serum is mediated by negatively-charged serum proteins and inactivation can be overcome by increasing the positive charge of the lipoplex. As shown in these *in vitro* studies, a cationic lipoplex can attract a variety of blood components, which results in alterations in the overall physicochemical properties of the lipoplex. Therefore, such an interaction should alter the tissue distribution of lipoplex following intravenous injection. Li *et al.* (1998) has demonstrated that lipidic vectors become negatively charged after exposure to mouse serum and increase in size. The choice of a helper lipid plays an important role in determining the potential of the lipoplex. In particular, when dioleoylphosphatidylethanolamine (DOPE) is used as a helper lipid, the *in vivo* transfection activity of the lipoplex is much lower than that achieved with a formulation containing cholesterol as a neutral helper lipid. One reason for this is that the cationic lipidic vectors containing DOPE recruit large amounts of serum proteins. Interactions of lipoplex with serum components lead to disintegration of the lipoplex, followed by the release and degradation of plasmid DNA (Li *et al.*, 1999). Release of plasmid DNA from the lipoplex might be due to the interaction of lipoplex with highly negatively charged molecules in serum, as proposed by Xu and Szoka (1996). A DOPE-containing formulation, in spite of its initial efficient accumulation in the lung, is poorly retained there because of its rapid disintegration. This, together with the rapid degradation of plasmid DNA, explains why DOPE-containing formulations do not result in satisfactory transfection of cells *in vivo*.

It is still not known which serum proteins are the major components that are responsible for vector disintegration. Li *et al.* (1998, 1999) have reported that a cationic lipid-protamine-DNA complex was enriched with a protein with a molecular weight corresponding to serum albumin and that further interaction of the complex with serum proteins led to its disintegration, and the release and degradation of plasmid DNA. DOPE-containing formulations disintegrated more rapidly when incubated in serum than cholesterol-containing ones. Barron *et al.* (1998) have shown that a lipoplex interacts with serum complements upon intravenous injection in mice, but this interaction does not affect the transfection efficiency in the lung nor the tissue distribution of the lipoplex. In addition to serum proteins, erythrocytes are also involved in the interaction with lipoplex *in vivo* (Sakurai *et al.*, 2001a,b), an interaction which is also highly dependent on the helper lipid within the lipoplex, DOPE or cholesterol. Lipoplexes without DOPE, in which lipids have a stable and rigid lamellar structure, do not induce fusion between erythrocytes. Although these complexes bind to erythrocytes, a fraction of the erythrocyte-bound complex could still be active as far as transfection is concerned. During its passage through the lung capillaries, some complexes may dissociate from erythrocytes without any loss of lipid components and change in their structure. Then, the complex released may be transferred to the lung endothelial cells and internalized by endocytosis. On

the other hand, lipids in the N-[1-(2,3-dioleyloxy)propyl]-n, n, n-trimethylammonium chloride (DOTMA)/DOPE complex have a highly curved structure with high fluidity, and induce fusion of erythrocytes within a short period. Following fusion, the lipid components of the liposomes are extracted by erythrocytes, resulting in a reduced potential to bind to endothelial cells. These observations also apply to the reduction in transfection activity. Following *in vivo* administration, we also demonstrated that lipoplex binds to blood cells and the interaction between the lipoplex and erythrocytes is important for efficient *in vivo* gene transfer (Sakurai *et al.*, 2001a). DOPE-containing lipoplex forms aggregates with erythrocytes, which will embolize in the capillaries of the lung following intravenous injection. However, erythrocytes bound to cholesterol-containing lipoplex dissociate so that the free lipoplex is able to accumulate in the lung.

To compensate for the lack of specificity of the electrostatic interaction of lipoplex, ligands are introduced into cationic liposomes and sugars are the most extensively investigated ligands so far (Kawakami *et al.*, 2002). The incorporation of sugars into lipoplex formulations increases the amount of the lipoplexes delivered to the cells having the corresponding receptors, such as hepatocytes and liver nonparenchymal cells (Kawakami *et al.*, 2000a,b). However, hepatocyte-targeted delivery of galactosylated lipoplex was achieved only by its intraportal injection, probably due to the nonspecific interaction of the galactosylated lipoplex with serum components as described above.

B. Polyplex

Cationic polymers are another class of non-viral vectors that can be used to increase gene delivery and transfer to target cells *in vivo*. Various types of polymers have been examined with respect to their ability to offer protection from nuclease degradation, deliver to target cells, and increase the transfection efficiency of plasmid DNA. Cationic polymers can condense plasmid DNA more efficiently than cationic liposomes, and this would be beneficial in controlling the tissue distribution of plasmid DNA complexes. These include: poly-L-lysine (PLL), poly-L-ornithine, polyethyleneimine (PEI), chitosan, starburst dendrimer and other novel synthetic polymers. Plasmid DNA/cationic polymer complex, or polyplex, is believed to be taken up by cells via an endocytotic pathway, so its transfection efficiency depends on the release of plasmid DNA into the cytoplasm after cellular uptake. The amount of transgene expression depends on a number of variables including the nature of the polymer used and the amount of plasmid DNA delivered. We demonstrated that the amount of ^{111}In-plasmid DNA delivered to the lung was proportional to the transgene expression in the organ when PEI/plasmid DNA was injected into mice using polyplexes at different N/P ratios (Nishikawa *et al.*, 2003).

As observed with lipoplexes, formation of a cationic complex can increase the interaction of plasmid DNA with various cells. When non-targeted, cationic polyplex is injected intravenously, the interaction with the lung endothelial cells is most marked. Then, transgene expression is also high in these cells. If large aggregates are formed after intravenous injection of polymer/plasmid DNA complex, they will also be trapped by the lung. In some cases, the mixing of plasmid DNA and a cationic carrier in vitro results in very large aggregates with a size close to that of the capillaries (Finsinger *et al.*, 2000; Nishikawa *et al.*, 1998). To prevent aggregation, the surface of the complex can be modified with hydrophilic compounds such as polyethylene glycol. Ogris *et al.* (1999) have shown that when incubated with plasma, the transferrin-PEI/plasmid DNA complex undergoes aggregation, which leads to reduced delivery to the target. PEGylation appears to be a useful method for prolonging the blood circulation of a polyplex after systemic administration, and PEGylated polyplex has resulted in gene transfer to a tumor without significant toxicity after intravenous injection into tumor-bearing mice (Kircheis *et al.*, 2001). In addition to PEG, other hydrophilic polymers have been shown to extend the circulation time of polyplex (Oupicky *et al.*, 2002; Verbaan *et al.*, 2004). However, it might also shield the cationic charge of the complex that is the driving force for interaction with target cells. To compensate for the loss of affinity for the target cell, the use of ligands is a good way to achieve efficient delivery of polyplex.

