Expert Opinion

Telomerase-specific virotherapy for human squamous cell carcinoma

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Background: Replication-selective tumor-specific viruses present a novel approach for treatment of neoplastic disease. They are designed to induce lysis after propagation within the tumor. Human telomerase is active in over 85% of primary cancers and its activity correlates closely with human telomerase reverse transcriptase (hTERT) expression. *Objectives*: Oncolytic viruses, Telomelysin and TelomeScan, that combine the specificity of hTERT promoter-based expression systems with the lytic efficacy of replicative viruses were developed. The goal was to confirm the efficacy of the viruses for human squamous cell carcinoma. *Results/conclusion*: Squamous cell carcinoma of the head and neck (SCCHN) is characterized by locoregional spread, and is clinically accessible, making it an attractive target for intratumoral virotherapy. The viruses replicated efficiently and induced killing in a panel of human cancer cell lines including SCCHN cells *in vitro* and *in vivo*. These results illustrate the potential of telomerase-specific oncolytic viruses for treatment of human SCCHN.

Keywords: adenovirus, GFP, hTERT, imaging, SCCHN, telomerase

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1. Introduction

Oncolytic virotherapy has become a reality on the basis of the safety of many types of viral vectors used for human gene therapy. Viruses are the simplest form of life, carry genetic materials and are capable of entering host cells efficiently. Because of this property, many viruses have been adapted as gene transfer vectors [1-7]. Adenoviruses have been studied extensively and are well characterized. Adenoviruses are large, double-stranded DNA viruses with tropism for many human tissues such as bronchial epithelia, hepatocytes and neurons. Furthermore, they are capable of transducing nonreplicating cells and can be grown to high titers *in vitro*, which allows for their potential use clinically. High titers of replication-defective adenoviruses can be produced and have been successfully used in eukaryotic gene expression [1,8,9]. Numerous studies using *in vitro* and animal models have tested a wide variety of adenoviral gene therapy agents and reported potential beneficial effects for different target diseases, and their tolerability and safety [10-13].

Gene and vector-based therapies for cancer encompass a wide range of treatment types that all use genetic material to modify cancer cells and/or surrounding tissues to make them exhibit antitumor properties. One of the most common approaches to emerge from the concept of gene therapy is the introduction of foreign therapeutic genes into target cells. A number of genes of interest with different functions such as tumor suppressor genes [14,15], proapoptotic genes [16,17], suicide genes that cause cellular death with prodrugs [13,18], and genes that inhibit angiogenesis [19] have been proposed for this type of therapy. In fact, the author's group and others have completed clinical trials of a replication-deficient adenoviral

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vector that delivers normally functioning *p53* tumor suppressor gene to cancer cells (Ad5CMV-p53, Advexin). It has been reported that multiple courses of intratumoral injection of Ad5CMV-p53 are feasible and well tolerated in patients with advanced head and neck squamous cell carcinoma and non-small cell lung cancers and appear to provide clinical benefits [20-24]. Another rapidly growing area of gene therapy for cancer is the use of oncolytic vectors for selective tumor cell destruction. Since viruses infect cells and then induce cell lysis through their propagation, they can be used as anticancer agents by genetic engineering that causes them to replicate selectively in cancer cells while remaining innocuous to normal tissues [25]. Clinical trials of intratumoral injection of Onyx-015, which is an adenovirus with the E1B 55-kDa gene deleted, engineered to selectively replicate in and lyse p53-deficient cancer cells [26], alone or in combination with cisplatin/5-fluorouracil have been conducted in patients with recurrent head and neck cancer [27,28]; however, the study afterwards has clarified that the capacity of Onyx-015 to replicate independently of the cell cycle does not correlate with the status of p53 but is determined by yet unidentified mechanisms [29].

The optimal treatment of human cancer requires improvement of the therapeutic ratio to increase the cytotoxic efficacy on tumor cells and decrease that on normal cells. This may not be an easy task because the majority of normal cells surrounding tumors are sensitive to cytotoxic agents. Thus, to establish reliable therapeutic strategies for human cancer, it is important to seek genetic and epigenetic targets present only in cancer cells. One of the targeting strategies has involved the use of tissue-specific promoters to restrict gene expression or viral replication in specific tissues. Telomerase is a ribonucleoprotein complex responsible for the addition of TTAGGG repeats to the telomeric ends of chromosomes, and contains three components: the RNA subunit (known as hTR, hTER, or hTERC) [30], the telomerase-associated protein (hTEP1) [31], and the catalytic subunit (hTERT, human telomerase reverse transcriptase) [32,33]. Both hTR and hTERT are required for the reconstitution of telomerase activity *in vitro* [34] and, therefore, represent the minimal catalytic core of telomerase in humans [35]. However, while hTR is widely expressed in embryonic and somatic tissues, hTERT is tightly regulated and is not detectable in most somatic cells. The hTERT proximal promoter can be used as a molecular switch for the selective expression of target genes in tumor cells, since almost all advanced human cancer cells express telomerase while most normal cells do not [36,37].

An estimated 500,000 patients worldwide are diagnosed with squamous cell carcinoma of the head and neck (SCCHN) annually [38]. This aggressive epithelial malignancy is associated with a high mortality rate and severe morbidity among the long-term survivors [39]. Current treatment strategies for advanced SCCHN include surgical resection, radiation and cytotoxic chemotherapy. Although a combination of these modalities can improve survival, most patients eventually experience disease progression that leads to death; disease progression is often the result of intrinsic or acquired resistance to treatment [40,41]. A lack of specificity for tumor cells is the primary limitation of radiotherapy and chemotherapy. To improve the therapeutic index, there is a need for anticancer agents that selectively target only tumor cells and spare normal cells. This review looks at recent developments in this rapidly evolving field, cancer therapeutic and cancer diagnostic approaches using the hTERT promoter, and highlights some very promising advances for the treatment of human SCCHN.

2. Telomerase-specific oncolytic virotherapy for human SCCHN

2.1 hTERT promoter-driven oncolytic adenovirus

The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for treatment of cancer. One approach to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication such as *E1A* or *E4*. As described above, telomerase, especially its catalytic subunit hTERT, is expressed in the majority of human cancers and the hTERT promoter is preferentially activated in human cancer cells [42]. Thus, the broadly applicable hTERT promoter might be a suitable regulator of adenoviral replication. Indeed, it has been reported previously that the transcriptional control of E1A expression via the hTERT promoter could restrict adenoviral replication to telomerase-positive tumor cells and efficiently lyse tumor cells [43-46]. Furthermore, Kuppuswamy *et al.* have recently developed a novel oncolytic adenovirus (VRX-011), in which the replication of the vector targets cancer cells by replacing adenovirus E4 promoter with the hTERT promoter [47]. VRX-011 could also overexpress the adenovirus death protein (ADP) (also known as E3-11.6K), which is required for efficient cell lysis and release of virions from cells at late stages of infection.

The adenovirus *E1B* gene is expressed early in viral infection and its gene product inhibits E1A-induced p53-dependent apoptosis, which in turn promotes the cytoplasmic accumulation of late viral mRNA, leading to a shut down of host cell protein synthesis. In most vectors that replicate under the transcriptional control of the *E1A* gene including hTERT-specific oncolytic adenoviruses, the *E1B* gene is driven by the endogenous adenovirus E1B promoter. However, Li *et al.* [48] have demonstrated that transcriptional control of both *E1A* and *E1B* genes by the α-fetoprotein (AFP) promoter with the use of internal ribosome entry sites (IRES) significantly improved the specificity and the therapeutic index in hepatocellular carcinoma cells. Based on the above information, Telomelysin (OBP-301) was developed, in which the tumor-specific hTERT promoter regulates both the *E1A* and *E1B* genes (**Figure 1**). Telomelysin is expected to control

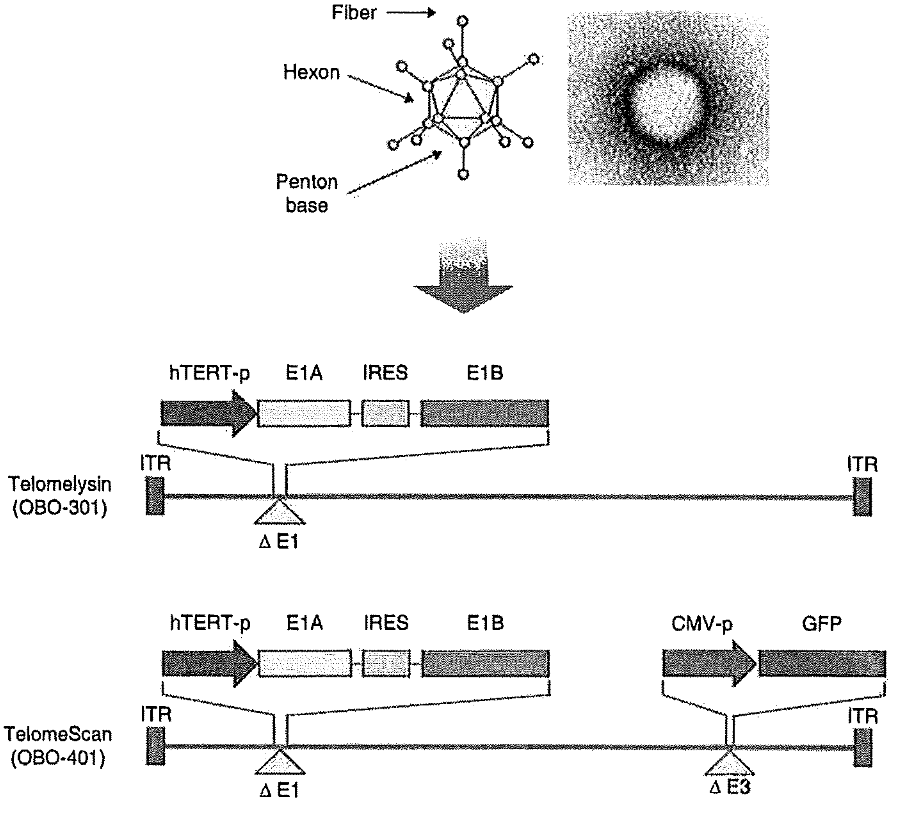

Figure 1. Structures of telomerase-specific oncolytic adenoviruses. Telomelysin (OBP-301), in which the humantelomerase reverse transcriptase (hTERT) promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES). TelomeScan (OBP-401) is a telomerase-specific replication-competent adenovirus variant, in which the green fluorescent protein (*GFP*) gene is inserted under the control of the cytomegalovirus (CMV) promoter into the E3 region for monitoring viral replication. Upper panel, schematic representation depicting major structural components of Telomelysin (hexon, penton base and fiber) and transmission electron microscopic image.

viral replication more stringently, thereby providing better therapeutic effects in tumor cells as well as attenuated toxicity in normal tissues [49].

