Asialoganglioside Enhances the Efficiency of Gene Transfection Mediated by Cationic Liposomes with a Cationic Cholesterol Derivative

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We investigated the transfection efficiency mediated by asialoganglioside-containing cationic liposomes. Previously, we reported that monosialoganglioside GM\textsubscript{1a} (GM\textsubscript{1a}) enhanced transfection efficiency. In this study, we investigated the effects of sialic acid in gangliosides on transfection efficiency. Two mammalian culture cell lines HeLa and HepG2 were transfected with luciferase plasmids (pGL3) using cationic liposomes which contain monosialoganglioside GM\textsubscript{1a} (GM\textsubscript{1a}) or its asialic counterpart, asialoganglioside GM\textsubscript{1a} (GA\textsubscript{1}). Both GM\textsubscript{1a} and GA\textsubscript{1} enhanced the efficiency of transfection mediated by cationic liposomes, and GA\textsubscript{1} exhibited higher efficiency than GM\textsubscript{1a} in both cell lines. Transfection efficiency of ganglioside-containing liposomes was also assessed by the effects of antisense oligonucleotides (AS-ODN) for bcl-2 gene, which suppresses apoptotic cell death. Western blotting analysis revealed that the expression of Bcl-2 was decreased by AS-ODN, and the reduction of protein expression in cells treated with GA\textsubscript{1}-containing liposomes was more remarkable than that with GM\textsubscript{1a}-containing liposomes. Furthermore, the induction rate of apoptosis was higher in cells treated with AS-ODN with GA\textsubscript{1}-containing liposomes. Together with the results obtained by luciferase assay mentioned above, the removal of sialic acid from ganglioside causes the enhancement of efficiency of transfection mediated by cationic liposomes.

Key words gene transfection; asialoganglioside; cationic cholesterol; sialic acid; antisense oligonucleotide; bcl-2

Cationic liposomes are commonly used for delivery of foreign genes into target cells.\textsuperscript{1, 2, 3} Various kinds of cationic liposomes for gene delivery have been developed and some have exhibited very high transfection efficiency.\textsuperscript{4–8} However, their transfection efficiency was not satisfactory compared to viral vectors. In parallel with efforts to elucidate the mechanism of gene transfection with cationic liposomes,\textsuperscript{9–13} all sorts of molecules ranging from synthetic compounds to biomolecules were introduced to liposome-based gene transfer systems to obtain higher efficiency. For example, the co-introduction of peptides with an NLS-like sequence\textsuperscript{14} or the cationic polymer polyethylenimine\textsuperscript{15} facilitated nuclear transport, resulting in higher efficiency. Biomolecules such as transferrin\textsuperscript{16} enhance efficiency by facilitating receptor-mediated endocytosis. We also reported that various kinds of molecules enhance transfection efficiency. Inhibitors of microtubules,\textsuperscript{11} biosurfactants (surface-active compounds produced by microorganisms),\textsuperscript{17} and growth factors\textsuperscript{18} increase the efficiency of gene delivery when used with cationic liposomes.

Recently, we found that monosialoganglioside GM\textsubscript{1a} enhances efficiency by facilitating receptor-mediated endocytosis.\textsuperscript{19} In addition, it was reported that GM\textsubscript{1a}-containing liposomes effectively reduced the splenic uptake of large GM\textsubscript{1a}-containing liposomes for drug delivery and maintained long-term expression of the gene in mice.\textsuperscript{20} These findings led us to further study gangliosides, especially as a transfection agent complexed with cationic liposomes.

In the present study, we investigated the effects of sialic acid in gangliosides on transfection efficiency. We compared the efficiency of transfection mediated by cationic liposomes with monosialoganglioside and asialoganglioside in which an N-acetylneuraminic acid group (sialic acid group) was removed from the ganglioside. Many kinds of proteins have glycochains and some have sialic acid groups. However, sialic acid groups are removed by sialidase in the bloodstream\textsuperscript{13} and this glycoprotein without sialic acid (asialoglycoprotein) is internalized into liver cells by receptor-mediated endocytosis. Therefore, it is expected that an exogenous gene complexed with asialoganglioside-containing liposomes is targeted to liver cells, since liver cells express receptors specific for asialoglycoprotein. We found that asialoganglioside showed higher transfection efficiency than monosialoganglioside when used with cationic liposomes. Furthermore, this enhancement was observed not only in HepG2 cells (hepatocytoma) but also in HeLa cells.