The tissue distribution of polyplex is more easily controlled than that of lipoplex, because there is less interaction between the cationic polymers and serum components. Therefore, active targeting to a specific population of cells in the body was attempted as early as 1988 by Wu and Wu. Polymers such as PLL and PEI have been covalently modified with targeting ligand, and these include asialoglycoproteins (Wu and Wu, 1988), carbohydrates (Perales *et al.*, 1994), transferrin (Kircheis *et al.*, 1999), folate (Ward *et al.*, 2002), and antibody (Li *et al.*, 2000). We carried out pharmacokinetic evaluations of the tissue distribution of galactosylated PLL (Gal-PLL)/plasmid DNA complex following intravenous injection in mice (Nishikawa *et al.*, 1998). As mentioned above, naked plasmid DNA is rapidly taken up by the liver. Cell fractionation and confocal imaging of fluorescein-labeled pDNA following intravenous injection into mice showed that plasmid DNA is mainly taken up by sinusoidal cells such as Kupffer cells and liver sinusoidal endothelial cells (Kawabata *et al.*, 1995; Kobayashi *et al.*, 2001). Because the uptake by these cells seems to be mediated by the strong negative charge of plasmid DNA and its clearance is very high, it is very important to mask the negative charge of plasmid DNA in order to control its tissue distribution. After intravenous injection of Gal-PLL/^{32}P-plasmid DNA complex, the hepatic uptake clearance was much greater than that by any other tissue. However, the physicochemical properties of

Gal-PLL used for the complexation markedly affected the pharmacokinetics of the polyplexes. The clearance values demonstrate that polyplexes with a large Gal-PLL (13 or 29 kD for the molecular weight of PLL) have a greater hepatic (target) clearance than those with a small Gal-PLL (1.8 kD), which failed to achieve efficient delivery of plasmid DNA to hepatocytes probably due to complex dissociation before reaching the target. Similar effects of molecular size have also been obtained with galactosylated PEI (Gal-PEI) (Morimoto *et al.*, 2003). Although the transfection potential was highest for PEI with the smallest molecular weight of 1.8 kD, the polyplex composed of Gal-PEI_{1800} and plasmid DNA was the least effective as far as *in vivo* transgene expression was concerned.

These polyplexes with cationic vectors are internalized by cells via endocytosis resulting in lysosomal degradation. This intracellular pathway greatly limits the efficiency of gene transfer by this approach. In addition to controlling the *in vivo* pharmacokinetics by using a carrier molecule like Gal-PLL, the control of intracellular sorting of plasmid DNA is a good approach to increasing gene transfer at the target. Wagner *et al.* (1992) demonstrated increased transgene expression in cultured cells following the addition of fusogenic peptides, derived from influenza virus hemmaglutinin subunit HA-2, to plasmid DNA complexes. We attached a fusogenic peptide to hepatocyte-targetable polymer and obtained improved transgene expression in the liver, indicating that the peptide also works in whole animals to, at least partially, avoid intracellular degradation (Nishikawa *et al.*, 2000).

V. PHARMACOKINETIC FEATURES AFTER TISSUE INJECTION

Direct tissue injection of plasmid DNA or its complex is used to obtain the expression of a secretion protein that will exhibit its therapeutic activity after entering the blood circulation, or an intracellular protein that will work within the cells in the injected tissue. As stated above, the tissue distribution of 'vector' is only important in the latter case. Because of extensive degradation of naked plasmid DNA entering into the blood circulation, transgene expression after tissue injection is normally highly tissue-specific.

The distribution of plasmid DNA within the injected tissue is a major factor that should be considered as far as the efficacy of *in vivo* gene transfer after tissue injection is concerned. A needle injection of naked plasmid DNA into various tissues, including skeletal muscle (Wolff *et al.*, 1990), heart (Lin *et al.*, 1990), liver (Hickman *et al.*, 1994), brain (Ono *et al.*, 1990), skin (Raz *et al.*, 1994), thyroid (Sikes *et al.*, 1994), urological organs (Yoo *et al.*, 1999), and tumors (Nomura *et al.*, 1999; Plautz *et al.*, 1993), results in significant transgene expression in the tissue injected. However, cells with transgene expression were normally found only in close proximity to the track of the needle injection

(Hickman *et al.*, 1994; O'Hara *et al.*, 2001). Therefore, dispersion of the injected plasmid DNA within the tissue is an important issue in the delivery of naked plasmid DNA. Compared with conventional, low-molecular weight drugs, plasmid DNA is a huge molecule with a molecular weight of about 2,000 kD or more. This huge size greatly restricts its diffusion within the tissue where plasmid DNA is injected, because the diffusion as well as the absorption of an injected compound into the circulation is governed by its molecular weight (Nara *et al.*, 1992). Furthermore, the complex formation of plasmid DNA with cationic liposomes limits the diffusion within tissues due to the increased size and net charge (Nomura *et al.*, 1997, 1999) Therefore, plasmid DNA locally injected into tissues, such as muscle and skin, may only transfect cells around the injection site, which makes *in vivo* gene transfer tissue-specific. A local injection of lipoplex has been shown to be an effective approach to achieve transgene expression in the lung, brain, tumor and skin (Brigham *et al.*, 1989; Ono *et al.*, 1990; Plautz *et al.*, 1993; Raz *et al.*, 1994).

Some polymers, which do not form condensed complexes with plasmid DNA, have been reported to exhibit an enhancing effect on the transgene expression of naked plasmid DNA in skeletal muscle. Polyvinyl pyrrolidone or polyvinyl alcohol has been used to increase the area and level of transgene expression following intramuscular injection of plasmid DNA (Alila *et al.*, 1997). Use of these polymers resulted in widespread transgene expression within the injected tissues, suggesting altered distribution of plasmid DNA by the presence of such polymers.

VI. CONCLUSION

The key issue for successful *in vivo* gene therapy is to develop a vector and optimize its delivery, so that significant transgene expression *in vivo* can be achieved. Understanding the tissue distribution of plasmid DNA and its complex is a prerequisite for designing an effective approach to improve their efficacy *in vivo*. Pharmacokinetic analysis will give us information about the events occurring in the body following administration and, therefore, it is a very powerful tool for developing a strategy to improve the poor results obtained in gene therapy trials to date.

