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Suppression of TNFα production in LPS induced liver failure in mice after intravenous injection of cationic liposomes/NFκB decoy complex

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Received August 8, 2005, accepted September 6, 2005

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Pharmazie 61: 144–147 (2006)

NFκB decoy, double stranded oligonucleotides containing NFκB binding sequences, inhibits NFκB-mediated production of inflammatory cytokines, and therefore NFκB decoy has been applied to several diseases. However, naked NFκB decoy, which is quickly cleared from the circulation in mice after intravenous injection, is readily absorbed into the systemic circulation. In order to deliver enough NFκB decoy for a therapeutic effect, it is necessary to develop a carrier, which enables much more NFκB decoy to transfer to the target cells. In this study, using *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA)/cholesterol (1 : 1) liposomes, the therapeutic effect of NFκB decoy was investigated in an LPS induced acute hepatitis model mice. The mean diameter of the cationic liposomes/NFκB decoy complex was about 70.9 nm and the zeta potential of complex was about 37.4 mV. Tissue distribution was determined by measuring the radioactivity of a cationic liposomes/[^{32}P] NFκB decoy complex after intravenous injection. The cationic liposomes/[^{32}P] NFκB decoy complex was rapidly accumulated in the lung and gradually moved to the liver. The therapeutic effect was determined by the serum concentration of TNFα in LPS treated mice. The production of TNFα was significantly inhibited by cationic liposomes/NFκB decoy complex but not by cationic liposomes/random decoy complex or naked NFκB decoy. These results suggested that NFκB decoy therapy could be achieved using cationic liposomes. This information is of great value for the design of NFκB decoy carrier systems.

1. Introduction

Endotoxin syndrome is a particularly serious complication because bacteriologically proven infection occurs in up to 80% of patients with hepatic failure (Rolando et al. 1990, 2000). Binding of lipopolysaccharide (LPS) by toll-like receptor 4 expressed on macrophages causes the activation of transcriptional factor nuclear factor kappa B (NFκB), triggering the rapid release of cytokines TNFα, IL-1, IL-2, IL-6, IL-8, IL-12, and IFN-β, etc. by macrophages (Morrison et al. 1987; Heinzel, 1990; Dinarello et al. 1993; Essani et al. 1996; Pahl et al. 1999; Han et al. 2002). Therefore, prevention of NFκB activation would be a critical therapeutic goal of fetal liver injury caused by the endotoxin syndrome. Several recent reports indicate that NFκB decoy, double stranded oligonucleotides containing NFκB binding sequences, inhibits NFκB-mediated production of inflammatory cytokines (Morishita et al. 1997, Tomita et al. 2000a); therefore, NFκB decoy could be used in various diseases (Tomita et al. 2000, Yoshimura et al. 2001, Azuma et al. 2003). Considering that naked NFκB decoy is easily digested by DNase in serum and hardly taken up by cells, it is necessary to develop a non-invasive form of NFκB decoy delivery, which would be safe for repeated use and provide reproducible therapeutic effects, for wider clinical application.

In spite of the high transfection efficiency of viral vectors, safety concerns have been raised in clinical trials because of their highly toxic nature; therefore, the use of non-viral vectors has attracted great interest for *in vivo* gene delivery because they are free from some of the risks inherent in these systems (Mahato et al. 1997; Kawakami et al. 2002; Hashida et al. 2005). We previously reported that rapid clearance of pDNA from the circulation was observed with extensive accumulation in the lung and liver after intravenous injection of pDNA complexed with cationic liposomes (Mahato et al. 1995a, 1995b). As regards intrahepatic distribution, pDNA complexed with cationic liposomes was predominantly taken up by liver non-parenchymal cell (NPC), composed of Kupffer cells, sinusoidal endothelial cells etc., via a phagocytic process. NFκB decoy is a double stranded oligonucleotide which could interact with cationic liposomes. Because NPC is a main target for NFκB decoy inhibition of NFκB-mediated production of inflammatory cytokines, it would be expected that NFκB decoy delivery to liver NPC by cationic liposomes would efficiently suppress cytokine production by the prevention of NFκB activation. However, little information is available on the use of cationic liposomes/NFκB decoy complex against inflammatory disease after intravenous administration.

