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# Evaluation of Proinflammatory Cytokine Production Induced by Linear and Branched Polyethylenimine/Plasmid DNA Complexes in Mice

Shigeru Kawakami, Yoshitaka Ito, Pensri Charoensit, Fumiyoshi Yamashita, and Mitsuru Hashida

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

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## ABSTRACT

The purpose of this study was to evaluate the cytokine response induced by linear and branched polyethylenimine (PEI)/plasmid DNA (pDNA) complex (polyplex) in relation to the ratio of PEI nitrogen and DNA phosphate (N/P ratio) of the polyplex, dose of pDNA, and structure and molecular weight of PEI, which are important for transfection efficacy of PEI polyplex. As a control, a $N$-[1-(2, 3-dioleyloxy) propyl]-$n,n,n$-trimethylammonium chloride/cholesterol liposome/pDNA complex (lipoplex) was selected for its high transfection efficacy in vivo. The concentration of proinflammatory cytokines such as tumor necrosis factor (TNF)-$\alpha$ were much lower after the administration of polyplex than lipoplex irrespective of the N/P ratio, dose of pDNA, or structure and molecular weight of PEI, although these factors affected the transfection efficacy in vivo. We demonstrated that the amount of activated nuclear factor-$\kappa$B, which contributes substantially to the production of cytokines, was comparable with the control (no treatment) level, and significantly less than that obtained with lipoplex. Although the production of proinflammatory cytokines (TNF-$\alpha$, interferon-$\gamma$, and interleukin-12) was reduced on the administration of the linear PEI polyplex, serum alanine aminotransferase levels were significantly enhanced by pDNA in a dose-dependent manner, suggesting that such hepatic damage is not induced by proinflammatory cytokines.

The success of gene therapy largely depends upon the development of delivery vehicles or vectors, which can selectively and efficiently deliver therapeutic genes to target cells with minimal toxicity (Ross et al., 1996). Viral vectors, although highly efficient, have inherent drawbacks such as immunogenicity; therefore, nonviral vectors have increasingly been receiving attention (Yang et al., 1994; Knowles et al., 1995). Of the various types of nonviral vectors, polyethylenimine (PEI) and cationic liposomes are most effective vectors in transfecting pDNA into target cells in vivo (Boussif et al., 1995; Boletta et al., 1997; Goula et al., 1998; Tranchant et al., 2004; Neu et al., 2005). PEI and cationic liposome-mediated gene transfer efficiently delivers a gene to the pulmonary endothelium after an i.v. administration. The

factors that enhance the transfection efficacy have been well studied, but it is also important to analyze the side effects of nonviral vectors for clinical applications.

As for the cationic liposome/pDNA complex (lipoplex), side effects have been documented. The CpG motifs in the pDNA sequence up-regulate the expression of transcription factors such as nuclear factor (NF)-$\kappa$B, which contributes substantially to the production of cytokines (Krieg et al., 1995; David et al., 2000; Klinman, 2004). Consequently, lipoplex could induce the production of large quantities of proinflammatory cytokines, such as tumor necrosis factor (TNF)-$\alpha$, interferon (IFN)-$\gamma$, and interleukin (IL)-12 (Freimark et al., 1998; Li et al., 1999; Yew et al., 1999; Sakurai et al., 2002). It was suggested that these cytokines cause liver damage (Tan et al., 2001). In addition, these cytokines cause gene inactivation-inducing transient gene expression after a single injection and a refractory period on repeated dosing (Li et al., 1999; Tan et al., 2001). Therefore, these studies demonstrated that the immune response could influence the hepatic toxicity as well as the gene expression period.

To date, the transfection efficacy of the PEI/pDNA complex

**ABBREVIATIONS:** PEI, polyethylenimine; pDNA, plasmid DNA; lipoplex, cationic liposome/pDNA complex; NF$\kappa$B, nuclear factor $\kappa$B; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; N/P ratio, the ratio of PEI nitrogen and DNA phosphate; polyplex, PEI/pDNA complex; DOTMA, $N$-[1-(2, 3-dioleyloxy) propyl]-$n,n,n$-trimethylammonium chloride; −:+, charge ratio; ALT, alanine aminotransferase.

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(polyplex) has been determined based on the N/P ratio of PEI polyplex, the dose of pDNA, and the structure and molecular weight of PEI (Goula et al., 1998; Bragonzi et al., 2000; Zou et al., 2000; Wightman et al., 2001). In contrast, not much has been done to clarify the cytokine response to PEI polyplex after its i.v. administration. Therefore, these three factors need to be related with cytokine levels for the optimization of gene therapy using PEI polyplex.

In this study, the transfection efficacy, production of cytokines, and toxicity in the liver after the i.v. administration of linear and branched PEI polyplexes were evaluated in relation to the N/P ratio of PEI polyplex, the dose of pDNA, and the structure and molecular weight of PEI. As a control, a $N$-[1-(2, 3-dioleyloxy) propyl]-$n,n,n$-trimethylammonium chloride (DOTMA)/cholesterol liposome-based lipoplex was selected because of its high transfection efficacy in vivo (Song et al., 1997; Sakurai et al., 2001).

## Materials and Methods

**Materials.** DOTMA was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The branched PEI (10, 25, and 70 kDa) and linear PEI (25 kDa) were obtained from Polysciences, Inc. (Warrington, PA). Cholesterol and Clear-sol I were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was purchased from PerkinElmer, Inc. (Wellesley, MA). [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) was obtained from Amersham Biosciences Co. (Piscataway, NJ). QIAGEN Endofree Plasmid Giga Kit was purchased from QIAGEN GmbH (Hilden, Germany). All other chemicals were of the highest purity available.

**Animals.** Five-week-old female ICR mice (20–23 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Kyoto University.

**Preparation of pDNA.** pCMV-Luc was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from a pGL3-control vector (Promega Co., Madison, WI) into the polylinker of a pcDNA3 vector (Invitrogen, Co., Carlsbad, CA). pDNA was amplified in the *Escherichia coli* strain DH5$\alpha$, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit. The concentration of DNA was determined by measuring UV absorption at 260 nm. The pDNA was labeled with [$\alpha$-$^{32}$P]dCTP by nick translation.

**Preparation of PEI Polyplex.** PEI polyplex was prepared as reported (Morimoto et al., 2003). In brief, linear PEI or branched PEI was dissolved in a 5% dextrose solution and adjusted to pH 7.4. PEI polyplex was formed by adding an equal volume of PEI to pDNA in 5% dextrose at various ratios and left at 37 °C for 30 min. The ratio of PEI to pDNA was expressed as the N/P ratio, which is the molar ratio of PEI nitrogen to DNA phosphate (Goula et al., 1998).

**Preparation of Cationic Liposomes.** DOTMA/cholesterol liposomes were prepared as reported (Kawakami et al., 2000a). DOTMA and cholesterol were dissolved in chloroform at a molar ratio of 1:1. The mixture was vacuum-desiccated and resuspended in 5% dextrose. After hydration, the suspension was sonicated on ice for 3 min, and the resulting liposomes were extruded through a 220-nm polycarbonate filter.

**Preparation of Lipoplex.** Lipoplex was prepared as reported (Kawakami et al., 2000a,b; Sakurai et al., 2001). In brief, it was formed by adding an equal volume of cationic liposomes to pDNA in 5% dextrose at a mixing ratio (−:+) of 1.0:3.1 and stored at room temperature for 30 min. The ratio of liposomes to pDNA was expressed as a charge ratio (−:+), which is the molar ratio of cationic lipids to DNA phosphate (Yang and Huang, 1997).

**Measurement of $\zeta$ Potential and Particle Size.** PEI and pDNA were mixed in 5% dextrose as above and concentrated for i.v. administration. After 30 min, the $\zeta$ potential and size of PEI polyplex were measured using Nano ZS (Malvern Instruments, Ltd., Malvern, Worcestershire, UK).

**Gene Expression Experiments.** Gene expression was measured as described previously (Liu et al., 1997; Kawakami et al., 2000a,b). Mice were administered i.v. with 300 $\mu$l of lipoplex or PEI polyplex. At specific time points, mice were sacrificed, the lung and liver were harvested, and homogenates were prepared by adding lysis buffer (0.05% Triton X-1000, 2 mM EDTA, and 0.1 M Tris, pH 7.8) using homogenizer (OMNI TH; Yamato Scientific Co. Ltd., Tokyo, Japan) at 4°C. The volume of lysis buffer added was 4 $\mu$l/mg for lung and 5 $\mu$l/mg for liver. To lyse cells, the homogenates were treated with three cycles of freezing and thawing. The homogenates were centrifuged at 12,000$g$ for 7 min at 4°C. Twenty microliters of each supernatant was analyzed for luciferase activity with 100 $\mu$l of luciferase assay buffer (Picagene; Toyo Ink Mfg. Co. Ltd., Tokyo, Japan), using a luminometer (Lumat LB 9507; Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany).

**Measurements of Cytokines and ALT.** Serum was prepared as outlined in our previous study (Sakurai et al., 2001). At specific time points after the i.v. administration of PEI polyplex and lipoplex,
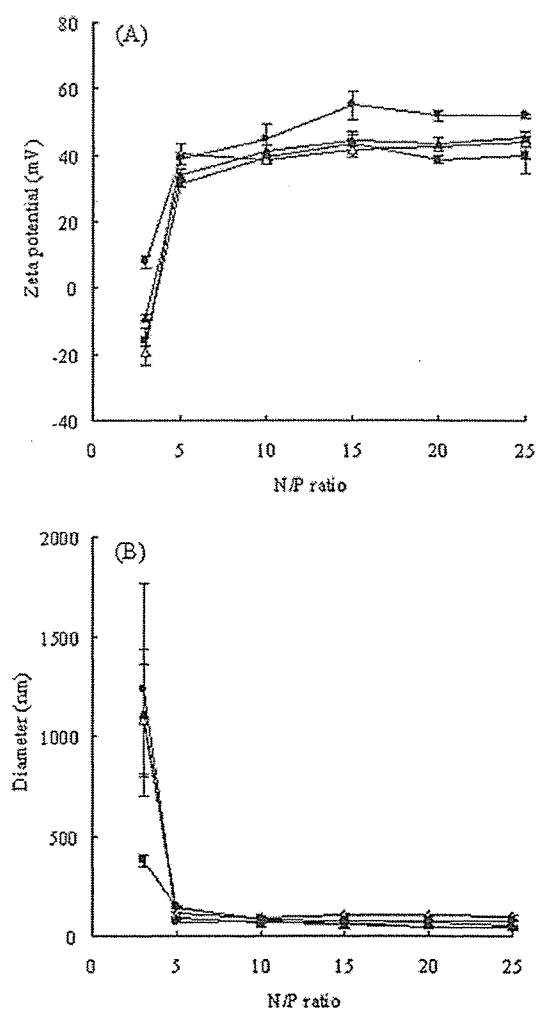


**Fig. 1.** $\zeta$ Potential (A) and particle size (B) of PEI polyplex at various N/P ratios. Linear PEI$_{25}$ polyplex (open triangles), branched PEI$_{10}$ polyplex (filled squares), branched PEI$_{25}$ polyplex (filled triangles), and branched PEI$_{70}$ polyplex (filled circles) were mixed with pDNA in 5% dextrose for 30 min at room temperature. Results are the mean ± S.D. of three measurements.

blood was collected from the vena cava and left to stand for 3 h at 37°C and then overnight at 4°C. Samples were centrifuged, and the supernatants were collected for serum. Serum TNF-α, IFN-γ, and IL-12 concentrations were determined with enzyme-linked immunosorbent assay kits according to the manufacturer's (Genzyme Co., Cambridge, MA) instructions. The serum ALT concentration was measured with kits using the UV-Rate method according to the manufacturer's direction (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Measurement of NFκB.** Three hours after the administration of PEI polyplex and lipoplex, mice were scarified, and their livers were collected. A nuclear extract of liver cells was prepared using the Nuclear/Cytosol Fractionation Kit (BioVision, Inc., CA). To analyze the extract, protein concentrations were prepared at 0.25 $\mu g/\mu l$. The amount of activated NFκB was measured by using an Enzyme Immunoassay for NFκB (human, mouse and rat) (Oxford Biomedical Research, Inc., Oxford, MI).

