

Fig. 1. Schematic DNA structures of telomerase-specific viruses and selective cytopathic effect in human SCCHN cell lines *in vitro*. **A**, OBP-301 is a telomerase-specific replication-competent adenovirus containing the hTERT promoter sequence inserted into the adenovirus genome to drive transcription of the E1A and E1B bicistronic cassette linked by the IRES. OBP-401 is a variant of OBP-301, in which the GFP gene is inserted under the cytomegalovirus (CMV) promoter into the E3 region for monitoring viral replication. **B**, human SCCHN cell lines were infected with OBP-301 at the indicated MOI values, and surviving cells were quantitated over 5 d by the XTT assay. The cell viability of mock-treated cells on day 0 was considered 1.0, and the relative cell viability was calculated. Points, mean of triplicate experiments; bars, SD. **C**, effects of various concentrations of OBP-301 on SAS-L and HSC-3 cells assessed 5 d after the XTT assay. Results are expressed as the relative cell viability of untreated control cells.

To confirm the specificity of telomerase activity in human SCCHN cells, we next measured the expression of *hTERT* mRNA in a panel of human SCCHN cell lines and normal cell lines by using a real-time reverse transcription-PCR method. Although the levels of expression varied widely, all SCCHN cell lines expressed detectable levels of *hTERT* mRNA, whereas human fibroblast cells such as NHLF and WI38 were negative for *hTERT* expression (Supplementary Fig. S1A). We also examined the expression levels of coxsackievirus and adenovirus receptor on the cell surface of each type of cell by flow cytometric analysis. Apparent amounts of coxsackievirus and adenovirus receptor expression were detected on SAS-L and HSC-3 human SCCHN cells (Supplementary Fig. S1B).

To assess whether viral replication was restricted to tumor cells, we next examined the replication ability of OBP-301 by measuring the relative amounts of E1A DNA. SAS-L human SCCHN cells and MRC-5 human fibroblasts were harvested at indicated time points over 72 h after infection with OBP-301 and subjected to quantitative real-time PCR analysis. The ratios were normalized by dividing the value of cells obtained 2 h after viral infection. OBP-301 replicated 3 to 4 logs within 48 h after

infection; the viral replication, however, was attenuated up to 2 logs in normal MRC-5 cells (Supplementary Fig. S2).

The response of tumor cells to DNA-damaging stimuli such as chemotherapeutic drugs and ionizing radiation is predetermined by the functional status of their *p53* gene (15); however, the *p53* status of human SCCHN cell lines (wild-type *p53* [SAS-L], mutant *p53* [SCC-4, HSC-2, HSC-3, HSC-4], and deleted *p53* [SCC-9]) is not related to their sensitivity to OBP-301. Indeed, OBP-301 similarly killed parental SAS-L cells and cells stably transfected with the mutant *p53* gene (Supplementary Fig. S3), suggesting that OBP-301 induces cell death in a *p53*-independent manner.

Selective replication of OBP-401 in human SCCHN cell lines *in vitro*. OBP-401 is a genetically engineered adenovirus that expresses GFP by inserting the GFP gene under the control of the cytomegalovirus promoter at the deleted E3 region of OBP-301 (Fig. 1A). To determine whether OBP-401 replication is associated with selective GFP expression in human SCCHN cells, cells were analyzed and recorded by using a time-lapse fluorescent microscope after OBP-401 infection. Representative images at the indicated time points are shown (Fig. 2A). SAS-L

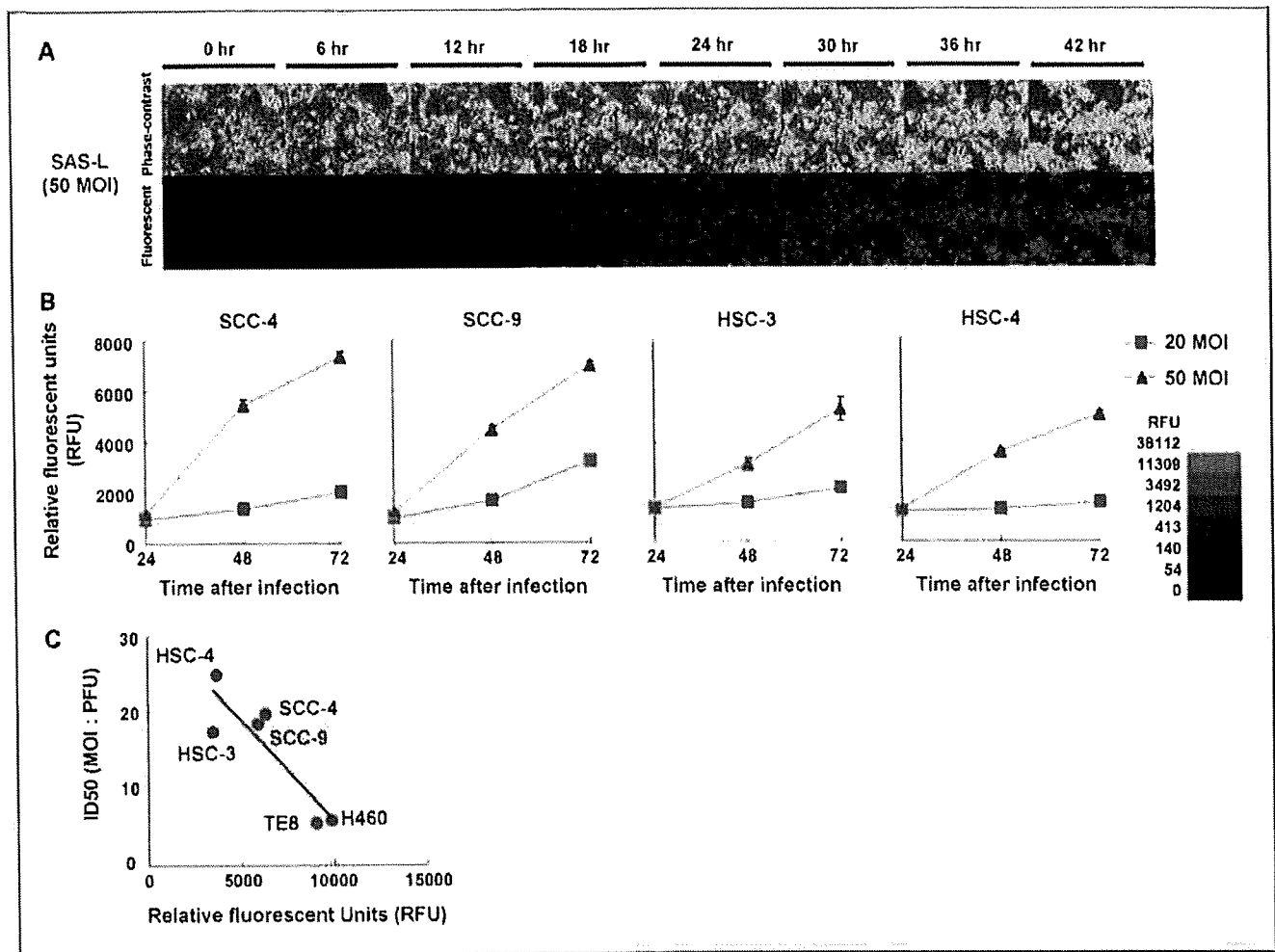


Fig. 2. Selective visualization of human SCCHN cells *in vitro* by OBP-401. A, time-lapse images of SAS-L cells were recorded for 42 h after OBP-401 infection at a MOI of 50. Representative images taken at the indicated time points show cell morphology by phase-contrast microscopy (top) and GFP expression under fluorescence microscopy (bottom). Magnification, $\times 200$. B, quantitative assessment of GFP labeling by OBP-401 in human SCCHN cell lines. Cells were infected with OBP-401 at the indicated MOI values, and GFP fluorescence was measured over 72 h by the fluorescence microplate reader. The intensity of green fluorescence was evaluated based on the brightness determinations used as relative fluorescence units (RFU). The relative fluorescence unit and time after infection were plotted on the ordinate and abscissa, respectively. A green color calibration bar for the indicated relative fluorescence unit is shown on the right. C, relationship between GFP fluorescence after OBP-401 infection and ID₅₀ values after OBP-301 infection in human cancer cell lines, including SCCHN cells. Relative GFP fluorescence was measured by the fluorescence microplate reader 72 h after OBP-401 infection at a MOI of 50. The ID₅₀ values of OBP-301 on cell viability at 5 d after infection were calculated and expressed as ID₅₀ values. The slope represents the inverse correlation between these two factors ($R^2 = 0.7839$).

human SCCHN cells expressed bright GFP fluorescence as early as 12 h after OBP-401 infection at a MOI of 50. The fluorescence intensity gradually increased in a dose-dependent manner, followed by rapid cell death due to the cytopathic effect of OBP-401, as evidenced by floating, highly light-refractile cells under phase-contrast photomicrographs.

We also quantified GFP expression in human SCCHN cells following OBP-401 infection by using a fluorescence plate reader. Relative expression levels of GFP gradually increased in a dose-dependent manner (Fig. 2B). Moreover, we found an apparent inverse correlation between relative GFP expression at 72 h after OBP-401 infection and the ID₅₀ values of OBP-301 in various human cancer cell lines including SCCHN cell lines (Fig. 2C), indicating that the outcome of OBP-301 treatment could be predicted by measuring GFP expression following OBP-401 infection.

In vivo antitumor effect of intratumoral injection of OBP-301 in an orthotopic nude mouse model of human SCCHN. To assess the effect of OBP-301 on SCCHN *in vivo*, we used an orthotopic animal model for SCCHN in which SAS-L cells were implanted into the tongues of BALB/c *nu/nu* mice. Histopathologic examination of the excised primary tumors showed a tumor formation composed of implanted SAS-L cells with a solid architecture (Fig. 3A). Mice bearing palpable SAS-L tumors with a diameter of 3 to 5 mm received three courses of intratumoral injections of 10^8 pfu of OBP-301 or PBS (mock treatment) every 3 days beginning on the 7th day (regimen 1) or 10th day (regimen 2) after the initial tumor inoculation (Fig. 3B). Representative images from each group showed that tumors treated with OBP-301 starting on day 7 after tumor inoculation were consistently smaller than those of mock-treated mice 28 days after the first viral injection (Fig. 3C).

Tumor growth at the primary site and body weight were continuously monitored. Intratumoral injection of OBP-301 in both regimens induced a gradual reduction in tumor volumes compared with mock-treated mice. Mice with tumor shrinkage significantly recovered body weight starting on day 10 (regimen 1) or day 15 (regimen 2) after the last virus injection ($P < 0.05$), although there was a decrease in body weight in the control group (Fig. 3D). This antitumor effect could be observed in mice orthotopically implanted with HSC-3 cells; the appearance of the effect, however, was ~4 to 5 days slower than that of SAS-L tumor-bearing mice (Supplementary Fig. S4).

