Silencing of Exogenous DNA in Cultured Cells

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The intranuclear disposition of exogenous DNA is highly important for the therapeutic effects of the administrated DNA. Naked luciferase-plasmid DNA was transfected into cultured cells including HeLa by electroporation, and the amounts of intranuclear plasmid DNA and luciferase were quantitated at various time points. Decrease in expression efficiency from one copy of the exogenous DNA over time occurred as the case of mouse liver, and its degrees varied between cell lines. These results suggest that exogenous DNA is 'silenced' in the cultured cells as well as in mouse hepatocytes.

Key words exogenous DNA; silencing; intranuclear disposition

Nonviral vectors using plasmid DNA are quite attractive in gene therapy due to their excellent safety profile, albeit their low transgene expression efficiency in comparison to viral vectors.[1—5] It is obvious that the intracellular DNA trafficking, particularly the nuclear entry, is a very important issue for high transgene expression. Moreover, we previously proposed that the 'controlled intranuclear disposition' of the delivered DNA would also be highly important for achieving practical gene therapy.[5] For example, transgene expression with nonviral vectors is generally transient. Thus, the intranuclear disposition of the exogenous DNA is of great interest.

Recently, we examined the intranuclear disposition of plasmid DNA delivered into mouse liver by a hydrodynamics-based injection. We have demonstrated that expression efficiency from one copy of the exogenous DNA dramatically decreases over time ('silencing') without increased promoter methylation.[6] These findings raise the question of whether silencing of exogenous DNA occurs similarly in cultured cells.

In this study, we transfected naked luciferase-plasmid DNA into HeLa cells by electroporation, and the intranuclear disposition of the plasmid DNA was examined. Our results revealed relatively slow silencing of exogenous DNA in cultured cells.

MATERIALS AND METHODS

Oligodeoxyribonucleotides Oligodeoxyribonucleotides (ODNs) were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms.

Plasmid The pYK-CMV-luc plasmid containing the CMV promoter and the luciferase gene[6] was amplified in the *E. coli* strain DH5α and purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit. The absorbance at 260 nm and the Hoechst 33258 fluorescence were measured to quantify the DNA concentrations.

Electroporation The pYK-CMV-luc plasmid (8 μg) was introduced into HeLa cells (2×10^7 cells) by electroporation (0.3 kV, 950 μF, 0.8 cm gap) with a Gene Pulser II (Bio-Rad, Hercules, CA, U.S.A.). The treated cells were suspended in 40 ml of prewarmed D'MEM medium with 10% fetal calf serum and 2 ml of each was transferred into a 10-cm dish. The cells were incubated under 5% CO_2/air at 37 °C and harvested at various time points, and the luciferase activity was measured with a Luciferase Assay System with a Reporter Lysis Buffer kit (Promega, Madison, Wisconsin, U.S.A.). The amount of the plasmid DNA was measured as described below.

Isolation of Nuclear DNA and Quantitative PCR The cells were washed with phosphate-buffered saline (PBS) twice and trypsinized. After centrifugation at 800 ***g*** for 5 min at 4 °C, the pellet was washed twice with PBS. The pellet was resuspended in DNA Lysis Buffer (10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (w/v) IGEPAL-CA630, pH 7.4). After centrifugation at 1400 ***g*** for 5 min at 4 °C, the pellet was washed three times with DNA Lysis Buffer. The intranuclear DNA was extracted with SepaGene (Sanko Junyaku, Tokyo, Japan).

Quantitative polymerase chain reaction (Q-PCR) was performed using an ABI 7500 real time PCR system and SYBR-Green chemistry. A portion of the recovered DNA was analyzed by Q-PCR. The luciferase gene in the cells was detected using the following primers: Luc (+), 5′-dGGTCCTATGATTATGTCCGGTTATG; Luc (−), 5′-dATGTAGCCATCCATCCTTGTCAAT. Data were expressed as a ratio to the β-actin gene which was determined using the following primers: β-actin (+), 5′-dAGAGGGAAATCGTGCGTGAC; β-actin (−), 5′-dCAATAGTGATGACCTGGCCGT.