MATERIALS AND METHODS

Chemicals A cationic cholesterol derivative, cholosteryl-3β-carboxymido ethylene-N-hydroxyethylamino was described in our previous paper.\textsuperscript{5} Monosialoganglioside GM\textsubscript{1a}, GM\textsubscript{2}, GM\textsubscript{3}, asialoganglioside GM\textsubscript{1} (GA\textsubscript{1}), 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) 4,4′-dicarboxy-2,2′-biquinoline (BCA), and polyoxyethylene sorbitan monolaurate (Tween 20) were purchased from Sigma (St. Louis, MO, U.S.A.). Luciferase plasmid (pGL3) was obtained from Promega (Madison, WI, U.S.A.). The sequence of antisense bcl-2 phosphorotioate oligodeoxyribonucleotide (AS-ODN) was 5′-TCTCCACGGTGCCCAT-3′, which directs the translation initiation site of bcl-2 transcripts. The sequence of nonsense phosphorotioate oligodeoxyribonucleotide was 5′-TGCATCTACGTCGGCCT-3′, which was the scrambled-sequence to bcl-2 AS-ODN. All ODNs were purchased from Japan Gene Research Co. (Sendai, Japan).

Cell Culture HeLa and HepG2 cells were obtained from the Riken Gene Bank (Tsukuba, Japan). HeLa cells were cultured in Eagle's MEM (Nissui, Tokyo, Japan) supplemented with 2 mM l-glutamine, 0.1 mM nonessential amino acid solutions, 1.5 mg/ml NaHCO\textsubscript{3}, and 10% fetal calf serum (Boehringer Mannheim). HepG2 cells were cultured in Eagle's MEM supplemented with 2 mM l-glutamine, 0.1 mM nonessential amino acid solutions, 1.5 mg/ml NaHCO\textsubscript{3}, and

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Preparation of Liposomes with Ganglioside DOPE (20 nmol) was mixed with cholesteryl-3β-carboxyamido ethylene-N-hydroxyethylamine (30 nmol) and ganglioside in chloroform/methanol (1:1), and the mixture was evaporated under reduced pressure to give a thin film of lipids. To the thin film, phosphate-buffered saline (PBS) was added, and the mixture was allowed to stand for 60 min in the dark. The samples were then sonicated using a bath-type sonicator (Bransonic Model B1200, Branson) for 5 min, followed by sonication with a probe-type sonicator (Sonifier 250, Branson) for 10 min to form small unilamellar vesicles (SUVs).

The cationic liposomes were mixed with plasmid DNA (5 μg) to form liposome/DNA complexes. Liposome–DNA complexes were incubated with the cells (1×10^6 cells/dish) in SFM-101 in a culture dish (60×15 mm) for 4 h at 37 °C, and then the cells were washed and cultured in growth medium (MEM) for another 24 h at 37 °C, followed by a luciferase assay.

Luciferase Assay The luciferase assay was carried out using a picagene luciferase assay kit (Toyo Ink, Tokyo). The transfected cells were washed three times with PBS and lysed in a cell lysis buffer. The lysate was centrifuged at 12000×g at 4 °C for 3 min and the supernatant was subjected to a luciferase assay. The intensity of chemiluminescence was measured with a luminometer (TD-20/20, Turner Designs; Sunnyvale, CA, U.S.A.) and values were normalized to the amount of protein determined by BCA assay.

Western Blotting Western blotting analysis of Bcl-2 was carried out following procedures described previously. In brief, cells were harvested and lysed with NP-40 lysis buffer (50 mM Tris–HCl, pH 7.9, 150 mM NaCl, 1% (V/V) NP-40, 3 M urea, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After sonication for 10 s and centrifugation at 15000 rpm at 4 °C for 5 min, the supernatants were used as samples for immunoblot analysis. Mouse monoclonal antibody for Bcl-2 (c-2) and secondary antibody were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody for β-actin (AC-15) was purchased from Sigma. Samples were electrophoresed by sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were visualized by enhanced chemiluminescence using an ECL™ kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and measured with a luminimage analyzer (LAS-1000, FUJIFILM, Japan). Relative protein levels were quantified by analysis software (Image Gauge, FUJIFILM).

Measurement of Apoptosis Induction of apoptosis was evaluated by the propidium iodide method using flow cytometry. In treatment of cells with oligonucleotide, cells were washed and stained with propidium iodide in PI solution (50 μg/ml propidium iodide, 3.4 mM sodium citrate, 0.1% Triton-X100, 0.1 mM EDTA, 1 mM Tris–HCl, pH 7.9) for 30 min at 4 °C. After washing, the cells were subjected to flow cytometry (FACSort, Becton Dickinson, NJ, U.S.A.).

