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13. ABSTRACT (Maximum 200 Words)
The goal of the proposed study is to develop a genetic immunotherapy strategy that can compliment and enhance current prostate cancer radiotherapy. The specific strategy that will be adopted in this proposal is to test the capacity of an adenovirus encoding immunostimulatory genes IL-12 and B7 to enhance the therapeutic effects of ionizing radiation in an experimental murine prostate tumor model TrampC. A substantial progress has been made towards this goal. We have now conducted experiments where we combined radiotherapy and genetic immunotherapy in the TrampC1 model. The results indicate synergistic effects in controlling subcutaneous tumor growth. In addition, we have discovered that our immunogene therapy approach had a limited efficacy in controlling metastatic tumor growth. We explored the possibility that this limited success is due the in-efficiency of gene transfer by the viral vectors. Our results indicated that a novel, telomerase-targeted replicative gene therapy approach can significantly enhance the efficacly of the gene transduction into prostate cancer cells. We will explore this novel and exciting vector approach in the next year for our prostate cancer gene therapy approach.
Table of Contents

Cover......................................................................................................................... 1

SF 298...................................................................................................................... 2

Table of contents...................................................................................................... 3

Body............................................................................................................................ 4-7

Key Research Accomplishments............................................................................. 7

Reportable Outcomes................................................................................................ 7

Conclusions................................................................................................................ 7

References................................................................................................................. 8

Appendices................................................................................................................
Year 2 progress report

The long-term goal of the proposed study remains the same: the development of a genetic immunotherapy strategy that can compliment and enhance current prostate cancer radiotherapy. We have planned the following three specific aims:

**Task 1.** To test the efficacy of an adenovirus encoding immunostimulatory IL12 and B7 genes (AdIL12.B7) to enhance the radiation treatment of primary tumors in a mouse prostate cancer model. (Months 1-18).

**Task 2.** To examine the potential capacity of combined radiotherapy and AdIL12.B7 mediated genetic immuno therapy to control distant metastases (Months 12-24).

**Task 3.** To elucidate the mechanisms of potential anti-tumor effects of the AdIL12.B7 (Months 18-36).

We are continuously making steady progress towards these tasks. So far we have achieved the following:

**For task 1,** we have finished the experiments to test the efficacy of the combined genetic immunotherapy with radiation therapy. Our results indicated the following:

1) There is synergistic efficacy when AdIL12.B7 is combined with radiation therapy. The growth delay achieved when the two modalities were combined were greater than the sum of growth delay achieved when each modality was applied individually (Figure 1);

2) AdIL12.B7 based immunogene therapy can only enhance radiation therapy when the gene therapy vectors were injected 1 day after the completion of the radiation therapy. When the virus vectors were injected before radiation therapy, no significant increase in growth delay was seen at all (Figure 1). These results may be explained by the sensitivity of the immuno effector cells to radiation. This was clear Figure 1.

In summary, the data in Figure 1 therefore suggest that adenovirus mediated immunogene therapy can indeed enhance the efficacy of prostate cancer radiotherapy.

**For task 2,** in order to monitor tumor metastasis non-invasively, we have developed a novel firefly luciferase-based model for observing metastatic tumor growth. We have established a stable prostate cancer cell line that expresses the luciferase gene (TrampC-luc). Using this cell line, a preliminary experiment has been carried out to examine whether our combined radiation/immunogene therapy can suppress metastasis. In this experiment, subcutaneously established TrampC tumors were treated with ionizing radiation and subsequently injected with AdIL12.B7.
When the treated tumors were in remission, TrampC-luc cells were injected intravenously through tail vein injection. Luciferase expression in the mice were then examined periodically to monitor metastatic TrampC tumor growth. Figure 2 shows the results. These data, although preliminary in nature, do indicate that combined radiation/immunogene therapy may have a suppressive effect on tumor growth at distant loci. These experiments are now being repeated to ensure its reproducibility.

Figure 1. Significant enhancement of the efficacy of radiation by adenovirus-mediated immunogene therapy. TrampC prostate tumors were established subcutaneously. When the tumor diameter is reach 5-7 mm, the tumors were then subject to different treatments (irradiation, adenovirus injection, and combined therapy). Shown in this figure are growth delay data. The time to reach 3 times the initial volume were used to measure tumor growth delay. IR, irradiation alone. AdIL12.B7, virus vector clone. Ad(pre)+IR, combined group with virus injection given before radiation. Ad(post)+IR, combined group with virus given after irradiation. The combined group (Ad(post)+IR) never reaches 3 times the initial volume during the entire duration of observation (90 days). The adenovirus dosage were 3E8 pfu/tumor while the irradiation dosage were 3 times at 6 Gray each, given on Monday, Wednesday, and Tuesday.

