Modulation of Telomerase Promoter Tumor Selectivity in the Context of Oncolytic Adenoviruses

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Abstract

The telomerase RNA (hTR) and reverse transcriptase (hTERT) promoters are active in most cancer cells, but not in normal cells, and are useful for transcriptional targeting in gene therapy models. Telomerase-specific conditionally replicating adenoviruses (CRAd) are attractive vectors because they should selectively lyse tumor cells. Here, we compare CRAds, in which either the hTR or hTERT promoter controls expression of the adenovirus E1A gene. In replication-defective reporter adenoviruses, the hTERT promoter was up to 57-fold stronger in cancer cells than normal cells and up to 49-fold stronger than hTERT. In normal cells, hTERT promoter activity was essentially absent. Doses of telomerase-specific CRAds between 1.8 and 28 infectious units per cell efficiently killed cancer cells, but normal cells required higher doses. However, CRAd DNA replication and E1A expression were detected in both cancer and normal cells. Overall, tumor specificity of the CRAds was limited compared with non-replicating vectors. Surprisingly, both CRAds expressed similar E1A levels and functional behavior, despite known differentials between hTR and hTERT promoter activities, suggesting that the promoters are deregulated. Rapid amplification of cDNA ends analysis of hTR-/hTERT-E1A transcripts ruled out cryptic transcription from the vector backbone. Blocking E1A translation partially restored the hTR-/hTERT-E1A mRNA differential, evidencing feedback regulation by E1A. [Cancer Res 2007;67(3):1299–307]

Introduction

Telomerase is a ribonucleoprotein reverse transcriptase minimally composed of RNA (hTR) and reverse transcriptase (hTERT) subunits, which counteracts cell division–associated telomere attrition and is a causative factor underlying immortalization of the majority of human cancer cells (1, 2). Telomerase is essential in most human cancers but is not expressed in most normal tissues (3–7). Therefore, it is widely regarded as a highly attractive target in cancer therapy.

Selective telomerase expression in cancer cells is mainly the result of aberrant transcription of hTR and hTERT; both transcripts are easily detectable in cancer cells but are either absent or at low levels in normal cells when assayed by appropriate methodologies (8–13). Interestingly, the cloned hTR and hTERT promoters also show selective activity in cancer cells with the hTR promoter usually stronger than hTERT (14–19). Several groups have used the hTR and hTERT promoters for broad spectrum transcriptional targeting of cancer cells in gene therapy models with encouraging results (refs. 14–25; reviewed in ref. 26).

However, hTR and hTERT promoter activities are relatively weak in some cancer cells, which could prove limiting for approaches, such as enzyme/prodrug therapy, in which there is a good correlation between transgene expression levels and therapeutic effect (15, 17). Thus, there is a theoretical requirement to improve the efficiency of telomerase-specific targeting while retaining the specificity (14, 27).

Cancer gene therapy is also limited by poor biodistribution of vector, even within tumor models (15, 28, 29). It has been proposed that conditionally replicating adenoviruses (CRAd) may provide a solution: CRAds enable virus replication and cytolysis to be targeted specifically in tumor cells allowing viral progeny to be released into the tumor milieu and theoretically spread throughout target tumors. Wild-type (WT) adenovirus displays a limited oncolytic activity and has been tested in early cancer clinical trials (30). However, it is not selective enough to be considered a viable therapy, and more recent studies have attempted to improve the specificity through engineering CRAds. Most commonly, CRAds have been constructed by harnessing expression of the multifunctional adenovirus E1A gene, which is essential for the virus transcriptional program and for replication (31, 32). Several groups have reported recently the development of hTERT-specific CRAds (examples can be found on refs. 33–38; reviewed in ref. 26). No study has yet reported the development of an hTR-specific CRAd. In this context, the aim of this study was to compare the efficacy of CRAds, in which the selectivity of replication is conferred by either the hTERT or the hTR promoter.

hTERT and hTR promoters showed predictable activities comparable with those obtained in other model systems in normal and cancer cells when cloned in a nonreplicating reporter virus. Importantly, the hTR promoter was always stronger than hTERT. Surprisingly, however, in the oncolytic system, both hTR- and hTERT-specific CRAds showed similar cytopathic effects, had similar DNA replication kinetics, and expressed similar levels of E1A. Critically, E1A expression, replication, and cytotoxicity were detected in both normal and cancer cells. We show here that E1A expression mediates feedback regulation of both promoters in the CRAd context, resulting in altered promoter activities.

