RAPID COMMUNICATION Jpn. J. Cancer Res. **89**, 352–354, April 1998

Improvement of Transduction Efficiency of Recombinant Adeno-associated Virus Vector by Entrapment in Multilamellar Liposomes

Masaaki Mizuno and Jun Yoshida[1]

Department of Neurosurgery, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550

Recombinant adeno-associated virus (AAV) has attracted considerable interest as a potential vector for human gene therapy, but its transduction efficiency is quite low. The present study demonstrated AAV vector-associated liposomes to be more effective for *in vitro* gene transfer to human glioma cells than are liposomes containing plasmid DNA. Using vector-associated liposomes increased the transduction efficiency more than 10-fold compared to liposomes containing plasmid DNA and more than 6-fold compared to AAV alone. From these results, AAV vector-associated liposomes appear to be a good candidate for *in vivo* gene delivery to human gliomas.

Key words: AAV — Liposome — Gene transfer — Glioma

Adeno-associated virus (AAV) is a defective parvovirus with a genome of 4675 bp, requiring co-infection with either adenovirus or herpes simplex virus for replication.[1–4] When no helper virus is available, AAV infection results in efficient integration of the AAV genome into host cell genomic DNA.[5, 6] Viral integration has no apparent effect on cell growth or morphology. Therefore, recombinant AAV has emerged as an attractive candidate vector for human gene therapy. Recombinant AAV vectors lack cell and tissue specificity, resulting in ability to transduce foreign genes into a wide range of cell types but difficulty in targeting gene transfer.

On the other hand, liposomes are synthetic lipid vesicles able to entrap drugs or genes within their aqueous compartment and/or lipid bilayer. Like AAV vectors, liposomes have been regarded as a useful delivery system; their potential for *in vivo* gene transfer has been reported.[7, 8] Importantly, cell or tissue specificity can be conferred on liposomes by conjugation or association with specific proteins or antibodies (immunoliposomes). We have confirmed efficient *in vivo* gene delivery by cationic immunoliposomes prepared from *N*-(α-trimethylammonioacetyl)didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidylethanolamine (DOPE). In the present study we investigated whether AAV vector-associated liposomes could afford more efficient *in vitro* gene transfer to human glioma cells than liposomes containing plasmid DNA or AAV alone.

We used three human astrocytoma-glioblastoma cell lines, U251-SP, U251-MG and SK-MG-1. Recombinant AAV vector including the *Lac*Z gene derived from the CMV promoter (AAV-*Lac*Z) and AAV-plasmid DNA including the *Lac*Z gene derived from the CMV promoter (pV*Lac*Z), which has AAV-ITR sequences, were produced. For preparation of AAV-*Lac*Z-associated liposomes, TMAG, DLPC, and DOPE in a molar ratio of 1:2:2 (total amount, 1 μmol) were dissolved in 0.5 ml of chloroform, and the solvent was evaporated. The lipid film was wetted with 0.2 ml of phosphate-buffered saline (PBS) containing 10^3 to 10^9 particles of AAV-*Lac*Z, and then mixed with a vortex agitator for 2 min. The volume of the suspension was adjusted to 0.5 ml with PBS. After preparation, unentrapped AAV-*Lac*Z vector was removed by flotation on a Ficoll gradient.[8] Lipid concentration was assessed by determination of phosphatidylcholine using a Wako PC determination kit (Osaka), and concentrations of liposomes were adjusted accordingly. Preparation of pV*Lac*Z-associated liposomes has been described in previous papers.[9, 10] We applied the following agents to the three human glioma cell lines (5×10^3 cells/well): empty liposomes (15 nmol of lipids/ml), liposomes containing pV*Lac*Z (15 nmol of lipids and 0.3 μg of DNA/ml), AAV-*Lac*Z (1.5×10^6 particles/ml), a mixture of AAV-*Lac*Z and empty liposomes in the same amounts as above, and AAV-*Lac*Z-associated liposomes (1.5×10^6 particles and 15 nmol of lipids/ml). The mixtures were incubated for 2 or 4 days, then the transduction efficiency was evaluated by measurement of β-galactosidase activity according to the protocol provided by Promega Co. (Madison, WI). Protein was measured by a modified Lowry method.[11]

We first examined the relationship between the amount of AAV-*Lac*Z in AAV-*Lac*Z-associated liposomes and the β-galactosidase activity of the transductants. As shown in Fig. 1, the β-galactosidase activity increased with increase in the amount of AAV-*Lac*Z up to 10^8 particles per micromole of lipid. For subsequent experiments, we prepared AAV-*Lac*Z-associated liposomes containing 10^8 particles

[1] To whom requests for reprints should be addressed.

of AAV-*Lac*Z per micromole of lipid. The quantity of liposomes added corresponded to 15 nmol of lipid and 1.5×10^6 particles/ml, since the liposomes induced toxicity when more than 20 nmol/ml of lipid was added to the human glioma cells.

