Pharmaceutical Research, Vol. 22, No. 6, June 2005 (© 2005)
DOI: 10.1007/s11095-005-4583-2

Research Paper

Tissue-Specific Characteristics of *in Vivo* Electric Gene: Transfer by Tissue and Intravenous Injection of Plasmid DNA

Oranuch Thanaketpaisarn,[1] Makiya Nishikawa,[2,3] Fumiyoshi Yamashita,[1] and Mitsuru Hashida[1]

Received January 14, 2005; accepted March 9, 2005

Purpose. To evaluate the tissue-specific characteristics of electric gene transfer after tissue and intravenous injection of naked plasmid DNA (pDNA).
Methods. pDNA encoding firefly luciferase was injected directly into the liver, kidney, spleen, skin and muscle, or into the tail vein of mice, and electric pulses were then applied to one of these organs. The distribution of transgene expressing cells was evaluated using pDNA encoding β-galactosidase.
Results. Tissue injection of pDNA produced a significant degree of transgene expression in any tissue with the greatest amount in the liver, followed by kidney and spleen. The expression in these organs decreased quickly with time, and muscle showed the greatest expression at 7 days. Electroporation significantly increased the expression, and the expression level was comparable among the organs. Intravenous injection of pDNA followed by electroporation resulted in a significant expression in the liver, spleen, and kidney but not in the skin or muscle.
Conclusions. Electric gene transfer to the liver, kidney, and spleen can be an effective approach to obtain significant amounts of transgene expression by either tissue or intravenous injection of pDNA, whereas it is only effective after tissue injection as far as skin- or muscle-targeted gene transfer is concerned.

KEY WORDS: electroporation; gene transfer; intravascular injection; plasmid DNA; tissue distribution.

INTRODUCTION

Of the nonviral gene transfer methods developed thus far, an injection of plasmid DNA (pDNA) is the simplest. Significant amounts of transgene products are produced after the injection of pDNA into tissues such as skeletal muscle (1), skin (2), and liver (3). Intramuscular injection of pDNA encoding hepatocyte growth factor or vascular endothelial growth factor is reported to be therapeutically effective in treatments for peripheral arterial disease in animal models as well as in clinical trials (4,5). However, a simple injection of pDNA into tissues generally gives less amount of transgene expression than that required for therapeutic benefit. A reliable approach developed so far to increase the expression by tissue-injected pDNA is the application of controlled electric pulses to the injection site: *in vivo* electroporation. It is able to facilitate both interstitial and intracellular transport of pDNA by the formation of transient pores on cell membranes and by electrophoresis (6). There have been several reports on successful applications of electroporation for pDNA-based gene transfer in tissues including tumor (7–10). Although the application of electric pulses appears to increase the area of transfected cells (11,12), the distribution of these cells is still limited due to the large molecular size of pDNA.

Another pDNA-based gene transfer has been attempted by its intravenous injection. Because of digestion by nucleases and extensive clearance, a simple injection of pDNA into blood circulation did not lead to significant expression (13). On the other hand, a large volume injection of pDNA with high velocity, first described by Liu *et al*. (14) and Zhang *et al.* (15), has become an important experimental tool to produce very high transgene expression in the liver. This pressure-based gene transfer has also been applied locally, and significant amounts of transgene expression were obtained in skeletal muscle (16,17). As demonstrated in a previous paper (18), the administration of pDNA into blood circulation is theoretically superior to its local injection as far as the number of transfected cells is concerned. However, the harshness of the administration procedure has halted its clinical application. Again, electroporation can be used as a driving force for pDNA in the circulation to get into the inside of target cells. We and others have shown that the application of electric pulses to the surface of liver can induce significant transgene expression after intravenous injection of pDNA (19,20). However, the applicability of this approach to tissues other than the liver has not been examined so far.

When a protein encoded in pDNA exhibits its activity after being secreted into the blood circulation, such as blood

[1] Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.
[2] Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.
[3] To whom correspondence should be addressed. (e-mail: makiya@pharm.kyoto-u.ac.jp)

coagulation factors, the level and persistence of transgene expression are very important, although the type of cells as source of production would be much less significant (21). In the case of gene therapy approaches for hemophiliacs, not only hepatocytes (22) that produce the coagulation factors in healthy subjects, but also other cells, such as fibroblasts (23) and muscle cells (24), have been investigated as target cells producing these factors. The properties of cells and tissues, such as the ease of transfection, the ability to synthesize proteins, location, life span of the cells, and tissue blood flow, are important factors determining the efficacy of gene therapy. Because of these different properties of tissues, it is necessary to investigate the differences in transgene expression in a variety of tissues in order to choose the most appropriate target for a specific disease.

In the present study, therefore, we examined the level of transgene expression in five different tissues, that is, liver, kidney, spleen, dorsal skin, and skeletal muscle, after tissue or intravenous injection of pDNA. The effect of electroporation was examined following both modes of administration. Here, we demonstrate that the level of transgene expression was dependent on the tissue and route of pDNA administration. The application of electric pulses to the injection site increases the expression level to almost an identical value, irrespective of the tissue examined. In contrast, intravenous injection of pDNA followed by electroporation was found to be a promising approach only for gene transfer to the liver, kidney and spleen.

MATERIALS AND METHODS

Animals

ICR mice (female, 18–20 g) were purchased from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan) and were maintained on a standard diet and water under conventional housing conditions. All animal experiments were carried out in accordance with the guidelines for Animal Experiments of Kyoto University.

Plasmid DNA

pDNA encoding firefly luciferase cDNA under the control of CMV-IE promoter was constructed by subcloning the *Hind*III/*Xba*I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) as previously reported (11). pCMV.SPORT-β-gal containing a CMV promoter upstream of the β-gal gene, followed by the SV40 t-intron and polyadenylation signal were purchased from GibcoBRL (GibcoBRL, Carlsbad, CA, USA). Both pDNAs were amplified in the *E. coli* strain DH5∝, then isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN, Hilden, Germany). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide straining and the DNA concentration was measured by UV absorption at 260 nm. For biodistribution experiments, pDNA was labeled with [∝-^{32}P]dCTP (Amersham, Tokyo, Japan) by nick translation (25).

