A Phase I Study of Telomerase-specific Replication Competent Oncolytic Adenovirus (Telomelysin) for Various Solid Tumors

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A phase I clinical trial was conducted to determine the clinical safety of Telomelysin, a human telomerase reverse transcriptase (hTERT) promoter driven modified oncolytic adenovirus, in patients with advanced solid tumors. A single intratumoral injection (IT) of Telomelysin was administered to three cohorts of patients (1×10^{10}, 1×10^{11}, 1×10^{12} viral particles). Safety, response and pharmacodynamics were evaluated. Sixteen patients with a variety of solid tumors were enrolled. IT of Telomelysin was well tolerated at all dose levels. Common grade 1 and 2 toxicities included injection site reactions (pain, induration) and systemic reactions (fever, chills). hTERT expression was demonstrated at biopsy in 9 of 12 patients. Viral DNA was transiently detected in plasma in 13 of 16 patients. Viral DNA was detectable in four patients in plasma or sputum at day 7 and 14 post-treatment despite below detectable levels at 24 h, suggesting viral replication. One patient had a partial response of the injected malignant lesion. Seven patients fulfilled Response Evaluation Criteria in Solid Tumors (RECIST) definition for stable disease at day 56 after treatment. Telomelysin was well tolerated. Evidence of antitumor activity was suggested.

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INTRODUCTION

Conditionally replicative oncolytic viruses are engineered to replicate selectively in cancer cells with specified oncogenic phenotypes. Multiple viral backbones have been employed, although the most commonly utilized is derived from the adenovirus serotype 5.

Two different approaches have been used to limit adenoviral replication to cancer cells. One approach is to delete components of viral genes (*E1A*, *E1B*) that function in part to neutralize normal cell defense (p53, Rb) mechanisms. Loss of function of the cell defense genes in cancer cells renders the virus cytotoxic to tumor cells but incapable of replication in normal cells, as exemplified by ONYX-015 or Δ24.[1] Alternatively, native viral promoters that govern the initiation of viral replication can be replaced with a promoter region for genes that are active and/or overexpressed in cancer cells.[2,3] The resulting constructs display viral cytolytic activity that is confined to cancer cells but at a level that approaches that of wild-type adenovirus.[2] Numerous studies have confirmed that administration of live, wild-type adenovirus to healthy, adult humans is safe.[3]

Telomelysin is a novel, replication-competent adenovirus serotype 5-based adenoviral construct that incorporates a human telomerase reverse transcriptase gene (*hTERT*) promoter. *hTERT* encodes for the catalytic protein subunit of telomerase, a polymerase that acts to stabilize telomere lengths and is highly expressed in tumors but not in normal, differentiated adult cells.[4,5]

Additional modifications of Telomelysin include the replacement of the normal transcriptional element of viral *E1B* gene by an IRES (Internal Ribosomal Entry Site) sequence to minimize "leakiness" further enhancing specificity. Furthermore, Telomelysin is the first replication-competent adenovirus that retains a fully functional viral E3 region.[6]

In vitro studies have validated the selective infectivity and direct cytolysis of Telomelysin in cancer cells but not nonmalignant cells.[5] In animal experiments, intratumoral injection (IT) of Telomelysin demonstrated antitumor activity without significant toxicity to normal organs. Additionally, distant viral uptake was observed following IT evidenced by the presence of adenoviral protein identified in noninjected tumor following intratumoral treatment of the contralateral tumor.[5]

These encouraging preclinical findings of safety and directed antitumor activity form the basis of our phase I study, which is designed to validate safety, response and pharmacodynamics of Telomelysin in advanced cancer patients.

RESULTS

Patient profile

Sixteen patients were entered into trial: three each into cohorts 1 and 2 and 10 into cohort 3. The age, sex, histological diagnosis, and prior treatments of the evaluated patients are shown in **Table 1**.

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Table 1 Patient demographics

Patient	Age	Sex	Histological diagnosis	Prior treatments
1 (101)	57	F	Squamous cell ca unknown primary	XRT, carboplatin, docetaxel, anastrozole
2 (102)	54	M	Melanoma	Interferon, dacarbazine
3 (103)	34	F	Melanoma	Lenalidomide, dacarbazine, vinblastine, cisplatin, IL-2, interferon
4 (204)	60	M	Salivary gland tumor	XRT, perifosine
5 (205)	69	M	Squamous cell ca base of tongue	Cisplatin, XRT
6 (206)	60	F	Leiomyosarcoma	Doxorubicin, ifosfamide, gemcitabine, docetaxel, perifosine
7 (307)	52	F	Neuroendocrine tumor	Irinotecan, cisplatin, topotecan, docetaxel, pemetrexed, CT 2103, XRT
8 (308)	78	F	Melanoma	Interferon
9 (309)	54	M	NSCLC	Paclitaxel, carboplatin, pemetrexed, XRT
10 (310)	49	M	Squamous cell ca base of tongue	Paclitaxel, carboplatin, cisplatin, fluorouracil, cetuximab, XRT
11 (311)	60	M	Squamous cell ca floor of mouth	Cisplatin, XRT
12 (312)	48	M	Melanoma	Interferon, melphalan, actinomycin-D
13 (313)	54	F	Sarcoma	None
14 (314)	38	M	Basal cell carcinoma	Cisplatin, fluorouracil
15 (315)	54	F	Squamous cell ca of gall bladder	Capecitabine, gemcitabine
16 (316)	46	F	Breast cancer	Doxorubicin, cyclophosphamide, paclitaxol, herceptin, tamoxifen, anastrozole, capecitabine, docetaxel

M, male; F, Female.

Table 2 List of common[a] adverse events

	Grade 1	Grade 2	Grade 3, 4	Overall (*N* = 16)
Cardiac arrhythmia				
Supraventricular and nodal arrhythmia—sinus tachycardia	2	1	0	3
Gastrointestinal				
Nausea	3	1	0	4
Constitutional Sypmtoms				
Chills	1	5	0	6
Fatigue	7	2	0	9
Edema peripheral	1	2	0	3
Fever	3	3	0	6
Pain				
Bone	2	1	0	3
Muscle	0	3	0	3
Extremity	2	1	0	3
Pain	3	1	0	4
Headache	3	1	0	4
Pulmonary/upper respiratory				
Nasal cavity/paranasal reactions	2	1	0	3
Dermatology/skin				
Erythema	0	0	3	3
Injection site bruising	3	0	0	3
Injection site erythema	1	4	0	5
Injection site pain	4	2	0	6

[a]Occurring in >15% of patients (*n* = 16).

Adverse events

No clinically significant grade 3 or 4 treatment related toxic events were experienced by any patients. There were multiple grade 1 and 2 adverse events, with the most common being fever, chills, fatigue, and injection site pain (Table 2). Thirteen patients developed asymptomatic transient lymphocyte decreases, seven grade 2, five grade 3 and one grade 4, 24 hours after Telomelysin injection with complete recovery by day 7 following injection.

Clinical response

Eleven patients satisfied Response Evaluation Criteria in Solid Tumors (RECIST) criteria for stable disease response to the injected lesion at Day 28, three had progressive disease and two more unevaluable. Seven of the day 28 stable disease patients had stable disease at day 56, two had progressive disease and two were unevaluable. One patient (pt 308) had 33% reduction of injected lesion at day 28 and 56.7% reduction of injected lesion at day 56 (see Figure 1).

Postinjection biopsies performed at day 28 on four of the patients with stable disease revealed necrosis that may or may not be treatment induced. Three of these patients had melanoma. Survival of all patients ranged from 1 to 21 months (median 10).

Viral pharmacokinetics analysis

Systemic dissemination of Telomelysin was evaluated by collection of patient plasma, urine, sputum, and saliva at time points before and after IT. Quantitative real-time PCR analysis was carried out with primers that were specific for the Telomelysin E1A and IRES regions. We detected the presence of viral DNA in 13 of 16 patient plasma samples tested, including 9 of 10 patients in cohort 3 (Table 3). Plasma viral DNA was detected between 30 minutes and 6 hours in most patients, at concentrations that ranged from 2.1×10^3 to 1.5×10^7 viral copies/ml. We detected the presence of plasma viral particles in two cohort 3 patients. Viral DNA copies detected on day 7 (pt 312: 3.7×10^3; pt 316: 2×10^4 viral copies/ml, respectively) were ~10–50-fold higher than detection threshold (400 vp/ml). Viral DNA was also detected in one cohort 2 patient on days 7 and 14 [pt 205: 3.7×10^3 (day 7), 6.0×10^3 (day 14)] but not at (Figure 2). No viral DNA was detected at 24 hours post-treatment for these patients, suggesting that detectable levels of viral DNA at days 7 and 14 may constitute a second

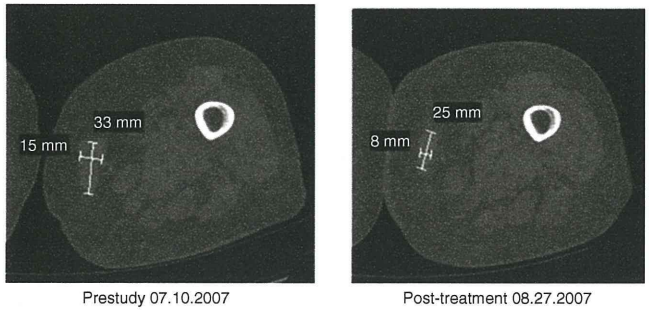

Figure 1 Patient 308: Initial response of the largest of three metastatic melanoma lesions involving the right thigh.

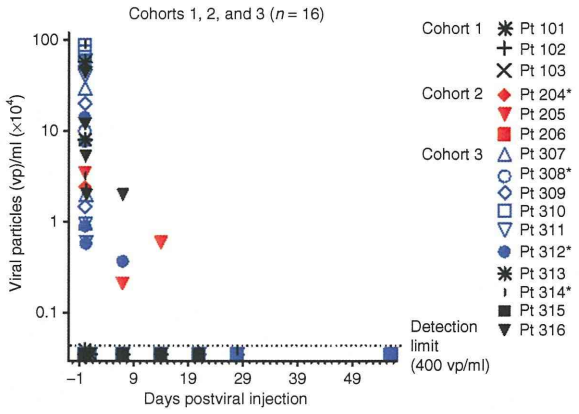

Figure 2 Detection of Telomelysin viral DNA in patient plasma samples on various days post-treatment. Data represented at day 1 constituted peak values determined at up to 6 hours post-treatment. All patients exhibited below detection levels of plasma viral particles (≤400vp/ml) at day 1 post-treatment.

wave of viremia from replication. Viral DNA was detected in two cohort 3 sputum specimens on day 1 (pt 310: 8.2×10^4 viral copies/ml) and day 7 (pt 307, 5×10^3 viral copies/ml) but not at earlier time points post-treatment. Viral DNA was not detected in any other body fluid compartments examined. The systemic detection of viral DNA at these extended time points is suggestive of viral replicative activity.

Viral E1A and hexon expression in treated tumors

Immunohistochemical evaluation of adenoviral hexon protein expression in treated tumor biopsies was carried out as a surrogate indicator of viral replicative activity at days 28 and 56 postinjection. Viral hexon protein expression was not detected in Telomelysin treated tumor biopsies collected at days 28 and 56 from 15 of 16 patients (Table 3), whereas one patient displayed an equivocal reaction at day 56 but not day 28. Viral E1A expression was uniformly negative from all 16 patients. The negative findings indicate that viral replicative activity did not extend to these time points, despite suggestion of viral dissemination for up to day 7–14 after the single viral injection.

Neutralizing antibody response

To identify systemic immune-activating events from intratumoral Telomelysin treatment, a functional assay with Telomelysin-infected HEK 293 cells was used to determine the neutralizing antibody (NAb) titer of patients entered into trial. Blocking activity of graded concentrations of the patient's pre- and post-treatment plasma was determined by light microscopy. An elevated NAb titer was observed in 14 of 14 plasma samples collected at day 28 (Table 3). Two patients (pt 313 and pt 316) did not have samples collected. The increase in titer ranged from 8- to 512-fold (Figure 3). However, the magnitude of titer increase did not correlate either with dose or with the presence or absence of a pre-existing NAb titer (Figure 3).

Table 3 Pharmacokinetics and immune response assessments

	No. positive[a]/No. tested			
Analyses performed[a]	Total	Cohort 1	Cohort 2	Cohort 3
Viral DNA in plasma	13/16	2/3	2/3	9/10
Viral DNA in sputum	2/16	0/3	0/3	2/10
Viral DNA in urine and saliva	0/16	0/3	0/3	0/10
Endogenous hTERT expression	9/12[b]	2/3	1/1	6/8
ADV neutralizing antibody (D28)	14/14	3/3	3/3	8/8[c]
Viral plaque assay	3/16	0/3	0/3	3/10
Serum IL-6	8/9[d]	3/3	2/3	3/3
Serum IL-10	7/9[d]	2/3	2/3	3/3
Serum IFN-γ	2/9[d]	2/3	0/3	0/3
In situ viral hexon (D56)	1/16	0/3	0/3	1/10
Lymphocyte subset alterations by immunophenotyping analysis	0/10	0/0	0/0	0/10

[a]Positive at any post-treatment time point tested. [b]12 of 15 patients with adequately recovered RNA were analyzed. [c]d28 plasma samples were not collected from patients 313 and 316 in cohort 3. [d]Only first 3 patients per cohort were analyzed, per protocol.

