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放射線感受性ナノバイオ・ウイルス製剤の開発と難治性固形癌に対する臨床応用の検討

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主任研究者　　藤原　俊義

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放射線感受性ナノバイオ・ウイルス製剤の開発と難治性固形癌に対する臨床応用の検討

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【研究要旨】
最近のゲノム科学やナノテクノロジーの進歩により、癌の悪性形質の発現に関わる分子機構が明らかとなり、癌に特異的な標的分子を定めることが可能となってきた。p53はヒト悪性腫瘍で最も高頻度に異常がみられる癌抑制遺伝子であり、その正常型の遺伝子導入により放射線感受性増強をはじめとする多彩な作用機序を介した抗腫瘍効果が認められる。しかし、非増殖型ウイルスベクターでは腫瘍内へのウイルス拡散や遺伝子導入効率に限界があり、根治を目指した治療には至っていない。ウイルスは本来、ヒトの細胞に感染して複製・増殖することで細胞を破壊する。その増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを殺傷する抗癌剤として用いることが可能となる。本研究では、テロメラーゼ依存性に増殖し、細胞死を誘導するOBP-301（Telomelysin）をさらに武装化（arming）し、ウイルス増殖による細胞死とともにアポトーシス誘導分子p53を発現することで強力な抗腫瘍活性を発揮する新規ナノバイオ・ウイルス製剤OBP-702を開発する。平成19年度は、まず癌細胞で選択的に増殖するウイルス製剤OBP-301 (Telomelysin)の構造を基盤としてp53遺伝子を組み込む遺伝子改変を行い、次に作成したOBP-702ウイルスを用いて*in vitro*における機能解析と抗腫瘍活性の検討を行った。

分担研究者：
浦田　泰生
（オンコリスバイオファーマ・代表取締役社長）

A．研究目的

難治固形癌に対する新たな抗癌剤開発は、分子標的薬剤の開発などにより積極的に進められており、その治療成績の向上も現実のものとなってきている。しかし、副作用や耐性の出現など解決すべき問題点は多く、新たな治療戦略の開発は必須と考えられる。本研究の目的は、ベクターとして多くの遺伝子治療で使用され、その安全性が確認されてきたアデノウイルスのゲノムを改変し、より強力な抗腫瘍活性を有する武装化（armed）ナノバイオ・ウイルス製剤を開発することである。

ウイルスは本来ヒトの細胞に感染して、その構造蛋白質を産生することで複製・増殖する。その増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを殺傷する抗癌剤として用いることが可能となる。「かぜ」症状の原因となるアデノウイルス5型を基本骨格とし、80-90%のヒト悪性腫瘍で極めて高い活性がみられる不死化関連酵素テロメラーゼの構成分子であるhTERT (human telomerase reverse transcriptase)遺伝子のプロモーターでウイルス増殖に必須のE1AおよびE1B遺伝子を制御することで、癌細胞のみで増殖する腫瘍融解アデノウイルス（Oncolytic adenovirus）を構築する。さらに、放射線感受性プロモーターEgr-1で強力なアポトーシス誘導機能を持つ癌抑制遺伝子p53を駆動する発現カセットを、ウイルスのE3遺伝子領域に搭載する。

このナノバイオ・ウイルス製剤OBP-702は、癌細胞で選択的に増殖することにより標的細胞死を引き起こす機能を有すると同時に、体外からの放射線照射に反応してp53遺伝子を発現し、強力なアポトーシス誘導を介した抗腫瘍効果を発揮する。また、放射線によるアポトーシス細胞でもp53は重要なシグナル伝達経路であり、OBP-702によるp53の過剰発現により放射線感受性自体も増強されると考えられる。さらに、原発腫瘍内に局所投与されたOBP-702は、周辺のリンパ節にも到達してリンパ節転移巣でも増殖するため、臨床的には所属リンパ節を含めた放射線治療との強力な相乗効果が期待される。

B．研究方法

1）テロメラーゼ（hTERT）特異的p53遺伝子発現腫瘍融解ウイルス製剤（開発コード：OBP-702）の作成

テロメラーゼ構成分子であるhTERT遺伝子のプロモーターとIRES配列を間置したアデノウイルスE1A遺伝子およびE1B遺伝子から成る増殖カセット、

および放射線感受性プロモーターEgr-1とヒト正常型p53遺伝子を有するp53発現カセットを、アデノウイルス5型ゲノムのE1領域とE3領域にそれぞれ挿入した。このウイルスゲノムのプラスミドをE1遺伝子によるトランスフォームされたヒト腎臓293細胞にトランスフェクションし、上清中に産生されたウイルスを抽出精製、放射線感受性プロモーターEgr-1でp53遺伝子発現を制御するhTERT選択的ナノバイオ・ウイルス製剤OBP-702を作成した。さらに、高品質ウイルスストック作成のために、OBP-702を大量培養した293細胞に感染させ、細胞融解にてウイルスを回収、塩化セシウム濃度勾配にて分離・精製した。

2）OBP-702と放射線照射によるp53遺伝子発現の検討

H1299ヒト肺癌細胞にOBP-702を感染させ、経時的にp53蛋白質発現をウエスタンブロット法にて確認した。また、各種ヒト癌細胞（H1299、H460、H358ヒト肺癌細胞、SW620、LoVoヒト大腸癌細胞、HepG2、Huh7ヒト肝癌細胞）にOBP-702を感染させ、24時間後に10Gyの放射線照射を行い、感染から48時間後にp53蛋白質発現を確認した。

