Sustained Exogenous Expression of Therapeutic Levels of IFN-γ Ameliorates Atopic Dermatitis in NC/Nga Mice via Th1 Polarization

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The short in vivo half-life of IFN-γ can prevent the cytokine from inducing immunological changes that are favorable for the treatment of Th2-dominant diseases, such as atopic dermatitis. To examine whether a sustained supply of IFN-γ is effective in regulating the balance of Th lymphocyte subpopulations, plasmid vector encoding mouse IFN-γ, pCpG-Muγ, or pCMV-Muγ was injected into the tail vein of NC/Nga mice, a model for human atopic dermatitis. A single hydrodynamic injection of a CpG motif reduced pCpG-Muγ at a dose of 0.14 μg/mouse resulted in a sustained concentration of IFN-γ in the serum, and the concentration was maintained at >300 pg/ml over 80 d. The pCpG-Muγ–mediated IFN-γ gene transfer was associated with an increase in the serum concentration of IL-12, reduced production of IgE, and inhibition of mRNA expression of IL-4, -5, -10, -13, and -17 and thymus and activation-regulated chemokine in the spleen. These immunological changes were not clearly observed in mice receiving two injections of 20 μg pCMV-Muγ, a CpG-replete plasmid DNA, because of the transient nature of the expression from the vector. The mice receiving pCpG-Muγ showed a significant reduction in the severity of skin lesions and in the intensity of their scratching behavior. Furthermore, high transepidermal water loss, epidermal thickening, and infiltration of lymphocytes and eosinophils, all of which were obvious in the untreated mice, were significantly inhibited. These results indicate that an extraordinary sustained IFN-γ expression induces favorable immunological changes, leading to a Th1-dominant state in the atopic dermatitis model. ***The Journal of Immunology*****, 2010, 184: 2729–2735.**

The number of patients with allergies, including those with asthma, pollinosis, and atopic dermatitis, has been increasing in recent decades, especially in developed countries. It is believed that these disorders result from the imbalance of Th lymphocyte subpopulations (Th1 and Th2), which play major roles in the immune response (1). Under normal conditions, the differentiation of naive T cells to Th1 and Th2 lineages is regulated by cytokines that are secreted from various cells, including themselves, and the Th1/Th2 balance is maintained. However, in atopic dermatitis, which is one of the most common type I allergic diseases, the balance shifts to Th2 dominance; this eventually leads to excessive Th2 cytokine production. Th2-like immune responses play an important role in the pathogenic mechanism of atopic disorders, because Th2 cytokines mediate excessive IgE production, a major cause of atopic inflammation (2–5).

IFN-γ, a Th1 cytokine, inhibits the differentiation of naive T cells to Th2 cells, as well as the production of Th2 cytokines from Th2 cells. Thus, IFN-γ has been considered to be capable of correcting the Th1/Th2 imbalance and is effective in the treatment of diseases in which the balance is impaired, such as atopic dermatitis (6). Despite such positive features, few attempts have been made to use IFN-γ as a pharmaceutical agent for the treatment of atopic dermatitis (7–10). This is mainly due to the fact that IFN-γ, as well as other IFNs, has a short half-life in vivo, and multiple injections are required to maintain its concentration at levels high enough to prevent dermatitis (11).

Several approaches have been developed to extend the duration of the therapeutic effects of biologically active proteins. Extension of the in vivo half-life of proteins can be achieved by using controlled- or sustained-release systems (12–15) or by chemical modification of proteins (16–18). Pepinsky et al. (19) demonstrated that the high clearance of IFN-β-1a was reduced by PEGylation, and its increased systemic exposure resulted in better antiviral effects. PEGylated IFN-α in combination with an antiviral drug, ribavirin, is now a standard treatment for hepatitis C virus-induced chronic hepatitis. The increased half-life of IFNs obtained by PEGylation has greatly increased their therapeutic efficacy. In addition to these challenges, gene delivery is an option to increase the in vivo half-life of therapeutic proteins, including IFNs. In previous studies, we proved that the depletion of CpG motifs in plasmid vectors is an effective approach for extending the duration of transgene expression (20, 21). We also succeeded in developing a murine IFN-γ–expressing plasmid DNA, pCpG-Muγ, which contains no CpG motifs except for those in the cDNA region (22). A single i.v. injection of pCpG-Muγ resulted in a high and sustained IFN-γ concentration in the serum over 1 mo after hydrodynamic injection into healthy ICR mice. However, little is

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Abbreviations used in this paper: SPF, specific pathogen-free; TARC, thymus and activation-regulated chemokine; TEWL, transepidermal water loss; TNCB, 2,4,6-trinitrochlorobenzene.

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known about how such a sustained transgene expression of IFN-γ influences the Th1/Th2 balance under Th2-dominant conditions.

In this study, we injected pCpG-Muγ, a murine IFN-γ–expressing plasmid DNA, into a human atopic dermatitis model (NC/Nga mice) (23), to achieve a sustained transgene expression of IFN-γ. A conventional CpG replete plasmid vector expressing IFN-γ, pCMV-Muγ (21, 24), was also used for comparison to examine the importance of the duration of transgene expression on the immunological changes induced by IFN-γ gene transfer. The expression profile of IFN-γ was first examined in NC/Nga mice, and the effect of the expression on the level of IL-4, -5, -10, -12, -13, -17, and thymus and activation-regulated chemokine (TARC) was evaluated. Then, skin lesions, the intensity of scratching behavior, transepidermal water loss (TEWL), the thickness of the epidermis, and the infiltration of the skin by inflammatory cells were evaluated. In this study we showed that sustained, but not transient, gene expression of IFN-γ can induce favorable immunological changes in a human atopic dermatitis model, which allows the prevention of the development of atopic dermatitis-like skin lesions.

