An oncolytic adenovirus controlled by a modified Telomerase promoter is attenuated in telomerase-negative cells, but shows reduced activity in cancer cells

Authors: Bortolanza S.[1], Qian C.[2], Kramer MG.[2], Gomar C.[2], Prieto J.[2], Farinati F.[1], Hernandez-Alcoceba R[2].

1. Padua University Surgical and Gastroenterological Sciences, Padua, 35128 Italy

2**.** Gene Therapy Unit. Center for Applied Medical Research (CIMA). Pamplona 31008, Spain.

Running title: Telomerase promoter in oncolytic adenoviruses

Corresponding Author: Ruben Hernandez-Alcoceba. Gene Therapy Unit. Center for Applied Medical Research. Pio XII, 55. Pamplona 31008, Spain.

Phone: +34-948104700x4032

Fax: +34-948194717

rubenh@unav.es

Acknoledgements of funding and grants:

This work was supported by grant SAF2003-08385 from the Spainsh Department of Science and Technology and the UTE project CIMA. RHA is a recipient of a Ramon y Cajal research contract. MGK is a recipient of a Fondo de Investigacion Sanitaria (FIS) research contract.

ABSTRACT

The promoter for human telomerase reverse transcriptase (hTERTp) is preferentially active in malignant cells. Recently, it has been used to control the expression of the adenoviral E1A gene for the development of oncolytic adenoviruses. In order to ensure maximal repression in normal cells, the inclusion of additional E-boxes in the proximal region of the core promoter has been described. We found that the transcriptional activity of this artificial sequence (T-255-4DEB) is minimal in normal cells, but it is also reduced in all the cancer cell lines tested. The cancer specificity of a new oncolytic adenovirus based in this promoter (AdTE1) was evaluated by direct comparison with wild type adenovirus type 5 (AdWT) in vitro and in vivo. In all the parameters tested, AdTE1 was attenuated in normal cells, but the efficacy in cancer cells showed a parallel reduction, suggesting a lack of specificity. However, the cytotoxicity of AdTE1 was repressed in senescent cells compared to AdWT. Therefore, we conclude that AdTE1 is preferentially attenuated only in cells that are permanently devoid of telomerase expression such as senescent cells. Further modifications in the telomerase-based promoters should be introduced in order to combine maximal attenuation of oncolytic adenoviruses in normal tissues and enhanced activity in tumors.

Keywords: oncolytic adenovirus; telomerase promoter; cancer; gene therapy.

INTRODUCTION

Adenoviruses that preferentially replicate and destroy cancer cells (oncolytic or conditionally replicating adenoviruses, CRADs) constitute an emerging modality of experimental treatment against cancer. One of the strategies used to obtain specificity is the transcriptional control of genes that are necessary for viral replication. Among them, the early gene E1A is the key regulator of adenovirus infective cycle [1]. Many CRADs have been produced by replacement of the natural E1A promoter with tissue or tumor-specific promoters [2, 3]. One of the problems for the clinical application of this approach is the heterogeneity of cancers in humans and the fact that some promoters do not retain their specificity in the context of the adenoviral genome. Since E1A is a potent trans-activator that can trigger the expression of a cascade of early viral genes, the promoters used to control it must be tightly repressed in normal cells.

In the past few years, special attention has focused on the upstream regulatory sequence of the human telomerase reverse transcriptase (hTERT) as a broad-spectrum tumor-specific promoter. The hTERT is the catalytic subunit of telomerase, a ribonucleoprotein complex that uses RNA as a template to amplify the sequence TTAGGG at the end of the chromosomes (telomeres) [4-7]. It is believed that this function compensates the shortening of telomeres that occurs in each cell division and keeps the integrity of the chromosomes [8, 9]. More than 85% human cancers exhibit telomerase activity as a requisite for their unrestricted cell proliferation, whereas in normal somatic cells this activity is virtually undetectable [10-13]. The sharp difference in telomerase expression observed between normal and cancer cells is determined mostly at the transcriptional level [14, 15]. Several mechanisms may cooperate in the

repression/activation of the hTERT promoter [16-21], including DNA methylation [22, 23] and histone acetylation [24-26]. In addition, the study of the 5' region of the hTERT gene using transient transfection assays in cell culture identified a GC-rich promoter lacking canonical TATA and CCAAT boxes [27-29]. A core promoter retaining full activity in cancer cells has been identified comprising approximately nucleotides –208 to +5, being +1 the initiation of transcription. It contains five binding sites for the ubiquitous transcription factor Sp1, and one E-box at positions –187 to –182, where activators like c-Myc or repressors like Mad1 can bind (Fig. 1) [28]. The key sequences involved in transcriptional repression in normal cells are still not clearly defined. They may be located in areas distant from the defined core promoter, and they will probably be different depending on the cellular environment [30].

In addition, new data emerging from the study of the telomerase regulation raises concerns about the use of the hTERT promoter in the context of a replication-competent adenovirus. First, the adenoviral E1A protein can activate the telomerase promoter, probably by an indirect mechanism involving binding to the Sp-1 motifs [31]. This activation can lead to a beneficial positive feed back loop between E1A expression and telomerase activation in cancer cells, as previously reported [31]. On the other hand, it also means that any leakage of the regulatory system in normal cells would be amplified and could trigger viral replication. Second, normal human fibroblasts express low levels of hTERT when they undergo cell division in order to keep the integrity of the 3' telomeric overhang, and they are only permanently negative for telomerase expression when they enter cellular senescence [32]. In fact, Won et al. recently showed that

Hystone Deacetylase (HDAC) bound to the E2F-pocket protein complex plays a central role in the regulation of hTERT expression [26].

