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RESEARCH ARTICLE

TLR9-dependent systemic interferon-β production by intravenous injection of plasmid DNA/cationic liposome complex in mice

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Abstract

Background The type I interferon (IFN) response to DNA/cationic liposome complex, or lipoplex, has been reported in cultured cells, but little is known about the response *in vivo*. Studies of the pro-inflammatory cytokine response to lipoplex have shown the importance of the unmethylated CpG dinucleotide (CpG motif) and its receptor, Toll-like receptor (TLR)-9.

Methods CpG- and non-CpG lipoplex consisting of CpG- or non-CpG plasmid DNA, respectively, and *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride/cholesterol liposomes were intravenously injected into mice. IFN-β and interleukin (IL)-6 in the serum and organs were measured by the enzyme-linked immunosorbent assay. The involvement of TLR9, phagocytic cells and the spleen in the responses was evaluated using $TLR9^{-/-}$, clodronate liposome-treated-, and splenectomized mice, respectively. Accumulation of blood cells in the lung was evaluated histologically.

Results CpG lipoplex induced a large increase in the levels of IFN-β and IL-6 in the serum, liver, spleen, lung and kidney, whereas non-CpG lipoplex scarcely had any effect. Neither formulation led to significant cytokine production in $TLR9^{-/-}$ mice. Clodronate liposome-treated mice showed a large reduction in both IFN-β and IL-6 levels. Splenectomized mice receiving CpG lipoplex also showed a significantly low production of IL-6 but a similar level of IFN-β production to that of unsplenectomized mice. A large number of monocytes were found in the capillary vessels around the pulmonary alveoli of mice receiving lipoplex.

Conclusions These findings indicate that, in contrast to the production of IL-6 from splenic macrophages, IFN-β is produced from phagocytic cells other than splenic macrophages after the injection of CpG lipoplex through the TLR9-dependent pathway. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords CpG motif; interferon-β; lipoplex; plasmid DNA; splenectomy; Toll-like receptor-9

Introduction

Genomic DNA, a major component of organisms, is released into the circulation when cells undergo apoptosis and necrosis. Self-DNA released from cells is immunologically inert in healthy subjects. Although vertebrates and

bacteria use the same DNA bases for storing genomic information, there is a slight, but immunologically very important, difference between vertebrate and bacterial DNA. Mammalian immunocompetent cells, such as dendritic cells (DCs) and macrophages, express Toll-like receptor (TLR)-9, which recognizes unmethylated CpG dinucleotides (CpG motifs), comprising a unique feature frequently found in bacterial and viral DNA [1–3]. The interaction of CpG-TLR9 results in the release of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12 and interferon (IFN)-γ.

Another response to DNA, which is not dependent on the abundance of CpG motifs in DNA, has recently been identified. When mammalian immunocompetent cells are mixed with DNA/cationic liposome complex, or lipoplex, cells produce a large amount of inflammatory cytokines irrespective of the number of CpG motifs in DNA [4–7]. We have previously reported that human macrophages respond to mammalian DNA-containing lipoplex and secret TNF-α [8,9]. Moreover, we have demonstrated that this immune response even occurs in macrophages from TLR9$^{-/-}$ mice [7]. Several hallmarks have been previously reported for this DNA-inducible, TLR9-independent cellular activation: (i) significant production of type I IFN, such as IFN-α and IFN-β; (ii) the requirement of double-stranded B-form DNA in the cytosol; and (iii) activation of immune competent and noncompetent cells, including resident macrophages, bone marrow-derived DCs, mouse embryonic fibroblasts and HEK293 cells [10–12]. Because type I IFNs are reported to potently suppress the gene expression of plasmid DNA in target cells [13], it is important to obtain information about the profiles of type I IFN production to allow effective DNA-based therapies to be carried out.

The tissue distribution of lipoplex after systemic administration has been thoroughly investigated, and it has been demonstrated that lipoplex accumulates in the lung and plasmid DNA-encoded gene is mainly expressed there [14–16]. Separately, it has been reported that lipoplex is also distributed in the liver and spleen, and resident macrophages (i.e. hepatic Kupffer cells and splenic macrophages) are found to play a pivotal role in the uptake of lipoplex [16–19]. Kupffer cells, splenic macrophages and other resident macrophages express TLR9 mRNA and protein [20]. Functional depletion of Kupffer cells and splenic macrophages significantly reduces the lipoplex-induced production of inflammatory cytokines [17,21], suggesting that these resident macrophages are the major source of these cytokines.

Zhao *et al.* [22] used wild-type and TLR9$^{-/-}$ mice to demonstrate that serum inflammatory cytokines, excluding granulocyte colony-stimulating factor, such as TNF-α, IL-6, IL-12p40 and IFN-γ, were not significantly induced by lipoplex in TLR9$^{-/-}$ mice [22]. Meanwhile, intravenous injection of lipoplex containing plasmid DNA with no CpG motifs induced very little TNF-α or IL-12 production, but induced a significant level of serum IL-6 [23]. Such findings suggest that the immune response to DNA within the body is a complicated process.

Despite many reports of DNA-induced immune response, little is known about the lipoplex-induced IFN production *in vivo*, which comprises a possibly important reaction to DNA administered as a pharmaceutical agent intended for gene therapy or DNA vaccination. In the present study, we used two types of plasmids, one with many CpG motifs and the other with no motifs, and prepared CpG- and non-CpG lipoplex, respectively, by mixing each DNA with *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)/cholesterol liposome. Each lipoplex was injected into mice via the tail vein and the production of IFN-β and IL-6 was evaluated as a representative type I IFN and an inflammatory cytokine, respectively. The involvement of TLR9, phagocytic cells and the spleen in the response was evaluated using TLR9$^{-/-}$, clodronate liposome-treated-, and splenectomized mice, respectively.

Materials and methods

Chemicals

DOTMA was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan). Dichloromethylenediphosphonic acid disodium salt (clodronate) was purchased from Sigma (St Louis, MO, USA). All other chemicals used were of the highest purity available.

DNA

Plasmid vector pCMV-Luc, which encodes firefly *luciferase* gene, was constructed based on pcDNA3 as described previously [24]. pCMV-Luc has 33 Pur-Pur-CpG-Pyr-Pyr sequences, including two GACGTT, a most potent CpG motif for mice [25]. pCpG-ΔLuc was constructed by inserting the firefly *luciferase* cDNA fragment from pORF-LucSh (Invivogene, San Diego, CA, USA) into the *Bgl*II/*Nhe*I site of pCpG-mcs vector (Invivogene). pCMV-Luc was amplified in the *Escherichia coli* DH5α and pCpG-ΔLuc was amplified in *E. coli* GT115. Then, plasmid DNA was isolated and purified using a JETSTAR 2.0 Plasmid GIGA Filter Purification Kit (GENOMED GmbH, Löhne, Germany). Purified pCpG-ΔLuc was sequenced by the Shimadzu Genomic Research Laboratory (Shimadzu, Kyoto, Japan), and it was confirmed that the plasmid has no CpG motifs.

Purification of DNA

To minimize the activation by contaminated lipopolysaccharide (LPS), DNA samples were extensively purified with Triton X-114, a non-ionic detergent. Extraction of LPS from plasmid DNA samples was performed according to a previously described method [26]. The level of contaminated LPS was checked by a Limulus amebocyte

lysate assay using the Limulus F Single Test kit (Wako Pure Chemical Industries, Osaka, Japan). The level of contaminated LPS was over 1 EU/μg plasmid DNA before extraction, and it was reduced to below the detection limit of 0.01 EU/μg plasmid DNA by Triton X-114 extraction.

Preparation of cationic liposomes and lipoplex

In the present study, DOTMA/cholesterol liposomes were used to prepare plasmid DNA complexes because of their high transfection efficiency after intravenous injection [27,28]. Cationic liposomes consisting of DOTMA and cholesterol in a 1:1 molar ratio were prepared by allowing the lipids to dry as a thin film in a round-bottomed flask using a rotary evaporator, and then hydrating in 5% w/v dextrose by gentle mixing. After hydration, the dispersions were sonicated for 2.5 min and passed through a Minisart® 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako (Wako Pure Chemical Industries). Cationic liposomes and two types of plasmid DNA (i.e. pCMV-Luc and pCpG-ΔLuc) in 5% dextrose were mixed at a charge ratio of +2.24 and the final concentration of lipoplex was adjusted to 25 μg of DNA and 179.5 μg of cationic liposome/300 μl 5% dextrose solution. The mixtures were left for at least 30 min at 37 °C before use as CpG lipoplex or non-CpG lipoplex, respectively.

Measurement of zeta potential and particle size

The zeta potential and size of lipoplex were measured using a Nano ZS (Malvern Instruments, Ltd, Malvern, Worcestershire, UK) and shown in Table 1.

Animals

TLR9$^{-/-}$ mice were purchased from the Oriental Yeast Company (Tokyo, Japan). C57BL/6 wild-type mice and Institute for Cancer Research (ICR) mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and maintained on a standard food and water diet under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Institutional Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

Table 1. Properties of plasmid DNA and lipoplex used in the present study

	CpG lipoplex	Non-CpG lipoplex
Plasmid name	pCMV-Luc	pCpG-ΔLuc
Plasmid size (base pair)	7041	3604
Number of CpG motifs	846	0
Particle size (nm)	127 ± 2	133 ± 2
Zeta potential (mV)	49.3 ± 0.5	41.8 ± 1.3

Cytokine assay of serum and tissue samples

Lipoplex was injected into the tail vein of mice at a dose of 1 mg DNA/kg body weight. After injection, blood was collected in plastic tubes from the inferior vena cava under anaesthesia or the tail vein of mice, and allowed to stand for 3 h at 4 °C. Then, the samples were centrifuged at 15 500 *g* for 20 min at 4 °C and the serum obtained was used for cytokine assay. Organs such as lung, liver, spleen and kidney were isolated at 2 h after the injection of plasmid DNA, washed with ice-cold saline and blotted dry. Isolated organs were homogenized in 2 ml (liver) or 1 ml (other organs) of phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors for assay. The cytoplasmic fractions were isolated as the supernatant after centrifugation at 15 500 *g* for 20 min at 4 °C. The levels of IL-6 in the serum and cytoplasmic fractions of organs were determined by the OptEIA™ set (BD Biosciences, San Diego, CA, USA). In addition, IFN-β was determined by a two-site enzyme-linked immunosorbent assay (ELISA), using a rat anti-mouse IFN-β antibody (Seikagaku Biobusiness Corporation, Tokyo, Japan) as a capture antibody and a rabbit anti-mouse IFN-β polyclonal antibody (PBL Biomedical Laboratories, New Brunswick, NJ, USA) as a detection antibody, followed by a donkey anti-rabbit horseradish peroxidase antibody (Jackson ImmunoResearch, West Grove, PA, USA). Recombinant mouse IFN-β (PBL Biomedical Laboratories) was used to construct a standard curve for quantification.