Materials and Methods

Animals

Five-week-old male C57BL/6 mice and 6-wk-old male NC/Nga mice that were raised under conventional conditions, but had not developed dermatitis, were purchased from Japan SLC (Hamamatsu, Japan) and maintained on a standard food-and-water diet under conventional housing conditions. The protocol for the animal experiments was approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

Plasmid DNA

Two types of IFN-γ–expressing plasmid vectors developed in our laboratory were used: pCMV-Muγ, which was constructed by inserting a murine IFN-γ cDNA fragment into the BamHI site of pcDNA3 (Invitrogen, Carlsbad, CA) (24), and pCpG-Muγ, which was constructed by inserting the BglII/NheI IFN-γ cDNA fragment amplified by PCR from the pCMV-Muγ into the BglII/NheI site of pCpG-mcs (Invivogen, San Diego, CA) (22).

In vivo gene transfer of IFN-γ

Naked plasmid DNA dissolved in isotonic saline solution was injected into the tail vein of mice over 5 s on day 0 (25, 26). To adjust the peak level of the IFN-γ concentration after gene transfer, the plasmid dose was fixed at 0.14 and 20 μg/mouse for pCpG-Muγ and pCMV-Muγ, respectively, based on preliminary experiments. pCMV-Muγ was injected twice at an interval of 1 wk (days 0 and 7). The body weight and temperature of mice were measured to assess the adverse effects of IFN-γ gene transfer.

Measurement of concentration of IFN-γ, IgE, and IL-4, -12, and -13

Blood samples were obtained from the tail vein at indicated times after gene transfer, incubated at 4°C for 2 h to allow clotting, and centrifuged to obtain serum. Dorsal skin tissue was homogenized in PBS containing protease inhibitors (protease inhibitor mixture; Sigma-Aldrich, Munich, Germany) and then centrifuged for 30 min at 12,000 × *g*. The concentration of IFN-γ, IgE, and IL-4, -12, and IL-13 in the serum or supernatant of skin homogenate was measured using ELISA kits (Ready-SET-Go! Mouse IFN-γ and IL-13 ELISA, eBioscience, San Diego, CA; OptEIA set Mouse IL-12, IgE and IL-13, BD Biosciences, San Jose, CA).

mRNA quantification

Total RNA was extracted from ~100 mg spleen or skin sample using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). The total RNA was cleaned up using an RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed using a SuperScript II (Invitrogen) and oligo (dT) primer, according to the manufacturer's protocol. For a quantitative analysis of mRNA expression, real-time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The oligonucleotide primers used for amplification are listed in Table I. Amplified products were detected online via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics, Indianapolis, IN). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 s, 60°C for 5 s, and 72°C for 20 s. All cycling reactions were performed in the presence of 3.5 mM $MgCl_2$. Gene-specific fluorescence was measured at 72°C. The mRNA expression of genes of interest was normalized using the mRNA level of β-actin.

Scoring skin lesions

Skin lesions were scored at indicated times after gene transfer, according to the criteria of Matsuda et al. (23). The scoring was based on the severity of eczema, erosion/excoriation, scaling, erythema/hemorrhage, inflammation of the face, and inflammation of the ear. The total clinical skin severity score was defined as the sum of each of the six signs (none = 0; mild = 1; moderate = 2; and severe = 3).

Observation of scratching behavior

On days 7, 14, and 35, scratching behavior was monitored using SCLABA Real (Noveltec, Kobe, Japan), an automated system to analyze the scratching behavior of small animals. Each mouse was put into an acrylic cage, and the behavior of the mice was recorded for 30 min. The number of episodes and the duration of scratching behavior were automatically quantified. In a different set of mice, the scratching behavior was recorded on video for 1 h on days 7, 14, 35, and 84. The videotape was played back at a later time, and the number of scratching episodes was counted manually. A series of scratching behaviors, starting with the stretching of the hind paws to the head, face, or back and ending with the set-back of the paws, was counted as one bout of scratching (27).

Measurement of TEWL

TEWL was measured using a VAPO SCAN (AS-VT 100RS, Asahi Biomed, Yokohama, Japan) on the shaved back of mice.

Analysis of skin sections

The dorsal skin of the mice was excised, fixed in 4% paraformaldehyde, and embedded in paraffin. Then, 4 μm-sections were made using a microtome and stained with H&E for histological evaluation or with toluidine blue to detect mast cells. The numbers of lymphocytes, eosinophils, and mast cells on H&E-stained sections (lymphocytes and eosinophils) or toluidine blue-stained sections (mast cells) were manually counted under a microscope and expressed as the number per unit length of skin section.

Effect of IFN-γ gene transfer on 2,4,6-trinitrochlorobenzene-induced dermatitis

To assess whether IFN-γ gene transfer increases the risk for contact dermatitis, C57BL/6 mice were sensitized by painting 25 μl 3% 2,4,6-trinitrochlorobenzene (TNCB) in acetone/olive oil (4:1) on the shaved abdomen (day 0). pCpG-Muγ was injected by the hydrodynamic injection method to TNCB-treated mice 1 d before or 7 d after the TNCB treatment, at a dose of 0.14 μg/mouse. On day 5, the thickness of the ear was measured with a Quick Mini thickness gauge (Mitutoyo, Tokyo, Japan). Then, 20 μl 1.5% TNCB solution was applied to the surface of the ear. The thickness of the ear was measured again 24 h after the second application (challenge), and ear swelling was evaluated by the difference in the thickness before and after the challenge.

