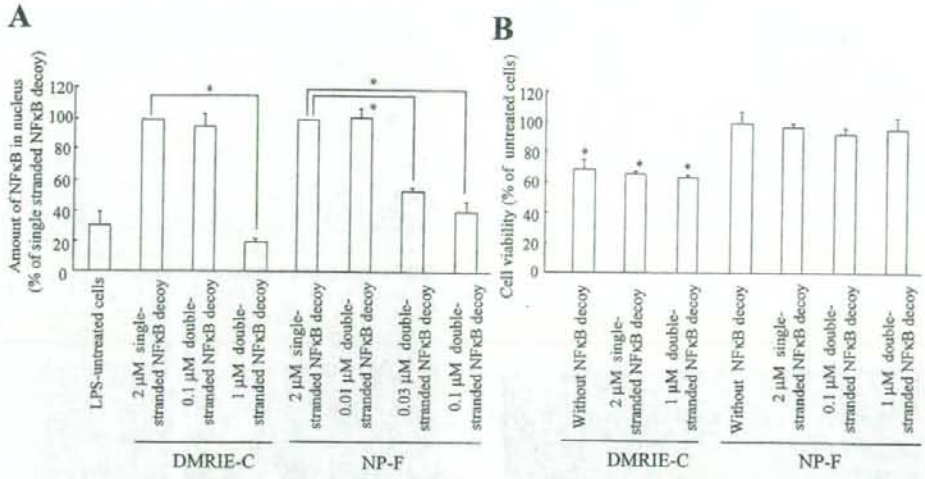

Fig. 3. (A) Inhibition of the Translocation of NFκB in LPS-Treated RAW264.7 Cells by the NFκB Decoy Transfected by NP-F or DMRIE-C Reagent
Cells were incubated with NP-F nanoplex or DMRIE-C lipoplex of either single- or double-stranded decoy for 4 h and then treated with 100 ng/ml of LPS for 1 h.
(B) Cytotoxicity of NP-F Nanoplex and DMRIE-C Lipoplex of NFκB Decoy
Each result represents the mean±S.D. ($n=3$). Statistically significant difference ($*p<0.05$) from untransfected cells.

decoy could not inhibit the translocation of NFκB. The double-stranded NFκB decoy transfected by DMRIE-C did not inhibit the translocation of NFκB even at 0.1 μM, however, it well inhibited it at 1 μM. The double-stranded NFκB decoy transfected by NP-F, inhibited the translocation of NFκB at 0.03 and 0.1 μM, indicating that NP-F could achieve the same degree of inhibition at a 10 times lower concentration of decoy. The cellular uptake of the NFκB decoy was much greater with NP-F than the DMRIE-C reagent (Fig. 2); therefore, the inhibition of NFκB's translocation into the nucleus may be more effective than that obtained with DMRIE-C.

Finally, we investigated the cytotoxicity of the transfection by NP-F or the DMRIE-C reagent. Either the NP-F nanoplex or DMRIE-C lipoplex was formed at various concentrations of the NFκB decoy (0.1 and 1 μM), and the cytotoxity was assessed by the WST-8 assay after incubation of the cells with the nanoplex or lipoplex in the medium with serum for 24 h. No cytotoxicity was observed without the NFκB decoy using NP-F. However, significant toxicity was observed using DMRIE-C compared with untransfected cells (Fig. 3B). When the cells were transfected with the NP-F nanoplex containing 0.1 and 1 μM double-stranded NFκB decoy or 2 μM single-stranded NFκB, no cytotoxicity was observed (Fig. 3B). A liposomal formulation containing DC-Chol has been administered to patients in clinical trials for treating cancer because of its strong transfection activity and low toxicity.[13] The DC-Chol-based folate-linked nanoparticle used in this study showed little cytotoxicity in RAW264.7 cells (Fig. 3B). It will be important to develop applications *in vivo*.

Antohe *et al.* reported that systemically administered folate-fluorescent conjugates accumulated in protrusions of atherosclerotic plaques populated by macrophages, suggesting that activated macrophages can be efficiently targeted *in vivo* by using a folate conjugate.[14] Paulos *et al.* reported that folate-linked imaging agents could be selectively delivered to arthritic joints, allowing the visualization of RA.[7] These reports suggested that a folate-linked therapeutic agent could be selectively delivered to activated macrophages *in vivo*, allowing the treatment of RA. We also confirmed that the folate moiety promoted the association of FITC-f-BSA with RAW264.7 cells treated with LPS (Figs. 1C, D). Therefore, the FR expression and folic acid can be utilized to target activated macrophages in humans and mice.

In conclusion, we have demonstrated that NP-F effectively delivered a NFκB decoy into the cytoplasm, which inhibited the translocation of NFκB into the nucleus of LPS-activated macrophages, compared with commercial transfection reagent, DMRIE-C. To our knowledge, this is the first report that folate-linked vector is effective for DNA delivery into activated macrophage-targeting. This information is of value for the design of carriers for NFκB decoys that will enable therapy for rheumatoid arthritis to be carried out.

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Two-step transcriptional amplification–lipid-based nanoparticles using PSMA or midkine promoter for suicide gene therapy in prostate cancer

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A two-step transcriptional amplification system (TSTA) was used to enhance the efficacy of suicide gene therapy for treatment of prostate cancer. We designed a TSTA system and constructed two types of plasmid: one containing GAL4–VP16 fusion protein under the control of a tumor-specific promoter, the other containing luciferase or herpes simplex virus thymidine kinase (HSV-tk) under the control of a synthetic promoter. The TSTA systems using nanoparticles based on lipids were evaluated by measuring the amount of induced luciferase activity as a function of prostate-specific membrane antigen (PSMA) and midkine (Mk) promoters, specific for LNCaP and PC-3 prostate cancer cells, respectively. In LNCaP cells that were PSMA-positive, the TSTA system featuring the PSMA enhancer and promoter exhibited activity that was 640-fold greater than a system consisting of one-step transcription with the PSMA promoter. In contrast, this difference in activity did not occur in PSMA-negative PC-3 cells. In Mk-positive PC-3 cells, the TSTA system with the Mk promoter exhibited a five-fold increase in activity over one-step transcription, but such activity was not induced in Mk-negative LNCaP cells. When using HSV-tk for suicide gene therapy, TSTA systems featuring the PSMA or Mk promoter inhibited *in vitro* cell growth in the presence of ganciclovir. Furthermore, the TSTA system featuring the Mk promoter suppressed *in vivo* growth of PC-3 tumor xenografts to a greater extent than one-step transcription. These findings show that TSTA systems can enhance PSMA and Mk promoter activities and selectively inhibit PC-3 cell growth in tumors. This suggests that TSTA systems featuring tumor-specific promoters are suitable for cancer treatment by gene therapy. (*Cancer Sci* 2006; 97: 787–798)

Currently, many kinds of gene therapy research are being carried out, especially in the field of cancer treatment.[1] The most difficult aspect of developing an *in vivo* approach is correctly targeting cancer cells. The random delivery of a therapeutic gene damages normal cells in essential organs such as the liver, lung, kidney and spleen, and can cause death. Many vectors target tumors for gene delivery, including viral and synthetic vectors (liposome and emulsion),[2] whereas others that use tumor-specific promoters to regulate expression transcriptionally in target cancer cells have also shown promise. It is essential to use a strong and tissue-specific promoter region if a suicide gene is to be expressed selectively in the cancer cells.

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of death in men in the USA, after lung cancer.[3] The prostate-specific antigen (PSA) test is carried out routinely in men to detect the presence of prostate cancer by immunoassaying the level of PSA in serum. Currently, androgen deprivation is the most effective treatment for advanced prostate cancer, but it reduces PSA serum levels,[4] affecting the utility of PSA as a prostate tumor-specific promoter. Like PSA, prostate-specific membrane antigen (PSMA) has elevated expression in prostate cancer[5] and has been reported to accumulate under conditions of androgen deprivation,[6] potentially making it a more useful tool when tracking a patient's response during prostate cancer treatment. This suggested that the PSMA promoter appears to be highly suitable for gene therapy.[7] Midkine (Mk) is a heparin-binding growth factor whose expression is regulated developmentally.[8,9] Its biological function during tumorigenesis remains unclear but Mk is expressed in various types of human cancer, including prostate cancer.[10–12] In contrast, its expression in adult tissues is strictly limited.[10] Several groups have reported that Mk promoter-mediated suicide gene therapy effectively produces cytotoxic effects in cancer cells.[13]

Both the PSMA and Mk genes have been identified as factors expressed specifically in cancer cells; however, the promoter region has one disadvantage in that it does not have strong promoter activity, which, in turn, limits the cells' ability to express the suicide gene. Suicide gene therapy with a PSA enhancer and promoter in the LNCaP model also had no significant effect with a one-step transcription system.[14] It is essential to find a way to enhance the transcriptional activity of such promoters. Several methods can potentially be used to increase levels of reporter or therapeutic proteins in prostate cancer.[15–18] One of the amplification approaches, referred to as a two-step transcriptional amplification system (TSTA), can potentially be used to improve the transcriptional activity of cellular promoters with the GAL4–VP16 fusion protein, which comprises the DNA-binding domain of the yeast transcriptional activator GAL4 and the activation domain of the herpes simplex virus 1 activator VP16 (Fig. 1). GAL4 is a transcriptional factor that regulates gene transcription tightly by binding its responsive elements. A potent transcriptional activator, GAL4–VP16, which is driven by the cell-specific promoter of an effector plasmid, acts on the promoter of a second expression plasmid (reporter plasmid), which encodes the reporter or therapeutic protein (Fig. 1). For temporally regulated expression, a tetracycline-repressible transactivator system for inducible gene expression was developed using tet-repressor fused to VP16.[19] TSTA

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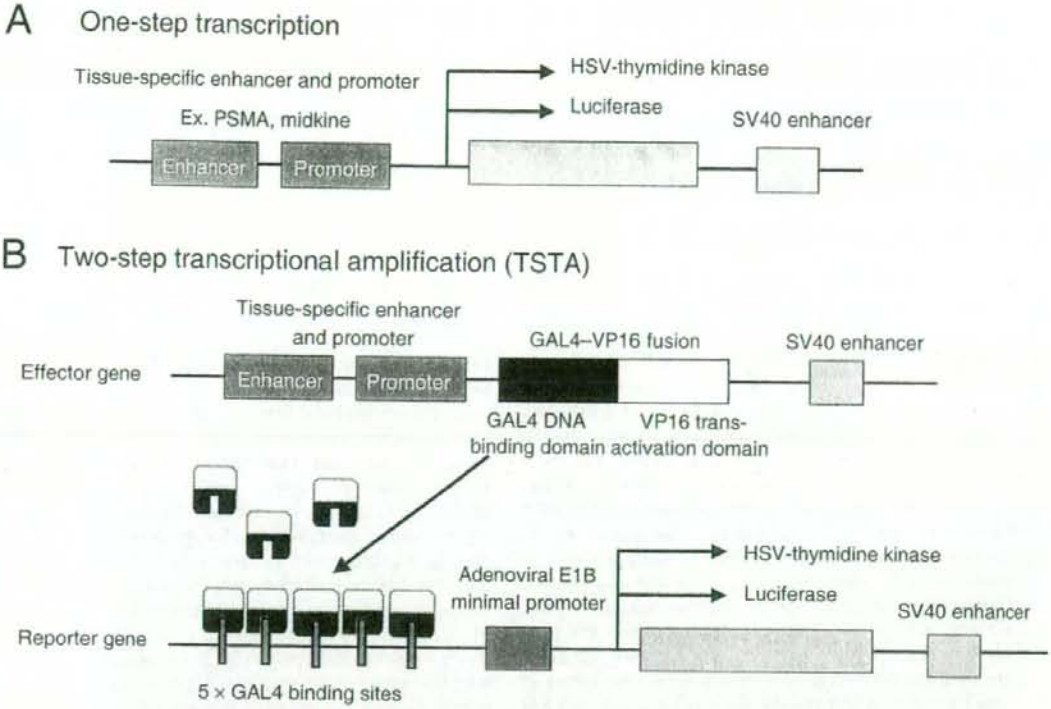

Fig. 1. Schematic diagram of (A) one-step transcription and (B) two-step transcriptional amplification (TSTA system) used in this study.[16] In the TSTA system, the first step involves the tissue-specific (e.g. prostate-specific membrane antigen [PSMA], midkine) expression of the GAL4–VP16 fusion protein. In the second step, GAL4–VP16 drives the target gene expression under the control of GAL4-binding sites and the adenoviral E1B minimal promoter. Transcription of the reporter gene, either herpes simplex virus (HSV) thymidine kinase or luciferase, leads to reporter protein. The use of the GAL4–VP16 fusion protein can potentially lead to amplified levels of the reporter protein.

systems for the amplification of tumor-specific gene expression has been reported in PSA promoter for prostate cancer,[15,16] Muc-1 promoter for colon carcinoma[20] and caricinoembryonic antigen (CEA) promoter for lung cancer and colon adenocarcinoma, respectively.[21] However, TSTA systems with PSMA and Mk promoters have not been reported, and the TSTA system has not been applied to suicide gene therapy with herpes simplex virus thymidine kinase (HSV-tk).

