Neurol Med Chir (Tokyo) 36, 141~144, 1996

Repeated Exposure to Cationic Immunoliposomes Activates Effective Gene Transfer to Human Glioma Cells

Masaaki MIZUNO and Jun YOSHIDA

Department of Neurosurgery, Nagoya University School of Medicine, Nagoya

Abstract

The use of whole immunoglobulin G (IgG) and $F(ab')_2$ of the G-22 monoclonal antibody associated with cationic liposomes (immunoliposomes) and the effect of repeated exposure were investigated for the transfection of the LacZ gene to various glioma cell lines. Immunoliposomes associated with either whole IgG or $F(ab')_2$ monoclonal antibody caused an about 2-fold increase in β-galactosidase activity compared with liposomes associated with no antibody in glioma cell lines expressing the CD44 antigen. β-Galactosidase activity was further increased by about 2-fold by repeated exposure compared with single exposure. A glioma cell line not expressing the CD44 antigen showed no such increase in β-galactosidase activity. These results indicate that repeated exposure of cationic immunoliposomes achieves a higher transfection efficiency and is a potentially effective method of gene therapy for patients with malignant glioma.

Key words: cationic immunoliposomes, gene transfer, repeated exposure, glioma

Introduction

Clinical application of gene therapy requires a deoxyribonucleic acid (DNA) delivery system which can effectively transfer the gene both *in vitro* and *in vivo*. There are many DNA delivery systems, including calcium phosphate precipitation, diethylaminoethyl dextran, electroporation, virus, and liposomes. However, few procedures for *in vivo* gene transfer are applied to patients with formidable congenital metabolic disorders or neoplastic diseases, except the use of viruses and liposomes. Methods of human gene therapy used since 1990 in the United States have usually been based on viral vectors, especially retroviruses and adenoviruses. However, such procedures may result in unexpected responses in the cells or patients infected with viral vectors, because viral vectors can infect normal cells or integrate into chromosomal DNA at random in the case of retroviruses. To prevent these problems, we developed a new non-viral DNA delivery system using cationic liposomes associated with tumor-specific monoclonal antibody.[5,6,8] The present work investigated the effectiveness of whole immunoglobulin G (IgG) and the $F(ab')_2$ fragment of G-22 glioma-specific monoclonal antibody associated with cationic liposomes (immunoliposomes), and the effect of repeated exposure of these immunoliposomes for the transfer of a plasmid expressing the LacZ gene into various glioma cell lines.

Received May 26, 1995; Accepted September 19, 1995

Materials and Methods

I. Cell lines

Rapid growing subclones (U251-MG-S and U251-SP-S) of human astrocytoma-glioblastoma cell lines, U251-MG and U251-SP, and human ependymoma cell lines, KMS-II104, were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 0.1 mM non-essential amino acids, 5 mM L-glutamine, and antibiotics (streptomycin 100 μg/ml, penicillin 100 U/ml).

II. Antibody

A G-22 monoclonal antibody (mouse IgG monoclonal antibody) which specifically reacts with a surface antigen (G-22) of human glioma cells was prepared in our laboratory. This monoclonal antibody reacted with 18 of 20 glioma cell lines and two

of two melanoma cell lines. Extensive screening of other cell lines revealed no cross reactivity except for three cell lines for lung cancer.[9] Recently, we confirmed that the antigen for the G-22 monoclonal antibody is CD44, which is overexpressed in human glioma cells.[7] The U251-MG-S and U251-SP-S cell lines possess the G-22 antigen, but the KMS-II104 cell line does not.

III. Preparation of cationic liposomes and immunoliposomes containing plasmid

Cationic liposomes containing plasmid were prepared by dissolving the positively charged lipid, N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (Sogo Pharmaceutical Co., Ltd., Tokyo), dilauroyl phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo., U.S.A.), and dioleoyl phosphatidylethanolamine (Avanti Polar-Lipids, Inc., Pelham, Ala., U.S.A.) in the molar ratio of 1:2:2 (total amount 1 μmol) in 0.5 ml of chloroform, and then evaporating the solvent. The lipid film was wetted with 0.2 ml of phosphate-buffered saline (PBS) containing 20 μg of plasmid DNA (pCH110; Pharmacia Co., Uppsala, Sweden), and then vortexed for 2 minutes. The volume of the suspension was adjusted to 0.5 ml with PBS.[10,11] Immunoliposomes were prepared by a simplified method of monoclonal antibody-liposome association. Briefly, G-22 monoclonal antibody (2 μg per 1 μmol lipids) was added together with the plasmid DNA before vortexing. Details of the preparation and characterization of cationic liposomes associated with G-22 monoclonal antibody has been reported elsewhere.[12] Uncaptured plasmid or antibody was removed by floatation on a Ficoll gradient.[3]