Materials and Methods

Cell lines, viruses, and reagents. The cells used in this study were A2780 ovarian adenocarcinoma cells (p53 WT), W138 normal fibroblasts (p53 WT), C33A cervical carcinoma cells (p53 mutant), 5637 bladder carcinoma cells (p53 mutant), HT29 colon carcinoma cells (p53 mutant), A549 lung adenocarcinoma cells (p53 WT), HCT116 colon cancer cells
(designated HCT116+/+, p53 WT), and their derivatives HCT116−/− (homozygous p53 knockout). Cycloheximide was obtained from Merck Biosciences Ltd. (Nottingham, United Kingdom) and used at a concentration of 10 μg/mL.

Ad-hTR-Luc and Ad-hTERT-Luc contain luciferase cDNA from pGL3 (Promega, Madison, WI) and 867- and 572-bp fragments of the hTR and hTERT promoters, respectively, which have been shown to direct expression of transgenes in tumor cells (15, 17). The expression cassettes were subcloned into pShuttle using XhoI/XhoI digest. Ad-hTR-E1A and Ad-hTERT-E1A contain the same promoter fragments inserted upstream of E1A. The expression cassettes were subcloned in pShuttle using XhoI/BglII digest. All cassettes were cloned in a left-to-right orientation, and viruses were constructed using the AdEasy system (Qbiogene, Livingstone, United Kingdom; ref. 39). Ad-CMV-LacZ was purchased from Qbiogene. All vectors were amplified on 293 cells and purified and quantified using BD Biosciences (Oxford, United Kingdom) AdenoX virus purification and rapid titre kits according to the manufacturer's instructions.

**Infectivity assays.** The LacZ reporter assay has been described previously (15). Briefly, cells were pulse infected (1 h) with a titration of Ad-CMV-LacZ and then incubated for 24 h before fixation and LacZ staining using 20 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 2.5 mM/L K3Fe(CN)6, and 2.5 mM/L K4Fe(CN)6. Proportions of blue cells were assessed by counting 500 to 1,000 cells. All experiments were repeated at least twice.

For analysis of viral DNA uptake, all cell lines were pulse infected (1 h) in parallel with 1 infectious unit (i.u.) per cell of each of four independently titred viruses (Ad-hTR-Luc, Ad-hTERT-Luc, Ad-WT, and a nonreplicating control). Following infection, cells were incubated to allow nuclear trafficking of intracellular virus. At 1 h of postinfection, cells were rinsed extensively and trypsinized to remove extraneous virus, and genomic DNA was isolated. The presence of the adenovirus penton and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences were isolated. The presence of the adenovirus penton and cellular GAPDH genomic DNA was detected with the primers 5′-CTACCGGTGTTCTGGTTTC-3′ and 5′-TTTGATCTCTTGATCACTGAA-3′. A1 and E1A mRNA was monitored using the primers 5′-AGAAGGCGGACGTCAGAC-3′ and 5′-CACCACACTCTCAGGCAACTC-3′. Reactions were done in triplicate and normalized to GAPDH mRNA levels. Cycloheximide did not affect GAPDH levels (data not shown). All experiments were repeated four times, and the QPCRs were done twice.

**5′ Rapid amplification of cDNA ends.** Amplification of transcript 5′ ends from C33A cells pulse infected with 100 i.u. per cell Ad-hTR-E1A or Ad-hTERT-E1A was accomplished using the Smart-RACE kit obtained from Takara Bio Europe (Saint Germain-en-Laye, France) according to the manufacturer’s instructions. The PCR step used the universal primer included in the kit, together with the E1A-specific primer 5′-ACAGTCTGT-GAAGGGTTAGTGGG-3′, for 5′ end amplification. Reaction products were subcloned in a TOPO-TA cloning vector (Invitrogen) and sequenced using the M13 primer set provided.

**Results**

**Infectivity of cell lines.** We first determined the infection efficiency of all cell lines using the replication-defective reporter adenovirus Ad-CMV-LacZ. All cells were easily transduced by adenovirus, with several cell lines showing 100% infection efficiency at 100 i.u. per cell (Fig. 1A). At lower doses of 10 i.u. per cell and 1 i.u. per cell, WI38 cells were more easily infected than the other cells, whereas A2780 cells were quite refractory. None of the mock-infected cells showed any staining (data not shown).

Because the LacZ reporter assay does not take account of the amount of virus construct internalized in cell lines, which may directly contribute to transgene expression levels in a cell population, we also did QPCR to determine relative uptake of adenovirus penton DNA after parallel pulsed infections with four independent adenoviruses. Uptake of individual constructs was similar in individual cell lines, and the combined (mean) results for all four adenoviruses. All experiments were repeated twice.

**Promoter activity assays.** Cells were seeded in quadruplicate in 96-well luminometer plates and pulse infected (1 h) with titrations of Ad-hTR-Luc or Ad-hTERT-Luc. Postinfection, cells were incubated for a further 48 h before luciferase assay (Promega). All experiments were repeated at least thrice. Promoter activities were normalized to adenosiral DNA uptake in each cell line.