**Experiments on in Vivo Distribution.** Radioactivity was measured as reported previously (Kawakami et al., 2000a). Mice received an i.v. injection of 10 kBq [$^{32}$P]pDNA and pDNA (30 $\mu g$) in a complex with linear PEI$_{25}$ or cationic liposomes in 5% dextrose (300 $\mu l$) and were killed at a given time point. The liver and lung were removed, washed with saline, blotted dry, and weighed. Ten microliters of blood and 20 to 30 mg of each tissue were digested with 700 $\mu l$ of Soluene-350 by incubation overnight at 45 °C. After the digestion, 200 $\mu l$ of isopropanol, 200 $\mu l$ of 30% hydrogen peroxide, 100 $\mu l$ of 5 N HCl, and 5.0 ml of Clear-sol I were added. The samples were stored overnight, and radioactivity was measured in a scintillation counter (LSA-500; Beckman, Tokyo, Japan).

**Statistical Analysis.** Statistical analysis was performed using Student's paired $t$ test for two groups. Multiple comparisons among different groups were performed with the Turkey-Kramer test. $P < 0.05$ was considered to be indicative of statistical significance.

## Results

**ζ Potential and Particle Size.** Figure 1A shows the ζ potential of the linear and branched PEI with various molecular weights and N/P ratios. PEI polyplex was negatively charged at an N/P ratio of 3 except the branched PEI$_{70}$ polyplex which was positive, and increasing the N/P ratio from 5 resulted in a positive charge for all polyplexes (approximately 30–40 mV). Increasing the N/P ratio between 5 and 25 hardly changed the ζ potential of PEI polyplex (approximately 30–50 mV). As for the size of particles, it was approximately 400 nm at an N/P ratio of 3, except for the branched PEI$_{10}$ polyplex (Fig. 1B). At an N/P ratio of from 5 to 25, the size of all PEI polyplexes appeared to be reduced to approximately 80 to 90 nm, and this value was constant as the N/P ratio increased. In the present study, the ζ potential and particle size of lipoplex (−:+ of 1.0:3.1) were 60 ± 3.1 mV ($n$ = 3) and 103 ± 0.78 nm ($n$ = 3), respectively.

**Effect of the N/P Ratio of Polyplex and Structure and Molecular Weight of PEI on Cytokines.** After the i.v. administration of PEI polyplex in mice, the gene expression was much stronger in lung than liver, heart, spleen, or kidney (data not shown). Figure 2 shows the gene expression in lung and liver after the i.v. administration of the PEI polyplex and lipoplex preparations. The higher the N/P ratio of the branched PEI polyplex and molecular weight, the higher the transfection efficiency observed (Fig. 2, A–C). As far as the branched PEI polyplex is concerned, an N/P ratio of 10 yielded the highest level of expression in lung and liver, but
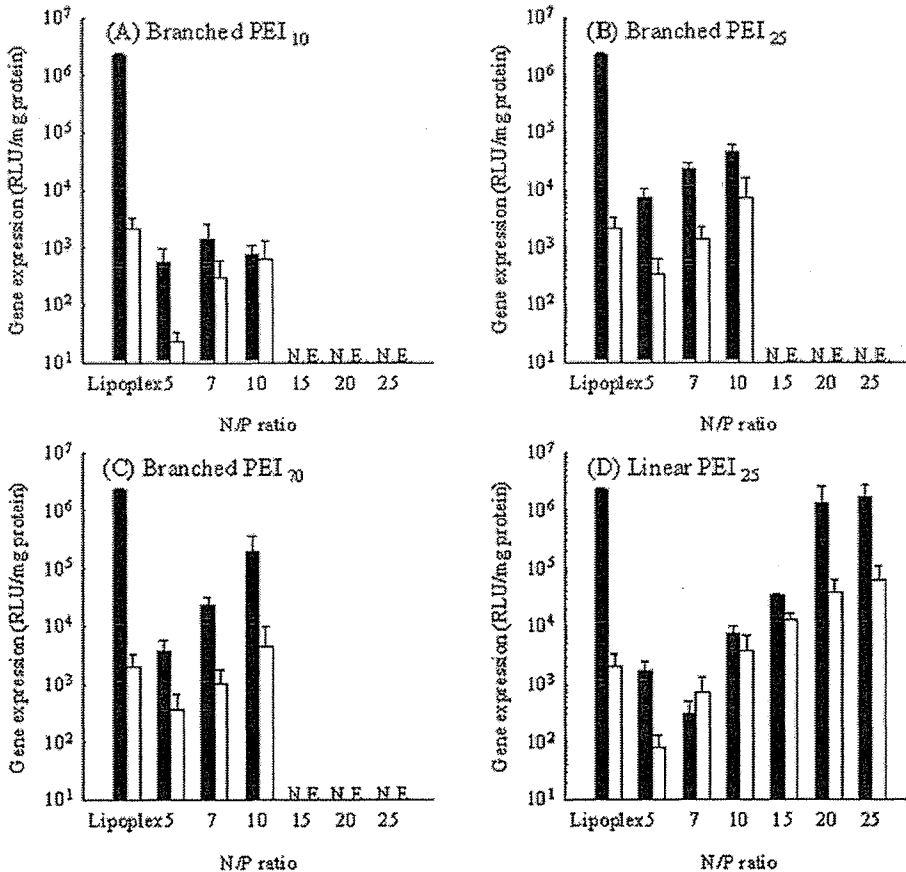


**Fig. 2.** Effect of N/P ratio of PEI polyplex and molecular weight of PEI on gene expression after the i.v. administration of branched and linear PEI polyplexes delivering 30 $\mu g$ of pDNA per mouse. Branched PEI$_{10}$ (A), PEI$_{25}$ (B), and PEI$_{70}$ (C), and linear PEI$_{25}$ (D) were used to produce polyplexes with an N/P ratio of 5, 7, 10, 15, 20, and 25, respectively. The lipoplex had a −:+ of 1.0:3.1 and delivered 30 $\mu g$ of pDNA per mouse. Mice were sacrificed at 6 h after the injection, and levels of luciferase activity in the lung (filled bars) and liver (open bars) were measured. N.E., not evaluated. Each value represents the mean + S.D. for at least three mice.

the gene expression was much lower than that obtained with lipoplex. However, this was the maximal tolerated N/P ratio since a further increase to 15 was lethal for 6-h evaluation. In contrast, the linear PEI polyplex yielded similar levels to lipoplex at N/P ratios of 20 and 25 (Fig. 2D). The maximal tolerated N/P ratio of the linear PEI polyplex was 30.

Intravenously injected lipoplex induced the production of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-12 (Whitmore et al., 1999; Sakurai et al., 2002). Among these cytokines, TNF-α is the primary source of toxicity because it induces septic shock in animals at high concentrations (Tan et al., 2002). Then, the response to an i.v. administration of a preparation of PEI polyplex varying in N/P ratio and the structure and molecular weight of PEI was investigated. After the injection, a significantly high TNF-α concentration was observed in serum (Fig. 3). The branched PEI was not lethal at an N/P ratio of up to 15 as of 3 h after the injection. After the i.v. injection of PEI polyplex, however, the TNF-α concentration was compatible with the control value (no treatment) and was significantly lower than that after the injection of lipoplex ($P < 0.01$). Moreover, this reduction in response was independent of the N/P ratio of the polyplex or structure and molecular weight of PEI (Fig. 3, A–D), although these factors could affect the gene expression in the lung and liver. When a linear PEI polyplex was prepared at an N/P ratio of 20, the efficacy of transfection increased without lethal toxicity. Accordingly, a linear PEI polyplex with an N/P ratio of 20 was selected for further investigation.

**Effect of pDNA Dose and Time Course of PEI Polyplex on Cytokine Response.** The transfection efficiency of the linear PEI polyplex was enhanced by the increasing of the pDNA dose (Fig. 4A). However, the TNF-α concentration obtained with PEI polyplex at a pDNA dose of 30, 50, and 80 μg was significantly lower than that obtained with lipoplex at a pDNA dose of 30 μg (Fig. 4B). To investigate the cytokine response in detail, serum levels of not only TNF-α but also

IFN-γ and IL-12 were measured for 12 h (Fig. 5, A–C). These proinflammatory cytokines were significantly induced by the administration of lipoplex. This characteristic was consistent with the previous results about lipoplex (Whitmore et al., 1999; Sakurai et al., 2002). However, TNF-α, IFN-γ, and IL-12 concentrations were much lower after the injection of PEI polyplex than that of lipoplex (Fig. 5).

**Serum ALT Activity Triggered by PEI Polyplex.** To evaluate the toxicity in the liver, the serum ALT level was determined. Raising the dose of pDNA (30 and 50 μg) in lipoplex increased the serum ALT level, and 80 μg of pDNA was lethal (Fig. 6A). Increasing the dose of pDNA (30, 50, and 80 μg) in the linear PEI polyplex also increased the serum ALT level (Fig. 6B). When we checked the liver surface after abdominal operation, the damage of hepatic lobule was observed after mice administered the linear PEI$_{25}$ polyplex at a pDNA dose of 50 and 80 μg.

**Amount of Hepatic NFκB Activated by Linear PEI Polyplex.** To investigate the mechanism of the cytokine response by linear PEI polyplex, the amount of activated NFκB was measured. After i.v. administration of lipoplex, significantly more activated NFκB was detected ($P < 0.05$). In contrast, the amount activated by the PEI polyplex was compatible with the control (no treatment group) and was significantly lower than that in response to lipoplex ($P < 0.05$) (Fig. 7).

**Biodistribution of Linear PEI Polyplex.** Biodistribution was examined to determine the difference in cytokine response to lipoplex versus the linear PEI polyplex (Fig. 8). [32P] Linear PEI polyplex mostly accumulated in the liver after the i.v. administration, whereas [32P] lipoplex accumulated in the lung. Blood concentration profiles of [32P] linear PEI polyplex and [32P] lipoplex did not differ.

