厚生労働科学研究費補助金

医療機器開発推進研究事業

テロメラーゼ依存性蛍光発現ナノバイオ・ウイルス製剤を標識薬剤とする高感度リアルタイム微小癌転移イメージングシステムの開発

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研究代表者　　藤原　俊義

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【研究要旨】

天然に存在する生物由来の蛍光タンパク質は、至適な波長の励起光を吸収することにより強い蛍光を発し、導入した細胞を生きたままの状態で可視化することができる。Green Fluorescent Protein（GFP）をはじめとする蛍光タンパク質を用いた分子イメージングは、最先端の生命科学の研究や技術開発には広範囲に利用されているが、医療への応用は未だ研究段階であり、実際にヒトに臨床応用された事例はない。平成17-19年度の厚生労働科学研究費にて、標識薬剤としてテロメラーゼ活性依存性に癌細胞で選択的に増殖してGFP遺伝子を発現するウイルス製剤TelomeScan（開発コード：OBP-401）を作成し、携帯用接触プローブ型蛍光検出装置の有用性を明らかにしてきた。本研究では、新たに鏡視下手術用の高感度蛍光感知機能を付与したビデオスコープを試作し、大動物でその操作性と有用性を評価することで、最近の腹腔鏡・胸腔鏡手術の普及に対応した、より実践的な低侵襲治療の確立を目指す。

本年度は、近赤外蛍光を感知できる高感度蛍光感知ビデオスコープを試作し、組織透過性の高い近赤外蛍光*Katushka*遺伝子を挿入した非増殖型アデノウイルスベクター（Ad-Katushka）を感染させたヒト癌細胞を大動物の消化管や近傍のリンパ節に投与して、赤色蛍光が明瞭に観察可能であることを明らかにした。

A．研究目的

天然に存在する生物由来の蛍光タンパク質は、至適な波長の励起光を吸収することにより強い蛍光を発し、導入した細胞を生きたままの状態で可視化することができる。GFP（Green Fluorescent Protein）をはじめとする蛍光タンパク質を用いた分子イメージングは、最先端の生命科学の研究や技術開発には広範囲に利用されているが、医療への応用は未だ研究段階であり、実際にヒトに臨床応用された事例はない。われわれは、テロメラーゼ活性（hTERT遺伝子発現）に依存して癌細胞で選択的に増殖し細胞死を誘導する改変アデノウイルス製剤Telomelysin（開発コード：OBP-301）を開発し、米国にて臨床試験を行い、その安全性と臨床効果を確認した。このウイルスのゲノム配列は完全に明らかになっており、癌選択的ベクターとして外来遺伝子を搭載することができる。すなわち、蛍光タンパク質をコードする遺伝子を組み込み、癌細胞で選択的に蛍光発現を誘導することができる。

本研究では、テロメラーゼ活性依存性に癌細胞で選択的に増殖してGFP遺伝子を発現するナノバイオ・ウイルス製剤TelomeScan（OBP-401）を標識薬剤とし、鏡視下手術用の高感度蛍光感知ビデオスコープを用いたリアルタイム微小癌転移診断用の外科ナビゲーション・システムを開発する。また、GFPの蛍光波長より長く、組織透過性の高い近赤外蛍光を発する新しい蛍光タンパク質遺伝子を搭載した新規ウイルス標識薬剤を開発し、鏡視下手術時における有用性や汎用性をTelomeScanと比較検討する。術前に原発病巣や体腔に投与されたナノバイオ・ウイルス製剤は、微小転移巣で癌細胞に感染・増殖して選択的に蛍光を発するので、高感度蛍光検出ビデオスコープを用いてリアルタイムにハイビジョン・モニター上で可視化することができる。

本年度は、近赤外蛍光を観察可能な高感度蛍光検出ビデオスコープを試作し、近赤外蛍光*Katushka*遺伝子を発現する非増殖型アデノウイルス（Ad-Katushka）を感染させたヒト癌細胞が、大動物（ミニブタ）の消化管や近傍のリンパ節で可視化できることを確認した。

