Development of Gene Therapy Using Prostate-specific Membrane Antigen Promoter/Enhancer with Cre Recombinase/LoxP System for Prostate Cancer Cells under Androgen Ablation Condition

Shusei Ikegami,1 Takushi Tadakuma,2, 3 Satoshi Suzuki,1 Ichiro Yoshimura,1 Tomohiko Asano1 and Masamichi Hayakawa1

1Department of Urology and 2Department of Parasitology and Immunology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513

To enhance the efficacy of suicide gene therapy for prostate cancer under androgen deprivation, we designed a promoter system that consists of the prostate-specific membrane antigen (PSMA) promoter/enhancer (PEPM) and Cre-loxP DNA recombination system. We constructed two kinds of plasmids. One plasmid contains a Cre recombinase (Cre) under the control of PEPM and the other expresses CMV-lox-luciferase/herpes simplex virus thymidine kinase (TK). In PSMA-positive LNCaP cells, the promoter activity of the PEPM-Cre plus CMV-lox-luciferase demonstrated 800-fold greater activity compared with that of the PSMA promoter alone. However, no enhancement of the promoter activity was observed in the PSMA-negative cells. Furthermore, in contrast to prostate specific antigen promoter/enhancer (PP), the promoter activity of PEPM did not decrease when the LNCaP cells were cultured in charcoal-stripped fetal bovine serum (CFBS). In an in vitro gene therapy model with LNCaP cells, the cell growth inhibition in the presence of ganciclovir (GCV) was more evident in the cells transfected with the PEPM-Cre plus CMV-lox-TK than in the cells with the PP-TK, and the difference in efficacy between the two plasmids was more remarkable when the cells were maintained in CFBS medium. The therapeutic effect of PEPM-Cre plus CMV-lox-TK was also observed in xenografted LNCaP cells on nude mice when the plasmids were directly injected into tumors and GCV was administered intraperitoneally. These findings indicate that the combination of the PSMA promoter/enhancer and the Cre-loxP system can enhance the PSMA promoter activity even under androgen ablation conditions and can exert its anti-tumor effect both in vitro and in vivo.

Key words: Prostate specific membrane antigen — Cre loxP system — Prostate cancer — Androgen — Gene therapy

For gene therapy on prostate cancer cells, it is essential to use a strong and tissue-specific promoter/enhancer region for suicide genes to be selectively expressed on the cancer cells. Furthermore, in the case of prostate cancer, it is important to note that most patients have already received androgen ablation therapy. Prostate-specific antigen (PSA) is expressed in the normal and hyperplastic prostate as well as in prostate cancer, and it has a strong enhancer activity. The transcriptional regulatory region is, therefore, suitable for gene therapy, and various studies have been published to demonstrate this fact.1–3 However, the expression of PSA is strictly regulated by androgen, and the PSA promoter activity is greatly reduced under androgen ablation conditions.4 It therefore appears to be more practical to devise a promoter system which is independent of androgen. The prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein of Mr~10 000.5 The protein has a high specificity to prostate epithelial tissue and is expressed in benign secretory acinar epithelium, and prostate cancer,6–9 and tumor neovasculature.10 The expression of PSMA is independent of androgen. Moreover, previous observations have demonstrated that the level of PSMA expression in prostate cancer was upregulated after chemical or surgical androgen ablation therapy.11, 12 As a result, the PSMA promoter appears to be highly suitable for gene therapy under androgen ablation conditions. However, the usage of the PSMA promoter/enhancer region (PEPM) has one disadvantage in that it does not have as strong promoter activity as that of PSA.

The Cre-loxP DNA recombination system consists of two elements; one plasmid has a Cre recombinase (Cre) cDNA gene while the other has a pair of loxP sequences. Cre recombinase derived from bacteriophage P1 is a MW 38 000 protein, and mediates the site-specific excisional deletion of the DNA sequence that is flanked by a pair of loxP sites.13 In the presence of the Cre, the DNA sequence downstream of the pair of loxP sites can be transcribed and expressed. We applied this DNA recombination sys-
Gene Therapy with PSMA Promoter/Enhancer

tem to suicide gene therapy. We designed two plasmids; one was a tissue-specific promoter to drive the Cre gene while the other had a stop signal flanked with a pair of loxP sequences between the CMV promoter-suicide gene, such as the herpes simplex virus thymidine kinase (TK) gene. The co-transfection of these plasmids can induce an enhancement of the expression of the TK gene by the CMV promoter in the presence of the Cre. As a result, the Cre-loxP system can contribute to the targeting by plasmids with a tissue-specific promoter to drive the Cre and amplify the tissue-specific promoter activity by means of a strong, universal promoter linked loxP sequence. A previous report showed that the Cre-loxP system could provide a ~100-fold increase in the tissue-specific promoter activity.\(^{14}\)

