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Research Paper

Construction of Smac gene-containing and human prostate specific antigen promoter-regulated vector and its expression☆

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Abstract

Objective: To construct an eukaryotic expression vector containing Smac gene and study the expression efficiency and specificity of prostate specific antigen(PSA) enhancer/promoter in a possible targeted gene therapy scheme for prostate cancer. **Methods:** PSA enhancer (PSAE) and promoter (PSAP) sequences were amplified using PCR method. CMV and T7 promoters were deleted from pcDNA3.1-Smac and replaced by the two specific fragments to generate pPSAE-PSAP-Smac. After transfection into different cell lines, the status of cells was observed. And then, we determined the relative concentration of Smac mRNA in RT-PCR. **Results:** The recombinant plasmid of pPSAE-PSAP-Smac was successfully constructed. And only the prostate cancer cell line PC-3 was suppressed after transfection with pPSAE-PSAP-Smac. However, other nonprostate lines were not. Moreover, the concentration of Smac mRNA regulated by PSA promoter and enhancer was higher in comparison to the CMV promoter-driven control vectors. **Conclusion:** An expression vector containing the Smac gene (based on elements of the PSA gene regulatory sequences) has been developed and shown to function in prostate cancer cell lines which provides a solid platform for launching clinical studies.

Keywords: prostate specific antigen; enhancer; promoter; Smac; gene therapy

INTRODUCTION

Prostate cancer is one of the common malignant tumors in the urogenital system. The increased use of PSA testing is resulting in a rapid rise in the numbers of diagnosed cases and is producing a marked change in case-mix toward early disease. To date, curative therapy for prostate cancer exists only for early stages and nonmetastatic disease, whereas patients with metastatic prostate cancer have a poor survival rate. Although androgen ablation at least leads to a partial remission in about 70-80% of prostate cancer patients, tumor recurrence is very likely after several months or years [1-3]. Therefore, alternative treatment strategies for prostate cancer are urgently needed.

Cancer gene therapy is the transfer to(and expression of) genetic material in malignant human cells for a therapeutic purpose[4]. The novel approaches to prostate cancer are attractive because they hold the possibility of selective targeting therapy to the affected tissue, thereby avoiding toxicities associated with treatments such as cytotoxic chemotherapy, which has thus far proved of limited benefit in prostate cancer[5-7]. PSA is a protein expressed exclusively by benign, hyperplastic, and malignant prostatic epithelium. Rising levels in serum are indicative of prostate disease, and this has allowed PSA to be used as a diagnostic marker [1,8-10]. In addition, PSA is expressed in prostatic tissue in tissue specific fashion under tight control by tissue specific promoters. These promoters provide a means of targeting the expression of therapeutic transgenes selectively to

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prostate cancer.

Recently, Smac, the second mitochondria-derived activator of caspase (a mitochondrial protein that is released together with cytochrome c from the mitochondria in response to apoptotic stimuli) was found to promote caspase activation by binding and neutralizing the IAPs(Inhibitors of apoptosis proteins)[11,12]. In this study, we attempted to construct and evaluate a prostate tissue-specific vector containing Smac. PCR was used to clone the upstream fragments of the PSA 5′-flank sequences. DNA isolated from a patient with prostate cancer served as the PCR template. Two 1.6 kb (PSA enhancer, named PSAE) and 0.5 kb (PSA promoter, named PASP) DNA fragments were obtained and subcloned into a plasmid containing Smac gene. DNA transfection and RT-PCR were performed to test the concentration of Smac. Our results demonstrate that the PSA promoter and enhancer can dramatically increase gene expression while preserving tissue specificity.

MATERIALS AND METHODS

Reagents and supplies

Super high fidelity PCR kit was bought from Bio Basic Inc(Ontario, Canada). Genomic DNA extraction kit, PCR purification and gel extraction kit, and plasmid minipreparation kit were supplied by Tiangen Biotech (Beijing, P.R. China). Restriction endonucleases, T4 DNA ligase and DNATrizol were bought from TakaRa Biotech (DaLian, P.R. China). Transfection reagent Lipofectamine 2000 was offered by Invitrogen (California, USA). Plasmid pEGFP-N1, engineering bacteria JM109 were routinely preserved in the Urology Laboratory of Union Hospital. Plasmid pEGFP-1 was presented from Dr. Wenzhuang Shen and pcDNA3.1-Smac was from Dr. Xiaodong Wang as a gift.