Serum cytokines

Non specific systemic immune activation from intratumoral Telomelysin treatment was observed as evidenced by an elevated increase in serum cytokine levels, in particular, interleukin-6 (IL-6) and IL-10 in all cohorts (Table 3). An elevated IL-6 level (>50%) was observed in 8 of 9 patients tested, as early as 30 minutes after treatment. Increased IL-10 level was also observed in 7 of 9 patients, whereas two patients had elevated interferon-γ.

Peripheral blood lymphocyte immunophenotyping

There were no demonstrable trends of altered post-treatment changes in the frequency distribution of $CD4^+$ T, $CD8^+$ T, B, and NK cells that correlated with viral treatment (Table 3) in 10 tested patients.

hTERT mRNA

To validate viral replication permissiveness of injected tumor specimens, real time, quantitative real-time-PCR assays were carried out retrospectively using tumor biopsy specimens collected before treatment, using total RNA from frozen patient tumor biopsy and primers and a TaqMan probe specific to hTERT or the

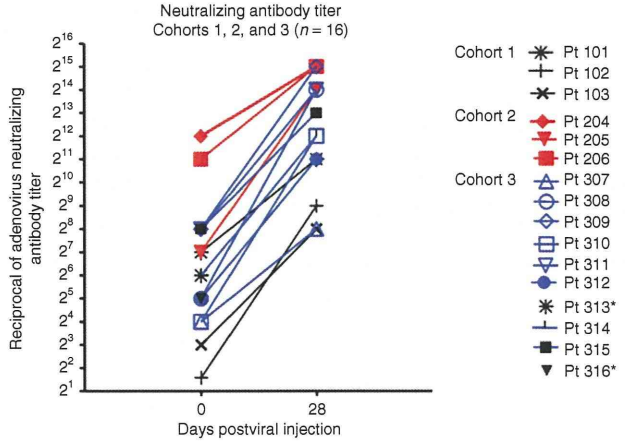

Figure 3 Neutralizing antibody titer. Change in neutralizing antibody titer on day 28 after injection compared to baseline. *Patients 313 and 316 did not have day 28 plasma samples to determine post-treatment neutralizing antibody titer.

housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Tumor hTERT expression was carried out in 12 of 15 tumor biopsies that yielded adequate RNA. Tumor biopsy was not available from pt 316. Endogenous hTERT expression was detected in 9 of the 12 tumor biopsy specimens (Table 3). These included two with high hTERT (>10^4 copies/μg) mRNA expression (one in cohort 2 and one in cohort 3), four with moderate expression (10^3–10^4 copies/μg) (one in cohort 1, three in cohort 3), and three with low expression (≤10^3 copies/μg) (one in cohort 1, two in cohort 3). hTERT was below detection limit in three other patients tested (one in cohort 1, two in cohort 3). Of the three patients with prolonged, detectable plasma viral DNA at day 7 post-treatment, pt 205 displayed a high level of endogenous hTERT mRNA, whereas tumor samples were either unavailable (pt 316) or inadequate (pt 312) for assessment. These limited findings confirm hTERT expression in the majority of human tumors.

DISCUSSION

Telomelysin administration in this Phase I safety trial demonstrated safety with no treatment related grade 3/4 adverse effects. Further, we observed the encouraging findings of one patient with partial response at day 56 after a single IT. The transient presence of systemic Telomelysin dissemination following IT was documented early after IT injection. Immune activation was observed, with cytokine upregulation of IL-6 and IL-10 and the induction of viral neutralizing antibodies. Limited suggestive evidence of vial replication was observed at day 7 post-treatment in three patients, for whom plasma viremia was not detected on day 1. One of these three patients had elevated malignant tissue hTERT expression with a significant clinical response. However, these limited findings require additional confirmation as we cannot completely exclude the unlikely possibility of delayed viral clearance. Immunohistochemical analysis of viral E1A and hexon was negative 28 days after injection suggesting rapid clearance. In Galanis' Phase II osteosarcoma trial with ONYX-015, 5 of 6 patients had detectable viral DNA on Day 5 of the first cycle.[7] In Makower's hepatobiliary tumor trial with ONYX-015, no viral DNA was detected in plasma following intralesional injection.[8] In our previous work with ONYX-015 we showed that 41% of patients had detectable viral DNA at days 5 and 6, and 9% had circulating DNA at day 10.

Adenoviral immunogenicity can be affected by viral structural modification (E3 region function), physical properties (temperature), other agents (enbrel, steroids), serotype status, removal of neutralizing antiviral antibodies (plasmapheresis) or presence of antibody producing cells (B-cell inhibition secondary to Ribavirin, Rituxan), use of physical shields (liposome, polymer, cellular delivery), and/or alteration of neutralizing surface epitopes (hexon, knob, fiber).[9–32]

Evidence of clinical efficacy has previously been demonstrated with a *E1B-55* kd deleted oncolytic adenoviral therapeutic (ONYX-015); however, the opportunity to move towards systemic administration was hampered by efficacy results and the limitations imposed by rapid viral clearance and low replication capacity. These data were insufficient for advancement of phase III development with ONYX-015. Telomelysin was designed with a structure to enhance tumor selective viral gene expression (*hTERT* promoter) thereby allowing the opportunity to consider systemic administration in tandem with masked delivery approaches.[33,34] The adenovirus early transcription unit (E3) encodes for polypeptides (14.7 k, 10.4, 14.5),[35–39] which function to directly block tumor necrosis factor–α activation as well as apoptotic pathways shared by tumor necrosis factor–α and *fas*.[38,40,41] The E3 gp19k protein functions to bind and retain MHC class I molecules within the endoplasmic reticulum, thus preventing surface presentation of viral antigens, thereby limiting class I-restricted CTL clearance of virally infected cells.[35,36,42–45] The expression of the E3 gene region products may, therefore, decrease viral clearance, increase the expression of those viral genes that suppress immune recognition and enhance viral replication.[40,46]

In conclusion, both activity and safety of a single injection approach for Telomelysin has been demonstrated. However, despite activity in a subset of patients, limited clinical relevant responses were observed in others. This may be attributed to the single viral treatment administered to each patient. An increase in viral NAb titer in all patients tested is indicative of systemic immune sensitization following IT. We and others have shown previously that systemic viremia can be maintained at 3–6 days after second intravenous or intra-arterial treatments in spite of the presence of high levels of NAb titers and antiviral cytokines.[47,48] Thus repeat intratumoral or intranvenous injection of Telomelysin is a viable treatment option to achieve an improved clinical response. Alternatively, artificial envelopment of Telomelysin with bilamellar cationic liposomes for "stealth" systemic delivery may be applicable for improving systemic pharmacokinetics and coxsackie and adenovirus receptor-independent tropism.[33,34] With these considerations, data support further clinical assessment of a multi treatment schedule.

MATERIALS AND METHODS

Test article. Telomelysin is manufactured at Introgen Therapeutics, Houston, TX. Telomelysin was reconstituted using aseptic technique in a Biocontainment Level 2 ISO Class 5 Biosafety Cabinet.

Study design. This was a dose escalation study in patients with advanced solid tumors. A single IT of virus particles (vp) was administered through a single injection site using a radial method of distribution in order to evenly

distribute material to both peripheral and central sites of growing tumor without removing the needle completely from the tumor. Most of the viral dose was administered at the tumor periphery and at the interface between normal tissue and tumor; prior studies have indicated improved efficacy with this administration approach.[49] Attempts were made to distribute the virus uniformly along the needle tracks by gradually depressing the syringe plunger during withdrawal of the needle. Each patient was enrolled into one of the following cohorts: Cohort 1: 1×10^{10} vp/tumor ($n = 3$); Cohort 2: 1×10^{11} vp/tumor ($n = 3$); Cohort 3: 1×10^{12} vp/tumor ($n = 10$). Patients in cohorts 1 and 2 remained on study for 28 days after injection. Cohort 3 patients were followed until day 56 post-treatment.

Viral DNA was monitored using quantitative PCR (Q-PCR) technique. After the first patient was enrolled into Cohort 1, each of the remaining patients (*i.e.*, pt 2 and pt 3) was enrolled. In Cohort 1, clearance of viral DNA in all body fluid specimens including blood, saliva, sputum, and urine of the preceding patients by two consecutive negative Q-PCR results at least 3 days apart was required. Enrollment of the first patient in Cohort 2 began when viral DNA results on the last patient in Cohort 1 were negative on two consecutive tests at least 3 days apart.

If a dose-limiting toxicity was observed in one of three patients related to Telomelysin, an additional three patients were enrolled. If only one of the six total patients experienced a dose-limiting toxicity, then the dose escalation would be continued to the next cohort. If two or more of the six patients experienced a dose-limiting toxicity, the maximum tolerated dose would be defined as exceeded and an additional three patients would be treated at the dose level below. Toxicities were graded and reported according to the National Cancer Institute common terminology criteria for adverse events, version 3.0. Response was evaluated in this study using the international criteria proposed by the RECIST Committee.

Study population. Patients with superficial accessible cancer who had failed at least one prior therapeutic regimen and for whom effective conventional therapy was not available were eligible for the study. All patients were required to be at least 18 years old, have histologically confirmed carcinoma and a Karnofsky performance status of at least 70%. Inclusion was also predicated on normal laboratory assessment. All patients were required to provide written consent according to local institutional review board–approved guidelines. Women and men of reproductive potential were required to use contraception.

Baseline assessments included: concomitant medications, interval history, physical examination, performance status, tumor assessment, medical laboratory studies, adenoviral NAb, urinalysis, tumor biopsy, viral DNA in blood, saliva, sputum, and urine. Viral plaque forming titer in serum, cytokine levels (IL-6, IL-10, INF-γ). Peripheral blood immunephenotype analyses were performed for Cohort 3 patients.

Assessments were performed using samples collected as follows: plasma viral DNA: pretreatment and at 30 minutes, 1 hour, 3 hours, 6 hours and on days 1, 7, 14, 21, 28, and 56 post-treatment; viral DNA in sputum, urine, and saliva: pretreatment and days 1, 7, 14, 21, 28, and 56 postinjection; endogenous hTERT expression: assessed with pretreatment tumor biopsy; adenovirus NAb: pretreatment and day 28 post-treatment; cytokine: pretreatment and 30 minutes, 1 hour, 6 hours, and on days 1, 14, and 28 post-treatment for first three patients per cohort only; immunohistochemistry for viral hexon: tumor biopsies collected pretreatment and on days 28 and day 56 post-treatment; immunephenotyping analysis: pretreatment and days 7, 14, and 28 post-treatment. Viral plaque assay was performed only on patient plasma samples that yielded $\geq 1 \times 10^5$ vp/ml by Q-PCR analysis.

Detection of viral DNA. Patient samples were collected previral infusion and on day 0 (1 hour, 3 hours, 6 hours post-treatment), day 1, 7, 14, 21, 28, and 56 post-IT. DNA extraction was carried out from patient's archived, frozen tumor biopsy specimen, plasma, sputum, saliva, and urine specimens. Viral DNA was quantified by real-time Q-PCRs. Briefly, DNA was extracted with the Qiagen QIAmp DNA Mini Kit (plasma and saliva samples) or QIAamp Viral RNA Mini Kit (urine and sputum samples). Plasma, saliva, sputum, and urine samples from normal donors were used for protocol validation, with or without "spiking" with known amounts of Telomelysin immediately prior to DNA extraction. Q-PCRs were carried out on the iQ5 Q-thermal cycler (BioRad, Hercules, CA), using Telomelysin-specific primers for the E1A and IRES region and the 2 × Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The amounts of detectable viral particles were quantified by extrapolation with a standard curve, generated with serially diluted (1:10) DNA templates with predetermined copy numbers (10 to 1×10^6 copies) of pure Telomelysin viral DNA. A positive response is based on the detection of both IRES- and E1A amplification products with an assay threshold of 4×10^2 vp/ml for plasma and saliva; 1×10^3 vp/ml for urine; 2×10^3 vp/ml for sputum samples for both reactions.

Primer sequences

IRES-Forward 5′-GAT TTT CCA CCA TAT TGC CG
IRES-Reverse 5′-TTC ACG ACA TTC AAC AGA CC
E1A-Forward 5′-CCT GTG TCT AGA GAA TGC AA
E1A-Reverse 5′-ACA GCT CAA GTC CAA AGG TT.