2.2 *In vitro* cytopathic efficacy of Telomelysin in human SCCHN cell lines

The majority of human cancer cells including human SCCHN cells acquire immortality and unregulated proliferation by expression of hTERT [42] and, therefore theoretically, hTERT-specific Telomelysin can possess a broad-spectrum antineoplastic activity against a variety of human tumors [49,50]. Indeed, although the levels of expression varied widely, it was confirmed by using a real-time RT-PCR method that all SCCHN cell lines expressed detectable levels of *hTERT* mRNA, whereas human fibroblast cells were negative for *hTERT* expression. The author's group also examined the expression levels of coxsackievirus and adenovirus receptor (CAR) on the cell surface of each type of cell by flow cytometric analysis. Appreciable amounts of CAR expression were detected on human SCCHN cells. Thus, Telomelysin infection could efficiently induce cell death in a variety of human SCCHN cell lines such as SAS-L, SCC-4, SCC-9, HSC-2, HSC-3 and HSC-4 in a dose-dependent manner; the sensitivity, however, varied among different cell lines [51]. Telomelysin induced selective E1A and E1B expression in these SCCNH cells, which resulted in viral replication at 4 logs by 24 h after infection; on the other hand, Telomelysin replication was attenuated up to 2 logs in cultured normal cells. These data clearly demonstrate that Telomelysin exhibits desirable features for use as an oncolytic therapeutic agent for human SCCHN.

2.3 *In vivo* antitumor effect of Telomelysin in human SCCHN xenografts

The *in vivo* antitumor effect of Telomelysin was also investigated by using athymic mice carrying xenografts. Intratumoral injection of Telomelysin into human tumor xenografts resulted in a significant inhibition of tumor growth and enhancement of survival [49,50]. Macroscopically, massive ulceration was noted on the tumor surface after injection of high-dose Telomelysin, indicating that Telomelysin induced intratumoral necrosis due to direct lysis of tumor cells by virus replication *in vivo* [52,53].

To further explore the *in vivo* antitumor effects of telomerase-specific virotherapy for SCCHN, we used an orthotopic nude mouse model of human tongue squamous cell carcinoma. An orthotopic nude mouse model to investigate the cellular and molecular mechanisms of metastasis in human neoplasia was first described by Fidler *et al.* [54,55] and Killion *et al.* [56]. The orthotopic implantation of tumor cells restores the correct tumor–host interactions, which do not occur when tumors are implanted in ectopic subcutaneous sites [54]. In our preliminary experiments, we inoculated tumor cells into the tongue of BALB/c *nu/nu* mice and confirmed the formation of tumors with a diameter of 3 – 5 mm after 7 days and the development of metastases in neck lymph nodes after 35 days. Intratumoral injection of Telomelysin significantly shrunk the tongue tumor volumes, which in turn increased the body weight of mice by enabling oral ingestion. Since the body weight loss due to a feeding problem in this orthotopic SCCHN model resembles the disease progression in SCCHN patients, the finding that Telomelysin increased the body weight of mice suggests that telomerase-specific virotherapy could potentially improve the quality of life in advanced SCCHN patients (**Figure 2**).

3. Telomerase-specific oncolytic adenovirus for SCCHN diagnostics

3.1 hTERT promoter-driven *GFP*-expressing oncolytic adenovir

The green fluorescent protein (GFP), which was originally obtained from the jellyfish *Aequorea victoria*, is an attractive molecular marker for imaging of live tissues because of the relatively non-invasive nature of the fluorescence [57]. To label target tumor cells efficiently and uniformly with green fluorescence, we modified Telomelysin to contain the *GFP* gene driven by the cytomegalovirus (CMV) promoter in the E3 deleted region. The resultant adenovirus was termed TelomeScan or OBP-401 (**Figure 1**) [58,59]. Similar to Telomelysin, TelomeScan replicated in human cancer cell lines and coordinately induced GFP expression; TelomeScan replication, however, was attenuated in normal human fibroblasts without GFP expression.

Human SCCHN cells also expressed bright GFP fluorescence after TelomeScan infection. The fluorescence intensity gradually increased in a dose-dependent manner, followed by rapid cell death due to the cytopathic effect of TelomeScan, as evidenced by the presence of floating, highly light-refractive cells under phase-contrast photomicrographs. We also quantified GFP expression in human SCCHN cells following TelomeScan infection by using a fluorescence plate reader. Relative expression levels of GFP gradually increased in a dose-dependent manner. Moreover, we found an apparent inverse correlation between relative GFP expression at 72 h after TelomeScan infection and cell killing effects of Telomelysin in monolayer cultures (defined as ID_{50}) in various human cancer cell lines including SCCHN cell lines, indicating that the outcome of Telomelysin treatment could be predicted by measuring GFP expression following TelomeScan infection. For example, when the biopsy tissue samples of the tumor are exposed to TelomeScan for a certain amount of time *ex vivo*, the levels of GFP expression may be of value as a positive predictive marker for the outcome of Telomelysin virotherapy (**Figure 2**).

3.2 *In vivo* imaging of SCCHN micrometastasis with Telomescan

Improvements in methods of external imaging such as computed tomography (CT), MRI and ultrasound techniques have increased the sensitivity for visualizing tumors and metastases in the body [60]. Positron emission tomography (PET) using the glucose analogue ^{18}F-2-deoxy-D-glucose (FDG), was the first molecular imaging technique to be widely applied for cancer imaging in clinical settings [61]. Although FDG-PET has high detection sensitivity, it has some limitations such as difficulty in distinguishing between proliferating tumor cells and inflammation, and its unsuitability for real-time detection of tumor tissues. Therefore, tumor-specific imaging is of considerable value in the treatment of human cancer because it can define the location and area of tumors without microscopic analysis. In particular, if tumors too small for direct visual detection and therefore not detectable by direct inspection could be imaged *in situ*, surgeons could precisely excise tumors with appropriate surgical margins. This paradigm requires an appropriate 'marker' that can facilitate visualization of physiological or molecular events that occur in tumor cells but not normal cells.

Lymphatic invasion is one of the major routes for cancer metastasis, and adequate resection of locoregional lymph nodes is required for curative treatment in patients with advanced malignancies. Indeed, SCCHN patients with metastases to regional lymph nodes have a poorer prognosis than patients without nodal metastases [62]. Therefore, the utility of TelomeScan, which can be used for real-time imaging of tumor tissues *in vivo*, offers a practical, safe and cost-effective alternative to the traditional, cumbersome procedures of histopathological examination. We have previously demonstrated that TelomeScan could be delivered into human tumor cells in regional lymph nodes and replicate with selective GFP fluorescence after injection into the primary tumor in an orthotopic rectal tumor model [63]. In the orthotopic SCCHN model, TelomeScan also spread into the neck lymph nodes after injection into the primary tongue tumor and selectively replicated in metastatic nodules. Although the virus replication can not catch up in tumors with an extremely rapid progress, leading to the incomplete tumor eradication, these results suggest

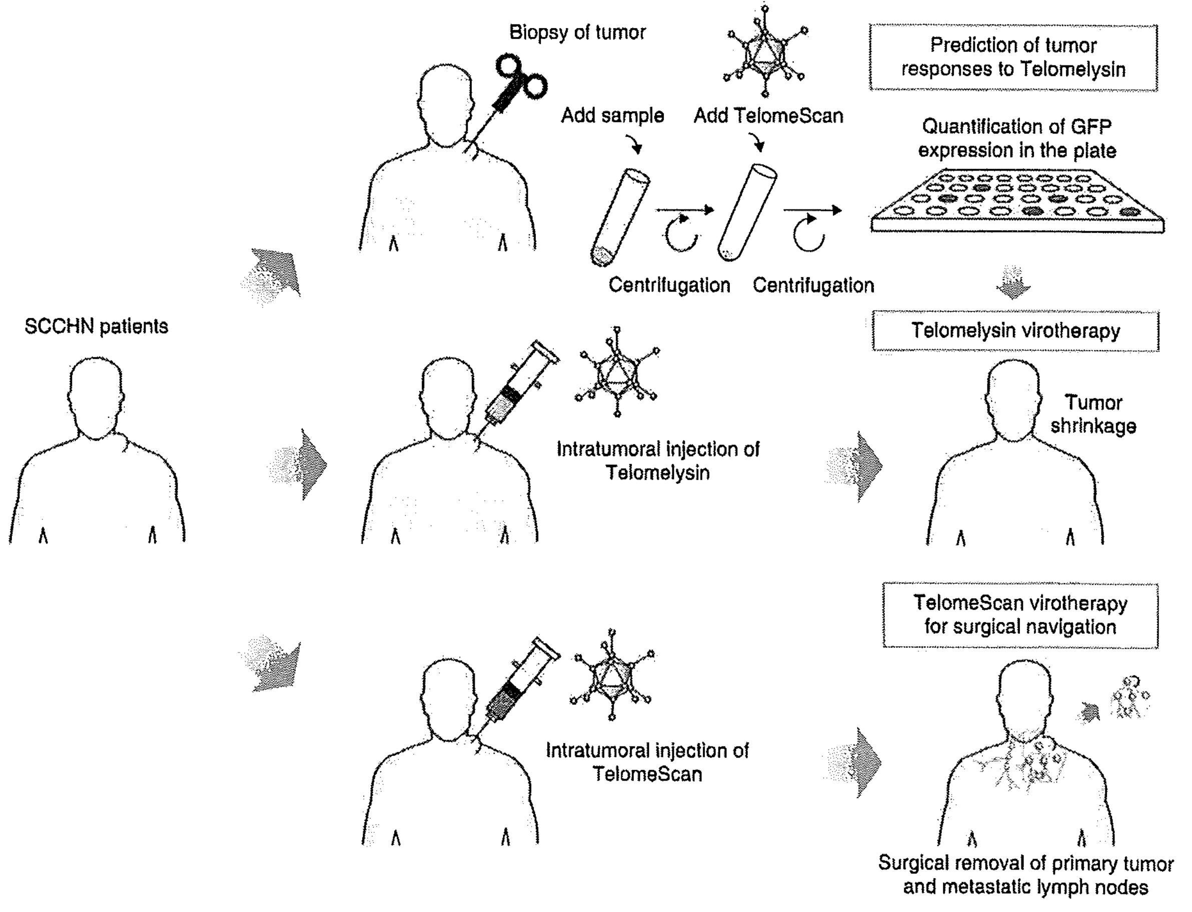

Figure 2. A schematic representation of diagnostic and therapeutic approaches using telomerase-specific oncolytic adenoviruses for human squamous cell carcinoma of the head and neck (SCCHN) patients. Top row: The outcome of Telomelysin treatment can be predicted by measuring GFP expression following TelomeScan infection. When the biopsy tissue samples of the tumor are exposed to TelomeScan *ex vivo*, the levels of GFP expression may be of value as a positive predictive marker for the outcome of Telomelysin virotherapy. Middle row: Intratumoral injection of Telomelysin may reduce the tumor volumes, which could potentially improve the quality of life in advanced SCCHN patients. Bottom row: TelomeScan can spread into the neck lymph nodes after injection into the primary tumors and selectively express GFP fluorescence in metastatic nodules. Surgeons may be able to excise primary tumors as well as metastatic lymph nodes precisely with appropriate margins by using this novel surgical navigation system with TelomeScan.

that surgeons may be able to excise primary tumors as well as metastatic lymph nodes precisely with appropriate margins by using this novel surgical navigation system with TelomeScan (**Figure 2**).