3）*In vitro*におけるOBP-702の抗腫瘍効果の検討

上記細胞株を含む11種の癌細胞株において、OBP-702あるいはOBP-301感染後、経日的にXTTアッセイにて生細胞数比率を測定し、それぞれの抗腫瘍効果を比較検討した。

（倫理面への配慮）

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）テロメラーゼ（hTERT）特異的p53遺伝子発現腫瘍融解ウイルス製剤（開発コード：OBP-702）の作成

hTERT遺伝子のプロモーターとE1A／E1B遺伝子から成る増殖カセット、およびEgr-1プロモーターとヒト正常型p53遺伝子を有するp53発現カセットを、アデノウイルス5型ゲノムのE1領域とE3領域にそれぞれ挿入してOBP-702を作成した。

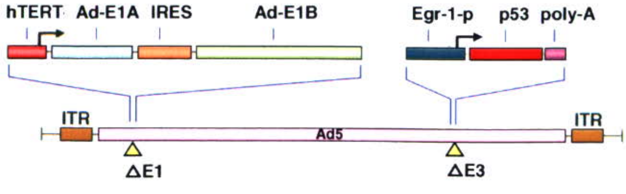

図１　OBP-702の構造

高品質ウイルスストック作成では、最終的に1.9x10e10 plaque forming units (PFU)の濃度のOBP-702ウイルスの調製が可能であった。また、コントロール・ウイルスとして、Egr-1プロモーターでGFP蛍光遺伝子を発現するOBP-704も作成し、3.9x10e10 PFUのストックを調製した。

2）OBP-702と放射線照射によるp53遺伝子発現の検討（*in vitro*）

H1299ヒト肺癌細胞において、OBP-702感染後12時間からp53蛋白質発現がみられ、少なくとも72時間後まで発現は維持された。

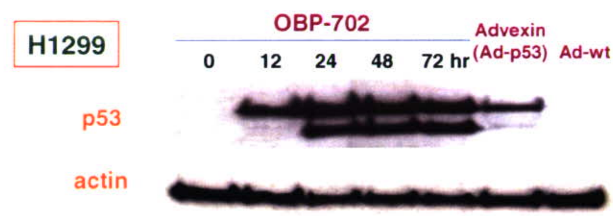

図2　OBP-702によるp53蛋白質発現

また、ヒト癌細胞（H1299、H460、H358ヒト肺癌細胞、SW620、LoVoヒト大腸癌細胞、HepG2、Huh7ヒト肝癌細胞）において、OBP-702を感染48時間後にp53蛋白質発現を確認されたが、放射線照射によってもp53蛋白質発現レベルの明らかな増強は認められなかった。これらの結果から、ウイルス増殖自体がストレスとしてEgr-1プロモーターを駆動したと考えられる。

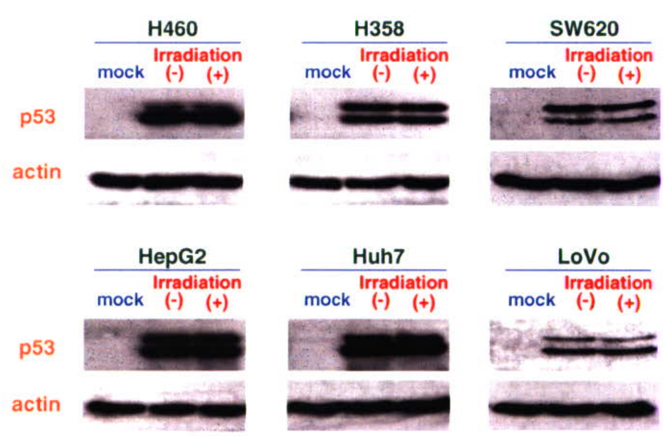

図3　放射線のOBP-702によるp53発現への影響

3）*In vitro*におけるOBP-702の抗腫瘍効果の検討(*in vitro*)

11種のすべての癌細胞株において、OBP-301よりもOBP-702で強い抗腫瘍活性がみられ、OBP-702の方が低いID50を示した。特に、OBP-301に完全に耐性のT.Tn食道癌細胞でOBP-702の抗腫瘍効果が認められ、その有用性が示唆された。

