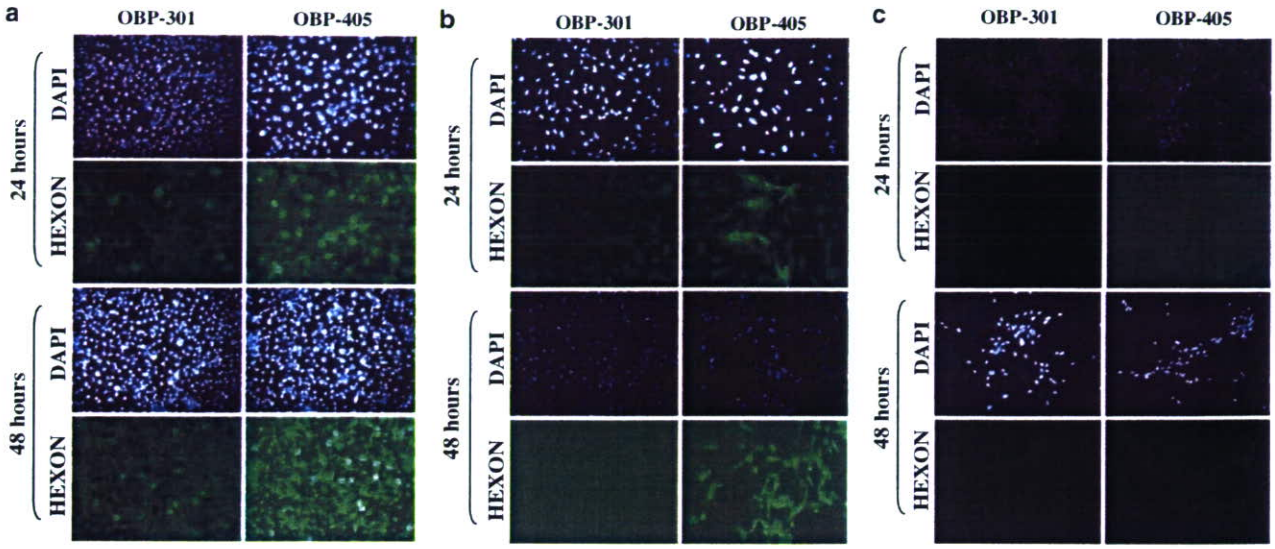


Figure 3 *In vitro* virus spread in CAR-positive H1299 **(a)**, CAR-negative LN444 **(b)**, and NHLF **(c)** cells. Cells cultured in chamber slides were infected with OBP-301 or OBP-405 at an MOI of 1. Cells stained with FITC-labeled goat anti-hexon antibody to monitor the replication of viruses 24 and 48 h after infection are shown. Cell nuclei were counterstained with DAPI. Virus replication was assessed with fluorescence microscopy, and the blue and green fluorescence correspond to cell nuclei and adenovirus hexon, respectively. Original magnification, ×200

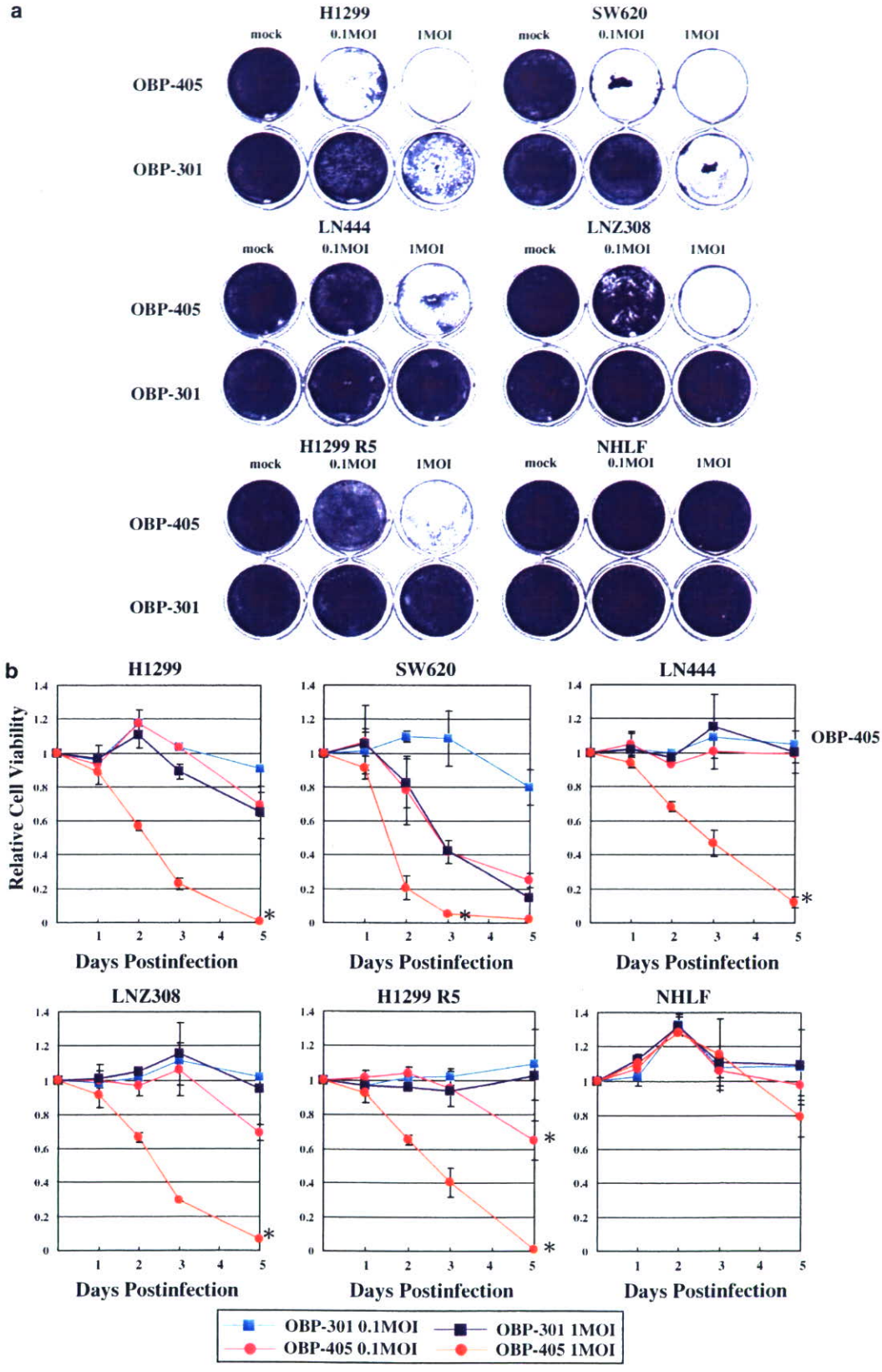
H1299-R5 tumor treated with OBP-405 had completely disappeared, and the level of E1A copy number of OBP-301 was almost consistent with that at 14 day postinfection. These results suggest that OBP-405 could more efficiently replicate in both injected and uninjected tumors, when CAR-negative H1299-R tumors were treated. Notably, no E1A DNA could be detected in the blood of mice treated with OBP-301 or OBP-405, indicating that viral replication in tumors dose not correlate with the level of viruses in the blood circulation (Figure 7a and b).

Moreover, we histologically confirmed a profound replication of OBP-405 in untreated H1299-R5 tumors. As shown in Figure 7c, immunohistochemical analysis for the detection of adenoviral hexon demonstrated that the percentage of positive-staining cells was apparently higher in uninjected tumors of OBP-405-treated mice than those of OBP-301-treated mice. Hematoxylin/eosin analysis revealed apparent tumor cell death at the central portions of the tumors; morphological changes, however, that are associated with the apoptotic phenotype such as nuclear fragmentation and chromatin condensation were not evident (data not shown), suggesting that oncolysis by viral replication might be nonapoptotic cell death.

Discussion

Viral replication generally results in tissue destruction. Oncolytic viruses have been developed as an anticancer agent, because controlled replication in the tumors causes selective killing of tumor cells and minimizes the undesired effects on normal cells (Kirn *et al*., 2001). Amplified viruses can infect adjacent tumor cells as well as reach distant metastatic tumors with the blood circulation. Therefore, oncolytic viruses can amplify the administered dose as a result of *in vivo* viral replication. This might be one of the potential advantages of oncolytic viruses compared with conventional cancer therapies. We previously reported that hTERT promoter-specific replication-competent adenovirus OBP-301 could replicate and eventually lyse the telomerase-expressing tumors cells, leading to the viral spread to adjacent cells (Kawashima *et al*., 2004). OBP-301 could infect both normal and tumor cells, but the virus would only replicate in those cells that have robust telomerase activity. OBP-301 induced oncolysis in a variety of human cancer cell lines; tumors that lost CAR expression, however, might be refractory to infection with OBP-301, because subgroup C adenoviruses, including serotypes 2 and 5, rely on CAR as the primary binding

Figure 4 Oncolytic effect of OBP-301 and OBP-405 *in vitro* on human cancer and normal cell lines. **(a)** CAR-positive (H1299 and SW620) and CAR-negative (LN444, LNZ308, and H1299-R5) cell lines and normal cells (NHLF) were stained with Coomassie brilliant blue 5 day after infection with OBP-301 or OBP-405. Blue areas indicate viable cells; white areas show loss of cells through cell lysis. **(b)** Cells were infected with OBP-301 or OBP-405 at the indicated MOI values, and surviving cells were quantitated over 5 days by XTT assay. Statistical analysis was performed using Student's *t*-test for differences among groups. Statistical significance (*) was defined as $P<0.01$


a
H1299
SW620
LN444
LNZ308
H1299 R5
NHLF
mock
0.1MOI
1MOI
OBP-405
OBP-301
b
Relative Cell Viability
Days Postinfection
OBP-405
OBP-301 0.1MOI
OBP-301 1MOI
OBP-405 0.1MOI
OBP-405 1MOI

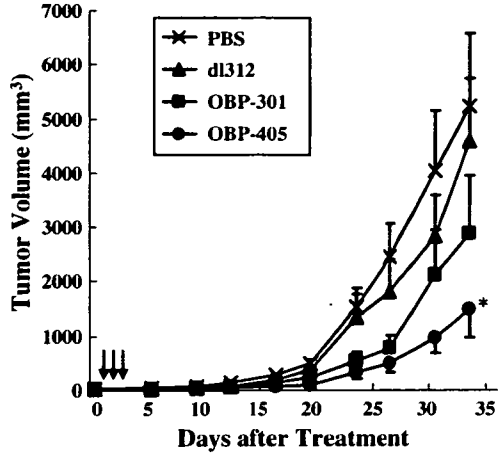


Figure 5 Antitumor effects of intratumorally injected OBP-301 or OBP-405 against established flank H1299-R5 xenograft tumors in *nu/nu* mice. PBS and replication-deficient dl312 were used as a control. Six mice were used for each group. The tumor growth was expressed by the tumor mean volume ±s.e. Statistical significance (*) was defined as $P<0.01$ (Student's *t*-test)

site on the target cells (Bergelson *et al.*, 1997). In fact, CAR deficiency in primary tumors has been reported (Miller *et al.*, 1998; Li *et al.*, 1999). Here, we demonstrate that the modification of the adenovirus fiber knob by the addition of an RGD-containing peptide in the HI loop increased its infectious efficiency and enabled the virus to kill CAR-negative tumor cells. The fiber-modified new oncolytic adenovirus OBP-405 was more effective to inhibit the growth of CAR-negative tumors *in vivo*, comprised of OBP-301.

A wide spectrum of CAR levels exists among many types of human cancer lines (Figure 1), although the regulation as well as the function of this transmembrane protein are poorly understood. If expression of CAR on target cells could be increased, this could potentially yield improved efficacy of adenovirus-based therapies. We previously established H1299-R5 human lung cancer cell line refractory to adenovirus infection by five-time repeated infections (Tango *et al.*, 2004). The observation that CAR expression markedly diminished as the cells are repeatedly infected with adenovirus suggests that the levels of CAR expression could be altered. Indeed, It has been reported that the chemotherapeutic agents are effective in increasing CAR expression (Hemminki *et al.*, 2003); in our preliminary experiments, however, CAR expression could not be modified in H1299-R5 cells by any chemotherapeutic agents tested, including the EGF receptor-tyrosine kinase inhibitor ZD1839 (Gefitinib, 'Iressa') (data not shown). Therefore, it seems to be difficult to consistently upregulate CAR expression in various types of human cancer cells.