In Vivo Gene Expression Experiments

Four-week-old ICR female mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Then, a pDNA solution in saline or dextrose water (20 μg/20 μl) was injected directly into an injection site (1 injection/mouse). For injection into the liver, spleen or left kidney, a midline incision was made on the abdomen. Then, pDNA was injected and the incision was closed with metal clips. pDNA was injected into the gastrocnemius muscle of right hind leg or shaved dorsal skin. Square-wave electric pulses were applied with forceps-type electrodes to the injection site of the pDNA. In the case of intravenous injection, pDNA solution in saline (25 μg/200 μl) was injected into the tail vein following electroporation in the same manner as the cases of tissue injection. At optimal time points after injection, mice were killed and the tissue or organ receiving electroporation was excised, homogenized in a lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8), and subjected to three cycles of freezing in liquid nitrogen (−190°C) and thawing (37°C). Then, the homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C and 10 μl of the supernatant was mixed with 100 μl of luciferase assay buffer and the chemiluminescence produced was measured in a luminometer. The luciferase activity was expressed as relative light units (RLU) per tissue (RLU/tissue). The average weight (g) and protein content (mg protein/g tissue) of the tissues sampled were listed in Table I.

Electrodes and Electric Pulse Delivery

Electric pulses were delivered to tissue using a pair of 1-cm^2 forceps-type electrodes connected to a rectangular direct current generator (CUY-21, Nepagene, Chiba, Japan). The parameters of the electric pulses were: 5 ms/pulse, 12 pulses, 4 Hz, and a variable electric field from 50 to 1,000 V/cm. Electric pulses were delivered to target tissue using the electrodes 30 sec after tissue or intravascular injection of plasmid DNA.

Biodistribution Experiments After Intravenous Administration of ^{32}P-pDNA

^{32}P-pDNA was added with unlabeled pDNA to adjust the injection dose to 25 μg pDNA/mouse. Each mouse was injected with ^{32}P-pDNA in saline. A set of electric pulses (500 V/cm for liver, kidney and muscle or 1,000 V/cm for

Table I. Average Weight and Protein Content of the Tissues Sampled

Tissue	Weight (g)	Protein Content (mg protein/g tissue)
Liver	0.99 ± 0.13 (100)	325.0 ± 43.9 (100)
Kidney	0.12 ± 0.01 (12)	93.0 ± 16.6 (29)
Spleen	0.09 ± 0.02 (9)	95.4 ± 12.1 (29)
Skin	0.15 ± 0.03 (15)	51.2 ± 8.4 (16)
Muscle	0.11 ± 0.02 (11)	58.4 ± 8.0 (18)

Values are expressed as the mean ± SD of tissues collected in this study (n = 50 to 60).
The values in parentheses represent relative values to the liver.

spleen and skin, 5 ms/pulse, 12 pulses, 4 Hz) was applied to target tissues 30 sec after systemic injection. At 1, 3, 5, 10, and 30 min after injection, groups of 3–4 mice each were anesthetized with ether, blood was collected from the vena cava to obtain plasma by centrifugation. We examined the pDNA disposition only up to 30 min after injection since the disposition of pDNA after longer periods would not represent that of intact pDNA due to its rapid degradation (26). The liver, kidney, spleen, skin and muscle were excised, rinsed with saline and weighed. These organs were homogenized with 0.05% Triton X-100 solution and each sample was dissolved in soluene-350 (Packard, Netherlands), then Scintillation medium (Clear-sol I, Nacalai Tesque, Kyoto, Japan) was added and then ^{32}P-radioactivity was measured in an LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan). Radioactivity derived from plasma in each tissue was corrected for as previously reported (27).

Characterization of LacZ pDNA Expression After Tissue or Intravenous Injection Followed by Electroporation

LacZ pDNA was administered by either tissue or intravenous injection as described above. After pDNA injection, at 24 h for liver, kidney, spleen and skin and at 7 days for muscle, these whole organs were collected and then stained using an X-gal histochemistry method to evaluate β-galactosidase activity.

X-gal Histochemistry

X-gal 5-bromo-4 chloro-3-indolyl-β-D-galactoside is a chromogenic substrate for the β-galactosidase gene product expressed in the transgenic hepatocytes used. X-gal turns blue on exposure to β-galactosidase present in those cells. X-gal histochemistry was performed on the whole liver: fresh samples were placed in organ fixing solution (4% paraformaldehyde), 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% IGEPAL C-630 (Sigma) for 60 min at 4°C, rinsed three times for 30 min with wash buffer [0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% IGEPAL C-630], then incubated in X-gal staining solution [1 mg/ml X-gal (Sigma), 5 mM ferricyanide, and 5 mM ferrocyanide at pH 7.3–7.6 in wash buffer] for 16–24 h at 37°C, and fixed for 24–48 h in 10% formalin (28).

Statistical Analysis

Experimental data were analyzed by Student's *t* test (see Fig. 3) or one-way ANOVA followed by the LSD multiple comparison test (see Fig. 2) using SPSS software. *p* values for significance were set at 0.05.

RESULTS

Increased Transgene Expression by Electroporation After Tissue Injection of pDNA

Naked pDNA was injected into one of the mouse tissues under the same condition: 20 μg pDNA in 20 μl saline solution was injected using an insulin syringe with a 29G-needle. Figure 1A shows the transgene expression in the tissues at 6 h after injection of pDNA. The liver gave the greatest expression at this earliest time point, followed by the kidney and spleen. The expression in skin and muscle was about 160- and 270-fold less than that in the liver. At 7 days, however, muscle showed the greatest transgene expression among the tissues examined, which was followed by skin, the liver, spleen and kidney (Fig. 1B). Figure 1C shows the time-courses of transgene expression in these organs. The expression in the liver, spleen and kidney reached a peak at 6 h, then decreased with time at a similar rate with an apparent half-life of 16 h. On the other hand, the expression in skeletal muscle was relatively low at 6 h but continuously increased with time and leveled off after 3 days. The expression in the

