Telomerase-Specific Replication-Selective Virotherapy for Human Cancer

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ABSTRACT

Purpose: **Replication-selective tumor-specific viruses present a novel approach for treating neoplastic disease. These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor. Telomerase activation is considered to be a critical step in carcinogenesis, and its activity is closely correlated with human telomerase reverse transcriptase (hTERT) expression. We investigated the antitumor effect of the hTERT-specific replication-competent adenovirus on human cancer cells.**

***Experimental Design*: We constructed an adenovirus 5 vector [tumor- or telomerase-specific replication-competent adenovirus (TRAD)], in which the hTERT promoter element drives expression of *E1A* and *E1B* genes linked with an internal ribosome entry site, and we examined the selective replication and antitumor effect in human cancer cells *in vitro* and *in vivo*.**

Results: **TRAD induced selective *E1A* and *E1B* expression in human cancer cells, but not in normal cells such as human fibroblasts. TRAD replicated efficiently and induced marked cell killing in a panel of human cancer cell lines, whereas replication as well as cytotoxicity was highly attenuated in normal human fibroblasts lacking telomerase activity. In *nu/nu* mice carrying s.c. human lung tumor xenografts, intratumoral injection of TRAD resulted in a significant inhibition of tumor growth. No evidence of TRAD was identified in tissues outside of the tumors, despite the presence of TRAD in the circulation. Moreover, TRAD replication in the distant, noninjected tumors was demonstrated.**

Conclusions: **Our results suggest that the hTERT promoter confers competence for selective replication of TRAD in human cancer cells, an outcome that has important implications for the treatment of human cancers.**

INTRODUCTION

Recent advances in molecular biology have fostered remarkable insights into the molecular basis of neoplasia, and cancer gene therapy has emerged as a promising new modality. Replication-defective, E1-deleted adenoviral vectors facilitate the efficient delivery of a variety of transgenes to target tissues and have demonstrated clear therapeutic benefits and safety in a variety of clinical studies (1); a significant obstacle, however, is the limited distribution of the vectors within the tumor mass even after direct intratumoral administration. To confer specificity of infection and increase viral spread to neighboring tumor cells, the notion of using replication-competent adenoviruses has become a reality (2).

Telomerase is a ribonucleoprotein complex that is responsible for the complete replication of chromosomal ends (3). Many studies have demonstrated that the majority of malignant tumors express telomerase activity (4), whereas most normal cells do not (5). Three major components associated with telomerase activity in humans have been identified: (*a*) the RNA component [hTER (6)]; (*b*) the telomerase-associated protein [hTEP1 (7)]; and (*c*) the telomerase catalytic unit or human telomerase reverse transcriptase [hTERT (8, 9)]. Only hTER and hTERT, however, are required for the reconstitution of telomerase activity *in vitro* (10) and therefore represent the minimal catalytic core of telomerase in humans (11). The promoter region of hTERT has been cloned and characterized previously (12). Telomerase-specific expression of cytotoxic or proapoptotic genes such as the diphtheria toxin A-chain, FADD, caspases, and Bax by the hTERT promoter has been successfully achieved and reported in various gene transfer systems [*e.g.*, plasmid and adenovirus (13–17)]. Although adenovirus-mediated *Bax* gene expression via the hTERT promoter elicited therapeutic effects on tumor cells and could prevent toxic effects on normal cells (17), the viral spread might be less than ideal after intratumoral administration.

We hypothesized that an adenovirus containing hTERT promoter-driven *E1* genes could be used to target a variety of tumor cells and kill them efficiently by viral replication because >85% of human cancers display telomerase activity (6). In the present study, we have constructed tumor- or telomerase-specific replication-competent adenovirus (TRAD) and then explored the selective replication of TRAD in tumor cells as well

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as the antitumor effects such as *in vitro* oncolysis and a therapeutic benefit *in vivo*.

