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-sulfophenyl]-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

chemotherapeutic agents could improve the antitumor effects and minimize the toxic side effects of the latter by reducing the concentrations of anticancer drugs. To test our hypothesis, we examined the therapeutic effects of OBP-301 combined with gemcitabine both *in vitro* and *in vivo*. The results showed that combination therapy with OBP-301 and gemcitabine produced therapeutic benefits over either individual modality.

Materials and Methods

Cell Lines and Cell Cultures

The human large cell lung cancer cell line H460, the bronchioloalveolar carcinoma cell line H322, and the bronchioloalveolar carcinoma cell line H358 were propagated in monolayer culture in RPMI 1640 supplemented with 10% FCS.

Chemotherapeutic Agents and Viruses

Gemcitabine (Gemzar) was obtained from Eli Lilly Co. Stock solution was prepared in 0.9% NaCl and the agent was further diluted in growth medium immediately before use. OBP-301 is a telomerase-specific replication-competent adenovirus variant, in which the hTERT promoter element drives the expression of *E1A* and *E1B* linked with internal ribosomal entry site. The virus was purified by ultracentrifugation in cesium chloride step gradients and titer was determined by plaque assay in 293 cells, as described previously (2–5).

Cell Viability Assay

2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay was done to assess the viability of tumor cells. H460, H322, and H358 cells at 1,000 per well were seeded onto 96-well plates at 18 to 20 h before viral infection. Cells were then infected with OBP-301 at low to high concentrations and were treated with fresh medium containing gemcitabine at various concentrations at 24 h after OBP-301 infection. Cell viability was determined at 4 d after treatment with OBP-301 and gemcitabine by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

In vitro Replication Assay

H460, H322, and H358 cells were seeded in six-well plates at 10^5 per well at 12 h before infection. Cells were infected with OBP-301 at a multiplicity of infection (MOI) of 10, 25, and 20 plaque-forming units (pfu)/cell, respectively, and fresh medium containing gemcitabine at 70 nmol/L for H460 cells, 100 nmol/L for H322 cells, and 3 nmol/L for H358 cells was then added at 24 h after infection. Cells were incubated at 37°C, trypsinized, and harvested for intracellular replication analysis at 2, 24, 48, 72, 96, and 108 h after OBP-301 infection. DNA purification was done using QIAmp DNA Mini kit (Qiagen, Inc.). The E1A DNA copy number was determined by quantitative real-time PCR using a LightCycler instrument and LightCycler-DNA Master SYBR Green I (Roche Diagnostics).

Assessment of E1A Expression by Western Blotting

H460, H322, and H358 cells infected with OBP-301 at an MOI of 10, 25, and 20, respectively, were collected at 5 d after infection, lysed in lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 400 mmol/L NaCl, 1 mmol/L DTT, 5 mmol/L NaF, 1 mmol/L EDTA, 0.5% Na_3VO_4, 10% glycerol, 0.5% NP40, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, 1 mg/mL aprotinin] for 30 min on ice, and centrifuged at 15,000 rpm for 30 min. Protein concentration was measured by means of the Bradford assay. Equal amounts of protein-containing sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol] were boiled for 5 min and electrophoresed under reducing conditions on 12% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to Hybond polyvinylidene difluoride transfer membranes (Amersham) and incubated with primary antibody against E1A (BD Pharmingen) or rabbit anti-human β-actin monoclonal antibody (Sigma-Aldrich) followed by peroxidase-linked secondary antibody. An enhanced chemiluminescence Western system (Amersham) was used to detect secondary probes.

Cell Cycle Analysis

H460, H322, and H358 cells were infected with OBP-301 at 40, 100, and 80 MOI, respectively, for 24 h. The cells were then harvested and suspended in 1.5 mL PBS before fixing with ice-cold 70% ethanol for 30 min. Fixed samples were centrifuged for 5 min, and cell pellets were resuspended in 700 μL PBS containing RNase (0.25 mg/mL) followed by incubation for 30 min at 37°C. The volume was increased to 1 mL with PBS containing 1% bovine serum albumin and propidium iodide (50 μg/mL) and the suspensions were incubated at 4°C for 30 min. Stained cells were analyzed by FACScan (Becton Dickinson) and by WinMDI v2.8 software (Scripps Institute).

