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Development of fusogenic liposomes and its application to gene therapy

Hiroyuki Mizuguchi[a], Shinsaku Nakagawa[a], Mahito Nakanishi[b,c], Tadanori Mayumi[a]

[a] Faculty of Pharmaceutical Science, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan.
[b] Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan.
[c] PRESTO, Research Development Corporation of Japan, 4-1-8, Honmachi, Kawaguchi, Saitama, 332, Japan.

We have developed fusogenic liposomes which could deliver their encapsulated materials such as DNA and proteins into the cytoplasm through the membrane fusion activity of Sendai virus. Characteristics of fusogenic liposomes as a gene transfer vector was examined in comparison with cationic liposomes (Lipofectin^{TM})-DNA complex, a common nonviral gene transfer vector. Fusogenic liposomes could transfer genes efficiently even when incubated with L cells as short as one minutes, while cationic liposomes required the incubation for at least 30 min to get the same level of gene expression. Transfection efficiency by fusogenic liposomes was much higher than that by cationic liposomes. Especially, in gene transfer into Sarcoma-180 ascitis tumor of the mouse *in vivo*, fusogenic liposomes were over 2,000-fold more efficient than cationic liposomes. We conclude that fusogenic liposomes should introduce the genes efficiently and directly into the tissue cells *in vitro* and *in vivo*.

The introduction of foreign genes into mammalian cells has become increasing important for the investigations of gene expression and function in target cells and the clinical studies of gene therapy. Many viral and nonviral vectors are available for the transfer of foreign genes into the target cells [1,2]. Most viral vectors have a high transfection efficiency and are suitable for *in vivo* use. However, they have the disadvantages that the structure and expression of genes are restricted by the character of the virus genome. On the other hand, nonviral vectors, such as cationic liposomes, are safe and dose not restrict the character of the genes, but are low efficiency and are unsuitable for *in vivo* use. It is the most important to develop the novel and efficient vector for gene transfer.

In this article, we describe the development of fusogenic liposomes which have membrane fusion activity of Sendai virus and the characteristics of fusogenic liposomes as a gene transfer vector.

1. DEVELOPMENT OF FUSOGENIC LIPOSOMES

1.1 Preparation of fusogenic liposomes

Fusogenic liposomes were prepared by the fusion of unilamellar simple liposome with UV (2,000 J/cm^2)-inactivated Sendai virus. Unilamellar liposomes were prepared by a reverse-phase evaporation method [3] with some modifications using egg phosphatidylcholine, L-α-dimyristoryl phosphatidic acid, and cholesterol (5 : 1 : 4, molar ratio) and sized by extrusion through a 0.2 μm polycarbonate membrane. Then fusogenic liposomes were purified by sucrose step centrifugation (10-50 %) (24,000 rpm, 2 h) to remove free liposomes and Sendai virus.

Purified fusogenic liposomes had an average diameter of 379 nm, while unilamellar simple liposomes and Sendai virus had that of 304 and 334 nm, respectively [4]. When the fusogenic

liposomes were examined by electron microscopy, they were unilamellar and had spike structure on their surface similar to Sendai virus particles [4].

1.2 Fusion activity of fusogenic liposomes

Fusion activity of fusogenic liposomes was examined by the introduction of fragment A of diphtheria toxin (DTA) into the cytoplasm. DTA is known to kill cells by inactivating elongation factor 2 even when only one molecule of this protein is introduced into the cytoplasm, while it is absolutely non-toxic even if it is taken up by endocytosis, because it cannot reach the cytoplasm due to the degradation by lysosomal enzymes [5]. Therefore, DTA is the excellent marker protein to estimate liposome-cell fusion.

When fusogenic liposomes containing DTA were incubated with L cells, they killed the cells with dose dependent manner (Fig.1). Neither simple liposomes containing DTA nor UV-inactivated Sendai virus killed the cells. These results suggest that fusogenic liposomes containing DTA can kill L cells efficiently *in vitro* by introducing DTA into the cytoplasm.

Furthermore, fusogenic liposomes could deliver DTA to tissue cells *in vivo*, and proved to fuse the cells both *in vitro* and *in vivo* [6,7].

