厚生労働科学研究費補助金

萌芽的先端医療技術推進研究事業

細胞内動態制御機能を有する新規細胞選択型ナノ遺伝子キャリアの開発と遺伝子治療への応用

平成１６年度　総括研究年度終了報告書

主任研究者　　川上　茂

平成１７（２００５）年　４月

目　　次

厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）
研究報告書

細胞内動態制御機能を有する新規細胞選択型ナノ遺伝子キャリアの開発と遺伝子治療への応用 (H16-ナノ-008)

主任研究者　川上　茂　京都大学大学院薬学研究科
分担研究者　奥田竜也　流動研究員

研究要旨　*In vivo* 遺伝子治療の実現には効率的に標的細胞に遺伝子を送達する技術の開発が不可欠であり、世界中において独自のベクター開発研究の推進・振興が強く望まれている。非ウイルスベクターによる *in vivo* 遺伝子導入は、ウイルスベクターと比較して、その安全性、低免疫原性、調製の容易さなどから今後の遺伝子治療において有望と考えられる。非ウイルスベクターの中でもカチオン性リポソームを用いた導入法は、高い遺伝子導入効率を示すことが知られており将来の遺伝子治療ベクターとして注目されているものの、正電荷を介した非特異的な機構による細胞への取り込みを利用しているため、特定の細胞への選択性を有しておらず、肺以外の臓器に対する遺伝子導入効率は低いことが知られている。申請者は、第３世代のナノ・遺伝子キャリア開発の先駆け研究として、肝臓を構成する各細胞が有する糖鎖認識機構は細胞に固有で比較的厳密な基質認識性を利用できるように分子設計した各種糖修飾カチオン性リポソームを開発し、*in vivo* での肝細胞選択的な遺伝子導入に既に成功し、遺伝子導入効率を制御する製剤学的因子も明らかにしている。本研究では、過去の遺伝子デリバリーシステム開発で培ってきた設計技術と実験評価法を駆使しつつ、特に細胞質内へ遺伝子を送達し得る新規機能性脂質を合成し、既存の *in vivo* レベルでの細胞選択的デリバリーシステム組み込むことで新しい多機能性標的指向型（体内/細胞内動態制御型）ナノ・遺伝子キャリアの創製・開発を目指す。本年度は、標的細胞へ取り込まれた後の細胞内動態制御させることを目的に、脂質のスペーサー部に pH 緩衝能を有するヒスチジンを組み込んだ新規ガラクトースまたはマンノース/ヒスチジン修飾コレステロール誘導体（Gal-または Man-His-C4- Chol）を設計・合成し、これを含有する新規細胞内動態制御型カチオン性リポソームを調製した。また、ホタルルシフェラーゼ (Luc) をコードしたプラスミド DNA (pCMV-Luc)を用いて複合体を種々の混合比率で調製し、複合体の粒子径ならびに複合体をマウス腹腔内投与後の遺伝子導入能に関する評価をおこなった。

A. 研究目的

本研究では、特に細胞質内へ遺伝子を送達し得る新規機能性脂質を合成し、既存の *in vivo* レベルでの細胞選択的デリバリーシステム組み込むことで新しい多機能性標的指向型（体内/細胞内動態制御型）ナノ・遺伝子キャリアの創製・開発を目指す。本年度は、標的細胞へ取り込まれた後の細胞内動態制御させることを目的に、これまでの脂質のスペーサー部に pH 緩衝能を有するヒスチジンを組み込んだ新規ガラクトースまたはマンノース/ヒスチジン (His) 修飾コレステロール誘導体（Gal/Man-His-C4-Chol）を設計・合成し、これを含有する機能性カチオン性リポソーム(DOTMA/Chol/Gal/Man-His-C4-Chol (1:0.5:0.5))を調製した。また、ホタルルシフェラーゼ (Luc) をコードしたプラスミド DNA (pCMV-Luc)を用いて複合体を種々の混合比率で調製し、複合体をマウス腹腔内投与後の遺伝子導入能に関する評価をおこなった。本機能性カチオン性リポソームの体内/細胞内動態制御機能を評価するための対照として、未修飾カチオン性リポソーム（DOTMA/Chol(1:1)リポソーム）ならびにマンノース修飾カチオン性リポソーム（DOTMA/Chol/Man-C4-Chol(1:0.5:0.5)リポソーム）を用いた。

B. 研究方法

Man/Gal-His-C4-Chol 合成: Cholesterol chlorofarmate を出発物質として、N-(4-aminobuthyl)carbamic acid *tert*-butyl ester とクロロホルム中で反応させる。クエン酸緩衝液で洗浄後、有機層を evaporation

し、Boc-C4-Chol を得た。その後、トリフルオロ酢酸を用いて脱保護し、末端にアミノ基を有する C4-Chol を得た。さらに、Boc-His(Boc)-OH を反応させ、トリフルオロ酢酸を用いて脱保護し、His-C4-Chol を得た。この His-C4-Chol のアミノ基と 2-imino-2-methoxy-1-thiomannosyde と反応させ、反応物に精製水を加えミセルを形成させた後、透析法により不純物を除き、凍結乾燥によりマンノース/His 修飾コレステロール誘導体、Man-His-C4-Chol を得た。同様の合成法にて、ガラクトース/His 修飾コレステロール誘導体、Gal-His-C4-Chol を得た。リポソームの調製: DOTMA/Chol/Man-His-C4-Chol をモル比 1:0.5:0.5 の割合で混合し、vortex 法により調製した。複合体調製ならびに平均粒子径の測定: pCMV-Luc ならびに各種リポソームを電荷のモル比 (-:+) 1.0:1.6 で混合し 30 分間室温で静置することで調製した。平均粒子径は動的光散乱法により測定した。*In vivo* 遺伝子導入実験: 5 週齢雌性 ICR マウス（22-25g）の腹腔内へ各種複合体 (p-CMV-Luc; 50 μg)を投与した。6 時間後にマウスを安楽死させ、腹腔滲出細胞 (APCs)、大網、リンパ節を回収し、それぞれの細胞における遺伝子発現レベルを測定した。

C. 結果

Mass spectrum より Man-His-C4-Chol ならびに Gal-His-C4-Chol の合成を確認した。最初に、Man-His-C4-Chol に関して評価をおこなったところ、Man-His-C4-Chol 含有リポソーム複合体の平均粒子径は、約 120 nm と対照のリポソーム複合体とほぼ同じ程度であった。*In vivo* における遺伝子導入能を評価したところ、Man-His-C4-Chol 含有リポソーム複合体では、標的細胞である免疫担当細胞が多数存在する APCs ならびにリンパ節において、対照のリポソーム複合体群に比べ有意に高い遺伝子発現を示すことが明らかとなった。

D. 考察

Man-His-C4-Chol 含有リポソーム複合体が *in vivo* において高い遺伝子導入能を有していることが明らかとなり、His による細胞内動態の改善の可能性が示された。また、Man-His-C4-Chol 含有リポソーム複合体では、より低い電荷比において高い遺伝子発現能を有する特長を有しており、したがって生体に投与後の正電荷による非特異的な吸着はより少なく、標的細胞選択性も改善できると推察される。来年度は、複合体の電荷、Man-His-C4-Chol 量、分子内 His 量の最適化により更なる *in vivo* 遺伝子発現増強を試み、遺伝子・核酸医薬品の体内/細胞内動態制御理論の構築を行っていく予定である。

E. 結論

新規機能性脂質、Man-His-C4-Chol 含有リポソームが、*in vivo* における有効な多機能性標的指向型（体内/細胞内動態制御型）ナノ・遺伝子キャリアとなり得る可能性が示された。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

1) Y. Hattori, S. Kawakami, S. Suzuki, F. Yamashita, and M. Hashida: Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice, *Biochemical and Biophysical Research Communications*, 317(4): 992-999 (2004).

2) K. Sakanaka, K. Kawazu, M. Tomonari, T. Kitahara, M. Nakashima, S. Kawakami, K. Nishida, J. Nakamura, and H. Sasaki: Ocular pharmacokinetic/pharmacodynamic (PK/PD) modeling for bunazosin after instillation into rabbits, *Pharmaceutical Research*, 21(5): 770-776 (2004).

3) M. Teshima, S. Kawakami, K. Nishida, J. Nakamura, T. Sakaeda, H. Terazono, T. Kitahara, M. Nakashima, and H. Sasaki: Prednisolone retention in integrated liposomes by chemical approach and pharmaceutical approach, *Journal of Controlled Release*, 97, 211-218 (2004).

4) S. Kawakami, A. Harada, K. Sakanaka, K. Nishida, J. Nakamura, T. Sakaeda, N. Ichikawa, M. Nakashima, and H. Sasaki: In vivo gene transfer to the eye using the cationic liposomes after intravitreal injection in rabbits, *International Journal of Pharmaceutics*, 278(2): 255-262 (2004).

5) E. Ishida, C. Managit, S. Kawakami, M. Nishikawa, F. Yamashita, and M. Hashida: Development of galactosylated emulsions for hepatocyte-selective targeting of lipophilic drug

in mice, *Pharmaceutical Research*, 21 (6): 932-939 (2004).
6) S. Kawakami, Y. Hattori, Y. Lu, Y. Higuchi, F. Yamashita, and M. Hashida: Effect of cationic charge on receptor-mediated transfection using mannosylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice, *Pharmazie*, 59 (5): 405-408 (2004).
7) M. Yamada, M. Nishikawa, S. Kawakami, Y. Hattori, T. Nakano, F. Yamashita, and M. Hashida: Tissue and intrahepatic distribution and subcellular localization of mannosylated cationic liposome/plasmid DNA complex after intravenous administration in mice, *Journal of Controlled Release*, 98 (1): 157-167 (2004).
8) S. Fumoto, S. Kawakami, T. Ito, K. Shigeta, F. Yamashita, and M. Hashida: Enhanced hepatocyte-selective in vivo gene expression by stabilized galactosylated liposome/plasmid DNA complex using sodium chloride on complex formation, *Molecular Therapy*, 10 (4): 719-729 (2004).
9) Y. Higuchi, M. Nishikawa, S. Kawakami, F. Yamashita, and M. Hashida: Uptake characteristics of mannosylated and fucosylated bovine serum albumin in primary cultured rat sinusoidal endothelial cells and Kupffer cells, *International Journal of Pharmaceutics*, 287 (1-2): 147-154 (2004).