Locoregional spread of virus following virotherapy in an orthotopic human SCCHN model. SCCHN patients with metastases to regional lymph nodes have a poorer prognosis than patients without nodal metastases (16). To verify whether adenoviruses could traffic to regional lymph nodes through the lymphatics, we injected 1×10^8 pfu of OBP-401 into SAS-L tumors implanted into the tongues of mice. Five days after virus injection, primary tongue tumors

as well as lymph node metastases could be detected as light-emitting spots with GFP fluorescence under the optical charge-coupled device imaging (Fig. 5A). We also found that OBP-401 could infect and replicate in SAS-L cells trafficking in lymphatic vessels (Fig. 5B). These results suggest that although adenoviruses could effectively drain to regional lymph nodes, OBP-401 replicated only in metastatic lymph nodes, which was confirmed by a histopathologic analysis. Metastatic SCCHN cells were mostly observed in the lymph nodes with fluorescence emission, whereas most of GFP-negative lymph nodes contained no tumor cells (Fig. 5C). The optical imaging detected 13 lymph nodes labeled in spots with GFP fluorescence in 14 metastatic nodes (sensitivity of 92.9%). Among 21 metastasis-free lymph nodes, 3 nodes were GFP positive (specificity of 85.7%). In another orthotopic model implanted with HSC-3 human SCCHN cells, we could also detect GFP signals in one or two metastatic lymph nodes but not in other nonmetastatic nodes and salivary glands (Fig. 5; Supplementary Fig. S5).

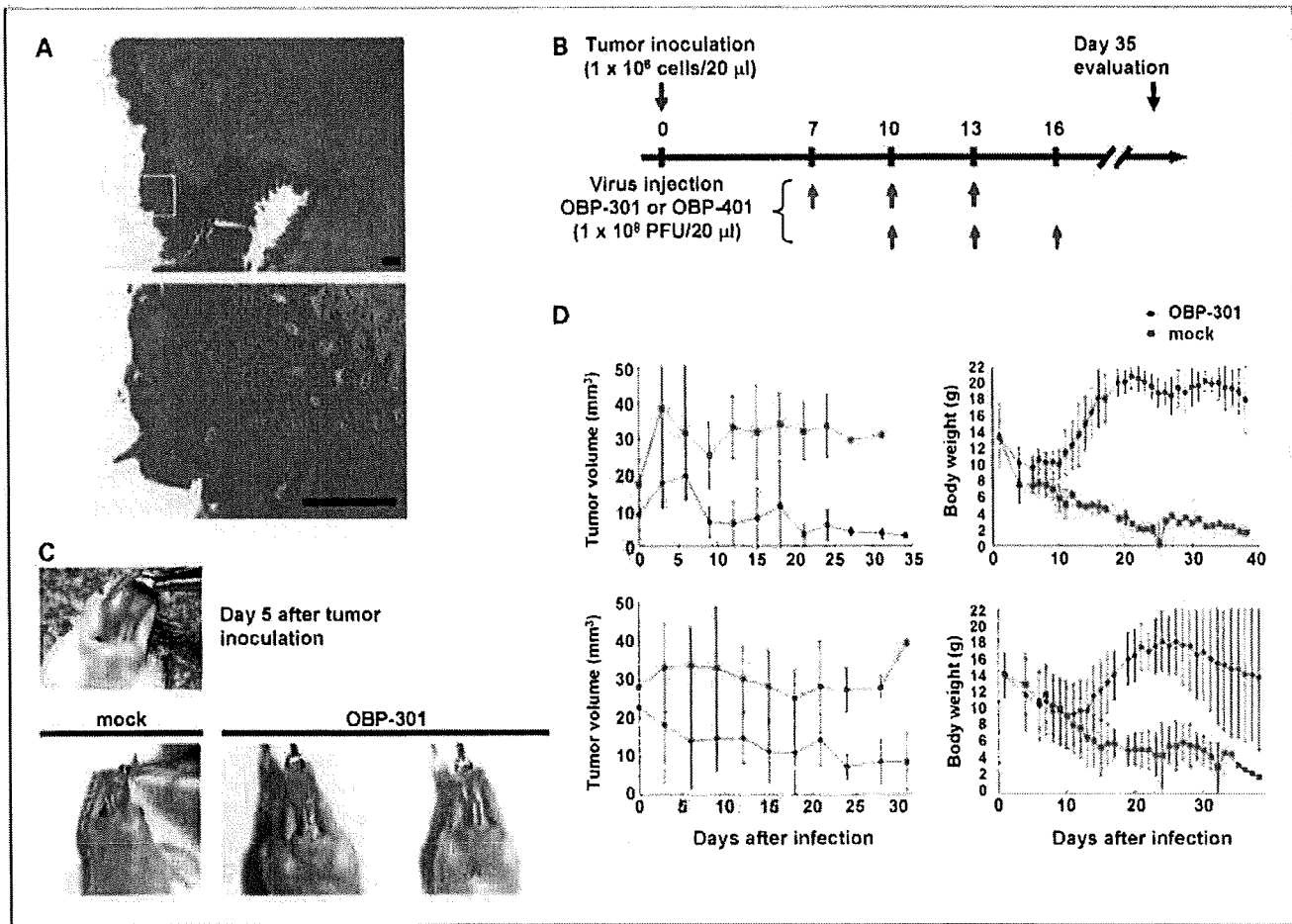


Fig. 3. Antitumor effects of OBP-301 *in vivo* in an orthotopic SCCHN model. **A**, tumor sections were obtained 35 d after tumor cell implantation. Paraffin-embedded sections of SAS-L tongue tumors were stained with H&E. Scale bar, 100 μm . Top, $\times 40$ magnification; bottom, detail of the boxed region of the top panel; magnification, $\times 400$. **B**, orthotopic animal experiment regimens. The tongues of BALB/c *nu/nu* mice were inoculated with 1×10^5 SAS-L human SCCHN cells. Orthotopic tumor-bearing mice received three courses of intratumoral injection of 1×10^8 pfu of viruses every 3 d starting on day 7 (regimen 1) or day 10 (regimen 2) after tumor cell inoculation. Eight mice were used in each group. **C**, macroscopic appearance of SAS-L tongue tumors on BALB/c *nu/nu* mice 5 d (top) or 35 d (bottom) after tumor cell inoculation. Representative tumors treated with PBS or OBP-301 are shown. Note the eradicated tumors in mice that received OBP-301 injection. Green arrowhead, SAS-L tumors. **D**, orthotopic tumor-bearing mice received three courses of intratumoral injection of 1×10^8 pfu of viruses every 3 d starting on day 7 (regimen 1, top) or day 10 (regimen 2, bottom) after tumor cell inoculation. The tumor volume (left) and the body weight (right) were monitored and plotted. Point, mean; bars, SD. Statistical significance was defined as $P < 0.05$.

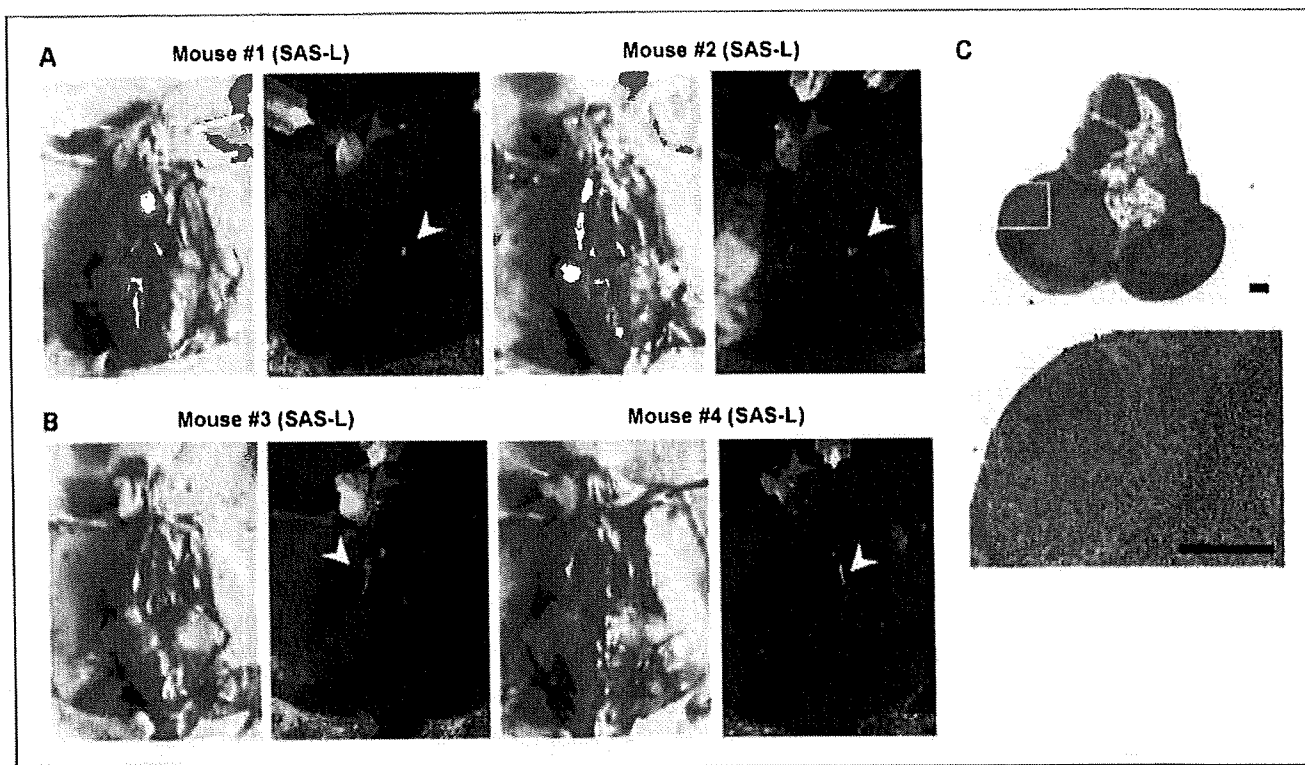


Fig. 4. Virus spread of OBP-401 via lymphatics to regional lymph nodes on SAS-L tumor-bearing mice. *A*, selective visualization of lymph node metastasis in orthotopic xenografts of SAS-L human SCCHN cells. Mice received intratumoral injection of OBP-401 (1×10^9 pfu) 24 d after tumor inoculation and were assessed for lymph node metastasis 5 d later under charge-coupled device imaging. Left, gross appearance; right, fluorescence image. Red arrowhead, primary tumor; white arrowhead, metastatic tumor cells. *B*, selective visualization of lymph node metastasis and lymphatic dissemination in orthotopic xenografts of SAS-L cells. Note the GFP-expressing disseminated tumor cells in lymphatics. Red arrowhead, primary tumor; white arrowhead, metastatic tumor cells in lymphatics. *C*, sections of GFP-positive lymph nodes were obtained 35 d after tumor cell implantation. Paraffin-embedded sections of lymph nodes were stained with H&E. Scale bar, 100 μ m.