Clodronate liposomes

Phosphatidylcholine and cholesterol were dissolved in chloroform and a thin lipid film was formed by low-vacuum rotary evaporation. This film was dispersed in 5 ml of PBS in which clodronate was dissolved. The suspension was maintained at room temperature for 2 h followed by sonication for 3 min. After another 2 h at room temperature, the suspension was centrifuged at 22 000 *g* for 1 h at 10 °C to remove free clodronate, and then washed four times using centrifugation at 22 000 *g* for 25 min at 10 °C. The liposomes were then resuspended in 2 ml of PBS and stored at 4 °C until use.

Transient depletion of macrophages

Macrophages were transiently depleted by intravenous injection of clodronate liposomes as previously reported

[29]. Clodronate liposomes or PBS (200 μl) was injected into the tail vein 48 h prior to the injection of lipoplex.

Spleen removal

Under anaesthesia, 4-week-old ICR mice were placed on a surgical board in the prone position. An incision of approximately 1 cm in length was made in the left flank, and the spleen was dissected and removed after the splenic vein and artery were ligated using silk sutures. In the sham-operated group, the flank incision was made, but the spleen was not removed. The wounds were closed using Diener clips (Diener, Tuttlingen, Germany). Mice were kept on warm pads until they recovered from anaesthesia. After a 1-week recovery period, these mice were used for cytokine assay.

Immunohistochemistry and immunofluorescence of lung tissue

Lipoplex was intravenously injected into mice. Thirty minutes, 2 h or 24 h later, mice were sacrificed and 4% paraformaldehyde in PBS was injected via the trachea followed by incubation for 24 h at 4 °C. The fixed lung tissues were embedded in paraffin and sectioned into 5-μm slices. The paraffin sections were stained with hematoxylin and eosin to evaluate the infiltration of blood cells. Separately, to evaluate the infiltration of monocytes, the lung sections were immunostained with flurescein isothiocyanate (FITC)-labeled F4/80 antibody. In brief, the paraffin sections were deparaffinated with xylene and washed twice with ethanol. Then, the sections were treated with 0.1% trypsin in PBS for 30 min at 37 °C. Nonspecific binding sites were blocked with 3% bovine serum albumin in PBS for 15 min at room temperature. Then, FITC-labeled rat anti-mouse F4/80 monoclonal antibody (Antibodies Direct, Oxford, UK; 1 : 500 dilution) was added. After 1 h of incubation at 37 °C, the sections were washed five times with PBS and observed under a fluorescent microscope (Biozero BZ-8000; Keyence, Osaka, Japan).

IFN-β release from peripheral blood mononuclear cells (PBMCs)

Peripheral blood was obtained via the inferior vena cava under anaesthesia from ICR mice using a heparinized syringe. The blood was diluted with an equal volume of PBS and separated by centrifugation on Ficoll-Paque Plus (GE Healthcare UK Ltd, Bucks, UK). The middle layer cells were recovered as PBMCs. Contaminating red blood cells were lysed by the addition of a hypo-osmotic solution of 0.1% ammonium chloride. After washing with PBS, PBMCs were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.2 μg/ml), and then plated on round-bottom 96-well culture plates (Greiner Bio-One, Kremsmunster, Austria) at a density of 2×10^5 cells/well. PBMCs were incubated with lipoplex or DOTMA/cholesterol liposome for 2 h. Then, cells were washed with RPMI-1640 and incubated with growth medium for an additional 10 h, and the supernatants were collected for ELISA and maintained at −80 °C. The level of IFN-β in the supernatant was determined by the Verikine™ Mouse Interferon-Beta ELISA Kit (PBL Biomedical Laboratories).

Statistical analysis

Differences were statistically evaluated by Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

CpG motif-dependent systemic production of IL-6 and IFN-β by lipoplex

IL-6 was measured as an indicator of systemic inflammation [23]. Figure 1a shows the IL-6 production in mice 2 h after intravenous injection of CpG lipoplex, non-CpG lipoplex or liposomes only. In preliminary experiments, a peak level of serum IL-6 was observed at this time point of 2 h after the injection of CpG lipoplex (data not shown), which was in a good agreement with a previous study [22]. The CpG lipoplex-treated mice showed a significantly higher amount of IL-6 production than the liposome-treated control mice in the serum and all organs that we measured (Figure 1a). Non-CpG lipoplex induced IL-6 production in the serum and spleen, even though the levels were much less than those induced by CpG lipoplex. The profiles of TNF-α, another pro-inflammatory cytokine, production were almost identical to those of IL-6 (data not shown). Therefore, we selected IL-6 as a representative pro-inflammatory cytokine in the subsequent experiments.

Figure 1b shows the time-course of IFN-β production after intravenous injection of lipoplex. A large amount of IFN-β was detected 2 h after the injection of CpG lipoplex, and then the cytokine gradually decreased to a very low level at 24 h. The level of IFN-β produced by non-CpG lipoplex was significantly lower than that by CpG lipoplex. In addition, non-CpG lipoplex showed a different profile of IFN-β production from CpG lipoplex. A large amount of IFN-β was also detected in the liver, spleen and lung 2 h after the injection of CpG lipoplex, but not after non-CpG lipoplex (Figure 1c). Similar results were observed for IL-6 production (Figure 1a). These results indicate that lipoplex induces IFN-β production in a CpG-motif dependent manner after intravenous injection into mice.

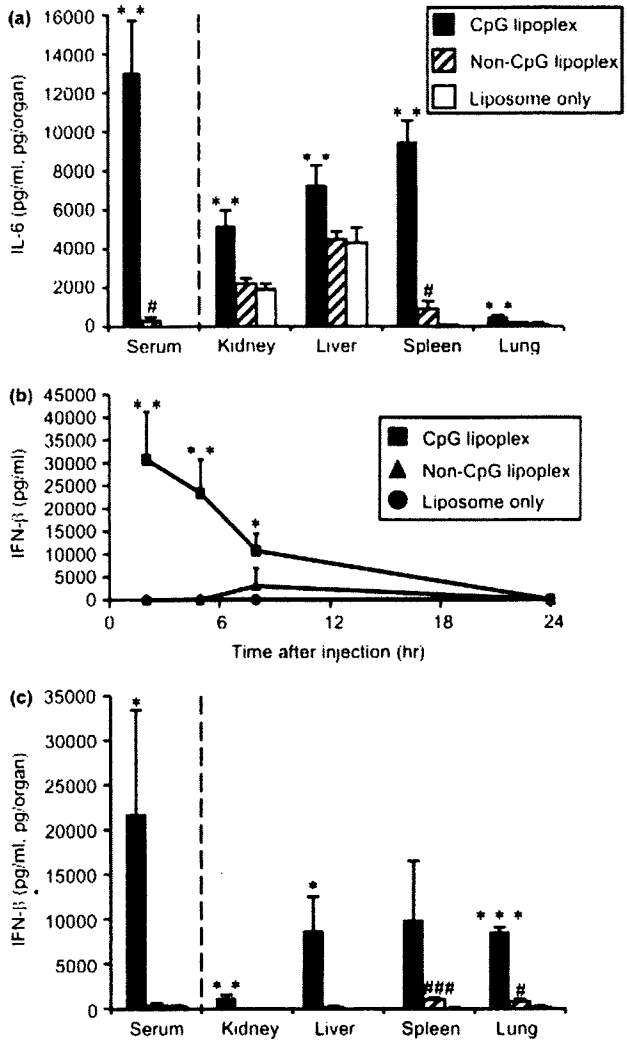

Figure 1. IL-6 and IFN-β levels in serum and organs after intravenous injection of lipoplex into mice. ICR mice were intravenously injected with CpG- or non-CpG lipoplex (1 mg DNA and 7.18 mg lipids/kg) or cationic liposomes (7.18 mg/kg). Serum and organs were collected and the cytokine levels were measured by ELISA: (a) IL-6 level in serum and organs at 2 h after injection; (b) time-course of IFN-β level in serum; (c) IFN-β level in serum and organs at 2 h after injection. The results are expressed as the mean ± SD of three or four mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from non-CpG lipoplex-treated mice; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, significantly different from liposome-treated mice

TLR9-dependent cytokine production by lipoplex

The involvement of TLR9 in the lipoplex-induced cytokine production in mice was directly examined using TLR9$^{-/-}$ mice. As reported in a previous study [22], CpG lipoplex-induced IL-6 production in serum was quite low in TLR9$^{-/-}$ mice, and less than 1% of the activity detected in wild-type mice (Figure 2a). In addition, the levels of IL-6 in all the organs examined were also very low (data not shown). Non-CpG lipoplex induced scarcely any IL-6 production in the serum of TLR9$^{-/-}$ mice.

No significant IFN-β production was observed in TLR9$^{-/-}$ mice after intravenous injection of CpG lipoplex (Figure 2b). Neither wild-type nor TLR9$^{-/-}$ mice produced IFN-β after the injection of non-CpG lipoplex. These results suggest that lipoplex-induced IFN-β production is almost completely mediated by TLR9 and the TLR9-independent or CpG-independent pathway makes little contribution to IFN-β production.

Decreased cytokine production in mice pretreated with clodronate liposomes

To examine the contribution of tissue macrophages to cytokine production by lipoplex, mice received a tail vein injection of clodronate liposomes, followed by an injection of lipoplex. Clodronate liposome-pretreated mice exhibited a marked reduction in CpG lipoplex- and non-CpG lipoplex-induced IL-6 production in the serum and all organs except for the lung (Figures 3a and 3b). The CpG lipoplex-induced IFN-β production in the serum and all organs was also significantly reduced by pretreatment with clodronate liposomes (Figure 3c). These results indicate the possibility that tissue macrophages and other phagocytic cells depleted by clodronate liposomes contribute not only to IL-6, but also to IFN-β production in mice receiving lipoplex.