In this study, the physicochemical properties and distribution characteristics of a NFκB decoy/cationic liposomes complex were evaluated. In mice with LPS induced hepatitis, TNFα concentration was measured to evaluate the prevention of NFκB activation after intravenous administration of NFκB decoy/cationic liposomes complex. *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA)/cholesterol (1 : 1) liposomes were selected as the cationic liposomes because of the many reports about their use for *in vivo* pDNA delivery with high transfection efficacy (Song et al. 1997; Kawakami et al. 2000, 2001). Results were compared with naked NFκB decoy.

2. Investigations, results and discussion

The table summarizes data on mean diameter and zeta potential of cationic liposomes and the cationic liposomes/NFκB decoy complex. It has been reported that mixing complexes at low ion strength prevents aggregation, although large complexes resulting from aggregation showed high transfection efficiency *in vitro* in the case of DNA/transferring-PEI complexes (Ogris et al. 1998). Having regard to this report, the cationic liposomes/NFκB decoy complexes were prepared with 5% dextrose solution. The mean diameter of the cationic liposomes or cationic liposomes/NFκB decoy complex was about 73.8 and 70.9 nm, respectively. There was no significant difference in the mean diameter of empty liposomes and complex.

The zeta potential of cationic liposomes or cationic liposomes/NFκB decoy complex was about 56.7 and 37.4 mV, respectively. The zeta potential of the cationic liposomes/NFκB decoy complex was slightly smaller than that of empty liposomes. This result suggested that NFκB decoy would form a complex with cationic liposomes because attaching NFκB decoy with anionic charge to cationic liposomes would decrease the zeta potential of the cationic liposomes.

The liver and lung accumulation of the cationic liposomes/[^{32}P] NFκB decoy complex was compared with that of naked [^{32}P] NFκB decoy (Fig. 1). Both liver and lung accumulation of the cationic liposomes/[^{32}P] NFκB decoy complex were significantly higher than for naked [^{32}P] NFκB decoy at 1, 10, and 60 min after injection. Focusing on the cationic liposomes/[^{32}P] NFκB decoy complex, the complex accumulated initially in the lung at 1 min after injection, then moved gradually to the liver. At 60 min, 60% of the dose of the cationic liposomes/[^{32}P] NFκB decoy complex had accumulated in the liver (Fig. 1B). In our previous study, we demonstrated that [^{32}P] pDNA complexed with DOTMA/cholesterol liposomes rapidly accumulated in the lung and gradually accumulated in the liver (Mahato et al. 1995a, 1995b). Our findings concur that distribution of the cationic liposomes/NFκB decoy complex agrees with the distribution of the cationic liposomes/pDNA complex.

In order to determine the therapeutic effect of NFκB decoy on acute hepatitis *in vivo*, the serum concentration of TNFα in LPS treated mice was measured after intravenous injection of NFκB decoy (Fig. 2). Cationic liposomes/NFκB decoy complex suppressed the increase of TNFα in the serum, although naked NFκB decoy did not show an inhibitory effect on the production of TNFα. In endotoxin-induced liver failure, it is known that Kupffer cells, resident macrophages in the liver, play a major role in producing inflammatory cytokines (Arai et al. 1993; Iimuro et al. 1994; Mochida et al. 1996). Considering that the liver accumulation of the cationic liposomes/NFκB decoy complex was significantly higher than that of naked NFκB decoy (Fig. 1), this raises the possibility that some part of the NFκB decoy taken up by Kupffer cell might suppress NFκB mediated TNFα production induced by LPS.

To examine whether the inhibitory effect of NFκB decoy depended on the sequence of NFκB binding site, a similar size of double stranded oligonucleotide without NFκB binding site and also without any effective sequence, which was called random decoy, was designed and the inhibitory effect of its complex with cationic liposomes on TNFα production investigated. As shown in Fig. 2, the cationic liposomes/random decoy complex had no effect on TNFα production; therefore, the inhibitory effect on TNFα production was observed to depend on the sequence of NFκB decoy.