A variety of strategies have been devised to increase adenovirus infection to cells with low or absent CAR. For our study, we have chosen to alter the tropism of oncolytic virus by the modification of the fiber. Making the fiber-modified oncolytic adenovirus, we supposed that the virus could infect not only by CAR-dependent entry but also by CAR-independent, RGD-integrin ($\alpha v\beta 3$ and $\alpha v\beta 5$)-dependent entry. As expected, OBP-405 was taken up efficiently by both CAR-positive and CAR-negative human cancer cells; the infectivity of OBP-405 was 10- and 1000-fold higher in CAR-positive and CAR-negative human cancer cell lines, respectively, than that of OBP-301 (Figure 2a). In contrast, the replication yields of OBP-301 and OBP-405 were persistent in both cell lines (Figure 2b), indicating that the tropism modification is not anticipated to alter fundamental aspects of the viral replication cycle. The increased initial virus entry into the cells results in earlier detection (data not shown) and augmented yields of OBP-405 compared with unmodified OBP-301 (Figure 3). Enhancing the infection efficiency of OBP-405 translated into increased oncolytic effects (Figure 4). OBP-301 showed complete oncolysis at as low as 1 MOI in H299 and SW620 cells, suggesting that OBP-301 is sufficient to treat CAR-positive human cancer cells; OBP-301, however, could not kill CAR-negative cell lines at all. Notably, OBP-405 did effectively kill LN444, LNZ308, and H1299-R5 cells at an MOI of 1, indicating that the infection enhancement of OBP-405 contributed to its efficacy on CAR-negative cancer cells. Another important finding is that OBP-405 elicited no increased infectivity as well as cytopathic effect to normal cells despite of CAR expression (Figures 3 and 4).

We also demonstrated the superior oncolytic effect of OBP-405 in the subcutaneous xenograft model of CAR-negative H1299-R5 cells. Intratumoral injection of OBP-405 for three consecutive days resulted in the significant inhibition of H1299-R5 tumor growth (Figure 5) and selective spread of viruses throughout the tumor tissues (Figure 6b). Although the RGD fiber knob modification of selectively replicating adenoviruses, such as AdΔ24 containing the Rb-binding mutation in E1A (Lamfers *et al.*, 2002) and the cyclooxygenase-2 (Cox-2) promoter-based adenovirus (Davydova *et al.*, 2004), has been previously reported to reduce tumor size *in vivo*, the major advantage of OBP-405 is the broad applicability for many types of human cancers because of the telomerase-specific hTERT promoter. In fact, many studies have reported that telomerase is present in nearly all immortal cell lines and ~90% of human tumors but seldom in normal somatic cells (Kim *et al.*, 1994; Shay and Wright, 1996). In addition to the antitumor effect, when the tropism of the virus is modified, it has to be addressed whether a pattern of biodistribution could be affected. We observed that OBP-405 showed a tumor-restricted pattern of biodistribution in mice after intratumoral administration (Figure 6a) and no hepatotoxicity despite of high levels of CAR and αv integrin expression in the liver (Tomko *et al.*, 1997; Fechner *et al.*, 2000) (Table 1). Viral replication and spread of OBP-405 could be detected at least for 4 weeks (Figure 7b), whereas OBP-405 was negative in any normal specimen throughout the period (data not shown). A limitation of our biodistribution data is that the hTERT promoter is not expected to function in mice as it does in humans. Indeed, some studies have reported that mouse and rat tumors do not support efficient replication of human

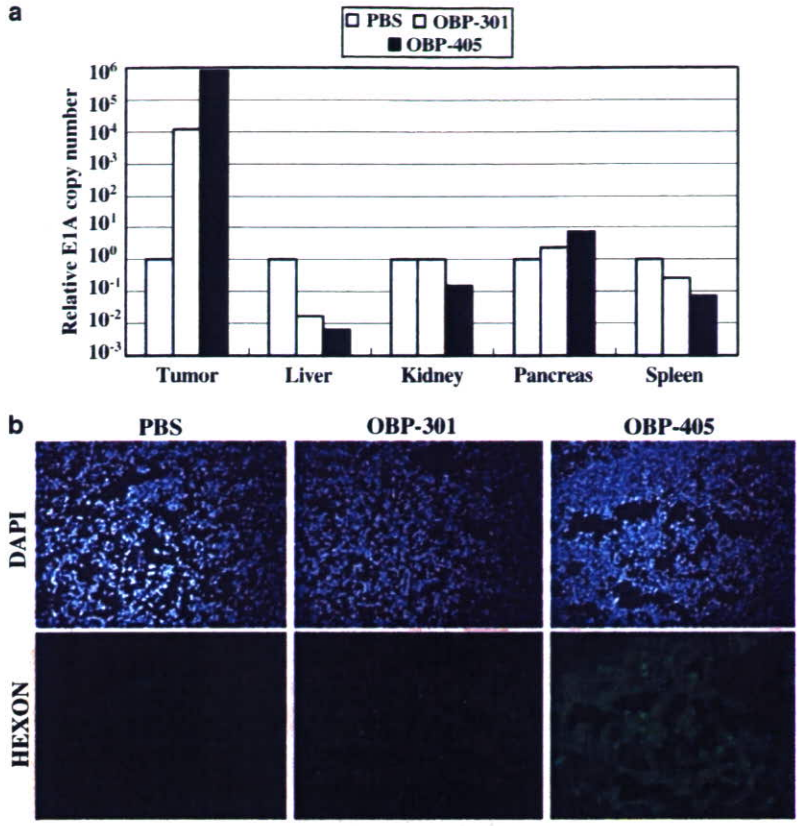


Figure 6 Spread and replication of OBP-301 or OBP-405 following intratumoral administration in *nu/nu* mice transplanted with H1299-R5 tumor cells. **(a)** DNA was extracted from subcutaneous tumor and various tissues in *nu/nu* mice at 7 day postinfection. Viral DNA was detected by quantitative PCR amplification of the adenoviral E1A sequence. The amounts of viral E1A copy number are defined as the fold increase for each sample relative to that with PBS (PBS equals 1). The results are representative of three separate experiments. **(b)** Sections of H1299-R5 tumors were immunofluorescently stained with anti-hexon antibody 7 days after virus injection, followed by counterstaining with DAPI. Magnification, × 200

Table 1 Assessment of hepatotoxicity in *nu/nu* mice intratumorally injected with OBP-301 or OBP-405

	T-Bil (mg/dl)	*AST (IU/l)*	*ALT (IU/l)*	*LDH (IU/l)*	*GGT (IU/l)*	*ALP (IU/l)*
PBS	0.4	87	31	258	< 10	475
OBP-301	0.3	118	40	388	< 10	382
OBP-405	0.4	69	27	190	< 10	492

Blood was obtained from H1299-R5 tumor-bearing mice 7 days after intratumoral injection of PBS or 1×10^7 PFU of OBP-301 or OBP-405, and the levels of liver enzymes were analysed

adenoviruses (Ginsberg *et al.*, 1991; Prince *et al.*, 1993). However, as preliminary data, we confirmed that OBP-405 could infect and efficiently lyse murine adenocarcinoma cell line Colon-26 (data not shown). Therefore, OBP-405 is considered to be specific and safe within its therapeutic window.

To treat distant, metastatic tumors, an infusion of chemotherapeutic drugs by intravenous administration will need to distribute a sufficient quantity of agents to the tumor sites; oncolytic viruses, however, could replicate in the tumor, cause oncolysis, and then release virus particles that will reach to the distant metastatic lesions. Therefore, intratumoral administration that causes the release of newly formed virus from infected tumor cells might be theoretically suitable for oncolytic virus rather than systemic administration. OBP-405 cleared rapidly from the body after intravenous administration (data not shown). This is one of the reasons why we also used intratumoral injection of OBP-405 for the toxicity analysis. In fact, a phase I clinical study demonstrated PSA-specific oncolytic virus shedding in the blood after intraprostatic delivery (DeWeese *et al.*,

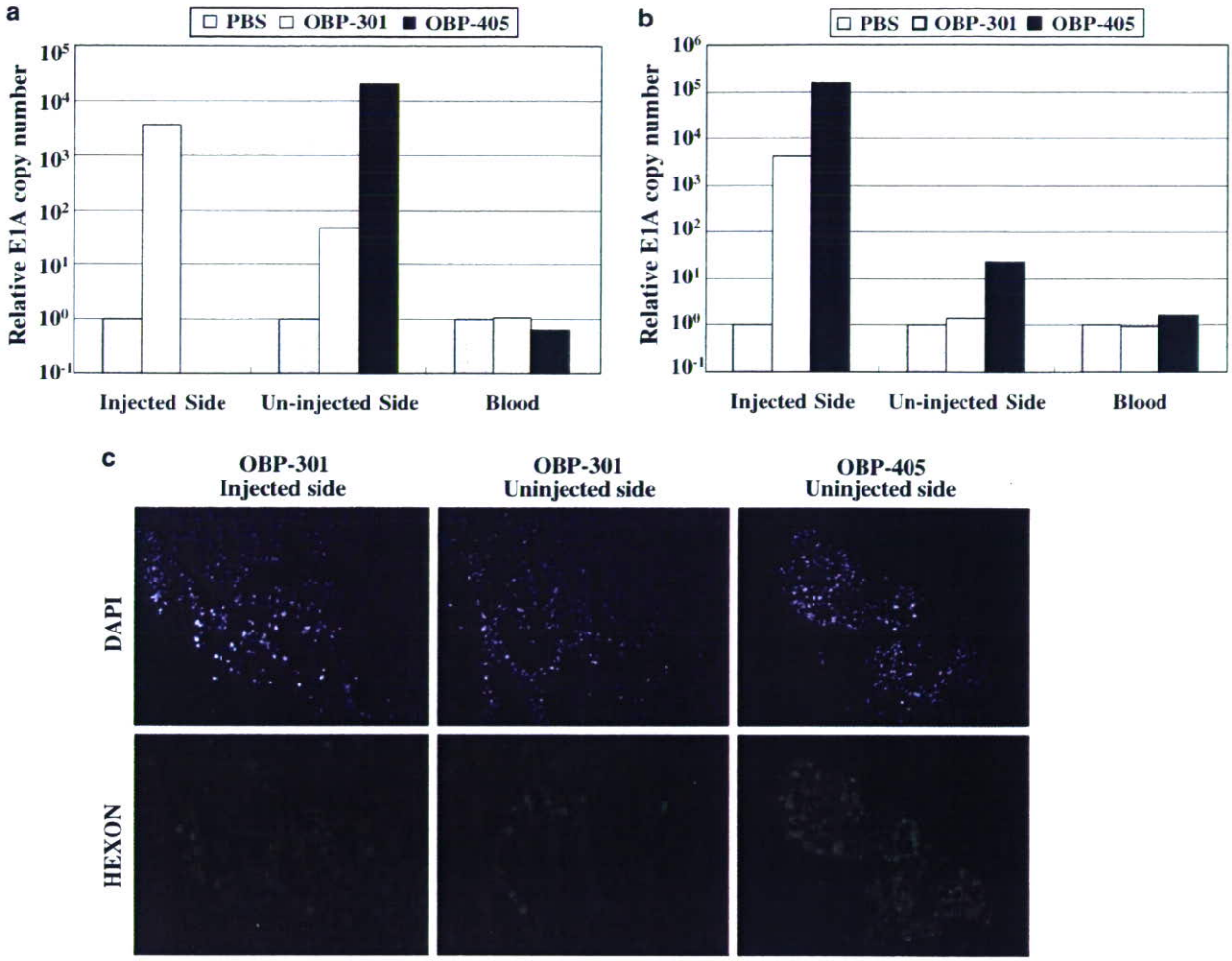


Figure 7 Replication of OBP-301 and OBP-405 in the tumor distant from the site of virus inoculation. Bilateral H1299-R5 tumors were implanted in *nu/nu* mice, and the right-sided tumors received intratumoral injection of PBS or 1×10^7 PFU of OBP-301 or OBP-405. The results are representative of three separate experiments. **(a, b)** Quantitative real-time PCR amplification of the E1A gene with DNA isolated from the right (injected) and left (uninjected) H1299-R5 tumors at 2 weeks **(a)** and 1 month **(b)** post-treatment. **(c)** Sections of H1299-R5 tumors were immunofluorescently stained with anti-hexon antibody 1 month after virus injection, followed by counterstaining with DAPI. Magnification, $\times 200$

2001). These findings indicate that the intratumorally administered virus that reached the circulation could potentially replicate and lyse metastatic tumors. We observed evidence of OBP-405 replication in the distant, uninjected H1299-R5 tumors after its intratumoral administration into the contralateral tumors by quantification of virus DNA (Figure 7). Moreover, OBP-405 continued to replicate in the distant tumors even after the injected tumors disappeared, although the presence of OBP-405 in the blood circulation could not be detected over time. One possible explanation for this result is that the amount of OBP-405 in the circulation might be quite small due to its short half-life (approximately 2 min) (Huard *et al*., 1995; Wood *et al*., 1999), but sufficient to initiate replication once it reached the distant tumors.