Endogenous hTERT expression in patient tumor. To validate viral replication, real time, quantitative real-time-PCR assays were carried out with total RNA from patient tumor biopsy. Briefly, Q-PCR assays were carried out on iQ5 Q-PCR machine (BioRad), using primers and a TaqMan probe specific to hTERT or GAPDH (Sigma/Proligo, St Louis, MO), and TaqMan Core PCR reagents (Applied Biosystems). Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs were generated according to manufacturer's instructions (RETROscript kit; Ambion, Foster City, CA). PCR standard curves for determination of gene copy number in the reaction template were generated with triplicate reactions, using 1:10, serially diluted samples of either the hTERT or GAPDH PCR amplification products.

Primer sequences

hTERT Forward primer: 5′-GCACTGGCTGATGAGTGTGT-3′
hTERT Reverse primer: 5′- CTCGGCCCTCTTTTCTCTG-3′
hTERT TaqMan probe: 5′-(FAM) TTGCAAAGCATTGGAATCAGA CAGCACT-(TAMRA)-3′
GAPDH Forward primer: 5′-GAAGGTGAAGGTCGTAGTC-3′
GAPDH Reverse primer: 5′-GAAGATGGTGATGGGATTTC-3′
GAPDH TaqMan probe: 5′-(FAM) CAAGCTTCCCGTTCTCAGCC (TAMRA)-3′.

Immunohistochemical analysis. A previously described automated immunoperoxidase staining technique was used to characterize viral protein expression.[50] Briefly, viral E1A and hexon expression was determined with the avidin-biotin-complexed immunoperoxidase reaction (*i*VIEW DAB Detection kit; Ventana Medical Systems, Tucson, AZ) following initial incubation with antibodies specific to viral E1A (prediluted mouse monoclonal adenovirus type 5 E1A antibody, GeneTex, Irvine, CA), or hexon (goat antiadenovirus polyclonal antibody (Millipore, Billerica, MA), using the Ventana 320ES System (Ventana Medical Systems, Tucson, AZ).

Flow cytometric immunophenotype analysis. Peripheral blood immunophenotype analysis was carried by a two color immunofluorescence reaction and flow cytometric analysis as described previously.[50] The frequency distribution of T, B, and NK cell subsets: CD45-FITC/CD14-PE, CD3-FITC/CD19-PE, CD4-FITC/CD8-PE, CD13-FITC [CD16 CD56]-PE (all from BD Biosciences, San Jose, CA) were determined.

Serum cytokine analysis. ELISA assays (R&D Quantikine kits, Minneapolis, MN) were used to quantify patient serum cytokine levels.[50] Serial serum samples were analyzed simultaneously, using cytokine-specific immunoassay reagents. The colorimetric reaction was quantified as a function of optical density absorbance at 450 nm with the correction wavelength set at 540 nm

(SpectraMax 340; Molecular Devices, Sunnyvale, CA). The minimal detectable concentration was as follows: interferon-γ: <16 pg/ml; IL-10: <8 pg/ml; IL-6: <1 pg/ml. The percent increase in cytokine level at any time point post-treatment was determined through comparison with serum harvested before Telomelysin injection. Based on inter- and intra-sample variations, increases in cytokine level of ≥50% over baseline were considered significant.

Antiadenovirus antibodies. Adenovirus-NAb titer in patient plasma samples was measured as a function of blocking human adenovirus infection of 293 cells. Briefly, twofold serially diluted patient plasma samples were added to 293 cells that were infected with Telomelysin virus. The plates were evaluated microscopically for the percentage of cells that lysed in presence of patient plasma samples at 72 and 96 hours postinfection. The adenovirus-NAb titer for a given sample was the highest dilution of the plasma that showed a blocking effect (>60% 293 cells intact and attached as monolayer).

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ORIGINAL ARTICLE

A novel translational approach for human malignant pleural mesothelioma: heparanase-assisted dual virotherapy

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Malignant pleural mesothelioma (MPM) is a highly aggressive tumor that is related to asbestos exposure. MPM is characterized by rapid and diffuse local growth in the thoracic cavity, and it has a poor prognosis because it is often refractory to conventional therapy. Although MPM is an extraordinarily challenging disease to treat, locoregional virotherapy may be useful against this aggressive disease because of the accessibility by intrapleural virus delivery. In this study, we show that telomerase-specific, replication-selective adenovirus OBP-301 can efficiently infect and kill human mesothelioma cells by viral replication. Intrathoracic administration of virus significantly reduced the number and size of human mesothelioma tumors intrathoracically implanted into *nu/nu* mice. A high-definition, fluorescence optical imaging system with an ultra-thin, flexible fibered microprobe clearly detected intracellular replication of green fluorescent protein-expressing oncolytic virus in intrathoracically established mesothelioma tumors. As the extracellular matrix (ECM) may contribute to the physiological resistance of a solid tumor by preventing the penetration of therapeutic agents (including oncolytic viruses), we also examined whether the co-expression of heparanase, an endoglucuronidase capable of specifically degrading heparan sulfate, that influences the physiological barrier to macromolecule penetration, can modify the permeability of the ECM, resulting in profound therapeutic efficacy. Co-injection of OBP-301 and a replication-defective adenovirus (Ad-*S/hep*)-expressing heparanase resulted in more profound antitumor effects without apparent toxicity in an orthotopic pleural dissemination model. Our results suggest that intrathoracic dual virotherapy with telomerase-specific oncolytic adenovirus in combination with heparanase-expressing adenovirus may be efficacious in the prevention and treatment of pleural dissemination of human malignant mesothelioma.
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Introduction

Malignant pleural mesothelioma (MPM) is an uncommon neoplasm with an annual estimated incidence of 2000–3000 new cases in the United States (Connelly *et al.*, 1987; Price, 1997). In more than 70% of patients, the origin of the tumor is linked to a history of exposure to asbestos fibers (Chahinian *et al.*, 1982; Chailleux *et al.*, 1988). The use of asbestos in Japan increased rapidly after the 1950s and remained at a high level even as the worldwide use of asbestos decreased substantially after the 1980s, therefore, the mortality rate for MPM is expected to continuously increase in Japan (Murayama *et al.*, 2006). MPM is characterized by progressive local tumor invasion and poor median survival ranging from 9 to 16 months (Ruffie *et al.*, 1989). MPM is notoriously refractory to treatment, and neither surgery nor radiotherapy alone results in increased survival (Ball and Cruickshank, 1990; Rusch *et al.*, 1991). Although many chemotherapeutic regimens have been suggested, a standard treatment strategy for MPM remains elusive (Alberts *et al.*, 1988; Ryan *et al.*, 1998). Therefore, the development of novel therapeutic options is required.

Clinical trials of patients with MPM have established the safety of the intrapleural delivery of replication-deficient adenoviral vectors expressing the suicide gene, herpes simplex thymidine kinase, followed by the administration of ganciclovir, an antiviral drug. Some evidence indicates that this approach induces an effective antitumor immune response (Sterman *et al.*, 1998, 2005; Molnar-Kimber *et al.*, 1998). Moreover, intrapleural interferon-ß gene transfer with a replication-defective adenoviral vector may potentially be a useful approach for the generation of antitumor immune responses in MPM patients (Sterman *et al.*, 2007). A significant obstacle to these approaches is the limited distribution of the non-replicative vectors within the tumor mass, even after direct intratumoral administration. Histopathological analyses have shown that these vectors transduce only a few tumor cells,

despite the successful antitumor responses. Therefore, more efficient strategies for the virus to spread within tumors may be required to increase the clinical benefit.

Replication-selective, tumor-specific viruses present a novel approach for the treatment of neoplastic diseases. These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor. Telomerase activation is a critical step in carcinogenesis, and it correlates closely with human telomerase reverse transcriptase (hTERT) expression. We constructed an attenuated adenovirus 5 vector (OBP-301, Telomelysin), in which the hTERT promoter element drives expression of the *E1A* and *E1B* genes linked with an internal ribosome entry site. OBP-301 replicated efficiently and induced marked cell killing in a panel of human cancer cell lines, whereas replication as well as cytotoxicity was highly attenuated in normal human cells lacking telomerase activity (Kawashima *et al.*, 2004; Taki *et al.*, 2005). In this study, we examined the therapeutic potential of intrapleural delivery of OBP-301 against human MPM tumors intrathoracically implanted into *nu/nu* mice. As the extracellular matrix (ECM) may contribute to the physiological resistance of a solid tumor by preventing the penetration of therapeutic agents (including oncolytic viruses), we also examined whether the co-expression of heparanase, an endoglucuronidase capable of specifically degrading heparan sulfate, that influences the physiological barrier to macromolecule penetration, can modify the permeability of the ECM, resulting in profound therapeutic efficacy.

Results

Expression of CAR and hTERT levels in human mesothelioma cell lines

To examine the biological characteristics of human mesothelioma cells, we first used flow cytometry to determine the cell surface expression of coxsackie and adenovirus receptor (CAR). CAR was expressed in all four cell lines tested, although the expression levels varied (Figure 1b). H2052 and H2452 cells showed low,

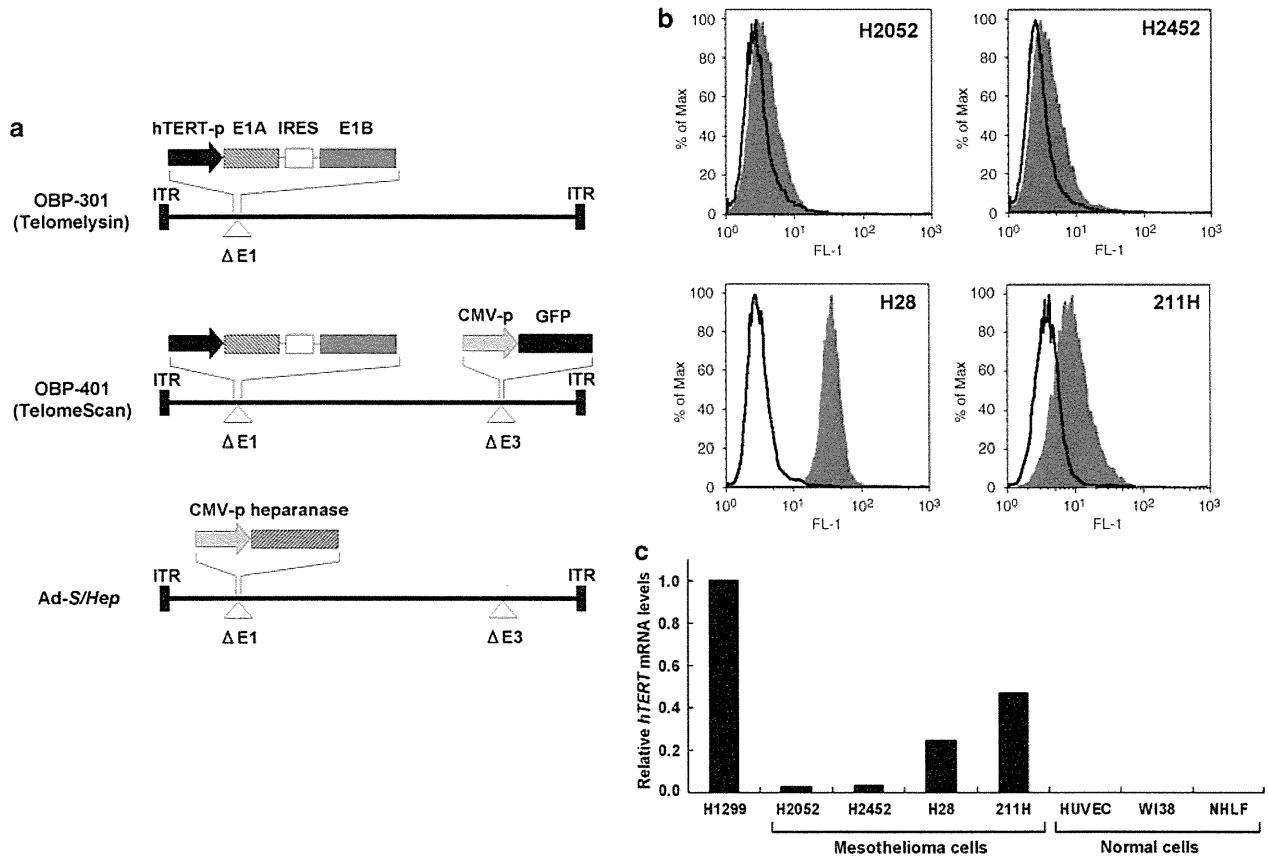

Figure 1 Schematic DNA structures of telomerase-specific viruses and characteristics of human mesothelioma cell lines. (**a**) OBP-301 is a telomerase-specific, replication-competent adenovirus that contains the human telomerase reverse transcriptase (hTERT) promoter sequence inserted into the adenovirus genome to drive transcription of the E1A and E1B bicistronic cassette linked by internal ribosome entry site (IRES). OBP-401 is a variant of OBP-301 and contains the green fluorescent protein *(GFP)* gene inserted under the cytomegalovirus (CMV) promoter into the E3 region for monitoring viral replication. Ad-*S/hep* vector contains human heparanase complementary DNA (cDNA) driven by the CMV promoter. (**b**) Flow cytometric analysis of coxsackie and adenovirus receptor (CAR) expression in human mesothelioma cell lines. Cells were incubated with anti-CAR monoclonal antibodies followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (gray area). An isotype-matched normal mouse IgG conjugated to FITC was used as a control (black line). (**c**) Relative *hTERT* messenger RNA (mRNA) expression in human mesothelioma cell lines and normal cell lines was determined by real-time reverse transcription (RT)–PCR analysis. The *hTERT* mRNA expression of H1299 human lung cancer cells was considered 1.0, and the relative expression level of each cell line was calculated against that of H1299 cells.