**Gene Expression Characteristics of Linear PEI Polyplex.** Previous studies suggested that proinflammatory cytokines cause gene inactivation such as transient gene expres-
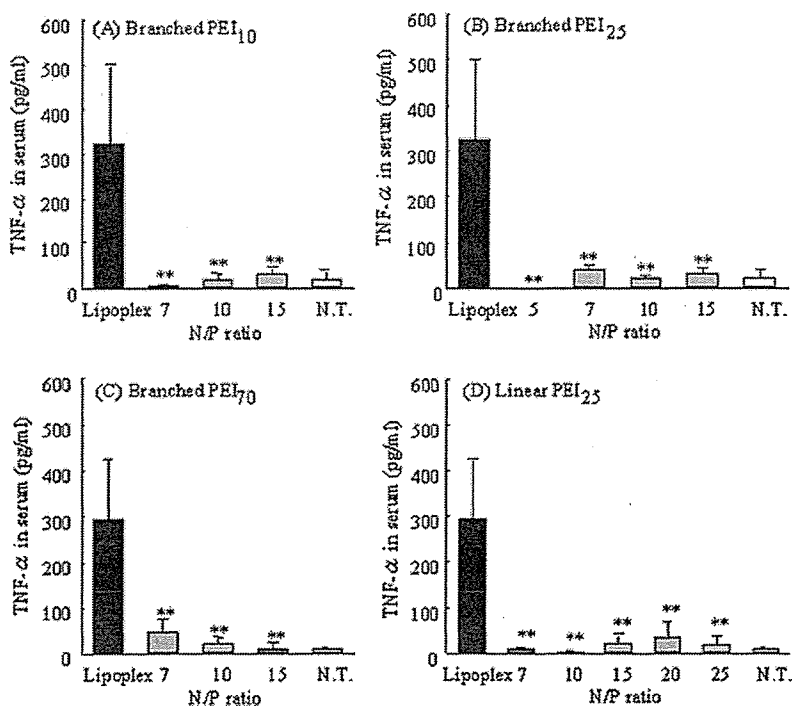


Fig. 3. Effect of N/P ratio of PEI polyplex and molecular weight of PEI on TNF-α release after the i.v. administration of branched and linear PEI polyplexes delivering 30 μg of pDNA per mouse. Branched PEI$_{10}$ (A), PEI$_{25}$ (B), and PEI$_{70}$ (C), and linear PEI$_{25}$ (D) were used to produce polyplexes with an N/P ratio of 7, 10, 15, or 20 and 25, respectively. Lipoplex had a −:+ of 1.0:3.1 and delivered 30 μg of pDNA per mouse. Mice were sacrificed at 3 h after the administration, and the concentration of TNF-α was measured. N.T., no treatment. Each value represents the mean + S.D. for at least three mice. **, statistically significant difference from the control group ($P < 0.01$).
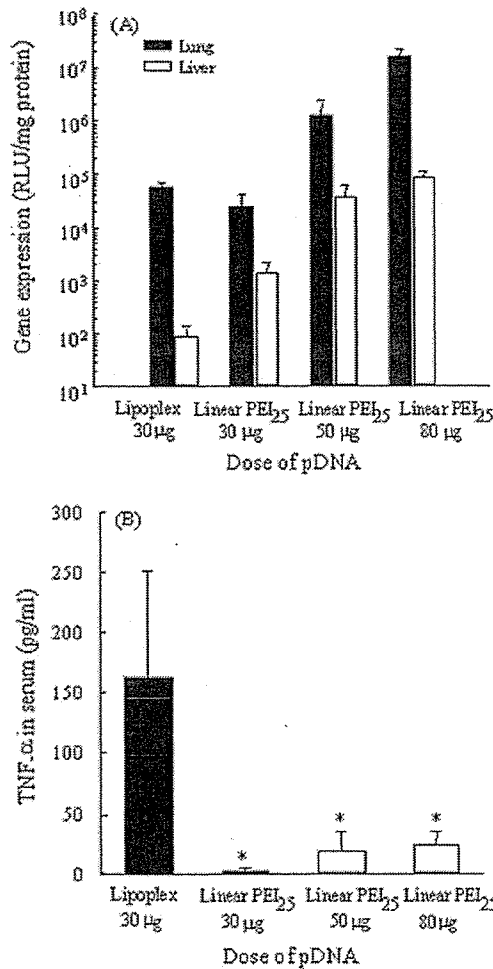
Fig. 4. Effect of the dose of pDNA on gene expression (A) and release of TNF-α (B) after the i.v. administration of linear PEI polyplex delivering 30, 50, and 80 μg of pDNA per mouse, respectively. Linear PEI polyplex was prepared at an N/P ratio of 20. Lipoplex had a −:+ of 1.0:3.1 and delivered 30 μg of pDNA per mouse. Mice were sacrificed at 6 h after administration, and levels of luciferase activity in the lung (filled bars) and liver (open bars) were measured. Mice also were sacrificed at 3 h after administration, and TNF-α concentrations were measured. *, statistically significant difference between the groups indicated (P < 0.05). Each value represents the mean + S.D. of at least three mice.

sion on a single injection of lipoplex and a refractory period on repeated dosing (Li et al., 1999; Tan et al., 2001). To investigate whether the linear PEI polyplex provides long-term gene expression, its effect was compared with that of lipoplex at various time points. However, there was little difference in gene expression between linear PEI polyplex and lipoplex (Fig. 9).

## Discussion

To clarify the relationship between gene expression and cytokine production in response to PEI polyplex, first, gene expression characteristics after the i.v. administration of the polyplex were evaluated based on the N/P ratio of PEI polyplex, the dose of pDNA, and the structure and molecular weight of PEI. After the i.v. administration in mice, the highest level of gene expression was observed in the lung. The gene expression characteristics were affected by the N/P ratio of polyplex and the structure and molecular weight of
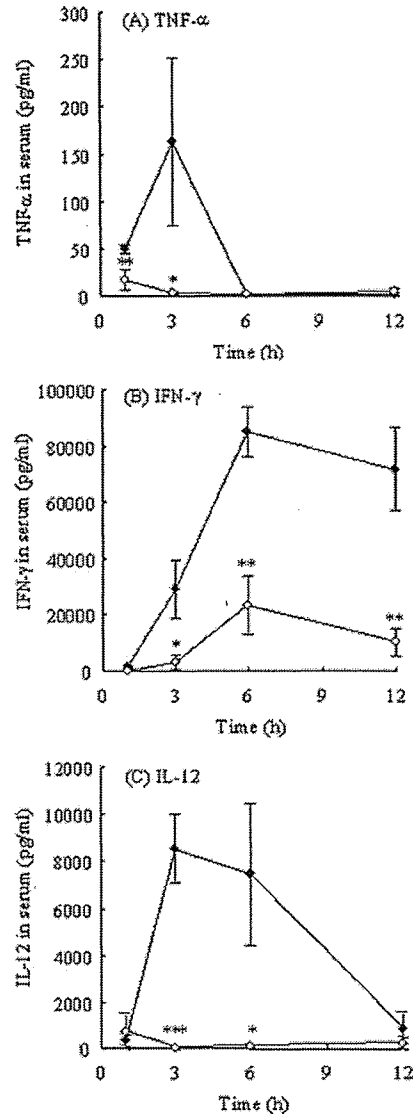


Fig. 5. Serum TNF-α (A), IFN-γ (B), and IL-12 (C) concentrations after the i.v. administration of linear PEI polyplex. Lipoplex (closed circles) was prepared with a −:+ of 1.0:3.1 and delivered 30 μg of pDNA per mouse. Linear PEI polyplex (open circles) was prepared at an N/P ratio of 20 with a pDNA dose of 30 μg per mouse. At the indicated time points after the administration of lipoplex or PEI polyplex, serum samples were collected from mice, and concentrations of TNF-α, IFN-γ, and IL-12 were measured. Each value represents the mean ± S.D. for at least three mice. Statistically significant difference from lipoplex (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

PEI (Fig. 2) as well as the dose of pDNA (Fig. 4A). These observations are consistent with previous reports (Goula et al., 1998; Bragonzi et al., 2000; Wightman et al., 2001; Zou et al., 2001). In this study, we selected DOTMA/cholesterol liposomes as cationic liposomes to prepare the lipoplex because of their high transfection efficacy in vivo (Song et al., 1997; Kawakami et al., 2000a; Sakurai et al., 2001). As shown in Fig. 2, the linear PEI polyplex induced the highest level of gene expression among the polyplexes under optimized conditions and was equal in efficacy to lipoplex. These observations are consistent with those of Bragonzi et al. (2000), who found that the gene expression efficacy of linear PEI polyplex was equal to lipoplex under optimal conditions.
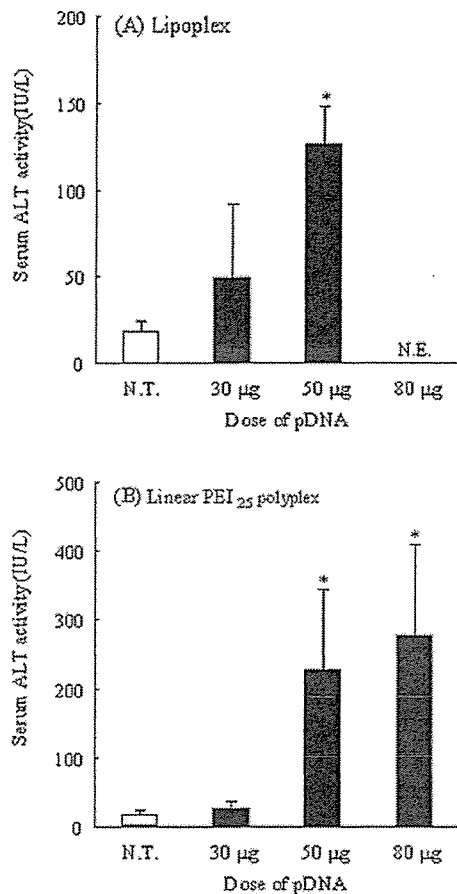
**Fig. 7.** Amount of activated NFκB in the liver after i.v. administration of linear PEI polyplex. Linear PEI polyplex was prepared at a pDNA dose of 30 μg per mouse. Lipoplex was prepared at −:+ of 1.0:3.1 and a pDNA dose of 30 μg per mouse. Mice were sacrificed at 3 h after the administration, and the amount of activated NFκB in the liver was measured. N.T., no treatment. *, statistically significant difference from the untreated group ($P < 0.05$). Each value represents the mean + S.D. for at least three mice.



**Fig. 6.** Serum ALT activity after the i.v. administration of linear PEI polyplex. Lipoplexes were prepared with a −:+ of 1.0:3.1 and a pDNA dose of 30, 50, and 80 μg per mouse (A). Linear PEI polyplexes were prepared with a pDNA dose of 30, 50, and 80 μg per mouse (B). Mice were sacrificed at 24 h after the administration, and the ALT level in serum was measured. N.E., not evaluated. N.T., no treatment. *, statistically significant difference from untreated groups ($P < 0.05$). Each value represents the mean + S.D. for at least three mice.

