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We have developed a prostate-specific vector to deliver the diphtheria toxin A gene into cancer cells. Using liposome-mediate DNA transfection, the vector specifically destroys prostate cancer cells. Enhanced by the by-stander effect, the specific expression of the diphtheria toxin A gene causes significant cell death in prostate cancer cell culture, with very low background in the control cell lines. The highly specific and efficient cytopathicity combined with low immunogenicity of the diphtheria toxin A gene vector will potentially be useful for systemic treatment of patients with metastatic prostate cancer.

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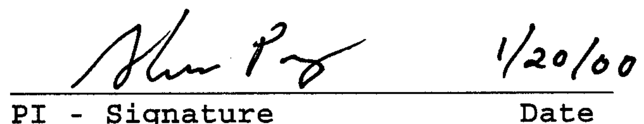

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Progress Report (Apr. 1, 1999 to Dec 31, 1999):

Introduction

The most difficult aspect of developing an *in vivo* approach for human cancer gene therapy is correctly targeting the cells of concern. Random delivery of a therapeutic gene damages normal cells in essential organs such as the liver, bone marrow, kidney and intestine and may subsequently cause death of the treated subjects (1). Using a tissue-specific promoter to transcriptionally target cancer cells is promising (2-4). To apply this strategy to prostate cancer gene therapy, a tissue-specific promoter is required. Although there are several genes that have been identified to be specifically expressed in the prostate cells, the most extensively studied gene is the prostate-specific antigen (PSA) gene. The PSA protein is exclusively expressed in the prostate cells and has been used for diagnosis of prostate cancer. The specific expression of the PSA protein suggests that the promoter of this gene may be useful for tissue specific expression of a therapeutic gene.

The promoter and the upstream sequences of the PSA gene were cloned by a few laboratories (5-7). However, the cloned sequences in most cases did not show high transcriptional activity (5-8). The low levels of activity may be related to the sources of the promoter and the enhancer that located upstream of the promoter. The low levels of activity hinders the use of the value of the PSA promoter and enhancer in a gene delivery vector. We cloned the PSA promoter and the upstream regulatory sequences from cancer cells of a prostate patient with extremely high level of blood PSA concentration, about 2 to 8,000-fold higher than normal. Sequence analysis revealed that there are mutations in the promoter and the upstream regulatory region (9). In our previous studies we found that the combination of the 550 base pair (bp) PSA promoter and the 822 bp PSA regulatory sequences together provide highest activity in driving the expression of transgenes. The activity of the patient-derived sequences is 5-50% of the activity of the promoter of cytomegalovirus early genes (CMV promoter) in prostate cell line LNCaP. Using electroporation transfection, the constructed promoter has show about 1000-fold tissue specificity (9). We hypothesize that with such a tissue-specific promoter to drive a therapeutic gene, such as a potent toxic gene, we will be able to specifically kill prostate cancer cells. To increase gene delivery efficacy, we tested five different liposomes for transfection. We tested a modified thymidine kinase (TK) gene and a potent cytotoxic gene, the *diphtheria toxin* A (DTA) gene as the therapeutic gene. Our results demonstrated that the DTA gene driven by the prostate-specific promoter can efficiently and very specifically eradicate the prostate cancer cells.

Results (Body)

1. Methods:

Plasmid constructs containing the prostate-specific promoters and the luciferase gene was described previously (9). The Plasmid containing the CMV promoter and the enhanced green fluorescent protein gene was purchased from Clontech (Palo Alto, CA). The plasmid containing the PSAR-PCPSA promoter and the EGFP gene was constructed by following steps: 1) the promoter was recovered from the plasmid PSAR-PCPSAP-Luc (9) by endonucleases Xho I and Bam HI digestion; 2) the resulting fragment was inserted into multi-restriction endonuclease sites of plasmid pNLDAT1RUEGFP (a gift from Dr. Irvin Chen, UCLA School of Medicine) digested with Xho I and Bam HI. The plasmid containing the DTA gene driven by the PSAR-PCPSA promoter was constructed by replacing the Lux gene in the plasmid PSAR-PCPSAP-Luc by the DTA gene. Briefly, the DTA gene was recovered by PCR amplification with plasmid pDT201 (purchased from American Type Culture Collection (ATCC)) as the template. The sequence coding the N-terminal 193 amino acid residues was amplified by the primers containing restriction sites for endonucleases Hind III in the 5' and Asp 718 in the 3' ends. The

resulting fragment was trimmed by digestion with Hind III and Asp 718 endonucleases before insertion into the plasmid PSAR-PCPSAP-Luc digested by Hind III and Asp 718.

