Efficient Gene Transfer to Hepatoblastoma Cells through Asialoglycoprotein Receptor and Expression under the Control of the Cyclin A Promoter

Yukihiro Aramaki, a Insugi Lee, a Hidetoshi Arima, a Takatoshi Sakamoto, a Yasushi Magami, b Takayuki Yoshimoto, b Fuminori Moriyasu, b Junichiro Mizuguchi, d Yasuhisa Koyanagi, b, c Toshio Nikaido, f and Seishi Tsuchiya a, b

Tokyo University of Pharmacy and Life Science; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan: b Division of Gene Therapy, Intractable Disease Research Center, Tokyo Medical University; 6–1–1 Shinjuku, Shinjuku-ku, Tokyo 160–8402, Japan: c Fourth Department of Internal Medicine, Tokyo Medical University; 6–7–1 Nishishinjuku, Shinjuku-ku, Tokyo 160–0023, Japan: d Department of Surgery, Tokyo Medical University; 6–1–1 Shinjuku, Shinjuku-ku, Tokyo 160–8402, Japan: e Department of Obstetrics and Gynecology, Shinshu University School of Medicine; 3–1–1 Asahi, Matsumoto, Nagano 390–8621, Japan. Received September 30, 2002; accepted December 9, 2002

Specific gene delivery into hepatoma cells by liposomes and specific gene expression under the control of the cyclin A promoter were examined in HepG2 cells, a hepatoblastoma cell line that overexpresses cyclin A. A plasmid carrying the luciferase gene under the cyclin A promoter sequence was condensed with poly-L-lysine and encapsulated into anionic asialofetuin-labeled liposomes (AF-liposomes), which were preferentially taken up by hepatocytes through the action of the asialoglycoprotein receptor (AgpR). AF-liposomes delivered plasmids to the hepatoma cells by receptor-mediated endocytosis through the AgpR, and transgene expression could be achieved under the control of the cyclin A promoter. Furthermore, a fusogenic lipid, DOPE, as a liposomal component was required for the enhancement of transfection efficiency of AF-liposomes.

Key words: liposome; asialoglycoprotein; gene delivery; gene expression; cyclin A promoter

The development of specific delivery vectors for gene transfer is a prerequisite for the clinical application of gene therapy in vivo. A variety of methods has been developed to transfer genes into eukaryotic cells. Although several highly efficient virus vectors are available, they have certain drawbacks such as immunogenicity, lack of tissue specificity, and difficulty of large-scale production, and there is the potential risk of inducing tumorigenic mutations and/or the generation of active viral particles through recombination. Synthetic vectors such as liposomes composed of cationic lipid derivatives, poly-L-lysine (PLL), and polyethyleneimine have therefore become an attractive alternative due to their lack of immunogenicity and restraints on the DNA they can carry.

However, these cationic carriers do not show the potential for tissue- or cell-specific targeting. The development of systems for tissue- or cell-specific delivery of genetic materials is required for the use of these cationic carriers in vivo. Ligand-labeled cationic carriers have been developed to deliver plasmid DNA to specific tissues or cells. We also reported that asialofetuin (AF)- and epidermal growth factor (EGF)-labeled cationic liposomes associated with plasmid DNA were efficiently taken up and showed efficient transfection in the human hepatoblastoma cell line HepG2 and EGF receptor-overexpressing cancer cells through the asialoglycoprotein receptor (AgpR) and EGF receptor, respectively. Such an approach will also lead to expression in normal cells expressing AgpR and EGF receptors when plasmid DNA constructed with a viral promoter is used. It is necessary to develop cell-specific gene expression systems, and cell-specific promoters are expected to be useful in tumor gene therapy.

During the cell cycle progression, cyclin A is involved in the onset of DNA replication and is required for the G2-M transition in mammalian cells. Overexpression of cyclin A would contribute to the high proliferative activity of hepatomas. Therefore we constructed a plasmid carrying the luciferase gene under the control of the human cyclin A promoter sequence (pCyclin A-Luc). To achieve gene delivery to hepatoma cells by AF-liposomes through the AgpR and specific gene expression under the control of the cyclin A promoter, pCyclin A-Luc was compacted by interaction with PLL and encapsulated into AF-labeled anionic liposomes (AF-liposomes) and then transfected into HepG2 cells. The advantages of the pCyclin A-Luc and AF-liposome system for hepatoma cell-specific gene expression are discussed in this paper.