In conclusion, we demonstrate that the fiber-modified telomerase-specific replication-selective adenovirus OBP-405 permits CAR-independent cell entry and effective destruction of tumors lacking the primary CAR. The feasibility of original OBP-301 (Telomelysin) for human cancer therapy will be confirmed in clinical trials in the near future; some CAR-negative tumors, however, may be refractory to OBP-301. Under such circumstances, OBP-405 is a powerful way of overcoming low infectivity and increasing antitumor activity. Our data may be consequential for the development of virotherapy for human cancers.

Materials and methods

Cells and culture conditions

The H1299 and H1299-R5 human non-small-cell lung cancer cell lines and the SW620 human colon cancer cell line were cultured in RPMI 1640 medium supplemented with 10% FCS. H1299-R5 is a subline of H1299 that are refractory to adenovirus infection due to the decreased CAR expression

(Tango *et al.*, 2004). The human glioma cell lines LN444 and LNZ308 (kindly provided by Dr N Ishi, Hokkaido University, Hokkaido, Japan), and the transformed embryonic kidney cell line 293 were cultured in DMEM containing high glucose (4.5 g/l) and supplemented with 10% FCS. The normal human lung fibroblast cell line NHLF was purchased from TaKaRa Biomedicals (Kyoto, Japan) and cultured in the medium recommended by the manufacturer.

Recombinant adenoviruses

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 ('Telomelysin') was previously constructed and characterized (Kawashima *et al.*, 2004; Umeoka *et al.*, 2004). OBP-405 ('Telomelysin-RGD') that has mutant fiber containing the RGD peptide, CDCRGDCFC, in the HI loop of the fiber knob was created using the method developed by Mizuguchi *et al.* (2001). OBP-301 and OBP-405 viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titer and infectious titer was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and by the method of Kanegae *et al.* (1994), respectively.

Flow cytometry

The cells (2×10^5 cells) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Biotechnology, NY, USA), anti-human integrin $\alpha v\beta 3$ (LM609; Chemicon International, Temecula, CA, USA), or anti-human integrin $\alpha v\beta 5$ (P1F6; Chemicon International, Temecula, CA, USA). Then, the cells were incubated with FITC-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco, USA) and analysed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA).

Quantitative real-time PCR assay

DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA), and quantitative real-time PCR assay for the E1A gene was performed using a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN, USA). The sequences of specific primers used for E1A were as follows: sense: 5′-CCT GTG TCT AGA GAA TGC AA-3′ and antisense: 5′-ACA GCT CAA GTC CAA AGG TT-3′). PCR amplification began with a 600-s denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 8 s. Data analysis was performed using LightCycler Software (Roche Molecular Biochemicals). The ratios normalized by dividing the value of untreated cells were presented for each sample.

Viral spread assay

H1299, LN444, and NHLF were cultured in two-well chamber slides and infected with OBP-301 or OBP-405 at an MOI of 1. The slides were fixed with 4% paraformaldehyde 24 or 48 h after infection, blocked, incubated with FITC-labeled goat anti-hexon polyclonal antibody (25 μg/ml; Chemicon Inc., Temecula, CA, USA), and counterstained with DAPI (1 μg/ml; Molecular Probes, Eugene, OR, USA). The slides were photographed under the fluorescence microscopy and then analysed using the software (Viewfinder; Pixera, CA, USA).

Cell killing assay

Cells were plated at 100 000 cells/well on 12-well plates and infected either with OBP-301 or OBP-405 at an MOI of 0, 0.1, or 1 for 2 h. The medium with 10% FBS was then added following the removal of viruses. Coomassie brilliant blue staining was performed on day 5.

Cell viability assay

An XTT assay was performed to measure cell viability. Cells were plated on 96-well plates at 5×10^3/well, 24 h before infection and infected either with OBP-301 or OBP-405 at an MOI of 0, 0.1, or 1. Cell viability was determined at the indicated times by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the manufacturer's protocol.

In vivo *human tumor model*

Human lung cancer H1299-R5 cells (1×10^7 cells/mouse) were subcutaneously injected into the flank of 5–6-week-old female BALB/c *nu/nu* mice and permitted to grow to approximately 5–6 mm in diameter. At that time, the mice were randomly assigned into four groups, and a 100 μl solution containing 1×10^7 PFU of dl312, OBP-301, or OBP-405, or PBS was injected into the tumor on days 1, 2, and 3. Tumors were measured for perpendicular diameters every 3 or 4 days, 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 to calculate the volume of an ellipsoid. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry.

In vivo *toxicity study*

Mice bearing H299-R5 tumors received intratumoral injection of 1×10^7 PFU of OBP-301 or OBP-405, or PBS. At 1 week after treatment, blood samples were obtained and the serum levels of total bilirubin (T-Bil), aspartate amino transferase (AST), alanine amino transferase (ALT), lactate dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP) were determined by automated colorimetric assays to assess the hepatotoxicity.

In vivo *viral replication*

OBP-301 or OBP-405 at 1×10^7 PFU/100 μl, or PBS were intratumorally injected into H1299-R5-bearing mice. After 1 week, the tumors and organs were harvested and DNA was extracted from each tissue. To compare the viral replication in the tumor and other normal organs, quantitative real-time PCR for the E1A gene was performed with a LightCycler instrument. The tumors and organs were immediately embedded in Tissue Tek (Sakura, Tokyo, Japan), cut into 5 μm-thick sections, and assessed by immunofluorescence detection of the adenoviral hexon protein using a goat anti-hexon polyclonal antibody (Chemicon, Temecula, CA, USA). To assess the viral replication on distant, uninjected tumors, H1299-R5 cells (1×10^7 cells/mouse) were injected subcutaneously into bilateral flanks of mice. At 2 weeks or 1 month after intratumoral inoculation of OBP-301 or OBP-405 at 1×10^7 PFU/100 μl into tumors in the right flank, the bilateral tumors and blood were collected from mice and DNA was extracted. Quantitative real-time PCR as well as immunofluorescence staining for the hexon protein were performed.

Statistical analysis

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

Abbreviations

hTERT, human telomerase reverse transcriptase; IRES, internal ribosome entry site; CAR, Coxsackie-adenovirus receptor; NHLF, normal human lung fibroblasts; MOI, multiplicity of infection; PFU, plaque-forming units.

Acknowledgements

We thank Yoshiko Shirakiya, Nobue Mukai, and Yuri Hashimoto (Oncolys BioPharma, Inc.) for their excellent technical support. This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan; and by grants from the Ministry of Health and Welfare, Japan.

References

Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL and Finberg RW. (1997). *Science*, **275**, 1320–1323.

Collins K and Mitchell JR. (2002). *Oncogene*, **21**, 564–579.

Davydova J, Le LP, Gavrikova T, Wang M, Krasnykh V and Yamamoto M. (2004). *Cancer Res.*, **64**, 4319–4327.

DeWeese TL, van der Poel H, Li S, Mikhak B, Drew R, Goemann M, Hamper U, DeJong R, Detorie N, Rodriguez R, Haulk T, DeMarzo AM, Piantadosi S, Yu DC, Chen Y, Henderson DR, Carducci MA, Nelson WG and Simons JW. (2001). *Cancer Res.*, **61**, 7464–7472.

Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N and Curiel DT. (1998). *J. Virol.*, **72**, 9706–9713.

Fang B and Roth JA. (2003). *Curr. Opin. Mol. Ther.*, **5**, 475–482.

Fechner H, Wang X, Wang H, Jansen A, Pauschinger M, Scherubl H, Bergelson JM, Schultheiss HP and Poller W. (2000). *Gene Ther.*, **7**, 1954–1968.

Ginsberg HS, Moldawer LL, Sehgal PB, Redington M, Kilian PL, Chanock RM and Prince GA. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 1651–1655.

Greider CW and Blackburn EH. (1985). *Cell*, **43**, 405–413.

Gu J, Kagawa S, Takakura M, Kyo S, Inoue M, Roth JA and Fang B. (2000). *Cancer Res.*, **60**, 5359–5364.

Gu J, Zhang L, Huang X, Lin T, Yin M, Xu K, Ji L, Roth JA and Fang B. (2002). *Oncogene*, **21**, 4757–4764.

Hemminki A, Kanerva A, Liu B, Wang M, Alvarez RD, Siegal GP and Curiel DT. (2003). *Cancer Res.*, **63**, 847–853.

Huard J, Lochmuller H, Acsadi G, Jani A, Massie B and Karpati G. (1995). *Gene Ther.*, **2**, 107–115.

Kanegae Y, Makimura M and Saito I. (1994). *Jpn. J. Med. Sci. Biol.*, **47**, 157–166.

Kawashima T, Kagawa S, Kobayashi N, Shirakiya Y, Umeoka T, Teraishi F, Taki M, Kyo S, Tanaka N and Fujiwara T. (2004). *Clin. Cancer Res.*, **10**, 285–292.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL and Shay JW. (1994). *Science*, **266**, 2011–2015.

Kirn D, Martuza RL and Zwiebel J. (2001). *Nat. Med.*, **7**, 781–787.

Koga S, Hirohata S, Kondo Y, Komata T, Takakura M, Inoue M, Kyo S and Kondo S. (2000). *Hum. Gene Ther.*, **11**, 1397–1406.

Kohn EC, Lu Y, Wang H, Yu Q, Yu S, Hall H, Smith DL, Meric-Bernstam F, Hortobagyi GN and Mills GB. (2004) **31**, 39–53.

Komata T, Kondo Y, Kanzawa T, Hirohata S, Koga S, Sumiyoshi H, Srinivasula SM, Barna BP, Germano IM, Takakura M, Inoue M, Alnemri ES, Shay JW, Kyo S and Kondo S. (2001). *Cancer Res.*, **61**, 5796–5802.

Krasnykh V, Dmitriev I, Mikheeva G, Miller CR, Belousova N and Curiel DT. (1998). *J. Virol.*, **72**, 1844–1852.

Lamfers ML, Grill J, Dirven CM, Van Beusechem VW, Geoerger B, Van Den Berg J, Alemany R, Fueyo J, Curiel DT, Vassal G, Pinedo HM, Vandertop WP and Gerritsen WR. (2002). *Cancer Res.*, **62**, 5736–5742.

Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z and Hsieh JT. (1999). *Cancer Res.*, **59**, 325–330.

Maizel JVJ, White DO and Scharff MD. (1968). *Virology*, **36**, 115–125.

McCormick F. (2001). *Nat. Rev. Cancer*, **1**, 130–141.

Miller CR, Buchsbaum DJ, Reynolds PN, Douglas JT, Gillespie GY, Mayo MS, Raben D and Curiel DT. (1998). *Cancer Res.*, **58**, 5738–5748.

Mizuguchi H, Koizumi N, Hosono T, Utoguchi N, Watanabe Y, Kay MA and Hayakawa T. (2001). *Gene Ther.*, **8**, 730–735.

Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB and Cech TR. (1997). *Science*, **277**, 955–959.

Prince GA, Porter DD, Jenson AB, Horswood RL, Chanock RM and Ginsberg HS. (1993). *J. Virol.*, **67**, 101–111.

Shay JW and Wright WE. (1996). *Curr. Opin. Oncol.*, **8**, 66–71.

Tango Y, Taki M, Shirakiya Y, Ohtani S, Tokunaga N, Tsunemitsu Y, Kagawa S, Tani T, Tanaka N and Fujiwara T. (2004). *Cancer Sci.*, **95**, 459–643.

Tomko RP, Xu R and Philipson L. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 3352–3356.

Umeoka T, Kawashima T, Kagawa S, Teraishi F, Taki M, Nishizaki M, Kyo S, Nagai K, Urata Y, Tanaka N and Fujiwara T. (2004). *Cancer Res.*, **64**, 6259–6265.

Wickham TJ, Mathias P, Cheresh DA and Nemerow GR. (1993). *Cell*, **73**, 309–319.

Wickham TJ, Tzeng E, Shears II LL, Roelvink PW, Li Y, Lee GM, Brough DE, Lizonova A and Kovesdi I. (1997). *J. Virol.*, **71**, 8221–8229.

