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Direct gene transfer into rat liver cells by in vivo electroporation

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Abstract In vivo electro-transfection efficiency and manner of transferred gene expression were investigated by fluorescence microscopic image analysis. Green fluorescent protein (GFP) gene was used as the genetic marker. Electroporation was carried out on the liver of live rats by use of disk electrodes mounted in the tips of tweezers, which were directly pressed onto the surface of a liver lobe in situ. Electroporation with eight electric pulses of 50 ms in duration at 50 V gave a good efficiency of transfection as judged by the induced GFP expression. Bright fluorescence of GFP appeared as dots, which were scattered around the area damaged by electroporation. The transfection efficiency increased as the amount of injected DNA was increased. The results indicate that the amount of induced gene expression can be controlled. Estimation of the efficiency of electro-gene transfer using the fluorescence of GFP and digital analysis of microscopic images was useful to determine the optimum conditions for local gene therapy in tissues and organs.

Key words: Electroporation; In vivo; Green fluorescent protein; Rat liver; DNA dosage; Electric pulse; Local gene therapy

1. Introduction

Methods for introduction of foreign genes into the cells of living animal tissues in vivo, which have been carried out by the use of agents such as viral vectors and cationic liposomes, are powerful tools for research into the function and behavior of biological macromolecules in the living body and have a possible application in gene therapy [1–9]. Although these techniques have been successfully used, they exhibit a variety of shortcomings, and the ideal techniques applicable for all types of living tissues have not yet been established [10].

The method using adenoviral vectors can introduce foreign genes into differentiated non-dividing cells in living animal tissues [1–3,6,7,9]. However, the preparation of recombinant viral granules is cumbersome, and the efficiency of recombinant formation is sometimes very low. Moreover, the foreign genes introduced with adenoviral vectors are not integrated into the genomic DNAs of the host cells, and are basically only expressed transiently. Retroviral vectors are widely used for transfection of cultured cells, since they are simple to construct and integrate the foreign genes efficiently into the host genome. Such vectors, however, can infect only propagating cells, and are inactivated by complements in the blood stream [10]. Therefore the practical application of these vectors for in vivo transfection is considerably restricted.

As for the non-viral delivery of DNA, lipofection with cationic liposomes associated with anionic plasmid DNA was shown to deliver foreign genes efficiently into cells in vitro, and recently in vivo by endocytotic machinery [4,5,11–13]. Endocytosed plasmid DNAs are, however, liable to be digested in the lysosome, and cationic liposomes have some cytotoxicity [10]. A particle-gun powered either by high-pressure gas or by a gunpowder explosion successfully introduced metallo-particles coated with foreign plasmid DNAs into living cells. Recently, a handy particle-gun that does not require a decompression chamber was introduced, making the direct gene transfer into living animal tissue cells much easier [14]. With this technique, however, metallo-particles only reach the cells on the surface of organs. Therefore, the target tissue cells must be exposed by surgical operation, and this technique seems to be useful in only restricted tissue cells.

Electroporation efficiently introduces foreign genes into living cells [15–17], but the use of this technique had been restricted to suspensions of cultured cells only, since the electric pulses are administered in a cuvette-type electrode. Recently, various types of electrodes have been developed, and electroinjection of chemicals or foreign genes was applied in vivo [18–25]. Whereas the electroporation with a cuvette-type electrode is carried out with an electric pulse administered at several kilovolts for several microseconds, multiple pulses under much lower voltage for several milliseconds are used for in vivo electroporation. The optimum conditions of electro-gene transfer, however, have not yet been generalized.

In the present study, the effects of variation of electric voltage, pulse time, and injected plasmid DNA dosage on electrotransferred gene expression were investigated by in vivo electroporation of living rat liver with the green fluorescent protein (GFP) gene. The efficiency of electro-gene transfer was evaluated from the autofluorescence of GFP and digital analysis of microscopic images.

