Adenoviral-mediated Transfer of a Heat-inducible Double Suicide Gene into Prostate Carcinoma Cells

Robert V. Blackburn, Sandra S. Galoforo, Peter M. Corry, and Yong J. Lee

Department of Radiation Oncology, William Beaumont Hospital, Royal Oak, Michigan 48073

Abstract

Tumor cells that express a fusion gene comprised of Escherichia coli cytosine deaminase (CD) and herpes simplex virus type 1 thymidine kinase (TK) sequences exhibit activation of and subsequent killing by the normally innocuous prodrugs 5-fluorocytosine and ganciclovir (Rogulski et al., Hum. Gene Ther., 8: 73–85, 1997). To target localized expression of this therapeutic gene, we have constructed a recombinant adenovirus containing the CD-TK fusion gene under the control of a human inducible heat shock protein 70 promoter sequence. Strong expression of the fusion gene product was induced by heating at 41°C for 1 h. Expression levels obtained were dependent on the multiplicity of infection used and the incubation time after heat shock. Heat-induced expression of the CD-TK protein significantly reduced the survival of PC-3 cells in the presence of both 5-fluorocytosine and ganciclovir. These studies represent a novel form of gene therapy for the transduction and regulation of a double suicide gene in tumor cells and may provide a unique application for hyperthermia in cancer therapy.

Introduction

Although early detection and new surgical and radiotherapy regimens have contributed to improved survival and quality of life for prostate cancer patients, approximately 25–60% of patients demonstrate elevated prostate-specific antigen, which is indicative of future recurrence, by 5 years after treatment (1–3). This translates to roughly 100,000 patients/year facing the possibility of recurrent prostate cancer after initial treatment (4). Obviously, greater intervention will be required to significantly enhance primary local control of prostate cancer. A relatively recent approach to cancer treatment involves the use of gene therapy or gene transfer techniques to correct aberrant characteristics of cancerous cells or to specifically eliminate the cells through toxic gene expression (5, 6). The use of viral vectors for the transfer of so-called “suicide genes,” specifically the Escherichia coli CD (7, 8) and HSV-1 TK (9–12) genes, has been previously examined for use in gene therapy techniques. Cells transduced with the HSV-1 TK gene can convert the clinically well-tolerated prodrug GCV into its monophosphorylated toxic form. The antifungal compound 5-FC can be converted into the cytotoxic chemotherapeutic agent 5-fluorouracil by CD-expressing cells. In addition to direct cytotoxicity to the transduced cells, it has been demonstrated that significant toxicity from these two converted prodrugs can be transmitted to adjacent cells, a process known as bystander killing (13, 14). Rogulski et al. (15) have reported the construction and use of a fusion gene of the E. coli CD gene and the HSV-1 TK sequences. Transduced cells that constitutively express this fusion gene product exhibit acute supra-additive sensitivity to combined GCV and 5-FC treatment as well as enhanced radiosensitivity in the presence of both drugs (15). To date, use of this double suicide gene in the highly efficient adenoviral gene transfer systems (16–18) has not been reported.

Heating of tumor tissue (hyperthermia) has been successfully used to enhance the effectiveness of several forms of anticancer treatment, including chemotherapy and radiation therapy (19). It has been demonstrated that human prostate carcinoma cells are relatively sensitive to hyperthermic treatment (20). Through the use of heat-inducible regulatory sequences, expression of the virally transduced therapeutic gene(s) could theoretically be targeted to cells of the prostate tumor through localized infection and heating, thereby limiting normal tissue toxicity. The clinically achievable range of uniform prostate tumor heating before overt heat-associated toxicities arise is 41°C–42°C (18). We therefore chose to develop and examine a system through which heat-inducible expression of the CD-TK fusion protein could be conferred on prostate cancer cells by infection with recombinant, replication-deficient adenovirus. Evidence from our in vitro experiments indicates that we can achieve hyperthermia-inducible expression of the CD-TK protein in infected PC-3 prostate cancer cells. In addition, expression of this protein significantly sensitizes cells to the cytotoxic effects of 5-FC and GCV.