Cytokine production in splenectomized mice

The spleen, which is an organ containing many tissue macrophages, has been reported to be responsible for the production of inflammatory cytokines after intravenous injection of lipoplex. To evaluate the involvement of the spleen in lipoplex-induced cytokine production, we injected lipoplex into mice whose spleens were removed. Upon injection of lipoplex, splenectomized mice showed profiles of IL-6 and IFN-β production in the kidney, liver and lung that were similar to those of unsplenectomized mice (data not shown). The serum level of IFN-β in splenectomized mice injected with CpG lipoplex was slightly, but insignificantly, lower than that in control mice (Figure 4c). On the other hand, the serum level of IL-6 in splenectomized mice was significantly lower than that in control mice (Figure 4a). IL-6 induced by non-CpG lipoplex was not affected by splenectomy (Figure 4b). These results suggest that the spleen is a very important organ for the production of serum IL-6 after the injection of CpG lipoplex, but the spleen made little contribution to CpG lipoplex-induced serum IFN-β or non-CpG lipoplex-induced serum IL-6.

Accumulation of blood cells in the vessels of the lung

The results obtained indicating that the level of IFN-β, but not of IL-6, in the lung was increased by

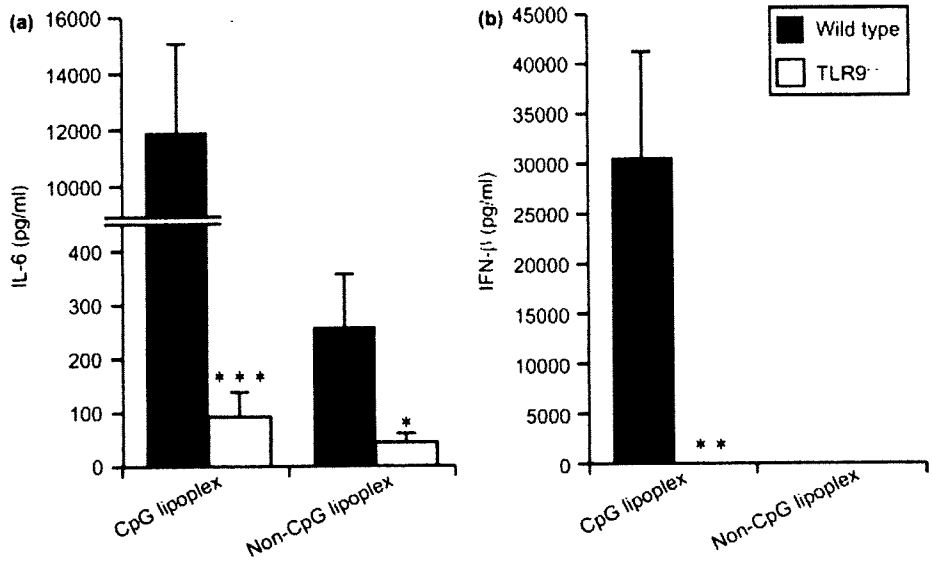

Figure 2. IL-6 and IFN-β levels in serum after intravenous injection of lipoplex into wild-type- or TLR9-/- mice. Wild-type (C57BL/6)- or $TLR9^{-/-}$ mice were intravenously injected with CpG- or non-CpG lipoplex (1 mg DNA/kg). At 2 h after injection, serum was collected and the cytokine levels were measured by ELISA: (a) IL-6 level and (b) IFN-β level. The results are expressed as the mean ± SD of three or four mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from wild-type mice

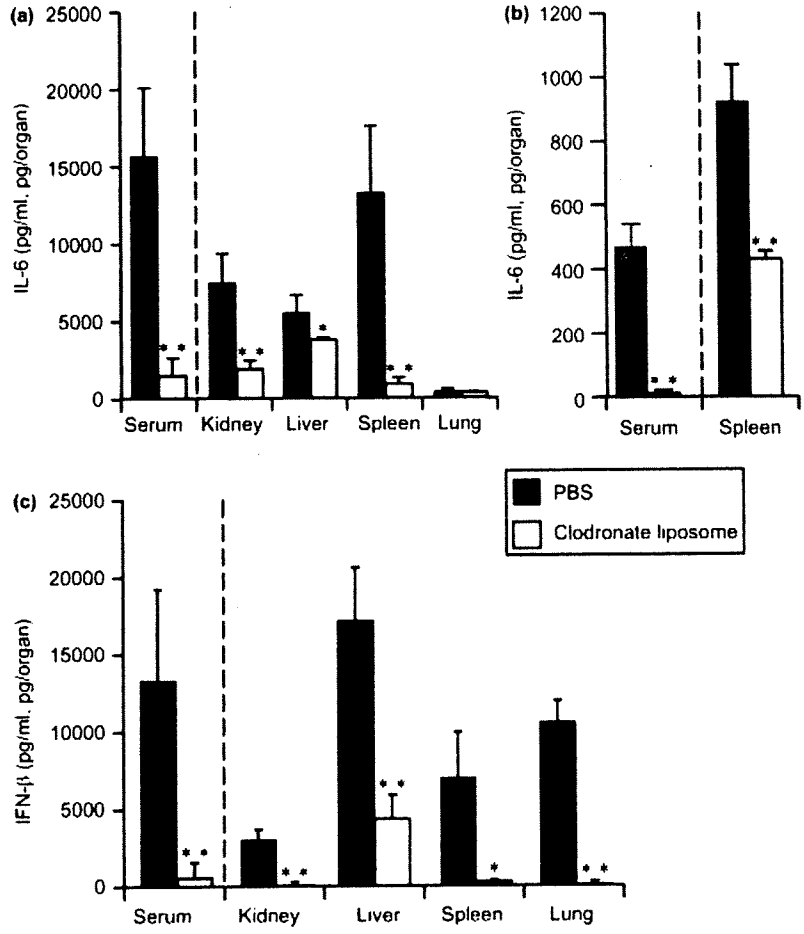

Figure 3. Effect of clodronate liposomes on cytokine production after intravenous injection of lipoplex into mice. ICR mice were injected first with PBS (vehicle) or clodronate liposomes, and then with CpG- or non-CpG lipoplex (1 mg DNA/kg) after an interval of 48 h. At 2 h after the injection of lipoplex, serum and organs were collected and the cytokine levels were measured by ELISA: (a) IL-6 level after the injection of CpG lipoplex; (b) IL-6 level after the injection of non-CpG lipoplex; and (c) IFN-β level after the injection of CpG lipoplex. The results are expressed as the mean ± SD of three or four mice. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from PBS-treated mice

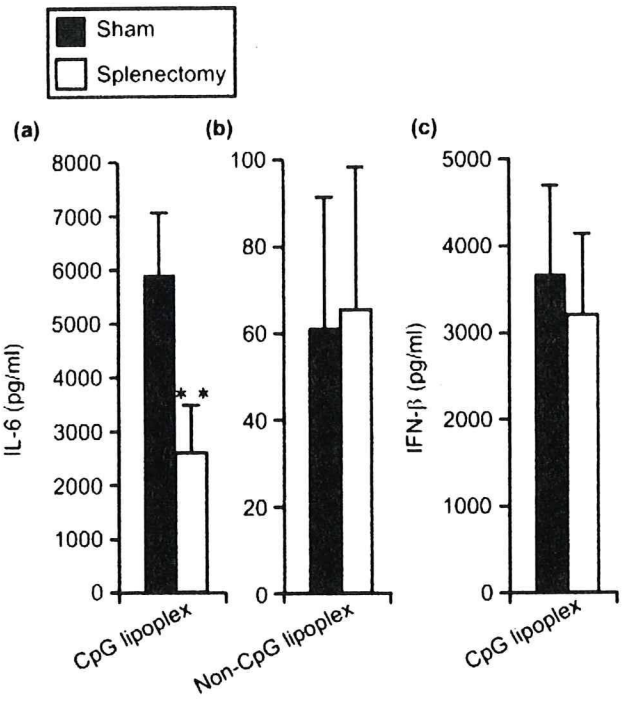

Figure 4. Effect of splenectomy on cytokine production in serum after intravenous injection of lipoplex into mice. ICR mice underwent a sham operation or splenectomy. One week after surgery, the mice were injected with CpG- or non-CpG lipoplex (1 mg DNA/kg). At 2 h after the injection of lipoplex, serum was collected and the cytokine levels were measured by ELISA: (a) IL-6 level after the injection of CpG lipoplex; (b) IL-6 level after the injection of non-CpG lipoplex; and (c) IFN-β level after the injection of CpG lipoplex. The results are expressed as the mean ± SD of four or five mice. $^{**}p < 0.01$, significantly different from the sham-operated mice

intravenous injection of CpG lipoplex suggest that the cells in the lung contribute to the release of IFN-β into the circulation. Accordingly, we evaluated the accumulation of immunocompetent cells in the pulmonary vessels after intravenous injection of CpG lipoplex. Without injection of lipoplex, there were only a few neutrophils (Figure 5a) and macrophages (Figure 5e) in the capillary vessels around the pulmonary alveoli. Thirty minutes after intravenous injection of lipoplex, a large number of neutrophils were found in the vessels (Figure 5b). The neutrophils had fallen rapidly by 2 h after the injection of lipoplex (Figure 5c), but a few were still detected even at 24 h after injection (Figure 5d). Similarly, F4/80-positive cells (i.e. monocytes and pulmonary macrophages) were found in the capillary vessels at 30 min after the injection of CpG lipoplex and remained at the same level for at least up to 2 h (Figure 5g).

IFN-β release from PBMCs

To examine whether monocytes are involved in the CpG lipoplex-induced IFN-β production, the release of IFN-β from PBMCs was examined. The addition of CpG lipoplex resulted in a significant release of IFN-β from PBMCs (Figure 6). On the other hand, no significant IFN-β was secreted from cells by the addition of non-CpG lipoplex. These results support the hypothesis that monocytes and other cells in PBMCs infiltrating the lung are responsible for the IFN-β production after intravenous injection of CpG lipoplex.

Discussion

Although many studies have reported lipoplex-induced production of type I IFNs in cultured cells [10–12,30–32], the production has not been examined in detail in animals.