Assessment of Cell Cycle Regulator Protein Expression by Western Blotting

H460, H322, and H358 cells were infected with OBP-301 at 40, 100, and 80 MOI, respectively, before harvesting 24 h later. Collected cells were analyzed for expression of E2F1, p53, and E1A and phosphorylation of Akt. Primary antibodies were purchased from Santa Cruz Biotechnology (E2F1), Calbiochem Co. (p53), and Sigma Co. (β-actin). Protein expression was quantified by densitometric scanning using NIH Image software.

In vivo Human Tumor Model

H358 cells (5×10^6 per mouse) were injected s.c. into the backs of 5- to 6-wk-old female BALB/c *nu/nu* mice and were permitted to grow to 5 to 10 mm in diameter. At that time, mice were randomly assigned into four groups: mock, OBP-301, gemcitabine, and OBP-301 plus gemcitabine. Next, 50 μL of solution containing OBP-301 at a dose of 1×10^7 pfu/body or PBS were injected into the tumors. Simultaneously, each mouse in the combination group and gemcitabine group received an i.p. injection of 100 μL gemcitabine at a dose of 70 mg/kg every 3 d for three cycles starting at day 0. The perpendicular

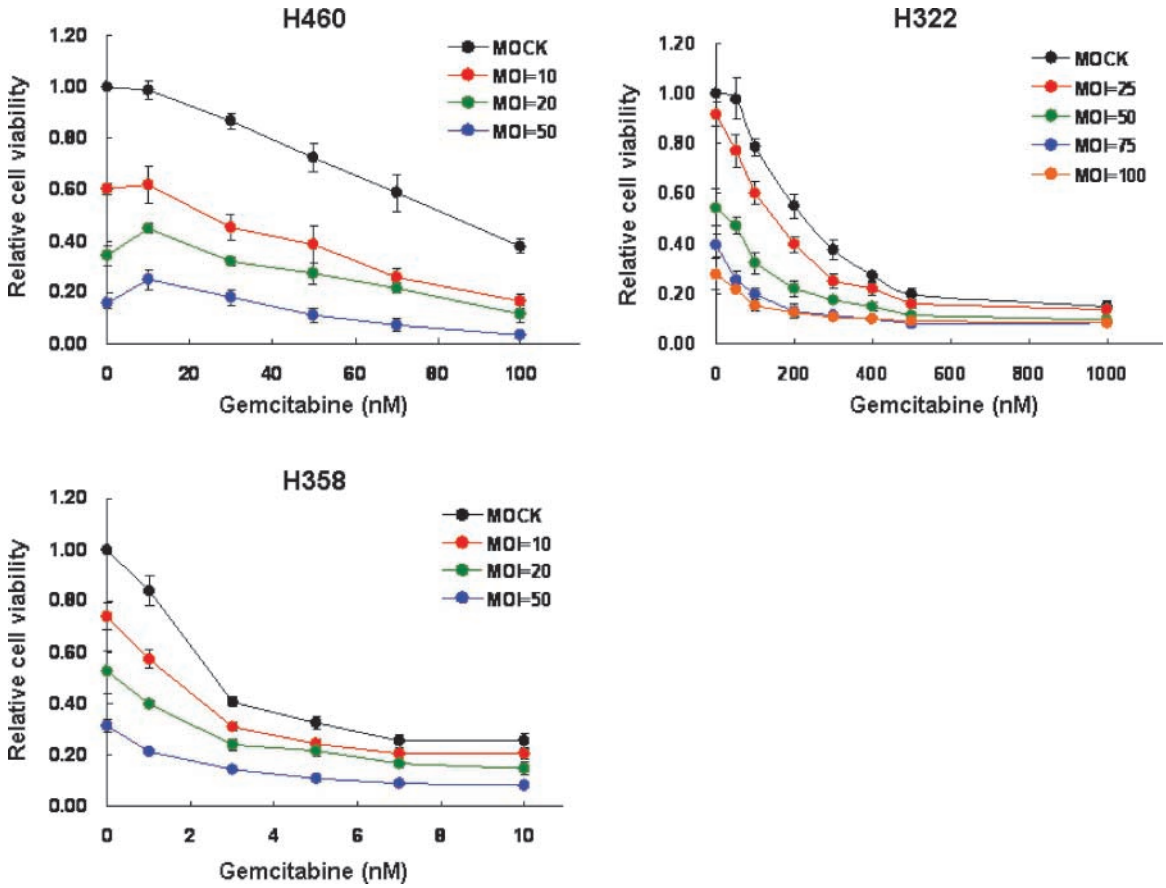

Figure 1. Combination efficiency of OBP-301 and gemcitabine on human lung cancer cell lines. H460, H322, and H358 cells were infected with OBP-301 at the indicated MOIs and then exposed to gemcitabine at the indicated concentrations at 24 h after infection. Cell viability was assessed by XTT assay at 5 d after OBP-301 infection. *Bars,* SD.

diameter of each tumor was measured every 3 d, and tumor volume was calculated using the following formula: tumor volume (mm^3) = $a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant used to calculate the volume of an ellipsoid. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University.

Statistical Analysis

Determinations of significant differences in mean tumor size among groups were assessed by calculating the value of Student's t using the original data analysis.

Results

Antitumor Efficacy of OBP-301 Combined with Gemcitabine in Human Lung Cancer Cell Lines *In vitro*

