

Inhibition of Growth of Human Prostate Cancer Xenograft by Transfection of *p53* Gene: Gene Transfer by Electroporation¹

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Abstract

To date, there is no effective therapy for hormone-independent prostate cancer. Therefore, as a new strategy for refractory cancer, gene therapy is showing increasing promise. In this study, we attempted to use a nonviral gene transfer system, *in vivo* electroporation, in prostate cancer cell PC-3 xenografts with the wild-type *p53* (*wt-p53*) gene, as gene therapy for hormone-independent prostate cancer. To evaluate this *in vivo* gene transfer method, the β -galactosidase gene was transfected into xenografts by electroporation. Then, the efficiency of transfection of exogenous *p53* gene by electroporation was confirmed by reverse transcription-PCR, which indicated that *p53* mRNA was present in samples from xenografts. Next, to estimate the reduction of prostate cancer xenografts by this method, we measured the size of PC-3 xenografts in nude mice after electroporation with the *wt-p53* gene. The growth of tumors was markedly suppressed by *wt-p53* gene transfection by electroporation compared with transfection of mutated type *p53* gene ($P = 0.0027$) or vector only ($P = 0.0015$). Furthermore, histological specimens revealed increased apoptotic cell death in *p53*-transfected tumors. These results suggest that it is possible to transfer *wt-p53* into prostate cancer xenografts using electroporation and to suppress the growth of tumors; they, furthermore, suggest that this system might be used for local advanced hormone-independent prostate cancer.

Introduction

Prostate cancer is the most common malignant disease in men and the second most frequent cause of cancer death in the United States (1). Recently, in Japan, patients with prostate cancer have been increasing. To date, effective therapy for prostate cancer is possible only when the disease has been diagnosed early enough to be still localized within the

prostate gland. In contrast, the survival of patients with metastatic prostate cancer remains poor. Although endocrine treatment is initially highly effective and most cases experience at least partial remission for several years, treatment failure and tumor recurrence are almost inevitable several years later (2). Once prostate cancer becomes resistant to hormone therapy, there is no effective therapy at present. Some chemotherapeutic protocols have been tried; however, they are far from effective. As a new strategy for hormone-resistant prostate cancer, gene therapy is expected to be an effective therapeutic option.

The tumor suppressor *p53* gene is one of the most frequently mutated genes detected in a variety of human cancers (3). Normal function of *p53* includes the regulation of two critical cellular pathways: (a) the G_1 checkpoint in response to DNA damage; and (b) apoptosis induced by certain stimuli, such as radiation or chemotherapeutic agents (4, 5). Therefore, *p53* appears to play a key role in maintaining the integrity of the cellular genome, and on the other hand, the inactivation of *p53* function by mutations or other mechanisms occurs in the process of tumorigenesis.

The *p53* gene also appears to be involved in the pathogenesis of prostate cancer (6). *p53* mutations are associated with metastatic prostate cancer, especially hormone-refractory cancer (7, 8). It has been demonstrated that transfection of *wt-p53*³ into prostate cancer cell lines deficient in *wt-p53* can inhibit growth and suppress tumorigenicity (9).

Gene therapy using the *p53* gene has been proposed for cancers characterized by inactivation of *p53* function, and successful therapy will require efficient and safe gene delivery. To date, many investigations and clinical trials using transfer of viral genes encoding *wt-p53* have been performed (10–16). However, viral gene transfer at present has some problems in clinical use. For example, the strong antigenicity of adenovirus is a serious problem. Furthermore, the quality control of viral particles for *in vivo* administration is laborious and expensive (17).

On the other hand, as *in vivo* nonviral gene transfer systems, calcium phosphate, lipofection, particle bombardment, and electroporation have been developed. Electroporation of cell membranes has been developed for the purpose of achieving highly efficient *in vitro* gene and/or drug transfer. This method has been demonstrated to have higher efficiency of gene transfer compared with other nonviral transfer systems (18). This method was introduced into *in vivo* systems (18–23), and might be adapted to clinical treatment, as gene therapy.

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³ The abbreviations used are: *wt-p53*, wild-type *p53* (gene); *mt-p53*, mutated *p53* (gene); CMV, cytomegalovirus; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; TdT, terminal deoxynucleotidyltransferase; TUNEL, TdT-mediated nick end labeling.