**Western blotting.** Cells were mock infected or were pulse infected (1 h) with 100 i.u. per cell of Ad-hTR-E1A, Ad-hTERT-E1A, Ad-WT, or the nonreplicating vector Ad-SV40-Luc. Postinfection, the cells were incubated for a further 48 h before harvesting and protein extraction. Fifty micrograms of protein equivalent were separated on 10% Bis-Tris gels and transferred to nitrocellulose membranes. The membranes were blocked in PBS-Tween 20 containing 5% nonfat dried milk. Filters were probed with antibodies raised against E1A (1:1,000), extracellular signal-regulated kinase (ERK) 1/2 (1:3,000), and adenovirus hexon protein (1:2,000) obtained from Autogen Bioceil UK Ltd. (Wiltshire, United Kingdom).

**Cytotoxicity assays.** Cells were seeded in triplicate wells and seeded with 96-well plates with titrations of Ad-hTR-E1A, Ad-hTERT-E1A, Ad-WT, or Ad-SV40-Luc added to the cell suspension at the time of seeding and incubated for 7 days before 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Dorset, United Kingdom) assay. Virus was not removed during the incubation period. At the end of the incubation, the MTT reduction assays were done using Softmax Pro 4.6 software (Molecular Devices Ltd., Wokingham, United Kingdom). All experiments were repeated at least thrice.

**QPCR analysis.** To quantify viral DNA replication, cells were infected with 1 i.u. per cell of CRAds, Ad-WT, or Ad-SV40-Luc for a period of 0 to 5 days. Postinfection, cells were rinsed extensively and trypsinized to remove extraneous virus. Genomic DNA was prepared from cell pellets, and QPCR was done in triplicate using GRI Opticon monitor equipment and software (Genetic Research Instrumentation, Essex, United Kingdom) as described previously (40). Penton DNA was monitored using the primers 5′-GAGG-CAAGCACAGACACATC-3′ and 5′-GCTTTCCTTGCTCCTGTC-5′, and cellular GAPDH genomic DNA was detected with the primers 5′-ACACGTCATGCATCAC-3′ and 5′-TCCACACCCGTGCTCTGTA-5′. Penton DNA was normalized with GAPDH at each time point. All experiments were repeated twice.

For analysis of E1A and luciferase mRNA levels, cells were pretreated with cycloheximide for 1 h and then pulse infected for 1 h with Ad-hTR-E1A, Ad-hTERT-E1A, Ad-hTR-Luc, or Ad-hTERT-Luc. Postinfection, the cycloheximide block was replenished for a further 24 h before RNA extraction. Luciferase mRNA was monitored using the primers 5′-CTACCGGTGTTCTGGTTTC-3′ and 5′-TTTGATCTCTTGATCACTGAA-3′, and E1A mRNA was monitored using the primers 5′-AGAAGGCGGACGTCAGAC-3′ and 5′-CACCACACTCTCAGGCAACTC-3′. Reaction products were done in triplicate and normalized to GAPDH mRNA levels. Cycloheximide did not affect GAPDH levels (data not shown). All experiments were repeated four times, and the QPCRs were done twice.

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Most of the cells showed similar uptake of adenovirus. In contrast, C33A cells internalized 7-fold more adenovirus DNA than the HCT116−/− cells, and WI38 cells were the most efficiently infected cells, internalizing >21-fold more adenovirus DNA than HCT116−/− cells. Thus, all cell lines were easily infected with adenovirus, although WI38 and C33A cells took up substantially more construct than the other cell lines.

**Telomerase promoter activities of cell lines.** We next determined the relative activities of the hTR and hTERT promoters in all cell lines by luciferase assay using pulsed infection of a titration of the replication-defective reporter viruses Ad-hTR-Luc and Ad-hTERT-Luc (Fig. 1C). Data shown are the means and SEs recorded at 1 i.u. per cell derived from three independent experiments normalized to viral DNA uptake.

As reported previously, the hTR promoter had strong activity in cancer cells but was weak in WI38 cells. The hTERT promoter was
also significantly stronger than the hTERT promoter in all cancer cell lines analyzed. Within the panel, hTR promoter activity ranged from 8-fold greater than that of hTERT in C33A cells to 49-fold greater in 5637 cells. These results were similar to those obtained in our previous analysis of the ratio of activities of the two promoters using transfected plasmid DNA vectors (17). Therefore, the relative activities of hTR and hTERT promoters in cancer cells were predictable compared with previous results when cloned in the deleted E1 region.