Effect of IFN-γ gene transfer on TEWL in tape stripping- and TNCB-induced dermatitis models

C57BL/6 mice were shaved on their dorsal skin on day 0 and injected with pCpG-Muγ (0.14 μg/mouse) or saline by the hydrodynamic injection method on the same day. Mice were repeatedly treated with 50 μl 1% TNCB dissolved in acetone/olive oil (4:1) to the shaved skin on days 1, 8, 11, and 13. In a separate experiment, the dorsal skin of C57BL/6 mice was tape stripped on days 1 and 8 and injected with pCpG-Muγ (0.14 μg/mouse) or saline by the hydrodynamic injection method on day 0. TEWL from the stripped or shaved skin was measured as described above.

Statistical analysis

Differences were evaluated by the Student *t* test, and the level of statistical significance was $p < 0.05$.

Results

IFN-γ concentration in the serum of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

Fig. 1 shows the time courses of the concentration of IFN-γ in the serum after i.v. injection of pCpG-Muγ or pCMV-Muγ. A very high and sustained concentration of IFN-γ was detected in the

serum of mice receiving pCpG-Muγ at a dose of 0.14 μg/mouse: >300 pg IFN-γ/ml was detected ~3 mo after a single injection. However, the concentration of IFN-γ had decreased very quickly below the detection limit (25 pg/ml) 3 d after the first injection of pCMV-Muγ (20 μg/mouse). The plasmid was injected again 1 wk later, but the peak level was lower than that after the first injection, and the concentration decreased quickly again.

IL-12 concentration in the serum of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

It would be expected that a persistent expression of IFN-γ would induce the expression of IL-12, a typical Th1 cytokine that promotes the differentiation of naive T cells into the Th1 phenotype. Therefore, the serum concentration of IL-12 was measured (Fig. 2*A*). The IL-12 concentration in the untreated group was not constant during the experimental period; it fluctuated, reflecting the acute and chronic phases of the disease (28). The concentration of IL-12 was significantly increased in the pCpG-Muγ–treated group from days 3 to 42 after injection. The concentration in the pCMV-Muγ–treated group was significantly greater 3 d after the first injection compared with that in the untreated group; thereafter, the profile became superimposed on that of the untreated group. The second injection of pCMV-Muγ on day 7 barely affected the serum concentration of IL-12.

IgE concentration in the serum of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

Next, the concentration of IgE, a diagnostic marker of atopic dermatitis (29), was measured in the serum, because increased IgE production is a hallmark of Th2 immune responses. Fig. 2*B* shows the concentration of IgE in the serum of the untreated mice or mice treated with pCpG-Muγ or pCMV-Muγ. The concentration at the onset of treatment was ~10 μg/ml, and it increased with time to >100 μg/ml in the untreated group. It also increased in the treated groups, but the increase was significantly inhibited in the pCpG-Muγ–treated group at ≥14 d after the treatment. In addition, we measured the serum concentrations of IL-4 and -13, which play important roles in the regulation of IgE synthesis (30). However, the concentrations of IL-4 and -13 in the serum of all groups were below the detection limit (4 pg/ml) of the analysis.

mRNA expression of cytokine and chemokine in spleen cells of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

The effect of IFN-γ gene transfer on the expression of cytokines/chemokines was evaluated by measuring the mRNA expression of IL-12, Th2 cytokines (IL-4, -5, -10, and -13), IL-17, and a Th2 chemokine (TARC) in spleen cells collected from mice 14 d after gene transfer using the primers listed in Table I. The mRNA expression of these genes in spleen cells was normalized to that of β-actin, and the ratios were compared between the untreated and the pCpG-Muγ–treated mice (Fig. 3). The differences between the groups were very large for IL-5, -10, -12, and -13 and TARC, although they were not statistically significant because of the limited number of samples. The IL-12 mRNA expression was increased by injection of pCpG-Muγ, which was in good agreement with the serum level of IL-12 (Fig. 2*A*). The mRNA expression of IL-4, -5, -10, -13, and -17 and TARC in the pCpG-Muγ–treated group was lower than that in the untreated group. The mRNA expression in spleen cells 40 d after gene transfer showed no significant differences (data not shown). In addition, the expression of these cytokines in the skin was examined by ELISA and RT-PCR 40 d after gene transfer. However, no significant differences were detected among the groups (data not shown).

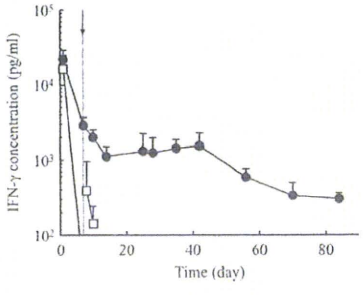

FIGURE 1. Time course of the concentration of IFN-γ in mouse serum after injection of IFN-γ–expressing plasmid DNA. NC/Nga mice were injected i.v. with 0.14 μg pCpG-Muγ (●) or 20 μg pCMV-Muγ (□) by the hydrodynamic injection method. The pCMV-Muγ–injected group received a second injection of 20 μg pCMV-Muγ 7 d after the first injection (indicated by a dashed line and an arrow). The results are expressed as the mean ± SD of five mice.