PCR cloning and construction of plasmids

According to the PSA 5′-flank region sequences (GenBank Accession Number U37672.1) and the principles of primer design, several primers were obtained. *Bgl*II/*EcoR*I were ligated onto the end of the PSA enhancer (from −5325 bp to −3704 bp) primers, whereas *Sal*I/*Apa*I were added onto the end of the PSA promoter (from −534 bp to −13 bp) primers to amplify sequences with different restriction enzyme sites. Primers were shown as below: PSAE sense (5′-GAA GAT CTC CCT CTA GAA ATC TAG CTG AT-3′), PSAE antisense (5′-CGG AAT TCT CAA ACC CAG AAG TTC TGA T-3′), PSAP sense (5′-GCG TCG ACT TTA TGA TGA CAG TAG CAA TG-3′), PSAP antisense (5′-ATT GGG CCC CAG GAG CCC TAT AAA ACC-3′). Primers were synthesized by Shanghai Sagon Biological Engineering Technology & Services Co., Ltd. DNA isolated from a patient with prostate cancer was used as the template. PCR for PSAP was performed with 30 cycles of denaturing for 30 seconds at 94℃, annealing for 30 seconds at 57℃, and allowed primer extension for 1 minute at 72℃. PCR for PSAE was performed with 30 cycles of denaturing for 30 seconds at 94℃, annealing for 30 seconds at 57℃, and given a primer extension of 2 minutes at 72℃.

Plasmid DNA was harvested from Escherichia coli strain JM109 with the plasmid minipreparaiton kit. The purified PSAP was subcloned as a *Sal*I/*Apa*I fragment into the pEGFP-1 vector, to construct recombinant plasmid pPSAP-EGFP. An equivalent construct was made using purified PSAE with *Bgl*II/*EcoR*I sites in similar fashion, to give plasmid pPSAE-PSAP-EGFP. Moreover, the constructed product pPSAE-PSAP-EGFP and pcDNA3.1-Smac were digested with restriction enzyme *Bgl*II/*Apa*I. After that, the *Bgl*II/*Apa*I fragment of PASE-PSAP was inserted into the same site of pcDNA3.1-Smac to generate pPSAE-PSAP-Smac. All of recombinants were identified by double enzymatic digestion and DNA sequencing.

Cell lines culture and maintenance

Human prostate cancer cell line PC-3 and human bladder cancer cell line EJ were preserved in our department. Other nonprostate lines HepG2 (human hepatoma cell line), A375 (human melanoma cell line) were gifts from Dr. Wenzhuang Shen. All the cells were routinely grown in RPMI 1640 medium (Gibco, USA) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2.

DNA transfection and trypan blue assay

Cells of PC-3, EJ, HepG2, A375 were washed once in PBS, trypsinized, and then washed. Then, cells were resuspended with RPMI 1640 and plated out in 6-well plates at 5×10^5/well. Cells were grown until 80 - 90% confluent and then transfected with the use of Lipofectamine 2000 according to the manufacturer's instructions. Each cell line was divided into four groups: a blank group (no transfection), the control group (pPSAE-PSAP-EGFP), transfection group 1(pcDNA3.1-Smac) and transfection group 2 (pPSAE-PSAP-Smac). The cells were overlaid with DNA/ Lipofectamine 2000 complexes (4 μg plasmids with 10 μl liposomes) in a total

volume of 2.5 ml of RPMI1640 without FBS. Cells were incubated at 37℃ in a humidified incubator with 5% CO_2. Six hours later, we changed the medium RPMI1640(now containing 10% FBS). The growth of cells was observed throughout the course of the experiment with an inverse phase-contrast microscope. The number of damaged PC-3 cells was estimated by trypan blue staining.

RT-PCR

After transfection for 24 hours, PC-3 cells were collected. Target gene expression was measured by an RT-PCR assay of Smac mRNA. Forward primer (5′-ATG CTC GAG GCG TTG ATT GAA GCT ATT ACT GAA TAT-3′) and reverse primer (5′-AGC CGG ATC CTC AAA TGG GTA AGA GCA GCT GTA CAG AGT-3′) were designed. Amplification conditions were 3 minutes at 94℃ followed by 30 cycles of 30 seconds at 94℃, 30 seconds at 55℃, 1 minute at 72℃, and then 5 minutes at 72℃. Product concentrations were analyzed by a gel imaging system with β-actin as an internal control.

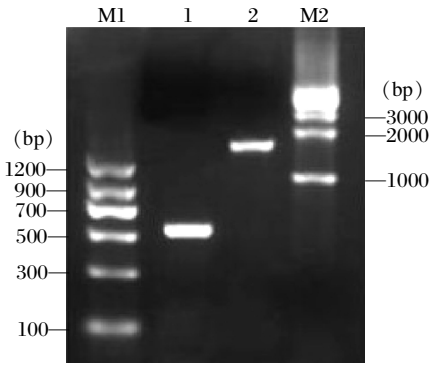
Statistical analysis

All the data were analyzed by ANOVA and *t* test with SPSS software.