Then, cytokine response characteristics after the i.v. administration of PEI polyplex were evaluated based on the N/P ratio of PEI polyplex, the dose of pDNA, and the structure and molecular weight of PEI. Interestingly, serum TNF-α levels were low irrespective of the N/P ratio of PEI polyplex and structure and molecular weight of PEI (Fig. 3) or the dose of pDNA (Fig. 4B), although these factors affected the transfection efficacy in vivo (Figs. 2 and 4A). These findings are partly supported by the report that the serum TNF-α level was much lower in mice administered linear PEI₂₂ polyplex (N/P ratio, 6; pDNA dose, 25 μg/mouse) than those given lipoplex (Tan et al., 2001). To confirm whether this response is specific to TNF-α at 3 h, TNF-α, IFN-γ, and IL-12 concentrations were measured until 12 h; consequently, TNF-α, IFN-γ, and IL-12 concentrations by linear PEI polyplex were also much lower than that by lipoplex at various time points (Fig. 5). All of these results provide evidence that PEI polyplex hardly induced the production of proinflammatory cytokines irrespective of the N/P ratio of PEI polyplex, dose of pDNA, or structure and molecular weight of PEI, although these are important factors for transfection efficacy in vivo (Figs. 2 and 4A).
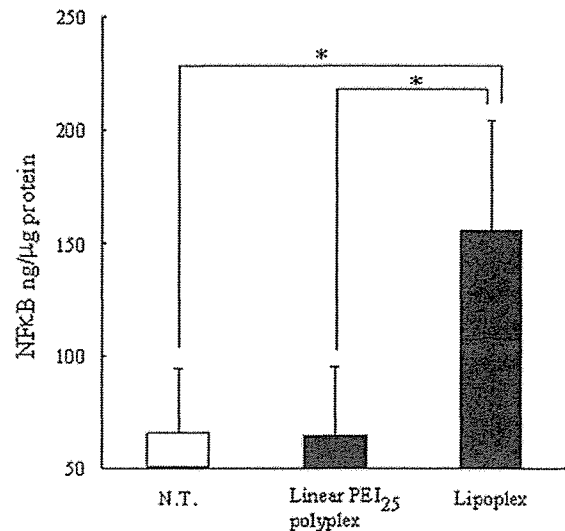
NFκB is a central regulator of inflammatory and immune

responses (Barnes and Karin, 1997) and is crucial for the transcription of multiple proinflammatory molecules, including TNF-α, IL-1β, IL-2, IL-6, IL-8, IL-12, and IFN-β (Lenardo and Baltimore, 1989). To investigate further the production of cytokines in response to PEI polyplex, the amount of activated NFκB in liver was measured. As shown in Fig. 7, we demonstrated that the amount of NFκB activated by linear PEI polyplex was comparable with the control level (untreated group) and was significantly lower than that activated by lipoplex ($P < 0.05$). In contrast, the amount of hepatic NFκB activated by lipoplex was significantly enhanced. These results are well consistent with the concentrations of proinflammatory cytokines produced when lipoplex and linear PEI polyplex were administered (Fig. 5). Thus, these observations lead us to conclude that lower levels of proinflammatory cytokines are produced in response to PEI polyplex after i.v. administration.

The immunostimulatory response observed in mammalian cells has been shown to arise in part from the recognition of the unmethylated CpG dinucleotides present in bacterial DNA or pDNA. Yi et al. (1998) reported that the activation of leukocyte by CpG DNA might occur in association with the acidification of endosomes since chloroquine, which is an inhibitor of endosomal acidification, blocks CpG DNA-induced IκBα and IκBβ degradation and the subsequent activation of NFκB; consequently, the response to produce proinflammatory cytokines is reduced. Likewise, Yew et al. (2000) demonstrated that two such inhibitors, chloroquine and quinacrine, greatly reduced the production of IL-12 by mouse spleen cells in vitro and inhibited cytokine production in the lung by approximately 50% without affecting gene expression. It has been reported that the transfection efficiency of PEI polyplex is due to its capacity to buffer the endosome (proton sponge effect) (Boussif et al., 1995; Kichler et al., 2001; Akinc et al., 2005); therefore, such a property might
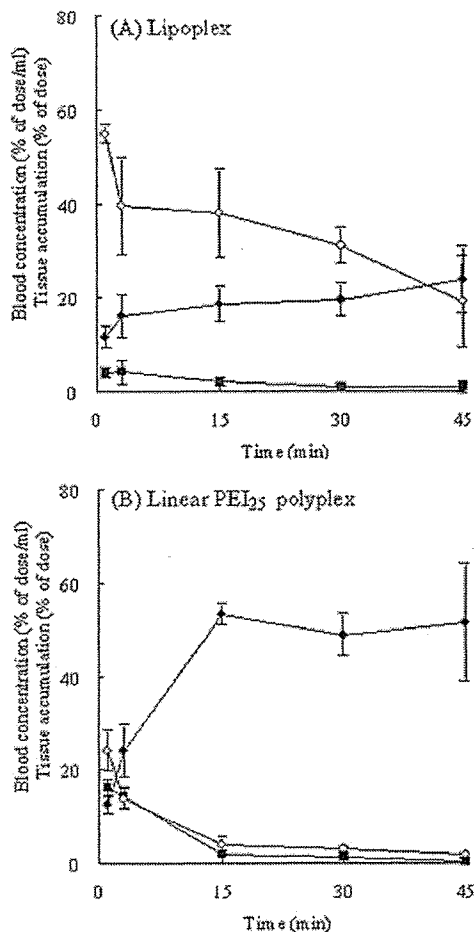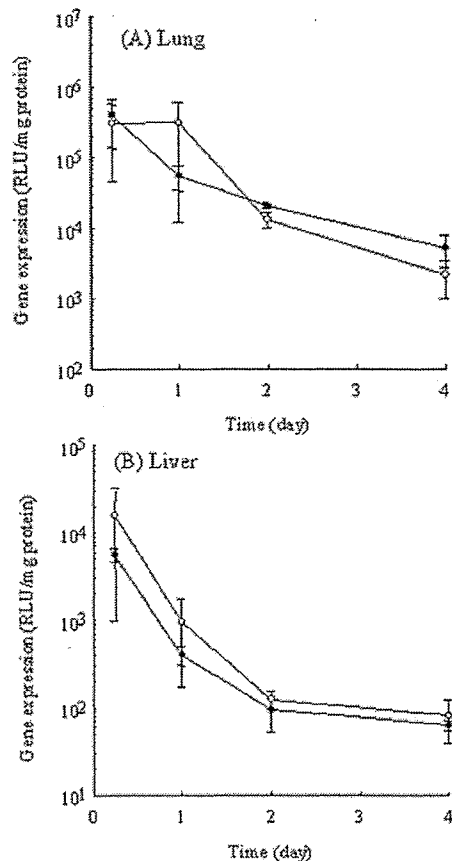
**Fig. 8.** Biodistribution after the i.v. administration of [$^{32}$P] linear PEI polyplex. Linear PEI polyplex was prepared at a pDNA dose of 30 μg per mouse. Lipoplex was prepared at −:+ of 1.0:3.1 at a pDNA dose of 30 μg per mouse. Blood (filled squares) was collected from the vena cava, liver (filled circles), and lung (open circles) at 1, 3, 15, 30, and 45 min, and levels of radioactivity in lung (A) and liver (B) were measured. Each value represents the mean ± S.D. for at least three mice.



**Fig. 9.** Time course of gene expression in the lung (A) and liver (B) after the i.v. administration of linear PEI polyplex. Linear PEI polyplex (open circle) was prepared at a pDNA dose of 30 μg per mouse. Lipoplex (closed circle) was prepared at −:+ of 1.0:3.1 at a pDNA dose of 30 μg per mouse. At the indicated time point, mice were sacrificed, and luciferase activity was measured. Each value represents the mean ± S.D. for at least three mice.

abolish the pDNA (CpG DNA)-induced activation of NFκB (Fig. 7).

We previously investigated the distribution of lipoplex and demonstrated that when i.v. injected, it was predominantly taken up by Kupffer cells via the phagocytic process that is responsible for the production of proinflammatory cytokines (Sakurai et al., 2002). Taking this into consideration, we hypothesized that the hepatic uptake of PEI polyplex is less than that of lipoplex. However, the biodistribution study demonstrated that much more [$^{32}$P] linear PEI polyplex than [$^{32}$P] lipoplex accumulates in the liver (Fig. 8). These results suggested that the difference in the response induced by PEI polyplex and lipoplex could not explain their distribution.

As far as the pulmonary accumulation of lipoplex is concerned, we and other groups have reported that lipoplex-induced hemagglutination is an important factor in the localization of lipoplex to the lung (Sakurai et al., 2001; Fumoto et al., 2005). It should be considered that the hemagglutination is caused by electrostatic interaction between the erythrocytes and lipoplex. In the distribution study, the ζ potential of PEI polyplex (approximately 40 mV) (Fig. 1) was lower than that of lipoplex (approximately 60 mV), suggest-

ing less electrostatic interaction between the erythrocytes and linear PEI polyplex. This hypothesis may be partly supported by the report that the aggregation of erythrocytes caused by linear PEI polyplex was minimal (Kircheis et al., 2001). Such ζ potential characteristics may reflect a more hepatic-selective distribution of the linear PEI polyplex (Fig. 8).

To evaluate hepatic damage, serum ALT activity was measured. As shown in Fig. 6A, raising the dose of pDNA in the lipoplex preparation increased the serum level of ALT. This finding regarding toxicity was consistent with the previous reports (Tousignant et al., 2000; Loisel et al., 2001). Recently, Tan et al. (2001) suggested that such hepatic damage was caused by the proinflammatory cytokines secreted when lipoplex was injected i.v. In this study, we demonstrated that PEI polyplex hardly induced the production of any proinflammatory cytokines (Figs. 3–5), but hepatic toxicity was observed (Fig. 6B). These observations provide evidence that the hepatic damage is not mediated by the proinflammatory cytokines, suggesting that the mechanisms behind the toxicity of lipoplex and linear PEI polyplex are different. Recently, Moghimi et al. (2005) reported PEI polyplex (branched and linear), by using calf thymus DNA, induced the cytotoxicity (necrosis and/or apoptosis) in several cultured human cell

lines. These results also suggested hepatic damage might be induced by PEI itself.

In this study, we examined the effect of the dose of pDNA in the PEI polyplex on hepatic damage. As shown in Fig. 6B, serum ALT activity at 30 $\mu$g of pDNA was compatible with the control (no treatment). However, at higher doses (50 and 80 $\mu$g), the serum ALT level increased, suggesting that i.v. injected PEI polyplex causes hepatic damage in a pDNA dose-dependent manner. These observations are consistent with those of Chollet et al. (2002), whose histological analysis revealed necrosis in the liver after the i.v. administration of a linear polyplex containing 100 $\mu$g of pDNA.

As shown in Fig. 6, lipoplex at pDNA dose of 80 $\mu$g was lethal. This observation corresponded with Hofland et al. (1997), who reported lipoplex at the pDNA dose of approximately 80 $\mu$g was the maximal tolerated dose in mice. It is expected that the hepatic toxicity between lipoplex and polyplex at pDNA dose of 80 $\mu$g is to the same extent since hepatic toxicity was nearly similar between lipoplex and PEI polyplex at pDNA dose of 30 and 50 $\mu$g (Fig. 6). Therefore, the lethal effect of lipoplex at pDNA dose of 80 $\mu$g might be explained by cytokine response, hematologic and serologic changes typified by leukopenia and thrombocytopenia (Tousignant et al., 2000).

Goula et al. (1998) reported that the branched $PEI_{25}$ polyplex was lethal within a few minutes even when used at a low N/P ratio, although how it was prepared is not clear. As shown in Fig. 2, the maximal tolerated N/P ratio of branched $PEI_{10}$, $PEI_{25}$, and $PEI_{70}$ was 10 since a further increase to 15 was lethal in 6 h. Thus, this observation is consistent with the report by Goula et al. (1998). Our results also suggested that the lethal toxicity of the branched PEI polyplex does not depend on the molecular weight of PEI.

In conclusion, the concentration of proinflammatory cytokines, such as TNF-$\alpha$, produced were much lower when PEI polyplex rather than lipoplex was administered irrespective of the N/P ratio of the polyplex, dose of pDNA used, or structure and molecular weight of PEI, although these factors affected the transfection efficacy in vivo. We demonstrated that the amount of NF$\kappa$B activated by the linear PEI polyplex was comparable with the control (untreated group) and was significantly lower than that when lipoplex was administered. Although the production of proinflammatory cytokines (TNF-$\alpha$, IFN-$\gamma$, and IL-12) was reduced by the administration of the linear PEI polyplex, serum ALT levels were significantly enhanced by pDNA in a dose-dependent manner, suggesting that the hepatic damage is not induced by proinflammatory cytokines. This information will be valuable for the development of nonviral vectors for clinical applications.