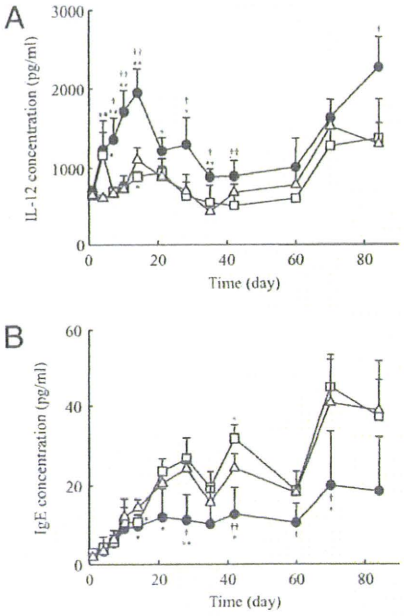

FIGURE 2. Time course of the concentration of IL-12 (*A*) and IgE (*B*) in mouse serum after injection of IFN-γ–expressing plasmid DNA. NC/Nga mice were injected i.v. with 0.14 μg pCpG-Muγ or 20 μg pCMV-Muγ, as described in the legend of Fig. 1. Blood samples from untreated (△), pCpG-Muγ–injected (●), or pCMV-Muγ–injected (□) mice were collected from the tail vein at the indicated times after gene transfer. The results are expressed as the mean ± SD of at least three mice. $*p < 0.05$ compared with the untreated group; $**p < 0.01$ compared with the untreated group; $^{\dagger}p < 0.05$ compared with the pCMV-Muγ–treated group; $^{\dagger\dagger}p < 0.01$ compared with the pCMV-Muγ–treated group.

Table I. *Primer sequences for quantitative RT-PCR*

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
Mouse β-actin	gcaccacaccttctacaatgag	ttggcatagaggtctttacgga
Mouse IL-12	catcgatgagctgatgcagt	cagatagcccatcaccctgt
Mouse IL-4	gcttttcgatgcctggattc	gctttccaggaagtctttcagtg
Mouse IL-5	agagaagtgtggcgaggagaga	cattgcccactctgtactcatca
Mouse IL-10	ttgccaagccttatcgga	ttctgggccatgcttctct
Mouse IL-13	cagctccctggttctctcac	ccacactccataccatgctg
Mouse IL-17	tccagaaggccctcagacta	agcatcttctcgaccctgaa
Mouse TARC	agtggagtgttccagggatg	gtcacaggccgctttatgtt

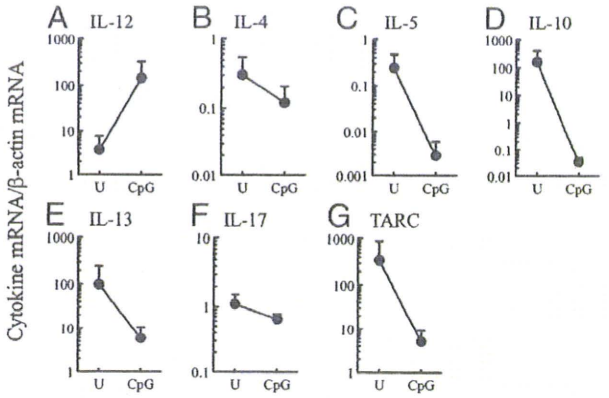

FIGURE 3. mRNA expression of cytokines and TARC in spleen cells. Spleens from NC/Nga mice receiving 0.14 μg pCpG-Muγ (CpG) were collected 14 d after gene transfer, and the mRNA expression of cytokine and chemokine genes was measured by real-time PCR. The mRNA expression of genes was normalized using the mRNA level of β-actin. In comparison, spleens from untreated mice (U) were treated as above. *A*, IL-12. *B*, IL-4. *C*, IL-5. *D*, IL-10. *E*, IL-13. *F*, IL-17. *G*, TARC. The results are expressed as the mean ± SD of three mice.

Skin lesions of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

The results indicated that the Th1/Th2 balance can be shifted to Th1 in the atopic dermatitis model by sustained, but not transient, transgene expression of IFN-γ. The effects of gene transfer on atopic dermatitis were examined in NC/Nga mice. Fig. 4 shows the typical images of the back skin of mice 35 d after the start of treatment. Clinical signs and symptoms were clearly seen on the skin of the untreated mice (Fig. 4*A*) and the pCMV-Muγ–treated mice (Fig. 4*C*), indicating that these mice developed a severe dermatitis. The severity of skin damage was scored using a clinical skin score (23). The untreated and the pCMV-Muγ–treated groups developed dermatitis within a week after the start of the experiment, and the severity of the dermatitis increased with time (Fig. 4*D*). In contrast, the pCpG-Muγ–treated mice developed much less severe skin inflammation throughout the experimental period (Fig. 4*B*), and the clinical skin score of the group was significantly lower than that of the untreated or the pCMV-Muγ–treated group (Fig. 4*D*).

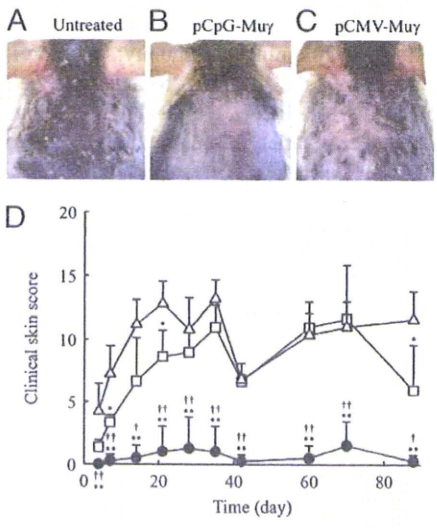

FIGURE 4. Typical images of the back skin of NC/Nga mice (*A–C*) and the time course of the skin clinical score (*D*). Photographs were taken 35 d after the start of treatment. *A*, Untreated mice. *B*, pCpG-μγ–treated mice. *C*, pCMV-μγ–treated mice. *D*, Clinical features of dermatitis were scored at indicated periods of time after the start of treatment. The results are expressed as the mean ± SD of at least three mice. $*p < 0.05$ compared with the untreated group; $**p < 0.01$ compared with the untreated group; $^{\dagger}p < 0.05$ compared with the pCMV-Muγ–treated group; $^{\dagger\dagger}p < 0.01$ compared with the pCMV-Muγ–treated group.