Wood M, Perrotte P, Onishi E, Harper ME, Dinney C, Pagliaro L and Wilson DR. (1999). *Cancer Gene Ther.*, **6**, 367–372.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Experimental Cell Research 312 (2006) 256 – 265
Experimental Cell Research
www.elsevier.com/locate/yexcr

Research Article

Histone deacetylase inhibitor FR901228 enhances the antitumor effect of telomerase-specific replication-selective adenoviral agent OBP-301 in human lung cancer cells

Takanori Watanabe[a], Masayoshi Hioki[a], Toshiya Fujiwara[a], Masahiko Nishizaki[a], Shunsuke Kagawa[a,b], Masaki Taki[a], Hiroyuki Kishimoto[a], Yoshikatsu Endo[a], Yasuo Urata[c], Noriaki Tanaka[a], Toshiyoshi Fujiwara[a,b,*]

[a]*Division of Surgical Oncology, Department of Surgery, Okayama University, Graduate School of Medicine and Dentistry, Okayama, Japan*
[b]*Center for Gene and Cell Therapy, Okayama University Hospital, Okayama, Japan*
[c]*Oncolys BioPharma, Inc., Tokyo, Japan*

Received 9 June 2005, revised version received 17 October 2005, accepted 21 October 2005

Abstract

Replication-competent oncolytic viruses are being developed for human cancer therapy. We previously reported that an attenuated adenovirus OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of E1A and E1B genes linked with an internal ribosome entry site, could replicate in and causes selective lysis of human cancer cells. Infection efficiency in target cancer cells is the most important factor that predicts the antitumor effects of OBP-301. The objectives of this study are to examine the effects of the histone deacetylase inhibitor FR901228 on the level of coxsackie and adenovirus receptor (CAR) expression and OBP-301-mediated oncolysis in human non-small cell lung cancer cell lines. Flow cytometric analysis revealed up-regulated CAR expression in A549 and H460 cells following treatment with 1 ng/ml of FR901228, which was associated with increased infection efficiency as confirmed by replication-deficient β-galactosidase-expressing adenovirus vector. In contrast, neither CAR expression nor infection efficiency was affected by FR901228 in H1299 cells. To visualize and quantify viral replication in the presence of FR901228, we used OBP-401 (Telomelysin-GFP) that expresses the green fluorescent protein (GFP) reporter gene under the control of the cytomegalovirus promoter in the E3 region. Fluorescence microscopy and flow cytometry showed that FR901228 increased GFP expression in A549 and H460 cells following OBP-401 infection in a dose-dependent manner, but this effect did not occur in H1299 cells. In addition, OBP-301 and FR901228 demonstrated a synergistic antitumor effect in A549 cells in vitro, as confirmed by isobologram analysis. Our data indicate that FR901228 preferentially increases adenovirus infectivity via up-regulation of CAR expression, leading to a profound oncolytic effect, which may have a significant impact on the outcome of adenovirus-based oncolytic virotherapy.

Keywords: Oncolytic virus; HDAC inhibitor; Adenovirus; CAR; Lung cancer

Introduction

Replication-selective, oncolytic viruses provide a new platform to treat a variety of human cancers [1,2]. Promising clinical trial data have shown the antitumor potency and safety of mutant or genetically modified adenoviruses [3–6]. We previously constructed an adenovirus vector (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase (hTERT) promoter element drives expression of E1A and E1B genes linked with an internal ribosome entry site (IRES). We showed that OBP-301 caused efficient selective killing in human cancer cells, but not in normal cells [7]. Although OBP-301 demonstrated a broad-spec-

* Corresponding author. Center for Gene and Cell Therapy, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Fax: +81 86 235 7884.
E-mail address: toshi_f@md.okayama-u.ac.jp (T. Fujiwara).

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2005.10.026

trum antitumor activity, infection efficiency of the presently available adenoviral agent, which is derived from human adenovirus serotype 5, varies widely depending on the expression of coxsackie-adenovirus receptor (CAR) [8,9]. To overcome the limitation of low levels of CAR in certain tumors, we further modified the fiber of OBP-301 to contain RGD peptide. We demonstrated that this fiber-modified OBP-405 permits CAR-independent cell entry and effective destruction of tumors lacking the primary CAR [10]. This strategy has been commonly used to alter adenovirus infectivity [11,12]; the genetic modification, however, might not always be successful. An alternative approach is to modify CAR expression in target tumor cells.

FR901228 (Depsipeptide, FK228) is a novel anticancer agent isolated from the fermentation broth of *Chromobacterium violaceum*. FR901228 has been identified as a potent histone deacetylase (HDAC) inhibitor, although it has no apparent chemical structure that interacts with the HDAC

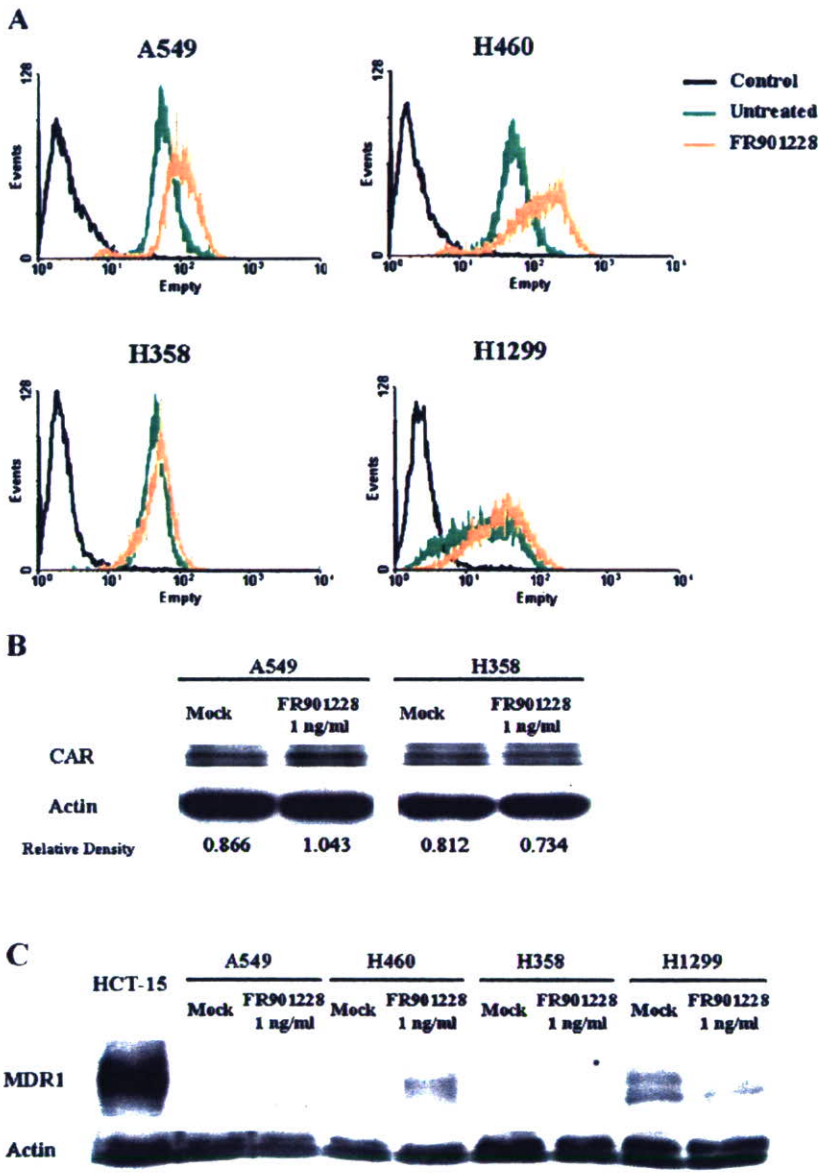


Fig. 1. (A) Expression of CAR on human NSCLC cell lines after FR901228 treatment. Cells were treated with 1 ng/ml of FR901228 for 48 h and then subjected to flow cytometric analysis. Both treated and untreated cells were incubated with mouse monoclonal anti-CAR (RmcB) followed by detection with FITC-labeled secondary antibody. An isotype-matched normal mouse IgG1 conjugated to FITC was used as a control in all experiments. The rightward shift of the histogram after exposure to FR901228 indicates increased CAR expression. (B) Western blot analysis of CAR and actin in A549 and H358 cells. Cells were treated same as above. Equivalent amounts of protein obtained from whole cell lysates were loaded in each lane, probed with anti-CAR antibody, and then visualized by using an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-actin antiserum. CAR protein expression was quantified by densitometric scanning using NIH Image software and normalization by dividing the actin signal. (C) Western blot analysis for MDR1 expression. Equivalent amounts of protein obtained from whole cell lysates were loaded in each lane and probed with anti-MDR1 antibody with or without FR901228 treatment. The cell lysate obtained from HCT-15 human colorectal cancer cells was used as a positive control.

active-site pocket. Thus, FR901228 is structurally distinct from other known HDAC inhibitors such as the trichostatins and trapoxins [13–15]. Histone deacetylation is an important component of transcriptional control, and it has been reported that FR901228 can increase CAR gene expression in several different cancer cell lines [16–20]. Moreover, FR901228 is known to increase viral and transgene expression following adenovirus infection [16]. These findings led us to examine whether FR901228 could augment the antitumor activity of OBP-301 against human cancer cells.

In the present study, we show that FR901228 treatment up-regulates CAR levels on target tumor cells, which in turn increases the amount of cellular viral replication, thereby promoting a synergistic antitumor effect. These findings suggest that treatment with OBP-301 in combination with FR901228 is a promising strategy for human cancer.

Results

Effect of FR901228 on CAR expression in human non-small cell lung cancer (NSCLC) cell lines

To explore the combination effect of OBP-301 and FR901228, we first used flow cytometry to determine if FR901228 has an effect on the cell surface expression of CAR. CAR was expressed in all four cell lines tested: the percentages of CAR-positive cells were 99.4%, 99.4%, 99.8%, and 86.8% in A549, H460, H358, and H1299 cells, respectively. As shown in Fig. 1A, CAR expression levels apparently increased in A549 and H460 cells following 48-h exposure to 1 ng/ml of FR901228, whereas H358 and H1299 cells showed a similar expression pattern of CAR before and after FR901228 treatment. The data were calculated as the relative mean fluorescent intensity (MFI), in which the MFI from FR901228-treated cells is divided by the MFI of cells without FR901228 treatment, thereby giving a fold enhancement of expression. In A549 and H460 cells, there were 1.6- and 2.15-fold higher MFI after FR901228 treatment compared to those before treatment, respectively (MFI: 61.13 to 97.88 [A549] and 59.51 to 127.94 [H460]). Western blot analysis for CAR expression also demonstrated that FR901228 treatment resulted in a 1.2-fold increase in the CAR protein level in A549 cells, whereas CAR protein expression level in H358 cells was consistent even after FR901228 treatment (Fig. 1B).

FR901228 has been previously reported to be a substrate for multidrug resistance protein (MDR1), which mediates FR901228 resistance in human cancer cells [21]. We next examined whether FR901228 treatment could alter MDR1 protein expression in the cell lines we used. As shown in Fig. 1C, MDR1 expression was not detected in these cell lines by Western blot analysis and could not be induced even after FR901228 treatment.

Adenovirus infectivity after FR901228 treatment in human NSCLC cell lines

To evaluate the effect of FR901228 on the infectious efficiency, we compared β-galactosidase-positive cells by X-gal staining 24 h after infection with recombinant replication-deficient adenovirus carrying the β-galactosidase gene under the control of cytomegalovirus (CMV) promoter (Ad-LacZ) in the presence or absence of FR901228. A549 and H1299 cells were treated with FR901228 at the indicated concentration for 48 h. Then, cells were infected with Ad-LacZ at multiplicities of infection (MOI) of 1 or 10 after removing medium containing FR901228. The X-gal staining 24 h after infection demonstrated that FR901228 treatment increased the percentage of blue-stained β-galactosidase-positive A549 cells in a dose-dependent manner; FR901228,

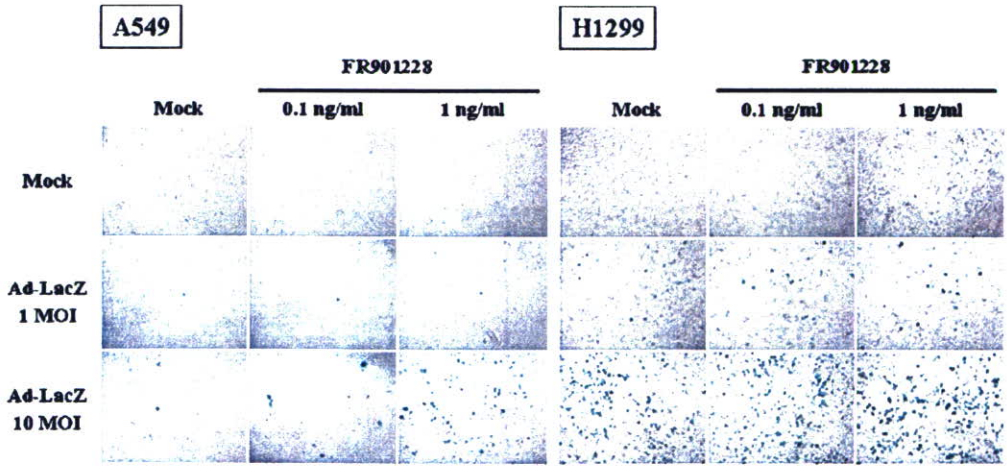


Fig. 2. Expression of β-galactosidase after Ad-LacZ infection in FR901228-treated cells. A549 and H1299 cells were treated with Ad-LacZ and FR901228 at the indicated concentrations, grown for 24 h, and then stained for β-galactosidase activity.

