厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

光感受性ROS産生蛍光タンパク質を発現する遺伝子改変アデノウイルス製剤を用いた新たな癌の光線力学療法システムの開発

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研究代表者　**藤原　俊義**

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【研究要旨】

テロメライシンは岡山大学で開発された国産の抗癌アデノウイルス製剤であり、テロメラーゼ構成成分であるhTERT遺伝子のプロモーターにより、癌細胞のみで選択的に増殖して腫瘍融解を引き起す。本研究では、テロメライシンに光感受性蛍光タンパク質をコードする遺伝子を搭載した改変アデノウイルス製剤を開発する。腫瘍選択的に光感受性蛍光タンパク質を発現させ、治療用デバイスにて励起光照射することで癌細胞のみで活性酸素（reactive oxygen species：ROS）を産生して細胞死を誘導する新たな癌の光線力学療法を確立する。

本研究の最終到達目標は、テロメラーゼ依存性増殖機能を持つアデノウイルス製剤テロメライシンをベクターとして癌細胞選択的に光感受性蛍光タンパク質KillerRed遺伝子を発現する次世代型武装化アデノウイルス製剤を作成し、新たな癌の光線力学療法としての臨床応用を行うことである。

本年度は、光感受性蛍光タンパク質KillerRed遺伝子を発現するプラスミドを作成し、各種培養ヒト癌細胞株に遺伝子導入を行って励起・蛍光スペクトルを確認した。ヒト肺癌、子宮頸癌、骨肉腫細胞においてKillerRed遺伝子を発現させ励起光照射を行ったところ、赤色蛍光は急速に退色し、約1時間以内にKillerRed発現細胞は死滅した。

A．研究目的

テロメライシン（Telomelysin、開発コード：OBP-301）は岡山大学で開発された国産の抗癌アデノウイルス製剤であり、癌細胞で選択的に増殖・複製する機能を有しており、自立的に癌細胞を破壊することで増殖したウイルスが拡散していく。したがって、テロメライシン自体が新しい作用機構に基づく新規の画期的な医薬品となるが、ゲノム構造が明らかなアデノウイルスを基本骨格としているため、テロメライシンはベクターとして応用可能であり、導入遺伝子の癌選択的かつ長期的発現が期待できる。

最近、GFPをはじめとする蛍光タンパク質は分子イメージングのマーカーとして注目されているが、マーカー機能では細胞毒性が低いものが選択される。研究代表者らも、GFP遺伝子を搭載したテロメスキャン（TelomeScan、OBP-401）を診断用医薬品として開発してきたが、GFP自体の細胞毒性は極めて低い（Kishimoto et al., Nature Med., 12, 1213-1219, 2006）。その発想を転換し、本研究では診断と治療を融合した新しい研究分野「Theranostics」の医薬品として、光誘導細胞死を生じる新規蛍光赤色タンパク質KillerRed遺伝子を用いた光感受性武装化アデノウイルス製剤を開発する。標的癌細胞でウイルス増殖に伴う強い蛍光タンパク質発現により癌細胞の可視化を可能とし、また同時に励起光照射で産生されるROSによる強力な細胞傷害活性が発揮される。すなわち、遺伝子改変ウイルス製剤を基盤とする本技術は、分子イメージングと分子手術による全く新しい独創的な治療戦略と言える。

本研究では、テロメラーゼ依存性増殖機能を持つアデノウイルス製剤テロメライシンをベクターとして癌細胞選択的に光感受性蛍光タンパク質遺伝子を発現する次世代型武装化アデノウイルス製剤を作成し、新たな癌の光線力学療法としてその機能解析、小動物・大動物を用いた有効性・安全性の検討を終了することを目指す。

B．研究方法

1）KillerRedおよびEGFP発現プラスミドの作成

KillerRedは*Anthomedusae*クラゲの発色タンパク質であるanm2CPを改変して開発された新しい赤色蛍光タンパク質であり、540～580nmの緑色光照射によって活性酸素（reactive oxygen species：ROS）を産生して細胞死を誘導することができる。今回、細胞死の誘導効率を確認し、光線力学療法としての有用性を検証するために、KillerRed遺伝子発現プラスミドを準備した。さらに、標的細胞でKillerRedと*Aequorea victoria*由来のGreen Fluorescent Protein (GFP)を同時に発現するために、KillerRedとEGFPをIRES配列で連結した発現カセットを挿入したプラスミドも作成した。