In the present study, we tried to enhance the PSMA promoter/enhancer activity under androgen ablation conditions by combining it with the Cre-loxP DNA recombination system. We also applied this system to TK suicide gene therapy with ganciclovir (GCV) in vitro and in vivo in order to examine the anti-tumor effect.

MATERIALS AND METHODS

Cell lines Two human adenocarcinoma cell lines of the prostate, LNCaP and DU145, and a human transitional cell carcinoma cell line of the bladder, T24 were obtained from American Type Culture Collection (Manassas, VA). LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) or 10% charcoal-stripped FBS (CFBS; HyClone Laboratories). T24 cells in RPMI 1640 medium supplemented with 10% FBS, and DU145 cells in Eagle’s MEM medium containing 10% FBS, respectively.

Plasmid construction We cloned the PSMA promoter and enhancer fragment by PCR amplification from normal human genomic DNA.\(^{15}\) We designed three pairs of oligonucleotide primers incorporating artificially introduced restriction enzyme sites for PCR amplification. The primer sets were determined according to the findings reported by O’Keef et al.,\(^{16,17}\) and the sets were 5′-GGGGTACCCCTACTCAGCTGGCCCA-3′ and 5′-GAAAGATCTTGCCTGCTGCTCTACTGC-3′ to amplify a DNA fragment for PSMA promoter (PM) located between −1283 and −39. The amplified PCR fragment of the PSMA promoter, ~1.2 kb in length, was subcloned into the KpnI and BglII restriction enzyme sites of the pGL3 Basic vector (PM-Luc), or into the KpnI and BamHI restriction enzyme sites of the pUC19 vector (New England Biolabs, Beverly, MA). The PSMA enhancer fragment was cloned by nested-PCR amplification. The first PCR primer sets were 5′-GATGGGCAAGTCTGAGTTGTGTTGATTGTGTTGTTGTTGTTGTT-3′ and 5′-GCTGTGACGACAATTGACGACGACTGACA-3′. The nested-PCR primer sets were 5′-cgcggtagacccCTTTCTAAAATGAGTGGGG-3′ and 5′-cggtagacccGCTACGCTACTCATAGTATAAGTAC-3′ to amplify DNA fragment for PSMA enhancer (PE) located between +11 958 and +13 606. The amplified PCR fragment of the PSMA enhancer, ~1.6 kb in length, was cloned into pCR2.1 vector (pCR-PE; Invitrogen, Carlsbad, CA). The plasmid PSMA-Cre (PM-Cre) contains PSMA promoter to drive Cre recombinase. The Cre recombinase cDNA was obtained by restriction enzyme digestion with XhoI and XbaI from pBS185 (Life Technologies, Carlsbad, CA), and the Cre fragment was subcloned into the XhoI and XbaI restriction enzyme sites of pGL3 Basic (pGL-Cre; Promega, Madison, WI). The PSMA promoter fragment was obtained from pUC19 vector by digestion with KpnI and SalI, and after restriction enzyme digestion by KpnI and XhoI of pGL-Cre, the PSMA promoter fragment was inserted upstream to Cre cDNA of pGL-Cre to construct PM-Cre. We also constructed the plasmid PSMA enhancer/promoter-Cre (PEPM-Cre) by obtaining a fragment after enzyme digestion of pCR-PE with KpnI, and thereafter inserted it upstream to the PSMA promoter of PM-Cre. To prepare the plasmids, CMV-lox-TK or CMV-lox-luciferase (CMV-lox-Luc), the fragment of a stop signal flanked with a pair of loxP sequences (loxP) was prepared by the restriction enzyme digestion with EcoRI and Sphi from pbS302 (Life Technologies), and then, the loxP fragment was inserted into CMV-TK or CMV-Luc. The plasmid PSA promoter/enhancer-TK (PP-TK), PM-TK, PEPM-TK, CMV-TK, and CMV-Luc were constructed in a similar manner, as reported previously.\(^{15}\) The constructed plasmids are shown in Fig. 1. All of the plasmids we used were confirmed by restriction enzyme digestion

Fig. 1. The plasmids used in this study. The PSMA promoter (PM) and PSMA enhancer (PE) fragments were obtained by PCR amplification. The positions were relative to the translation initiation codon of the PSMA gene. The positions refer to the Genbank accession number AF007544.
and sequencing analysis with an ABI PRISM 310 Gene Analyzer by the Big Dye Termination Method (PE Applied Biosystems, Foster City, CA).

