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NFκB decoy delivery using dendritic poly(L-lysine) for treatment of endotoxin-induced hepatitis in mice

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ABSTRACT

Mono-dispersed, 6th generation dendritic poly(L-lysine) (KG6) forms a stable complex with plasmid DNA and this complex can circulate in vivo for extended times before the DNA finally accumulates in the liver. In this study, we attempted to use KG6 as a carrier of NFκB decoy oligonucleotide to the liver to treat hepatitis, induced by lipopolysaccharide and D-galactosamine. KG6 formed a complex with the NFκB decoy. Serum aspartate aminotransferase and alanine aminotransferase were dramatically suppressed in the hepatitis mouse model after intravenous injection of KG6/NFκB decoy complexes. Expression levels of several cytokines and proteins related to the inflammatory reaction were also suppressed by intravenous administration of KG6/NFκB decoy complexes. Because [^{32}P] NFκB decoy was found in non-parenchymal cells after intravenous injection, KG6 has been shown to be a promising carrier molecule of various oligonucleotides to non-parenchymal liver cells, including Kupffer cells.

1. Introduction

Oligonucleotide-based therapeutic techniques have attracted much attention because they provide a rational way to design antisense oligonucleotides, decoy oligonucleotides that bind to DNA-binding regulatory proteins and siRNA/miRNA that suppress specific gene expressions. The development of efficient delivery systems is necessary to establish oligonucleotide therapies. In particular, when oligonucleotides are administered systemically, their stability in circulation, targeted delivery to specific sites and efficient cellular uptake are key issues for successful oligonucleotide therapies. Many studies into efficient oligonucleotide delivery systems have been reported.[1–7]

Previously, we reported that mono-dispersed, 6th generation dendritic poly(L-lysine) (KG6) formed complexes with plasmid DNA and showed high transfection efficiency into several cultivated cells, with low cytotoxicity.[8,9] The transfection efficiency was not significantly reduced even when 50% serum was added to the transfection medium. The zeta potential of DNA complexes with KG6 remained neutral even when the charge ratio (+/−) was increased to 8, indicating their stability in the presence of serum at high concentration. KG6 also enabled efficient delivery of siRNA into rat hepatoma cells and reduced the gene expression for gluconeogenesis.[10] In addition, the complex could circulate in vivo for extended times, for at least 3 h after intravenous injection into mice, before the DNA finally accumulated in the liver.[11]

We have therefore attempted to use dendritic poly(L-lysine), which delivers nucleic acids to the liver, as a carrier for delivery of oligonucleotides, in particular decoy oligonucleotides, to liver cells. We focused on lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced hepatitis as a model disease.[12] NFκB decoy oligonucleotide (NFκB decoy) is known as an inhibitor of the inflammatory reactions that produce cytokines, such as TNF-α, IL-1β, IFNγ and IL-12 that are induced by activators such as LPS.[13–18] NFκB decoy binds to activated NFκB and suppresses the protein expressions that take part in the inflammatory reaction under the control of the NFκB promoter. Therefore, NFκB decoy is expected to act as a drug for hepatitis.[19] In this study, we examined the ability of KG6 to act as a carrier of NFκB decoy to the liver for treatment of LPS/D-GalN-induced hepatitis in mice.

Abbreviations: AST, aminotransferase; ALT, alanine aminotransferase; D-GalN, D-galactosamine; ELISA, enzyme-linked immunosorbent assay; iNOS, inducible nitric oxide synthase; KG6, dendritic poly(L-lysine) of the 6th generation; LPS, lipopolysaccharide; PEI, polyethyleneimine; PLL, poly(L-lysine); qRT-PCR, quantitative real-time PCR.

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2. Results and discussion

2.1. Establishment of LPS/D-GalN-induced hepatitis in model mice

Induction of hepatitis in ddY mice was confirmed by measuring serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as an index of liver damage.[20,21] Their activities increased to 7000 and 10,000 IU/L, respectively, at 7 h after intraperitoneal administration of LPS/D-GalN (Fig. 1). At 9 h after the injection, their levels were slightly decreased. We employed data at 7 h after the injection to measure the anti-hepatitis activity of the decoy oligonucleotide.