MATERIALS AND METHODS

Materials HepG2 cells, a human hepatoblastoma cell line, were provided by Riken Cell Bank (Ibaraki, Japan). Phosphatidylycholine (PC) was purchased from Nippon Oil and Fat Co. Ltd. (Tokyo, Japan). Phosphatidylserine (PS), cholesterol (Chol), PLL, and AF were obtained from Sigma Co. Ltd. (St. Louis, MO, U.S.A.).

Plasmid Purification pCyclin A-Luc was constructed as previously described, isolated from Escherichia coli by the alkaline lysis method and purified using a plasmid Midi Kit (Qiagen).

Complex Formation of Plasmid DNA and PLL Complexes of the pCyclin A-Luc DNA plasmid (1 μg) and various amounts of PLL were prepared by rapid mixing in 50 μl of distilled water as reported by Lee and Huang. The formation of the DNA–PLL complex (polyplex) was evaluated in the gel retardation assay: samples (10 μl) were elec-
trophoresed through a 2% agarose gel and DNA was visualized by staining with 0.1 μg/ml of ethidium bromide.

Preparation of AF-Liposomes and Encapsulation of the Complex The net positive polypeptide of pCyclin A-Luc and PLL was encapsulated in negatively charged AF-liposomes composed of PC:PS:DOPE:Chol=3:2:3:2 by a combination of the detergent removal and freeze-thaw methods as described by Hara et al. Briefly, palmitoyl-AF (125 μg as AF) dissolved in 50 μl of 10 mM Tris–HCl buffer (pH 7.5) containing 2% sodium deoxycholate (DOC) was added to 1 μmol of liposomal lipid film and mixed micelles were prepared by vigorous vortexing. DOC was dialyzed against 10 mM Tris–HCl buffer (pH 7.5) for 24 h, and empty AF-liposomes were obtained. Following sonication for 5 min in a bath-type sonicator (Branson B-2000), AF-liposomes were then mixed with 10 μg of pCyclin A-Luc complexed with PLL in 10 mM Tris–HCl buffer (pH 7.5) and lyophilized. After rehydration with 20 μl of 10 mM Tris–HCl buffer (pH 7.5) containing 0.9% NaCl, freeze-thaw cycling was repeated twice to encapsulate the plasmid–PLL complex into AF-liposomes. AF unlabeled liposomes (N-liposomes) were also prepared by the same method without palmitoyl-AF. These liposomes encapsulating plasmid DNA (lipopolyplex) were purified by metrizamide-gradient centrifugation as described previously. Phospholipid concentrations in each fraction were measured as inorganic phosphorus by the method of Chen et al. Following the addition of 2% SDS to each fraction to degrade the lipopolyplex, the samples were subjected to agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide to visualize plasmid DNA, and then the encapsulation efficiency of DNA was evaluated using NIH images.

Transfection Protocol HepG2 cells were plated at a density of about 5×10^5 cells/60 mm culture dish (Corning) in 3 ml of DM-160AU medium containing 10% fetal calf serum (FCS) and antibiotics (100 units/ml of penicillin and 100 units/ml of streptomycin). The cells were grown to half confluence at 37°C in a humidified atmosphere containing 5% CO₂. In a typical experiment, HepG2 cells were transfected in 1.5 ml of medium without FCS by the addition of 0.5 ml of Hanks’ balanced salt solution (HBSS) containing 10 μg of plasmid encapsulated in AF-liposomes. Five hours later, the medium was replaced with fresh medium containing 10% FCS. Following an additional 24-h incubation, the cells were washed with 0.5 ml of phosphate-buffered saline and then lysed using Cell Culture Lysis Reagent (Promega). The cell lysate was then centrifuged and the supernatant was assayed for luciferase activity using a commercial kit from Promega on a luminometer (Lumi-Counter 1000, Nichion, Tokyo, Japan) and expressed in relative light units (RLU, photocounts/min/μg protein). Protein concentrations were determined using a Bradford microprotein assay kit (Bio-Rad).