However, in the last two years, a number of CRADs using the hTERT promoter for the control of the E1A gene have been described [33-41]. In this context, the length of exogenous DNA that can be introduced without deleting viral genes is less than 2 Kb. This has forced researchers to engineer variations of the promoter with different modifications intended to preserve the maximum cancer specificity in a limited space. Lanson et al. [33] used the upstream 1.7 kb region, where most of the regulatory elements of the hTERT promoter have been identified. Other groups opted for smaller sequences, most of them based on the core promoter. In order to increase the efficacy, Wirth et al.[37] introduced the E1A-TATA box, whereas Kim et al.[40] duplicated the core promoter. It is important to note that a proximal E-box (+22 to +27, Fig. 1a) has been identified as a key determinant in the specificity of the hTERT promoter [42], suggesting that in normal cells, transcriptional repressors bind selectively to this element. Interestingly, the inclusion of 3 extra E-boxes in the proximal region of the core promoter (T-255-4DEB) further decreased its activity in normal cells, which makes this construct a good candidate for the design of improved CRADs. However, it was also noted that the repression mediated by the downstream E-box was also present in same of the cancer cell lines tested [42], and this could compromise the performance of the promoter. Recently, Su et al. [38] demonstrated that an oncolytic adenovirus based on a similar promoter is highly attenuated in normal cells. Since no comparison with wild type adenovirus is available, the degree of cancer specificity conferred by the T-255-4DEB promoter is unclear.

In order to study all these issues in detail, we have tested the influence of E1A on different versions of the hTERT promoter. Then, we have produced a new replication-competent adenovirus with the E1A gene under the control of the T-255-4DEB sequence (AdTE1), and have compared its properties with a wild type adenovirus type 5 (AdWT). Our data confirms that the inclusion of 3 extra E-boxes in the proximal region of the promoter reduces the activity in non-transformed cells. The replication and cytotoxicity of AdTE1 are in fact diminished in normal cells compared to AdWT. However, the virus was also drastically attenuated in all the malignant cells analyzed, which suggests that, at least in vitro, the T-255-4DEB promoter does not confer significant cancer specificity to a replication-competent adenovirus. Only in senescent cells the AdTE1 was preferentially attenuated, which may reflect the influence of the telomerase promoter on the replication of the virus in normal non-senescent human cells. Finally, although AdTE1 showed some anti-tumor effect on human cancer xenografts, our data indicate that the use of telomerase promoter-based sequences for the regulation of adenovirus replication may obtain CRADs with relatively low oncolytic activity.

MATERIALS AND METHODS

Cell Culture

N52.E6, a cell line derived from primary human amniocytes stably transfected by adenoviral E1 [43], was obtained from S. Kochanek, Ulm, Germany. These cells were cultured in MEM α supplemented with 10% FBS. The transformed embryonic kidney cell line 293 (ATCC#CRL-1573), the lung cancer cell line A549 (ATCC# CCL-185), the colorectal adenocarcinoma cell line HT29/219 (ECCC# 85061109), the hepatocellular carcinoma cell lines Hep3B (ATCC#HB-8064), HepG2 (ATCC#HB-8065), Huh7 (obtained from Prof. Brechot, INSERM, Paris, France) and PLC/PRF/5 (ATCC#CRL-8024), were maintained in DMEM supplemented with 10% FBS. The pancreatic adenocarcinoma cell line AsPC-1 (ATCC#CRL-1682) was cultured in RPMI 1640 with Glutamax and supplemented with 20% FBS. The normal human fibroblasts BJ (ATCC#CRL-2522), IMR90 (ATCC#CCL-186), and the primary dermal fibroblasts FJD (a kind gift from J. Dotor) and NHF were cultured in MEM supplemented with 10%FBS, 1 mM sodium pyruvate and 0,1mM non essential amino acids (BioWhittacker). The dermal microvascular endothelial cells, HMVEC (Cascade Biologics #C-011-5C), were maintained in Medium 131 (Cascade Biologics), supplemented with Microvascular growth supplement (Cascade Biologics) as recommended by the provider. All culture media were supplemented with 100 U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. Except as noted, tissue culture reagents were obtain from Gibco-BRL. All cells were maintained at 37°C with 5% CO_2 in a humidified incubator.

Plasmids.

The E1 region, corresponding to the nucleotides 504-3510 of the adenovirus genome, was amplified by PCR from the plasmid pXCI (Microbix, Toronto, Canada). We used two sets of primers: Ad19 (5'-GAGTGCCAGCAGTAGAGTT-3') with Ad20 (5'-GAAAATCCAGCAGGTACCCC-3'), and Ad21 (5'-GGGGTACCTGCTGGATTTTC-3') with Ad22 (5'-GAATTCTCAATCTGTATCTTCATC-3'). PCR products were cloned in tandem in the pGEM-T-Easy plasmid (Promega) to generate pGEM-T-E1, containing the whole E1 region. The fidelity of the clone was verified by sequencing. The E1 fragment was excised from the plasmid pGEM-T-E1 and cloned into the NotI site of pcDNA3 (Invitrogen), to generate the plasmid pCMV-E1A. From this plasmid the E1 fragment was extracted and inserted into the EcoRI site of a modified pBlueScript KS plasmid (Stratagene) containing two BGH polyA sequences in the BamHI site (pBS-E1pA). The plasmids pBT-255 and pBT-255-4DEB were a kind gift of Dr. I. Horikawa (National Institutes of Health, NCI) [28, 42]. pBT-255 was digested with ClaI and HindIII to liberate the hTERT promoter fragment from -255 to + 40 nt, and it was inserted in the EcoRV site of pBS-E1pA. This plasmid was named pBST-255-E1A. pBT-255-4DEB was used as a template to amplify the hTERT promoter fragment from -255 to + 40 plus three extra E-boxes. We used the primers 5'-GTGCAGGTGCCAGAACATTTCT-3', and 5'-CAGTACCGGATATCCAAGCTCCCA-3' that contains an EcoRV site (underlined). The fragment was digested with ClaI and EcoRV and inserted in the EcoRV site of pBS-E1-pA to generate pBST-255-4DEB-E1A. The fidelity of the clone was verified by

sequencing. The plasmid pGL3basic was purchased from Promega and the pCMV-Luc was constructed as previously described [44].

