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 (S.K., Y. I., P.C., F.Y., M.H.)
a) Running title:

Proinflammatory cytokine production induced by PEI polyplex

b) Address correspondence to:

Mitsuru Hashida, Ph.D., Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshidashimoadachi-cho, Sakyoku, Kyoto 606-8501, Japan. E-mail: hashidam@pharm.kyoto-u.ac.jp

TEL: +81-75-753-4525, FAX: +81-75-753-4575

c) Number of:

- text pages; 38
- tables; 0
- figures; 9
- references; 40
- words in the Abstract; 187
- words in the Introduction; 475
words in the Discussion; 1601

d) Abbreviations:

PEI, polyethylenimine; DOTMA, N-[1-(2, 3-dioleyloxy) propyl]- n, n, n -trimethylammonium chloride; N/P ratio, the ratio of PEI nitrogen and DNA phosphate; pDNA, plasmid DNA; polyplex, PEI/pDNA complex; lipoplex, cationic liposome/pDNA complex; TNF-α, tumor necrosis factor-α; IFN-γ interferon-γ; IL-12, interleukin-12; NF-κB, nuclear factor-κB; ALT, alanine amino transferase

e) A recommended section assignment:

Chemotherapy, Antibiotics, & Gene Therapy
ABSTRACT

The purpose of this study was to evaluate the cytokine response induced by linear and branched polyethylenimine (PEI)/pDNA complex (polyplex) in relation to i) the N/P ratio of the polyplex, ii) dose of pDNA, and iii) structure and molecular weight of PEI, which are important for transfection efficacy of PEI polyplex. As a control, a DOTMA/cholesterol liposome/pDNA complex (lipoplex) was selected for its high transfection efficacy in vivo. The concentration of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) 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-κB (NF-κB), which contributes substantial the production of cytokines was comparable to the control (no treatment) level, and significantly less than that obtained with lipoplex. Although the production of proinflammatory cytokines (TNF-α, IFN-γ, and IL-12) was reduced on the administration of the liner PEI polyplex, serum ALT levels were significantly enhanced by pDNA in a dose-dependent manner, suggesting that such hepatic damage is not induced by proinflammatory cytokines.
INTRODUCTION

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, non-viral vectors have increasingly been receiving attention (Yang et al., 1994; Knowles et al., 1995). Of the various types of non-viral 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 intravenous administration. The factors that enhance the transfection efficacy have been well studied, but it is also important to analyze the side-effects of non-viral vectors for clinical applications.

As for the cationic liposome/pDNA complex (lipoplex), side-effects have been documented. The CpG motifs in the pDNA sequence upregulate the expression of transcription factors such as nuclear factor-κB (NF-κB), which contributes substantially to the production of cytokines (Krieg et al., 1995; David et al., 2000; Klinman et al., 2004). Consequently, lipoplex could induce the production of large quantities of proinflammatory...
cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-12 (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; Tousignant et al., 2003). In addition, these cytokines cause gene inactivation inducing transient gene expression following 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 (polyplex) has been determined based on i) the N/P ratio of PEI polyplex, ii) the dose of pDNA, and iii) the structure and molecular weight of PEI (Goula et al., 1998; Bragonzi et al., 1999; 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 intravenous 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 following the intravenous administration of linear and branched PEI polyplexes were evaluated in relation to i) the N/P ratio of PEI polyplex, ii) the dose of
pDNA, and iii) 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, USA). Cholesterol and Clear-sol I were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was purchased from PerkinElmer, Inc. (Wellesley, MA, USA). [α-32P]dCTP (3000 Ci/mmol) was obtained from Amersham Biosciences Co. (Piscataway, NJ, USA). 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 Hind III/Xba I
Firefly luciferase cDNA fragment from a pGL3-control vector (Promega Co., Madison, WI, USA) into the poly linker of a pcDNA3 vector (Invitrogen, Co., Carlsbad, CA, USA). pDNA was amplified in the E.coli strain DH5α, 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 \([\alpha^{-32}\text{P} ]\text{dCTP} \) by nick translation.