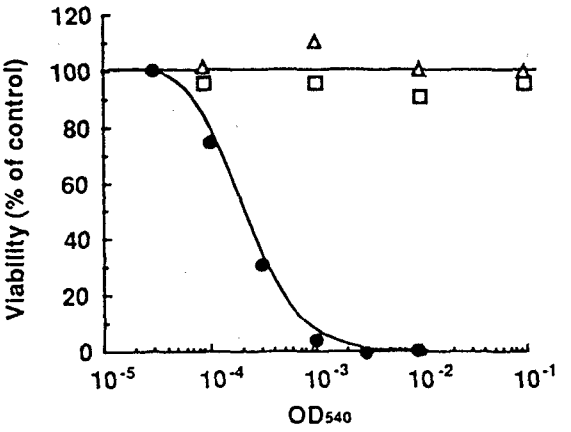

Fig.1 Effect of fusogenic liposomes containing DTA on colony formation of L cells
Five hundred L cells were seeded in 35-mm dishes. Ten hours later, the cells were treated with various concentrations of fusogenic liposomes containing DTA (●), liposomes containing DTA (△), UV-inactivated Sendai virus (□) suspended with balanced salt solution (BSS; 150 mM NaCl, 10 mM Tris, pH 7.6) at 4 °C for 30 min, and at 37 °C for 30 min. Then the cells were cultured in fresh medium for one week. Number of colonies are counted to examine the cell viability. Each point is shown as average of duplicate assays.

2. CHARACTERISTICS OF FUSOGENIC LIPOSOMES AS A GENE TRANSFER VECTOR

2.1 Transfection efficiency *in vitro*

We used a luciferase expression plasmid, pCAL2, which contained the chicken β-actin promoter, cytomegalovirus enhancer and SV40 early poly(A) signal, as the model gene, and encapsulated these plasmids into fusogenic liposomes. One milliliter of fusogenic liposomes suspension at OD_{540} of 1.0 contained 2.0 μg DNA. Transfection efficiency of fusogenic liposomes containing pCAL2 was examined under various conditions in comparison with cationic liposomes (Lipofectin™)-pCAL2 complex, a common nonviral gene transfer vector.

When L cells were treated with fusogenic liposomes containing pCAL2, the cells showed transient luciferase expression and maximal activity was observed at 2 days after the transfection. Luciferase expression at 8 days was one-fourth lower than that at 2 days (data not shown).

Fig.2 shows the effect of the length of the exposure of the vectors to the cells on the gene transfer. L cells were incubated with fusogenic liposomes containing pCAL2 for 1 to 30 min. With increasing time of incubation, higher luciferase activity was observed. Fusogenic liposomes transferred

genes efficiently into the cells even when they were incubated with the cells for 1-10 min, while cationic liposomes required the incubation for at least 30 min to get the same level of gene expression. When HeLa and bovine aortic endothelial cells were treated with fusogenic liposomes or cationic liposomes, the pattern of transfection efficiency was similar to L cells (data not shown).

Moreover, fusogenic liposomes had no cytotoxicity at the highest concentration examined (DNA 6.0 μg/ml, OD_{540}=3.0), whereas cationic liposomes were acutely cytotoxic at higher concentration of DNA (DNA 2.0 μg/ml, cationic liposomes 10.0 μg/ml) (data not shown).

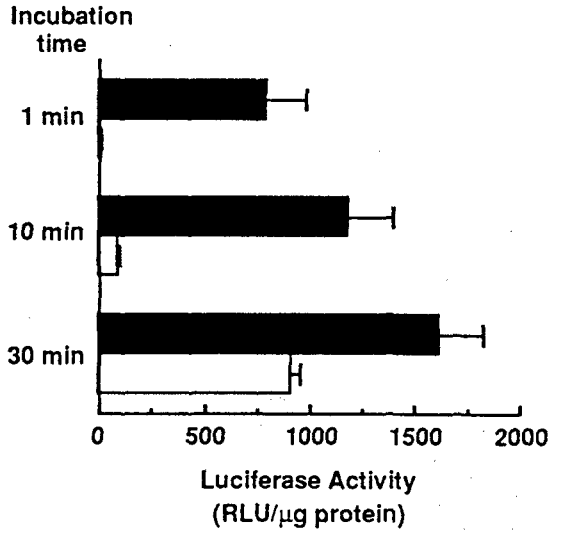

Fig.2 Effect of the time for incubating cells with fusogenic liposomes or cationic liposomes on gene transfer
L cells were incubated with fusogenic liposomes containing pCAL2 (■) with OD_{540} of 0.25 (DNA 0.5 μg/ml) or complexes of cationic liposomes and pCAL2 (cationic liposomes 2.5 μg/ml, DNA 0.5 μg/ml) (□) at 37 °C for 1, 10, or 30 min. After 48 h in culture, luciferase activity was determined. Each point represents the mean ± S.D. of three to four experiments.