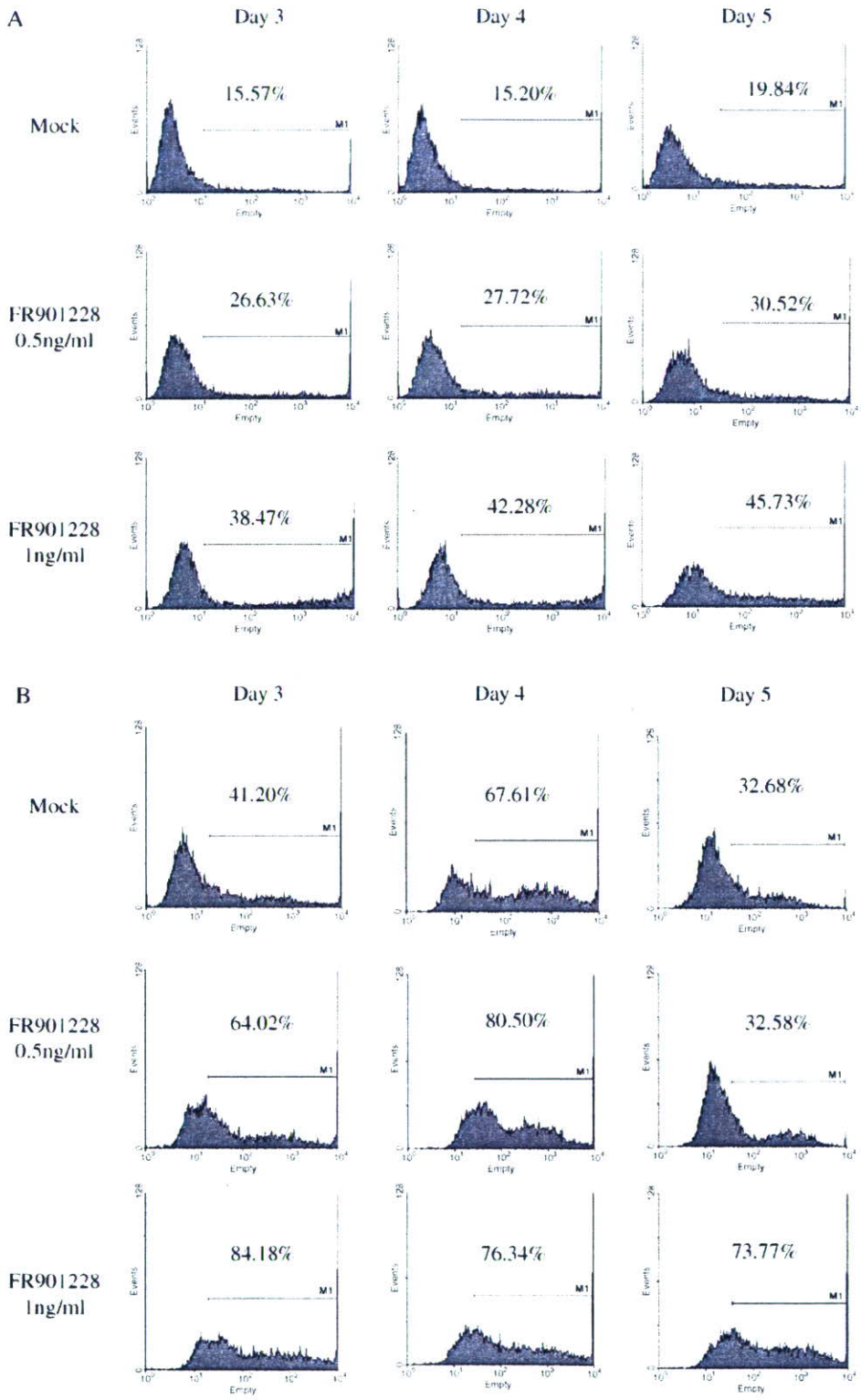


Fig. 3. Flow cytometric analysis of viral-replication-associated GFP expression following OBP-401 and FR901228 treatment. A549 (A), H460 (B), and H1299 (C) cells were infected with 0.1 MOI of OBP-401 and simultaneously treated with FR901228 at the indicated concentrations. The percentages of GFP-positive cells were assessed at different time points after treatment.

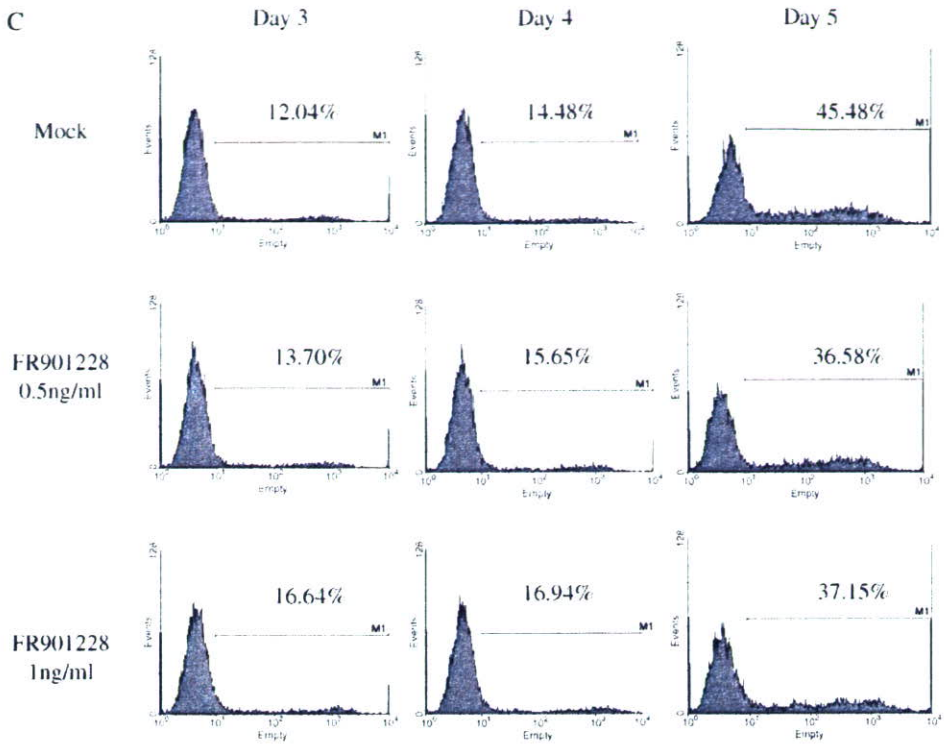


Fig. 3 (*continued*).

however, had no apparent effect on adenovirus infectious efficiency in H1299 cells (Fig. 2). These results were compatible with the effect of FR901228 treatment on CAR levels.

Oncolytic virus replication after FR901228 treatment in human NSCLC cell lines

We next examined the effect of FR901228 on oncolytic virus replication by flow cytometric analysis using telomerase-specific oncolytic adenovirus containing the GFP gene (OBP-401). The proportion of GFP-positive A549 cells increased from 15.57% (day 3) to 19.84% (day 5) after OBP-401 infection because OBP-401 can replicate in telomerase-positive tumor cells. However, the percentage of GFP-expressing cells was 45.73% in the presence of 1 ng/ml of FR901228 compared with 19.84% in cells without FR901228 at 5 days post-infection with 0.1 MOI of OBP-401 (Fig. 3A). Similarly, treatment with 1 ng/ml of FR901228 increased the percentage of GFP-expressing cells from 32.68% to 73.77% in H460 cells 5 days after infection with 0.1 MOI of OBP-401 (Fig. 3B). In contrast, there was no increase in the fraction of GFP-expressing cells after FR901228 treatment in H1299 cells (Fig. 3C). Moreover, the percentage of GFP-expressing H1299 cells decreased in the presence of 1 ng/ml of FR901228 5 days after OBP-401 infection, presumably due to the toxicity of FR901228.

To visualize viral replication in vitro, OBP-401-infected cells were photographed under a fluorescent microscope. As shown in Fig. 4, FR901228 increased the GFP fluorescence intensity in a dose-dependent manner on A549 and H460 cells 4 days after infection with 0.1 MOI of OBP-401. These findings are consistent with the results of flow

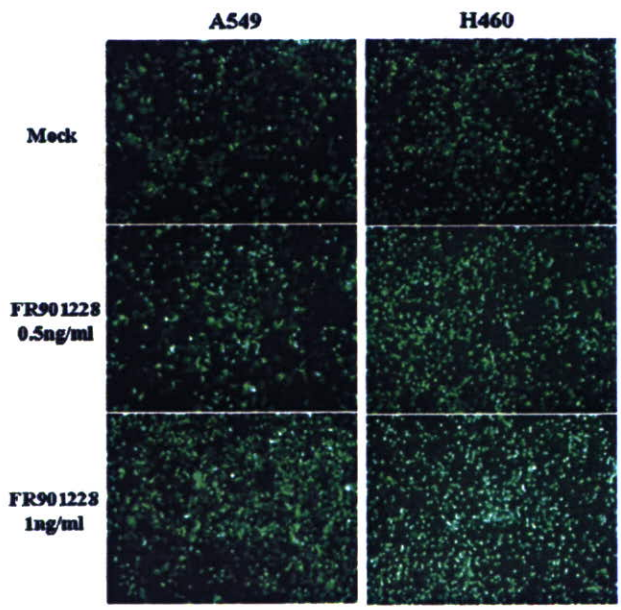


Fig. 4. Visualization of viral-replication-associated GFP expression following OBP-401 and FR901228 treatment. A549 and H460 cells were treated as described in the legend for Fig. 3. Cells were assessed for GFP expression with fluorescence microscopy 4 days after treatment.

cytometric analysis. The microphotographs also demonstrated that the number of GFP-positive cells increased in the presence of FR901228, indicating that OBP-401 could efficiently spread to the neighboring tumor cells. These results suggest that FR901228-induced up-regulation of CAR expression resulted in increased replication of oncolytic virus.

Cell cycle analysis after oncolytic virus infection and FR901228 treatment

To examine whether OBP-301 infection and FR901228 treatment result in cell cycle arrest, apoptosis, or a combination of both processes, the cell cycle distribution was determined by flow cytometric analysis of propidium-iodide-stained cells, a measure of DNA content. As shown in Table 1, neither OBP-301 infection nor FR901228 treatment affected the cell cycle distribution or the fraction of sub-G_0–G_1 apoptotic population in A549 and H460 cell lines.

Synergistic antitumor effect of OBP-301 and FR901228 in human NSCLC cells

We finally examined whether concurrent addition of FR901228 had an effect on the antitumor effect of OBP-301 in A549 cells. The cell viability with 8 doses of OBP-301 or 6 doses of FR901228 was assessed by MTT assay 4 days after treatment with concentrations that resulted in 0 to 100% cell kill when either drug was given alone (Fig. 5A). To determine whether the interaction between the two drugs exhibited synergistic cytotoxic effects, the data from the dose–response curves were examined by constructing isobolograms. Isobologram analysis indicated that the combination was synergistic across all dose levels tested, with all of the points lying into the area below the envelope of additivity (Fig. 5B). The combination index (CI) value of observed data points was 0.6823 ± 0.0761, which was smaller than 1. Thus, telomerase-specific oncolytic adenovirus OBP-301 in combination with FR901228 produces synergistic cell cytotoxicity in A549 human NSCLC cells.

Table 1
Cell cycle analysis after OBP-301 and FR901228 treatment

Cell line	Treatment	Sub-G_0/G_1 (%)	G_1 (%)	S (%)	G_2/M (%)
A549	Pre-treatment	2.04	68.74	15.35	14.18
	FR901228	1.13	74.58	10.43	13.88
	OBP-301	0.78	79.64	10.55	9.17
	FR901228 + OBP-301	2.24	73.08	11.58	13.26
H460	Pre-treatment	18.70	51.79	14.82	15.15
	FR901228	11.28	51.71	11.80	25.33
	OBP-301	1.07	69.70	14.72	14.56
	FR901228 + OBP-301	15.10	47.08	13.60	23.74

A549 and H460 cells were treated with 1 ng/ml of FR901228, 0.1 MOI of OBP-301, or both. The DNA content was determined by propidium iodide staining and flow cytometric analysis at 72 h after treatment. The percentage of cells in each stage of the cell cycle is shown.