In the present study, we modified the TSTA system using a reporter plasmid with a combination of the adenoviral E1B minimal promoter, SV40 enhancer and an effector plasmid with the PSMA enhancer and promoter or Mk promoter to achieve novel tumor-specific transcriptional amplification for prostate cancer, and evaluated selectiveness to drive gene expression in LNCaP and PC-3 cancer cells. In PSMA-positive LNCaP cells and Mk-positive PC-3 cells, the TSTA system with each promoter and enhancer showed greater activity than one-step transcription with each promoter, as confirmed by growth inhibition of the cells and PC-3 tumor xenografts on suicide gene therapy.

Materials and Methods

Plasmid construction

pGL3-control, pGL3-enhancer and pGL3-basic plasmids were purchased from Promega (Madison, WI, USA). pFR-luc plasmid for expression of the luciferase gene controlled by a synthetic promoter that contains the yeast GAL4-binding sites in front of the E1B minimal promoter was obtained from Stratagene (La Jolla, CA, USA). The HSV-tk cDNA fragment was amplified as described previously.[22] This cDNA was then subcloned into the *Nco*I and *Xba*I restriction enzyme sites of the pGL3 enhancer vector, and pGL3-tk was constructed.

For amplification of the GAL4–VP16 fusion protein, the cDNA encoding the GAL4–VP16 fusion protein was generated as follows by site-directed mutagenesis by overlap extension using polymerase chain reaction (PCR).[23] The yeast GAL4 cDNA was amplified by PCR using the primer set GAL4-forward primer (FW), 5′-ATCCATGGaccATGAAGCTACTGTCTTCTAT-3′ and GAL4-reverse primer (RW), 5′-CGGTCGGGGGGGCCGTCGAGACAGTCAACTGTCT-3′. The GAL4-FW contained a 3-bp optimal Kozak sequence (in lowercase letters) together with a *Nco*I restriction site (underlined). The GAL4-RW coded for the N-terminal region of the activation domain of VP16 (underlined), followed by the C-terminal region of GAL4. The cDNA coding for the activation domain of VP16 was amplified by PCR using the primer set VP16-FW, 5′-ACAGTTGACTGTATCGACGGCCCCCCCGACCGAT-3′ and VP16-RW, CATATAGACTATCCCGGACCCGGGGAATCC-3′. The forward primer, VP16-FW, coded for the C-terminal

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region of GAL4 (underlined), followed by the N-terminal region of the activation domain of VP16. The reverse primer, VP16-RW, coded for the VP16 sequence with an *Xba*I restriction site (underlined). The amplified PCR fragment of the GAL4–VP16 fusion protein by overlap extension (~0.6 kb in length) was subcloned into the *Nco*I and *Xba*I restriction enzyme sites of the pGL3 enhancer vector, and pGL3-GAL/VP was constructed.

For the construction of plasmids containing the PSMA promoter and enhancer, we cloned the DNA fragments by PCR amplification from normal human genomic DNA (Seegene, Seoul, Korea). The primer sets were determined according to the findings reported by Ikegami *et al.*,[17] and were PSMA(P)-FW, 5′-GAACTCGAGCCTACTCAGCTGGCCCA-3′ and PSMA(P)-RW, 5′-CTTAAGCTTGCTGCTGCTCTACTGCGCGC-3′ to amplify a DNA fragment for the PSMA promoter located between −1283 and −39. The forward and reverse primers, respectively, contained *Xho*I and *Hind*III restriction sites (underlined).

The PSMA enhancer fragment was cloned by nested-PCR amplification. The first PCR primer set was PSMA(E)1-FW, 5′-GGATGTGGCAAGTCGTAGTTGATTTGGT-3′, and PSMA(E)1-RW, 5′-GCTGTGTACCAATTGACAAGCAGTGACA-3′. The nested-PCR primer set was PSMA(E)2-FW, 5′-GAAGGTACCCTTCTAAAATGAGTTGGG-3′, and PSMA(E)2-RW, 5′-GAACTCGAGGGCTACTACATAAGTATAAGT-3′, to amplify the DNA fragment for the PSMA enhancer located between +11 958 and +13 606. The forward and reverse primers, respectively, contained *Kpn*I and *Xho*I restriction sites (underlined). The amplified PCR fragment of the PSMA promoter (~1.2 kb in length) was subcloned into the *Xho*I and *Hind*III restriction enzyme sites of the pGL3 enhancer, pGL3-tk and pGL3-GAL/VP vectors, and pPSMA(P)-luc, pPSMA(P)-tk and pPSMA(P)-GAL4-VP, respectively, were constructed. Subsequently, the amplified PCR fragment of the PSMA enhancer (~1.6 kb in length) was cloned into the *Kpn*I and *Xho*I restriction sites of the above plasmids, and pPSMA(EP)-luc, pPSMA(EP)-tk and pPSMA(EP)-GAL/VP, respectively, were constructed.

For the construction of plasmids containing the PSA promoter and enhancer, the primer sets were determined according to the findings of Pang *et al.*[24] One primer set was PSA(P)-FW, 5′-GAACTCGAGTTGGAATTCCACATTGTTG-3′ and PSA(P)-RW, 5′-GTTCCATGGTGACACAGCTCTCCGGGTGC-3′ for the PSA promoter located between −636 and +46. The forward and reverse primers, respectively, contained *Xho*I and *Nco*I restriction sites (underlined). The other set was PSA(E)-FW, 5′-GAAGGTACCCTGCAGAGAAATTAATTGTG-3′ and PSA(E)-RW, 5′-GAACTCGAGAATTCTCCATGGTTCTGTCA-3′ to amplify the DNA fragment for the PSA enhancer located between −4757 and −3928. The forward and reverse primers, respectively, contained *Kpn*I and *Xho*I restriction sites (underlined). The amplified PCR fragment of the PSA promoter (~0.6 kb in length) was subcloned into the *Xho*I and *Nco*I restriction sites of the pGL3 enhancer vector, and pPSA(P)-luc was constructed. Subsequently, the amplified PCR fragment of the PSA enhancer (~0.8 kb in length) was subcloned into the *Kpn*I and *Xho*I restriction sites of pPSA(P)-luc, and pPSA(EP)-luc was constructed.

For the construction of plasmids containing a Mk promoter, we designed a pair of primers incorporating artificially introduced restriction enzyme sites for PCR amplification according to findings reported previously.[13] The primer set was Mk(P)-FW, 5′-GAAGGTACCGCTTCCCTGCCCACCCGCGG-3′ and Mk(P)-RW, 5′-GTTCCATGGGCTTCGCTCCCTCCCGCGCC-3′ to amplify a DNA fragment for the Mk promoter stretching from bp −559 of the 5′ upstream flanking region to bp 27 of exon 1 of the human Mk gene. The forward and reverse primers, respectively, contained *Kpn*I and *Hind*III restriction sites (underlined). The amplified PCR fragment of the Mk promoter (~0.6 kb in length) was subcloned into the *Kpn*I and *Hind*III restriction sites of the pGL3 enhancer and pGL3-tk and pGL3-GAL/VP, and pMk(P)-luc, pMk(P)-tk and pMk(P)-GAL/VP, respectively, were constructed.

For the construction of reporter plasmids, the primer set was Syn-FW, 5′-CCAAGCTTGCATGCCTGCAG-3′ and Syn-RW, 5′-ATCCATGGTACCAACAGTACCGGAA-3′ to amplify a DNA fragment for a synthetic promoter that contained the yeast GAL4-binding site in front of the E1B minimal promoter of adenovirus. The forward and reverse primers, respectively, contained *Hind*III and *Nco*I restriction sites (underlined). The amplified PCR fragment of the synthetic promoter from pFR-luc (~0.2 kb in length) was subcloned into the *Hind*III and *Nco*I restriction sites of the pGL3 enhancer and pGL3-tk vector, and pRep-luc and pRep-tk, respectively, were constructed.

The plasmid pCMV-tk encoding HSV-tk under the control of the cytomegalovirus (CMV) promoter was constructed as described previously.[22] The plasmid pCMV-luc, encoding luciferase under the CMV promoter, was constructed by insertion of the CMV promoter into the pGL3 enhancer, as described previously.[22] A protein-free preparation of the plasmid was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany).

Cell culture

LNCaP cells were supplied by the Department of Urology, Keio University Hospital (Tokyo, Japan). PC-3 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). Human cervix carcinoma HeLa cells were kindly provided by Toyobo (Osaka, Japan). All of the cell lines used in this study were grown in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and kanamycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was isolated from LNCaP, PC-3 and HeLa cells, using NucleoSpin RNA II (Macherey-Nagel, Düren, Germany). Total RNA from normal and malignant prostate cells were purchased from Ambion (First Choice Tumor/Normal Adjacent Prostate Total RNA and First Choice Prostate Tumor Total RNA; Austin, TX, USA). First-strand cDNA was synthesized from 5 μg of total RNA as described previously.[22] Reverse transcription (RT)-PCR was carried out in a 25-μL reaction volume containing the following: 1 μL of synthesized cDNA, 10 pmol of each specific primer pair, and 0.25 units of Ex *Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan) with a PCR buffer containing 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. The

profile of PCR amplification consisted of denaturation at 94°C for 0.5 min, primer annealing at 58°C for 0.5 min, and elongation at 72°C for 1 min for 25 cycles. PCR of the housekeeping gene β-actin, Mk and PSMA were carried out during the same cycle run for all samples. The PCR products for Mk, PSMA and β-actin were analyzed by 1.5% agarose gel electrophoresis in a Tris-Borate-ethylenediamine tetraacetic acid (TBE) buffer. The products were visualized by ethidium bromide staining.

Real-time PCR was carried out on the corresponding cDNA synthesized from each sample described above. The optimized settings were transferred to real-time PCR protocols on iCycler MyiQ detection systems (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR Green I assay (iQ SYBER Green Supermix, Bio-Rad Laboratories) was used for quantification. Samples were run in triplicate and the expression level of Mk and PSMA mRNA was normalized for the amount of β-actin in the same sample. The difference of one cycle was calculated as a two-fold change in gene expression.