RESULTS

Transfection Efficiency Mediated by Cationic Liposomes Containing Gangliosides Transfection efficiency was measured in two cell lines, HeLa and HepG2 cells. HepG2 is derived from human hepatocytes and expresses receptors for asialoglycoproteins. Cells were transfected with pGL3, which codes luciferase protein, by cationic liposomes with ganglioside or asialoganglioside. We used monosialo-ganglioside GM_1a and asialoganglioside GM_1 (GA_1) which is an asialo-counterpart of monosialoganglioside GM_1a. The transfection efficiency was dependent on the amount of ganglioside in liposomes and the efficiency was highest when the concentration of ganglioside in cationic liposomes was 0.5 mol% (data not shown). Therefore, the concentration of ganglioside was fixed at 0.5 mol% in the following experiments. Both GM_1a and GA_1 enhanced the transfection efficiency in HeLa cells, while only GA_1 exhibited significant effects in HepG2 cells (Fig. 2). As expected, GA_1 was more effective than GM_1a in HepG2 cells which express receptors for asialoglycoprotein. Interestingly, GA_1 exhibited a greater ability than GM_1a to deliver a foreign gene in HeLa cells. This might imply that asialogangliosides have a greater abil-
ity for gene delivery than monosialogangliosides through the ubiquitously expressed recognition system for sugar other than asialoglycoprotein receptors expressed in HepG2 cells specifically. Other monosialogangliosides without a galactose group, GM2 and GM3, did not enhance transfection efficiency in either cell line (Fig. 2), suggesting galactose at the terminal of a glycochain is essential for the enhancement of transfection efficiency.

Reduction of Expression of Bcl-2 by Antisense Oligonucleotide

To investigate transfection efficiency at the functional level, we introduced antisense-oligonucleotide (AS-ODN) for \( bcl-2 \). Bcl-2 was discovered as one of the first mammalian regulators of apoptosis, and blocking expression of this protein is thought to be an important target for antisense therapy. Introduction of AS-ODN of \( bcl-2 \) into target cells is expected to reduce the expression of Bcl-2 and induce apoptosis of the target cells. In this study, we transferred AS-ODN of \( bcl-2 \) into HeLa cells. As revealed by Western blotting analysis shown in Fig. 3A, a reduction in expression of Bcl-2 was observed in HeLa cells treated with AS-ODN. The results of Western blotting are summarized in Fig. 3B. As in the case of luciferase assay shown in Fig. 2A, the reduction rate was larger in the case of GA1-containing liposomes than in the case of GM1a-containing liposomes (Fig. 3B).

Furthermore, similar and consistent results were obtained at the functional level of Bcl-2. Figure 4 shows the induction rate of apoptosis in cells treated with AS-ODN of \( bcl-2 \). Apoptotic cell death was induced in larger number of cells when the cells were treated with AS-ODN complexed with GA1-containing liposomes. Apoptosis was also observed morphologically with differential interference microscopy and confocal laser scanning microscopy, and the enhancement of apoptosis in cells treated with AS-ODN was confirmed by a reduction in cell size, fragmentation of the nucleus, and membrane blebbing (data not shown).

Transfection in the Presence of Galactose

Several lines of evidence suggest that ganglioside is recognized by its specific receptor through galactose at the glycochain terminal.24,25 We previously reported that an excess amount of galactose decreased the transfection efficiency mediated by cationic liposomes with GM1a.19) To examine the mechanism of enhancement of transfection efficiency, HepG2 cells were transfected in the presence of an excess amount of galactose. As shown in Fig. 5, transfection efficiency mediated by cationic liposomes with ganglioside was greatly decreased in the presence of 100 mM galactose when the cells were transfected with GA1-containing liposomes, while galactose did not affect the efficiency when the cells were transfected with

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Fig. 2. Efficiency of Transfection Mediated by Cationic Liposomes with Various Gangliosides

Transfection efficiency was evaluated by measuring luciferase activity of cell lysates prepared from HeLa and HepG2 cells transfected with luciferase gene pGL3. Error bars stand for mean±S.D. and \( p \) values were obtained by Student’s \( t \)-test.