Luciferase and LacZ reporter gene expression assay
The promoter activities examined by the luciferase (Luc) reporter gene expression assay have all been previously reported. Briefly, LNCaP, DU145 and T24 cells were plated at 1 × 10^5 cells/well (LNCaP) or 4 × 10^4 cells/well (DU145 and T24) in 24-well plates in adequate medium containing 10% FBS or 10% CFBS the day before transfection. The plasmids were transfected into the cells with cationic liposomes, LipofectAMINE Plus (Life Technologies). We co-transfected the two plasmids for the Cre-loxP system. Based on preliminary experiments, the optimized ratio of the co-transfected plasmids was determined as 1:1. Pre-complexed 0.8 µg of DNA plasmid and 2.5 µg of Plus Reagent (Life Technologies) in 25 µl of OPTI-MEM (Life Technologies) and 3 µg of LipofectAMINE (Life Technologies) in 25 µl of OPTI-MEM were gently mixed and incubated at room temperature for 30 min. Then the plasmid-liposome complex was poured into each well. Forty-eight hours after incubation the cells were washed with PBS and then were lysed with Luc cell lysis buffer (Promega). The Luc activity of each lysate was measured using the Luc assay system (Promega) by Tropix TR717 Microplate Luminometer (PE Applied Biosystems).

Luc activities were indicated as percentages of the activity obtained with pGL3 Control vector which has the SV40 promoter (Promega). Each experiment was repeated twice in our cancer cell lines. Moreover, we examined transfection efficiency in LNCaP, DU145 and T24 cells with the X-Gal staining method. We transfected the plasmid CMV-LacZ in these cells in the same fashion. After 48 h of incubation, the transfected cells were fixed with 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min at 4°C, washed three times with PBS and incubated for 6 h in a solution containing X-Gal (1 mg/ml), 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, and 1 mM MgCl2, in PBS. The positively stained cells were detected by light microscopy and were evaluated by two pathologists. Under our experimental conditions, about 20% of cells were stained in all of the three cell lines used here (data not shown).

GCV-mediated cell growth inhibition in vitro
LNCaP cells (4 × 10^4, 10% FBS; 6 × 10^4, 10% CFBS), DU145 and T24 cells (1 × 10^4) per well were seeded into 24-well plates in a suitable medium containing 10% FBS or 10% CFBS. The plasmids were transfected in the same fashion as in Luc reporter gene expression assay. The cells were cultured in a medium containing 10 µg/ml of GCV (Sigma, St. Louis, MO), and half of the medium was changed on days 1, 3, 5 of cultivation in the medium with 10% FBS or on days 1, 3, 6 in the medium with 10% CFBS. The number of recovered viable cells in each well was counted by means of a trypan blue dye exclusion test and the results were expressed as the mean cell number ± SD of three independent wells. Each experiment was repeated at least three times in our cancer cell lines.