MATERIALS AND METHODS

Construction of TRAD. A 897-bp fragment of the *E1A* gene was amplified by reverse transcription-PCR from total cellular RNA of 293 cells using the primers E1A-S (5′-ACA-CCG-GGA-CTG-AAA-ATG-AG-3′) and E1A-AS (5′-CAC-AGG-TTT-ACA-CCT-TAT-GGC-3′). A 1822-bp fragment of the *E1B* gene was amplified by PCR from genomic DNA of 293 cells using the primers E1B-S (5′-CTG-ACC-TCA-TGG-AGG-CTT-GG-3′) and E1B-AS (5′-GCC-CAC-ACA-TTT-CAG-TAC-CTC-3′). The amplified products were subcloned into the pTA plasmid according to the instructions provided by the manufacturer (Invitrogen, Carlsbad, CA). After confirmation by DNA nucleotide sequencing, the *E1A* gene (911 bp) and the *E1B* gene (1836 bp) were digested with *Eco*RI and then cloned into the pIRES vector (Clontech, Palo Alto, CA) at the *Mlu*I and *Sal*I restriction sites, respectively (pE1A-IRES-E1B). A 455-bp fragment of the hTERT promoter was digested with *Mlu*I and *Bgl*II restriction enzymes from pGL3–378, which contains a 378-bp region upstream of the transcription start site, and then ligated into the *Xho*I site of the pE1A-IRES-E1B (phTERT-E1A-IRES-E1B). The 3828-bp fragment was digested from phTERT-E1A-IRES-E1B with *Nhe*I and *Not*I restriction enzymes and then cloned into pShuttle after deletion of the cytomegalovirus promoter. The resultant shuttle vector was used to generate replication-competent adenovirus under control of the hTERT promoter using the Adeno-X Expression System (Clontech) according to the protocol provided by the manufacturer. Recombinant adenovirus was isolated from a single plaque and expanded in 293A cells. PCR was performed using several primers specific for adenovirus *E1* sequences, and the PCR products were digested with restriction enzymes to confirm the structure. The E1A-deleted adenovirus vector d1312 was used as the control vector. Recombinant viruses were purified by ultracentrifugation in cesium chloride step gradients, and their titers were determined by plaque assay in the 293 cells.

Cell Lines and Antibody. The human non-small cell lung cancer cell lines H1299, H358, and H226Br and the human colorectal carcinoma cell lines SW620, LoVo, and DLD-1 were routinely propagated in monolayer culture in RPMI 1640 supplemented with 10% FCS. The human non-small cell lung cancer cell line A549 was cultured in DMEM containing Nutrient Mixture (Ham's F-12). The transformed embryonic kidney cell line 293 was grown in DMEM containing high glucose (4.5g/liter) and supplemented with 10% FCS. The normal human lung diploid fibroblast cell line WI38 (JCRB0518) was obtained from the Health Science Research Resources Bank (Osaka, Japan), and grown in Eagle's MEM with 10% FCS. The normal human lung fibroblast cell line NHLF was purchased from TaKaRa Biomedicals (Kyoto, Japan) and cultured in the medium recommended by the manufacturer. For Western blot analysis, primary antibody against E1A (PharMingen International), peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL), and an Amersham enhanced chemiluminescence Western system (ECL; Amersham, Tokyo, Japan) were used. For immunohistochemistry, tumor cryosections were fixed and immunostained with anti-E1A antibody (14161A; PharMingen) followed by a avidin-biotin-peroxidase complex.

PCR Analysis. After reverse transcription or extraction of DNA, PCR was performed with specific primers for 30 cycles using the GeneAmp PCR system 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences of specific primers used in this experiment were as follows: E1A, 5′-GTA-TGA-TTT-AGA-CGT-GAC-GG-3′ (sense) and 5′-GAT-AGC-AGG-CGC-CAT-TTT-AG-3′ (antisense); E1B, 5′-GGC-TAA-AGG-GGG-TAA-AGA-GGG-3′ (sense) and 5′-CCT-TAC-ATC-GGT-CCA-GGC-TTC-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase, 5′-CAG-CCG-AGC-CAC-ATC-3′ (sense) and 5′-TGA-GGC-TGT-TGT-CAT-ACT-TCT-3′ (antisense); and β-actin, 5′-GCC-ATC-CTG-CGT-CTG-GAC-CTG-3′ (sense) and 5′-CAT-TTG-CGG-TGC-ACG-ATG-GAG-3′ (antisense). The amplification reactions involved denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The *hTERT* mRNA copy number was determined by real-time quantitative RP-PCR using a LightCycler instrument and a LightCycler DNA TeloTAGGG Kit (Roche Molecular Biochemicals, Indianapolis, IN). PCR amplification began with a 60-s denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 10 s, and extension at 72°C for 9 s.