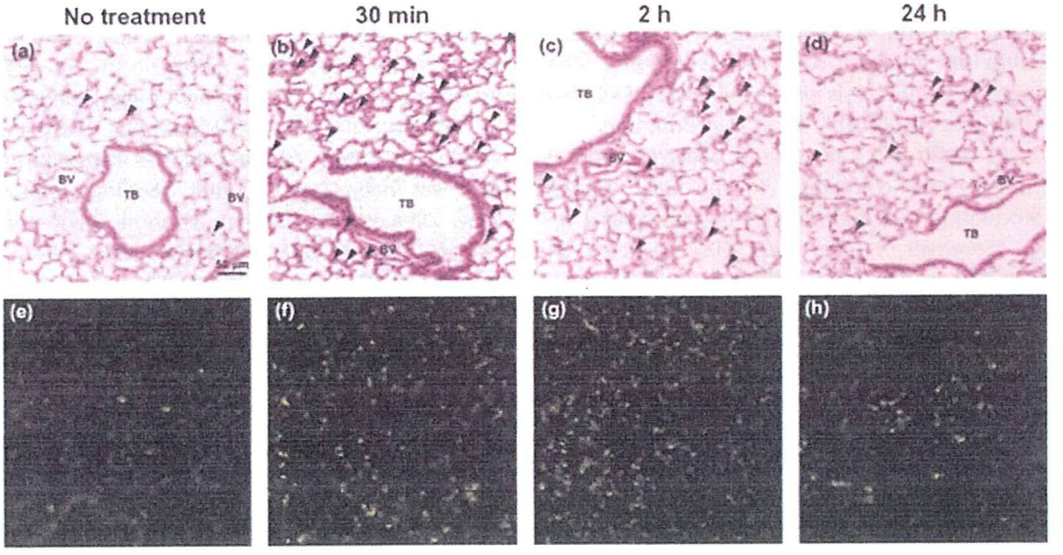

Figure 5. Lung sections of mice receiving an intravenous injection of CpG lipoplex. (a–d) Hematoxylin and eosin-stained sections of (a) untreated mice and (b–d) CpG-lipoplex-injected mice: (b) 30 min (c) 2 h and (d) 24 h after injection. (e–h) F4/80-positive cells of (e) untreated mice and (f–h) CpG-lipoplex-injected mice: (f) 30 min (g) 2 h and (h) 24 h after injection. Original magnification × 100. Arrowheads indicate neutrophils. BV, blood vessel; TB, terminal bronchiole

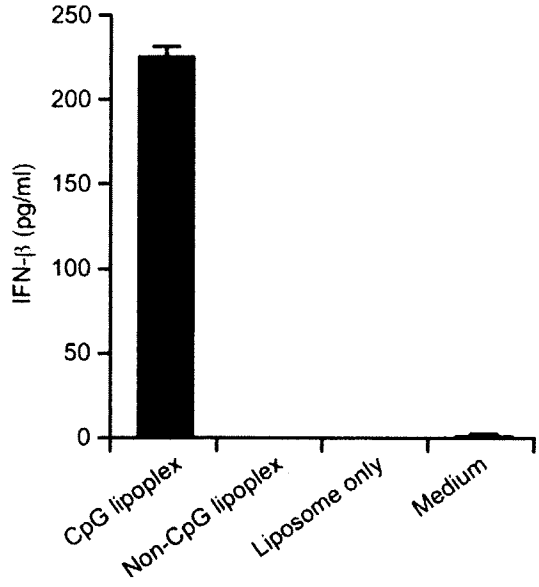

Figure 6. IFN-β production in PBMCs isolated from ICR mice. CpG lipoplex containing 1 μg pDNA/ml was added to cells and incubated for 12 h. Then, supernatants were collected and the level of IFN-β was determined by ELISA. Each result represents the mean ± SD of triplicate values

Discrepancies are known to exist in the DNA-induced cytokine production between cultured cells and whole animals, so that it is difficult to speculate about the *in vivo* immune responses to lipoplex based on the results obtained with cultured cells. In the present study, we have demonstrated that a large amount of IFN-β is produced in mice after intravenous injection of CpG lipoplex, and that this production is completely dependent on TLR9. Production was very low when non-CpG lipoplex was injected into mice instead of CpG lipoplex, suggesting that IFN-β production can be successfully avoided by using non-CpG plasmid DNA for cationic liposome-mediated *in vivo* gene delivery in mice. Unlike IFN-β production, a small, but significant, amount of IL-6 production was detected in serum after the injection of non-CpG lipoplex. These results suggest that IFN-β is produced by a mechanism differing from that responsible for IL-6 production.

It has been confirmed that splenic macrophages and hepatic Kupffer cells contribute to the CpG lipoplex-induced production of inflammatory cytokines and IFN-γ [17,21]. Moreover, Lones *et al.* [33] reported that the kinetics of serum TNF-α induced by intravenous injection of CpG lipoplex was comparable with the kinetics of intracellular TNF-α in the spleen but not in the liver. Our previous study has also shown that splenic macrophages responded to CpG lipoplex and induced TNF-α, whereas other resident macrophage-like cells, such as peritoneal macrophages, hepatic nonparenchymal cells and mesangial cells, did not [20]. The results obtained in the present study demonstrate that the spleen is involved in the production of the inflammatory cytokine, IL-6, which is in a good agreement with previous studies. However, the splenectomized mice also produced a significant amount of IL-6 (Figure 4), so that other cells or organs than the spleen could contribute to the CpG lipoplex-induced IL-6 production. Further studies are required to identify these cells.

On the other hand, the serum level of IFN-β induced by CpG lipoplex was not affected by splenectomy (Figure 4c), suggesting that the spleen scarcely contributes to the CpG lipoplex-induced IFN-β production. Instead, a large amount of IFN-β was detected in the lung after the injection of CpG lipoplex (Figure 1c). In addition, a large number of monocytes were found in the capillary vessels around the pulmonary alveoli after the injection of lipoplex. Pretreatment of mice with clodronate liposomes, which was reported to deplete not only resident macrophages, but also monocytes in the peripheral blood [34], significantly reduced the amount of IFN-β in the lung (Figure 3). Finally, primary cultured PBMCs including monocytes induced IFN-β by the addition of CpG lipoplex (Figure 6). Such experimental evidence strongly suggests that the infiltrating monocytes are a major source of IFN-β after the administration of CpG lipoplex.

Preclinical studies have shown that plasmid DNA-induced production of pro-inflammatory cytokines is greatly reduced by removal of CpG motifs from plasmid DNA [21,35,36]. On the basis of these findings, CpG-reduced or non-CpG plasmid DNA has been used in some clinical trials of *in vivo* gene therapy [37]. The results obtained suggest that the replacement of CpG-replete conventional plasmid DNA with CpG-free plasmid DNA comprises a universal approach for achieving safe *in vivo* gene transfer. However, recent studies using cultured cells have reported that Z-DNA binding protein-1 (ZBP1) and/or TANK-binding kinase-1 (TBK1), both of which are suggested to be cytosolic sensors for double stranded DNA, triggers the production of type I IFNs when DNA enters the cytosol, irrespective of the presence of CpG motifs [30,31]. Separately, Yasuda *et al.* [38] have reported that flt3-ligand-induced dendritic cells from $TLR9^{-/-}$ mice produced IL-6 when added to non-CpG lipoplex. Moreover, a recent study demonstrated that DNA sugar backbone 2′ deoxyribose activates the TLR9 pathway, suggesting that DNA bases, including CpG, are not required for the TLR9-mediated cytokine production [39]. In addition to these studies using cultured cells, it has also been reported that lipoplex composed of non-CpG DNA or mammalian genome DNA with few CpG motifs induced IL-6 production after administration to mice [23,40]. Such findings obtained in previous studies suggest the possibility that lipoplex-induced cytokine production occurs irrespective of the presence of CpG motifs in plasmid DNA or TLR9 in cells. In the present study, however, we clearly showed that very little IFN-β is produced when non-CpG plasmid DNA is used for gene transfer. In addition, the lipoplex-induced IFN-β production was almost completely prevented in $TLR9^{-/-}$ mice. Therefore, it can be concluded that the recognition of the CpG motif by TLR9 triggers the production of IFN-β after the administration of CpG lipoplex and that ZBP1- and/or TBK1-mediated IFN-β production was not induced or was negligible after intravenous injection of

non-CpG lipoplex in mice. On the other hand, we detected CpG-independent, but TLR9-dependent, production of IL-6, both in the spleen and in serum. The level of IL-6 in the circulation was reduced in mice pre-injected with clodronate liposomes but not in splenectomized mice. These results suggest that IL-6 production by non-CpG-lipoplex is induced by phagocytic cells sensitive to clodronate liposomes in organs other than the spleen. Further studies are needed to identify the cells responsible for the production of IL-6 after the administration of non-CpG lipoplex.

In conclusion, the present study demonstrates that IFN-β, a representative type I IFN, is induced in the systemic circulation in a CpG motif- and TLR9-dependent manner when lipoplex is systemically administered. The results obtained indicate that lipoplex-induced IFN-β production can be circumvented by using non-CpG plasmid DNA because the lipoplex-induced IFN-β production was shown to be almost completely mediated by the interaction between CpG motifs and TLR9. Monocytes infiltrating the lung would be a major source of IFN-β produced after the administration of lipoplex. Thus, CpG-independent production of type I IFNs, which has been reported in cultured cells, does not occur when lipoplex is systemically injected into mice, so that the depletion of CpG motifs from plasmid DNA is an effective approach for achieving *in vivo* gene transfer with little production of type I IFNs.

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Nonviral vector-mediated RNA interference: Its gene silencing characteristics and important factors to achieve RNAi-based gene therapy ☆

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ABSTRACT

RNA interference (RNAi) is a potent and specific gene silencing event in which small interfering RNA (siRNA) degrades target mRNA. Therefore, RNAi is of potential use as a therapeutic approach for the treatment of a variety of diseases in which aberrant expression of mRNA causes a problem. RNAi can be achieved by delivering siRNA or vectors that transcribe siRNA or short-hairpin RNA (shRNA). The aim of this review is to examine the potential of nonviral vector-mediated RNAi technology in treating diseases. The characteristics of plasmid DNA expressing shRNA were compared with those of siRNA, focusing on the duration of gene silencing, delivery to target cells and target specificity. Recent progresses in prolonging the RNAi effect, improving the delivery to target cells and increasing the specificity of RNAi in vivo are also reviewed.