Scratching behavior of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

Fig. 5 shows the number of episodes of scratching and the cumulative time of scratching for a 30-min period. The number and duration of scratching episodes increased with time in the untreated group. The pCpG-Muγ– and pCMV-Muγ–treated groups had significantly fewer episodes and shorter duration of scratching than the untreated group, with significantly better results for pCpG-Muγ–treated mice. Similar results were obtained in a different set of mice whose scratching episodes were counted manually after videotaping (data not shown).

TEWL of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

Dry skin is a common symptom of atopic dermatitis, which is characterized by extensive water loss through the skin. Thus, the TEWL of the skin was measured on the back (Fig. 6). The TEWL value at day 0 was ~10 g/h/m^2 in all groups. In the untreated mice, the value increased to >50 g/h/m^2 by day 14, and an almost constant value was observed in the period that followed. The elevation of TEWL was significantly inhibited in the pCpG-Muγ–treated group throughout the experimental period. The TEWL of the pCMV-Muγ–treated mice was as low as that of the pCpG-Muγ–treated ones for the first 14 d, but it was significantly higher at days 70 and 84.

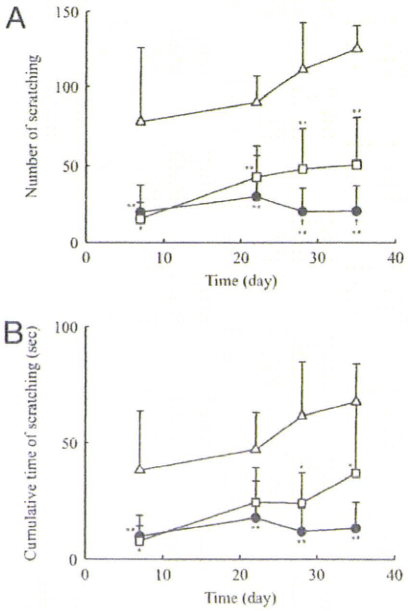

FIGURE 5. Number (*A*) and cumulative time (*B*) of scratching episodes. Scratching behavior of NC/Nga mice treated as described in the legend of Fig. 1 were automatically evaluated using SCLABA-Real. △, untreated mice; ●, pCpG-Muγ–treated mice; □, pCMV-Muγ–treated mice. The results are expressed as the mean ± SD of at least three mice. $*p < 0.05$ compared with the untreated group; $**p < 0.01$ compared with the untreated group; $^{\dagger}p < 0.05$ compared with the pCMV-Muγ–treated group.

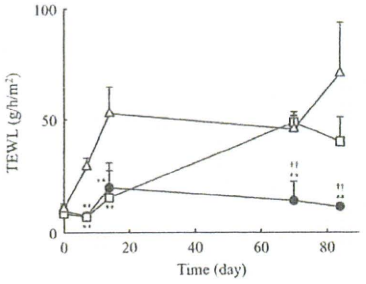

FIGURE 6. Time course of the TEWL of the back skin of mice. TEWL was measured on the back of untreated mice (△), pCpG-Mug–treated mice (●), and pCMV-Mug–treated mice (□). The results are expressed as the mean ± SD of at least three mice. $^{**}p < 0.01$ compared with the untreated group; $^{\dagger\dagger}p < 0.01$ compared with the pCMV-Muγ–treated group.

Histological examination of skin sections of NC/Nga mice after injection of IFN-γ–expressing plasmid DNA

Fig. 7 shows the H&E sections of the back skin of treated and untreated mice at day 14 after the start of treatment. NC/Nga mice maintained under specific pathogen-free (SPF) conditions were used as control mice with healthy skin; the skin sections from the SPF control mice showed no pathological features (Fig. 7*A*). In marked contrast, there was clear hyperplasia of the epidermis (acanthosis) in the untreated group (Fig. 7*B*). The sections from the untreated mice also showed an extensive infiltration of lymphocytes and eosinophils. These characteristic features of inflamed skin tissues were not as apparent in the skin sections from the pCpG-Muγ–treated mice (Fig. 7*C*), which were indistinguishable from the skin sections from the SPF control mice. Compared with the sections from the pCpG-Muγ–treated mice, the ones from pCMV-Muγ–treated mice showed less significant improvement (Fig. 7*D*). Fig. 8 shows the skin sections in which mast cells were stained with toluidine blue. Again, a significant infiltration of mast cells was observed in the untreated mice (Fig. 8*B*), but not in the SPF control (Fig. 8*A*) or the pCpG-Muγ–treated mice (Fig. 8*C*). The numbers of lymphocytes, eosinophils, and mast cells in skin sections were counted (Fig. 9). The numbers of these cells were significantly less in mice receiving IFN-γ–expressing plasmid DNA compared with untreated mice.

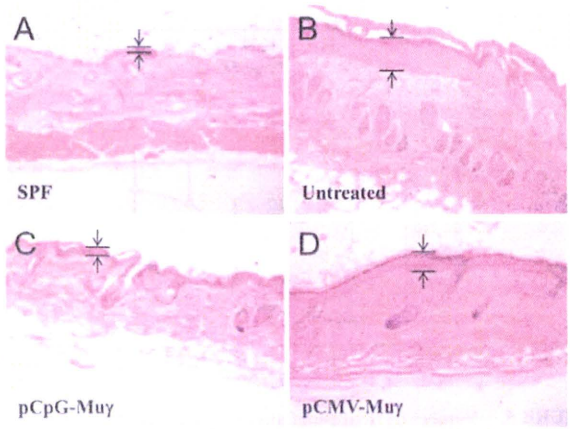

FIGURE 7. H&E sections of the back skin of NC/Nga. Dorsal skin of mice maintained under SPF conditions (*A*), untreated mice (*B*), pCpG-μγ–treated mice (*C*), and pCMV-μγ–treated mice (*D*) were collected at day 14. Skin sections were stained with H&E for histological evaluation and detection of inflammatory cells. Arrows and bars indicate the thickness of the epidermis. Original magnification ×400.

