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

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Abbreviations: ALT, alternative lengthening of telomeres; CAR, Coxsackie and adenovirus receptor; hTERT, human telomerase reverse transcriptase; IRES, internal ribosome entry site; Ad5, wild-type adenovirus serotype 5; MOI, multiplicity of infection; PFU, plaque forming units; MFI, Mean fluorescent intensity; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; XTT, sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

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 non-epithelial malignant cells show low telomerase activity, suggesting lower effectivity 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-derived-OBP-405 through virus-integrin binding. Thus, a telomerase-specific oncolytic adenovirus would greatly improve the clinical outcome of young patients with advanced sarcomas.
Abstract

Purpose: Tumor-specific replication-selective oncolytic virotherapy is a promising antitumor therapy for induction of cell death in tumor, 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 non-epithelial malignant cells, which have low telomerase activity, maintain telomere length through alternative lengthening of telomeres (ALT). However, it remains unclear if OBP-301 is cytopathic for non-epithelial 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 OUUMS-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, (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, 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.
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 multi-agent 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 in order 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 - 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 (hTERT) (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) (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 two known telomere-maintenance mechanisms in human malignant tumors (15, 16); telomerase activation (4-6) and telomerase-independent alternative lengthening of telomeres (ALT) (17-19). The ALT-type mechanism is more prevalent in tumors arising from non-epithelial tissues than in those of epithelial origin (20, 21). Therefore, ALT-type non-epithelial 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 if OBP-301 can exert an antitumor effect on human non-epithelial malignancies as well as 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 target 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-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 \textit{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 \textit{in vivo} antitumor effect of OBP-301 was also confirmed using orthotopic animal models. Finally, the anti-tumor effect of OBP-405 against OBP-301-resistant sarcoma cells was evaluated \textit{in vitro} and \textit{in vivo}.

Materials and Methods

Cell lines

The human osteosarcoma (HuO9) (32), chondrosarcoma (OUUMS-27) (33) and synovial sarcoma (SYO-1) (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 lines CCS were 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; Manassas, VA) and was grown in McCoy’s 5a medium. The human osteosarcoma cell line MNNG/HOS was purchased from DS Pharma Biomedical (Osaka, Japan) and was maintained in Eagle’s minimum essential medium containing 1% non-essential 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 was maintained in DMEM. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂.

Recombinant adenoviruses

The recombinant tumor-specific, replication-selective adenovirus OBP-301 (Telomelysin), in which the promoter 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 type 5 (Ad5) were used as the control vectors. Recombinant viruses were purified by ultracentrifugation using cesium chloride step gradients, their titers were determined by a plaque-forming assay 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 × 10³ cells/well 20 h before viral infection. All cell lines were infected with OBP-301 or OBP-405 at multiplicity of infections (MOIs) 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, Indianapolis, IN) 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 of CAR expression**

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

**Quantitative real-time PCR analysis**

U2OS cells, seeded on 6-well plates at a density of 5 × 10$^5$ cells/well 20 h before viral infection, were infected with Ad5, OBP-301 or dl312 at an MOI of 10 or 100 PFU/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 h before viral infection and were infected with OBP-301.
at an MOI of 100 PFU/cell. Total RNA was extracted from the cells 2 days after virus infection by using the RNA-Bee™ reagent (Tel-Test Inc., TX). 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, Foster, CA) 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-infected and Ad5-infected U2OS cells, U2OS cells, seeded on 6-well plates at a density of $5 \times 10^6$ cells/well 20 h before viral infection, were infected with OBP-301 or Ad5 at an MOI of 10 PFU/cell. Genomic DNA was extracted from serially diluted viral stocks and tumor cells were infected with OBP-301 or Ad5 using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA). E1A copy number was also determined using TaqMan real-time PCR systems (Applied Biosystems).

**In vivo orthotopic 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 ($5\times10^6$ cells per site) were inoculated into the tibia or the flank of female athymic nude mice aged 6 to 7 weeks (Charles River Laboratories, Wilmington, MA). Palpable tumors developed within 14-21 days and were permitted to grow to approximately 5-6 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 using calipers. The volumes of OUMS-27 tumors were calculated using the 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, Tokyo, Japan) and an X-ray system (SOFTEX TYPE CMB, SOFTEX co, Kanagawa, Japan).

**Histopathological analysis**

Tumors were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections were stained with hematoxylin/eosin and analyzed using 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 ver. 18 software (SPSS Inc., Chicago, IL). 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 \textit{in vitro} cytopathic effect of OBP-301 against non-epithelial malignant cells, 14 tumor cell lines 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 using the XTT assay. OBP-301 infection induced cell death in 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 non-specific 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**

Since adenovirus infection efficiency depends mainly on cellular CAR expression (22), we determined the expression level of CAR on the 14 sarcoma cell lines using flow cytometry. The 12 OBP-301-sensitive sarcoma cell lines showed CAR expression, determined as mean fluorescent intensities (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 if 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 using quantitative real-time 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).