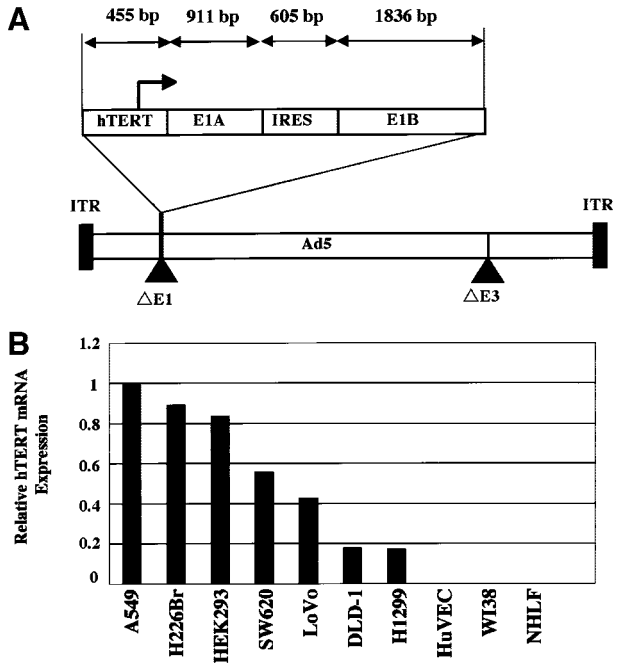

Fig. 1 *A*, a schematic DNA structure of tumor- or telomerase-specific replication-competent adenovirus. Tumor- or telomerase-specific replication-competent adenovirus contains the human telomerase reverse transcriptase (hTERT) promoter sequence inserted into the E3-deleted adenovirus genome to drive transcription of the E1A and E1B bicistronic cassette linked by the internal ribosome entry site structure. *B*, relative *hTERT* mRNA expression in human tumor and normal cells as determined by real-time reverse transcription-PCR analysis. The *hTERT* mRNA expression of A549 human lung cancer cells was considered 1.0, and the relative expression level of each cell line was calculated against that of A549 cells.

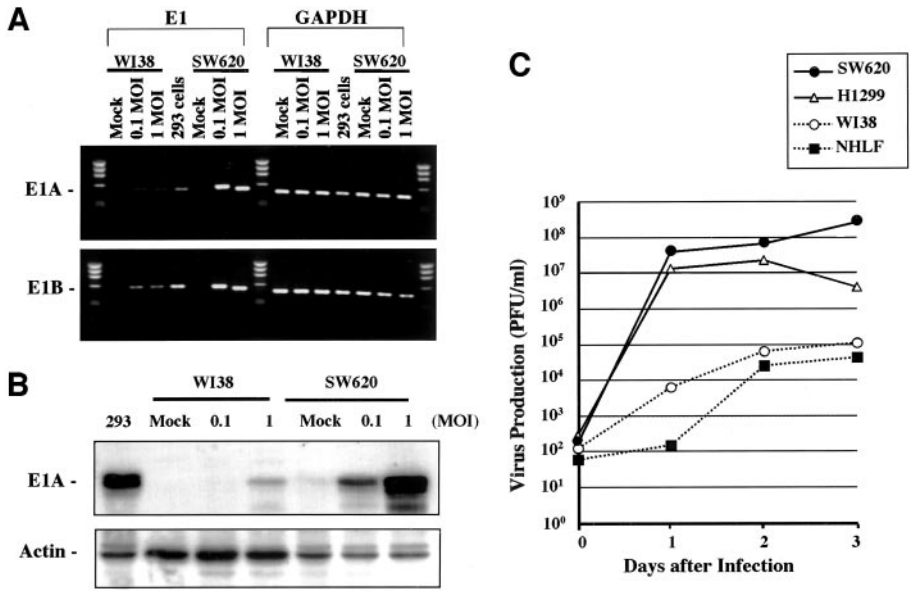

Fig. 2 Selective expression of *E1* genes and replication selectivity of tumor- or telomerase-specific replication-competent adenovirus (TRAD) in human cancer cells. *A*, reverse transcription-PCR analysis showing selective expression of *E1A* (502 bp) and *E1B* (543 bp) genes in human cancer cells. Telomerase-positive SW620 human colorectal cancer cells and telomerase-negative WI38 human fibroblasts were used. 293 cells were also used as positive controls. *B*, selective expression of E1A protein in human cancer cells. Representative immunoblot analysis of SW620 and WI38 cells with anti-E1A and anti-actin antibodies. The *top panel* represents the 45-kDa adenoviral E1A protein, and the *bottom panel* represents the 42-kDa actin loading control. *C*, replication efficiency of TRAD in human cancer (SW620 and H1299) and normal cells (WI38 and NHLF). Virus production was assessed by plaque assays after TRAD infection at a multiplicity of infection of 1.0 plaque-forming unit/cell.

Cell Killing Assay and Cell Viability Assay. Human tumor and normal cells were plated on 24-well plates at 5×10^4 cells/well 24 h before viral infection. Cells were then infected with TRAD at a multiplicity of infection (MOI) of 0.01, 0.1, 1, 2, or 5 plaque-forming units (PFU)/cell and maintained for an additional 96 h, and photomicrographs were taken at a magnification of ×100. Cells were then fixed with formalin and stained with Coomassie Brilliant Blue. A 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay was performed to measure cell viability. Briefly, cells were plated on 96-well plates at 5×10^3 cells/well 24 h before infection and infected with TRAD at a MOI of 0.01, 0.1, and 1 PFU/cell. Cell viability was determined at the times indicated by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the manufacturer's protocol.

***In Vitro* Replication Assay.** Twenty four-well plates were seeded with 5×10^4 cells/well 24 h before infection. Cells were infected with TRAD at a MOI of 1 PFU/cell. Virus inocula were removed after 2 h. The cells were washed twice with medium, and 1 ml of the medium was added to each well. The cells were incubated at 37°C for varying periods of time, scraped into culture medium, and then lysed by five freeze/thaw cycles. Serial dilutions of the lysates were titered on 293 cells.