B．研究方法

1）近赤外蛍光*Katushka*遺伝子発現アデノウイルスベクター（Ad-Katushka）の構造と機能

GFPより深部の微小癌組織を高感度に検出するために、組織透過性の高い近赤外蛍光を発する蛍光タンパク質を搭載した新たなウイルス標識薬剤を作成した。GFPの蛍光波長505 nmより長く組織透過

性の高い635 nmの近赤外蛍光を発するイソギンチャク*Entacmaea quadricolor*由来の新しい蛍光タンパク質Katushkaをコードする遺伝子を、幼児の「かぜ」症状の原因となるアデノウイルス5型のE1欠失領域にサイトメガロウイルス・プロモーターとともに挿入した。

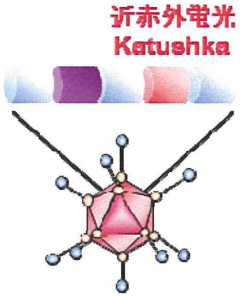

図１　**Ad-Katushkaアデノウイルスベクター**

2）近赤外蛍光を観察可能な高感度蛍光検出ビデオスコープの試作

近赤外蛍光を可視化することができる高感度蛍光検出ビデオスコープを試作した。光源、スコープ、カメラアダプタ、カメラ、プロセッサ、モニターから成り、励起波長570～590nm、蛍光波長600～655nmで観察する。

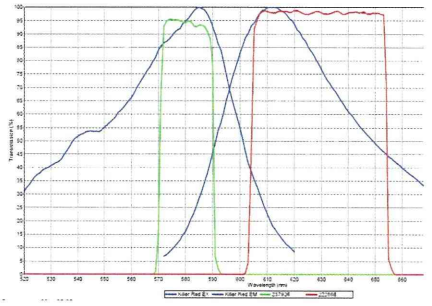

図2　励起波長と蛍光波長プロファイル

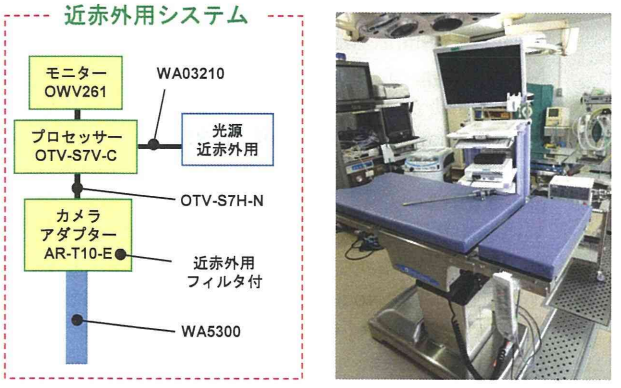

図3　近赤外蛍光感知ビデオスコープシステム

3）大動物における*Katushka*遺伝子発現ヒト癌細胞の可視化の試み

非増殖型Ad-KatushkaアデノウイルスをH1299ヒト肺癌細胞に感染させ、24-48時間後に遠心にて回収、濃縮懸濁液にして、全身麻酔下のミニブタに開腹にて胃壁や近傍リンパ節に注入する。気腹下に近赤外蛍光観察用ビデオスコープを用いて静止画、動画撮影を行った。

（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）大動物における*Katushka*遺伝子発現ヒト癌細胞の可視化の試み

全身麻酔下のミニブタに*Katushka*遺伝子発現H1299ヒト肺癌細胞を濃縮懸濁液にして開腹にて胃壁漿膜下や近傍リンパ節に注入し、高感度近赤外蛍光検出ビデオスコープにて腹腔内を観察したところ、近赤外蛍光が明瞭に検出可能であった。