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1. Introduction

Small interfering RNA (siRNA) can degrade mRNA which has a complementary sequence to the siRNA by the mechanism called RNA interference (RNAi) [1–3]. Soon after its discovery, siRNA began to be widely used as an experimental tool to investigate the function of target genes because of its convenient, specific and potent gene silencing effect compared with conventional techniques such as antisense oligodeoxynucleotides and homologous recombination-based knockout strategy [4,5]. Moreover, therapeutic application of siRNA targeting the gene of interest has been actively investigated. In addition to siRNA, DNA vectors that transcribe siRNA or short hairpin RNA (shRNA) are also available to induce RNAi [6–8]. A number of viral and nonviral vectors have been developed, but the safety concerns of

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viral vectors have not been resolved yet. Therefore, the nonviral vector-based approach using plasmid DNA (pDNA) is expected to be a safer method to induce RNAi compared with any approaches using viral vectors. Vector-based approaches and siRNA share the same RNAi pathway, but have different properties that affect the efficacy of RNAi-based therapy. In this review, we describe the current status of the development of pDNA vectors for siRNA expression, compare the profiles of gene silencing by either shRNA-expressing pDNA or siRNA and discuss the advantages and limitations of RNAi-based gene silencing in therapeutic applications at the present time.

2. Development of DNA vectors for siRNA expression

Intracellular transcription of siRNA can be achieved by introducing the vector containing a siRNA template under the control of a promoter. In this section, the current status of the development of pDNA vectors for siRNA expression in mammalian cells is summarized.

2.1. Vector construct

Two approaches have been developed for expressing siRNA inside cells: one is a tandem-type vector which transcribes a pair of sense and antisense transcripts from individual promoters; the other is a hairpin vector which transcribes a single strand RNA that forms short hairpin RNA (shRNA), which is processed into siRNA inside cells [9]. As shRNA requires only one DNA template, shRNA-expressing vectors are more convenient than those expressing two templates as far as the construction of vectors is concerned. Moreover, the knockdown efficiency of shRNA-expressing vectors is generally higher than that of tandem-type vectors [8,10,11]. Therefore, shRNA-expressing vectors have been used much more frequently. Here we will focus on shRNA-expressing vectors. In addition to shRNA, micro RNA (miRNA), an endogenous RNA molecule that regulates gene expression, has also been reported to be used for inducing RNAi. miRNA has some advantages over siRNA or shRNA, because single miRNA transcript is processed into multiple siRNAs and relatively long miRNA transcripts can be transcribed by Pol II promoter [12,13].

A pDNA vector for shRNA expression normally consists of a promoter, a shRNA template and necessary components for pDNA amplification (i.e., replication origin and selection marker genes). Gene therapy studies have demonstrated that a variety of factors affect the level and duration of transgene expression from the pDNA [14]. Of such factors, the promoter has been shown to be the most important for determining the profile of transgene expression. Therefore, the effect of the type of promoter on shRNA expression is discussed first, followed by those of other components of shRNA-expressing pDNA.

2.2. Promoter

The promoter that drives shRNA expression is an important factor for determining the RNAi effect produced by shRNA-expressing pDNA. RNA polymerase III (Pol III) promoters, such as small nuclear RNA U6 (U6) and human RNase P RNA H1 (H1), have been frequently used for shRNA expression because these promoters are suitable for the transcription of short RNA in large quantities and their sites of transcription initiation and termination are well defined. Lots of studies including our own have demonstrated that the knockdown efficiency of shRNA-expressing pDNA depends on the type of promoter. We found that shRNA-expressing pDNA driven by U6 promoter has more sustained effects than those driven by H1 or tRNA promoter [15]. However, the rank order of promoter strength varies among studies. For example, the results reported by Boden et al. have shown that tRNA-driven shRNA-expressing pDNA induced HIV-1-specific RNAi more efficiently than those driven by other promoters, such as U6, H1 and CMV promoters [16]. Differences in experimental conditions, such as the type of target cells, delivery methods and shRNA sequence, would explain the discrepancy among the studies, as demonstrated in a recent paper in which the effective promoter was a function of the type of cell line [17]. These results would suggest that a suitable promoter should be selected on a case-by-case basis.

Inducible expression systems provide further benefits to vectors with pol III promoters. The expression of shRNA from such vectors has been shown to be induced by chemical reagents, such as tetracycline [18–20]. This inducible system will be advantageous not only as experimental tools but also as therapeutics.

In addition to Pol III promoters, Pol II promoters are also available to transcribe shRNA [21,22]. As gene silencing in non-target cells may cause undesired effects, target cell specific gene silencing is effective in reducing possible side effects. Pol II promoters can provide cell- or tissue-specific expression of shRNA, which would be a feasible approach to achieve target cell-specific gene silencing. Cell type-specific gene silencing has already been demonstrated by using cell-specific promoters, such as telomerase reverse transcriptase promoter (tumor cells) [23], glial fibrillary acidic protein promoter (hepatic stellate cell) [24], human α_1-antitrypsin (hAAT) promoter (hepatocyte) [25] and prostate specific membrane antigen promoter/enhancer (prostate cancer) [26]. Grimm et al. reported a successful gene silencing of the envelope surface antigen (sAg) of hepatitis B virus (HBV) in the liver of HBV-transgenic mice by U6 promoter-driven shRNA targeting the gene, but they found that the ubiquitous expression of an excess amount of shRNA produced toxic effects in the mice [27]. Recently, Giering et al. have shown a solution for this toxicity by using a hepatocyte-specific promoter (hAAT promoter) to express the shRNA [25]. Hepatocyte-specific expression of shRNA was found to be effective not only in inhibiting HBV replication in HBV transgenic mice but also in avoiding shRNA-mediated toxicity.

2.3. Components of shRNA-expressing pDNA

Jenke et al. inserted a scaffold/matrix attachment region into an shRNA-expressing pDNA targeting hepatitis B virus (HBV) in order to retain the plasmid as an episome in the cells [28]. They found that the vector was effective in suppressing HBV replication for at least 8 months after the transfection of shRNA-expressing pDNA to HBV-replicating HepG2.2.15 cells. Because no in vivo results have been reported and the effect of the scaffold/matrix attachment region on the duration of the RNAi effect has not been reported, additional studies are required to confirm the importance of the insertion of the region on the duration of the knockdown effect. In addition, use of a transposon system, which has an ability to insert pDNA into genomic DNA of target cells, has been reported to be effective for long-term expression [29,30]. As the random insertion of pDNA into genome DNA carries a risk of mutagenesis, which is the same problem as that of retroviral vectors, further improvements in safety is required for the application of such transposon systems as therapeutic treatments.

In our gene therapy studies aiming to achieve sustained transgene expression, we investigated the effect of the number and position of unmethylated CpG dinucleotides (CpG motifs) in pDNA on the duration of transgene expression in vivo [31] (Mitsui et al., in press). In these studies, we have found that reducing the number of CpG motifs in pDNA is effective in prolonging the duration of transgene expression. Recently, Escoffre et al. reported the time-course of gene silencing in mice after intramuscular injection of shRNA-expressing pDNA followed by electroporation [32]. Here, one of the two types of shRNA-expressing pDNA with different numbers of CpG motifs was co-administrated with a pDNA encoding reporter gene (target for shRNA). The authors found little difference in the gene silencing effects between the two types of shRNA-expressing pDNAs. As they did not investigate the gene silencing effects on any endogenous genes, further studies are required to confirm the effect of CpG motifs in shRNA-expressing pDNA on its gene knockdown effect.

3. Comparison of shRNA-expressing vectors and siRNA

Although the characteristics of gene silencing by shRNA-expressing pDNA are affected by a variety of factors, such as the type of promoter, shRNA-expressing pDNAs share common characteristics which are distinct from those of siRNA. Both shRNA-expressing pDNAs and siRNA have relative advantages and disadvantages. In this section, the advantages and disadvantages of these nonviral RNAi methods are discussed.

3.1. Molecular characteristics

siRNA is a short (usually 19–30 bp) double strand RNA while shRNA-expressing pDNA is a relatively large (usually more than 2000 bp) double strand DNA. Therefore, shRNA-expressing pDNA usually has a molecular weight that is about 100-fold greater than siRNA. Both of them are negatively charged hydrophilic molecules so that they have difficulty in crossing negatively charged hydrophobic cellular membranes.

siRNA, a double strand RNA, is relatively stable compared with single strand RNAs [33]. When incubated with serum, both siRNA and pDNA can be detected by agarose gel electrophoresis for about 2 h [34,35], although there are some differences in the stability among experiments [36,37]. The results of previous studies suggest that there are small differences in the stability of siRNA and shRNA-expressing pDNA in biological environments. Chemical modification of siRNA is an effective approach to greatly increasing its stability without decreasing knockdown efficiency [38–40].

3.2. Duration of knockdown effect

As the gene-silencing effect of siRNA and shRNA-expressing pDNA is transient, attention should be paid to the duration of the RNAi effect. For a quantitative comparison of the duration of effect of siRNA and shRNA-expressing pDNA, we analyzed the time-course of the gene silencing effects of siRNA and shRNA-expressing pDNA using moment analysis [41]. Moment analysis of the time-course data showed that the gene-silencing effect induced by shRNA-expressing pDNA was significantly longer than that induced by siRNA [15]. McAnuff et al. compared the potency of siRNA and shRNA-expressing pDNA-mediated gene-silencing in vivo by co-administration of siRNA or shRNA-expressing pDNA with pDNA encoding a target reporter gene [42]. The extent of the reduction in the target gene expression was almost identical between siRNA and shRNA-expressing pDNA at 1–3 days after administration. The expression of the reporter gene seemed to be transient, so that it is difficult to conclude whether these compounds are effective and comparable with each other for longer than 3 days. To our knowledge, there are no studies in which the time-courses of gene silencing of endogenous genes by siRNA and shRNA-expressing pDNA are compared. Therefore, further research regarding the comparison of the duration of effect between siRNA and shRNA-expressing pDNA needs to be performed.