Liposomes purchased from Gibco-BRL (Gaitherburg, MD), Promega (Madison, WI) and Boehringer Mannheim (Indianapolis, IN) were tested following the methods suggested by the manufacturers.

Prostate cell line LNCaP and DU145 were purchased from ATCC and oral tumor cell line T139 was a gift from Dr. NH Park, UCLA School of Dentistry. Other cells were described previously (9). Cells were maintained in RPMI medium with 10% fetal bovine serum from GIBCO-BRL

Luciferase assay was performed using the kit from Promega (Madison, WI).

2. Obtained results

Although electroporation-mediated DNA transfection is efficient in delivering the vectors into the prostate cells (9), it may not be suitable for *in vivo* gene delivery. Alternatively, liposome-mediated gene delivery has been shown to be feasible for clinical trials and *in vivo* gene delivery (10-12). Since no virus is involved, this method will not induce an immune response against re-injection of the vector. To achieve a high transfection, we used luciferase and green fluorescent protein genes as reporters to test liposomes from several sources, and a high efficient liposome was selected. With the optimized liposome-mediated gene delivery method, we transfected the prostate cancer cell line LNCaP and control cell lines derived from renal carcinoma, non-PSA-producing prostate cancer and oral cancers.

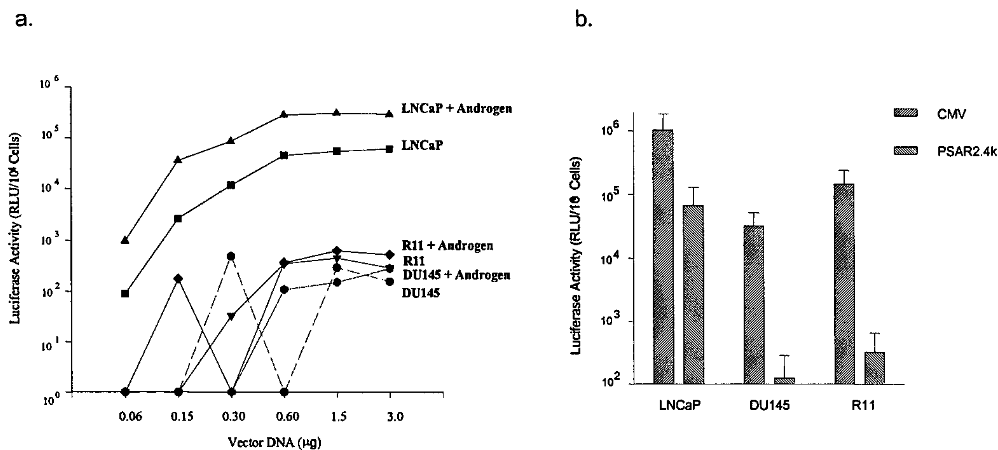

Fig. 1. Transfection by a prostate-specific vector containing the luciferase gene. Panel A) Plasmid PSAR2.4k-PCPSA-P-Lux (9) was utilized to transfect the PCA-producing prostate cell line, LNCaP, and control cell lines, R11 and DU145. Cells with the number of 5×10^4 were plated to each well in 24-well plates 16 hours prior to lipofection. Androgen dihydrotestosterone (DHT) (50 nM) was added to one set of transfected cell lines one hour post-transfection. Penal B) Comparison of the prostate specific vector with vector containing the CMV promoter.