Figure 2. Significant effect of combined AdIL12.B7 and radiation treatment on the growth of intravenously injected TRAMP-C cells stably expressing the luciferase gene. TrampC tumors were initially established subcutaneously in the hind leg. When tumor sizes reached 5-7 mm in diameter, combined AdIL12.B7 and radiation were initiated as described in Figure 1. When tumors were in remission, about 1E6 trampc-luc cells were injected intravenously. The mice were then followed every 3-5 days by use of the Xenogen system for luciferase imaging. A. Luciferase imaging at 21 days after cell injection. Notice the significantly brighter image on the left, which is indicative of significant tumor growth. B. The average of values of total light output as measured in relative light units. Each data point represents the average of three mice. Higher light output is indicative of larger tumor growth.
In addition, through our past experience, we realize that within the dose range that were using, only a small fraction (<10%) of the tumor actually got transduced with the adenovirus vector. This prompts us to develop a more efficient approach for adenovirus. We therefore constructed a telomerase-targeted adenovirus vector (Figure 3), which in theory only replicates in telomerase-positive cancer cells. Because telomerase was positive in over 90% of all solid tumors and negative in the majority of normal cells. This vector should specifically target most tumor cells. This vector was then tested in a xenograft model of human prostate cancer line. Results indicate that our newly constructed vector could efficiently replication in vivo in human prostate cancer cells (Figure 4). This advancement greatly improves the potential to deliver therapeutic genes to real-life tumors. A paper describing this new approach for targeted tumor gene delivery and accompanying editorial were published in Clinical Cancer Research (see attached paper).

![Figure 3](image_url)

**Figure 3. A)** A schematic diagram of the telomerase-selective adenovirus vector. TERT-P, the TERT promoter. E1A and E1B, early E1 genes of the adenovirus. dsRed2, a gene encoding the red fluorescent protein. ITR, inverted repeats of the adenovirus genome. ΔΕ3, deletion of the E3 region.

![Figure 4](image_url)

**Figure 4. A)** Robust replication and virus spread of AdTERT-E1a-dsRed2 in a LNCap tumor vs the limited gene expression and virus spread for non-replicative Ad-dsRed in the same tumor. Tumors were sectioned 5 days after initial viral injections. The sections were then observed under a fluorescence microscope equipped with a rhodamine filter. The scale bars represent 2 mm. **B** Virus titers in tumors after injection. About 5x10^7 pfu of Ad-dsRed2 (solid bar) and Ad-TERT-E1a-dsRed2 (empty bars) were injected into established tumors at around 10 mm in diameter. Tumors were then sacrificed and lysed at day 1 and day 3 after infection. The lysates were then evaluated for virus titer in 293 cells.
For task 3, work has just begun. A preliminary experiment was carried out in athymic nude mice, which do not possess T cells. Our results indicate that administration of immunostimulatory IL12 and B7 genes are still effective in suppressing prostate tumor growth, where significant growth delay is observed in nude mice. However, this growth delay is smaller than those observed in immunocompetent mice (a delay of 11 days vs 21 days). This indicates that the observed anti-tumor efficacy in task 1 is not only dependent on T cells. Other factors, such as NK cells or B cells may also play significantly roles. More experiments will be conducted to further dissect the role of individual immuno-effectector cell types.

Key Research Accomplishments:

♦ We have conducted experiments that indicated a synergistic interaction between radiation therapy and genetic immunotherapy. Administration of immunostimulatory IL12 and B7-encoding adenovirus after radiation therapy can significantly boost the efficacy of radiation therapy in a murine prostate cancer model. We further discovered that the enhancement is at the highest level if the adenovirus vectors were injected after the completion of radiation therapy.

♦ By use of a novel non-invasive tumor model, we have conducted experiments to examine the effect of combined radiation/immunogene therapy on distant tumor growth and our initial results were encouraging.

♦ A novel, conditionally replicative oncolytic adenovirus vector targeted to telomerase was successfully constructed and tested. It can potentially target most prostate and other cancer cells.

Reportable Outcomes: We have, with partial support of this grant, evaluated a novel strategy for gene delivery based on telomerase. We have shown that with this strategy, it is possible to deliver therapeutic genes into the tumor mass with greatly enhanced efficacy. Our paper describing these results was published in Clinical Cancer Research with an accompanying editorial(1, 2).