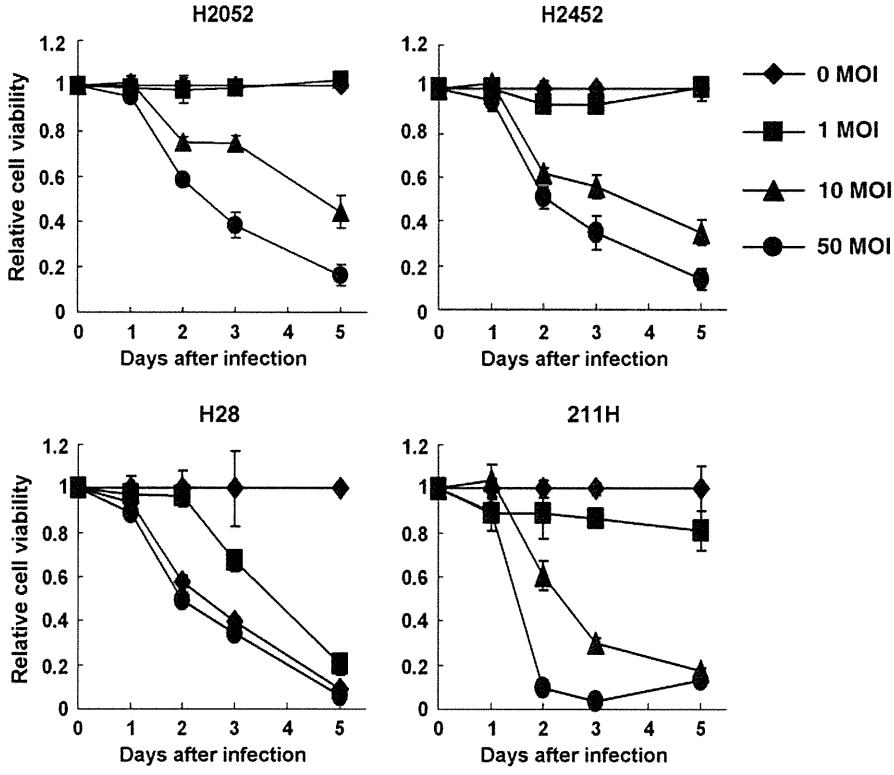

Figure 2 Selective cytopathic effect of OBP-301 in human mesothelioma cell lines *in vitro*. Cells were infected with OBP-301 at the indicated multiplicity of infection (MOI) values, and the surviving cells were quantitated over 5 days by XTT assay. The cell viability of mock-treated cells on day 1 was considered 1.0, and the relative cell viability was calculated. Values represent the mean ± s.d. of triplicate experiments.

but detectable CAR expression compared with CAR-negative cell lines such as LN444, LNZ308 and H1299R5 that we reported earlier (Tango *et al.*, 2004; Taki *et al.*, 2005). A real-time reverse transcription–PCR method showed that all cell lines expressed detectable levels of *hTERT* messenger RNA (mRNA), suggesting that the hTERT promoter element can be used to target human mesothelioma cells (Figure 1c).

In vitro *cytopathic efficacy of OBP-301 on human mesothelioma cell lines*

To determine whether OBP-301 infection induces selective cell lysis, mesothelioma cells were infected with OBP-301 at various multiplicity of infections (MOIs), and then the XTT cell viability assay was performed over 5 days. All mesothelioma cell lines were efficiently killed by OBP-301 in a dose-dependent manner (Figure 2). Infection at an MOI of 10 was sufficient to induce cell lysis within 3 days. To visually confirm the viral replication and spread, we modified OBP-301 to express the green fluorescent protein (*GFP*) reporter gene under the control of the cytomegalovirus promoter in the E3 region (modified virus, OBP-401) (Figure 1a). We have confirmed earlier that the propagation and yields of OBP-301 and OBP-401 are equivalent (Kawashima *et al.*, 2004; Kishimoto *et al.*, 2006). After OBP-401 infection, phase-contrast images showed a rapid loss of viability because of massive cell death, as evidenced by ballooning and floating cells. We observed a strong and persistent GFP fluorescence expression in these mesothelioma cells under a fluorescence microscope, indicating the viral replication and spread into the neighboring tumor cells (Figure 3a).

Intrathoracic virus spread and infection in an orthotopic pleural human mesothelioma model

We also evaluated the viral infection and replication in human mesothelioma cells growing intrathoracically in athymic *nu/nu* mice. When H2052 and H2452 mesothelioma cells were inoculated into the thoracic space, disseminated tumor nodules were detected in the visceral pleura, parietal pleura, diaphragmatic pleura and mediastinum. We used H2452 cells with low CAR and *hTERT* mRNA expression that were considered to be most refractory to OBP-301 for the further *in vivo* experiments. Tumor weights at autopsy more than 40 days after tumor cell inoculation were significantly greater than tumor weights at <30 days, indicating the tumor growth in the thoracic cavity (Supplementary Figure 1). Optical charged-coupled device imaging detected GFP-labeled tumors at the gross level during a midsternal thoracotomy 6 days after intrathoracic injection of 1×10^8 plaque-forming units (PFU) of OBP-401. Moreover, GFP expression in macroscopically invisible tumors could be detected at the microscopic level with a hand-held flexible probe inserted through

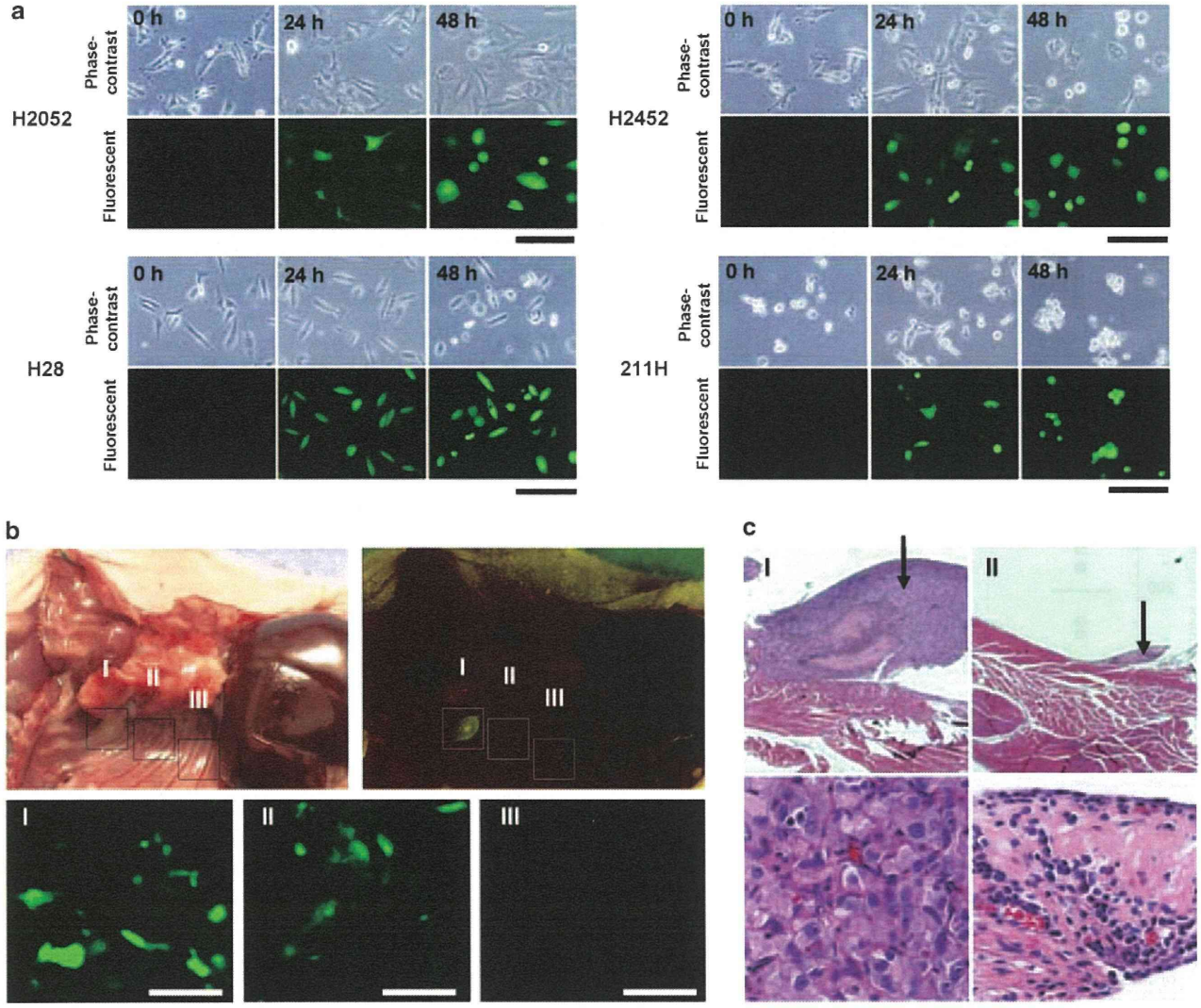

Figure 3 Visualization of human mesothelioma cells *in vitro* and *in vivo* by OBP-401 infection. (**a**) H2052, H2452, H28 and 211 H cells were infected with OBP-401 at an multiplicity of infection (MOI) of 10. Cell morphology and green fluorescent protein (GFP) expression were evaluated by fluorescence microscopy at the indicated time. Bar = 200 μm. (**b**) Internal images of pleural mesothelioma dissemination visualized by intrathoracic injection of OBP-401. Six weeks after intrathoracic inoculation of 5×10^6 H2452 cells, mice received an intrathoracic injection of 1×10^8 plaque-forming units (PFU) of OBP-401. The GFP fluorescence expression was detected 6 days after virus administration by a 3-charged-coupled device (CCD) camera (top panels) and an *in situ* molecular imaging system (bottom panels). Top-left panel, gross appearance of disseminated H2452 tumors; top-right panel, fluorescent detection. Bottom panels, I, II and III represent the boxed regions of the top panels. Bar = 30 μm. (**c**) Histologic sections stained with hematoxylin and eosin showing local growth of H2452 mesothelioma cells (arrows) in the thoracic spaces. Top panels, × 40 magnification; bottom panels, × 400 magnification. I and II represent the boxed regions of (**b**).

the intercostal small incision (Figure 3b and Supplementary Figure 2). Histological analysis confirmed the presence of disseminated tumors in the sites of fluorescence emission (Figure 3c). These results suggest that intrathoracically injected oncolytic virus can infect and selectively replicate in disseminated tumor tissues.