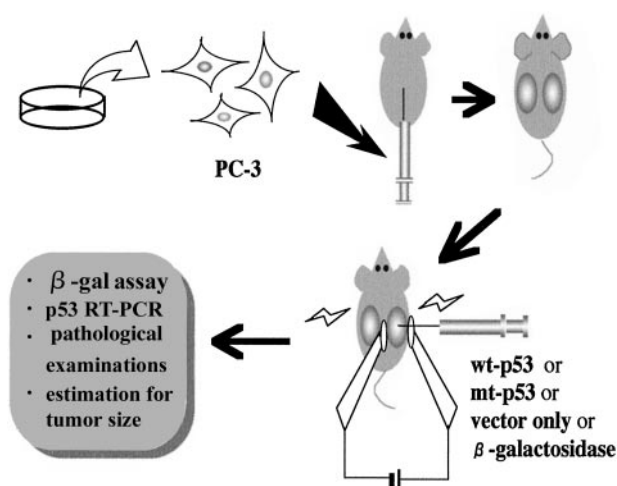


Fig. 1. Schema of electroporation for gene transfer. A pair of disc-shaped electrodes was used to nip the tumor nodule through the skin, and a series of eight electrical pulses with pulse length of 99 μ s was delivered with a standard square wave electroporator BTX T820 (BTX, Inc., San Diego, CA). The delivered voltage was 1.0 kV/1.0-cm diameter of each xenograft.

In the present study, we evaluated the antitumor activity of p53 gene therapy in human prostate cancer xenografts using this electroporation system. Our results demonstrated that *in vivo* electroporation had high efficiency of gene transfer and induced inhibition of tumor growth. These results suggest that gene therapy using an electroporation system could be applicable to clinical gene therapy for human prostate cancer.

Materials and Methods

Cell Lines and Establishment of Tumors in Nude Mice.

The human prostate cancer cell line PC-3 was cultured in F12 with 10% fetal bovine serum at 37°C in 3.5% CO₂. These cells were trypsinized, washed with F12, and suspended in saline solution at 1×10^7 cells/0.1 ml; a 0.1-ml cell suspension was injected into each nude mouse (BALB/c, male) s.c. in the flank area bilaterally. Formation of a tumor nodule 5 mm in diameter was observed at about 1 week after cell inoculation. Each group was given injections with pC53-SN3, pC53-SCX3 or pCMV-Neo-Bam vector after electroporation. The number of mice injected with pC53-SN3 or pCMV-NEO-BAM vector was 12. The number of mice injected with pC53-SCX3 was 15.

Electroporation and Plasmid Injection. A pair of disc-shaped electrodes was used to nip the tumor nodule through the skin, similar to using forceps, and a series of eight electrical pulses with pulse length of 99 μ s was delivered with a standard square wave electroporator BTX T820 (BTX, Inc., San Diego, CA; Fig. 1). To determine the optimal voltage of electroporation, we delivered various levels of voltage to the xenografts. Low voltages (range, 50–300 V) were not effective for gene transfection by electroporation, whereas high voltages (>1.2 kV) were fatal in nude mouse. The final voltage delivered was 1.0 kV/1.0 cm diameter of xenografts. Then, we delivered an appropriate pulse length and fre-

