Key Physiological Phenomena Governing Transgene Expression Based on Tissue Pressure-Mediated Transfection in Mice

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It is generally recognized that in vivo gene transfection is one of the most important techniques used in the post-genome era. Above all, naked plasmid DNA transfection has attracted much attention because of its advantages including convenience of preparation and handling and lack of toxicity associated with the transfection agents. We have investigated tissue pressure-mediated transfection performed by light and controlled pressure of the target tissue after normal intravenous injection of plasmid DNA. So far, we have demonstrated that plasmid DNA and small-interfering RNA (siRNA) are very efficiently transfected into murine kidney, liver and spleen without causing marked tissue damage. In this study, in order to understand the key physiological phenomena affecting transgene expression, we performed a set of experiments involving tissue pressure-mediated transfection, including the biodistribution and cellular transport of plasmid DNA and activation of transcriptional factors and obtained the following results: i) plasmid DNA transfer to the target tissue and its cells increased although the transferred fraction was small compared to the total administered plasmid DNA, ii) a transient increase in cellular translocation of plasmid DNA was induced, and iii) transcriptional factors were activated. Taking all these results into consideration, it would appear that tissue pressure-mediated transfection enhances plasmid DNA transfer to the target tissue and its cells and also activation of the transcriptional process. This information will allow a better understanding of in vivo transgene expression based on naked plasmid DNA transfection involving tissue pressure-mediated transfection.

Key words transgene expression; tissue pressure-mediated transfection; naked plasmid DNA

It is recognized that in vivo gene transfection is one of the most important techniques used in biological research, drug therapy development and clinical treatment in the post-genome era. Above all, naked plasmid DNA transfection without viral vectors, synthetic carriers and special physical devices has attracted attention because it is versatile, and has a number of advantages including convenience of preparation and handling and a lack of toxicity associated with the transfection agents. In 1990, muscle cells were transfected with plasmid DNA by direct injection of naked plasmid DNA solution into the muscle tissue in mice4 and then this technique has been used in some gene therapy applications based on plasmid DNA injection encoding secretory proteins.2,3 Using the same strategy, various organs were transfected with plasmid DNA, including the liver and tumors.4,5 Gene expression obtained by the direct injection into the target tissues is limited to the injection site while gene transfection via blood flow is superior to direct injection from the point of view of transfection to many cells in the target tissue. Although normal intravenous injection of plasmid DNA solution offers low transfection efficiency,6 relatively high gene expression in many hepatic cells has been reported to be obtained by rapid intravenous injection of large-volume plasmid DNA solution.7,8 This very simple and convenient method, hydrodynamics-based transfection, has attracted much attention and has been used for a variety of purposes.9 Regrettably, its application is limited to the liver and also the method can cause severe liver injury, including marked increase in the levels of serum aspartate and alanine transaminases.10

We have investigated tissue pressure-mediated transfection in order to develop a naked plasmid DNA transfection method with a high gene transfection efficiency and which causes little tissue injury.11,12 This transfection is performed by light and controlled pressure of the target tissue after normal intravenous injection of plasmid DNA. It is a development that is based on the phenomena described by Liu and Huang in their pioneering report where liver-specific gene expression could be obtained without liver toxicity by massaging the abdomen after intravenous injection of plasmid DNA in mice.10 We have been successful in quantitatively controlling the degree of pressure on tissues, and have suggested that plasmid DNA and small-interfering RNA (siRNA) are very efficiently transfected into the murine kidney, liver and spleen without marked tissue damage and this includes an increase in biomarker and pro-inflammatory cytokine production.11,12

Understanding the key physiological phenomena affecting transgene expression is very important for the proper use of this method and to allow improvements to be made. In general, there are many barriers to gene expression following administration of plasmid DNA: biodistribution, cellular and nuclear translocation of plasmid DNA and transcription and translation.13 Biodistribution and cellular translocation of plasmid DNA are difficult to control because of its rapid hepatic accumulation by the reticuloendothelial system14 and weak interaction with cell membranes by electrostatic repulsion13 and, therefore, these are important processes for the development of gene transfection methods. Also, with regard to the transcription process, Hama et al. developed an evaluation system of administered DNA localization in culture cells using confocal microscopy and real-time polymerase chain
reaction (PCR) and then suggested that transcription is a crucial process determining the difference in the level of gene expression between adenovirus and LipofectAMINE-mediated transfection. However, the role of each factor in transgene expression with regard to tissue pressure-mediated transfection has not been well documented.

In this study, we investigated and discussed the biodistribution, distribution at a tissue level and cellular transport of administered plasmid DNA and also the activation of transcriptional factors from the point of view of affecting transgene expression based on tissue pressure-mediated transfection.