2.2. Formation of KG6/NFκB decoy complexes and suppression of serum AST and ALT activities after intravenous injection of the complexes

Formation of KG6/NFκB decoy complexes was examined by their electrophoretic mobility on a polyacrylamide gel at various C/A (cations of KG6/anions of the decoy) ratios (Fig. 2A). KG6 completely inhibited the migration of the decoy oligonucleotide on the agarose gel at a C/A ratio of 2.0. When plasmid DNA was used instead of the decoy oligonucleotide, the migration was inhibited at a C/A ratio of 1.0,[9] indicating that the binding ability of KG6 with oligonucleotide was weaker than that with plasmid DNA, as previously reported by Keller's group.[22,23]

We examined suppression of serum AST and ALT activities after intravenous injection of KG6/NFκB decoy complexes. The KG6/NFκB decoy complexes at various C/A ratios were injected 1 min after intraperitoneal injection of LPS/D-GalN. At C/A ratios of 2.0 or more, where complexes were completely formed, there was significant suppression of the serum AST and ALT activities (Fig. 2B). The suppression reached a maximum at a C/A ratio of 8.0. At this C/A ratio, the size of the complex was 164 ± 3 nm and its zeta-potential was +40.8 ± 0.6 mV. Clearly, the size and positive surface charge of the complexes were advantageous to the uptake by liver cells, especially macrophages, that is, Kupffer cells that participate in the inflammatory reaction in the liver.[24] On the contrary, at a C/A ratio of 16, the suppression was weakened. At such a high C/A ratio, the excess amount of KG6 would interfere with uptake of the complex by liver cells. Although KG6/scrambled oligonucleotide complexes slightly reduced serum AST and ALT activities, their residual activities were significantly higher than in the case of KG6/NFκB decoy complexes. The reduction observed for the case

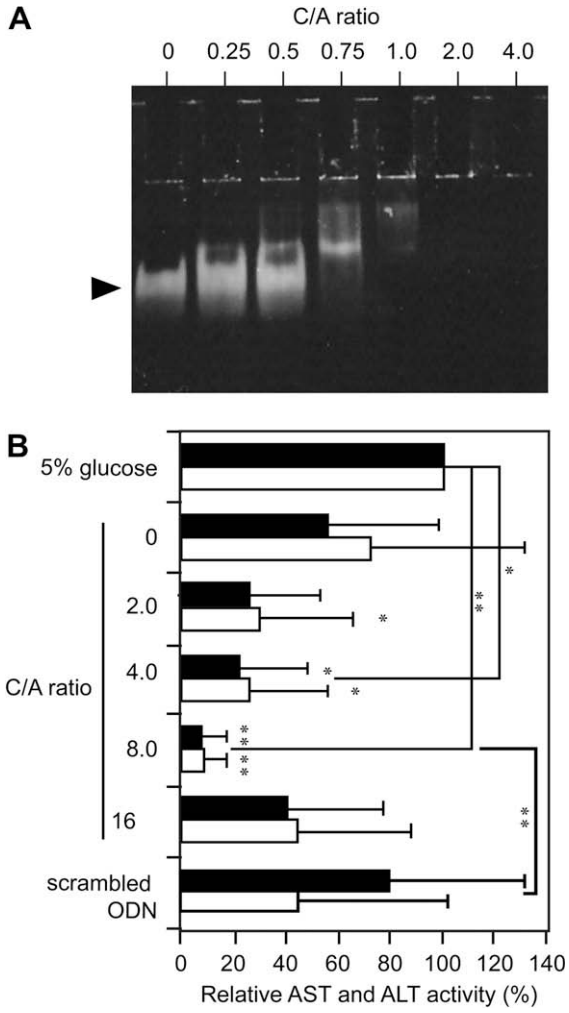

Figure 2. Effect of C/A ratio on binding ability of KG6 with the decoy (A) and suppression of the serum AST and ALT activities in vivo (B). (A) NFκB decoy (100 ng) and KG6 at various C/A ratios were mixed, then the complexes were applied to polyacrylamide gel. After electrophoresis, the gel was stained with SYBR Gold. The arrowhead on the left of the gel indicates the position of the naked NFκB decoy. (B) At 1 min after intraperitoneal injection of LPS/D-GalN, KG6/NFκB decoy complexes at the various C/A ratios were intravenously injected. A scramble oligonucleotide (ODN) complex with a C/A ratio of 8.0 was also injected. After 7 h, the serum AST and ALT activities were measured. *Closed bars* and *open bars* indicate AST and ALT activities, respectively. Each value represents the mean ± SD ($n > 3$). Significance of differences from 5% glucose are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