Conclusions:

We have made steady progress in the proposed project. In addition, we have added some novel elements (e.g. luciferase-based imaging) so that planned experiments can be conducted with better monitoring approach. We expect to accomplish the goals stated in the proposal on time.
References


The Biology Behind

Targeting Cancer with Telomerase


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During the past 50 years, the development and widespread use of antibiotics, vaccines, and other antimicrobial agents has dramatically reduced the mortality caused by infectious agents. These therapeutic successes were achieved, in large part, by the identification and development of approaches that target molecules expressed exclusively by microbes. This paradigm not only continues to drive efforts to develop new antimicrobials and vaccines but has also stimulated efforts to apply this approach to the treatment of cancer.

Indeed, concerted efforts from many laboratories have identified specific molecular alterations unique to cancer cells. However, unlike microbes, cancer cells originate from normal precursors, and the majority of proteins expressed by such malignant cells are also found in normal cells. Moreover, many of the molecular changes associated with human tumors are subtle amino acid substitutions or alterations in the level of protein expression. This overall similarity of normal and malignant cells complicates the application of this paradigm that has worked so well in infectious disease to cancer treatment. In addition, because some cancer-associated molecules are not required for continued malignant potential, selection of cancer cells that have down-regulated or lost expression of the intended target represents a major mechanism of acquired resistance.

Despite these challenges, recent work indicates that this model for drug development will succeed in cancer. Approaches using small molecules or antibodies such as Gleevec, Herceptin, and Rituximab provide excellent examples of how targeting cancer-associated molecules, even those expressed by some normal cells, can lead to therapies that are not only effective but also less toxic than traditional chemotherapy. The challenge remains to identify and exploit other molecules critical to tumor maintenance.

Several lines of evidence now implicate telomeres and telomerase as potential anticancer therapeutic targets. Telomeres are nucleoprotein structures composed of arrays of repeated G-rich DNA (TTAGGG in mammals) and specific binding proteins. The integrity of these terminal structures protects chromosome ends from recognition as a broken fragment of DNA and thus promotes genome stability (1). In addition, recent evidence indicates that dysfunctional telomeres limit cell replicative life span and, in this manner, serve as a barrier to immortalization, a hallmark of the malignant state (2). Disruption of these two telomere functions contributes in essential ways to cancer initiation and progression.

Telomeres are maintained by the reverse transcriptase telomerase, which is composed of essential RNA (hTERC) and protein (hTERT) components. In mammalian cells, hTERC is ubiquitously expressed, whereas the expression of hTERT is tightly regulated, indicating that hTERT is the rate-limiting component of the telomerase holoenzyme (4, 5). Indeed, in normal human cells, hTERT is expressed at levels that are insufficient to maintain overall telomere length (6). As a consequence, telomere attrition occurs in such cells with successive cell divisions, eventually leading to telomere dysfunction, chromosome instability, and a proliferative arrest (7). However, if telomere shortening occurs in the setting of inactivation of the retinoblastoma (pRB) and p53 tumor suppressor pathways, rare cells will survive this period called crisis induced by dysfunctional telomeres and exhibit high levels of telomerase activity, stabilized telomere lengths, and replicative immortality (8). These observations suggest that constitutive expression of hTERT plays a critical role in malignant transformation. In consonance with this notion, most human tumors constitutively express hTERT and telomerase activity (9), and the experimental transformation of human cells requires telomerase activation (10).

Taken together, these findings suggest that strategies that target telomerase hold promise as novel anticancer agents. Indeed, genetic (11), antisense (12, 13), and pharmacological (14) strategies that inhibit telomerase activity in human cancer cell lines induce cell death by apoptosis, suggesting that clinically useful telomerase inhibitors may eventually be identified. In addition, several groups have reported that telomerase also serves as an excellent target for immunotherapeutic strategies (15), and Phase I clinical trials testing these strategies have recently been completed (15). Despite these promising developments, additional work is necessary to fully understand the usefulness and potential toxicities of these telomerase-specific approaches.