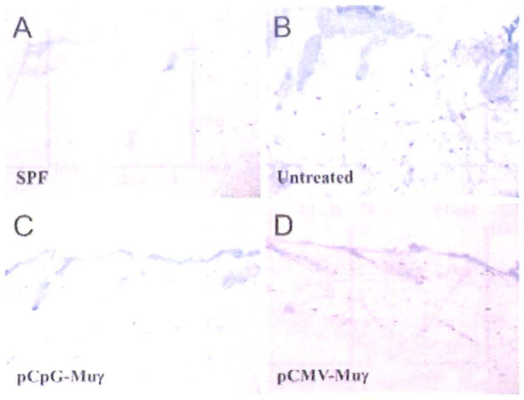

FIGURE 8. Toluidine blue sections of the back skin of NC/Nga mice. Dorsal skin of mice maintained under SPF conditions (*A*), untreated mice (*B*), pCpG-Muγ–treated mice (*C*), and pCMV-Muγ–treated mice (*D*) were collected at day 14. Skin sections were stained with toluidine blue to detect mast cells. Original magnification ×400.

Adverse effects of IFN-γ gene transfer

There were no significant differences in the body weight or temperature between the saline-injected mice and the pCpG-Muγ–treated mice. To examine whether IFN-γ gene transfer increases the risk for contact dermatitis, the ear thickness was measured in a mouse model of TNCB-induced contact dermatitis. The challenge with TNCB significantly increased the thickness, but the injection of pCpG-Muγ had no significant effects on the change (data not shown). Fig. 10 shows the time courses of the TEWL from the dorsal skin of tape stripped (Fig. 10*A*) or TNCB-treated mice (Fig. 10*B*). Again, no IFN-γ gene transfer-induced increase was observed in any case examined.

Discussion

Because of the multiple functions of cytokines and their complicated network, the effects of externally administered cytokines, including IFN-γ, depend on their pharmacokinetics. Atopic dermatitis, a chronic inflammatory disease with skin inflammation, is characterized by Th2-dominant immunity; therefore, any treatment that normalizes the Th1/Th2 balance can be useful for treatment of the disease. IFN-γ, a typical Th1 cytokine, has been considered to induce a variety of immunological changes, leading to a Th1-dominant state, but its effects on the Th1/Th2 balance in patients with Th2 predominance is not fully understood. The outcome from the previous clinical experiments in which IFN-γ was administered to patients with atopic dermatitis suggested that

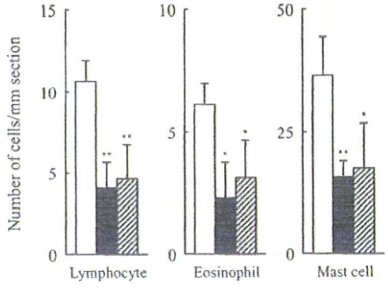

FIGURE 9. Number of lymphocytes, eosinophils, and mast cells in skin sections. Lymphocytes and eosinophils in H&E-stained sections were counted under a microscope. The toluidine blue sections were used for the counting of mast cells. The number of cells was expressed as the mean ± SD of three sections: untreated mice (open bars), pCpG-Muγ–treated mice (filled bars), and pCMV-Muγ–treated mice (striped bars).

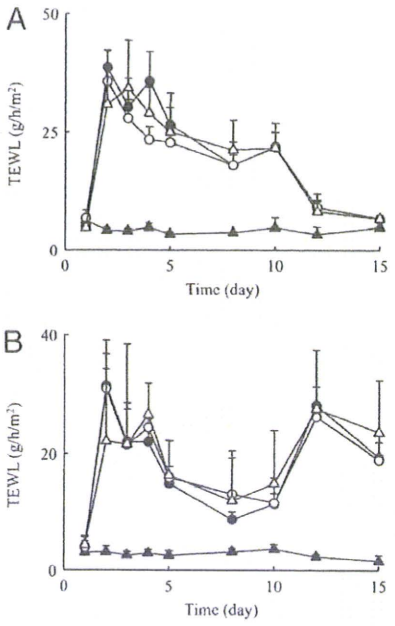

FIGURE 10. Time course of the TEWL of the back skin of mice in a stripped skin and a TNCB-induced dermatitis model. *A*, The back skin of C57BL/6 mice was shaved and tape stripped on days 1 and 8. ▲, untreated mice; △, tape-stripped mice; ○, tape-stripped, saline-treated mice; ●, tape-stripped, pCpG-Muγ–treated mice. *B*, The back skin of C57BL/6 mice was shaved, and TNCB was applied on days 1, 8, 11, and 13. ▲, untreated mice; △, TNCB-treated mice; ○, TNCB-treated, saline-treated mice; ●, TNCB-treated, pCpG-Muγ–treated mice. Results in *A* and *B* are expressed as the mean ± SD of four mice.

there would be few significant changes in the immune system of the patients receiving such treatments (7–10). This can primarily be attributed to the unfavorable pharmacokinetic properties of IFN-γ, which easily passes through the glomerulus of the kidney because it is smaller than the threshold of glomerular filtration, and it disappears from the systemic circulation very quickly (11).