In vitro transfection

Cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol) was synthesized as reported previously.[25] The nanoparticle (NP) as a gene transfection reagent was prepared with lipids (OH-Chol : Tween 80 [NOF, Tokyo, Japan] 95 : 5, molar ratio = 10 : 1.3, weight) in 10 mL of water using a modified ethanol injection method as described previously.[22,25] Based on preliminary experiments with the cotransfected plasmids, the optimized ratio (w/w) of effector : reporter plasmid was determined as 1 : 1. The nanoplex at a charge ratio (+/–) of cationic lipid to DNA of 3/1 was formed by addition of NP to 2 μg of DNA (e.g. 1 μg of effector plasmid and 1 μg of reporter plasmid in the TSTA system) in 50 mM NaCl with gentle shaking and left at room temperature for 10 min. For transfection, the nanoplex was diluted in 1 mL of medium supplemented with 10% serum and then incubated for 24 h. Androgen stimulation of transfected cells was carried out by adding 10 nM dihydrotestosterone (DHT; Sigma, St Louis, MO, USA) to the culture medium.

Luciferase assay

Cell cultures were prepared by plating cells in a 35-mm culture dish 24 h prior to each experiment. The cells at 70% confluence were transfected as described above. Luciferase expression was measured as counts per s (cps)/μg protein using the luciferase assay system (Pica gene; Toyo Ink Manufacturing, Tokyo, Japan) and bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA) as reported previously.[22]

In vitro sensitivity to the ganciclovir assay

LNCaP, PC-3 and HeLa cells were seeded separately at a density of 1×10^4 cells per well in 96-well plates and maintained for 12 h before transfection in RPMI-1640 medium supplemented with 10% serum. The cells were transfected with the nanoplexes at 0.2 μg plasmid/well. After 12 h incubation, the culture medium was replaced with medium containing various concentrations of ganciclovir (GCV; Glaxo Smith Kline, Helis, UK) ranging from 0.1 to 1000 μg/mL. The number of surviving cells was determined with a WST-8 assay (Dojindo Laboratories, Kumamoto, Japan) after 3 days' exposure to GCV as described previously.[22]

Immunoblotting

LNCaP, PC-3 and HeLa cells were transfected with various plasmids and then incubated for 24 h. The cells were suspended in lysis buffer (0.5% Triton-X 100 in phosphate-buffered saline pH 7.4), then centrifuged at 15 000 r.p.m. (20 000 × g) for 10 min. The supernatants were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (FluoroTrans W; PALL Gelman Laboratory, Ann Arbor, MI, USA). HSV-tk was identified using a specific rabbit antiserum (kindly provided by the Department of Virology, Toyama Medical and Pharmaceutical University) with antirabbit IgG peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the secondary antibody and detected with peroxidase-induced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Pierce).

Assessment of PC-3 xenograft tumor growth *in vivo*

To generate PC-3 tumor xenografts, 1×10^7 cells suspended in 50 μL of RPMI-1640 medium containing 60% reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA, USA) were inoculated subcutaneously into the flanking region of male BALB/c nu/nμ mice (6 weeks of age; CLEA Japan, Tokyo, Japan). The tumor volume was calculated using the formula:

$$\text{tumor volume} = 0.5ab^2,$$

where a and b are the larger and smaller diameters, respectively. When the average volume of PC-3 xenograft tumors reached 150 mm^3 (day 0), these mice were divided into four groups: group I, pGL3-basic (10 μg) as a control; group II, pMk(P)-tk (10 μg); group III, pMk(P)-GAL/VP (5 μg) plus pRep-tk (5 μg); and group IV, pCMV-tk (10 μg) Each experimental group consisted of four tumors. Based on a preliminary experiment of gene expression by intratumoral injection, the optimized ratio of cationic lipid to DNA was determined as 1 : 1. The nanoplex at a charge ratio (+/–) of 1/1 of cationic lipid to DNA was formed by addition of NP (15.8 μL) to 10 μg of DNA with gentle shaking and incubation at room temperature for 10 min. The nanoplexes of 10 μg of plasmid per tumor were injected directly into xenografts on days 0, 3 and 6. GCV at a dose of 25 mg/kg was administered intraperitoneally 12, 24 and 36 h after the injections of nanoplexes. The tumor volume was measured at days 0, 3, 6, 8, 10, 12 and 14.

Statistical analysis

The statistical significance of differences between mean values was determined using Welch's *t*-test. Multiple measurement comparisons were carried out by analysis of variance followed by the Bonferroni/Dunn test. For the animal study, statistical comparisons were carried out using Fisher's exact test. *P*-values less than 0.05 were considered significant.

Results

Expression of midkine and PSMA mRNA

First, we investigated the expression of Mk and PSMA in human prostate malignant biopsy and LNCaP, PC-3 and HeLa cells using the quantitative PCR and RT-PCR method (Fig. 2). HeLa cells were used as a Mk-positive control.[26] In

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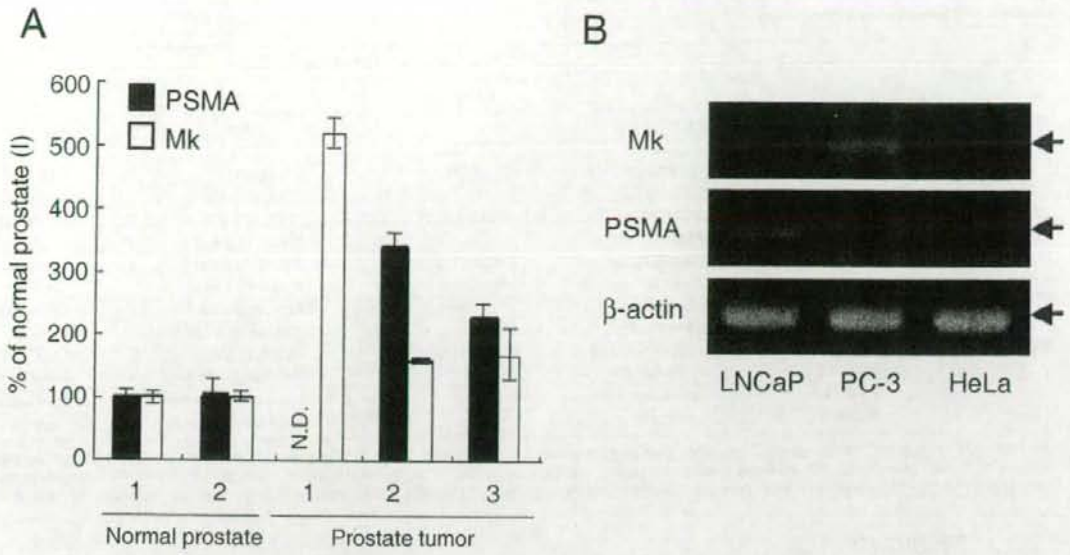

Fig. 2. (A) Relative amount of midkine (Mk) and prostate-specific membrane antigen (PSMA) mRNA in normal and malignant prostate was compared by SYBR Green I-based quantitative polymerase chain reaction analysis. The *y*-axis indicated the fold induction of gene expression. The expression level of Mk and PSMA mRNA was normalized for the amount of β-actin in the same sample. Each result represents the mean ± SD ($n = 3$). (B) Expression of Mk and PSMA mRNA in LNCaP, PC-3 and HeLa cells detected by reverse transcription–polymerase chain reaction.

the human prostate biopsy, two kinds of RNA from normal prostate and three prostate tumors were used. Elevated expression of PSMA and/or Mk mRNA was observed in prostate tumor (Fig. 2A), indicating that upregulated expression of PSMA and Mk could be utilized as a marker for prostate tumor. Mk mRNA was expressed strongly in PC-3 cells and weakly in HeLa cells, but was not detected in LNCaP cells (Fig. 2B). Quantitative PCR analysis showed that the amount of Mk mRNA in PC-3 cells was 31-fold higher than in HeLa cells (data not shown). PSMA mRNA was detected in LNCaP cells, but not in PC-3 or HeLa cells. This result suggested that PC-3 and HeLa cells could be utilized with the Mk promoter for tumor-specific expression, and LNCaP cells could be utilized with the PSMA promoter.

Analysis of PSMA and PSA enhancer/promoter activity

In a preliminary study, we evaluated the activities of the PSA and PSMA promoters as they have been well characterized and determined to be tissue specific. PSMA promoter activity was increased with a combination of SV40 enhancer in LNCaP cells.[27] Therefore, we used the combination of PSA or PSMA enhancer and promoter and SV40 enhancer to enhance prostate-specific gene expression (Fig. 1). To assess the transcriptional activity of our cloned promoter and enhancer regions, we constructed two PSA promoter-based plasmids, pPSA(P)-luc and pPSA(EP)-luc coding for the luciferase gene under the control of the PSA promoter and PSA enhancer and promoter, respectively, and two PSMA promoter-based plasmids, pPSMA(P)-luc and pPSMA-(EP)-luc coding for the luciferase gene under the control of the PSMA promoter and PSMA enhancer and promoter, respectively (Table 1). Four kinds of plasmids (2 μg) were transfected into LNCaP cells cultured in the absence or presence of DHT or into PC-3 cells, and a luciferase assay was carried out 24 h after transfection. We normalized each experiment using SV40 constructs (pGL3-control) (Fig. 3). It has been reported that LNCaP cells are androgen responsive, showing a decrease in PSMA mRNA levels and increase in PSA mRNA levels with increasing androgen concentrations in culture media.[28–30] In the medium without DHT, PSA-related plasmids, pPSA(P)-luc and pPSA(EP)-luc, exhibited weak transfectional activity (11 and 24% of the luciferase activity with pGL3-control) in LNCaP cells. However, in the medium with DHT, pPSA(EP)-luc, but not pPSA(P)-luc, increased the luciferase activity 6.3-fold compared to that in medium without DHT (151% of pGL3-control). Among the PSMA promoter-based plasmids, pPSMA(P)-luc showed weak transfection activity (28.8% and 45.9% of pGL3-control in medium without or with DHT, respectively). In contrast, pPSMA(EP)-luc induced relatively strong transfection activity (153% of pGL3-control) in medium without DHT and a small decrease of 36% in medium with DHT in LNCaP cells. In PC-3 cells, pPSA(P)-luc, pPSA(EP)-luc, pPSMA(P)-luc and pPSMA(EP)-luc drove only 2.3%, 13.1%, 17.8% and 22.5%, respectively, of the luciferase activity of pGL3-control. We confirmed that our cloned promoter and enhancer regions of PSA and PSMA had prostate-specific transcriptional activity consistent with findings reported previously.[28,29,31,32]

Two-step transcriptional amplification system using the PSMA promoter

The PSMA promoter appeared to be suitable for gene therapy because the expression of PSMA was not strongly affected

Table 1. Plasmids used in this study

Transcription		Plasmid	Upstream of expression gene		Expression gene
			Enhancer	Promoter	
One-step		pGL3-control	–	SV40	Luciferase
		pCMV-luc	CMV	CMV	Luciferase
		pCMV-tk	CMV	CMV	HSV-tk
		pPSA(P)-luc	–	PSA (0.6 kb)	Luciferase
		pPSA(EP)-luc	PSA (0.8 kb)	PSA (0.6 kb)	Luciferase
		pPSMA(P)-luc	–	PSMA (1.2 kb)	Luciferase
		pPSMA(EP)-luc	PSMA (1.6 kb)	PSMA (1.2 kb)	Luciferase
		pPSMA(EP)-tk	PSMA (1.6 kb)	PSMA (1.2 kb)	HSV-tk
		pMk(P)-luc	–	Midkine (0.6 kb)	Luciferase
		pMk(P)-tk	–	Midkine (0.6 kb)	HSV-tk
Two-step	Effector Plasmid	pPSMA(EP)-GAL/VP	PSMA (1.6 kb)	PSMA (1.2 kb)	GAL4/VP16
		pMk(P)-GAL/VP	–	Midkine (0.6 kb)	GAL4/VP16
	Reporter Plasmid	pRep-luc	–	5 × GAL4 bs + Minimal	Luciferase
		pRep-tk	–	5 × GAL4 bs + Minimal	HSV-tk

All plasmids contained SV40 enhancer in downstream of expression gene. GAL4 bs, GAL4 binding site; HSV-tk, herpes simplex virus thymidine kinase; minimal, adenoviral E1B minimal promoter; Mk(P), midkine promoter; PSA, prostate-specific antigen; PSA(EP), PSA enhancer and promoter; PSA(P), PSA promoter; PSMA, prostate-specific membrane antigen; PSMA(EP), PSMA enhancer and promoter; PSMA(P), PSMA promoter.