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RESEARCH ARTICLE

Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and in vivo electroporation

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To increase transgene expression in the liver, electric pulses were applied to the left lateral lobe after intravenous injection of naked plasmid DNA (pDNA) or pDNA/liver targeting vector complex prepared with galactosylated poly(L-lysine) or galactosylated polyethyleneimine. Electroporation (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) after naked pDNA injection dramatically increased the expression up to 200 000-fold; the expression level obtained was significantly greater than that achieved by the combination of pDNA/vector complex and electroporation. We clearly demonstrated that the expression was dependent on the plasma concentration of pDNA at the time when the electric pulses were applied. Separation of liver cells revealed that the distribution of naked pDNA as well as transgene expression was largely selective to hepatocytes in the electroporated lobe. The number of cells expressing transgene product using vascularly administered naked pDNA followed by electroporation was significantly ($P < 0.01$) greater and more widespread than that obtained by local injection of naked pDNA. These results indicate that the application of in vivo electroporation to vascularly administered naked pDNA is a useful gene transfer approach to a large number of hepatocytes.
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Introduction

The success of *in vivo* gene therapy relies on the development of vectors that can selectively and effectively deliver a therapeutic gene to the target with minimal toxicity. Nonviral vectors such as naked plasmid DNA (pDNA) and its complex with a cationic vector, with or without a specific ligand, has advantages over viral vectors in terms of the simplicity of use, ease of large-scale production and lack of a specific immune response.[1] However, the low efficiency of transgene expression needs to be improved. We have developed various delivery systems for pDNA, incorporating galactose[2,3] or mannose[4] to achieve cell-specific targeting to hepatocytes or liver nonparenchymal cells (NPC), respectively. These vectors were highly effective in delivering pDNA to the cells, but the transfection efficiency still needs to be improved.

A major barrier for successful *in vivo* gene transfer is the poor membrane permeability of pDNA as well as its lysosomal degradation within cells after entrance via endocytosis. Recently, various physical approaches such as electroporation,[5] sonoporation,[6] and hydrodynamic or hydrostatic pressure by large-volume injection[7,8] have been developed for improving transgene expression by nonviral vectors. Among these approaches, *in vivo* electroporation, which is suggested to transiently create pores on cell membranes[9] and has been tested in clinical trials for cancer chemotherapy,[10,11] has been extensively studied as a method to increase transgene expression by naked pDNA after local injection into the interstitial spaces of tissues. *In vivo* electroporation has been applied to various tissues including skin,[5] liver,[12,13] melanoma,[14] and muscle.[15] Generally speaking, electroporation increases transgene expression up to 1000-fold compared with that obtained with simple naked pDNA injection. However, the application of *in vivo* electroporation has been limited to locally injected pDNA, where the distribution of transgene-expressing cells is limited to a narrow area around the injection site.[16] Although the application of electric pulses to locally injected pDNA can increase the area of those cells, the spread is still limited due to the huge size of pDNA.

Vascularly administered pDNA can be distributed to a greater number of cells than locally injected pDNA. Therefore, the application of electroporation to vascularly administered pDNA could achieve transgene expression in a large target cell population. Gene therapy for the deficiency of an intracellular enzyme or structural protein requires functional restoration at the cell level, so the number of cells producing the transgene would be a major factor determining the therapeutic efficacy of the gene transfer approach. Although the application of electroporation after systemic administration of pDNA

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can be a promising technique to achieve this, it has received little attention so far. Recently, Liu and Huang[17] published a report on electroporation-mediated gene transfer to the liver after intravenous injection of naked pDNA. They found that electroporation combined with intravenous naked pDNA can be a good method of achieving gene transfer to a larger number of liver cells compared with locally injected pDNA. However, the characteristics of gene transfer and its underlying mechanism need to be investigated to optimize this technique, in which electroporation is applied to a tissue after vascularly administered pDNA. Due to its strong negative charge, naked pDNA is rapidly taken up by the liver NPC after intravenous injection,[18,19] which results in no significant transgene expression in the organ. This uptake rapidly reduces the concentration of pDNA within the circulation. Although the results obtained by Liu and Huang[17] suggest that the distribution of pDNA would be altered by electroporation, little is known about the tissue distribution of pDNA. Targeted delivery of pDNA to hepatocytes can be achieved by galactosylated vectors, but the combined use of targeted delivery of pDNA using vectors and electroporation has not been examined so far.

We hypothesized that the application of electric pulses alters the distribution of pDNA at the site of electroporation. The liver is composed of various types of cells including hepatocytes, sinusoidal endothelial cells and Kupffer cells, and, in many genetic deficiencies such as ornithine transcarbamylase deficiency, hepatocytes can be the target of gene transfer. Therefore, we tried to achieve efficient gene transfer to hepatocytes by applying electroporation to intravenously administered pDNA. We injected pDNA into the tail vein of mice in the free form (naked pDNA) or in the complex form with a hepatocyte-targeted vector: galactosylated poly(L-lysine) (Gal-PLL)[20] or galactosylated polyethyleneimine (Gal-PEI).[3] First, the effect of electroporation was examined on the whole-body distribution of naked pDNA and pDNA/Gal-PLL using ^{32}P-labeled pDNA. Then transgene expression in the liver was evaluated after administering intravenous pDNA or its complex followed by electroporation onto the liver. As naked pDNA showed the greatest transgene expression among the vectors examined, the characteristics of the expression by naked pDNA with electroporation were investigated in detail in terms of the distribution of pDNA and transfected cells within the electroporated lobe. We report here that intravenous naked pDNA injection followed by hepatic electroporation is a good way of achieving selective gene transfer to a number of hepatocytes with a higher transfection efficiency than that offered by vector-targeted gene transfer. Hepatocytes are found to be more susceptible to the effects of electroporation than other liver NPC, and the delivery of pDNA and transgene expression are selectively improved in hepatocytes by electroporation.

Results

Effect of electroporation on pharmacokinetics of pDNA and its complex

After intravenous injection, ^{32}P-pDNA is preferentially taken up by liver NPC,[18,19] whereas a large fraction of ^{32}P-pDNA/Gal-PLL is delivered to hepatocytes through asialoglycoprotein receptor-mediated endocytosis.[20] Without electroporation, both naked ^{32}P-pDNA and ^{32}P-pDNA/Gal-PLL showed similar pharmacokinetic profiles to those reported previously; ^{32}P-radioactivity quickly disappeared from the blood circulation and accumulated in the liver (Figures 1 and 2). When naked ^{32}P-pDNA was injected, the radioactivity in the liver quickly decreased with time (Figure 1), reflecting its degradation by the organ. A set of electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) was applied to the left lateral lobe of the liver at 30 s after intravenous injection of either naked ^{32}P-pDNA (Figure 1) or ^{32}P-pDNA/Gal-PLL (Figure 2). No significant changes in the plasma concentration and liver accumulation of ^{32}P-radioactivity were detected in either case.