![Figure 1](image-url)

**Figure 1.** Infectivity and telomerase promoter activities in cell lines. A, Ad-CMV-LacZ infectivity assay. Cells were pulse infected with a titration of Ad-CMV-LacZ (1, 10, or 100 i.u. per cell). Twenty-four hours postinfection, cells were fixed and stained overnight for LacZ expression. Infectivity was assessed by counting the percentage stained cells in five random fields (500–1,000 cells). The experiment was repeated twice. Columns, mean of both experiments; bars, SE. B, uptake of adenovirus DNA in cell lines. Cells were pulse infected with 1 i.u. per cell of Ad-WT, Ad-hTR-E1A, Ad-hTERT-E1A, and the nonreplicating vector Ad-SV40-Luc in parallel. Penton and cellular GAPDH DNA levels were monitored by QPCR at 1 h postinfection. For an independent experiment, relative uptake in each cell line was the mean penton value calculated for all viruses. The experiment was repeated twice. Columns, mean of both experiments; bars, SE. C, hTR and hTERT promoter activities in cell lines. Cells were pulse infected in quadruplicate with a titration Ad-hTR-Luc or Ad-hTERT-Luc (0.01–100 i.u. per cell). Luciferase activities were determined at 48 h postinfection and normalized to adenoviral DNA uptake in each cell line. All experiments were repeated thrice. Results were obtained at 1 i.u. per cell. Columns, mean of three independent experiments; bars, SE. D, cytotoxicity of Ad-hTR-E1A and Ad-hTERT-E1A in cancer cell lines at 10 i.u. per cell. MTT assays were done on Ad-hTR-E1A– and Ad-hTERT-E1A–infected cells at 7 d postinfection. The surviving cell fraction was estimated relative to mock-infected cells for MOI of 10, at which concentration effects on the normal fibroblasts WI38 were limited. Columns, mean of three independent experiments; bars, SE.

| Table 1. IC50 values at 7 d postinfection |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell line       | Ad-hTR-E1A, mean (SE) | Ad-hTERT-E1A, mean (SE) | AD-WT, mean (SE) | Nonreplicating, mean (SE) |
| A2780           | >100             | >100             | 4.01 (0.29)     | >100             |
| C33A            | 8.64 (4.28)      | 7.12 (3.07)      | <0.01           | 43.3 (17.01)     |
| A549            | 16.65 (2.09)     | 10.78 (1.87)     | 0.09 (0.13)     | >100             |
| 5637            | 19.54 (1.36)     | 16.22 (1.0)      | 0.53 (0.04)     | >100             |
| HT29            | 28.28 (9.99)     | 15.34 (3.74)     | <0.01           | 19.29 (6.44)     |
| HCT116−/−       | 2.15 (0.59)      | 1.77 (0.37)      | <0.01           | 51.8 (4.0)       |
| HCT116+/+       | 14.06 (1.76)     | 13.13 (1.21)     | <0.01           | >100             |
| WI38            | 88.2 (1.84)      | 75.36 (4.72)     | 1.35 (0.07)     | >100             |

NOTE: Cells were mock infected or incubated in the presence of a titration of each virus for 7 d. IC50 values were determined by MTT assay relative to mock-infected cells. Each experiment was done thrice, and IC50 values reported were mean ± SE derived from the three independent experiments.
luciferase activities showed a linear increase with increasing multiplicity of infection (MOI) in all cells, including WI38 (data not shown). Therefore, at high dose, above-background hTERT promoter activity was also detected in WI38 cells, although the overall pattern of hTR/hTERT promoter activities across the cell panel was similar to Fig. 1C. Thus, hTR and hTERT promoters retain their desirable properties (that is, specificity for cancer cells) in the context of an E1 deleted adenoviral genome.

**Cytotoxicity of replicating vectors.** To examine cytotoxic effects mediated by telomerase-specific expression of E1A, we infected cells with a titration of Ad-hTR-E1A, Ad-hTERT-E1A, Ad-WT, or a nonreplicating control vector. Cells were incubated in the presence of each virus for 7 days before analysis of cell survival by MTT assay. Figure 1D shows cell survival at 10 i.u. per cell. Figure 2 shows kill curve results for all cell lines, and Table 1 shows IC50 values. All experiments were repeated thrice and each data point represents the mean of three independent experiments.