To determine the effect of dose of NFκB decoy, the inhibitory effect on LPS induced TNFα production in serum was investigated at different doses of NFκB decoy com-

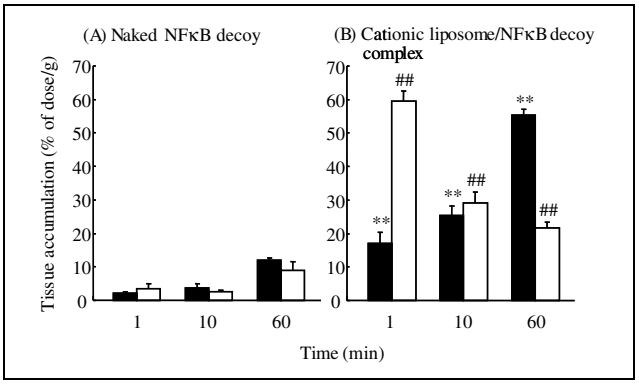

Fig. 1: Tissue accumulation of naked [^{32}P] NFκB decoy (A) or cationic liposomes/[^{32}P] NFκB decoy complex (B) after intravenous injection into mice. Radioactivity was determined in liver (■) and lung (□). Each value represents the mean + S.D. (n = 3). Significant difference ** $P < 0.01$ vs. liver accumulation of naked NFκB decoy and ## $P < 0.01$ vs. lung accumulation of naked NFκB decoy

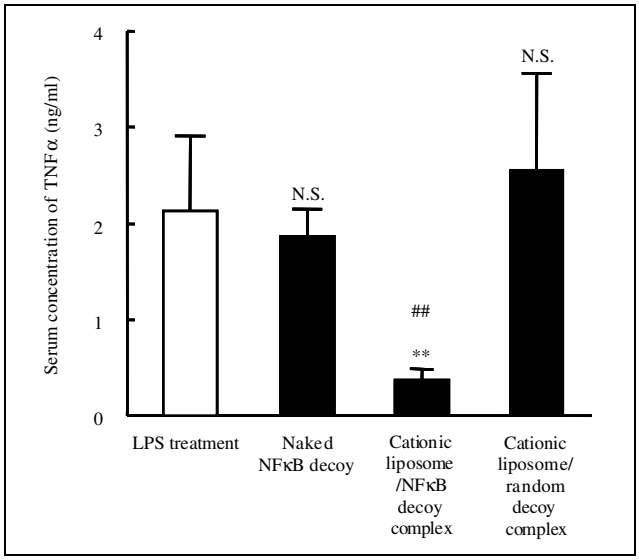

Fig. 2: Effect of complex formation and sequence of NFκB decoy on prevention of the TNFα production after intravenous injection of naked NFκB decoy, cationic liposomes/NFκB decoy complex or cationic liposomes/random decoy complex into mice. Each value represents the mean + S.D. (n = 3). Significant difference ** $P < 0.01$ vs. naked NFκB decoy, ## $P < 0.01$ vs. random decoy complex and N.S. not significant

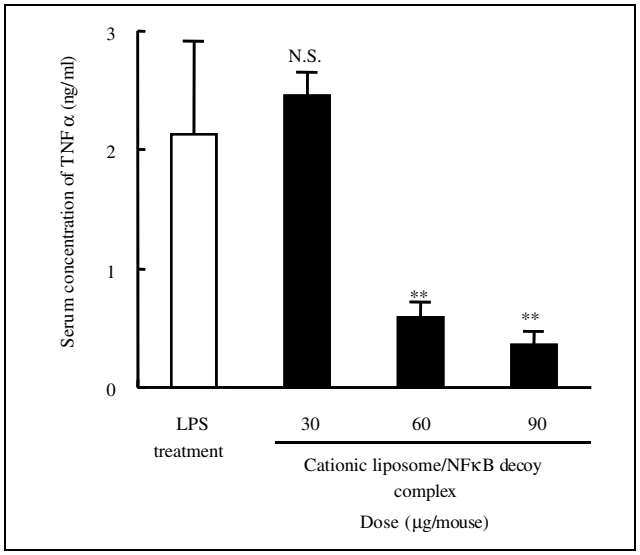

Fig. 3: Effect of dose on the prevention of TNFα production after intravenous injection of cationic liposomes/NFκB decoy complex into mice. Each value represents the mean + S.D. (n = 3). Significant difference ** $P < 0.01$ vs LPS treatment and N.S. not significant