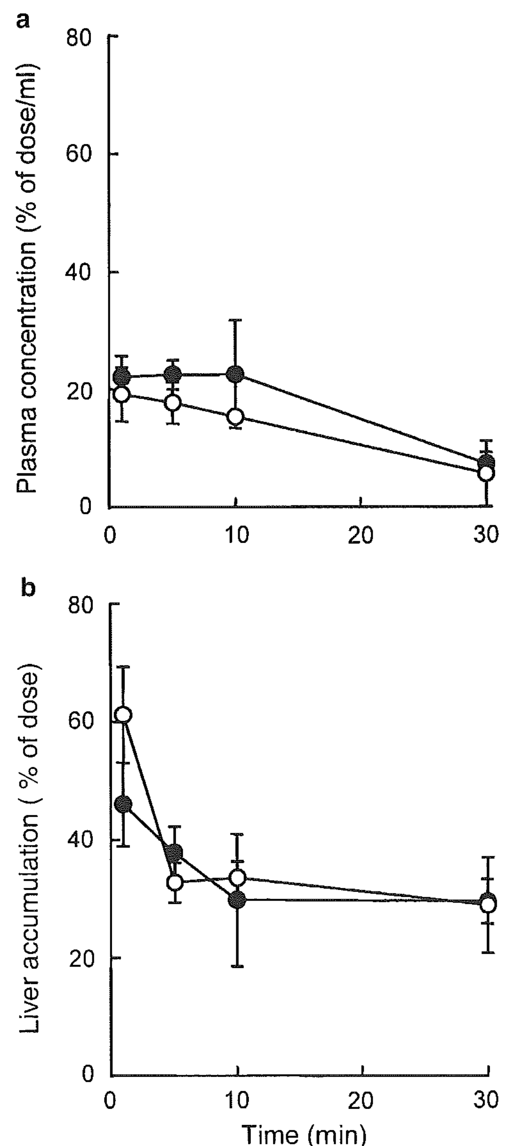

Figure 1 Plasma concentration (a) and liver accumulation (b) of ^{32}P-radioactivity after injection of naked ^{32}P-pDNA with or without electroporation. Naked ^{32}P-pDNA was injected into the tail vein at a dose of 25 μg/mouse, and electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) were applied to the left lateral lobe of the liver at 30 s after injection. Results are expressed as the mean ± s.d. of three mice. (○) injection only; (●) electroporated.

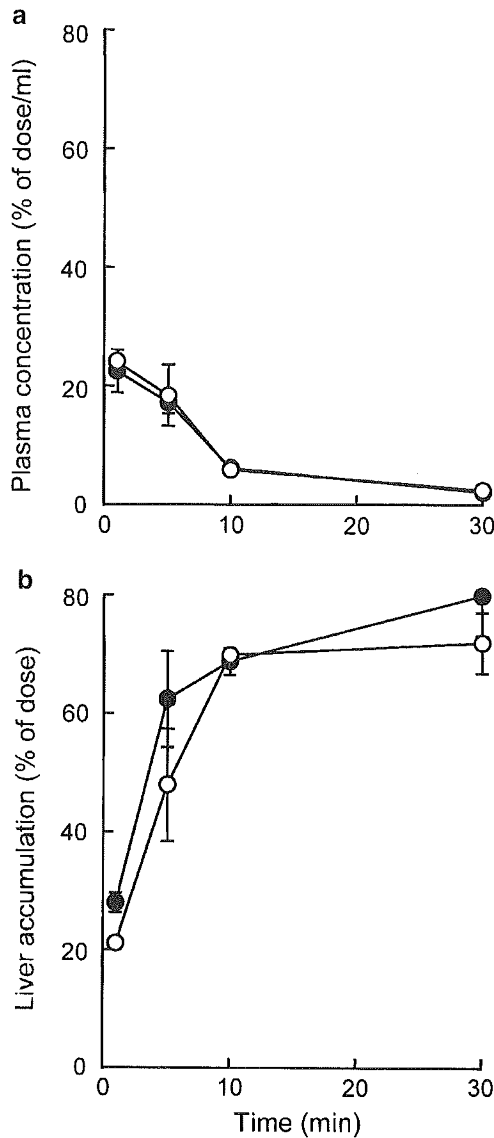

Figure 2 *Plasma concentration (a) and liver accumulation (b) of ^{32}P-radioactivity after injection of ^{32}P-pDNA/Gal-PLL complex with or without electroporation. ^{32}P-pDNA/Gal-PLL complex (N/P ratio of 2.4) was injected into the tail vein at a dose of 25 μg/mouse, and electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) were applied to the left lateral lobe of the liver at 30 s after injection. Results are expressed as the mean ± s.d. of three mice. (○) Injection only; (●) electroporated.*

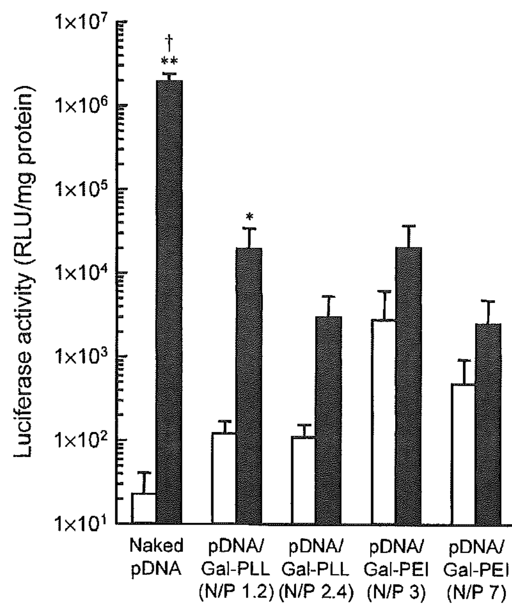

Figure 3 *Transgene expression after injection of naked pDNA, pDNA/Gal-PLL or pDNA/Gal-PEI complex with or without electroporation. pDNA complex was injected into the tail vein at a dose of 25 μg/mouse, and electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) were applied to the left lateral lobe of the liver at 30 s after injection. Results are expressed as the mean ± s.d. of at least three mice. (open bar) injection only; (closed bar) electroporated. *,**Statistically significant difference compared to the expression without electroporation (*$P<0.01$, **$P<0.001$). †Statistically significant difference between naked pDNA and all other groups ($P<0.001$).*

Effect of electroporation on transgene expression by vascular pDNA and its complex

Transgene expression was detected at 6 h after injection of 25 μg pDNA or its complex in the following experiments. The electroporation parameters were fixed as follows unless otherwise indicated: electric field, 250 V/cm; duration of each pulse, 5 ms; number of pulses, 12; frequency of pulses, 4 Hz; timing of pulses, 30 s after injection; site of electroporation, the left lateral lobe.