In all cell lines, Ad-WT was the most potent cytotoxic agent (Fig. 2). In contrast, the nonreplicating vector had little or no effect in most cell lines, although both HCT116 subclones and C33A cells showed toxicity at 100 i.u. per cell, likely indicating an intrinsic sensitivity to infection by high-dose adenovirus. A2780 cells were most resistant to killing by the CRAds, but WI38 cells were also refractory at low dose (Table 1). HCT116+/− and C33A cells were the most sensitive to killing by this approach, showing IC50 values of 2.2 and 1.8 i.u. per cell for hTR and hTERT (HCT116+/−) and 8.6 and 7.1 i.u. per cell (C33A). IC50 values for the other cancer cells were in the range 10 to 28 i.u. per cell, averaging 16.7 i.u. per cell.

At 10 i.u. per cell, no toxicity was observed after infection of WI38 cells with the CRAds (Fig. 1D). In contrast, the cancer cells showed a range of mild to severe toxicity. For Ad-hTR-E1A, HCT116+/− and C33A cells were most sensitive, showing only 10% and 44% survival, respectively. HCT116+/− and A549 cells were moderately affected, showing 64% and 67% survival, whereas HT29 and 5637 cells were only mildly affected, showing 80% and 82% survival. A2780 cells were totally unaffected at this dose. Similar results were obtained with Ad-hTERT-E1A. Therefore, most cancer cells were killed more efficiently by the telomerase-specific CRAds than WI38 cells, but the differential was lost at higher MOI.

Interestingly, no differential was observed between cytotoxicity of Ad-hTR-E1A and Ad-hTERT-E1A in any of the cancer cell lines (Fig. 2). Therefore, the relative activities and desirable properties of the hTR and hTERT promoters in the nonreplicating system do not correlate quantitatively with the cell killing effect mediated by the CRAd system. Because E1A itself can exhibit antineoplastic effects (31, 41), it was conceivable that both CRAds achieved a low threshold level of E1A expression, sufficient to result in cell killing independently of viral replication. We therefore examined replication of viral DNA in infected cells.

**Replication rates of viral vectors.** To determine the replication kinetics of the CRAds, accumulation of the penton gene sequence in cellular genomic DNA over time was monitored by real-time PCR. Cells were initially infected with 1 i.u. per cell of Ad-hTR-E1A, Ad-hTERT-E1A, Ad-WT, or a nonreplicating control and incubated continuously in the presence of viral constructs until harvesting at 1 h of postinfection (T0) or at 24-h intervals thereafter for a further 4 to 5 days. The experiment was repeated twice with similar results, and Fig. 3 shows a representative plot for each cell line.

Within individual cell lines, similar quantities of each vector were detected at T0. In most cells, the amount of penton in the cellular genomic DNA increased slightly between T0 and T1 for all vectors, including the nonreplicating control, presumably indicating that the infection efficiency was better after 24 h of infection than 1 h. Replication of Ad-WT was rapid, generally reaching plateau between 2 to 3 days postinfection, at which time all cells showed a 3 to 4 log increase in penton DNA. Post-T1, the E1-deleted vector showed no significant accumulation of penton DNA in most cells (<1.8-fold increase). However, in HCT116+/− and HT29 cells, mild increases were observed. In contrast, penton DNA accumulated over the time course, albeit relatively inefficiently, in most of the Ad-hTR-E1A and Ad-hTERT-E1A infected cells.

CRAd DNA replication was most efficient in HCT116+/− cells, with 186- and 363-fold increases of Ad-hTR-E1A and Ad-hTERT-E1A DNA, respectively, evident between T1 and T4. More modest replication in the range of 7- to 17-fold increases of each vector were detected between T1 and T4.5 in HCT116+/−, A549, C33A, and HT29 cells. Interestingly, WI38 cells also showed clear evidence of relatively efficient CRAd DNA replication, with Ad-hTERT-E1A (69-fold increase), surprisingly, replicating to greater levels than Ad-hTR-E1A (13-fold increase). No significant replication of either CRAd was observed over the period T1 to T5 in A2780 or 5637 cells (<1-fold), although mild toxicity had been noted in 5637 cells.

**Expression of viral proteins in cell lines.** We next determined the expression levels of E1A and hexon in cells pulse infected by 100 i.u. per cell WT adenovirus, nonreplicating virus, or Ad-hTR-E1A by Western blot analyses. Western blots of cellular lysates from cell lines infected with Ad-WT, Ad-hTR-E1A, or Ad-hTERT-E1A were probed with anti-E1A or anti-hexon antibodies. As shown in Fig. 4, both the adenoviral E1A and hexon proteins were expressed in all the cell lines. The level of E1A expression was highest in the Ad-hTR-E1A-infected cells, followed by Ad-hTERT-E1A and Ad-WT-infected cells. The hexon protein was expressed at lower levels in all the cell lines, with the highest expression in Ad-hTR-E1A-infected cells.
Therefore, the efficiencies of hTR- or hTERT-mediated transgene in some cell lines, hTERT-specific E1A expression appeared slightly E1A expressed similar levels of E1A in all cancer cell lines, although prerequisite for efficient replication. Ad-hTR-E1A and Ad-hTERT-E1A. 293 cell extracts and denatured purified hexon expression in Ad-hTERT-E1A–infected WI38 cells. Consequently, Ad-hTR-E1A– and Ad-hTERT-E1A–infected cells frequently expressed both fast- and slow-migrating 12s species, whereas Ad-WT–infected cells usually expressed only fast-migrating 12s (e.g., HCT116(−)). Whether altered distribution of these species mediates a significant functional effect on behavior of the CRAd system is unclear at this time.