Materials and Methods

The human prostate cell line PC-3 was used to examine the cytotoxic effects of CD-TK transduction and prodrug exposure. The cells were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% FCS. Infections with recombinant adenovirus were performed in DMEM (Life Technologies, Inc.) supplemented with 5% heat-inactivated horse serum for 24 h before heat shock and incubation in maintenance medium containing the prodrug(s). Cells were heated by total immersion in a water bath (Heto) maintained within ± 0.05°C of the desired temperature.

A schematic drawing of the cloning strategy used to construct the recombinant virus is shown in Fig. 1. A 400-bp fragment of the inducible human hsp 70B gene heat shock promoter (21) was removed from plasmid pD3SX (StressGen, Victoria, Canada) by XhoI/SalI restriction digestion, gel-purified, and cloned into the adenovirus shuttle vector pAE1sp1A (Microbix, Toronto, Canada) at the XhoI and EcoRV sites, creating pAA/HS. Plasmid PWZLneo-CDglyTK containing the CD-TK fusion gene was obtained from the laboratory of Dr. Svend O. Freytag (Henry Ford Hospital, Detroit, MI) and has been characterized previously (15). The CD-TK gene was isolated by restriction digestion with BamHI and EcoRI enzymes and cloned into the identical sites in the eukaryotic expression vector pCDNA3 (Invitrogen, Carlsbad, CA). The clone obtained was digested with HindIII and PvuII enzymes to remove the contiguous CD-TK gene and bovine growth hormone polyadenylation sequences and cloned into pAA/HS at the HindIII site by blunt ligation to create pAA/HS/CD-TK. Similar procedures were used to clone the 3.7-kb β-gal gene sequence from plasmid pSVβ-gal vector (Promega, Madison, WI) into pAA/HS to create plasmid pAA/HS/β-gal for examining viral infection efficiency. The completed shuttle vectors were cotransfected (CaP01 method; Ref. 22) into 293 cells with viral plasmid pM17 to obtain replication-defective...
HEAT-INDUCIBLE GENE THERAPY IN PROSTATE CELLS

To examine the efficiency of adenoviral transfer of heat-inducible heterologous gene expression, PC-3 cells were infected with Ad.HS-β-gal (20 pfu/cell) for 24 h, followed by L h of heat shock at 41°C. The cells were then incubated for an additional 24 h before staining for β-gal activity. Infected cells that did not receive heat shock (Fig. 2, left panel) failed to express β-gal (i.e., dark blue color was not detected). However, virally infected cells exposed to heat (Fig. 2, right panel) expressed high levels of β-gal activity. Because the recombinant virus used for these studies is defective in replication (i.e., is unable to package replicated DNA for subsequent infection to adjacent cells), distribution of β-gal activity directly corresponds to the efficiency of the initial infection. Approximately 90–100% of the cells examined expressed varying levels of β-gal activity.

We next examined the parameters of heat-inducible expression in cells infected with the Ad.HS-CD-TK virus. PC-3 cells were exposed to Ad.HS-CD-TK virus (MOI = 20 pfu/ml) for 24 h, followed by heat shock at 41°C for 1 h. The infected cells were harvested at various time points (shown in hours) after heat shock for total cellular protein. Expression of CD-TK protein in adenovirus-infected clones and tumor tissue was detected using SDS-PAGE gradient gels (10–18%) and immunoblotting, as described previously (24), using a human HSV-1 TK monoclonal antibody (4C8) provided by Dr. W. C. Summers (Yale University, New Haven, CT). Proteins were visualized using the nonradioactive enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) and Fuji X-ray film.

Results

It has recently been demonstrated that transduced cells that constitutively express a fusion gene of the E. coli CD and HSV-1 TK sequences exhibit greatly enhanced cytoxicity in the presence of the prodrugs GCV and 5-FC, both in vitro and in vivo (15). We chose to investigate the use of adenoviral vectors to transfer heat-inducible expression of this fusion gene. The cloning strategy used to construct the recombinant shuttle vectors containing either the CD-TK fusion gene or the β-gal reporter gene and HS is shown in Fig. 1. The bovine growth hormone polyadenylic acid tail sequence (Fig. 1, BGH-pA) is shown at the end of CD-TK sequences. The viruses obtained were designated Ad.HS-CD-TK and Ad.HS-β-gal. One clone of each virus type was selected for the following studies.