No significant transgene expression was detected in the liver after intravenous injection of naked pDNA (Figure 3). pDNA/Gal-PLL or pDNA/Gal-PEI complex showed detectable transgene expression in the liver. However, the expression level obtained was not very high, probably due to the small dose of pDNA injected. The application of electric pulses increased the expression in the electroporated lobe of the liver against any vector used (Figure 3). However, the enhancement ratio and the final level of transgene expression varied among the vectors. Naked pDNA showed the greatest enhancement ratio in transgene expression and the greatest expression level. The expression in the electroporated lobe increased up to 200 000-fold. The level of expression (about 2×10^6 RLU/mg protein) was about 100-fold greater than that obtained after the injection of pDNA complex followed by electroporation.

To confirm the effect of electroporation on the receptor-mediated gene transfer, transgene expression by naked pDNA or pDNA/Gal-PLL was examined in HepG2 cells, a human hepatoma cell line expressing asialoglycoprotein receptors. Again, electroporation increased the expression to 4500-fold for naked pDNA and nine-fold for pDNA/Gal-PLL (data not shown), suggesting that the free form of pDNA is more effective than its complexed form for transgene expression once it enters into the cytoplasm of cells.

Effect of strength of electric field on transgene expression by vascular naked pDNA

Figure 4 shows the transgene expression in the electroporated lobe and other lobes after intravenous injection of naked pDNA followed by electroporation with various electric fields from 50 to 500 V/cm. At any

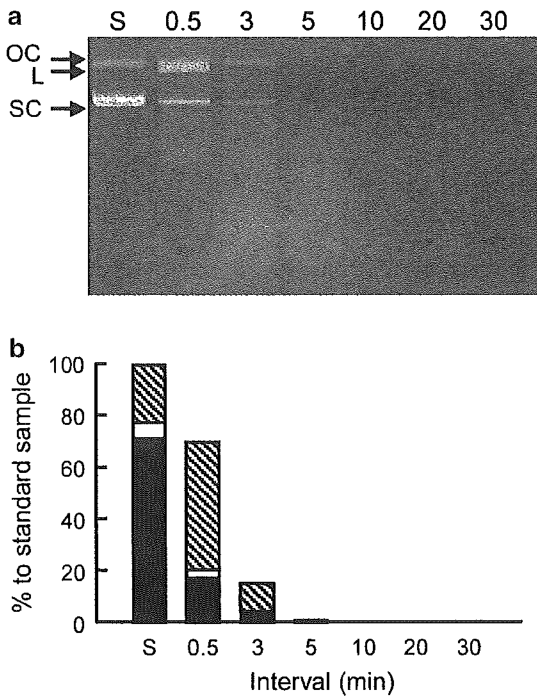

Figure 7 *Stability of naked pDNA after injection into mice. (a) Naked pDNA was injected into the tail vein at a dose of 25 μg/mouse, and pDNA was recovered from mouse blood at indicated time points after injection, electrophoresed on agarose gel and visualized with ethidium bromide. (b) The amounts of pDNA in supercoiled, open circular and linear forms were measured by densitometric analysis. SC (closed bar), supercoiled; OC (open bar), open circular; L (striped bar), linear; S, standard pDNA (0.5 μg pDNA).*

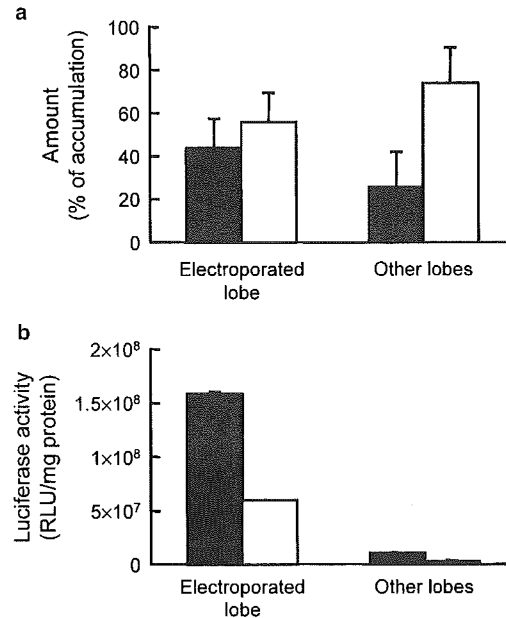

Figure 9 *Distribution of ^{32}P-radioactivity (a) and luciferase activity (b) in the electroporated and nonelectroporated lobes after injection of naked pDNA followed by electroporation. Naked ^{32}P-pDNA or pDNA was injected into the tail vein at a dose of 25 μg/mouse, and electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) were applied to the left lateral lobe of the liver at 30 s after injection. PC (closed bar) and NPC (open bar) were separated and ^{32}P-radioactivity (a) or luciferase activity (b) in these cell fractions was assayed. Results are expressed as the mean ± s.d. of at least three mice.*

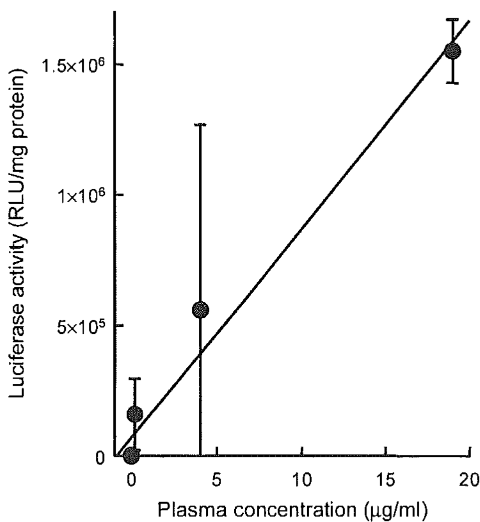

Figure 8 *Relation between transgene expression and the plasma concentration of pDNA at electroporation. Transgene expression in the electroporated lobe of the liver (Figure 6) was plotted against the total concentration of pDNA (Figure 7b). Experimental details are described in the legends of Figures 6 and 7.*

Cellular distribution of radioactivity and transgene expression in mouse liver after injection of naked pDNA followed by electroporation

Naked pDNA is extensively taken up by liver NPC via mechanism(s) like scavenger receptors.[18,21] In the nonelectroporated lobes of the liver, ^{32}P-radioactivity preferentially distributed to NPC rather than hepatocytes (parenchymal cells, PC) (Figure 9a). NPC uptake was about three-fold greater than PC uptake on a cell number basis, with a PC/NPC ratio of 0.35. The PC/NPC ratio increased in the electroporated lobe to 0.78, indicating that the distribution of pDNA shifted from NPC to PC by electroporation.