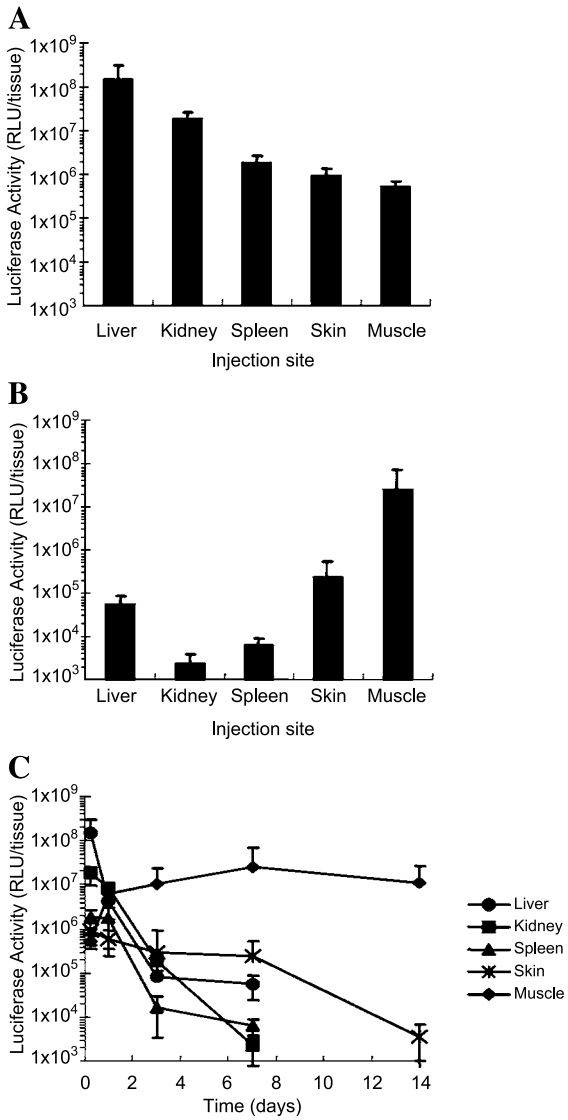

Fig. 1. Transgene expression after tissue injection of pDNA. ICR mice were injected directly with naked pDNA (20 μg/20 μl) into the liver, kidney, spleen, dorsal skin, or gastrocnemius muscle (1 injection/mouse). The total luciferase activity per tissue receiving an injection of pDNA is expressed in RLU (relative light units)/tissue as mean ± SD of four mice: (A) 6 h, (B) 7 days, and (C) time course after injection.

skin declined with time at a slower rate than that of the internal organs with an apparent half life of 44 h.

Twelve electric pulses of 5 ms-length, 4 Hz, with varying electric fields were applied to the injection site of pDNA 30 sec after injection using forceps-type electrodes. Figure 2A–E shows the transgene expression in the tissues examined. The electric field was changed from 50 to 1,000 V/cm. The expression was only evaluated at the time points when the expression in each tissue was at its peak: 6 h for the liver, kidney, spleen and skin, and 7 days for muscle. The application of electric pulses increased the expression in most cases, but the response to the pulses depended on the type of tissue. The expression in the liver was increased by the electric pulses of any given electric field by about 17-fold, with the greatest value of 2.15×10^9 RLU/liver being obtained at 500 V/cm (Fig. 2A). A similar tendency was observed in the case of the kidney (Fig. 2B), but the greatest enhancement at an electric field of 500 V/cm was only seven-fold. The expression in muscle was also the greatest at 500 V/cm, and up to a 119-fold increase with a value of 6.06×10^9 was obtained (Fig. 2E).

On the other hand, the expression in the spleen and skin simply increased as the electric field increased and the greatest expression was obtained at 1,000 V/cm (Fig. 2C and D). In skin, the increase in the expression was about 144-fold. Among the tissues examined, the spleen exhibited the greatest enhancement (997-fold) in the expression with the greatest value of 6.16×10^9 RLU. These results show that the transgene expression in any tissue can be significantly improved by the application of electric pulses with parameters that need to be optimized depending on the target tissue.

Increased Transgene Expression by Electroporation After Intravenous Injection of pDNA

A normal intravenous injection of pDNA resulted in no significant transgene expression in any tissue examined (Fig. 3). To obtain detectable transgene expression, electric pulses that were optimized as above were applied to each of the tissues at 30 s after intravenous injection of pDNA. Electroporation significantly increased the expression in all