Prolonged survival following OBP-301 virotherapy in an orthotopic human SCCHN model. Finally, we assessed the effect of intratumoral injection of OBP-301 on survival time of SAS-L-bearing mice. Mice treated with OBP-301 beginning either on the 7th day (regimen 1) or the 10th day (regimen 2) after tumor implantation survived significantly longer (mean = 27.4 or 33.7 days) than mice without treatment (mean = 14.7 or 24.3 days; regimen 1, $P = 0.017$; regimen 2, $P = 0.016$; Fig. 6). The prolonged survival might reflect an antitumor effect of oncolytic adenoviruses spreading into the locoregional area, including regional lymph nodes.

Discussion

The present study illustrates the potential application of replication-selective oncolytic adenoviruses as an anticancer agent in human SCCHN patients. We found that intratumoral administration of telomerase-specific oncolytic adenovirus induced tumor volume reduction as well as the recovery of weight loss by enabling oral ingestion in an orthotopic xenograft model, in which human SCCHN cells were implanted into the tongues of BALB/c *nu/nu* mice. Oncolytic virotherapy also prolonged the survival of SCCHN tumor-bearing mice, presumably due to the locoregional antitumor effect against primary tumors and lymph node metastases with viruses spreading into the lymphatics.

Telomerase-specific oncolytic adenovirus OBP-301 exhibits a broad cytopathic effect against human cancer cell lines of different tissue origins (8–10). In a panel of human SCCHN cell lines, OBP-301 also showed apparent antitumor effects *in vitro* in a dose-dependent manner (Fig. 1B), although the sensitivity varied greatly between cell lines despite hTERT and coxsackievirus and adenovirus receptor expression (Supplementary Fig. S1). We have previously found that the process of oncolysis is morphologically distinct from apoptosis and necrosis (17). The cell death machinery triggered by OBP-301 infection is still under the investigation, although autophagy is partially involved in this effect (17, 18). OBP-301 has been developed based on the ability of the hTERT promoter to control replication of the virus in the tumors, leading to selective killing of tumor cells and minimal undesired effects on normal cells; the ID₅₀ values of OBP-301 in various human cancer cell lines, however, were not related to the levels of hTERT mRNA expression (8, 10). Indeed, HSC-3 and HSC-4 human SCCHN cells expressing high levels of hTERT mRNA were less sensitive to OBP-301 than SCC-4 and SCC-9 cells with low levels of hTERT expression. Thus, neither hTERT expression nor coxsackievirus and adenovirus receptor expression could be useful for predicting the outcome of OBP-301 treatment.

Biomarkers have been extensively studied and often used to predict the potential therapeutic benefit of new agents, including molecular-targeted therapies (19). There is a widely recognized need for biomarkers that could improve the

clinician's ability to select suitable drugs for appropriate patients. We found that the levels of GFP expression following OBP-401 infection were highly associated with ID_{50} values of OBP-301 in individual cell lines *in vitro* (Fig. 2C). This correlation may be an expected result, because OBP-301 and OBP-401 have the same genomic backbone except for the GFP expression cassette. Although it is necessary to establish the assay procedures for GFP-based fluorescence measurement in more detail, we propose the diagnostic application of OBP-401 to predict tumor responses to OBP-301. For example, when the biopsy tissue samples of the tumor are exposed to OBP-401 for a certain amount of time *ex vivo*, the levels of GFP expression may be of value as a positive predictive marker for the outcome of OBP-301 virotherapy. Further prospective clinical studies are required to confirm the direct correlation between the GFP expression in biopsy samples following *ex vivo* OBP-401 infection and the clinical responses to OBP-301 in patients with SCCHN.

An orthotopic nude mouse model to investigate the cellular and molecular mechanisms of metastasis in human neoplasia was first described by Fidler et al. (20, 21) and Killion et al. (22). The orthotopic implantation of tumor cells restores the

correct tumor-host interactions, which do not occur when tumors are implanted in ectopic subcutaneous sites (20). To further explore the *in vivo* antitumor effects of telomerase-specific virotherapy for SCCHN, we used an orthotopic nude mouse model of human tongue squamous cell carcinoma. In our preliminary experiments, we inoculated tumor cells into the tongue of BALB/c *nu/nu* mice and confirmed the formation of tumors with a diameter of 3 to 5 mm after 5 days and the development of metastases in neck lymph nodes after 35 days. We also identified the presence of disseminated tumor cells in the regional lymph nodes at least 10 days after tumor cell implantation by using GFP-expressing SAS-L human SCCHN cells (data not shown). Intratumoral injection of OBP-301 done 7 or 10 days after tumor inoculation significantly shrunk the tongue SAS-L tumor volumes, which in turn increased the body weight of mice by enabling oral ingestion (Fig. 3D). Moreover, HSC-3 cells were relatively resistant to OBP-301 *in vitro*; intratumoral injection of OBP-301 was, however, effective for recovering the body weight in mice bearing HSC-3 tongue tumors after a long-term observation (Supplementary Fig. S4). These results suggest that although the appearance of the effect may be slower, the *in vivo* antitumor activity could be

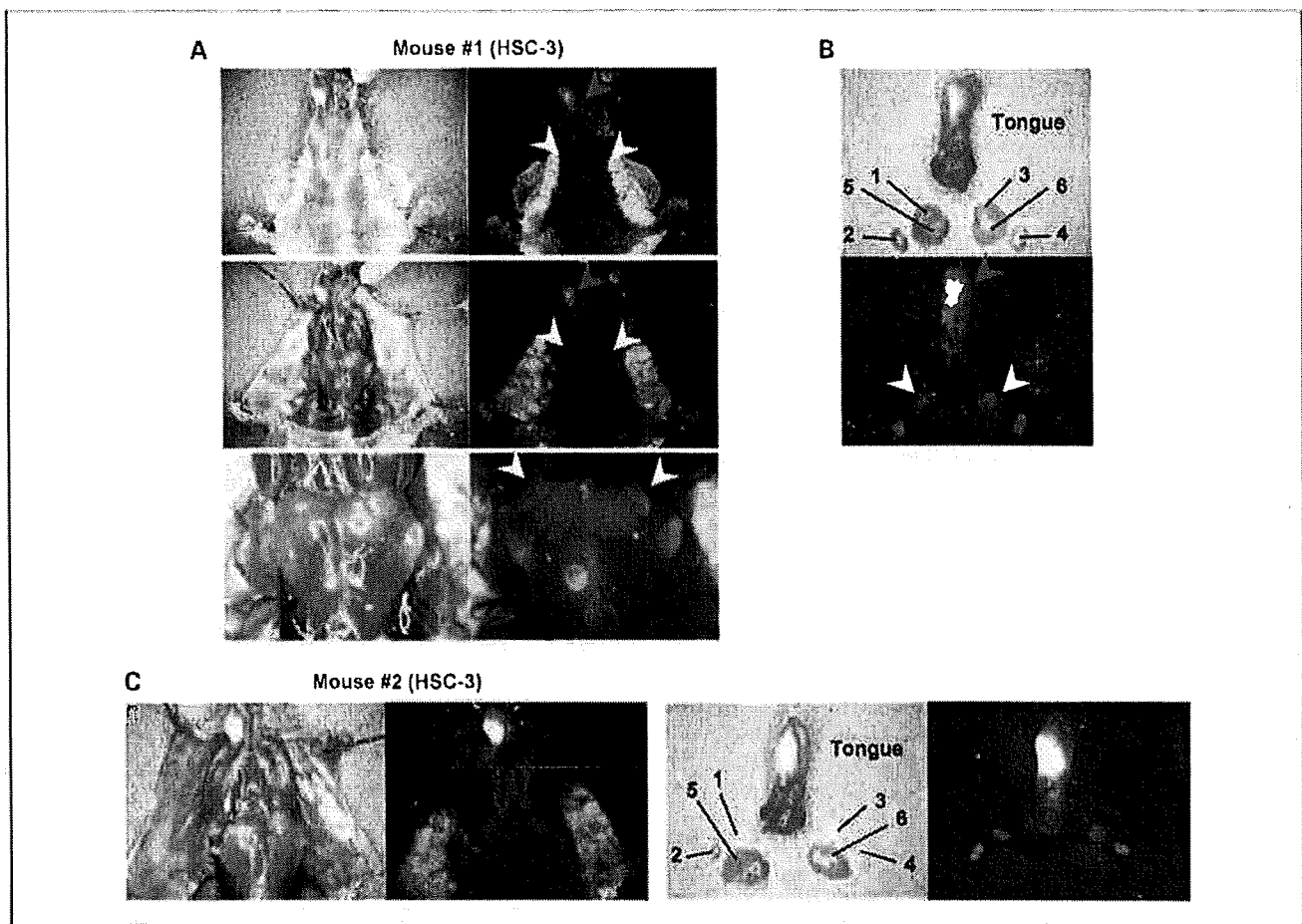


Fig. 5. Virus spread of OBP-401 via lymphatics to regional lymph nodes on HSC-3 tumor-bearing mice. **A**, selective visualization of lymph node metastasis in orthotopic xenografts of HSC-3 human SCCHN cells. Mice received intratumoral injection of OBP-401 at the concentration of 1×10^8 pfu after 24 d of tumor inoculation and were assessed for lymph node metastasis 5 d later under fluorescence stereomicroscope. **B**, HSC-3 primary tumor, salivary glands, and lymph nodes were excised 5 d after OBP-401 injection and then assessed for GFP fluorescence. 1 to 4, lymph nodes; 5 and 6, salivary glands. **C**, other HSC-3 tumor-bearing mice. Excised primary tumors, salivary glands, and lymph nodes were assessed for GFP fluorescence.