Administration of TelomeScan offers an additional advantage in cancer therapy. TelomeScan, like Telomelysin, is an oncolytic virus, and selectively kills human tumor cells by viral replication; the process of cell death by TelomeScan, however, is relatively slow compared with apoptosis-inducing chemotherapeutic drugs, because the virus needs time for replication. Therefore, tumor cells infected with TelomeScan express GFP fluorescence, followed by loss of viability, allowing the timing of detection. Thus, TelomeScan can spread into the regional lymph nodes after intratumoral injection, express GFP signals in tumor cells by virus replication, and finally kill tumor cells even if the surgeon failed to remove all nodes containing micrometastasis.

4. Clinical application of Telomelysin

Preclinical models suggested that Telomelysin could selectively kill a variety of human cancer cells *in vitro* and *in vivo* via intracellular viral replication regulated by the hTERT transcriptional activity. Pharmacological and toxicological studies in mice and cotton rats demonstrated that none of the animals treated with Telomelysin showed signs of viral distress (e.g., ruffled fur, weight loss, lethargy or agitation) or histopathological changes in any organs at autopsy. These promising data led us to design a Phase I clinical trial of Telomelysin as a monotherapy.

The protocol 'A Phase I dose-escalation study of intratumoral injection with telomerase-specific replication-competent oncolytic adenovirus, Telomelysin (OBP-301) for various solid tumors' sponsored by Oncolys BioPharma, Inc. is an open-label, Phase I, three-cohort dose-escalation study [52]. The trial commenced following approval by the FDA in October, 2006. The study to assess the safety, tolerability, and feasibility of intratumoral injection of the agent in patients with advanced solid cancer has almost been completed. The author and colleagues also analyzed the humoral immune response to Telomelysin, and obatined tissue biopsies to evaluate the pharmacokinetics and pharmacodynamics of Telomelysin in the injected tumor. The therapeutic responses were assessed by measuring changes in tumor dimensions, comparative analysis of tumor biopsies, and cytokine and/or viral measurements. Patients selected for this trial have histologically or cytologically proven non-resectable solid tumors and have failed to respond to conventional therapies such as primary external beam radiation or systemic chemotherapy. All patients had a disease that is measurable and accessible to direct injection of Telomelysin. The doses of Telomelysin were escalated from low to high virus particles (VP) in one log increments. Patients were treated with a single dose intratumoral injection of Telomelysin and then monitored over one month.

Although the final report is not available yet, the data on pharmacokinetics and biodistribution of Telomelysin may be of interest. Clinical trials of intratumoral and intravenous administration of CG7870, a replication-selective oncolytic adenovirus genetically engineered to replicate preferentially in prostate tissue, demonstrated a second peak of the virus genome in the plasma [64,65], suggesting active viral replication and shedding into the bloodstream. Therefore, it is anticipated that intratumorally administered Telomelysin can spread into the lymphatic vessels as well as the blood circulation, and potentially kill metastatic tumor cells in regional lymph nodes and distant organs and tissues. Theoretically, Telomelysin can replicate continuously in the injected tumors and releases virus particles unless all tumor cells are completely eliminated, indicating that a single intratumoral injection should be sufficient to induce an antitumor effect. The preclinical study, however, showed that multiple injections of Telomelysin resulted in a profound inhibition of tumor growth in xenograft models [49,50,59]. Thus, we also evaluated the feasibility of the multi-cycle treatment with Telomelysin.

5. Expert opinion

There have been very impressive advances in our understanding of the molecular aspects of human cancer and in the development of technologies for genetic modification of viral genomes. Nevertheless, many ethical and technical hurdles remain to be tackled and must be solved before virotherapy, including virus-mediated gene therapy, ever reaches routine clinical application. The safety considerations in the virus manufacture and clinical protocols are among the most important issues to be studied. Another important issue is to find ways to selectively deliver viruses into a high percentage of malignant cells in an existing tumor mass. The use of tissue- or cell-type-specific promoters could perhaps achieve specificity of virus-mediated antitumor effect. The hTERT promoter-based transcriptional targeting in adenoviral constructs is a powerful tool for cancer diagnosis and therapy. In particular, the hTERT-specific oncolytic adenovirus achieves a more strict targeting potential due to the amplified effect resulting from viral replication, and is a promising therapeutic alternative to replication-deficient gene therapy vectors. Several independent studies that used different regions of the hTERT promoter and different sites of the adenoviral genome responsible for viral replication, have shown that the hTERT promoter allows adenoviral replication as a molecular switch and induces selective cytopathic effects in a variety of human tumor cells including SCCHN cells [43-45,49-51]. Among these viral constructs, to the best of our knowledge, Telomelysin seems to be the first hTERT-dependent oncolytic adenovirus that has been used in a clinical trial based on preclinical pharmacological and toxicological studies.

SCCHN accounts for 5% of newly diagnosed adult cancers in the United States and 8% of cancers worldwide [66]. Most patients are treated with various combinations of surgery, radiotherapy and systemic agents [67]. Despite major advances in the treatment of locoregionally advanced SCCHN such as the introduction of novel chemotherapy regimens, treatment fails in about half of the patients [68]. The median survival of patients with recurrent or metastatic SCCHN who undergo chemotherapy is 6 – 9 months [69]. A considerable number of patients with SCCHN need additional treatment as the disease progresses. Targeted therapies such as the anti-EGFR monoclonal antibody cetuximab and other small-molecule EGFR-tyrosine kinase inhibitors have been developed for SCCHN. Although a Phase III trial demonstrated a survival benefit with cetuximab and standard platinum-based therapy in SCCHN patients [70], some patients are exquisitely sensitive to these drugs and can develop particular and severe toxicities [71]. An interim analysis of a Phase I study of Telomelysin for histologically proven non-resectable solid tumors including SCCHN patients indicates that Telomelysin virotherapy is well-tolerated without any severe adverse events [52], suggesting that Telomelysin may be much more potent than other targeted therapies for human SCCHN in terms of specificity, efficacy and toxicity.

Although Telomelysin showed a broad and profound antitumor effect in human SCCHN cells, one weakness of Telomelysin is that virus infection efficiency depends on CAR expression, which may not be highly expressed on the cell surface of some types of human SCCHN cells. Thus, tumors that lost CAR expression might be refractory to infection with Telomelysin. Since modification of fiber protein is an attractive strategy for overcoming the limitations imposed by the CAR dependence of Telomelysin infection, we modified the fiber of Telomelysin to contain RGD (Arg-Gly-Asp) peptide, which binds with high affinity to integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) on the cell surface, on the HI loop of the fiber protein. The resultant adenovirus, termed Telomelysin-RGD or OBP-405, mediated not only CAR-dependent virus entry but also CAR-independent, RGD-integrin-dependent virus entry [50]. Telomelysin-RGD had an apparent oncolytic effect on human cancer cell lines with extremely low CAR expression. Intratumoral injection of Telomelysin-RGD into CAR-negative tumor xenografts in mice resulted in significant inhibition of tumor growth and long-term survival. These data suggest that fiber-modified Telomelysin-RGD exhibits a broad target range by increasing infection efficiency, although one needs to be cautious about increased toxicity since hematopoietic cell population such as dendritic cells can be efficiently infected with RGD-modified adenovirus [72].

Possible future directions for Telomelysin include combination therapy with conventional therapies such as chemotherapy, radiotherapy, surgery, immunotherapy and new modalities such as antiangiogenic therapy. Since clinical activities observed with intratumoral injection of Telomelysin suggest that even partial elimination of the SCCHN tumor could be clinically beneficial, the combination approaches may lead to the development of more advanced biological therapy for human SCCHN. The combination of systemic chemotherapy and local injection of Telomelysin has been previously shown to be effective [58,59]. In addition, we found that oncolysis induced by Telomelysin infection could be the most effective stimulus for immature dendritic cells to induce specific activity against human cancer cells [73]. Therefore, Telomelysin can be effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that induces specific cytotoxic T-lymphocytes against the remaining antigen-bearing tumor cells. We also confirmed that Telomelysin seems to have antiangiogenic properties through the stimulation of host immune cells to produce endogenous antiangiogenic factors such as IFN-γ and interleukin 12. Peri- or postoperative administration of Telomelysin may be valuable as adjuvant therapy in areas of microscopic residual disease at tumor margins to prevent recurrence or regrowth of SCCHN tumors.

The field of telomerase-specific gene- and vector-based therapies is progressing considerably and is rapidly gaining medical and scientific acceptance. Although many technical and conceptual problems remain to be solved, ongoing and future clinical studies will no doubt continue to provide important clues that may allow substantial progress in the treatment of human SCCHN.

Declaration of interest

The author is a Chief Scientific Officer of Oncolys BioPharm, Inc.

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ORIGINAL ARTICLE

Virus-mediated oncolysis induces danger signal and stimulates cytotoxic T-lymphocyte activity via proteasome activator upregulation

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Dendritic cells (DCs) are the most potent antigen-presenting cells and acquire cellular antigens and danger signals from dying cells to initiate antitumor immune responses via direct cell-to-cell interaction and cytokine production. The optimal forms of tumor cell death for priming DCs for the release of danger signals are not fully understood. OBP-301 (Telomelysin) is a telomerase-specific replication-competent adenovirus that induces selective E1 expression and exclusively kills human cancer cells. Here, we show that OBP-301 replication produced the endogenous danger signaling molecule, uric acid, in infected human tumor cells, which in turn stimulated DCs to produce interferon-γ (IFN-γ) and interleukin 12 (IL-12). Subsequently, IFN-γ release upregulated the endogenous expression of the proteasome activator PA28 in tumor cells and resulted in the induction of cytotoxic T-lymphocytes. Our data suggest that virus-mediated oncolysis might be the effective stimulus for immature DCs to induce specific activity against human cancer cells.
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Keywords: adenovirus; telomerase; dendritic cell; uric acid; danger signal

Introduction

Dendritic cells (DCs) are the most important professional antigen-presenting cells and play a critical role in the induction of primary immune responses against tumor-associated antigens. Mature DCs express high levels of major histocompatibility complex (MHC) class I, II and co-stimulatory molecules such as CD80 and CD86, and secrete T-helper type-1 (Th1) cytokines such as interleukin (IL)-12 and interferon (IFN)-γ. DCs acquire endogenous maturation stimuli from dying cells as a danger signal when they capture cellular antigens. Lack of danger signals delays maturation of DCs and causes active suppression of DCs stimulatory capacity, leading to the induction of T-cell tolerance (Steinman *et al.*, 2000). Shi *et al.* (2003) have previously identified uric acid as a novel endogenous warning molecule capable of alerting the immune system within cell lysates. The uric acid activates DCs following relocation from the inside to the outside of injured cells and converts immunity from non-protective to protective. In fact, it has been reported that uric acid levels are elevated in tumors undergoing immune rejection and that the inhibition of uric acid production delays tumor regression (Hu *et al.*, 2004).