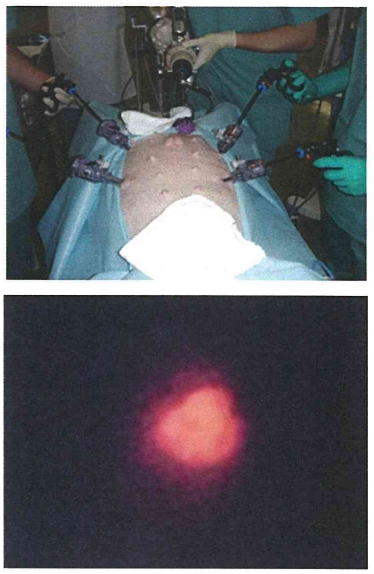

図4　蛍光観察ビデオスコープによる蛍光検出

また、陽性コントロールとして赤色ビーズを同様に胃漿膜下やリンパ節に投与して同様に観察可能であることを確認した。さらに、胃漿膜下にインジゴカルミン色素を注入し、リンパ流が近傍リンパ節に流入することを検証し、病変近辺に投与したウイルス製剤がリンパ節まで到達できることを確認した。

D．考察

蛍光タンパク質を用いた*in vivo*蛍光イメージングは、最先端の生命科学の研究に重要な技術であるが、医療の現場で実用化された事例はまだない。テロメラーゼ活性に反応して増殖する遺伝子改変アデノウイルスは80-100 nmの天然のバイオ・ナノマシーンであり、癌細胞で選択的に蛍光遺伝子を発現するために適したベクターと成り得る。投与されたウイルス標識薬剤はリンパ流や血流に乗っ

て拡散し、微小転移病巣で増殖するとともに蛍光発現を生じる。高感度蛍光感知ビデオスコープを組み合わせることで鏡視下手術用外科ナビゲーション・システムの臨床応用が実現すれば、手術中にリアルタイムにリンパ節転移などの微小癌組織を同定することができ、必要最小限の領域を切除する超縮小手術の施行が可能となる。

たとえば早期消化器癌の場合、本技術による診査腹腔鏡や経管腔的内視鏡手術（NOTES、Natural Orifice Translumenal Endoscopic Surgery）で確実にリンパ節転移や播種がないことを確認できれば、原発巣は内視鏡的粘膜下切開術（ESD、Endoscopic Submucosal Dissection）のみで切除可能なケースも増えてくる。すなわち、本研究成果によって、外科切除範囲を最小限に留めることで大部分の臓器を温存したり、あるいはリンパ節郭清そのものを省略したりすることができれば、画期的な機能温存が可能となり、治療後の患者の生活の質の著しい向上に貢献することができる。

初年度である平成21年度は、GFPを可視化することができる高感度蛍光感知ビデオスコープの第1号試作機、および小型・軽量化した第2号試作機を作成した。カメラヘッドのスイッチにて明視野モードと蛍光モードのフィルター切り替えがワンタッチでできる、その切り替えに連動して光源フィルターとモニター表示が切り替わり、明視野と蛍光視野を切り替えて表示するシステムとした。全身麻酔下のミニブタで、経口的に挿入した消化管内視鏡で胃粘膜下にTelomeScanと同一の蛍光特性を持つ蛍光ビーズを注入したところ、腹腔内から第2号試作機にて極めて良好に胃から所属リンパ節へのリンパ流をリアルタイムに確認することができた。

研究2年目には、高感度蛍光感知ビデオスコープ第2号試作機によるミニブタの組織における*GFP*遺伝子発現の検出を試みた。Ad-GFPの投与では蛍光発現はみられなかったが、細胞レベルでGFP発現していると高感度に検出可能であることが明らかとなった。このGFP蛍光は、平成19年度までに本研究課題にて試作した携帯型蛍光検出プローブにおいても同様に検出することができた。

また、近赤外蛍光*Katushka*遺伝子を挿入した非増殖型アデノウイルスベクター（Ad-Katushka）を作成し、各種ヒト癌細胞において近赤外蛍光の発現を確認した。当初、近赤外蛍光遺伝子を発現する癌特異的制限増殖型ウイルスの作成を試みる予定であったが、非担癌動物での発現は期待できないため、まず基盤研究としてAd-Katushkaを構築した。