The transfection efficiency in an in vivo tumor model
We examined the transfection efficiency in vivo with Luc or LacZ reporter gene expression assay. Ten million LNCaP cells suspended in 50 µl of Matrigel (Becton Dickinson Labware, Lincoln Park, NJ) were inoculated subcutaneously in the flank region of male BALB/c nu/nu mice of 6–8 weeks of age (Clea Japan, Tokyo). When the volume of the inoculated tumors reached 80 mm³, the plasmids, CMV-LacZ, GL3 control vector, PEPM-Luc, or the paired PEPM-Cre plus CMV-lox-Luc, were transfected into the inoculated tumors with cationic liposomes, DMRIE-C (Life Technologies). Three micrograms of DNA plasmid and 3 µg of DMRIE-C (Life Technologies) in 50 µl of OPTI-MEM (Life Technologies) were gently mixed and incubated at room temperature for 30 min. Next, the plasmid-liposome complex was directly injected into the inoculated tumor. After 48 h, tumors were excised and X-Gal staining and luciferase expression assay were performed. For X-Gal staining in vivo,26 frozen sections (5 µm) were fixed for 10 min in 0.25% glutaraldehyde and 2% paraformaldehyde in PBS at room temperature, washed three times with PBS and incubated for 6 h in a solution containing X-Gal (1 mg/ml), 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, and 1 mM MgCl2 in PBS. After X-Gal staining, all samples were counter-stained with hematoxylin-eosin solution. The positively stained cells were detected by light microscopy and were evaluated by the two pathologists. In vivo luciferase expression assay was reported previously. Briefly, the excited tumors were immediately frozen in liquid nitrogen and homogenized after adding 1× lysis buffer (Promega) with a volume (µl) equivalent to five times the tissue weight (mg). The tissue homogenate was centrifuged at 14 000 rpm for 20 min after undergoing three freeze-thaw procedures. The resulting supernatants were assayed with the luminometer and the Luc activities were indicated in the same manner as in vitro. These in vivo experiments were repeated twice.

Assessment of LNCaP xenograft tumor growth in vivo
When the average volume of xenograft tumors reached 80 mm³ on average (day 0), the mice were divided into four groups including; group I PBS, group II PEPM-TK, group III PEPM-Cre plus CMV-lox-TK, group IV CAG-TK. Each experimental group consisted of six mice. In vivo, the plasmids were transfected into the inoculated tumors with cationic liposomes, DMRIE-C (Life Technologies) on days 1, 4, and 8. Three micrograms of DNA plasmid and 3 µg of DMRIE-C (Life Technologies) in 50 µl of OPTI-MEM (Life Technologies) were gently mixed and incubated at room temperature for 30 min. Next the plasmid-liposome complex was directly injected into the inoculated tumor model.
tumor. GCV at a dose of 50 mg/kg was administered i.p. twice a day for 12 days. The tumor volume was measured every 4 days and calculated by using the formula: length × width × depth × 0.526. All mice were sacrificed at day 24, and the tumor weight and the serum concentration of PSA were measured. The serum concentration of PSA was determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.1 µg/liter (MEDICORP, Montréal, Canada) according to the manufacturer’s protocol. All data are shown as the mean values ± SD. Each animal experiment was repeated twice. Statistical analysis The in vivo growth-inhibitory effects on the tumor volume, the weight of xenografted tumors, and the serum concentration of PSA of individual groups were analyzed using a repeated-measures ANOVA model. The level of statistical significance was set at \( P < 0.05 \). All statistical calculations were performed using the Statview 4.5 software package (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Enhancement of PSMA promoter activity by the Cre-loxP system We examined the promoter activity with or without the Cre-loxP system in prostate cancer cell lines. The plasmids were transfected into the PSMA-expressing human prostate cancer cell line LNCaP, the PSMA-non-expressing human prostate cancer cell line DU145, and the human bladder cancer cell line T24. In LNCaP cells, the luciferase activity obtained by the paired plasmids, PEPM-Cre and CMV-lox-Luc, was higher than that by the plasmid PEPM-Luc, and the activity was 800-fold higher compared with that by the plasmid of PSMA promoter (PM-Luc) alone (Fig. 2). This luciferase activity was not reduced when the cells were cultured in the medium with CFBS and still showed a 250-fold increase. In contrast, PSA promoter/enhancer (PP-Luc) demonstrated a strong luciferase activity in the medium with FBS, but the activity decreased to the background level when the medium was changed from FBS to CFBS. Subsequently, we investigated whether or not the combination of PEPM-Cre and CMV-lox-Luc works selectively in PSMA-positive cells (Fig. 3). In PSMA-negative DU145 and T24 cells, the luciferase activities by PP-Luc and PEPM-Luc plasmids remained at the background level. However, a small increase in the luciferase activities was observed when the CMV-lox-Luc system was used, particularly when it was paired with PM-Cre. It appears that PEPM-Cre will be more suitable for selective gene therapy (see also Fig. 6). In vitro suicide gene therapy model in PSMA-expressing LNCaP cells with GCV As shown above, we demonstrated that the paired plasmids, PEPM-Cre and CMV-lox-Luc, enhanced not only the promoter activity, but also the tissue specificity. We therefore applied these plasmids to suicide gene therapy with GCV. We transfected the plasmids, CMV-lox-TK, PP-TK, PM-TK, PEPM-TK, the

Fig. 2. The enhancement of the PSMA promoter activity by the Cre-loxP system as determined by a luciferase expression assay in LNCaP cells cultured with FBS (■) or CFBS (□). The 100% relative luciferase activity is taken as that of the GL3 control vector. The actual 100% mean luciferase activity was 1.0×10^5. The results were expressed as the mean value ± SD of three independent wells.