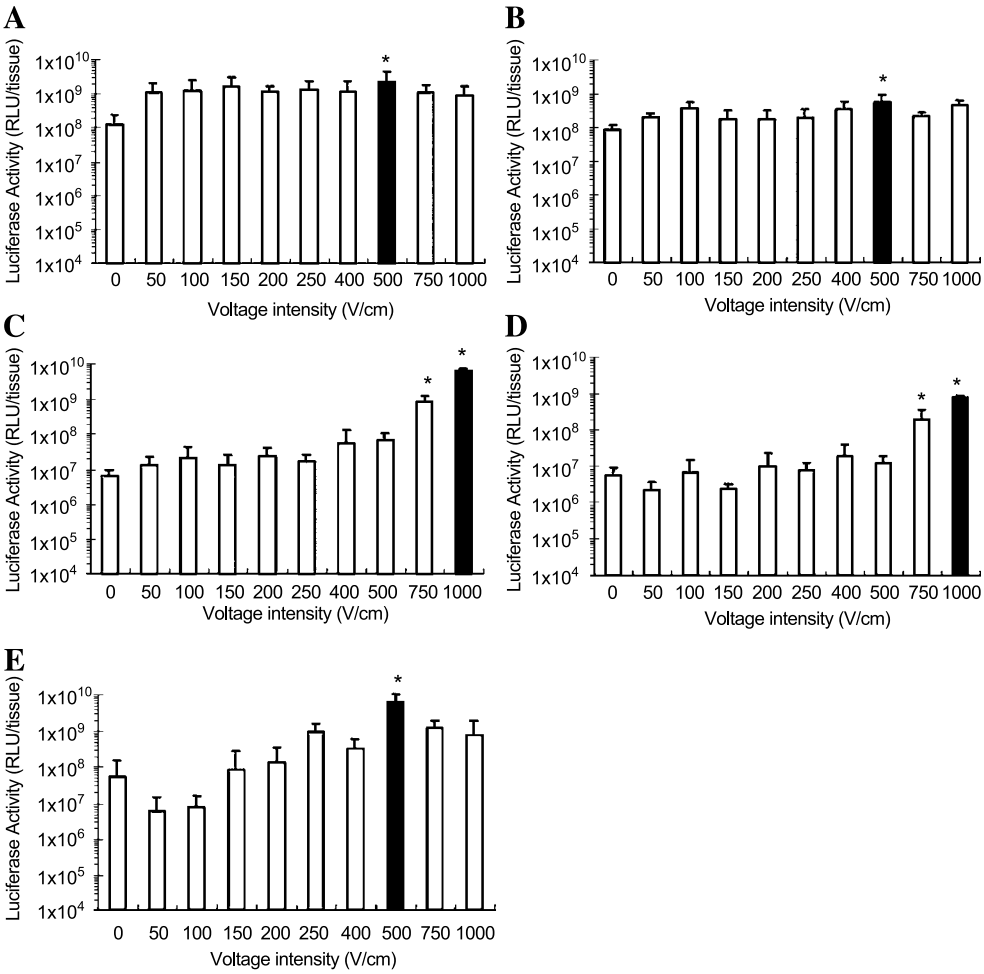

Fig. 2. Effect of the electric field strength on trangene expression after tissue injection of pDNA followed by electroporation the tissue: (A) liver, (B) kidney, (C) spleen, (D) dorsal skin, and (E) gastrocnemius muscle. ICR mice were injected directly with pDNA (20 μg/20 μl) into each tissue and received electric pulses: 12 pulse, 4 Hz, 5 ms/pulse, and variable electric field from 50 to 1,000 V/cm. After administration, mice were killed and the luciferase activity was measured at 7 days for gastrocnemius muscle and at 6 h for other organs. The results are expressed in RLU/tissue as mean ± SD of four mice. *A statistically significant difference against the control group ($p < 0.05$).

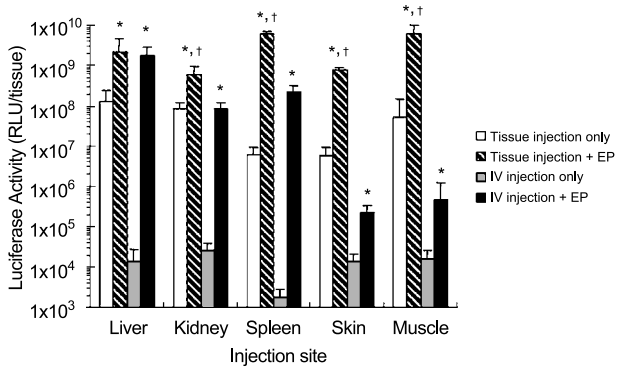

Fig. 3. Transgenic expression after intravascular (IV) injection of pDNA with or without subsequent electroporation (EP). ICR mice were injected with pDNA solution into the tail vein (25 μg/200 μl). Electric pulses were applied at 30 s after pDNA injection under the optimal conditions: 500 V/cm for liver, kidney and gastrocnemius muscle and 1,000 V/cm for spleen and dorsal skin, 12 pulse, 4 Hz, 5 ms/pulse. After administration, the luciferase activity was determined at 7 days for gastrocnemius muscle and at 6 h for other organs. The results are expressed in RLU/tissue as mean ± SD of four mice. *A statistically significant difference ($p < 0.05$) against the control (without electroporation) group; †a statistically significant difference ($p < 0.05$) against the tissue injection group. The expression data after tissue injection (Figs. 1 and 2) are also shown for comparison.

tissues receiving electric pulses. However, no significant expression was observed in tissues that did not receive electric pulses. The enhancement ratios of transgene expression by electroporation were 130,000, 125,000 and 3,300 for the liver, spleen and kidney, respectively. The liver showed the greatest level of expression among the tissues, and this was almost identical to that achieved by the tissue injection of pDNA followed by electroporation. The expression in the spleen and kidney was also efficient, but that in the skin and muscle was significantly lower than that obtained by the tissue injection followed by electroporation. These findings indicate that intravenous injection of pDNA followed by electroporation is a suitable approach for tissue-selective gene transfer to the liver, kidney and spleen, but it is much less effective in achieving skin- and muscle-targeted gene transfer.

Effect of Electroporation on the Tissue Distribution of pDNA After Intravascular Injection of pDNA

Figure 4 shows the time courses of the concentration in the plasma (upper panel) and the amounts of radioactivity in tissues (lower panel) in the electroporated tissues, that is, the liver (A), kidney (B), spleen (C), dorsal skin (D), and muscle (E). A set of electric pulses (500 V/cm for liver, kidney and muscle or 1,000 V/cm for spleen and skin, 5 ms/pulse, 12 pulses, 4 Hz) was applied to each tissue 30 s after intravascular injection of ^{32}P-pDNA. As reported previously (22,23), ^{32}P-radioactivity derived from ^{32}P-pDNA quickly disappeared from the blood circulation and about 40% of the injected dose accumulated in the liver (Fig. 4A). The delivery of ^{32}P-pDNA to the tissues receiving electric pulses was increased by electroporation. However, the differences in the distribution were not so significant and did not explain the great increase in transgene expression in these organs receiving electroporation.

Distribution of Transgene Expressing Cells

X-gal histochemistry staining was used to assess the gross three-dimensional pattern of LacZ expression in whole organs at 24 h for the liver, kidney, spleen and skin and at 7 days for muscle after tissue or intravenous injection of pDNA encoding LacZ (Fig. 5). The patterns of β-galactosidase activity corresponded to the quantitative results of the firefly luciferase activity described above. After direct tissue injection, transgene products were limited to the area around the injection site. Electroporation expanded the area, but the transgene expression was still limited. In addition, electric gene transfer clearly showed its potential as a driving force for pDNA in the blood circulation to get into the inside of the target cells, not only in the liver but also in the spleen and kidney. The patterns of β-galactosidase activity in the liver indicate the possibility of obtaining an increased number of transfected cells when pDNA was injected into the blood circulation compared with that obtained after its tissue injection. However, this approach was less effective for skin and muscle-targeted gene transfer.

DISCUSSION

In vivo gene transfer appears to be a promising technology to treat various diseases including monogenic diseases such as Duchenne muscular dystrophy, hemophilia, and OTC deficiency, as well as cancers and viral infection. Except for lysosomal enzymes, intracellular proteins such as dystrophin can hardly be supplemented in the protein form, but they can be easily delivered to the site of action by using the gene-based approach. The supplementation of intracellular proteins by means of gene delivery, therefore, requires tissue-specificity as far as the delivery and expression of the target gene is concerned. In a marked contrast, extracellular or plasma proteins, including blood coagulation factors, cytokines, and growth factors, do not need to be delivered to the target tissue to exhibit their pharmacological activity. The target tissue for gene transfer should be optimized to obtain the best result as far as therapeutic efficacy is concerned. The parameters that need to be considered are: the efficacy of gene delivery, transfection efficiency, and duration of transgene expression. In the present study, therefore, we examined the tissue-specific characteristics of gene transfer after tissue and intravenous injection of pDNA.