Ad-WT–infected cells showed stronger expression of hexon than Ad-hTR-E1A– and Ad-hTERT-E1A–infected cells. Hexon expression therefore correlated more closely with the cytotoxic effect of each virus in individual cell lines than did E1A expression. For example, A2780 and 5637 cells showed no evidence of hexon expression after Ad-hTR-E1A and Ad-hTERT-E1A infection (in A2780 cells, a very weak hexon band was present in Ad-WT–infected cells after prolonged exposure; data not shown). Consistent with this observation, A2780 and 5637 also showed no evidence of DNA replication after Ad-hTR-E1A and Ad-hTERT-E1A infection and A2780 were extremely resistant to cytotoxicity induced by the CRAds, although 5637 showed mild effects (Table 1).

By contrast, C33A, A549, and HCT116(−) cells showed strong expression of hexon after Ad-hTR-E1A and Ad-hTERT-E1A infection and also showed relatively efficient DNA replication. Weaker hexon expression was also detected in HCT116(−) and WI38, both of which also showed evidence of DNA replication. In-keeping with lower E1A expression from the hTERT promoter in WI38 cells, hexon expression in Ad-hTERT-E1A–infected WI38 cells was weaker than that in Ad-hTR-E1A–infected cells. However, replication of the Ad-hTERT-E1A genome was more efficient than Ad-hTR-E1A in these cells. Therefore, the ability to replicate the CRAd genomes was qualitatively, but not quantitatively, correlated with the ability to express late gene products, although E1A expression alone did not guarantee late gene expression, replication, or cytotoxicity (as in 5637 and A2780).

Regulation of the telomerase promoters in the CRAd background. Surprisingly, the hTR- and hTERT-specific CRAds expressed similar levels of E1A and behaved similarly in all functional assays despite large differences in hTR and hTERT promoter activity in the nonreplicating system. To rule out the possibility that hTR and hTERT promoter differentials are lost as functional assays despite large differentials in hTR and hTERT transcription initiation sites that have been mapped previously in the CRAd system is unclear at this time.
promoters (Fig. 5f; references contained in ref. 15). Furthermore, gel electrophoresis of each 5’ RACE PCR product showed a single band of the correct size with no significant larger species (data not shown). Together, these data argue against the presence of a cryptic upstream promoter activity.

The discrepancies between observed transgene expression levels from the hTR and hTERT promoters in the CRAd versus nonreplicating systems suggested that the lack of differential E1A expression could be specifically related to E1A expression itself. We reasoned that if feedback regulation by E1A alters the behavior of the promoters in these CRAds, then E1A mRNA levels should be similar in Ad-hTR-E1A– and Ad-hTERT-E1A–infected cells. Additionally, blockage of the expression of E1A protein with cycloheximide should result in recovery of the hTR/hTERT–specific differential expression of E1A mRNA detectable by QPCR.

As shown in Fig. 5B, cycloheximide efficiently blocked expression of E1A protein in CRAd-infected A549 cells. Figure 5C shows the expression of E1A or luciferase mRNA 24 h postinfection with 50 i.u. per cell Ad-hTR-E1A and Ad-hTERT-E1A or Ad-hTR-Luc and Ad-hTERT-Luc in the presence or absence of cycloheximide (data are presented relative to Ad-hTR-E1 or Ad-hTR-Luc vectors in the absence of cycloheximide). In the absence of cycloheximide, E1A expression by Ad-hTERT-E1A was 157% of the level of Ad-hTR-E1A, in agreement with the Western data, which showed a slightly stronger E1A protein signal for Ad-hTERT-E1A than Ad-hTR-E1A (Fig. 4 and 5f). In the presence of a cycloheximide block, hTR-E1A mRNA expression increased by ∼3-fold, suggesting that the hTR promoter is suppressed in this system, whereas hTERT-E1A expression decreased to 72% of that detected in the absence of cycloheximide (Fig. 5C). Thus, when cells were infected with the CRAds in the presence of a cycloheximide block, Ad-hTERT-E1A–specific expression of E1A mRNA decreased from 157% to 42% of the level of Ad-hTR-E1A (Fig. 5D). The shift in ratio hTERT/hTR of about 4-fold was highly significant (P < 0.01).