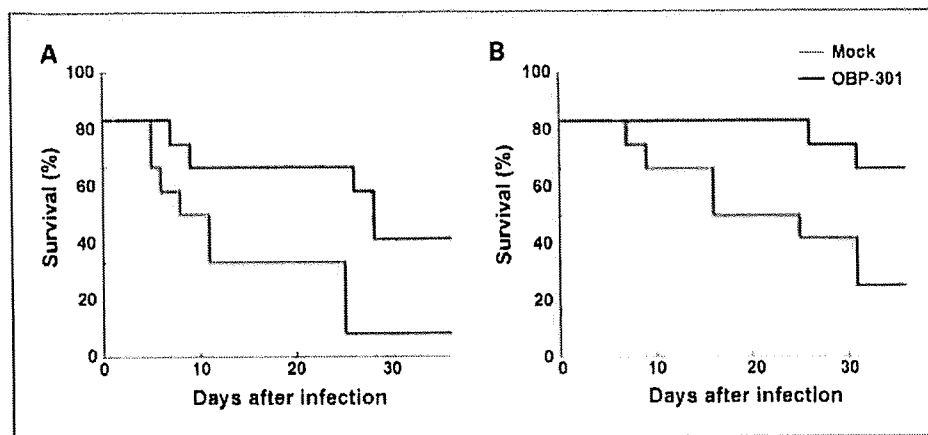


Fig. 6. Prolonged survival of SAS-L tumor-bearing mice treated with OBP-301. Mice bearing SAS-L xenografts were treated starting on day 7 (regimen 1; A) or day 10 (regimen 2; B) after tumor cell inoculation as described in Fig. 3A. Survival was monitored over time after virus injection and plotted as a Kaplan-Meier plot.

expected even in resistant SCCHN tumors. Because the body weight loss due to a feeding problem in this orthotopic SCCHN model resembles the disease progression in SCCHN patients, the finding that OBP-301 increased the body weight of mice suggests that OBP-301 virotherapy could potentially improve the quality of life in advanced SCCHN patients.

Amplified viruses can infect adjacent tumor cells as well as reach metastatic lymph nodes via the lymphatic circulation. We have previously shown that the telomerase-specific OBP-401-expressing GFP could be delivered into human tumor cells in regional lymph nodes and replicate with selective GFP fluorescence after injection into the primary tumor in an orthotopic rectal tumor model (11). In the orthotopic SCCHN model, OBP-401 spread into the neck lymph nodes after injection into the primary tongue tumor and selectively replicated in metastatic nodules (Figs. 4 and 5; Supplementary Fig. S5). The sensitivity and specificity of this imaging strategy for SAS-L tumors are 92.9% and 85.7%, respectively, which are sufficiently reliable to support the concept of this approach. These results suggest that surgeons may be able to excise primary tumors as well as metastatic lymph nodes precisely with appropriate margins by using this novel surgical navigation system with OBP-401. Moreover, the therapeutic profiles of OBP-401 and OBP-301 are considered similar, and a histopathologic analysis showed the destruction of micrometastases by virus in metastatic lymph nodes. This regional antitumor effect of oncolytic viruses could have a significant effect on the prolongation of the survival of mice bearing orthotopic tumors (Fig. 6).

Targeted therapies such as the anti-epidermal growth factor receptor monoclonal antibody cetuximab and other small-molecule epidermal growth factor receptor-tyrosine kinase inhibitors have been developed for SCCHN. Although a phase III trial showed a survival benefit with cetuximab and standard platinum-based therapy in SCCHN patients (23), some patients are exquisitely sensitive to these drugs and can develop

particular and severe toxicities (24). A phase I study is currently under way in the United States to determine the feasibility and to characterize the pharmacokinetics of OBP-301 in patients with histologically proven nonresectable solid tumors (25). An interim analysis of the first 12 patients, including four SCCHN patients treated with escalating doses of OBP-301, indicates that OBP-301 virotherapy is well tolerated without any severe adverse events, suggesting that OBP-301 may be much more potent than other targeted therapies for human SCCHN in terms of specificity, efficacy, and toxicity.

In conclusion, our data clearly indicate that telomerase-specific oncolytic adenoviruses have a significant therapeutic potential against human SCCHN *in vitro* and *in vivo*. Moreover, these viruses can be used in an *ex vivo* diagnostic assay to predict the therapeutic potential of the virus in SCCHN patients. The combination of a diagnostic assay with a therapeutic entity is termed theranostics (26). Telomerase-specific oncolytic viruses can be used to treat the patients and to identify the patients who will likely benefit from virotherapy (Supplementary Fig. S6). In addition, telomerase-specific *in situ* imaging strategy has a potential of being widely available in humans as a navigation system in the surgical treatment of SCCHN. Thus, our oncolytic virus-based approach might be a novel "virotheranostics" for SCCHN. Phase II studies of telomerase-specific virotheranostics in advanced SCCHN patients are warranted.

Disclosure of Potential Conflicts of Interest

H. Onimatsu and Y. Urata are employed by Oncolys BioPharma, Inc. T. Fujiwara is a consultant to Oncolys Biopharma, Inc.

Acknowledgments

We thank Daiju Ichimaru and Hitoshi Kawamura for their helpful discussions and Tomoko Sueishi for her excellent technical support.

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Telomerase-specific virotherapy in an animal model of human head and neck cancer

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Abstract

Telomerase-specific replication-competent adenovirus, Telomelysin (OBP-301), has a human telomerase reverse transcriptase promoter that regulates viral replication and efficiently kills human cancer cells. The objectives of this study are to examine the effects of OBP-301 in squamous cell carcinoma of the head and neck cells *in vitro* and in xenografted animals *in vivo*. OBP-301 was found to be cytotoxic to the YCUT892, KCCT873, KCCT891, KCCL871, YCUM862, HN12, and KCCOR891 cell lines *in vitro*. However, the level of cytotoxicity is not correlated with the expression levels of adenoviral receptors, which may be required for adenoviral infection in squamous cell carcinoma of the head and neck cells. OBP-301 shows remarkable antitumor activity against established s.c. KCCT873 tumors in immunodeficient animals in a dose-dependent manner. In addition, no significant toxicity was observed in animals receiving treatment. These results suggest that OBP-301 is a novel therapeutic agent with promise for the treatment of human head and neck cancers. [Mol Cancer Ther 2009;8(1):171–7]

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) accounts for 5% of newly diagnosed adult cancers in the United States and 8% of cancers worldwide (1). Most patients are treated with various combinations of surgery,

radiotherapy, and systemic agents (2). Despite major advances in the treatment of locoregionally advanced SCCHN, such as the introduction of novel chemotherapy regimens and inhibitors of the epidermal growth factor receptor, treatment fails in about half of the patients (3). The median survival of patients with recurrent or metastatic SCCHN who undergo chemotherapy is 6 to 9 months (4). Therefore, a considerable number of patients with SCCHN need additional treatment as the disease progresses.

Virotherapy, the approach to treat cancer with virus, has been done in some clinical trials; for example, clinical trials primarily using *p53* gene replacement (INGN-201; a replication-competent adenoviral-based vector expressing wild-type *p53*) have provided the basis for the design of ongoing randomized gene therapy clinical trials in SCCHN patients in the United States (5). Although systemic administration is probably required in the case of micro-metastatic disease, virotherapy has some promise when tumor is limited to the head and neck. SCCHN is a particularly attractive model because most primary and recurrent lesions are easily acceptable to direct injection (6). Potential usage of virotherapy may include the perioperative application in the surgical wound and the addition of intratumoral (i.t.) virotherapy to current standard options, such as radiotherapy and/or chemotherapy.

Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosomal ends (7). Many studies have shown the expression of telomerase activity in >85% of human cancers (8) but only in a few normal somatic cell types (9). Telomerase activation is considered to be a critical step in carcinogenesis, and its activity is closely correlated with human telomerase reverse transcriptase (hTERT) expression (10). Therefore, the hTERT proximal promoter can be used as a molecular switch for selective expression of target genes in tumor cells. Replication-selective tumor-specific adenoviruses are being developed as novel anticancer therapies (11–14). In this context, an adenoviral vector that drives E1A and E1B genes under the hTERT promoter has been developed, termed Telomelysin or OBP-301 (15). OBP-301 can replicate in and lyse only cancer cells but not normal cells, and its strong cytotoxic activity were shown in a variety human cancer cells (15–17). Also, OBP-301-mediated oncolysis induces uric acid production as a danger signal and stimulates CTL activity via proteasome activator up-regulation (18).

The infection efficiency of recombinant adenoviral vectors varies widely depending on the expression of the primary receptor, the coxsackie adenovirus receptor (CAR); the secondary receptors, integrin $\alpha_v\beta_3$ and integrin $\alpha_v\beta_5$; and the tertiary receptor, heparan sulfate glycosaminoglycans (HSG; refs. 19, 20). The first step is the attachment of

Received 7/1/08; revised 9/26/08; accepted 10/23/08.

Grant support: Japanese Ministry of Health, Labour and Welfare grant-in-aid KH33332 (K. Kawakami).

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doi:10.1158/1535-7163.MCT-08-0620

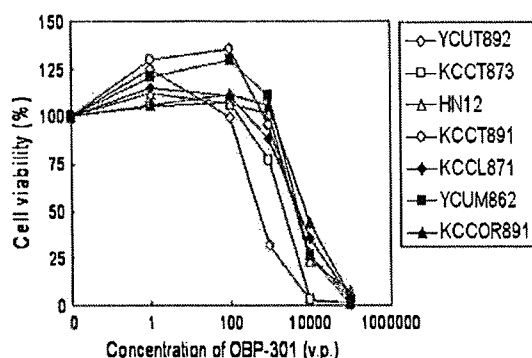


Figure 1. Sensitivity of SCCHN cells to OBP-301 *in vitro*. Cytotoxic activity of OBP-301 on 13 SCCHN cell lines was evaluated by XTT assay. Cells were cultured with various concentrations of OBP-301 (0-100,000 vp/mL). Mean \pm SD of quadruplicate determinations. The assay was repeated three times.

the virus to the cell surface through CAR (20). Following attachment, the internalization of the virus into cells occurs through the integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$ that are expressed in most cell types (19).

Previously, OBP-301 has been reported to induce cell death of human non-small cell lung, colorectal, and prostate cancers *in vitro* and *in vivo* (15, 17). The present study investigates the cytotoxic activity of OBP-301 in 13 SCCHN cell lines and the association between cytotoxic activity and adenoviral receptor expression. We also assessed the *in vivo* antitumor activity and toxicity and tolerability of OBP-301 in an athymic nude mouse model with KCCT873 SCCHN tumors.

Materials and Methods

Adenovirus

The recombinant replication-selective, tumor-specific adenoviral vector OBP-301 was provided by Oncolys Biopharma. The hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (15). The virus particle (vp) titer-to-infection titer (plaque-forming units) ratios were 110:3.

Cells

The human non-small cell lung cancer cell line H1299 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L HEPES (Nacalai Tesuque), 100 μ g/mL penicillin, and 100 μ g/mL streptomycin (Nacalai Tesuque). The SCCHN cell line HN12 was grown in MEM containing 10% fetal bovine serum, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin. The SCCHN cell lines YCUT892, KCCT873, KCCT891, KCCL871, YCUM862, KCCOR891, YCUL891, YCUM911, YCUMS861, YCUT891, 012SCC, and Wmm-SCC (21) were cultured in RPMI 1640 containing 10% fetal bovine serum, 1 mmol/L HEPES, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin.