2. Materials and methods

2.1. Plasmid for GFP expression

The expression vector pEGFP-C1, harboring a red-shifted variant of wild-type GFP and having been optimized for brighter fluorescence and higher expression in mammalian cells under the control of the CMV promoter, was from Clontech Laboratories (Palo Alto, CA). Plasmid DNA was multiplied by culturing the host bacteria JM109 and isolated with a Qiagen plasmid isolation kit (Qiagen, Santa Clarita, CA).

2.2. Electric pulse generator and electrode

Electric pulses were generated with an Electro Square Porator (T820; BTX, San Diego, CA). Pulses were given to the organ by a pair of electrode disks (1 cm diameter) rigged on the tips of tweezers (Pinsettes-Type electrode 449-10PRG; Meiwa Shoji, Tokyo, Japan).

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Abbreviations: GFP, green fluorescent protein; PBS, phosphate-buffered saline; CMV, cytomegalovirus

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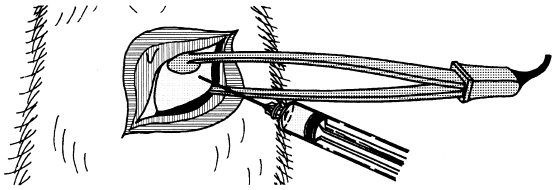


Fig. 1. A scheme showing in vivo electroporation in the living rat liver in situ. The center of the lobe of the liver was placed between the tweezer-type electrode disks, and the plasmid DNA was injected into the liver tissue just midway between the two electrode disks. Immediately after the DNA injection, electric pulses were administered.

One to eight electric pulses were administered at a rate of one pulse per second. Pulses were delivered to each rat liver, which was placed between the electrode disks separated by a distance of approximately 2 mm (Fig. 1). Electric pulses were 25, 50, or 99 ms in duration at voltages of 25, 50, or 100 V; the resistance was monitored with a graphic pulse analyzer (Optimizer 500, BTX, San Diego, CA).

2.3. Electric gene transfer

Male 6-week-old Wistar rats were anesthetized with diethyl ether. The left median or left lateral lobe of the liver was exposed by making a transverse incision starting from the mid-sagittal position, approximately 5 mm under the xiphoid process, and extending 2.0–2.5 cm toward the left-lateral surface of the rat. The center of the lobe was caught between the tweezer-type electrode disks (Fig. 1), and the electric resistance of the tissue was measured. The plasmid DNA in 100 μl of phosphate-buffered saline (PBS) was injected with a 27-gauge needle into the tissue halfway between the two electrode disks (Fig. 1). Immediately after the DNA injection, electric pulses were administered. Electric resistance of the tissues was measured once again just after the electric administration. The abdominal wound was closed by suturing the muscles together and then stapling the skin incision. Rats were killed 48 h after the electroporation, by cervical dislocation under ether anesthesia, and the treated lobes of the liver were sampled.

2.4. Measurement of GFP expression

Liver specimens were sliced at a thickness of 1.73 mm with an array of razor blades, and fixed in 3% paraformaldehyde in PBS for 2 h on ice. Fixed liver slices were mounted on glass slides with mounting medium (5% 1,4-diazabicyclo[2,2,2]octane, 100 mM Tris-HCl, pH 8.0, 90% glycerol). Green fluorescence showing expressed GFP was captured with an AX-70 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a cooled-CCD camera (PXL1400, Photometrics, Tucson, AZ) and processed with IPLab image analyzing software (IPLab Spectrum, Signal Analytics, Vienna, VA). The GFP fluorescence signals were observed with a U-MNIBA filter-dichroic mirror cube set (Olympus, Tokyo, Japan) under a 4× objective (UPlanApo 4×/0.16, Olympus, Tokyo, Japan). Signal intensities and areas were measured with NIH image digital image analyzing software.

2.5. Laser confocal microscopy

Fresh liver specimens were embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen and stored at −80°C until use. Frozen sections (10 μm thick) were cut with a cryostat and affixed onto glass slides. The sections were fixed with 3% paraformaldehyde at room temperature for 5 min, rinsed with PBS, and mounted in a drop of the mounting medium containing 2 μM YO-PRO-1 for cell nuclear counterstaining [26,27], and examined with a BX-50 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a MRC-1024 laser confocal system (Bio-Rad Laboratories, Hercules, CA) utilizing a 15-mW krypton/argon laser.