Fig. 3. The specificity of PSMA promoter activity determined by a luciferase expression assay in PSMA-negative cell lines, DU145 and T24. The 100% relative luciferase activity is taken as that of the GL3 control vector. In DU145 cells (A), the actual mean 100% luciferase activity was 5.5×10^4 and that in T24 cells (B) was 9.6×10^4. The results were expressed as the mean value ± SD of three independent wells.
paired PM-Cre and CMV-lox-TK, and the paired PEPM-Cre and CMV-lox-TK into three cell lines. The inhibitory effects of these plasmids on the cell growth was investigated in the presence of GCV. The plasmid CAG-TK was used as a positive control, and the plasmid CAG-GS as a negative control.4)

In LNCaP cells in the medium with 10% FBS, a considerable inhibitory effect was observed with PP-TK plasmid. However, the paired plasmids, PEPM-Cre and CMV-lox-TK, could inhibit cell growth more effectively and the observed cell growth inhibition was closely equivalent to that observed with plasmid CAG-TK (Fig. 4).

In contrast, when LNCaP cells were maintained in the medium with CFBS, a clear-cut cell growth inhibition was only observed with the plasmids PEPM-TK, PEPM-Cre plus CMV-lox-TK, and CAG-TK (Fig. 5). These results strongly indicate that PSMA promoter/enhancer could work even under conditions of androgen ablation. The therapeutic effects of various plasmids were also tested in PSA- and PSMA-negative DU145 and T24 cells (Fig. 6). PP-TK and PEPM-TK plasmids are expected to exert their effects selectively on PSA- or PSMA-positive cells, respectively, and actually no cell growth inhibition was observed with these plasmids. We did observe, however, that CMV-lox-TK plasmid always exerted some inhibitory effects, especially, when the plasmid was combined with PM-Cre plasmid. Again, it appears that the combination of PEPM-Cre and CMV-lox-TK will be better for gene therapy against PSMA-positive cancer cells.

![Fig. 4. Cell growth inhibition of LNCaP cells cultured with 10% FBS by the pair plasmids of PSMA promoter/enhancer and Cre-loxP system. LNCaP cells were transfected with various plasmids and incubated in the medium containing 10 µg/ml of GCV. The results were expressed as the mean cell number ±SD of three independent wells. The plasmids used were: Control (○), CAG-TK (●), CAG-GS (□), CMV-lox-TK (■), PP-TK (△), PM-TK (▽), PEPM-TK (▲), and PEPM-Cre+CMV-lox-TK (◇).](image)

![Fig. 5. Cell growth inhibition of LNCaP cells cultured with 10% CFBS by the pair plasmids of PSMA promoter/enhancer and Cre-loxP system. LNCaP cells in CFBS were cultured in medium containing 10 µg/ml of GCV. The results were expressed as the mean cell number ±SD of three independent wells. The plasmids used were: Control (○), CAG-TK (●), CAG-GS (□), CMV-lox-TK (■), PP-TK (△), PM-TK (▽), PEPM-TK (▲), and PEPM-Cre+CMV-lox-TK (◇).](image)

![Fig. 6. In vitro suicide gene therapy with GCV in cancer cell lines without PSMA expression. DU145 (A) or T24 (B) cells were transfected with various plasmids and maintained in the medium containing 10 µg/ml of GCV. The results were expressed as the mean viable cell number ±SD of three independent wells. The plasmids used were: Control (○), CAG-TK (●), CAG-GS (□), CMV-lox-TK (■), PP-TK (△), PEPM-TK (▲), PEPM-Cre+CMV-lox-TK (◇), and PM-Cre+CMV-lox-TK (◆).](image)
The transfection efficiency in LNCaP xenograft tumors on nude mice We evaluated the transfection efficiency in xenograft tumors on nude mice with the X-Gal staining method and luciferase expression assay. The rate of the positive cells was 2–7% in the tumor (Fig. 7A), and we estimated that the transfection efficiency by single injection in vivo would be 3.5% on average. Moreover, the luciferase expression assay in vivo demonstrated that the paired plasmids, PEPM-Cre and CMV-lox-Luc had fourteen times higher promoter activity than the plasmid GL3 control vector, while PEPM-Luc demonstrated only two-fold greater activity (Fig. 7B).

