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

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Received December 26, 2005; accepted March 3, 2006

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)-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, 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 concentration of proinflammatory cytokines (TNF-α, interferon-γ, 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)-κB, 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)-α, interferon (IFN)-γ, 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...
(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 lipopipid 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 were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was purchased from PerkinElmer, Inc. (Wellesley, MA). [α-32P]dCTP (300 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, Carlsbad, CA). pDNA was amplified in the Escherichia 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 [α-32P]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 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 ζ Potential and Particle Size. PEI and pDNA were mixed in 5% dextrose as above and concentrated for i.v. administration. After 30 min, the ζ 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 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. To lyse cells, the homogenates were treated with three cycles of freezing and thawing. The homogenates were centrifuged at 12,000g for 7 min at 4°C. Twenty microliters of each supernatant was analyzed for luciferase activity with 100 μl of luciferase assay buffer (Picagen; 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,
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 (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 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).

Experiments on in Vivo Distribution. Radioactivity was measured as reported previously (Kawakami et al., 2000a). Mice received an i.v. injection of 10 kBq [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 to 30 mg of each tissue were digested with 700 μl of Soluene-350 by incubation overnight at 45 °C. After 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

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 PEI70 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 PEI10 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
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 a pDNA dose of 30 μg (Whitmore et al., 1999; Sakurai et al., 2002). Among these factors, the transfection efficiency of branched and linear PEI polyplexes delivering 30 μg of pDNA per mouse. Branched PEI10 (A), PEI25 (B), and PEI70 (C), and linear PEI25 (D) were used to produce polyplexes with an N/P ratio of 7, 10, 15, or 20 and 25, respectively. Lipoplex had a N/P 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).

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 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-
ation 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 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 N/P 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.

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 N/P ratio 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).

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.
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).

NFкB 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 NFкB in liver was measured. As shown in Fig. 7, we demonstrated that the amount of NFкB 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 NFкB 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 NFкB; 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
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 [32P] linear PEI polyplex than [32P] 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), 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 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
In conclusion, the concentration of proinflammatory cytokines (TNF-α, IFN-γ, 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.

References


Yang JP and Huang L (1997) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther 4:950–960.


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