3.3. In vitro transfection efficiency

siRNA forms an RNA-induced silencing complex (RISC) in the cytosol to degrade target mRNA, so that siRNA can exert its gene-silencing effect as long as siRNA reaches the cytosol of target cells [43]. However, shRNA-expressing pDNA needs to be delivered to the nucleus of target cells in order to transcribe shRNA, which is transported to the cytosol where it is processed to siRNA (Fig. 1). Gene therapy studies have demonstrated that nuclear delivery is one of the biggest challenges in transfection [44]. Therefore, siRNA has a great advantage in delivery efficiency compared with shRNA-expressing pDNA. Although there have been few studies directly comparing the transfection efficiency of siRNA and shRNA-expressing

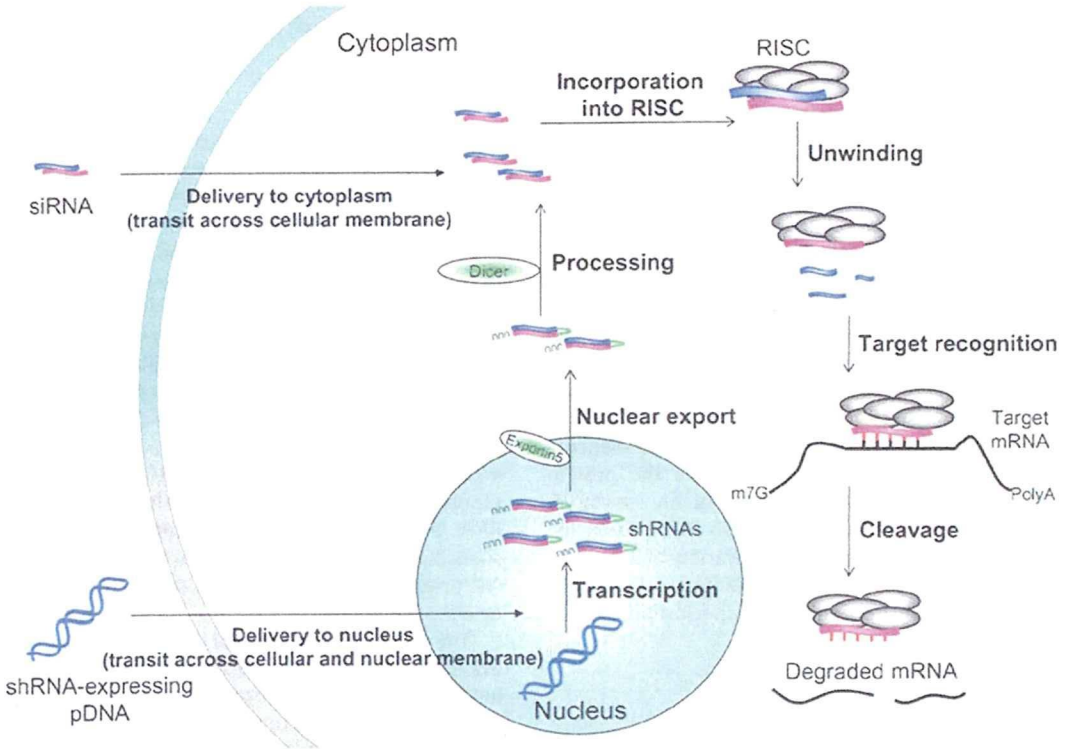

Fig. 1. Schematic image of gene silencing by siRNA and shRNA-expressing pDNA. As the RNAi effect is limited in the cells that have siRNA, both siRNA and shRNA-expressing pDNA should be delivered to the target cells. shRNA-expressing pDNA should be delivered to the nucleus to transcribe shRNA while siRNA does not have to be delivered to the nucleus but should be delivered to the cytoplasm. shRNA-expressing pDNA that reaches the nucleus transcribes multiple shRNAs, which are transported from the nucleus to the cytoplasm by a transporter protein exportin 5. shRNA transported to the cytoplasm is processed by Dicer to produce siRNA. siRNA in cytoplasm is incorporated into RNA-induced silencing complex (RISC). RISC releases the passenger strand (complementary strand to the guide strand which is complementary to target mRNA) and the released passenger strand is degraded. Then, RISC recognizes target mRNA which has a complementary sequence to guide the strand and cleave the mRNA.

pDNA, it should be pointed out that siRNA has a great advantage in being able to transfect poorly proliferating cells in which the nuclear entry of shRNA-expressing pDNA is limited by the nuclear envelope. For example, successful target gene knockdown in cells such as bone marrow-derived dendritic cells [45] and primary T lymphocytes [46], for which transfection with pDNA is difficult because of low permeability in their nuclear envelope, has been reported with siRNA.

Even in the case of tumor cells, which are actively proliferating cells, their nuclear envelope is still a major barrier. In our study investigating the effectiveness of RNAi in suppressing the expression of P-glycoprotein, multi drug resistance (MDR) protein, we transfected a colon cancer cell line with synthetic siRNA or shRNA-expressing pDNA [47]. In that study, transfection of siRNA suppressed MDR mRNA expression more than shRNA-expressing pDNA did. As the target sites for siRNA and shRNA-expressing pDNA used in this study are the same, this difference in knockdown efficiency is mainly due to the difference in transfection efficiency.

3.4. In vivo transfection efficiency

Several studies regarding the delivery efficiency of siRNA and shRNA-expressing pDNA to the liver by the hydrodynamic injection method, which is a highly efficient in vivo delivery method to the liver, reported that fluorescence-labeled siRNA is delivered to about 60–70% of hepatocytes [48,49]. On the other hand, hydrodynamic injection of pDNA encoding β-galactosidase gene resulted in the expression of the reporter gene in 20–40% of liver cells [50,51]. Therefore, the delivery efficiency of siRNA seems to be higher than that of shRNA-expressing pDNA as far as delivery of these compounds to the liver by the hydrodynamic injection method is concerned. We examined the gene-silencing of MDR genes in mouse liver after hydrodynamic injection of siRNA and shRNA-expressing pDNA on MDR expression in the liver [47]. Administration of siRNA and shRNA-expressing pDNA reduced MDR mRNA expression to about 30% and 50% of the control value, respectively. As the gene-silencing effect cannot exceed 100%, this result indicates that at least 70 and 50% of cells in the liver received siRNA and shRNA-expressing pDNA, respectively. Therefore, the delivery efficiency of siRNA is higher than that of shRNA-expressing pDNA. These results indicate that the delivery efficiency of siRNA might be slightly higher than that of shRNA-expressing pDNA in vivo.

4. Factors affecting therapeutic effectiveness of RNAi-based gene-silencing

To apply RNAi-based gene-silencing as a therapeutic treatment, the target genes and target cells should be primarily selected on the basis of the target disease. Then, properly designed siRNA or shRNA-expressing pDNA should be delivered to the target cells. To obtain a biological effect (i.e. therapeutic effect) by RNAi, the gene-silencing effect should be maintained long enough to reduce the protein expression. Although RNAi-based gene-silencing is highly specific, it may exert non-specific effects, such as inflammation and cell toxicity. In addition, if the silenced gene product exhibits a variety of functions, RNAi-based gene-silencing may result in unwanted side effects. Therefore, these factors are very important in performing RNAi-based therapeutic treatments.

4.1. Selection of target genes and cells

The selection of target genes and cells is the key factor that determines what happens following RNAi-based gene-silencing. The effectiveness of RNAi-based gene-silencing has been proven in a variety of experimental therapeutic models by targeting many types of genes and cells. In such models, cancer is the most frequently treated disease. In this section, we will discuss how to select target genes and target cells for performing RNAi-based therapy, taking cancer as a model disease.

In the process of cancer development and progression, many genes play a variety of roles, and are targeted to perform RNAi-based cancer therapy. Most studies have targeted the genes that are expressed in tumor cells and that are related to tumor cell survival, proliferation or metastasis. Inhibition of tumor growth in vivo has been achieved by administration of shRNA-expressing pDNA targeting tumor genes such as β-catenin, hypoxia-inducible factor (HIF)-1, epidermal growth factor receptor (EGFR), and human ether-à-go-go (eag) related gene (herg) [52–54]. In these studies, shRNA-expressing pDNA was delivered to tumor cells and the growth of tumor tissue in vivo was suppressed by silencing the expression of target genes that are related to tumor cell survival and proliferation. As shown by these studies, tumor cells are typical target cells when performing RNAi-based cancer therapy.

In addition to this basic strategy, we have been recently demonstrated that not only tumor cells but also normal cells can be target cells in cancer therapy if an appropriate gene is selected as a target gene [55]. In that study, HIF-1, a ubiquitously expressed heterodimeric transcription factor composed of a constitutively expressed β subunit and an oxygen-regulated α subunit, was selected as a target gene to suppress tumor growth in the liver. Activation of HIF-1 transcribes the genes that are related to tumor cell metastasis and angiogenesis such as vascular endothelial growth factor and matrix metalloproteinases. As we found that HIF-1α expression was increased in normal liver cells as well as cancerous cells in the process of tumor metastasis to the liver, HIF-1α expression in not only tumor cells but also normal liver cells was investigated. As a result, silencing HIF-1α expression in normal liver cells as well as in tumor cells in the liver by shRNA-expressing pDNA significantly reduced the number of tumor cells after the establishment of a hepatic metastasis model. Our study demonstrated that both tumor and normal cells can be targets for RNAi-based anti-cancer treatment if we choose an appropriate target gene, which can be one approach used to increase the efficiency of cancer therapy.

4.2. Delivery to target cells

The RNAi-mediated gene-silencing effect is limited in the cells reached by RNAi effectors, which makes the delivery of RNAi effectors to target cells important in achieving RNAi-based therapeutic treatment. Lots of in vivo delivery methods of RNAi effectors have been developed, including those developed and optimized for the delivery of pDNA for gene therapy. Although these methods allow the delivery of not only pDNA but siRNA to target cells, such as liver cells, special attention should be paid to how many cells are delivered or transfected with shRNA-expressing pDNA or siRNA, something which is not so important for gene delivery of secretory proteins [56]. Therefore, efficient delivery methods developed in gene therapy studies cannot always be applicable to the delivery of shRNA-expressing pDNA or siRNA. Early studies on in vivo RNAi often used the expression of reporter genes that were expressed from vectors co-administered with RNAi effectors [10,57,58]. In these models, the evaluated gene-silencing effects reflect only those in the cells that are reached by the vector-expressing reporter gene, so that the extent of the delivery may be over-estimated.

The extent of delivery of RNAi effectors can be quantitatively evaluated in transgenic mice that express reporter genes, such as firefly luciferase, or in cells that stably express reporter genes. In a previous study, we developed a tumor cell clone that stably expresses firefly and renilla luciferases and constructed a system to allow quantitative evaluation of the efficiency of gene-silencing [59]. This system enables us to estimate the gene-silencing effect by simple luciferase assay. This system was useful in developing methods for delivery of RNAi effectors to tumor cells in vivo. We found that target gene expression in subcutaneous tumor tissues was suppressed by an intratumoral

injection of RNAi effectors followed by electroporation to about 20% of the control value. Similar to our study, many other studies have demonstrated suppression of tumor cell growth by efficient RNAi induction in tumor tissue by intratumoral injection [60–63].