As shown in Figure 4, transgene expression was very high in the electroporated lobe. Separation of the cells in the lobe clearly demonstrated that the expression in PC was significantly ($P<0.01$) greater than that in NPC (Figure 9b).

Analysis of the number of cells expressing transgene product

Figure 10b and c shows typical liver tissues receiving an intravenous injection of naked β-galactosidase-expressing pDNA followed by electroporation. For comparison, naked pDNA was directly injected into the left lateral lobe of the liver (Figure 10a), which resulted in a very localized distribution of β-galactosidase-positive cells near the injection site (arrow). Greater numbers of hepatocytes in a large area around the site of electroporation were transfected by intravenous naked pDNA followed by electroporation (Figure 10b and c).

To quantitatively evaluate the number of β-galactosidase-positive cells, we isolated hepatocytes from the left lateral lobe of the liver after administration of β-galactosidase-expressing pDNA to mice (Figure 10d).

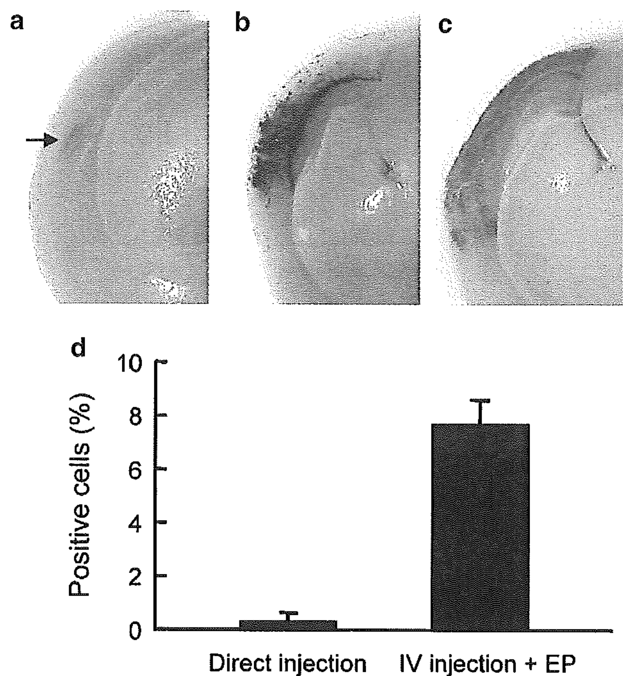

Figure 10 *X-gal staining of the liver of mice receiving β-galactosidase-expressing pDNA. (a) Naked pDNA was injected directly into the left lateral lobe. (b, c) Naked pDNA was injected into the tail vein and electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) were applied to the left lateral lobe at 30 s after injection. (d) Hepatocytes of the left lateral lobe were isolated by collagenase perfusion and stained with X-gal as described in Materials and methods. More than 100 individual cells per sample were observed under a microscope, and the number of blue cells, that is, β-galactosidase-expressing cells, was counted. Results are expressed as the mean ± s.d. of three measurements of each sample from two mice per group.*

The number of hepatocytes expressing β-galactosidase using vascularly administered naked pDNA followed by electroporation was significantly ($P<0.01$) and about 25-fold greater than that obtained by local injection of naked pDNA.

Discussion

Naked pDNA injection is the simplest method of the nonviral gene delivery approaches. However, no significant gene expression can be detected in any tissue when naked pDNA is intravenously injected by a normal, conventional technique because of its rapid degradation and low membrane permeability. Therefore, pDNA needs to be protected from nuclease-mediated degradation by complex formation with a vector such as cationic polymer or cationic liposome.[1] Cationic vectors can increase transfection efficiency by pDNA, but the expression level seems to be still low as far as therapeutic applications are concerned. As a different challenge involving *in vivo* gene transfer, electroporation has been applied to various tissues after local injection of naked pDNA, and great improvements in transgene expression have been reported.[9] However, direct tissue injection of pDNA has the disadvantage of the limited distribution of transfected cells,[16,22] which would greatly restrict the efficacy of gene replacement therapy of intracellular protein.[23] These two different approaches, that is, pDNA delivery via the intravascular route and *in vivo* electroporation, could be used to achieve significant transgene expression in a large number of target cells. In a pioneer work by Liu and Huang,[17] this approach was found to be effective as far as achieving transgene expression in a greater number of liver cells was concerned. However, it was not clear how electroporation alters the distribution of pDNA within the liver. Transgene expression occurs only in the cells that take up intact pDNA into the cytoplasm. Therefore, in the present study, we focused on the changes in the distribution of pDNA and examined the option involving liver-targeted gene transfer by injecting naked or hepatocyte-targeted pDNA followed by electroporation to the organ.

As hepatocytes uniquely express asialoglycoprotein receptors on their sinusoidal surface, asialoglycoproteins or galactosylated polymers have been used to deliver a variety of pharmaceutical agents ranging from anticancer drugs to genes. Attempts to deliver pDNA to hepatocytes by conjugating or mixing it with galactose-containing vectors, however, sometimes resulted in failure because of problems in tissue distribution. We have been trying to overcome these problems by controlling the physicochemical properties of the pDNA/galactosylated vector complex.[2,3,20] In these studies, we successfully delivered pDNA to the liver, especially to hepatocytes, in amounts up to about 60% of the injected dose of pDNA. Although the pDNA complex taken up by asialoglycoprotein receptor-mediated endocytosis undergoes lysosomal degradation, a fraction is believed to be released from the endosome/lysosome pathway into the cytoplasm, then being transported into the nucleus. Some structural features have been introduced onto vectors to increase gene transfer by facilitating the release of pDNA into the cytoplasm. They are fusogenic peptides, which create pores on the plasma and/or endosomal membrane,[2,24] and polymers having buffering capacity like PEI.[25] In the present study, pDNA/galactosylated vector complexes showed greater transgene expression in the liver than naked pDNA. Of the complexes used, Gal-PEI was superior to Gal-PLL in terms of transfection efficiency to mouse liver, reflecting the buffering ability of PEI. Electroporation of a set of fixed parameters (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) increased the expression in the electroporated lobe of the liver after intravenous injection of both pDNA/Gal-PLL and pDNA/Gal-PEI complexes. As the tissue distribution profile of ^{32}P-pDNA was hardly altered by electroporation (Figure 2), the enhanced expression would be mediated by changes in the intrahepatic and/or intracellular distribution of pDNA. Assuming that electroporation creates pores on biological membranes,[9] it is reasonable to accept that it can increase the amount of pDNA delivered to the cytoplasm prior to degradation. The pDNA/galactosylated vector complex is believed to bind to cell-surface receptors, captured in endosomes and then transported into lysosomes where it is degraded. In the present study, electric pulses were applied to a mouse at 30 s after injection of the pDNA/vector complex. However, the interval between the injection and electroporation may affect the results. The application of electric pulses at later times such as 5 or 10 min after injection did not increase the expression in the liver (data not shown). Furthermore, an increase in the amount of Gal-PLL or Gal-PEI in each complex