IV. Transfection of LacZ gene

Gene transfection into glioma cells was achieved by incubating the cells with the pCH110-entrapping cationic liposomes or immunoliposomes (15 nmol/ml of lipids and 0.3 μg/ml of DNA) once or several times for a specific time. In one experiment, immunoliposomes were prepared using whole IgG or $F(ab')_2$ of the G-22 monoclonal antibody to evaluate any difference between whole IgG and $F(ab')_2$. The cells were exposed once to the cationic liposomes or immunoliposomes and then were incubated for 6 days. In another experiment, cationic liposomes and immunoliposomes prepared using $F(ab')_2$ were added to the cells on Day 1 only, Days 1 and 2, or Days 1, 2, 3, and 4, and then incubated until Day 6, to investigate the effect of repeated exposure. The cells were then scraped from the dish, collected by centrifugation, lysed by freeze-thawing three times in buffer (0.25 M sucrose, 10 mM Tris HCl, 10 mM ethylenediaminetetra-acetic acid, pH 7.4), and centrifuged to remove the debris. The β-galactosidase activity in the cells was measured using *o*-nitrophenyl-β-D-galactopyranoside according to the protocol provided by Promerga Co., Madison, Wis., U.S.A. Proteins were measured by modified Lowry methods.[4]

Results

Table 1 shows the results of the first experiment assessing the difference between use of whole IgG and $F(ab')_2$ of G-22 monoclonal antibody. Association of G-22 monoclonal antibody increased the activity of β-galactosidase in G-22 positive cells (U251-MG-S and U251-SP-S cells), but there was no significant difference between immunoliposomes made with whole IgG and $F(ab')_2$. In contrast, no increase of β-galactosidase activity was observed in G-22 negative cells (KMS-II104). Table 2 shows the effect of single and repeated exposure of cationic liposomes or immunoliposomes. The β-galactosidase activity after repeated exposure was greater than after a single exposure and reached about 1.5- to 2-fold by the fourth exposure.

Discussion

Liposomes containing drugs or high molecular weight substances within the aqueous phase and/or in the lipid bilayer are regarded as a useful delivery

Table 1 β-Galactosidase activity in glioma cells transfected with pCH110 by a single exposure to liposomes or immunoliposomes

Additives	U251-MG-S	U251-SP-S	KMS-II104
No addition	0.11 ± 0.03	0.07 ± 0.04	4.6 ± 1.3
Empty lip	0.03 ± 0.02	0.01 ± 0.01	3.5 ± 1.0
Empty immunolip			
whole IgG	0.08 ± 0.04	0.10 ± 0.02	4.7 ± 2.1
$F(ab')_2$	0.07 ± 0.02	0.05 ± 0.04	4.5 ± 1.8
Liposomes	88.3 ± 14.7	53.5 ± 10.7	12.2 ± 4.0
Immunolip			
whole IgG	133.4 ± 21.0*	92.1 ± 12.8*	11.7 ± 3.3
$F(ab')_2$	124.3 ± 24.1*	90.5 ± 15.0*	12.1 ± 4.1

All values are β-galactosidase activity (mU/mg protein, n = 3). *$p < 0.05$ *vs.* Liposomes. Empty lip: empty liposomes (15 nmol lipids/ml), Immunolip: immunoliposomes containing pCH110 (15 nmol/ml of lipids, 0.3 μg/ml of DNA, and G-22 monoclonal antibody 2 μg per 1 μmol lipids), Liposomes: liposomes containing pCH110 (15 nmol/ml of lipids and 0.3 μg/ml of DNA).

Table 2 β-Galactosidase activity in glioma cells transfected with pCH110 by repeated exposure to liposomes or immunoliposomes

(1) U251-MG-S cells

Additives	Frequency of exposure		
	Once	Twice	Fourth
No addition	0.10 ± 0.02	0.09 ± 0.06	0.07 ± 0.03
Empty lip	0.04 ± 0.01	0.08 ± 0.02	0.01 ± 0.01
Liposomes	80.7 ± 13.0	101.8 ± 14.6	122.3 ± 23.0#
Immunolip	124.3 ± 24.1*	149.6 ± 17.7*	213.5 ± 28.4*,##

(2) U251-SP-S cells

Additives	Frequency of exposure		
	Once	Twice	Fourth
No addition	0.07 ± 0.01	0.14 ± 0.08	0.09 ± 0.02
Empty lip	0.08 ± 0.04	0.10 ± 0.08	0.05 ± 0.03
Liposomes	54.6 ± 12.7	94.5 ± 12.5##	74.6 ± 17.0
Immunolip	88.4 ± 25.6	89.6 ± 30.4	114.8 ± 29.0

(3) KMS-II104 cells

Additives	Frequency of exposure		
	Once	Twice	Fourth
No addition	5.4 ± 1.5	4.1 ± 1.4	3.5 ± 2.6
Empty lip	4.8 ± 1.7	5.1 ± 2.9	5.0 ± 3.6
Liposomes	12.6 ± 4.4	23.1 ± 4.8	28.1 ± 8.4
Immunolip	13.6 ± 3.8	19.7 ± 3.5	24.3 ± 3.7