Using the liposome we tested, satisfactory transfection efficiency was achieved. Several different liposomes were tested and the liposome FuGENE 6 from Boehringer Mannheim (Indianapolis, IN) showed best results. In the PSA-producing prostate cell line LNCaP, the activity of luciferase was 200 to 1000-fold higher than that in the renal cell line, R11 or in the non-PSA-producing prostate cell line DU145 (Fig. 1). It is not surprising that the non-PSA-producing prostate cell line DU145 does not support our PSAR-PCPSA promoter since this cell line does not support the expression of endogenous PSA gene either. The transcriptional activity and the tissue specificity is comparable to our previous result using electroporation[9]. With the addition of 50 nM androgen dihydrotestosterone (DHT) to the medium, the luciferase

expression increased about 10-fold while no significant increase of luciferase expression was seen in the controls (Fig. 1a). We compared the prostate-specific promoter side by side with the well-characterized CMV promoter, and the results indicated that the transcriptional activity of the prostate-specific promoter in the LNCaP prostate cell line was about 10-50% of the activity of the CMV promoter (Fig. 1b).

We have inserted the modified thymidine kinase gene (13) into the prostate-specific vector. Although the modified thymidine kinase gene should be at least 10-fold potent than the wild-type thymidine kinase gene, no significant cytopathic effects developed in the infected cells (data not shown). Alternatively, we tested the toxic gene, diphtheria toxin A (DTA). The coding region of the amino terminal 193 amino acid residues was recovered by polymerase chain reaction (PCR) with the additions of start and termination codons. Since the DTA can block protein synthesis, the effect of DTA gene expression can be evaluated by the inhibition of luciferase gene expression it a luciferase gene is present. We co-transfect the DTA vector with the luciferase vector into the PSA-producing prostate cell line LNCaP. Significant decreases in luciferase expression was demonstrated (Fig. 2) suggesting that the DTA expression in the prostate cells blocks protein synthesis.

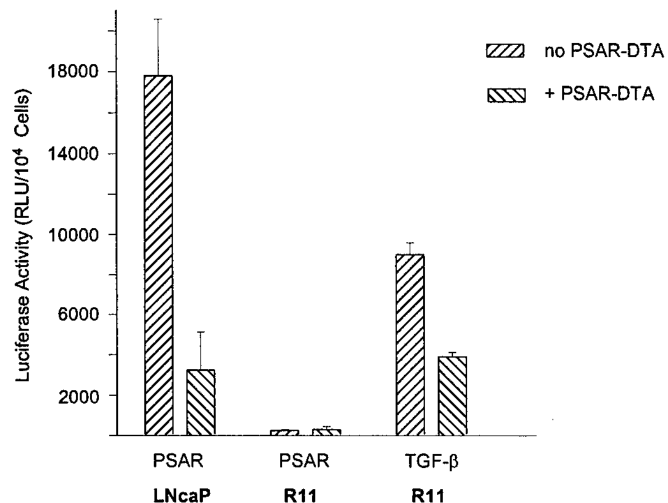

Fig. 2. Inhibition of protein synthesis by expression of DTA gene. The plasmid containing PSAR-PCPSA promoter and the DTA gene was co-transfected with either the plasmid PSAR2.4k-PCPSA-P-Lux or the plasmid containing transforming growth factor β (TGFβ) promoter and the luciferase gene. Cell cultures were set up by the method described in Fig. 1. DNA from plasmids PSAR2.4k-PCPSA-P-Lux or TGFβ-P-Lux DNA (1.2 μg) with or without 0.5-0.6 μg PSAR-PCPSA-P-DTA plasmid were used to transfect cultured cells. Luciferase assay were performed 2 days post-transfection.

The DTA peptide inhibits protein synthesis by binding to elongation factor 2 (EF-2). Although the toxicity of DTA has been widely recognized, the cytopathic kinetics of DTA is not well-characterized. If only the transfected cells can be killed by the expression of DTA, the liposome mediated method will be less significant, since only a fraction of cells can be transfected and among the transfected cells, some of them may not support the prostate-specific promoter. In such case, only a small fraction of cells can be killed and most cancer cells may continue growing and become resistant to the expression of DTA. To evaluate the transfection efficiencies and characterize the expression profile in prostate cells, we inserted the enhanced green fluorescent protein gene (EGFP) (purchased from Clontech, Palo Alto, CA) into the prostate-specific vector. Four days post-transfection, we counted the positive cells under a UV microscope. We found that 5-10% cells of LNCaP were transfected with detectable expression (Fig. 3). CMV vector showed higher expression in the transfected LNCaP cells and 25-50% cells were shown detectable level of gene expression. In the control cells such as R11 renal, DU145 non-PSA-producing prostate, and T139 oral cancer cell lines, expression of the enhanced green fluorescent protein was also very high with transfection by the CMV vector, while no expression was detected in the cell culture transfected by the prostate-specific vector.