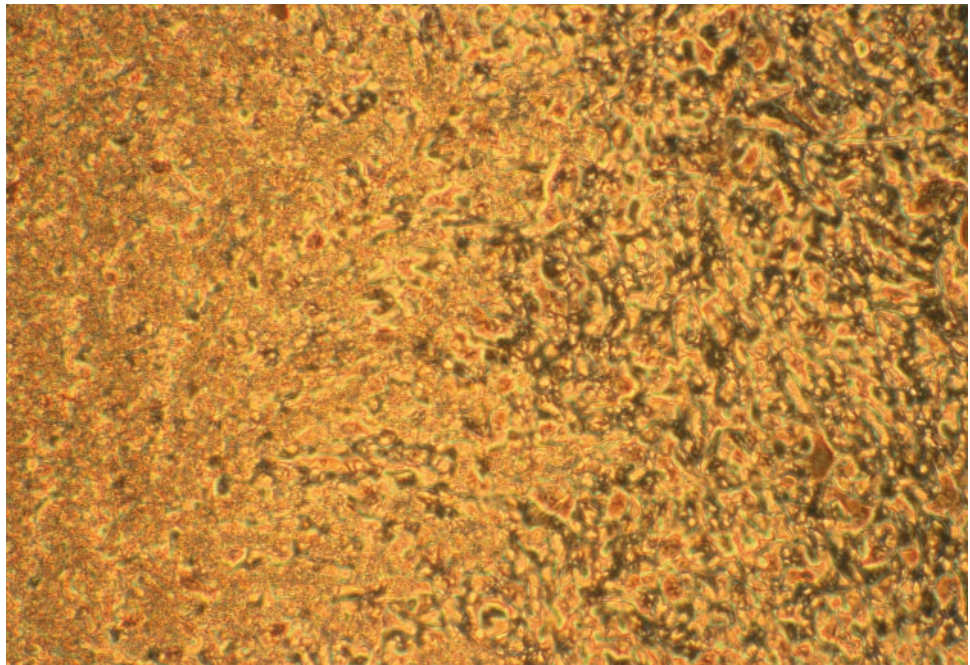
quency of pulses according to previous reports (20, 21). Immediately after electrical pulsing, 20 μ g of plasmid DNA, *wt-p53* or *mt-p53* dissolved in 50 μ l of Tris EDTA buffer [10 mM Tris-HCl (pH 8)-1 mM EDTA (pH 7.5)] was directly injected into the tumor nodule. Previous data indicated that uptake by electroporated cells through membrane pores is not affected by whether the molecules are present during, or are added after, the electroporating pulse (24, 25). Moreover, to avoid the problem of leakage of DNA plasmid from the needle holes, we performed electroporation before DNA plasmid injection. For *wt-p53* gene transfection, we used plasmid DNA of pC53-SN3 (26), which contains *wt-p53* in the sense orientation. This gene was inserted into the pCMV-Neo-Bam vector [pCMV-neo-Bam vector (6.6 kb); Ref. 26], which contains the CMV promoter. As a *mt-p53*, we used pC53-SCX3 (26). pC53-SN3 and pC53-SCX3 differed by a single nucleotide (Cysteine to Threonine), resulting in a substitution of alanine for valine at p53 codon 143 in pC53-SCX3 (26).

This electroporation and injection were performed a total of three times at 3-day intervals. Tumor volume was measured every 3 days from the time electroporation started until the animals were killed when they exhibited signs of distress. The tumor volume was determined by measuring the largest (L) and smallest (S) diameters of the tumor and calculated as $V = (L \times S^2)/2$. The growth curve of each treatment group was plotted as the mean relative tumor volume \pm SE. We continued to observe the tumor size over a period of 2 months. Because PC-3-inoculated mice became extremely exhausted or died, it was impossible to compare the tumor sizes statistically after 15 days from the first electroporation. We used dispersion analysis for statistical analysis with Fisher's Protected Least Significance Difference as a post-hoc test.

Detection of Transgene Expression *in Vivo*. To evaluate the efficiency of gene transfection by electroporation, PC-3 xenografts were subjected to electroporation three times with the β -galactosidase expression vector under the control of the human CMV gene promoter. On day 2 after electroporation, nude mice were killed. Tumors were embedded in O.C.T. compound (Miles Inc., Elkhart, IN), frozen, and sectioned on a cryostat. Frozen sections were fixed [2% (v/v) phosphate-buffered formalin, 2% (v/v) glutaraldehyde, and 2 mM MgCl₂, in PBS] at room temperature for 45 min. Sections were then placed in rinse solution [2 mM MgCl₂, 0.1% (w/v) sodium deoxycholate, and 0.2% (v/v) NP40, in PBS] and incubated overnight at 4°C. Each section was stained in X-gal solution for 6–8 h at room temperature and observed under a microscope.

Detection of p53 Gene Expression. The xenografts transfected with pC53-SN3, pC53-SCX3, or pCNB vector by electroporation were removed and immediately stored at -80°C . Total RNA of these xenografts was extracted using ISOGEN (Nippon Gene Co., Tokyo, Japan) with DNase, and then converted into cDNA (cDNA) by Moloney murine leukemia virus (MMLV) reverse transcriptase. Polymerase chain reamplification of *wt-p53*, *mt-p53*, and β -actin was performed under the following conditions: denaturing for 30 s at 95°C, annealing for 30 s at 55°C, and elongation for 30 s at 72°C.

Fig. 2. Efficiency of gene transfer by electroporation. The β -galactosidase expression vector under the control of the human CMV gene promoter was injected after electroporation, and the xenografts were stained in X-gal solution. Transgene expression was clearly evident in the center of xenografts, showing a dark cytosol, with 25–30% of tumor cells expressing β -galactosidase in the sections.