In vivo *antitumor effect of intrathoracic delivery of OBP-301 in an orthotopic pleural human mesothelioma model*
To examine the therapeutic effect of telomerase-specific oncolytic virus, mice received an injection of 1×10^7 or 1×10^8 PFU of OBP-301, 1×10^8 PFU of replication-defective control adenovirus (dl312), or phosphate-buffered saline into the thoracic spacethe injections were administered twice at a 1-week interval beginning 24 h after tumor cell inoculation. Injection of 10^8 PFU of OBP-301 significantly reduced the incidences of tumor cell dissemination and the total weights of tumor nodules as compared with mice that received dl312 or phosphate-buffered saline injection, although 10^7 PFU of OBP-301 had no apparent effect (Figures 4a and b). Next, we examined treatment schedules with different starting points. Two injections of 1×10^8 PFU of OBP-301 administered at a 1-week interval starting on day 1, 8, 22 or 29 after tumor inoculation showed statistically significant antitumor effects when mice were killed on day 43 (Figure 4c and Supplementary Figure 3). These results suggest that oncolytic virotherapy could be

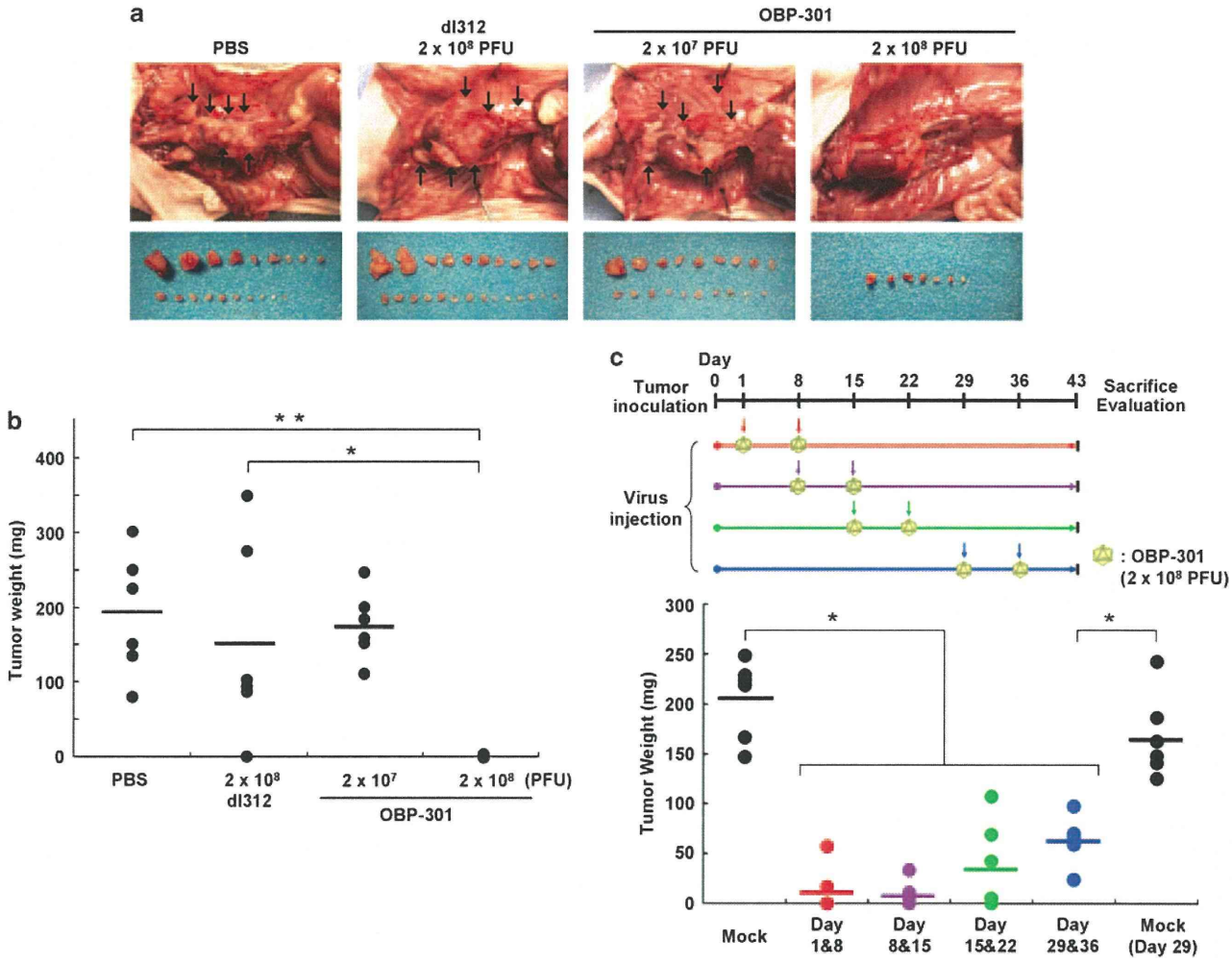

Figure 4 *In vivo* antitumor effect of OBP-301 on pleural dissemination of H2452 human mesothelioma cells. (**a**) Gross appearance of H2452 tumors grown orthotopically in the thoracic spaces. H2452 cells (5×10^6) were inoculated into the thoracic space of athymic *nu/nu* mice. After 24 h, either 1×10^7 plaque-forming units (PFU)/100 µl or 1×10^8 PFU/100 µl of OBP-301, 1×10^8 PFU/100 µl of dl312 (replication-deficient adenovirus), or phosphate-buffered saline (PBS) were injected into the thoracic space twice at a 1-week interval (total dose: 2×10^7 or 2×10^8 PFU). Eight weeks after tumor cell inoculation, the mice were killed, and the pleural dissemination of the thoracic spaces was assessed. (**b**) The weight of each tumor nodule found in the thoracic spaces was determined. Closed circles: individual tumor weights. Bars: mean weight. $^*P<0.05$, $^{**}P<0.01$. (**c**) The antitumor effect of OBP-301 administered in different treatment schedules was also assessed on an orthotopic pleural dissemination model. Top panel, treatment schedule. Bottom panel, tumor weight of each tumor nodule found in the thoracic spaces after treatment. The treated mice were killed and assessed for pleural dissemination 43 days after tumor inoculation. Closed circles: individual tumor weights. Bars: mean weight. $^*P<0.05$.

effective for preventing the dissemination of mesothelioma cells as well as shrinking established tumors; complete eradication of disseminated nodules, however, was not achieved.

Enhanced antitumor effect of OBP-301 in combination with heparanase-expressing adenovirus in an orthotopic pleural human mesothelioma model

To further enhance the *in vivo* therapeutic potential of telomerase-specific virotherapy, we examined the combination effect of OBP-301 and a replication-defective adenovirus vector expressing the human *heparanase* gene (Ad-*S/hep*) (Uno *et al.*, 2001). Heparan sulfate is a major constituent of the ECM that is responsible for a barrier to macromolecular diffusion in tumors. Thus, heparanase-mediated ECM degradation may be a critical requisite for virus penetration and distribution into tumor tissues. Western blot analysis revealed the expression of both proheparanase (*Mr* 65 000) and cleaved, active heparanase (*Mr* 50 000) in H2542 cells after Ad-*S/hep* infection expression of these proteins was not affected by the presence of OBP-301 (Figure 5a). In addition, an *in vitro* XTT analysis showed that co-infection of Ad-*S/hep* at various MOIs did not affect OBP-301-mediated cytotoxicity on human mesothelioma cells (Supplementary Figure 4).

We next examined whether heparanase expression enhanced the virus penetration into three-dimensional tumor structures using a human mesothelioma spheroid model. Tumor spheroids provide an excellent *in vitro* three-dimensional model resembling *in vivo* tumor masses for visualizing the dynamics of the virus and

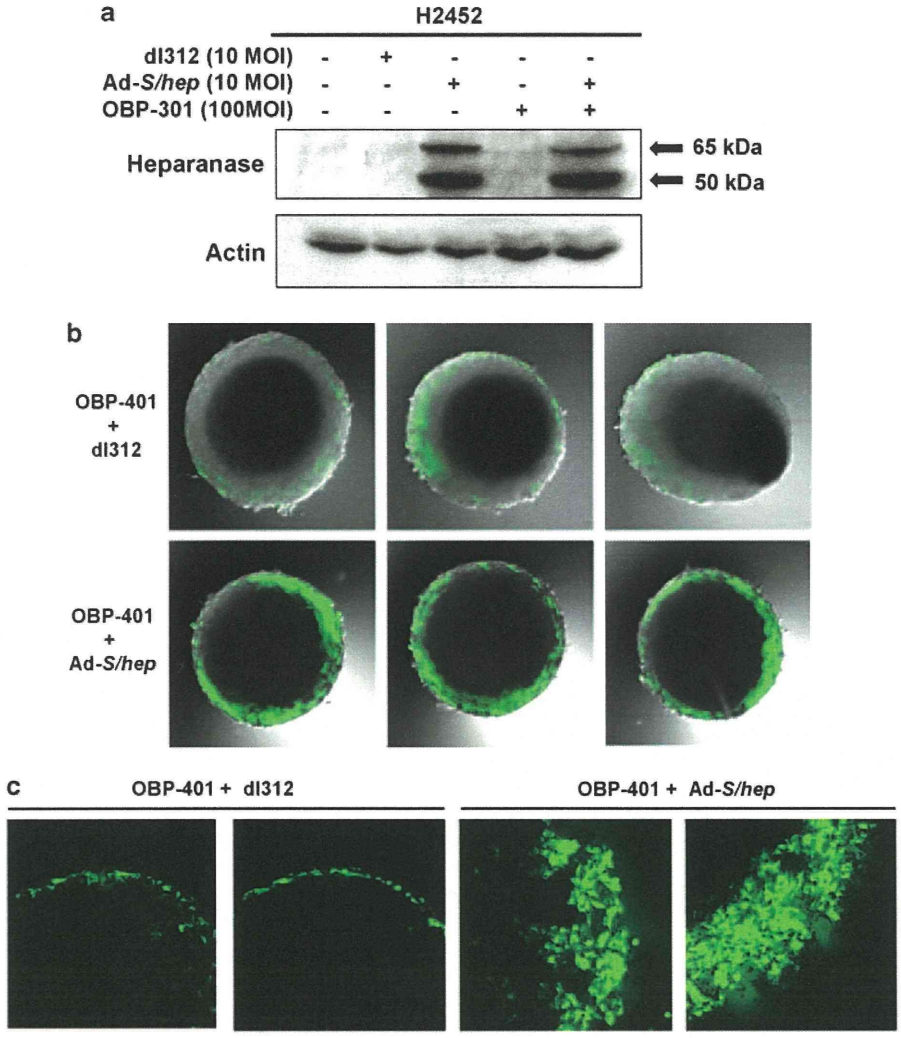

Figure 5 Enhanced penetration of the virus into tumor spheroids by heparanase expression. (**a**) Western blot analysis of human heparanase protein expression in H2452 cells. Cells were infected with either dl312, Ad-*S/hep*, OBP-301 or OBP-301 in combination with Ad-*S/hep* at different multiplicity of infections (MOIs), as indicated. Equivalent amounts of protein obtained from whole cell lysates 30 h after infection were separated by electrophoresis, probed with primary antibodies, and then visualized by using an ECL detection system. Equal loading of samples was confirmed by reprobing with anti-actin antiserum. Both inactive (*Mr* 65 000) and active (*Mr* 50 000) forms of heparanase proteins were detected. (**b**, **c**) Transduction efficiency and viral spread of OBP-401 in combination with Ad-*S/hep* in H2452 tumor spheroids. H2452 tumor spheroids were infected with dl312 (replication-deficient adenovirus) or Ad-*S/hep* at 1×10^3 plaque-forming units (PFU), followed by infection with OBP-401 at 1×10^4 PFU 48 h later. Green fluorescent protein (GFP) expression in each tumor spheroid was assessed with a laser-scanning confocal fluorescent microscope 48 h later. (**b**) Gross imaging of H2452 tumor spheroids. (**c**) Higher magnification to show the surface area of the spheroids.

assessing the levels of virus penetration. Sequential confocal fluorescent microscopy showed that OBP-401 could penetrate and express GFP fluorescence in H2452 spheroids; GFP expression, however, could be detected in the deeper areas of the spheroids in the presence of Ad-*S/hep* (Figure 5b, c). High-magnification images showed that GFP signals were detected only at the spheroid surface after OBP-401 and control dl312 exposure, whereas co-infection of Ad-*S/hep* enhanced the OBP-401 penetration, leading to GFP expression in multiple layers.

Finally, we assessed the combination effect of OBP-301 and Ad-*S/hep* in an orthotopic pleural human mesothelioma model. Intrathoracic injection with 1×10^8 PFU of OBP-301 plus 1×10^7 PFU of Ad-*S/hep* on days 8 and 15 resulted in a significant reduction of tumor weights on day 43 (Figure 6a). This combination therapy showed greater antitumor effects than the therapy with 10^8 PFU of OBP-301 alone. The administration of Ad-*S/hep* alone did not affect tumor weights as compared with the tumors in the mock-treated group. Moreover, only one of the seven (14.3%) mice injected with OBP-301 alone survived over a 12-week observation period, whereas five of the seven (71.4%) mice treated with OBP-301 plus Ad-*S/hep* remained alive (Figure 6b).