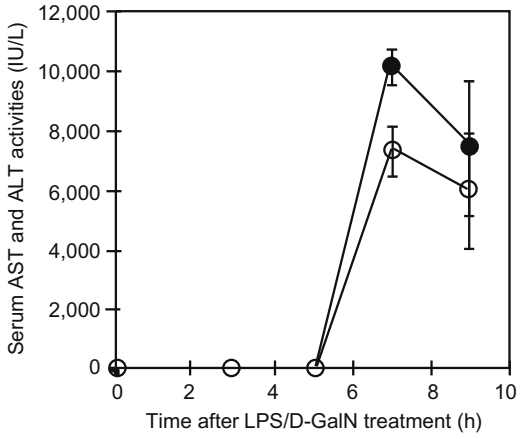

Figure 1. Serum AST and ALT activities at various times after LPS/D-GalN treatment. *Closed circles* and *open circles* indicate AST and ALT activities, respectively. Each value represents the mean ± SD ($n > 3$).

of KG6/scrambled oligonucleotide complexes would be due to a non-specific effect by the phosphorothioate oligonucleotides used in this study. However, it can be concluded that the suppressions of AST and ALT activities were sequence specific.

Next, we examined the dose dependency of the suppression effect by KG6/NFκB decoy complexes on the inflammatory reaction (Fig. 3). At a C/A ratio of 8.0, the serum AST and ALT activities were decreased with increasing doses of NFκB decoy, reaching about 5% of the original activity of AST and ALT.

2.3. Comparison of KG6 with other polymeric carrier molecules

The conventional polymeric carrier molecules, linear poly(L-lysine) (PLL) (Mw 15,000–30,000) and polyethyleneimine (PEI) (in vivo JetPEI purchased from Polyplus-transfection SA, Illkirch, France) were compared with KG6 (Fig. 4). Linear PLL showed a moderate suppression effect on the serum AST and ALT activities. In the case of PEI, data varied widely compared with the cases of

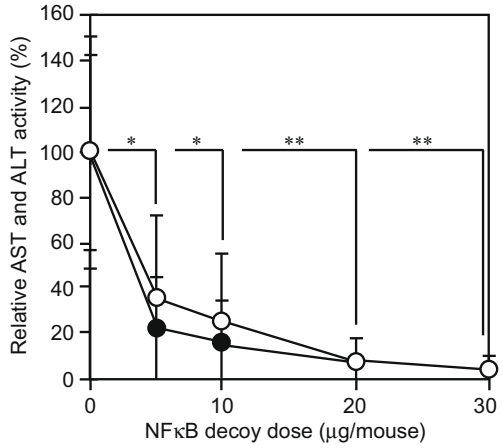

Figure 3. Effect of the NFκB decoy dose on the serum AST and ALT activities. At 1 min after intraperitoneal injection of LPS/D-GalN, KG6/NFκB decoy complexes at a C/A ratio of 8.0 were intravenously injected. After 7 h, the serum AST and ALT activities were measured. *Closed circles* and *open circles* indicate AST and ALT activities, respectively. Each value represents the mean ± SD ($n > 3$). Significances of difference from C/A = 0 (5% glucose) are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

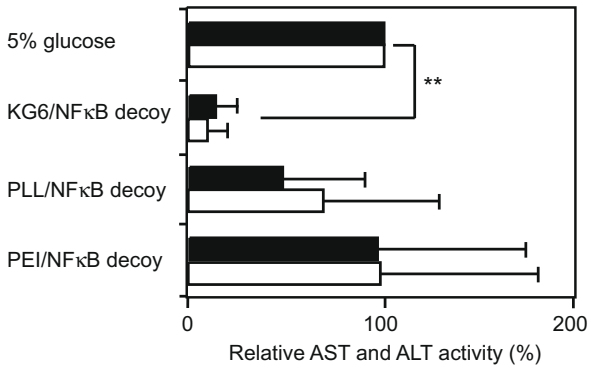

Figure 4. Comparison of polymeric carrier molecules. At 1 min after intraperitoneal injection of LPS/D-GalN, NFκB decoy complexes at a C/A ratio of 8.0 were intravenously injected. After 7 h, the serum AST and ALT activities were measured. *Closed bars* and *open bars* indicate AST and ALT activities, respectively. Each value represents the mean ± SD ($n > 3$). Significance of difference from C/A = 0 (5% glucose) is indicated by ** for $p < 0.01$.