2. 学会発表

1) 服部芳幸、呂 言、川上 茂、山下富義、橋田 充: マンノース修飾リポソームを用いたマクロファージに対する *in vivo* 遺伝子導入における投与経路の影響、*日本薬学会第124年会*、2004年3月29-31日、大阪.
2) 麓伸太郎、川上 茂、重田耕佑、山下富義、橋田 充: ガラクトース修飾リポソーム/pDNA電荷制御複合体の *in vivo* 遺伝子導入効率改善機構の解析、*日本薬学会第124年会*、2004年3月29-31日、大阪.
3) Y. Ito, S. Kawakami, F. Yamashita, and M. Hashida: Influence of physicochemical properties of lipoplex on acute toxicity and gene expression after intravenous administration, *Pharmaceutical Sciences World Congress (PSWC 2004)*, May 30-June 3, 2004, Kyoto.
4) Y. Lu, Y. Hattori, S. Kawakami, F. Yamashita, and M. Hashida: Efficient gene transfection to macrophages following the intraperitoneal injection of mannosylated lipoplex in mice, *Pharmaceutical Sciences World Congress (PSWC 2004)*, May 30-June 3, 2004, Kyoto.
5) K. Shigeta, S. Fumoto, S. Kawakami, Y. Ito, F. Yamashita, and M. Hashida: Effect of blood components on in vivo gene expression by galactosylated liposome/plasmid DNA complex, *Pharmaceutical Sciences World Congress (PSWC 2004)*, May 30-June 3, 2004, Kyoto.
6) Y. Hattori, Y. Lu, S. Kawakami, F. Yamashita, and M. Hashida: Enhanced cytotoxic T lymphocytes (CTL) activity of DNA vaccine using novel mannosylated cationic liposomes, *Pharmaceutical Sciences World Congress (PSWC 2004)*, May 30-June 3, 2004, Kyoto.
7) Y. Higuchi, M. Nishikawa, S. Kawakami, F. Yamashita, and M. Hashida: Uptake of NFκB decoy and inhibition of NFκB activation in RAW 264.7 cells, *Pharmaceutical Sciences World Congress (PSWC 2004)*, May 30-June 3, 2004, Kyoto.
8) S. Kawakami, S. Fumoto, K. Shigeta, Y. Ito, F. Yamashita, and M. Hashida: Enhanced hepatocyte-selective in vivo gene transfer by stabilized galactosylated lipoplex using sodium chloride on complex formation, *Pharmaceutical Sciences World Congress (PSWC 2004)*, May 30-June 3, 2004, Kyoto.
9) 服部芳幸、呂 言、川上 茂、山下富義、橋田 充: マンノース修飾リポソームを用いた DNA ワクチンの細胞選択的遺伝子導入による細胞障害性 T 細胞 (CTL) 誘導効果の増強、*第20回日本DDS学会*、2004年7月15-16日、東京.
10) 伊藤佳孝、波々伯部美幸、川上 茂、山下富義、橋田 充: Lipoplex の静脈内投与による IL-12 の肺選択的遺伝子導入、*第20回日本DDS学会*、2004年7月15-16日、東京.
11) 鈴木幸子、川上 茂、山下富義、橋田 充: カチオン性リポソームによる全トランスレチノイン酸 (ATRA) の肺癌細胞株 A549 に対する抗癌作用の増強、第 20 回日本 DDS 学会、2004年7月15-16日、東京.
12) Y. Ito, S. Kawakami, F. Yamashita, and M. Hashida: In vivo cytokine response against polyethyleneimine/plasmid DNA complexes after intravenous administration, *第19回日本薬物動態学会*, 2004年11月17−19日, 金沢.

H. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Y. Hattori S. Kawakami S. Suzuki F. Yamashita M. Hashida	Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administaration in mice	Biochemical and Biophysical Research Communications	317(4)	992-999	2004
S. Fumoto S. Kawakami Y. Ito K. Shigeta F. Yamashita M. Hashida	Enhanced hepatocyte-selective in vivo gene expression by stabilized galactosylated liposome/plasmid DNA complex using sodium chloride for complex formation	Molecular Therapy	10(4)	719-729	2004
M. Yamada M. Nishikawa S. Kawakami Y. Hattori T. Nakano F. Yamashita M. Hashida	Tissue and intrahepatic distribution and subcellular localization of a mannosylated lipoplex after intravenous administration in mice	Journal of Controlled Release	98(1)	157-167	2004
S. Kawakami Y. Hattori Y. Lu Y. Higuchi F. Yamashita M. Hashida	Effect of cationic chrge on receptor-mediated transfection using mannosylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice	Pharmazie	59(5)	405-408	2004
Y. Higuchi M. Nishikawa S. Kawakami F. Yamashita M. Hashida	Uptake characteristics of mannosylated and fucosylated bovine serum albumin in primary cultured rat sinusoidal endothelial cells and Kupffer cells	International Journal of Pharmaceutics	287(1-2)	147-154	2004
E. Ishida C. Managit S. Kawakami M. Nishikawa F. Yamashita M. Hashida	Biodistribution characteristics of galactosylated emulsions and incorporated probucol for hepatocyte-selective targeting of lipophilic drugs in mice	Pharmaceutical Research	21(6)	932-939	2004
S. Kawakami A. Harada K. Sakanaka K. Nishida J. Nakamura T. Sakaeda N. Ichikawa M. Nakashima H. Sasaki	In vivo gene transfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits	International Journal of Pharmaceutics	278	255-262	2004
M. Teshima S. Kawakami K. Nishida J. Nakamura T. Sakaeda H. Terazono T. Kitahara M. Nakashima	Prednisolone retention in integrated liposomes by chemical approach and pharmaceutical approach	Journal of Controlled Release	97	211-218	2004

K. Sakanaka K. Kawazu M. Tomonari T. Kitahara M. Nkashima S. Kawakami K. Nishida J. Nakamura H. Sasaki	Ocular pharmacokinetic/ pharmacodynamic modeling for bunazosin after instillation into rabbits	Pharmaceutical Research	21(5)	770-776	2004

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 317 (2004) 992–999
BBRC
www.elsevier.com/locate/ybbrc

Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice

Yoshiyuki Hattori, Shigeru Kawakami, Sachiko Suzuki, Fumiyoshi Yamashita, and Mitsuru Hashida*

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Received 27 January 2004

Abstract

The present study investigated the potency of the mannosylated cationic liposomes (Man liposomes) that we have developed in novel DNA vaccine carrier. Ovalbumin (OVA) was selected as a model antigen for vaccination; accordingly, OVA-encoding pDNA (pCMV-OVA) was constructed to evaluate DNA vaccination. The potency of the Man liposome/pCMV-OVA complex was compared with naked pCMV-OVA and that complexed with DC-Chol liposomes. In cultured mouse peritoneal macrophages, MHC class I-restricted antigen presentation of the Man liposome/pCMV-OVA complex was significantly higher than that of naked pCMV-OVA and that complexed with DC-Chol liposomes. After intravenous administration, OVA mRNA expression and MHC class I-restricted antigen presentation on $CD11c^+$ cells and inflammatory cytokines, such as TNF-α, IL-12, and IFN-γ, that can enhance the Th1 response of the Man liposome/pCMV-OVA complex were higher than that of naked pCMV-OVA and that complexed with DC-Chol liposomes. Also, the spleen cells from mice immunized by intravenous administration of the Man liposome/pCMV-OVA complex showed the highest proliferation response and IFN-γ secretion. These findings suggest that the targeted delivery of DNA vaccine by Man liposomes is a potent vaccination method for DNA vaccine therapy.

Keywords: DNA vaccine; Gene delivery; Mannosylated liposome; Cationic liposome; Plasmid DNA; Targeting

DNA vaccination, the administration of DNA-encoding antigen gene into the body, is of great interest in gene therapy as a means of immunotherapy against cancer and infectious diseases. Animal studies have shown that DNA immunization induces not only an antibody response but also a potent cell-mediated immune response against the encoding antigen [1–3]. This cell-mediated immune response plays a crucial role in the immune response against cancer and infectious diseases [4]. Recent immunological studies have demonstrated that gene transfection and subsequent activation of dendritic cells are key events in the development of immunity following DNA vaccination [5]. Antigen-presenting cells (APCs), especially dendritic cells (DCs), process peptide epitopes in the expressed antigen in the context of both class I and class II MHC molecules resulting in the induction of cytotoxic T lymphocytes (CTL) with the help of $CD4^+$ T cells [6,7]. Consequently, high transfection efficiency into DCs is essential for efficient DNA vaccination. In the early 1990s, Wolff et al. [8] demonstrated that sustained and efficient gene transfection could be achieved following the intramuscular administration of naked plasmid DNA (pDNA). Therefore, many attempts have been made to use naked pDNA-based immunization to produce humoral and cellular immunity and demonstrate its potency in non-human primates. However, recent clinical trials have shown that the immune response following topical injection of naked pDNA solution is insufficient [9,10], indicating that further improvements in the transfection efficiency involving some pharmaceutical modification are needed for DNA vaccine therapy.

* Corresponding author. Fax: +81-75-753-4575.
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.141

So far, many kinds of vectors, both viral and non-viral, have been developed to enhance transfection activity to various cells [11–15]. Although virus-based vectors are known to exhibit high transfection efficiency to DCs, their immunogenicity and toxicity restricts their application to ex vivo DC therapy. Non-viral vectors, such as cationic polymers and cationic liposomes, have seen a great advance clinically due to their low toxicity, low immunogenicity, and ease of preparation.

Perrie et al. [16] reported that cationic liposomes enhance gene expression in the draining lymph node, which contains many APC populations, after intramuscular administration as well as enhancing the antigen-specific antibody response. Furthermore, other investigators have also demonstrated that cationic liposomes enhance the CTL response to antigens, such as hepatitis C virus, human immunodeficiency virus, and influenza antigen [17–19]. Although the adjuvant effect of cationic liposomes has been widely accepted, the lack of cell-specificity of cationic liposomes after in vivo application is regarded as limiting their transfection efficiency to APCs and the resulting adjuvant efficiency. For efficient gene therapy, non-viral vectors offer the major advantages of sustained effect, high-level transgene expression with minimal toxicity, and immunological side effects. It is well known that APCs express high levels of mannose receptors having a high capacity for the uptake of antigens that have mannose residues. Therefore, one promising approach for efficient gene delivery to APCs is attachment of mannose residues to cationic liposomes for cell-selective gene transfection.

Recently, we synthesized a novel mannosylated cationic cholesterol derivative{(cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosyl-ethyl)amino)butyl)formamide) (Man-C4-Chol)} for the preparation of mannosylated liposomes (Man liposomes) [20,21]. Man-C4-Chol exhibits bi-functional properties, i.e., an imino group for binding to pDNA via electrostatic interaction and a mannose residue for the cell surface receptors in APCs. Therefore, a high density of mannose residues can be provided on the liposome surface without affecting the binding ability of the cationic liposomes to DNA in these mannosylated cholesterol derivatives. In fact, we have reported that Man-C4-Chol mixed with dioleoylphosphatidylethanolamine (DOPE), which is a pH-sensitive lipid that accelerates the endosomal escape of pDNA [22], results in liposomes that can deliver firefly luciferase-encoding pDNA (pCMV-Luc), a conventional model gene for evaluating gene transfection. This delivery is selective for APCs via mannose receptor-mediated endocytosis after intravenous or intraportal injection into mice [20,23].

The Man liposome formulation allows development of a DNA vaccine with suitable pharmaceutical properties for APC targeting under in vivo conditions; therefore, this carrier system is expected to improve the immune response of this novel DNA vaccine. We selected ovalbumin (OVA) as a model antigen for DNA vaccination; accordingly, OVA encoding pDNA (pCMV-OVA) was constructed for the evaluation of DNA vaccination. The potency of the Man liposome/pCMV-OVA complex was compared with that of naked pCMV-OVA and that complexed with 3β [*N*-(*N'*-*N'*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) liposomes [24].