In the current study, a sustained level of IFN-γ in the serum was successfully achieved by injecting pCpG-Muγ. An IFN-γ concentration >300 pg/ml, which is greater than the half maximal effective concentration of the protein needed to inhibit virus replication (100 pg/ml), was obtained throughout the experimental period of 84 d. This profile of IFN-γ in the serum was in marked contrast to that obtained by the injection of pCMV-Muγ, which resulted in a very transient IFN-γ concentration in the serum. Such a large difference in the duration of transgene expression is most likely due to the number of CpG motifs in plasmid DNA (21, 31). A detailed discussion on this sustained expression of IFN-γ from pCpG-Muγ was presented elsewhere (22).

Sustained expression of IFN-γ from pCpG-Muγ induced a variety of immunological changes in NC/Nga mice. In summary, the level of Th1 cytokines IFN-γ and IL-12 (Fig. 2*A*) was increased and that of IL-4, -5, -10, -13, and -17 and TARC tended to be decreased (Fig. 3); thus, the Th1/Th2 balance shifted toward Th1 dominance. In addition, the level of IgE, a hallmark of allergic reactions, was also significantly decreased. These changes can be considered to be due to the biological activity of IFN-γ, which promotes the production of IL-12, a typical Th1 cytokine important for differentiation to the Th1 subset. IL-12 is produced from macrophages or NK cells stimulated with IFN-γ. The reduced expression of the Th2 cytokines and TARC also resulted from the expression of IFN-γ, as reported using PBMCs, keratinocytes, or Langerhans cells (32–34).

A major finding of the current study is that the immunological changes induced by IFN-γ gene transfer differ, depending on the vector used (i.e., on the pharmacokinetics of IFN-γ). A variety of changes, including the increase in the IL-12 concentration and the decrease in the IgE level, were observed only in the mice receiving pCpG-Muγ. These results clearly indicate the importance of a sustained concentration of IFN-γ at a level high enough to allow it to modulate the Th1/Th2 balance. In addition, these results may explain the fact that most challenges using IFN-γ protein for the treatment of atopic dermatitis are not very effective, because IFN-γ protein quickly disappears from the systemic circulation (11).

The modulation of Th1/Th2 imbalance by sustained expression of IFN-γ was significantly effective in preventing the onset of symptoms of atopic dermatitis in NC/Nga mice. Rash, scratching, bleeding on the back or ear, acanthosis, and infiltration of lymphocytes, eosinophils, and mast cells were not very apparent in the pCpG-Muγ–treated mice. Inhibition of cellular infiltration into skin would be the consequence of the changes induced by the sustained concentration of IFN-γ; this directly inhibits the infiltration of eosinophils (35, 36), and it could inhibit Th2 cell migration into skin promoted by Th2 chemokines (37, 38). Because Th2 lymphocytes are the cells that release Th2 cytokines (39), our findings strongly suggest that IFN-γ gene transfer results in the inhibition of the differentiation of Th2 lymphocytes. Based on the results obtained, a mechanism of inhibition of atopic dermatitis by sustained expression of IFN-γ is proposed as follows. A sustained concentration of IFN-γ promotes the production of IL-12 from macrophages and NK cells, and these two Th1 cytokines inhibit the production of the Th2 cytokines/chemokines. These changes inhibit the recruitment of immune cells to the skin and prevent the onset of symptoms typical of atopic dermatitis. It is important to prove whether sustained IFN-γ gene transfer is also effective in individuals with dermatitis. A few NC/Nga mice that developed dermatitis were injected with pCpG-Muγ, and the clinical skin score and TEWL were measured. These parameters were reduced by IFN-γ gene transfer, although the reduction was less significant than in mice that did not develop dermatitis. Further studies are needed to conclude that IFN-γ gene transfer provides efficacy for patients with atopic dermatitis.

There would be concerns about sustained delivery of IFN-γ, because it is a highly potent Th1 cytokine. However, we observed no significant adverse effects of IFN-γ gene transfer on physiological parameters, such as body weight and body temperature. Also, contact dermatitis, a Th1-associated allergic dermatitis, was not aggravated by the injection of pCpG-Muγ. In addition, the improvement in TEWL observed in NC/Nga mice receiving pCpG-Muγ would not be due to the direct effects of IFN-γ gene transfer, because TEWL was minimally affected by IFN-γ gene transfer in other models (Fig. 10).

The hydrodynamic injection method used for gene transfer in the current study is one of the most efficient methods; using this method, >99% of transgenes were expressed in the liver, followed by kidneys, spleen, lung, and other internal organs (25, 40). Although there is concern about the toxicity related to the mode of gene delivery (25, 26, 41, 42), a recent report showed that this method of gene delivery can be applied to humans with few toxic effects when plasmid DNA is delivered to a lobe using a balloon catheter (43). Computer-assisted hydrodynamic gene delivery would also be a less invasive method (44). Other modes for gene delivery could be used to achieve sustained transgene expression of IFN-γ at a relatively high level, because the dose of pCpG-Muγ used was as low as 0.14 μg/mouse (i.e., ~7 μg/kg body weight). In general, greater amounts of plasmid DNA up to 100 μg/mouse (5 mg/kg) have been administered, and an increase in the dose

may compensate for the low efficiency of other gene-delivery methods, such as intradermal injection of naked plasmid DNA.

In conclusion, it was proved that an extraordinarily sustained IFN-γ expression induces a variety of immunological changes, leading to a Th1-dominant state in the atopic dermatitis model. A short expression of IFN-γ was not effective in inducing such changes, indicating the importance of a sustained concentration of IFN-γ. These results provide a novel strategy for the treatment of atopic dermatitis, in which biologically active IFN-γ protein is supplied to patients from cells transduced with plasmid vector expressing the protein for a long period of time.