2）各種培養ヒト癌細胞株における KillerRed遺伝子発現とタイムラプス観察

ヒト肺癌、子宮頸癌、骨肉腫細胞（H1299、HeLa、OST）にLipofectamin、FuGeneHD等の試薬を用いてKillerRedあるいはKillerRed/EGFP発現プラスミドを導入し、G418（ネオマイシンアナログ）添加でstable cloneを樹立した。540-580nmの緑色励起光を照射し、蛍光顕微鏡下でタイムラプス観察を行うことで細胞死誘導を比較検討した。

3）テロメライシンへのKillerRed遺伝子搭載

テロメライシンのゲノムにKillerRed遺伝子を組み込み、次世代型光感受性武装化アデノウイルス製剤の作成を試みた。

（倫理面への配慮）

制限増殖型ウイルス製剤を用いる本研究は「大臣確認実験」となるため、「第二種使用等拡散防止措置確認申請書」を作成、学内の担当部署での検討の後に文部科学省に申請し、研究計画実施の承認を得ている。

C．研究結果

1）KillerRedおよびEGFP発現プラスミドの作成

KillerRed遺伝子発現には、Evrogen社細胞膜局在ベクターpKillerRed-memを使用した。また、EGFPを共発現するベクターは以下の手順で作成した。

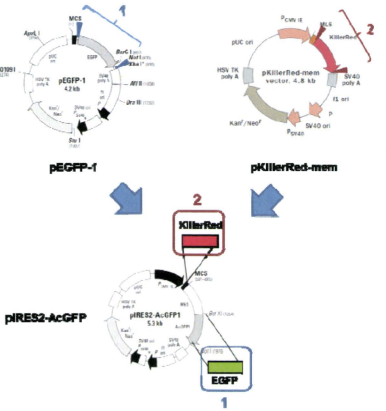

図1　KillerRed/EGFP遺伝子発現ベクターの作成

2）各種培養ヒト癌細胞株における KillerRed遺伝子発現とタイムラプス観察

ヒト肺癌、骨肉腫細胞においてKillerRed遺伝子を発現させ580nmの緑色励起光を照射したところ、KillerRedタンパク質発現による赤色蛍光は急速に退色がみられ、約1時間以内にKillerRed遺伝子発現細胞は死滅した（図2）。

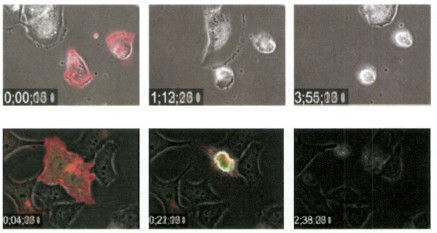

図2　KillerRed発現細胞のタイムラプス観察
（上段：H1299細胞、下段：OST細胞）

一方、KillerRed/EGFP発現させた子宮頸癌細胞にそれぞれの励起光を照射したところ、EGFPの緑色蛍光は比較的維持されたが、やはり赤色蛍光は早期に退色して細胞死が誘導された（図3）。

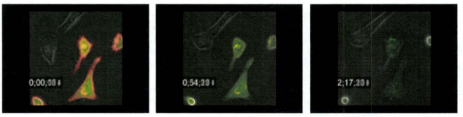

図3　KillerRed/EGFP遺伝子共発現HeLa細胞のタイムラプス観察

3）テロメライシンへのKillerRed遺伝子搭載

テロメライシンのアデノウイルスゲノムのE3領域に挿入するための発現カセットを構築した。まず、KillerRedとEGFPの共発現を得るためにKillerRed/IRES/EGFPを作成し、H1299ヒト肺癌細胞に一過性に導入して発現を確認したが、KillerRed遺伝子発現は十分認められたにもかかわらず、IRESの影響のためか低GFP発現しか得られなかった（図4）。そこで、KillerRed遺伝子のみをサイトメガロウイルス・プロモーターにて駆動する発現カセットも作成し、現在、両者のテロメライシンへの組み込みを行っている。