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**Address correspondence to:** Dr. Mitsuru Hashida, Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: hashidam@pharm.kyoto-u.ac.jp

# Novel PEG-matrix metalloproteinase-2 cleavable peptide-lipid containing galactosylated liposomes for hepatocellular carcinoma-selective targeting

Takeshi Terada, Mieko Iwai, Shigeru Kawakami, Fumiyoshi Yamashita, Mitsuru Hashida *

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

## Abstract

In order to obtain an HCC-selective drug delivery system, a novel functional lipid, which is cleaved by the protease activity of matrix metalloproteinase-2 (MMP-2), was developed. The amino group of dioleoylphosphatidylethanolamine (DOPE) was conjugated with PEGylated MMP-2 substrate peptide (Gly–Pro–Leu–Gly–Ile–Ala–Gly–Gln), and MMP-2-cleavable PEG-Peptide-DOPE (PEG-PD) was synthesized. When PEG-PD was incorporated in galactosylated liposomes (Gal-PEG-PD-liposomes), we expected that Gal-PEG-PD-liposomes would not be taken up by normal hepatocytes due to the steric hindrance effect, but would be activated around HCC cells by secreted MMPs. In the pretreatment by hMMP2 (1, 5, and 10 μg/ml), an hMMP2 concentration-dependent higher uptake of Gal-PEG-PD-liposomes was observed in HepG2 cells, suggesting PEG-PD cleavage. In the presence of an excess of galactose, the uptake of Gal-PEG-PD-liposomes with hMMP2 was significantly inhibited, suggesting asialoglycoprotein receptor-mediated uptake of Gal-PEG-PD-liposomes following the PEG-PD cleavage. Pretreatment of Gal-PEG-PD-liposomes with the conditioned medium of B16BL6, which contained secreted MMPs, enhanced the binding to HepG2 cells, as in the case of hMMP-2 treatment. Moreover, the cytotoxicity of $N^4$-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC) incorporated Gal-PEG-PD-liposomes was enhanced by hMMPs (5 μg/ml) and its cytotoxicity was significantly reduced by the presence of an excess of galactose in HepG2 cells. In conclusion, Gal-PEG-PD-liposomes were successfully developed for novel HCC-selective targeting.
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*Keywords:* Matrix metalloproteinase-2; Liposomes; Targeting; PEG-Peptide-DOPE

## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and is frequently a terminal complication of chronic inflammatory and fibrotic liver disease [1]. HCC frequently shows early invasion into blood vessels as well as intrahepatic metastases, followed later by extrahepatic metastases. Intrahepatic metastasis is one of the modalities of reoccurrence within the liver in HCC. The process of invasion and metastasis of cancer cells involves several important steps: (i) detachment of the tumor cell from the primary site; (ii) invasion into blood vessels with degradation of the extracellular matrix (ECM); (iii) adhesion to blood vessels at a distant site; and (iv) invasion of the distant organs. Among them, degradation of the surrounding ECM (step (ii)) is an important

step in tumor invasion and metastasis [2], and MMPs are considered to play a crucial role in this step [3,4]. In cancer research, much interest has been devoted recently to a gelatinase subgroup of MMPs that include MMP-2 (gelatinase A, $M_r$ 72,000 type IV collagenase) and MMP-9 (gelatinase B, $M_r$ 92,000 type IV collagenase) [5–7]. In particular, MMP-2 plays a critical role in tumor progression, angiogenesis, and metastasis [8–11]. Overexpression of MMP-2 in hepatocellular carcinoma has been shown in a number of preclinical as well as clinical investigations [12,13].

In a series of investigations, we have synthesized a novel galactosylated cholesterol derivative, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) form-amide (Gal-C4-Chol), and demonstrated that the liposomes containing Gal-C4-Chol (Gal-liposomes) were efficiently taken up by hepatocytes via the asialoglycoprotein receptor-mediated mechanism after intravenous injection [14–16]. In the case of HCC therapy, however, although HCC cells highly express

* Corresponding author. Tel.: +81 75 753 4525; fax: +81 75 753 4575.
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

asialoglycoprotein receptors, the help of Gal-liposomes is required for HCC-selective delivery; this is because galactosylated liposomes can deliver incorporated antitumor drugs not only to HCC cells but also to normal liver cells.

In the present study, therefore, we tried to develop a more HCC-selective drug delivery system in the liver. In order to achieve such a system, a novel functional lipid, which is cleaved by the protease activity of MMP-2, was exploited to activate the liposomes. The amino group of dioleoylphosphatidylethanolamine (DOPE) was conjugated with PEGylated MMP-2 substrate peptide (Gly–Pro–Leu–Gly–Ile–Ala–Gly–Gln) [17,18], and MMP-2-cleavable PEG-Peptide-DOPE (PEG-PD) was synthesized. Since the PEG chains cover the galactose ligands on the surface of the liposomes, the uptake by normal hepatocytes of Gal-liposomes containing PEG-PD (Gal-PEG-PD-liposomes) could be inhibited by steric hindrance [19]. Furthermore, the PEG chain reduces liposome uptake by macrophages of the reticuloendothelial system (RES) and extends the liposome half-time of the circulation [20]. In the HCC cells, on the other hand, the PEG-PD in Gal-PEG-PD-liposomes can be cleaved by MMP-2, exposing the galactose moieties on the liposomal surface; consequently, they can be selectively recognized by asialoglycoprotein receptors on HCC cells.

We describe here the uptake characteristics of Gal-PEG-PD-liposomes by cultured HepG2 cells after pretreatment with purified human MMP-2 (hMMP-2) and conditioned medium of mouse melanoma cells (B16BL6), known to highly express MMPs [21]. Furthermore, $N^4$-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC), which is a lipophilic derivative of ara-C [22–24], was incorporated in Gal-PEG-PD-liposomes, and its cytotoxicity against cultured HepG2 cells was evaluated.

## 2. Materials and methods

### 2.1. Materials

N-(4-aminobutyl) carbamic acid tert-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). Distearoyl phosphatidylcholine (DSPC), DOPE, cholesteryl chloroformate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ara-C, and NOAC were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Chol and Clear-Sol I was obtained from Nacalai Tesque Inc. (Kyoto, Japan), and Soluene 350 was purchased from Packard Bioscience Co. (Groningen, Netherlands). [³H] CHE was purchased from NEN Life Science Products Inc. (Boston, MA, USA). L-α-Phosphatidylethanolamine-N-fluorescein (PE-fluorescein) was purchased from Avanti Polar Lipids Inc. (Alabama, AL, USA). Two-imino-2-methoxyethyl-L-thiogalactoside was synthesized as reported previously [25]. MMP-2-cleavable peptide (Gly–Pro–Leu–Gly–Ile–Ala–Gly–Gln) was custom-made by Toray Research Center Inc. (Tokyo, Japan). Methoxy-poly (ethylene glycol)-N-hydroxysuccinimide (mPEG-NHS) was purchased from NOF Co. Inc. (Tokyo, Japan). All other chemicals were reagent grade products obtained commercially.

### 2.2. Synthesis of PEG-PD

PEG-PD was synthesized by reacting the peptide (Gly–Pro–Leu–Gly–Ile–Ala–Gly–Gln) containing MMP-2-cleavable sequences with each chain length of mPEG-NHS and then with DOPE. Briefly, the peptide and each chain length of mPEG-NHS with each molecular weight (1.2 : 1, molar ratio) were mixed and stirred in carbonate buffer (pH 8.5) at 4 °C overnight. After dialysis and lyophilization, the reacted powder (mPEG-peptide) and DOPE (1 : 1, molar ratio) in chloroform : tetrahydrofuran (1 : 4, v/v) were reacted overnight by adding 1.2-equal mol of 1,3-dicyclohexyl-carbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) at room temperature. TLC analysis and ninhydrin assay showed that the reaction had gone to completion. After filtration of the precipitate, a powder of PEG-PD was obtained after evaporation of the solvent. After dialysis in distilled water and lyophilization, the material was used in the next step.

MMP-2-uncleavable PEG-DOPE (PEG-D) was synthesized by reacting mPEG-NHS with DOPE. The purity of each synthesized PEG-lipid was calculated by phosphate assay and was >80% on the basis of DOPE.

### 2.3. Synthesis of Gal-C4-Chol

Gal-C4-Chol was synthesized according to the method reported previously [26]. Cholesteryl chloroformate and N-(4-aminobutyl)carbamic acid tert-butyl ester were reacted in chloroform for 24 h at room temperature. After the reaction mixture was incubated with trifluoroacetic acid for 4 h at 4 °C, the solvent was evaporated to give N-(4-aminobutyl)-(cholesten-5-yloxyl)formamide, which was reacted with an excess of 2-imino-2-methoxyethyl-1-thiogalactoside [25] in pyridine containing triethylamine for 24 h at room temperature. After the reaction mixture was evaporated, the resultant material was suspended in water and dialyzed against distilled water for 48 h using a dialysis membrane (12-kDa cutoff).

### 2.4. Preparation of liposomes

Each lipid mixture (DSPC, Chol, Gal-C4-Chol, and PEG-PD) was dissolved in chloroform and evaporated to dryness. [³H] CHE or PE-fluorescein (1 mol %) was added to the liposomes for the uptake study and confocal microscopy, respectively. The dried lipid films were hydrated in HBS (50 mM HEPES, 150 mM NaCl, pH 7.4) containing 5 mM CaCl₂ and sonicated at 65 °C for 3 min. The lipid concentration was monitored by phosphate assay. The mean vesicle diameters of the liposomes were determined using a laser light scattering size analyzer (LS-900, Otsuka Electronics Co. Ltd., Osaka, Japan).

### 2.5. Gelatin zymography assay

B16BL6 cells ($1 \times 10^6$) were seeded onto 100 mm tissue culture dishes and allowed to adhere in the presence of serum. Subsequently, medium was replaced by 10 mL serum-free

DMEM per well. After 72 h of incubation, the conditioned medium was collected and ultracentrifuged to remove the cell debris. The supernatants concentrated by 10-kDA cutoff VIVA SPIN (Vivascience, Hannover, Germany) were used for MMP-2 detection by zymography [27]. Purified hMMP-2 (Chemicon, California, USA) was used as a migration standard for the assays.

## 2.6. PEG-PD cleavage assay in PEG-PD-liposomes

PEG-PD cleavage by MMP-2 was checked by evaluating the amino residues appearing following digestion of PEG-PD. TLC detection and the TNBS method were used for PEG-PD cleavage assay of hMMP-2 and the conditioned medium of B16BL6 cells, respectively. In the hMMP-2 digestion, 4 mM liposome samples were incubated with 5 μg/ml active hMMP-2 (activated with $p$-aminophenylmercuric acetate for 1 h) in 1 ml HBS containing 5 mM $CaCl_2$ at 37 °C for 24 h. After ultracentrifugation at 50 000 ×$g$ for 10 min at 4 °C in a Himac CS100EX ultracentrifuge (Hitachi, Ltd., Tokyo, Japan) and washing 3 times with saline, the lipid pellets were resuspended in chloroform and spotted onto TLC plates. TLC was carried out with a chloroform:methanol (75:25) mobile phase, followed by air drying, and visualization by ninhydrin. In the conditioned medium, B16BL6 cells were cultured with serum-free DMEM for 72 h. Subsequently, the supernatants were collected and ultracentrifuged to remove the cell debris. The supernatants concentrated by 10-kDa cutoff VIVA SPIN were used for MMP-2 detection by zymography and PEG-PD cleavage assay. Four mM samples of liposomal suspensions were diluted twice with the conditioned medium of B16BL6 cells in the absence or presence of 25 nM GM6001 (Chemicon, California, USA) then incubated at 37 °C. At each time point, the liposomal suspensions were centrifuged and washed as described for the TLC detection; also, 4 mM samples of the liposomal suspensions were analyzed by the TNBS method [28].