Luciferase Assays.

For Luciferase assays, cells were transfected using Fugene 6 (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In brief 24-well plates were seeded with 3 to 20 x10^4 cells/well and transfected with 1 μg of reporter plasmid the following day. When different plasmids were co-transfected, the amount of reporter plasmids was half the amount of plasmid carrying E1 gene. pCDNA3 was included to adjust the total DNA. In order to normalize transfection efficiency, 25 ng of pRL-SV40 (Promega) were added to each reaction. After 48 h, cells were lysed and analyzed for luciferase activity, using the Dual-Luciferase Reporter Assay System (Promega) as indicated by the manufacturer.

Adenoviruses

pBST-255-4DEB-E1A was digested with SalI and SpeI and the cassette hTERT promoter-E1-pA was cloned in pTGC1960, obtaining the plasmid pTE1. pTGC1960 is a plasmid derived from pmV60 [45] where the sequence of Adenovirus from 3328 to 3528 nt was substituted with a multi cloning site. In summary, pTE1 contains the Ad5 1-452 nt, 3528-5788 nt, and the E1 region under the control of hTERT promoter (-255 to + 40 plus three extra E-box). The E1 transcription unit is placed in reverse orientation. The oncolytic Adenovirus AdTE1 was produced by standard homologous recombination techniques using the pTE1 and pBGH10 plasmids (Adenoviral packaging plasmid

Microbix, Ontario, Canada) in N52.E6 cells. Recombinant adenovirus was isolated by limiting dilution, and expanded in N52.E6. Control AdWT (wild type adenovirus Ad5) was purchased from ATCC (#VR5) and expanded in A549 cells. Recombinant viruses were purified on CsCl gradients and viral particles titers determined by limiting dilution in 293 cells and confirmed by a Adeno-X Rapid Titer Kit (BD Bioscience). The titer is indicated as infectious units (iu). AdLacZ (replication-defective Ad5 devoid of E1 and E3 containing the immediate early cytomegalovirus promoter controlling *LacZ* gene) was produced as reported previously [46]. The shuttle vector used to produce AdGFP (replication-defective Ad5 devoid of E1 and E3, containing the immediate early cytomegalovirus promoter controlling GFP gene) was generate by a homologous recombination in bacteria using the plasmid pAdTrack-CMV and pAdEasy-1, kind gift of Dr. B. Volgestein (John Hopkins Oncology Center, Baltimore MD). The recombinant Adenovirus was produced according to the protocol [47].

Reverse transcriptase-PCR analysis

Total RNA was isolated from cells using TriReagent (Sigma) according to the manufacturer's protocol. Complementary DNA was synthesized from 2 μg of RNA. After the reserve transcriptase reaction, the hTERT cDNA, was amplified in a PCR reaction using the primers 5'-GAGTGTCTGGAGCAAGTTGCA-3' (forward) and 5'-CTCGTAGTTGAGCACGCTGAA-3' (reverse) designed according to complete hTERT mRNA (GenBank accession no. NM_003219). As a control, we amplified the β-actin cDNA (GenBank accession no. NM_001101) with the following primers: 5'-AGCCTCGCCTTTGCCTG-3' (forward) and 5'-CTGGCCTGGGGCG-3' (reverse).

Progeny assay (in vitro viral replication assay)

Cells ($2x10^4$ /well) were cultured in 24-well plates overnight and infected with AdWT and AdTE1 at a MOI of 1 iu/cell for the tumor cell lines and of 10 iu/cell for the normal cells. Adenoviruses were removed after 12 h. Then cells were washed once with PBS and incubated at 37°C for 5 days. The cellular pellets were lysed with three cycles of freezing and thawing and titered by limiting dilution in 293 cells.

Cytotoxicity assay

Cytotoxicity of adenoviral vectors was tested using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation assay as described by the manufacturer (MTS assay; Promega, Madison, WI). Briefly, cells were seeded in 96-well tissue culture plates at $5x10^3$ cells/well. The next day, serial 4-fold dilutions of AdWT and AdTE1 were added to the wells. When at least the first three doses of AdWT killed the cells, the MTS assay was performed. The data were analyzed using the Prism GraphPad program and LD_{50} values were calculated (MOI at which the virus reduced the viability to 50%).

Complementation

Tumor cells (Hep3B and HepG2) and normal cells (IMR90, BJ and NHF) were infected with AdGFP alone or in combination with either AdTE1 or AdWT. After 5 days the percentage of GFP-positive cells was analysed by flow cytometry (FACScalibur, Becton Dickinson).

SA-β-galactosidase Staining

Late passages BJ cells were checked for senescence by a SA-β gal staining as described [48]. Briefly, cells were washed in PBS, fixed for 10 min in 0,5% glutaraldehyde, washed, and incubated for 12-14 h at 37°C with SA-β gal stain solution containing 1 mg/ml X-Gal, 40 mM citric acid, 10mM sodium phosphatase pH 6.0, 5mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 150mM $NaCl_2$ and 2mM $MgCl_2$. The cells were photographed under bright field at 100X magnification.