**Materials and Methods**

**Plasmid DNAs** The previously constructed cytomegalovirus (CMV) early immediate promoter-driven plasmid encoding the complementary DNA luciferase (pCMV-Luc) was used. Mercury Pathway Profiling Luciferase system 1 (pTAL-Luc, pAP-Luc, plasmid nuclear factor (pNF)xB-Luc, pCRE-Luc and pSRE-Luc; No. 631911) was purchased from BD Biosciences Clontech (Palo Alto, CA, U.S.A.). pCMV-Luc and plasmid DNAs of the Mercury Pathway Profiling Luciferase system were amplified in the *Escherichia coli* strain DH5α (Toyobo, Co., Ltd., Osaka, Japan), then isolated, and purified using a JETSTAR2.0 Plasmid Giga Kit (GENOMED, GmbH, Löhne, Germany). [32P] pCMV-Luc was obtained by nick translation using [α-32P]-dCTP (Perkin-Elmer, Inc., Boston, MA, U.S.A.), deoxyribonuclease I, DNA polymerase I, dATP, dGTP and dTTP (Takara Bio, Inc., Otsu, Japan), as described previously. TM-Rhodamine labeled pCMV-Luc was synthesized using the Label IT Nucleic Acid Labeling Kit, TM-Rhodamine (Mirus Bio LLC, Madison, WI, U.S.A.).

**Animals** Female ICR mice (5 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the U.S. National Institutes of Health and the guideline for animal experiments of Kyoto University.

**Plasmid DNA Transfection by Tissue Pressure-Mediated Transfection and Hydrodynamics-Based Transfection**

In a typical case of tissue pressure-mediated transfection, 100 μg pCMV-Luc in 200 μl saline was intravenously injected into anesthetized mice and the target tissue (the right kidney or left lobe of the liver) exposed by a midline incision in injected into anesthetized mice and the target tissue (the right portion of each tissue was dissolved in 0.7 ml Soluen-350 (Packard Instrument Co., Ltd., Meriden, CT, U.S.A.), followed by addition of 0.2 ml isopropanol, 0.2 ml 30% H2O2, 0.1 ml 5% HCl and 5 ml Creasol I (Nakalai Tesque Co., Ltd., Kyoto, Japan) and the samples were allowed to stand overnight. The radioactivity was determined using Beckman Model LS5000TA (Beckman Coulter, Inc., Fullerton, CA, U.S.A.). Contamination of the plasma in tissue sample was corrected using distribution data of [111In] bovine serum albumin at 10 min after intravenous injection.

**Immunofluorescence Microscopy** Mice were given 10 μg TM-Rhodamine labeled pCMV-Luc by tissue pressure-mediated transfection against the right kidney, which was then excised following blood removal by slow saline injection from the left ventricle of the heart 10 min after administration. Prepared cryostat sections (5 μm) were counterstained by Alexa-fluor488-phalloidin (for F-actin) and examined under a fluorescence microscope (Biozero; Keyence, Corp., Osaka, Japan).

**Luciferase Assay** Luciferase assay was performed as described previously. Relative light units were normalized to milligrams of protein and converted to ng using a standard curve generated with a luciferase enzyme standard (Toyko Ink, Co., Ltd., Tokyo, Japan).

**Evaluation of the c-fos and c-jun mRNA Level by Real-Time PCR** Mice were euthanized and total RNA was extracted from the right kidney or left lobe of liver samples using a Gen Elute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Co., Ltd., St. Louis, MO, U.S.A.). Subsequently, reverse transcription was performed using a PrimeScript RT reagent Kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer’s protocol. For a quantitative analysis of mRNA expression, real-time PCR was carried out according to total cDNA using a Light-Cycler instrument (Roche Diagnostics, Basle, Switzerland). The oligodeoxynucleotide primers used for amplification were as follows: c-fos, forward 5’-CCAGTCAAGAGCATAGC3’, reverse 5’-AAGTAGTCAGCGCCGGATG-3’, c-jun, forward 5’-TCTCCTATGCACATGGAGTC-3’, reverse 5’-TGAAGTGCCGACCACCCTGTTA-3’, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5’-TCTCCCTGACATGGAGTC-3’, reverse 5’-GCTGTAGGCTTCATTG3’. Amplification products were detected on-line via intercalation of the fluorescent dye SYBR green (SYBR Premix Ex Taq; Takara Bio, Inc., Otsu, Japan). The cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 20 s. Gene specific fluorescence was measured at 60 °C.