Before we tested the combination efficacy, sensitivity to gemcitabine and OBP-301 was evaluated in a variety of human lung cancer cell lines by the XTT method, and we selected three cell lines, H460, H322, and H358, for further experiments. From the XTT experiments with gemcitabine alone or OBP-301 alone (Supplementary Fig. S1),[5] the optimal concentrations of gemcitabine and OBP-301 were determined for each cell line. To examine the potential interaction between gemcitabine and OBP-301 *in vitro*, cell viability with six to eight different doses of OBP-301 and four to five doses of gemcitabine was then assessed by XTT assay at 5 days after treatment. Representative dose-response curves are shown in Fig. 1. All cell lines treated with OBP-301 and gemcitabine showed reduced viability when compared with cells treated with single agents.

We then used software to analyze the combination efficiency in these three cell lines (Table 1). In H358 cells, OBP-301 and gemcitabine were apparently synergistic at most doses, whereas the effect of the combination was mostly additive in H322 cells. In H460 cells, the effect was additive when the concentration of gemcitabine was 50 nmol/L; with the increasing of the concentration, however, a clear synergistic effect was seen. When the concentration was 100 nmol/L, synergism was apparent. These

[5] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Table 1. Combination index value analysis by CalcuSyn software (version 2) of combination efficiency in human lung cancer cells

Cells	Gemcitabine (nmol/L)	OBP-301 (MOI)	Combination index	Synergy
H460	10	10	1.261	−−
		20	1.405	−−
		50	1.688	−−
	30	10	0.996	±
		20	1.106	−
		50	1.349	−−
	50	10	1.037	±
		20	1.104	−
		50	0.998	±
	70	10	0.87	+
		20	1.037	±
		50	0.793	++
	100	10	0.793	++
		20	0.785	++
		50	0.531	+++
H358	1	10	0.952	±
		20	0.812	++
		50	0.772	++
	3	10	0.713	++
		20	0.674	+++
		50	0.641	+++
	5	10	0.792	++
		20	0.828	++
		50	0.613	+++
	7	10	0.88	+
		20	0.812	++
		50	0.596	+++
	10	10	1.178	−
		20	0.948	±
		50	0.693	+++
H322	50	25	1.028	±
		50	0.941	±
		75	0.874	++
		100	1.033	±
	100	25	0.953	±
		50	0.842	++
		75	0.848	++
		100	0.938	±
	200	25	0.944	±
		50	0.856	+
		75	0.82	++
		100	0.979	±
	300	25	0.912	±
		50	1.024	±
		75	0.887	+
		100	0.887	+
	400	25	1.005	±
		50	0.975	±
		75	1.093	±
		100	0.938	±
	500	25	0.977	±
		50	0.97	±
		75	0.946	±
		100	1.154	−

NOTE: Range of combination index symbol descriptions: 0.3 to 0.7, +++, synergism; 0.7 to 0.85, ++, moderate synergism; 0.85 to 0.90, +, slight synergism; 0.90 to 1.10, ±, additive; 1.10 to 1.20, −, slight antagonism; 1.20 to 1.45, −−, moderate antagonism.

results suggest that combination treatment with OBP-301 plus gemcitabine was effective in all cell lines tested.