Human tumor xenografts

Human HCC xenografts were established by subcutaneous inoculation of $7.5x10^6$ Hep3B cells in the right flank of female nude mice, in a volume of 50 µl saline. Tumor progression was monitored at least once a week by measuring length and width with a caliper. The volume was calculated using the following formula: $V=Width^2xlenght/2$. When the tumors reached a volume of approximately 150 mm^3, animals were separated in control a control group receiving saline injection and a treatment group receiving adenovirus injection. Mice were monitored and sacrificed when tumors exceeded 2 cm in diameter, or if suffering was evident. The orthotopic HCC model was established by intrahepatic injection of $1.5x10^6$ Hep3B cells in female nude mice. The cells were resuspended in 100 µl saline and injected using a 29G needle in laparotomized mice. One month later, a new laparotomy was performed in order to inject the oviruses into the tumors, dissolved in a volume of 50 µl saline. Four weeks later, the animals were sacrificed and tumor volumes were calculated at necropsy. All procedures were performed following the local guidelines for animal care.

RESULTS

The T-255-4DEB promoter is tightly repressed in telomerase-negative cells, but the activity in telomerase-positive cell lines is low.

In order to determine the expression of hTERT in panel of cells in culture, we isolated RNA from cellular extracts and performed Reverse Transcriptase-PCR (RT-PCR) with specific primers. As expected, we were unable to detect hTERT expression in any of the normal human cells using this method. These cells included primary fibroblasts (FJD, IMR-90, BJ, NHF) and microvascular endothelial cells (HMVEC). On the other hand, all cancer cell lines from liver (HepG2, Hep3B, PLC/PRF/5, Huh-7), colon (HT-29), lung (A549) and pancreas (AsPC-1) showed reproducible telomerase expression (Fig. 2a).

Next, we studied the correlation between the transcriptional activity of the endogenous telomerase promoter revealed by the RT-PCR assay, and the activation of two different regulatory sequences based on this promoter (Fig. 1a). The T-255 construct (-255 to +40) includes 5 Sp-1 binding sites and both distal and proximal E-boxes, whereas the T-255-4DEB has 3 additional E-boxes downstream of the endogenous proximal E-box. Both fragments were cloned in luciferase reporter plasmids (pBT-255 and pBT-255-4DEB, respectively) and transfected into telomerase-positive and negative cells. For each kind of cell, a plasmid that contains the luciferase gene under the control of the viral CMV promoter (pCMVLuc) was used to normalize the transcriptional activity. As shown in figure 2b, both of the telomerase promoter-based constructs had lower activity than the

CMV promoter in cancer cells. However, the T-255 was in the same range for most of them, with a maximal activity around 30% of CMV for HepG2 and A549 cells. Interestingly, the activity was only 10% in those cancer cells with relatively low telomerase expression like Hep3B and Huh-7 cells (Fig. 2a). In the telomerase-negative cells, the T-255 promoter was drastically attenuated, with relative activities around 1% for IMR-90 cells, and even lower for BJ and NHF cells. The T-255-4DEB promoter followed the same trend, but with reduced intensity. This means that in normal cells, there was a 2-3-fold reduction in luciferase activity compared to the T-255 promoter, but the same or even stronger reduction was observed in all cancer cells tested. Based on these data, a "cancer-specific index" can be calculated for this panel of cells. This is obtained as the ratio between the transcriptional activity (normalized by the CMV promoter) in cancer cells and normal cells. The average index is 32 for T-255 and 15 for T-255-4DEB, which means that the promoters are 30 and 15 times more potent in cancer cells than in normal cells, respectively. Since the T-255-4DEB promoter is attenuated both in normal and cancer cell lines, no increase in specificity was observed. However, taking into account that the attenuation of the tumor-specific promoters in normal cells is considered a key issue in the development of CRADs, we decided to investigate if the effect of the additional E-boxes was beneficial for this application.

The addition of proximal E-boxes attenuates the influence of E1A on the telomerase core promoter.

It is know that the adenoviral E1A protein activates the telomerase promoter [31], especially if it lacks the binding sites for the WT1 transcriptional repressor (-423 to –

307), as is the case for the T-255 core promoter. In order to analyze if the T-255-4DEB suffers the same influence, we co-transfected Hep3B, IMR90 and BJ cells with pBT-255 or pBT-255-4DEB and a plasmid that contains the E1A gene under the control of the constitutive CMV promoter (pCMV-E1A). As shown in figure 3a, E1A significantly increased the activity of the promoters in IMR90 and BJ cells, although the enhancement was lower for the T-255-4DEB promoter in BJ cells (30 fold versus 6 fold). As expected, the effect of E1A was less pronounced in Hep3B cells because the promoters were constitutively activated. These data indicate that if the E1A protein is produced inside normal cells, both T-255 and T-255-4DEB promoters can be activated, although they do not reach the levels observed in malignant cells. If E1A is placed under the control of the telomerase promoters, as will be the case in a CRAD, then a small amount of E1A produced in normal cells could trigger a positive feedback. We hypothesized that the lower activity of the T-255-4DEB would be an advantage to avoid this phenomenon. In order to mimic this situation, we co-transfected the cells with the luciferase reporter plasmids pBT-255 or pBT-255-4DEB, plus a plasmid that contains the E1A gene under the control of the same promoters (pBST-255-E1A and pBST-255-4DEB-E1A, respectively). In figure 3b we show that the activation takes place in the case of the T-255 promoter, whereas no significant enhancement of the luciferase signal is observed if the T-255-4DEB controls the expression of E1A (Fig. 3c). Again, the repression was stronger in BJ than in IMR90 cells.

Evaluation of the T-255-4DEB promoter for the regulation of E1A expression in a replication-competent adenovirus (AdTE1).

The results described above suggested that T-255-4DEB is a weak promoter that could achieve a tight control of E1A in the context of an adenovirus. Therefore, we used this strategy for the construction of a CRAD (AdTE1). As shown in figure 1b, the viral E1A promoter is deleted and substituted by T-255-4DEB. The E1B maintains the endogenous promoter, but both E1 transcription units are placed in reverse orientation in order to prevent the influence of the E1A enhancer located in the packaging signal.