2.6. Measurement of the necrotized area in electric-administered tissues

For estimation of the damage caused by electric pulses, the area of necrosis was measured in each liver slice used for the measurement of GFP expression. Color images of these samples on slides were captured as digital image files with a digital film scanner (QuickScan 35, Minolta, Osaka, Japan), and the area was measured with NIH image software.

2.7. Statistical analysis

Comparisons of values were analyzed by Student's *t*-test or Cochran-Cox's test with $P < 0.05$ considered to indicate statistical significance.

3. Results

To observe the expression pattern of the in vivo electrotransferred GFP gene, we injected pEGFP-C1 plasmid DNA (40 μg in 100 μl PBS) into the living rat liver in situ and administered eight pulses of 50 μs duration at 50 V. Liver

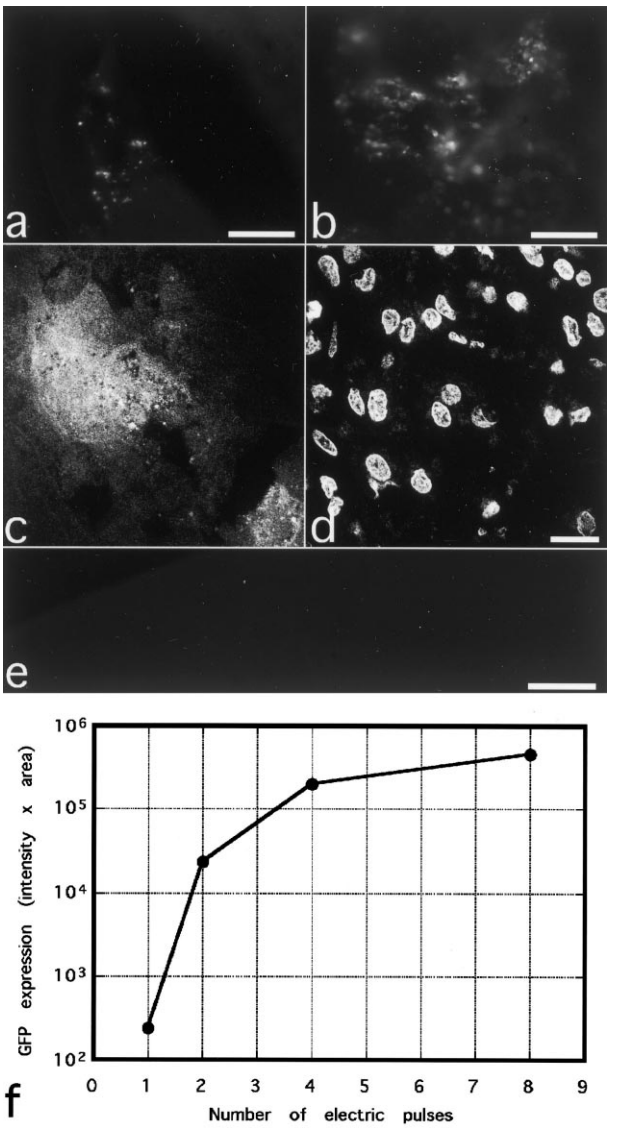


Fig. 2. Fluorescence images of the expressed GFP in the rat liver. The rat liver received 40 μg of pEGFP-C1 plasmid DNA and eight pulses of 50 ms duration at 50 V in situ (a–d). Slices of the liver specimens were observed with a fluorescence microscope (a,b) and a laser confocal microscope (c,d). a: Survey view. Dots of GFP fluorescence are seen. Scale bar = 1 mm. b: GFP signal dots are of various shapes and sizes. Scale bar = 500 μm. c,d: High-magnification laser confocal images of a GFP signal dot (c) and the cell nuclei in the same area (d). Note that each signal dot as seen in b consists of a few to several tens of GFP-expressing cells. Scale bar = 20 μm. e: The liver received 40 μg of pEGFP-C1 without electric administration. Scale bar = 500 μm. f: Effects of the number of electric pulses in the electroporation on the efficiency of gene transfer.