RESULTS

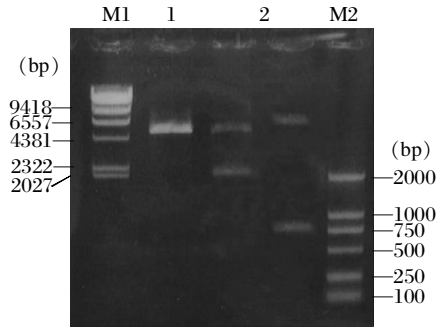
Identification of PCR products and recombinant plasmid

PCR products of PSA promoter and enhancer were initially identified by electrophoresis. Amplified bands were clear and specific at 537 bp and 1637 bp, respectively(***Fig 1***). The recombinant plasmid of pPSAE-PSAP-Smac was digested using several restriction endonucleases. A 2.1-kb fragment (PSAE-PSAP with *Bgl*II/*Apa*I) and a 0.7-kb fragment (Smac with *Apa*I/*Kpn*I) were obtained respectively (***Fig 2***). And then, the fragments were confirmed by DNA sequencing in Takara Biotechnology Co, Ltd, Dalian.



M1, M2: DNA marker; 1: PSAP (537bp); 2: PSAE (1637 bp)

Fig 1 Agarose gel electrophoresis results of PASP and PSAE



M1, M2: DNA marker; 1: pPSAE-PSAP-Smac; 2: pPSAE-PSAP-Smac (*Bgl*II/*Apa*I); 3: pPSAE-PSAP-Smac(*Apa*I/*Kpn*I)

Fig 2 Restriction enzyme digestion analysis of recombinant plasmids

Growth inhibition

No marked change was observed on the growth velocity of all cells transfected with pPSAE-PSAP-EGFP as compared to the blank group. However in transfection group 1 (pcDNA3.1-Smac), we found the growth of four cell lines was inhibited significantly($P < 0.05$). And we also determined that only the PC-3 cell line was suppressed after transfection with pPSAE-PSAP-Smac($P < 0.05$) (***Fig3***,***4***).

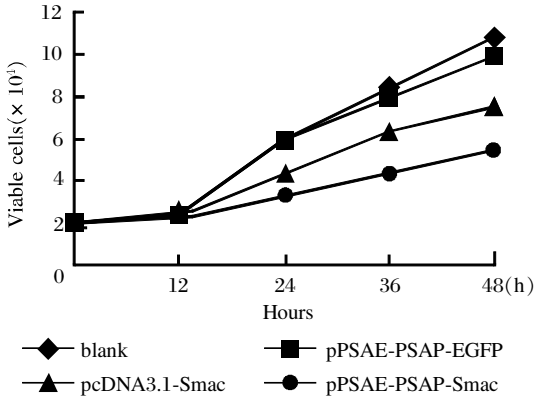


Fig 3 Growth inhibition of PC-3 after different plasmids transfection

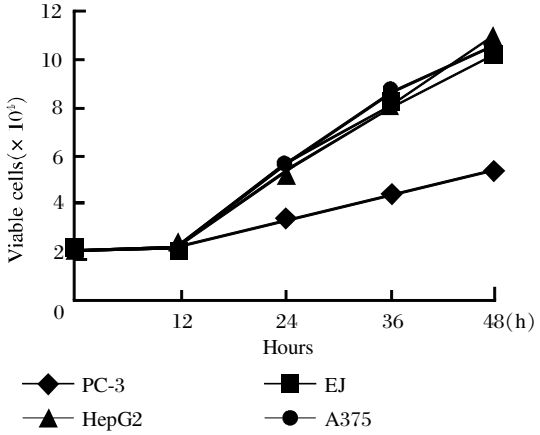
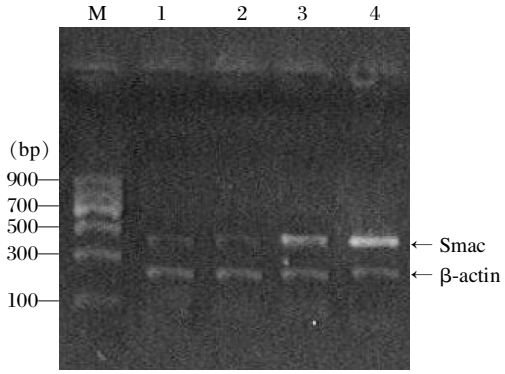


Fig 4 Growth inhibition of different cell lines after pPSAE-PSAP-Smac transfection

RT-PCR

The PCR products were clearly visible after 2% agarose gel electrophoresis and ethidium bromide staining. The RT-PCR product's amounts of Smac mRNA were standardized relative to that of β-actin mRNA in the same sample via densitometric analysis by the autogel-ananlysis system. The results were expressed as the relative level of mRNA expression (ratio of Smac/β-actin). As shown in ***Fig 5***, the products of RT-PCR for human β-actin mRNA and Smac mRNA were 227 bp and 437bp respectively.