Viruses have evolved to infect, replicate in and kill human cells through diverse mechanisms such as direct cell death machinery and fairly brisk immune responses. We reported previously that telomerase-specific replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase (hTERT) promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES), induced selective E1 expression and efficiently killed human cancer cells, but not normal human fibroblasts (Kawashima *et al.*, 2004; Umeoka *et al.*, 2004; Taki *et al.*, 2005; Watanabe *et al.*, 2006). Although the precise molecular mechanism of OBP-301-induced cell death is still unclear, the process of oncolysis is morphologically distinct from apoptosis and necrosis. These findings led us to examine whether tumor cells killed by OBP-301 infection could stimulate DCs, thus enhancing the immune response.

In the present study, we compared three types of tumor preparations as a source of cell-derived antigen for the priming of DCs: virus-induced oncolysis, chemotherapeutic drug-induced apoptosis and necrosis by freeze/thaw. We also explored the cytokine signature and activating property of these cells for antitumor immune response against human cancer cells.

Results

We first examined whether OBP-301 infection affects the viability of human cancer cells using the XTT assay.

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OBP-301 infection induced death of human cancer cell lines (H1299 human lung cancer and SW620 human colorectal cancer cells) in a dose-dependent manner (Figure 1). Although autophagy, or type II programmed cell death, partially involved in the cell death machinery triggered by OBP-301 infection, oncolytic cells are distinct from apoptotic cells (Supplementary Figure 1).

We next examined whether OBP-301 infection modulated intracellular concentrations of uric acid that might act as a danger signal in tumor cells. Uric acid levels increased in H1299 cells following OBP-301 infection in a time-dependent fashion, although docetaxel slightly upregulated the uric acid concentration 72 h after treatment (Figure 2a). Thus, tumor cells undergoing oncolysis can produce significantly greater amounts of uric acid when compared with apoptotic tumor cells. The uric acid elevation pattern of OBP-301-infected cells almost paralleled that of cells infected with Onyx-015, an E1B 55 kDa-deleted adenovirus engineered to selectively replicate in and lyse p53-deficient cancer cells, and wild-type adenovirus type 5 (Figure 2b), indicating a general effect of adenovirus infection in the regulation of intracellular uric acid levels.

Uric acid is produced during the catabolism of purines and is the end product of this process. Adenoviral replication facilitates the purine catabolism to stimulate the synthesis of progeny DNA, which in turn may increase intracellular uric acid levels by the purine degradation process. In fact, OBP-301 infection significantly increased the amount of uric acid in the cells, whereas replication-deficient dl312 infection had no apparent effect on the levels of uric acid. OBP-301-induced elevation of uric acid levels could be inhibited in the presence of cidofovir (CDV), an acyclic nucleoside phosphonate having potent broad-spectrum anti-DNA virus activity (Figure 2c). CDV has been approved for the treatment of many types of viruses including cytomegalovirus and adenovirus (Lenaerts and Naesens, 2006). We confirmed that CDV at 100 μM could significantly inhibited replication of OBP-301 in H1299 cells by the real-time quantitative PCR analysis (Supplementary Figure 2). Moreover, as OBP-301 replication was attenuated in telomerase-negative cells, the levels of uric acid could not be altered in normal human lung fibroblasts (NHLF) after OBP-301 infection (Figure 2d). These results suggest that viral replication is required to produce uric acid in infected cells.

Xanthine oxidoreductase (XOR) is a member of the molybdoflavoenzyme family that catalyses the formation of uric acid from xanthine and hypoxanthine (Glantzounis *et al.*, 2005). A strand-specific reverse transcriptase PCR assay demonstrated that XOR mRNA expression gradually decreased in OBP-301-infected cells presumably due to the negative feedback of increased uric acid levels, whereas docetaxel-treated cells yielded consistent bands of the XOR transcripts (Figure 2e). Thus, adenoviral replication could directly stimulate the catalytic DNA turnover, which enables cells to produce more uric acid.

We then examined the ability of OBP-301-infected cells to stimulate immature DCs *in vitro*. DCs generated from HLA-A24$^+$ healthy volunteers were co-cultured with HLA-matched H1299 cells (HLA-A32/A24) treated with OBP-301 or docetaxel for 72 h, or freeze thawed. The production of Th1 cytokines such as IFN-γ and IL-12 in the supernatants was then explored by enzyme-linked immunosorbent assay (ELISA) analysis 48 h after the co-culture. DCs incubated with OBP-301-infected cells secreted large amounts of IFN-γ and IL-12, whereas stimulation with docetaxel-treated apoptotic cells induced their secretion at low levels (Figure 3a). The level of cytokine production from DCs incubated with freeze-thawed necrotic cells was similar to that of untreated immature DCs. Moreover, we confirmed that addition of OBP-301 alone without target tumor cells did not affect the cytokine secretion of DCs into the supernatant, indicating that infection of OBP-301 itself had no apparent effect on DCs. Thus, DCs stimulated with oncolytic tumor cells preferentially secrete high-level Th1 cytokines. Flow cytometry demonstrated that the increase in the expression of CD83, which is expressed on mature DCs, was slightly higher on DCs incubated with oncolytic cells than those with apoptotic or necrotic cells, indicating that oncolytic tumor cells seems to have a positive influence on DC maturation (Supplementary Figure 3).

In the next step, we investigated the effects of oncolytic tumor cells on T-cell activation in the presence of DCs. H1299 cells were infected with OBP-301 over 72 h, and then co-incubated with HLA-matched HLA-A24$^+$ peripheral blood mononuclear cells (PBMCs) for another 48 h in mixed lymphocyte tumor culture (MLTC). In other tests, H1299 cells were exposed to docetaxel for 72 h or freeze thawed, and then co-cultured with PBMCs. We examined the secretion of IFN-γ and IL-12 into the supernatants after MLTC for 7 days. Stimulation with OBP-301-infected cells induced the secretion of high levels of IFN-γ and IL-12 into MLTC supernatants, which was significantly higher

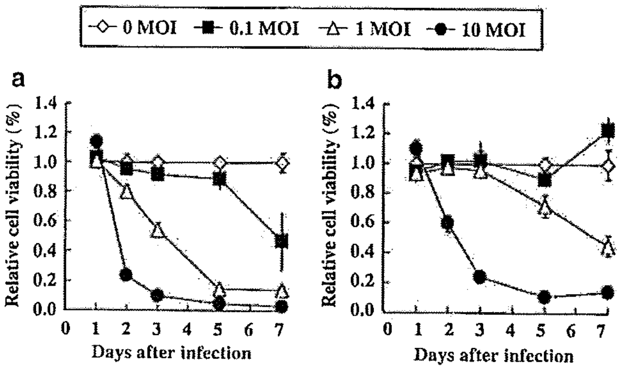

Figure 1 *In vitro* cytopathic effects of OBP-301 on human cancer cells. H1299 human non-small cell lung cancer (**a**) and SW620 human colorectal cancer cells (**b**) were infected with OBP-301 at indicated multiplicity of infection (MOI) values, and surviving cells were quantitated over 7 days by XTT assay. The cell viability of mock-treated cells on day 1 was considered 1.0, and the relative cell viability was calculated. Each data represent the mean±standard deviation (s.d.) of triplicate experiments.

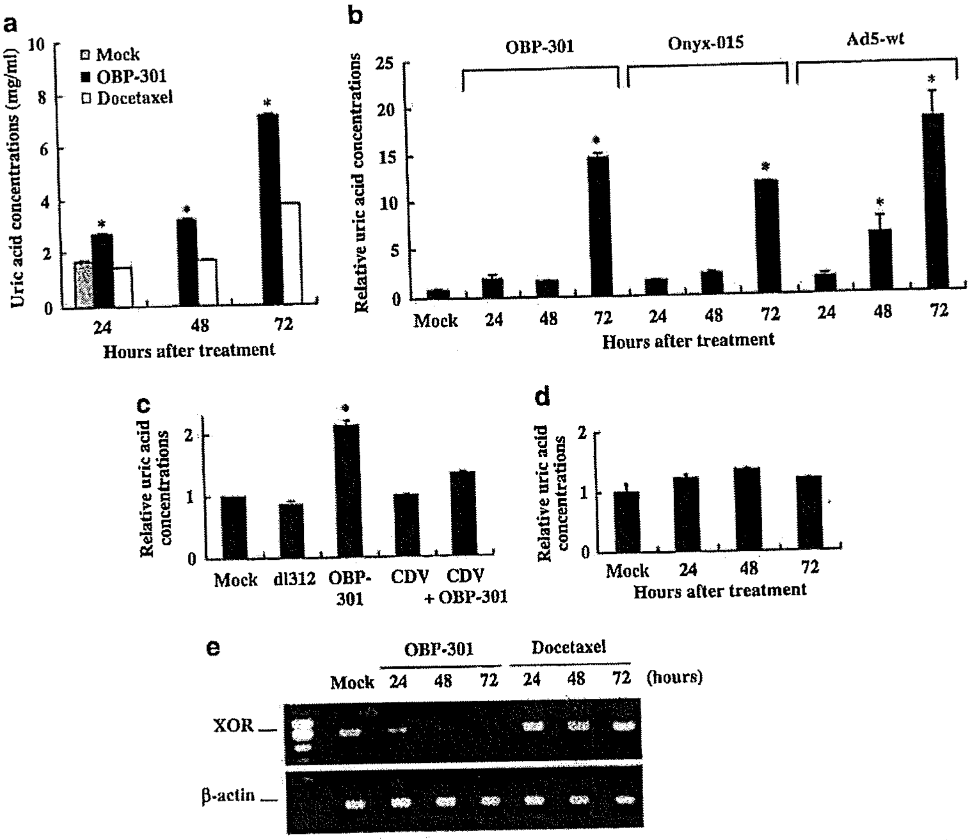

Figure 2 (a) Uric acid concentrations in H1299 cells treated with OBP-301 or docetaxel. H1299 cells were infected with 1.0 MOI of OBP-301 or treated with 10 nM of docetaxel for indicated time periods, and uric acid concentrations were determined enzymatically in the cell homogenates. Single asterisk indicates $P < 0.01$, significantly different from docetaxel-treated cells. (b) Uric acid levels in H1299 cells treated with OBP-301, Onyx-015 or wild-type adenovirus. H1299 cells were harvested at indicated time points over 72 h after infection with 10 MOI of viruses, and subjected to the measurement of uric acid concentrations. The levels of uric acid concentration are defined as the fold-increase for each sample relative to that of mock-treated cells (mock equals 1). Single asterisk indicates $P < 0.01$, significantly different from mock-treated cells. (c) Uric acid concentrations in H1299 cells infected with 1.0 MOI of OBP-301 or replication-deficient dl312 adenovirus were measured 24 h after infection. Uric acid production was also assessed in H1299 cells infected with 1.0 MOI of OBP-301 in the presence of 100 μM of anti-virus agent cidofovir (CDV). H1299 cells treated with 100 μM of CDV were subjected to the assay as a control. All uric acid levels are normalized to that of mock-treated cells (mock equals 1). (d) Uric acid levels in NHLF infected with OBP-301. NHLF cells were infected with 1.0 MOI of OBP-301 for indicated time periods, and uric acid concentrations were measured. The uric acid levels are normalized to that of mock-treated cells. (e) Detection of xanthine oxidoreductase (XOR) mRNA expression in OBP-301-infected H1299 cells by RT–PCR analysis. Cells were infected with 1.0 MOI of OBP-301 or treated with 10 nM of docetaxel, and then collected at the indicated time points. First-strand DNA generated from RNA was amplified using either the primers specific for XOR sequence or the primers that recognize β-actin sequences as an internal control.

than that with docetaxel-treated or freeze-thawed H1299 cells (Figure 3b). Thus, oncolytic tumor cells can accelerate the cleavage of tumor antigen peptides that can be associated with MHC class I molecules via IFN-γ secretion by immune cells.