Cell Viability Assay

The XTT assay was done to measure cell viability. Briefly, cells were plated on 96-well plates at 1×10^3 per well 24 h

before viral infection. Cells were then infected with 1 to 1×10^5 multiplicity of infection (vp) of OBP-301 and further cultured for 120 h. Cell viability was determined using the Cell Proliferation Kit II (Roche Diagnostics) according to the protocol provided by the manufacturer.

Flow Cytometry

Cells (1×10^5) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Cell Signaling Solution), anti-integrin $\alpha_V\beta_3$ (Chemicon), anti-integrin $\alpha_V\beta_5$ (Chemicon), or anti-heparan sulfate (Seikagaku) for 60 min at 4°C, incubated with FITC-conjugated goat anti-mouse IgG secondary antibody (Chemicon), and analyzed by the FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Control cells were incubated with anti-mouse IgG primary antibody (BD Bioscience) and FITC-conjugated goat anti-mouse IgG secondary antibody. G-means were calculated by the following formula: (G-means of antibody-treated cells) - (G-means of control cells). Correlation coefficients were obtained between the expression levels of CAR, integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, HSG, and the ID₅₀ of OBP-301 in 7 SCCHN cell lines.

Quantitative Real-time PCR Analysis

Total RNA from cultured cells was obtained using the RNeasy Mini kit (Qiagen). Total RNA ($\sim 0.1 \mu$ g) was used for reverse transcription. Reverse transcription was done at 22°C for 10 min and then at 42°C for 20 min. The hTERT mRNA copy number was determined by real-time quantitative reverse transcription-PCR using a LightCycler instrument and a LightCycler DNA TeloTAGGG kit (Roche Diagnostics). PCR amplification was run with activation at 95°C for 15 s, annealing at 58°C for 10 s, and extension at 72°C for 9 s.

Athymic Nude Mouse Models of Human Head and Neck Cancer

Five- to 6-week-old female athymic nude mice (BALB/c *nu/nu*) were obtained from SLC. Animal care was in

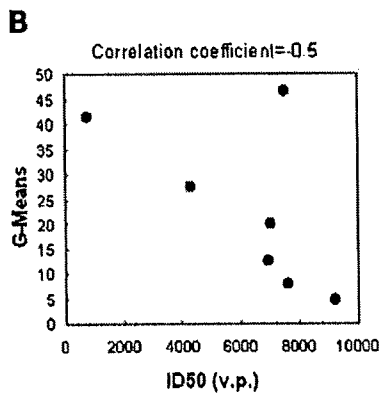
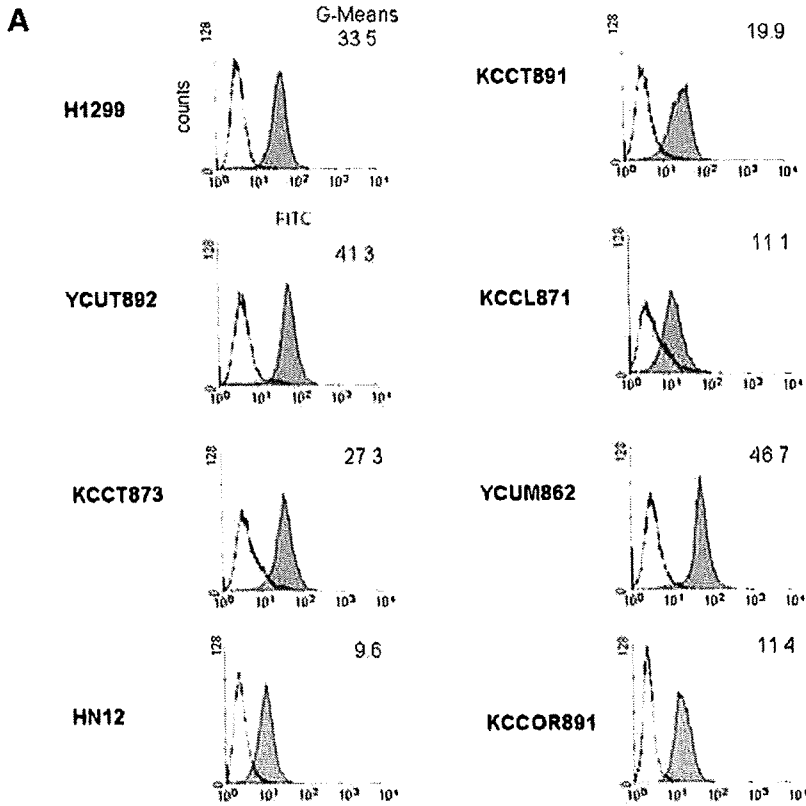
Table 1. Cytotoxic activity of adenoviral receptors on head and neck cancer cell lines

Cell line	Origin	ID ₅₀ (vp)*
YCUT892	Tongue	759
KCCT873	Tongue	4,279
HN12	Lymph node	6,943
KCCT891	Hypopharynx	7,025
YCUM862	Oropharynx	7,512
KCCL871	Larynx	7,599
KCCOR891	Oral floor	9,204
YCUL891	Larynx	ND
YCUM911	Oropharynx	ND
YCUMS861	Maxillary sinus	ND
YCUT891	Tongue	ND
012SCC	Unknown	ND
Wmm-SCC	Unknown	ND

Abbreviation: ND, not done.

*ID₅₀, infection dose of OBP-301 at which 50% inhibition of cell viability is observed compared with untreated cells.

Figure 2. Expression of the CAR in SCCHN cell lines. **A**, cells were incubated with mouse monoclonal anti-CAR (RmcB) followed by detection with FITC-labeled secondary antibody. *Gray histogram*, staining with anti-CAR antibody treatment. H1299 human lung cancer cells were used as a positive control. **B**, correlation between CAR expression in SCCHN cells and the ID₅₀ of OBP-301 for these cells. **C**, correlation between integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ and HSG expression in SCCHN cells and the ID₅₀ of OBP-301 for these cells. The experiment was repeated three times.



C

Cell line	$\alpha_V\beta_3$	$\alpha_V\beta_5$	HSG
H1299	5.16	9.6	27.1
YCUT892	0.5	7.7	102.4
KCCT873	31.1	10.8	9.6
HN12	1.9	4.4	7.0
KCCT891	1.6	5.5	7.7
KCCL871	2.9	4.6	105.4
YCUM862	1.5	1.3	20.7
KCCOR891	2.0	8.8	156.5
correlation coefficient	-0.2	-0.3	0.1

accordance with the guidelines of the Kyoto University School of Medicine. A SCCHN model was established in nude mice by s.c. injection of KCCT873 tumor cells (5×10^6) in 150 μ L PBS into the flank. Palpable tumors developed within 3 to 4 days. Tumors were measured by vernier calipers. Six to 7 mice were used for each group.

Toxicity Assessment

Blood samples and organs were collected from athymic nude mice at day 10 or 17 after i.t. administration of OBP-301 (3×10^{10} vp/d for days 5-9). Organs from the experimental animals were fixed in 10% formalin, and 5 μ m tissue sections were prepared and stained with H&E.

Statistical Analysis

Tumor volume on a given day was calculated by the following formula: (length of the tumor) \times (width of the tumor)² / 2. The statistical significance of tumor regression was calculated by the Student's *t* test.

Results

Cytotoxic Activity of OBP-301 to Various SCCHN Cell Lines

We first examined the effect of OBP-301 infection on the viability of SCCHN cell lines assessed by the XTT assay.

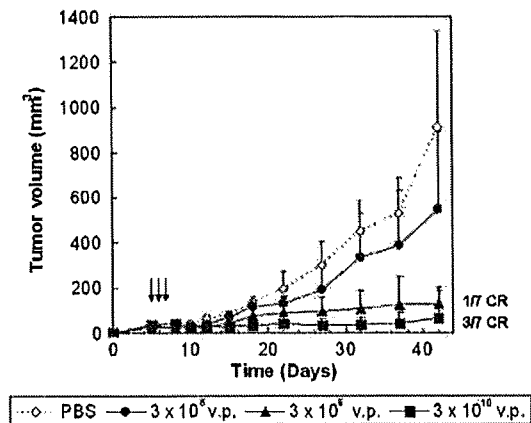


Figure 3. OBP-301 acted in a dose-dependent manner in KCCT873 tumor-bearing mice. Athymic nude mice received s.c. implantation of 5×10^6 KCCT873 cells on day 0. Animals then received injections of OBP-301 at the doses of 3×10^8 (●), 3×10^9 (▲), or 3×10^{10} vp (■) on days 5 to 7 (total of three injections). Each group had 7 animals, and the injection volume was 30 μ L in each tumor. Arrows, day of injections. Bars, SD. The experiment was repeated two times.

Because OBP-301 showed slightly cytotoxic activity against 7 of 13 cell lines at a dose of 1,833 vp (50 plaque-forming units/cell), we assessed the ID_{50} of OBP-301 using these 7 SCCHN cell lines (Fig. 1; Table 1). As shown in Table 1, OBP-301 shows modest to strong cytotoxic activity in the 7 cell lines tested, with the ID_{50} varying from 759 to 9,204 vp. The cytotoxic activity of OBP-301 in these cell lines shows dose dependence (Fig. 1). YCUT892 cells were most sensitive to OBP-301 followed by KCCT873, HN12, KCCT891, YCUM862, KCCL871, and KCCOR871 cells, suggesting that 2 of the SCCHN cell lines are most sensitive to OBP-301.

Expression of Adenovirus Receptors in SCCHN Cell Lines

Because the cytotoxic activity of OBP-301 was anticipated to be correlated with efficiency of adenoviral infection through CAR, integrin $\alpha v \beta_3$, integrin $\alpha v \beta_5$, or HSG receptors (22), we then assessed the expression levels of CAR on SCCHN cells using flow cytometry. As shown in Fig. 2A, all 7 SCCHN cell lines have been found to express detectable levels of CAR. However, as shown in Fig. 2B, correlation between the cytotoxic activity of OBP-301 and the expression level of CAR was not significant (correlation coefficient = -0.5). In addition, we assessed the expression of integrin $\alpha v \beta_3$, integrin $\alpha v \beta_5$, and HSG in SCCHN cell lines; however, the expression levels were not correlated with cytotoxic activity of OBP-301 (Fig. 2C). We also assessed *hTERT* expression using the quantitative PCR method and found that all of the SCCHN cell lines express detectable levels of *hTERT* mRNA; however, there was no correlation between the expression levels and ID_{50} of OBP-301 in these cells (data not shown). These results suggest that, although 7 of 13 SCCHN cell lines are sensitive to OBP-301, its ability to enter the cell is not necessarily correlated with the degree of cytotoxicity.