## 2.7. Cellar uptake of Gal-PEG-PD-liposomes in HepG2 cells

HepG2 cells were plated on 12-well culture plates at a density of $5 \times 10^5$ cells/well. After 24 h cultivation, cells were washed three times and preincubated for 20 min with Hanks' balanced salt solution (HBSS). Then, 4 mM samples of liposomes were incubated with various concentrations of hMMP-2 or conditioned medium of B16BL6 cells for 24 h as described above. After pre-incubation, the cells were incubated at 37 °C for 2 h with HBSS containing treated [$^3$H] liposomes (100 μM, 3.0 MBq/ml). For the inhibition study, 20 mM galactose was added to both solutions for the preincubation and liposome suspensions. At the end of the incubation period, the medium was removed and the cells were washed 5 times with cold HBSS. The cells were then solubilized with 0.5 ml 1 N NaOH solution overnight and then neutralized with 0.1 ml 5 N HCl solution. The radioactivity of the [$^3$H] CHE was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan). The protein content was also measured using a Protein Quantification Kit (Dojindo Molecular Technologies, Inc.,

Gaithersburg, MD, USA). In the case of the confocal microscopy study, the cells were incubated at 37 °C for 1 h with fluorescein-labeled liposomes and examined by confocal laser microscopy (MRC-1024; Bio-Rad, California, USA).

## 2.8. Cellar uptake of Gal-PEG-PD-liposomes in primary cultured macrophages

Each sample of liposomes was prepared in HBSS as described above. The prepared liposomes were extruded 5 times through 200 and 100 nm pore-size polycarbonate membranes at 65 °C. The mean vesicle diameters were confirmed to be about 100 nm using a laser light scattering size analyzer. Five-week-old male ICR mice were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). All animal procedures were examined by the Ethics Committee on Animal Experiment at the Kyoto University. Resident macrophages were collected from the peritoneal cavity of unstimulated mice with serum-free RPMI 1640 medium [29]. Washed cells were suspended in RPMI 1640 medium supplemented with serum and plated on 12-well culture plates at a density of $1 \times 10^6$ cells/well. After incubation for 2 h at 37 °C in 5% $CO_2$–95% air, adherent macrophages were washed three times with RPMI 1640 medium to remove nonadherent cells and then cultured under the same conditions. After 48 h cultivation, the cells were washed 3-times and incubated with HBSS containing treated [$^3$H] liposomes (200 μM, 3.0 MBq/ml) at 37 °C for 2 h. The radioactivity was measured as described above.

## 2.9. Stability of Gal-PEG-PD-liposomes in fetal bovine serum (FBS)

Liposome samples were prepared in HBSS and extruded as described above. Four mM liposome samples were diluted twice with fetal bovine serum (Flow Laboratories, Irvine, UK) and incubated at 37 °C. At each time point, the change in particle diameter was measured using a laser light scattering size analyzer.

## 2.10. Cytotoxicity assay of NOAC incorporated Gal-PEG-PD-liposomes in HepG2 cells

Four mM DSPC/Chol/Gal/PEG-PD/NOAC (60:24.5:5:0.5:10) liposome samples were incubated with 5 μg/ml hMMP-2 as described above. HepG2 cells were counted and seeded in 96-well plates ($5 \times 10^4$ cells/well). After 24 h incubation, cells were exposed to serum-free DMEM containing various concentration (0–400 μM) of ara-C, NOAC or empty PEG-PD-liposomes in the case of pretreatment or no treatment with hMMP-2 for 24 h at 37 °C. The cytotoxic effects were determined by MTT dye reduction assay as described by Mosmann [30]. In the inhibition study, the cells were exposed to each of the conditioned liposome samples in the presence or absence of 20 mM galactose for 2 h at 37 °C and then the medium was changed to DMEM supplemented with serum. After 24 h incubation, the cytotoxic effects were determined by MTT dye reduction assay (Table 1).

Table 1
Lipid composition and mean particle size of the tested liposomes

| Lipid composition (molar ratio) | Particle size (nm)[a] |
|---|---|
| Gal-liposomes (DSPC/Chol/Gal-C4-Chol=60:35:5) | 120.9±4.9 |
| Gal-PEG-PD-liposomes (DSPC/Chol/Gal-C4-Chol/PPD=60:34.99:5:0.01) | 110.4±6.6 |
| Gal-PEG-PD-liposomes (DSPC/Chol/Gal-C4-Chol/PPD=60:34.9:5:0.1) | 105.1±0.91 |
| Gal-PEG-PD-liposomes (DSPC/Chol/Gal-C4-Chol/PPD=60:34.5:5:0.5) | 107.1±3.2 |
| Gal-PEG-PD-liposomes (DSPC/Chol/Gal-C4-Chol/PPD=60:34:5:1) | 106.3±9.5 |

[a] The mean particle sizes of the liposomes were measured using a laser light scattering particle size analyzer. Results are expressed as the mean±S.D. of three experiments.

### 2.11. Statistical analysis

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

## 3. Results

### 3.1. Cleavage of PEG-PD-liposomes by preincubation with hMMP-2 or conditioned medium of B16BL6

Cleavage by hMMP-2 to release the PEG coating of PEG-PD-liposomes was tested by TLC assay. The spot of the PEG-PD-liposomes incubated with hMMP-2 on the TLC plate was visualized by ninhydrin reagent, suggesting that

$NH_2$-IAGQ-DOPE is produced by the cleavage (data not shown).

The preincubation of PEG-PD-liposomes with the conditioned medium of B16BL6, known to be cells that highly secrete MMPs, resulted in the cleavage of PEG-PD. Gelatin zymography was employed to confirm that the conditioned medium contained MMP-2 (Fig. 1A). The result of the TNBS assay demonstrated a time-dependent cleavage of PEG-PD and appearance of $NH_2$-IAGQ-DOPE (Fig. 1B). In contrast, MMP-2-uncleavable PEG-DOPE (PEG-D), which is synthesized by reacting mPEG-NHS with DOPE, was negative. In addition, inhibition of the cleavage by GM6001, known to inhibit MMPs, and the negative results of PD-liposomes suggested that the cleavage was specifically by MMPs present in conditioned medium of B16BL6 (Fig. 1C).

### 3.2. Activated binding of Gal-PEG-PD-liposomes with HepG2 cells by hMMP-2 or conditioned medium of B16BL6 cells

Gal-liposomes showed a high uptake via the asialoglycoprotein receptor-mediated process by HepG2 cells, but hMMP-2 treatment had no effect on this uptake (Fig. 2A). On the other hand, Gal-PEG-PD (0.1%)-liposomes after pretreatment with hMMP-2 exhibited a higher uptake by HepG2 cells, compared with the non-treatment group. In addition, inhibition of the higher uptake by 20 mM galactose suggested that the increase in the uptake was due to asialoglycoprotein receptor-mediated uptake by galactose ligands on the surface of the liposomes. Pretreatment of Gal-PEG-PD-liposomes with conditioned medium of B16BL6 enhanced the binding to HepG2 cells, as in the case of hMMP-2 treatment (Fig. 2B). In addition, the
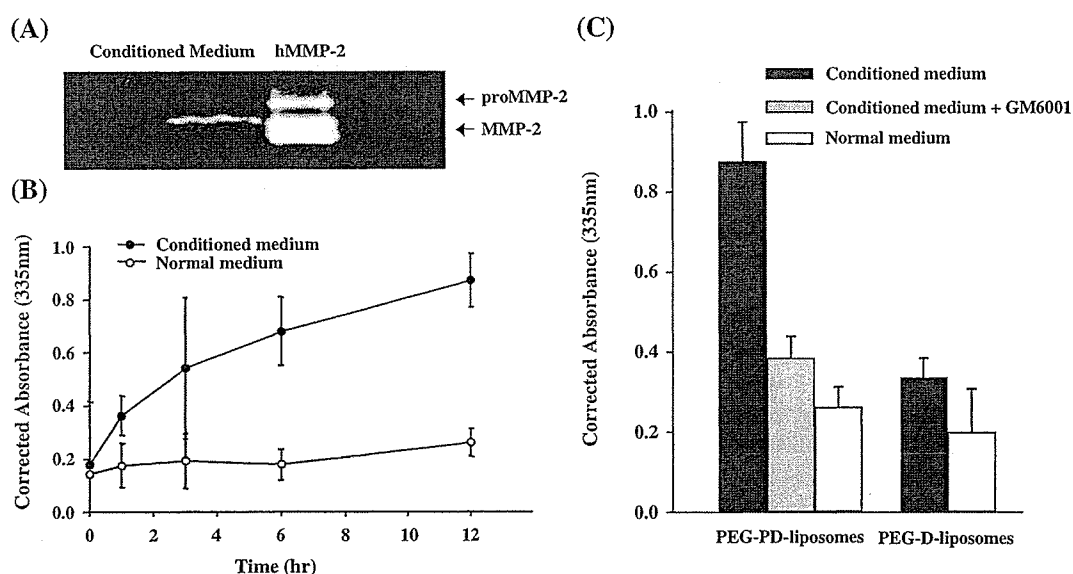


Fig. 1. Quantitation of the cleavage of PEG-PD by the conditioned medium of B16BL6 cells. (A), Zymography of secreted MMP-2 in the conditioned medium of B16BL6 cells. The right and left lanes show hMMP-2 as the standard and the conditioned medium, respectively. The amino residues appearing through digestion of PEG-PD were determined by the TNBS method. (B), Cleavage kinetics of DSPC/Chol/PEG-PD (60:35:5) liposomes incubated with the conditioned (●) and normal (○) medium. (C), Each liposome sample was incubated with the conditioned (filled) and normal (open) medium for 12 h at 37 °C. In the case of Gal-PEG-PD-liposomes, GM6001 was added to the conditioned medium as an MMP inhibitor (hatched). Each result represents the mean±S.D. of three experiments.
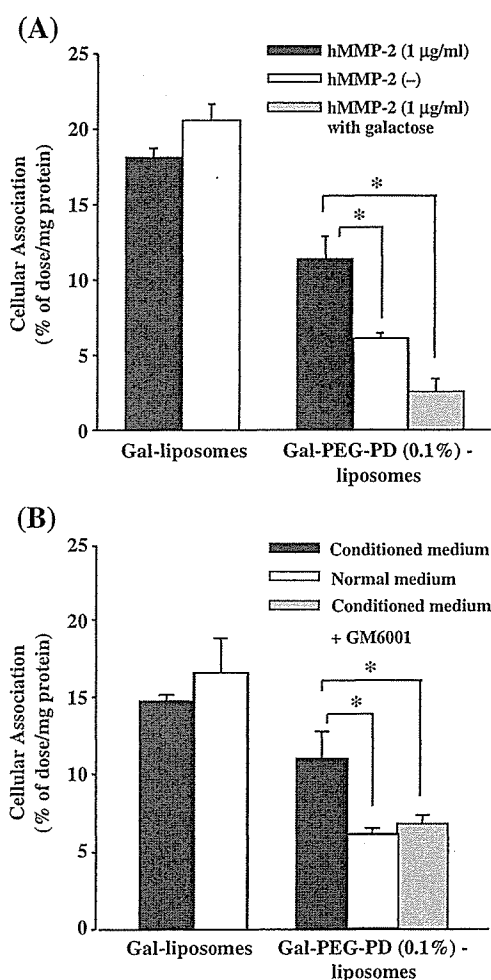
## (A)



## (B)



Fig. 2. Effect of MMP pretreatment on the uptake of liposomes (100 μM) by HepG2 cells. (A), [³H] liposomes were incubated with (filled) or without (open) 1 μg/ml hMMP-2 used in the uptake study. Then, galactose was used to inhibit the uptake (hatched). (B), [³H] liposomes incubated with the conditioned medium of B16BL6 cells (filled) or normal medium (open) used in the uptake study. GM6001 was added to the conditioned medium as an inhibitor of MMPs (hatched). Each result represents the mean±S.D. of three experiments. *Statistically significant differences ($P < 0.05$).

higher uptake of PEG-PD-liposomes after treatment with conditioned medium was inhibited in the presence of GM6001. This suggests that MMPs secreted by B16BL6 cells can cleave PEG-PD, and then the liposomes will be taken up via galactose ligands on the surface by HepG2 cells.