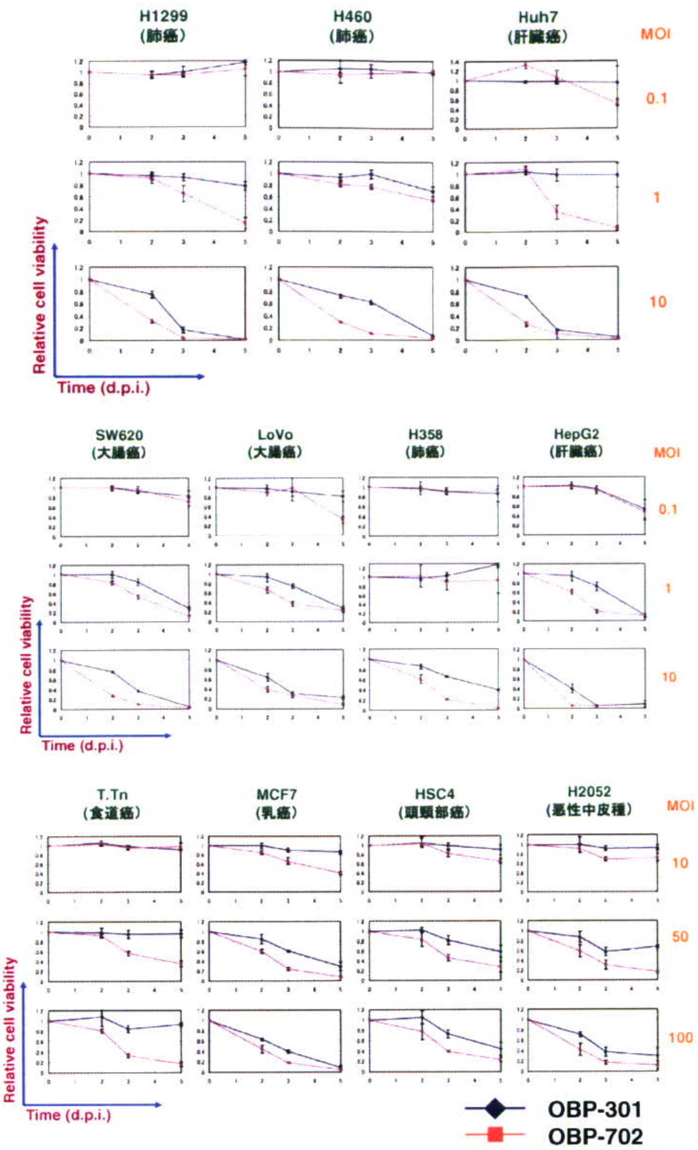

図4 OBP-702の抗腫瘍効果(*in vitro*)

表1 ヒト癌細胞におけるOBP-702の抗腫瘍活性
(ID50 value on day 3)

		OBP-301	OBP-702
肺癌	H1299	4.24	1.49
	H460	13.13	2.41
	H358	21.51	4.19
肝臓癌	Huh7	10.35	0.93
	HepG2	1.8	0.47
大腸癌	SW620	5.58	1.14
	LoVo	3.76	0.98
食道癌	T.Tn	ND (>100)	61.05
乳癌	MCF7	70.84	18.72
頭頸部癌	HSC4	ND (>100)	51.77
中皮腫	H2052	64.91	22.32

ND: not determined

また、アポトーシスの指標としてCaspase 3陽性細胞の比率をBD FACSArray バイオアナライザーにて比較したところ、OBP-301 10MOI感染後48時間で6.1%に対して、OBP-702では23.5%と明らかに多くのアポトーシス細胞が確認された。非増殖型p53遺伝子発現アデノウイルスAdvexinでは、100MOIで24.4%と10MOIのOBP-702と同等のアポトーシス誘導を示した。

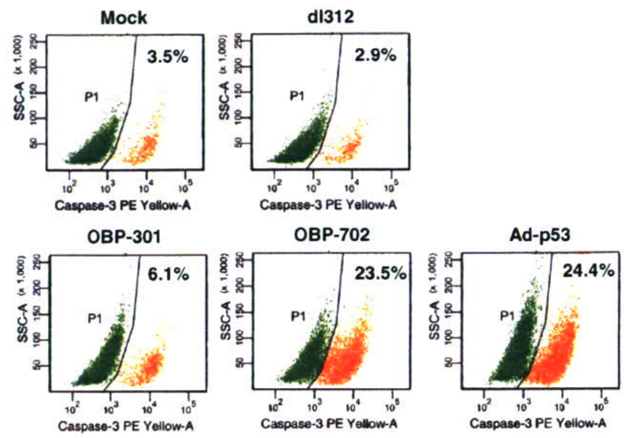

図5 OBP-702感染によるアポトーシス誘導能

D．考察

平成19年度は、癌細胞で選択的に増殖することによりナノバイオ・ウイルス製剤OBP-702は標的細胞死を引き起こすと同時に、p53遺伝子を発現して強力なアポトーシス誘導を介した抗腫瘍効果を発揮することが明らかになった。特に、従来のウイルス製剤OBP-301（Telomelysin）に耐性の癌細胞を傷害することが可能であり、その広範囲の抗腫瘍スペクトラムは、将来的な臨床応用には大きなメリットとなる。Egr-1プロモーターの放射線制御性の根拠は得られなかったが、サイトメガロウイルス・プロモーターなどに比べて活性が低いため、十分なウイルス増殖が可能となり、その相乗効果が顕著にみられたと考察できる。

平成20年度以降は、タイムラプス観察によりウイルス増殖によるoncolysisとp53遺伝子発現によるアポトーシス誘導が個々の細胞でどのように生じているのかを明らかにしたい。今までの経験から、p53 による急速な細胞死誘導からsurviveした細胞群がoncolysisで緩徐な細胞死に至ると推測され、分子イメージングによる細胞内蛋白質の局在変化などを標的として解析を進める予定である。また、放射線によるアポトーシス細胞死でもp53は重要なシグナル伝達経路であり、今後はOBP-702でのp53過剰発現による放射線感受性増強を検討する。さらに、*in vivo*でのOBP-702の抗腫瘍活性、および放射線療法との相互作用を検証し、前臨床研究としての毒性試験や体内動態の分析を進めることで、OBP-702によるトランスレーショナルリサーチの実