plexed with cationic liposomes (Fig. 3). At doses of 60 and 90 μg/mouse, the production of TNFα was significantly inhibited but not at 30 μg/mouse.

To date, several kinds of non-viral carriers have been applied to *in vivo* gene delivery. As far as NFκB decoy delivery targeting to the liver is concerned, Ogushi et al. (2003) have also demonstrated the inhibitory effect of NFκB decoy on the production of cytokines using HVJ liposomes given by intraportal injection. HVJ liposomes are well known as a gene carrier, which could rapidly fuse to cells and transfer the incorporated gene to the cells. However, HVJ liposomes are not suitable for intravenous injection because intravenously injected HVJ liposomes accumulate in the lung, spleen, kidney and liver; therefore, several researchers have reported that they could not achieve enough therapeutic effect or transfection efficiency (Hirano et al. 1998; Morishita et al. 2000; Yoshida et al. 2002; Ogushi et al. 2003). Conversely, we report here that intravenously injected cationic liposomes/NFκB decoy complex accumulates in the liver (Fig. 1).

Cationic liposome-based systemic pDNA delivery has been reported as a novel technology, however the uptake of cationic liposomes/pDNA by Kupffer cells triggers production of inflammatory cytokines and disturbs gene transfer efficiency, which is largely due to unmethylated CpG motif in the pDNA (Sakurai et al. 2002). Recently, Tan et al. have reported that NFκB decoy could inhibit TNFα induction by a cationic liposomes (DOTAP/cholesterol, 1 : 1)/pDNA complex, which was prepared by mixing liposome solution and DNA solution containing pDNA and NFκB decoy (Tan et al. 2002). In this study, we demonstrate that LPS induced TNFa production could be inhibited by cationic liposomes/NFκB decoy complex (Fig. 2). This result leads us to believe that the cationic liposomes/NFκB decoy complex in liver is mainly distributed in Kupffer cells after intravenous injection.

In this study, we demonstrate that intravenously injected cationic liposomes/NFκB decoy complex effectively inhibits TNFα production in LPS induced acute hepatitis in mice. This result will give useful information on NFκB decoy therapy using cationic liposomes. With more study and development of cellular targeting with cationic liposomes, it will be possible to establish NFκB therapy by intravenous injection.

Table: Mean particle sizes and zeta potentials of cationic liposomes and cationic liposomes/NFκB decoy complexes

	Particle size (nm)	Zeta potential (mV)
Cationic liposome	73.8 ± 5.46	56.7 ± 1.53
Cationic liposome/NFκB decoy complex	70.9 ± 1.07	37.4 ± 2.84

Results are expressed as the mean ± SD of three experiments

3. Experimental

3.1. Materials

NFκB decoy and control oligonucleotides used in this study are phosphodiester double stranded oligonucleotides. Their sequences are as follows: 20 mer NFκB decoy 5′-AGTTGAGGGGACTTTCCCAGGC-3′ 5′-TCAACTCCCCTGAAAGGGTCCG-3′ (B), control oligonucleotides 5′-TTGCCGTACCTGACTTAGCC-3′ 5′-AACGGCATGGACTGAATCGG-5′. These oligonucleotides were purchased from Operon Biotechnologies, Inc. (Tokyo, Japan). DOTMA was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cholesterol, Clear-Sol I and Soluen 350 were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Lipopolysaccharide (LPS) from Salmonella Minnesota Re 595 (Re mutant) was purchased from Sigma Chemicals Inc. (St. Louis, MO, USA). [γ-^{32}P] ATP and NAP 5^{TM} columns were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA). MEGALABELTM 5′-End Labeling Kit was purchased from Takara Bio Inc. (Shiga, Japan). OptiEIA^{TM} enzyme-linked immuno-sorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA). Enzyme Immunoassay for NFκB Product No.TF 01 was purchased from Oxford Biomedical Research, Inc. (Oxford, MI, USA). All other chemicals were of the highest purity available.