Antitumor Effect of OBP-301 in SCCHN-Bearing Animals

To assess the antitumor effect of OBP-301 in the animal model of human SCCHN, KCCT873 cells were implanted s.c. in athymic nude mice (6, 23) to examine the effect of OBP-301 at a variety of dosages *in vivo*. Mice received i.t. injections of OBP-301 at 3×10^8 , 3×10^9 , or 3×10^{10} vp for 3 days from days 5 to 7 after tumor implantation (Fig. 3). Tumors grew to mean tumor volume of 32.2 ± 4.5 mm³ at day 5. As shown in Fig. 3, the 3×10^8 dose of OBP-301 treatment was less effective against KCCT873 tumor growth. The mean tumor volume was 549 mm³ on day 42, which is comparable with control tumor volume (910 mm³). Higher doses of OBP-301 led to superior antitumor activity. The mean tumor volume of treated tumors was 130 mm³ at 3×10^9 vp and 67 mm³ at 3×10^{10} vp, which is significantly smaller compared with the control tumor at day 42 ($P < 0.0001$). Remarkably, in addition to a 93% inhibition in tumor volume in mice receiving a 3×10^{10} vp dosage, 3 of 7 tumors completely disappeared by day 37, which persisted through day 42. These results suggest that OBP-301 shows a remarkable antitumor effect in a dose-dependent manner in KCCT873 SCCHN tumors. Based on these findings, OBP-301 at a dosage of 3×10^{10} vp per injection shows the maximum tumor reduction effect.

Optimization of OBP-301 Injection Times in KCCT873 SCCHN Tumors

We next evaluated the treatment schedule of OBP-301 in s.c. xenografted KCCT873 tumor-bearing mice. Mice were treated i.t. with OBP-301 for 1, 3, or 5 subsequent days. The

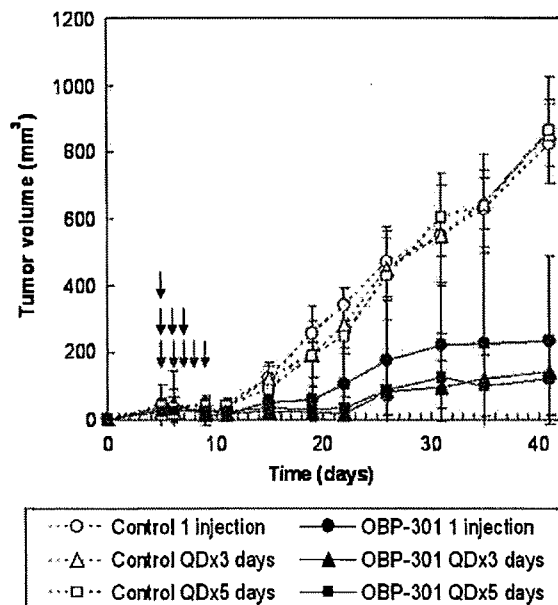


Figure 4. Regression of KCCT873 tumors by i.t. treatment of OBP-301. Athymic nude mice receiving s.c. KCCT873 implantation were treated with OBP-301 (3×10^{10} vp) for 1 (●), 3 (▲), or 5 (■) days. Injections were made on consecutive days (QD). Each group had 6 animals, and the injection volume was 30 μ L in each tumor. Arrows, day of injections. Bars, SD. The experiment was repeated two times.

OBP-301 treatment started on day 5 (mean tumor volume, $31.4 \pm 7.3 \text{ mm}^3$), as palpable tumors developed within 3 to 4 days. As shown in Fig. 4, i.t. administration of OBP-301 showed considerable antitumor activity in all groups. The mean tumor volume of animals receiving a one-time injection was 236 mm^3 at day 41, 71% smaller than excipient-only injected control tumors (823 mm^3 ; $P < 0.0001$). Interestingly, three or five injections of OBP-301 treatment showed superior antitumor activity. The mean tumor volume of animals in the group receiving a three-time treatment was 142 mm^3 at day 41, 83% smaller than control tumors (856 mm^3 ; $P < 0.0001$). Three of 6 tumors had completely regressed by day 27; however, later on, all of the tumors appeared and slowly started to grow again by day 41. Animals receiving OBP-301 for 5 days showed superior tumor response, including complete disappearance of tumors in 2 of 6 mice through day 41. The mean tumor volume measured on day 41 (121 mm^3) was 86% smaller than control tumors (863 mm^3 ; $P < 0.0001$). These results suggest that three- and five-time injections of OBP-301 treatment were equally effective in KCCT873 SCCHN tumor reduction.

Toxicity Profile in Mice Treated with OBP-301

Finally, to assess the toxicity and safety profile of OBP-301 treatment, blood and major organs including heart, liver, lung, kidney, and spleen were collected from KCCT873 tumor-bearing athymic nude mice receiving i.t. OBP-301 (3×10^{10} vp/d for 5 days) on day 10 or 17 after tumor implantation. As shown in Table 2, a blood serum chemistry analysis showed no remarkable changes in any variable in all the mice tested, except for a minor elevation of creatinine phosphokinase and aspartate aminotransferase in the OBP-301 treatment group. Similarly, no pathologic alterations were observed in any of the organs tested (data not shown). Although a slight necrosis was observed in livers from mice treated with i.t. OBP-301, all other organs from untreated control and OBP-301-treated mice did not show any evidence of toxicity. The result that all the treated mice tolerated therapy very well without any behavioral changes or toxicities in blood and pathology

suggests that OBP-301 treatment leads to considerable antitumor activity without unwanted safety or toxicity issues.

Discussion

Although it has been reported that OBP-301 showed a strong anticancer activity in colorectal, prostate, and non-small cell lung cancer *in vitro* and *in vivo*, the effect of OBP-301 in SCCHN has not been pursued (17, 18, 24). Therefore, in this study, we planned to assess the detailed antitumor and toxicity profile of OBP-301 in an animal model of SCCHN. OBP-301 induces cell death in 7 of 13 cell lines *in vitro* and shows dramatic antitumor effects in an animal model bearing KCCT873 tumors without significant toxicity.

OBP-301 showed cytotoxic activity in 7 of 13 SCCHN cell lines. Because the effect of OBP-301 against SCCHN cell lines was limited compared with that previously shown against human non-small cell lung, colorectal, and prostate cancer cell lines (14, 17), we hypothesized that the limitation came from the lower viral infection rate. However, it is of interest to note that the expression levels of adenoviral receptors including CAR, integrins, and HSG are comparable between SCCHN and non-small cell lung cancer H1299 cell lines (Fig. 2; data not shown). In addition, we did not find a significant correlation between *hTERT* mRNA expression and the cytotoxic activity of OBP-301. These results suggest that various factors such as replication speed of viruses and the existence of unknown receptors might be involved in the cytotoxic activity of OBP-301.

The i.t. three- or five-time administration of OBP-301 dramatically inhibited the growth of KCCT873 tumors *in vivo*. The antitumor effect was actually superior to what we expected from our *in vitro* results. Previously, we reported that adenovirus present in blood of mice exists for at least 1 week after i.t. treatment with OBP-301 (15, 17), and i.t. OBP-301 showed antitumor effects both in the injected primary tumor site and in tumors located at distant sites (17). From these results, it is conceivable that OBP-301 attacked the xenografted KCCT873 tumor over and over through the bloodstream for at least 1 week after injection.

Table 2. Changes in blood serum chemistry of mice receiving OBP-301 treatment

Profile	Untreated control	Day 10*		Day 17*	
		PBS	OBP-301	PBS	OBP-301
Sodium (mEq/L)	156	153	151	157	156
Potassium (mEq/L)	7.5	7.3	9.3	8.7	8.2
Creatinine phosphokinase (units/L)	4,007	6,895	8,790	4,907	6,508
Lactate dehydrogenase (units/L)	2,153	3,197	3,158	2,600	2,740
Aspartate aminotransferase (units/L)	195	274	445	349	536
Alanine aminotransferase (units/L)	38	48	64	62	71
Bilirubin (mg/dL)	0.1	0.1	0.1	0.1	0.1
Creatinine (mg/dL)	0.16	0.13	0.13	0.14	0.13

NOTE: Data are mean blood samples from 3 animals in each group.

*Blood samples were collected from athymic nude mice receiving five i.t. injections of OBP-301 (days 5-9).

It has been reported that oncolytic virus replication induces tumor-specific immune responses by stimulating uric acid production as a danger signal as well as accelerating tumor antigen cleaved by IFN- γ -inducible PA28 expression (18). Additionally, because it has been shown that telomerase is active in ~80% to 90% of SCCHN tumor tissues as assessed by immunohistochemistry (25), we speculate that SCCHN cancer preferentially responds to OBP-301 treatment. These results may be the reason why the antitumor activity of OBP-301 is more profound in KCCT873 tumors than expected from our *in vitro* results. Therefore, the strong anticancer effect shown in these animal studies suggests that OBP-301 could be an attractive agent to accomplish an *in situ* radical cure of SCCHN patients.

Although chemoradiotherapy, radiotherapy plus concurrent chemotherapy, has become the standard care for patients with unresectable SCCHN and organ preservation (26, 27), it has recently been reported that cisplatin and fluorouracil with docetaxel plus chemoradiotherapy has a greater effect (28). Because our previous study showed that OBP-401 containing a green fluorescent protein gene for monitoring viral replication (TelomeScan) showed enhanced antitumor efficacy in an *in vivo* human lung cancer model when given in combination with docetaxel, it is possible that combination of OBP-301 with conventional chemotherapy may be a powerful regimen for the treatment of SCCHN in the clinic (29). Additionally, as SCCHN is easily acceptable site for direct injection (6) and i.t. OBP-301 was emerged strong antitumor effect in the xenografted KCCT873 tumor, the i.t. OBP-301 may be a new tool for the treatment of head and neck cancer. Future directions of clinical exploration with OBP-301 are still being considered. Utilization of OBP-301 via i.t. injection appears to be associated with modest activity, although clinical utility of local regional therapy is limited. Further exploration via intrahepatic arterial infusion or i.v. infusion awaits discovery of methods to improve OBP-301 activity.