現を目指す。

E．結論

p53遺伝子を搭載したテロメラーゼ特異的増殖アデノウイルスOBP-702は作成可能であった。OBP-702製剤は、基盤となったp53遺伝子を持たないOBP-301製剤より強い抗腫瘍活性を示し、その有用性が示された。

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研究成果の刊行に関する一覧表

雑誌

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Endo, Y., Sakai, R., Ouchi, M., Onimatsu, H., Hioki, M., Kagawa, S., Uno, F., Watanabe, Y., Urata, Y., Tanaka, N., Fujiwara, T.	Virus-mediated oncolysis induces danger signal and stimulates cytotoxic-T-lymphocyte activity via proteasome activator upregulation.	**Oncogene**	(in press)		2007
Hashimoto, Y., Watanabe, Y., Shirakiya, Y., Uno, F., Kagawa, S., Kawamura, H., Nagai, K., Tanaka, N., Kumon, H., Urata, Y., Fujiwara, T.	Establishment of Biological and Pharmacokinetic Assays of Telomerase-Specific Replication-Selective Adenovirus (TRAD).	**Cancer Science**	99	385-390	2008
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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

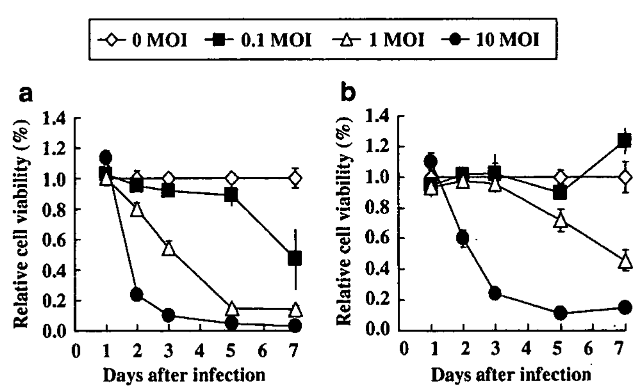

Figure 1 *In vitro* cytopathic effects of OBP-301 on human cancer cells. H1299 human non-small cell lung cancer (a) and SW620 human colorectal cancer cells (b) were infected with OBP-301 at indicated multiplicity of infection (MOI) values, and surviving cells were quantitated over 7 days by XTT assay. 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.

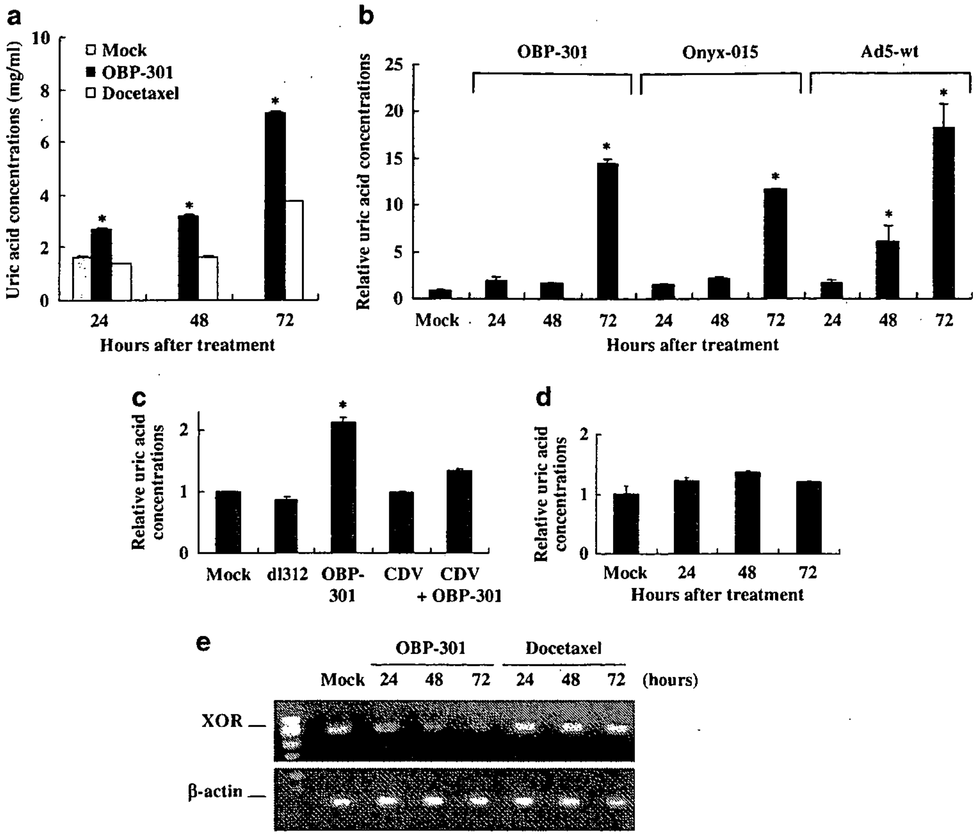

Figure 2 (**a**) Uric acid concentrations in H1299 cells treated with OBP-301 or docetaxel. H1299 cells were infected with 1.0 MOI of OBP-301 or treated with 10 nM of docetaxel for indicated time periods, and uric acid concentrations were determined enzymatically in the cell homogenates. Single asterisk 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