KG6 and PLL. As one of the reasons, it could be due to formation of heterogeneous complexes with NFκB decoy. The mean values of the AST and ALT in the case of PEI were still similar level to the control case (5% glucose injection), indicating that PEI/NFκB decoy complex could not work effectively. From this result, KG6 is concluded to be a uniquely effective polymeric carrier of the NFκB decoy for suppression of the inflammatory response induced by LPS/D-GalN.

2.4. mRNA levels of cytokines relating to the inflammation

TNF-α is a proinflammatory cytokine expressed in the early stage of the hepatitis induced by LPS/D-GalN.[25,26] Intravenous administration of KG6/NFκB decoy complexes decreased TNF-α expression in the liver at 2 h after the injection of LPS/D-GalN (Table 1). After 7 h, the mRNA of TNF-α was decreased in the control (5% glucose injection) compared with that at 2 h. The complexes of KG6 both with NFκB decoy and scrambled oligonucleotide showed further increases of TNF-α expressions. This could be due to long-term side effects of the phosphorothioate oligonucleotide. However, the increase in the case of the scrambled oligonucleotide was higher than that of NFκB decoy. It can be concluded that the NFκB decoy maintained the suppression of the expression of TNF-α even 7 h after stimulation by LPS/D-GalN. The amount of serum TNF-α was evaluated by enzyme-linked immunosorbent assay (ELISA). The protein expression was then correlated to the amount of mRNA evaluated by quantitative real-time PCR (qRT-PCR) (data not shown).

IL-1β and IFNγ are proinflammatory cytokines induced after TNF-α expression. Although IL-1β does not have the direct cytotoxicity to liver cells that TNF-α does, it activates T-cells, B-cells and NK-cells in combination with other interleukins and interferons, then induces production of TNF-α, IL-1, IL-6, IL-8, activates epithelial cells, enhances vascular permeability and induces production of adhesion molecules.[26,27] Measurement of IL-1β mRNA 2 h after induction by LPS/D-GalN, showed the mRNA level to be similarly decreased by the complexes of KG6 with both NFκB decoy and scramble oligonucleotide (Table 1). However, after 7 h, KG6/NFκB decoy complexes showed significantly higher suppression of IL-1β compared with both the control (5% glucose injection) and the KG6/scramble oligonucleotide complexes.

IFNγ is called type II interferon and is known to be produced by T-cells. IFNγ activates macrophages, induces IL-1 production and has functions against viral and intracellular bacterial infections.[26,28,29] It also increases activities of NK cells, cytotoxic T lymphocytes, macrophages and promotes NO production in macrophages. Intravenous administration of KG6/NFκB decoy complexes clearly showed effective reduction of IFNγ mRNA at 2 and 7 h (Table 1). Here, higher activation by the scrambled oligonucleotide was observed, as in the cases of TNF-α and IL-1β.

ICAM-1 is an intercellular adhesion molecule that belongs to the immunoglobulin superfamily and is induced by IL-1 and TNF-α. It is expressed by the vascular endothelium, macrophages and lymphocytes and participates in the inflammatory reaction by accelerating the migration of neutrophils to the inflammation site.[26,30] Intravenous administration of NFκB decoy complexes clearly showed effective reduction of ICAM-1 mRNA at both 2 and 7 h (Table 1). These results support the view that NFκB decoy can suppress ICAM-1 expression in cultured liver sinusoidal endothelial cells.[31]

In macrophages, inducible nitric oxide synthase (iNOS) is expressed to produce nitric oxide that damages pathogens.[32] Septic shock, caused by LPS/D-GalN, induces a large amount of nitric oxide

Table 1
mRNA expression of proinflammatory cytokines in mice livers

Intravenous injection	TNF-α		IL-1β		IFN-γ		ICAM-1		iNOS	
	2 h	7 h	2 h	7 h	2 h	7 h	2 h	7 h	2 h	7 h
5% glucose	40.1 ± 15.9	32.2 ± 21.7	35.2 ± 13.3	41.3 ± 19.6	10.5 ± 6.4	127.7 ± 45.7	19.1 ± 14.3	56.7 ± 70.8	130.1 ± 162.3	1832.3 ± 2555.0
KG6/NFκB decoy	9.5 ± 0.4	44.1 ± 15.3	8.4 ± 0.6	27.8 ± 3.7	1.0 ± 0.4	52.4 ± 31.5	5.5 ± 1.5	13.8 ± 5.5	3.0 ± 0.5	172.2 ± 26.6
KG6/scrambled ODN	21.4 ± 5.1	116.2 ± 76.5	10.6 ± 3.5	59.6 ± 9.1	14.9 ± 10.8	318.8 ± 283.5	25.1 ± 14.4	88.7 ± 19.2	135.9 ± 192.2	539.3 ± 139.0