In vivo suicide gene therapy of LNCaP xenograft tumors on nude mice We evaluated the anti-tumor effect of the paired plasmids, PEPM-Cre and CMV-lox-TK, in vivo. When the average volume of the inoculated tumors reached 80 mm³, the mice were grouped into 4 groups and were treated with the intratumoral injection of DNA-liposome complex on days 1, 4, and 8. From the day after the first DNA-liposome complex injection, GCV at a dose of 50 mg/kg was administered i.p. twice a day for 12 days. In our preliminary experiments, we did not observe any significant difference in effect between PBS and liposomes on xenograft growth, and in this experiment, we used PBS as a control group. Tumor regression was observed in all of the mice treated with DNA-liposome complex (group II, III, and IV vs. group I; P<0.01). However, the growth inhibition was more evident in the xenograft tumors transfected with the paired plasmids, PEPM-Cre and CMV-lox-TK, followed by GCV (group III) than in those transfected with PEPM-TK plasmids, and the inhibitory effect by the paired plasmids was similar to that of the positive control, CAG-TK (group IV) (Fig. 8).

A comparison of tumor weight and PSA concentration after excision also demonstrated that the tumor growth and PSA production were more attenuated in the mice treated with the paired plasmids, PEPM-Cre and CMV-lox-TK, than in the mice with the plasmid PEPM-TK (Table I).

DISCUSSION

The major problem of gene therapy is how to maintain tissue specificity and also to increase the anti-tumor effect. The PSMA promoter/enhancer has already been cloned, and suicide gene therapy with cytosine deaminase gene has also been reported. However, in general, the activity of tissue-specific promoters is low in comparison to a universal promoter such as CMV promoter. We, therefore, introduced the Cre-loxP system and at first we tried to combine the PSMA promoter (PM) with this system. In the Cre-loxP system, the DNA recombination can be performed only when Cre recombinase is produced. Therefore, the enhancement of the promoter activ-
ity by the Cre-loxP system will be extremely low when the PSMA promoter is inactive. However, the CMV promoter has a high promoter activity, and the CMV-lox-Luc itself showed about 5% of the promoter activity of the control vector with SV40 promoter. This nonspecific activity is considered to be responsible for the slight inhibition of cell growth observed in DU145 or T24 cells when the paired plasmids PM-Cre and CMV-lox-TK were transfected. We cloned a 1.6 kb fragment of PSMA enhancer and constructed PEPM-Cre and CMV-lox-Luc/TK. In the presence of PSMA enhancer, we could demonstrate an enhancement of both the tissue specificity and cytotoxic effect.

For gene therapy, PSA promoter/enhancer has been widely used, and we herein demonstrated that the activity of PSA promoter/enhancer is stronger than that of PSMA. However, the activation of PSA promoter/enhancer strictly depends on the presence of androgen, and in the medium with CFBS, the promoter activity was completely abolished. Clinically speaking, most patients with advanced prostate cancer tend to be treated with androgen ablation therapy. As a result, an ideal promoter of gene therapy must be independent of androgen. To satisfy this requirement, we recently introduced a genetically engineered androgen receptor (AR), called ARf, in which the first 201 amino acids and part of the ligand-binding domain were removed from AR. AR binds to androgen and changes to an activated form. The activated AR moves to the nucleus, binds with an androgen responsive element in androgen target genes, and thereby enhances transcription. In contrast ARf may work in an androgen-independent manner, and we demonstrated that the plasmid CMV-ARf-PP-TK exerts its cytotoxic effect even under androgen ablation conditions. As an alternative method, it is also reasonable to adopt a promoter/enhancer region from androgen-independent tumor-associated genes. In this respect, PSMA seems to be a particularly good candidate for gene therapy, PSMA is androgen-independent, and we demonstrated here that the combination of PSMA promoter/enhancer and the Cre-loxP system exerts its promoter activity selectively in PSMA-positive LNCaP cells even when the cells are maintained in the medium with CFBS. Furthermore, the expression of PSMA was reported to increase in androgen-independent prostate cancer cells, and Wright et al. have already indicated that PSMA is upregulated in the majority of prostate carcinomas after androgen deprivation therapy.