Fig. 3. Transfection Efficiency Evaluated by Expression of Bcl-2 in Cells Treated with Antisense Oligonucleotide

(A) Expression of Bcl-2 and \( \beta \)-actin in HeLa cells treated with AS-ODN of \( bcl-2 \) complexed with ganglioside-containing liposomes was evaluated by Western blotting. The signal intensity of lysate from intact cells (CT) was used as 100% expression. Treatment of cells with cationic liposomes without ODN, with AS-ODN and NS-ODN were expressed as CL, AS, and NS, respectively. (B) Reduction rates of Bcl-2 expression in HeLa cells are summarized. Each value was calculated as follows; reduction rate (%)=(1−\((I_{100}−I_b)/I_{100}\))×100, where \( I \) : signal intensity, \( I_b \) : background intensity, \( I_{100} \): signal intensity of 100% expression. White, black and gray bars represent treatments with no ODN, AS-ODN and NS-ODN, respectively. Error bars stand for mean±S.D. and \( p \) values were obtained by Student’s \( t \)-test.

Fig. 4. Induction of Apoptosis in HeLa Cells Treated with AS-ODN of \( bcl-2 \)

The induction rates of apoptosis were compared among various kinds of liposome–DNA complexes. The induction rate was measured and calculated by flow cytometry (FACSort). White, black and gray bars represent treatments with no ODN, AS-ODN and NS-ODN, respectively. A significant difference was observed between GA1 (+) and no ganglioside by Student’s \( t \)-test (\( p<0.01 \)).
cationic liposomes without GA1.

These results suggested that the enhancement of transfection efficiency was mainly mediated by receptor-mediated endocytosis, probably through receptors of asialoglycoprotein.

DISCUSSION

In this study, we showed that asialoganglioside GA1 promoted gene transfer into mammalian cultured cells with higher efficiency than monosialoganglioside GM1a. We previously reported that monosialoganglioside GM1a enhanced transfection efficiency mediated by cationic liposomes. We demonstrated that asialoganglioside GA1 had higher efficiency than monosialo-counterpart GM1a by two ways, one the expression of exogenously introduced protein (Fig. 2), the other a reduction in the expression of endogenous protein Bcl-2 by antisense oligonucleotides (Fig. 3). Furthermore, measurement of apoptosis induced by AS-ODN of bcl-2 supported our conclusion (Fig. 4). Small but not negligible apoptosis was observed in cells other than AS-ODN-treated cells. This apoptosis might reflect a reduction in the expression level of Bcl-2 observed in cells treated with AS-ODN and without ODN (Fig. 3). Since both apoptosis and a reduction in Bcl-2 were observed in cells treated with liposomes without ganglioside, the procedure for transfection, such as incubation of cells without serum, might have caused these phenomena.

Since the transfection efficiency decreased in the presence of an excess amount of galactose (Fig. 5), the enhancement of efficiency is mainly due to the receptor-mediated uptake of liposome–DNA complexes. This is consistent with the result that gangliosides without a galactose group at the terminal, GM2 and GM3, did not increase the transfection efficiency (Fig. 2). It is likely that GM2 is recognized by the asialoglycoprotein receptor since GM2 has N-acetylgalactosamine. In the present study, however, GM2-containing liposome did not enhance the transfection efficiency as shown in Fig. 2. There are several points to be taken into account when discussing the recognition of GM2 by asialoglycoprotein receptor through N-acetylgalactosamine. First, a glycochain with N-acetylgalactosamine in GM2 is connected to a sphingolipid and not a protein. Second, in this study GM2 exists in the complex of liposome. Third, since transfection efficiency was measured by expression of protein, recognition of GM2 by receptors does not always reflect the transfection efficiency directly. The results obtained in this study did not provide the direct evidence for the recognition of GM2 by the asialoglycoprotein receptor, but strongly suggested the involvement of specific receptors. Thus it is interesting that GA1 had higher efficiency than GM1a which had higher efficiency than GM2, especially from the point of structure–activity correlation of gangliosides with specific receptors.

The difference in the efficiency between GM1a and GA1 still needs to be clarified. It is unlikely that sialic acids of gangliosides affect the formation of liposome–DNA complexes since the concentration of ganglioside in cationic liposomes is only 0.5 mol%. It is more probable that the existence of sialic acid decreased the affinity of gangliosides to the specific receptor, resulting in a decrease in cellular uptake of exogenous DNA.

Interestingly, we found that GA1 enhanced transfection efficiency not only in HepG2 cells but also in HeLa cells. This suggests that GA1 has the ability to increase the efficiency even in the absence of receptors which express in HepG2 cells specifically. A ubiquitously expressed recognition system for sugar might be involved in the recognition of GA1 in HeLa cells. There was no significant difference in the excess amount of galactose between transfection with and without GA1 (Fig. 5). This result suggests that the recognition system which recognizes GA1 in HeLa cells is not nonspecific, but rather specific to sugar and blocked by excess galactose, although its specificity might not be high.

REFERENCES


