telomerase activity (12, 13). However, nonepithelial malignant cells often show low telomerase activity and instead maintain telomere length through an ALT mechanism (20, 21). The effect of OBP-301 on human bone and soft tissue sarcoma cells has not been extensively examined. In this study, we showed that OBP-301 induced cell death in 12 of 14 human bone and soft tissue sarcoma cell lines (Fig. 1) and that the cytopathic activity of OBP-301 significantly correlated with tumor CAR expression (Fig. 2A). Furthermore, 2 ALT-type sarcoma cells showed low *hTERT* mRNA expression (Fig. 2B) but a similar sensitivity to OBP-301 compared with non–ALT-type cells because of *hTERT* mRNA upregulation by OBP-301 infection (Fig. 3). In contrast, 2 OBP-301–resistant sarcoma cells that lack CAR expression were highly sensitive to OBP-405, which can infect cells by binding to surface integrin molecules (Fig. 5). On the basis of these results, and with future clinical application in mind, we established a therapeutic strategy for the use of telomerase-specific oncolytic adenoviruses to treat patients with bone and soft tissue sarcomas (Fig. 6). This strategy involves assessment of the expression levels of CAR, *hTERT*, and integrins on human sarcoma cells, which would then allow easy selection of the most effective protocol for the treatment of patients by using oncolytic adenoviruses. Furthermore, as OBP-301 and OBP-405 show the profound antitumor effect in the combination of various chemotherapeutic agents (43, 44), further evaluation for the strategy using OBP-301 and OBP-405 in combination with chemotherapy should be warranted.

The cytopathic activity of OBP-301 significantly correlated with CAR expression, but not with telomerase activity, of human sarcoma cells (Fig. 2). These results suggest that the cytopathic activity of OBP-301 depends primarily on infection efficiency rather than virus replication. Primary epithelial and nonepithelial malignant tumors frequently express CAR (23–30). However, CAR expression can often be downregulated by tumor progression (45, 46) or under hypoxic conditions (47), possibly leading to a low infection efficiency and resistance to OBP-301. Thus, for future clinical application of OBP-301, it may be necessary to overcome the resistance to OBP-301 that arises during tumor progression. A histone deacetylase (HDAC) inhibitor has been previously shown to enhance CAR expression on human cancer cells (48–50). Therefore, for the treatment of OBP-301–resistant sarcomas, it may be necessary to either upregulate CAR expression on tumor cells in combination with an HDAC inhibitor or use OBP-405 to kill tumor cells in an integrin-dependent manner (31).

ALT-type sarcoma cells that express a low level of *hTERT* mRNA showed sensitivity to OBP-301 that was similar to that of non–ALT-type sarcoma cells (Figs. 1 and 2). We further showed that OBP-301 infection upregulates *hTERT* gene expression and subsequently activates virus replication and cytopathic activity in ALT-type sarcoma cells (Fig. 3). These results suggest that the *hTERT* gene promoter is a useful tool for enhancement of the oncolytic adenoviruses not only because it induces tumor-specific virus replication but also because it enhances virus replication after infection. Indeed, the ALT-type sarcoma SaOS-2 cells that lack *hTERT* gene expression were relatively less sensitive to OBP-301 than the other ALT-type sarcoma U2OS cells that express low levels of *hTERT* mRNA (Figs. 1 and 2). We further observed that *hTERT* mRNA expression was not upregulated after OBP-301 infection of SaOS-2 cells (data not shown). These results suggest that if *hTERT* gene expression cannot be detected in tumor cells, then ALT-type sarcoma cells should be treated with high doses of OBP-301, or with OBP-405, to enhance OBP-301 infection efficiency (Fig. 6).

It is also worth noting in terms of future clinical application that an interval of more than 2 days between injections is necessary in order for repeated injections of OBP-301 to induce a strong antitumor effect in an SYO-1 animal xenograft model (Supplementary Fig. S5). We first expected that continuous injection of OBP-301 at intervals of 1 day, when tumors are of a minimum size, might be more effective in inducing an antitumor effect than injection at intervals of 2 days or 1 week. Surprisingly, continuous injection of OBP-301 at intervals of 1 day, for 3 days, could not induce an antitumor effect. There are 2 possible explanations for these results. The

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first possibility is that 3 days of continuous injections may not provide enough time for OBP-301 to replicate and reach the minimal dose required for induction of an antitumor effect within tumor tissues. The second possibility is that OBP-301 may be less effective against more slowly proliferating tumor cells than it is against rapidly proliferating tumor cells because its replication rate would be lower in the more slowly proliferating cells. Although it remains unclear why continuous injection of OBP-301 was less effective, it is clear that repeated infection with OBP-301 at intervals of more than 2 days would be sufficient to exert an antitumor effect against human sarcoma tissues.

In conclusion, we have clearly shown that OBP-301 has strong *in vitro* and *in vivo* antitumor effects against human bone and soft tissue sarcoma cells. Telomerase-specific replication-selective oncolytic virotherapy would provide a new platform for the treatment of patients with bone and soft tissue sarcomas.

Disclosure of Potential Conflict of Interest

Y. Urata is an employee of Oncolys BioPharma, Inc., the manufacturer of OBP-301(Telomelysin). The other authors disclosed no potential conflicts of interest.

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