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Protamine-Derived Synthetic Peptide Enhances the Efficiency of Sperm-Mediated Gene Transfer Using Liposome-Peptide-DNA Complex

Tomohiro YONEZAWA¹⁾, Yasufumi FURUHATA^{1#)}, Keiji HIRABAYASHI¹⁾, Masatoshi SUZUKI¹⁾, Keitaro YAMANOUCHI¹⁾ and Masugi NISHIHARA¹⁾

¹⁾Department of Veterinary Physiology, Veterinary Medical Science, The University of Tokyo, Tokyo 113-8657, Japan

[#]Present: Ajinomoto Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki 210-0801, Japan

Abstract. Sperm-mediated gene transfer (SMGT), which uses sperm cells as vectors for DNA delivery during fertilization, is one of the methods used to achieve transgenesis in animals as an alternative to pronuclear microinjection. Despite the convenience of SMGT, its application in producing transgenic animals, especially domestic animals, is still limited due to its low success rate. In this study, to increase the efficiency of SMGT, we tested liposome-peptide-DNA (LPD) complex, a new reagent known to stabilize transfection in cultured cells. Two peptides, one designed from the amino acid sequence of human histone H1 (Hs) and the other from human protamine (Pr), were used with a CMV/ β -actin/EGFP fusion gene to form LPD complex. Spermatozoa obtained from rat epididymis were incubated with LPD complex and used for artificial insemination, and the expression of EGFP in the morula-stage embryos was observed. Pr caused significantly more embryos to express EGFP, while Hs had no effect on the expression rate. Moreover, only Pr resulted in the production of offspring carrying foreign DNA. These results suggest that LPD complex with the aid of Pr could be useful in the transfer of foreign DNA by SMGT, probably by stabilizing liposome-DNA complex during fertilization.

Key words: Gene transfer, Sperm vector, Peptide, Liposome, EGFP

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Gene transfer in mammals has been achieved using different methodologies. The most widespread method has been microinjection of DNA into pronuclei of fertilized 1-cell stage eggs [1–4], even though this method requires special equipment and skills. One of the more convenient alternatives is sperm-mediated gene transfer (SMGT), which uses spermatozoa as vectors for DNA delivery [5]. A large number of transgenic animals including mice, pigs and cattle have been

produced by SMGT using sperm associated with naked DNA or liposome-DNA complex [6–12]. However, the efficiency and reliability of SMGT are still controversial [13, 14], though the capacity of spermatozoa capturing foreign DNA has been proven [15, 16].

When cultured cells are transfected with DNA, various cationic liposomes are now widely used due to their considerable efficacy in transfection [17, 18]. However, the efficacy of cationic liposomes for gene transfer *in vivo* is still limited and, therefore, several attempts have been made to

give DNA-liposome complexes a resistance to factors that decrease their stability. Among them, one successful strategy was the addition of short synthetic peptides derived from motifs of nuclear DNA-binding proteins to liposome-DNA complexes in an attempt to induce DNA compaction. The application of liposome-peptide-DNA (LPD) complexes has been demonstrated to enhance the efficiency of gene transfer both *in vivo* and *in vitro* [19, 20].

The aim of the present study was therefore to elucidate if the addition of synthetic peptides to liposome-DNA complexes could improve the efficiency of SMGT. Two peptides having amino acid motifs from histone and protamine, respectively, were used to form LPD complexes, and the expression of the transgene in early embryos as well as its integration into postpartum progeny were examined following SMGT.

Materials and Methods

DNA (CMV/ β -Actin/EGFP), liposome and peptides

The plasmid (pCX-EGFP), containing cytomegalovirus (CMV) enhancer, chicken β -actin promoter, EGFP cDNA and rabbit β -globin 3' flanking sequence, was generously donated by Dr. Okabe (Genome Information Research Center, Osaka University) [21]. The plasmid DNA was purified by a Plasmid Purification System (Qiagen, Hilden, Germany) and then linearized using *SalI*. After enzymatic digestion, the linearized plasmid DNA was purified by phenol-chloroform extraction and then ethanol-precipitated. The cationic liposome, SuperFect, was purchased from Qiagen (Hilden, Germany).