Stimulation of cells with IFN-γ is known to induce the expression of PA28, a proteasome activator that accelerates the *in vitro* processing of MHC class I ligands from their polypeptide precursors (Sun *et al.*, 2002). We investigated whether PA28 expression was upregulated in H1299 cells by adding the supernatants of co-cultures of PBMCs and OBP-301-infected H1299 cells. Western blot analysis for PA28 demonstrated that, following heat inactivation of residual OBP-301, MLTC supernatants with oncolytic tumor cells induced a strong endogenous PA28 expression in H1299 cells. In contrast, exposure to the supernatants of PBMCs alone, PBMCs with untreated H1299 cells, and PBMCs with oncolytic tumor cells without heat inactivation resulted in no apparent changes in the expression levels of PA28 (Figure 4).

Finally, the cytotoxic T-lymphocyte (CTL) response against human cancer cells was assessed by a standard 6-h ^{51}Cr release assay after a 7-day MLTC using various forms of H1299 cells. The lytic activity of CTLs induced by apoptotic or necrotic H1299 cells was comparable with that of human lymphokine-activated killer (LAK) cells; CTLs stimulated with oncolytic H1299 cells, however, more efficiently killed target H1299 cells (Figure 5). In contrast, LAK cells effectively lysed SW620 cells, whereas these cells were minimally killed by CTLs stimulated with apoptotic, necrotic or oncolytic H1299 cells. Furthermore, HLA-unmatched, HLA-A26/A30$^+$ A549 human lung cancer cells were not sensitive to oncolytic tumor cell-induced cytotoxicity (data not shown), suggesting that effector cells stimulated with

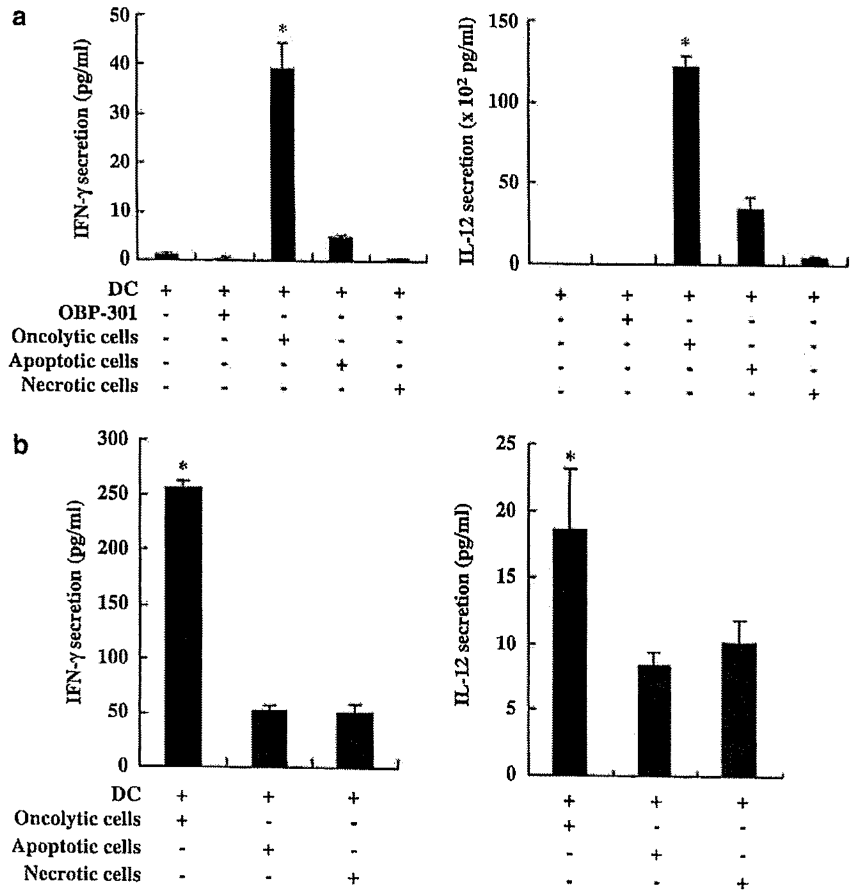

Figure 3 (a) Secretion of Th1-type cytokines by oncolytic, apoptotic or necrotic tumor cells. H1299 cells were treated with 1.0 MOI of OBP-301 or 50 nM of docetaxel for 72 h, or freeze thawed, and then co-cultured with immature dendritic cells (DCs) obtained from monocytes for additional 48 h. The culture supernatants were harvested and tested by ELISA for interferon (IFN)-γ (left) and interleukin (IL)-12 (right) concentrations. As a control, the supernatants of immature DCs alone or with OBP-301 at an MOI of 1.0 were also examined. Data are mean±s.d. of triplicate experiments. Single asterisk indicates $P<0.01$, significantly different from other groups. (b) Tumor-specific CTL induction in MTLC with oncolytic, apoptotic or necrotic tumor cells. IFN-γ (left) and IL-12 (right) concentrations in the supernatants of MLTC analysed by ELISA. H1299 cells were treated with 1.0 MOI of OBP-301 or 50 nM of docetaxel for 72 h, or freeze thawed, and then co-cultured with PBMCs obtained from HLA-A24$^+$ healthy volunteers for 48 h in MLTC. Data are mean±s.d. of triplicate experiments. Single asterisk indicates $P<0.01$, significantly different from other groups.

OBP-301-infected tumor cells exhibit MHC class I-restricted reactivity.

Discussion

In the present study, our goal was to determine whether oncolytic virus is effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that could induce specific CTL for the remaining antigen-bearing tumor cells. Several groups have debated whether necrotic or apoptotic cells can stimulate DCs to cross-present cell-derived peptides, with subsequent enhancement of tumor immunogenicity. Furthermore, it has been reported recently that the immunogenicity of tumors is not regulated by signals associated with apoptotic or necrotic cell death, but is an intrinsic feature of the tumor itself (Bartholomae *et al.*, 2004). Our data indicate that viral oncolysis could efficiently load tumor antigen on DCs, and then generate CTL response as judged from the production of cytokines. Moreover, the CTL activity against untreated tumor cells suggests that CTLs are specific to tumor antigens, but not to adenovirus proteins.

DCs are known to ingest dying tumor cells and initiate tumor-specific responses when associated with appropriate danger signals, which are endogenous activation signals liberated by dying cells. Recent studies have shown that some intrinsic biochemical factors, such as uric acid, bradykinin and heat shock protein (HSP110) act as danger signals through their interaction with DCs, and influence the subsequent immune response (Aliberti *et al.*, 2003; Shi *et al.*, 2003; Manjili *et al.*, 2005). Large amounts of uric acid can be produced following tissue injury *in vivo*, and activate the immune response against injured cells and dying tissues. We found that OBP-301 infection increased intracellular uric acid levels in human tumor cells compared with apoptosis- or necrosis-inducing stimuli, suggesting that viral replication itself can enhance tumorigenicity.

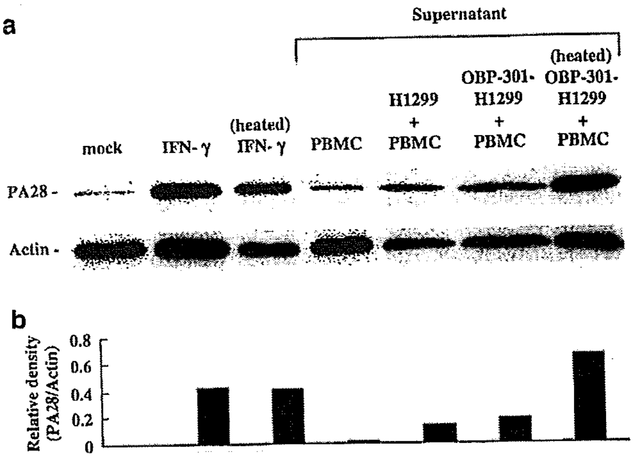

Figure 4 (a) Western blot analysis of PA28 in H1299 cells exposed to the supernatants of MLTC. peripheral blood mononuclear cells (PBMCs) were incubated with mock, untreated H1299 cells or H1299 cells treated with 10 MOI of OBP-301 for 72 h in MLTC, and the supernatants were harvested 48 h after the co-culture. H1299 cells were further incubated with the supernatants for 72 h with or without heat inactivation of residual virus (56 °C, 10 min). H1299 cells were also incubated with 5 ng ml^{-1} of interferon (IFN)-γ with or without heating for 72 h. Equivalent amounts of protein obtained from whole cell lysates were loaded in each lane, probed with anti-PA28 antibody and then visualized by using an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-actin antiserum. (b) PA28 protein expression was quantified by densitometric scanning using NIH Image software and normalization by dividing the actin signal.

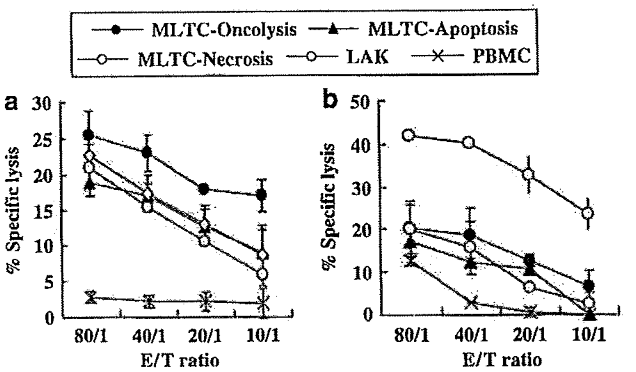

Figure 5 Cytolytic reactivity against H1299 (a) and SW620 (b) human cancer cells was assessed after 7-day mixed lymphocyte tumor culture (MLTC) with oncolytic, apoptotic or necrotic H1299 cells treated the same as above by 6-h standard ^{51}Cr-release assay. Lymphokine-activated killer (LAK) cells were generated from peripheral blood mononuclear cells (PBMCs) in the presence of interleukin (IL)-2 (100 U ml^{-1}) for 3 days. The CTLs were compared with LAK cells and untreated PBMCs, which served as positive and negative controls, respectively. Data represent the mean ± s.d. of three wells at four different effector-to-target (E/T) ratios.