Although these pharmacological and immunotherapeutic strategies focus on targeting telomerase in cancer cells, an alternative method to use telomerase therapeutically relies on using telomerase biology to direct therapy specifically to cancer cells. This approach exploits the observation that hTERT is tightly regulated in normal cells while constitutively active in cancer cells (2, 8). Over the past several years, several groups have created gene expression vectors in which the upstream regulatory sequences for the hTERT gene are used to drive the expression of cytotoxic genes such as caspase-8 (16), diphtheria...
toxin A-chain (17), herpes virus thymidine kinase (18), bacterial nitroreductase (19), and Bax (20). These investigators showed that introduction of vectors encoding these hTERT promoter-driven genes in cultured human cancer cell lines rapidly induce cell death. However, implementation of these types of vectors clinically will require significant technical advances that will allow efficient insertion of these vectors into spontaneously arising human cancers while sparing normal cells and tissues.

In this issue of *Clinical Cancer Research*, Huang et al. (21) report on a related strategy to use telomerase biology to direct cancer gene therapy. These authors used a small portion of the upstream regulatory region of the hTERT gene to create a conditionally replicating oncolytic adenoviral vector (21). By introducing a 400-bp portion of the hTERT promoter upstream of the E1A gene, which is required for viral replication, these authors created an adenoviral vector that should only replicate in cells in which the hTERT promoter is active. Indeed, when introduced into normal and malignant human cells, this adenoviral vector showed 1000-fold more lytic activity in human tumor cells that express hTERT constitutively both in culture and in xenograft models. These observations corroborate and extend similar experiments in which different portions of the hTERT promoter were used to create other conditionally replicative adenoviruses (22–25).

Although both these expression vectors and conditionally replicative, oncolytic viruses attempt to exploit the relative specificity of the hTERT promoter, hTERT-driven, replication competent adenoviruses present several potential advantages over other gene therapy strategies. Because adenoviruses infect human cells highly efficiently, this approach may permit effective introduction of such viruses into cancer cells in vivo. Moreover, once productively infected, this target cell will produce adenoviruses that can then infect and presumably kill surrounding cells through a bystander effect, potentially amplifying the cytotoxic effects.

Despite these promising findings, additional work is necessary to evaluate whether these viral vectors truly replicate in a conditional manner. Indeed, although Huang et al. (21) reported a 1000-fold difference in the replication rate measured in normal and malignant cell lines, they also observed replication in normal human cells, consistent with the observation that telomerase is transiently expressed in such cells (6). This finding raises the possibility that the use of such viruses may also replicate in normal tissues and induce unintended cell killing and toxicity. In addition, we still lack a comprehensive understanding of when telomerase is activated during malignant transformation. Only by determining which normal, premalignant, and malignant cells constitutively express hTERT will we be able to make rational decisions regarding which clinical scenarios represent the optimal situations for the testing and use of these agents.

Despite these concerns, additional work, both basic and translational, will certainly lead to a deeper understanding of telomere and telomerase biology and will define the parameters that will dictate the effectiveness of these approaches. Moreover, we can expect that advances in applied immunology and gene therapy technology will increase the efficiency of these approaches. Taken together, targeting telomerase continues to represent a promising avenue for the development of novel antineoplastic strategies or one or more of these approaches may allow us to add a new tool to our armamentarium to treat cancer.

### References


A Novel Conditionally Replicative Adenovirus Vector Targeting Telomerase-Positive Tumor Cells

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ABSTRACT

Purpose: To develop a novel conditionally replicative adenovirus vector that targets telomerase-positive cancer cells.

Experimental Design: A telomerase gene-derived promoter was used to control the expression of the E1a gene so that the E1a gene is only expressed in telomerase-positive tumor cells. In addition, a reporter gene was also engineered into the vector so that its infection and replication can be monitored easily.

Results: A novel recombinant adenovirus vector that could selectively replicate in telomerase-positive cancer cells was made successfully. This vector showed active replication in a panel of cancer cells and minimal replication in normal human fibroblast or epithelial cells. The recombinant vector could effectively lyse various cultured tumor cells even at very low multiplicity of infection. The replication efficiency in tumor cells is over 105-fold more than normal fibroblast and epithelial cells. In s.c. tumor models, the newly developed telomerase-selective adenovirus vectors exhibited significantly more virus replication and reporter gene expression.

Conclusions: The telomerase-targeted adenovirus vector has significant potential as an oncolytic virus as well as a tumor-specific therapeutic gene delivery vehicle.

INTRODUCTION

The most promising feature of the cancer gene therapy approach is the potential to exploit the genetic differences between normal and tumor cells so that effective killing of tumor cells is achieved without harming normal cells in the body. With this goal in mind, many efforts are under way to develop recombinant replication-competent virus vectors that can selectively replicate in tumor cells (1–3). The eradication of tumor cells is achieved by virus-mediated cell lysis and subsequent infection and killing of neighboring cells. These recombinant viruses are termed “oncolytic” virus vectors (4–7).