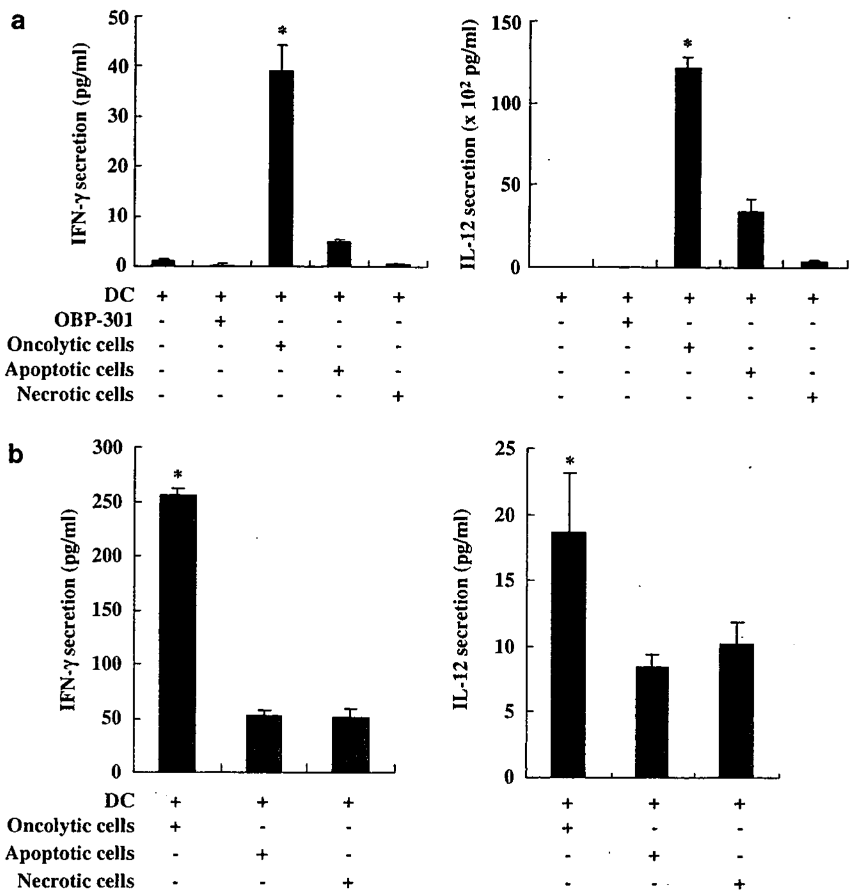

Figure 3 (**a**) Secretion of Th1-type cytokines by oncolytic, apoptotic or necrotic tumor cells. H1299 cells were treated with 1.0 MOI of OBP-301 or 50 nM of docetaxel for 72 h, or freeze thawed, and then co-cultured with immature dendritic cells (DCs) obtained from monocytes for additional 48 h. The 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.

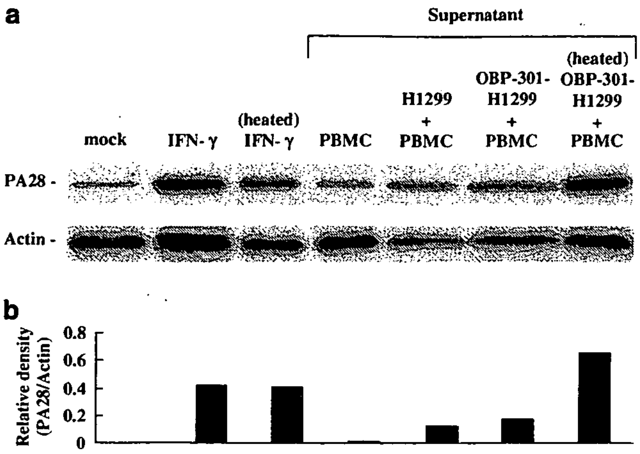

Figure 4 (a) Western blot analysis of PA28 in H1299 cells exposed to the supernatants of MLTC. peripheral blood mononuclear cells (PBMCs) were incubated with mock, untreated H1299 cells or H1299 cells treated with 10 MOI of OBP-301 for 72 h in MLTC, and the supernatants were harvested 48 h after the 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.