specimens were sliced at a thickness of 1.73 mm, and the green fluorescence signals of expressed GFP were examined. Clusters of dotty fluorescence were observed (Fig. 2a) with dots having various shapes and sizes (Fig. 2b). For further analysis of the expression pattern of the induced GFP, frozen sections of the liver were observed by laser confocal microscopy. A signal dot was resolved to a few to several tens of GFP-expressing cells (Fig. 2c,d). These results show that the plasmid DNA molecules were successfully introduced into the liver tissue cells. No GFP signals were observed in the experiment with plasmid DNA injection without electric administration (Fig. 2e). The GFP expression level was increased as the number of electric pulses per administration increased from one to four (Fig. 2f).

To investigate the effects of voltage and duration of pulse time on the efficiency of gene transfer, we injected 40 μg of plasmid DNA into the living rat liver and administered eight electric pulses, which were 25, 50, or 99 μs in duration at voltages of 25, 50, or 100 V. When the electroporation was carried out at 25 V, only weak signals were observed. The number of signal dots increased as the pulse duration increased without affecting the signal intensity and size of each dot (Fig. 3a–c). The electro-transfection efficiency was the highest with 50-V electric pulses (Fig. 3d–f, Fig. 4). The amount of GFP expression at 50 V was over 100-fold greater than that at 25 V. Among the experiments with 50 V, the 50-ms duration of the electric pulse showed the highest efficiency of gene transfer. The amount of GFP expression with the 50-ms pulse at this voltage was more than 3.5-fold larger than that with 25- or 99-ms pulses. The efficiency of gene transfer with 100 V decreased compared with that with 50 V (Fig. 4). The decrease at 100 V may have been due to necrosis of the cells damaged by the electric pulses. The 100-V pulses caused extreme damage to the liver tissue directly sandwiched between the electrode disks (Fig. 5a–c). The extent of tissue damage was measured in the same slices used for analysis of GFP expression. The necrotized area enlarged considerably as the administered voltage was increased, and also enlarged as the duration of each pulse time was increased (Fig. 5d). It was extremely enlarged with 100-V administration, about 5-fold more than that with the 50-V treatment; and most of the tissues sandwiched by electrode disks were damaged to the point of death. In either case the fluorescence signals of GFP were seen concentrated along the border between the