The sequences of oligonucleotides as forward or reverse primers of pC53-SN3 and β -actin were as follows: pC53-SN3: forward, 5'-TGACCTCCATAGAAGACACC-3', and reverse, 5'-AAGCGTGTCCACGTCGTGAAAGCTT-3'; β -actin: forward, 5'-TAATACGACTCACTATAGGGAGAGCGGAAA-TCGTGCGTGACATT-3', and reverse, 5'-GATGGAGTTGAA-GGTAGTTTCGTG-3'. The forward primer of pC53-SN3 corresponded to the sequence of a part of the pC53 vector, which was located downstream of the transcription initiation site. The reverse primer of pC53-SN3 was a part of *wt-p53*. PCR products were loaded on 2% agarose gel containing 0.5 mg/ml ethidium bromide.

Histology and TUNEL. The tumor tissue was fixed in 10% formalin and embedded in paraffin for histological examination. The paraffin sections were stained with H&E. To assess the incidence of apoptotic cell death, the sections were stained with an *in situ* cell death detection-Peroxidase kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's recommendations. Briefly, after deparaffinization, and dehydration and inactivation of intrinsic peroxidase activity, the sections were digested with 2 μ g/ml proteinase K at 37°C for 15 min. Then the sections were incubated with reaction buffer containing FITC-conjugated dUTP and TdT at 37°C for 1 h. The labeled DNAs were visualized by means of a signal converter followed by diaminobenzidine reaction. The sections were counterstained with methyl green.

Results

Detection of Transgene Expression in Xenografts. To evaluate the efficiency of gene transfer by electroporation, the β -galactosidase expression vector under the control of the human CMV promoter was injected after electroporation, and the xenografts were stained in X-gal solution. Transgene

expression was clearly evident in the center of xenografts, showing a dark cytosol, with 25–30% of tumor cells expressing β -galactosidase in the sections (Fig. 2). On the other hand, expression of β -galactosidase was not observed in xenografts injected with the *β -galactosidase gene* without electroporation, and tumors electroporated with empty plasmid showed no color staining of X-gal (data not shown). Then, to confirm *p53* transgene expression, we performed reverse transcription-PCR using total RNA extracted from xenografts. The mRNA of *p53* was detected in samples from xenografts treated with pC53-SN3. Especially, *p53* mRNA expression in extracts from xenografts 2 days after electroporation was detected more strongly than that after 7 days. Expression of the *p53* gene gradually declined after electroporation. A similar pattern of *p53* expression determined by PCR was shown in pC53-SCX3 transfection (data not shown). However, *p53* mRNA was not detected in samples from xenografts treated with pCMV-Neo-Bam vector by electroporation (Fig. 3).

Apoptosis Induced by *wt-p53*. To assess the incidence of apoptotic cell death, the sections were stained with H&E or an *in situ* cell death detection-POD kit. The histological appearance of the xenograft was similar in the control and experimental groups, but apoptotic cells were significantly increased in the latter (data not shown). To demonstrate apoptosis, we performed TUNEL staining, which clearly revealed increased apoptotic cells in pC53-SN3-transfected PC-3 xenografts (Fig. 4a, b, and c).

***In Vivo* Effect of *wt-p53* Transfection by Electroporation on Tumor Growth.** To investigate the effect of *wt-p53* on tumor growth, we set up three groups of BALB/c nude mice inoculated with human prostate cancer PC-3 cells. Each group was given injections of pC53-SN3, pC53-SCX3, or pCMV-Neo-Bam vector after electroporation. In each

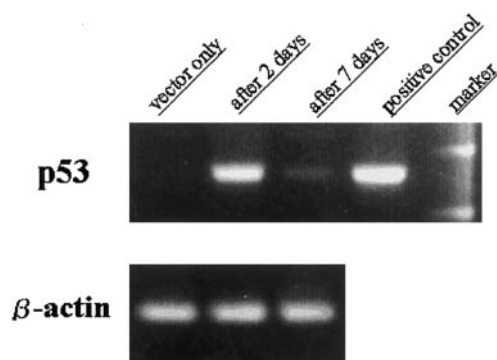


Fig. 3. Transgene expression of *p53* gene. Reverse transcription-PCR using total RNA extracted from xenografts. mRNA of *p53* was detected only in samples from xenografts treated with pC53-SN3. In addition, *p53* mRNA expression in the extract from xenografts was detected more clearly 2 days after electroporation than after 7 days.