Viral oncolysis increases the immunogenicity of tumor cells presumably by the release of proinflammatory cytokines (Lindenmann and Klein, 1967). We showed that OBP-301-infected oncolytic tumor cells efficiently stimulated immature DCs to produce greater amounts of IFN-γ and IL-12 than apoptotic and necrotic cells, and that such stimulation led to DC maturation. Viral infection itself has been reported to activate DCs to secrete pro- or anti-inflammatory cytokines, which can drive DCs to undergo the maturation process (Ho *et al.*, 2001); the observation that OBP-301 alone had no effect on cytokine production by DCs, however, indicates that OBP-301 itself may be less infective or stimulatory to DCs. The result is consistent with our finding that OBP-301 attenuated replication as well as cytotoxicity in human normal cells.

It will be of interest to more mechanistically define why viral oncolysis efficiently induces CTL activity against tumor cells. We hypothesized that viral replication itself or the released cytokines by immune cells positively influences tumor cell immunogenicity. The IFN-γ-inducible proteasome modulator complex PA28 participates in the generation of antigenic peptides required for MHC class I antigen presentation (Sijts *et al.*, 2002). As expected, the supernatants of MLTC with OBP-301-infected tumor cells, in which IFN-γ secretion was detected, induced a strong expression of endogenous PA28. Thus, oncolytic tumor cells can accelerate the cleavage of tumor antigen peptides that can be associated with MHC class I molecules via IFN-γ secretion by immune cells. In fact, it has been reported that restoration of PA28 expression in PA28-deficient melanoma cells rescues the melanoma antigen epitope presentation (Sun *et al.*, 2002); our preliminary experiments however demonstrated that human tumor cells transfected with PA28α expression vector were less sensitive to tumor-specific CTLs (data not shown). These observations suggest that antigen peptide production alone does not seem sufficient to enhance tumor immunogenicity.

In conclusion, we provide for the first time evidence that oncolytic virus replication induces tumor-specific immune responses by stimulating uric acid production as a danger signal as well as accelerating tumor antigen cleavage by IFN-γ-inducible PA28 expression. Since the induction of systemic immunity has rarely been observed in clinical trials with other conditionally replication-competent viruses, more *in vivo* experiments are clearly required to support the induction of antitumor immunity by OBP-301 treatment. Our data, however, suggest that the antitumor effect of OBP-301 might be potentially both direct and indirect as well as systemic rather than local.

Materials and methods

Cell lines and reagents

The human non-small lung cancer cell lines H1299 (HLA-A32/A24) and the human colorectal carcinoma cell lines SW620 (HLA-A02/A24) were maintained *in vitro* in RPMI 1640 supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. Recombinant human cytokines granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-4, TNF-α and IL-7 were purchased from Genzyme

Techne (Minneapolis, MN, USA), IFN-γ from Peprotech (Rocky Hill, NJ, USA) and IL-2 from Roche (Mannheim, Germany). [^{51}Cr] sodium chromate was obtained from NEN Life Science Products (Boston, MA, USA). Docetaxel (taxotere) was kindly provided by Aventis Pharma (Tokyo, Japan).

Adenovirus
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 IRES, was constructed and characterized previously (Kawashima *et al.*, 2004; Umeoka *et al.*, 2004; Taki *et al.*, 2005; Watanabe *et al.*, 2006). Onyx-015 (dl1520) is an E1B 55 kDa-deleted adenovirus engineered to selectively replicate in and lyse p53-deficient cancer cells, and kindly provided by Dr Frank McCormick (UCSF Comprehensive Cancer Center and Cancer Research Institute). The E1A-deleted adenovirus vector lacking a cDNA insert (dl312) was also used as a control vector. The viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation.

Cell viability assay
XTT assay was performed to measure cell viability. Briefly, cells were plated on 96-well plates at 5×10^3 per well 24 h before treatment and then infected with OBP-301 or exposed to docetaxel. Cell viability was determined at the times indicated by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

Reverse transcription (RT)–PCR
Total RNA was isolated from mock-, OBP-301- and docetaxel-treated cells using RNAzol (Cinna/BioTecx, Friendswood, TX, USA) in a single-step phenol-extraction method and used as templates. Reverse transcription was performed at 22 °C for 10 min and then 42 °C for 20 min using 1.0 μg of RNA per reaction to ensure that the amount of amplified DNA was proportional to that of specific mRNA in the original sample. PCR was performed with specific primers in volumes of 50-μl according to the s protocol provided by the manufacturer (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT, USA). The specific primers used for XOR were 5′-GCG AAG GAT AAG GTT ACT TGT-3′ (forward) and 5′-CTC CAG GTA GAA GTG CTC TTG-3′ (reverse); and for β-actin were 5′-ATG GTG GGA ATG GGT CAG AAG-3′ (forward) and 5′-GCA GCT CAT TGT AGA AGG-3′ (reverse). The reaction conditions were denaturing at 94 °C for 2 min followed by 30 cycles consisting of denaturing at 94 °C (30 s), annealing at 65 °C (15 s) and extension at 72 °C (10 s) using a thermal cycler (Perkin-Elmer, Foster City, CA, USA). The reactions were completed by a final 2-min extension at 72 °C. The PCR products were resolved on 1% agarose gels and visualized by SYBR Gold Nucleic Acid Gel Stain (Molecular Probes Inc., Eugene, OR, USA).

Preparation of tumor cells
For induction of oncolysis, tumor cells were infected with OBP-301 at a multiplicity of infection (MOI) of 1–10, and then collected 24–72 h after infection. Apoptotic tumor cells were obtained after 24–72-h exposure to 50–100 nM of docetaxel. For induction of necrosis, tumor cells suspended in phosphate-buffered saline (PBS) were subjected to rapid four freeze/thaw cycles using a 60 °C water bath and liquid nitrogen.

Measurement of uric acid concentration
Cultured cells were harvested after treatment and rinsed three times with PBS. These cells were resuspended in lysis buffer at a density of 200×10^6 cells per 100 μl. The buffer contained 10 mM Tris–HCl (pH 7.5), 150 nM NaCl, 50 mM NaF, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM Na_3VO_4, 10% glycerol, 0.5% NP-40 and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After 10-s homogenization, the resulting extracts were kept on ice for 30 min and then were centrifuged for 15 min at 2000 *g*. The supernatants from treated tumor cells were assayed for uric acid using Uric Acid C test (Wako, Osaka, Japan).

Preparation of DCs
Peripheral blood samples were obtained from normal HLA-A24 positive healthy volunteers and PBMC were isolated by sedimentation over Ficoll-Hypaque. They were subsequently allowed to adhere in culture flasks for 1 h at 37 °C at a density of 4.0×10^7 cells per plate. Non-adherent cells in the plate were removed and the remaining (adherent) cells were cultured for 7 days in AIM-V (Gibco, Rockville, MD, USA) containing 2% heated-inactivated autologous serum supplemented with GM-CSF (50 ng ml^{-1}) and IL-4 (50 ng ml^{-1}).

Cytokine production assay
DCs were co-cultured with treated tumor cells at a ratio of 3:1 (DC/tumor cell) in a culture medium containing GM-CSF (50 ng ml^{-1}) and IL-4 (50 ng ml^{-1}). After 24-h incubation, the supernatant was collected and stored at −80 °C until the assay. The concentrations of IFN-γ and IL-12 (p40 and p70) were measured with appropriate ELISA kits (BioSource, Camarillo, CA, USA).

MLTC and CTL assay
PBMCs were co-cultured with treated tumor cells at a ratio of 20:1 in the presence of IL-2 (Roche) (10 U ml^{-1}) and IL-7 (Genzyme Techne) (5 ng ml^{-1}) for 7 days. Cultured cells were then used as effector cells in a standard 4 h-^{51}Cr release assay and the percentage of lysed cells was calculated. Percent specific lysis = ((experimental cpm−spontaneous cpm)/(maximal cpm−spontaneous cpm) × 100). Supernatants from MLTC performed as above were also assayed for IFN-γ and IL-12 by ELISA assays (BioSource).

Western blot analysis
The primary antibodies against proteasome activator PA28 (ZMD353; Invitrogen, Carlsbad, CA, USA), actin (AC-40; Sigma Chemical Co., St. Louis, MO, USA) and peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL, USA) were used. Cells were washed twice in cold PBS and collected, then lysed in lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% glycerol and 0.5% NP40) containing proteinase inhibitors (0.1 mM PMSF and 0.5 mM Na_3VO_4). After 20 min on ice, the lysates were spun at 14 000 rpm in a microcentrifuge at 4 °C for 10 min. The supernatants were used as whole cell extracts. Protein concentration was determined using the Bio-Rad protein determination method (Bio-Rad, Richmond, CA, USA). Equal amounts (50 μg) of proteins were boiled for 5 min and electrophoresed under reducing conditions on 6–12.5% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to a Hybond-polyvinylidene difluoride transfer membranes (Amersham Life Science, Buckinghamshire, UK), and incubated with the primary antibody, followed by peroxidase-linked secondary antibody. An Amersham ECL

chemiluminescent western system (Amersham) was used to detect secondary probes.

Statistical analysis
Data are expressed as mean ± s.d. The Student's *t*-test was used to compare differences. Statistical significance was defined when P was <0.05.

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Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus

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The use of replication-selective tumor-specific viruses represents a novel approach for the treatment of neoplastic disease. We constructed an attenuated adenovirus, telomerase-specific replication-selective adenovirus (TRAD), in which the human telomerase reverse transcriptase promoter element drives the expression of the *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES). Forty-eight hours after TRAD infection at a multiplicity of infection of 1.0, the cell viability of H1299 human lung cancer cells was consistently less than 50% and therefore this procedure could be used as a potency assay to assess the biological activity of TRAD. We also established a quantitative real-time polymerase chain reaction (PCR) analysis with consensus primers for either the adenovirus E1A or IRES sequence. The linear ranges of quantitation with E1A and IRES primers were 10^3–10^8 and 10^2–10^8 plaque-forming units/mL in the plasma, respectively. The PCR analysis demonstrated that the levels of *E1A* in normal tissues were more than 10^3 lower than in the tumors of A549 human lung tumor xenografts in *nu/nu* mice at 28 days after intratumoral injection. Our results suggest that the cell-killing assay against H1299 cells and real-time PCR can be used to assess the biological activity and biodistribution of TRAD in clinical trials. (*Cancer Sci* 2008; 99: 385–390)

The emerging fields of functional genomics and functional proteomics provide an expanding repertoire of clinically applicable targeted therapeutics.[1] Replication-selective oncolytic viruses provide a new platform for treatment of a variety of human cancers.[2,3] Promising clinical trials have shown the antitumor potency and safety of mutant or genetically modified adenoviruses.[4,5] We constructed previously an adenovirus vector, TRAD, in which the hTERT promoter element drives the expression of the *E1A* and *E1B* genes linked with an IRES. We showed that TRAD caused efficient selective killing of human cancer cells, but not normal cells.[6] Many studies have demonstrated that the majority of malignant tumors express telomerase activity,[7] suggesting that TRAD can potentially kill most human cancer cells.