In order to study the regulation of E1A in AdTE1, and the functionality of the protein, we compared the ability of AdTE1 and AdWT to complement the E1A deficiency of a first-generation adenovirus expressing the Green Fluorescent Protein (AdGFP). Cancer cells (Hep3B, HepG2) and normal cells (IMR-90, BJ and NHF) were co-infected with AdTE1 or AdWT plus AdGFP. The amount of AdGFP was adjusted for each cell line in order to reach an initial infection of no more than 20% of the cells in the monolayers. The amount of the replication-competent viruses AdTE1 and AdWT were also increased in the normal fibroblasts in order to compensate for their lower susceptibility to adenovirus infection. Under these conditions, if a functional E1A is expressed, the replication of AdGFP will be activated inside the cell, and a new round of viruses capable of infecting additional cells will be released. In order to quantify this effect, we analyzed the number of GFP-positive cells by flow cytometry 5 days after infection, and the result is shown in figure 4. In Hep3B and HepG2 cells, AdWT caused close to a 20-fold increase in GFP-positive cells, whereas AdTE1 did not exceed a 4-fold increase. In BJ and NHF cells, the activation was about 5 fold for AdWT and 2 fold for AdTE1. In the case of IMR-90, the increase was modest for both viruses. Thus, in agreement with the activity described for the T-255-4DEB promoter (Fig 2b), the

complementation achieved by AdTE1 was reduced in both normal and cancer cell lines compared to AdWT.

AdTE1 is attenuated in normal cells, but also in cancer cells.

Next, we evaluated the performance of AdTE1 as an oncolytic virus using two key parameters: cytotoxicity and viral progeny production in a panel of cancer and normal cells, compared to AdWT. In figure 5 we represent the viruses produced five days after infecting the cells with a reduced MOI. A drastic reduction of AdTE1 viral progeny (more than 100 fold) was observed in NHF and FJD normal fibroblasts. However, this reduction of viral progeny was not confined to normal cells. In some telomerase-positive cells like AsPC-1, PLC/PRF/5 and HT29/219, the magnitude of the decrease was similar to the reduction observed in normal cells.

Finally, we evaluated the ability of AdTE1 to specifically destroy cancer cells, which is the main characteristic of oncolytic adenoviruses. In figure 6a we show the percentage of cells that survive several days after infection with AdTE1 or AdWT viruses at different MOIs, compared to non-infected cells. The viability of the cells was analyzed using the MTS assay, at least five days after infection. To verify that the cytotoxicity observed was mainly due to viral replication, we included a non-replicative adenovirus (AdLacZ) as a negative control. This virus only caused a modest reduction in viability at the highest MOI, and it is not represented in the graphics. The results show again that the effect of AdTE1 was reduced compared to AdWT, both in normal and cancer cell lines. In order to distinguish if this reduction was stronger in normal than cancer cells, we analyzed more cell types and calculated the MOI that causes a 50% reduction in the

viability of the monolayer (LD_{50}). In figure 6b we represent the ratio of LD_{50} for AdTE1 and AdWT in each experiment. The higher the ratio, the more intense is the attenuation of AdTE1 versus AdWT in each cell type. With this normalized measurement, we can conclude that the reduction in AdTE1 cytotoxicity is not specific for normal cells, at least in culture, since the ratio was elevated in all the cells, and there was not significant difference between normal and cancer cells.

AdTE1 is preferentially attenuated in senescent cells.

It has been recently described that normal human fibroblasts in culture are able to express low levels of telomerase when they proliferate, and that they only lose telomerase expression completely when they enter senescence. It is possible that this low activity of the telomerase promoter, not detected by conventional methods, is reducing the specificity of AdTE1. In order to study this possibility, we compared the LD_{50} ratio of AdTE1 and AdWT on human fibroblasts BJ and NHF that were either low passage (proliferation-competent) or entering senescence. The cells were maintained in culture until they ceased proliferating, and the appearance of senescent cells in the monolayer was demonstrated by β-gal staining as previously described [48] (Fig. 7a). As shown in figure 7b, there was a significant increase in the LD_{50} ratio of AdTE1 in the senescent cells, which means that the virus is further attenuated in this condition. This suggests that the CRADs controlled by telomerase-related promoters are very sensitive to the low basal activity present in normal cells in culture.

Therapeutic effect of AdTE1 in vivo.

The anti-tumor effect of AdTE1 was initially analyzed on human HCC xenografts established by subcutaneous inoculation of Hep3B cells in nude mice. The treatment consisted on daily intratumoral injections of $5x10^8$ iu during 4 consecutive days. As shown in figure 8a, AdTE1 achieved a significant reduction of tumor growth 6 weeks after the treatment. On the other hand, the same dose of AdWT caused a complete inhibition of tumor progression, confirming the stronger oncolytic effect observed in the in vitro experiments. Next, we used and orthotopic HCC model established by intra-hepatic inoculation of Hep3B cells in nude mice. A single intratumoral injection of 10^9 iu of either AdTE1 or AdWT was administered one month after the inoculation of the cells. Four weeks later, when some of the animals experienced weight loss, they were sacrificed and necropsies were performed in order to evaluate tumor progression. The tumor volume for each mouse is represented in figure 8b. This orthotopic HCC model confirms that AdTE1 is able to inhibit the growth of implanted liver tumors, but it does not reach the same antitumor effect observed with AdWT.

DISCUSSION

The transcriptional control of the E1A gene has been extensively used as a method to obtain oncolytic adenoviruses. The regulatory sequence must be small, tightly repressed in normal cells, and relatively active in cancer cells. The hTERT promoter is considered a broad-spectrum tumor-specific promoter and it is an interesting option for the construction of CRADs [33-41]. However, the original idea that telomerase expression is completely repressed in normal somatic cells has been challenged by recent discoveries, and the importance of the chromatin environment in the control of its expression is becoming evident [26, 32]. Therefore, the use of small fragments of the telomerase promoter in the context of a viral genome does not necessarily reproduce the situation observed in the natural setting.