We also assessed the morphologic changes in cells treated with either the combination modality or single agents. Phase-contrast images at 96 hours after OBP-301 infection showed the growth of cells to subconfluence without morphologic changes in the presence of gemcitabine, whereas a rapid loss of viability due to massive cell death, as evidenced by ballooning and floating cells, was evident when gemcitabine was combined with OBP-301 infection (Supplementary Fig. S2).[5]

Effects of Gemcitabine on Replication of OBP-301 in Human Lung Cancer Cells *In vitro*

We used quantitative real-time PCR and Western blotting to assess the effects of gemcitabine on replication of OBP-301 in the three lung cancer cell lines. H460, H322, and H358 cells were infected with OBP-301 at an MOI of 10, 25, and 20, respectively, and were then treated with 70, 100, and 3 nmol/L of gemcitabine at 24 hours after infection. Cells were harvested at the indicated time points after OBP-301 infection, and extracted DNA was subjected to assay. As shown in Fig. 2A, the increase in intracellular viral copy number of OBP-301 by 4 to 5 orders of magnitude was consistent with or without gemcitabine in both treatment regimens. A plateau was reached at ~48 hours after infection. Western blot analysis also showed that E1A expression following OBP-301 infection was not hindered by gemcitabine in three lung cancer cell lines (Fig. 2B). These results suggest that gemcitabine does not interfere with OBP-301 replication.

Cell Cycle Analysis following OBP-301 Infection in Human Lung Cancer Cells

To further explore the "greater than additive response" observed when cells were infected with OBP-301 followed by gemcitabine treatment, we carried out cell cycle analysis of these cells after OBP-301 infection by flow cytometric analysis of propidium iodide–stained cells, a measure of DNA content. As shown in Fig. 3A, the cell cycle distribution apparently changed compared with mock-infected cells at 24 hours after OBP-301 infection in all cell lines tested, although there was no increase in the sub-G_0-G_1 population indicating apoptotic cell death. The number 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 (Table 2). These results suggest that OBP-301 is able to accumulate infected cells in S phase, which may render cells more sensitive to gemcitabine.

Changes in Cell Cycle Regulator Protein Expression following OBP-301 Infection

To clarify the mechanisms of cell cycle regulation by OBP-301, we analyzed the expression of proteins that have a crucial role in the cell cycle. H460, H322, and H358 cells were infected with OBP-301 at an MOI of 40, 100, and 80, and Western blot analysis was then done 24 hours later. As shown in Fig. 3B, expression levels of E2F1, as well as phosphorylated Akt, greatly increased after OBP-301 infection compared with the mock-infected controls in all three cell lines. p53 protein expression was not detectable in H460