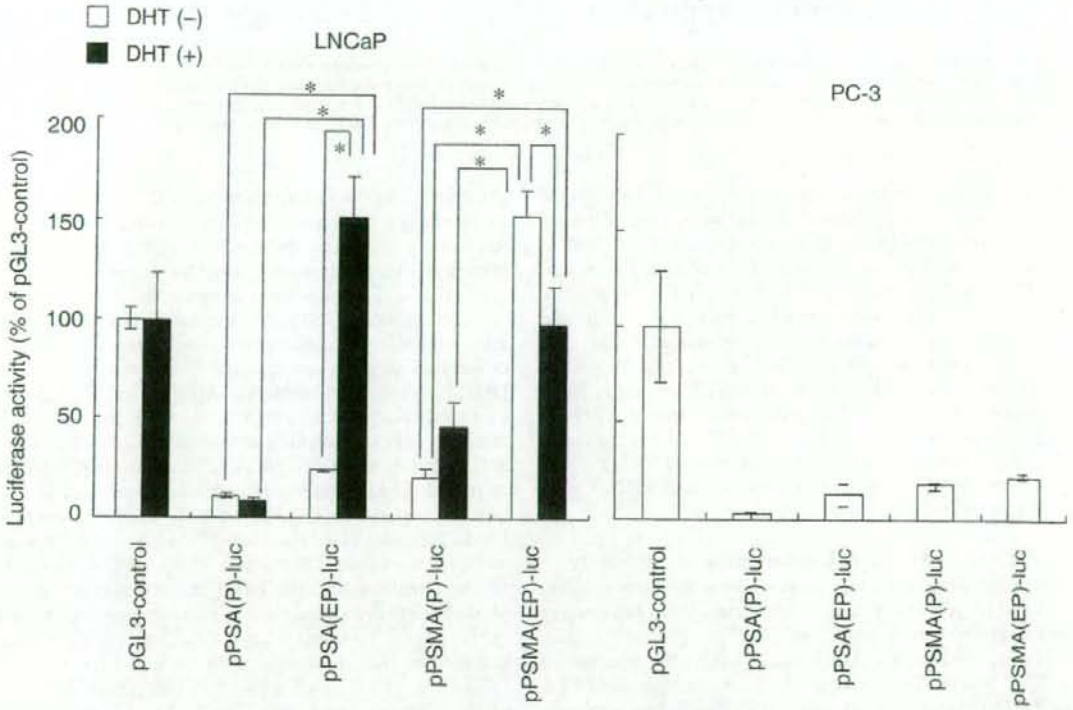

Fig. 3. Prostate-specific antigen (PSA) or prostate-specific membrane antigen (PSMA) enhancer and promoter activities in the absence or presence of dihydrotestosterone (DHT) in LNCaP and PC-3 cells. The prostate cancer cell lines, PSA-positive and PSMA-positive LNCaP cells and PSA-negative and PSMA-negative PC-3 cells were transfected with the plasmids indicated (2 μg). The promoter and enhancer activities were determined with a luciferase expression assay: 100% luciferase activity was taken as that of the pGL3-control. The results were expressed as the mean ± SD ($n = 4$). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. *$P < 0.05$.

by the presence or absence of androgen. However, the PSMA enhancer and promoter do not have strong promoter activity even when combined with the SV40 enhancer. Therefore, to enhance the PSMA promoter activity, we constructed the PSMA promoter-based effector plasmid pPSMA(EP)-GAL/VP coding for the GAL4–VP16 fusion protein under control of the PSMA enhancer and promoter, and a reporter plasmid, pRep-luc, coding for a luciferase gene under the control of a

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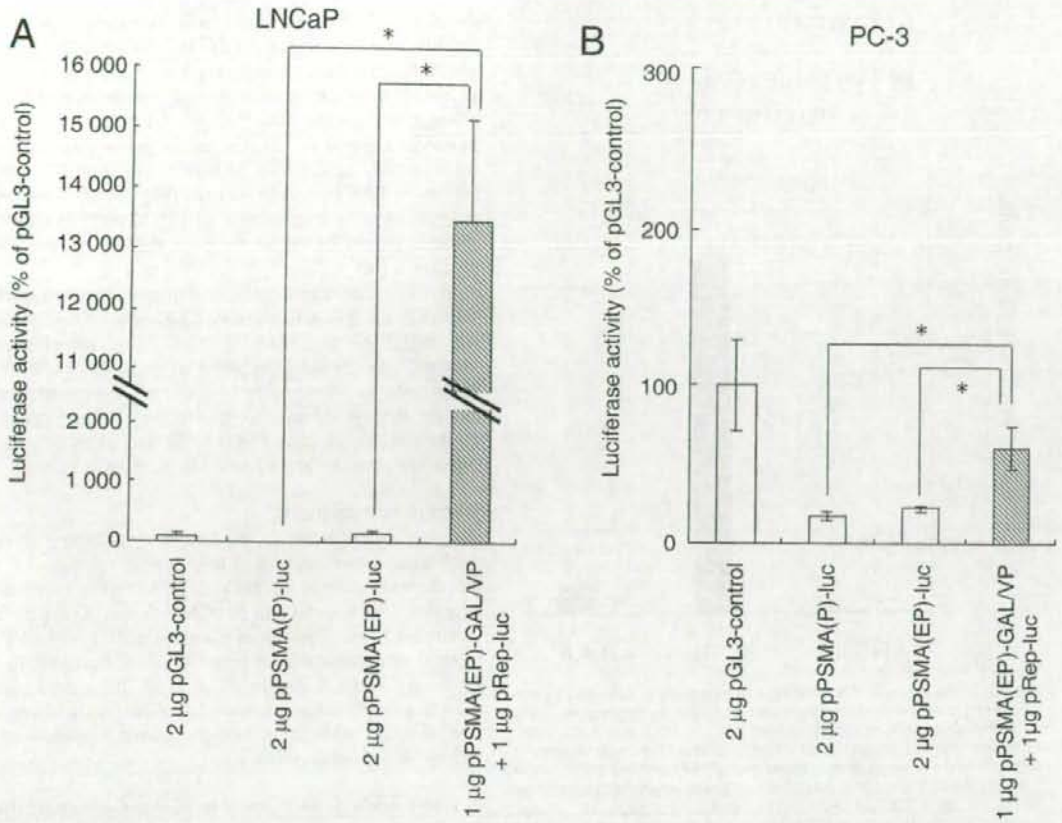

Fig. 4. Enhancement of the prostate-specific membrane antigen (PSMA) promoter activities by the two-step transcriptional amplification (TSTA) system determined using a luciferase expression assay in (A) LNCaP and (B) PC-3 cells. Comparison of transcriptional activity between one-step transcription (pPSMA[P]-luc or pPSMA[EP]-luc) and the TSTA system (pPSMA[EP]-GAL/VP plus pRep-luc). The luciferase activity of pGL3-control is taken as 100%. The results were expressed as the mean ($n = 4$). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. *$P < 0.05$.

synthetic promoter composed of 5 × GAL4-binding sites and the adenoviral E1B minimal promoter with the SV40 enhancer (Table 1), and evaluated luciferase activity by conducting cotransfection assays in LNCaP and PC-3 cells.

Strong luciferase activity was observed when the combination of pPSMA(EP)-GAL/VP and pRep-luc was transfected into LNCaP cells (Fig. 4A), but not when either pPSMA(EP)-GAL/VP or pRep-luc alone was transfected into the cell lines (1.7% and 3.8% of pGL3-control, respectively; data not shown). The paired plasmid, pPSMA(EP)-GAL/VP and pRep-luc (13 500% of pGL3-control, equivalent to approximately 45 000 cps/µg protein) showed 85-fold and 640-fold more luciferase activity than pPSMA(EP)-luc and pPSMA(P)-luc, respectively, in LNCaP cells (Fig. 4A), but showed comparatively less promoter activity, approximately 61% of pGL3-control in PC-3 cells (Fig. 4B). Luciferase activity by the paired plasmid, pPSMA(EP)-GAL/VP and pRep-luc, in LNCaP cells was 2.7% of that by CMV promoter (data not shown). This suggested that the TSTA system induced strong activity in PSMA-positive LNCaP cells but comparatively less activity in PC-3 cells.

Two-step transcriptional amplification system using the Mk promoter

To examine the TSTA system using another promoter, we constructed Mk promoter-based plasmids, pMk(P)-luc and pMk(P)-GAL/VP coding for the luciferase gene and GAL4–VP16 fusion protein, respectively, under control of the Mk promoter and SV40 enhancer (Table 1). We cloned a DNA fragment for the Mk promoter stretching from bp 27 of exon 1 to bp 559 of the 5′ flanking region of the human Mk gene. The 2.3-kb genomic fragment in the 5′ region of the Mk gene contained the elements responsible for promoter activity.[33] The Mk enhancer is composed of two elements, which are located between bp −1006 and −895 and between bp −901 and −794.[34] However, Yoshida *et al.* reported that the transcriptional activity mediated by a fragment spanning bp −559 to +50 was stronger than that mediated by a fragment stretching from bp −2285 to 50.[13] Therefore, this fragment would be suitable for the Mk promoter. In Mk-positive PC-3 and HeLa cells, the paired plasmid, pMk(P)-GAL/VP and pRep-luc, showed 5.0-fold and 2.6-fold higher luciferase

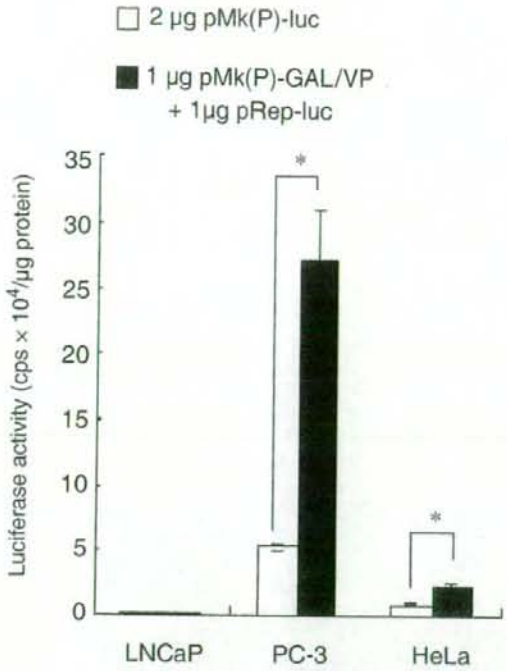

Fig. 5. Enhancement of the midkine (Mk) promoter activities by the two-step transcriptional amplification (TSTA) system determined using a luciferase expression assay among LNCaP, PC-3 and HeLa cells. Comparisonal of transcriptional activities of the Mk promoter among cell lines using one-step transcription (pMk[P]-luc) and the TSTA system (pMk[P]-GAL/VP and pRep-luc). The luciferase activity was expressed as counts per s (cps)/μg protein. The results were expressed as the mean ± SD ($n = 4$). Statistical significance of the data was evaluated using the Welch's *t*-test. *$P < 0.05$, compared with pMk(P)-luc.

activities, respectively (270 000 and 22 400 cps/μg protein, equivalent to approximately 9100% and 5500% of the pGL3-control), than the plasmid pMk(P)-luc (54 000 and 8500 cps/μg protein, equivalent to approximately 1800% and 2100% of the pGL3-control) (Fig. 5). Luciferase activities by the paired plasmid, pMk(P)-GAL/VP and pRep-luc, in PC-3 and HeLa cells were 22.1% and 3.7%, respectively, of that by the CMV promoter, (data not shown). However, strong luciferase activity was not observed when either pMk(P)-GAL/VP (0.1% and 4.2% of pGL3-control in PC-3 and HeLa cells, respectively) or pRep-luc alone (8.1% and 11.1% of pGL3-control, respectively) was transfected into the cells (data not shown). The paired plasmids did not induce luciferase activity in LNCaP cells (Fig. 5), suggesting that the TSTA system with the Mk promoter did not induce promoter activity in Mk-negative LNCaP cells.