![Graph showing tumor volume over time](image)

Fig. 8. *In vivo* suicide gene therapy of xenografted prostate cancer cells with GCV on nude mice. PBS (○), or the following plasmid-liposome complexes, CAG-TK (●), PEPM-TK (▲), PEPM-Cre plus CMV-lox-TK (◇) were injected directly into the tumor mass three times (day 1, day 4, and day 8). GCV (50 mg/kg) was administered i.p. twice a day for 12 days. Each experimental group consisted of six mice. The results were expressed as the mean volume ± SD. * P<0.01, ** P<0.05.

| Treatment group | Tumor weight (g) | PSA concentration (µg/liter) |
|-----------------|-----------------|-----------------------------|
| PBS             | 0.515±0.08      | 48.6±17.3                   |
| PEPM-TK         | 0.288±0.11      | 22.9±4.5                    |
| PEPM-Cre+       | 0.188±0.03      | 11.9±1.7                    |
| CMV-lox-TK      | 0.125±0.02      | 9.5±2.5                     |
| CAG-TK          |                 |                             |

All mice were sacrificed at day 24, and the tumor weight and serum concentration of PSA were measured. Each treatment group consisted of six mice. The data points are shown as the mean±SD. * P<0.01, ** P<0.05.
Thus, the PSMA promoter system seems to offer greater advantages under androgen ablation conditions, and the combination of PEPM-Cre and CMV-lox-TK is thus strongly recommended for performing gene therapy on prostate cancer. As the first step for application of our therapy in vivo, we demonstrated (Fig. 8) that the combination of PEPM-Cre and CMV-lox-TK followed by GCV treatment could inhibit the growth of LNCaP xenograft in nude mice. We are now planning to examine whether this combination therapy also works on an animal model with castration.

As a gene vector, we used cationic liposomes. In most experiments so far reported on prostate cancer cells, adenovirus vectors have been used for gene therapy. However, adenovirus can evoke nonspecific inflammation, and strong anti-virus immune responses are also easily induced. Therefore, repeated administration will not be practical. Furthermore, in 1999, there was an incident in which a patient receiving gene therapy with adenovirus vector had a severe complication of systemic inflammation response syndrome. The strategy of gene therapy with adenovirus vector has been reconsidered from the viewpoint of safety. We, therefore, used cationic liposomes in this study as a safe and reproducible system for gene therapy. In our study, we observed a rather high transfection efficiency in vitro, but a far lower efficiency in vivo with one shot of non-viral vectors. However, the growth-inhibitory effect was observed not only in vitro but also in vivo. Since the cytotoxic effect of the TK/GCV system is mediated by a bystander effect, we believe that three injections of cationic liposomes are sufficient to inhibit cell growth in vivo, as demonstrated here. Huber et al. reported that a 1–5% transfection efficiency in vivo could generate a significant anti-tumor effect in suicide gene therapy. Furthermore, Takakuwa et al. reported that a 1.1% transfection efficiency of liposomes in vivo could afford a significant anti-tumor effect.

In addition, liposomes have the advantage that monoclonal antibodies can be easily conjugated to them. PSMA protein is expressed on the cell membrane. The monoclonal antibody against PSMA has now become available, and recently the radiolabeled monoclonal antibody has been applied for targeting therapy. Monoclonal antibodies against PSMA can be conjugated to liposomes, and gene therapy using PSMA has the advantage of being able to provide not only transcriptional targeting by tissue-specific promoters, but also receptor-mediated targeting by monoclonal antibodies. Furthermore, recent immunohistochemical studies have revealed that PSMA protein is expressed on the endothelial cells of solid tumor neovasculature, but not in normal vessels. It is therefore conceivable that the PEPM-TK gene carried by monoclonal antibody-conjugated liposomes will first destroy the endothelial cells of the neovasculature, and thereafter prostate cancer cells will be attacked. Such a two-step destruction mechanism is considered to be ideal for the treatment of prostate cancer cells in vivo. We are now focusing on establishing a combination therapy modality using the PEPM-Cre-loxP system and targeting with monoclonal antibody-conjugated liposomes.

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