One of the most popular approaches to achieve tumor-specific virus replication is the use of tumor-specific gene promoters to control the expression of essential virus genes. In this approach, the higher the specificity of the promoter, the more specific the engineered vector will be. One of the most universal tumor-specific genes identified thus far is the telomere reverse-transcriptase (TERT) gene. TERT is a ribonucleoprotein enzyme that is responsible for the maintenance of the normal length of telomeres (8). In the overwhelming majority of somatic human cells, TERT is inactive (9). Therefore, these cells have a finite life span, dictated by the length of their telomeres. Only a few normal cell types, which included the embryonic cells, germ cells, stem cells, and hematopoietic cells, have active TERT activity to enable them to divide constantly (10). Cancer cells, on the other hand, have regained the ability to maintain a stable telomere length. This ability has been shown to be a result of the reactivation of the TERT transcription. Indeed, it was demonstrated that late stage tumors had high levels of telomerase activity. Several studies indicate that over 85% of all human tumors possess active TERT activities (11).

The TERT promoter has been shown to mediate tumor-specific expression of a variety of cytotoxic genes. In this study, we report the successful use of the human (h)TERT (telomerase) promoter (12, 13) to control adenovirus vector replication. The newly engineered vectors showed strong tumor-specific replication in tissue-cultured cells. Effective lysis of the tumor cells was observed. In addition, strong intratumoral replication of the vectors was observed.

MATERIALS AND METHODS

Cell Lines

The 293 cells for virus production were obtained from the American Type Culture Collection (Manassas, VA). The other cell lines used include prostate cancer cell lines, LNCaP and Du145; breast cancer cell lines, MD-MB-231 and MCF-7; ovarian cancer cell lines, OVCAR-3 and SKOV3; and colon cancer cell lines, HCT116 and HT29. These cell lines were obtained from the Cell Culture Facility of the Duke University Cancer Center and cultured in DMEM with supplementation of 10% fetal bovine serum. Two fibroblast cell strains from the Coriell Institute (Camden, NJ) were also used. They were also cultured in DMEM with 10% fetal bovine serum. In addition, prostate epithelial cells (PrECs) were used. They were purchased from Clonetics. Medium and supplements were obtained from the...
vendor to culture the cells. All of the tumor cell lines are known to possess telomerase activities (11).

**Engineering of Adenovirus Vectors**

To engineer the telomerase-selective adenovirus, a 400-bp promoter for the hTERT gene was first cloned into the plasmid pTOPO-1 (Clontech, Palo Alto, CA) by PCR according to information published previously (12, 13). The primers used for the PCR reaction were as follows: forward primer, 5'-TGGC-CCCTCCCTCCTGGTTTTACCC-3' and reverse primer, 5'-CGCG-GGGGTGGCCGCGGGCCAG-3'.

The DNA fragment that contains adenovirus E1 gene (kindly provided by Dr. Chinghai Kao of Indiana University) was subsequently ligated 3' to the cloned hTERT' promoter. The native E1a promoter was deleted so that the hTERT promoter now controls the expression of the E1a gene. The E1b gene remained under the control of its native promoter. The hTERT-E1 gene expression cassette was then transferred into a plasmid pAE1spl1A (MicrobiX, Toronto, Canada), which contains the adenovirus left-side-inverted terminal repeat and virus-packaging signal. The dsRed2 gene, obtained from Clontech, was excised from the plasmid pDsRed2-N1 and transferred 5' to the hTERT-E1 and 3' to the packaging signal. The derived plasmid, pAE1spl1A-hTERT-E1-dsRed2 was then cotransfected into 293 cells with pBHG10 (MicrobiX, Ontario, Canada; Ref. 14). The ligation mixture was then transfected into 293 cells (with the help of liposome) for virus packaging. Recombinant adenovirus appeared in 7–10 days in the form of "comet"-like structures (15).

A similar procedure was used to derive the nonreplicative adenovirus Ad-dsRed2, which encodes the dsRed2 protein under the control of the cytomegalovirus promoter.