最終年度には、近赤外蛍光感知ビデオスコープを試作し、Ad-Katushkaを*ex vivo*で感染させたヒト癌細胞を投与して空間的検出能を含めたビデオスコープの機能解析を行った。

今後は、診断用イメージング医薬品としてのウイルスの安全性と有効性、高感度蛍光感知ビデオスコープの医療機器としての薬事申請を計画し、外科ナビゲーション・システムとしての臨床試験の立案を行い、胃癌をはじめとする消化器癌治療現場への臨床展開を目指す。

E．結論

大動物において高感度蛍光検出ビデオスコープ試作機にて*Katushka*遺伝子発現による近赤外蛍光を可視化することができた。

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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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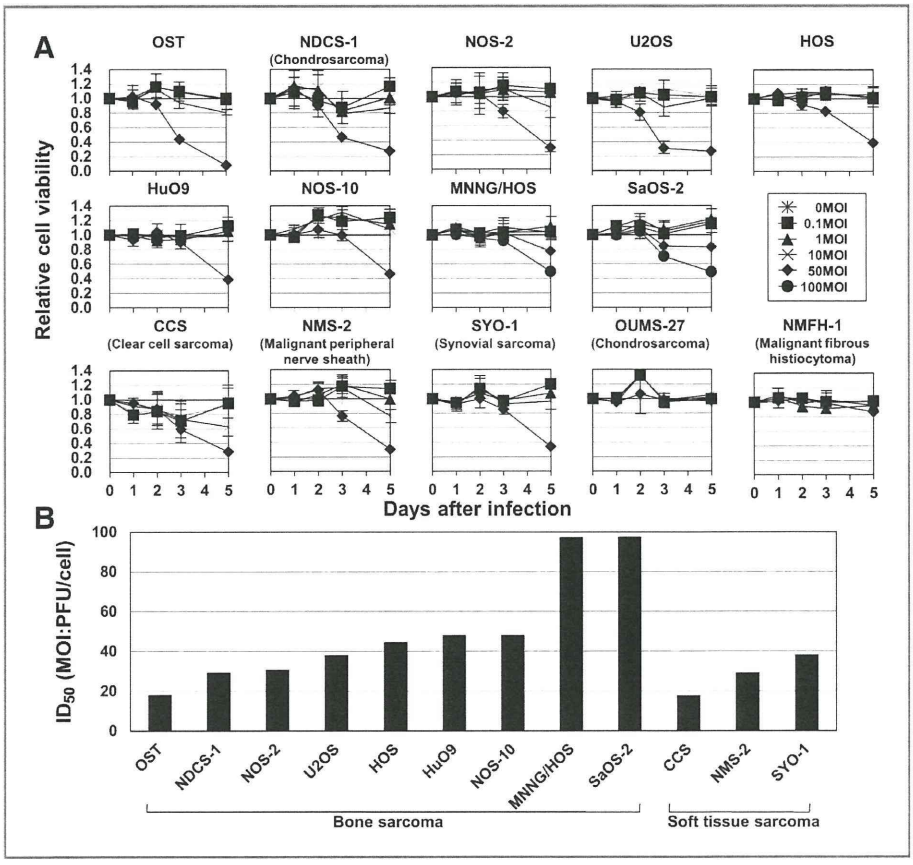