2.2 Transfection efficiency in the presence of serum

The vectors which can deliver the genes into the cells even in the medium containing serum are more desirable to efficient gene delivery, because those vectors can be used for the direct gene introduction into tissue cells *in vivo*. We examined the effect of serum on gene expression (Fig.3). Successful transfection with fusogenic liposomes could be achieved in the presence of serum. Even in the medium containing 40 % serum, higher transfection efficiency, which was about 70 % of the activity in serum-free conditions, was observed. In contrast, the presence of serum reduced cationic liposomes-mediated transfection. Cationic liposomes could not

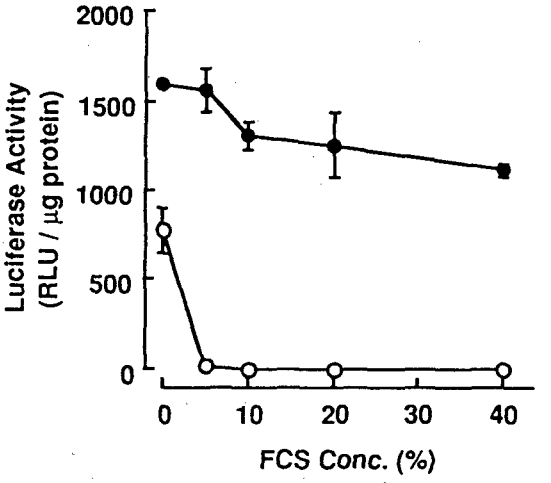

Fig.3 Effect of serum in the medium on gene transfer activity
L cells seeded onto 35-mm dish were treated with 0.5 ml of MEM supplemented with 10, 20, 40, or 80 % FCS, and were transfected with 0.5 ml of serum-free suspensions of fusogenic liposomes containing pCAL2 with OD_{540} of 0.5 (final OD_{540}=0.25, DNA 0.5 μg/ml) (●) or complexes of cationic liposomes and pCAL2 (cationic liposomes 5.0 μg/ml, DNA 1.0 μg/ml) (O) at 37 °C for 30 min (final FCS concentration is 5, 10, 20, or 40 %). After 48 h in culture, luciferase activity was measured. Each point represents the mean ± S.D. of four experiments.

show any gene expression even in the presence of 5 % serum. These results suggest that fusogenic liposomes are much superior vectors to cationic liposomes, when the cells are transfected in the medium containing serum.

2.3 Transfection efficiency *in vivo*

We next examined whether the direct *in vivo* gene transfer was possible using fusogenic liposomes. When fusogenic liposomes containing 1.5 μg of pCAL2 was i.p. injected into the mouse bearing Sarcoma-180 (S-180) ascites tumor, a high level of luciferase activity was detected in S-180 cells. In contrast, little luciferase activity was detected even when cationic liposomes containing 40 μg of pCAL2 as well as 1.5 μg of pCAL2 were administered (Table 1). Taking account of both gene expression and injected dose, fusogenic liposomes mediated over 2,000-fold higher *in vivo* gene transfer than cationic liposomes. This should be caused by the rapid and efficient gene transfer mediated by fusogenic liposomes, together with resistance to serum components.

In summary, we have developed fusogenic liposomes based on Sendai virus, and characterized these liposomes as a vector for the transfer of DNA and proteins. Fusogenic liposomes introduce their encapsulated materials such as DNA and proteins efficiently and directly into animal cells *in vitro* and *in vivo*. We conclude that fusogenic liposomes are a unique and efficient nonviral vector for gene and protein transfer.

Table 1 Comparison of *in vivo* gene transfer activity of fusogenic liposomes and cationic liposomes

Treatment	DNA Conc. (μg/mouse)	Luciferase Activity (RLU/10^7 cells)
Fusogenic liposome [a]	1.5	8013 ± 809
Cationic liposome [b]	1.5	4.9 ± 0.7
	40.0	92.2 ± 18.5

S-180 ($1X10^6$) cells were i.p. injected into male ddY mice at day 0. At 5 days, fusogenic liposomes containing pCAL2 or complexes of cationic liposomes and pCAL2 were given i.p. At 7 days, S-180 cells were recovered and luciferase activity was measured.
a); Fusogenic liposomes containing 1.5 μg of pCAL2 were injected i.p.
b); Complexes of cationic liposomes and 1.5 or 40.0 μg of pCAL2 (pCAL2 : cationic liposomes = 1 : 5 (W)) were injected i.p.
Means ± S.D. ; n=3

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