treatment group, the tumor volume just before transfection ranged from 40 to 1666 mm³ (mean, 420.1 mm³). Fig. 5a shows the mean relative volume (\pm SE) for PC-3 xenografts treated with an injection of pC53-SN3 or pCMV-Neo-Bam vector with *in vivo* electroporation. Fig. 5b shows the mean relative volume (\pm SE) for PC-3 xenografts treated with an injection of pC53-SN3 or pC53-SCX3 with *in vivo* electroporation. Significant differences in the mean relative volume (\pm SE) between the two groups (Fig. 5, a and b) were observed from 6 days after the first electroporation. The ratio of tumor volume at 15 days to that on the 1st day in the pC53-SN3 group ranged from 1.07 to 4.01 (mean, 3.02), and the ratio in the pCMV-Neo-Bam-vector group ranged from 1.30 to 6.08 (mean, 4.13; $P = 0.0012$; Fig. 5a). The ratio of tumor volume at 15 days to that on the first day in the pC53-SN3 group ranged from 1.06 to 6.72 (mean, 3.28), and the ratio in the pC53-SN3 group ranged from 1.65 to 18.75 (mean, 5.87; $P = 0.0027$; Fig. 5b). Accordingly, significant differences in the mean relative volume were shown between the *wt-p53*-transfected group and the control vector-transfected group or *mt-p53*-transfected group.

Discussion

In the present study, we attempted to use the *wt-p53* gene for transfection into prostate cancer PC-3 xenograft, which is androgen-independent and has deletion of *p53* protein with no function. We evaluated the antitumor growth activity of *p53* gene transfer on the growth of PC-3 xenografts.

For *in vivo* gene transfection, many methods and techniques have been developed, and some of them have already been applied in clinical trials. In most cases, genes are transferred into tissues through the infectivity of viral particles. The retroviral system can achieve highly efficient integration, providing the potential for permanent gene expression. However, the system has some major disadvantages, such as typically a low titer, instability of the viral vector obtained, and the requirement for target cell division for integration and expression (27). Although the adenovirus system can provide more efficient gene transfer and stability

of the virus, difficulties in the control of target cells and relating to readministration necessitated by the strong antigenicity of the virus are serious problems (28). Furthermore, there are some common drawbacks of viral gene transfer systems. Particularly, the quality control of viral particles for *in vivo* administration is laborious and expensive (17).

If an efficient and specific transfer method could be developed, the use of naked plasmid DNA might be an ideal system for gene transfer. A plasmid-mediated method would be more available and easy to handle, because the use of this system obviates the necessity to construct viral vectors, establish clones of producer cells, and assess viral titers and the presence of replication-competent helper virus, which is known to activate passive oncogenes. Also, the transfer procedure could be easily repeated because naked plasmid DNA has little antigenicity in the host body (29, 30).

Electroporation of cell membranes has been developed for the purpose of achieving highly efficient *in vitro* gene and/or drug transfer (31, 32). This system provides extremely high efficiency transfer compared with other nonviral transfer systems, including cationic liposomes and gene particle bombardment. Previously, although the application of electroporation to cultured cells has been well established, the use of *in vivo* electroporation has received little attention (18–20). However, in recent reports, an *in vivo* electroporation system was used to study the effect of cytokine gene transfection on the growth of experimental murine tumors (33, 34).

In the present study, we attempted to use electroporation with naked plasmid DNA, which is an effective gene transfer method *in vivo*. Furthermore, a *wt-p53* tumor suppressor gene was used as a transfection gene, and we evaluated its antitumor activity in human prostate cancer PC-3 xenografts that contained *mt-p53* alleles (9). From the results of our experiments, the efficiency of electroporation was thought to be about 25–30% of cells, determined by β -galactosidase expression as shown in Fig. 3. Also, it was demonstrated that the transfection of *wt-p53* by electroporation could induce apoptotic cell death as shown in Fig. 5, and inhibit the growth of prostate cancer xenografts. In this study, we performed *p53* gene injection and electroporation three times, and, finally, tumor growth was suppressed. With a greater number of applications, regression of tumors might become more obvious.

This report suggests that clinical application using this electroporation system for prostate cancer may be possible in the future. For example, we might be able to perform transurethral electroporation for local recurrence of prostate cancer, and also apply electroporation to the prostate or metastatic lymph nodes through laparoscopic surgery.