Figure 1. Cytopathic effect of OBP-301 on human bone and soft tissue sarcoma cell lines. A, cells were infected with OBP-301 at the indicated MOI, and cell survival was quantified over 5 days using the XTT assay. The cell viability of mock-treated group on each day was considered 1.0, and the relative 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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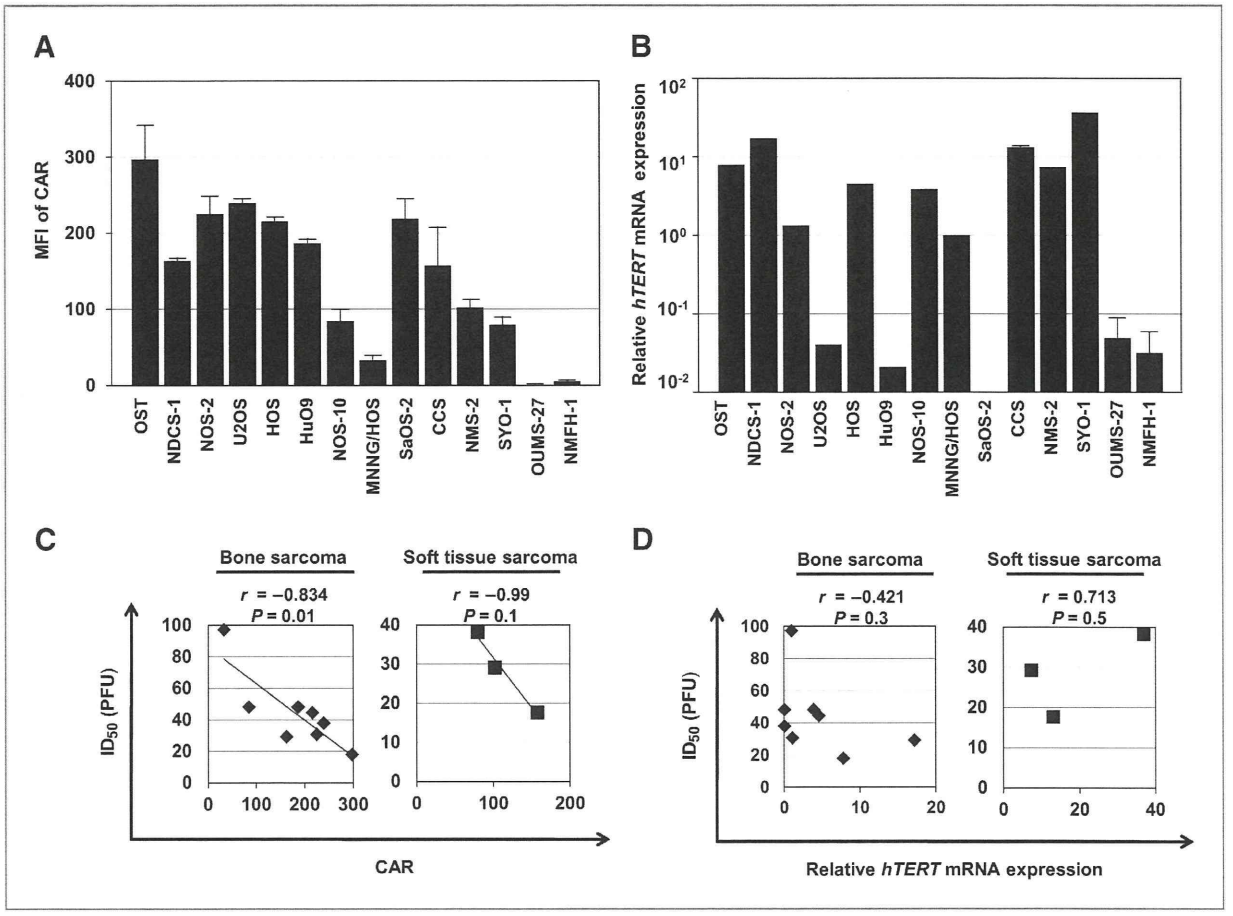