In contrast to relatively efficient in vivo RNAi induction after local administration of siRNA or shRNA-expressing pDNA, a few studies have reported successful induction of gene-silencing in target cells after systemic administration of shRNA-expressing pDNA. Zhang et al have reported that shRNA-expressing plasmids, which are encapsulated in the interior of 85 nm pegylated immunoliposomes (PILs), can suppress the gene expression in tumor cells intracranially inoculated into the brain [53, 64]. They found using tumor cells stably expressing luciferase reporter genes that their delivery method was effective in reducing luciferase expression in tumor cells in the brain. An intravenous injection of shRNA-expressing pDNA targeting EGFR encapsulated in PIL resulted in the reduction of EGFR expression in tumor cells and an 88% increase in the survival time of mice with advanced intracranial brain cancer. However, all the mice treated with EGFR knockdown died less than 35 days after tumor implantation. Such an incomplete therapeutic effect may be because the delivery efficiency was not high enough so that there was remaining EGFR expression in the brain or because tumor cells might be able to survive without EGFR expression.

To our knowledge, none of the published studies has achieved a radical cure for cancer by using the RNAi-based gene-silencing effect, and this may be because it is very difficult to deliver RNAi effectors to all the tumor cells in vivo. This can be a major problem in performing RNAi-based cancer therapy because tumor tissues can grow again even after most of cells die following the delivery of RNAi effectors as long as some tumor cells survive. Without solving the delivery issue, RNAi-based gene-silencing alone can hardly be an effective treatment.

4.3. Time-course of gene silencing

After efficient introduction of RNAi effectors to target cells, the expression of a protein of interest is reduced by degrading the targeted mRNA. As RNAi degrades target mRNA, not protein, the stability (half-life) of target protein is an important factor determining the gene silencing effect, which correlates with the amount of target protein [58]. As RNAi effectors are readily degraded and their effective concentration in the cells decreases with cell division, the expression level of the target gene will return to normal. Therefore, the gene-silencing effect by RNAi is temporary and its duration is determined by various factors, such as the stability of pDNA or siRNA and the proliferation rate of target cells [58,65]. When applying RNAi-based gene silencing as a therapeutic treatment, the gene-silencing effect needs to be maintained at least until the therapeutic effect is obtained. In addition, maintaining the gene-silencing effect after the cure can sometimes protect the patient from the recurrence of that disease. On the other hand, induction of RNAi should be stopped in the situation where it causes side effects. Therefore, regulation of the time-course of RNAi-based gene silencing is important to achieve effective and safe RNAi-based therapeutic treatment. Despite the importance of the time-course of gene silencing, few studies have investigated the duration of the RNAi effect. In particular, there are few reports describing the duration of the gene-silencing effect on the endogenous target gene compared with the relatively abundant number of studies investigating the time-courses of gene silencing of reporter transgenes [10,32,57,58,66,67]. In these studies, the gene-silencing effect was observed more than 100 days after co-administration of shRNA-expressing pDNA with a reporter construct targeted by RNAi [32,67]. In most of these studies, the silenced transgene expression did not return to the control level, which may be because of the fact that transgene expression decreases with time. As endogenous genes are actual target genes for RNAi-based therapy and their expression is much more stable than those of transgenes, additional studies should be performed regarding the time-course of the gene-silencing effect on endogenous gene expression in vivo.

4.4. Specificity of the effect

Even although RNAi-based gene silencing is reported to be highly specific, an RNAi-based therapeutic model has encountered unexpected problems involving by various mechanisms such as an inflammatory response including interferon response [68–70], saturation of endogenous micro RNA (miRNA) pathway [27,71] and undesirable effects of the target gene [72]. These undesirable effects can be a significant problem for RNAi-based therapy.

Since the discovery of siRNA, the inflammatory response against siRNA and its vector is one of the major concerns in using RNAi because such a non-specific response can lead to a misunderstanding of the results obtained by RNAi induction. To date, it has been reported that the inflammatory response against siRNA is dependent on a variety of factors including the sequence, length and amount of siRNA, the delivery method and the cell type [73,74]. Recently, Kleinman et al. demonstrated the sequence- and target-independent suppression of choroidal neovascularization (CNV) in an age-related macular degeneration model by siRNA [69]. In their study, they used siRNA targeting vascular endothelial growth factor-A (VEGFA) or its receptor VEGFR1 (also called FLT1) to suppress CNV progression. However, they found that all the siRNAs used, even negative control siRNAs, were effective in inhibiting CNV irrespective of their sequence. They found that this sequence non-specific inhibition was mediated by the recognition of siRNA by the Toll-like receptor-3 (TLR3). Another recent report by Robbins et al. also demonstrated that immunostimulatory siRNA can exert an effect that is independent of the target gene [68]. By using chemically modified siRNA which has no immunostimulatory effect, they showed that the antiviral effect of siRNA against influenza in vivo was not due to the sequence-specific degradation of viral RNA but mainly due to the immunostimulatory effect of siRNA, although the siRNA could exert antiviral effects in vitro independent of its immunostimulatory effect. Therefore, these reports indicate the necessity of anticipating, monitoring and preparing adequate controls for siRNA-mediated immune stimulation and that particular caution is required in interpretation of the results of therapeutic RNAi in vivo. As far as the plasmid DNA vector is concerned, the effect on RNAi induction has not been reported although inflammatory responses induced by CpG motif/TLR9-dependent and CpG motif-independent mechanisms could affect the RNAi effect by the vector [75–78]. When RNAi is being used to treat cancer or infectious diseases, innate immune response to RNAi effectors would be beneficial to obtain therapeutic effects. However, attention should be paid to such non-specific innate immune responses, because they may cause serious adverse reactions.

Regarding viral vector-based RNAi, fatalities in mice due to the saturation of the miRNA pathway by long-term expression has been reported using adeno-associated virus type 8 (AAV8) vector expressing shRNA [27]. The authors evaluated 49 distinct types of AAV/shRNA vectors, unique in length and sequence and directed against six targets, and found that 36 resulted in dose-dependent liver injury, with 23 ultimately causing death and found that morbidity was associated with the reduction in the amount of liver-deriver miRNA. Recent studies demonstrated that processing of shRNA is a saturable step. Overexpression of exportin-5 or Dicer, important molecules in the shRNA/miRNA processing, accelerates processing of shRNA, leading to high knock-down effects [79,80]. In addition, a recent study by Boudreau et al. reported that transfection of shRNA-expressing pDNA resulted in the accumulation of a large amount of immature shRNA in the cells [81]. As overexpression of shRNA competitively inhibits intracellular processing of miRNA, which could cause toxic side effects [27,71], saturation of the miRNA/shRNA pathway should be monitored carefully when shRNA-expressing vector is used. Even although RNAi is successfully induced without any non-specific effect, target-specific

gene silencing might cause unwanted side effects. β-Catenin regulates the activity of certain transcription factors, T cell factor/lymphoid enhancer factor in the Wnt pathway, which activates the transcription of genes related to cell growth and survival. As it has been shown that β-catenin plays an important role as an oncogene to promote tumor cell growth, β-catenin expression in tumor cells can be silenced to inhibit tumor growth [52,82–84]. However, in our recent study using shRNA-expressing plasmid vector, we found that silencing β-catenin gene expression promoted lung metastasis of melanoma cells despite the fact that it suppressed the growth of primary tumor tissue [71]. Detailed investigation suggested that silencing β-catenin gene expression promoted lung metastasis by increasing cell dissociation from the primary tumor tissue and cell mobility by reducing the amount of cadherin protein, a cell adhesion molecule which associates with β-catenin, in tumor cells. These findings raise a serious concern for the use of the suppression of β-catenin expression in tumor cells as an anticancer treatment, because the few cells surviving after treatment are likely to become more malignant as far as their metastatic properties are concerned. A similar problem might occur with other target genes. Therefore, the biological events following knockdown of the target gene should also be carefully evaluated when developing RNAi-based therapy.

5. Conclusion

RNAi has rapidly been established as an experimental tool and is expected to be used as a therapeutic treatment for various diseases. Besides siRNA, shRNA-expressing pDNA is also a promising candidate for RNAi-based therapeutic treatment. As shRNA-expressing pDNA and siRNA possess advantages and disadvantages, they should be chosen on a case-by-case basis. There are still difficulties in the successful therapeutic application of RNAi. However, considering the pace of new findings and developments in the application of RNAi, we believe that these problems will be solved and that RNAi will become a major therapeutic treatment in the near future.

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HCV Genetic Elements Determining the Early Response to Peginterferon and Ribavirin Therapy

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Key Words
Full open reading frame analysis · Hepatitis C virus · Peginterferon/ribavirin therapy

Abstract
The aim of this study was to search hepatitis C virus (HCV) genetic elements determining the early response to peginterferon/ribavirin therapy using HCV genome-wide analysis. From a total of 88 chronic hepatitis C patients with HCV-1b treated with peginterferon/ribavirin, the whole HCV amino acid sequence was determined and analyzed according to the viral response during the treatment. Mutations in NS5A-ISDR (interferon sensitivity-determining region) are associated with rapid viral response at week 4, and the core arginine70glutamine (R70Q) mutation is associated with no early viral response at week 12, revealing that core 70 and NS5A are the most important factors determining the virological kinetics during peginterferon and ribavirin therapy.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, and worldwide 170 million people are infected with HCV. With the introduction of the recent combination therapy of pegylated-interferon (PEG-IFN) and ribavirin (RBV), half of patients can eradicate the virus (sustained virological response, SVR). The SVR rate of HCV to the PEG-IFN/RBV therapy is dependent on HCV genotypes, and the viral kinetics during the treatment strongly affect the final viral clearance [1, 2]. It is generally considered that HCV structures affect the treatment response since the SVR rate to PEG-IFN/RBV therapy depends upon viral genotypes as described above. However, comprehensive analysis of the contribution of HCV structures to different responses has not yet been conducted. In the present study, in order to clarify the relationship between HCV sequences and viral responses, we have determined the complete HCV open reading frame sequences obtained from pretreatment patients' serum, and investigated their response by searching for HCV genetic elements determining the early response to PEG-IFN/RBV therapy using HCV genome-wide analysis.

Methods

A total of 88 chronic hepatitis C patients with HCV-1b treated with PEG-IFN/RBV were studied. From pretreatment sera, the whole HCV deduced amino acid sequence (3,010 amino acids) was determined in each patient by direct RT-PCR.