In vitro suicide gene therapy model in PSMA-positive LNCaP cells and Mk-positive PC-3 and HeLa cells

Next, we applied the TSTA system to suicide gene therapy with the HSV-tk gene. pPSMA(EP)-tk or a paired plasmid, pPSMA(EP)-GAL/VP and pRep-tk, was transfected into LNCaP and PC-3 cells, and the inhibitory effect on cell growth was investigated in the presence of various concentrations of GCV. pCMV-tk and pGL3-basic were used as positive and negative controls, respectively. In LNCaP cells, the paired plasmids significantly inhibited cell growth (Fig. 6A). However, pPSMA(EP)-tk did not inhibit cell growth, having a similar effect to pGL3-basic. The therapeutic effects were also tested in PSMA-negative PC-3 cells, and the paired plasmids showed no inhibitory effect (Fig. 6B). pCMV-tk exerted inhibitory effects on LNCaP and PC-3 cells (Fig. 6A,B). These results indicated that a combination of pPSMA(EP)-GAL/VP and pRep-tk would be better for gene therapy against PSMA-positive cells.

In PC-3 and HeLa cells, the paired plasmids pMk(P)-GAL/VP and pRep-tk showed significant inhibitory effects, but pMk(P)-tk did not (Fig. 7A,B). The paired plasmids showed very similar inhibition to pCMV-tk in PC-3 and HeLa cells. In Mk-negative LNCaP cells, the paired plasmids did not actually inhibit cell growth (Fig. 7C). It appears that a combination of pMk(P)-GAL/VP and pRep-tk would be better for gene therapy against Mk-positive cells.

Western blot analysis

We investigated whether the observed inhibitory effects in the TSTA system with the Mk promoter corresponded with the expression level of HSV-tk protein (Fig. 7D). HSV-tk expression on transfection of pCMV-tk was observed clearly in all cell lines. The paired plasmids pMk(P)-GAL/VP and pRep-tk more markedly expressed HSV-tk than pMk(P)-tk in PC-3 and HeLa cells. In PC-3 cells, the paired plasmid showed a similar expression level to pCMV-tk. In Mk-negative LNCaP cells, pMk(P)-tk and the paired plasmids did not induce HSV-tk expression.

In vivo suicide gene therapy in PC-3 tumor xenografts

The TSTA system with the Mk promoter in PC-3 cells (22% of CMV promoter) induced stronger luciferase activity than that with the PSMA promoter in LNCaP cells (2.7% of CMV promoter). Therefore, we evaluated the antitumor effect by direct injection of the nanoplexes into PC-3 tumor xenografts. The average growth rate of tumors was suppressed significantly in the mice treated with the nanoplex of the paired plasmid, pMk(P)-GAL/VP and pRep-tk, compared with the control mice (Fig. 8). The paired plasmid showed similar growth inhibition to pCMV-tk. These results indicated that the TSTA system with the Mk promoter could induce greater inhibition of tumor growth than one-step transcription with the Mk promoter.

Discussion

The activity of a prostate tumor-specific promoter is generally weak in comparison to that of a universal promoter such as the CMV promoter.[14,35] Therefore, in the present study, a TSTA system was used to enhance the efficacy of suicide gene therapy for treatment of prostate tumor. The TSTA systems showed greater activity than one-step transcription, as confirmed by the growth inhibition of PSMA-positive LNCaP cells and Mk-positive PC-3 cells, and PC-3 tumor xenografts on suicide gene therapy. This suggests that TSTA systems featuring tumor-specific promoters are suitable for cancer treatment by gene therapy.

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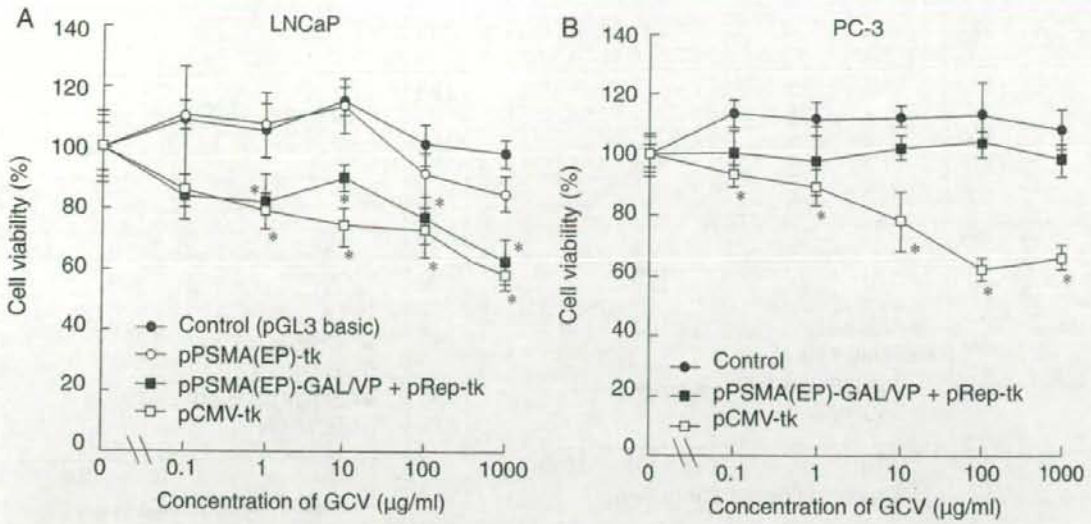

Fig. 6. *In vitro* suicide gene therapy using the two-step transcriptional amplification (TSTA) system with a prostate-specific membrane antigen (PSMA) promoter. (A) LNCaP and (B) PC-3 cells were transfected with various plasmids (0.2 μg). After 12 h incubation, the medium was changed to one containing ganciclovir (GCV). Data points indicate the mean ± SD ($n = 4$). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. $^*P < 0.05$, compared with control plasmid.

The metastatic prostate cancer within an individual is composed heterogeneously of clones of both androgen-dependent and androgen-independent cancer cells. At present, the androgen-dependent and androgen-independent prostate cancer cells available are LNCaP and PC-3, respectively.[36] PSMA mRNA was expressed in LNCaP cells, but not in PC-3 cells (Fig. 2B). Mk mRNA was expressed strongly in PC-3 cells, but not in LNCaP cells (Fig. 2B). These findings suggest that the PSMA promoter could be utilized with androgen-dependent prostate cancer (e.g. LNCaP cells), and the Mk promoter with androgen-independent prostate cancer (e.g. PC-3 cells) for tumor-specific gene therapy. The PSMA and Mk promoters have already been cloned and used for suicide gene therapy with the one-step transcription system.[13,37,38] However, usage of the TSTA system without the PSA promoter has not been reported in prostate cancer.

In the present study, we modified the TSTA system for use with the SV40 enhancer in reporter and effector plasmids to amplify the transcriptional activity, and tried to apply the PSMA or Mk promoter to the TSTA system. We confirmed that our cloned PSA or PSMA promoter and enhancer regions had prostate-specific transcriptional activity based on findings reported previously in which androgen-responsive LNCaP cells showed increasing PSMA promoter activity and decreasing PSA promoter activity when DHT was not added into the culture medium (Fig. 2).[28,29] Thus, the PSMA promoter system seems to offer an advantage under conditions of androgen ablation.

In PSMA promoter-related plasmids, the TSTA system with the paired plasmid, pPSMA(EP)-GAL/VP and pRep-luc, amplified the expression of the luciferase gene 640-fold and 85-fold more than one-step transcription with pPSMA(P)-luc and pPSMA(EP)-luc, respectively, in LNCaP cells. However, the paired plasmids showed weak promoter activity in PC-3 cells (Fig. 4B), whereas PSMA mRNA could not be detected by RT-PCR analysis (Fig. 2). Recently, Laidler *et al.* reported that basic fibroblast growth factor (bFGF) and estradiol (E2) induce weak expression of PSMA in PC-3 cells.[39] A growth factor such as bFGF is included in the serum for the culture medium. Therefore, in our study, the PSMA promoter activity in PC-3 cells may be induced weakly by bFGF or E2 in the culture medium and be amplified by the TSTA system. In Mk promoter-based plasmids, TSTA with the paired plasmids pMk(P)-GAL/VP and pRep-luc resulted in 5.0-fold and 2.6-fold higher luciferase activity than one-step transcription with pMk(P)-luc in PC-3 and HeLa cells (Fig. 5). The difference in the degree of amplification by the TSTA system between the PSMA and Mk promoters might be attributed to different transcriptional activity by the promoters.

In cancer gene therapy, the TSTA system with the PSA promoter used to drive the expression of expanded polyglutamine (ex-polyQ) for induction of apoptosis selectively eliminated PSA-positive LNCaP to an extent comparable with that of CMV promoter-driven ex-polyQ.[15] The TSTA system with the CEA promoter used to control the expression of Bax for induction of apoptosis can suppress *in vitro* and *in vivo* tumor growth in lung cancer and colon adenocarcinoma.[21] In the present study, we applied the TSTA system to suicide therapy with HSV-tk and demonstrated that the paired plasmids, pPSMA(EP)-GAL/VP and pRep-tk, and pMk(P)-GAL/VP and pRep-tk, enhanced cytotoxic activity in LNCaP cells, and in PC-3 and HeLa cells, respectively. In PSMA-positive LNCaP cells, the inhibitory effect was dependent on the concentration of GCV in the cells transfected with the paired plasmids, pPSMA(EP)-GAL/VP and pRep-tk (Fig. 6A). In PC-3 cells, the paired plasmids showed comparatively less