Figure 2. Relationship between the expression levels of CAR and *hTERT* mRNA and the cytopathic activity of OBP-301 against human bone and soft tissue sarcoma cell lines. A, the MFI of CAR expression on human bone and soft tissue sarcoma cells. The cells were incubated with a monoclonal anti-CAR (RmcB) 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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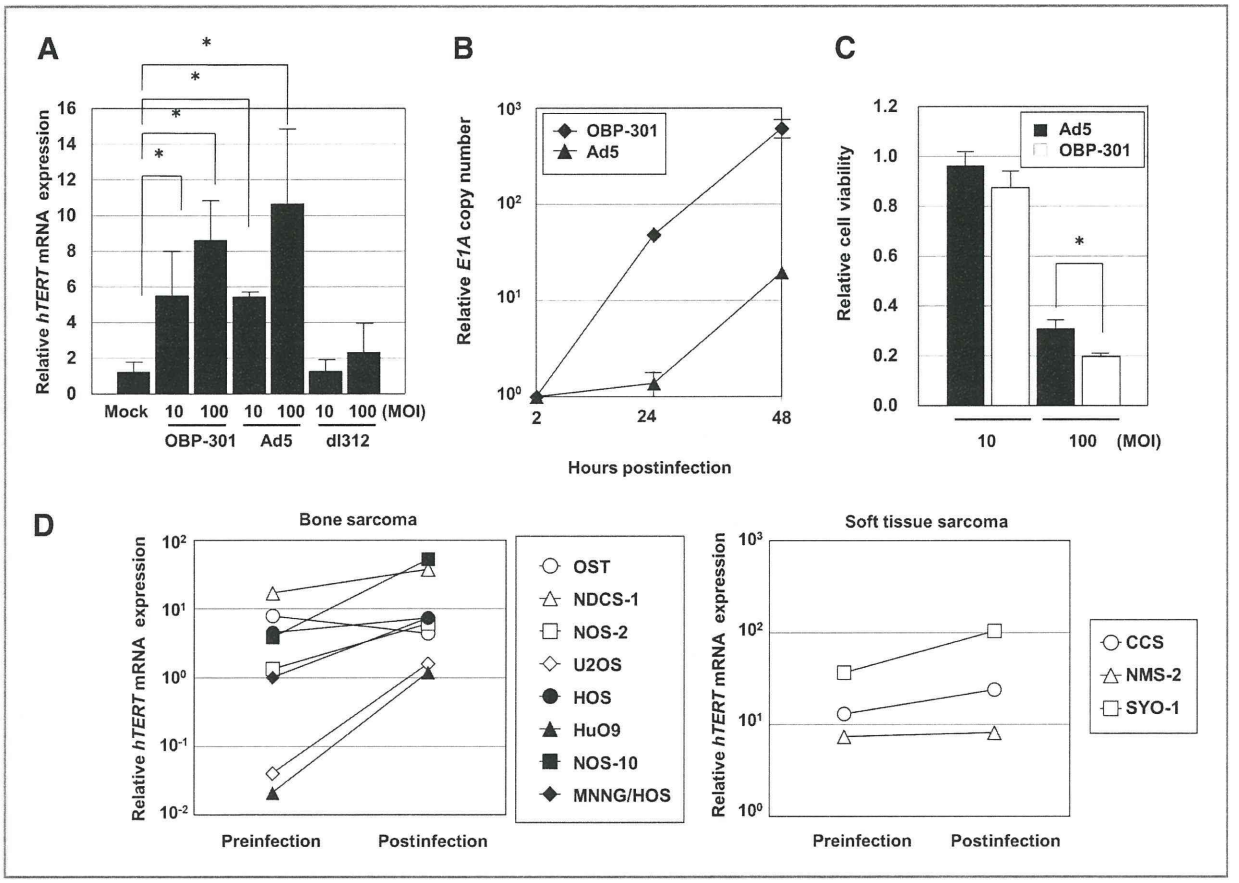