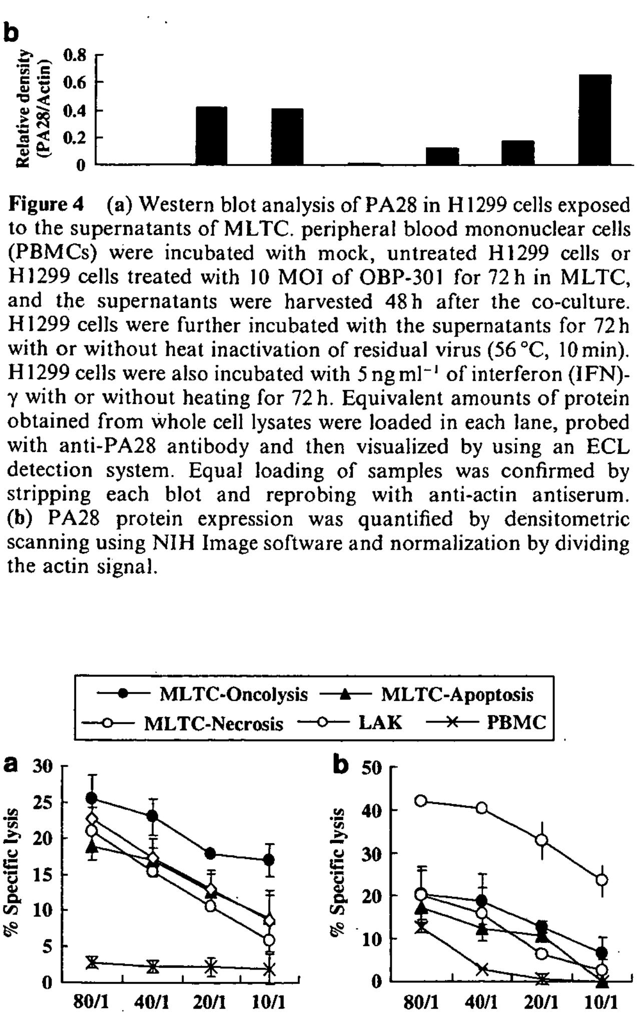

Figure 5 Cytolytic reactivity against H1299 (a) and SW620 (b) human cancer cells was assessed after 7-day mixed lymphocyte tumor culture (MLTC) with oncolytic, apoptotic or necrotic H1299 cells treated the same as above by 6-h standard ^{51}Cr-release assay. Lymphokine-activated killer (LAK) cells were 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/nµ* 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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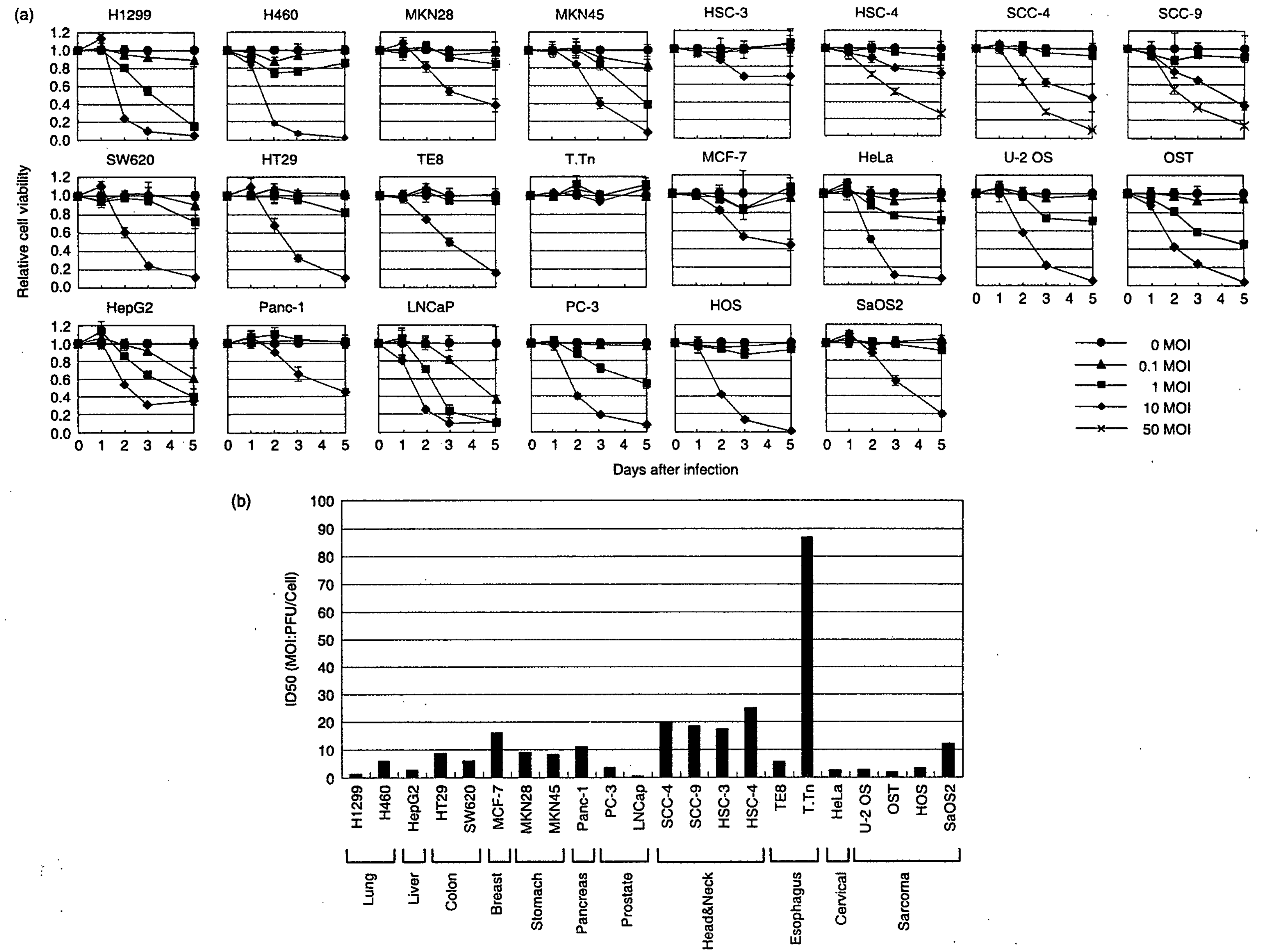

Fig. 1. Oncolytic effects of telomerase-specific replication-selective adenovirus (TRAD) *in vitro* on a variety of human cancer cell lines. (a) Cells were infected with TRAD at indicated multiplicity of infection (MOI) values, and surviving cells were quantitated over 5 days by XTT assay. Data are mean ± 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.