**Production of Adenovirus in 293 Cells**

A procedure similar to those described by Graham and Prevec (16) was followed to produce the adenovirus in large scale. Briefly, 293 cells were infected at a multiplicity of infection (MOI) of 5–10 using a total of 1 x 10^8 293 cells. After 3–4 days, the resultant viruses were purified by double CsCl banding. In general, about 3–10 x 10^10 plaque-forming units (pfu) were yielded at the end of the amplification procedure by use of 293 cells cultured in 20 150-mm Petri dishes.

**In Vitro Virus Infection Evaluation**

To evaluate the capacity of the newly made adenovirus vectors to infect and replicate in various tumor and normal cells, the cells were infected at various MOIs. Virus infection and replication were then evaluated in the following ways:

**DsRed2 Expression.** DsRed2 expression was followed by observing the cells under a fluorescence microscope (Axioskop; Zeiss). Infected cells were also subjected to flow cytometry analysis by use of the FACSscan apparatus from Becton Dickinson. It is part of the Duke University Cancer Center Core Facility.

**Virus Plaque Assays.** Virus plaque assays were carried by use of lysates from cells that have been infected with adenovirus vectors. The assays were carried out in 293 cells according to published protocols (16).

**Evaluation of Oncolytic Activity of the Virus Vector**

To evaluate the ability of AdTERT-E1a-dsRed2 to lyse tumor cells specifically, tumor and normal cells were infected with the virus vector at different MOIs. At days 3 and 5 after viral infection, the number of live cells was quantified by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which measures the activity of mitochondria enzymes in live cells. It was carried out by an established protocol (17). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma (St. Louis, MO). The catalogue number was M-5655.

**In Vivo Evaluation of Virus Infection**

To evaluate the ability of adenovirus vectors to infect and replicate in vivo, we established s.c. tumors by use of LNCaP prostate cancer cells (5 x 10^6 cells in 50 μl) and athymic Balb/C-derived nude mice (Charles River, Raleigh, NC). Virus vectors were injected into tumors when they reached sizes of 10 mm. About 5 x 10^7 pfu of virus vectors (in 50 μl of saline) were injected directly into the tumors by use of a 30-gauge needle. At different time points after injection, the tumor-bearing animals were sacrificed and their tumors excised. Some tumors were sectioned and observed under a fluorescence microscope for dsRed2 expression although others were ground up in a tissue homogenizer. The homogenates were spun down, and the supernatants were evaluated for virus titer. Virus titer was determined using the approach of Graham and Prevec (16). All animal procedures used in these experiments were approved by the Duke University Institutional Animal Use Committee.

**RESULTS**

**Successful Construction of a Telomerase-Selective Adenovirus Vector Encoding Reporter Genes.** To make a conditionally replicative adenovirus vector, we adopted a design as shown in Fig. 1. A hTERT was used to control the expression of the adenovirus E1 gene. In addition, a reporter gene, dsRed2 (a red fluorescent protein from Discosoma) was engineered into the vector. A cytomegalovirus promoter controls it. The vector, AdSERT-E1a-dsRed2, was successfully made. We were able to produce the vector with high titers (3–10 x 10^10 pfu/ml).

**Evaluation of AdSERT-E1a-dsRed2 in Various Normal and Malignant Cells.** A series of experiments were conducted to evaluate whether the newly made virus could replicate
Selective replication of AdTERT-E1a-dsRed2 in tumor cells. A, infection of AdTERT-E1a-dsRed2 in Du145 and prostate epithelial cells (PrECs). Both cells were infected at a multiplicity of infection (MOI) of 2 at day 0. Photomicrographs of the cells were then taken on days 3 and 8. Top panels are cells illuminated by transmitted white light. Bottom panels are the corresponding cells illuminated by UV light with a rhodamine filter. Notice the high percentage of dsRed2 expression in Du145 cells versus the sporadic pattern of dsRed2 expression in the PrECs at days 8 or 7 after viral infection. The cytopathic effects are also apparent in Du145 cells although it is absent in PrECs. The size bar represents 100 μm. B, Western blot analysis of E1a gene expression. Cells were infected with AdTERT-E1a-dsRed2 at a MOI of 2. Cells were harvested 3 days later, and the lysates were analyzed by Western blot analysis. Top panel, E1A expression. Bottom panel, β-actin expression, which serves as a loading control. C, active replication of AdTERT-E1a-dsRed in tumor cells and the absence of it in normal fibroblast cells GM8429 and PrEC. Each cell type was infected with AdTERT-E1a-dsRed2 and Ad-dsRed2 separately at an MOI of 0.5 plaque-forming units (pfu)/cell. Three days after infection, the cells were lysed and spun down in PBS. The supernatant was evaluated for virus titer in 293 cells. The bars represent the ratio of infectious virus particles in AdTERT-E1a-dsRed2-infected cells versus nonreplicative Ad-dsRed2-infected cells.