Figure 3. Upregulation of *hTERT* gene expression in ALT-type human sarcoma cell lines enhances the replication and the cytopathic effect of OBP-301. A, expression of *hTERT* mRNA in U2OS cells that were mock-infected or were infected with OBP-301, Ad5, or dl312. The cells were infected with OBP-301, 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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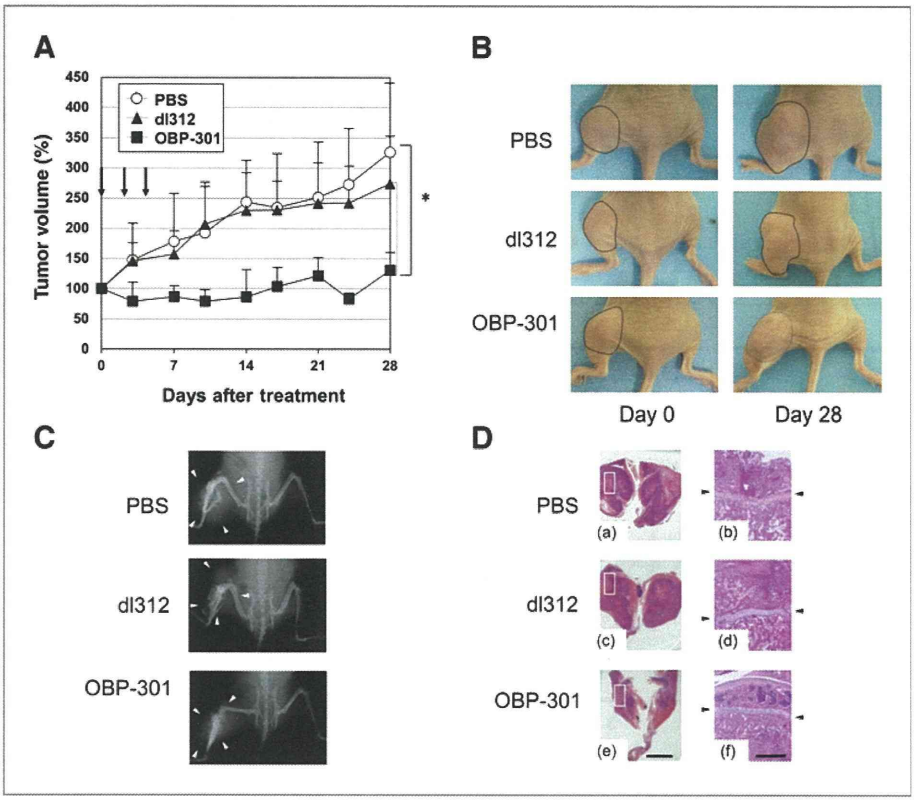