After tissue injection, pDNA can be taken up by cells adjacent to the injection site, which results in a substantial level of transgene expression. Among the tissues examined, we clearly demonstrated that the liver, kidney and spleen are good sites for the protein production when immediate expression is required. On the other hand, skeletal muscle is an attractive tissue for prolonged expression of therapeutic proteins. One explanation of this long-term stability and transcriptional activity in muscle is the terminally differentiated and long-living cells of myofibers including their postmitotic nature. However, this gene transfer approach involving simple pDNA injection has a relatively low efficiency of expression due to the limited distribution of plasmid DNA within the interstitial space of tissues only around the injection site (21). As first reported by Wolff *et al.* (29), *in vivo* electroporation can be used to improve the transfection efficiency of pDNA delivered

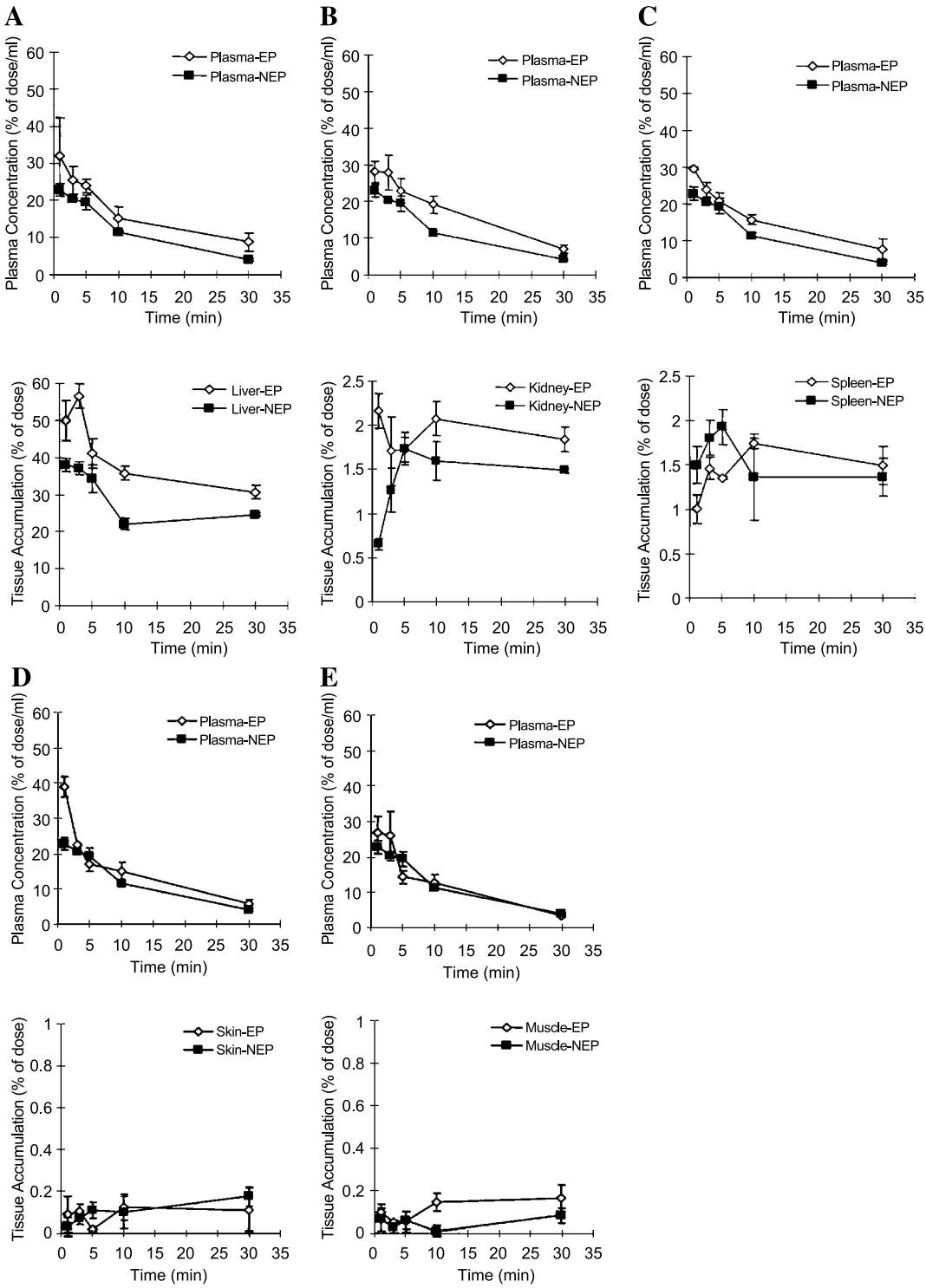

Fig. 4. Plasma concentration (upper panel) and tissue accumulation (lower panel) of ^{32}P-radioactivity after injection of naked ^{32}P-pDNA with electroporation (EP) or without electroporation (NEP). Naked ^{32}P-pDNA was injected into the tail vein at a dose of 25 μg/mouse, and electric pulses (500 V/cm for liver (A), kidney (B) and gastrocnemius muscle (E); 1,000 V/cm for spleen (C) and dorsal skin (D); 5 ms/pulse; 12 pulses; 4 Hz) were applied to the injection tissue at 30 s after injection. The results are expressed in % of dose/ml for plasma concentration or % of dose for tissue accumulation as mean ± SD of four mice.

by tissue injection. The application of an electric field can induce a transient structural reorganization and produce reversible permeability of the cell membrane, allowing direct entry of pDNA into cytoplasm via pores (6,21).