**Statistical Analysis** GraphPad InStat 3.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.) was used. Statistical significance was determined using a paired or unpaired Student’s *t*-test for two groups. All *p* values are two-tailed and *p*<0.05 was considered to be indicative of statistical significance.

**Results and Discussion**

**Biodistribution and Cellular Uptake of Plasmid DNA Administered by Tissue Pressure-Mediated Transfection** At first, the biodistribution of a plasmid DNA after tissue pressure-mediated transfection against kidney and liver was investigated and compared with a normal intravenous injec-
tion of plasmid DNA solution. We employed a method using a plasmid DNA labeled with [α-32P] dCTP by nick translation. Naked plasmid DNA is largely degraded after 5 min in plasma after following intravenous injection into mice. Therefore, we evaluated the initial biodistribution (up to 5 min) which affects gene expression. The plasmid DNAs were administered at a dose of 100 µg/mouse as a typical dose in this study, because the luciferase gene expression level was directly proportional to the dose of pCMV-Luc in the 10 to 200 µg range (data not shown) or the nonlinear saturation phenomenon was not observed. As shown in Fig. 1a, 20—30% of radioactivity was immediately distributed in liver after normal intravenous injection, although about half was retained in plasma. In contrast, in the other organs including kidney, lung, heart and spleen, very low radioactivity (no more than 2% of the dose) was observed. This biodistribution is characteristic of an anionic macromolecule, based on uptake by the reticuloendothelial system of the liver, and corresponds to the results obtained in previous reports. The biodistribution after tissue pressure-mediated transfection against kidney and liver was the same as that obtained by normal intravenous injection, and immediate liver distribution took place (Figs. 1b, c). In the case of targeting the kidney, a statistically significant increase in radioactivity was observed (Fig. 1d), however, it was not very marked. Regarding application of physical force to the target tissue after intravenous injection of plasmid DNA, biodistribution of plasmid DNA after electroporation against liver, kidney and spleen has been reported. Distribution in electric pulse-applied organs did not exhibit much of a significant increase, and our results corresponded to the finding that biodistribution does not exhibit a significant change. This indicates that the biodistribution of plasmid DNA at an individual level does not explain the high efficient transgene expression of tissue pressure-mediated transfection.

Next, we investigated the plasmid DNA distribution in pressed tissue at a tissue level. Mice were given TM-Rhodamine labeled plasmid DNA by renal pressure-mediated transfection, and then the pressed right kidney was excised after 10 min. Prepared frozen section was counterstained with Alexa-flour488-phalloidin (F-actin) and observed by fluorescence microscopy, and compared with the results obtained after normal intravenous injection. In this examination, we targeted renal pressure-mediated transfection, because the non specific accumulation of plasmid DNA after normal intravenous injection was very low in the kidneys and, consequently, it was hoped that the tissue migration of plasmid DNA would be easy to evaluate. As shown in Fig. 2 left, in the case of renal pressure-mediated transfection, fluorescence derived from TM-Rhodamine labeled plasmid DNA was widely observed at the cortex region, in particular the proximal and distal renal tubules, while no fluorescence was detected from the medulla region. This result corresponded to our previous report showing that gene expression was widely detected at the periphery of the kidney involving the cortex region following imaging of the excised organ after renal pressure-mediated transfection. However, in both sections of the cortex and medulla region prepared from mice given a normal intravenous injection, there was no significant fluorescence of TM-Rhodamine (Fig. 2 right). These results indicate that the increase in plasmid DNA transfer to the target tissue and its cells was caused by tissue pressure-mediated transfection although the transferred fraction was small compared to the total administered plasmid DNA.

Fig. 1. Biodistribution of Plasmid DNA after Administration by Tissue Pressure-Mediated Transfection, Compared with Intravenous Injection Alone