Fig. 1. Construction of recombinant adenoviral vectors containing heat-inducible elements. A 400-bp region of the human inducible hsp 70 gene was cloned into the adenovirus shuttle vector, pΔE1sp1A, with either the CD-TK fusion gene or the β-gal gene before obtaining recombinant virus by homologous recombination in 293 cells (see "Materials and Methods" for specific details).

adenovirus by homologous recombination (16–18). Initially, 10–20 adenovirus clones (plaques) were harvested and analyzed. Virus growth, titration, and purification (by CsCl step gradient) were performed as described previously (16–18).

Expression of the transduced β-gal gene was detected using the methods of Rosenthal (23). Cells were washed three times in HBBS and fixed in 0.25% glutaraldehyde solution. Cells that express β-gal activity appear blue in color after fixation and exposure to enzymatic substrate X-Gal (Promega). Expression of CD-TK protein in adenovirus-infected clones and tumor tissue was detected using SDS-PAGE gradient gels (10–18%) and immunoblotting, as described previously (24), using a human HSV-1 TK monoclonal antibody (4C8) provided by Dr. W. C. Summers (Yale University, New Haven, CT). Proteins were visualized using the nonradioactive enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) and Fuji X-ray film.

Virus, heat, and drug effects on PC-3 cells were quantitated using clonogenic survival assays, as described previously (24). PC-3 cells were exposed to virus at MOIs ranging from 1–20 pfu/cell. GCV and 5-FC were used at concentrations of 10 and 100 µg/ml, respectively. Hyperthermic treatment consisted of total immersion of flasks at 41°C for 60 min. Survival data were obtained from at least two independent experiments, and survival data were normalized for plating efficiency, drug, virus, and heat effects. TERs were expressed as the fold decrease in the mean normalized survival of heated cells versus unheated cell survival for each parameter of drug and virus (MOI) exposure.

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To examine the efficiency of adenoviral transfer of heat-inducible heterologous gene expression, PC-3 cells were infected with Ad.HS-β-gal (20 pfu/cell) for 24 h, followed by 1 h of heat shock at 41°C. The cells were then incubated for an additional 24 h before staining for β-gal activity. Infected cells that did not receive heat shock (Fig. 2, left panel) failed to express β-gal (i.e., dark blue color was not detected). However, virally infected cells exposed to heat (Fig. 2, right panel) expressed high levels of β-gal activity. Because the recombinant virus used for these studies is defective in replication (i.e., is unable to package replicated DNA for subsequent infection to adjacent cells), distribution of β-gal activity directly corresponds to the efficiency of the initial infection. Approximately 90–100% of the cells examined expressed varying levels of β-gal activity.

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Fig. 2. Heat-inducible expression of β-gal in virus-infected PC-3 cells. PC-3 cells were infected with recombinant Ad.HS-β-gal for 24 h at a MOI of 20 pfu/ml, followed by exposure to heating at 41°C for 60 min. Cells were stained for β-gal activity 24 h after heat shock. Left panel, virus without heat; right panel, virus and heat. Dark areas indicate β-gal activity.
CD-TK expression 24 h after heat shock is demonstrated in Fig. 3B. Heat-inducible expression of CD-TK protein was detected at MOIs of 10 pfu/cell and greater. An approximately 4-fold higher level of immunoreactive protein was detected when the MOI was increased from 10 to 25 pfu/ml. Higher MOIs of 50 and 100 pfu/cell did not significantly increase the amount of CD-TK detected, possibly due to increased toxicity related to extensive viral infection. In addition, exposure to higher MOIs altered cell morphology (rounding and detachment at highest levels; data not shown).