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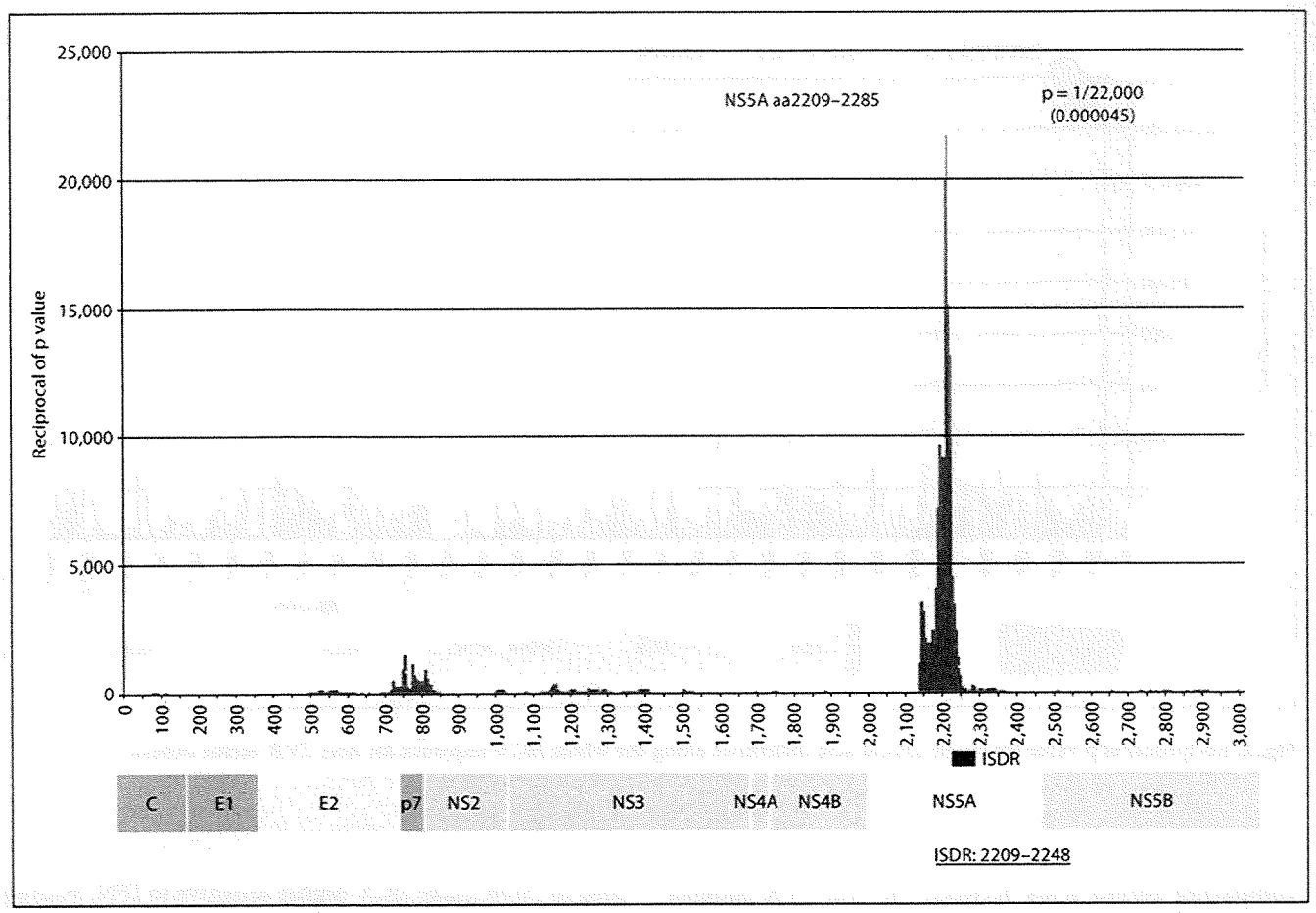

Fig. 1. Reciprocal of p value for sliding window analysis with 77 amino acid width for RVR versus others.

Amino acid usage of each of the 3,010 positions was compared according to the different virological response in order to identify the single amino acid differences determining the virological response. In addition, sliding window analyses were carried out in order to identify the amino acid region associated with the virological response. The number of the amino acid changes in the fixed stretch of the sequence (window: 2–100 amino acids) were compared according to the virological response, scanning the whole HCV amino acid sequence by sliding this window one by one.

Results

Of 88 patients studied, 9 showed rapid viral response (RVR; HCV-RNA undetectable at week 4) and 71 showed early viral response (EVR; over 2-log drop of HCV-RNA at week 12). The other 17 patients showed no EVR, indicating these patients are highly resistant to the treatment.

Mutations in the region overlapping NS5A-ISDR (interferon sensitivity-determining region, aa2209–2248) are associated with the good response to PEG-IFN/RBV therapy as shown in sliding window analysis comparing RVR patients at week 4 and others (fig. 1). In contrast, the core R(arginine)70Q (glutamine) mutation is associated with a poor response resulting in no EVR at week 12 by single amino difference analysis comparing non-EVR patients and the others (fig. 2).

Discussion

In the present study, using a sliding window analysis comparing all HCV amino acids, the amino acid region located in ISDR was extracted as the most significant region discriminating the RVR and non-RVR patients. By

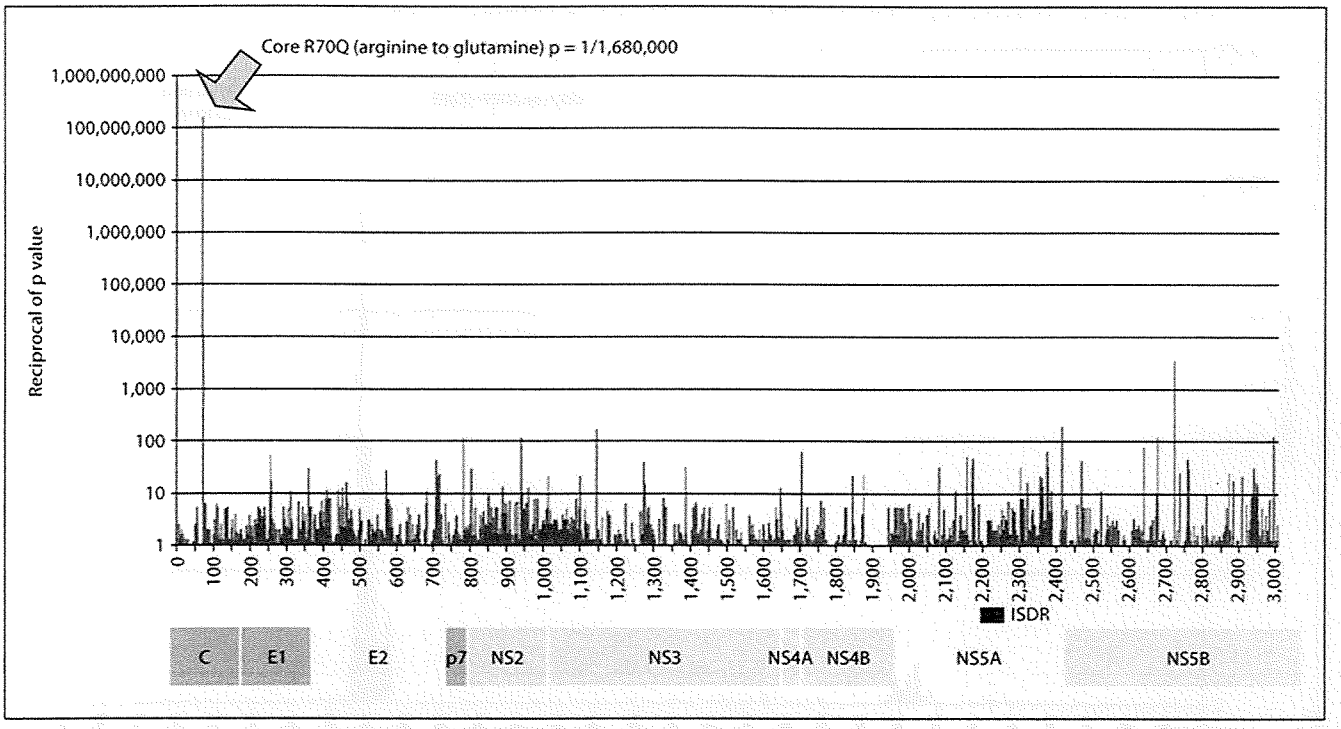

Fig. 2. Reciprocal of p value for single amino acid difference along the whole HCV sequence for non-EVR versus others.

comparing amino acids between the non-EVR patients and the others, remarkable differences were clustered in a single amino acid polymorphism in the core 70. Recent studies have proven that the initial viral response at week 4 and week 12 of the PEG-IFN/RBV therapy could be a useful predictor of the final outcome, indicating that the present findings are important for predicting treatment outcome and individualizing the treatment regimen for each patient as well as understanding the mechanism of diverse response to PEG-IFN/RBV therapy.

ISDR was first identified as the region significantly related to SVR in the era of IFN monotherapy in Japanese patients [3, 4]. 'Mutant type', meaning 4 or more mutations in the region, was associated with high SVR rate, while the rate was low in the 'intermediate type' (1–3 mutations) and wild type (no mutation). Though there were controversies as to the predictive value of ISDR, since studies in Europe and in North America did not necessarily reproduce evident correlation between ISDR and SVR, a recent meta-analysis proved its value by demonstrating a clear relationship all over the world, even in Western countries [5]. The present study reproduced the significance of ISDR in PEG-IFN/RBV therapy. Mutations in ISDR make HCV highly sensitive to IFN, leading to RVR. Current guidelines indicate that RVR patients with low viral load before treatment can be treated with 24 weeks instead of the standard 48 weeks of therapy. Since most ISDR mutant patients show low viral loads, these easy-to-treat patients in genotype 1b should be mainly infected with HCV with ISDR mutations, suggesting ISDR genotyping would identify the patients treatable with the abbreviated regimen.

On the other hand, in the present study, the polymorphism of core 70 was extracted as the most significant position to determine poor virological response in 12 weeks (non-EVR). The contribution of core region amino acid polymorphism in resistance to (PEG-)IFN/RBV therapy was previously reported by Akuta et al. [6], who first found that the polymorphisms in a combination of core 70 and 91 were closely related to the final outcome. The importance of core 70 polymorphism alone, however, was considered rather weak in their study for its smaller p value. Their end point was the final outcome of the treatment, which could be influenced by a variety of factors other than viral genetics, such as host factors (age, sex, fibrosis, body weight, etc.) and treatment (dose of