Materials and methods

Materials. Cholesteryl chloroformate, Hepes, and OVA were obtained from Sigma Chemicals (St. Louis, MO, USA). Diphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester was obtained from Tokyo Chemical Industry (Tokyo, Japan). Interleukin (IL)-2, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α ELISA kits were purchased from Genzyme Techne (Minneapolis, MN, USA). pVAX I, fetal bovine serum (FBS), and Opti-MEM I were obtained from Invitrogen (Carlsbad, CA, USA). Anti-CD11c monoclonal antibody (N418)-labeled magnetic beads were purchased from Miltenyi Biotec (Auburn, CA, USA). Alamar-Blue was purchased from TRECK Diagnostic Systems (West Sussex, UK). RPMI 1640 and thioglycollate medium were purchased from Nissui Pharmaceutical (Tokyo, Japan). Nucleic Acid Purification Kit MagExtractor-RNA- was purchased from TOYOBO (Osaka, Japan). 1st strand cDNA synthesis kit for RT-PCR(AMV), Lightcycler fast-start DNA master hybridization probes, and Lightcycler-Primer/Probes set for mouse β-actin were purchased from Roche diagnostics (Indianapolis, IN, USA). Primers/Probes for OVA were purchased from NIHON Gene Research Lab's (Sendai, Japan). All other chemicals were of the highest purity available.

Mice and cell line. Female ICR mice (4–5 weeks) and female C57BL/6 mice (6–8 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by US National Institutes of Health and the Guideline for Animal Experiments of Kyoto University. CD8OVA1.3 (kindly provided by Dr. C.V. Harding, Case Western Reserve University, Cleveland, OH, USA) [25] is a T cell hybridoma cell, which is specific for OVA (257–264)-kb.

Synthesis of Man-C4-Chol. Man-C4-Chol was synthesized as described previously [21]. In brief, *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide (C4-Chol) [26] was synthesized from cholesteryl chloroformate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester. The C4-Chol was reacted with 5 equivalents of 2-imino-2-methoxyethyl-1-thiomannoside [27] in pyridine containing 1.1 equivalents of triethylamine for 24 h. After evaporation of the reaction mixture in vacuo, the resultant material was suspended in water and dialyzed against water for 48 h and then lyophilized.

Construction and preparation of pCMV-OVA. pCMV-OVA was constructed by subcloning the *Eco*RI chicken egg albumin (ovalbumin) cDNA fragment from pAc-neo-OVA, which was kindly provided by Dr. M.J. Bevan (University of Washington, Seattle, WA, USA) into the polylinker of pVAX I. pCMV-OVA was amplified in the *Escherichia coli* strain, DH5α, then isolated, and purified using a Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). For immunization experiments, the endotoxin in pCMV-OVA solution was removed by the Triton X-114 method.

Preparation of DC-Chol and Man liposomes. Man-C4-Chol or DC-Chol was mixed with DOPE in chloroform at a molar ratio of 3:2 and the mixture was dried, vacuum desiccated, and resuspended in sterile 20 mM Hepes buffer (pH 7.8) or 5% dextrose solution in a sterile test

tube for in vitro and in vivo experiments, respectively. After hydration, the dispersion was sonicated for 5 min in a bath sonicator to form liposomes and sterilized by filtration with a 0.45 μm filter (Nihon-Millipore, Tokyo, Japan).

Preparation of pCMV-OVA/liposome complexes. pCMV-OVA/liposome complexes for in vitro and in vivo experiments were prepared as described previously [20]. Briefly, equal volumes of pCMV-OVA and stock liposome solution were diluted with Hepes buffer or 5% dextrose in 1.5 ml Eppendorf tubes for in vitro and in vivo experiments, respectively. Then, pCMV-OVA solution was added rapidly to the surface of liposome solution using a micropipette (PIPETMAN, Gilson, Villier-le Bel, France) and the mixture was agitated rapidly by pumping it up and down twice in the pipet tip. For in vivo experiments, pCMV-OVA/liposome complexes were incubated at 4 °C for 12 h before use and pCMV-OVA (50 μg) was complexed with DC-Chol or Man liposome at a charge ratio of 1.0:2.3 (−:+) that is optimized charge for cell-selective gene transfection [28]. The mean particle sizes were measured by dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan).

Collection of macrophages. Mouse elicited peritoneal macrophages were cultured according to our previous report [29]. Briefly, peritoneal macrophages were obtained from C57BL/6 mice 4 days after intraperitoneal injection by 1 ml of 3% thioglycollate medium. Collected macrophages were plated on 96-well cluster dishes at a density of 1.3×10^5 cells/cm^2, respectively.

In vitro antigen-presenting assay. Antigen-presenting assay was performed after 72 h in cultured mouse elicited macrophages. Naked pCMV-OVA and that complexed with DC-Chol or Man liposomes at the weight ratio of 1:5 in Opti-MEM I were added to adherent macrophage in 96-well plate and incubated at 37 °C for 6 h. Then, medium was replaced with RPMI 1640 medium supplemented with 10% FBS and incubated for another 18 h. The cells were washed with ice-cold RPMI 1640 medium and fixed with 1% paraformaldehyde solution. Finally, 1×10^5 CD8OVA1.3 T cell hybridoma was added to each well and was incubated for 20 h. The activation of CD8OVA1.3 T cell hybridoma was evaluated by measuring IL-2 secreted to culture medium by commercial mouse IL-2 ELISA kit.

Quantification of OVA mRNA in CD11c$^+$ cells after intravenous injection by Real Time PCR. ICR mice were injected pDNA (50 μg) or that complex with liposomes twice at 30 min intervals. Spleens were harvested 6 h after second injection and spleen cells were suspended in ice-cold RPMI 1640 medium on ice. Positive selection of CD11c$^+$ cells was carried out by using magnetic cell sorting with auto MACS (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's instructions. Total RNA was isolated from CD11c$^+$ cells using MagExtractor MFX-2000 (TOYOBO, Osaka, Japan) and MagExtractor-RNA- following the manufacturer's instructions. Reverse transcription of mRNA was carried out using 1st strand cDNA synthesis kit as follows: total RNA was added to the oligo(dT) primer (0.8 μg/μl) solution and incubated at 42 °C for 60 min with program temperature control system PC-808 (ASTEC, Fukuoka, Japan). Real time PCR was performed using Lightcycler quick system 350S (Roche diagnostics, Indianapolis, IN, USA) with hybridization probes. Primer and hybridization probes for OVA cDNA were constructed as follows; primer, 4′-GCGTCTCTGAATTTAGGG-3′ (forward) and 4′-TACCCCTGATACTACAGTGC-3′ (reverse); hybridization probes, 4′-CT TCTGTATCAAGCACATCGCAACCAACG-3′-Fluorescein isothiocyanate (FITC) and LightCycler-Red640 (LCRed)-4′-CGTTCTCTTC TTTGGCAGATGTGTTTCCCC-3′. The PCR for detection of OVA gene was carried out in a 20 μl final volume containing: (1) 2 μl DNA Master Hybridization Probes 10× (DNA Master Hybridization Probes Kit); (2) 1.6 μl of 25 mM $MgCl_2$; (3) 1.5 μl of forward and reverse primers (final concentration 0.75 μM); (4) 1 μl of 2 μM FITC-labeled hybridization probes and 2 μl of 2 μM LCRed-labeled Probes (final concentration 0.2 and 0.4 μM, respectively); (5) 5.4 μl H_2O; and (6) 5 ml cDNA or pCMV-OVA solution. As for mouse β-actin cDNA measurement, samples were prepared following instruction manuals. After initial denaturation step at 95 °C for 10 min, temperature cycling was initiated. Each cycle consisted of denaturation at 95 °C for 10 s, hybridization at 60 °C for 15 s, and elongation at 72 °C for 10 s. The fluorescent signal was acquired at the end of the hybridization step (F2/F1 channels). A total of 45 cycles were performed. The mRNA copy numbers were calculated for each sample from the standard curve by the instrument software ("Arithmetic Fit Point analysis" for the Lightcycler). Results were expressed in relative copy numbers calculated relative to β-actin mRNA (copy number of OVA mRNA/copy number of β-actin mRNA).

Collection of dendritic cells and antigen presentation assay after intravenous administration. C57BL/6 mice were immunized with pCMV-OVA or that complexed with DC-Chol or Man liposomes by intravenous administration. At indicated time period, mice were sacrificed and spleens were harvested. The spleen cells were suspended in ice-cold RPMI 1640 medium on ice. Positive selection of CD11c$^+$ cells was carried out by using magnetic cell sorting with auto MACS (Miltenyi Biotec, Auburn, CA, USA). The cell suspension was incubated with anti-CD11c$^+$ monoclonal antibody (N418) labeled magnetic beads. Positively collected cells with auto MACS were suspended in RPMI 1640 medium supplemented with 10% FBS. CD11c$^+$ cells were placed in 96-well plate at various number and co-cultured with 1×10^5 cells of CD8OVA1.3 T cell hybridoma for 20 h. Activation of CD8OVA1.3 T cell hybridoma was evaluated by measuring IL-2 secreted to culture medium by commercial IL-2 ELISA kit.

Lymphocyte proliferation assay after intravenous administration. C57/BL6 mice were immunized with naked pCMV-OVA or that complexed with DC-Chol or Man liposomes by intravenous administration 3 times at 2 weeks interval. Four weeks after last immunization, spleens of each group were harvested and suspended in ice-cold RPMI 1640 medium. After 3-times wash, spleen cells were suspended in RPMI 1640 medium supplemented with 10% FBS. Spleen cells (5×10^5 cells) were placed in 96-well plate and incubated for 72 h at 37 °C in the presence or absence of OVA (100 μg). Then spleen cells were incubated for another 6 h in 10% AlamarBlue containing RPMI 1640 supplemented with 10% FBS. Proliferation was evaluated by measuring absorbance (Abs) at 570 and 590 nm and calculated from equation as follows:

(i) Percentage of reduction of AlamarBlue = (Abs (570 nm) − Abs (590 nm) × A) × 100.
(ii) $A = Abs_{t=0}$ (570 nm)/$Abs_{t=0}$ (590 nm).
(iii) Proliferation index = Percentage of reduction (OVA (+))/ Percentage of reduction (OVA (−)).

Results represent means of five separate spleen cell suspensions.

OVA-specific cytokine secretion from spleen cells. C57BL/6 mice were immunized with naked pCMV-OVA or that complexed with DC-Chol or Man liposomes by intravenous administration three times at intervals of 2 weeks. Four weeks after the last immunization, the spleens of each group were harvested and suspended in ice-cold RPMI 1640 medium. After washing three times, spleen cells were suspended in RPMI 1640 medium supplemented with 10% FBS. Spleen cells (5×10^5 cells) were placed in 96-well plates and incubated for 72 h at 37 °C in the presence or absence of OVA (100 μg). IFN-γ and IL-4 in the culture medium were measured by the commercial IFN-γ and IL-4 ELISA kit, respectively. Results represent means of five separate spleen cell suspensions.

Evaluation of cytokines in serum after intravenous administration of pDNA/liposome complexes. C57BL/6 mice were immunized by intravenous administration of naked pCMV-OVA or that complexed with DC-Chol or Man liposomes. Serum was collected from tail vein at predetermined time-periods and the concentrations of TNF-α, IL-12 (p70), and IFN-γ were measured by commercial ELISA kits.

Statistical analysis. Statistical comparisons were performed by Student's t test for two groups and one-way ANOVA for multiple groups. A value of $P < 0.05$ was considered to be indicative of statistical significance.

Results

Particle size analysis of pCMV-OVA/liposome complexes

pCMV-OVA complexed with Man liposomes or DC-Chol liposomes was prepared in 5% dextrose. The mean particle size is important for effective in vivo cell-selective gene transfection [13], so that the mean particle sizes were evaluated. The mean particle sizes of constructed pCMV-OVA complexed with Man liposomes and DC-Chol liposomes were confirmed to be 182.3 ± 7.4 ($n = 3$) and 175.8 ± 5.2 ($n = 3$) nm, respectively. These values are comparable with our previous results obtained with pCMV-Luc complexed with cationic liposomes that produced APC-selective gene transfection [20].

Antigen presentation in cultured mouse peritoneal macrophages

The expressed antigen in APCs can be subsequently processed and presented as peptide epitopes on the MHC class I molecules; consequently, antigen epitope-specific $CD8^+$ T cells are stimulated. Therefore, the antigen presentation was evaluated using cultured macrophages. As shown in Fig. 1, the Man liposome/pCMV-OVA complex induces secretion of IL-2 from a CD8OVA1.3 T cell hybridoma more strongly than naked pCMV-OVA and that complexed with DC-Chol liposomes, indicating that the Man liposome/pCMV-OVA complex produces more efficient antigen epitope presentation on the MHC class I complex than cationic liposomes.

OVA mRNA expression on $CD11c^+$ cells in the spleen after intravenous administration

To evaluate OVA gene expression on $CD11c^+$ cells after intravenous administration, total RNA was extracted from $CD11c^+$ cells in the spleen after intravenous administration of naked pCMV-OVA and that complex with Man or DC-Chol liposome and mRNA expression was evaluated by 2-step quantitative RT-PCR. Relative copy number of mRNA of OVA in Man liposome/pCMV-OVA injected group was the highest of all (Fig. 2). This result shows that Man liposome/pCMV-OVA enhance gene expression on $CD11c^+$ cells.

Antigen presentation on MHC class I $CD11c^+$ cells in the spleen after intravenous administration

To evaluate the antigen presentation under in vivo conditions, $CD11c^+$ cells in the spleen were separated after intravenous administration of naked pCMV-OVA and that complexed with Man and DC-Chol liposome and co-cultured with a CD8OVA1.3 T cell hybridoma. The IL-2 release by the Man liposome/pCMV-OVA complex was the highest of all (Fig. 3), suggesting that targeted delivery of DNA vaccine via the intravenous

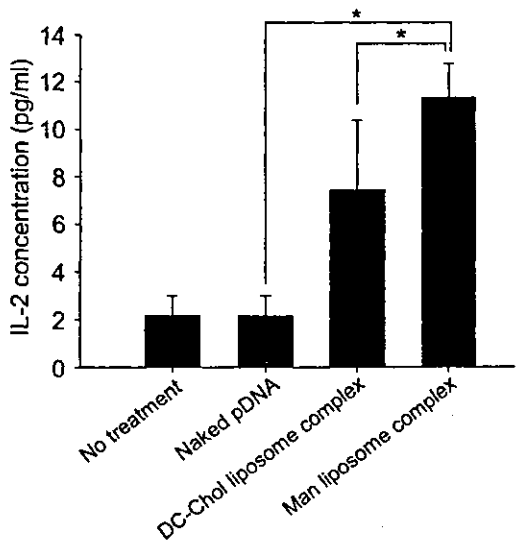

Fig. 1. Antigen presentation on MHC class I molecules in elicited mouse peritoneal macrophages after transfection with naked pCMV-OVA and that complexed with DC-Chol or Man liposomes. OVA peptide presentation was evaluated by IL-2 secretion from a CD8OVA1.3 T cell hybridoma during co-culture for 20 h with fixed macrophages. Each value represents mean + SD of five experiments. Statistical analysis was performed (*$P < 0.05$).

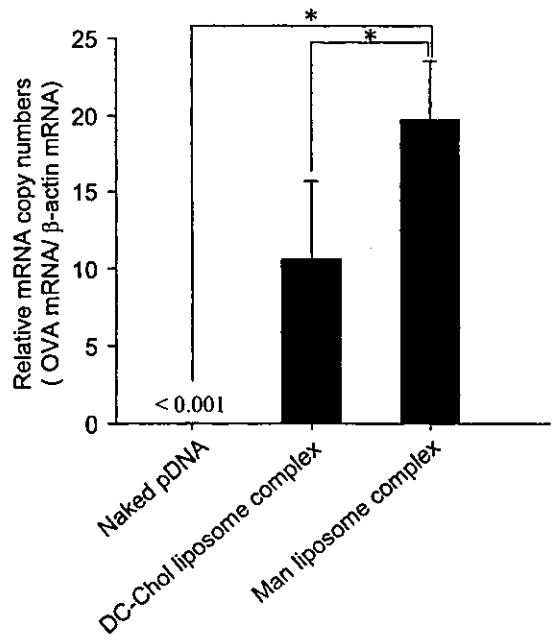

Fig. 2. Relative mRNA number of OVA gene in splenic $CD11c^+$ cells after intravenous administration of naked pCMV-OVA and that complexed with DC-Chol or Man liposomes. Each value shows the mean of relative copy number of OVA mRNA/β-actin mRNA value + SD of three experiments. Statistical analysis was performed (*$P < 0.05$).

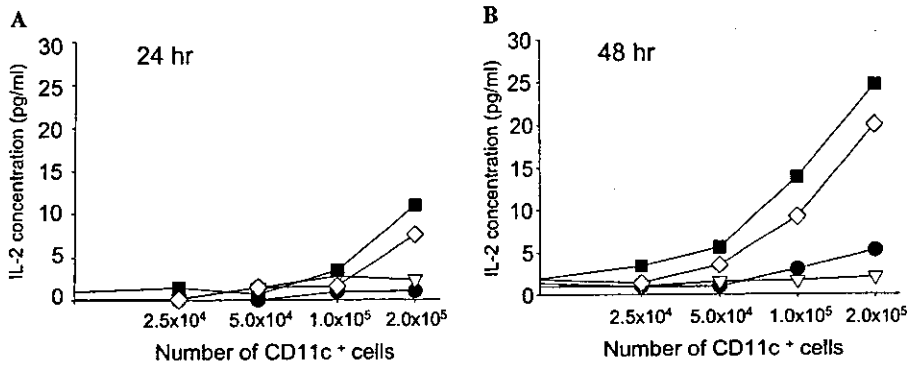

Fig. 3. Antigen presentation on MHC class I molecules in splenic CD11c$^+$ cells after intravenous administration of naked pCMV-OVA (▽) and that complexed with DC-Chol (◇) or Man (■) liposomes. (●) represents the no treatment group. OVA peptide presentation on MHC class I molecules in CD11c$^+$ cells was determined at 24 (A) and 48 (B) h with a CD8OVA1.3 T cell hybridoma. Each value represents the mean of pentapricated assay using a single pool of spleen cell suspension from 5 mice in each group.

route leads to CD8 T cell activation through enhancement of antigen epitope presentation on MHC class I molecules under in vivo conditions.

Induction of proinflammatory cytokine production after intravenous administration

The effect of liposome/pCMV-OVA complex injections on cytokine release was assessed by monitoring the cytokine levels in serum. Fig. 4 shows the time-course of TNF-α, IL-12, and IFN-γ concentrations in serum. After intravenous administration of naked pCMV-OVA, no cytokine release was detected. However, cytokine release was observed after intravenous administration of pCMV-OVA complexed with liposomes. The highest concentration of TNF-α, IL-12, and IFN-γ was observed at 3, 6, and 6 h, respectively. These observations regarding the cytokine release profiles by pDNA that complexed with cationic liposomes are in good agreement with other reports [30,31]. After intravenous administration of Man liposome/pCMV-OVA complex, a significantly higher induction of TNF-α, IL-12, and IFN-γ was observed, suggesting the higher uptake of pCMV-OVA to APCs by Man liposomes. Also, this observation is supported by our previous results showing that pCMV-Luc complexed with Man liposome could selectively transfect the luciferase, which is an encoded model gene, in APCs via mannose receptor-mediated endocytosis after intravenous administration [20].

Antigen (OVA)-specific proliferation of spleen cells and cytokine release after intravenous administration

To analyze the antigen-specific proliferation of spleen cells and cytokine release by spleen cells from immunized mice, suspensions of spleen cells were incubated in cultured medium containing OVA. The spleen cells from mice immunized with Man liposome/pCMV-OVA complex exhibited a higher proliferation response than those with naked pCMV-OVA and that complexed with DC-Chol liposomes (Fig. 5).

Spleen cells from mice immunized with Man liposome/pCMV-OVA complex produced the highest IFN-γ

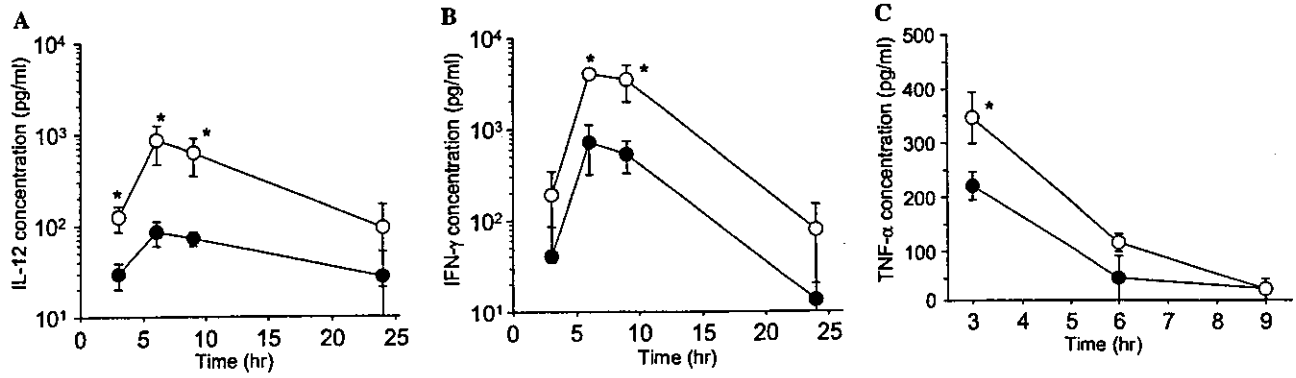

Fig. 4. Cytokine release profiles in serum after intravenous administration of pCMV-OVA complexed with DC-Chol (●) or Man (○) liposomes. IL-12 (A), IFN-γ (B), and TNF-α (C) in serum were determined by ELISA. Each value represents the mean ± SD of four experiments. Statistical analysis was performed (*$P < 0.05$).

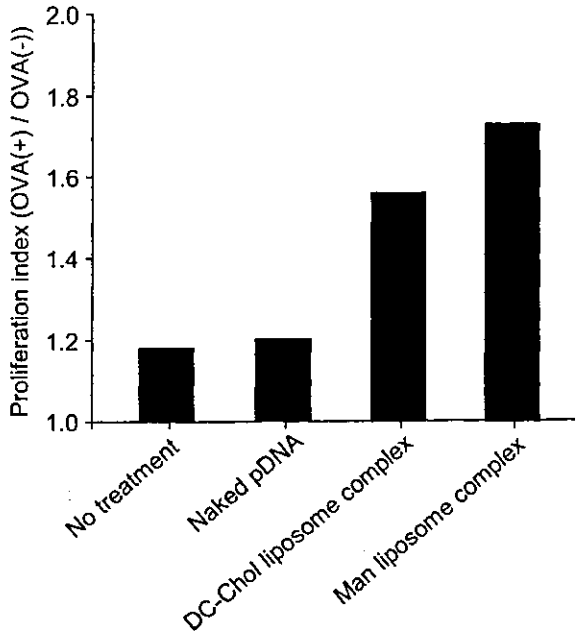

Fig. 5. Antigen (OVA)-specific proliferation response of spleen cells from mice immunized with naked pCMV-OVA and that complexed with DC-Chol or Man liposomes. After immunization by intravenous administration, spleen cells were cultured and stimulated with 100 μg OVA. Proliferation of spleen cells was evaluated by AlamarBlue. Each value represents mean of pentapricated assay using a single pool of spleen cell suspension from 5 mice in each group.

secretion of all (Fig. 6). In contrast, no IL-4 was detected in cultured medium of spleen cells in any of the experiments (data not shown).

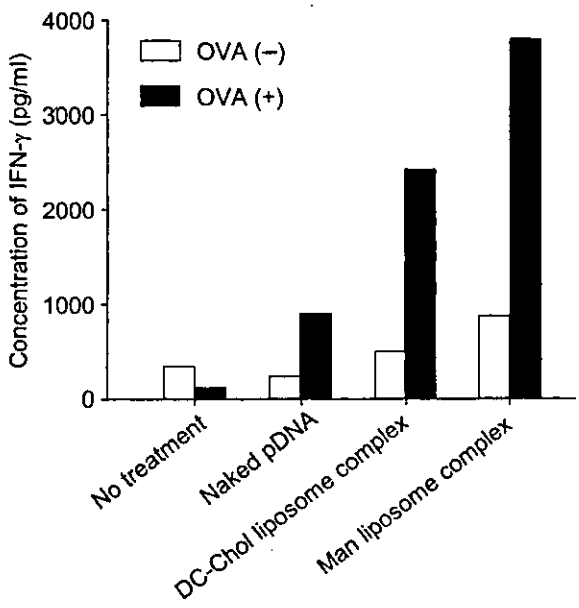

Fig. 6. Antigen-specific IFN-γ secretion by spleen cells from mice immunized with naked pCMV-OVA and that complexed with DC-Chol or Man liposomes. After immunization by intravenous administration, spleen cells were cultured in the absence (□) or presence (■) of 100 μg OVA. IFN-γ in the culture medium was evaluated by ELISA. Each value represents the mean of pentapricated assay using a single pool of spleen cell suspension from 5 mice in each group.

Discussion

This manuscript summarizes our initial efforts to investigate whether Man liposomes are able to act as potent carriers of DNA vaccine. Although topical application, intradermal, subcutaneous, and intramuscular injection of naked pDNA led to potent DNA vaccination in mice, leakage of pDNA from the injection site, trauma at the injection site, and limitations on the injection volume and/or dose are problems. In the present study, we selected the intravenous route for DNA vaccine immunization because it has many advantages compared with topical administration, including (i) firmness of administration, (ii) safety, (iii) relatively low restriction on administration volume, and (iv) the relatively large number of APCs in the spleen. In actual fact, an enhanced mRNA expression of OVA and MHC class I antigen presentation on APCs and an induced Th-1 polarized T cell response were observed after intravenous administration of the Man liposome/pCMV-OVA complex, suggesting that targeted delivery of pDNA to APCs offered an effective approach to DNA vaccine therapy.

DC maturation is crucial in the induction of an immune response in DNA vaccination [5]. In some animal models, it has been reported that pDNA itself possesses adjuvant properties determined by the presence of immunostimulatory sequences within the DNA vector backbone. To date, non-methylated, palindromic DNA-sequences containing CpG-oligonucleotides have been shown to activate dendritic cells, macrophages, monocytes, NK-cells, and B-cells, subsequently leading these cells to secrete proinflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-12 [32,33]. These cytokines, especially IL-12, serve as strong adjuvants for the forward differentiation of helper T cells to type 1 helper T cell (Th1), which have been reported to play a pivotal role in the activation of the CTL response [32,34]. As shown in Fig. 4, the Man liposome/pCMV-OVA complex produces a stronger induction of IL-12, IFN-γ, and TNF-α than the unmodified liposome complex. Several investigators, including ourselves, have already reported that Kupffer cells, splenic macrophages, and DCs play a crucial role in this cytokine release [30,31]. Thus, this observation supports our previous results showing that the Man liposome/pDNA complex can be efficiently transfected to APCs after intravenous administration [20]. Also, such enhancement of Th1 cytokine secretion led us to surmise that the DNA vaccine potency is enhanced by biasing helper T cells towards differentiation to Th1 cells when DNA vaccine is administered with Man liposomes, indicating the usefulness of Man liposomes as a DNA vaccine carrier. In fact, spleen cells from mice immunized with the Man liposome/pCMV-OVA complex following intravenous administration induced the splenic T cell proliferation response strongly and led to the highest IFN-γ secretion, while there was no IL-4

secretion after stimulation with OVA (Figs. 5 and 6). These results suggest that facilitated differentiation to Th1 cells resulted from the administration of pDNA complexed with Man liposomes. Thus, targeted delivery of DNA vaccine to APCs is an effective approach for enhancing the potency of DNA vaccination therapy.

To accomplish efficient DNA vaccine therapy without adverse effect, reduction of gene expression on non-APCs is considered to be important because transfected non-APCs can be target of evoked CTL and that may result in organ failure. Man liposome/pDNA has potency to enhance gene expression on mannose receptor-expressing non-APC, i.e., vascular endothelial cells. However, accumulation of Man liposome/pDNA complex to these cells seems to be limited because our previous biodistribution study demonstrated that Man liposome/^{32}P-labeled pDNA complex showed rapid accumulation in the liver non-parenchymal cells [35], implying that gene expression on these cells might to be low and diminish before induction of CTL response. As for intravenous injection of cationic liposome/pDNA complexes, on the other hand, the highest gene expression was observed in the lung [20,36,37] and this seems to have a much impact on adverse effect.

In the present study, no measurable CTL response was observed (data not shown). Our previous studies using pCMV-Luc as a model gene showed that the luciferase expression in the liver and spleen after intravenous injection of the Man liposome/pCMV-Luc complex reached a maximum at 6 h and then fell within 24 h [35]. However, it has been reported that priming of naive T cells occurred in the T cell region by APCs in lymphoid tissue, including spleen, and priming efficacy is correlated with the duration of antigenic presentation [38]. In this context, prolonged gene expression to APCs by Man liposomes might be an important issue for efficient vaccination; therefore, further studies are needed to obtain effective CTL activity and this may include (i) stabilizing the complex, (ii) designing a pDNA construct, and (iii) altering the administration route. In conclusion, the present study has demonstrated that Man liposomes enhance gene expression of antigen and MHC class I antigen presentation on APCs and Th1 T cell response. However, a higher immunogenicity needs to be attained by modifying this system. These findings may indicate that targeted delivery of DNA vaccine by Man liposomes is a potent vaccination method for DNA vaccine therapy. Hence, the obtained information will be of value for the future use, design, and development of Man liposomes for in vivo applications involving DNA vaccine therapy.

Acknowledgments

We are grateful to Dr. C.V. Harding (Department of Pathology, Case Western Reserve University, Cleveland, OH, USA) and Dr. M.J. Bevan (Department of Immunology, University of Washington, Seattle, WA, USA) for providing us with CD8OVA1.3 T cell hybridoma and pAc-neo-OVA, respectively. This work was supported in part by Grants-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health and Labor Sciences Research Grants for Research on Hepatitis and BSE from the Ministry of Health, Labor and Welfare of Japan and by the 21st Century COE Program "Knowledge Information Infrastructure for Genome Science".

References

[1] J.B. Ulmer, J.J. Donnelly, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Deck, C.M. DeWitt, A. Friedman, L.A. Hawe, K.R. Leander, D. Martinez, H.C. Perry, J.W. Shiver, D.L. Montgomery, M.A. Liu, Heterologous protection against influenza by injection of DNA encoding a viral protein, Science 259 (1993) 1745–1749.

[2] R. Schirmbeck, W. Bohm, K. Ando, F.V. Chisari, J. Reimann, Nucleic acid vaccination primes hepatitis B surface antigen specific cytotoxic T lymphocytes in non-responder mice, J. Virol. 69 (1995) 5929–5934.

[3] D.L. Doolan, M. Sedegah, R.C. Hedstrom, P. Hobart, Y. Charoenvit, S.L. Hoffman, Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8$^+$ cell-, interferon γ-, and nitric oxide-dependent immunity, J. Exp. Med. 183 (1996) 1739–1946.

[4] T. Nishimura, M. Nakui, M. Sato, K. Iwakabe, H. Kitamura, M. Sekimoto, A. Ohta, T. Koda, S. Nishimura, The critical role of Th1-dominant immunity in tumor immunology, Cancer Chemother. Pharmacol. 46 (2000) S52–S61.

[5] O. Akbari, N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, B. Stockinger, DNA vaccination: transfection and activation of dendritic cells as key events for immunity, J. Exp. Med. 189 (1999) 169–178.

[6] R.M. Steinman, The dendritic cell system and its role in immunogenicity, Annu. Rev. Immunol. 9 (1991) 271–296.

[7] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature 392 (1998) 245–252.

[8] J.A. Wolff, R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P.L. Felgner, Direct gene transfer into mouse muscle in vivo, Science 247 (1990) 1465–1468.

[9] M.J. Roy, M.S. Wu, L.J. Barr, J.T. Fuller, L.G. Tussey, S. Speller, J. Culp, J.K. Burkholder, W.F. Swain, R.M. Dixon, G. Widera, R. Vessey, A. King, G. Ogg, A. Gallimore, J.R. Haynes, D. Heydenburg Fuller, Induction of antigen-specific CD8$^+$ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine, Vaccine 19 (2000) 764–778.

[10] S.A. Rosenberg, J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, K.E. Morton, S.A. Mavroukakis, D.E. White, Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen, Hum. Gene Ther. 14 (2003) 709–714.

[11] L. Huang, S. Li, Liposomal gene delivery: a complex package, Nat. Biotechnol. 15 (1997) 620–621.

[12] M. Hashida, M. Nishikawa, F. Yamashita, Y. Takakura, Cell-specific delivery of genes with glycosylated carriers, Adv. Drug Deliv. Rev. 52 (2001) 187–196.

[13] S. Kawakami, F. Yamashita, K. Nishida, J. Nakamura, M. Hashida, Glycosylated cationic liposomes for cell-selective gene delivery, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 171–190.

[14] G. Gregoriadis, R. Saffie, B. de Souza, Liposome-mediated DNA vaccination, FEBS Lett. 402 (1997) 107–110.

[15] L.S. Klavinskis, L. Gao, C. Barnfield, T. Lehner, S. Parker, Mucosal immunization with DNA-liposome complexes, Vaccine 15 (1997) 818–820.
[16] Y. Perrie, P.M. Frederik, G. Gregoriadis, Liposome-mediated DNA vaccination: the effect of vesicle composition, Vaccine 19 (2001) 3301–3310.
[17] N. Ishii, J. Fukushima, T. Kaneko, E. Okada, K. Tani, S.I. Tanaka, K. Hamajima, K.Q. Xin, S. Kawamoto, W. Koff, K. Nishioka, T. Yasuda, K. Okuda, Cationic liposomes are a strong adjuvant for a DNA vaccine of human immunodeficiency virus type 1, AIDS Res. Hum. Retroviruses 13 (1997) 1421–1428.
[18] M. Tanaka, Y. Kaneda, S. Fujii, T. Yamano, K. Hashimoto, S.K. Huang, D.S. Hoon, Induction of a systemic immune response by a polyvalent melanoma-associated antigen DNA vaccine for prevention and treatment of malignant melanoma, Mol. Ther. 5 (2002) 291–299.
[19] S. Toda, N. Ishii, E. Okada, K.I. Kusakabe, H. Arai, K. Hamajima, I. Gorai, K. Nishioka, K. Okuda, HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-γ antibody, Immunology 92 (1997) 111–117.
[20] S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, M. Hashida, Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes, Gene Ther. 7 (2000) 292–299.
[21] S. Kawakami, J. Wong, A. Sato, Y. Hattori, F. Yamashita, M. Hashida, Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice, Biochim. Biophys. Acta 1524 (2000) 258–265.
[22] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer, Biochim. Biophys. Acta 1235 (1995) 289–295.
[23] S. Kawakami, A. Sato, M. Yamada, F. Yamashita, M. Hashida, The effect of lipid composition on receptor-mediated in vivo gene transfection using mannosylated cationic liposomes in mice, STP Pharma Sci. 11 (2001) 117–120.
[24] X. Gao, L. Huang, A novel cationic liposome reagent for efficient transfection of mammalian cells, Biochem. Biophys. Res. Commun. 179 (1991) 280–285.
[25] N.S. Potter, C.V. Harding, Neutrophils process exogenous bacteria via an alternate class I MHC processing pathway for presentation of peptides to T lymphocytes, J. Immunol. 167 (2001) 2538–2546.
[26] S. Kawakami, F. Yamashita, M. Nishikawa, Y. Takakura, M. Hashida, Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes, Biochem. Biophys. Res. Commun. 252 (1998) 78–83.
[27] Y.C. Lee, C.P. Stowell, M.J. Krantz, 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins, Biochemistry 15 (1976) 3956–3963.
[28] S. Kawakami, S. Fumoto, M. Nishikawa, F. Yamashita, M. Hashida, In vivo gene delivery to the liver using novel galactosylated cationic liposomes, Pharm. Res. 17 (2000) 306–313.
[29] T. Takagi, M. Hashiguchi, R.I. Mahato, H. Tokuda, Y. Takakura, M. Hashida, Involvement of specific mechanism in plasmid DNA uptake by mouse peritoneal macrophages, Biochem. Biophys. Res. Commun. 245 (1998) 729–733.
[30] D. Schattenberg, M. Schott, G. Reindl, T. Krueger, D. Tschoepe, J. Feldkamp, W.A. Scherbaum, J. Seissler, Response of human monocyte-derived dendritic cells to immunostimulatory DNA, Eur. J. Immunol. 30 (2000) 2824–2831.
[31] F. Sakurai, T. Terada, K. Yasuda, F. Yamashita, Y. Takakura, M. Hashida, The role of tissue macrophages in the induction of proinflammatory cytokine production following intravenous injection of lipoplexes, Gene Ther. 9 (2002) 1120–1126.
[32] T. Sparwasser, T. Miethke, G. Lipford, K. Borschert, H. Hacker, K. Heeg, H. Wagner, Bacterial DNA causes septic shock, Nature 386 (1997) 336–337.
[33] T. Sparwasser, E.S. Koch, R.M. Vabulas, K. Heeg, G.B. Lipford, J.W. Ellwart, H. Wagner, Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells, Eur. J. Immunol. 28 (1998) 2045–2054.
[34] M.K. Gately, U. Gubler, M.J. Brunda, R.R. Nadeau, T.D. Anderson, J.M. Lipman, U. Sarmiento, Interleukin-12: a cytokine with therapeutic potential in oncology and infectious diseases, Ther. Immunol. 1 (1994) 187–196.
[35] M. Yamada, M. Nishikawa, S. Kawakami, Y. Hattori, T. Nakano, F. Yamashita, M. Hashida, Tissue and intrahepatic distribution and subcellular localization of a mannosylated cationic liposome/plasmid DNA complex after intravenous administration in mice, J. Control Release (in press).
[36] S. Li, M.A. Rizzo, S. Bhattacharya, L. Huang, Characterization of cationic lipid–protamine–DNA (LPD) complexes for intravenous gene delivery, Gene Ther. 5 (1998) 930–937.
[37] Y.K. Song, F. Liu, S. Chu, D. Liu, Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration, Hum. Gene Ther. 8 (1997) 1585–1594.
[38] R.M. Zinkernagel, S. Ehl, P. Aichele, S. Oehen, T. Kundig, H. Hengartner, Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity, Immunol. Rev. 156 (1997) 199–209.

doi:10.1016/j.ymthe.2004.07.015

ARTICLE

Enhanced Hepatocyte-Selective *in Vivo* Gene Expression by Stabilized Galactosylated Liposome/Plasmid DNA Complex Using Sodium Chloride for Complex Formation

Shintaro Fumoto, Shigeru Kawakami, Yoshitaka Ito, Kosuke Shigeta, Fumiyoshi Yamashita, and Mitsuru Hashida*

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

To whom correspondence and reprints requests should be addressed. Fax: +81 75 753 4575. E-mail: hashidam@pharm.kyoto-u.ac.jp.

Available online 28 August 2004

In this study, we demonstrated that the presence of an essential amount of sodium chloride (NaCl) during the formation of cationic liposome/plasmid DNA complexes (lipoplexes) stabilizes the lipoplexes according to the surface charge regulation (SCR) theory. Fluorescence resonance energy transfer analysis revealed that cationic liposomes in an SCR lipoplex (5 and 10 mM NaCl solution in lipoplex) increased fusion. Also, aggregation of SCR lipoplexes was significantly delayed after exposure to saline (150 mM NaCl) as a model of physiological conditions. After intraportal administration, the hepatic transfection activity of galactosylated SCR lipoplexes (5 and 10 mM NaCl solution in lipoplex) was approximately 10- to 20-fold higher than that of galactosylated conventional lipoplexes in mice. The transfection activity in hepatocytes of galactosylated SCR lipoplexes was significantly higher than that of conventional lipoplexes, and preexposure to competitive asialoglycoprotein-receptor blocker significantly reduced the hepatic gene expression, suggesting that hepatocytes are responsible for high hepatic transgene expression of the galactosylated SCR lipoplexes. Pharmacokinetic studies both *in situ* and *in vivo* demonstrated a higher tissue binding affinity and a greater expanse of intrahepatic distribution by galactosylated SCR lipoplexes. Moreover, enhanced transfection activity of galactosylated SCR lipoplexes was observed in HepG2 cells, and investigation of confocal microscopic images showed that the release of plasmid DNA in the cell was markedly accelerated. These characteristics partly explain the mechanism of enhanced *in vivo* transfection efficacy by galactosylated SCR lipoplexes. Hence, information in this study will be valuable for the future use, design, and development of ligand-modified lipoplexes for *in vivo* applications.

Key Words: gene therapy, cationic liposomes, targeting, plasmid DNA, hepatocytes

INTRODUCTION

Gene delivery to hepatocytes is of great therapeutic potential, since the cells are responsible for the synthesis of a wide variety of proteins that play important biological roles both inside and outside the liver. Despite the high transfection efficiency of viral vectors, safety concerns have been raised in clinical trials because of their highly toxic nature [1]. The use of nonviral vectors has attracted great interest for in vivo gene delivery because they are free of some of the risks inherent in these systems. Furthermore, the characteristics of nonviral vectors can be more easily modified than those of viral vectors. To achieve targeted gene delivery, a number of receptor-mediated gene delivery systems have been developed [2–5], including our carriers [6–10]. As far as in vivo selective gene delivery to hepatocytes is concerned, galactose has been shown to be a promising targeting ligand to hepatocytes (liver parenchymal cells) because the cells possess a large number of asialoglycoprotein receptors that recognize the galactose units on the oligosaccharide chains of glycoproteins or on the chemically synthesized galactosylated carriers [11]. Recently, we have developed several types of macromolecular [6,7] and particulate [9] gene carriers for hepatocyte-selective gene transfection in vivo. Among them, cationic liposomes containing cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol)[1] are one of the potential gene transfection carriers [8,9] that can be efficiently recognized by asialoglycoprotein receptors in hepatocytes. More recently, however, we have demon-

1525-0016/$30.00

doi:10.1016/j.ymthe.2004.07.015

strated that the penetration of the galactosylated liposome/pDNA complex (lipoplex) through the hepatic fenestrated endothelium to the parenchymal cells was greatly restricted in perfused rat liver [12]. Taking this into consideration, the stabilization and/or size reduction of galactosylated lipoplex is expected to improve the transfection efficiency *in vivo* due to greater accessibility to hepatocytes.

Although there are some current methodologies for stabilizing and/or reducing the size of nonviral carriers, each method has a disadvantage involving the transfection efficiency. For example, some reports have shown that sonication of lipoplexes reduces the diameter [13,14]; however, only 40% of pDNA in the optimized formulation is unbroken as the sum of relaxed and supercoiled forms after a 3-min sonication, and a marked reduction in the supercoiled form is observed even after a 1-min sonication [13]. The detergent dialysis method using PEGylated lipids also produces an average diameter of 70 nm; however, the surface modification of liposomes with PEG results in a reduced interaction with cells so that the system exhibits a low transfection potential [15]. For the rational design of hepatocyte-targeted gene carrier systems, the lipoplex needs to be stabilized and/or reduced in the size without losing the transfection activity.

Lipoplexes are often prepared in a nonionic solution due to their well-known tendency to aggregate out of solution as the salt concentration is increased [9,16,17]. Aggregation during lipoplex formation in ionic solution might be due to neutralization of the surface positive charge of the lipoplex intermediate by the associated counterion. Taking into account neutralization by counterion, we hypothesized that the presence of an essential amount of sodium chloride (NaCl) during lipoplex formation might regulate repulsion between cationic liposomes and fusion of cationic liposomes in the lipoplex would be accelerated by partial neutralization of the positive charge. Consequently, pDNA in the lipoplex could be largely covered by cationic lipids while retaining enough positive charge to prevent aggregate formation. Such types of lipoplex are expected to be more stable than the conventional lipoplex, which is prepared using a nonionic solution. To evaluate this hypothesis, we investigated the physicochemical properties and *in vivo* gene transfection efficacy of galactosylated "surface charge regulated" (SCR) lipoplex, prepared in the presence of an essential amount of NaCl during lipoplex formation, as a novel approach to stabilization. Also, the intrahepatic and intracellular dispositions of galactosylated SCR lipoplexes were examined to prove the mechanism of *in vivo* gene transfection.

RESULTS

Physicochemical Characteristics and Stability after Mixing Galactosylated SCR Lipoplexes with Saline

As shown in Fig. 1A, the particle size of the lipoplex was dependent on the concentration of NaCl in the lipoplex solution. When we increased the NaCl concentration in the lipoplex solution from 0 to 5 mM, the mean particle size of the lipoplexes decreased from 140 to 120 nm. At NaCl concentrations more than 5 mM, the particle size of the lipoplexes increased. These results indicate that the

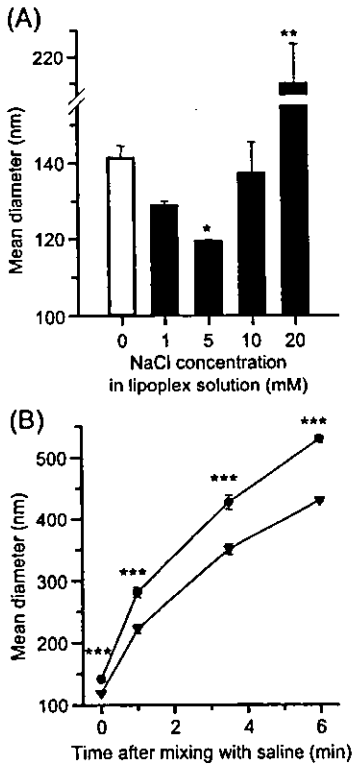

FIG. 1. (A) Difference in particle size of galactosylated lipoplexes. The particle size of galactosylated lipoplexes was measured by dynamic light scattering spectrophotometry. Each bar represents the mean diameter + SD of three experiments. Statistical comparisons with galactosylated conventional lipoplexes were performed using Dunnett's test at a significance level of 5 (*) and 1% (**). (B) Stability of galactosylated lipoplexes after mixing with saline as a model of physiological conditions. Each symbol represents the mean diameter ± SD of three experiments of galactosylated SCR lipoplexes (▼, 5 mM NaCl in lipoplex solution) or galactosylated conventional lipoplexes (●, 0 mM NaCl in lipoplex solution). Statistical comparisons were performed using an unpaired Student *t* test at each time point (***$P < 0.001$).

[1] Abbreviations used: pDNA, plasmid DNA; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride; Chol, cholesterol; Gal-C4-Chol, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl)-formamide; lipoplex, cationic liposome/pDNA complex; SCR lipoplex, surface charge-regulated lipoplex; Gal-BSA, galactosylated bovine serum albumin; PC, parenchymal cells; NPC, nonparenchymal cells.

particle size of the lipoplex can be controlled by the NaCl concentration in the lipoplex solution. The mean diameter of galactosylated SCR lipoplexes (5 mM NaCl) did not change for 2 weeks at 4°C, suggesting that the SCR lipoplex was stable in size during storage (data not shown). To assess the stability of the SCR lipoplex under physiological conditions, we measured the particle size–time profile of the lipoplex after mixing the lipoplexes with saline (150 mM NaCl) solution (Fig. 1B). Galactosylated SCR lipoplexes (5 mM NaCl) significantly delayed particle size enlargement of the galactosylated conventional lipoplexes. In contrast, the particle size of the cationic liposomes remained unchanged after mixing with saline (data not shown).

Fluorescence Resonance Energy Transfer (FRET) Analysis of SCR Lipoplex

We performed FRET analysis to evaluate further the formation of galactosylated SCR lipoplexes (Fig. 2). We evaluated the FRET effect using the peak ratio of nitrobenzoxadiazol (NBD) and rhodamine (Rh). First, we measured the changes in liposome–liposome interaction of the SCR lipoplexes using NBD- and Rh-labeled liposomes (Fig. 2A). We observed distinct energy transfer from NBD (emission 534 nm) to Rh (emission 590 nm) in galactosylated SCR lipoplexes (5 and 10 mM NaCl) and the NBD/Rh fluorescence intensity ratio (F534/F590) was 2.12 ± 0.18, 1.55 ± 0.13, and 1.39 ± 0.085 ($n = 3$) for 0, 5, and 10 mM NaCl solution, respectively, suggesting that each cationic liposome in the galactosylated SCR lipoplexes (5 and 10 mM NaCl ($P < 0.01$)) had significantly easier access to the others than the galactosylated conventional lipoplexes (0 mM NaCl).

It has been reported that cationic liposomes in the lipoplex are fused to one another [17–19]. To evaluate the fusion of cationic liposomes in the galactosylated SCR lipoplex, we mixed NBD–Rh double-labeled liposomes with unlabeled liposomes and measured the fusion of each of the cationic liposomes during lipoplex formation (Fig. 2B). Although the fluorescence intensities of NBD and Rh in liposomes before lipoplex formation were almost the same level (F534/F590 = 1.15), the fluorescence intensities of NBD in each of the galactosylated lipoplexes were greatly increased, suggesting the fusion of each of the cationic liposomes in these lipoplexes. The NBD/Rh ratio (F534/F590) was 2.04 ± 0.09, 2.26 ± 0.05, and 2.39 ± 0.02 for 0, 5, and 10 mM NaCl solution, suggesting that the cationic liposomes in the galactosylated SCR lipoplex are greatly fused compared with conventional lipoplex ($P < 0.01$ ($n = 3$)).

We evaluated the pDNA–cationic liposome interaction using PicoGreen-labeled pDNA and Rh-labeled liposomes (Fig. 2C). The fluorescence intensities at the PicoGreen emission wavelength (520 nm) were drastically increased by the galactosylated SCR lipoplexes, while no significant changes were observed at the Rh emission wavelength (590 nm). The PicoGreen/Rh ratio (F520/F590) was 0.66 ± 0.04, 0.88 ± 0.03, and 0.88 ± 0.02 for 0, 5, and 10 mM

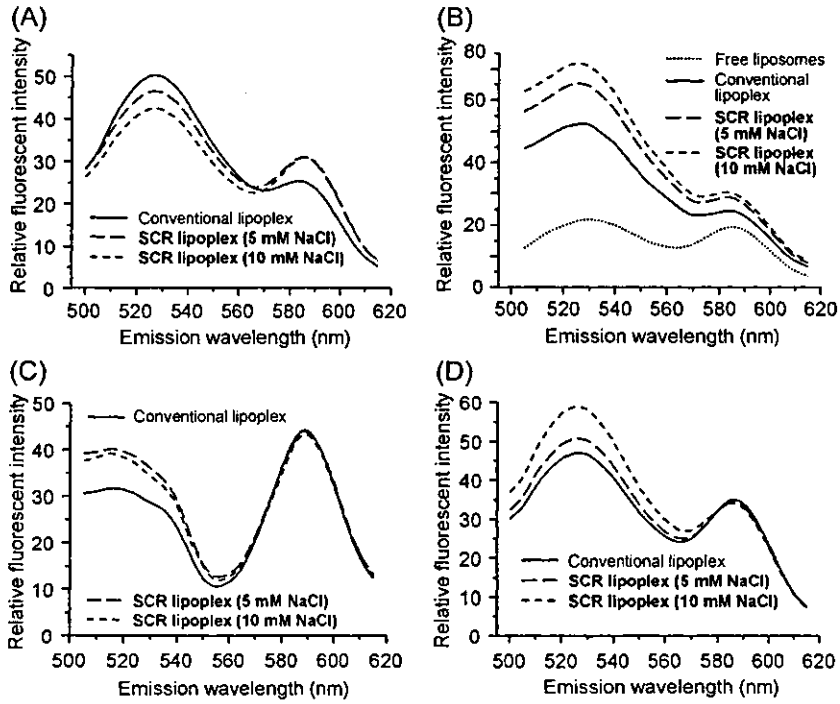

FIG. 2. FRET analysis of galactosylated SCR lipoplex formation. (A) Liposome–liposome interaction. NBD–liposomes and Rh–liposomes were mixed at a ratio of 1:1 prior to lipoplex formation. Thirty minutes after pDNA was mixed with a mixture of NBD- and Rh-labeled liposomes, the fluorescence intensity spectra were measured at an excitation wavelength of 460 nm. (B) Fusion among liposomes in the lipoplexes. NBD and Rh double-labeled liposomes were mixed with unlabeled liposomes at a ratio of 1:4 prior to lipoplex formation. Thirty minutes after lipoplex formation, the fluorescence intensity spectra were measured at an excitation wavelength of 460 nm. (C) pDNA–liposome interaction. Thirty minutes after Rh-labeled liposomes were mixed with PicoGreen-labeled pDNA, the fluorescence intensity spectra were measured at an excitation wavelength of 480 nm. (D) Spectral change after the galactosylated lipoplexes were mixed with saline. NBD–lipoplexes and Rh–lipoplexes were separately prepared. Thirty minutes after lipoplex formation, NBD- and Rh-labeled lipoplexes were mixed at a volume ratio of 1:1. One minute after the galactosylated lipoplexes were mixed with saline at a volume ratio of 3:17, the fluorescence intensity spectra were measured at an excitation wavelength of 460 nm.

doi:10.1016/j.ymthe.2004.07.015

NaCl solution, respectively, suggesting that the pDNA–liposome interaction with the galactosylated SCR lipoplexes was significantly weaker than that with conventional lipoplexes ($P < 0.01$ ($n = 3$)).

To support the stabilization effect of galactosylated SCR lipoplex after mixing with saline as shown in Fig. 1B, we performed FRET analysis following mixing with saline using NBD-labeled lipoplex and Rh-labeled lipoplex (Fig. 2D). We observed marked FRET in galactosylated conventional lipoplexes after mixing them with saline, while the FRET in galactosylated SCR lipoplexes was less than that in galactosylated conventional lipoplexes. The NBD/Rh ratio (F534/F590) was 1.32 ± 0.02, 1.41 ± 0.02, and 1.64 ± 0.02 for 0, 5, and 10 mM NaCl solution, respectively, suggesting that galactosylated SCR lipoplexes can significantly prevent aggregation after exposure to saline ($P < 0.01$ ($n = 3$)). Similar results were obtained when lipoplexes were diluted with phosphate-buffered saline (PBS) containing 10% fetal bovine serum (3.68 ± 0.21, 5.12 ± 0.23, and 5.51 ± 0.70 for 0, 5, and 10 mM NaCl solution, respectively ($n = 3$)), supporting the notion that SCR lipoplexes (5 and 10 mM NaCl) were more stable than conventional lipoplexes under physiological conditions.