The lower percentage of transfected positive cells by the prostate-specific vector in LNCaP cells suggests that the cell line contains heterogeneous prostate cells. Only a portion of cells support the expression of the prostate-specific vector. We postulate that same percentage of LNCaP cells was transfected by each of these two vectors; however, the CMV vector was expressed in all the transfected cells, while the prostate-specific vector only expressed in about one-third or less of the transfected cells.

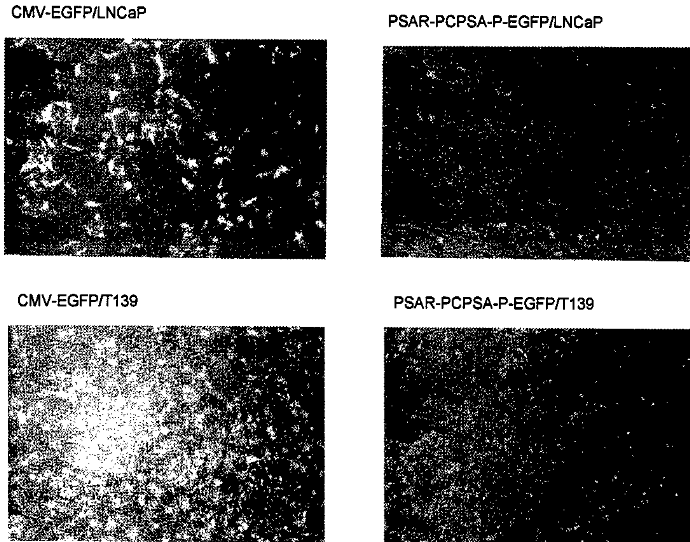

Fig. 3. Plasmids with the EGFP gene driven by either the CMV or the PSAR-PCPSA promoter were transfected into either LNCaP cell line or the oral cancer cell line T139. Three days post transfection, the expression of the EGFP gene could be detected under a UV microscope.

It is important to determine whether the expression of the DTA gene in the prostate-specific vector can cause significant pathogenic effects. We transfected the PSA-producing prostate cell line LNCaP and the control cell lines with the DTA vector. Significant pathogenic effects were demonstrated 7-8 days post-transfection (Fig. 4). By day 8 post-transfection, more than 95% cells died in the DTA transfected LNCaP cell culture compared with only about 10-20% cell deaths in the cell culture transfected by other plasmids or liposome only. Since only 5-10% transfected LNCaP cells actively express DTA protein, cell deaths may not be attribute only to the expression of the DTA gene. Most cell deaths may cause by the by-stander effect similar to what is seen with the expression of the thymidine kinase gene. It is also possible that the DTA or other toxic factors released from the transfected cells enter the not transfected cells, causing cell death. To ascertain such effect we set up cell cultures, using cell cultures in Transwell plates (purchased from Fisher). The LNCaP cell line in the lower chambers was transfected by the DTA vector and the R11 renal cell line or DU145 cell line were cultured on the upper chambers of the plates after the transfection of the LNCaP cells . Significant cell deaths occurred in the transfected LNCaP cell line, but not in the upper chamber cell cultures, suggesting that no cytotoxic factors were secreted from the transfected LNCaP cells. We performed a similar test