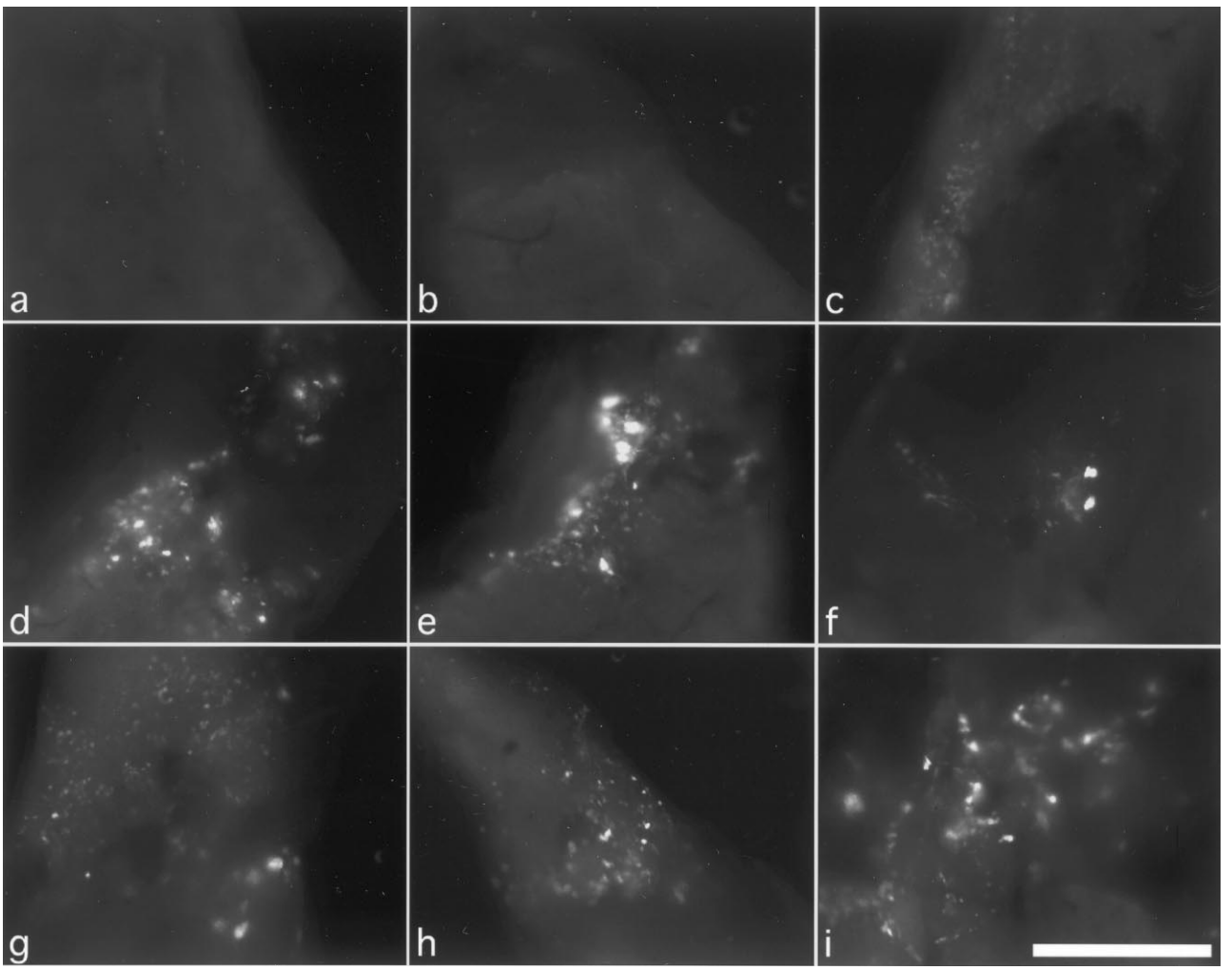


Fig. 3. GFP fluorescence images in rat livers which were injected with 40 μg of plasmid DNA and received eight electric pulses of 25 ms (a,d,g), 50 ms (b,e,h), and 99 ms (c,f,i) duration at 25 V (a,b,c), 50 V (d,e,f), and 100 V (g,h,i). Each image was captured and processed under the same conditions. Scale bar = 1 mm.

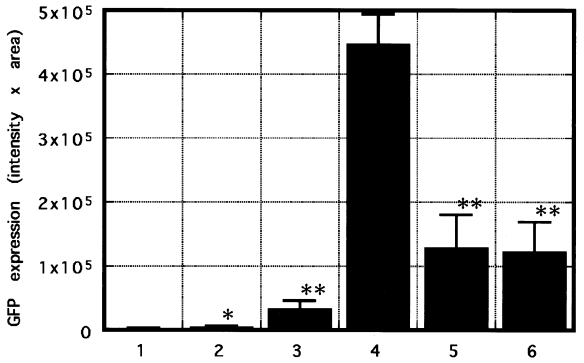
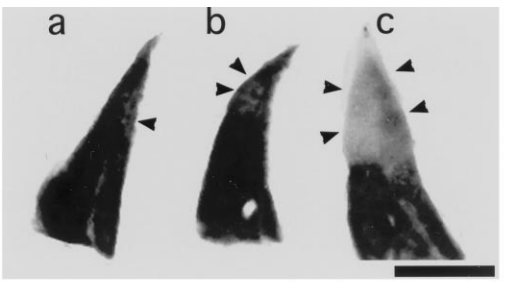


Fig. 4. Effects of voltage and duration of pulse time on the efficiency of gene transfer. Left-lateral lobes of the liver injected with 40 μg/100 μl of plasmid DNA (2–6) or 100 μl of PBS (1) were examined. Each GFP expression level was estimated from digital fluorescence-microscopic images of all slices of the lobe by integration of the signal intensity and total signal area. Each digital image was captured and processed under the same conditions. (1) 50 V, 50 ms, without DNA. (2) 25 V, 50 ms. (3) 50 V, 25 ms. (4) 50 V, 50 ms. (5) 50 V, 99 ms. (6) 100 V, 50 ms. Results are means ± S.E.M. of triplicate samples. The single and double asterisks indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively, as compared with 50 V at 50 ms (lane 4).

necrotized area and the surviving area, indicating that the voltage that just allowed the tissue to escape death was the most efficient for gene transfer.