In blank and control groups, the basic expression of Smac mRNA in PC-3 were no difference. But in pcDNA3.1-Smac group and pPSAE-PSAP-Smac group, the relative levels of Smac mRNA expression were 1.83, 2.64 folds higher than that in blank group respectively ($P < 0.05$). The transfection groups were significantly higher than the former. Furthermore, the concentration of Smac mRNA regulated by PSAE-PSAP was also higher comparing to the CMV promoter-driven control vectors($P < 0.05$).



M: DNA marker; 1: blank group; 2: control group; 3: pcDNA3.1-Smac group; 4: pPSAE-PSAP-Smac group

Fig 5 Agarose gel electrophoresis results of Smac

DISCUSSION

The ultimate goal of cancer therapy is to get the maximum of tissue-specific cytotoxicity with a minimum of toxic side effects in nonmalignant cells. Gene therapy using tissue-specific promoters provides a way of selectively targeting therapeutic genes to malignant cells[13,14]. In the case of prostate tissue, because the PSA gene is strictly regulated in a tissue-specific manner, the PSA promoter might be an ideal means for prostate cell-specific gene delivery [15]. We therefore set out to optimize tissue-specific control of expression using the PSA promoter as a model.

Earlier reports with the 632-bp proximal PSA promoter suggested that the region from -632 to -1 from the gene was sufficient to give tissue-specific regulation of gene expression[16]. And this was further supported by subsequent studies aimed at testing targeted expression constructs for gene therapy vectors[17]. However, it could not produce a marked effect. Subsequent research suggested that a 4 kb upstream enhancer was required. Schuur *et al*[18] described the location of enhancer sequences in the DNA upstream of the PSA gene. And the presence of a 1.6 kb core enhancer from −5322 to −3740 was a prerequisite for high activity [19]. In the present study, we cloned the 537 bp promoter and 1637 bp enhancer of PSA from a prostate cancer patient for our investigations aimed at developing promoter constructs for our gene therapy program. DNA sequencing confirmed that the two fragments were correspondent to the published sequences U37672.1. Then, in our hands, the CMV promoter in pcDNA3.1-Smac was deleted and the tandem PSA enhancer-PSA promoter configuration was introduced in an attempt to increase the level of target gene expression. This gave substantial increases in expression.

Clearly the first major challenge is that of efficient gene delivery. If this can be achieved, the next decision is the selection of the most appropriate target gene of therapy [20,21]. Smac (a mitochondrial molecule) has been documented to be a neutralizing inhibitor of IAP family proteins. Upon receiving a death signal, mitochondria release Smac into the cytoplasm, in addition to the release of cytochrome C. Cytoplasmic Smac binds to IAP family members. It subsequently relieves the IAP-mediated inhibition of caspases-9 and −3 and promotes the apoptosis of cells [11,12]. In this study, we transferred the Smac gene into different cell lines to observe its depressant effect. Trypan blue assay showed that Smac could inhibit the proliferation of cells, which is correspondent to earlier reports. And if the gene vector was tissue specific, only the homologous cells were suppressed. RT-PCR determined the mRNA expression of Smac. We found that the relative levels of expression of Smac mRNA in the pcDNA3.1-Smac group and pPSAE-PSAP-Smac group were higher than that in blank and control groups ($P < 0.05$). In addition, the concentration of Smac mRNA regulated by PSA promoter and enhancer was also higher comparing to the CMV promoter-driven control vectors($P < 0.05$). These demonstrated that the recombinant plasmid pPSAE-PSAP-Smac could improve the concentration of Smac in cells and preserve the characteristics of tissue restriction in the same time. In the near future, further studies will focus on the relative molecular mechanism involved in apoptosis pathway

of Smac and its protein function.

In conclusion, an expression vector based on elements of the PSA gene regulatory sequences has been developed and shown to function in prostate cancer cell lines. As is apparent from the large number of studies published in the last years, the pace of research into gene therapy for prostate cancer is likely to accelerate in the coming years. This research serves as a method of maximizing cytotoxicity to the target tissue where the gene of interest is expressed, while minimizing exposure to other cells (that do not express it) therefore providing a solid platform for launching further clinical analysis.

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