reduced the enhancement ratio produced by electroporation (Figure 3). These results, together with those obtained using naked pDNA, suggest that the polymers disturb to a certain degree the electroporation-mediated enhancement in transgene expression, although galactosylated vector-mediated gene transfer could be improved by *in vivo* electroporation. Different parameters involving the number, duration and electric field of electric pulses hardly improved the electroporation-mediated transgene expression by pDNA/vector complex (data not shown). The results of transgene expression in HepG2 cells strongly support the hypothesis that the free form of pDNA is more effective than its complexed form for transgene expression once it enters into the cytoplasm of cells.

In order to achieve liver-directed gene transfer, naked pDNA has been administered by various methods, including local tissue injection,[16] electroporation after local injection,[12,13] intraportal injection in a hypertonic solution,[26,27] gene gun,[28] and large-volume injection at a high velocity.[7,8] In some cases, transfection efficiency by naked pDNA exceeds that by the pDNA/vector complex. Electroporation generally increases the level of transgene expression up to 1000-fold, but the distribution of cells expressing the transgene product is still limited to around the injection site. It is expected that, when pDNA is injected into the vasculature instead of into the tissue parenchyma, it will be delivered to the vicinity of a large number of cells. Rapid injection of naked pDNA solution into the vasculature has resulted in high transgene expression in a large number of myotubes.[29] Furthermore, intraportal injection of pDNA in a hypertonic solution achieved widespread distribution of transfected cells throughout the liver.[26,27] These approaches, however, need to be studied to investigate the tissue damage induced by the procedures. Although electroporation might also induce tissue damage after its application onto the liver surface, we observed very little GPT leakage and little change in appearance, indicating that the conditions for electroporation used in this study do not result in severe tissue damage. This is marked contrast to the large volume injection of pDNA solution at high-velocity, which induced the leakage of enormous amounts of liver transaminases over a short period after injection.[30]

When intravenously injected, naked pDNA undergoes rapid degradation by nuclease and clearance by Kupffer cells, splenic macrophages, and sinusoidal endothelial cells in the liver.[18,19] No detectable transgene expression was observed in the liver. It is suggested that when electric pulses are applied to the surface of the liver, intravenously injected naked pDNA will pass through the discontinuous endothelium of the liver vasculature, reach the surface of hepatocytes, and then enter the cytoplasm through the pores created by electroporation. The application of electric pulses at different intervals clearly demonstrated that the electroporation-induced gene transfer is mediated by pDNA present in the circulation (Figure 6). There was a good correlation between the plasma concentration of undegraded pDNA and transgene expression (correlation coefficient: $r^2 = 0.97$). These findings suggest that any approach to increase the plasma retention, not to increase delivery to the liver, of pDNA could further improve electroporation-mediated gene transfer to the organ.

pDNA is significantly taken up by liver NPC due to its strong negative charge.[18,19] Electroporation did alter the distribution of ^{32}P-pDNA between hepatocytes (PC) and NPC: the distribution ratio of naked ^{32}P-pDNA to PC was greater in the electroporated lobe compared with that in the non-electroporated lobe (Figure 9a). These results suggest that electroporation selectively increases the delivery of pDNA to PC. Anatomical features do not explain the selectivity because PC are localized underneath the lining of the sinusoidal endothelial cells that should have an initial contact with vascularly injected pDNA. Canatella and Prausnitz[31] experimentally and theoretically derived an equation to predict the effects of electroporation on the uptake of solute by cells and their viability. According to this equation, the cell volume is a major factor determining the number of solute (eg pDNA) molecules delivered to the cell; liver PC, or hepatocytes, are much larger in size than liver NPC. Such a difference in size would explain the selective increase in the uptake of pDNA by hepatocytes. Transgene expression correlated well with the amount of pDNA delivered to each type of cells, and hepatocytes had a greater amount of expression than NPC. The efficiency of expression would depend on the cell type, and macrophages such as Kupffer cells undergo transfection with difficulty, which would broaden the difference between hepatocytes and other cells in terms of transgene expression.

In the present study, we successfully demonstrated that the total number of transfected cells was much greater in the liver of mice given an intravenous injection of naked pDNA followed by electroporation than in animals given a local injection of pDNA into the liver (Figure 10). A further increase in this number could be achieved by modification of the size and shape of the electrodes, because the cells between or around them were the major cells transfected.

In conclusion, a more than 200 000-fold increase in transgene expression in the liver was achieved by *in vivo* electroporation after intravenous injection of naked pDNA. pDNA in blood circulation is delivered to the inside of the cells, leading to transgene expression. Although the galactosylated vector is effective in delivering pDNA to hepatocytes through the receptor-mediated process, the vector itself can be an obstacle in the electroporation-mediated increase in transgene expression. These results indicate that the application of electroporation to vascularly administered naked pDNA is a useful gene transfer method for reaching a large number of hepatocytes, which is a key factor in determining the therapeutic efficacy of the *in vivo* gene therapy approach to treating hepatic diseases lacking any intracellular protein, liver cancer, viral hepatitis and allograft rejection. Clinical application of this approach may be achieved in combination with abdominal operation, or by the development of endoscope-type electrodes.

Materials and methods

Chemicals

[α-^{32}P]dCTP was obtained from Amersham (Tokyo, Japan). Poly(L-lysine) (PLL: average molecular weight 29 000) and branched polyethyleneimine (PEI: 70 000)

were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Biowhittaker (Walkersvile, MD, USA), respectively. All other chemicals were obtained commercially as reagent-grade products. Galactosylation of PLL and PEI was performed as reported previously,[3,18] by covalently binding 2-imino-2-methoxyethyl 1-thiogalactoside to each polymer. The numbers of galactose molecules per polymer were: 44 for Gal-PLL and 60 for Gal-PEI.