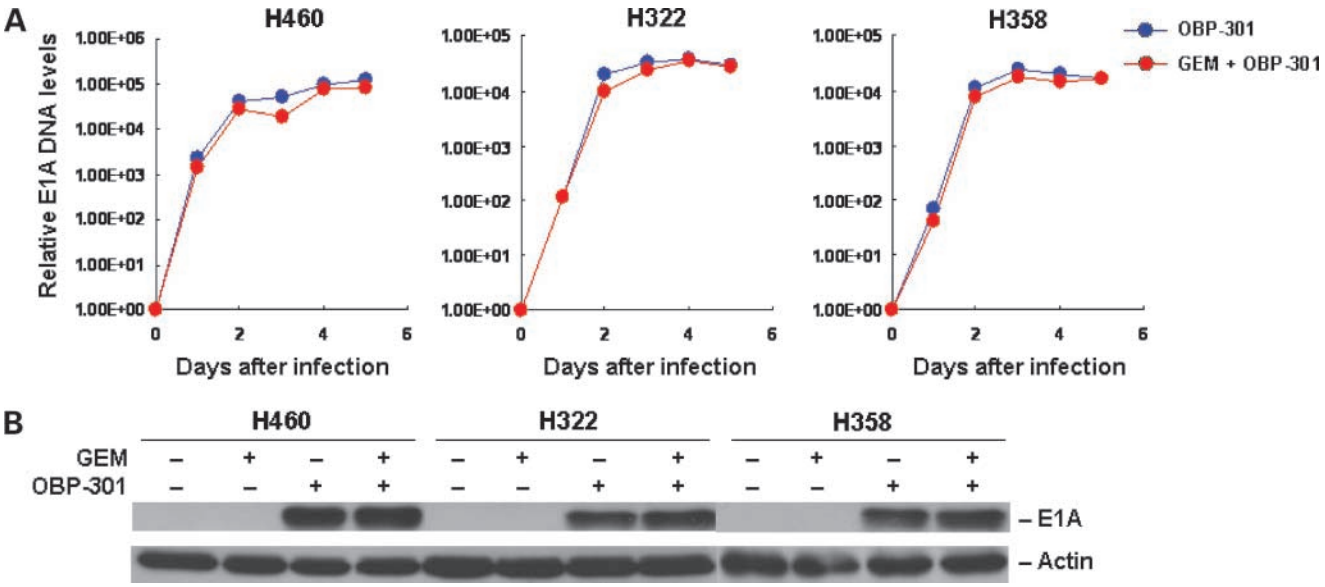

Figure 2. Assessment of viral DNA replication in human lung cancer cells. **A,** H460, H322, and H358 cells were infected with OBP-301 at an MOI of 10, 25, and 20, respectively, for 2 h as a baseline for virus DNA levels. Following the removal of virus inocula at 24 h after the infection, H460, H322, and H358 cells were further incubated with 70, 100, and 3 nmol/L of gemcitabine (*GEM*), respectively, for the indicated periods of time. Cells were then subjected to quantitative real-time PCR assay. Viral E1A copy number was defined as the fold increase for each sample relative to that at 2 h (2 h = 1). **B,** Western blot analysis of E1A expression in human lung cancer cells. Cells were treated with OBP-301, gemcitabine, or a combination of both, as described above, and then subjected to assay at 4 d after infection.

expressing the wild-type *p53* gene and p53-null H358 cells, whereas OBP-301 infection down-regulated mutant p53 expression in H322 cells.

Antitumor Effects of OBP-301 plus Gemcitabine in Human Lung Cancer Xenografts

Finally, we assessed the therapeutic efficacy of OBP-301 in combination with gemcitabine against H358 human lung cancer cells *in vivo*. H358 cells were implanted as xenografts into the hind flanks of *nu/nu* mice. Mice bearing palpable H358 tumors measuring 5 to 7 mm in diameter received simultaneous treatment of intratumoral injection of either 10^7 pfu OBP-301 or PBS plus i.p. administration of either 70 mg/kg gemcitabine or PBS every 3 days for three cycles starting at day 0. As shown in Fig. 4, administration of gemcitabine resulted in significant tumor growth suppression compared with mock-treated tumors for 34 days after initiation of treatment ($P < 0.05$); the combination of OBP-301 plus gemcitabine, however, produced a more profound and significant inhibition of tumor growth compared with mice treated with gemcitabine alone for at least 45 days ($P < 0.05$). The addition of OBP-301 clearly prolonged the antitumor effects of gemcitabine. Intratumoral injection of a replication-deficient adenovirus with or without systemic administration of gemcitabine had no apparent effect on the growth of H358 tumors (data not shown).

Discussion

Replication-competent oncolytic adenoviruses are promising as a novel anticancer therapy (9). In our laboratory, a tumor-specific replication-selective adenovirus, designated Telomelysin or OBP-301, is effective against human cancers (2–5). This virus was genetically designed to replicate under the control of hTERT promoter specifically in tumor cells, causing specific oncolysis. Despite the encouraging outcomes in animal experiments, combination chemotherapy and virotherapy are recommended in clinical treatment, as tumor progression is very rapid in most patients. In the current study, we explored the combination effects of OBP-301 and gemcitabine in human lung cancer cells *in vitro* and *in vivo*.