Discussion

Malignant pleural mesothelioma is an aggressive neoplasm with a dismal prognosis because of its resistance

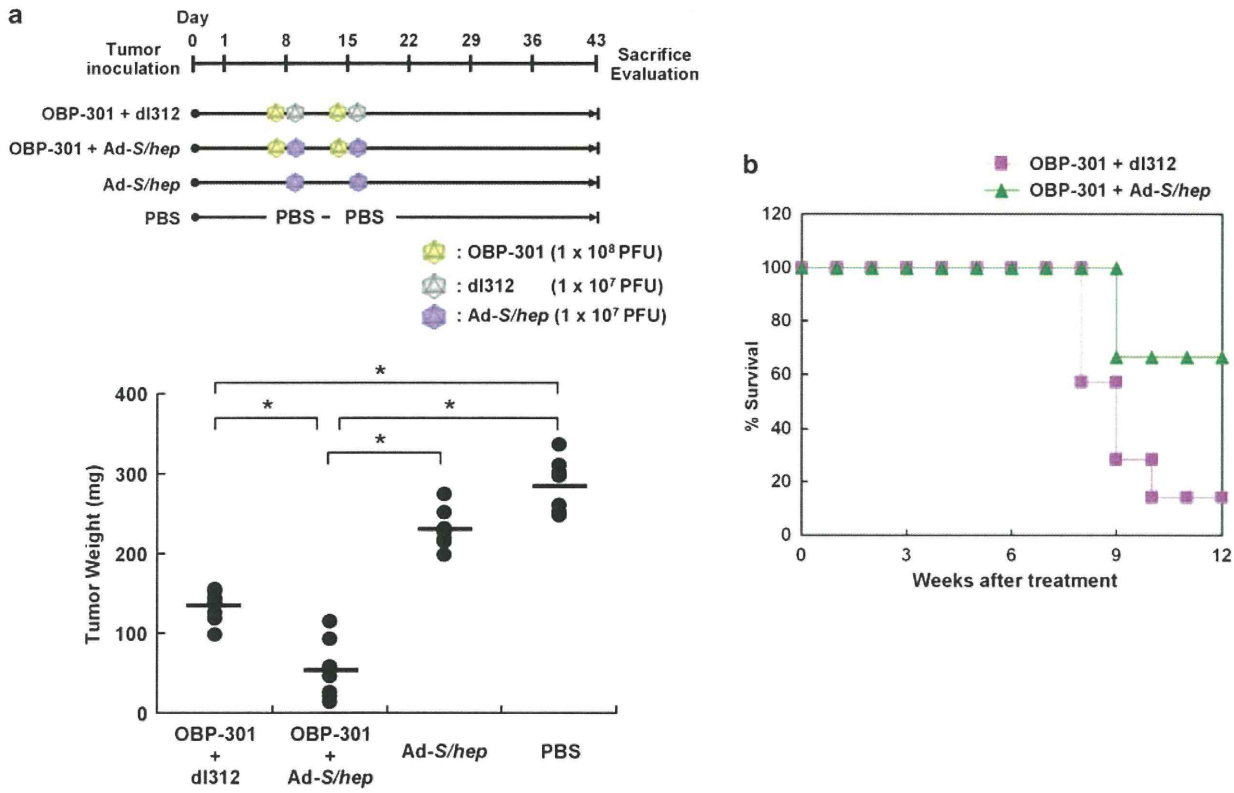

Figure 6 Enhanced antitumor effect of OBP-301 with Ad-*S/hep* in an orthotopic pleural dissemination model. (**a**) Top panel, treatment schedule. Bottom panel, tumor weight of each tumor nodule found in the thoracic spaces after treatment. Treated mice were killed and assessed for pleural dissemination 43 days after tumor inoculation. Closed circles: individual tumor weights. Bars: mean weight. $*P<0.05$. (**b**) Mice bearing H2452 xenografts in the thoracic spaces received intrathoracic administration of either OBP-301 plus dl312 or OBP-301 plus Ad-*S/hep*. Their post-treatment survival was monitored and plotted as a Kaplan–Meier plot.

to therapeutic modalities such as chemotherapy and radiotherapy. An alternative therapeutic option is the use of gene- and vector-based therapies. MPM is characterized by intrathoracic spread, and it is clinically accessible, making it an attractive target for locoregional delivery of genetically engineered viral agents. Replication-competent viral agents can confer specificity of infection and increase viral spread to neighboring tumor cells. Onyx-015, a conditional replication-competent adenovirus lacking the 55-kDa *E1b* gene, may be an effective treatment for human mesothelioma cells retaining wild-type p53 but lacking $p14^{ARF}$ (Ries *et al.*, 2000; Yang *et al.*, 2000, 2001), the targets of Onyx-015, however, are not general and its clinical trials for various types of human malignancies have been discontinued (Goodrum and Ornelles, 1998). In this study, we showed that intrathoracic administration of telomerase-specific oncolytic viruses induced significant antitumor effects against both pre-established and established pleural dissemination of human MPM. Moreover, we found that co-infection of oncolytic adenoviruses with non-replicative adenovirus expressing an ECM-digestive enzyme, heparanase, resulted in a virus distribution into the deeper areas of tumor spheroids, with substantial tumor weight reduction and enhanced efficacy in an orthotopic *in vivo* mesothelioma model.

For the success of gene- and vector-based therapies, it is critical to develop strategies to improve the vector distribution within tumors *in vivo*. Oncolytic viruses can mediate infected cell death, release viral progeny for propagation of infection and induce resultant lysis of neighboring tumor cells. Therefore, these viruses should have a more profound therapeutic efficacy even without particular therapeutic genes when compared with non-replicative viral vectors. Indeed, as human malignant mesothelioma cells express sufficient telomerase activity as well as CAR (Figure 1), most of the disseminated nodules were imaged with GFP fluorescence by intrathoracic administration of GFP-expressing, telomerase-specific OBP-401 in an orthotopic pleural mesothelioma model, which coincided with histologically confirmed mesothelioma (Figure 3). We have recently shown that this OBP-401-mediated GFP-labeling strategy is extremely sensitive to detect disseminated nodules and applicable for the surgical navigation (Kishimoto *et al.*, 2009). A confocal fluorescent imaging system with fibered microprobes showed that OBP-401 could also identify macroscopically invisible tumor tissues, suggesting that OBP-301 might be able to eliminate microscopic dissemination. In fact, local administration of OBP-301 into the thoracic cavity significantly suppressed the disseminated tumor growth (Figure 4). The treatment immediately after mesothelioma

cell inoculation resembles the state of a minimum residual disease after extended surgical excision. Most of the floating mesothelioma cells could be efficiently treated by locoregional OBP-301 administration, resulting in little disseminated tumor nodule formation. Tumor weights, however, increased gradually as the treatment time was delayed (Figure 4c), suggesting that some additional approaches are required to improve the therapeutic efficacy.

Extracellular matrix is a major barrier to macromolecular transport in the tumor interstitium, but digestive enzymes that degrade ECM may overcome the limited spread of viral agents within tumors. Previous studies have shown that protease that degrades multiple ECM components as well as collagenase that digests fibrillar collagen can mediate a broad distribution of virus particles within tumors, leading to enhanced therapeutic efficacy (Kuriyama *et al.*, 2001; McKee *et al.*, 2006). Non-replicating adenovirus vector expressing the matrix metalloproteinase-8 (MMP-8), which effectively degrades collagen-I, was also able to modify a fibrillar collagen substrate to allow oncolytic virus diffusion into tumors (Cheng *et al.*, 2007). More recent studies have also shown that relaxin-expressing, replication-competent adenovirus could increase the virus distribution and show a profound antitumor effect in mice (Kim *et al.*, 2006; Ganesh *et al.*, 2007). Although the most effective enzyme for the promotion of viral penetration into tumor masses has not been determined, we used heparanase, which has a hydrolytic mechanism to cleave glycosidic bonds in the heparan sulfate component of the ECM (McKenzie, 2007).

The expression of functional heparanase degrades the ECM, which in turn improves the uptake and distribution of biological agents including antibodies and viruses (Eikenes *et al.*, 2004). An advantage of heparanase is that other enzymes that are capable of digesting ECM and basement membrane components (such as MMP-2 and MMP-8) can be subsequently induced after heparanase expression. We reported earlier that the over-expression of the heparanase gene upregulated *MMP-2* mRNA expression in human lung cancer cells (Uno *et al.*, 2001). Arterial injury also increased heparanase activity in vascular endothelial cells, which was associated with MMP-2 and MMP-9 activation (Fitzgerald *et al.*, 1999). Therefore, a more prominent virus infiltration through broad ECM degradation with multiple enzymes can be expected by exogenous heparanase expression. The co-infection of Ad-*S/hep* considerably enhanced OBP-401 virus penetration into the multicellular spheroids, mimicking the *in vivo* biology of tumors (Figure 5b, c). Furthermore, combination therapy with OBP-301 and Ad-*S/hep* in an orthotopic murine model significantly reduced the tumor weights of disseminated plural mesothelioma as compared with tumors from mice treated with OBP-301 alone (Figure 6a), suggesting that heparanase-assisted broad virus distribution could mediate a more profound antitumor effect against human malignant mesothelioma.

Our data indicate that this dual virotherapy may be a promising therapeutic strategy for malignant pleural mesothelioma. However, the over-expression of ECM-digesting enzymes may potentially promote the metastasis of tumor cells. MMPs as well as heparanase were detected in many types of human cancer, and their expression has a very active role in tumor invasion and metastasis. Indeed, targeted inhibition of heparanase expression by antisense complementary DNA transfection showed a significant reduction in the invasive and metastatic properties of tumor cells in an animal model (Uno *et al.*, 2001). Short hairpin RNAs that mediated the attenuation of MMP expression also prevented the progression of human tumor cells *in vivo* (Blackburn *et al.*, 2007). Although there is a risk that the metastatic potential of tumor cells may be increased by heparanase expression, we found that the intrathoracic administration of 10^7 PFU of Ad-*S/hep* alone had no apparent effects on the growth of pleural mesothelioma, indicating that this particular dose of the virus appears to be safe (Figure 6a). In the dual-vector system, the two viral loads can be adjusted according to the function of each virus. We showed earlier that telomerase-specific oncolytic viruses and non-replicative adenovirus-expressing functional genes can successfully work together by determining the optimal doses of vectors (Umeoka *et al.*, 2004; Hioki *et al.*, 2008). A single oncolytic virus vector-expressing relaxin inhibits tumor growth and metastasis, however, it may be impossible to reduce the amount of relaxin expression when high doses of the virus are used. In contrast, our dual-vector system of telomerase-specific oncolytic adenovirus in combination with heparanase-expressing replication-deficient adenovirus can be used safely by a fine adjustment of the optimal doses.

In conclusion, our data clearly indicate that telomerase-specific oncolytic adenoviruses have significant therapeutic potential against human malignant pleural mesothelioma *in vitro* and *in vivo*. Moreover, the addition of heparanase-expressing adenovirus significantly enhanced the virus distribution and the antitumor effects of oncolytic adenoviruses. A phase I, dose-escalation study of telomerase-specific oncolytic adenovirus, OBP-301, is currently underway in the United States to assess the treatment feasibility and to characterize its pharmacokinetics in patients with advanced solid tumors (Fujiwara *et al.*, 2008). Phase II studies of telomerase-specific virotherapy in malignant pleural mesothelioma patients are warranted.

Materials and methods

Cell lines and culture conditions

The human mesothelioma cell lines H2052, H2452, H28 and 211H were purchased from American Type Culture Collection (Manassas, VA, USA). H2052 and H2452 cells were cultured as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. H28 and 211H were routinely propagated in monolayer culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 mg/ml streptomycin. The human non-small-cell lung cancer cell line H1299 was also cultured in RPMI

1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. The normal human lung diploid fibroblast cell line WI38 (JCRB0518) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and grown in Eagle's MEM with 10% fetal bovine serum. The normal human lung fibroblast and the human umbilical vascular endothelial cell line (TaKaRa Biomedicals, Shiga, Japan) were cultured according to the vendors' specifications.

Recombinant adenoviruses
OBP-301 is a telomerase-specific replication-compenent adenovirus variant, in which the hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with internal ribosome entry site (Figure 1a). OBP-301 was modified to create OBP-401 for monitoring viral replicationthe *GFP* gene was inserted under the cytomegalovirus promoter into the E3 region to create OBP-401. Ad-*S/hep* is a replication-deficient adenovirus expressing the human *heparanase* gene under the cytomegalovirus promoter. The E1A-deleted adenovirus dl312 was used as the control adenovirus.

Flow cytometric analysis
A total of 2×10^5 cells were labeled with mouse monoclonal anti-CAR (RmcBUpstate Biotechnology, Lake Placid, NY, USA) for 30 min at 4 °C. Then, the cells were incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, South San Francisco, CA, USA) and analysed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA, USA). An isotype-matched normal mouse IgG_1 conjugated to fluorescein isothiocyanate (Serotec, Oxford, UK) was used as a control.

Quantitative real-time PCR analysis of hTERT mRNA
Total RNA from the culture cells was obtained by using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Approximately 0.1 μg of total RNA was used for reverse transcription. Reverse transcription was performed at 22 °C for 10 min and then at 42 °C for 20 min. The *hTERT* mRNA copy number was determined by real-time quantitative reverse transcription–PCR using a LightCycler instrument and a LightCycler DNA TeloTAGGG hTERT Quantification Kit (Roche Molecular Diagnostics, Indianapolis, IN, USA). Data analysis was performed using the LightCycler software. The ratios normalized by dividing the value of untreated cells were presented for each sample.

Cell viability assay
The XTT assay was performed to measure cell viability. Briefly, cells were seeded at 1×10^3 cells/well in 96-well plates 16–20 h before viral infection and infected with OBP-301 at a MOI of 0, 1, 10 or 50 PFU/cell. Cell viability was determined at the indicated times by using a Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol.

Fluorescent microscopy
Human mesothelioma cell lines were infected with 10 MOI of OBP-401 *in vitro*. Expression of the *GFP* gene was assessed and photographed using an IX71 fluorescent microscope (Olympus, Tokyo, Japan) at indicated times.