Animals
Male ddY mice (5 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained on a standard food and water diet and housed under conventional conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Kyoto University.

Preparation of pDNA
pDNA encoding firefly luciferase cDNA under the control of CMV-IE promoter was prepared as previously reported.[2] pCMV.SPORT-βgal was purchased from GibcoBRL (GibcoBRL, Carlsbad, CA, USA). pDNA was amplified in DH5α, isolated and purified using a QIAGEN Plasmid Giga Kit (QIAGEN, Hilden, Germany). The purity of the pDNA was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining. The pDNA concentration was measured by UV absorption at 260 nm. pDNA was labeled with [α-^{32}P]dCTP by nick translation for biodistribution experiments.[19]

Formation of pDNA complex
pDNA complex was prepared in 5% dextrose solution. Various amounts of Gal-PLL or Gal-PEI were added to pDNA, and the mixture was tapped and then left for over 30 min at room temperature. The N/P ratio, the ratio of the concentration of total nitrogen atoms in the polymer to the phosphate group (P) in pDNA, was used as an index of formation. Based on the complex formation and previous studies, N/P ratios were set at 1.2 and 2.4 for the Gal-PLL complex, and 3 and 7 for the Gal-PEI complex.

Electrodes and electric pulse delivery
Electric pulses were delivered to the liver by a pair of 1-cm^2 forceps-type electrodes connected to a rectangular direct current generator (CUY21, Nepagene, Chiba, Japan).

Electric gene delivery after systemic administration of pDNA complex
Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). A midline incision was made on the abdomen and the liver was exposed. Then 5% dextrose solution of naked pDNA or pDNA complex was injected into the tail vein at a fixed dose of 25 μg pDNA/mouse. Unless otherwise indicated, electric pulses were delivered to the left lateral lobe of the liver through the electrodes at 30 s after injection. The electric pulse parameters were: 5 ms/pulse, 12 pulses, 4 Hz, and variable electric field from 50 to 500 V/cm. The incision was closed with metal clips or sutured. At 6 h after pDNA injection, the mice were killed and the liver was excised and divided into two parts: the electroporated lobe (the left lateral lobe) and the other lobes. The liver lobes were homogenized with a five-fold excess of lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). Then the homogenates were subjected to three cycles of freezing and thawing, and centrifuged at 10 000 *g* for 8 min at 4°C. The supernatant was used for the measurement of luciferase assay. The protein content of each supernatant was also determined using a Protein Quantification Kit (Dojindo Molecular Technologies, Kumamoto, Japan). The GPT level in plasma was measured using a GPT-UV test kit (Wako, Osaka, Japan).

In vivo distribution experiment
^{32}P-pDNA was added to unlabeled pDNA to give an injection dose of 25 μg pDNA/mouse. Each mouse was injected with ^{32}P-pDNA or its complex in 5% dextrose solution. Then, electric pulses of 250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz were applied to the left lateral lobe of the liver at 30 s after injection. At 1, 5, 10 and 30 min after injection, groups of three mice each were anesthetized with ether and blood was collected from the vena cava and plasma samples were obtained by centrifugation. The liver, kidney, spleen, lung and heart were excised, rinsed with saline and weighed. These organs were homogenized with 0.05% Triton X-100 solution. Each sample was dissolved in Soluene-350 (Packard, Netherlands), then scintillation medium (Clear-sol I, Nacalai Tesque, Kyoto, Japan) was added and the ^{32}P-radioactivity was counted in an LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan). Radioactivity derived from the plasma in each tissue was corrected for as previously reported.[2]

Isolation of liver PC and NPC
pDNA or ^{32}P-pDNA was injected and electric pulses were applied as above. At 10 min (for radioactivity measurement) or 6 h (for luciferase assay) after injection, liver cells were separated into hepatocytes (PC) and NPC as reported previously.[18] In brief, the liver was perfused with a buffer containing collagenase. The dispersed cells were separated into PC and NPC fractions by differential centrifugation. These cell fractions were subjected to ^{32}P-radioactivity measurement or luciferase assay.

Stability of pDNA in plasma after intravenous injection
Mice received 25 μg pDNA into the tail vein. At indicated time points, 500 μl blood was collected from the vena cava. pDNA was extracted and electrophoresed as reported previously.[19] The amount of pDNA was evaluated by computerized densitometry.

Assessment of transgene-positive cells by X-gal staining of whole liver
Naked pCMV.SPORT-βgal (25 μg/mouse) was administered as described above. The liver was removed at 24 h after injection, then placed in a 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, and rinsed three times with a 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). β-Galactosidase activity was detected by immersing the liver in 5-bromo-

4-chloro-3-indolyl-β-galactopyranoside (X-gal, Sigma) staining solution (1 mg/ml X-gal, 5 mM ferricyanide and 5 mM ferrocyanide at pH 7.3–7.6 in the wash buffer) for 16–24 h at 37°C. For comparison, the same amount of pDNA in 20 μl 5% dextrose solution was injected directly into the lobe and the X-gal staining was performed in a similar manner.

Quantification of β-galactosidase-expressing hepatocytes

At 48 h after administration of pCMV.SPORT-βgal, hepatocytes were isolated as above from the left lateral lobe of the liver, the lobe receiving the electric pulses after intravenous injection of naked pDNA (i.v. injection+EP group) or an injection of naked pDNA (direct injection group). The hepatocytes isolated and supplemented with William medium E containing 10% fetal bovine serum were seeded into culture plates. After 12-h incubation, the hepatocytes were washed, fixed with 0.25% glutaraldehyde for 20 min at 4°C, then stained with X-gal solution (1 mg/ml in phosphate-buffered saline, pH 7.4) for 16–24 h at 37°C. More than 100 individual cells per sample were observed under a microscope, and the number of blue cells, that is, β-galactosidase-expressing cells, was counted. Results are expressed as the percentage of β-galactosidase-expressing hepatocytes in the left lateral lobe of the liver.

Transfection to HepG2 cells

HepG2 cells, a human hepatoma cell line, were seeded to 2×10^6 cells in Dulbecco's minimum essential medium/10% fetal bovine serum in six-well plates for 24 h prior to transfection. Naked pDNA or pDNA/Gal-PLL was added to the cells and electric pulses (95 V, 5 ms/pulse, 12 pulses, 10 Hz) were delivered to the cells through an electrode for cultured cells. The luciferase activity was assayed at 24 h after transfection.

Statistical analysis

Data were statistically analyzed by Student's *t*-test.

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