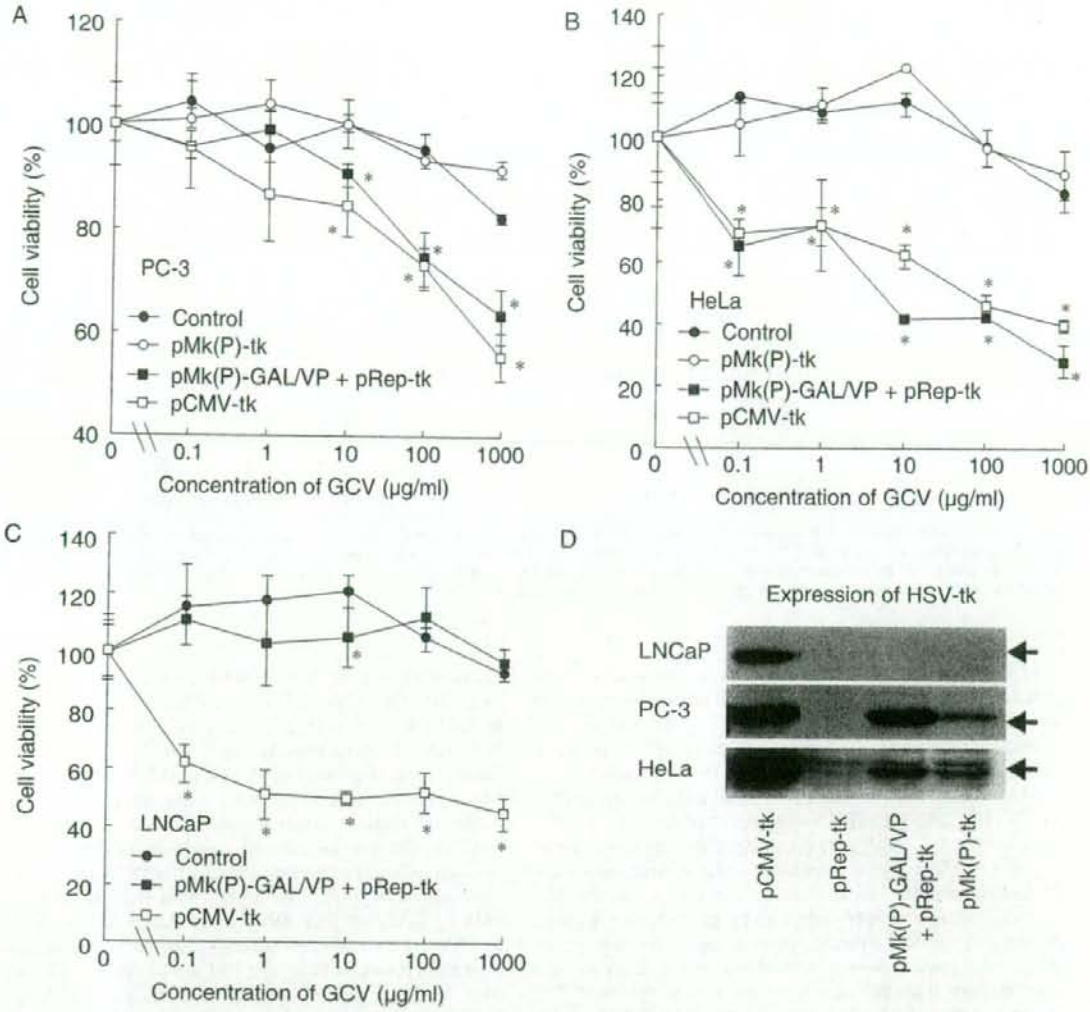

Fig. 7. (A–C) *In vitro* suicide gene therapy and (D) herpes simplex virus thymidine kinase (HSV-tk) expression using the two-step transcriptional amplification (TSTA) system with a midkine (Mk) promoter. (A) PC-3, (B) HeLa and (C) LNCaP cells were transfected with various plasmids (0.2 μg). After 12 h incubation, the medium was changed to one containing ganciclovir (GCV). Data points indicate the mean ± SD ($n = 4$). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. $^*P < 0.05$, compared with control plasmid. (D) Specific detection of HSV-tk protein expressed by one-step transcription and the TSTA system with the Mk promoter by western blotting.

promoter activity (Fig. 4B); however, the activity was apparently non-toxic as long as the level of HSV-tk expression by the paired plasmid remained low (Fig. 6B). In PC-3 and HeLa cells, inhibitory effects were observed with paired plasmids, pMk(P)-GAL/VP and pRep-tk (Fig. 7A,B), but not in Mk-negative LNCaP cells (Fig. 7C). HSV-tk expression with the TSTA system with the Mk promoter was stronger than that with one-step transcription in PC-3 and HeLa cells, being equivalent to that with one-step transcription with the CMV promoter in PC-3 cells (Fig. 7D). Furthermore, in *in vivo* suicide gene therapy, the TSTA system with the Mk promoter suppressed the growth of PC-3 tumor xenografts greatly, compared with one-step transcription (Fig. 8). These characteristics can be used most powerfully in prostate cancer when combining the TSTA system with the PSMA or Mk promoter, in which strong expression is restricted to target cells, and would minimize the side-effects in non-targeted cells.

The TSTA system has been applied to gene therapy mediated by adenovirus,[20,21,40,41] lentivirus[42] and cationic liposomes.[15,16] Adenoviruses and lentiviruses can evoke non-specific inflammation, and readily induce strong antivirus immune responses. The mixing of cationic liposomes with DNA often results in large aggregated lipoplexes, which cannot be injected into blood vessels and yield very low levels of transfection efficiency

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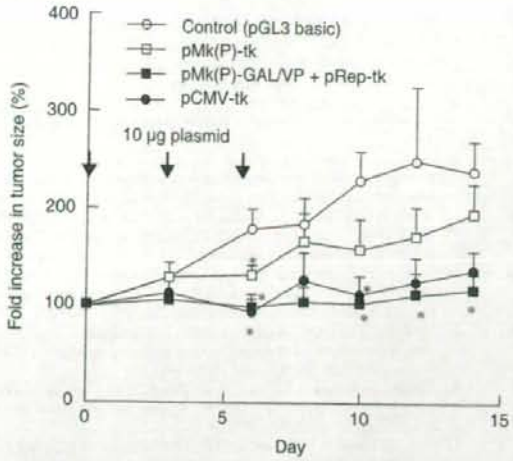

Fig. 8. *In vivo* suicide gene therapy of PC-3 tumor xenografts with ganciclovir (GCV) in mice. When the average volume of PC-3 xenograft tumors reached 150 mm^3 (day 0), mice were divided into four groups: group I, pGL3-basic (10 μg) as a control; group II, pMk(P)-tk (10 μg); group III, pMk(P)-GAL/VP (5 μg) plus pRep-tk (5 μg); group IV, pCMV-tk (10 μg). The nanoplexes of the plasmids were injected directly into the tumor three times (days 0, 3 and 6). GCV (25 mg/kg) was administered intraperitoneally at 12, 24 and 36 h after the injection of nanoplexes. The results indicate the mean volume ± SE ($n = 4$). Statistical significance of the data was evaluated using the Fisher's exact test. *$P < 0.05$, compared with control.

in vivo. Recently, we developed a lipid-based nanoparticle that formed injectable-sized nanoplexes (200–300 nm).[25] When the nanoplexes were injected directly into PC-3 xenografts, they showed approximately 6.5-fold higher luciferase activity than *in vivo*-Jet PEI-Gal, a commercially available cationic polymer transfection reagent (PolyPlus-transfection; ILLKIRCH, Illkirch, France; data not shown). Therefore, in the present study, we used this nanoparticle to induce strong expression in the TSTA system *in vitro* and *in vivo*. Furthermore, we reported recently that a folate-linked nanoparticle could deliver DNA with high transfection efficiency and selectivity into human nasopharyngeal and prostate cancer cells.[22,25] The combination of this folate-linked nanoparticle with the TSTA system has great potential as a tumor-specific vector for *in vivo* cancer gene therapy.

In conclusion, we developed an ideal gene expression amplification system with the PSMA and Mk promoters. This system is a promising tool with which to create targeted gene-based therapeutic applications.

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Biosurfactant MEL-A enhances cellular association and gene transfection by cationic liposome

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Abstract

Mannnosylerythritol lipid A (MEL-A), a biosurfactant produced by microorganisms, has many biological activities. To enhance the gene transfection efficiency of a cationic liposome, we prepared a MEL-liposome (MEL-L) composed of 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), dioleoyl phosphatidylethanolamine (DOPE) and MEL-A, and investigated its transfection efficiency in human cervix carcinoma Hela cells. MEL-L was about 40 nm in size, and the MEL-L/plasmid DNA complex (MEL-lipoplex) remained an injectable size (169 nm). MEL-A induced a significantly higher level of gene expression, compared to commercially available Tfx20 and the liposome without MEL-A (Cont-L). Analysis of flow cytometric profiles clearly indicated that the amount of DNA associated with the cells was rapidly increased and sustained by addition of MEL-A to the liposome. Confocal microscopic observation indicated that the MEL-lipoplex distributed widely in the cytoplasm, and the DNA was detected strongly in the cytoplasm and around the nucleus, compared with Cont-L. These results suggested that MEL-A increased gene expression by enhancing the association of the lipoplexes with the cells in serum. MEL-L might prove a remarkable non-viral vector for gene transfection and gene therapy.

Keywords: Biosurfactant; Cationic liposome; DC-Chol; Cellular association; Gene expression

1. Introduction

In gene therapy, the transport and delivery of DNA inside cells are key steps. Viral and non-viral vectors for transferring DNA have been developed intensively. Viral vectors are efficient but have various disadvantages such as immune recognition and potential virus-associated toxicity, including helper virus replication and insertional mutagenesis. In contrast, non-viral vectors are highly attractive due to their excellent safety profile despite their low transgene expression efficiency in comparison to viral vectors. The cationic liposome-mediated transfer of DNA is a particularly promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. Therefore, cationic lipids and improved formulations of liposome have been developed for the efficient delivery of DNA to cells [1,2]. Notably, cationic liposomes composed of 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) together with dioleoylphosphatidylethanolamine (DOPE) have been reported as an efficient vector for the transfection of DNA into cells [3–5] and in clinical trials [6,7].

Glycolipid biosurfactants (BSs) have received much attention as leading materials for drug-carrying microcapsules and artificial cells, owing to their stabilizing effect on liposomes [8,9]. BSs have numerous advantages, such as lower toxicity, higher biodegradability and unique biological activities. To further improve the transfection efficiency, a BS was used as a component of liposomes, resulting in increased transfection [10]. Mannosylerythritol lipid (MEL) is a BS abundantly produced by the yeast strain *Candida antarctica* T-34 [11]. MEL exhibits excellent surface-activity [12] (i.e., an efficient decrease in surface tension), remarkable cell differentiation and growth inhibition against human leukemia [13], mouse melanoma [3] and PC12 cells [14], and antimicrobial activity particularly against Gram-positive bacteria [13]. One MEL-related compound is MEL-A,

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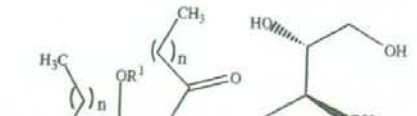

MEL-A; R_1=R_2=Ac

(n=6-10)

Mannosylerythritol lipid A (MEL-A)

Fig. 1. Chemical structure of biosurfactant MEL-A. MEL-A consists of 4-*O*-(di-*O*-acetyl-di-*O*-alkanoyl-β-D-mannopyranosyl)-erythritol esterified two fatty acids and two acetic acids.

which consists of 4,6-di-*O*-acetyl-2,3-di-*O*-alkanoyl-β-D-mannopyranosyl-(1→4)-*O*-erythritol esterified with two fatty acids and two acetic acids (Fig. 1). MEL-A increased remarkably the transfection efficiency of liposomes with a cationic cholesterol derivative such as DC-Chol bearing a tertiary amine head group and cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine (OH-Chol) bearing a secondary amine head group [10]. The mechanism of gene transfection by MEL-A with OH-Chol/DOPE liposome, but not that with DC-Chol/DOPE liposome, has been clarified [15]. We found that MEL-A added in DC-Chol/DOPE liposomes increased transfection efficiency in serum and did not show a similar mechanism of transfection increased with OH-Chol.