RESULTS AND DISCUSSION

We have recently reported that expression efficiency from one copy of the exogenous DNA dramatically decreases over time *in vivo*.[6] We delivered plasmid DNA into mouse liver by a hydrodynamics-based injection since cationic lipids and polymers could potentially affect the quantitation of the intranuclear exogenous DNA. To examine the relationship between the amount of exogenous DNA and the transgene expression in cultured cells, electroporation that enables the delivery of exogenous DNA without the aid of cationic lipids and polymers was employed. The transfected HeLa cells were harvested after 3, 6, 12, 24, 48, and 72 h, and the luciferase activity was measured. The amounts of exogenous DNA at the same time points were examined by quantitative polymerase chain reaction after isolation of the nuclei.

As shown in Fig. 1, the luciferase activity peaked at 12 h after the electroporation, and then decreased. As in mouse liver, the transgene expression was transient.[6] The luciferase activity at 72 h was 40-fold less than that at 24 h. On the

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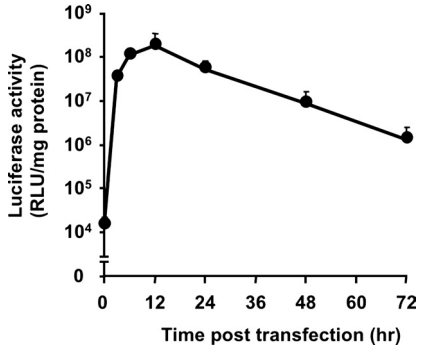

Fig. 1. Expression of the Luciferase Gene, Examined by Electroporation of Naked Plasmid

The pYK-CMV-luc plasmid (8 μg) was transfected into HeLa cells and the luciferase activities were measured. The values represent the averages of at least four separate experiments except for the data of 3 h (n=2). Bars indicate S.D. (standard deviation).

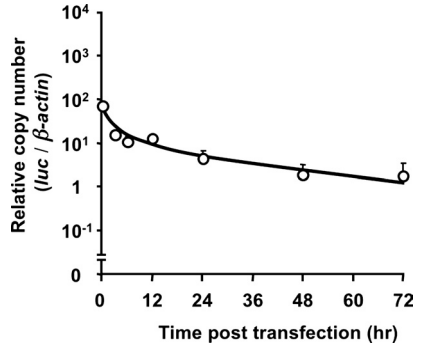

Fig. 2. Amounts of Luciferase DNA in the Nucleus

The pYK-CMV-luc plasmid (8 μg) was transfected into HeLa cells, and DNA was extracted from the nuclei, as described in the Materials and Methods section. The values represent the averages of at least four separate experiments except for the data of 3 h (n=2). Bars indicate S.D. (standard deviation).

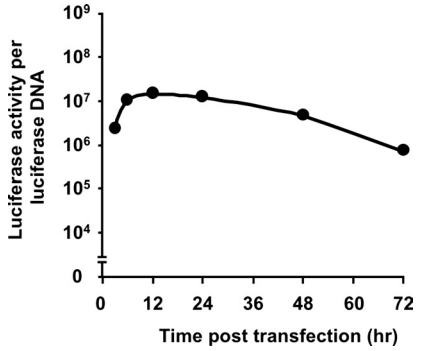

Fig. 3. Expression Efficiencies per Single Copy of Exogenous DNA in HeLa Cells

The luciferase activities shown in Fig. 1 were divided by the exogenous DNA amounts at the same time points shown in Fig. 2.

Table 1. Decrease of Luciferase Activity and Exogenous DNA from 24 to 72 h after Transfection[a)]

	HeLa	COS-7	NIH3T3	Mouse liver[b)]
Luciferase	0.025	0.16	0.043	0.014
Exogenous DNA	0.41	0.59	0.26	0.44
Ratio (luc/exogenous DNA)[c)]	0.062	0.27	0.17	0.031

a) The luciferase activity and amount of exogenous DNA at 72 h after transfection were divided by those at 24 h. *b*) Data from ref. 6. *c*) The luciferase activities at 24 and 72 h were divided by the amounts of exogenous DNA at the same time points. The quotient at 72 h was divided by that at 24 h.

other hand, the amount of exogenous DNA in the nuclei decreased gradually (Fig. 2). The amount of exogenous DNA at 72 h was 2.4-fold less than that at 24 h. Thus, the decrease in the expression (luciferase activity) was more than ten times as rapid as the decrease in the amount of exogenous DNA.