Adenovirus therapy combined with gemcitabine has been reported in the treatment of pancreatic cancer. Halloran et al. (9) reported that incubation of Panc-1 cells with either 5-fluorouracil or gemcitabine followed by adenovirus-mediated overexpression of $p16^{INK4A}$ resulted in a substantial reduction in cell viability under conditions where the drugs alone had minimal cytotoxicity. Although most studies reporting the combination effects of gemcitabine and adenoviral agents for pancreatic tumor used therapeutic genes critical for tumor growth inhibition, OBP-301 itself is an effective oncolytic virus and leads to infected cell destruction. Moreover, it has been reported that the type 5 adenoviral E1A sensitizes hepatocellular carcinoma cells to gemcitabine (10). These observations support the notion that oncolytic adenoviruses combined with gemcitabine are a rational modality for the treatment of human cancer.

The antitumor efficacy of OBP-301 was found to be enhanced when combined with gemcitabine in human lung cancer cells *in vitro* (Fig. 1; Table 1). Synergistic interaction was apparent in H460 and H358 cells; the combination

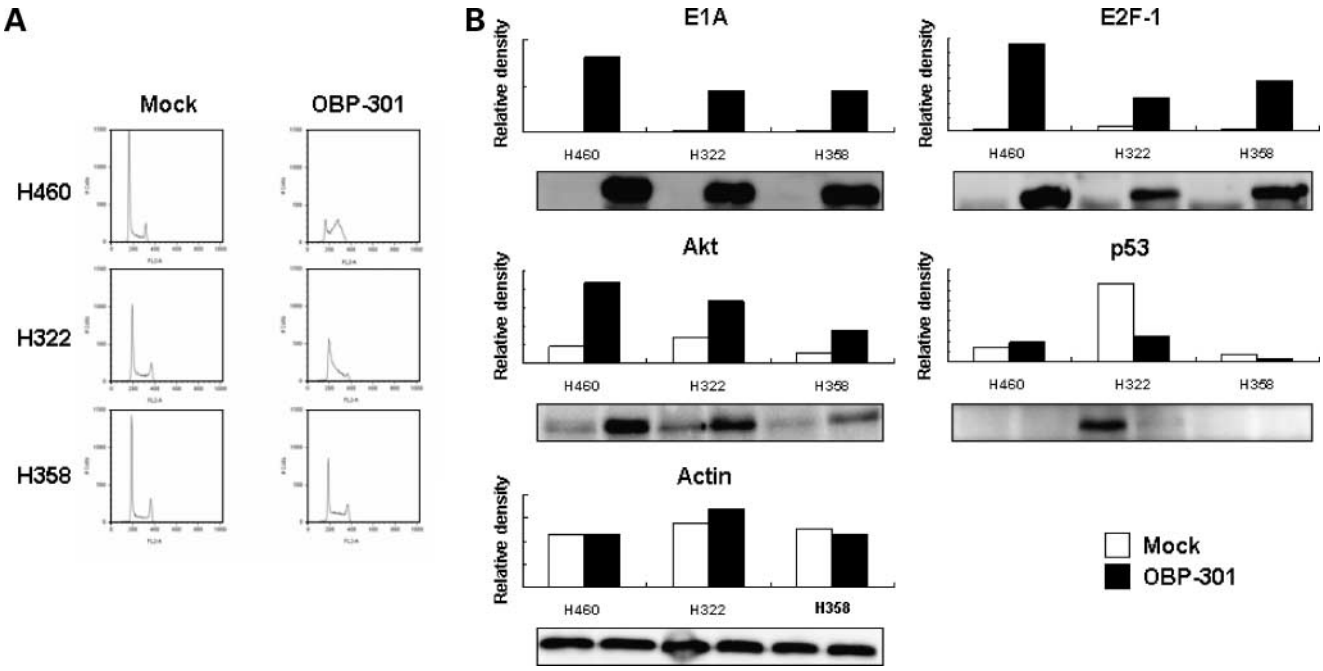

Figure 3. Cell cycle analysis and Western blotting of cell cycle regulator protein following OBP-301 infection in human lung cancer cells. **A,** H460, H322, and H358 cells were infected with OBP-301 at an MOI of 40, 100, and 80 MOI, respectively. DNA content was determined by propidium iodide staining and flow cytometric analysis at 24 h after OBP-301 infection. **B,** H460, H322, and H358 cells were either mock infected or infected with OBP-301 at an MOI of 40, 100, and 80 MOI, respectively. Following the removal of virus inocula, cells were collected at 24 h after infection and subjected to analysis. Equivalent amounts of protein obtained from whole-cell lysates were loaded into each lane, probed with primary antibodies, and then visualized using an enhanced chemiluminescence detection system. Equal loading of samples was confirmed by reprobing with antiactin antiserum. Protein expression was quantified by densitometric scanning using NIH Image software.