To evaluate if heat-induced expression of CD-TK could activate the cytotoxic activity of the prodrugs GCV and 5-FC, PC-3 cells were treated with the virus alone at various MOIs, GCV or 5-FC alone, or in combined treatments with heat exposure before plating for clonogenic survival determination. Fig. 4 shows that similar toxicities were observed for virally infected, heated cells in the presence of either GCV or 5-FC alone at all MOIs, with a greater extent of killing observed with increasing MOIs (Fig. 4A). However, a marked decrease in cell survival was observed when both GCV and 5-FC were added to heat- and virus-treated cells. Supra-additive killing in the presence of both prodrugs was observed at all MOIs examined. The extent of killing by combined drug exposure was also dependent on the MOI virus used. PC-3 cells have some inherent sensitivity to the hyperthermic treatment used, because approximately 19% killing was observed by heat treatment alone (data not shown). However, heating of virally infected cells resulted in significant enhancement of prodrug killing. Fig. 4B shows the enhancement of drug toxicity (fold decrease in heated cell versus unheated cell survival) in the presence of singular or combined drug treatments at various MOIs. The level of thermally enhanced killing was dependent on the specific drug treatment and MOI used. In the presence of both GCV and 5-FC, the thermal enhancement values were approximately additive for the individual prodrug values at MOIs of 1 and 5 pfu/cell. However, at a MOI of 10, a supra-additive TER of 7.6 was obtained for combined prodrug treatment (versus an additive TER of 4.6 for singular GCV and 5-FC treatment). Increasing the MOI to 20 pfu/cell elevated the TER to 51.1-fold killing by combined GCV/5-FC treatment, as compared to an additive TER of 7.7 for singular GCV and 5-FC treatments, indicating synergistic enhancement of combined prodrug killing. Killing due to virus alone was not a factor except at a MOI of 20 pfu/cell, where an approximately 26% loss of viability was observed. The survival and TER data shown in Fig. 4 were normalized for this factor and therefore did not affect the magnitude of the thermally enhanced killing shown.

Discussion

Although current gene therapy techniques have been shown to be potentially powerful therapeutic tools, practical clinical application of
these technologies remains to be demonstrated (25). Inherent limitations of viral-mediated gene transfer include: (a) efficient delivery of the virus into tumors; (b) the limited number of cells in a tumor that can actually be infected; (c) the limited number of cells that can be infected by the virus in vivo; (d) the limited number of cells that can be infected by the virus in vitro. Therefore, effective cancer therapies involving gene transfer may prove to be more effective through the use of several modalities that complement and interactively enhance the activity of each form of treatment. To address a novel approach to this issue, we have developed the present system and in vitro testing of a system that uses recombinant, defective adenovirus to mediate gene transfer of a heat-inducible therapeutic gene into prostate cancer cells. Primary prostate tumors exhibit the features of relatively slow and localized growth at early stages, creating optimal conditions for viral-mediated transfer of drug susceptibility or cytotoxic genes. Because adenoviral vector systems can express the therapeutic gene of interest in nondividing cells, they may have a distinct advantage over retroviral-based therapies for prostate carcinomas.

As Fig. 2 demonstrates, infection with the Ad.HS-β-gal virus effectively transduces heat-inducible expression of β-gal into PC-3 cells at a MOI of 20 pfu/cell, with greater than 90% efficiency. Infection of PC-3 cells with the Ad.HS-CD-TK virus allows for heat-inducible accumulation of the CD-TK protein with time- and MOI-dependent kinetics (Fig. 3). Therefore, this system offers strong regulatory control over expression of the heterologous gene carried by the adenovirus, via relatively moderate heating. Because the amount of heat exposure used to obtain these results corresponds to clinically achievable levels of uniform prostate heating (18), it is conceivable that similar levels of induction from infected cells could be obtained in vivo.

Our studies have demonstrated that prostate tumor cells transduced with the heat-inducible CD-TK fusion gene in vitro were sensitized to killing by GCV and 5-FC through mild hyperthermic treatment (Fig. 4). It has been shown previously that glioma cells constitutively expressing the CD-TK gene exhibited slightly synergistic cytotoxicity with combined exposure to GCV and 5-FC and were acutely sensitized to radiotherapy (15). Presumably, this supra-additive/synergistic activity may result from the accumulation of cells in early S phase and DNA strand breakage (CD-mediated; Ref. 26) combined with inhibited DNA synthesis and repair (TK mediated; Ref. 27). These concomitant cytotoxic factors may also contribute to the enhanced killing observed with infected PC-3 cells in the presence of both prodrugs (Fig. 4A). Additionally, the defective viral infection itself may contribute to the combinatorial toxicity through preferential synthesis of viral components and inhibition of host protein synthesis (28) before prodrug exposure. We would expect that the extent of interactive cytotoxicity may be cell type specific, due to the relative endogenous enzymatic activities. For example, after the conversion of GCV to the monophosphorylated form, additional activity by endogenous kinases is required to produce GCV-triphosphate, the final moiety that can inhibit RNA polymerase and DNA synthesis. Similarly, the conversion of 5-FC to 5-fluorouracil by CD by deamination reactions must be followed by further endogenous processing to produce 5-fluorouridine 5′-triphosphate, and 5-fluoro-dUMP before inhibition of RNA processing and DNA synthesis, respectively. Although the exact mechanism(s) of cooperative prodrug cytotoxicity remains to be determined, it is clear that the use of heat-inducible CD-TK expression increases the efficacy of cell killing in our gene delivery system.