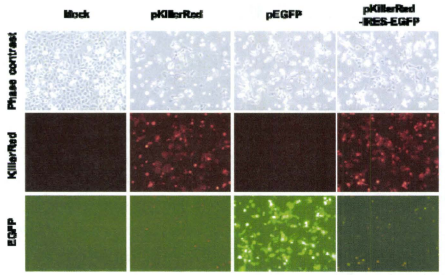

図4　各蛍光タンパク質の発現の比較

D．考察

光感受性物質と励起光照射を組み合わせて行う光線力学療法は、侵襲が少ない癌治療として一部の早期癌で臨床応用が試みられている。しかし、光感受性物質の標的とする生体内分子に対する選択性が小さいことや、光感受性物質の投与のために光線過敏症を引き起こすなどの問題点を残していた。本研究では、癌への選択性が強いテロメライシンをベクターとしてKillerRed遺伝子を発現するため、癌集積性は極めて高い。生物製剤、特にウイルス製剤と光照射を組み合わせた光線力学療法は、未だ世界で報告がなく、臨床応用に向けた試みももちろん存在しない。一方、蛍光タンパク質自体の発見や開発は最近のトピックスであり、これらを結びつけることで現実的な治療開発につながる可能性が強い。

テロメライシンはテロメラーゼ活性を標的とする癌治療を目的とした生物製剤であり、生体内で自律性を持って増殖することによる従来の抗癌剤や分子標的薬剤にない抗腫瘍効果の増強が期待できる。本研究では、米国の臨床試験で安全性が確認されたこのテロメライシンをベクターとして全く新たな光線力学療法への応用を目指しており、その安全性と効果に関する理論的根拠が得られれば、創薬シーズとしては極めて魅力的なものとなると思われる。中国では、すでに2種類の局所療法剤としてのウイルス製剤（Gendicine、H101）が中国FDAの承認を受けており、一般市場にて日常臨床に使用されている。しかし、遺伝子治療やペプチド医薬などに関しては中国以外の海外でも未だ実績がない分野だけに、新技術が製品の形で現実のものになれば、社会的インパクトは極めて大きく、抗癌剤市場はさらに高い伸びを示すと予測されている。

来年度は、現在作成中のKillerRed遺伝子発現カセットを搭載した光感受性武装化アデノウイルス製剤を用いた前臨床研究を実施する。各種培養ヒト癌細胞株にウイルスを感染させ、励起・蛍光スペクトルを確認するとともに、励起光照射による抗腫瘍効果の相関を検証する。また、オリンパス株式会社では、動物モデルで用いる励起光照射のための治療用デバイスを試作する。さらに、Good Laboratory Practice（GLP）規格のウイルス製剤を製造し、マウスを用いて光感受性武装化アデノウイルス製剤の毒性試験、薬理動態試験を実施する。大動物での励起光照射ビデオスコープを試作して、光線力学療法の有効性を検討する。

光感受性武装化アデノウイルス製剤は低侵襲な局所制御療法であり、光線力学療法への応用で選択的な抗腫瘍活性を増強することで、集学的治療として癌患者の生活の質（QOL）の向上に貢献する。また、有効な癌治療戦略の一つとなることで、国民の健康増進や医療経済の節減にも役立つと期待される。

E．結論

緑色励起光によりKillerRed遺伝子発現ヒト癌細胞を選択的に殺傷することができ、光感受性武装化アデノウイルス製剤を用いたKillerRedの光線力学療法への応用の可能性が示唆された。

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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,