Figure 4. Antitumor effect of OBP-301 in an orthotopic OST bone sarcoma xenograft model. A, athymic nude mice were inoculated intratibially with OST cells (5×10^6 cells/site). Fourteen days after inoculation (designated as day 0), OBP-301 (■) or OBP-405 (▲) was injected into the tumor, with 1×10^8 PFUs 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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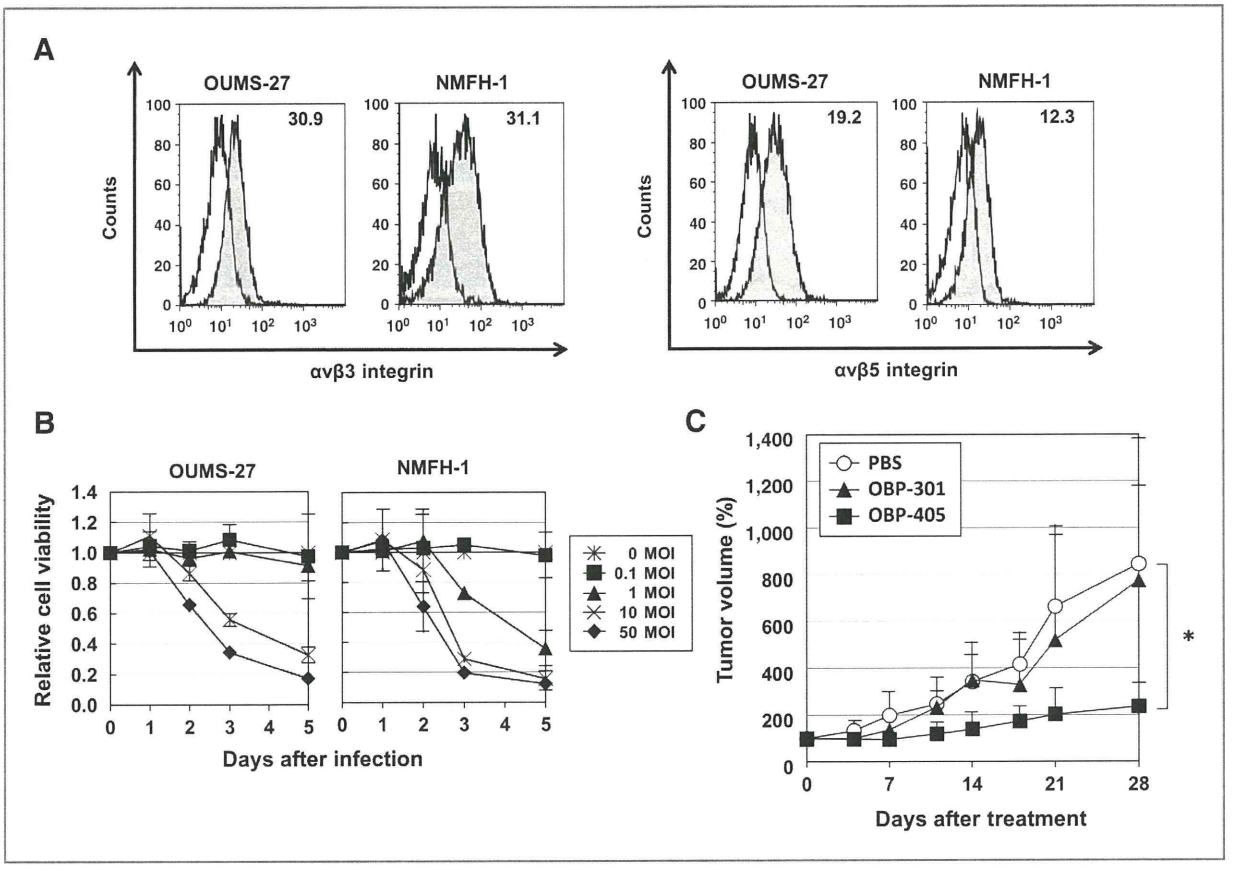

Figure 5. *In vitro* and *in vivo* antitumor effects of OBP-405 on OBP-301–resistant human sarcoma cell lines. A, expression of the integrins αvβ3 (left) and αvβ5 (right) on OUMS-27 and NMFH-1 cells. The cells were incubated with a monoclonal anti-αvβ3 integrin (LM609) or an anti-αvβ5 integrin (P1F6), 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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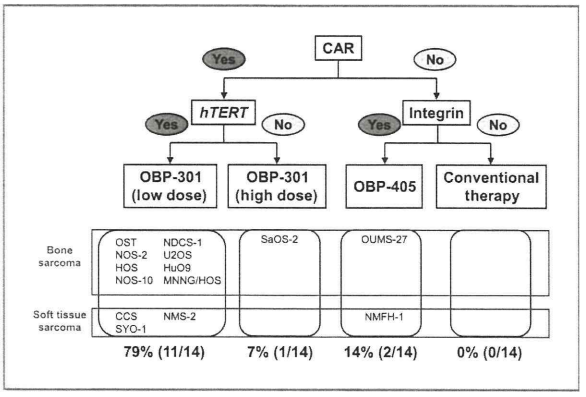

Figure 6. Outline of a therapeutic strategy for the use of telomerase-specific replication-selective oncolytic adenoviruses for human bone and soft tissue sarcoma cells. Assessment of CAR expression in tumor cells could serve as an indicator for OBP-301 or OBP-405 treatment. Of the 14 human sarcoma cell 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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