As listed in Table I, the tissues examined are largely diverse in the weight and protein content. Therefore, if the expression were expressed as the amount per tissue weight (RLU/g tissue) or protein content (RLU/mg protein), the

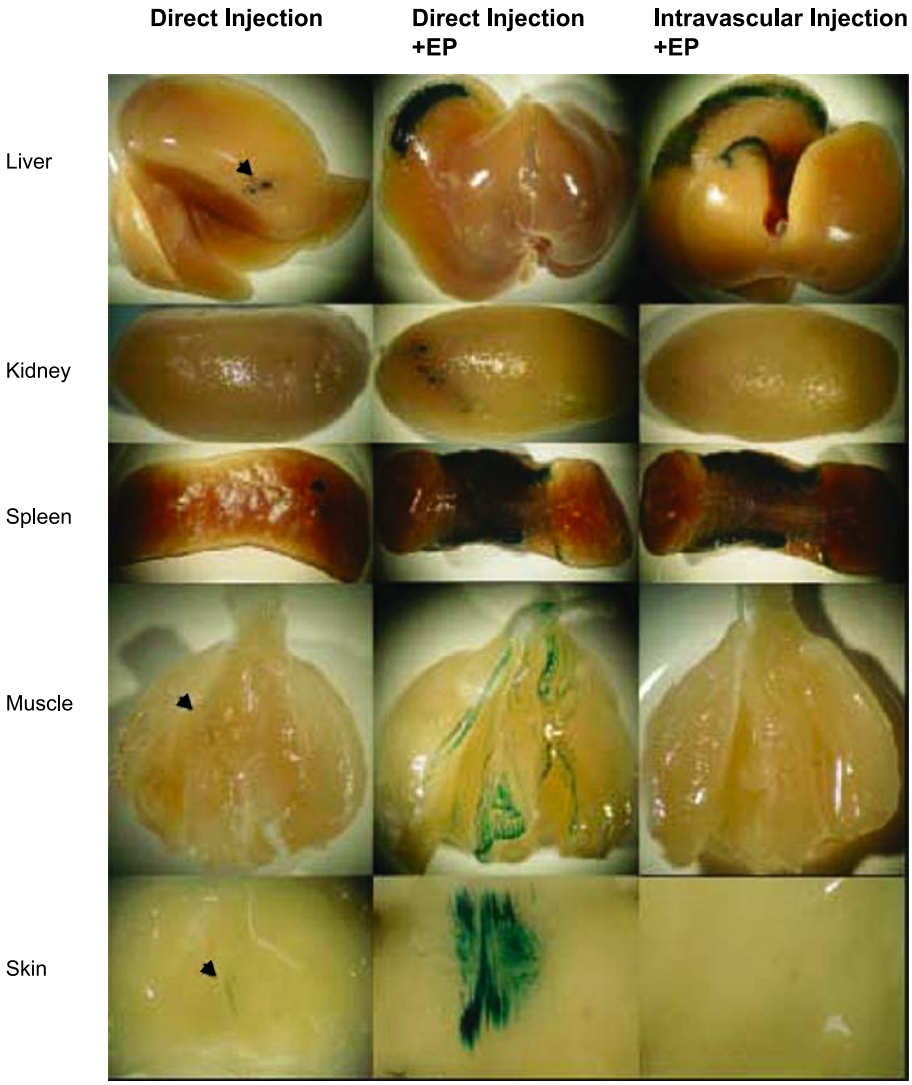

Fig. 5. The gross three-dimensional patterns of lacZ expression in whole organs after tissue or intravascular injection of lacZ DNA with electroporation (EP) or without electroporation. ICR mice were injected locally with pDNA solution (20 μg/20 μl) into target tissue or intravenously into the tail vein (25 μg/200 μl). Electric pulses were applied 30 s after pDNA injection at an optimum voltage (500 V/cm for liver, kidney and gastrocnemius muscle; 1,000 V/cm for spleen and dorsal skin) under the following conditions: 12 pulse, 4 Hz, 5 ms/pulse. After administration, X gal histochemistry staining was performed at 7 days for gastrocnemius muscle and at 24 h for other organs.

rank order of the tissues is different from that shown in Fig. 1; the kidney showed the greatest expression, followed by the liver. Although these parameters could be also important in considering the differences in transgene expression among tissues, the total amount of the expression should be used for the comparison, because it would directly correlate with the concentration of the transgene product in blood when secreted.

In the current study, the application of electric pulses to the injection sites greatly increased the amounts of transgene product in all tissues examined. However, at any electric field examined, the response to the pulses was dependent on the type of tissue, which is due to differences in the characteristics of cells and the relative conductivity of the extracellular matrix and interstitial fluid within each tissue (6,30). The greatest transgene expression was obtained at 500 V/cm for the liver, kidney and muscle, whereas the expression in the spleen and skin simply increased as the electric field increased and exhibited the greatest values at 1,000 V/cm. When compared using the peak levels of expression, electroporation gave the greatest expression in the spleen, followed by muscle, liver, skin and kidney in this order. In addition, the spleen showed the greatest enhancement by electroporation up to 998-fold with a value of 6.16×10^9 RLU. A recent study of electric gene transfer to the spleen indicated that transgene-expressing cells were found mostly in the white pulp of the spleen where the lymphocytes are located (31). Therefore, electric gene transfer can be an effective approach for spleen-targeted DNA vaccination to cancers and infectious diseases.

The patterns of β-galactosidase activity in the tissues confirmed that the application of electric pulses can signifi-

cantly increase the area of transfected cells after the injection of pDNA into the interstitial space of tissues. Therefore, electro-gene transfer can improve not only pDNA delivery to nucleus but also the distribution of transgene expressing cells.

As mentioned above, direct tissue injection of pDNA has the drawback of limited distribution of transfected cells (32). To overcome this limitation, intravascular administration of pDNA has been used to deliver pDNA to a greater number of target cells through the capillaries. Hydrodynamic pressure or electroporation was used as a driving force to deliver pDNA into cells, because pDNA itself lacks any ability to get into the cytoplasm in an active form for transgene expression. In an actual fact, no significant transgene expression was detected in any tissue examined after intravenous injection of naked pDNA. The application of electric pulses to the tissues significantly increased the amount of the transgene product in any case examined after systemic injection of pDNA. The assumption that electroporation creates pores on biological membranes makes it reasonable to speculate that it can increase the amount of pDNA delivered to the cytoplasm prior to degradation by nucleases within tissues and blood circulation. The longer time interval between the intravenous injection of pDNA and electroporation resulted in less transgene expression (20), indicating that the circulating intact pDNA is responsible for transgene expression.