***In Vivo* Human Tumor Model.** Human lung cancer H1299 cells (5×10^6 cells/mouse) were injected s.c. into the flank of 5–6-week old female BALB/c *nu/nu* mice and permitted to grow to approximately 5–6 mm in diameter. At that time, the mice were randomly assigned into three groups, and a 200-μl solution containing dl312, TRAD, or PBS was injected into the tumor. The H1299 tumors were measured for perpendicular diameters every 3 or 4 days, and tumor volume was calculated using the following formula: tumor volume (mm^3) = $a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine.

RESULTS

Construction of Adenovirus Containing hTERT-Controlled *E1A* and *E1B* Genes. To construct an adenovirus that replicates selectively in telomerase-positive cells, the *E1A* and *E1B* genes linked with the internal ribosome entry site (IRES) were placed under the control of the hTERT promoter. TRAD was constructed by inserting this expression cassette at the deleted E1 region of the replication-deficient adenovirus type 5 virus (Fig. 1*A*). We first examined a panel of tumors and normal cell lines for hTERT expression using a real-time reverse transcription-PCR method. All tumor cell lines including human non-small cell lung cancer A549, H226Br, and H1299 cell lines and human colorectal cancer SW620, LoVo, and DLD-1 cell lines, expressed detectable levels of *hTERT* mRNA, although the levels of expression varied widely (Fig. 1*B*). In contrast, human fibroblast cells, such as WI38 and NHLF, were negative for *hTERT* expression. Human vascular endothelial cells transformed with the SV40 large T-antigen gene transfer also exhibited no *hTERT* mRNA expression. 293 cells are known to be active for telomerase and were used as a positive control.

Replication Selectivity of TRAD in Human Cancer Cells. To assess the transcriptional activity of the hTERT promoter, SW620 and WI38 cells were infected with TRAD at the indicated MOI. A strand-specific reverse transcription-PCR assay demonstrated that TRAD-infected SW620 cells yielded strong bands of the E1A and E1B transcripts 24 h after infection, whereas the bands were extremely weak in WI38 cells infected with TRAD (Fig. 2*A*). Moderate levels of *hTERT* mRNA expression were seen in 293 cells. Densitometric quantification confirmed that TRAD infection at a MOI of 1 caused an ap-