Western blot analysis
H2452 cells were collected by trypsinization and washed twice in cold phosphate-buffered saline. Cells then were dissolved in lysis buffer containing 50 mM Tris–HCl (pH7.5), 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM 4-(2-aminoethyl)benzenesul-fonyl-fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 μg/ml aprotinin).The lysis was performed at 4 °C for 30 min, and then the reaction mixture was centrifuged at 15 000 revolutions per minute. The protein concentration of the supernatant was determined by using the Bio-Rad protein determination method (Bio-Rad, Hercules, CA, USA). Equal amounts (60 μg) of proteins were electrophoresed under reducing conditions on 12% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to Hy-bond-polyvinylidene diflouride transfer membranes (Amersham, Arlington Heights, IL, USA) and incubated with primary antibodies against heparanase or β-actin, and then peroxidase-linked secondary antibody. An enhanced chemiluminescence Western system (Amersham, Tokyo, Japan) was used to detect secondary probes.

Spheroid culture
Single-cell suspensions of H2452 cells were obtained by trypsinization of monolayer cultures that consisted of 1×10^4 cells seeded on SUMILON Celltight Spheroid (Sumitomo Bakelite Co, Tokyo, Japan) according to the manufacturer's protocol. After formation of small spheroidal aggregates, 1×10^3 PFU of Ad-*S/hep* or dl312 were added to the culture, followed by the addition of 1×10^4 PFU of OBP-401 48 h later. The GFP expression in each tumor spheroid was assessed under the laser-scanning confocal fluorescent microscope (Carl Zeiss, Jena, Germany) 48 h later.

Animal experiments
The experimental protocol was approved by the ethics review committee for animal experimentation of our institution. We used a 27-gauge needle to intrathoracically inject female BALB/c *nu/nu* mice with 100 μl of suspension containing 5×10^6 H2452 cells. The same technique was used for each viral injection into the thoracic space at the indicated time points. Mice were killed and their thoracic spaces were examined macroscopically. Tumor nodules in the thoracic spaces were removed and weighted. *In vivo* GFP fluorescence imaging was also acquired by using a Hamamatsu C5810 three-chip color cooled charged-coupled device camera (Hamamatsu Photonics Systems, Hamamatsu, Japan) and an *in situ* molecular imaging system (Cell~VIZIOMauna Kea Technologies, Paris, France).

Statistical analysis
We used the Student's *t*-test to determine statistically significant differences among the groups. *P*-values <0.05 were considered statistically significant.

Conflict of interest

Yasuo Urata is an employee of Oncolys BioPharma, Inc., the manufacturer of OBP-301 and OBP-401. Toshiyoshi Fujiwara is a consultant of Oncolys BioPharma, Inc.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

In Vivo Biological Purging for Lymph Node Metastasis of Human Colorectal Cancer by Telomerase-Specific Oncolytic Virotherapy

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Background/Objective: The aim of this study was to develop a less invasive way of targeting lymph node metastasis for the treatment of human gastrointestinal cancer. Lymphatic invasion is a major route for cancer cell dissemination, and adequate treatment of locoregional lymph nodes is required for curative treatment in patients with malignancies.

Methods: Human telomerase reverse transcription (hTERT) is the catalytic subunit of telomerase, which is highly active in cancer cells but quiescent in most normal somatic cells. OBP-301 (Telomelysin) is an attenuated adenovirus with oncolytic potency that contains the hTERT promoter element to regulate viral replication. We examined whether OBP-301 injected into the primary tumor might be useful for purging micrometastasis from regional lymph nodes in an orthotopic colorectal cancer model.

Results: OBP-301 was intratumorally injected into HT29 tumors orthotopically implanted into the rectum in BALB/c *nu/nu* mice. By using a highly sensitive quantitative PCR analysis that targets the human-specific *Alu* sequence, we showed that OBP-301 caused viral spread into the regional lymphatic area and selectively replicated in neoplastic lesions, resulting in tumor-cell-specific death in metastatic lymph nodes. Moreover, although the surgical removal of primary tumors increased the tendency of lymph node metastasis, preoperative intratumoral injection of virus significantly reduced lymph node metastasis.

Conclusions: Our results indicate that intratumoral injection of OBP-301 mediates effective in vivo purging of metastatic tumor cells from regional lymph nodes, which may help optimize treatment of human cancer, especially gastrointestinal malignancies.

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Lymph node status provides important information for both the diagnosis and treatment of human cancer. Lymphatic invasion is a major route for cancer cell dissemination, and lymph node metastasis is a frequent type of recurrence that is associated with a survival disadvantage in many types of cancers.[1–3] Therefore, adequate resection of the locoregional lymph nodes is required for curative treatment in patients with malignancies.[4,5] Extended lymphadenectomy, however, may greatly impair quality of life, especially for patients with early stage epithelial neoplasms in the gastrointestinal tract.[6] Their primary tumors can be removed by new endoluminal therapeutic techniques such as endoscopic submucosal dissection; however, patients with submucosal invasion, lymphovascular infiltration of cancer cells, or undifferentiated histology often become candidates for surgical organ resection with lymphadenectomy, because there is a risk of regional lymph node metastasis, although the frequency is relatively low.[7] Thus, a less invasive way to selectively treat lymph node metastasis would benefit these patients by allowing them to avoid a prophylactic surgery.

Oncolytic viruses that can selectively replicate in tumor cells and lyse infected cells have been extensively investigated as novel anticancer agents.[8–10] These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor cell. We previously developed an attenuated adenovirus designated OBP-301 (Telomelysin) that drives the *E1A* and *E1B* genes under the human telomerase reverse transcription (hTERT) promoter.[10–13] The clinical development of OBP-301 as a monotherapy for various solid tumors is currently underway in the United States.[14] We and others have reported that human adenovirus is capable of effective transport into the lymphatic circulation.[15–17] Injection of OBP-401 (TelomeScan), telomerase-specific, replication-competent adenovirus expressing green fluorescent protein (GFP) into primary tumors allows its lymphatic spread, which in turn induces viral replication in metastatic lymph nodes, allowing us to directly image the micrometastases.

In the present study, we explore whether viruses injected into the established primary tumors could traffic to regional lymph nodes and selectively kill metastatic tumor cells in a human colorectal tumor xenograft model. To measure virus-mediated therapeutic efficacy against lymphatic micrometastasis, we established a highly sensitive real-time PCR method targeting human *Alu* sequences.

MATERIALS AND METHODS

Cell Line and Viruses

The human colorectal cancer cell line HT29 was routinely propagated in monolayer culture in McCoy's medium. The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site, was previously constructed and

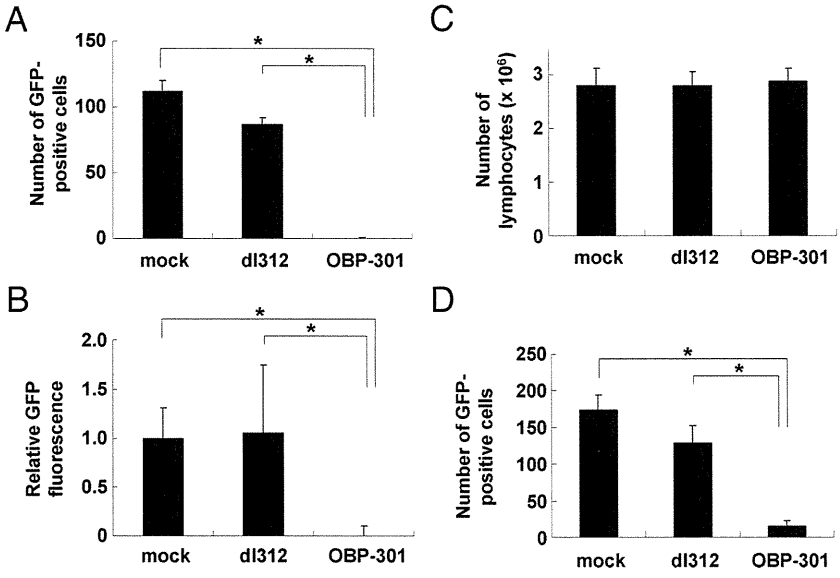

FIGURE 1. In vitro purging effect of OBP-301 infection on HT29 human colorectal cancer cells. We plated 5×10^6 PBMC or mouse splenocytes per well along with 2×10^5 HT29 human colorectal cancer cells. After 24 hours, the mixed culture was infected with 2×10^7 PFU of OBP-301 or dl312 (100 multiplicity of infection [MOI] for HT29 cells) for 96 hours, followed by infection with OBP-401 at 2×10^6 PFU (10 MOI for HT29 cells) to visualize viable HT29 cells (see Figure, Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A39, which illustrates the procedures for in vitro purging experiments). A, The number of GFP-positive, viable HT29 cells was counted in 3 random fields at a magnification of × 200 under the fluorescent microscope. Values represent means ± SEM, and a single asterisk indicates $P < 0.05$ as compared with the other groups. B, The intensity of GFP fluorescence in each treatment group was also measured by using a fluorescence microplate reader. Values are relative to mock (mock = 1) and represent means ± SEM. A single asterisk indicates $P < 0.05$. C, Toxicity of OBP-301 infection was assessed for human lymphocytes. A total of 5×10^6 PBMCs were exposed to 2×10^7 PFU of OBP-301 or dl312 for 96 hours, and their viability was then determined by trypan blue exclusion. D, An efficient purging effect of OBP-301 on HT29 cells in mouse splenocytes. To mimic the animal experiments in vitro, HT29 cells mixed with splenocytes from BALB/c *nu/nu* mice were exposed to OBP-301 or dl312 for 96 hours, followed by OBP-401 infection. The number of cells positive for GFP was counted as described above, and presented as the mean ± SEM. A single asterisk indicates $P < 0.05$.

characterized.[11] OBP-401 (TelomeScan) is a telomerase-specific, replication-competent adenovirus variant in which the replication cassette and *GFP* gene under the control of the cytomegalovirus promoter were inserted into the E3 region for monitoring viral replication[15] (see Figure, Supplemental Digital Content 1, online only, available at http://links.lww.com/SLA/A38, which illustrates schematic DNA structures of telomerase-specific viruses). The *E1A*-deleted adenovirus vector lacking a cDNA insert (dl312) was also used as a control vector.

In Vitro Purging Experiments

For in vitro purging studies, peripheral blood samples were drawn from healthy volunteers, and mononuclear cells were isolated by sedimentation over Ficoll-Hypaque. Mouse spleens were removed aseptically and gently crushed with the flat end of a sterile syringe. The cells were passed through nylon mesh and then placed in buffered ammonium chloride solution to produce osmotic lysis of erythrocytes. We plated peripheral blood mononuclear cells (PBMC) or mouse splenocytes per well along with HT29 cells. The purging effect was assessed with an Eclipse TS-100 fluorescent microscope (Nikon, Tokyo, Japan) by counting the number of GFP-positive cells 24 hours after OBP-401 infection (see Figure, Supplemental Digital Content 2, online only, available at http://links.lww.com/SLA/A39, which illustrates the procedures for in vitro purging experiments). GFP fluorescence was also measured by using a fluorescence microplate reader (DS Pharma Biomedical, Osaka, Japan) with excitation/emission at 485 nm/528 nm.

Xenograft Model of Lymph Node Metastasis

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our institution. The implantation procedures for human rectal cancer xenografts were described previously.[15] Cell suspensions of HT29 cells at a density of 5×10^6 cells in 100 μL of Matrigel (BD Biosciences, Bedford, MA) were slowly injected into the submucosal layer of the rectum by using a 27-gauge needle. For pathologic evaluation of lymph node metastasis, mice were killed and all para-aortic or iliac lymph nodes were isolated and stained with hematoxylin and eosin.

In Vivo Fluorescence Imaging

In vivo GFP fluorescence imaging was acquired by illuminating the animal with a Xenon 150 W lamp. The re-emitted fluorescence was collected through a long pass filter on a Hamamatsu C5810 3-chip color cooled charged-coupled device (CCD) camera (Hamamatsu Photonics Systems, Hamamatsu, Japan). Abdominal images were also obtained during laparotomy with the IVIS CCD camera and analyzed with Living Image 2.20.1 software (Xenogen/Caliper Life Sciences, Hopkinton, MA) for the quantification of lymph node metastasis.

Quantitative Real-Time PCR Analysis

To measure the amounts of human tumor cells in mouse lymph nodes, we applied a previously described quantitative PCR assay that uses primer sets to amplify human *Alu* sequences present in mouse lymph node DNA extracts. Genomic DNA was extracted

from mixed cell cultures or harvested tissues by using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). To detect human cells in the mouse tissues, a set of human *Alu* primers (sense: 5′-CTG AGG TCA GGA GTT CGA G-3′; and antisense: 5′-TCA AGC GAT TCT CCT GCC-3′) were designed. We performed the quantitative real-time PCR assay by using a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN). PCR amplification began with a 120-second denaturation step at 95°C and then 30 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 30 seconds. We also amplified the mouse *GAPDH* genomic DNA sequence with mouse *GAPDH* primers (sense: 5′-CCA CTC TTC CAC CTT CGA T-3′; and antisense: 5′-CAC CAC CCT GTT GCT GTA-3′) by using the same PCR conditions described for *Alu*. The amounts of *E1A* DNA of OBP-301 and OBP-401 were measured as previously described. 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 of genomic DNA extracted from mouse lymph nodes was performed with precycling heat activation of DNA polymerase at 95°C for 600 seconds, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 8 seconds. Data analysis was performed with LightCycler Software (Roche Molecular Biochemicals).