Intravenous injection of pDNA followed by electroporation was found to be a promising approach for gene transfer to the liver, spleen and kidney, but it was much less effective in achieving muscle- and skin-targeted gene transfer. *In vivo* gene expression will take place only at restricted cells that are reached by intact pDNA after systemic administration. After intravenous administration, pDNA distributes within the body in a manner that depends on its interaction with biological components. Due to the limitation in huge size of pDNA, only capillaries in the liver, spleen and bone marrow posses pores and intercellular gaps that allow pDNA to reach parenchymal cells (33). Therefore, gene transfer to parenchymal cells in tissues having continuous-type endothelial cells, such as skeletal muscle and skin, after intravascular injection of pDNA, may require the delivery of pDNA across the endothelium. Although electroporation was expected to open the intercellular gaps in the endothelium, the very low degree of distribution of ^{32}P-pDNA in muscle and skin even after electroporation would explain the inability to achieve enhanced extravasation of pDNA from the capillaries to the interstitial space in these tissues. β-galactosidase expression data also supported the poor transfection results of skin and muscle electric gene transfer via the systemic circulation. Although the location of endothelial cells makes them the most liable cells to be transduced by intravascular pDNA with electric pulses, the endothelial cells are generally difficult to transfect as clearly demonstrated after hydrodynamics-based or electric pDNA delivery (20,34).

Among the tissues examined, the liver gave the greatest expression after systemic injection combined with electroporation, and this was almost identical to that achieved by the tissue injection of pDNA followed by electroporation. The discontinuous-type capillaries in the liver may help pDNA distribute to the parenchymal cells. We found that electroporation selectively increases the delivery of pDNA to liver parenchymal cells and transgene expression in these cells, probably due to the difference in size of cells (20). This may also be applied to the spleen, which showed a high level of transgene expression after electroporation. The β-galactosidase activity in these tissues indicated that the cells expressing the transgene are still limited to the area around the injection site. This limited distribution of transfected cells could be simply explained by the limited area of the effects of the electric pulses applied. Therefore, improvements in electrode design should make it possible to increase the area of transgene-expressing cells. Although directly-injected pDNA in the liver might be present within the tissue in a greater amount than pDNA after intravenous injection, the levels of transgene expression suggest that directly-injected pDNA in the liver rapidly leaks out from the tissue through the well-developed vasculature. In addition, it is also suggested that only a tiny fraction of the pDNA seems to contribute to the final output of transgene expression.

Although electroporation could induce tissue damage, we found little leakage of transaminases such as glutamyl oxaloacetic transaminase and glutamyl pyruvic transaminase from liver parenchymal cells after electric gene transfer to the liver (20). In addition, we observed little changes in the appearance of any tissue after electroporation in the present study. These results suggest that the conditions of the electroporation used in this study do not induce severe tissue damage. In previous studies, no histological changes were observed in glomeruli and tubular epithelial cells of the kidney after electroporation at 100 V (35), and no obvious damages were found in skin tissue at 24 and 48 h after electroporation at 1,750 V/cm (12). An electroporation with a high voltage of 900 V resulted in the generation of some necrotic cells in skeletal muscle (36); however, the damaged tissue was replaced with muscle fibres with central nuclei by 2 weeks, indicating that the tissue damage can be regenerated in a short period of time.

In summary, *in vivo* electroporation increases the transgene expression in all the cases of both tissue and intravascular injection of pDNA. Intravenous injection of pDNA followed by electroporation has been shown to be a promising approach for tissue-selective gene transfer to the liver, kidney and spleen, but it is much less effective in achieving skin- and muscle-targeted gene transfer. The level of transgene expression was clearly demonstrated to be dependent on the tissue and the route of pDNA administration. Liver and spleen are the most interesting tissues for prompt supply of proteins because of the highest transgene expression in both modes of administration in electric gene transfer. To prolong the supply of therapeutic proteins, intramuscular administration followed by electroporation should be concerned especially its advantage in the easiness of application of the electric field. Initially, the clinical applicability of these approaches for internal organs was achieved in combination with abdominal surgery, or by the modification of endoscope-type electrodes. However, the use of chest electrodes for the introduction of pDNA into the lungs after inhalation has been reported in an animal model (37). This type of electrode can be used to apply electric pulses to internal organs without surgery after intravenous injection.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