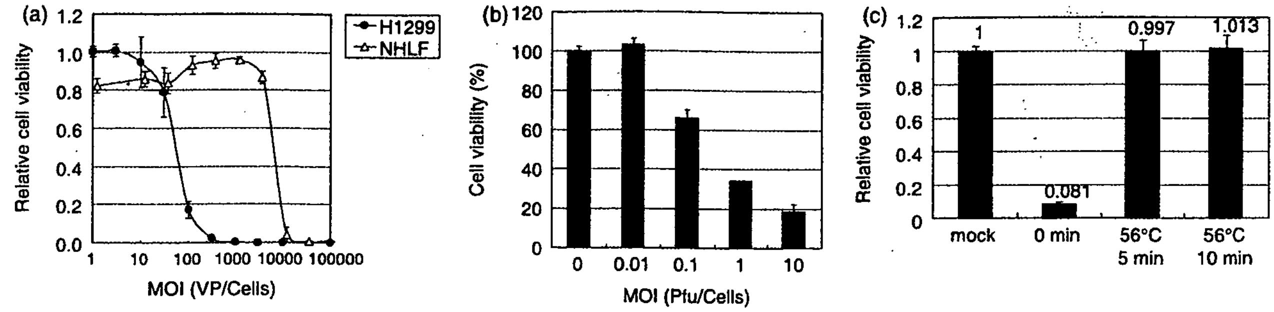

Fig. 2. Antitumor effects of telomerase-specific replication-selective adenovirus (TRAD) on H1299 non-small-cell lung cancer cells *in vitro.* (a) Effects of various concentrations of TRAD on H1299 cancer cells and normal human lung fibroblasts (NHLF) assessed at 5 days after treatment with XTT assay. 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.

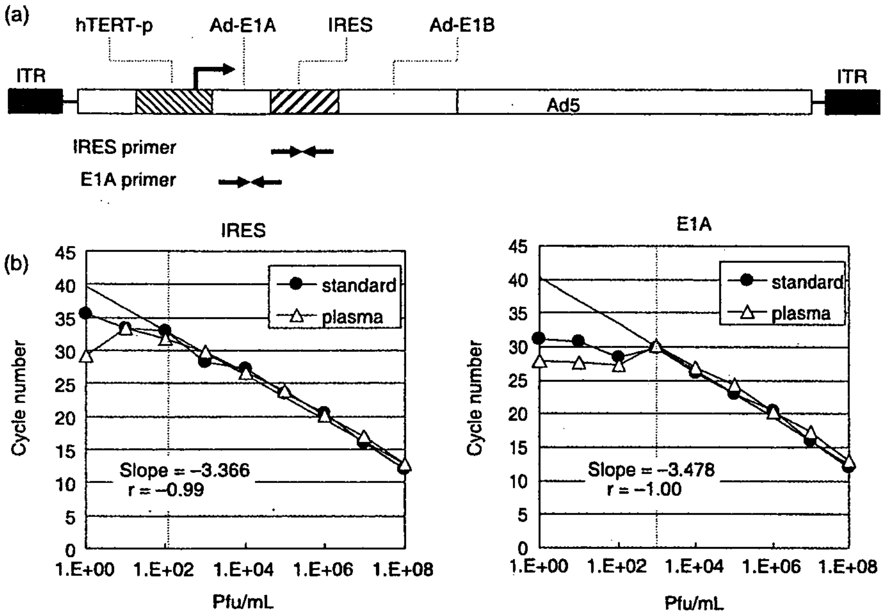

Fig. 3. Detection of normal human lung fibroblasts (TRAD) using quantitative polymerase chain reaction (PCR) assay. (a) Schematic diagram of the DNA structure of TRAD. TRAD contains the human telomerase reverse transcriptase (hTERT) promoter sequence inserted into the adenovirus genome to drive transcription 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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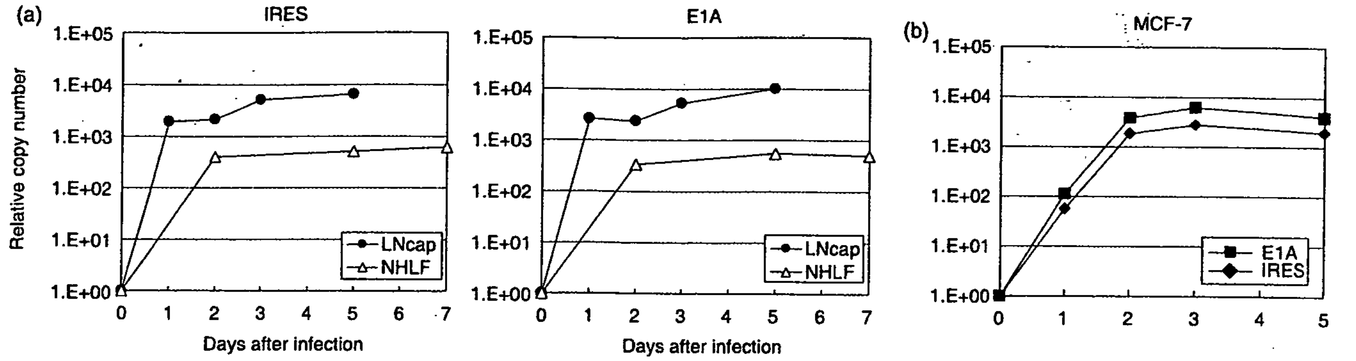