The effect of injected DNA dosage on the efficiency of gene transfer was examined by changing the amount of plasmid



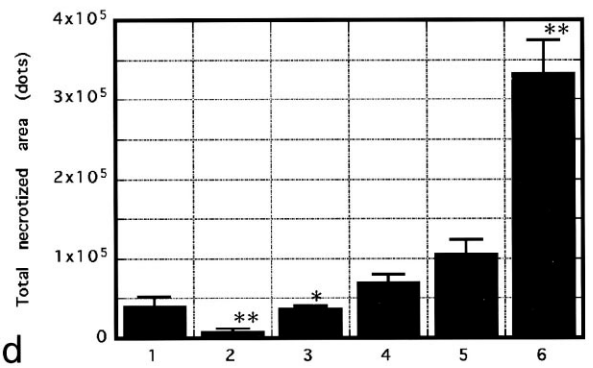


Fig. 5. Effect of voltage and duration of pulse time on cell damage. Electric pulses of 50 ms duration at 25 (a), 50 (b), and 100 (c) V were applied. Necrotized regions seen as bright areas are marked with arrowheads. Scale bar = 5 mm. d: Extent of tissue damage by electric administration. Each necrotized area was estimated as the sum of the discolored areas from digital color images of all the liver slices. Numbers on the abscissa are the same as in Fig. 4. Results are means ± S.E.M. of triplicate samples. The single and double asterisks indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively, as compared with 50 V at 50 ms (lane 4).

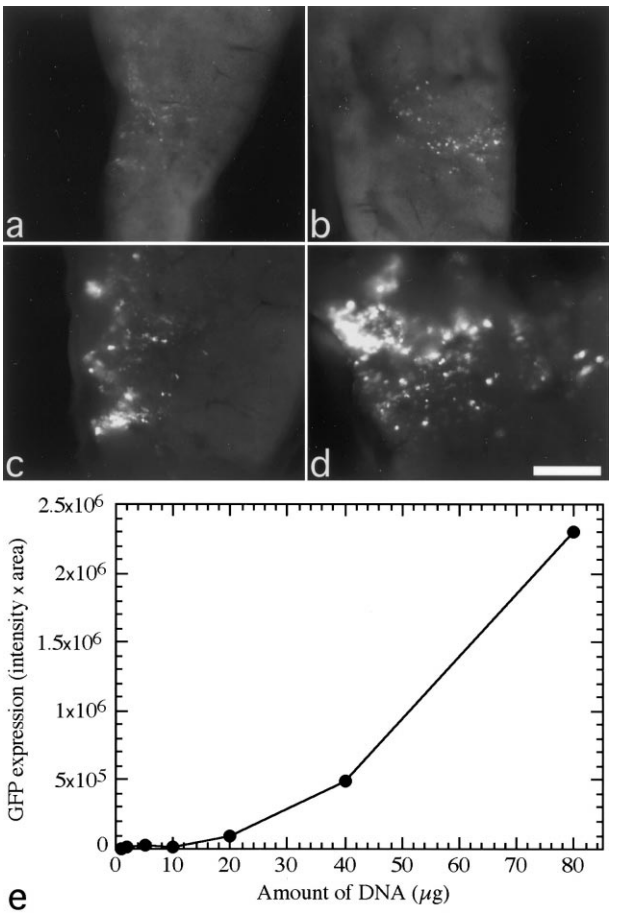


Fig. 6. Effect of injected DNA dosage on the efficiency of gene transfer. Various amounts of plasmid DNA were injected into the living rat liver in situ, which was then administered eight electric pulses of 50 ms at 50 V. a–d: Fluorescence images of expressed GFP with 1 (a), 10 (b), 40 (c), and 80 (d) μg of DNA injected. Scale bar= 500 μm. e: GFP expression levels at various amounts of DNA injection.