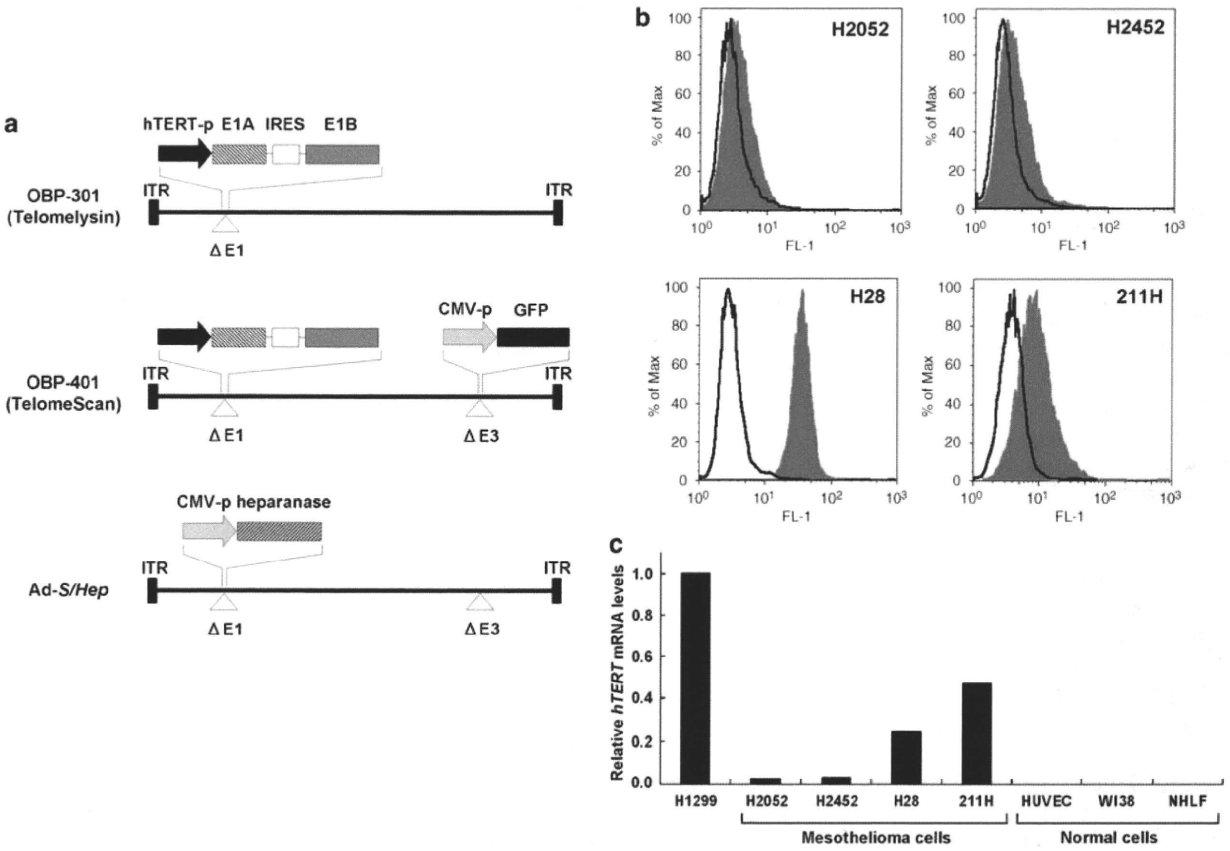

Figure 1 Schematic DNA structures of telomerase-specific viruses and characteristics of human mesothelioma cell lines. (**a**) OBP-301 is a telomerase-specific, replication-competent adenovirus that contains the human telomerase reverse transcriptase (hTERT) promoter sequence inserted into the adenovirus 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.

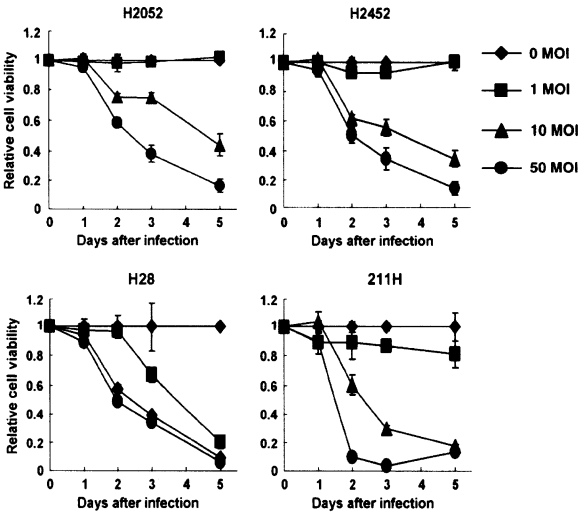

Figure 2 Selective cytopathic effect of OBP-301 in human mesothelioma cell lines *in vitro*. Cells were infected with OBP-301 at the indicated multiplicity of infection (MOI) values, and the surviving cells were quantitated over 5 days by XTT assay. The cell viability of mock-treated cells on day 1 was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