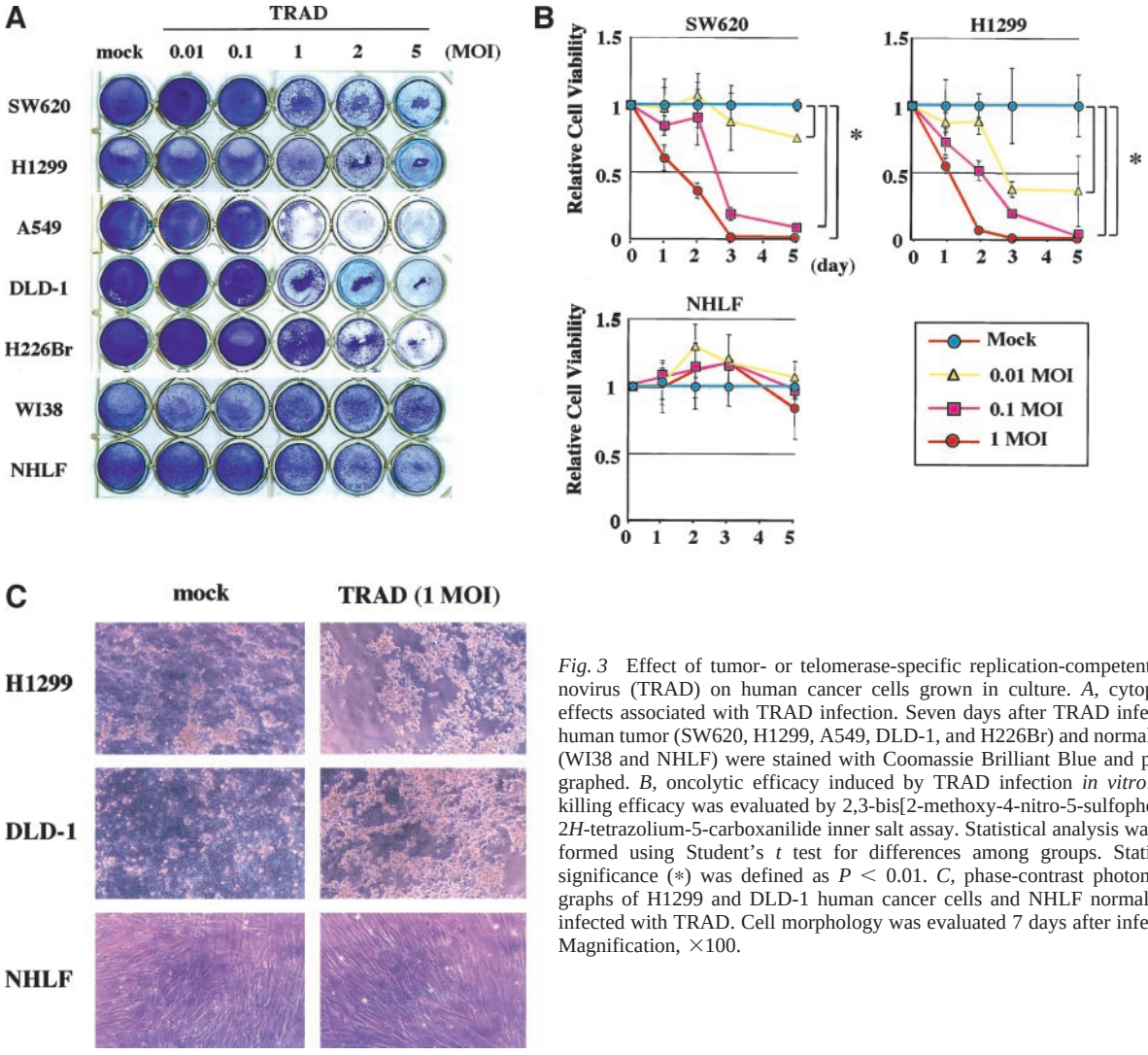

Fig. 3 Effect of tumor- or telomerase-specific replication-competent adenovirus (TRAD) on human cancer cells grown in culture. *A*, cytopathic effects associated with TRAD infection. Seven days after TRAD infection, human tumor (SW620, H1299, A549, DLD-1, and H226Br) and normal cells (WI38 and NHLF) were stained with Coomassie Brilliant Blue and photographed. *B*, oncolytic efficacy induced by TRAD infection *in vitro*. Cell killing efficacy was evaluated by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay. Statistical analysis was performed using Student's *t* test for differences among groups. Statistical significance (∗) was defined as $P < 0.01$. *C*, phase-contrast photomicrographs of H1299 and DLD-1 human cancer cells and NHLF normal cells infected with TRAD. Cell morphology was evaluated 7 days after infection. Magnification, ×100.

proximately 5- and 3-fold increase of *E1A* and *E1B* mRNA expression, respectively, in SW620 cells compared with the degree of expression in WI38 cells. To further confirm the transcriptional activity of the hTERT promoter, we conducted Western blot analysis using antibody against E1A protein. As shown in Fig. 2*B*, readily detectable E1A protein expression was achieved in a dose-dependent manner in SW620 cells 60 h after TRAD infection. In contrast, there was little, if any, detectable E1A expression in WI38 cells infected with TRAD at a MOI of 1. To evaluate the replication ability of TRAD in different cell lines, viral titers were assessed by measuring plaque formation. Human cancer cells (SW620 and H1299) and normal cells (WI38 and NHLF) were infected with TRAD at a MOI of 1 for 2 h, followed by incubation in the medium. Cells were harvested at various times during a 3-day period after infection, and the number of infectious virus particles was determined in 293 cells by standard plaque assay. In SW620 and H1299 cells, TRAD replicated 5–6 logs by 3 days after infection; TRAD replication, however, was attenuated up to 2 logs in normal WI38 and NHLF cells (Fig. 2*C*). These findings indicate that TRAD titer was reduced by 3–4 logs in normal cells compared with cancer cells.

***In Vitro* Cytotoxic Effects of TRAD in Human Cancer Cells.** To determine whether TRAD infection induces selective cell lysis, five tumor cell lines (SW620, H1299, A549, DLD-1, and H226Br) and two normal cell lines (WI38 and NHLF) were infected with TRAD at various MOIs and then stained with Coomassie Brilliant Blue 7 days after infection to visualize viable cells. All cancer cell lines were killed by TRAD in a dose-dependent fashion (Fig. 3*A*). Infection with TRAD at an MOI of 1 was sufficient to induce its lytic effects, although the sensitivity varied among the cell types. In contrast, no apparent cytopathic effects were observed in either normal cell line 7 days after TRAD infection. Cytotoxicity of TRAD was also assessed by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt cell viability assay. SW620, H1299, and NHLF cells were either mock-infected with culture medium or infected with TRAD at MOIs of 0.01, 0.1, or 1. As shown in Fig. 3*B*, in both SW620 and H1299 cells, TRAD