We compared the results of transduction with AAV-*Lac*Z-associated liposomes, with pV*Lac*Z-associated liposomes and with AAV-*Lac*Z alone by measuring β-galactosidase activity (Table I). Activity of β-galactosidase in human glioma cells transduced with AAV-*Lac*Z-associated liposomes was over 10 times that of cells incubated with pV*Lac*Z-associated liposomes. A similar increase was observed in all glioma cell lines tested. A slight increase in activity was also observed when AAV-*Lac*Z mixed with empty liposomes was used, but β-galactosidase expression remained substantially less than that conferred by AAV-*Lac*Z-associated liposomes. Production of β-galactosidase continued for over 2 months, but enzyme activity had decreased to about 10 to 20% by 2 months after transduction.

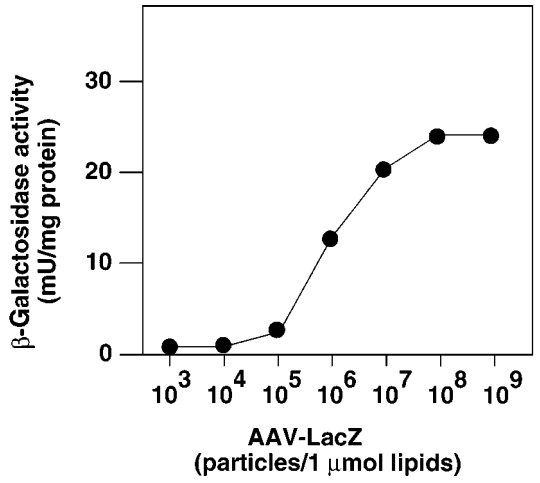

Fig. 1. β-Galactosidase activity generated by transduction with of AAV-*Lac*Z-associated liposomes. We prepared various AAV-*Lac*Z-associated liposomes (10^3 to 10^9 particles/μmol lipid) and added them to human glioma cell lines (U251-SP). Twenty-four hours later, we assayed the β-galactosidase activity and examined the relationship between the amount of AAV-*Lac*Z and the β-galactosidase activity.

Recombinant AAV vectors are currently viewed as a promising approach to gene therapy, since they are physically stable and elicit relatively little host immune response. However, their transduction efficiency is quite low, especially *in vivo*. For example, transduction efficiency was 20% at most when we injected AAV-*Lac*Z (1.82×10^{11} particles) into an experimental glioma prepared in a mouse brain. Therefore, a new delivery system with much higher transduction efficiency and target specificity is needed. In this report, we have demonstrated that the transduction efficiency of AAV vectors in human glioma cells was increased markedly by association with cationic liposomes. Philip *et al.* have reported that AAV plasmid DNA-complexed liposomes showed levels of expression several times higher than those produced by complexes with standard plasmids, and also resulted in long-term expression (>30 days) of the gene in primary T lymphocytes and primary and cultured tumor cells.[12] Zhou *et al.* combined adenovirus (Adv-5) capsid protein or the fiber protein of Adv with liposomes, termed adenosomes (adenovirus protein-cationic liposome complex), and demonstrated that this complex could carry AAV/CMV-*Lac*Z to endothelial cells and improve the efficiency of gene trans-

Table I. β-Galactosidase Activity in Human Glioma Cells Transfected with AAV-*Lac*Z-associated Liposomes

Culture additive[a)]	β-Galactosidase activity					
	U251-SP		U251-MG		SK-MG-1	
	2 days	4 days	2 days	4 days	2 days	4 days
1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
2	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
3	18.2±6.3	20.4±7.7	12.8±5.3	14.0±6.2	11.4±3.1	15.9±4.8
4	22.9±9.2	28.3±9.3	18.7±8.5	23.9±7.7	21.7±8.7	22.1±4.3
5	39.0±8.7	45.3±11.4	74.7±10.3	84.5±9.5	43.4±6.6	47.8±7.6
6	198.7±24.7	203.5±21.3	187.1±14.3	189.2±16.6	120.2±23.6	175.9±22.4

All values are the mean and SD of four experiments, expressed as β-galactosidase (mU/mg protein; *n*=4); moi, multiplicity of infection.

a) 1, control; 2, empty liposomes (15 nmol of lipids/ml); 3, liposomes containing pV*Lac*Z (15 nmol of lipids and 0.3 μg of DNA/ml); 4, AAV-*Lac*Z (1.5×10^6 particles/ml; moi=300); 5, AAV-*Lac*Z (1.5×10^6 particles/ml; moi=300) and empty liposomes (15 nmol of lipids/ml); 6, AAV-*Lac*Z-associated liposomes (1.5×10^6 particles and 15 nmol of lipids/ml; moi=300).

fer.[13] However, few reports have dealt with the combination of AAV vectors and liposomes. Further studies seem warranted.