TRAD can replicate and then lyse cancer cells, infect neighboring cancer cells, and subsequently induce oncolysis throughout the whole tumor mass *in vivo*. As preclinical models showed that TRAD could spread into the bloodstream, it is important to monitor carefully the amount of TRAD in the circulation after intratumoral injection of TRAD to avoid serious adverse events due to viremia. Although we used vector-specific primers that detected the p53 open reading frame–adenoviral DNA junction in a phase I clinical trial of a replication-deficient adenoviral vector expressing the wild-type *p53* gene (Advexin),[8] no appropriate method has been established to detect TRAD quantitatively. In addition, there is also a need for a procedure that can evaluate the biological activity of TRAD for clinical application.

In the present study, we characterized a potent antitumor viral agent, TRAD, to establish a biological assay and developed a single quantitative PCR method that can be used to assess the number of viral genomes present in the plasma as well as tissues.

Materials and Methods

Cells and culture conditions. H1299 (a human non-small-cell lung cancer cell line), H460 (a human large-cell lung cancer cell line), A549 (a human lung adenocarcinoma cell line), LNCap (a human metastatic prostate carcinoma cell line), MKN28 and MKN45 (human gastric adenocarcinoma cell lines), PC-3 (a human prostate adenocarcinoma cell line), SW620 (a human colorectal carcinoma cell line), and TE8 and T.Tn (human esophagus squamous carcinoma cell lines) were propagated to monolayer cultures in RPMI-1640 supplemented with 10% FBS, and 100 units/mL PG and 100 µg/mL SM. HeLa (a human cervical adenocarcinoma cell line), HepG2 (a human hepatocellular carcinoma cell line), Panc-1 (a human pancreatic epithelioid carcinoma cell line), and 293 (a transformed embryonic kidney cell line) were grown in DMEM containing high glucose (4.5 g/L) (high) with 10% FBS and PG/SM. HT-29 (a human colorectal adenocarcinoma cell line) was grown in McCoy's 5a with 10% FBS and PG/SM. MCF-7 (a human mammary gland adenocarcinoma cell line) was grown in Earle's Minimum Essential Medium with 10% FBS, PG/SM, and 2 mM L-glutamine. OST, SaOS2, and HOS (human osteosarcoma cell lines) were grown in DMEM (high) with 10% FBS and PG/SM. HSC-3 and HSC-4 (human tongue squamous carcinoma cell lines) were obtained from the Health Science Resources Bank (Osaka, Japan) and grown in DMEM (high) with 10% FBS and PG/SM. SCC-4 and SCC-9 (human tongue squamous carcinoma cell lines) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in DMEM containing Nutrient Mixture (Ham's F-12) with 10% FBS, PG/SM, and 400 ng/mL hydrocortisone. U-2OS (a human osteosarcoma cell line) was obtained from ATCC and grown in McCoy's 5a with 10% FBS and PG/SM. NHLF was purchased from Takara Biomedicals (Kyoto, Japan) and cultured in the medium recommended by the manufacturer.

Recombinant adenoviruses. The recombinant replication-selective tumor-specific adenovirus vector TRAD was constructed and

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Abbreviations: ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hTERT, human telomerase reverse transcriptase; ID_{50}, the multiplicity of infection that causes 50% growth inhibition; IRES, internal ribosome entry site; NHLF, normal human lung fibroblasts; MOI, multiplicity of infection; PCR, polymerase chain reaction; PFU, plaque-forming units; PG, penicillin; SM, streptomycin; TRAD, telomerase-specific replication-selective adenovirus; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

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characterized as described previously.[6,9–11] The virus was purified by $CsCl_2$ step-gradient ultracentrifugation followed by $CsCl_2$ linear-gradient ultracentrifugation. The virus particle titer and infectious titer were determined spectrophotometrically and by plaque assay, respectively, in 293 cells.

Cell-viability assay. The XTT assay was carried out to measure cell viability. Cells were plated on 96-well plates at 1×10^3 cells/well 20 h before viral infection. HSC-4, SCC-4, and SCC-9 cells were then infected with TRAD at MOI of 0, 1, 10, and 50 PFU/cell. Other cell lines were infected with TRAD at MOI of 0, 0.1, 1, and 10 PFU/cell. Cell viability was determined at 1, 2, 3, and 5 days after virus infection using Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the protocol provided by the manufacturer. Using the cell viability data at 3 days after virus infection, we determined the TRAD ID_{50} of each cell line.

Cell-killing assay. H1299 cells were plated at 5×10^4 cells/well on 24-well plates and infected with TRAD at MOI of 0, 0.01, 0.1, 1, and 10 PFU/cell. Forty-eight hours later, the number of cells in each well was counted. Experiments were carried out in triplicate for each MOI, and cell viability was assessed by the trypan blue dye exclusion assay.

Quantitative real-time PCR assay. Viral DNA from serially diluted viral stocks and tumor cells infected with TRAD were extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), and quantitative real-time PCR assay for either the *E1A* gene or the IRES sequence was carried out using a LightCycler instrument and a LightCycler DNA Master SYBR Green I kit (Roche Molecular Biochemicals). Typical amplification mixes (20 μL) contained 3 mM $MgCl_2$, 0.3 μM of each primer for IRES or 0.5 μM for *E1A*, and 2 μL of 10 × LightCycler FastStart DNA Master SYBR Green I. The sequences of the specific primers used in this experiment were: IRES, 5′-GAT TTT CCA CCA TAT TGC CG-3′ and 5′-TTC ACG ACA TTC AAC AGA CC-3′; E1A, 5′-CCT GTG TCT AGA GAA TGC AA-3′ and 5′-ACA GCT CAA GTC CAA AGG TT-3′. PCR amplifications were carried out in glass capillary tubes. PCR amplification for IRES began with a 10-min denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 6 s. PCR amplification for *E1A* began with a 10-min denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 8 s. Data analysis was carried out using LightCycler Software (Roche Molecular Biochemicals).

***In vivo* human tumor model.** A549 human lung cancer cells (5×10^6 cells/mouse) were injected subcutaneously into the flank of 7- to 9-week-old female BALB/c *nu/nu* mice and permitted to grow to approximately 5–6 mm in diameter. At that stage, a 100-μL solution containing 1×10^8 PFU of TRAD was injected into the tumor. The tumors and organs were harvested 28 and 70 days later and DNA was extracted from each tissue. To compare viral replication in the tumor and other normal organs, quantitative real-time PCR for the *E1A* gene was carried out using a LightCycler instrument. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine.

Statistical analysis. All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using Student's *t*-test. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

Results

***In vitro* cytopathic efficacy of TRAD in human cancer cell lines derived from different organs.** To determine whether TRAD infection induces broad-spectrum selective cell lysis, 23 tumor cell lines derived from 11 different organs (head and neck, lung, esophagus, stomach, colon, liver, pancreas, breast, prostate, uterus, and bone) were infected with TRAD at various MOI. Previous studies using a real-time reverse transcription-PCR method have demonstrated that these cell lines express detectable levels of hTERT mRNA.[6,9] Cytotoxicity was then assessed using the XTT cell-viability assay over 5 days after infection. As shown in Figure 1a, TRAD infection induced cell death in all cell lines except T.Tn esophageal cancer cells in a dose-dependent manner. Calculated ID_{50} values confirmed that all cell lines except T.Tn could be killed efficiently by TRAD at an MOI of less than 25 (Fig. 1b). These results suggest the broad-spectrum antitumor potency of TRAD.

Establishment of a standard assay to assess the biological activity of TRAD. H1299 human lung cancer cells and LNCap human prostate cancer cells were the most sensitive cell lines to TRAD-induced cell death (Fig. 1b). Accordingly, we used H1299 cells to evaluate the biological activity of TRAD. To test whether the selective replication of TRAD translates into selective oncolysis, we compared the cytopathic effects of TRAD in H1299 cells and NHLF at 5 days after infection. The dose–response curve of the relative cell viability in H1299 cells was shifted to the left compared to that in NHLF, suggesting that TRAD killed H1299 cells 10^2–10^3 more efficiently than NHLF (Fig. 2a).

We next determined the minimal dose of TRAD that could induce more than 50% of cell death in H1299 cells. As shown in Figure 2b, the cell viability of H1299 cells was less than 40% at 48 h after their infection with TRAD at a MOI of 1.0, but was 60% after infection with a MOI of 0.1. We also confirmed that H1299 cells at various passages (5th to 20th after purchase from ATCC) could be killed by TRAD in a similar fashion (data not shown). Therefore, TRAD could be considered biologically active, if TRAD at a MOI of 1 reduces the cell viability of H1299 cells by more than 50% at 48 h after infection. To estimate the utility of this assay, we examined the biological activity of heat-inactivated TRAD. Infection with intact TRAD at a MOI of 10 induced approximately 90% reduction in H1299 cell viability at 48 h after infection, whereas the antitumor activity was completely inhibited when it was preheated at 56°C for 5 or 10 min (Fig. 2c).

Development of quantitative PCR assay to detect copy numbers of TRAD. We used real-time PCR for quantitative detection of TRAD. Oligonucleotide primers were designed to achieve DNA amplification of the adenoviral E1A or IRES sequences in the TRAD genome (Fig. 3a). To generate accurate standard curves, TRAD at a known concentration was serially diluted and used as a template for real-time PCR analysis. Detection of IRES and E1A genome copies was achieved consistently and reproducibly by the PCR cycle values used. A linear relationship could be obtained between the number of cycles and the $\log_{10}$ dilution when 10^2–10^8 IRES copies and 10^3–10^8 E1A copies were assayed. Regression analysis of IRES and E1A curves resulted in very high correlation coefficients (0.99 and 1.00, respectively) for these concentration ranges (Fig. 3b). In addition, the dilution of TRAD virus in the plasma did not affect the sensitivity and dynamic ranges of quantification (Fig. 3b), suggesting that this method can be used to detect TRAD in the blood circulation.