RESULTS AND DISCUSSION

Receptor-mediated gene transfer is an attractive method for the treatment of genetic diseases since it permits the targeting of DNA to specific tissues or cells, and many ligand-labeled cationic carriers for the delivery of plasmid DNA into target tissues or cells have been developed. However, possible transgene expression in normal cells is a concern when plasmid DNA constructed with viral promoters such as cytomegalovirus and SV-40 is used. To avoid this, cell-specific gene expression systems using specific promoters, such as α-fetoprotein and carboxenonbrionic antigen, have recently been developed for use in tumor gene therapy.

Cyclin A is involved in the onset of DNA replication, and is required for the G2-M transition in the mammalian cell cycle. Overexpression of cyclin A contributes to the high proliferative activity of hepatoma, indicating that the cyclin A promoter is active in such cells. Therefore we examined the gene expression in the hepatoma cell line HepG2 under the regulation of the cyclin A promoter using AF-liposomes, which can deliver genes specifically to hepatic cells through the AgpR as a nonviral vector.

Polyplex Formation and Encapsulation into AF-Liposomes To encapsulate pCyclin A-Luc into AF-liposomes, DNA condensation was carried out by means of electrostatic interaction with PLL. Gel retardation is a result of electrostatic and steric effects and suggests the formation of a charged complex between anionic DNA and cationic carriers. Therefore we examined the complex formation of pCyclin A-Luc (6.5 kbp) and PLL (degree of polymerization [DP]: 92) by gel retardation using 2% agarose gel electrophoresis. As shown in Fig. 1, DNA mobility in the gel was retarded with increases in the ratio of PLL, and complete retardation was observed at a weight ratio of 1 : 0.75. Kukowska-Latallo et al. reported that ca. 1.7×10^15 negative charges are present per 1.0 μg of DNA, whereas PLL (DP: 92) has ca. 4.6×10^15 charges/1.0 μg. Thus the net charge of the complex with ratios above 1 : 0.5 may be positive if all of the phosphate groups in pCyclin A-Luc can bind to amino groups in PLL by electrostatic interaction. pCyclin A-Luc and PLL complex (polyplex) formed at a weight ratio of 1 : 0.75 with an excess positive charge was used for DNA condensation and AF-liposome incorporation.

Cationic pCyclin A-Luc–PLL complex was encapsulated into anionic AF-liposomes composed of PC:PS:DOPE:Chol=3:2:3:2 by the freeze-thaw method, and AF-liposomes encapsulating the pCyclin A-Luc–PLL complex (lipopolyplex) were purified by metrizamide-gradient centrifugation. AF-liposomes were floated at fraction 2 (Fig. 2B), and about 30% of added DNA was recovered in the same fraction (Fig. 2A). In the case of N-liposomes, almost the same recovery ratio was observed (data not shown).

Cationic liposomes show cytotoxicity, and it is necessary to take great care when cationic carriers are used as...
nonviral vectors. In this study, plasmid DNA condensed with a small amount of PLL was encapsulated in anionic AF-liposomes by electrostatic interaction, and thus the cytotoxicity caused by this lipopolyplex was negligible. The same strategy has been reported by Lee and Huang, and tumor cell-specific gene transfer was demonstrated using folate-labeled anionic liposomes in vitro.