**Preparation of PEI Polyplex.** PEI polyplex was prepared as reported (Morimoto et al., 2003). Briefly, 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 (Goura 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; Kawakami et al., 2000b; Sakurai et al., 2001). Briefly, 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 intravenous administration. After 30 min, the zeta potential and size of PEI polyplex were measured using Nano ZS (Malvern Instruments, Ltd., Malvern, WR, UK).

Gene Expression Experiments. Gene expression was measured as described previously (Liu et al., 1997; Kawakami et al., 2000a; Kawakami et al., 2000b). Mice were administered intravenously with 300 µl 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 µl/mg for lung and 5 µl/mg for liver. In order to lyse cell, the homogenates were treated with 3 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 µl of luciferase assay buffer (Picagene, Toyo Ink Mfg. Co. Ltd., Tokyo, Japan), using a luminometer (Lumat LB 9507; Berthold Technologies GmbH & 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 intravenous administration of PEI polyplex and lipoplex, 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 (ELISA) kits according to the manufacturer’s (Genzyme Co., Cambridge, MA, USA) instructions. The serum ALT concentration was measured with kits using the UV-Rate method according to the manufacturer’s (Wako Pure Chemical Industries, Ltd., Osaka, Japan) direction.
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, USA). To analyze the extract, protein concentrations were prepared at 0.25 µg/µl. The amount of activated NFκB was measured by using an Enzyme Immunoassay for NFκB (human, mouse and rat) (Oxford Biomedical Research, Inc., Oxford, MI, USA).

Experiments on In Vivo Distribution. Radioactivity was measured as reported previously (Kawakami et al., 2000a). Mice received an intravenous injection of 10kBq [32P]pDNA and pDNA (30 µg) in a complex with linear PEI25 or cationic liposomes in 5% dextrose (300 µl) 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 - 30 mg of each tissue were digested with 700 µl of Soluene-350 by incubation overnight at 45 °C. Following the digestion, 200 µl of isopropanol, 200 µl of 30% hydrogen peroxide, 100 µl 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

Zeta Potential and Particle Size. Fig. 1(A) shows the zeta potential of the linear and branched PEI with various molecular weights and N/P ratios. PEI polyplex was negatively charged at a 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 (about 30 - 40 mV). Increasing the N/P ratio between 5 and 25 hardly changed the zeta potential of PEI polyplex (about 30 - 50 mV). As for the size of particles, it was about 400 nm at a N/P ratio of 3, except for the branched PEI$_{10}$ polyplex (Fig. 1(B)). At an N/P ratio of from 5 to 25, the size of all PEI polyplexes appeared to be reduced to about 80 - 90 nm and this value was constant as the N/P ratio increased. In the present study, the zeta potential and particle size of lipoplex (charge ratio (-:+) 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 intravenous administration of PEI polyplex in mice, the gene expression was much stronger in lung than liver, heart, spleen, or kidney (data not shown). Fig. 2 shows the gene expression in lung and liver after the intravenous 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 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. 2(D)). 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 intravenous 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 a N/P ratio of up to 15 as of 3 h after the injection. After the intravenous injection of PEI polyplex, however, the TNF-α concentration was compatible with the control value (no treatment) and was significantly lower than that following 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 a N/P ratio of 20, the efficacy of transfection increased without lethal toxicity. Accordingly, a linear PEI polyplex with a 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. 4(A)). 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. 4(B)). In order 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 following 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. 6(A)). Increasing the dose of pDNA (30, 50 and 80 µg) in the linear PEI polyplex also increased the serum ALT level (Fig. 6 (B)). When we checked the liver surface after abdominal operation, the damage of hepatic lobule was observed after mice administered the linear PEI25 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 intravenous 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). [$^{32}$P] Linear PEI polyplex mostly accumulated in the liver after the intravenous
administration, whereas $[^{32}\text{P}]$ lipoplex accumulated in the lung. Blood concentration profiles of $[^{32}\text{P}]$ linear PEI polyplex and $[^{32}\text{P}]$ lipoplex did not differ.