Viral replication generally results in tissue destruction. In fact, interactions between adenovirus type 5 with CAR, integrin $\alpha_v\beta_3$, integrin $\alpha_v\beta_5$, or HSG and the fiber shaft of adenovirus type 5 are known to be involved in accumulation in the liver of mice and cynomolgus monkeys when administered (30–33). In this study, a significant therapeutic effect of i.t. OBP-301 treatment was achieved without any significant liver toxicity. Histologic analyses in the brain, lung, heart, kidney, and spleen showed no toxicity profile. Oncolytic viruses have been developed as anticancer agents because controlled replication in the tumors causes selective killing of tumor cells and minimizes the effect on normal cells (34). Thus, the current results are consistent with the mechanism of action of virotherapy with oncolytic viruses.

Notably, a phase I study of OBP-301 has been initiated in the United States to test the safety and tolerability of OBP-301 in patients with various types of progressive solid cancer including SCCHN. Results from current clinical trials may further show additional information on its safety and efficacy. As for the clinical use of OBP-301 in SCCHN,

the preliminary information obtained from our study is, based on the present results, considered to be useful for the planning of future clinical trials.

In conclusion, this study clearly shows that OBP-301 has remarkable *in vivo* anticancer effects against SCCHN. These findings suggest that the replication-selective oncolytic virus provides a new platform for treating patients with human head and neck cancer.

Disclosure of Potential Conflicts of Interest

Y. Urata and D. Ichimaru: employees of Oncolys Biopharma Inc. T. Fujiwara: consultant to Oncolys Biopharma Inc. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Hisashi Urushihara and Ritsuko Asai for technical support.

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Antiviral activity of cidofovir against telomerase-specific replication-selective oncolytic adenovirus, OBP-301 (Telomelysin)

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Toshiyoshi Fujiwara

Received: 15 June 2008 / Accepted: 30 July 2008 / Published online: 27 August 2008
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Summary We constructed a replication-competent oncolytic adenovirus, OBP-301 (Telomelysin), in which human telomerase reverse transcriptase (hTERT) promoter drives E1 genes. OBP-301 is currently being used in a phase-I clinical trial for various types of tumors. Under such conditions, anti-adenoviral agents should be available for safety use against OBP-301 since any adenoviral viremia could cause severe adverse effects. Cidofovir (CDV) is an acyclic nucleoside phosphonate that has a broad antiviral activity against DNA viruses. Here, we examined the antiviral effects of CDV against OBP-301. The *in vitro* cytopathic effects of OBP-301 were suppressed by CDV. Moreover, CDV decreased the adenoviral E1A gene copy number after OBP-301 infection. These results suggest that CDV is a potentially useful antiviral agent for OBP-301.

Keywords hTERT · Adenovirus · Cidofovir ·
Oncolytic virus · Clinical trial

Introduction

Oncolytic adenoviruses have been developed for treatment of human cancer. These viruses are designed to replicate and selectively kill cancer cells but to have minimum effect on normal cells [1]. Two major approaches to generate selective

replication of viruses within tumor cells have been used [2, 3]. One is to delete genes that are critical for replication of the virus in normal cells but are dispensable for cancer cells such as ONYX-015 or $\Delta 24$ [4]. The other approach is the replacement of the promoter region that initiates viral replication genes to the promoter region of the genes active in cancer cells [2, 3]. Various genetic or epigenetic targets limited to cancer cells have been investigated and used for constructing oncolytic adenoviruses.

Human telomerase reverse transcriptase (hTERT) is an enzymatic subunit of human telomerase [5]. Telomerase is expressed in almost all cancer cells but not in all normal cells [6]. Therefore, telomerase is an attractive target for treatment of cancer. We constructed previously the attenuated adenovirus, OBP-301 (Telomelysin), in which adenoviral E1A and E1B genes are linked with internal ribosomal entry site under the control of the hTERT promoter. We reported that OBP-301 induced selective expression of E1A and E1B genes in many cancer cell lines and selectively replicated and lysed cancer cells but not normal cells [7–9]. OBP-301 is currently being tested in a phase-I clinical trial that includes various types of solid tumors. Although patients receiving this type of therapy become positive for anti-adenoviral neutralizing antibodies, those treated with OBP-301 could develop adenoviral viremia with potentially severe adverse effects. Thus, there is a need for anti-adenoviral agents for treatment of potential viremia in clinical trials of OBP-301.

One of the antiviral compounds is phosphonyl acyclic nucleotides, (S)-9-(3-hydroxy-2-phosphonomethoxy propyl) cytosine dehydrate, also known as HPMPC (cidofovir, or CDV). CDV was developed for the treatment of viral infections and has a broad antiviral activity against DNA viruses, such as cytomegalovirus and adenoviruses (AdV). CDV exhibits potent inhibitory effects against several

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adenoviral serotypes in cell culture models [10]. Furthermore, CDV has been used clinically for AdV infections after bone marrow transplantation in immunodeficient patients [11]. Thus, we presumed that CDV could be a useful antiviral drug against OBP-301. In the present study, we examined the *in vitro* inhibitory effects of CDV against OBP-301 in human lung cancer cell lines.

Materials and methods

Cell culture, viruses, and chemicals

The human non-small lung cancer cell H1299 and lung cancer cell line A549 were purchased from American Type Culture Collection (ATCC). H1299 was cultured in RPMI 1640 medium supplemented with 10% FCS. A549 was cultured in DMEM F12 medium supplemented with 10% fetal calf serum (FCS). OBP-301 was constructed and characterized as described previously [7–9]. The human wild-type adenovirus type 5 (wt-Ad) was also used. VISTIDE™ (CDV injection) was purchased from Gilead Sciences (Foster City, CA).

Cell viability assay

Cells were seeded in 96-well plate at 1×10^3 cells per well and incubated at 37°C. After incubation, cells were infected with OBP-301 at a MOI of 1 (in H1299) and 5 (in A549) for 2 hours. The medium was aspirated and replaced with fresh medium containing 2% FCS and serially diluted CDV. Cell viability was determined by XTT assay 7 days after infection using Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the protocol recommended by the manufacturer. Protection was determined by the following formula: Protection (%) = $\frac{\text{OD (AdV(+):CDV(+))} - \text{OD (AdV(+):CDV(-))}}{\text{OD (AdV(-):CDV(+))} - \text{OD (AdV(+):CDV(-))}} \times 100$. CC₅₀ (50% cytotoxic concentration) was defined as CDV concentration that inhibited relative cell viability to 0.5 without OBP-301 infection. EC₅₀ (50% effective concentration) was defined as CDV concentration that archived 50% protection.

Quantitative real-time PCR analysis

Cells were seeded in six-well plate at 2×10^5 cells per well. After overnight incubation at 37°C, the medium was aspirated, and cells were infected with OBP-301 or wt-Ad at a MOI of 10 for 2 hours at 37°C with gentle shaking every 15 minutes. After incubation, the cells were washed with PBS and placed in a medium containing serially diluted CDV (100, 20, 4, 0.8, 0.16 and 0 μM). The cells were harvested 24 hours later with Trypsin/EDTA and total

DNA was extracted using QIAamp™ DNA Mini Kit (Qiagen, Hilden, Germany). Viral EIA copy number was measured using LightCycler instruments and LightCycler Faststart DNAMaster SYBR Green I (Roche, Mannheim, Germany). EC₅₀ (EIA) was defined as the CDV concentration that inhibits the EIA ratio (with CDV/no CDV) to 0.5. Primers for EIA gene were: forward: 5'- CCTGTGCTA GAGAATGCAA -3', reverse: 5'- ACAGCTCAAGTC CAAAGGTT - 3'. PCR amplification began with a 600-s of denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 8 s.

Statistical analysis

The Student's *t*-test was used to compare differences. Statistical significance was defined when *p* was <0.05.

Results

In vitro cytopathic effect of OBP-301 on lung cancer cell lines

We reported previously that OBP-301 exhibited oncolytic activity against many types of human cancer cells [7–9]. To confirm this, we tested its cytopathic effects in cancer cell line *in vitro*. Human lung cancer cell lines, A549 and H1299, were infected with OBP-301 at various MOIs and numbers of living cells were measured by XTT assay (Fig. 1). At 5 days after infection, the majority of H1299 cells were killed by OBP-301 at MOI of 1 and 10, and approximately 70% of A549 cells were killed by OBP-301 at MOI of 50. These results confirmed that OBP-301 induced cell death in A549 and H1299 cells.

Inhibitory effects of CDV on the cytopathic effect of OBP-301

Next, we tested whether the cytopathic effect by OBP-301 on these cancer cells could be inhibited by CDV treatment. A549 and H1299 cells were infected with OBP-301 then treated with CDV at various concentrations. Cell viability was also determined by XTT assay. In the presence of the drug and virus, relative cell viability significantly increased in the presence of CDV at > 30 μM in A549 cells and > 40 μM in H1299 cells (*p*<0.01) (Fig. 2). Furthermore, inhibition of cell growth of each cell line was observed in the presence of CDV at > 100 μM. The calculated EC₅₀ values of CDV were 20.4 μM for H1299 and 35.9 μM for A549 cells, while the calculated CC₅₀ values were 146.4 μM for H1299 cells and 106.9 μM for A549 cells. Similar results were obtained by using ONYX-015 (see

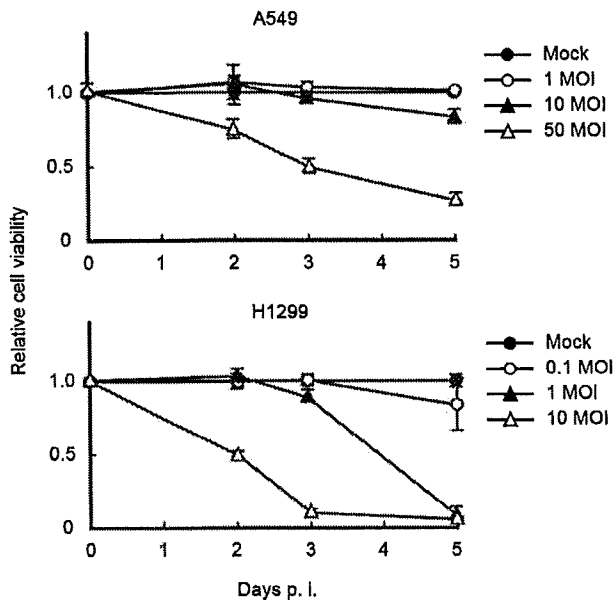


Fig. 1 Cytopathic effects of OBP-301 on H1299 and A549 lung cancer cell lines *in vitro*. Each cell was infected with OBP-301 at the indicated MOI and cell viability was evaluated by XTT assay (no virus=1.0). Data are mean±SD values

Introduction) and OBP-401, a modified OBP-301 that contains the GFP gene [12] (data not shown).