effect, however, was additive in H322 cells, suggesting that the effect of the combination is dependent on cell type. We also confirmed that this synergistic effect could be observed in human pancreatic cancer cells (Supplementary Fig. S3).[5] Gemcitabine is a deoxycytidine analogue and the incorporation of gemcitabine triphosphate into DNA causes chain termination, which is the major mechanism underlying the cytotoxicity of gemcitabine (11). Although there was concern over whether gemcitabine would interrupt the viral replication of OBP-301, quantitative real-time PCR analysis showed that intracellular replication of OBP-301 was not affected by gemcitabine (Fig. 2). The cytotoxic mechanisms of OBP-301 are distinct from those of gemcitabine, and therefore, combination effects could be observed provided that gemcitabine does not inhibit viral replication.

To clarify the mechanisms of the greater than additive response, cell cycle analysis was done following OBP-301 infection. Cells treated with OBP-301 tended to accumulate in the S phase at 24 hours after infection (Fig. 3A; Table 2). It has been reported that many DNA viruses can drive quiescent cells through G_1 into S phase by the expression of viral proteins (12–14). During the early phase of the adenovirus infection, the host cell is transformed into an efficient producer of the viral genome. The first gene that is transcribed in the viral genome is *E1A*, which can bind to numerous cellular proteins and acts as a multifunctional protein. Our data showed that OBP-301 infection increases the phosphorylation of Akt, as well as E2F1 expression, in all three human lung cancer cell lines (Fig. 3B). These effects are thought to be due to adenoviral E1A protein expression, as the dl312 adenovirus lacking the E1 genes did not phosphorylate Akt (data not shown).

Direct evidence of cell cycle promotion by Akt was seen when coexpression of Akt rescued cells from PTEN-induced cell cycle arrest (15). Retinoblastoma (Rb) protein restrains proliferation, in part, by modulating the activity of E2F

Table 2. Cell cycle analysis after OBP-301 infection in human lung cancer cells

Cell lines	Treatment	Cell cycle		
		G_1 (%)	S (%)	G_2 (%)
H460	Mock	43.54	43.85	8.61
	OBP-301	10.91	56.41	32.54
H322	Mock	40	46.72	10.85
	OBP-301	27.49	67.09	3.23
H358	Mock	45.89	38.22	14.29
	OBP-301	28.93	57.67	11.45

NOTE: H460, H322, and H358 cell lines were treated with OBP-301 at 40, 100, and 80 MOI, respectively. Cells were then subjected to cell cycle analysis at 24 h after treatment by the fluorescence-activated cell sorting method. The percentages of cells in the G_1, S, and G_2 phases are shown.