***In vitro* quantification and replication monitoring of TRAD in infected human tumor and normal cells.** We next examined the replication ability of TRAD in different cell lines by measuring the relative amounts of IRES and E1A copy numbers. LNCap and NHLF cells were harvested at the indicated time points over 5 and 7 days, respectively, after infection with TRAD, and subjected to quantitative real-time PCR analysis using IRES and E1A primers. The ratios were normalized by dividing the value of cells obtained at 2 h after viral infection. As shown in Figure 4a, TRAD replicated 10^3–10^4 by 5 days after infection; its replication, however, was attenuated to less than 10^3 in normal NHLF cells. We previously reported that TRAD could replicate 10^5–10^6 by 3 days after infection in H1299 cells;[6,10] however, as

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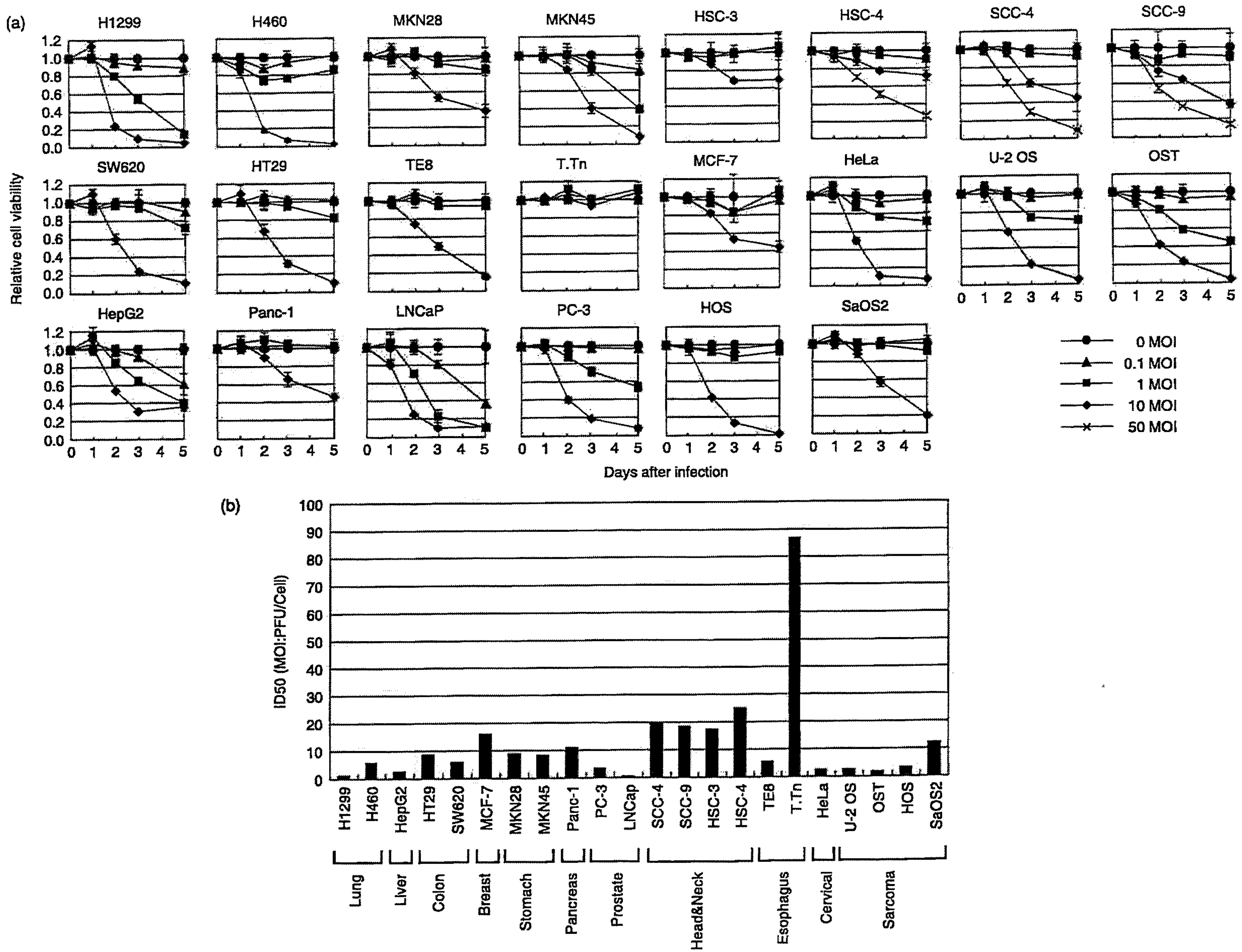

Fig. 1. Oncolytic effects of telomerase-specific replication-selective adenovirus (TRAD) *in vitro* on a variety of human cancer cell lines. (a) Cells were infected with TRAD at indicated multiplicity of infection (MOI) values, and surviving cells were quantitated over 5 days by XTT assay. Data are mean ± SD. (b) The 50% inhibiting doses of TRAD on cell viability at 3 days after infection were calculated and expressed as ID_{50} values. PFU, plaque-forming units; XXT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

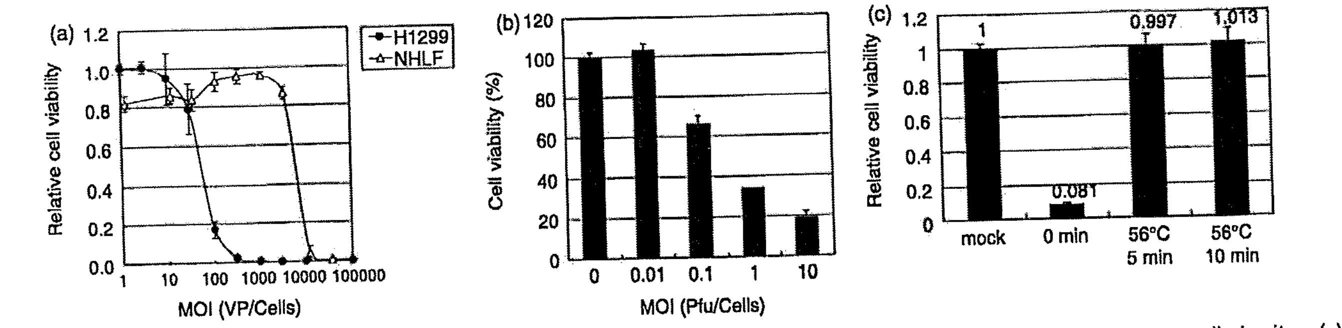

Fig. 2. Antitumor effects of telomerase-specific replication-selective adenovirus (TRAD) on H1299 non-small-cell lung cancer cells *in vitro*. (a) Effects of various concentrations of TRAD on H1299 cancer cells and normal human lung fibroblasts (NHLF) assessed at 5 days after treatment with XTT assay. Results are expressed as the percentage of untreated control. (b) H1299 cells were cultured as monolayers in triplicate in 24-well culture plates, infected with TRAD at the indicated multiplicities of infection (MOI), and assessed for cell viability 48 h after infection. Mock-infected cells were used as a control. (c) H1299 cells were plated on 96-well plates and infected with 10 MOI of TRAD heated at 56°C for 5 or 10 min, or non-treated TRAD. An XTT assay was carried out at 3 days after virus infection. Mock-infected cells were used as a control. Data represent the mean ± SD of triplicate experiments. PFU, plaque-forming units; XXT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate; VP, virus particles.

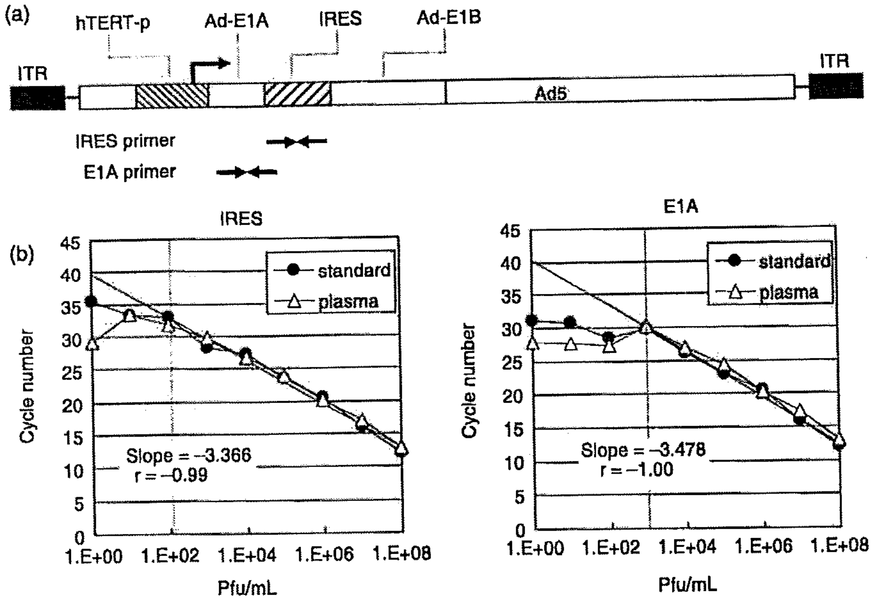

Fig. 3. Detection of normal human lung fibroblasts (TRAD) using quantitative polymerase chain reaction (PCR) assay. (a) Schematic diagram of the DNA structure of TRAD. TRAD contains the human telomerase reverse transcriptase (hTERT) promoter sequence inserted into the adenovirus genome to drive transcription of the *E1A* and *E1B* bicistronic cassette linked by the internal ribosome entry site (IRES) structure. Sites to which PCR primers (IRES and E1A) were targeted are indicated. Two primer pairs of IRES and E1A were designed to detect the TRAD genome. (b) Standard calibration curves of threshold cycle values and copy numbers are shown using serial dilution of TRAD virus stock. The coefficient of correlation (r^2) and slope are indicated for assays with IRES and E1A primers. ITR, inverted terminal repeats; PFU, plaque-forming units.

LNCap cells were more sensitive to TRAD-mediated cytotoxicity than H1299 cells (Fig. 1a), viral replication reached a plateau phase around 10^4 when LNCap cells started to die. Moreover, PCR targeting IRES and E1A showed similar replication profiles for TRAD in MCF-7 human breast cancer cells (Fig. 4b). To monitor the long-term viral replication, MCF-7 cells that were less sensitive to the cytopathic effect of the virus were used.

***In vivo* determination of TRAD genomes in tissue samples after intratumoral injection.** To evaluate selective replication of TRAD *in vivo*, we examined mouse tissues, including implanted tumors, for the presence of viral DNA by quantitative real-time PCR, following intratumoral viral injection. Mice with established subcutaneous A549 human lung tumor xenografts received a single intratumoral injection of 1×10^8 PFU of TRAD, and were killed 28 or 70 days after injection. To obtain the sufficient amounts of tumor tissues for analysis, we chose to use A549 cells. Our preliminary experiments demonstrated that intratumoral administration of TRAD suppressed tumor growth significantly compared with mock-treated tumors at 42 days after initiation of treatment ($P < 0.05$); however, the *in vivo* antitumor effect against A549 tumors was less than that against H1299 or LNCap tumors (data not shown). Although E1A DNA was detected in serum and some normal tissues examined (brain, heart, lung, ovary, liver, uterus, kidneys, bladder, colon, and axillary and mesenteric lymph nodes), tumors injected with TRAD contained at least 1000-fold more *E1A* copies (Fig. 5). These results suggest that quantitative real-time PCR allows detection and quantification of the number of TRAD genomes present in tissue samples after intratumoral injection of TRAD *in vivo*.

Discussion

Oncolytic viruses have been developed as anticancer agents based on the advantage of selective killing of tumor cells by controlled replication of the virus in the tumors, resulting in minimal undesired effects on normal cells.[2] Furthermore, amplified viruses can infect adjacent tumor cells as well as reach distant metastatic tumors through the blood circulation. Alhtough this might be a potential advantage of oncolytic viruses, systemic dissemination of large amounts of virus may induce virus-related symptoms including fever, diarrhea, pneumonia, and hepatitis, eventually leading to death. Therefore, virus shedding and distribution have to be evaluated by appropriate and suitable methods. In addition,

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