We have confirmed here that the addition of E-boxes in the proximal region of the hTERT promoter reduced the basal activity in normal cells. In addition, we found that it minimized the influence of E1A observed in the unmodified core promoter. However, an oncolytic adenovirus based on this promoter (AdTE1) is attenuated both in normal cells and cancer cells. This may reflect the low activity of the promoter observed in the in vitro studies, and is consistent with the results of Horikawa et al. [42] that observed how the repression mediated by E-boxes could take place also in cells derived from prostate and bladder human cancers. The specificity conferred to adenoviruses by some tumor-specific promoters can be overestimated if the natural preference of AdWT to infect and replicate in cancer cell lines versus primary fibroblasts is not taken into account. For instance, a MOI of 500 is necessary to infect 30% of BJ cells, whereas the same proportion of

Hep3B cells can be infected with a MOI of 20 (data not shown). Therefore, if a direct comparison with AdWT is not performed, the real cancer specificity achieved with the recombinant adenoviruses cannot be determined.

The influence of a basal, periodic expression of telomerase in normal cells such as human fibroblasts in culture can explain why AdTE1 is more attenuated than AdWT in senescent cells. The study of this phenomenon in other cell types and with different viruses will indicate if it has general implications for the use of this strategy.

In summary, we show here that using the telomerase promoter as the basis for controlling the viral E1A gene is not a straightforward method to obtain specific and efficient oncolytic adenoviruses. Therefore, although some oncolytic adenoviruses, including AdTE1, have shown anti-tumor effect in pre-clinical studies using different telomerase-based promoters [33-41], we suggest that there is room for improvement of these agents. Possible approaches include the design of hybrid regulatory sequences with elements that re-enforce the specificity and efficacy of the telomerase promoters; the simultaneous control of E1A, E4 or E2 transcription units; and the combination with specific deletions in E1A or E1B.

ACKNOWLEDGEMENTS

We thank Izumi Horikawa (National Cancer Institute, Bethesda, MD) for the pBT-255 and pBT-255-4DEB plasmids, Helena Villanueva for technical assistance, Javier Dotor for the FJD cells and Michel Centelles for assistance with flow cytometry.

REFERENCES

1. Shenk, T.(1996) Adenoviridae: The viruses and their replication., in Fields Virology, (B.N. Fields, D.M Knipe, and P.M. Howley, Eds), pp2111-2147. Lippincott-Raven Philadelphia, PA
2. Hallenbeck, P. L., Chang, Y. N., Hay, C., Golightly, D., Stewart, D., Lin, J., et al. (1999). A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum Gene Ther 10: 1721-33
3. Takahashi, M., Sato, T., Sagawa, T., Lu, Y., Sato, Y., Iyama, S., et al. (2002) E1B-55K-deleted adenovirus expressing E1A-13S by AFP-enhancer/promoter is capable of highly specific replication in AFP-producing hepatocellular carcinoma and eradication of established tumor. Mol Ther 5: 627-34
4. Greider, C. W. (1996). Telomere length regulation. Annu Rev Biochem 65: 337-65
5. Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., et al. (1997). Telomerase catalytic subunit homologs from fission yeast and human. Science 277: 955-9
6. Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., et al. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 90: 785-95
7. Kilian, A., Bowtell, D. D., Abud, H. E., Hime, G. R., Venter, D. J., Keese, P. K., et al. (1997). Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum Mol Genet 6: 2011-9
8. Cech, T. R. (2004). Beginning to understand the end of the chromosome. Cell 116: 273-9
9. Blackburn, E. H. (2001). Switching and signaling at the telomere. Cell 106: 661-73
10. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., et al. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011-5
11. Shay, J. W. and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. Eur J Cancer 33: 787-91
12. Wright, W. E., Tesmer, V. M., Huffman, K. E., Levene, S. D. and Shay, J. W. (1997). Normal human chromosomes have long G-rich telomeric overhangs at one end. Genes Dev. 11: 2801-9
13. Shay, J. W. and Wright, W. E. (2000). Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol 1: 72-6.
14. Ramakrishnan, S., Eppenberger, U., Mueller, H., Shinkai, Y. and Narayanan, R. (1998). Expression profile of the putative catalytic subunit of the telomerase gene. Cancer Res 58: 622-5
15. Takakura, M., Kyo, S., Kanaya, T., Tanaka, M. and Inoue, M. (1998). Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. Cancer Res 58: 1558-61
16. Cong, Y. S., Wright, W. E. and Shay, J. W. (2002). Human telomerase and its regulation. Microbiol Mol Biol Rev 66: 407-25, table of contents

17. Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsudo, M., et al. (2000). Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res 28: 669-77
18. Crowe, D. L., Nguyen, D. C., Tsang, K. J. and Kyo, S. (2001). E2F-1 represses transcription of the human telomerase reverse transcriptase gene. Nucleic Acids Res 29: 2789-94
19. Crowe, D. L. and Nguyen, D. C. (2001). Rb and E2F-1 regulate telomerase activity in human cancer cells. Biochim Biophys Acta 1518: 1-6
20. Lin, S. Y. and Elledge, S. J. (2003). Multiple tumor suppressor pathways negatively regulate telomerase. Cell 113: 881-9
21. Wang, S. and Zhu, J. (2003). Evidence for a relief of repression mechanism for activation of the human telomerase reverse transcriptase promoter. J Biol Chem 278: 18842-50
22. Dessain, S. K., Yu, H., Reddel, R. R., Beijersbergen, R. L. and Weinberg, R. A. (2000). Methylation of the human telomerase gene CpG island. Cancer Res 60: 537-41
23. Devereux, T. R., Horikawa, I., Anna, C. H., Annab, L. A., Afshari, C. A. and Barrett, J. C. (1999). DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. Cancer Res 59: 6087-90
24. Cong, Y. S. and Bacchetti, S. (2000). Histone deacetylation is involved in the transcriptional repression of hTERT in normal human cells. J Biol Chem 275: 35665-8
25. Takakura, M., Kyo, S., Sowa, Y., Wang, Z., Yatabe, N., Maida, Y., et al. (2001). Telomerase activation by histone deacetylase inhibitor in normal cells. Nucleic Acids Res 29: 3006-11
26. Won, J., Chang, S., Oh, S. and Kim, T. K. (2004). Small-molecule-based identification of dynamic assembly of E2F-pocket protein-histone deacetylase complex for telomerase regulation in human cells. Proc Natl Acad Sci U S A 101: 11328-33
27. Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., et al. (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res 59: 551-7
28. Horikawa, I., Cable, P. L., Afshari, C. and Barrett, J. C. (1999). Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res 59: 826-30
29. Cong, Y. S., Wen, J. and Bacchetti, S. (1999). The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum Mol Genet 8: 137-42
30. Ducrest, A. L., Szutorisz, H., Lingner, J. and Nabholz, M. (2002). Regulation of the human telomerase reverse transcriptase gene. Oncogene 21: 541-52
31. Kirch, H. C., Ruschen, S., Brockmann, D., Esche, H., Horikawa, I., Barrett, J. C., et al. (2002) Tumor-specific activation of hTERT-derived promoters by tumor suppressive E1A-mutants involves recruitment of p300/CBP/HAT and suppression of HDAC-1 and defines a combined tumor targeting and suppression system. Oncogene 21: 7991-8000
32. Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., et al. (2003). Telomerase maintains telomere structure in normal human cells. Cell 114: 241-53

33. Lanson, N. A., Jr., Friedlander, P. L., Schwarzenberger, P., Kolls, J. K. and Wang, G. (2003). Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis. Cancer Res 63: 7936-41
34. Huang, T. G., Savontaus, M. J., Shinozaki, K., Sauter, B. V. and Woo, S. L. (2003). Telomerase-dependent oncolytic adenovirus for cancer treatment. Gene Ther 10: 1241-7
35. Huang, Q., Zhang, X., Wang, H., Yan, B., Kirkpatrick, J., Dewhrist, M. W., et al. (2004). A novel conditionally replicative adenovirus vector targeting telomerase-positive tumor cells. Clin Cancer Res 10: 1439-45
36. Irving, J., Wang, Z., Powell, S., O'Sullivan, C., Mok, M., Murphy, B., et al. (2004). Conditionally replicative adenovirus driven by the human telomerase promoter provides broad-spectrum antitumor activity without liver toxicity. Cancer Gene Ther 11: 174-85
37. Wirth, T., Zender, L., Schulte, B., Mundt, B., Plentz, R., Rudolph, K. L., et al. (2003). A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. Cancer Res 63: 3181-8
38. Su, C. Q., Sham, J., Xue, H. B., Wang, X. H., Chua, D., Cui, Z. F., et al. (2004). Potent antitumoral efficacy of a novel replicative adenovirus CNHK300 targeting telomerase-positive cancer cells. J Cancer Res Clin Oncol 130: 591-603
39. Zou, W., Luo, C., Zhang, Z., Liu, J., Gu, J., Pei, Z., et al. (2004). A novel oncolytic adenovirus targeting to telomerase activity in tumor cells with potent. Oncogene 23: 457-64
40. Kim, E., Kim, J. H., Shin, H. Y., Lee, H., Yang, J. M., Kim, J., et al. (2003). Ad-mTERT-delta19, a conditional replication-competent adenovirus driven by the human telomerase promoter, selectively replicates in and elicits cytopathic effect in a cancer cell-specific manner. Hum Gene Ther 14: 1415-28
41. Kawashima, T., Kagawa, S., Kobayashi, N., Shirakiya, Y., Umeoka, T., Teraishi, F., et al. (2004). Telomerase-specific replication-selective virotherapy for human cancer. Clin Cancer Res 10: 285-92
42. Horikawa, I., Cable, P. L., Mazur, S. J., Appella, E., Afshari, C. A. and Barrett, J. C. (2002). Downstream E-box-mediated regulation of the human telomerase reverse transcriptase (hTERT) gene transcription: evidence for an endogenous mechanism of transcriptional repression. Mol Biol Cell 13: 2585-97
43. Schiedner, G., Hertel, S. and Kochanek, S. (2000). Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. Hum Gene Ther 11: 2105-16
44. Kramer, M. G., Barajas, M., Razquin, N., Berraondo, P., Rodrigo, M., Wu, C., et al. (2003). In vitro and in vivo comparative study of chimeric liver-specific promoters. Mol Ther 7: 375-85
45. Qian, C., Bilbao, R., Bruna, O. and Prieto, J. (1995). Induction of sensitivity to ganciclovir in human hepatocellular carcinoma cells by adenovirus-mediated gene transfer of herpes simplex virus thymidine kinase. Hepatology 22: 118-23
46. Mazzolini, G., Qian, C., Narvaiza, I., Barajas, M., Borras-Cuesta, F., Xie, X., et al. (2000). Adenoviral gene transfer of interleukin 12 into tumors synergizes with

adoptive T cell therapy both at the induction and effector level. Hum Gene Ther 11: 113-25
47. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95: 2509-14
48. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 92: 9363-7

FIGURE LEGENDS

Fig 1. Schematic diagram of hTERT promoter-based regulatory sequences and the replicative adenovirus AdTE1

a. The upper figure represents the endogenous hTERT promoter with the indicated binding sites for transcription factors: Sp-1, c-Myc/Mad (E-box), Willm´s tumor suppressor-1 (WT-1), Myeloid-specific Zinc finger protein-2 (MZF-2), and Estrogen Receptor (ER). The pBT-255 is a luciferase reporter plasmid containing a telomerase-based promoter (T-255) comprising bp -255 to +40, being +1 the initiation of transcription. The pBT-255-4DEB plasmid is identical to pBT-255, with three extra E-boxes in the proximal region of the core telomerase promoter (T-255-4DEB). **b.** The AdTE1 virus contains the E1A gene under the control of the T-255-4DEB promoter. Both E1A and E1B transcription units are placed in reverse orientation to avoid interference with the wild type E1A enhancer that overlaps with the packaging signal. The E3 region was removed. pA, polyadenylation sequence; ITR, inverted terminal repeat; Ψ, packaging signal.