On the other hand, some disadvantages of this method should be considered. In our study, although transfection by electroporation inhibited the growth of PC-3 xenografts, the target area was limited to local tumors. Therefore, the growth of multiple bone metastatic lesions cannot be the target for efficient suppression. Secondly, transgene expression in the peripheral part of the xenografts was lower than that in the central region. Therefore, this procedure should be repeated several times. If the *wt-p53* gene could be transfected to the peripheral part of xenografts, the suppression might be

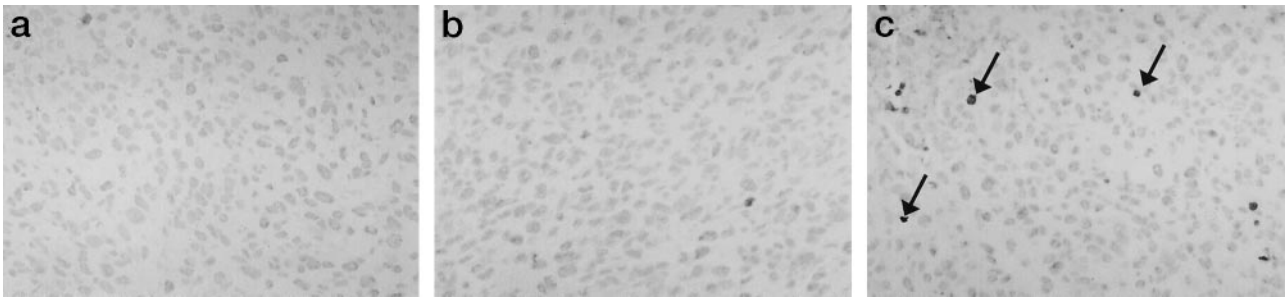
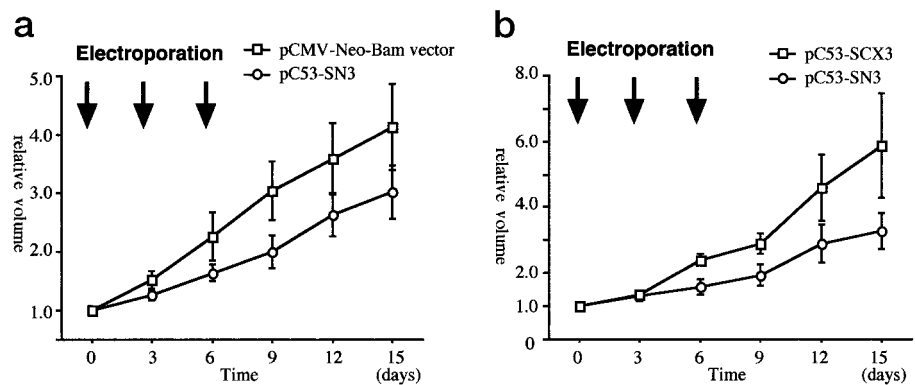


Fig. 4. TUNEL staining of PC3 xenotransplants. *a*, untreated tumor; *b*, empty vector-transfected tumor; *c*, p53-expression vector-transfected tumor. In the p53-transfected tumor (*c*), apoptotic cell death was markedly increased (arrows) compared with control tumors (*a* and *b*). TUNEL, $\times 66$.

Fig. 5. Ratio of tumor growth with pC53-SN3 versus pCMV-NEO-BAM vector (*a*) or pC53-SCX3 (*b*). Electroporation and injection of genes into PC-3 xenografts were performed a total of three times at 3-day intervals. Tumor size was measured every 3 days, until 15 days from the time of first electroporation. In *a*, there was a statistically significant difference in the growth curve between pC53-SN3-transfected tumors and pCMV-NEO-BAM vector-transfected tumors ($P = 0.0015$). In *b*, there was a statistically significant difference in the growth curve between pC53-SN3-transfected tumors and pC53-SCX3-transfected tumors ($P = 0.0027$).



greater than the present result. We have not arrived at a conclusion as to how many applications of electroporation are effective for the reduction of xenografts. At 3 days after the first electroporation, the volume of xenografts was no different between the *wt-p53* group and *mt-p53* group (Fig. 5). However, at 6 days after the first electroporation, a significant difference between them became apparent. Finally, the growth of xenografts treated with the *wt-p53* gene was retarded; nevertheless, the xenografts were not reduced in size after 3 weeks of electroporation. If we applied this electroporation many more times, the xenografts might be reduced in size.

With a view to obtaining more effective gene therapy using electroporation for prostate cancer, we plan to attempt gene transfer with several other genes, for instance, the apoptosis induction gene or herpes simplex virus-thymidine kinase, the so-called suicide gene. We also plan to use various anticancer agents in combination with gene transfection by this electroporation.

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