Tissue accumulation of radioactivity after administration of 32P-labeled pCMV-Luc (diluted with unlabeled pCMV-Luc to a total of 100 µg/mouse) by intravenous injection alone without pressure (a), renal tissue pressure-mediated transfection (b), and hepatic tissue pressure-mediated transfection (c). Key: plasma (closed circles), left lobe of liver (closed upper triangles), right kidney (open squares), left kidney (open upper triangles), lung (closed diamonds), heart (open circles), spleen (open diamonds). Right kidney (d) and left lobe of liver (e) accumulation by renal and hepatic tissue pressure-mediated transfection (open lower triangles), respectively, compared with normal intravenous injection (closed circles). *p<0.05, **p<0.01 vs. normal intravenous injection. Each value represents mean±S.D. (n=4).
In addition, in order to obtain information about the cellular uptake of plasmid DNA caused by tissue pressure-mediated transfection, we performed another experiment. In this, the target tissue was pressed for different periods, from 60 s before to 180 s after intravenous plasmid DNA injection into mice, followed by luciferase assay of the tissue. In the case of pressing the right kidney immediately and 180 s after plasmid DNA injection, the luciferase level of the kidney was 2—3 ng/mg protein (Fig. 3a). In contrast, the luciferase level for 10 s before injection was 0.004 ng/mg protein and that for 20—60 s before was almost zero (Fig. 3a). This result suggests that the period of transient increase in cellular translocation of plasmid DNA was approximately 10 s in the case of renal pressure-mediated transfection of mice. On another front, in the case of hepatic pressure-mediated transfection, little luciferase gene expression was obtained (0.02 ng/mg protein) when pressing at 60 s before injection (Fig. 3b) and, therefore, the period was slightly longer.

We should add that the period of transient increase in cellular translocation of plasmid DNA caused by tissue pressure-mediated transfection was quite short, compared with that of hydrodynamics-based transfection (15—30 min),20) although there were some tissue differences. Liu et al. showed a correlation between transgene expression and venous blood pressure and, therefore, argued that this gene expression is a physiological phenomenon like hydrodynamics-based transfection.21) These findings support the view that efficient transgene expression with little tissue damage obtained by tissue pressure-mediated transfection is related to a moderately short-duration increase in cellular translocation.

**Effect of Transcriptional Factors on Gene Expression of Tissue Pressure-Mediated Transfection**

Moreover, we investigated the effect of transcriptional factors on gene expression of tissue pressure-mediated transfection. Transcriptional factors are reported to participate in both the nuclear translocation of plasmid DNA and transcription process22) and, therefore, are important for transgene expression. We performed an experiment using luciferase expression plasmid DNAs carrying an enhancer element activated by different transcriptional factors, including activator protein-1 (AP-1), nuclear factor κB (NFκB), cyclic adenosine 3’,5’-monophosphate response element (CRE) and serum response element (SRE) (Pathway Profiling Luciferase system; pTAL-Luc (negative control), pAP-1-Luc, pNFκB-Luc, pCRE-Luc and pSRE-Luc). Mice were given these plasmid DNAs by tissue pressure-mediated transfection against the right kidney and the left lobe of the liver and then the luciferase level in the target tissue was evaluated. As shown in Figs. 4a, b, significantly-high luciferase gene expression was obtained after pAP-1-Luc and pNFκB-Luc administration in both the kidney and liver, compared with pTAL-Luc that has no binding site for transcriptional factors. Therefore, transgene expression of tissue pressure-mediated transfection is highly dependent on the binding of plasmid DNA to transcriptional factors.

The possibility that tissue pressure-mediated transfection, the activation of transcriptional factors, c-Fos and c-Jun, which are component proteins of AP-1 after performance of tissue pressure-mediated transfection against the kidney and liver, were evaluated at the mRNA level. The levels of c-fos and c-jun mRNA increased rapidly and about a 25- and 5-fold increase, respectively, was observed after 1 h compared with untreated mice (Figs. 4c, d). This increase in mRNA ex-
pression was about 20% that of liver after hydrodynamics-based transfection that has been reported to induce a similar transcriptional factor activation.\(^{(23)}\) (Figs. 4c, d). Then, the levels of both mRNAs fell and reached almost normal levels at 6 h. Similarly, the expressed luciferase protein level investigated in a time-dependent manner reached a peak at about 12 h and then fell gradually and became almost zero at 1 week after administration of pCMV-Luc that has binding elements for AP-1 and NF\(\kappa\)B.\(^{(24)}\) (Fig. 4e). Combined with the time lag between c-fos and c-jun expression and AP-1 mediated gene expression (for example, about 6—12 h in the case of inducible NO synthase)\(^{(25,26)}\) and the half-life of luciferase (about 3 h),\(^{(27)}\) these results support the view that tissue pressure-mediated transfection activates transcriptional factors, thereby producing a high level of gene expression.

The importance of the transcriptional process has received greater recognition as an important factor affecting transgene expression efficiency and persistence, because reactivation of silenced transgene expression by administration of histone deacetylase or re-application of physical force has been reported.\(^{(23,27,28)}\) Especially, in the case of hydrodynamics-based transfection that has been reported to induce a similar transient increase in cellular translocation of plasmid DNA was induced, and iii) transcriptional factors (AP-1 and NF\(\kappa\)B) were activated. This information will provide a better understanding of in vivo transgene expression based on naked plasmid DNA transfection involving tissue pressure-mediated transfection in mice.

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