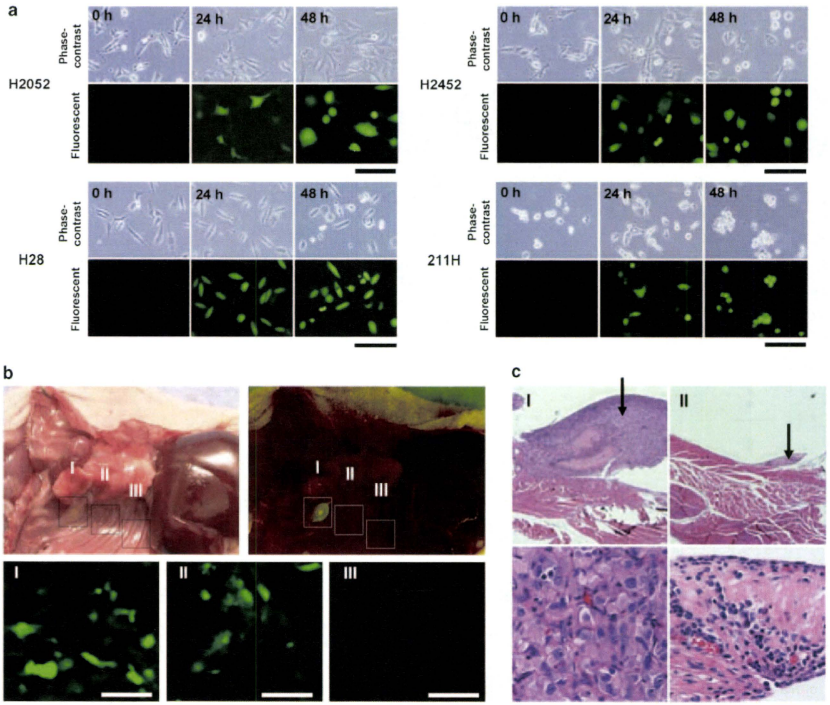

Figure 3 Visualization of human mesothelioma cells *in vitro* and *in vivo* by OBP-401 infection. (**a**) H2052, H2452, H28 and 211 H cells were infected with OBP-401 at an multiplicity of infection (MOI) of 10. Cell morphology and green fluorescent protein (GFP) expression were evaluated by fluorescence 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 1×10^9 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

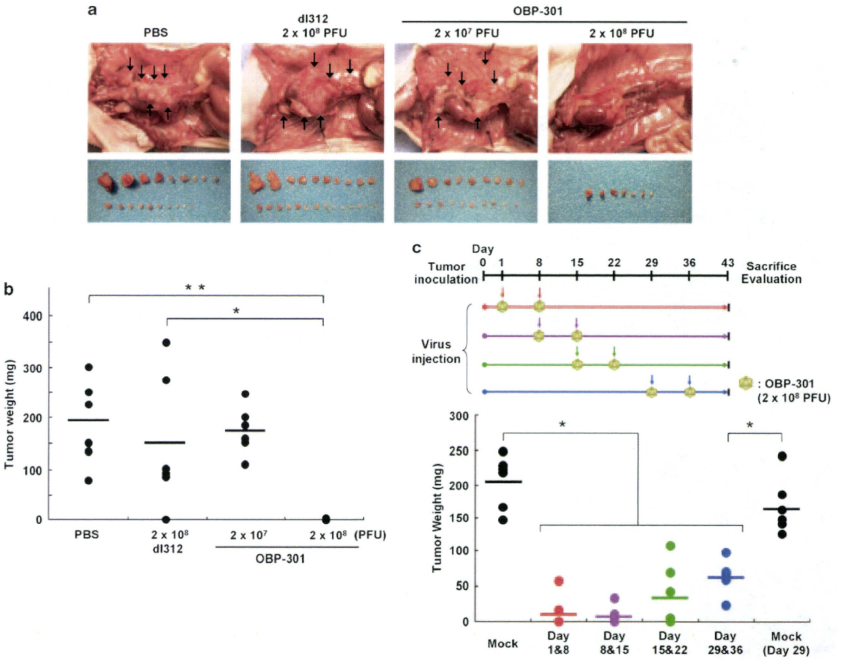

Figure 4 *In vivo* antitumor effect of OBP-301 on pleural dissemination of H2452 human mesothelioma cells. (**a**) Gross appearance of H2452 tumors grown orthotopically in the thoracic spaces. H2452 cells (5×10^6) were inoculated into the thoracic space of athymic *nu/nu* mice. After 24 h, either 1×10^7 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