### 3.3. The effect of PEG-PD content and its hMMP stability on the binding of Gal-PEG-PD-liposomes in HepG2 cells

Since the steric hindrance effect by PEG-PD is expected to depend on the amount of PEG-PD in Gal-PEG-PD-liposomes, the uptake of Gal-PEG-PD-liposomes with various amounts of PEG-PD was examined in HepG2 cells (Fig. 3). In Gal-PEG-PD-liposomes with 0.5% and 1% PEG-PD, the binding to HepG2 was significantly inhibited, suggesting a steric hindrance effect. Therefore, at least 0.5% PEG-PD is required to

avoid recognition by asialoglycoprotein receptors on normal hepatocytes.

In the HCC-selective targeting system, the uptake by normal hepatocytes of Gal-PEG-PD-liposomes needs to be reduced, that is, the steric hindrance effect by PEG-PD in the blood circulation is very important. A previous study has reported that the serum MMP-2 concentrations in HCC patients are higher than those in healthy subjects, and the value has been reported to be about 1 μg/ml [13]. Therefore, we evaluated the effect of 1 μg/ml MMP-2 on the uptake by HepG2 cells. In the presence of 1 μg/ml hMMP-2, the cellular uptake of Gal-PEG-PD-liposomes with 0.01% and 0.1% PEG-PD was significantly higher than that in the absence of hMMP-2, suggesting the degradation of PEG-PD in Gal-PEG-PD-liposomes. However, the cellular uptake of Gal-PEG-PD-liposomes with 0.5% and 1% PEG-PD was not enhanced by treatment with 1 μg/ml hMMP-2, suggesting that Gal-PEG-PD (0.5 and 1%)-liposomes are stable in blood. Such steric characteristics of Gal-PEG-PD (0.5 and 1%)-liposomes can enhance the HCC-selectivity. Thus, Gal-PEG-PD (0.5%)-liposomes were selected for the following experiments.

### 3.4. The effect of hMMP-2 concentration on the binding of Gal-PEG-PD-liposomes in HepG2 cells

In our HCC-selective targeting system, PEG-PD in Gal-PEG-PD (0.5%)-liposomes needs to be degraded by a higher concentration of hMMP-2, i.e. >1 μg/ml (serum MMP-2 concentration of HCC patients). Thus, the effect of hMMP-2 concentration on the binding of Gal-PEG-PD-liposomes to HepG2 cells was evaluated. The binding of Gal-PEG-PD-liposomes to HepG2 cells was significantly enhanced in an hMMP-2 concentration-dependent manner (Fig. 4). Especially, the treatment with 5 and 10 μg/ml hMMP-2 significantly enhanced the uptake of Gal-PEG-PD (0.5%)-liposomes by HepG2 cells. The confocal microscopy study also showed that
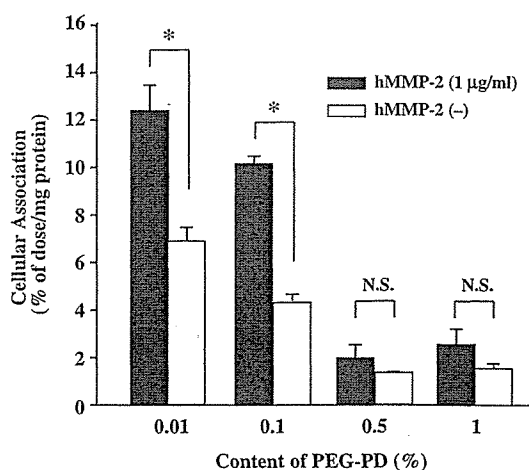


Fig. 3. Effect of PEG-PD content and hMMP-2 stability on the uptake of Gal-PEG-PD-liposomes by HepG2 cells. [³H] Gal-PEG-PD-liposomes were incubated with 1 μg/ml hMMP-2. Each result represents the mean±S.D. of three experiments.
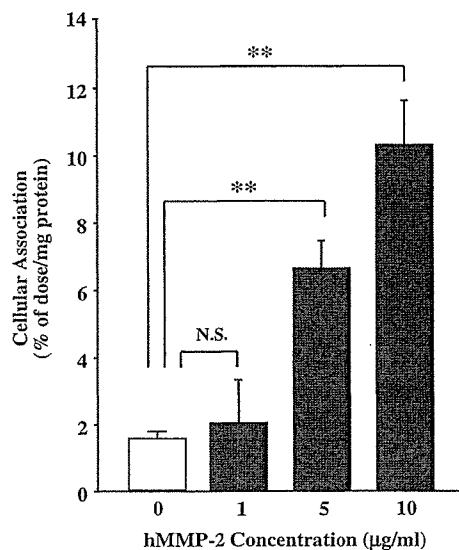
Fig. 4. Effect of hMMP-2 concentration on the uptake of Gal-PEG-PD-liposomes by HepG2 cells. [$^3$H] Gal-PEG-PD-liposomes were incubated with 1, 5, and 10 μg/ml hMMP-2. Each result represents the mean±S.D. of three experiments.

the uptake of Gal-PEG-PD (0.5%)-liposomes by HepG2 was enhanced by the treatment of 5 μg/ml hMMP-2 (Fig. 5A) and completely inhibited in the case of no treatment (Fig. 5B). These results suggest that the cleavage by 5 μg/ml hMMP-2 is required to achieve the significant uptake of Gal-PEG-PD (0.5%)-liposome by HepG2 cells.

### 3.5. The effect of PEG-PD on the binding of Gal-PEG-PD-liposomes in primary cultured macrophages and the stability of liposomal size in FBS

To evaluate the non-specific uptake of Gal-PEG-PD-liposomes by RES, mouse peritoneal macrophages were used as a model of mononuclear phagocytes in the RES. The uptake of Gal-liposomes by peritoneal macrophages was significantly inhibited by the incorporation of PEG-PD, and the effect was dependent on the amount of PEG-PD (Fig. 6A), suggesting the reduced non-specific uptake of Gal-PEG-PD-liposomes by RES.

Moreover, PEG-PD enhanced the stability of liposomes in the serum (Fig. 6B). The particle size of Gal-liposomes quickly increased after incubation with FBS. On the other hand, the size of Gal-PEG-PD-liposomes could sustain a particle size of 100 nm. In particular, the size of Gal-PEG-PD-liposomes with 0.5% PEG-PD was constant even 24 h after incubation, suggesting that Gal-PEG-PD (0.5%)-liposomes are stable in the body.

### 3.6. The cytotoxicity of NOAC incorporated in Gal-PEG-PD-liposomes in HepG2 cells

In order to evaluate the cytotoxicity of Gal-PEG-PD (0.5%)-liposomes in HepG2 cells, NOAC, a lipophilic derivative of ara-C, was incorporated into Gal-PEG-PD-liposomes (Fig. 7). The

cytotoxicity of NOAC incorporated into Gal-PEG-PD (0.5%)-liposomes with the hMMP-2 (5 μg/ml) was significantly more cytotoxic than that without hMMP-2, suggesting that the higher cytotoxicity resulted from the degradation of PEG-PD. In the presence of 20 mM galactose, the cytotoxicity of NOAC incorporated into Gal-PEG-PD-liposomes with hMMP-2 treatment was significantly inhibited, suggesting that asialoglycoprotein receptor-mediated uptake is involved in the cytotoxicity of NOAC incorporated into Gal-PEG-PD (0.5%)-liposomes.

Fig. 8 shows the concentration-dependent cytotoxicity of NOAC, Gal-PEG-PD (0.5%)-liposomes, NOAC incorporated into Gal-PEG-PD (0.5%)-liposomes with or without hMMP-2 in HepG2 cells. In these NOAC concentration ranges, the cytotoxicity of NOAC incorporated into Gal-PEG-PD (0.5%)-liposomes was higher than that of NOAC and/or Gal-PEG-PD (0.5%)-liposomes. Furthermore, hMMP-2 treatment enhanced the cytotoxic effect of NOAC incorporated into Gal-PEG-PD (0.5%)-liposomes, suggesting that the

**(A)**        hMMP-2 (5 μg/ml)
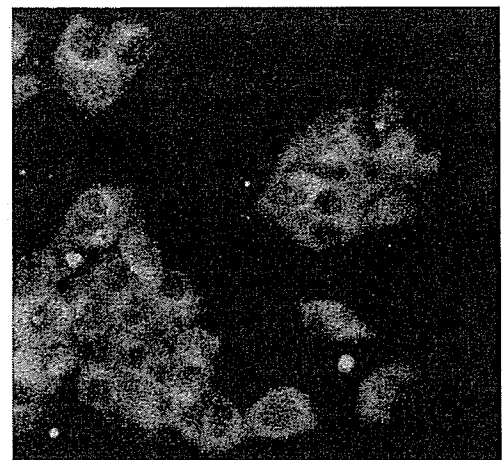


**(B)**        hMMP-2 (–)



Fig. 5. Confocal microscopic image of Gal-PEG-PD-liposomes with (A) or without (B) hMMP-2 (5 μg/ml) in HepG2 cells. Conditioned Gal-PEG-PD-liposomes containing 1% PE-fluorescein were incubated with HepG2 cells for 1 h. (A), treatment with 5 μg/ml hMMP-2. (B), no treatment.
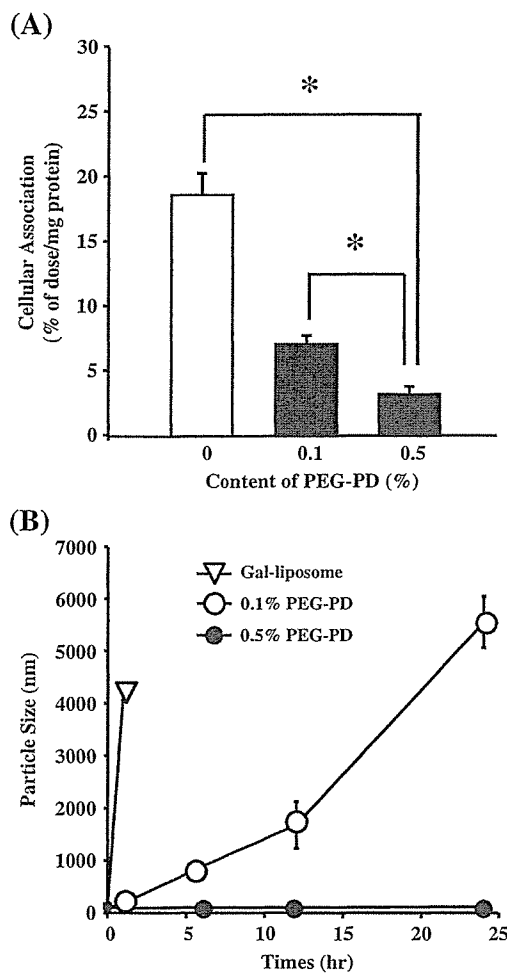
**(A)**



**(B)**



Fig. 6. Effect of PEG-PD content in Gal-liposomes on the uptake by peritoneal macrophages and the stability in FBS. (A), The uptake study of [$^3$H] Gal-PEG-PD-liposomes with 0.1, and 0.5% PEG-PD or without PEG-PD (Gal-liposomes) in peritoneal macrophages was carried out. Each result represents the mean±S.D. of three experiments or three wells. *Statistically significant differences ($P<0.05$). (B), The particle size of each Gal-PEG-PD-liposomes with 0.1 (O), and 0.5 (●) % PEG-PD or without PEG-PD (▽) was measured at each time point after incubation with an equal volume of FBS.

higher cytotoxicity resulted from the cleavage of PEG-PD. Thus, these results suggest that NOAC incorporated into Gal-PEG-PD (0.5%)-liposomes with hMMP-2 is taken up by asialoglycoprotein receptor-mediated endocytosis following the cleavage of PEG-PD.