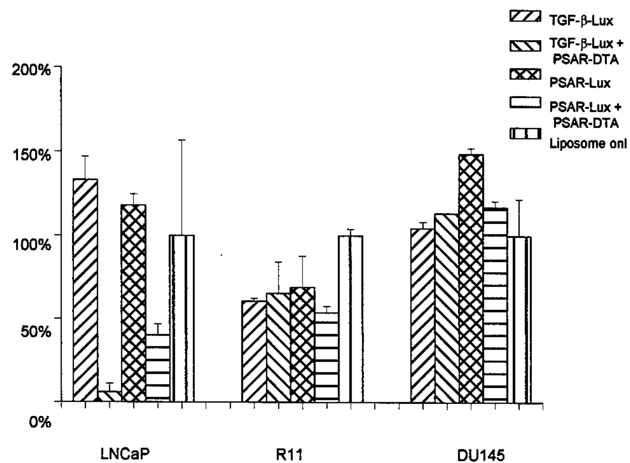

Fig. 4. Tissue-specific pathogenic effects of the prostate-specific vector carrying the diphtheria toxin A gene. DNA transfection and the cell culture were set up by methods described in the legend for Fig. 2. Culture medium was changed every 3 days. Cell numbers were counted under microscope with trypan blue staining. The number of surviving cells from the plates transfected by liposome only was counted as 100% surviving.

with LNCaP cell line cultured in the upper chamber, but the cells did not grow well in the upper chamber plates with pores. We added condition medium from the DTA vector transfected LNCaP cell culture to the untransfected LNCaP cell culture, but no significant cell death occurred. The results suggest that contact of the transfected cells to the untransfected cells is required for inducing the death of the untransfected cells.

Previous reports suggest that inhibition of protein synthesis by proteins such as diphtheria toxin activates the machinery of cell apoptosis (14-15). The pathogenic effects of DTA may involve a chain reaction and the inhibition of protein synthesis may activate the reaction chain. Since the prostate cancer cells grow slowly, the thymidine kinase gene combined with Ganciclovir (GCV), is not very potent in such environment. In contrast the DTA gene that is independent of DNA synthesis is more potent in inducing cancer cell deaths.

Since DTA kills cells via apoptosis and the dead cells can induce immune response specifically against the cancer cells (19,20), potentially, the delivery of the vector into patients will not only eradicate cancer cells by direct effect such as apoptosis and the by-stander effect, but also indirectly though activation of the immune system against the metastatic cancer cells.

Key Research Accomplishments

During the period from Apr. 1, 1999 to Dec. 31, 1999, we complete the tasks described in the our grant proposal as following :

Task 1. To complete the construction of the Lux gene expression vector (months 1-6)

a. Using the PCR amplification method to obtain the AAV ITR sequences and to insert them into the plasmid pPSAR-PSAR-Lux.
b. Insertion of the EBNA-1 and oriP sequences recovered from the pREP10 plasmid into the gene vector.
c. Checking the resulting vector by DNA sequencing and restriction mapping.

- We have not completed task 1, a. due to thedifficulty involved in obtaining AAV ITR plasmid clones. Recently, we obtained an AAV plasmid clone and we are now working on this task. We have cloned EBNA-1 and oriP sequences from pREP10 to Lux gene expression vector (Task 1, b.) and has checked the clones by restriction mapping (Task 1, c.).

Task 2. To test the Lux expression vector in cell culture (months 7-9)

a. Using the Lux expression vector to transfect the LNCaP prostate cell line and control cell lines including the renal cell carcinoma line R11, the breast cancer cell line MCF-7, non-PSA-producing cell line DU145 and HeLa cells and to assess the tissue specificity of the constructed vector by luciferase assay.
b. Characterizing the responsiveness of the Lux vector to androgen stimulation. The cell lines to be transfected by the Lux vector will be maintained in the medium containing only charcoal stripped fetal bovine serum for three days before the transfection. After transfection, varying concentrations of dihydrotestosterone (DHT) will be added to the transfected cells. Two days post transfection the cells will be collected for luciferase assay.
c. Testing more liposomes to identify one with higher gene delivery efficiency.

- We tested our Lux plasmid vector using LNCaP prostate cell line and control cell lines, R11, MCF-7, DU145 and an oral cancer cell line TU139 and found that the cloned Lux plasmid vector is highly specific to prostate tissue (Task 2 a.). We also tested the effect of androgen stimulation. After plasmid transfection, varying concentrations of androgen, dihydrotestosterone (DHT), were added to the transfected cell cultures. A significant increase of luciferase gene expression was demonstrated in prostate cancer cell line LNCaP but not the control cell lines (Task 2 b.). We tested 8 different liposomes and found that a liposome from Roche Molecular Biochemicals transfected prostate cells with high efficacy (Task 2, c.).