In the present study, we investigated the mechanism by which gene transfection was enhanced by cationic liposomes composed of DC-Chol, DOPE and MEL-A (MEL-L) in Hela cells, measuring the cellular uptake and the intracellular localization of liposome/DNA complexes by flow cytometry and confocal laser scanner microscopy, compared to cationic liposomes without MEL-A (Cont-L). The results suggested that MEL-A enhanced the association of lipoplexes with the cells, delivered it widely into the cytoplasm and increased gene expression.

2. Materials and methods

2.1. Materials

MEL-A and 7-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled MEL-A were supplied by Dr. Kitamoto (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). DOPE was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). DC-Chol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tfx20 was purchased from Promega (Madison, WI, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

2.2. Preparation of oligonucleotide and plasmid DNA

The FITC-labeled 20-mer randomized oligodeoxynucleotide (5′-CGAGTGCACACGCCTCTCAG-3′, FITC-ODN) was synthesized with a phosphodiester backbone (Sigma Genosys Japan, Hokkaido, Japan). pCMV-luc was constructed using a cDNA fragment (589 bp) coding for a cytomegalovirus (CMV) promoter amplified by PCR with a pEGFP-C1 plasmid (Clontech, CA, USA) containing a green fluorescent protein (GFP) reporter gene under the control of the CMV promoter as a template, and the following CMV promoter-specific primers: CMV promoter forward primer (5′-ATGGTACCTAGTTATTAATAGTAATCAA-3′) and CMV promoter reverse primer (5′-TCAAGCTTGATCTGACGGTTCACTAAAC-3′). The forward and reverse primers, respectively, contained *Kpn*I and *Hind*III restriction sites (underlines). After the amplification, the cDNA was digested with *Kpn*I and *Hind*III and ligated into a *Kpn*I/*Hind*III-digested pGL3-enhancer (Promega, Madison, WI, USA).

A protein-free preparation of the plasmid was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany).

2.3. Cell culture

A human cervix carcinoma cell line, Hela 229, was supplied by the Department of Virology, Toyama Medical and Pharmaceutical University. The cells were grown in Minimal Essential Medium (MEM, Life Technologies, Inc., Grand Island, NY, USA) supplemented with kanamycin and 5% fetal bovine serum. Cells were grown at 37 °C in a 5% CO_2/air incubator.

2.4. Preparation of liposomes

Two liposomal formulae were used: DC-Chol, DOPE and MEL-A in a 3:2:2 molar ratio for MEL-L; and DC-Chol and DOPE in a 3:2 molar ratio for Cont-L were prepared by a modified ethanol injection method. Briefly, DC-Chol, DOPE and MEL-A were dissolved and mixed in ethanol and the solution was evaporated leaving behind about 2 ml of the solution. Next, a constant volume of water was added to the ethanol solution, and the mixture was evaporated again until only the ethanol was left. After sonication for 15 min in a bath type sonicator, the solution was filtrated through 0.45-μm Millex-HA filters (Millipore, Cork, Ireland) at once for sterilization. The particle size distributions and the ζ-potentials were measured by the dynamic light scattering method and the electrophoresis light scattering method, respectively (ELS-800, Otsuka Electronics Co., Ltd., Osaka, Japan), at 25 °C after the dispersion was diluted to an appropriate volume with water. In 1,1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate (DiI) (Lambda Probes & Diagnostics, Graz, Austria)-labeled liposomes, DiI was incorporated at 0.04 mol% of all the lipids. In NBD-labeled MEL-L, NBD-labeled MEL-A was added at 24% of all the MEL-A.

2.5. Transfection

Cell cultures were prepared by plating cells in a 35-mm culture dish 24 h prior to each experiment. The cells at confluences of 70% in the well were transfected with each lipoplex. Lipoplex at a charge ratio (+/−) of 3/1of cationic lipid to DNA was formed by addition of liposome to DNA (3.8 μl MEL-L (6.9 mg total lipid/ml) or Cont-L (4.8 mg total lipid/ml) suspension in water to 2 μg of DNA, MEL- or Cont-lipoplex) with gentle shaking and leaving at room temperature for 10–15 min. The lipoplexes were diluted with MEM containing 5% serum to a final concentration of 2 μg of DNA per 1 ml of medium, and incubated with the cells for 24 h in the medium. In effect of inhibitor of endocytosis on the transfection activity, the cells were treated with 50 μM chloroquine for 2 h and were then incubated with the lipoplexes for 24 h. The cell toxicity by transfection with the lipoplexes was determined with a WST-8 assay (Dojindo Laboratories, Kumamoto, Japan).

2.6. Luciferase assay

The pCMV-luc plasmid was transfected into cells by MEL-L and Cont-L, respectively. After 24 h of incubation, the cells were washed twice with PBS (pH 7.4) and harvested with 125 μl of cell culture lysis reagent (Toyo Ink, Tokyo, Japan). Luciferase expression was quantified with 10 μl of centrifuged lysate supernatant using a picagene luciferase assay kit (Toyo Ink, Tokyo, Japan) as described previously [16]. Light emission, expressed in counts per second (cps), was normalized to the protein concentration of each sample, determined using BCA protein assay reagent.

2.7. Flow cytometry

The cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each liposome (3.8 μl) was mixed with 2 μg of FITC-ODN or DiI-labeled liposome mixed with 2 μg of ODN, and then diluted in 1 ml of the medium. The cells were incubated with the lipoplex for 1, 2 and 6 h. After incubation, the dishes were washed 2 times with 1 ml of PBS (pH 7.4) to remove any unbound lipoplexes. The cells were detached with 0.25% trypsin and centrifuged at 1500*g*. The supernatant was discarded and the cells were resuspended with PBS containing 0.1% BSA and 1 mM EDTA. The suspended cells were directly introduced into a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm argon ion laser. Data for 10,000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC), and green (530/30 nm) fluorescence.

2.8. Confocal laser scanning microscopy

Hela cells were prepared by plating cells in a 35-mm culture dish 24 h prior to each experiment. DiI-labeled, NBD-labeled or non-labeled liposome (3.8 μl) was mixed with 2 μg of FITC-ODN or plasmid DNA and then diluted in 1 ml of the medium. After incubation for the periods indicated in the figures, the cells were washed twice with PBS and fixed with 4% formaldehyde in PBS for 15 min at room temperature. For staining the nucleus, the fixed cells were washed with PBS and incubated with 0.5 mg/ml of RNase in PBS for 20 min at 37 °C. Subsequently, the cells were washed with PBS and incubated with propidium iodide (PI) for 15 min at room temperature. Examinations were performed with a Radiance 2100 confocal laser-scanning microscope (BioRad, CA, USA) as previously described [16].

2.9. Statistical analysis

The statistical significance of the data was evaluated with Student's *t*-test. A p value of 0.05 or less was considered significant.

3. Results and discussion

3.1. Preparation and characterization of MEL-L and Cont-L

The success of gene therapy is largely dependent on the development of vectors as DNA carriers. Cationic liposomes composed of DC-Chol as a cationic lipid and DOPE as a helper lipid are generally used. A molar ratio of 3:2 has been used for DC-Chol /DOPE [5]. In a preliminary study, the addition of MEL-A to DC-Chol /DOPE liposomes increased transfection efficiency in serum, and the ratio of MEL-A to DC-Chol /DOPE liposome was optimized at a molar ratio of 3:2:2 (DC-Chol, DOPE and MEL-A). Therefore, in this study, we prepared two kinds of liposomes; Cont-L consisting of DC-Chol and DOPE at a molar ratio of 3:2 and MEL-L containing 28 mol% MEL-A in a Cont-L formulation. MEL-L and Cont-L were about 43 nm and 156 nm in size, and the lipoplexes about 169 nm and 265 nm in size, respectively (Table 1). Although the concentration of MEL-A and the method used to prepare the liposomes were not the same as those reported by Inoh et al. [10], our observation was consistent with their finding that the addition of MEL-A to liposomes makes them smaller. This suggested that MEL-A kept liposomes small via the biosurfactant's effect.

3.2. Effects of MEL-L on transfection efficiency

Next, we compared the efficiency of transfection into Hela cells between MEL-L, Cont-L and the commercially available transfection reagent Tfx20 in the presence of 5% serum (Fig. 2). MEL-L showed significantly greater transfection activity than

Table 1
Formulation of liposomes

Liposome	Formulation (molar ratio)	Particle size[a] (nm)	ζ-potential (mV)	Lipoplex size[b] (nm)
MEL-L	DC-Chol/DOPE/MEL=3/2/2	43.4±2.8	52.9±2.0	168.6±3.7
Cont-L	DC-Chol/DOPE=3/2	156.4±2.4	70.5±1.2	265.3±101.4

[a] In water.
[b] A change ratio (+/−) of 3/1 in water.

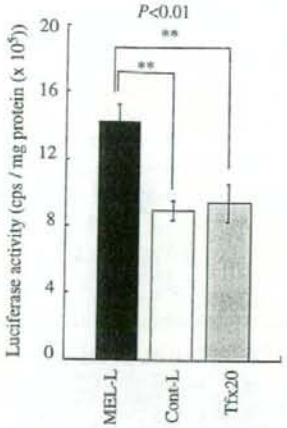

Fig. 2. Effect of MEL-A on transfection efficiency of MEL-L, Cont-L and Tfx20 in Hela cells. Lipoplexes were diluted in medium with serum to a final concentration of 2 μg of DNA in 1 ml of medium per well, and each cell was incubated for 24 h. The charge ratio (+/−) of liposome to plasmid DNA was 3:1. Each result represents the mean±S.D. (n=3).

Cont-L and Tfx20. This suggested that MEL-A enhanced the transfection efficiency of the DC-Chol/DOPE liposome. We also confirmed that MEL-L had no toxicity when the MEL-lipoplex was incubated with Hela cells at the dose used (data not shown). These results were consisted with the finding that cationic liposomes containing MEL-A promoted the efficiency of gene transfection into mammalian cultured cells although MEL-A itself did not increase transfection efficiency [15]. The effect on transfection efficiency might be because MEL-A minimized the aggregation induced by DNA and reduced particle size [10,17].