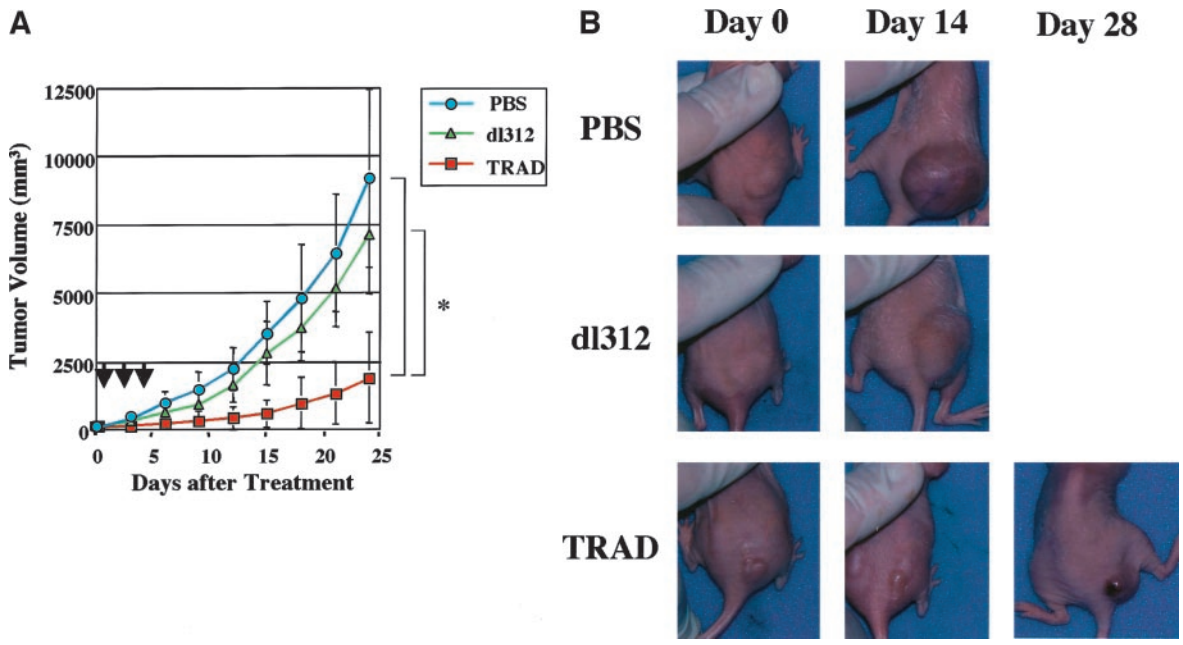

Fig. 4 Effect of tumor- or telomerase-specific replication-competent adenovirus on human cancer cells grown in *nu/nu* mice. *A*, antitumor effects of intratumorally injected tumor- or telomerase-specific replication-competent adenovirus against established flank H1299 xenograft tumors in *nu/nu* mice. Replication-deficient dl312 was used as a control. Eight mice were used for each group. Tumor growth was expressed as mean tumor volume ± SD. Statistical significance (∗) was defined as $P < 0.05$ (Student's *t* test). *B*, macroscopic appearance of H1299 tumors in *nu/nu* mice at 0, 14, and 28 days after treatment.

infection induced rapid cell death even at a MOI of 0.1 by 3 days after infection; TRAD infection, however, demonstrated less profound cytotoxicity against NHLF cells, although these cells were killed by wild-type adenovirus infection (data not shown). Phase-contrast photomicrographs also demonstrated that SW620 and H1299 cells infected with TRAD at a MOI of 1 underwent a rapid loss of viability due to massive cell death, as evidenced by floating, highly light-refractile cells, 7 days after infection, whereas NHLF cells infected with TRAD did not show any morphological changes (Fig. 3*C*).

Treatment of Human Tumor Xenografts with Intratumoral Injection of TRAD. We next assessed the therapeutic efficacy of TRAD against human tumor cells *in vivo*. H1299 cells were implanted as xenografts into the hind flank of *nu/nu* mice. Mice bearing palpable H1299 tumors with a diameter of 5–6 mm received three daily courses of intratumoral injection of 10^7 PFU of TRAD or replication-deficient adenovirus (dl312) or PBS (mock treatment). Compared with dl312 or PBS, tumor growth was significantly suppressed by TRAD injection (Fig. 4*A*). The macroscopic appearance showed that tumors treated with TRAD were consistently smaller than those of other cohorts of mice 14 days after treatment (Fig. 4*B*). Furthermore, a reddish area was noted on the tumor surface 28 days after TRAD injection, indicating that TRAD induced intratumoral necrosis of tumor cells due to direct lysis by virus replication *in vivo*.