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 three 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 protein can activate the promoter activity of the hTERT gene (41, 42). Therefore, to determine if 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 PFU/cell (Fig. 3A). Compared to mock-infected U2OS cells, OBP-301-infected U2OS cells showed a 6-8 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 if 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 out 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 a 50.0 fold increase (Fig. 3D and Supplementary Table 1). 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.

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

To evaluate the in vivo antitumor effect of OBP-301 against human bone and soft tissue sarcomas, we used two 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 more than 10⁷ PFU) (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 using an MOI of 10⁸ PFU 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 to 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 if OBP-301-treated tumors were less destructive to surrounding normal tissues compared to control tumors, using X-ray and histological analyses (Fig. 4C and 4D). X-ray examination revealed that OBP-301-treated tumors resulted in less bone destruction compared to dl312- or PBS-treated tumors. Histological 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 OBP301-treated tumors.

With future clinical application in mind, we sought to establish a suitable protocol for repeated intratumoral injection of OBP-301 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 (greater than $10^8$ PFU) were determined in a similar manner to their determination for OST bone sarcoma cells (data not shown). OBP-301 was injected three times into the tumor, using an MOI of $10^9$ PFU and using intervals of either 1 day, 2 days, or 1 week between injections (Supplementary Fig. S5). A total of three OBP-301 injections, with intervals of 2 days or 1 week between injections, induced a significant suppression of tumor growth, whereas 1 day intervals 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 using flowcytometry (Fig. 5A). Both 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 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 if OBP-405 has an in vivo antitumor effect by assaying the effect of three intratumoral injections of OBP-301 or OBP-405, using an MOI of 10⁸ PFU or of control PBS, into subcutaneous OUMS-27 tumor xenografts. As shown in Fig. 5C, administration of OBP-405 resulted in significant suppression of tumor growth compared to 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.

**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, non-epithelial 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 demonstrated that OBP-301 induced cell death in 12 out 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, two OBP-301-resistant sarcoma cells that lack CAR expression were highly sensitive to fiber-modified OBP-405, which can infect cells by binding to surface integrin molecules (Fig. 5). Based on 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 treatment of patients 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 non-epithelial 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 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 to use fiber-modified 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 (Fig. 1 and Fig. 2). We further demonstrated 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 (Fig. 1 and Fig. 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 fiber-modified 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 1 day-intervals, when tumors are of a minimum size, might be more effective in inducing an antitumor effect than injection at 2 day- or 1 week-intervals. Surprisingly, continuous injection of OBP-301 at 1-day intervals, for 3 days, could not induce an antitumor effect. There are two possible explanations for these results. The first possibility is that 3 days of continuous injections may not provide enough time for OBP-301 to replicate and to 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, since 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.

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Figure legends

Fig. 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 multiplicity of infection (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. B, The 50% inhibiting doses of OBP-301 on cell viability 5 days after infection were calculated and are expressed as ID_{50} values. PFU, plaque-forming units; XTT, sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

Fig. 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 mean fluorescence intensity (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 flowcytometric detection using a FITC-labeled secondary antibody. B, Expression of hTERT mRNA in human bone and soft tissue sarcoma cells using 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.
Fig. 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 h, 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 PFU/cell and E1A copy number was analyzed over the following 2 days using quantitative real-time PCR. The value of the E1A copy number at 2 h 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 using an XTT assay. D, Expression of hTERT mRNA after infection of human bone (left panel) and soft tissue (right panel) sarcoma cell lines with OBP-301 at an MOI of 100 PFU/cell. Statistical significance (*) was determined as $P < 0.05$ (Student’s t test).

Fig. 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 x 10^6 cells/site). Fourteen days after inoculation (designated as day 0), OBP-301(▲) or OBP-405 (■) was injected into the tumor using an MOI of 1 x 10^8 PFU on day 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**, Histological analysis of the OST tumors. Tumor sections were obtained 28 d 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 \( \mu \)m.

**Fig. 5.** *In vitro* and *in vivo* antitumor effects of OBP-405 on OBP-301-resistant human sarcoma cell lines. **A**, Expression of the integrins \( \alpha v \beta 3 \) (left panel) and \( \alpha v \beta 5 \) (right panel) on OUMS-27 and NMFH-1 cells. The cells were incubated with a monoclonal anti-\( \alpha v \beta 3 \) integrin (LM609), or an anti-\( \alpha v \beta 5 \) integrin (P1F6), followed by flowcytometric detection using a FITC-labeled secondary antibody. The gray histogram represents integrin antibody staining. The number at the top right hand corner of each graph is the mean fluorescence intensity (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 \( (5 \times 10^6 \) cells/site). Fourteen days after inoculation (designated as day 0), OBP-301(▴) or OBP-405(■) was injected into the tumor using an MOI of \( 1 \times 10^8 \) PFU 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).

**Fig.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.
Fig. 3
# Clinical Cancer Research

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

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