**Gene Expression Characteristics of Linear PEI Polyplex.** Previous studies suggested that proinflammatory cytokines cause gene inactivation such as transient gene expression 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 intravenous administration of the polyplex were evaluated based on i) the N/P ratio of PEI polyplex, ii) the dose of pDNA, and iii) the structure and molecular weight of PEI. After the intravenous 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 PEI (Fig. 2) as well as the dose of pDNA (Fig. 4 (A)). These observations are consistent with previous reports (Goula et al. 1998; Bragonzi et al. 1999; Zou et al., 2001; Wightman 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. (1999) who found that the gene expression efficacy of linear PEI polyplex was equal to lipoplex under optimal conditions.

Then, cytokine response characteristics after the intravenous administration of PEI
polyplex were evaluated based on i) the N/P ratio of PEI polyplex, ii) the dose of pDNA, and iii) 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. 4 (B)), although these factors affected the transfection efficacy in vivo (Fig. 2 and Fig. 4 (A)). 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 and pDNA dose: 25 µg/mouse) than those given lipoplex (Tan et al., 2001). In order 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 liner 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 (Fig. 2 and 4 (A)).

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 to the control level (un-treated 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 intravenous 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. Similarly, 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 polypeptide is due to its capacity to buffer the endosome (proton sponge effect) (Bousseif et al., 1995; Kichler et al., 2001; Akinc et al., 2005); therefore, such a property might abolish the pDNA (CpG DNA)-induced activation of NFκB (Fig. 7).

We previously investigated the distribution of lipoplex and demonstrated that when intravenously 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 polypeptide is less than that of lipoplex. However, the biodistribution study demonstrated that much more [32P] linear PEI polypeptide than [32P] lipoplex accumulates in the liver (Fig. 8). These results suggested that the difference in the response induced by PEI polypeptide 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 is consider that the hemagglutination is caused by electrostatic interaction between the erythrocytes and lipoplex. In the distribution study, the zeta potential of PEI polypeptide
(about 40 mV) (Fig. 1) was lower than that of lipoplex (about 60 mV), suggesting 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 zeta potential characteristics may reflect a more hepatic-selective distribution of the linear PEI polyplex (Fig. 8).

In order to evaluate hepatic damage, serum ALT activity was measured. As shown in Fig. 6 (A), 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 intravenously. In this study, we demonstrated that PEI polyplex hardly induced the production of any proinflammatory cytokines (Fig. 3, 4, and 5), but hepatic toxicity was observed (Fig. 6(B)). 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. 6(B), serum ALT activity at 30 µg of pDNA was compatible with the control (no treatment). However, at higher doses (50 and 80 µg), the serum ALT level increased, suggesting that intravenously 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 intravenous administration of a linear polyplex containing 100 µg of pDNA.

As shown in Fig. 6, lipoplex at pDNA dose of 80 µg was lethal. This observation was corresponding with Hofland et al. (1997) that reported lipoplex at the pDNA dose of approximately 80 µg was the maximal tolerated dose in mice. It is expected that the hepatic toxicity between lipoplex and polyplex at pDNA dose of 80 µg is same extent since hepatic toxicity was nearly similar between lipoplex and PEI polyplex at pDNA dose of 30 and 50 µg (Fig. 6). Therefore, the lethal effect of lipoplex at pDNA dose of 80 µg 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 PEI25 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\textsubscript{10}, PEI\textsubscript{25}, and PEI\textsubscript{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 \textit{in vivo}. We demonstrated that the amount of NF\(\kappa\)B activated by the linear PEI polyplex was comparable to the control (un-treated 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 non-viral vectors for clinical applications.
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*Proc Natl Acad Sci USA* **91**: 4407-4411.


Footnotes:

This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by Health and Labour Sciences Research Grants for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan.
Legends for Figures

Fig. 1. Zeta potential (A) and particle size (B) of PEI polyplex at various N/P ratios. Linear PEI\textsubscript{25} polyplex (open triangles), branched PEI\textsubscript{10} polyplex (filled squares), branched PEI\textsubscript{25} polyplex (filled triangles) and branched PEI\textsubscript{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.

Fig. 2. Effect of N/P ratio of PEI polyplex and molecular weight of PEI on gene expression after the intravenous administration of branched and linear PEI polyplexes delivering 30 μg of pDNA per mouse. Branched PEI\textsubscript{10} (A), PEI\textsubscript{25} (B), and PEI\textsubscript{70} (C), and linear PEI\textsubscript{25} (D) were used to produce polyplexes with a N/P ratio of 5, 7, 10, 15, 20 and 25. The lipoplex had a charge ratio (-:+) of 1.0:3.1 and delivered 30 μg 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 (opened bars) were measured. N.E. means not evaluated. Each value represents the mean ± S.D. for at least three mice.