The luciferase activities at various time points were divided by the amounts of the exogenous DNAs at the same time points (Fig. 3). As shown in Fig. 3, the ratio of the luciferase activity (amount of the encoded protein) to the amount of the exogenous DNA peaked at 12 h, and then decreased. At 72 h, the ratio was 1/16 of that at 24 h. Thus, the expression from the exogenous DNA was suppressed over time. In this study, the amounts of exogenous DNA were determined by Q-PCR. The results would reflect amounts of fragmented DNA as well as the intact plasmid. The interpretation, the expression suppression from the exogenous DNA over time, would be valid, since (i) the luciferase protein would be produced from the luciferase gene on the partially fragmented DNA and (ii) similar decrease in expression efficiency per a single copy of exogenous DNA was observed in both cases of the intact plasmid and the total exogenous DNA in mouse liver.[6)]

Similar analyses were carried out with COS-7 and NIH3T3 cells. As shown in Table 1, decrease in the luciferase activity was more rapid than that in the amount of the exogenous DNA. In COS-7 cells, the luciferase activity at 72 h was six-fold less than that at 24 h, and the amount of the exogenous DNA at 72 h was only 1.7-fold less than that at 24 h. In NIH3T3 cells, the luciferase activity and the exogenous DNA dropped to 23- and 4-fold, respectively, from 24 h to 72 h after the electroporation. As a result, 'silencing' of the transfected DNA was observed in common in the three cultured cells. As compared to in the mouse liver, the degree of the silencing was smaller in the three cell lines. The silencing occurred more slowly in COS-7 and NIH3T3 cells than in HeLa cells.

We studied the intranuclear disposition of exogenous DNA in cultured cells, by administering naked plasmid DNA. As expected, the transgene expression was transient (Fig. 1). As in mouse liver,[6)] the silencing and the exogenous DNA elimination occurred in the three cultured cells (Table 1). This exogenous DNA elimination might be due to degradation by endonuclease(s) associated with intranuclear proteins involved in DNA repair and replication.[7)] Excretion of exogenous DNA from the nucleus would not be the major reason.[8)] These findings raise the question of what triggered the silencing of exogenous DNA in cells. Previously, we found that production of histones at the S phase might be a key of transgene expression (Akita *et al.*, unpublished results). In dividing cells, histones are synthesized in harmony with replication. The interaction of exogenous DNA and histones should be different in the (dividing) three cultured cells and mouse hepatocytes. This might explain slower silencing of exogenous DNA in the three cultured cells than in mouse liver. Histone modification might be involved in this silencing of exogenous DNA.[9—13)] Further studies are required to know the silencing mechanisms of the exogenous DNA and to understand differences in the silencing rates between HeLa *versus* COS-7 and NIH3T3 cells and between the cultured

cells and mouse hepatocytes.

Previously, we chased luciferase production in NIH3T3 and HeLa cells after transfection with cationic liposomes.[14] Elimination constant (k_{el}) values based on luciferase activity per well are 0.042 and 0.015 h^{-1} in NIH3T3 and HeLa cells, respectively. When we calculated k_{el} values based on luciferase activity per dish, these were 0.031 and 0.054 h^{-1} in NIH3T3 and HeLa cells, respectively. It seems that the luciferase activity in HeLa cells decreased more slowly when plasmid DNAs were introduced as a complex with cationic lipids than as in the naked from (by electroporation). This might suggest that the release from the complex proceeds gradually, supplying the 'active DNA'[5] in HeLa cells. At present, separate quantitation of the complexed exogenous DNA and released exogenous DNA is highly difficult. Further studies with a novel quantitation method are required to examine this interpretation.

In this study, we observed the silencing and elimination of exogenous DNA in cultured cells. The intranuclear disposition of the exogenous DNA should be examined in more detail, and both the exogenous DNA and carrier molecules should be designed and modified to control the intranuclear disposition.

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