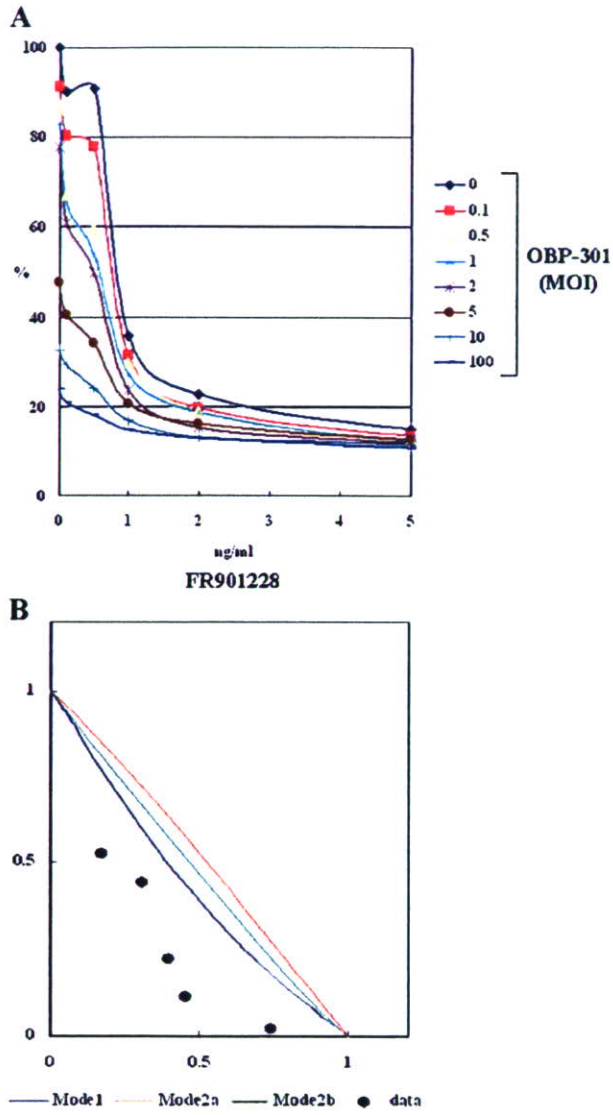


Fig. 5. Antitumor effect of OBP-301 and FR901228 on A549 NSCLC cells in vitro. (A) Effect of OBP-301 and FR901228 at various concentrations was assessed by MTT assay 4 days after treatment. Results are expressed as the percentage of untreated control. (B) The data from dose–response curves were subjected to isobologram analysis. Shown is the isobologram at IC50 based on the results of MTT assays for the A549 cell line treated with combinations of OBP-301 and FR901228 added simultaneously. Points below the envelope indicate a synergistic effect.

Discussion

Oncolytic adenovirus can efficiently kill a variety of human cancer cells; the death process, however, is morphologically distinct from apoptosis that is characterized by chromosome condensation and nuclear shrinkage and fragmentation [22]. In contrast, most of conventional chemotherapeutic drugs trigger apoptosis in human cancer

cells via caspase activation. These two types of anticancer agents that use different cytotoxic machinery have been shown to induce a combination effect [23–25], although the precise mechanism is still unclear. In an attempt to establish multidisciplinary therapeutics based on the rationale, we explored the antitumor effect of oncolytic virus in combination with an HDAC inhibitor. The HDAC inhibitor is a new class of anticancer agents that can modulate transcriptional activity [13,14]. As a result, in addition to the induction of apoptosis, HDAC inhibitors are able to block the cell cycle and angiogenesis, promote differentiation, and mediate additional unknown effects in human cancer cells. In the present study, we found that the most advanced therapeutic candidate, FR901228, had a synergistic antitumor effect with telomerase-specific oncolytic adenovirus OBP-301 through up-regulation of CAR expression in certain types of human NSCLC cells. This may be a novel therapeutic intervention of HDAC inhibitors.

Gene expression is considered to be regulated by chromatin remodeling. Chromatin is intrinsically modified by histone acetyltransferase or HDAC, and alterations in these mechanisms may lead to altered gene expression [26]. It has been reported that the activation of the CAR gene promoter is modulated by histone acetylation and that FR901228 increases CAR RNA levels through histone H3 acetylation in human cancer cells, leading to an increased adenovirus transgene expression [16–18]. Compatible with these observations, we demonstrated that FR901228 treatment led to increased levels of membrane-associated CAR protein in A549 and H460 human NSCLC cell lines (Figs. 1A, B). The reason why FR901228 did not affect CAR expression in H358 and H1299 cells is unclear. As all cell lines were negative for MDR1 expression that could mediate FR901228 resistance [21] (Fig. 1C), other mechanisms such as the Raf-MEK-ERK signal transduction pathway may be involved in CAR gene expression in H358 and H1299 cell lines [27].

We also confirmed increased infection efficiency of adenovirus following FR901228 treatment using Ad-LacZ vector (Fig. 2). One of the advantages of replication-competent oncolytic adenoviruses is that less virus particles are required for treatment because viruses can be produced in tumor tissues by replication. To directly evaluate the reliability of increased virus infectivity by FR901228 for oncolytic virus, we used Ad-LacZ at an MOI of 1 or 10, although 100 MOI of the vector was commonly used in previous studies. We previously reported that the fiber-modified oncolytic adenovirus (OBP-405) containing an RGD motif in the HI loop of the fiber knob showed increased initial virus entry into the target tumor cells and resulted in the augmented viral replication [10]. Thus, FR901228 treatment is expected to enhance virus infectivity as well as replication in A549 and H460 cells. In fact, the percentages of GFP-positive cells were more than 2-fold higher in A549 and H460 cells treated with GFP-expressing OBP-401 in combination with FR901228 than those infected with OBP-401 alone (Figs. 3 and 4). Studies using a non-viral plasmid vector have found that FR901228 enhances luciferase transgene expression at the transcriptional level [28]. Therefore, it is possible that increased GFP expression reflects transcriptionally enhanced transgene expression, but not viral replication. However, the fact that FR901228 treatment did not affect OBP-401-induced GFP expression in H1299 cells suggests that FR901228 could not alter the levels of transgene expression without CAR up-regulation and that GFP expression is dependent on virus infectivity and replication. Taken together, FR901228 increases CAR expression, which in turn facilitates virus infection, thereby leading to increased viral replication in certain types of human NSCLC cells.

Adenovirus replication within a target tumor cell can cause cell destruction by several mechanisms such as direct cytotoxicity due to viral proteins and augmentation of antitumoral immunity [2]. As expected, OBP-301 in combination with FR901228 demonstrated a profound antitumor effect in vitro in A549 cells, presumably because of enhanced viral replication. Isobologram and CI analyses, which are the two most popular methods for evaluating drug interactions in combination cancer chemotherapy [29], consistently identified OBP-301 infection and FR901228 treatment as a synergistic combination (Fig. 5). Theoretically, some chemotherapeutic drugs could act to inhibit viral replication because they might affect the cell cycle; our data, however, demonstrated that FR901228 with or without OBP-301 had no effect on the cell cycle distribution (Table 1), indicating that FR901228 may be an appropriate partner for oncolytic adenovirus because it does not affect the virus life cycle. Moreover, phase I clinical trials for advanced cancer as well as leukemia have shown that FR901228 can be safely administered without any life-threatening toxicities or cardiac toxicities, although the appropriate administration schedule has to be further examined [30,31]. These observations indicate that clinical trials of OBP-301 in combination with FR901228 are warranted.

In conclusion, our data demonstrate a possible interaction between a telomerase-specific oncolytic adenoviral agent and an HDAC inhibitor. Delineating specific virus/drug combinations that are tailored to be particularly effective in human cancer may have the potential to greatly improve the already encouraging results seen in the field of oncolytic virotherapy.

Materials and methods

Cells and culture conditions

The NSCLC cell lines A549, H460, H358, and H1299 were propagated in monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Compounds

FR901228 was kindly provided by the Fujisawa Pharmaceutical Company (Tokyo, Japan).

Recombinant adenoviruses

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin) was previously constructed and characterized [7,32]. Replication-selective OBP-401 (Telomelysin-GFP) containing GFP cDNA under the control of the CMV promoter and replication-deficient adenoviral vector containing β-galactosidase cDNA (Ad-LacZ) were also used [33]. These viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titer and infectious titer was accomplished spectrophotometrically by the method of Maizel et al. [34] and by the method of Kanegae et al. [35], respectively.

Flow cytometry

The cells (2×10^5 cells) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Biotechnology, NY) for 30 min at 4°C, incubated with FITC-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco), and analyzed by the FACSCalibur (Becton Dickinson, Mountain View, CA) using CELL Quest software. The window was set to exclude dead cells and debris. Expression of the GFP gene was also assessed by the FACSCalibur.

Western blot analysis

The primary antibodies against CAR (RmcB; Upstate Biotechnology) and MDR1 (DAKO, Carpinteria, CA) and peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL) were used. Cells were washed twice in cold PBS and collected then lysed in lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% glycerol, 0.5% NP40] containing proteinase inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4). After 20 min on ice, the lysates were spun at 14,000 rpm in a microcentrifuge at 4°C for 10 min. The supernatants were used as whole cell extracts. Protein concentration was determined using the Bio-Rad protein determination method (Bio-Rad, Richmond, CA). Equal amounts (50 μg) of proteins were boiled for 5 min and electrophoresed under reducing conditions on 6–12.5% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to a Hybond-polyvinylidene difluoride (PVDF) transfer membranes (Amersham Life Science, Buckinghamshire, UK) and incubated with the primary followed by peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent western system (Amersham) was used to detect secondary probes.

X-gal staining

Cells were seeded in 6-well plates (2×10^5 cells/well) and infected with Ad-LacZ at indicated MOIs. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed 24 h after infection according to the protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO).

MTT assay, isobologram analysis, and CI analysis

The cytotoxicity of FR901228 and OBP-301 was determined by measurement of cell viability by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 96-well tissue culture plates 24 h before treatment. Then, cells were treated with FR901228 and OBP-301 concurrently at the indicated concentrations and MOIs, respectively. Four days later, a medium containing 0.5 mg/ml MTT (Sigma) was added to each well after the wells were rinsed with PBS. After a 4-h incubation at 37°C, an equal amount of solubilization solution (0.04 N HCl in isopropyl alcohol) was added to each well and mixed thoroughly to dissolve the crystals of MTT formazan. Results were quantified using a Labsystems Multiskan MS at 540 nm wave length. Control absorbance was designated as 100%, and cell survival was expressed as a percentage of control absorbance. MTT results were analyzed quantitatively and statistically by plotting the observed experimental data onto the isobologram [29]. When the observed data points for a combination fell mainly within the envelope of additivity, the effect of the combination was considered as having an additive effect. When the observed data points for a combination fell into the area below the envelope of additivity, the combination effect was regarded as supra-additive (synergism). When the observed data points for a combination fell above the envelope but within the square or the cube, the combination's effect was considered as sub-additive. When the observed data points for a combination fell outside the square or the cube, the effect of the combination was considered protective. Both sub-additive and protective interactions were considered as antagonistic. CI analysis, similar to isobologram analysis, provides qualitative information on the nature of drug interaction. CI is a numerical value calculated as described previously. CIs of less than, equal to, and more than 1 indicate synergy, additivity, and antagonism, respectively. All of the analyses were performed using Statistica software (StatSoft Inc., Tulsa, Oklahoma).

Acknowledgments

We thank the Fujisawa Pharmaceutical Company for providing FR901228. We thank Yoshiko Shirakiya, Yuuri Hashimoto, and Nobue Mukai for their excellent technical

assistance. This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan and grants from the Ministry of Health and Welfare, Japan.