Task 3. To complete the construction of the Diphtheria toxin A (DTA) expression vector (month 10-11) PCR cloning method will be used to generate appropriate restriction sites in the ends of the DTA gene. The DTA fragment will be recovered by agarose gel electrophoresis and will be used to replace the Lux gene in the vector.

- We have cloned the DTA gene into our Lux vector

Task 4. To test the DTA vector using cell culture (months 12-18)

a. Defining the efficiency of the DTA vector in inhibition of protein synthesis. The DTA vector will cotransfect cells with the plasmid containing the Lux gene driven by the CMV promoter. The expression of the DTA gene in LNCaP cells will efficiently inhibit the generation of the Lux protein, thus only low Lux activity will be detected. In other cells, the DTA will not be efficiently expressed, thus the Lux activity in these cells will be high.
b. Quantitation of the bystander effect by the DTA. Cells transfected with the DTA vector will be mixed with the cells transfected with the CMV-Lux vector. If the DTA can be transported from the transfected cells to the adjacent cells that are transfected with the CMV-Lux vector, the Lux expression in the CMV-Lux vector transfected cells will be inhibited, thus lower Lux activity will be demonstrated.
c. Assessing the tumor cell eradication effect by the DTA vector. The inhibition of protein synthesis by DTA will ultimately kill the transfected cells. The prostate specific promoter will only permit the DTA to be actively transcribed in the PSA-producing prostate cells. Thus significant cell eradication will only be demonstrated in these cells.

- We have cotransfected our DTA vector with a plasmid vector containing the Lux gene. Significant inhibition of the lux gene was demonstrated in LNCaP prostate cell line but not the control cell lines (Task 4, a.). We have encountered some difficulties in performing the experiments described in Task 4, b. The major problem is that after transfection, LNCaP becomes very sensitive to trypsin, so most cells died when the two transfected cells mixed and plated to a new plate. However, we tested the by-stander effect by comparing the transfection efficiency determined by using a PSAP-EGFP (enhanced green fluorescent protein gene driven by the PSA promoter) vector and the percentage of pathogenic cells in the culture wells transfected by PSAP-DTA vector. Thus we used a different approach to investigate the effect of DTA in prostate cells (Task 4, b.). We also transfected a prostate cell line, LNCaP, and the control cell lines by the prostate specific DTA vector. Significant cell pathogenic effects were demonstrated only in the prostate cell line LNCaP (Task 4, c.).

- In summary, we have completed task 1 to 4 except task 1, a. We actually not only obtained the results we planed on time, but also have done additional researches related to this project including clone a prostate specific EGFP plasmid vector. completed

Reportable Outcomes

Some results from these studies have been used as preliminary results for applying a grant submitted to California Cancer Research Program. Please see Appendices, page 12.

Conclusions

We have developed a prostate-specific vector to deliver the diphtheria toxin A gene into cancer cells. Using liposome-mediate DNA transfection, the vector specifically destroys prostate cancer cells. Enhanced by the by-stander effect, the specific expression of the diphtheria toxin A gene causes significant cell death in prostate cancer cell culture, with very low background in the control cell lines. The highly specific and efficient cytopathicity combined with low immunogenicity of the diphtheria toxin A gene vector will potentially be useful for systemic treatment of patients with metastatic prostate cancer.

Compared with the research tasks we proposed, we found the researches go very smooth and we predict that we will be able to follow the schedule to accomplish the expected goals in this funding year.

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Appendices

Some results were used as Preliminary Studies in a grant application submitted to California Cancer Research Program. The grant is under pending circle.

Project Number (Principal Investigator):	
Source:	California Cancer Research Program (CRP),
Title of Project (*and/or Subproject*):	Cloning Genes Required for Prostate Cancer Growth
Dates of Approved/Proposed Project:	07/01/2000 -06/30/2003
Annual Direct Costs / Percent Effort:	$150,477 / 25%

The Specific Aims are to:

1) Clone the genes that positively regulate the expression of the PSA promoter;
2) Identify the genes specifically regulating prostate cancer cell growth, and generate antisense RNA and dominant mutants of these cloned genes to inhibit the growth of tumor cells.