In contrast, hTERT promoter-specific expression of luciferase mRNA in the nonreplicating system was 3.5% (±0.8%) that of the hTR promoter (in good agreement with the luciferase assay data in Fig. 1C). When a cycloheximide block was applied to Ad-hTR-Luc– or Ad-hTERT-Luc–infected cells, the luciferase mRNA expression levels of both promoters decreased by 2.6- and 3.6-fold, respectively (Fig. 5C). Interestingly, hTERT-Luc mRNA decreased much more sharply than hTERT-E1A mRNA (3.6-fold versus ∼1.3-fold), suggesting that residual E1A may continue to activate the hTERT promoter even under the blockade. Because both luciferase mRNAs decreased, the ratio of Ad-hTR-Luc/Ad-hTR-Luc–specific expression was not significantly altered in cycloheximide-treated cells (2.4 ± 0.5%; Fig. 5D). Therefore, the shift in the hTR/hTERT ratio of E1A expression in the CRAd-infected cells in the presence of cycloheximide cannot be attributed to inhibition of the expression of a cellular protein and must reflect inhibition of feedback regulation by E1A.

Discussion

Telomerase-specific gene therapy is an extremely promising anticancer approach because it should provide a relatively safe and selective approach to cancer cell killing. However, like other gene therapy strategies, targeting with telomerase-specific constructs may be limited by factors, such as poor construct biodistribution and transgene expression, in target tumors. CRAds are an attractive solution because both the levels and the biodistribution of therapeutic construct increase over time within target cells and tumors.

This is the first report of an hTR-specific CRAd, although several previous studies have examined hTERT-specific CRAds (examples can be found on refs. 33–38; reviewed in ref. 26). In nonreplicating systems, the stronger hTR promoter is able to confer greater transgene expression than hTERT in target cells, resulting in more efficacious cell killing (15, 17, 19). Our main objective in this study was to directly compare hTR- and hTERT-specific expression of

**Figure 4.** Western blot analysis of E1A and hexon expression in infected cells. Cells were mock infected or pulse infected with 100 i.u. per cell of Ad-hTR-E1, Ad-hTERT-E1, Ad-WT, or nonreplicating control. Forty-eight hours postinfection, cells were harvested for protein extraction. Blots were probed for expression of E1A, hexon, or ERK (as loading control). Each blot included the following internal controls: 15 μg HEK293 cell extracts and 10⁶ i.u. purified recombinant adenoviruses (Ad). Representative blot for each cell line. All experiments were repeated at least twice.
E1A to determine whether either promoter confers an advantage in the CRAd system.

Using nonreplicating Ad-hTR-Luc and Ad-hTERT-Luc vectors, we first showed that both promoters are active in cancer cells but are inactive or extremely weak in normal fibroblasts and that the hTR promoter is substantially stronger than hTERT. Importantly, the observed hTR/hTERT differential in each cell line was consistent with the known behavior of these promoters using plasmid or adenoviral vectors expressing luciferase, Cre recombinase, bacterial nitroreductase, tumor necrosis factor-α (TNF-α), or the sodium iodide symporter (14–17, 19). Taken together, all these results strongly suggest that under normal conditions, the hTR and hTERT promoters have predictable and specific activity irrespective of delivery method or linked transgene.

We next infected cells with the telomerase-specific CRAds and analyzed replication, cytotoxicity, and viral gene expression. Both vectors caused dose-dependent cytotoxicity in all cells tested, including the telomerase-negative normal fibroblast strain WI38.

However, cytotoxicity was limited, although not abrogated, in the fibroblasts. A dose resulting in little or no toxicity in WI38 showed a range of relatively mild to powerful cytotoxicity in cancer cells at 7 days postinfection. Therefore, both vectors seemed to exhibit some selectivity for cancer cells at low dose; importantly, however, all specificity was lost at high MOI. Notably, the dose/response profiles of both CRAds were similar in all cells.

Replication or lack thereof correlated well with cytotoxicity in most cell lines, although the toxicity profile observed in the current cell panel most probably involves complex, cell-specific responses both to replication and to the other cellular effects of E1A overexpression. Interestingly, CRAd DNA also replicated in normal fibroblasts, arguing against tumor specificity. Furthermore, replication kinetics of Ad-hTR-E1A and Ad-hTERT-Luc were similar in all cells.