All values are β-galactosidase activity (mU/mg protein, n = 3). *$p < 0.05$, **$p < 0.01$ *vs.* Liposomes. #$p < 0.05$, ##$p < 0.01$ *vs.* Once. Empty lip: empty liposomes (15 nmol lipids/ml), Immunolip: immunoliposomes containing pCH110 (15 nmol/ml of lipids, 0.3 μg/ml of DNA, and G-22 monoclonal antibody 2 μg per 1 μmol lipids), Liposomes: liposomes containing pCH110 (15 nmol/ml of lipids and 0.3 μg/ml of DNA).

system. Liposomes used as DNA delivery systems are expected to be non-toxic and non-immunogenic, and to achieve a high transfection efficiency. However, the transfection efficiency of ordinary liposomes is very low. Felgner *et al.*[1,2] demonstrated that cationic lipids (Lipofectin) facilitate the functional delivery of DNA into cells. We[5,6,8,11] and Yagi *et al.*[10] also found that cationic liposomes composed of N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroyl phosphatidylcholine, and dioleoyl phosphatidylethanolamine in the molar ratio of 1:2:2 have a high gene transfection efficiency to various cell types. In general, cationic liposomes have a significant toxicity when a large amount of cationic liposomes are applied to the cells. The toxicity varies with cell types, the duration of exposure, and the density of the cell culture. Our cationic liposomes are less toxic compared with Lipofectin. Therefore, cationic liposomes should be used within optimal concentration.

Selective and efficient transfer of the gene to target cells is very important for clinical application. We associated the liposomes with a monoclonal antibody (G-22 glioma-specific monoclonal antibody). However, the G-22 monoclonal antibody is a mouse IgG monoclonal antibody, and is difficult to apply repeatedly to humans. Use of the $F(ab')_2$ fragment would reduce the immunogenicity of the antibody. Fortunately, we found no significant difference between immunoliposomes containing whole IgG and $F(ab')_2$, and the association of the $F(ab')_2$ to cationic liposomes markedly increased the transfection efficiency. Furthermore, the transfection efficiency increased about 2-fold by repeated exposure. These results indicate that repeated exposure of immunoliposomes has a high potential for the transfer of genes in clinical applications.

References

1) Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JR, Ringold GM, Danielson M: Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 84: 7413–7414, 1987
2) Felgner RL, Ringold GM: Cationic liposome-mediated transfection. *Nature* 337: 387–388, 1989
3) Fraley R, Subramani S, Berg P, Papahadjopoulos D: Introduction of liposome-encapsulated SV40 DNA into cells. *J Biol Chem* 255: 10431–10435, 1980
4) Hess HH, Lees MB, Derr JE: A linear Lowry-Folin assay for both water-soluble and sodium dodecyl sulfate-solubilized proteins. *Anal Biochem* 85: 295–300, 1978
5) Mizuno M, Yoshida J, Sugita K, Inoue I, Seo H, Hayashi Y, Koshizaka T, Yagi K: Growth inhibition of glioma cells transfected with the human β-interferon gene by liposomes coupled with a monoclonal antibody. *Cancer Res* 50: 7826–7829, 1990
6) Mizuno M, Yoshida J, Sugita K, Yagi K: Growth inhibition of glioma cells of different cell lines by human interferon-β produced in the cells transfected with its gene by means of liposomes. *J Clin Biochem Nutr* 7: 185–192, 1990
7) Okada H, Yoshida J, Seo H, Wakabayashi T, Sugita K, Hagiwara M: Anti-glioma surface antigen monoclonal antibody G-22 recognizes overexpressed CD44 in glioma cells. *Cancer Immunol Immunother* 39: 313–317, 1994
8) Tashiro T, Yoshida J, Mizuno M, Sugita K: Reinforced cytotoxicity of lymphokine-activated killer cells toward glioma cells by transfection with the

tumor necrosis factor-α gene. *J Neurosurg* 78: 252–256, 1993

9) Wakabayashi T, Yoshida J, Seo H, Kato K, Murata Y, Matsui N, Kageyama N: Characterization of neuroectodermal antigen by a monoclonal antibody and its application in CSF diagnosis of human glioma. *J Neurosurg* 68: 449–455, 1988

10) Yagi K, Noda H, Kurono M, Ohishi N: Efficient gene transfer with less than cytotoxicity by means of cationic multilamellar liposomes. *Biochem Biophys Res Commun* 196: 1042–1048, 1993

11) Yoshida J, Mizuno M: Simple method to prepare cationic multilamellar liposomes for efficient transfection of human interferon-β gene to human glioma cells. *J Neurooncol* 19: 261–274, 1994

12) Yoshida J, Mizuno M: Simple preparation and characterization of cationic liposomes associated with a monoclonal antibody against glioma-associated antigen (immunoliposomes). *Journal of Liposome Research* 5: 981–995, 1995

Address reprint requests to: J. Yoshida, M.D., Department of Neurosurgery, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan.