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

化学修飾によるプラスミドDNAのナノ粒子化とDDSに関する研究

平成16年度　総括研究報告書

主任研究者　　西川 元也

平成17（2005）年　4月

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主任研究者　　西川元也　京都大学大学院薬学研究科助教授

研究要旨　ポリエチレングリコール（PEG）修飾を施したプラスミドDNA（pDNA）と、カチオン性ポリマーの1種polyethyleneimine（PEI）とを複合体化することによりpDNAのナノ粒子化を行い、抗癌剤ドキソルビシン（DXR）をナノ粒子化pDNAに担持させることによる腫瘍ターゲティングを試みた。まず、pDNAに4-[p-azidosalicylamido]butylamine（ASBA）を導入し、これにアミノ基反応型PEG誘導体を結合することでPEG修飾pDNA（pDNA-PEG）を合成した。この際、構造（1本鎖、2本鎖）ならびに分子量の異なるPEG誘導体を用い、平均分子量約10,000のPEIと混合することで得られる複合体の粒子特性について比較検討した。その結果、pDNA-PEG(5,000)$_2$またはpDNA-PEG(20,000)を用いて調製したPEI複合体では、平均粒子径が有意に小さいことが示された。そこで、pDNA-PEGを用いたマウス皮下腫瘍へのDXRターゲティングを試みた。PEI/pDNA-PEG/DXR複合体は、DXRを徐放し、マウスsarcoma 180細胞に対してDXR単独とほぼ同程度の細胞増殖抑制効果を示した。PEI/pDNA-PEG/DXRをsarcoma 180担癌マウスに静脈内投与したところ、DXRが腫瘍組織に高濃度に集積することが示され、高い抗腫瘍効果が得られた。以上、化学修飾によりpDNAのナノ粒子化を実現し、ナノ粒子化pDNAを用いた抗癌剤DDSの開発に成功した。

A. 研究目的

代表的な非ウイルスベクターの1種であるプラスミドDNA（pDNA）は、in vivo遺伝子治療を目的とした検討において汎用されるが、そのサイズならびに強い負電荷のために標的細胞内への取り込みが制限を受け、十分な遺伝子発現が得られない。一方、カチオン性ベクターとの複合体化は、培養細胞に対しては遺伝子導入効率を大幅に改善可能であるものの、混合の結果得られる複合体は200 nm以上の凝集体である場合が多く、in vivoでの遺伝子デリバリーには適さない。これは複数分子のpDNAが1粒子を形成することが原因と考えられ、少分子のpDNAから構成される複合体を形成することがpDNAのナノ粒子化には必須と考える。前年度までの検討において、遺伝子発現活性を十分保持した形でのpDNAへの直接化学修飾法の開発に成功した。そこで本年度は、ポリエチレングリコール（PEG）をpDNAに導入し、カチオン性ベクターとの複合体調製時の凝集体形成を抑制することでpDNAのナノ粒子化を試みた。また、得られたナノ粒子化pDNA複合体を用い、抗癌剤の腫瘍ターゲティングによる抗腫瘍効果の増強についても検討した。

B. 研究方法

(1) PEG修飾pDNAの合成：検討には、ホタルルシフェラーゼのcDNAをコードしたpDNAを用いた。易反応性アミノ基を有する4-[p-azidosalicylamido]butylamine（ASBA）を紫外線照射によりpDNAに導入し、そのアミノ基に分子量および構造の異なる3種のアミノ基反応型PEG誘導体を結合した。即ち、平均分子量約5,000または20,000の

N-hydroxysuccinimidyl succinate of methoxy-polyethylene glycol、あるいは1分子中に平均分子量約 5,000 の PEG 鎖を 2 本有する 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine を用いた。PEG 誘導体はそれぞれ PEG(5,000)、PEG(20,000)、PEG$(5,000)_2$と表す。反応後、エタノール沈殿ならびに限外ろ過により未反応 PEG を除去し、PEG 修飾 pDNA（pDNA-PEG）を得た。pDNA-PEG 中の PEG を barium-iodide complex 法により比色定量することで、pDNA への PEG 結合数を算出した。別途、pDNA-PEG の残存遺伝子発現活性について、TNT® T7 Quick Coupled Transcription/Translation Systems により評価した。

(2) PEI/pDNA-PEG 複合体の調製：平均分子量約 10,000 の分枝型ポリエチレンイミン（PEI）を用い、PEI 中のアミノ基数と pDNA 中のリン酸基数の比（N/P 比）を指標に複合体を調製した。粒子径を動的光散乱法により、また表面電荷をゼータ電位計により測定した。

(3) PEI/pDNA-PEG/DXR の調製および複合体からの DXR 放出：ドキソルビシン（DXR）を pDNA または pDNA-PEG と室温で 1 時間静置することにより DXR 結合体を得た。その後 PEI と混合し、PEI/pDNA/DXR または PEI/pDNA-PEG/DXR 複合体を調製した。別途、PBS 中での複合体からの DXR 放出を評価した。

(4) PEI/pDNA-PEG/DXR の癌細胞増殖抑制効果：マウス肉腫 sarcoma 180 細胞に DXR あるいは DXR 複合体を添加し、MTT assay 法により 24 時間後の細胞数を測定した。

(5) PEI/pDNA-PEG/DXR の皮下腫瘍への集積：1×10^6個の sarcoma 180 細胞を皮下移植した ICR 雌性マウスを担癌マウスとして用いた。移植 1 週間後に DXR あるいは DXR 複合体を DXR 換算 20 μg の投与量で静脈内投与し、一定時間後に摘出した腫瘍組織中 DXR 量を測定した。

(6) 担癌マウスにおける抗腫瘍効果：移植後 1 週間の時点で担癌マウスの尾静脈内に各種 DXR 複合体（DXR 換算 20 μg）を投与し、経時的に固形腫瘍の大きさを測定した。

C. 研究結果

(1) PEG 修飾 pDNA の合成：pDNA への PEG 結合数はいずれの PEG 誘導体を用いた場合にもほぼ等しく、pDNA あたり 16～20 個であった。また、アガロースゲル上での泳動度には PEG 修飾による影響は認められず、pDNA 構造には修飾による顕著な変化がないことが示唆された。いずれの修飾体も、未修飾 pDNA の 60 %以上の遺伝子発現活性を保持していることが明らかとなった。これは、ASBA を介した PEG 修飾が pDNA の 2 本鎖間を架橋しないためであると推察された。

(2) PEI/pDNA-PEG 複合体の調製：いずれの PEG 修飾体の場合にも、PEI/pDNA-PEG 複合体の粒子径は、N/P 比の増大とともに減少した。N/P 比が 10 または 20 において PEI/pDNA-PEG(20,000) および PEI/pDNA-PEG$(5,000)_2$の平均粒子径は 50 nm 程度となり、PEI/pDNA の場合（80～90 nm）と比較して有意に小さい粒子が得られた。このときの表面電荷にも大きな相違が認められ、PEI/pDNA で観察された N/P 比の増大によるゼータ電位の増大（N/P 比 20 で約 54 mV）は、pDNA-PEG では有意に抑制され、特に PEI/pDNA-PEG$(5,000)_2$では約 10 mV であった。以降の検討には、ナノ粒子化が実現された pDNA-PEG$(5,000)_2$、pDNA-PEG(20,000)を選択し、N/P 比 10 で調製した複合体を用いた。

(3) PEI/pDNA-PEG/DXR の調製および複合体からの DXR 放出：pDNA 1 分子当り 280～330 分子の DXR の結合（インターカレー

ション）が確認された。PEIと混合することで調製したDXR含有PEI/pDNA-PEG複合体（PEI/pDNA-PEG/DXR）の平均粒子径はDXR非含有の場合とほぼ同等であった。PBS中でDXRはPEI/pDNA-PEG/DXRから徐々に放出された。

(4)癌細胞増殖抑制：PEI/pDNA-PEG(20,000)/DXRおよびPEI/pDNA-PEG(5,000)$_2$/DXRは、sarcoma 180細胞に対しDXR単独とほぼ同等の細胞増殖抑制効果を示した。DXR非含有の複合体では全く細胞増殖に影響が認められなかったことから、PEI/pDNA-PEG/DXRによる増殖抑制効果はDXRによることが示唆された。

(5) 皮下腫瘍への集積：PEI/pDNA-PEG(20,000)/DXRまたはPEI/pDNA-PEG(5,000)$_2$/DXRを投与した場合は、DXR単独あるいはPEI/pDNA/DXR投与と比較して高い腫瘍組織中DXR濃度が得られた。別途、DXR非結合体投与後のルシフェラーゼ活性を測定したところ、PEI/pDNA-PEG投与群では若干ではあるものの腫瘍組織中にルシフェラーゼ活性が検出された。

(6) 担癌マウスにおける抗腫瘍効果：PEI/pDNA-PEG(20,000)/DXRまたはPEI/pDNA-PEG(5,000)$_2$/DXRの抗腫瘍効果は、DXR単独あるいはPEI/pDNA/DXRと比較して有意に高く、また長期間持続した。

D. 考察

ドキソルビシン（DXR）は、DNAにインターカレートしDNAおよびRNAの合成を抑制することで、悪性リンパ腫などの癌に対して抗腫瘍効果を示す強力で使用頻度の高い抗癌剤である。しかしながらその使用に際しては、心毒性や骨髄抑制などの強い副作用が問題とされる。治療係数を増大させることを目的にDXRの腫瘍組織へのターゲティングに関する検討が行われ、これまでにDoxil®あるいはNK911などのDDS製剤が開発されてきた。これらはDXRと比較すると高い治療係数を示すものの、製剤の血中安定性や薬物放出性などに改善すべき点が残されている。近年、マクロファージなど免疫担当細胞の活性化が、癌免疫療法に有望であることが報告され、これら既存製剤で用いられるリポソームや高分子ミセルなどの生物学的に非活性なキャリアではなく、活性を有するキャリアを用いることで、抗癌剤による抗腫瘍効果の増強が期待される。

そこで本研究では、DXRのキャリアとしてpDNAの利用を試みた。pDNAは均一な化合物として大量調製が容易であり、DDSキャリアとして有用と考えられる。しかしながら、これまでに遺伝子（cDNA）の運び屋（ベクター）としての利用に関しては膨大な量の検討が行われてきたが、その薬物キャリアとしての利用については殆ど検討されてこなかった。pDNAはDXRとの親和性を有することから、共有結合することなくDXRを担持可能である。またpDNAにはCpGモチーフと呼ばれるバクテリアDNAに特徴的な配列が多く存在することから、これが自然免疫を賦活化することで抗腫瘍効果の増強が期待できると考えた。別途行った検討では、pDNAあるいはCpGオリゴヌクレオチド複合体により抗腫瘍サイトカインが産生され、癌転移・増殖を抑制可能であることを明らかにしている。

一般に、腫瘍組織への薬物デリバリーにおいては、解剖学的特徴などからナノサイズの粒子が有効であるとされる。そこで本研究では、pDNAを基盤とする新規ナノ粒子化技術の開発に取り組んだ。カチオン性ベクターを用いる複合体化は、pDNAの安定性を増大しpDNAを凝縮（compaction）する有用な方法である。しかしながら複合体間での凝集（aggregation）が起こり、デリバリーに有利

なサイズの小さい粒子を得ることは困難である。そこで本研究では、pDNA に PEG 修飾を施し、カチオン性ベクターを用いて pDNA を凝縮するとともに、複合体間の凝集を抑制することで pDNA 粒子サイズの縮小化を試みた。その結果、pDNA-PEG(20,000) あるいは pDNA-PEG$(5,000)_2$ を用いることで、PEI 複合体の粒子サイズを約 50 nm にまで縮小することに成功した。これは、pDNA に導入された PEG 分子が、PEI と pDNA の静電的相互作用を適度に阻害することで、PEI を介する pDNA の凝集が抑制された結果と考えられる。一方、pDNA-PEG(5,000)では十分な縮小化が達成されず、複合体化において PEG の構造ならびに分子量の重要性が示唆された。また、PEG 鎖の存在により粒子の表面電位が低く抑えられたが、これは生体内での非特異的な相互作用を回避・抑制する上で重要であると考える。

室温で静置することで DXR は効率よく pDNA-PEG に担持された。PEI/pDNA-PEG/DXR 複合体は、DXR を徐放し DXR 単独と同程度の細胞増殖抑制効果を示した。DXR 非含有複合体では効果が認められなかったことから、少なくとも培養癌細胞に対する増殖抑制効果は DXR によることが示唆された。一方、PEI/pDNA-PEG 複合体が腫瘍組織中に浸潤してきた免疫担当細胞を活性化することで抗腫瘍サイトカインが産生され、DXR による抗腫瘍効果との相乗効果が得られるかについては今後の検討課題と考える。担癌マウスに静脈内投与した際には、腫瘍組織中に DXR が高濃度に検出され、それを反映した高い抗腫瘍効果が認められた。10 倍量の DXR を投与した場合にも有意な抗腫瘍効果は認められなかったことから、ナノ粒子化 pDNA を用いたデリバリーにより少なくとも 10 倍以上の効果増大が得られた。

E. 結論

ASBA を pDNA に導入し、これにアミノ基反応型 PEG 誘導体を結合することで新規化学修飾体－pDNA-PEG－の開発に成功し、これにより PEI 複合体の粒子サイズの縮小化が実現された。PEI/pDNA-PEG には、pDNA 1 分子当り約 300 分子の DXR を担持可能であり、得られた DXR 複合体は DXR 単独と同等の細胞増殖抑制効果を示した。担癌マウスへの尾静脈内投与により、PEI/pDNA-PEG/DXR は有意に高い抗腫瘍効果を示し、本ナノ粒子化 pDNA を基盤とした DDS が腫瘍組織への抗癌剤デリバリーに有効であることが証明された。

F. 健康危険情報

なし

G. 研究発表

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H. 知的所有権の出願・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

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GENE DELIVERY

Tissue and intrahepatic distribution and subcellular localization of a mannosylated lipoplex after intravenous administration in mice

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Abstract

We have previously reported that, unlike a lipoplex and mannosylated (Man) lipoplex underwent gene transfer to liver nonparenchymal cells (NPC) that possess mannose receptors after intravenous administration in mice. In this study, the tissue, intrahepatic distribution, and subcellular localization of the lipoplex after intravenous administration were investigated. DC-Chol liposome was selected as a cationic liposomes. After administration of lipoplex and Man lipoplex, the high gene expression was observed in the lung and liver, respectively. After administration of [^{32}P]Man lipoplex, about 80% of [^{32}P]plasmid DNA (pDNA) was accumulated in the liver. As for the intrahepatic distribution, the NPC/parenchymal cells (PC) ratio of [^{32}P]Man lipoplex was 9.64, whereas the NPC/PC ratio of [^{32}P]lipoplex was 1.93. The radioactivity in the cytosolic fraction of liver homogenate of [^{111}In]Man lipoplex was two-fold higher than that of [^{111}In]lipoplex, indicating that Man liposomes facilitate the release of pDNA into the cytosolic space. However, a rapid sorting of the radioactivity from endosomes to lysosomes was observed with the [^{111}In]Man lipoplex. Also, amplification of pDNA by PCR suggested that the Man lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex. These results suggested that modulation of its intracellular sorting could improve the transfection efficiency of Man lipoplex.

Keywords: Gene delivery; Mannose receptor; Mannosylated liposomes; Cationic liposomes; Plasmid DNA

1. Introduction

The success of in vivo gene therapy relies on the development of a vector that achieves target cell-specific, efficient, and prolonged transgene expression following its application. Nonviral vectors are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are, therefore, attractive vectors for clinical application. One of the most promising class of nonviral vectors developed so far is the cationic liposome-based transfection system. The lipoplex formation via electrostatic interaction of cationic liposomes and plasmid DNA (pDNA) facilitates the interaction of pDNA with cell membranes, leading to transgene expression in the cells [1]. In an attempt to increase the efficiency of transgene expression as well as to reduce cytotox-

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icity, several kinds of cationic lipids, such as quaternary ammonium detergents, cationic derivatives of cholesterol [2], diacylglycerol [1,3], and alkyl derivative of polyamines [4] have been developed. Among them, some lipoplex have been used in clinical trials for the treatment of cancer and cystic fibrosis [5,6].

The lipoplex is a useful nonviral vector, but it lacks specificity in delivery and transfection after systemic administration. Although the levels of gene expression vary from study to study, the lung invariably shows the highest gene expression. The attachment of a ligand that can be recognized by a specific mechanism would endow a vector with the ability to target a specific population of cells. In the search for macromolecule-based nonviral vectors, several ligands including galactose [7,8], mannose [9,10], transferrin [11], and antibodies [12] have been used to improve the delivery of pDNA to target cells. Therefore, the incorporation of such ligands into cationic liposomes would improve the cell specificity of in vivo gene transfer by lipoplex.

Mannose receptor-mediated targeting is a promising approach to achieve cell-specific delivery after systemic administration because (i) the expression of mannose receptors is restricted to the liver NPC and other macrophages, (ii) a complex entering the systemic circulation has easy access to the liver NPC, and (iii) the liver has a high blood flow. These physical and biological features give a mannosylated vector an opportunity to deliver pDNA to the liver NPC via mannose receptor-mediated endocytosis. Liver nonparenchymal cells (NPC), including sinusoidal endothelial cells and Kupffer cells, can be the targets for gene therapy because they have been implicated in a wide variety of diseases [13,14].

In a previous study, we developed a novel mannosylated derivative of cholesterol, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino) butyl)formamide (Man-C4-Chol), and used it to prepare a cationic liposome formulation (Man liposome) [10]. Man-C4-Chol possesses multi-functional properties, that is, (i) a lipophilic anchor moiety (cholesterol) for stable incorporation into liposomes, (ii) a mannose moiety for recognition by the mannose receptors, and (iii) an imino group for binding to pDNA via electrostatic interaction [15]. Furthermore, low-molecular-weight glycolipids are more promising due to their low immunogenicity, high reproducibility, and ease of mass production. Although, a high gene expression in the liver and spleen after intravenous injection was observed for Man lipoplex via mannose receptor-mediated endocytosis compared with the lung, its transfection efficiency was relatively low and, consequently, further improvements in the efficiency of transgene expression are required.

In order to obtain a theoretical strategy to develop an efficiently targetable gene carrier to the liver by mannosylation, therefore, detailed information on the distribution of a Man lipoplex needs to be obtained. In the present study, we studied the tissue, intrahepatic distribution, and subcellular localization of a [^{32}P]- or [^{111}In]-labeled Man lipoplex after intravenous administration. The results were compared with those for a 3β[*N*′,*N*′,*N*′-dimethylaminoethane]-carbamoyl]cholesterol liposomes (DC liposome), which is a cationic cholesterol derivative, based lipoplex [2].

2. Materials and methods

2.1. Chemicals

N-(4-Aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate was obtained from Sigma (St. Louis, MO, USA), dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar-Lipids (Alabaster, AL, USA). [α-^{32}P]dCTP was obtained from Amersham (Tokyo, Japan). ^{111}Indium chloride ([^{111}In]$InCl_3$) was supplied by Nihon Medi-Physics (Hyogo, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride and 4-[*p*-azidosalicylamido]butylamine (ASBA) were purchased from Dojindo (Kumamoto, Japan) and Pierce Biotechnology (Rockford, IL, USA). Man-C4-Chol [10] and DC-Chol [2] were synthesized as reported previously. Mannosylated bovine serum albumin (Man-BSA) was synthesized and radiolabeled with [^{111}In]$InCl_3$ as reported previously [16]. All other chemicals were obtained commercially as reagent-grade products.

2.2. Construction and preparation of pDNA

pCMV-Luc was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from

pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the E coli strain DH5α, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN, Hilden, Germany). Purity was confirmed by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm.

2.3. Animals

Female ICR mice (5-week-old, 20–25 g) were obtained from the Shizuoka Agricultural Co-operative 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 the US National Institutes of Health and with the Guidelines for Animal Experiments of Kyoto University.

2.4. Preparation of liposomes

Man-C4-Chol (8.1 μmol) or DC-Chol (10.0 μmol) was mixed with DOPE (5.4 μmol for Man-C4-Chol and 6.7 μmol for DC-Chol) in chloroform (5.0 ml) and evaporated to dryness in a round-bottomed flask. Then, the lipid film was vacuum desiccated to remove any residual organic solvent and resuspended in 5% (w/v) dextrose (2.5 ml). After hydration, the dispersion was sonicated for 5–10 min in a bath sonicator to produce liposomes. The liposome formulations were passed through a polycarbonate membrane filter (0.22 μm) for sterilization. The liposomal lipid concentration was determined by phosphorous analysis [17] and was adjusted to 3 mg/ml.

2.5. Lipoplex formation

The mixing ratio of liposomes with pDNA was expressed as a charge ratio, which is the molar ratio of the cationic lipids to the pDNA phosphate residues [18]. The charge ratio (+: −) of these liposomes and pDNA complex was adjusted to 2.3:1.0 for cell-selective gene transfection [19,20]. A solution of cationic liposomes (0.2 ml) was added to an equal volume of pDNA solution (0.2 ml and pDNA concentration ≅ 0.33 μg/μl) in a polyethylene tube. Then, the lipoplex was agitated rapidly by pipetting it up and down twice using a micropipette (PIPETMAN®, GILSON, Villier-le Bel, France) and left to stand for 30 min. The particle size and zeta potential of the lipoplex were measured using a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan) and a laser electrophoresis zeta-potential analyzer (LEZA-500T, Otsuka Electronics), respectively.

2.6. pDNA radiolabeling methods

pDNA was radiolabeled with [α-^{32}P]dCTP by nick translation [21]. In a separate preparation, pDNA was radiolabeled with ^{111}In using a newly developed method [22]. Briefly, a dimethylsulfoxide solution of ASBA was added to DTPA anhydride under darkroom conditions, and the mixture was incubated at room temperature for 1 h. Then, pDNA solution was added to the mixture, and the mixture was immediately irradiated under an UV lamp at room temperature for 15 min to obtain DTPA-ASBA coupled pDNA (DTPA-ASBA-pDNA). The product was purified by precipitation twice with ethanol, and labeled with [^{111}In]$InCl_3$. The purity of each pDNA was checked by Sephadex G-25 column chromatography and 1% (w/v) agarose gel electrophoresis.

2.7. Gene expression experiments

Five-week-old ICR mice were injected intravenously with 300 μl of lipoplex using a 30-gauge syringe needle. Three or six hours after injection, mice were killed and lung, liver, kidney, spleen, and heart were removed and assayed for gene expression. The organs were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). The lysis buffer was added in a weight ratio of 5 μl/mg for liver samples or 4 μl/mg for other organ samples. After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000 × g for 10 min at 4 °C and 20 μl supernatant was analyzed to determine the luciferase activity using a luminometer (Lumat LB9507, EG and G Berthold, Bad Wildbad, Germany). The protein

concentration of each tissue extract was determined by the modified Lowry method. Luciferase activity in each organ was normalized to relative light units (RLU) per mg extracted protein.

2.8. Tissue distribution of the [^{32}P]lipoplex

The [^{32}P]lipoplex was intravenously injected into mice. At predetermined time periods after injection, the blood was collected from the vena cava under ether anesthesia. The mice were then killed and the liver, spleen, kidney, lung, and heart were excised, rinsed with saline, and weighed. Tissue samples were digested in 0.7 ml Soluene-350 (Packard, Merdin, CT, USA) by overnight incubation at 45 °C. Then, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5 M HCl, and, finally, 5 ml Clear-Sol I (Nacalai Tesque, Kyoto, Japan) were added to the digested samples. The radioactivity of the samples was assayed in a Beckman Model LS5000TA liquid scintillation counter (Beckman, Tokyo, Japan).

2.9. Intrahepatic distribution of the [^{32}P]lipoplex

Ten minutes after intravenous injection of the [^{32}P]lipoplex, the liver of each mouse was perfused with a buffer [16] and the liver cells were separated into parenchymal cells (PC) and nonparenchymal cells (NPC) by differential centrifugation. Then, the ^{32}P radioactivity of the cell suspensions was assayed as above.

2.10. Fractionation of liver homogenate

After intravenous injection of [^{111}In]Man-BSA, naked [^{111}In]pDNA or [^{111}In]both lipoplex into mice, the liver was sampled at intervals. HEPES–sucrose buffer (250 mM sucrose, 20 mM HEPES, 2 mM EDTA, pH 7.2) was added to the liver in a ratio of 3 μl buffer/mg tissue, then the liver was homogenized in a Potter-type homogenizer. The homogenate was centrifuged at 4 °C for 10 min at 800 × *g* to remove nuclei and debris. The supernatant was subsequently centrifuged at 4 °C for 30 min at 100,000 × *g* and the resulting supernatant was collected as the cytosol fraction. The pellet obtained was resuspended with 0.5 ml HEPES–sucrose buffer using a syringe and needle. Part of the suspension was mixed with HEPES–sucrose buffer and Percoll (Amersham Biosciences, Piscataway, NJ, USA) in a centrifuge tube. The final concentration of Percoll was adjusted to 35% (v/v). The sucrose solution (65% (w/v), 0.5 ml) was layered with a syringe and needle at the bottom of the centrifuge tube. The gradient was centrifuged at 4 °C for 25 min at 50,000 × *g*. Fractions (approximately 0.4 ml each) were collected from the bottom of the tube using a peristaltic pump and the radioactivity or enzyme activity of each fraction was measured.

2.11. Organelle marker detection

The activity of a fluid phase endocytosis marker, horseradish peroxidase (HRP), was detected using its substrate *o*-phenylenediamine dihydrochloride to identify the endosome fractions. Five minutes after injection of HRP, the liver was taken and fractionated by Percoll density gradient centrifugation as described above. Twenty microliters of each fraction was added to 150 μl 0.04% (w/v) *o*-phenylenediamine dihydrochloride, 0.012% (w/v) hydrogen peroxide in 0.1 M citrate–phosphate buffer (pH 5.0). The reaction was stopped by the addition of 20% (w/v) H_2SO_4 (50 μl). The activity of a lysosomal marker enzyme, β-hexosaminidase, was determined with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. Twenty microliters of each fraction was added to 50 μl 1.2 mM 4-methylumbelliferyl-β-D-galactoside solution and subsequently incubated at 37 °C for 30 min. The reaction was stopped by the addition of 150 μl 0.5 M glycine in 0.5 M carbonate buffer (pH 10.0). The fluorescence intensity of each sample was measured (excitation wavelength 365 nm; emission 460 nm).

2.12. PCR amplification of pDNA

pDNA in the subcellular fractions of the liver was purified by GenElute Plasmid Miniprep Kit (Sigma). Then, aregion of about 2.8 kbp of the pDNA containing the luciferase gene was amplified by PCR using a forward (5′-GTATCTGCTCCCTGCTTGTG-3′) and reverse (5′-TCCGCCTCAGAAGCCATAGA-3′) primer under standard conditions for 20 cycles.

3. Results

3.1. Physicochemical properties and gene expression of the lipoplex

The zeta potential of lipoplex and Man lipoplex was 9.78 ± 3.5 (n=3) and 12.5 ± 4.11 (n=3), respectively. The mean particle size of lipoplex and Man lipoplex was 287.2 ± 1.2 nm (n=3) and 285.4 ± 18.3 nm (n=3), respectively. Thus, physicochemical properties of both lipoplex were almost the same.

Fig. 1A demonstrates the gene expression after intravenous administration of Man lipoplex at 3 and 6 h. High gene expression was observed in the liver and spleen, which is expressed mannose receptor on cell surface. Fig. 1B shows the gene expression after intravenous administration of lipoplex at 6 h. Among these organs, the highest gene expression was observed in the lung.

3.2. Tissue distribution of the [^{32}P]lipoplex

Fig. 2 shows that the tissue radioactivity after intravenous administration of [^{32}P]lipoplex and Man lipoplex up to 6 h. Both [^{32}P]lipoplex rapidly disappeared from the blood circulation. [^{32}P]Lipoplex accumulated in the lung and liver, whereas [^{32}P]lipoplex accumulated largely in the liver.

3.3. Intrahepatic distribution of the [^{32}P]lipoplex between liver PC and NPC

After intravenous administration of the [^{32}P]Man lipoplex, the radioactivity in the liver was preferentially recovered from the NPC fractions, with the radioactivity ratio of NPC to PC (NPC/PC ratio on a cell-number basis) in the liver being approximately 9.6 (Fig. 3). On the other hand, the [^{32}P]lipoplex had an NPC/PC ratio of 1.9.

3.4. Subcellular localization of the [^{111}In]lipoplex

Fig. 4 shows the radioactivity of the cytosolic fractions in liver homogenate after intravenous administration of the [^{111}In]lipoplex and Man lipoplex up to 60 min. In both cases, the radioactivity gradually increased with time. Thirty and sixty minutes after intravenous administration, however, the cytosolic amount of the [^{111}In]Man lipoplex was always greater than that of the [^{111}In]lipoplex, suggesting that Man liposomes facilitate the release of pDNA from intracellular vehicles into the cytosol.

To characterize the gradient of liver homogenate using Percoll-gradient centrifugation, the enzymatic activity of β-hexosaminidase, a lysosome marker, was used for the determination of the endosomal fractions. As shown in Fig. 5A, β-hexosaminidase activity was predominantly detected in the lower

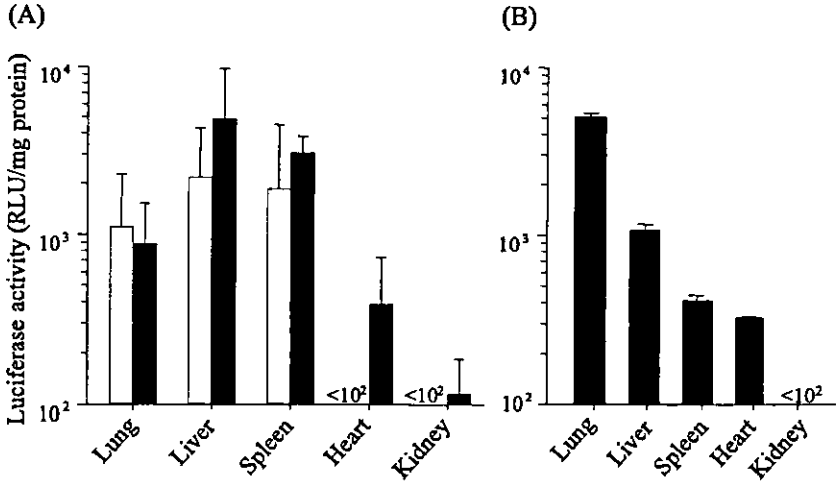

Fig. 1. Gene expression after intravenous administration at 3 (□) and 6 h (■) of Man lipoplex (A) and at 6 h of lipoplex (B) in mice. Each value represents the mean + S.D. values of three experiments.

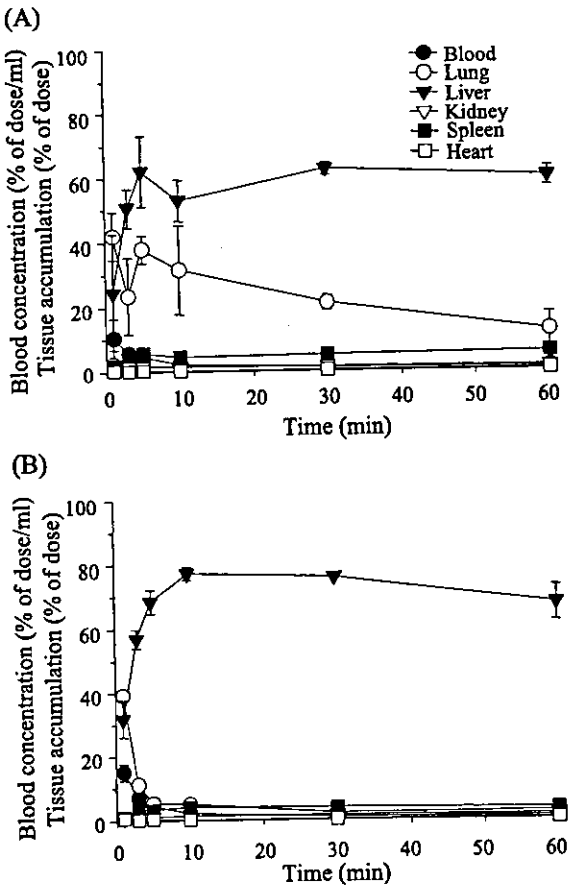

Fig. 2. Radioactivity in blood and tissues after intravenous administration of [^{32}P]lipoplex (A) or Man lipoplex (B) in mice. Each value represents the mean ± S.D. values of three experiments.

seven fractions. In a similar manner, the activity of horseradish peroxidase (HRP) injected intravenously into mice was used to confirm the lysosomal fractions. HRP was predominantly recovered in the upper seven fractions (data not shown). Therefore, the upper seven fractions and the lower seven fractions were identified as the endosomal and lysosomal fractions, respectively. To validate this system for the subcellular sorting of externally internalized compounds, the localization of [^{111}In]Man-BSA, a well-known ligand for mannose receptors [23], was then examined. At 15 min after intravenous administration, the radioactivity was largely recovered in the endosomal fractions, then the majority was transferred to the lysosomal fractions with time (Fig. 5B). These

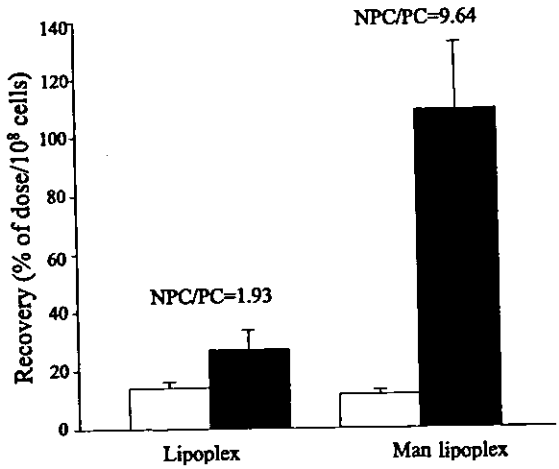

Fig. 3. Cellular localization of radioactivity between PC (□) and NPC (■) 30 min after intravenous administration of [^{32}P]lipoplex and Man lipoplex in mice. Each value represents the mean + S.D. values of three experiments.

results for [^{111}In]Man-BSA were in good agreement with previously reported characteristics of these ligands as far as mannose receptors [24] were concerned, indicating the validity of the detection method.

Fig. 6 shows the endosomal and lysosomal localization of radioactivity after intravenous admin-

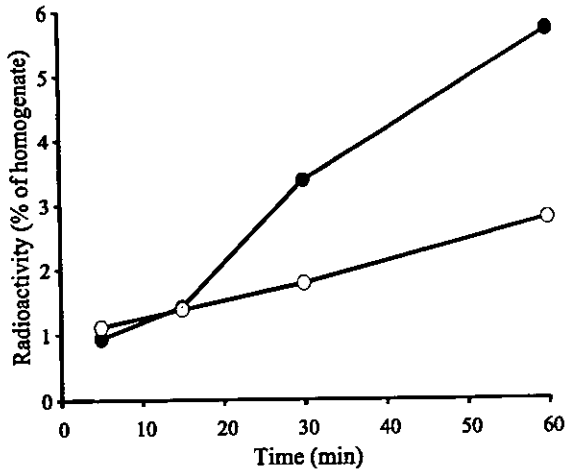

Fig. 4. Radioactivity in cytoplasmic fraction of mouse liver homogenates 5, 15, 30, and 60 min after intravenous administration of [^{111}In]lipoplex (○) or Man lipoplex (●) in mice. Similar results were obtained in two other independent runs.

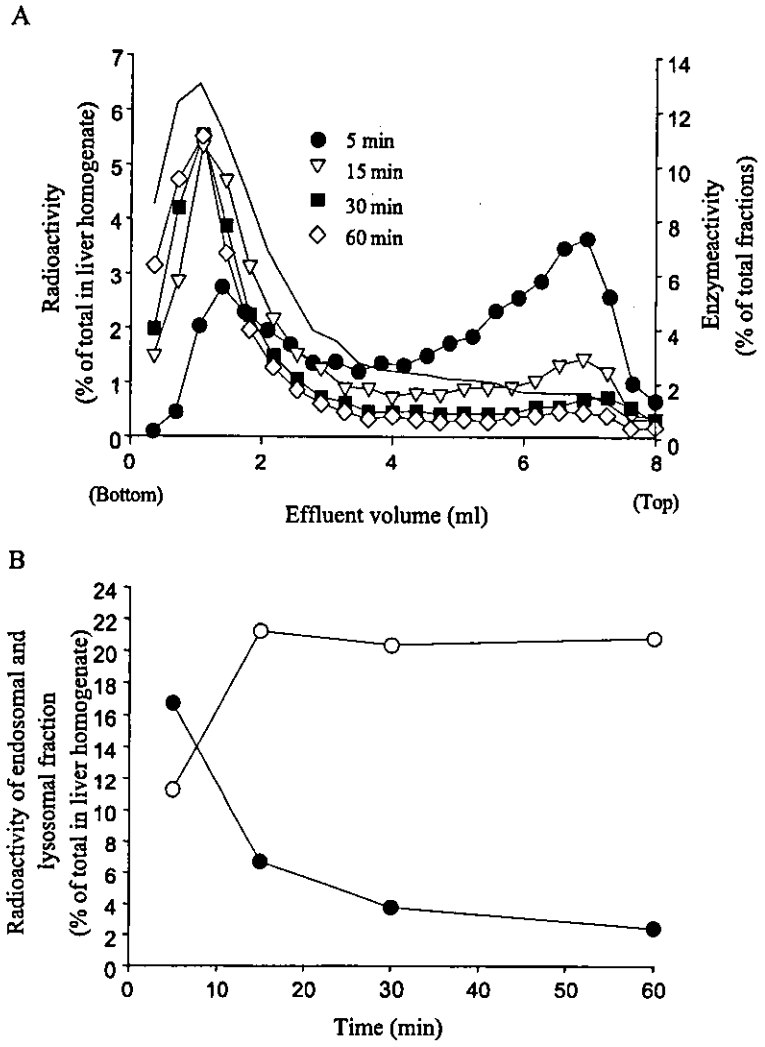

Fig. 5. Each fraction (A) and calculated endosomal (●) or lysosomal (○) localization (B) of radioactivity 5, 15, 30, and 60 min after intravenous administration of [^{111}In]mannosylated bovine serum albumin in mice. Liver homogenate was separated by Percoll density gradient centrifugation. Solid line in A represents the localization of β-hexosamidase activity. Similar results were obtained in two other independent runs.

istration of [^{111}In]lipoplex (B) or Man lipoplex (D). Sixty minutes after intravenous administration, the [^{111}In]Man lipoplex showed a larger distribution to the lysosome fractions than the [^{111}In]lipoplex. For comparison, naked [^{111}In]pDNA, which is extensively accumulated in the liver after intravenous administration but exhibits little gene expression [25], was also subjected to this assay (Fig. 6F). Naked [^{111}In]pDNA showed faster transfer to the lysosome fractions than both the [^{111}In]lipoplex and Man lipoplex.

3.5. PCR amplification

To determine whether pDNA within the subcellular fractions retained its structure, the luciferase sequence of pDNA was amplified by PCR (Fig. 7). When the Man lipoplex was intravenously adminis-

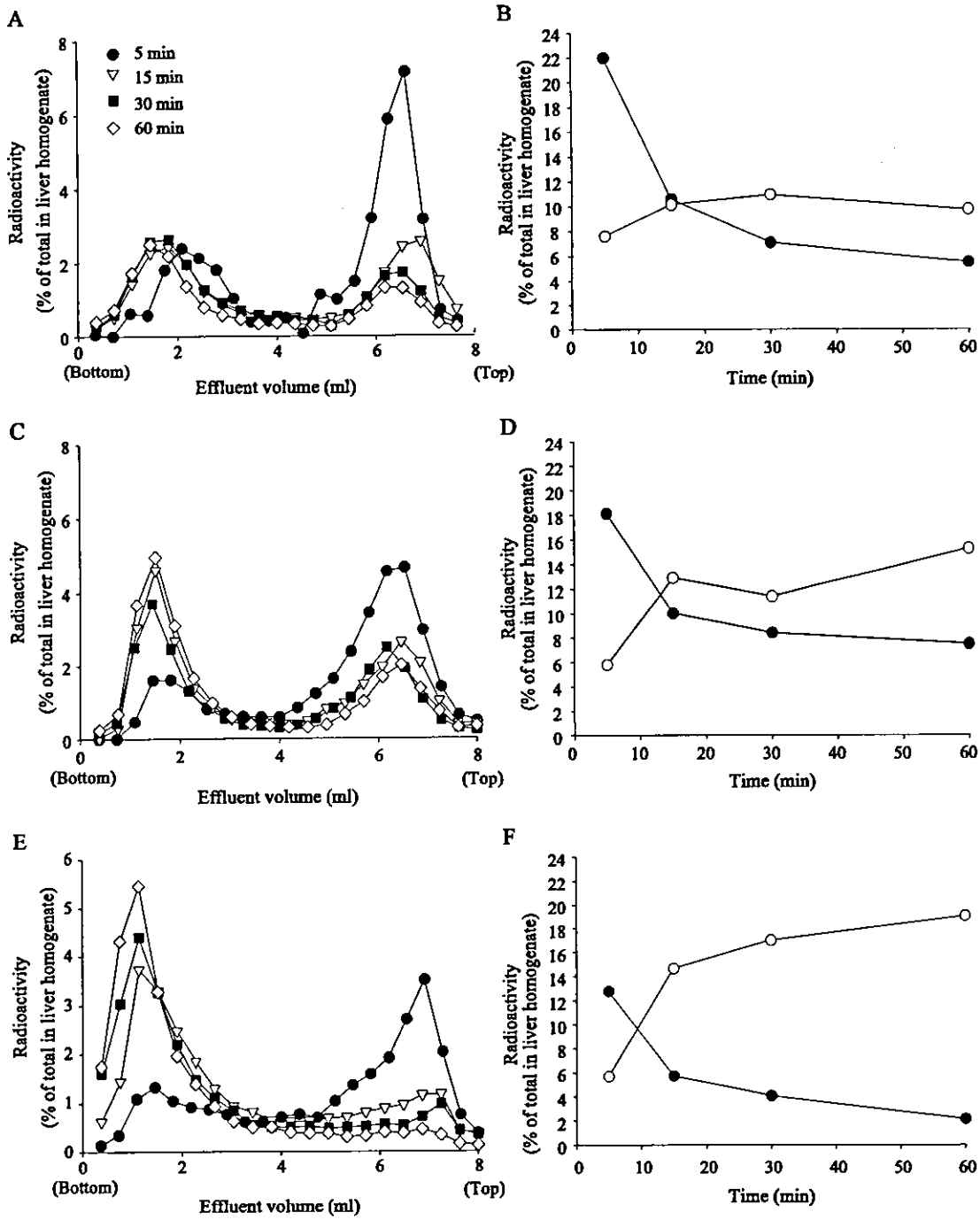

Fig. 6. Each fraction (A, C, and E) and calculated endosomal (●) and lysosomal (○) localization (B, D, and F) of radioactivity 5, 15, 30, and 60 min after intravenous administration of [^{111}In]lipoplex (A, B), Man lipoplex (C, D), or naked [^{111}In]pDNA (E, F) in mice. Liver homogenate was separated by Percoll density gradient centrifugation. Similar results were obtained in two other independent runs.

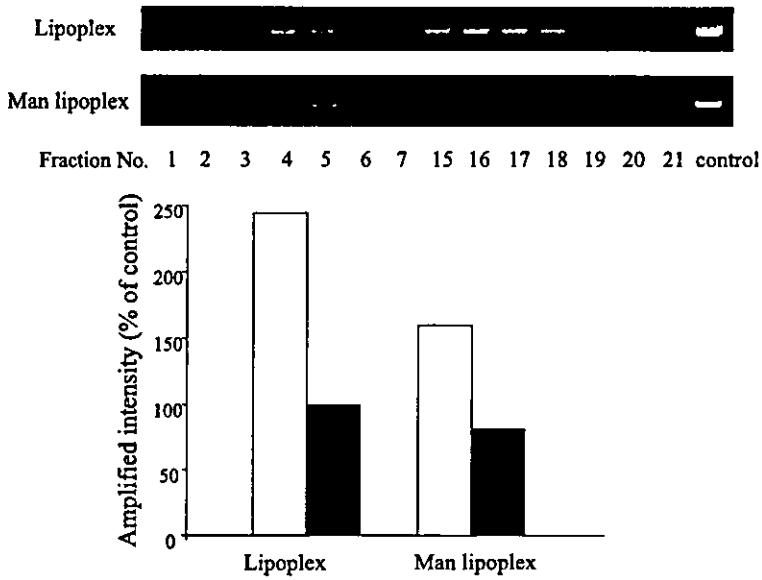

Fig. 7. Amplification of pDNA in endosomal (□) and lysosomal (■) fractions 30 min after intravenous administration of lipoplex and Man lipoplex in mice. Liver homogenate was separated by Percoll density gradient centrifugation. pDNA (1 ng/μl) was used as a control. Similar results were obtained in two other independent runs.

tered, the amounts of DNA amplified from the endosome and lysosome fractions were smaller than those after administration of the lipoplex.

4. Discussion

Transgene expression in target cells after intravenous administration of the Man lipoplex involves a number of distribution processes for pDNA: delivery to the target cells (tissue distribution), internalization, intracellular sorting, and nuclear entry [26]. In particular, the data presented in this study show the importance of intracellular sorting for efficient gene transfection of the Man lipoplex.

Since lipoplex was taken up the cell by the mechanism of endocytosis, pDNA needs to avoid degradation in lysosomes for improving the transfection efficiency. Thus, understanding of the intracellular fate of pDNA will help in the development of better transfection carrier systems. However, there are only a few studies that quantitatively investigate the intracellular fate of pDNA under in vivo conditions. Although so far several tracing methods of pDNA have been used such as ^{32}P-label by nick translation, however, radioactive metabolite, which are generated before and after the cellular uptake of radiolabeled pDNA, often make it extremely difficult to quantitative analyze the tissue distribution and pharmacokinetics of pDNA. In our preliminary experiment, when the fate of internalized [^{32}P]pDNA in liver homogenate was investigated, we found that [^{32}P]pDNA was not suitable for subcellular distribution studies because the radioactivity derived from [^{32}P]pDNA rapidly diminishes due to degradation during the preparation of subcellular fractions. More recently, we have developed [^{111}In]pDNA, an alternative radiolabeling method for pDNA [22], in a similar manner to the preparation of ^{111}In-labeled proteins [27,28], and demonstrated that the radioactivity of [^{111}In]pDNA is slowly released from cells after internalization. Therefore, [^{111}In]pDNA is considered the suitable method for analyzing the tissue and intrahepatic distribution of Man lipoplex. In fact, we observed that both [^{32}P]pDNA and [^{111}In]pDNA mainly accumulated in the liver after intravenous administration, but the radioactivity of ^{32}P gradually decreased (Fig. 2). In contrast, ^{111}In remained at a high level for 2 h after administration (data not shown). Such differences were explained by the poorer membrane permeability

of radioactive metabolites due to the attachment of DTPA for chelation of ^{111}In. Taking this into consideration, [^{111}In]pDNA was effective rebelling method for the subcellular distribution study of lipoplex.

As shown in Fig. 4, the cytoplasmic radioactivity was increased with time after intravenous injection of the [^{111}In]lipoplex and Man lipoplex; accordingly, pDNA is considered to be efficiently released into cytoplasm. On the other hand, the shift of radioactivity from the endosomal to the lysosomal fractions after intravenous administration of the [^{111}In]lipoplex and Man lipoplex suggests that both DC and Man liposomes promote pDNA transfer to lysosomes in the cell (Fig. 6). We previously reported that mannosylated proteins are internalized faster than cationic proteins, which are internalized by the liver via adsorptive endocytosis [16]. Receptor-mediated uptake of the Man lipoplex would explain its faster transport to the lysosome fractions. When naked [^{111}In]pDNA was injected intravenously, however, it showed more rapid transfer to lysosomes than both pDNA complexes. The lysosomes are where internalized substances are degraded, and it can be considered that lysosomal delivery is not suitable for transgene expression. Amplification of pDNA by PCR supported that the Man lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex (Fig. 7). Therefore, these results suggested that modulation of its intracellular sorting could improve the transfection efficiency of Man lipoplex.

After administration into the blood circulation, the lipoplex interacts with various cells and molecules, such as serum proteins and erythrocytes [29,30]. The cationic nature of the lipoplex attracts negatively charged cells and molecules, which eventually leads to an alteration in the physicochemical properties of the complex. Generally, cellular uptake of a lipoplex is considered to be a nonspecific process based on the interaction of its excess positive charge and the negatively charged cell membrane. Thus, high accumulation of radioactivity was observed both in lung and liver after intravenous administration of the [^{32}P]lipoplex (Fig. 2). On the other hand, the [^{32}P]Man lipoplex did not accumulate in the lung to any great extent compared with the [^{32}P]lipoplex, suggesting that nonspecific interaction could be reduced by mannosylation of cationic liposomes. This distribution study may be partly supported by the fact that our previous observation involved the liver NPC selective gene transfection after intravenous administration of Man lipoplex [10].

In conclusion, the Man lipoplex showed specific accumulation in NPC and achieved higher gene expression than the lipoplex after intravenous administration. It was shown that pDNA delivered by Man liposomes, which is taken up by the mannose receptor, was more susceptible to intracellular degradation than that delivered by conventional cationic liposomes, and this would impair higher gene expression. Also, this observation leads us to believe that further carrier development studies are needed for improving the intracellular sorting of pDNA to avoid degradation.

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