Two peptides were designed from the report by Schwartz *et al.* [20], and chemically synthesized and purified by the Peptide Institute (Osaka, Japan). One peptide was the dimeric sequence (KTPKKAKKP)₂ (subsequently "Hs") corresponding to the amino acid residues of 152–160 of human histone H1. This highly conserved sequence is repeated in the carboxyl-terminus of histones H1 of various species and its dimeric structure had been reported to alter the conformation of short double-stranded linear DNA fragments [22], resulting in an increase in the half life of DNA. The other peptide was a protamine-derived amino acid sequence,

SRSRYRQRQRSRRRRRR (subsequently "Pr"), which corresponds to the amino acid residues of 11–28 of human protamine 1. These two peptides were shown to induce DNA compaction when mixed with DNA, and thereby protected DNA from degradation by DNase [20]. The usefulness of these two peptides in increasing the efficiency of transfecting cultured cells or *in vivo* gene transfer was demonstrated previously [20].

Sperm Vector by LPD complex and Artificial Insemination

The medium used for artificial insemination (AM) was modified from Whittingham medium [23]: 120 mM NaCl, 25 mM NaHCO₃, 5 mM Glucose, 2.5 mM KCl and 0.15 mM Na₂HPO₄ (pH 7.4). Linearized plasmid DNA (10 μ g) dissolved in 500 μ l of filter-sterilized AM was mixed with 10 μ g of each peptide and incubated for 15 min at room temperature. Then the DNA-peptide solution was mixed SuperFect (50 μ l) dissolved in 500 μ l of AM with and incubated for more than 15 min.

Wistar-Imamichi strain rats at the age of 7–8 weeks old were purchased from Animal Reproduction Laboratory (Omiya, Japan). Male rats were sacrificed by decapitation and epididymides were excised. Spermatozoa (approx. 1–2 million) were prepared by squeezing each cauda epididymis into 1 ml of AM containing LPD complex. The sperm suspension was incubated with LPD complex for 1 h at 37 C under air/CO₂ (19:1) for capacitation and DNA-binding to the sperm.

The uterine cervix of female rats was stimulated with a glass rod to induce pseudopregnancy on the proestrous evening. After preparation of the sperm suspension with LPD complex, the female rats were anesthetized by pentobarbital. An incision was made on the skin to expose the uterus and then 500 μ l of suspended sperm/LPD complex was inseminated into each oviductal end of the uterus.

Observation of EGFP expression in early embryos

Some of the artificially inseminated female rats were killed by decapitation 4 days after the insemination, and uteri were excised. The uteri were flushed with saline to collect the embryos, and they were collected in a microdrop of saline. Expression of EGFP in the embryos was examined under a fluorescence microscope (E800, Nikon) with a GFP excitation light (490 nm).

PCR analysis of the offspring

Detection of transferred DNA in the progeny was done by PCR analysis. Offspring from two female rats of each experimental condition were used (no peptide, Hs and Pr, respectively). Genomic DNA was extracted from tails of 1-month-old pups. The tissue was lysed in 500 μ l of lysis buffer (30 mM Tris-Cl, 15 mM EDTA, 0.3% SDS, 0.2 mg/ml proteinase K) at 37 C overnight. The DNA was purified by phenol-chloroform extraction and then ethanol-precipitated.

The PCR primers designed to amplify a 548-bp fragment from the promoter sequence of pCX-EGFP were as follows: 5'-CTGCTAACCATGTTTCATGCC-3' (5' primer) and 5'-CGTTGTGGCTGTTGTAGTTG-3' (3' primer). The 25 μ l of PCR mixture consisted of reaction buffer (10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, Bio-Rad, Hercules, CA, USA), 200 μ M of each dNTP, 50 pM of each PCR primer and 0.1 μ l of Ampli Taq Gold polymerase (5 U/ μ l, Perkin-Elmer, Branchburg, NJ, USA) and 0.25 μ g of the extracted DNA. PCR amplification was carried out for 35 cycles of 1 min at 94 C, 1 min at 60 C and 1 min at 75 C. The reaction was then analyzed on a 1.5% agarose gel, followed by ethidium bromide staining. The amplified DNA fragments were visualized by ultraviolet transillumination.

Statistical analysis

The data were analyzed by Duncan's multiple range test or chi-square test, and differences were considered significant at $P < 0.05$.

Results

Expression of EGFP in transfected embryos

A total of 13 female rats were artificially inseminated (Hs, 4; Pr, 6; no peptide, 3 females).

Fig. 1. Representative microscopic pictures of the morula-stage embryos derived from SMGT using LPD complexes. Spermatozoa suspended with LPD complexes were inseminated artificially. At day 4 p.i., the uterus was flushed to collect embryos. The embryos in a microdrop of saline were observed under a fluorescence microscope. Fluorescent images were taken under an EGFP excitation light (490 nm). A, intact embryo; B, embryo with uniform expression (Pr was used); C, embryo with mosaic expression (Pr was used).