A

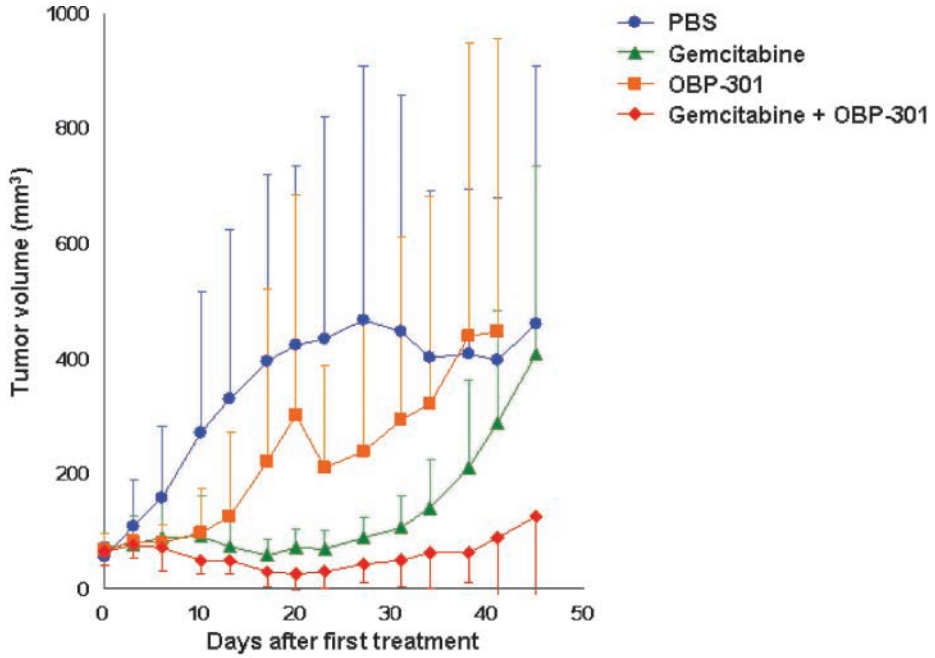

Figure 4. Antitumor effects of intratumorally injected OBP-301 and i.p. administered gemcitabine against established back H358 xenograft tumors in *nu/nu* mice. **A,** H358 tumor cells (5×10^6/each) were s.c. injected into the right flanks of mice. OBP-301 (1×10^8 pfu/body) and gemcitabine (70 mg/kg) were administered intratumorally and i.p., respectively, for three cycles every 3 d. PBS was used as the control. Eight mice were used for each group. Tumor growth is expressed as mean volume ± SE. **B,** statistical analysis was done using Student's *t* test for differences among indicated groups. Statistical significance (*red number*) was defined as $P < 0.05$.

B

	Day 23	Day 27	Day 31	Day 34	Day 38	Day 41	Day 45
PBS vs OBP-301	0.116	0.172	0.354	0.588	0.870	0.788	0.774
PBS vs Gemcitabine (GEM)	0.015	0.025	0.028	0.021	0.073	0.336	0.773
PBS vs GEM + OBP-301	0.009	0.014	0.014	0.005	0.004	0.008	0.051
OBP-301 vs GEM	0.034	0.068	0.101	0.155	0.198	0.377	0.594
OBP-301 vs OBP-301 + GEM	0.011	0.024	0.021	0.050	0.044	0.054	0.080
GEM vs OBP-301 + GEM	0.013	0.007	0.022	0.029	0.015	0.012	0.033

transcription factors. In quiescent cells, Rb associates with several E2Fs, resulting in the repression of proliferation-associated genes. As cells progress into the cell cycle, cyclin-dependent kinases phosphorylate Rb, freeing E2F and allowing it to directly transactivate genes required for S-phase entry (16). In fact, replication-deficient adenovirus-mediated *E2F1* gene transfer into human cancer cells resulted in accumulation of an S-phase cell population (Supplementary Fig. S4).[5] Thus, OBP-301 infection expressed E1A protein, which in turn up-regulated the expression of phosphorylated Akt and E2F1, leading to cell cycle promotion and S-phase entry presumably by the deactivation of Rb. Indeed, we confirmed that OBP-301 infection decreased Rb protein expression in H460 cells (data not shown). The accumulation of the tumor cells in S phase increases the cytotoxicity of gemcitabine, which kills cells in S phase.

In summary, our data show that telomerase-specific oncolytic adenovirus infection increases the sensitivity of human lung cancer cells to gemcitabine due to S-phase accumulation. The combination of OBP-301 and gemcitabine efficiently inhibits human cancer cell growth both *in vitro* and *in vivo*, an outcome that has important implications for tumor-specific oncolytic chemovirotherapies for human lung cancer.

Disclosure of Potential Conflicts of Interest

M. Ouchi, H. Onimatsu, and Y. Urata: employees of Oncolys BioPharma, Inc. T. Fujiwara: consultant for Oncolys BioPharma, Inc. No other potential conflicts of interest were disclosed.

Acknowledgments

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

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Mol Cancer Ther 2009;8:980-987.