To determine whether the virus spread beyond the tumor mass, we analyzed the tumor, distant tissues, and the blood for the presence of TRAD by PCR amplification of the *E1A* gene. When mice were sacrificed 7 days after intratumoral injection of 10^7 PFU of TRAD, *E1A* DNA was confined to the tumor; no *E1A* gene was detected in any tissue examined, including liver, kidney, spleen, small intestine, brain, heart, pancreas, ovary, and adrenal gland (Fig. 5*A*). In contrast, *E1A* DNA was amplified from blood obtained 7 or 14 days after intratumoral injection (Fig. 5*B*). These results suggest that TRAD replication is restricted to the target tumor cells *in vivo*, despite the presence of TRAD in the circulation. This biodistribution indicates the potential for TRAD to reach and then replicate in distant tumors. We also tested whether intratumoral injection of TRAD could mediate a therapeutic benefit on distant, noninjected H1299 tumors in a dual tumor model. H1299 tumors were established in the left and right flanks of *nu/nu* mice, and viral replication in the left tumors was assessed after intratumoral inoculation of TRAD into tumors in the right flank. As shown in Fig. 5*C*, TRAD replication in the tumor tissues in both flanks was demonstrated by PCR amplification of the *E1A* gene on postinfection day 14, although the amount of virus in the distant tumors was apparently less than that in injected tumors. Immunohistochemical analysis for the detection of adenoviral E1A protein also showed marked staining in both injected and noninjected tumors 14 days after treatment (Fig. 5*D*). Thus, the antitumor suppression by intratumoral injection of TRAD might be systemic rather than local.

DISCUSSION

The present study illustrates the potential application of replication-selective adenovirus as an anticancer agent. Based on knowledge of the adenoviral replication cycle, there are two