## 4. Discussion

We have described a novel PEGylated liposome approach, in which the protease activity of MMP-2 is exploited to release PEG coating and expose the tumor targeting ligand on the surface, as a drug delivery system for tumor therapy. Until now, an MMP cleavable linker has often been used as an effective targetable strategy to trigger the targeting of antitumor drugs or retroviral vectors to tumor tissues [31–34]. Recently, some groups have applied the MMP cleavable linker to dextran and hydrogel as MMPs triggered drug delivery carrier [35,36]. On
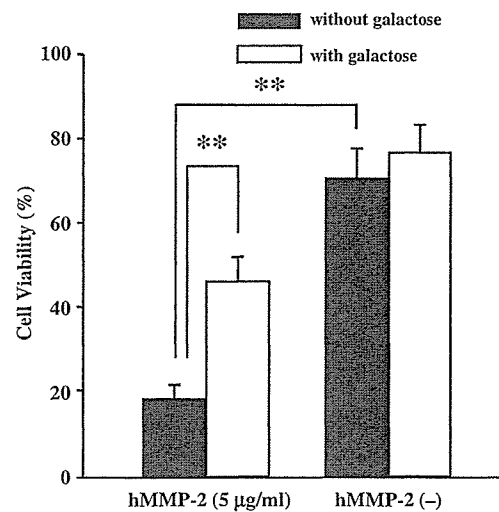


Fig. 7. Cytotoxicity of NOAC incorporated Gal-PEG-PD-liposomes by activation of hMMP-2 (5 μg/ml) in HepG2 cells. HepG2 cells were incubated with hMMP-2 treated liposomes, no treated liposomes in the absence (■) or presence (□) of galactose. Cytotoxicity was evaluated by MTT assay. Each result represents the mean±S.D. of three wells. **Statistically significant differences ($P<0.01$).

the other hand, liposomes are the most extensively studied of all particulate carriers, and several commercial products are now available. This is because liposomes are easily prepared and have a large capacity even for hydrophobic drugs. Our present report is the first study of MMP activated delivery via liposomes.

MMP-2 mediated cleavage of PEG-PD contained in liposomes is the essential step to success in our strategy. Therefore, the step was confirmed by using TLC assay and the TNBS method (Fig. 1). MMP-2 treatment hydrolyzed the peptide between DOPE and PEG and resulted in the conversion of PEG-PD to NH$_2$-Ile-Ala-Gly-Gln-DOPE. The core of the
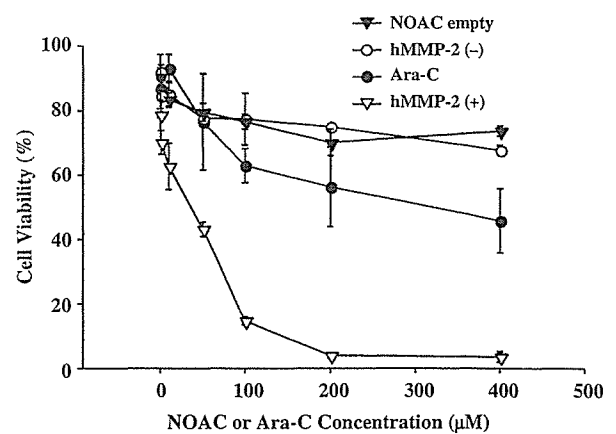


Fig. 8. Effect of NOAC concentration on the cytotoxicity of NOAC incorporated Gal-PEG-PD-liposomes in HepG2 cells. HepG2 cells were incubated with hMMP-2 treated NOAC incorporated Gal-PEG-PD-liposomes (▽), no treated NOAC incorporated Gal-PEG-PD-liposomes (O), Ara-C (●) and empty Gal-PEG-PD-liposomes (▼) at a concentration ranging from 0.1 to 400 μM NOAC. After incubation, cytotoxicity was evaluated by MTT assay. Each result represents the mean±S.D. of three experiments.

cleavage site (PLGI) is also cleaved by MMP-3, MMP-7, MMP-8, and MMP-9 [37]. The cleavage of PEG-PD by MMPs secreted by tumor cells was also confirmed. The conditioned medium of B16BL6 cells, generally known as cells over-expressing MMPs, was used for this study. It was confirmed that the conditioned medium contained MMP-2 by using gelatin zymography (Fig. 1A). The cleavage of PEG-PD was promoted time-dependently following treatment of the B16BL6 conditioned medium (Fig. 1B). The cleavage seemed to occur at the MMP-2-cleavable peptide contained in PEG-PD, since PEG-D without the peptide underwent no cleavage following treatment with the conditioned medium. Moreover, since co-incubation with enough GM6001, a known MMP inhibitor [38], led to a reduction in the cleavage, this suggests that the cleavage is due to MMPs contained in the conditioned medium (Fig. 1C).

Gal-PEG-PD-liposomes subjected to the cleavage step of PEG-PD by MMP-2 need specific uptake via the asialoglyco-protein receptors on HCC cells. The higher uptake of PEG-PD (0.1%)-Gal-liposomes incubated with hMMP-2 compared with that of the no hMMP-2 treatment group by HepG2 cells, was inhibited in the presence of 20 mM galactose, suggesting that the increase in the uptake is due to an asialoglycoprotein receptor-mediated uptake mechanism via the galactose ligands exposed on the surface of the liposomes after the cleavage of PEG-PD by hMMP-2 (Fig. 2A). The increasing uptake was not due to bridging between the liposomes and cells by hMMP-2, because the degree of uptake of Gal-liposomes without PEG-PD, incubated and not incubated with hMMP-2, was almost identical. A similar effect was confirmed using conditioned medium (Fig. 2B). The added GM6001 reduced the uptake of Gal-PEG-PD-liposomes by HepG2 cells, as the reagent was able to inhibit the cleavage of PEG-PD by MMPs.

Though the serum MMP-2 concentration of HCC patients has been reported to be about 1 μg/ml [13], it is difficult to accurately determine the local concentration of MMP-2 in tumor tissues. However, some groups have demonstrated the strong expression of MMP-2 in HCC tumor tissues [13,39], and it has reported that active form of MMP-2 is more strongly expressed by HCC than by noncancerous tissues. In addition, Murawaki et al. have reported that the elevation of serum MMP-2 concentration in the HCC patients is considered to be derived from the underlying liver cirrhosis [13]. Therefore, we considered that the intratumoral concentration of MMPs might be some times higher than the serum concentration (1 μg/ml), and the studies using the higher concentrations (5 and 10 μg/ml) were also conducted. Gal-PEG-PD (0.01 or 0.1%)-liposomes treated for 24 h by 1 μg/ml hMMP-2 exhibited a higher uptake, compared with no treatment groups (Fig. 3). On the other hand, Gal-PEG-PD (0.5%)-liposomes completely masked the galactose ligands on the surface and inhibited the uptake by HepG2 cells, and treatment for 24 h with 1 μg/ml hMMP-2 was not enough to release a significant amount of PEG-PD and lead to the subsequent uptake via exposed galactose ligands on the surface by HepG2 cells. However, treatment for 24 h with high concentrations of hMMP-2 (5 and 10 μg/ml) led to a significantly high uptake (Figs. 4 and 5). Our results led us to surmise that, in the therapy of HCC patients,

Gal-PEG-PD (0.5%)-liposomes can completely mask the galactose ligands on the surface under the circulation in the body other than MMP-2 concentrated tumor tissues, that is the condition of low MMP-2 concentration, suggesting that the non-expectant uptake by normal liver PC would be maximally reduced. The lipophilic derivatives of antitumor drugs are stably retained in liposomes. In particular, NOAC is a new lipophilic derivative able to overcome the disadvantage of rapid degradation of ara-C to the biologically inactive metabolite 1-β-D-arabinofuranosyluracil (ara-U) and it has potent antitumor activity against solid tumors [21–23]. In our studies, although Gal-PEG-PD-liposomes containing 10% NOAC had no cyto-toxic effect on HepG2 cells, the hMMP-2 pretreatment group exhibited a significant cytotoxic effect (Fig. 7). These results suggest that MMP-2 can release the PEG coating preventing the uptake of NOAC, and the subsequently exposed galactose ligand on the surface can induce the uptake in the same way as in the binding study of Gal-PEG-PD-liposomes and enhance the antitumor effect. The inhibition study in presence of galactose supports this theory. A dose-dependent cytotoxic effect was found in the hMMP-2 pretreatment group, but not in the no treatment group and for Gal-PEG-PD-liposomes without NOAC (Fig. 8). The higher cytotoxic effect of the hMMP-2 pretreatment group compared with that of ara-C in this in vitro study confirmed the report that $N^4$-alkyl-ara-C derivatives are active at lower concentrations compared with ara-C [40].

The PEGylation of liposomes leads to a reduction in the non-specific uptake by RES and subsequent prolongation of the circulation lifetime. In practical terms, our study demonstrated that 0.5% of PEG-PD, which undergoes cleavage by MMP-2, significantly reduced the uptake of Gal-liposomes by peritoneal macrophages, compared with 0% or 0.1% PEG-PD (Fig. 6A). In addition, Gal-PEG-PD (0.5%)-liposomes exhibited a higher retention of the particle size in the presence of serum (Fig. 6B). The smaller particle size (~200 nm) of liposomes reduces the unspecific uptake by RES. These results would lead to prolonged circulation and tumor-selective enhanced permeabil-ity and retention of Gal-PEG-PD (0.5%)-liposomes. Some recent studies have reported that, although PEG can act directly as a steric barrier in the interaction of the liposomes with macrophages and offer a prolonged lifetime, the reduction effect against adsorption of plasma proteins could not be obtained by modification [41,42]. Those studies support our strategy that secreted MMP-2 around HCC tissues can recognize the cleavable side of the novel PEGylated liposomes obtaining prolonged circulation and release the PEG coating.

The successful transfer of Gal-PEG-PD-liposomes into HepG2 cells by MMP-2 treatment is likely to be very useful for the delivery of antitumor drugs to HCC tissues. On the other hand, it has been reported the overexpression of MMP-9 in addition to MMP-2 in HCC tissues [43]. Since the conjugated peptide could be also cleaved by MMP-9 [18], Gal-PEG-PD-liposome is capable of HCC-selective drug delivery via the PEG-PD cleavage not only by MMP-2, but also by MMP-9. Recently, some novel MMP-2 substrates have been investigated using peptide libraries and peptide phage libraries [32,44]. In our strategy, the more specific substrates for MMP-2 are capable

of enhancing the efficiency of drug delivery to tumor tissues. Moreover, many reports have described the successful development of drug delivery systems using specific ligands for the receptors overexpressed by some tumor types, such as folate and transferrin [45,46]. These may represent further applications for our strategy.

## Acknowledgments

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