3.3. Localization of liposome and DNA transfected into Hela cells

To investigate the intracellular localization of the MEL-lipoplex, we prepared lipoplexes of either DiI-labeled MEL-L or Cont-L with FITC-ODN and transfected them into Hela cells (Fig. 3). After 2 or 24 h of incubation, the intracellular localization of the DiI-labeled liposome and FITC-labeled DNA were confirmed by changing the Z-axis of the observed area

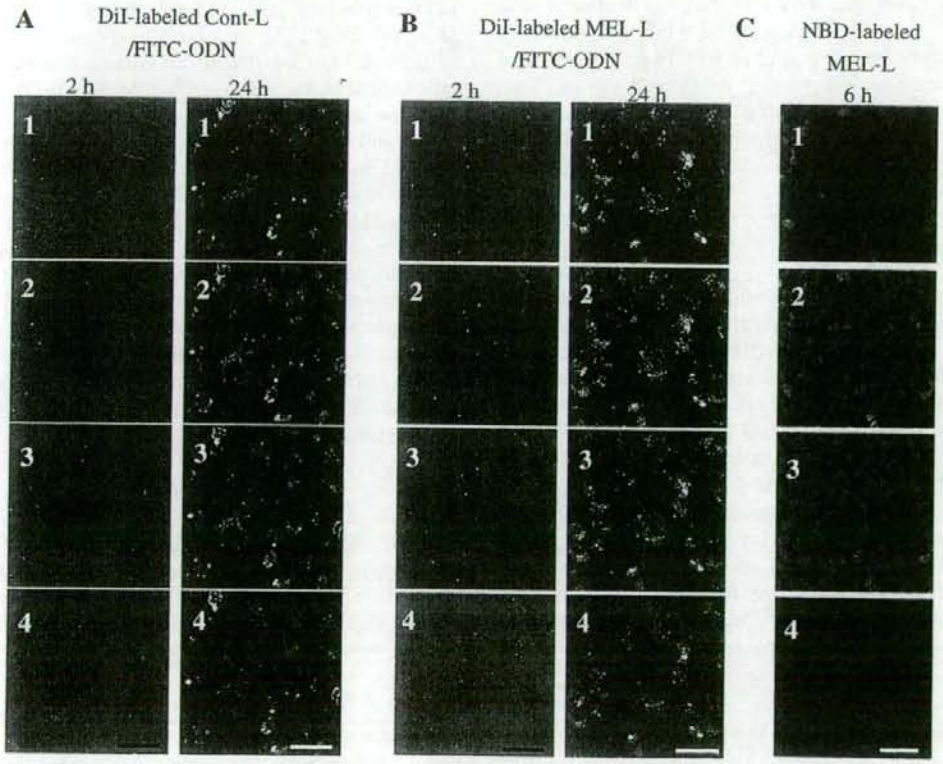

Fig. 3. The localization of lipoplexes of DiI-labeled liposome and FITC-ODN at 2 and 24 h after incubation (A and B). FITC-ODN was mixed with DiI-labeled Cont-L (A) and MEL-L (B), respectively. The localization of lipoplexes of MEL-L with NBD-labeled MEL-A and plasmid DNA at 6 h after incubation (C). Plasmid DNA was mixed with NBD-labeled MEL-L. The lipoplexes were transfected into Hela cells, and observed under a confocal laser microscope by changing the Z-axis. Images 1–4 represent regular intervals of 3 μm on the Z-axis from bottom to top of cells, respectively. In A and B, the red signals show the location of the liposome, and the green signals, that of the FITC-ODN. In C, the red signals show the location of the nucleus, and the green signals, that of the MEL-A. Scale bar=50 μm.

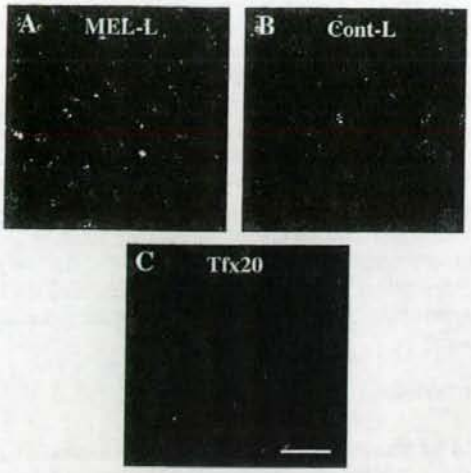

Fig. 4. Intracellular distribution of FITC-ODN by liposomes in Hela cells. FITC-ODN was transfected by MEL-L (A), Cont-L (B) and Tfx20 (C), respectively, into Hela cells. After 6 h of incubation, nuclei were stained by PI, and the cells were examined under a fluorescent microscope using a filter for green-fluorescence (FITC-ODN) and red-fluorescence (nucleus). Scale bar = 50 μm.

with 3 μm. DiI-fluorescence was weakly detected in the cytoplasm at 2 h incubation with MEL-L (Fig. 3B), but hardly detected with Cont-L (Fig. 3A). This indicated that MEL-L was rapidly internalized into the cells. At 24 h, DiI-fluorescence and FITC-fluorescence in both MEL-L and Cont-L were widely observed in the cytoplasm.

To confirm the distribution of the DiI-fluorescence of MEL-L by MEL-A, we prepared lipoplexes using NBD-labeled MEL-L with NBD-labeled MEL-A and transfected them into Hela cells. After 6 h of incubation, NBD-labeled MEL-A was widely observed in the cytoplasm but not detected in the nucleus (Fig. 3C). This localization was similar to that of DiI-labeled MEL-L (Fig. 3B) and suggested that MEL-A of MEL-L enhanced the association of MEL-lipoplex with the cells and induced the distribution of liposome and DNA into the cytoplasm. This might explain the enhancement of transfection efficiency by MEL-A. Inoh et al. reported that lipoplexes containing MEL-A and OH-Chol were temporarily located on the plasma membrane of target cells [15]. However, our results showed that MEL-A was located throughout the cytoplasm. We cannot explain this discrepancy, but the difference in cationic lipids might affect the distribution of MEL-L in the target cell.

To confirm the localization of DNA in the cells, lipoplexes of FITC-ODN were formed with MEL-L, Cont-L and Tfx20, respectively, and transfected into Hela cells. The fluorescence of FITC-ODN in the cells was more strongly observed in MEL-L than in Cont-L at 6 h of incubation (Fig. 4A and B). In MEL-L, FITC-ODN was distributed around the nucleus and cytoplasm, but in Cont-L, it was mostly localized to the cytoplasm. In Tfx20, little fluorescence was observed (Fig. 4C). This suggested that MEL-L could deliver DNA into the cytoplasm and nucleus better than Cont-L or Tfx20.

3.4. Association of MEL-, Cont- and, Tfx20-lipoplexes with the cells

To compare the cellular association of the DNA transfected by MEL-L, Cont-L and Tfx20, respectively, we examined the

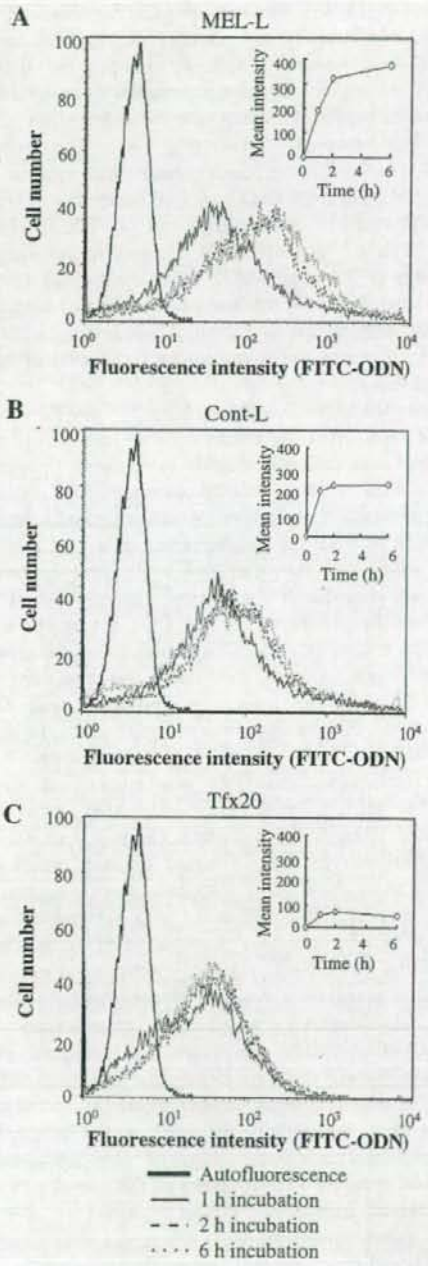

Fig. 5. The cellular association with lipoplexes of FITC-ODN. The kinetics of the cellular association of FITC-ODN transfected with MEL-L (A), Cont-L (B) and Tfx20 (C) was evaluated by flow cytometry. Each lipoplex was incubated with cells for 1, 2 and 6 h.

amount of DNA associated with the cells at different time points by flow cytometry. Analysis of flow cytometric profiles and mean intensities clearly indicated that the kinetics of the amount of DNA was remarkably different among MEL-L, Cont-L and Tfx20 (Fig. 5). The fluorescent intensity of FITC-ODN was stronger in MEL-L than in Cont-L (Fig. 5A and B). The fluorescence intensity of Cont-lipoplex increased rapidly, but reached a maximum at 1 h. In contrast, the fluorescence intensity of the MEL-lipoplex increased with time for up to 6 h. This might be corresponding with the observation of lipoplex using NBD-labeled MEL-L (Fig. 3C). The fluorescence intensity in Tfx20 was much weaker than these in MEL-L and Cont-L (Fig. 5C). The kinetics of the amount of DiI-labeled liposome was also similar with that of FITC-labeled DNA. After 24 h of incubation, the fluorescent intensity of the lipoplexes of DiI-labeled MEL-L was maintained whereas that of DiI-labeled Cont-L was decreased compared with that after 2 h of incubation (data not shown). These findings indicated that MEL-A enhanced and sustained the association of liposomes with cells.

The results of confocal laser microscopy and flow cytometry showed that MEL-A enhanced the interaction between lipoplexes and cells and quickly internalized lipoplexes and widely distributed them into the cytoplasm. MEL-A may have improved transfection activity by enhancing and sustaining the interaction between the lipoplexes and cells.

The entry into the cytoplasm is the first important step for liposome-mediated transfection. The addition of MEL-A to cationic liposomes with either DC-Chol or OH-Chol had a similar enhancing effect on transfection efficiency [15]. Confocal laser scanning microscopy revealed that MEL-A increased membrane fusion by liposomes with OH-Chol [15], but not that by liposomes with DC-Chol. In liposomes with OH-Chol, both MEL-A and DNA distributed on the plasma membrane, and DNA was internalized via fusion [15]. In MEL-L with DC-Chol, MEL-A and DNA distributed through the cytoplasm (Figs. 3 and 4), and the transfection activity was decreased by addition of endocytosis inhibitor, chloroquine (data not shown), suggesting that MEL-L was internalized into the cells via endocytosis. Our DC-Chol/DOPE liposomes were different from their DC-Chol/DOPE and OH-Chol/DOPE liposomes: in our case, the preparation involved a modified ethanol injection; the molar ratio of DC-Chol/DOPE was 3:2, the concentration of MEL-A in DC-Chol/DOPE liposomes was different, and the lipoplexes were formed by mixing the liposome with DNA directly in water without pre-incubation. In both cases, we can say that the addition of MEL-A to DC-Chol/DOPE liposomes decreased the size of the liposomes and lipoplexes, induced a rapid uptake of DNA in the cytoplasm, and increased transfection efficiency. However, the mechanism of enhancement by MEL-A was not similar with OH-Chol/DOPE liposomes [15], suggesting that cationic lipids also play an important role in facilitating the transfection. The role of DC-Chol in the formation of lipoplexes is presently unknown, but must be important in determining the final transfection efficiency.

MEL-L increased the association of lipoplexes with cell membranes, not by aggregation of lipoplexes. This better association results in better penetration by the lipoplexes. Biosurfactants of MEL-A have many excellent properties (low toxicity, biodegradability, etc.) compared to synthetic surfactants [18,19]. The combination of MEL-A and a cationic lipid might increase transfection efficiency via a synergetic effect. Furthermore, MEL-lipoplexes remained small in size and enhanced transfection efficiency in serum, which is important for applications in vivo. These findings suggested that MEL-L is a remarkable non-viral vector for gene transfection and gene therapy.

4. Conclusions

In the present study, we studied the transfection efficiency of MEL-L and investigated the localization and kinetics of lipoplexes to understand the mechanism of the enhancement of transfection efficiency by MEL-A. The transfection efficiency of MEL-L was significantly higher than that of Cont-L or Tfx20. We found that MEL-L increased the association with the cells in the experiment with flow cytometry, and the lipoplexes distributed widely in the cytoplasm and around the nucleus by confocal laser microscopy. These findings may be one of the reasons why MEL-L enhanced the transfection efficiency of MEL-A. These findings indicate that cationic liposomes containing MEL-A have potential as an effective vector in gene therapy.

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