KG6/NFκB decoy was administered at 1 min after the LPS/D-GalN treatment, then TNF-α, IL-1β, IFNγ, ICAM-1 and iNOS mRNA levels relative to β-actin mRNA at 7 h after the LPS/D-GalN treatment were evaluated by qRT-PCR analyses. Data mean fold increases were normalized to values from healthy mice, without any treatment.
Each value represents the mean ± SD ($n > 3$).

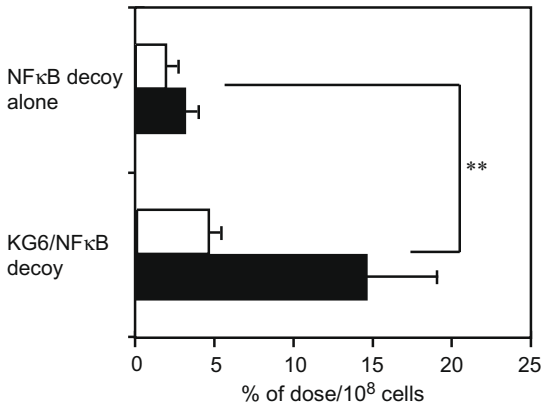

Figure 5. Intrahepatic distribution of [^{32}P] labeled NFκB decoy. Naked [^{32}P] NFκB decoy and KG6/[^{32}P] NFκB decoy complexes at a C/A ratio of 8.0 were intravenously injected into mice. After 10 min, the liver cells were divided into parenchymal and non-parenchymal cell fractions and the radioactivity of each fraction was measured. *Closed bars* and *open bars* indicate non-parenchymal cells and parenchymal cells, respectively. Each value represents the mean ± SD ($n > 3$). Significance of difference from NFκB decoy alone is indicated by ** for $p < 0.01$.

in macrophages and the macrophages then become cytotoxic and induce vasodilation. In this case, a significant reduction effect on iNOS mRNA resulted from administration of the complexes of KG6 with NFκB decoy. This was sequence specific at 2 and 7 h, although a slight non-sequence specific effect was observed in the case of the KG6/scramble oligonucleotide complexes at 7 h (Table 1).

2.5. Intrahepatic distribution of NFκB

Intrahepatic distribution of NFκB decoy, delivered by KG6, was evaluated. Complexes of [^{32}P] NFκB decoy with KG6 at a C/A ratio of 8.0 were intravenously injected into mice. After 10 min, the liver cells were divided into parenchymal and non-parenchymal cell fractions and the radioactivity of each fraction was measured. Here, we employed 10 min as a period for the measurement. The reason why is that since [^{32}P] phosphate was labeled on the terminus of 5′-end of the oligonucleotide, enzymatic cleavage of [^{32}P] phosphate and its redistribution would be occurred at longer time period.[33] The amount of NFκB decoy detected in non-parenchymal liver cells after intravenous injection of KG6/NFκB decoy complexes was 5 times higher than that after intravenous injection of NFκB decoy alone. Moreover, a threefold higher amount of NFκB decoy was detected in the non-parenchymal cells compared with parenchymal cells, whereas equal amounts of NFκB decoys were detected in both cells when naked NFκB decoy was injected (Fig. 5). Accumulation into the non-parenchymal cells would be mainly due to uptake by Kupffer cells in the liver. Since Kupffer cells have important roles in LPS induced hepatitis,[34] this indicates that KG6 may have great potential for oligonucleotide delivery, including NFκB decoy for the treatment of hepatitis.

3. Conclusion

Dendritic poly(L-lysine) (KG6) formed a complex with NFκB decoy oligonucleotide. After intravenous injection of the KG6/NFκB decoy complexes into mice with hepatitis induced by LPS/D-GalN, AST and ALT activities were specifically suppressed. KG6 complexes suppressed these activities with higher efficiency than linear poly(L-lysine) and PEI complexes. The expressions of several cytokines and proteins related to the inflammatory reaction were also suppressed by the intravenous administration of KG6/NFκB decoy complexes. KG6 has been shown to be a promising carrier molecule of oligonucleotides to the liver, especially to Kupffer cells. Furthermore, applications could be extended to the in vivo delivery of various oligonucleotides, such as a siRNA, into the liver.