AAV vector-associated liposomes exhibited markedly higher transduction efficiency than pV*Lac*Z-associated liposomes or AAV vector alone. In general, liposomal gene transduction involves two processes, delivery of plasmid DNA and transgene expression. Aspects of delivery include uptake of liposomes into cells, release of liposomes from endosomes to the cytosol, and entry of the vector into the nucleus. As the liposomes we used are multilamellar vesicles, AAV vectors are thought to be entrapped within the multiple layers. Therefore, it seems unlikely that the observed increase in transduction efficiency resulted from interaction between AAV surface proteins and cell membranes or from enhanced release of the liposomes from the endosomes to the cytosol. Enhanced expression of the successfully delivered transgene seems even less likely because the expression unit on AAV vector-associated liposomes and AAV vector alone is the same. Most likely, uptake of liposomes is increased on the basis of enhancement of endocytosis by the mixture of AAV vector and cationic lipid.

Having confirmed a great increase in gene transfer when we combined the AAV vector with cationic liposomes, we plan to investigate whether cell or tissue specificity can be obtained by associating the AAV vector with liposomes bearing cell or tissue-specific proteins or antibodies (immunoliposomes).

(Received January 8, 1998/Revised February 16, 1998/Accepted March 2, 1998)

REFERENCES

1) Berns, K. I. and Bohensky, R. A. Adeno-associated virus: an update. *Adv. Virus Res.*, **32**, 243–306 (1987).
2) Srivastava, A., Lusby, E. W. and Berns, K. I. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.*, **45**, 555–564 (1983).
3) Atchinson, R. W., Castro, B. C. and Hammond, W. M. Adeno-associated defective particles. *Science*, **149**, 754–756 (1965).
4) Buller, R. M., Janik, J. E., Sebring, E. D. and Rose, J. A. Herpes simplex virus types 1 and 2 completely help adeno-associated virus replication. *J. Virol.*, **40**, 241–247 (1981).
5) Samulski, R. J., Zhu, X., Xiao, X., Brook, J. D., Housman, D. E., Epstein, N. and Hunter, L. A. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.*, **10**, 3941–3950 (1991).
6) Linden, R. M., Ward, P., Giraud, C., Winocour, E. and Berns, K. I. Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA*, **93**, 11288–11294 (1996).
7) Fraley, R. T., Fornari, C. S. and Kaplan, S. Entrapment of a bacterial plasmid in phospholipid vesicles: potential for gene transfer. *Proc. Natl. Acad. Sci. USA*, **76**, 3348–3352 (1979).
8) Fraley, R., Subramani, S., Berg, P. and Papahadjopoulos, D. Introduction of liposome-encapsulated SV40 DNA into cells. *J. Biol. Chem.*, **255**, 10431–10435 (1980).
9) Yagi, K., Noda, H., Kurono, M. and Ohishi, N. Efficient gene transfer with less cytotoxicity by means of cationic multilamellar liposomes. *Biochem. Biophys. Res. Commun.*, **196**, 1042–1048 (1993).
10) Yoshida, J. and Mizuno, M. Simple preparation and characterization of cationic liposomes associated with a monoclonal antibody against glioma-associated antigen (immunoliposomes). *J. Liposome Res.*, **5**, 981–995 (1995).
11) Hess, H. H., Lees, M. B. and Derr, J. E. A linear Lowry-Folin assay for both water-soluble and sodium dodecyl sulfate-solubilized proteins. *Anal. Biochem.*, **85**, 295–300 (1978).
12) Philip, R., Brunette, E., Kilinski, L., Murugesh, D., McNally, M. A., Ucar, K., Rosenblatt, J., Okarma, T. B. and Lebkowski, J. S. Efficient and sustained gene expression in primary T lymphocytes and primary and cultured tumor cells mediated by adeno-associated virus plasmid DNA complexed to cationic liposomes. *Mol. Cell. Biol.*, **14**, 2411–2418 (1994).
13) Zhou, H., Zeng, G., Zhu, X., Tang, J., Chen, G., Huang, Q., Peng, T. and Hu, B. Enhanced adeno-associated virus vector expression by adenovirus protein-cationic liposomes complex. A novel and high efficient way to introduce foreign DNA into endothelial cells. *Chin. Med. J.*, **108**, 332–337 (1995).