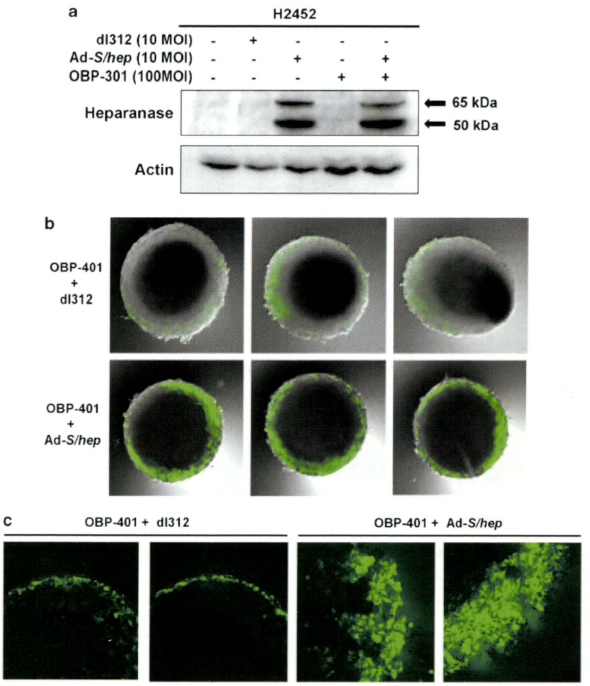

Figure 5 Enhanced penetration of the virus into tumor spheroids by heparanase expression. (**a**) Western blot analysis of human heparanase protein expression in H2452 cells. Cells were infected with either dl312, Ad-*S/hep*, OBP-301 or OBP-301 in combination with Ad-*S/hep* at different multiplicity 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 1×10^5 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

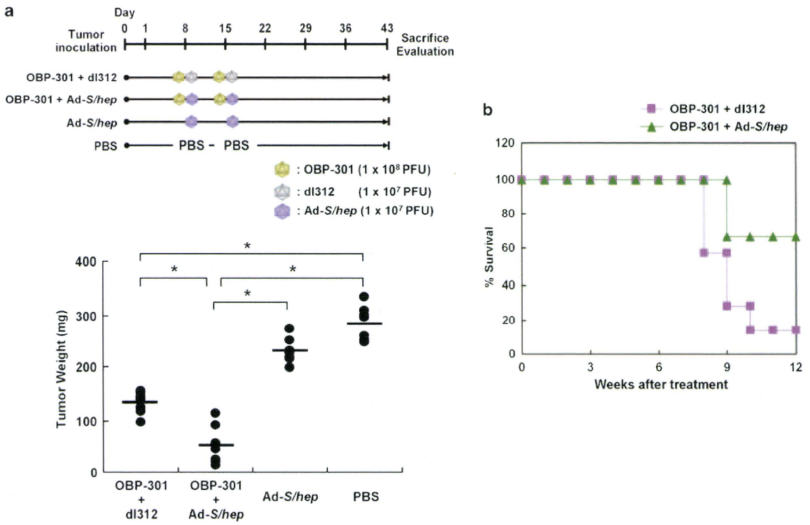

Figure 6 Enhanced antitumor effect of OBP-301 with Ad-*S/hep* in an orthotopic pleural dissemination model. (**a**) Top panel, treatment schedule. Bottom panel, tumor weight of each tumor nodule found in the thoracic spaces after treatment. Treated mice were killed and assessed for pleural dissemination 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

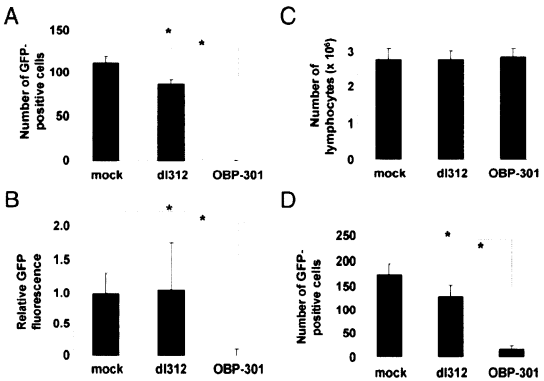

FIGURE 1. In vitro purging effect of OBP-301 infection on HT29 human colorectal cancer cells. We plated 5×10^6 PBMC or mouse splenocytes per well along with 2×10^5 HT29 human colorectal cancer cells. After 24 hours, the mixed culture was infected with 2×10^7 PFU of OBP-301 or dl312 (100 multiplicity 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