Transgene Expression HepG2 cells were transfected with liposomes encapsulating 10 µg of pCyclin A-Luc–PLL complex at a weight ratio of 1 : 0.75, and luciferase expression was examined. As shown in Fig. 3, luciferase activities were observed when HepG2 cells were transfected with AF- and N-liposomes, and a significantly high level of expression was observed in the former. To confirm the contribution of the AgpR-mediated mechanism to gene transfer by AF-liposomes, the effects of free AF on the luciferase expression were examined. The addition of free AF significantly decreased luciferase expression in HepG2 cells transfected with AF-liposomes. In the case of N-liposomes, luciferase activity also decreased upon the addition of free AF, but no significant difference was observed. These findings suggest that the AgpR contributes to the high level of expression of luciferase. Previously, we demonstrated that rat hepatocytes have a binding site for negatively charged liposomes composed of PS or phosphatidic acid, and the binding affinity of negatively charged liposomes to this site is about 1/140 that of AF-liposome binding to the AgpR. The luciferase activity observed in N-liposomes may have been due to binding to this site. The effects of the ratio of plasmid DNA and liposomal lipids in lipopolyplex on luciferase expression were also examined, and the highest level of expression was observed at a weight ratio of 1 : 10 (data not shown).

We next examined whether gene expression could be regulated under the control of the cyclin A promoter. As shown in Fig. 4, no luciferase expression was observed in HepG2 cells transfected with AF-liposomes encapsulating the cyclin A promoterless plasmid. This result indicates that the cyclin A promoter is active in HepG2 cells, as reported by Chao et al. and hepatoma cell-specific gene expression could be achieved using plasmid DNA under the control of the cyclin A promoter in combination with AF-liposomes.

To enhance the level of gene expression, the effects of DOPE and chloroquine were examined. DOPE is a hexagonal II phase-forming lipid under physiological conditions and is able to promote membrane fusion. Therefore DOPE has been widely used in various cationic liposomes for transfection such as Lipofectin. As shown in Fig. 5, the highest level of luciferase expression was observed in cells transfected with AF-liposomes containing DOPE 20 mol%.
may be attributable to the fusogenic activity of DOPE, and the importance of liposome-cell fusion, whether with the plasma membrane or lysosome/endosome membrane, was suggested in the efficient transfection using AF-liposomes. In AF-liposomes with DOPE concentrations higher than 20 mol%, luciferase expression decreased with increasing DOPE level. The hexagonal II phase formed by DOPE increased the instability of liposomes and resulted in a low level of luciferase expression in cells transfected with AF-liposomes with high concentrations of DOPE.

It has been reported that ligands endocytosed through the AgpR are transferred to lysosomes and degraded. The effects of chloroquine, a lysosomotropic agent and inhibitor of lysosomal function, on the transfection efficiency of AF-liposomes encapsulating the pCyclin A-Luc–PLL complex were investigated. Pretreatment of HepG2 cells with chloroquine 100 μM for 30 min resulted in a significant increase in luciferase expression following transfection with AF-liposomes with different concentrations of DOPE (Fig. 5). In the presence of chloroquine, lysosomal function is inhibited and plasmid DNA transferred to lysosomes may escape lysosomal degradation. Thus DNA could be released into the cytoplasm without degradation in lysosomes, resulting in high transfection efficiency. This strongly suggests that intracellular trafficking of plasmid DNA transfected with AF-liposomes through the AgpR was responsible for the high levels of luciferase expression seen with chloroquine treatment. Surprisingly, a high level of luciferase activity was also observed when HepG2 cells were transfected with DOPE-free AF-liposomes following treatment with chloroquine. This finding suggests that plasmid DNA transfected with DOPE-free AF-liposomes is taken up by receptor-mediated endocytosis through the AgpR and is degraded by DNase in lysosomes, resulting in a low level of luciferase expression in HepG2 cells transfected without chloroquine treatment. Thus DOPE is an important lipid component of liposomes for efficient transfection by means of receptor-mediated endocytosis.

In conclusion, specific gene delivery to hepatoma cells by AF-liposomes though the AgpR and specific gene expression under the control of the cyclin A promoter were examined in the hepatoblastoma cell line HepG2, which shows overexpression of cyclin A. AF-liposomes delivered plasmids to hepatoma cells by receptor-mediated endocytosis through the AgpR, and transgene expression was achieved under the regulation of the cyclin A promoter. The fusogenic lipid DOPE is necessary to enhance the transfection efficiency.

Acknowledgments The authors thank Miss A. Muraoka for technical assistance.

REFERENCES