References

[1] L.K. Hawkins, N.R. Lemoine, D. Kirn, Oncolytic biotherapy: a novel therapeutic platform, Lancet Oncol. 3 (2002) 17–26.
[2] E.A. Chiocca, Oncolytic viruses, Nat. Rev., Cancer 2 (2002) 938–950.
[3] J. Nemunaitis, F. Khuri, I. Ganly, J. Arseneau, M. Posner, E. Vokes, J. Kuhn, T. McCarty, S. Landers, A. Blackburn, L. Romel, B. Randlev, S. Kaye, D. Kirn, Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer, J. Clin. Oncol. 19 (2001) 289–298.
[4] T.L. DeWeese, H. van der Poel, S. Li, B. Mikhak, R. Drew, M. Goemann, U. Hamper, R. DeJong, N. Detorie, R. Rodriguez, T. Haulk, A.M DeMarzo, S. Piantadosi, D.C. Yu, Y. Chen, D.R. Henderson, M.A. Carducci, W.G. Nelson, J.W. Simons, A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy, Cancer Res. 61 (2001) 7464–7472.
[5] T. Reid, E. Galanis, J. Abbruzzese, D. Sze, L.M. Wein, J. Andrews, B. Randlev, C. Heise, M. Uprichard, M. Hatfield, L. Rome, J. Rubin, D. Kirn, Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints, Cancer Res. 62 (2002) 6070–6079.
[6] O. Hamid, M.L. Varterasian, S. Wadler, J.R. Hecht, A. Benson, E. Galanis, M. Uprichard, C. Omer, P. Bycott, R.C. Hackman, A.F. Shields, Phase II trial of intravenous CI-1042 in patients with metastatic colorectal cancer, J. Clin. Oncol. 21 (2003) 1498–1504.
[7] T. Kawashima, S. Kagawa, N. Kobayashi, Y. Shirakiya, T. Umeoka, F. Teraishi, M. Taki, S. Kyo, N. Tanaka, T. Fujiwara, Telomerase-specific replication-selective virotherapy for human cancer, Clin. Cancer Res. 10 (2004) 285–292.
[8] J.M. Bergelson, J.A. Cunningham, G. Droguett, E.A. Kurt-Jones, A. Krithivas, J.S. Hong, M.S. Horwitz, R.L. Crowell, R.W. Finberg, Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5, Science 275 (1997) 1320–1323.
[9] R.P. Tomko, R. Xu, L. Philipson, HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 3352–3356.
[10] M. Taki, S. Kagawa, M. Nishizaki, H. Mizuguchi, T. Hayakawa, S. Kyo, K. Nagai, Y. Urata, N. Tanaka, T. Fujiwara, Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD'), Oncogene 24 (2005) 3130–3140.
[11] H. Mizuguchi, T. Hayakawa, Enhanced antitumor effect and reduced vector dissemination with fiber-modified adenovirus vectors expressing herpes simplex virus thymidine kinase, Cancer Gene Ther. 9 (2002) 236–242.
[12] A.V. Borovjagin, A. Krendelchtchikov, N. Ramesh, D.C. Yu, T.J. Douglas, D.T. Curiel, Complex mosaicism is a novel approach to infectivity enhancement of adenovirus type 5-based vectors, Cancer Gene Ther. 12 (2005) 475–486.
[13] H. Ueda, H. Nakajima, Y. Hori, T. Fujita, M. Nishimura, T. Goto, M. Okuhara, FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968: I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity, J. Antibiot. (Tokyo) 47 (1994) 301–310.
[14] H. Nakajima, Y.B. Kim, H. Terano, M. Yoshida, S. Horinouchi, FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor, Exp. Cell Res. 241 (1998) 126–133.
[15] R. Furumai, A. Matsuyama, N. Kobashi, K.H. Lee, M. Nishiyama, H. Nakajima, A. Tanaka, Y. Komatsu, N. Nishino, M. Yoshida, S. Horinouchi, FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases, Cancer Res. 62 (2002) 4916–4921.
[16] M. Kitazono, M.E. Goldsmith, T. Aikou, S. Bate, T. Fojo, Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228, Cancer Res. 61 (2001) 6230–6328.
[17] M. Kitazono, V.K. Rao, R. Robey, T. Aikou, S. Bates, T. Fojo, M.E. Goldsmith, Histone deacetylase inhibitor FR901228 enhances adenovirus infection of hematopoietic cells, Blood 99 (2002) 2248–2251.
[18] M.E. Goldsmit, M. Kitazono, P. Fok, T. Aikou, S. Bates, T. Fojo, The histone deacetylase inhibitor FK228 preferentially enhances adenovirus transgene expression in malignant cells, Clin. Cancer Res. 9 (2003) 5394–5401.
[19] R.C. Pong, Y.J. Lai, H. Chen, T. Okegawa, E. Frenkel, A. Sagalowsky, J.T. Hsieh, Epigenetic regulation of coxsackie and adenovirus receptor (CAR) gene promoter in urogenital cancer cells, Cancer Res. 63 (2003) 8680–8686.
[20] A. Hemminki, A. Kanerva, B. Liu, M. Wang, R.D. Alvarez, G.P. Siegal, D.T. Curiel, Modulation of coxsackie-adenovirus receptor expression for increased adenoviral transgene expression, Cancer Res. 63 (2003) 847–853.
[21] J.J. Xiao, Y. Huang, Z. Dai, W. Sadee, J. Chen, S. Liu, G. Marcucci, J. Byrd, J.M. Covey, J. Wright, M. Grever, K.K. Chan, Chemoresistance to depsipeptide FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]-tricos-16-ene-3,6,9,22-pentanone] is mediated by reversible MDR1 induction in human cancer cell lines, J. Pharmacol. Exp. Ther. 314 (2005) 467–475.
[22] E.I. Abou, M.A. Hassan, I. van der Meulen-Muileman, S. Abbas, F.A. Kruyt, Conditionally replicating adenoviruses kill tumor cells via a basic apoptotic machinery-independent mechanism that resembles necrosis-like programmed cell death, J. Virol. 78 (2004) 12243–12251.
[23] C. Heise, A. Sampson-Johannes, A. Williams, F. McCormick, D.D. Von Hoff, D.H. Kirn, ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents, Nat. Med. 3 (1997) 639–645.
[24] D.C. Yu, Y. Chen, J. Dilley, Y. Li, M. Embry, H. Zhang, N. Nguyen, P. Amin, J. Oh, D.R. Henderson, Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel, Cancer Res. 61 (2001) 517–525.
[25] J. Zhang, N. Ramesh, Y. Chen, Y. Li, J. Dilley, P. Working, D.C. Yu, Identification of human uroplakin II promoter and its use in the construction of CG8840, a urothelium-specific adenovirus variant that eliminates established bladder tumors in combination with docetaxel, Cancer Res. 62 (2002) 3743–3750.
[26] G.J. Narlikar, H.Y. Fan, E.C. Kingston, Cooperation between complexes that regulate chromatin structure and transcription, Cell 108 (2002) 475–487.
[27] M. Anders, C. Christian, M. McMahon, F. McCormick, W.M. Korn, Inhibition of the Raf/MEK/ERK pathway up-regulates expression of the coxsackievirus and adenovirus receptor in cancer cells, Cancer Res. 63 (2003) 2088–2095.
[28] T. Yamano, K. Ura, R. Morishita, H. Nakajima, M. Monden, Y. Kaneda, Amplification of transgene expression in vitro and in vivo using a novel inhibitor of histone deacetylase, Mol. Ther. 1 (2000) 574–580.
[29] M. Nishizaki, R.E. Meyn, L.B. Levy, E.N. Atkinson, R.A. White, J.A. Roth, L. Ji, Synergistic inhibition of human lung cancer cell growth by adenovirus-mediated wild-type p53 gene transfer in combination with docetaxel and radiation therapeutics in vitro and in vivo, Clin. Cancer Res. 7 (2001) 2887–2897.

[30] J.L. Marshall, N. Rizvi, J. Kauh, W. Dahut, M. Figuera, M.H. Kang, W.D. Figg, I. Wainer, C. Chaissang, M.Z. Li, M.J. Hawkins, A phase I trial of depsipeptide (FR901228) in patients with advanced cancer, J. Exp. Ther. Oncol. 2 (2002) 325–332.

[31] J.C. Byrd, G. Marcucci, M.R. Parthun, J.J. Xiao, R.B. Klisovic, M. Moran, T.S. Lin, S. Liu, A.R. Sklenar, M.E. Davis, D.M. Lucas, B. Fischer, R. Shank, S.L. Tejaswi, P. Binkley, J. Wright, K.K. Chan, M.R. Grever, A phase I and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia, Blood 105 (2005) 959–967.

[32] T. Umeoka, T. Kawashima, S. Kagawa, F. Teraishi, M. Taki, M. Nishizaki, S. Kyo, K. Nagai, Y. Urata, N. Tanaka, T. Fujiwara, Visualization of intrathoracically disseminated solid tumors in mice with optical imaging by telomerase-specific amplification of a transferred green fluorescent protein gene, Cancer Res. 64 (2004) 6259–6265.

[33] Y. Tango, M. Taki, Y. Shirakiya, S. Ohtani, N. Tokunaga, Y. Tsunemitsu, S. Kagawa, T. Tani, N. Tanaka, T. Fujiwara, Late resistance to adenoviral p53-mediated apoptosis caused by decreased expression of Coxsackie-adenovirus receptors in human lung cancer cells, Cancer Sci. 95 (2004) 459–463.

[34] J.V.J. Maizel, D.O. White, M.D. Scharff, The polypeptides of adenovirus: I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12, Virology 36 (1968) 115–125.

[35] Y. Kanegae, M. Makimura, I. Saito, A simple and efficient method for purification of infectious recombinant adenovirus, Jpn. J. Med. Sci. Biol. 47 (1994) 157–166.

Int. J. Cancer: **119**, 432–440 (2006)

Enhanced antitumor efficacy of telomerase-selective oncolytic adenoviral agent OBP-401 with docetaxel: Preclinical evaluation of chemovirotherapy

Toshiya Fujiwara[1], Shunsuke Kagawa[1,2], Hiroyuki Kishimoto[1], Yoshikatsu Endo[1], Masayoshi Hioki[1], Yoshihiro Ikeda[1], Ryo Sakai[1], Yasuo Urata[3], Noriaki Tanaka[1] and Toshiyoshi Fujiwara[1,2]*

[1]*Division of Surgical Oncology, Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*
[2]*Okayama University Hospital, Center for Gene and Cell Therapy, Okayama, Japan*
[3]*Oncolys Biopharma Inc., Tokyo, Japan*

Oncolytic adenoviruses are being developed as novel anticancer therapeutics and currently undergoing clinical trials. We previously demonstrated that telomerase-specific replication-competent adenovirus (Telomelysin: OBP-301), in which the human telomerase reverse transcriptase (hTERT) promoter regulates viral replication, efficiently killed human tumor cells. We further constructed OBP-401 (Telomelysin-GFP) that expresses the green fluorescent protein (*GFP*) reporter gene under the control of the cytomegalovirus promoter in the E3 region to monitor viral distribution. Here, we examined the feasibility of a single-agent therapy with OBP-401 as well as of combining OBP-401 with chemotherapeutic agents. Infection of OBP-401 alone or followed by the treatment of a chemotherapeutic drug, docetaxel (Taxotere), resulted in a profound *in vitro* cytotoxicity and GFP expression in various human cancer cell lines originating from different organs (lung, colon, esophagus, stomach, liver and prostate), although the magnitude of antitumor effect varied among the cell types. Other chemotherapeutic drugs such as vinorelbine (Navelbine) and SN38 (the potent active metabolite of irinotecan) combined with OBP-401 also inhibited the growth of human cancer cells. Quantitative real-time PCR analysis demonstrated that docetaxel did not affect viral replication. For *in vivo* evaluation, *nu/nu* mice xenografted with H1299 human lung tumor received intratumoral injection of OBP-401 and intraperitoneal administration of docetaxel. Analysis of growth of implanted tumors showed a significant, therapeutic synergism, although OBP-401 alone and docetaxel alone showed modest inhibition of tumor growth. Thus, OBP-401 in combination with docetaxel efficiently enhances the antitumor efficacy both *in vitro* and *in vivo*, and the outcome has important implications for tumor-specific oncolytic chemovirotherapies for human cancers.

Key words: oncolytic adenovirus; combination therapy; docetaxel; GFP

Lack of restricted selectivity for tumor cells is the primary limitation of common cancer therapeutics such as chemotherapy and radiotherapy. To improve the therapeutic index, there is a need for anticancer agents that selectively target only tumor cells and spare normal cells. Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosomal ends.[1] Many studies have demonstrated the expression of telomerase activity in more than 85% of human cancers,[2] but only in few normal somatic cells.[3] Telomerase activation is considered a critical step in carcinogenesis and its activity is closely correlated with human telomerase reverse transcriptase (hTERT) expression.[4] Replication-selective tumor-specific adenoviruses are being developed as novel anticancer therapies.[5–9] We previously developed an adenovirus vector that drives E1A and E1B genes under the hTERT promoter, namely Telomelysin: OBP-301,[10–12] and showed its selective replication as well as profound cytotoxic activity in a variety of human cancer cells. Although the development of OBP-301 as a monotherapy is currently underway clinically based on promising results of preclinical studies, multimodal strategies to enhance antitumor efficacy *in vivo* is essential for successful clinical outcome. In fact, most of the clinical trials for oncolytic viruses have been conducted in combination with chemotherapy or radiotherapy.[13–16]

Docetaxel is an antineoplastic agent and a member of the taxane family, which are novel antimicrotubule agents that promote the assembly of microtubules from tubulin dimers and stabilization of such microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions. In addition, the taxanes induce abnormal arrays of microtubules throughout the cell cycle and multiple asters of microtubules during mitosis and arrest cells especially in G2 and M phases.[17,18] The effectiveness of any chemotherapy against malignant tumors may be increased by dose intensification; however, such strategy is often associated with the appearance of drug-induced toxicities. Ideally, the strategy for effective treatment should include maximizing the dose enhancement while minimizing systemic toxicity.

In the present study, we hypothesized that combination of oncolytic adenoviral agents (with novel mechanisms of action) with chemotherapeutic agents could minimize the toxic side effects of the latter by reducing the concentrations of anticancer drugs. To test our hypothesis, we used a novel oncolytic adenovirus named OBP-401, which expresses the green fluorescent protein (GFP) reporter gene under the control of the cytomegalovirus promoter in the E3 region to monitor viral distribution by its fluorescence. The therapeutic effects of OBP-401 combined with docetaxel were tested both *in vitro* and *in vivo*. The results showed that the combination therapy of OBP-401 and docetaxel produced additive therapeutic benefits over either individual modality. In addition, docetaxel is considered innocuous for replication of OBP-401 because this agent does not inhibit DNA synthesis.