An initial series of experiments were conducted to observe virus replication in cancer cells. Because our virus vector has a red fluorescent protein (dsRed2), virus infection and replication can be easily monitored by observation of dsRed2 expression under a fluorescent microscope.

Cells were infected with AdSTRE-E1a-dsRed2 at a MOI of 0.05 pfu/cell, 0.1 pfu/cell, 0.5 pfu/cell, 1.0 pfu/cell, and 5 pfu/cell. As a control, they were also infected with Ad-dsRed2, which was a replication-deficient virus encoding the dsRed2 gene under the control of the cytomegalovirus promoter. The infected cells were then examined under a fluorescence microscope. A rhodamine filter was used. Active virus replication was seen in all tumor cell lines but was absent in all normal cell lines. The kinetics of virus replication was different among the tumor cell lines despite robust virus replication in all tumor lines. LNCaP, Du145, HCT116, and SKOV3 exhibited vary fast virus infection and replication kinetics whereas MCF-7 and MD-MB231 exhibited somewhat slower kinetics. The evidence for Du145 is shown in Fig. 2A, where the level of red fluorescent protein is reflective of the extent of virus replication. Among the normal fibroblast and epithelial cells, none showed the same level of virus replication as the tumor cells. AdSTRE-E1a-dsRed showed roughly the same level of fluorescence as Ad-dsRed2 (data not shown), which indicated no significant virus replication in normal cells. Flow cytometry analysis was also conducted and the results confirmed the observations with fluorescence microscopy.

E1a gene expression in the infected cells would provide an independent verification of virus replication because it is an essential protein that was placed under the control of the hTERT promoter. Therefore, Western blot analysis was carried out to examine the level of E1a expression in AdSTRE-E1a-dsRed2-infected normal and malignant cells. Fig. 2B shows the results. It was clear that detectable levels of E1 proteins were only
produced in tumor cells, confirming the tumor-specific nature of Ad5ERT-E1a-DsRed2 replication.

A third assay that was used to confirm tumor-specific virus replication by use of a virus plaque-forming assay. This assay was aimed at quantifying the amount of active virus replication in infected normal and tumor cells. A nonreplicative Ad5-DsRed2, which encoded the dsRed2 gene under the control of a cytomegalovirus promoter, was used as a control. Seventy-two h after infection, the infected cells were harvested, and the amount of active viruses from the freeze-thawed cell lysates was evaluated by plaque-forming assays. The ratios of virus plaques in Ad5ERT-E1a-dsRed2-infected cells versus Ad-dsRed2-infected cells were then plotted. The results were shown in Fig. 2. These results indicate that Ad5ERT-E1a-dsRed2 is at least three orders of magnitude more effective than Ad-dsRed2 in all of the tumor lines tested whereas the difference between the two is negligible in normal fibroblast cells and PrECs. These results essentially confirmed the observations made in the previous section.

A further assay was conducted to evaluate the tumor-specific oncolytic ability of Ad5ERT-E1a-dsRed2. After infection with Ad5ERT-E1a-dsRed2 at different MOI and incubation for different periods of time, the amount of viable cells were evaluated by staining with crystal violet or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Fig. 3 shows the results. It is clear that Ad5ERT-E1a-dsRed2 has very strong antitumor lytic capability and very weak activities in normal fibroblast or PrECs.

**In Vivo Replication of Ad5ERT-E1a-dsRed2.** To test the capacity of the newly synthesized vectors to replicate in vivo, Ad5ERT-E1a-dsRed2 was injected into xenograft LNCaP tumors established in nude mice. To do this, about $5 \times 10^6$ LNCaP cells were used to establish s.c. xenograft tumors in the flanks of nude mice. Tumors with diameters of 1 cm were
injected with $5.0 \times 10^7$ pfu of AdTERT-E1a-dsRed2 or the control nonreplicative Ad-dsRed2. Injections were carried out by use of 30-gauge needles to the center of tumors. Five days after injection, the tumors were excised and sectioned for examination. DsRed2 expression was examined. As shown in Fig. 4A, most of the tumor mass (over 90%) was infected with AdTERT-E1a-dsRed2. This was in comparison with <10% for Ad-dsRed2, a nonreplicative adenovirus that was used as a control. In addition, the level of dsRed2 expression was >100-fold higher (from quantitative fluorescence analysis) in AdTERT-E1a-dsRed2-infected tumors than in Ad-dsRed2-infected tumors. Virus replication in the tumors was also quantified by carrying out plaque-forming capability assays with excised tumor lysates 3 and 5 days after initial injection. Fig. 4B showed the results. It is clear that AdTERT-E1a-dsRed2 replicated significantly after injection whereas control (Ad-dsRed2) virus showed minimal replication. The differential is $>10^3$-fold. This is an impressive result considering that it is done in vivo. Clearly, more experiments need to be carried out to verify the applicability of this differential in vivo.