DNA injected. Equal volumes (100 μl) of DNA solution containing 1–80 μg of pEGFP-C1 plasmid DNA were injected into the living rat liver in situ, and eight electric pulses of 50 V and 50 ms were applied. When the electroporation was carried out with 1 μg of DNA, a low level expression of GFP was observed (Fig. 6a). The amount of GFP signal with 1 μg DNA injection was less than 1% of that with the 40-μg injection (Fig. 6e). When 10 μg or less of DNA was injected, the number and size of GFP signals were very small (Fig. 6b). The GFP signals increased as the amount of injected DNA increased; GFP signals with the 80-μg injection were 600- and 4.6-fold greater than those with 1 μg and 40 μg, respectively (Fig. 6c–e). These results indicate that the foreign DNA was transferred into the liver cells more efficiently as the amount of injected DNA was increased, suggesting that the amount of induced gene expression is controllable.

4. Discussion

The results of this study demonstrate that electro-transfer of foreign DNA in situ in a living rat can be successfully achieved using disk-shaped electrodes. The electro-transferred GFP expression efficiency was dependent on the amount of

DNA injected into the tissue. Heller et al. [23] used three pairs of electrodes, which consisted of six needles arrayed at even intervals around a 1 cm diameter circle; and six pulses were administered with rotation of the pairs and direction of the electrodes. An apparent maximum expression of the marker gene was observed when 25 μg of plasmid DNA was injected, which stands in marked contrast to the dose-dependent increase in the efficiency of electro-transfer that we observed. Our results, however, are in good agreement with those of in vitro electroporation studies, in which dose-dependent expression was observed [17,28]. In the present study, the liver lobe was sandwiched between a pair of electrode disks. This type of electrode has a close resemblance to the commonly used cuvette-type electrode used in in vitro electroporation. Furthermore, we administered eight pulses with the same electric current direction per electroporation. The shapes of the electric field and the effects of electrophoresis on the injected DNA might be different for these two methods, resulting in the difference in dose dependence [17]. When only 1 μg of DNA was injected, a small but sufficient amount of GFP expression signal for microscopic analysis of induced gene products was observed (Fig. 6a). The sizes of the signal dots were larger when 20 μg of plasmid DNA was injected, and the intensity of each signal dot increased drastically with the 40-μg injection. In vivo electroporation with 20–40 μg of plasmid DNA injected produced many signals dots of various sizes and intensities. When 80 μg of DNA was injected, many large signal dots with bright fluorescence were observed. Massive DNA injection, however, may result in over-expression, and the consumption of a large amount of plasmid DNA is of no practical use.

It was previously reported that foreign genes were uniformly transferred into the administered tissue cells by in vivo electroporation [23], but the morphological investigation was carried out insufficiently. Our observations covering the entire lobe clearly showed that the foreign genes were successfully transferred into the cells scattered around the administered region and that the expression of the foreign gene was concentrated at the surviving area close to the necrotized regions. These results suggest that the plasmid DNA molecules were borne by the blood stream and accumulated at the specific regions of the tissue. They were then transferred into the neighboring cells passing through the holes opened by the electric pulses.

Electroporation with tweezer-type electrodes gave successful gene transfer in situ. However, improvement of the electrode shape for minimum tissue damage and maximum gene transfer efficiency is desired. In vivo electroporation, which enables efficient gene transfer and expression in designated regions of living organs, is being watched as an attractive approach for local gene therapy. Our results, however, indicate that the injected plasmid DNAs were transferred into cells scattered around the administered area, and thus caution should be exercised in practical use. Furthermore, the optimum conditions for in vivo electroporation may vary depending on tissues, organs, and animal species. In addition, estimation of the efficiency of electro-gene transfer using GFP and digital analysis of fluorescence microscopic images seems to be useful to examine the optimum conditions for local gene transfer in situ.

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