Fig 2. Expression and characterization of hTERT promoter in malignant and normal cells. a. RNA was isolated from the indicated cells, and the hTERT expression was detected by RT-PCR using 2 ug of total RNA. RT-PCR for β-actin mRNA was used as a control. **b.** Cells were transfected with the plasmids pGL3basic (pGL3b), pBT-255 (p255-Luc), and pBT-255-4DEB (p4DEB-Luc), as indicated. The luciferase activity of cellular extracts was quantified 48 h later. The activity of the pCMV-Luc plasmid was used as a reference (activity=1) for each cell line. In order to normalize transfection efficiency, 25 ng of pRL-SV40 (Promega) were added to each reaction. A representative experiment performed in triplicate is shown. The experiment was repeated at least two times with similar results.

Fig 3. Influence of E1A protein on expression of telomerase promoter. a. Cells were transfected with pBT-255 (p255-Luc) or pBT-255-4DEB (p4DEB-Luc) luciferase

reporter plasmids (0.7 μg for Hep3B and 0.4 μg for IMR90 and BJ), alone or in combination of pCMV-E1A (1.4 μg for Hep3B and 0.8 μg for IMR90 and BJ). Luciferase activity was measured 48 h after transfection. **b** and **c**, the pBT-255 (B) or pBT-255-4DEB (C) plasmids were co-transfected with plasmids expressing E1A under the control of the same promoters (pBST-255-E1A, indicated as p255-E1A, and pBST-255-4DEB-E1A, indicated as p4DEB-E1A, respectively). The transfection was performed as described for A. Statistical significance is indicated with one asterisk ($p<0.05$) or two asterisks ($p<0.01$).

Fig 4. Analysis of replication of the E1A-deleted adenovirus AdGFP in the presence of AdTE1. Cancer cell lines (HepG2 and Hep3B) and normal fibroblasts (BJ, IMR90 and NHF) were infected with AdGFP, alone or in combination with AdTE1 or AdWT. The MOI was 10 for AdGFP and 0.02 for AdTE1 and AdWT in cancer cells, 50 for each virus in IMR-90 cells and 350 for each virus in BJ cells. After 5 days the percentage of GFP-positive cells was analyzed by flow cytometry. The values in the Y-axis indicate the increase in GFP-positive cells compared with AdGFP alone.

Fig 5. AdTE1 viral replication in cancer and normal human cells. Cells were infected with AdTE1 or AdWT at a MOI of 1 for cancer cells and 10 for normal cells. The infective medium was removed 2 h later, and 5 days post infection the cells were harvested and lysed by 3 cycles of freezing and thawing. The viral yield was quantified by limiting dilution in 293 cells. The graphic shows the average of two independent experiments performed in quadruplicate.

Fig 6. Cytotoxic effect of AdTE1. a. Human cancer cells (Hep3B, HepG2, PLC/PRF/5, Huh7, AsPC-1 and HT29/219) and normal cells (BJ, IMR90 and NHF) were infected with AdTE1 (black triangles) and AdWT (black squares) at different MOIs. When at least the first three doses of AdWT caused cytophatic effect, an MTS cytotoxicity assay was performed. The viability is expressed as a percentage versus uninfected controls. A sigmoidal dose-response curve was fit to the data and the lethal dose 50 (LD_{50}) was calculated for each virus. Each graphic is a representative experiment performed in

quadruplicate. **b.** The ratios (LD_{50} AdTE1/ LD_{50}AdWT) obtained in different experiments on an extended panel of cells are summarized. Ratios above 1 indicate attenuation of AdTE1 in each cell type.

Fig 7. Cytotoxic effect of AdTE1 in senescent cells. a. BJ cells were maintained in culture until they ceased proliferating, and the appearance of senescence was assessed by SA-β-galactosidase Staining. Cells were photographed at 200X magnification 12 h after staining. **b.** Senescent and non-senescent BJ cells were infected with AdWT or AdTE1 at different MOIs. The viability of the cells was analyzed 11 to 16 days after infection by MTS assay. The values represent the mean of the relative LD_{50} (LD_{50}AdTE1/ LD_{50}AdWT) of 4 independent experiments.

Fig 8. Therapeutic effect in vivo. a. Subcutaneous tumors were induced by injection of $7.5x10^6$ Hep3B cells in the flank of nude mice. Once tumors were evident, daily intratumoral injections of AdTE1 or AdWT ($5x10^8$ iu) were administered during 4 consecutive days. The control animals were injected with vehicle alone (saline). The average tumor volume for each treatment group is indicated. **b.** Orthotopic liver tumors were induced by intrahepatic injection of $1.5x10^6$ Hep3B cells in nude mice. One month later, a single intratumoral administration of AdTE1 or AdWT (10^9 iu) was administered. The tumor volume was measured four weeks later. Black squares represent individual control mice. Mice treated with AdTE1 and AdWT are represented by triangles and inverted triangles, respectively. The average tumor volume is indicated by horizontal lines.