3.2. Preparation of liposomes

DOTMA was mixed with cholesterol, each in chloroform at a molar ratio of 1 : 1 and the mixture was dried, vacuum desiccated, and resuspended in sterile 5% dextrose. After hydration, the dispersion was sonicated for 10 min in a bath sonicator and then for 3 min in a tip sonicator to form liposomes. The preparation method for liposomes/NFκB decoy complexes for *in vivo* use has been reported previously (Kawakami et al., 2000b, 2004). Briefly, equal volumes of NFκB decoy and stock liposome solution diluted with 5% dextrose to produce various ratios of liposomes/NFκB decoy were mixed in 1.5 ml tubes at room temperature. Then, the NFκB decoy solution was added rapidly to the liposomes solution using a Pipetman pipet and the mixture was agitated rapidly by pumping it up and down twice in the pipet tip. The mixing ratio of liposomes and NFκB decoy was expressed as a +/− charge ratio, which is the molar ratio of cationic lipids to NFκB decoy phosphate residue (Huang et al. 1997).

3.3. Measurement of particle size and zeta potentials

The mean diameters of cationic liposomes or cationic liposomes/NFκB decoy complex were measured by dynamic light-scattering spectrophotometric methods using an LS-900 (Otsuka Electronics, Osaka, Japan). The zeta potentials of cationic liposomes or cationic liposomes/NFκB decoy complex were measured by laser-Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK).

3.4. Radiophosphorylation of decoy oligonucleotides

Oligonucleotides were labeled with [γ-^{32}P] ATP using a MEGALABELTM 5′-End Labeling Kit. Briefly, oligonucleotides, [γ-^{32}P] ATP and T4 polynucleotide kinase were mixed in phosphorylation buffer. After 30 min incubation at 37 °C, the mixture was incubated for 10 min at 70 °C in order to inactivate T4 polynucleotide kinase. Then, the mixture was purified by gel chromatography using a NAP 5^{TM} column and eluted with 10 mM Tris-Cl and 1 mM EDTA (pH 8.0). The fractions containing derivatives were selected on the basis of their radioactivity.

3.5. In vivo distribution

[^{32}P] NFκB (20 μg) decoy complexed with liposomes in 300 μl of 5% dextrose solution was injected intravenously to female five-week-old ICR mice (19–22 g). The mice were killed at 1, 10 and 60 min. Liver and lung were removed, washed with saline, blotted dry, and weighed. A small amount of each tissue was digested with 0.7 ml of Soluene-350 by incuba-

tion overnight at 54 °C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5 N HCl, and 5.0 ml Clear-Sol I were added. The samples were stored overnight, and radioactivity was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan).

3.6. Cytokine secretion

Mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University. The indicated amount of NFκB decoy or random decoy complexed with liposomes or the indicated amount of naked NFκB decoy in 300 µl of 5% dextrose solution was intravenously injected to the mice. Blood was collected from the mice 1 h after intravenous injection of LPS. The blood was allowed to coagulate for 3 h at 4 °C and serum was isolated as the supernatant fraction following centrifugation at 17000 × g for 20 min. The serum samples were immediately stored at −80 °C. The amounts of TNFα were analyzed using an OptiEIA™ ELISA kit according to the manufacturer's protocol.

3.7. Statistical analysis

Statistical comparisons were performed by Student's t test for two groups, one-way ANOVA for multiple groups, and Scheffe's post hoc test after ANOVA.

Acknowledgement: This work was supported in part by Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by Health and Labor Sciences Research Grants for Research on Advanced Medical Technology from the Ministry of Health, Labor and Welfare of Japan.

This research paper was presented during the 5th Conference on Retrometabolism-Based Drug Design and Targeting, May 8–11, 2005, Hakone, Japan.

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