E1A expression was observed in all cells infected with 100 i.u. per cell Ad-hTR-E1A and Ad-hTERT-E1A and both CRAds were more cytotoxic than nonreplicating adenovirus in all cells, providing...
evidence for a correlation between E1A expression and toxicity. However, expression levels were unrelated to the promoter activities measured in the nonreplicating system. Indeed, some cell lines showed higher expression of E1A protein after infection with the hTERT-specific CRAd than with Ad-hTR-E1A. In general, the cell lines with highest E1A expression after CRAd infection also showed hexon expression and DNA replication, providing further evidence for a correlation between E1A expression and CRAd function. However, the high E1A levels in CRAd-infected cells relative to Ad-WT infections suggest that high level of E1A expression is not necessarily essential for productive infection. By comparison, levels of hexon were a good marker of the relative cytotoxicity and DNA replication.

In agreement with the functional assays, we did not observe an hTR/hTERT differential in viral gene expression. To rule out the possibility of transcriptional run-through from upstream viral promoter elements, we did 5’RACE on the telomerase-specific E1A transcripts. The largest Ad-hTR-E1A RACE product initiated correctly at the previously described transcriptional start site (TSS; 46 bp upstream of the template sequence), whereas Ad-hTERT-E1A initiated at position T-695, relative to the translational start. Both transcriptional start sites conformed to expected results and no larger products were observed, suggesting the absence of significant upstream cryptic promoter activity.

Finally, blocking the translation of E1A protein in the CRAd system with cycloheximide partially restored the differential expression of E1A mRNA by the hTR/hTERT promoters in A549 cells by increasing hTR-E1A mRNA and decreasing hTERT-E1A mRNA. In contrast, both luciferase transcripts expressed by the nonreplicating system decreased after cycloheximide, although the ratio of hTR-Luc and hTERT-Luc transcripts was unaffected by cycloheximide. Therefore, the shift in E1A mRNA levels in the CRAd system cannot result from inhibition of a normal cellular protein regulator of the hTR and hTERT promoters and must reflect a feedback effect of E1A. Furthermore, the decrease of hTERT-E1A was small compared with hTERT-Luc, suggesting that residual E1A may continue to deregulate both promoters under the block, which likely accounts for the incomplete recovery.

Several groups have developed previously hTERT-specific CRAds for cancer gene therapy. However, some studies have also reported the detection of off-target hTERT-specific viral gene expression in normal cells, and several groups have attempted to improve the selectivity of hTERT-specific CRAds for cancer cells (33, 35, 44). The comparative approach we adopted in this study has allowed us to identify subtle deregulation of both promoters in the CRAd system. We reported recently that both hTR and hTERT promoters are regulated by E1A, raising the possibility that even very low level cryptic E1A expression in nontarget cells could establish a feedback loop acting on the promoters (45).

In summary, the current data indicate that E1A expression alone can be sufficient to result in Ad-hTR-E1A and Ad-hTERT-E1A replication, but on a cell-specific basis. Critically, the vectors described here show limited selectivity for cancer cells, particularly at high doses, although lower doses of a more efficient agent observed over longer periods of incubation may show greater selectivity. The inclusion of more of the viral backbone in future vectors, such as the E1B genes or the E3 11.6K gene, in particular, which promotes cytolysis, would be predicted to enhance the overall cytopathic effect (46). However, effective CRAd therapeutics must balance both specificity and efficacy. Thus, deregulation of telomerase promoters by E1A or other downstream viral genes is potentially of major concern in other telomerase-specific CRAds and should be thoroughly assessed in the particular tumor target if human applications are envisaged.

It is conceivable that telomerase-specific control of other viral genes with E1A under WT regulation may yield better results because E1A expression is relatively short lived in Ad-WT–infected cells (47). Currently, the selectivity achieved using nonreplicating approaches seems to be more robust. We have described previously telomerase-specific plasmid vectors expressing the noradrenaline transporter gene for targeted radiotherapy; plasmid vectors encoding Cre recombinase for site-specific recombination; plasmid and adenoviral vectors expressing bacterial nitroreductase for enzyme/prodrug therapy; adenoviral vectors expressing the sodium iodide symporter for radiotherapy and imaging; and plasmid vectors encoding TNF-α delivered by a novel polypropyleneimine dendrimer for cytotoxic therapy (14–17, 19, 20).

Although each potential system has a unique profile of advantages and disadvantages, telomerase specificity of transgene expression is highly reproducible between systems, with the hTR promoter frequently producing better results. In particular, polypropyleneimine dendrimer mediated delivery results in highly efficient and selective uptake in xenografts with very favorable biodistribution (19). Such delivery systems could also have the advantage over adenovirus of greater cost efficiency and reproducibility in manufacturing for clinical trials. As delivery remains a major issue in gene therapy, it is sensible to continue to evaluate different approaches to find the most promising combinations of reagents for progression to clinical testing of telomerase-specific targeting.

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References


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