The hyperthermic treatment used for our studies (although only mildly cytotoxic for PC-3 cells) has a profound effect on the induction of prodrug susceptibility in the virally infected cells, particularly at higher MOI exposure (Fig. 4B). Presumably, the predominant effect of heating in this system resides in the induction of the CD-TK protein expression and subsequent prodrug activation. However, the pleiotropic and selective effects of hyperthermia alone on malignant cells may also contribute to the observed prodrug sensitization. These heat-induced alterations include changes in membrane dynamics, macromolecule (DNA, RNA, and protein) biosynthesis and stability, and energy metabolism (see Refs. 29–31 for reviews). Mild heating (41°C) has been shown to significantly enhance other forms of tumor therapies, such as irradiation, possible through the inhibition of DNA repair (32). Therefore, in addition to the roles of localizing and initiating the therapeutic effect in our gene delivery system, hyperthermia may directly contribute to the overall supra-additive cytotoxicity of the combined treatments.

For potential clinical applications of this system, three separate forms of therapy could be used to safely deliver and target the overall anticancer activity to cells of a prostate tumor. These therapies are as follows: (a) localized infection of prostate tumor tissue with a defective (nonreplicating) recombinant virus carrying a DNA sequence coding for a functional therapeutic gene; (b) systemic delivery of two nontoxic, clinically approved compounds (inactive or prodrugs); and (c) local heating of prostate tissue to activate expression of the suicide gene. With this system, only infected prostate tumor cells that express the virus-encoded therapeutic gene could convert the prodrugs to the active, toxic forms that will eliminate the infected cells. In addition, tumor tissue immediately surrounding the infected cell should also be destroyed through bystander toxicity (18, 19), thereby obviating the need to infect all cells of the tumor. Each singular form of therapy to be used would be relatively nontoxic alone, and the anticancer (toxic) effects will only be manifested as a result of the combined therapeutic activities within the confines of the tumor tissue.

The inflammatory response resulting from immunogenicity of the transgene and low level early and late viral protein expression is considered to be a major detriment to adenoviral gene therapy systems (33). However, immune response to the viral infection may prove to be beneficial in this system, because the virus would be cleared by the immune system after induction of the anticancer activity has been completed. Because the therapeutic effect could theoretically be achieved within 8–48 h after heat induction (see the time course of CD-TK induction; Fig. 3A), inflammatory responses and immune clearing of the remaining virally infected cells could subsequently reduce the risk of recombinatory events between the defective and endogenous adenovirus. For applications requiring extended heat-inducible expression of the transferred therapeutic gene, a number of second-generation adenoviral vectors have been developed (33), involving further deletions in the adenoviral genomes in the E1, E2, and E4 regions (34). In addition, new viral packaging cell lines are being developed to efficiently replicate the second-generation defective adenovirus vectors (35).

The major objective of these studies was to examine the feasibility of using heat-inducible sequences, specifically, a region of the inducible hsp 70B gene promoter, to obtain high levels of heterologous gene induction by adenoviral transfer. As with all viral based therapies, in vivo studies may differ significantly with in vitro findings, and timing of virus, heat, and drug delivery would need to be carefully examined to optimize this system in animal models. However, we believe that the use of hyperthermia-activated elements to initiate expression of the therapeutic gene could confer an additional level of stringency for the localization and specificity of future gene delivery systems and may provide unique applications for hyperthermia in the treatment of cancer.
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References

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