Statistical Analysis

We used the Student 2-tailed *t* test to identify statistically significant differences between groups. Results are reported as mean ± SEM. *P* values less than 0.05 were considered statistically significant.

RESULTS

In Vitro Purging of Human Colorectal Cancer Cells by Telomerase-Specific Oncolytic Adenovirus

To examine whether telomerase-specific oncolytic adenovirus can selectively kill human tumor cells among the millions of lymphocytes in lymph nodes, HT29 human colorectal cancer cells were mixed with PBMC from healthy donors and purged in vitro with OBP-301 for 3 days. Viable HT29 cells were then visualized with GFP fluorescence by OBP-401 infection for 24 hours (see Figure, Supplemental Digital Content 2, online only, available at http://links.lww.com/SLA/A39, which illustrates the procedures for in vitro purging experiments). No GFP-positive viable HT29 cells were detected at 3 days postpurging with OBP-301, whereas infection with replication-deficient control adenovirus dl312 had no significant effects on the viability of HT29 cells (Fig. 1A) (see Figure, Supplemental Digital Content 2, online only, available at: http://links.lww.com/SLA/A39, which demonstrates in vitro purging effect of OBP-301 infection). The purging efficacy of OBP-301 was also confirmed by measuring the relative GFP expression of samples by using a fluorescence microplate reader (Fig. 1B). Neither OBP-301 nor dl312 infection affected the viability of PBMC, confirming the safety of OBP-301 to normal human lymphocytes (Fig. 1C). Next, we determined if human tumor cells mixed with mouse lymphocytes obtained from the spleen would be sensitive to OBP-301 treatment. As expected, purging with OBP-301 significantly reduced the number of viable HT29 cells in mouse splenocytes compared with mock- or dl312-treated samples (Fig. 1D).

In Vivo Lymphatic Spread of Virus on Regional Lymph Nodes

To verify that oncolytic adenoviruses traffic through the lymphatics to the regional lymph nodes, we used an orthotopic mouse model of human rectal cancer with spontaneous lymph node metastasis. We first determined whether the regional lymphatic system, including lymphatic vessels and lymph nodes, could be assessed by injecting dye into the primary tumors. Intense blue staining was detected in regional lymph nodes as early as 1 minute after injection of indigo carmine blue dye into the primary rectal tumors, indicating that the injected solution could rapidly enter the intratumoral lymphatics, which provides a route from the primary tumor to draining lymph nodes (Fig. 2A). Five days after OBP-401 injection into the primary tumors, we also detected GFP expression in both primary rectal tumors and metastatic lymph nodes under the laparotomy by using a 3-chip CCD optical imaging system (Fig. 2B).

To further evaluate the selective replication ability of telomerase-specific oncolytic adenovirus in metastatic lymph nodes, we measured the relative amounts of *E1A* DNA by quantitative real-time PCR analysis. The metastatic GFP-positive lymph nodes con-

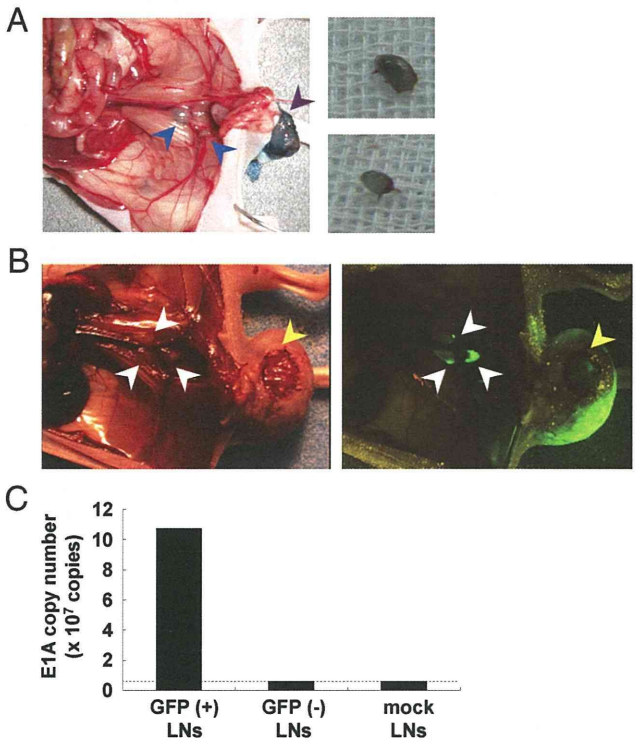

FIGURE 2. In vivo lymphatic spread of virus on regional lymph nodes. A, A lymphatic drainage pattern in an orthotopic xenograft of human HT29 cells. The abdominal cavity was photographed 1 minute after injection of 1% indigo carmine blue dye into an established orthotopic HT29 tumor. Arrowheads indicate the lymph nodes stained with blue dye. Gross appearance of the abdominal cavity (left) and excised lymph nodes (right). B, Five days after intratumoral injection of 1×10^8 PFU of OBP-401, HT29 tumor-bearing *nu/nu* mice were assessed for lymph node metastasis during laparotomy. Macroscopic (left) and fluorescent (right) images. Primary tumor (yellow arrowhead) and metastatic lymph nodes (white arrowheads) are shown. C, Assessment of viral replication in metastatic lymph nodes. GFP-positive and -negative lymph nodes were harvested from mice with HT29 tumor xenografts 5 days after injection of 1×10^8 PFU of OBP-401 and then subjected to real-time quantitative PCR assay to quantify the amounts of viral *E1A* copy number. The value in mock-infected lymph nodes is indicated with a dotted line as a baseline level.

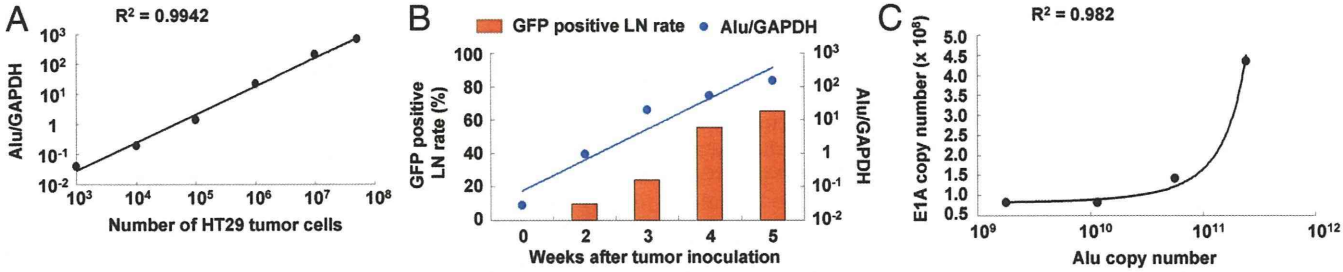

FIGURE 3. Detection and quantification of human cancer cells in mouse tissues with a quantitative real-time *Alu*PCR analysis. A, Generation of a standard curve. Mouse splenocytes (5×10^7 cells) were spiked with serially diluted HT29 human colorectal cancer cells. Genomic DNA was extracted from the mixtures and then subjected to the quantitative amplification (see Figure, Supplemental Digital Content 4A, available at: http://links.lww.com/SLA/A41, which illustrates schematic procedures of detection and quantification of human cancer cells in mouse splenocytes). The *Alu/GAPDH* ratios *versus* the numbers of spiked HT29 cells are presented. Regression analysis yielded a correlation coefficient of $R^2 = 0.9942$. The data are representative of 3 separate experiments. B, Quantitative analysis of spontaneous lymph node metastasis. Genomic DNA was extracted from the lymph nodes of mice bearing human HT29 tumor xenografts at the indicated time points after tumor inoculation and analyzed with the quantitative *Alu*PCR assay (see Figure, Supplemental Digital Content 4B, available at: http://links.lww.com/SLA/A41, which illustrates schematic procedures of detection and quantification of human cancer cells in mouse tissues). Metastatic lymph nodes were simultaneously visualized by injecting 1×10^8 PFU of OBP-401 into the primary tumors 5 days before lymph node isolation. C, The viral *E1A* copy numbers were also determined in genomic DNA extracted from isolated lymph nodes by the quantitative real-time PCR method and plotted *versus* the *Alu* copy numbers.

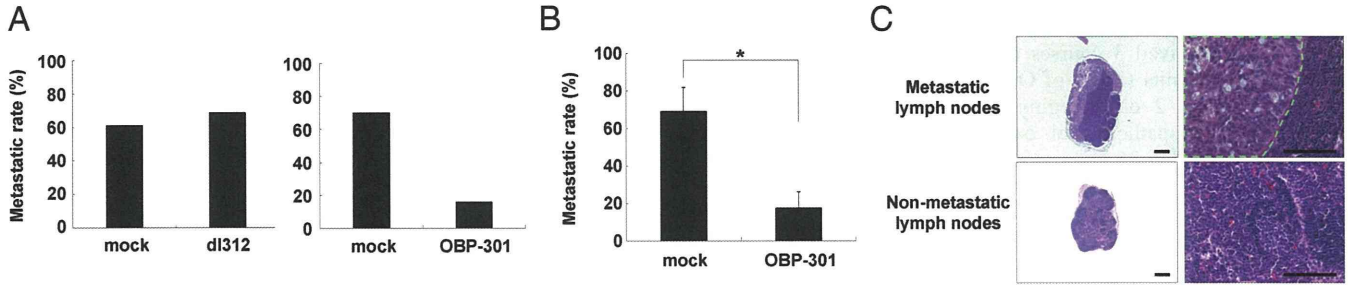

FIGURE 4. Histologic evaluation of selective antitumor effect of OBP-301 delivered into primary tumors on lymphatic metastasis in a colorectal cancer xenograft model. Mice bearing orthotopic HT29 tumors received 3 courses of intratumoral injections of 1×10^8 PFU of OBP-301 or dl312 every 2 days beginning on day 14 after the tumor inoculation. A, On day 35, we harvested a total of 23 to 29 lymph nodes from 5 to 8 mice per group, stained them with hematoxylin and eosin, and calculated metastatic rates. B, In a separate experiment, metastatic rates were histologically determined in individual mice and averaged with S.E.M. ($n = 8$). A single asterisk denotes statistical significance ($P < 0.05$) as compared with the mock group. C, Paraffin-embedded sections of lymph nodes with or without metastatic foci were obtained 35 days after tumor cell implantation and stained with hematoxylin and eosin. Left, ×40 magnification; right, ×200 magnification. Scale bar, 100 μm. The area with metastatic HT29 cells is indicated with the green dotted line.

tained more than 10^8 copies of OBP-401, whereas the number of viral genomes was at the baseline level in nonmetastatic GFP-negative lymph nodes (Fig. 2C). The trafficking and replication ability of the virus was also confirmed in an orthotopic head and neck cancer xenograft model (see Figure, Supplemental Digital Content 3, online only, available at: http://links.lww.com/SLA/A40, which demonstrates OBP-401 virus spread delivered into primary tumors).

Establishment of Highly Sensitive Quantitative Detection Assay for Lymph Node Metastasis

To achieve the highest sensitivity for detecting metastatic human tumor cells in mouse lymph nodes, we applied a novel quantitative real-time PCR assay that uses primer sets to amplify the consensus human *Alu* sequence.[18–20] *Alu* repeat sequences are specific to all human cells and are completely absent in mouse tissues.[21] To test the sensitivity and range of the assay, mouse splenocytes obtained from athymic *nu/nu* mice were spiked with variable numbers of HT29 human colorectal cancer cells in vitro (see Figure, Supplemental Digital Content 4A, online only, available at: http://links.lww.com/SLA/A41, which illustrates schematic procedures of detection and quantification of human cancer cells in mouse splenocytes). The total genomic DNA was extracted from the mixtures and subjected to quantitative *Alu*PCR. We also used the human *GAPDH* gene as an internal control to normalize the *Alu* signal in each sample. By plotting the *Alu/GAPDH* ratio as a function of the number of spiked tumor cells, a standard curve could be generated with a linear range between 10^3 and 10^8 cells, and regression analysis of the *Alu/GAPDH* ratio *versus* the number of expected tumor cells yielded a correlation coefficient of $R^2 = 0.9942$ ($P < 0.01$) (Fig. 3A). These results suggest that the *Alu/GAPDH* ratio reflects the actual human tumor cells among mouse splenocytes.

To verify the accuracy and sensitivity of the assay in vivo, we extracted DNA from total lymph nodes at different time points after the orthotopic implantation of HT29 cells and subjected the DNA to the *Alu*PCR analysis (see Figure, Supplemental Digital Content 4B,