Material and methods

Cell lines and cell cultures

The human non-small cell lung cancer cell lines H1299 and H226Br, the human colorectal carcinoma cell lines SW620 and DLD-1, the human gastric cancer cell line MKN28, the human esophageal cancer cell lines T.Tn and TE8 and the human prostate cancer cell line LNCaP were propagated in monolayer culture in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The human non-small cell lung cancer cell line A549 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing Nutrient Mixture (Ham's F-12). The transformed embryonic kidney cell line 293 and human hepatic cancer cell line HepG2 were grown in DMEM containing high glucose (4.5 g/l) and supplemented with 10% FCS.

Grant sponsors: Ministry of Education, Science, and Culture, Japan; Ministry of Health and Welfare, Japan.
***Correspondence to:** Center for Gene and Cell Therapy, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan.
Fax: +81-86-235-7884. E-mail: toshi_f@md.okayama-u.ac.jp
Received 2 August 2005; Accepted 14 December 2005
DOI 10.1002/ijc.21846
Published online 13 February 2006 in Wiley InterScience (www.interscience.wiley.com).

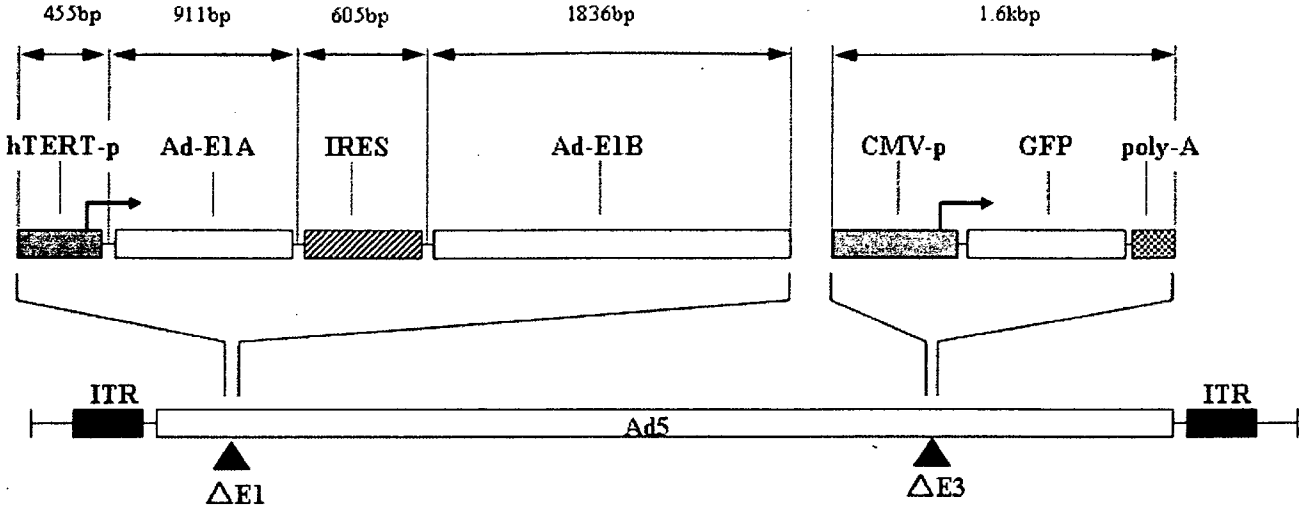


FIGURE 1 – Schematic diagrams of OBP-401DNA structures. OBP-401 is a telomerase-specific replication-competent adenovirus variant, in which the hTERT promoter element drives the expression of E1A and E1B genes linked with an IRES, and GFP gene is inserted under CMV promoter into E3 region for monitoring viral replication.

Chemotherapeutic agents and viruses

Docetaxel (taxotere), SN-38 (topotecin) and vinorelbine (navelbine) were kindly provided by Aventis Pharma (Tokyo, Japan), Daiichi Pharmaceutical Co. (Tokyo) and Kyowa Hakko Kogyo Co. (Tokyo), respectively. These agents were diluted with the respective medium just before use for *in vitro* studies and with phosphate-buffered saline (PBS) for *in vivo* studies. OBP-401 is a telomerase-specific replication-competent adenovirus variant, in which the hTERT promoter element drives the expression of E1A and E1B genes linked with an IRES, with the GFP gene inserted under cytomegalovirus (CMV) promoter into E3 region (Fig. 1). Construction of OBP-401 was described elsewhere. The virus was purified by ultracentrifugation in cesium chloride step gradients, and the titer was determined by plaque assay in 293 cells, as described previously.[19]

Cell viability assay

XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay was performed by seeding human tumor cells at 1,000 cells/well in 96-well plates 18–20 hr before viral infection. The cells were then infected with OBP-401 at a multiplicity of infection (MOI) of 0.1 and 1 plaque-forming units (PFU)/cell and subsequently treated with chemotherapeutic agents 24 hr after infection. Cell viability was determined at the indicated times by using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the protocol provided by the manufacturer.

Cell killing assay

Human non-small cell lung cancer cell line H1299 and human colorectal carcinoma cell line SW620 were plated on 25-cm^2 flasks at 1×10^5 cells at 48 hr before viral infection. Cells were then infected with OBP-401 at an MOI of 0.1 PFU/cell and the culture medium was replaced with freshly prepared medium containing docetaxel at 10 nM at 2 hr after infection, and photomicrographs were taken every day at a magnification of ×100.

In vitro *replication assay*

H1299 and SW620 cells were seeded on 25-cm^2 flasks at 5×10^4 cells at 48 hr before infection. Cells were infected with OBP-401 at an MOI of 0.1 PFU/cell and a fresh medium containing docetaxel at 10 nM was added 24 hr later. Alternatively, cells were pretreated with 10 nM of docetaxel for 24 hr, infected with 0.1 PFU/cell of OBP-401 for 2 hr, and then cultured in the presence of 10 nM of docetaxel following the removal of viruses. The cells were incubated at 37°C, trypsinized and harvested for intracellular replication analysis at 2, 12, 24, 36, 48 and 60 hr. The supernatants of H1299 and SW620 cells were also harvested for viral progenies release analysis at the indicated time points over 120 hr. DNA purification was performed using QIAmp DNA mini kit (Qiagen Inc., Valencia, CA). 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).

In vivo *human tumor model*

H1299 cells (1×10^7 cells/mouse) were injected subcutaneously into the flank of 5–6-week-old female BALB/c *nu/nu* mice and permitted to grow to ~5–10 mm in diameter. At that time, the mice were randomly assigned into 4 groups, and a 50-μl solution containing OBP-401, at a dose of 1×10^7 PFU/body or PBS was injected into the tumor. Simultaneously, each mouse received an intraperitoneal injection of 100 μl of docetaxel at a dose of 12.5 mg/kg every 2 days starting at day 1. The perpendicular diameter of each tumor was measured every 3 days, 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 to calculate the volume of an ellipsoid. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine.

Histopathological study

Mice were sacrificed 6 days after the final dose of OBP-401 and docetaxel, and histochemical examination was performed. Tumors and liver tissues were fixed in 10% Formalin, embedded in paraffin, and then cut into 4-μm thick sections. Liver tissue samples and tumor sections were stained with hematoxylin and eosin.

Results

Antitumor efficacy of OBP-401 plus docetaxel in human cancer cell lines in vitro

The tumor-specific replication-competent GFP-expressing adenovirus OBP-401 was constructed by inserting the GFP gene under the control of the CMV promoter at the deleted E3 region of

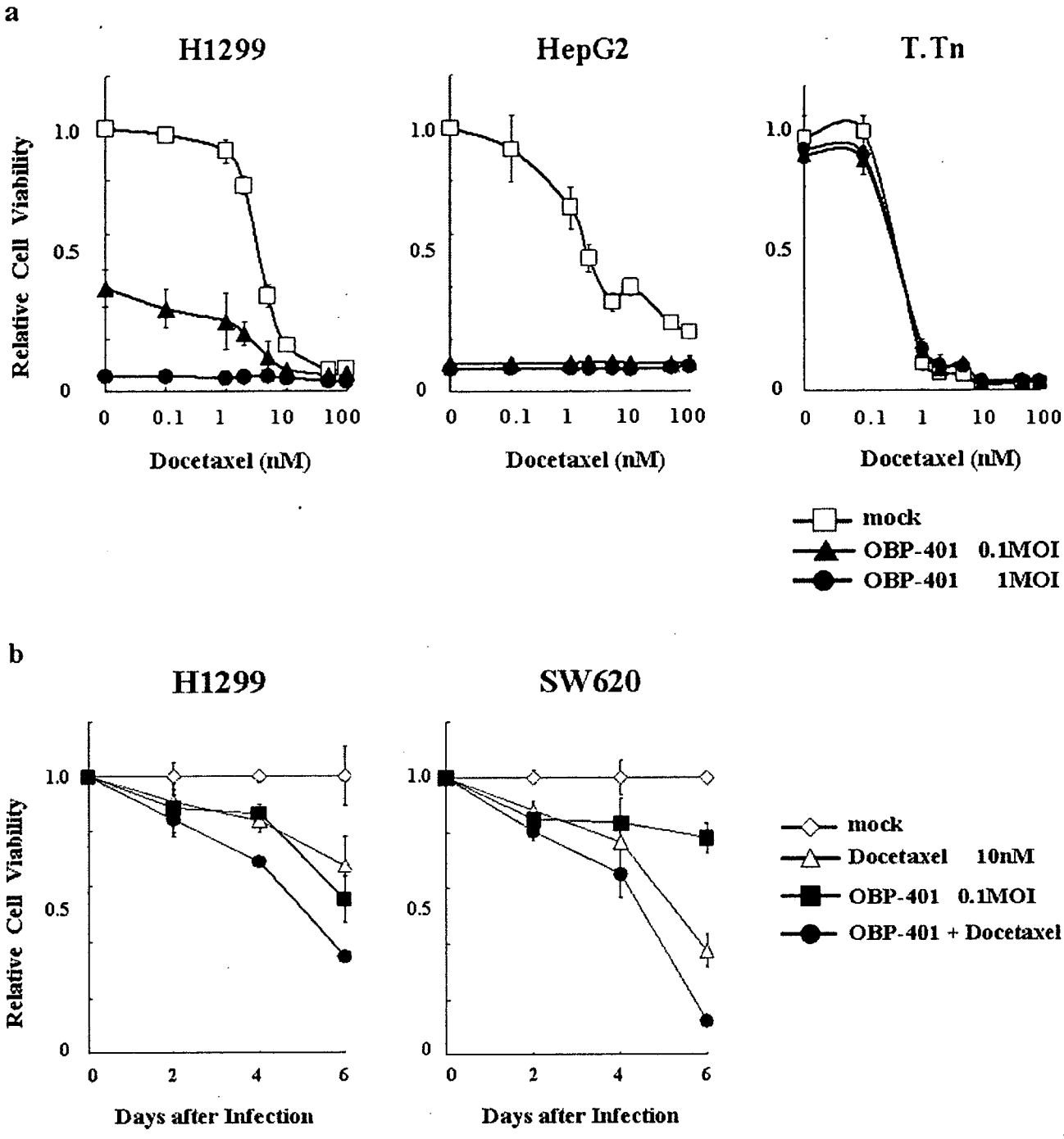


FIGURE 2 – Combination effect of OBP-401 and docetaxel on human cancer cell lines. (*a*) Cells were infected with 0.1 or 1 MOI of OBP-401, and then exposed to docetaxel at the indicated concentrations at 24 hr after infection. Cell viability was assessed by XTT assay at 5 days after OBP-401 infection. The results of H1299 (lung), HepG2 (liver) and T.Tn (esophagus) cells were shown as representative of 10 cell lines. Bars, standard deviation (SD). (*b*) Time course of combined effect of OBP-401 plus docetaxel on H1299 and SW620 cells. Cells were treated with 0.1 MOI of OBP-401, 10 nM of docetaxel, or a combination of both, and cell killing efficacy was evaluated by XTT assay over 6 days. Docetaxel was added to the medium 24 hr after OBP-401 infection (day 1).

the telomerase-specific replication-selective type 5 adenovirus OBP-301 (Fig. 1). To examine the potential interaction between OBP-401 and docetaxel *in vitro*, we first evaluated the effect of the 2 modalities at various doses in 10 human cancer cell lines originating from different organs (lung [H1299, A549 and H226Br], colon [SW620 and DLD-1], esophagus [TE8 and T.Tn], stomach [MKN28], liver [HepG2] and prostate [LNCaP]). The cell viability with 3 different doses of OBP-401 and 8 doses of docetaxel was assessed by XTT assay 5 days after treatment. To optimize the experimental design, the concentrations of docetaxel that resulted in 0–100% cell kill when given alone were chosen. The addition of 0.1 MOI of OBP-401 increased the cell killing ac-