Fig. 4. Quantitative measurement of viral DNA replication in human cancer and normal cells *in vitro* by quantitative polymerase chain reaction (PCR) assay. (a) LNCap human prostate cancer cells and normal human lung fibroblast (NHLF) cells were infected with telomerase-specific replication-selective adenovirus (TRAD) at a multiplicity of infection (MOI) of 1 for 2 h. Following the removal of virus inoculum, cells were further incubated for the indicated periods of time, and then subjected to the real-time quantitative PCR assay. The amounts of viral internal ribosome entry site (IRES) and E1A copy number was defined as the fold increase for each sample relative to that at 2 h (2 h equals 1). (b) MCF-7 human breast cancer cells were infected with TRAD at a MOI of 1 and subjected to the PCR assay at the indicated time points. The relative TRAD DNA levels detected by IRES and E1A primers were plotted.

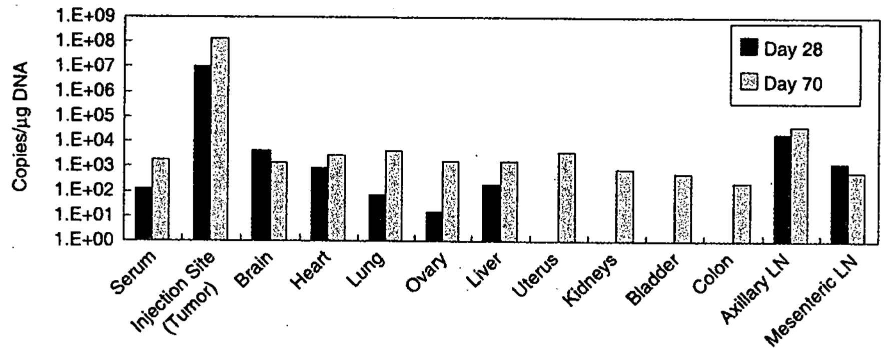

Fig. 5. Spread and replication of telomerase-specific replication-selective adenovirus (TRAD) following intratumoral administration in *nu/nu* mice transplanted with A549 tumor cells. A549 tumor cells were injected subcutaneously into the right flank of mice at 5×10^6 cells/mouse. Mice received intratumoral injection of 1×10^8 plaque-forming units of TRAD when the tumor reached a size of approximately 5–6 mm in diameter. DNA was extracted from the subcutaneous tumor and various tissues of *nu/nu* mice at 28 or 70 days after infection. Viral DNA was detected by quantitative polymerase chain reaction amplification of the adenoviral E1A sequence. The amounts of TRAD genome were defined as viral E1A copy number per µg DNA. LN, lymph nodes.

to avoid unexpected infectious disease due to viral overdose, we need assays that accurately detect the biological activity of viruses. In the present study, for clinical trials of TRAD, we developed an assay designed to estimate the biological activity of TRAD and to detect the copy number of TRAD in the plasma as well as tissues.

Although telomerase-specific TRAD exhibited a broad cytopathic effect against human cancer cell lines of different tissue origins, a human non-small-cell lung cancer cell line, H1299, was chosen for the biological assay of TRAD. H1299 was one of the most sensitive cell lines to TRAD-mediated cell death (ID_{50} = 0.94 MOI) and could be killed efficiently by TRAD infection in a dose-dependent fashion (Fig. 1). Because H1299 cells can be obtained from ATCC, they can be used in clinical laboratories to assess the biological activity of TRAD with a qualified standard protocol. In addition, although adenoviral E1B-55 kDa protein is known to bind to the tumor suppressor p53 protein,[12] H1299 cells are p53-null and therefore the interaction of E1B-55 kDa with p53, which in turn results in transcriptional modulation, can be ignored in this cell line. Thus, H1299 is considered an appropriate cell line for assessment of TRAD activity in certain preparations. In the present study, we considered TRAD to be active when the viability of H1299 cells was reduced by more than 50% at 48 h after TRAD infection at an MOI of 1. Using this biological assay, we confirmed that heat treatment of aliquots of TRAD at 56°C for 5 min is sufficient to inactivate its antitumor potential (Fig. 2c). These results advocate the use of the H1299 cell-based cytotoxicity assay as a standard method for quantitative assessment of the biological activity of TRAD in virus stocks for clinical trials.

Various biological methods, such as determination of infectious units in plaque assays, have been used routinely in clinical trials to monitor viral loads in the peripheral circulation.[8] These methods are useful for evaluating safety because the viral titers directly reflect the infectivity of viruses. However, because the plaque assay consists of labor-intensive and time-consuming steps, real-time monitoring of the biodistribution of the virus might be difficult. Here we described the development of a quantitative real-time PCR assay that can accurately quantify genome copy numbers of TRAD over a large linear range. Using primers targeting TRAD-specific sequences, such as adenoviral E1A and IRES, real-time PCR could accurately detect the number of TRAD genomes in the plasma as well as in the cells (Figs 3,4). The assay showed that TRAD replicated even in NHLF, although the level was much lower than that in tumor cells. It is usually difficult to maintain the normal cells primarily isolated from human tissues such as human hepatocytes in the culture; however, commercially available NHLF could be cultured for several passages, suggesting that NHLF may have some characteristics different from primary isolated normal cells, including