An important remaining question is whether the current vectors will have antitumor efficacies when injected intratumorally. These experiments should be done in a variety of tumor lines to examine the relationship between the ability of the vector to replicate in vitro and the ability of the vectors to control tumor growth.

DISCUSSION

Specificity and efficiency of viral replication in target tumor cells are the two most important parameters used to evaluate the potential of any novel oncolytic vectors. In this study, we evaluated the merits of a novel oncolytic adenovirus strategy that can potentially be targeted to telomerase activities, which is present in over 90% of all tumor types. Our results, although limited in nature (more tumor lines need to be evaluated to test the ability of the virus to replicate in vivo), proved that this vector can indeed replicate efficiently in vitro and in vivo in tumor cells. More importantly, in normal cells where telomerase activities are absent, there was minimal virus replication. Therefore, our results demonstrated the great potential of the telomerase-targeted virus approach.
The prototype oncolytic adenovirus vector is dl1520, or Onyx-015. It is an adenovirus vector that can selectively replicate in any cells that have lost the p53 tumor suppressor gene (1, 18). This is based on the fact that adenovirus replication requires the inactivation of the p53 gene by the viral E1b protein. Therefore, Onyx-015, which has a mutation in the E1b gene that completely knocks out its p53 inactivation capability, can replicate in cells that have already lost p53 function but not in cells with wild-type p53. Because p53 is lost in over 50% of all tumors (70% for some tumors such as colon cancer), Onyx-015 can in theory be applied for the treatment of more than half of all tumors. To date, Onyx-015 has progressed into a phase III clinical trial (19). Synergistic efficacy with chemotherapy has also been seen in patients in a phase I and a phase II trial (20).

Another promising strategy involves the use of tumor or tissue-specific promoters to control the expression of genes required for viral replication. A typical example is the Calydon CN706, where the prostate-specific antigen gene promoter drives the expression of adenovirus E1a gene (21). This virus was shown to have selective toxicity in prostate cancer cells. Even higher specificity was seen in another virus CN786, where the rat prostate-specific probasin promoter drives the expression of E1a although the prostate-specific antigen promoter drives the expression of E1b (22, 23). Synergistic interaction with radiotherapy has also been reported (13). Similar promotor-driven approaches have been reported by many other groups aimed at targeting different tumor types (24–27).

The TERT promoter as tested in our study demonstrated high specificity for tumor cells and high efficiency in mediating tumor cell-specific virus replication. These two characteristics make a telomerase-selective adenovirus vector very potent and promising as an oncolytic/gene delivery vector for cancer treatment. Compared with other types of conditionally replicative adenovirus vectors, such as Onyx-015, which can selectively replicate in p53-negative tumor cells (1, 18), and Calydon CN706, which preferentially replicates in prostate-specific antigen-positive prostate cancer, the telomerase-based conditionally replicative adenovirus can, in theory, replicate in almost any tumor cells with telomerase activity. Because telomerase activity is by far the most common tumor marker (>90% in all tumor types), the telomerase-selective adenovirus vector can be adapted to almost all solid tumor types. Therefore, compared with other types of conditionally replicative adenovirus vectors, it has a much wider range for tumor therapy. For example, p53 defect is only detected in 65% of all tumors whereas the prostate-specific antigen expression is only specific for prostate cancer patients. In addition, telomerase activity is likely correlated with the malignancy of tumors (28, 29). It is also known that the myc oncogene is a powerful regulator of telomerase activities (30, 31). Therefore, a telomerase-selective gene therapy vector may have the potential of replicating in more malignant tumor types, such as those expressing higher levels of the myc oncogene, although this has to be proven experimentally.

Taken together, the telomerase-selective replicative adenovirus vector reported here may indeed warrant additional study as a novel treatment approach to achieve significant tumor cell killing with minimal normal tissue damage.

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