4. Experimental

4.1. Animals

Male ddY mice (19–22 g, Kyudo Co., Ltd, Fukuoka, Japan) were used in the LPS/D-GalN-induced hepatitis model. The experiments with ddY mice were performed according to the guidelines of the Animal Care and Use Committee, Kyushu University. Female ICR mice (22–24 g, Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan) were used in intrahepatic distribution assays of the NFκB decoy. The experiments with ICR mice were carried out in accordance with the Principles of Laboratory Animal Care, as adopted and promulgated by the US National Institutes of Health and with the Guidelines for Animal Experiments of Kyoto University.

4.2. Materials

Organic solvents used in all synthesis procedures and ethylenediamine and transaminase CII-test Wako were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). *N*-Boc-protected β-alanine and lysine were purchased from Novabiochem, Merck Ltd (Tokyo, Japan). The coupling reagents, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) and 1-hydroxy-benzotriazol (HOBt) were purchased from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Trifluoroacetic acid (TFA) was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). NFκB decoy (5′-AGTTGAGGGGACTTTCCCAGGC-3′, 5′-GCCTGGGAAAGTCCCCTCAACT-3′) was kindly gifted by AnGes MG, Inc. (Osaka, Japan). Scrambled ODN (5′-TTGCCGTACCTGACTTAGCC-3′, 5′-GGCTAAGTCAGGTACGGCAA-3′) was purchased from Hokkaido System Science Co., Ltd (Hokkaido, Japan). RNA iso Plus, SYBR PrimeScript RT-PCR Kit II (Perfect Real Time), primers and MEGALABEL™ 5′-End Labeling Kit were purchased from Takara Bio Inc. (Shiga, Japan). Poly(L-lysine) hydrochloride (Mw 15,000–30,000) and collagenase were purchased from Sigma–Aldrich Inc. (St Louis, MO, USA). In vivo JetPEI was purchased from Polyplus-transfection SA (Illkirch, France). Ethylene glycol-bis (β-aminoethylether)- *N,N,N′,N′*-tetraacetic acid (EGTA) and Clear-Sol I were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was purchased from Perkin Elmer, Inc. (Boston, MA, USA). A NAP-5 column was purchased from GE Healthcare Corp. (Piscataway, NJ, USA).

4.3. Synthesis of dendritic poly(L-lysine)

Dendritic poly(L-lysine) was synthesized as previously reported by Ohsaki et al.[8] In brief, for the initial core synthesis, *N*-Boc-protected β-alanines were coupled with ethylenediamine in DMF by the HBTU–HOBt method and then deprotection was performed by TFA treatment. For the synthesis of the first and higher generations, the coupling reaction between the amino group-free previous generation of dendrimers and *N*-Boc-protected lysines was performed in DMF by the HBTU–HOBt method, then *N*-Boc-groups were removed by TFA. We synthesized dendrimers up to the sixth generation (KG6) by repetition of these coupling and deprotection procedures. The molecular weights of the synthesized dendrimers were measured by MALDI-TOF-MS. Observed molecular weights of the dendrimers were *m/z* 459.2 (1st generation; calcd $[M+H]^+$; *m/z* 459.55), 971.5 (2nd generation; calcd $[M+H]^+$; *m/z* 971.15), 1996.2

(3rd generation; calcd $[M+H]^+$; m/z 1996.35), 4048.7 (4th generation; calcd $[M+H]^+$; m/z 4047.75), 8192.5 (5th generation; calcd. $[M+H]^+$; m/z 8148.55) and 16,384.1 (6th generation (KG6); calcd $[M+H]^+$; m/z 16,350.15).

4.4. Preparation of KG6/NFκB decoy complexes

Complex formation between KG6 and NFκB decoy was examined by the electrophoretic mobility of the complex on a polyacrylamide gel at the various C/A ratios. The tests were performed by mixing 100 ng of NFκB decoy with KG6 in 10 μL of 5% glucose solution, in which C/A ratios were 0, 0.25, 0.50, 0.75, 1.0, 2.0 and 4.0, respectively. Gel electrophoresis sample buffer was added to each sample and it was electrophoresed on a 20% (w/v) polyacrylamide gel for 30 min at 100 V. TBE (45 mM Tris–borate and 1 mM EDTA, pH 8.0) buffer was used as an electrophoresis buffer. The gel was stained with SYBR Gold and illuminated with an UV illuminator to show the location of the DNA.