Fig. 3. Effect of N/P ratio of PEI polyplex and molecular weight of PEI on TNF-α release
after the intravenous administration of branched and linear PEI polyplexes delivering 30 µg of pDNA per mouse. Branched PEI\textsubscript{10} (A), PEI\textsubscript{25} (B), and PEI\textsubscript{70} (C), and linear PEI\textsubscript{25} (D) were used to produce polyplexes with a N/P ratio of 7, 10, 15, or 20 and 25. Lipoplex had a charge ratio (−:+) 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. stands for no treatment. Each value represents the mean + SD 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 intravenous administration of linear PEI polyplex delivering 30, 50, and 80 µg of pDNA per mouse. Linear PEI polyplex was prepared at a N/P ratio of 20. Lipoplex had a charge ratio (−:+) 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 + SD of at least three mice.
Fig. 5. Serum TNF-α (A), IFN-γ (B), and IL-12 (C) concentrations after the intravenous administration of linear PEI polyplex. Lipoplex (closed circles) was prepared with a charge ratio (-:+) of 1.0:3.1 and delivered 30 µg of pDNA per mouse. Linear PEI polyplex (opened circles) was prepared at a 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).

Fig. 6. Serum ALT activity after the intravenous administration of linear PEI polyplex. Lipoplexes were prepared with a charge ratio (-:+) 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. stands for not evaluated. N.T. indicates no treatment. *, Statistically significant difference from un-treated groups (P < 0.05). Each value represents the mean ± S.D. for at least three mice.
Fig. 7. Amount of activated NFκB in the liver after intravenous administration of linear PEI polyplex. Linear PEI polyplex was prepared at a pDNA dose of 30 µg per mouse. Lipoplex was prepared at charge ratio (-:+) 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. stands for no treatment. *, Statistically significant difference from the un-treated group (P < 0.05). Each value represents the mean ± S.D. for at least three mice.

Fig. 8. Biodistribution after the intravenous administration of [32P] linear PEI polyplex. Linear PEI polyplex was prepared at a pDNA dose of 30 µg per mouse. Lipoplex was prepared at charge ratio (-:+) 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 (opened 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 intravenous 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 charge ratio
(-:+) 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.
Fig. 1

(A) Zeta potential (mV)

(B) Diameter (nm)

N/P ratio
Fig. 2

(A) Branched PEI<sub>10</sub>

(B) Branched PEI<sub>25</sub>

(C) Branched PEI<sub>70</sub>

(D) Linear PEI<sub>25</sub>

Gene expression (FLU/mg protein)

Lipoplex 5 7 10 15 20 25

N/P ratio

Gene expression (FLU/mg protein)

Lipoplex 5 7 10 15 20 25

N/P ratio
Fig. 3

(A) Branched PEI_{10}

(B) Branched PEI_{25}

(C) Branched PEI_{70}

(D) Linear PEI_{25}
Fig. 4

(A) Gene expression (RLU/mg protein) for Lung and Liver with different doses of pDNA.

(B) TNF-α in serum (pg/ml) with different doses of pDNA.

Lipoplex and Linear PEI25 at various doses (30 µg, 50 µg, 80 µg) are compared.
Fig. 5

(A) TNF-α

(B) IFN-γ

(C) IL-12

Graphs showing the levels of TNF-α, IFN-γ, and IL-12 in serum over time (h).
Fig. 6
Fig. 7

![Bar chart showing NFκB levels in different conditions.](chart)

- **N.T.** (Normal Control)
- **Linear PEI<sub>25</sub> polyplex**
- **Lipoplex**

The chart illustrates a comparison of NFκB levels (ng/μg protein) across the three conditions. Significant differences are indicated by asterisks.
Fig. 8

(A) Lipoplex

(B) Linear PEI25 polyplex

Blood concentration (% of dose/ml)

Tissue accumulation (% of dose)

Time (min)

0 15 30 45