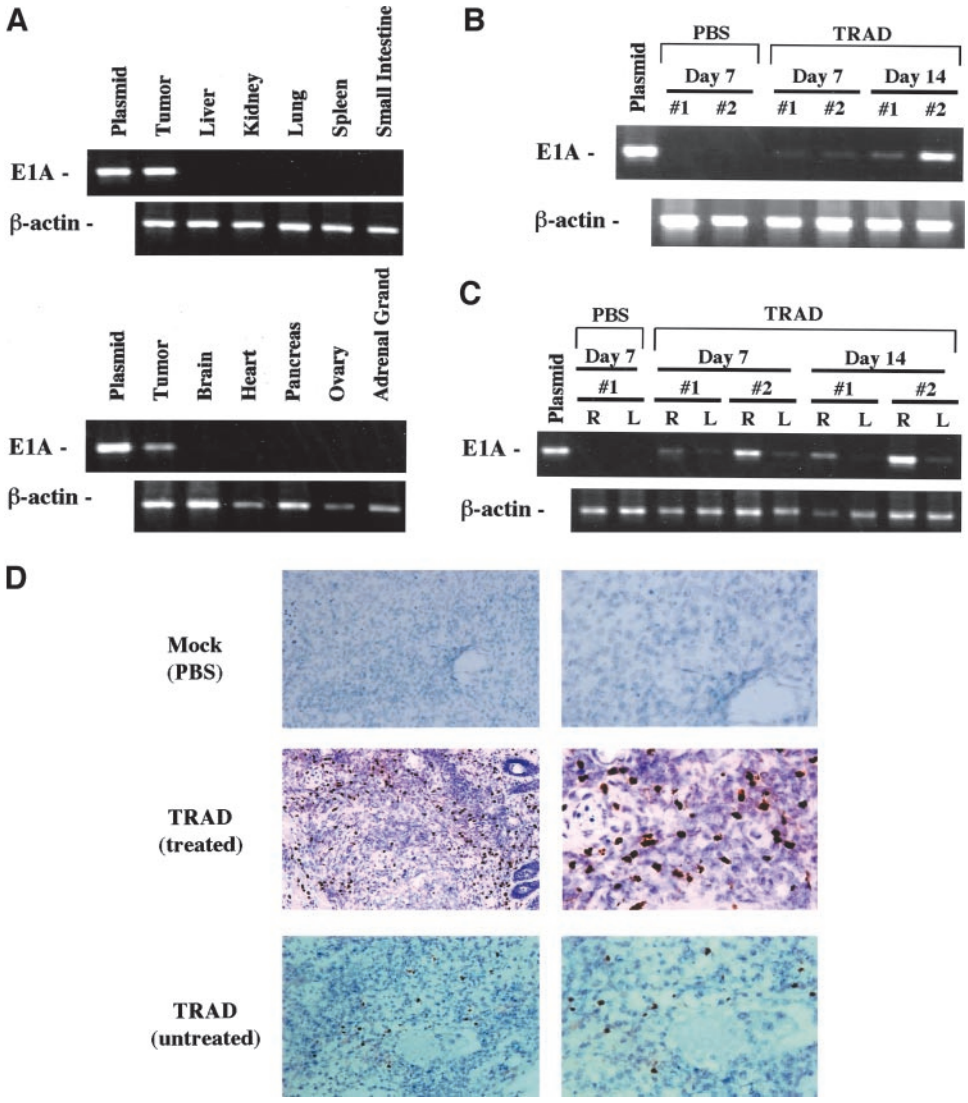

Fig. 5 Spread and replication of tumor- or telomerase-specific replication-competent adenovirus (TRAD) after intratumoral administration in *nu/nu* mice. *A* and *B*, detection of viral DNA by PCR amplification of the adenoviral E1A sequence (502 bp). *A*, DNA was prepared from s.c. tumor and various tissues in *nu/nu* mice 7 days after infection. *B*, a blood sample was also obtained 7 and 14 days after intratumoral TRAD injection and subjected to DNA extraction. A plasmid containing the adenoviral *E1A* sequence was used as a positive control. The *top panel* represents the *E1A* gene, and the *bottom panel* represents the β-actin loading control. *C* and *D*, replication of TRAD in the tumor distant from the site of virus inoculation. *C*, PCR amplification of the *E1A* gene with DNA isolated from the right flank (injected) and left flank (noninjected) H1299 tumors. *D*, sections immunostained with anti-E1A antibody show amplified E1A protein expression in both the right flank (injected) and left flank (noninjected) H1299 tumors 14 days after TRAD injection. Magnification, ×100.

types of approaches to restrict its replication to tumor cells. ONYX-015 is an adenovirus with a deleted *E1B* 55-kDa gene, which replicates and causes cell killing in p53-deficient tumor cells, but not in normal cells (18). However, many studies have demonstrated that replication of ONYX-015 is not strictly linked to the deficiency of p53 and that ONYX-015 also replicates in normal human primary cells (19, 20). An alternate strategy to obtain tumor-specific adenoviral replication has been developed by using heterologous promoters that regulate transcription of the *E1A* gene. In this context, the promoters from the prostate-specific antigen (21), MUC1 (22), osteocalcin (23), L-plastin (24), midkine (25), and E2F-1 (26) genes have been used to drive *E1A* expression. These vectors replicate preferentially in tumor cells that express each targeted tumor marker; their therapeutic window, however, is relatively narrow because only part of the tumor is positive for each tumor marker.

Telomerase is expressed in a majority of human cancers, and its activation plays a critical role in tumorigenesis by sustaining cellular immortality (4), suggesting that the hTERT promoter is preferentially activated in most human cancer cells. Thus, the broadly applicable hTERT promoter might be a suitable regulator of adenoviral replication. In fact, it has been reported recently that transcriptional control of E1A expression via the hTERT promoter could restrict adenoviral replication to telomerase-positive tumor cells and efficiently lyse tumor cells (27). The *E1A* gene has been shown to have tumor-suppressive activity including transcriptional repression of the HER-2/neu proto-oncogene and induction of apoptosis, suggesting that *E1A* gene transfer alone might be sufficient to kill host cells (28). 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 shutoff of host cell protein synthesis. In most vectors that replicate under the transcriptional control of the *E1A* gene, the *E1B* gene is driven by the endogenous adenovirus E1B promoter. Recent studies have demonstrated that transcriptional control of both *E1A* and *E1B* genes by the α-fetoprotein promoter with the use of IRES significantly im-

proved the specificity and therapeutic index in hepatocellular carcinoma cells (29). These observations indicate that the TRAD we developed, in which the hTERT promoter regulates both the *E1A* and *E1B* genes, may control viral replication more stringently, thereby providing profound therapeutic effects in tumor cells as well as attenuated toxicity in normal tissues.

Our data clearly demonstrated that TRAD exhibited desirable features for use as an oncolytic therapeutic agent. TRAD displayed selective attenuation in normal cells and potent lytic activity in tumor cells both *in vitro* and *in vivo*. The finding that all human cancer cell lines examined in our study were susceptible to TRAD infection *in vitro* suggests that the proportion of cancers potentially treatable by TRAD is high. Although the transduction efficiency of adenovirus is less efficient in normal cells compared with tumor cells, the observation that wild-type adenovirus infection effectively killed normal fibroblasts (data not shown) suggests that attenuated cytotoxicity of TRAD in normal cells is due to tumor-specific replication, but not due to the low transduction. Moreover, we found that, when injected intratumorally, TRAD replicated within tumors, spread into the bloodstream, and then replicated in distant tumor sites. Although modest growth inhibition compared with mock-treated tumors was observed in noninjected, distant tumors, the difference was not significant (data not shown). These results suggest that microscopic tumors that mimic the clinical situation of residual or micrometastatic tumors might be a better target for TRAD treatment. Indeed, systemic administration of TRAD would be a more clinically relevant approach for metastatic disease. The biodistribution of TRAD as assessed by PCR amplification provides evidence that TRAD replication is highly specific for tumors despite its presence in the circulation. None of the mice treated with TRAD showed signs of viral distress (ruffled fur, weight loss, lethargy, or agitation) or histopathological changes in any organs at autopsy (data not shown).

In conclusion, our data clearly indicate that TRAD displays an acceptable toxicity profile as well as a therapeutic oncolytic activity not only for primary tumors but also for metastatic tumors. Additional clinical studies will be required to address the issues of safety and efficacy of TRAD-mediated virotherapy for human cancers.

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