For in vivo NFκB decoy delivery, 20 μg of NFκB decoy was mixed with KG6 at a C/A ratio of 8.0, in 5% glucose. The particle size and zeta potential of KG6/NFκB decoy complexes were measured by Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, WR, UK).

4.5. LPS/D-GalN-induced hepatitis

ddY mice were injected intraperitoneally with LPS (50 μg/kg) and D-GalN (1.0 g/kg) in saline. Blood was collected from mice 7 h after the LPS and D-GalN treatment. The blood was allowed to coagulate for 1 h at room temperature and serum was isolated as the supernatant fraction following centrifugation at 2000*g* for 20 min. Serum AST and ALT were measured with transaminase C II test Wako according to the manufacturer's protocol.

4.6. Evaluation of NFκB activity against hepatitis

KG6/NFκB decoy complexes were intravenously injected into mice 1 min after LPS/D-GalN treatment. After 7 h, blood samples were collected, then allowed to coagulate for 1 h at room temperature. Serum was isolated as the supernatant fraction following centrifugation at 2000*g* for 20 min. Activities of AST and ALT were measured with transaminase C II test Wako, according to the manufacturer's protocol.

Livers were harvested from mice at 2 and 7 h after LPS/D-GalN treatment. The livers were flash-frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from the livers using RNA iso Plus and reverse transcribed to cDNA using a PrimeScript RT reagent kit, according to the manufacturer's protocol. Gene expressions were assayed by quantitative real-time polymerase chain reaction (qRT-PCR), using a SYBR PrimeScript RT-PCR Kit II, according to the manufacturer's protocol. qRT-PCR was performed with a LightCycler ST300 (Roche Diagnostics, Basel, Switzerland). Cytokine mRNA levels were standardized by β-actin mRNA levels.

4.7. Radiophosphorylation of NFκB decoy

Annealed NFκB decoy was labeled with [γ-^{32}P] ATP using a MEGALABELTM 5′-End Labeling Kit with some modifications, as reported previously by Kawakami et al.[35] 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 to inactivate T4 polynucleotide kinase. Then, the mixture was purified by gel chromatography using a NAP 5 column and eluted with 10 mM Tris–HCl and 1 mM EDTA (pH 8.0). The fractions containing derivatives ([^{32}P] NFκB decoy) were selected based on their radioactivity.

4.8. Intrahepatic distribution of NFκB decoy

Intrahepatic distribution of [^{32}P] NFκB decoy complexed with KG6 was determined as in previous reports.[19,36] Ten minutes after intravenous injection of naked NFκB decoy or KG6/NFκB decoy complexes, each mouse was anesthetized with diethyl ether and the liver perfused with pre-perfusion buffer (Ca^{2+}, Mg^{2+}-free Hanks buffer, pH 7.4 containing 1000 units/L heparin and 0.19 g/L EGTA) for 6 min at 5 mL/min, followed by Hanks buffer containing 5 mM $CaCl_2$ and 220 units/mL collagenase (Type I) (pH 7.4) for 6 min at 5 mL/min. After stopping perfusion, the liver was excised and liver cells were dispersed in ice-cold Hanks-HEPES buffer. The cell suspension was filtered through cotton gauze, followed by centrifugation at 50 *g* for 1 min at 4 °C. The pellet, containing parenchymal cells (PC), was washed four times with ice-cold Hanks-HEPES buffer. The supernatant, containing nonparenchymal cells (NPC), was collected and purified by centrifugation at 50 *g* for 1 min at 4 °C (4 times). PC and NPC suspensions were then centrifuged at 340 *g* for 10 min and then re-suspended separately in ice-cold Hanks-HEPES buffer (final volume 2 mL for PC, 0.5 mL for NPC). The cell number and viability were determined by the trypan blue exclusion method. 300 μL of each cell suspension was digested with Soluene-350 (0.7 μL for blood, urine and tissues) by incubation overnight at 54 °C. Following digestion, 0.2 mL isopropanol, 0.2 mL 30% hydroxyperoxide, 0.1 mL 5 M 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).

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