REFERENCES

1. I. Danko, P. Williams, H. Herweijer, G. Zhang, J. S. Latendresse, I. Bock, and J. A. Wolff. High expression of naked plasmid DNA in muscles of young rodents. *Hum. Mol. Genet.* **6**:1435–1443 (1997).
2. S. Chesnoy and L. Huang. Enhanced cutaneous gene delivery following intradermal injection of naked DNA in a high ionic strength solution. *Molec. Ther.* **5**:57–62 (2002).
3. M. A. Hickman, R. W. Malone, K. Lehmann-Bruinsma, T. R. Sih, D. Knoell, F. C. Szoka, R. Walzem, D. M. Carlson, and J. S. Powell. Gene expression following direct injection of DNA into liver. *Hum. Gene Ther.* **5**:1477–1483 (1994).
4. Y. Taniyama, R. Morishita, M. Aoki, H. Nakagami, K. Yamamoto, K. Yamazaki, K. Matsumoto, T. Nakamura, Y. Kaneda, and T. Ogihara. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hindlimb ischemia models: preclinical study for treatment of peripheral arterial disease. *Gene Ther.* **8**:181–189 (2001).
5. N. Sarkar, A. Rück, G. Källner, S. Y-Hassan, P. Blomberg, K. B. Islam, J. Van Der Linden, D. Lindblom, A. T. Nygren, B. Lind, L. Å. Brodin, V. Drvota, and C. Sylvén. Effects of intramyocardial injection of phVEGF-A165 as sole therapy in patients with refractory coronary artery disease-12-month follow-up: angiogenic gene therapy. *J. Intern. Med.* **250**:373–381 (2001).
6. S. Somiari, J. Glasspool-Malone, J. J. Drabick, R. A. Gilbert, R. Heller, M. J. Jaroszeski, and R. W. Malone. Theory and *in vivo* application of electroporative gene delivery. *Molec. Ther.* **2**:178–187 (2000).
7. D. A. Zaharoff, R. C. Barr, C. Y. Li, and F. Yuan. Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery. *Gene Ther.* **9**:1286–1290 (2002).
8. H. Maruyama, K. Ataka, N. Higuchi, F. Sakamoto, F. Gejyo, and J. Miyazaki. Skin-targeted gene transfer using *in vivo* electroporation. *Gene Ther.* **8**:1808–1812 (2001).
9. T. Suzuki, B. C. Shin, K. Fujikura, T. Matsuzaki, and K. Takata. Direct gene transfer into rat liver cells by *in vivo* electroporation. *FEBS Lett.* **425**:436–440 (1998).
10. M. Bettan, F. Emmanuel, R. Darteil, J. M. Caillaud, F. Soubrier, P. Delaere, D. Branelec, A. Mahfoudi, N. Duverger, and D. Scherman. High-level protein secretion into blood circulation after electric pulse-mediated gene transfer into skeletal muscle. *Molec. Ther.* **2**:204–210 (2000).
11. I. Mathiesen. Electropermeabilization of skeletal muscle enhances gene transfer *in vivo*. *Gene Ther.* **6**:508–514 (1999).
12. J. Glasspool-Malone, S. Somiari, J. J. Drabick, and R. W. Malone. Efficient nonviral cutaneous transfection. *Molec. Ther.* **2**:140–146 (2000).
13. M. Hashida, M. Nishikawa, F. Yamashita, and Y. Takakura. Cell-specific delivery of genes with glycosylated carriers. *Adv. Drug Deliv. Rev.* **52**:187–196 (2001).
14. F. Liu, Y. Song, and D. Liu. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**:1258–1266 (1999).
15. G. Zhang, V. Budker, and J. A. Wolff. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10**:1735–1737 (1999).
16. V. Budker, G. Zhang, I. Danko, P. Williams, and J. A. Wolff. The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Ther.* **5**:272–276 (1998).
17. K. W. Liang, M. Nishikawa, F. Liu, B. Sun, Q. Ye, and L. Huang. Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA. *Gene Ther.* **11**:901–908 (2004).
18. H. Herweijer and J. A. Wolff. Progress and prospects: naked DNA gene transfer and therapy. *Gene Ther.* **10**:453–458 (2003).
19. F. Liu and L. Huang. Electric gene transfer to the liver following systemic administration of plasmid DNA. *Gene Ther.* **9**:1116–1119 (2002).
20. M. Sakai, M. Nishikawa, O. Thanaketpaisarn, F. Yamashita, and M. Hashida. Hepatocyte-targeted gene transfer by combination of vascularly delivery plasmid DNA and *in vivo* electroporation. *Gene Ther.* **12**:607–616 (2005).
21. M. Nishikawa and M. Hashida. Nonviral approaches satisfying various requirements for effective *in vivo* gene therapy. *Biol. Pharm. Bull.* **25**:275–283 (2002).
22. C. H. Miao, A. R. Thompson, K. Loeb, and X. Ye. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer *in vivo*. *Molec. Ther.* **3**:947–957 (2001).
23. D. A. Roth, N. E. Tawa Jr., J. M. O'Brien, D. A. Treco, and R. F. Selden. Non-viral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N. Engl. J. Med.* **344**:1735–1742 (2001).
24. J. M. Wang, H. Zheng, M. Blaivas, and K. Kurachi. Persistent systemic production of human factor IX in mice by skeletal myoblast-mediated gene transfer: feasibility of repeat application to obtain therapeutic levels. *Blood* **90**:1075–1082 (1997).
25. N. Kobayashi, T. Kuramoto, K. Yamaoka, M. Hashida, and Y. Takakura. Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures. *J. Pharmacol. Exp. Ther.* **297**:853–860 (2001).
26. K. Kawabata, Y. Takakura, and M. Hashida. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* **12**:825–830 (1995).
27. M. Nishikawa and L. Huang. Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum. Gene Ther.* **12**:861–870 (2001).
28. D. Guo, T. Fu, J. A. Nelson, R. A. Superina, and H. E. Soriano. Liver repopulation after cell transplantation in mice treated with retrorsine and carbon tetrachloride. *Transplantation* 73:1818–1824 (2002).
29. J. A. Wolff, J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* **1**:363–369 (1992).
30. E. Fattori, N. L. Monica, G. Ciliberto, and C. Toniatti. Electrogene-transfer: a new approach for muscle gene delivery. *Somat. Cell Mol. Genet.* **27**:75–83 (2002).
31. E. Tupin, B. Poirier, M. F. Bureau, J. Khallou-Laschet, R. Vranckx, G. Caligiuri, A. T. Gaston, J. P. Duong, V. Huyen, D. Scherman, J. Bariéty, J. B. Michel, and A. Nicoletti. Nonviral gene transfer of murine spleen cells achieved by *in vivo* electroporation. *Gene Ther.* **10**:569–579 (2003)
32. A. J. O'hara, J. M. Howell, R. H. Taplin, S. Fletcher, F. Lloyd, B. Kakulas, H. Lochmuller, and G. Karpati. The spread of transgene expression at the site of gene construct injection. *Muscle Nerve* **24**:488–495 (2001).
33. Y. Takakura, R. I. Mahato, and M. Hashida. Extravasation of macromolecules. *Adv. Drug Deliv. Rev.* **34**:93–108 (1998).
34. N. Kobayashi, M. Nishikawa, K. Hirata, and Y. Takakura. Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery. *J. Gene Med.* **6**:584–592 (2004).
35. M. Tsujie, Y. Isaka, H. Nakamura, E. Imai, and M. Hori. Electroporation-mediated gene transfer that targets glomeruli. *J. Am. Soc. Nephrol.* **12**:949–954 (2001).
36. J. M. Vicat, S. Boisseau, P. Jourdes, M. Laine, D. Wion, R. Bouali-Benazzouz, A. L. Benabid, and F. Berger. Muscle transfection by electroporation with high-voltage and short-pulse currents provides high-level and long-lasting gene expression. *Hum. Gene Ther.* **11**:909–916 (2000).
37. D. A. Dean, D. Machado-Aranda1, K. Blair-Parks, A. V. Yeldandi, and J. L. Young. Electroporation as a method for high-level nonviral gene transfer to the lung. *Gene Ther.* **10**:1608–1615 (2003).