The number of collected embryos per uterus was not different among experimental groups (Hs, 9.0 ± 1.7 ; Pr, 11.2 ± 2.6 ; no peptide, 11.2 ± 1.2 embryos). EGFP expression in embryos of day 4 post-insemination (p.i.) was examined under a fluorescence microscope. The representative pictures of EGFP expressing morula-stage embryos are shown in Fig. 1. As shown in Table 1, the ratio of EGFP expressing embryos to total embryos examined was about 75% when Pr was used, and this percentage was significantly higher than in the other conditions. Some of the embryos positive for EGFP showed mosaic expression. The ratios of the number of mosaic embryos to total EGFP-expressing ones were not significantly different among the peptide conditions.

Detection of Transgene in the Offspring by PCR

Females delivered 9–13 offspring in each experimental group. The existence of foreign DNA in 1-month-old offspring was examined by PCR. The results of PCR analysis are summarized in Table 2. Twenty-one pups (12 from one female, and 9 from the other) were obtained when Pr was used

Table 1. EGFP expression in the embryos

Day 4 p.c.	Hs +/n (%)	Pr +/n (%)	No peptide +/n (%)
Incidence of expression ^a	18/36 (50.0)	50/67* (74.6)	17/35 (48.6)
Incidence of mosaicism ^b	11/18 (61.1)	26/50 (52.0)	12/17 (70.6)

^a Number of EGFP-expressing samples/number of samples examined.

^b Number of mosaic samples/number of EGFP-expressing samples.

* $p < 0.05$ vs. Hs and No peptide.

Table 2. DNA Detection in the Progeny

Hs +/n ^a	Pr +/n	No peptide +/n
0/27	1/21	0/32

^a Number of positive samples/number of samples examined.

to make LPD complex, and one pup was shown to carry the foreign DNA. In the other conditions, no pups were positive for foreign DNA. EGFP expression in pups was not observed in any of the experimental groups.

Discussion

The present study was undertaken to test if the addition of peptides into liposome-DNA complex could improve the expression rate of foreign gene transferred by SMGT in embryos. The use of LPD complex with lysine- or arginine-rich cationic peptides derived from histone or protamine has been shown to negate an inhibitory effect of extra environment, which surrounds LPD complex and affects its performance [20]. However, of the two peptides tested in the present study, Hs in the LPD complex did not affect the expression rate of EGFP in the embryos. The precise reason for this ineffectiveness of Hs is currently unknown. Previous *in vivo* gene transfer with LPD complex was carried out by injecting the complex intravenously [19, 24], while in the present SMGT experiment, the complex was exposed to an intra-uterine/oviductal environment. Therefore, it is possible that factor in the uterus/oviduct inhibiting the efficiency of SMGT might be different from those involved in the intravenous gene delivery approach.

On the other hand, more than 70% of the SMGT-derived embryos expressed EGFP when Pr was used. Since DNAs in spermatozoa are compacted by protamines [25], but not by histones as in somatic cells, it is possible that Pr might mimic the *in vivo* environment of DNAs present in spermatozoa, and protect foreign DNA from potential exposure to DNase activity during

fertilization.

In the present study, no progeny were shown to carry foreign DNA except in the case when Pr was used to prepare LPD complex. This result appears to contradict the high expression rate of EGFP (more than 70%) observed in day 4 p.i. embryos, and suggests that most of the foreign DNA was excluded in the course of embryonic development without integration into the innate chromosomes. In other words, the expression of EGFP in most of the early embryos might result from the transient transcription of extrachromosomal DNA, as has been commonly observed in other methods utilizing sperm vector [8, 26]. However, it should be noted that one pup still preserved the foreign DNA in its tail DNA at one month after birth when Pr was used, suggesting that integration occurred at least in this particular animal.

In our previous study, we reported that exogenous DNA injected into testes as a liposome complex could be transferred into the egg via sperm by natural mating and integrated into the genome, and this method is called testis-mediated gene transfer (TMGT) [26–28]. We speculate that, if the Pr is added to liposome-DNA complex injected into testes in TMGT, greater transfer efficiency might be achieved.

In summary, the present study demonstrated that Pr, a peptide derived from human protamine, could enhance the efficiency of SMGT when it was applied to liposome-DNA complex. The newly employed SMGT method with LPD complex could be suitable for generating transgenic animals, especially transgenic domestic animals. Further study is required to clarify the mechanism by which Pr in LPD complex potentiates the efficiency of gene transfer.

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