Enhanced Hepatocyte-Selective *in Vivo* Gene Expression and Intrahepatic Distribution of Galactosylated SCR Lipoplex

Fig. 3 shows the hepatic transfection activity of galactosylated SCR lipoplexes after intraportal administration in mice. The hepatic transfection activity of galactosylated SCR lipoplexes was markedly enhanced and ranged from 2- to 20-fold higher than that of the conventional lipoplexes; in particular, SCR lipoplexes at 5 mM NaCl solution exhibited the highest transfection activity (Fig. 3A). To assess organ selectivity, we compared the luciferase activities in the liver with those in the lung, which exhibited secondary high expression by lipoplex [9]. The liver/lung gene expression ratio (liver selectivity index) was 5.4 and 42 for galactosylated conventional and SCR lipoplexes, indicating that the liver selectivity of the galactosylated SCR lipoplexes was much higher (Fig. 3B). At doses ranging from 15 to 30 μg, we also observed an enhanced hepatic gene expression by galactosylated SCR lipoplexes (Fig. 3C). Furthermore, galactosylated SCR lipoplexes (5 mM NaCl) maintained significantly higher transfection activity for 6, 12, and 24 h than the galactosylated conventional lipoplexes (Fig. 3D).

To evaluate the hepatocyte selectivity in liver cells, we performed inhibition experiments using galactosylated bovine serum albumin (Gal-BSA) and separation of the hepatocytes (the target cells) from other liver cells (liver nonparenchymal cells) (Fig. 4). When we administered an excess amount of Gal-BSA, which is a ligand of the asialoglycoprotein receptor, intravenously 5 min prior to the intraportal administration of galactosylated SCR lipoplexes, the gene expression in the liver was significantly inhibited by the preadministration of Gal-BSA, suggesting involvement of receptor-mediated endocytosis (Fig. 4A). Furthermore, the transgene expression in hepatocytes of galactosylated SCR lipoplexes was significantly higher than that of conventional lipoplexes (Fig. 4B) and the hepatocytes/liver nonparenchymal cells transgene expression ratio of galactosylated SCR lipoplexes (ratio = 3.84) was higher than that of conventional lipoplexes (ratio = 1.86). These results suggest that hepatocytes are responsible for high hepatic transgene expression of the galactosylated SCR lipoplexes.

To confirm the hepatic distribution of the lipoplexes, we labeled pDNA fluorescently and then evaluated the intrahepatic distribution by observation of frozen liver sections. As shown in Fig. 5, the fluorescence intensity of the galactosylated SCR lipoplexes was more marked and extensive than that of the galactosylated conventional lipoplexes. We obtained similar results using galactosylated lipoplexes containing NBD-labeled liposomes (data not shown). The *in vivo* intrahepatic distribution results partly support the enhanced hepatic *in vivo* transfection activity of the galactosylated SCR lipoplexes.

As SCR lipoplexes without galactosylation (5 mM NaCl) were intraportally injected in mice, transfection activity of SCR lipoplexes in the liver (0.39 ± 0.047 pg luciferase/mg protein) was 6-fold higher than that of conventional lipoplexes without galactosylation (0.069 ± 0.0087 pg luciferase/mg protein, $P < 0.001$ ($n = 3$)). On the other hand, transfection activity of SCR lipoplexes in the lung (0.58 ± 0.41 pg luciferase/mg protein) was slightly higher than that of conventional lipoplexes (0.34 ± 0.067 pg luciferase/mg protein, not significant); consequently the liver/lung gene expression ratio (liver selectivity index) of SCR lipoplexes without galactosylation (0.67) was 3.3-fold higher than that of conventional lipoplexes (0.20). Although enhancing effects of galactosylated SCR lipoplexes on hepatic transfection activity (20-fold increase) and liver/lung selectivity (7.8-fold increase) were more remarkable, these results of lipoplexes without galactosylation suggested that the SCR hypothesis might be commonly applicable for other gene delivery systems using liposomes.

When we injected galactosylated lipoplexes intravenously, transfection activity of galactosylated SCR lipoplexes in the liver ($9.3 \times 10^{-2} \pm 5.9 \times 10^{-2}$ pg luciferase/mg protein ($n = 3$)) was 4.1-fold higher than that of galactosylated conventional lipoplexes ($2.3 \times 10^{-2} \pm 0.7 \times 10^{-2}$ pg luciferase/mg protein ($n = 3$)). As background of tissue light emission was $0.3 \times 10^{-2} \pm 0.2 \times 10^{-2}$ pg luciferase/mg protein, intravenously administered galactosylated SCR lipoplexes exhibited hepatic transfection activity high enough to detect ($P < 0.05$), while hepatic transfection activity of galactosylated conventional lipoplexes was barely higher than the background (not significant). Therefore, liver selectivity of galactosylated SCR lipoplexes could be improved to some extent. How-

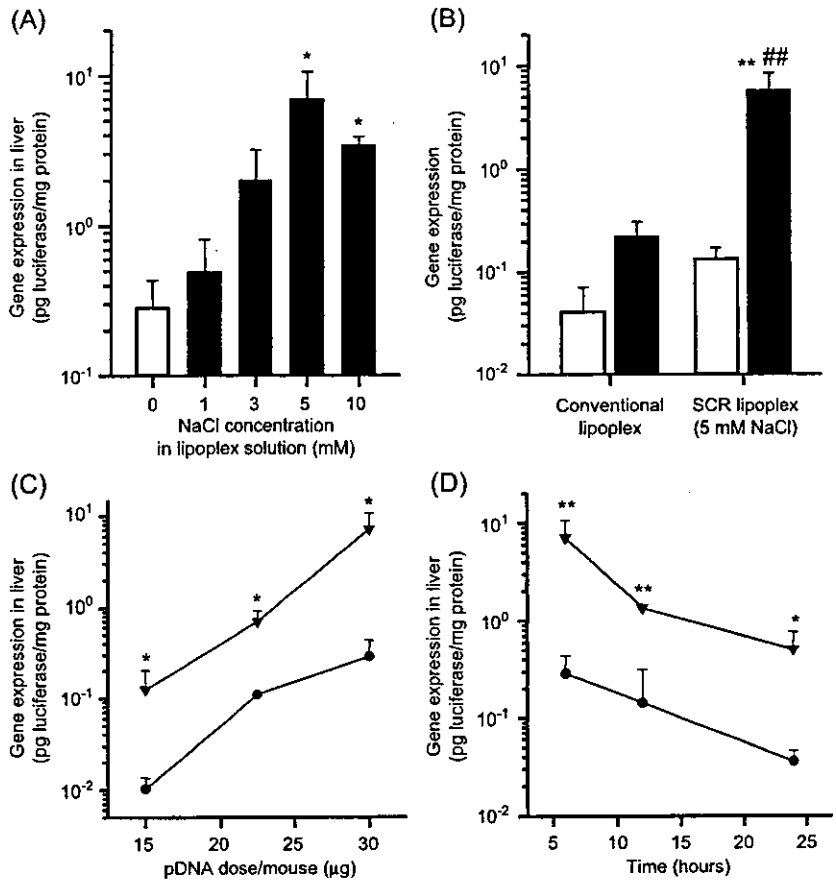

FIG. 3. Enhanced hepatic transfection activity of galactosylated SCR lipoplexes after intraportal administration in mice. Luciferase activity was determined 6 h post-injection of lipoplexes. Each value represents the mean + SD of at least three experiments. (A) Effect of NaCl concentration in the lipoplex solution on the hepatic gene transfection activity by galactosylated SCR lipoplexes. Statistical comparisons with galactosylated conventional lipoplexes were performed using Dunnett's test at a significance level of 5% (*). (B) Comparison of gene expression in liver and lung after intraportal administration of galactosylated SCR lipoplexes. Open and filled bars represent the lung and liver, respectively. Statistical comparisons were performed using Tukey's test. (** indicates comparison with galactosylated SCR lipoplexes in lung, ## indicates comparison with galactosylated conventional lipoplexes in liver: $P < 0.01$.) (C) Effect of pDNA dose on hepatic transfection activity of galactosylated SCR lipoplexes. Circles and inverted triangles represent galactosylated conventional and SCR lipoplexes (5 mM NaCl in lipoplex solution), respectively. Statistical comparisons were performed using an unpaired Student t test at each injected dose ($^*P < 0.05$). (D) Duration of gene expression of galactosylated SCR lipoplexes. Circles and inverted triangles represent galactosylated conventional and SCR lipoplexes (5 mM NaCl in lipoplex solution), respectively. Statistical comparisons were performed using an unpaired Student t test at each injected dose ($^*P < 0.05$, $^{**}P < 0.01$).

ever, transfection activity of galactosylated SCR lipoplexes in the lung (1.85 ± 0.34 pg luciferase/mg protein ($n = 3$)) was also 2.6-fold higher than that of conventional lipoplex (0.72 ± 0.25 pg luciferase/mg protein ($n = 3$)). After intravenous administration, the lung gene expression was still higher than that of liver. It is well known that most of injected cationic liposome/pDNA complexes are trapped in lung capillaries during the first passage. Therefore, further studies might be needed to prevent entrapment of lipoplexes by lung for cell-selective gene delivery after intravenous administration.

In Situ Rat Liver Perfusion Evaluation of Galactosylated SCR Lipoplex

To characterize the hepatic disposition profiles of galactosylated SCR lipoplexes, we analyzed the outflow profiles in the liver perfusion experiment based on a two-compartment dispersion model [12,20]. A liver perfusion system allows us to delineate the uptake characteristics of various molecules and complexes while keeping the structure of the liver intact. Table 1 summarizes each kinetic parameter obtained from the outflow data. Assuming that lipoplexes are bound to the tissue surface and internalized (and/or sequestrated) into the liver, the k_{12}/k_{21} value represents the binding affinity for the liver tissue, while the k_{int} value represents the efficiency of internalization (and/or sequestration) of lipoplex bound to the tissue. While the forward partition coefficient (k_{12}) of the galactosylated SCR lipoplexes was similar to that of the galactosylated conventional lipoplexes, the backward partition coefficient (k_{21}) of the galactosylated SCR lipoplexes was significantly lower than that of the galactosylated conventional lipoplex. As a result, the tissue binding affinity (k_{12}/k_{21}) of the galactosylated SCR lipoplexes was 5.5-fold greater than that of the galactosylated conventional lipoplexes. The k_{int} value of the galactosylated SCR lipoplexes was comparable with that of the galactosylated conventional lipoplexes. These results suggest that the binding affinity to the liver of the galactosylated SCR lipoplexes was greatly enhanced compared with that of the galactosylated conventional lipoplexes. We investigated the hepatic cellular localization of ^{32}P-radiolabeled lipoplexes following bolus injection into perfused rat liver. When we measured the radioactivity associated with hepatocytes (liver parenchymal cells, PC) and other cells (nonparenchymal cells, NPC) per unit cell number, the PC/NPC ratio for the galactosylated SCR lipoplexes was 1.9, which was higher