Inhibitory effects of CDV on viral replication of OBP-301

Finally, we examined whether CDV inhibits the replication of OBP-301 *in vitro*. We previously used two methods to

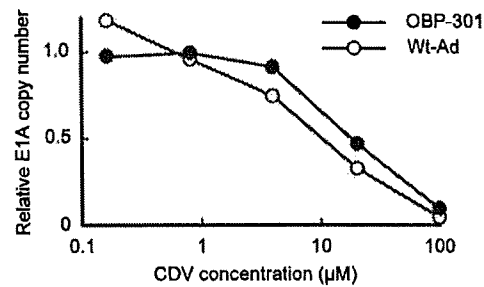


Fig. 3 Inhibition of OBP-301 replication in H1299 cells by CDV. Cells were infected with OBP-301 or wild-type AdV at MOI of 10, followed by the addition of CDV at the indicated concentrations. Cells were collected after 24 hours infection, total DNA was extracted, and viral E1A copy number was determined by quantitative real-time PCR analysis (with virus/no CDV=1.0)

quantify viral replication, biological plaque forming assay using 293 cells [7] and real-time PCR assay targeting adenoviral E1A sequence [8, 9], and found that both assays could detect viral replication similarly. H1299 cells were infected with OBP-301 or wt-Ad, followed by treatment with CDV. Wt-Ad was used for positive control in this assay since it had been reported that CDV had antiviral activity against wt-Ad. To measure the viral DNA, we quantified E1A copy number of cells infected with OBP-301 or wt-Ad by real-time PCR assay. CDV reduced the relative E1A copy number in both wt-Ad and OBP-301-infected cells and the effect was concentration-dependent, indicating that CDV inhibited viral replication of OBP-301 and wt-Ad in H1299 cells (Fig. 3). The calculated EC_{50} (E1A) value for OBP-301 was 19.55 μ M.

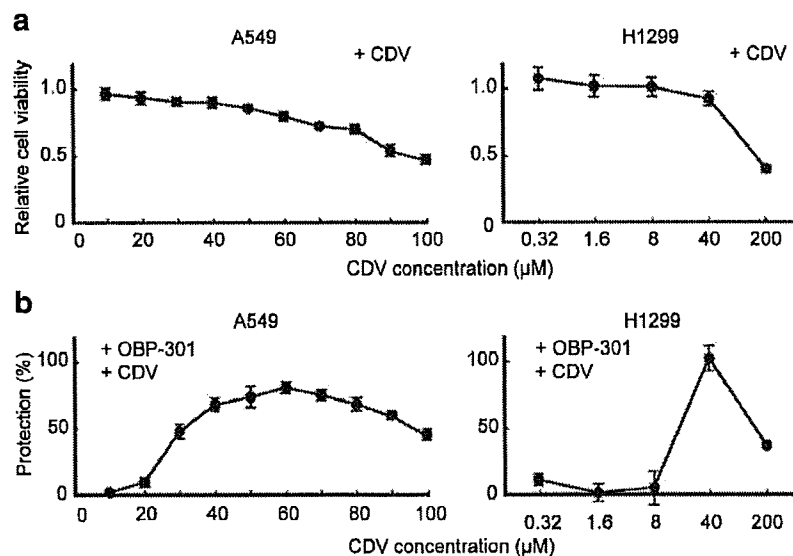


Fig. 2 Inhibition of cytopathic effects of OBP-301 by CDV in human lung cancer cell lines. (a) Cells were treated with CDV at the indicated concentrations and incubated for 7 days. The relative cell viability was evaluated by XTT assay. Data are mean±SD values. (b) Cells were

infected with OBP-301 (1 MOI in H1299 and 5 MOI in A549, PFU/cell), followed by the addition of CDV at the indicated concentrations. Protection was calculated as described in "Material and methods". Data are mean±SD values

Discussion

OBP-301 has been developed as an oncolytic viral agent for the treatment of human cancer and is currently used in a phase-I clinical trial. Although replication of OBP-301 is limited in normal cells evaluated *in vitro* and *in vivo* mouse model, the effect of OBP-301 in human is still unknown. In the phase-II clinical trial of ONYX-015, an E1B-55 kDa-deleted adenovirus mutant, adenoviral viremia occurred even in the presence of neutralizing antibodies and antiviral cytokines [13]. Several antiviral drugs are used for other DNA viruses, e.g. aciclovir, a synthetic acyclic purine-nucleoside analogue, for Herpes simplex virus (HSV) [14], and ganciclovir (GCV) for Cytomegalovirus infections [15]. For AdV infections, it has been reported that CDV exhibits potent inhibitory effects against several adenoviral serotypes in cell culture models [10, 16]. We considered that CDV can be used as antiviral drug for OBP-301.

The purpose of using CDV clinically is to avoid toxic effects of OBP-301 in normal tissues, when viral replication becomes uncontrollable. However, it is difficult to examine the inhibitory effect of CDV on cytopathic effect of OBP-301 in normal cells, because OBP-301 replicates and lyses only in cancer cells [7–9]. Therefore, we used human cancer cell lines to assess the potential antiviral activity of CDV. We showed that the cytopathic effects of OBP-301 were efficiently suppressed by CDV treatment at concentrations that did not affect cell growth (Fig. 2). Despite the high susceptibility of H1299 cells to OBP-301 infection (Fig. 1), CDV inhibited the cytopathic effects of OBP-301, suggesting that CDV has potent antiviral activity against OBP-301. The 50% effective concentration of CDV in A549 cells was in agreement with the published data using human wt-Ad [17], indicating that the inhibitory activity against the cytopathic effect of OBP-301 was equivalent to that of wt-Ad.

The mechanism of the antiviral effect of CDV is that of inhibition of viral replication by targeting the viral DNA polymerase [18]. The anti-adenoviral effect of CDV is quantified by evaluating the viral progeny in adenovirus-infected cells using quantitative PCR analysis [16]. We demonstrated that the replication of OBP-301 was inhibited by CDV in a concentration-dependent manner (Fig. 3). In addition, the 50% effective concentration on viral DNA copy number was almost the same as the 50% effective concentration on cell death by OBP-301 infection, suggesting that CDV inhibited the cytopathic effect of OBP-301 by inhibiting the replication of OBP-301. Recently, antiviral effect of CDV against wt-Ad in immunosuppressed Syrian hamster model was reported [19]. The 50% inhibitory concentration of CDV on viral DNA copy number in OBP-301 was slightly higher than that of wt-Ad (Fig. 3). Differences at E1A region between OBP-301 and wt-Ad may

affect of CDV activity on viral replication. It has been reported that CDV-resistant human Ad mutants were isolated by continuous passage *in vitro* condition [20]. Quality assurance and quality control of the master virus bank have been intensively performed for OBP-301 used in the current clinical trials; emergence of CDV-resistant OBP-301 variant, however, should be considered and long-term susceptibility of CDV against OBP-301 will be studied in the future clinical trials.

In conclusion, our *in vitro* data indicate that CDV can effectively inhibit the oncolytic activity of OBP-301 by inhibiting the replication of OBP-301. CDV may be a potential antiviral agent for OBP-301 in clinical trial.

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Preclinical evaluation of synergistic effect of telomerase-specific oncolytic virotherapy and gemcitabine for human lung cancer

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Abstract

A phase I dose-escalation study of telomerase-specific oncolytic adenovirus, OBP-301 (Telomelysin), is now under way in the United States to assess feasibility and to characterize its pharmacokinetics in patients with advanced solid tumors. The present preclinical study investigates whether OBP-301 and a chemotherapeutic agent that is commonly used for lung cancer treatment, gemcitabine, are able to enhance antitumor effects *in vitro* and *in vivo*. The antitumor effects of OBP-301 infection and gemcitabine were evaluated by 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay. *In vivo* antitumor effects of intratumoral injection of OBP-301 in combination with systemic administration of gemcitabine were assessed on *nu/nu* mice s.c. xenografted with human lung tumors. OBP-301 infection combined with gemcitabine resulted in very potent synergistic cytotoxicity in human lung cancer cells. The three human lung cancer cell lines treated with OBP-301 for 24 hours tended to accumulate in S phase compared with controls. The proportion of cells in S phase increased from 43.85% to 56.41% in H460 cells, from 46.72% to 67.09% in H322 cells, and from 38.22% to 57.67% in H358 cells. Intratumoral injection of OBP-301 combined

with systemic administration of gemcitabine showed therapeutic synergism in human lung tumor xenografts. Our data suggest that the combination of OBP-301 and gemcitabine enhances the antitumor effects against human lung cancer. We also found that the synergistic mechanism may be due to OBP-301-mediated cell cycle accumulation in S phase. These results have important implications for the treatment of human lung cancer. [Mol Cancer Ther 2009;8(4):980–7]

Introduction

Lung cancer is the most common cause of cancer-related mortality. In current clinical practice, chemotherapy is used in combination with radiotherapy as an adjuvant or neoadjuvant therapy. Moreover, combination chemotherapy is regarded as the standard care in the treatment of unresectable locally advanced (stage IIIB), metastatic (stage IV), or recurrent disease. Although there have been major improvements over recent decades in surgical techniques and the role of chemotherapy-radiotherapy in the treatment of non-small cell lung cancer, the long-term outlook for such patients has not changed significantly. The median survival for patients with advanced-stage non-small cell lung cancer treated with platinum-based chemotherapy is a disappointing 8 to 10 months (1). Clearly, new therapies are needed that are capable of treating such advanced cancers in addition to preventing their formation.

One type of cancer therapy that has been extensively investigated is virotherapy, which uses oncolytic viruses engineered to selectively replicate within tumor cells, killing them. We previously developed an adenovirus vector that drives the *E1A* and *E1B* genes under the hTERT promoter, designated OBP-301 (Telomelysin), and showed its selective replication, as well as its profound cytotoxic activity, in a variety of human cancer cells (2–5). Although the development of OBP-301 as a monotherapy is currently under way clinically based on the promising preclinical results, multimodal strategies to enhance antitumor efficacy *in vivo* are essential for successful clinical outcome. In fact, most clinical trials for oncolytic viruses have been conducted in combination with chemotherapy or radiotherapy (6).

Gemcitabine (2,2-difluorodeoxycytidine) is a third-generation agent that has been developed in the past decades. Gemcitabine is a deoxycytidine analogue that has shown efficacy as a treatment for many solid tumors and is now extensively used in the treatment of patients with various tumor types (7, 8), but inherent and acquired resistance has resulted in low response rates. In the present study, we hypothesized that combination of oncolytic adenoviral agents (with novel mechanisms of action) with

Received 9/19/08; revised 12/15/08; accepted 1/20/09.

Grant support: Ministry of Education, Science, and Culture, Japan (T. Fujiwara); Ministry of Health and Welfare, Japan (T. Fujiwara); and Japan China Medical Association (D. Liu).

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doi:10.1158/1535-7163.MCT-08-0901