Disclosures

The authors have no financial conflicts of interest.

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Development of a novel Hsp70-based DNA vaccine as a multifunctional antigen delivery system

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ABSTRACT

DNA vaccination is a simple and effective method to induce immune responses against a variety of tumors as well as infectious diseases. Vaccination with major histocompatibility complex (MHC) class I tumor peptide has been carried out to induce an antigen-specific and tumor-reactive cytotoxic T lymphocytes (CTLs) response *in vivo*. In this study, we describe a novel DNA vaccine based on heat shock protein 70 (Hsp70), which can chaperon antigenic peptides and initiate innate and adaptive immune responses, to induce a more effective immune response. Ovalbumin (OVA) MHC class I epitope peptide ($OVA_{257-264}$: SIINFEKL) was selected as a model antigen and polyhistidine was used to facilitate the cytosolic delivery of the antigen-Hsp70 after endocytic uptake. A novel plasmid DNA vector encoding polyhistidine, Hsp70 and $OVA_{257-264}$ (pHis-Hsp70-pep) was designed. When mice were immunized with pHis-Hsp70-pep by intradermal injection in combination with electroporation, strong antigen-specific CTL responses were generated. pHis-Hsp70-pep also showed a significant protective effect against tumor challenge with an OVA-expression EL4 tumor line. These results indicate that the Hsp70-based DNA vaccine is useful as a multifunctional antigen delivery system to induce the antigen-specific immune response.

1. Introduction

Heat shock protein 70 (Hsp70), is a member of the heat shock protein family, which is induced under stress conditions. Hsp70 also plays important roles in immune responses against tumor, bacterial and viral targets [1–5]. It can form complexes with various antigenic peptides through its polypeptide binding domain [6], to stimulate adaptive and innate immune responses [7]. Recent studies have indicated that the targeting of antigen to APCs through Hsp70 is a useful strategy to induce efficient cross-presentation, and it has been investigated for cancer immunotherapy [8].

The factors favoring the development of immunization strategies with plasmid DNA include the relative easy and inexpensive nature of preparation compared with peptide and protein vaccines, as well as its stability and no-infectious nature compared with viral vaccines [9–11]. In addition, DNA vaccines can also be designed or modified so that antigens encoded by plasmid DNA can be controlled to target specific cellular sites by using additional peptides or proteins, subsequently initiating the desired intensity and type of immune responses, including both humoral and cellular immune responses [12].

Polyhistidine, the imidazole-containing polymers, followed by Hsp70-antigen peptide is capable of escaping from the endosome/lysosome for minimizing degradation under acidic conditions, the so-called 'proton sponge' effect [13–16]. Enhanced cytosolic delivery of Hsp70-associated antigen would increase the entry of antigens into the class I presentation pathway, resulting in improved induction of specific $CD8^+$ T cells. In our previous study, we have designed a novel protein vaccine, Hsp70-based antigen delivery system, Hsp70-associated antigen fused to polyhistidine. The fusion protein of His-Hsp70-pep, which was expressed in *E. coli* and purified, exhibited a significantly improved efficacy of MHC class I-restricted presentation of antigen *in vitro*, and also generated strong antigen-specific CTL responses and antitumor activity in mice [17]. However, based on a number of advantages over protein vaccines, DNA vaccination may be an attractive alternative for delivering His-Hsp70-pep vaccines, which can induce stronger immune responses because of the presence of immunostimulatory unmethylated CpG motifs in the structure of plasmid DNA. There are reliable evidences showing that antigens can be taken up and presented by antigen presenting cells (APCs) through two distinct pathways [18–20]: the direct presentation pathway and the cross-presentation pathway [21–23]. In cross-presentation, professional APCs acquire antigens from other cell types such as keratinocytes or myocytes following intradermal [24] or intramuscular DNA vaccination [25]. It is likely that the cross-presentation pathway is the major mechanism for T cell priming by DNA vaccine [26] because of the relatively low quantity of APCs, especially dendritic cells (DCs), present at the sites for administration [27]. Accordingly, induction of cross-presentation is expected to be more effective for DNA vaccination. Based on this consideration, we have

Abbreviations: Hsp70, heat shock protein 70; OVA, ovalbumin; His, histidine; CTL, cytotoxic T lymphocytes; PAGE, polyacrylamide gel electrophoresis.

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designed a novel plasmid DNA vector expressing the fusion protein, His-Hsp70-pep, as a secretory protein from the cells transfected in order to increase the probability of cross-presentation. Then, we investigated whether the vaccination with this novel plasmid DNA vector could enhance the antigen-specific CTL responses and antitumor activity in mice.

2. Materials and methods

2.1. Cells and animals

DC2.4 cells, a cell line of murine dendritic cells (haplotype H-2^b) [28], were kindly provided by Dr. K. L Rock (University of Massachusetts Medical School, Worcester, MA). CD8OVA1.3 cells, T hybridoma cells against SIINFEKL-K^b [29], were a generous gift from Dr. C. V. Harding (Case Western Reserve University, Cleveland, OH). EL4 cells, C57BL/6 T lymphoma, and EG7 cells, an OVA transfect clone of EL4 [30], were purchased from American Type Culture Collection (Manassas, VA). DC2.4 cells were cultured in RPMI 1640 medium (Nissui Pharmaceuticals Pharmaceuticals, Tokyo, Japan) supplemented with 10% heat-inactive fetal bovine serum (Equitedh-Bio, Kerrville, TX), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, antibiotics (all from Invitrogen, Carlsbad, CA). CD8OVA1.3 and EL4 cells were cultured in Dulbecco's modified Eagle medium (Nissui) supplemented as described for RPMI 1640 medium. EG7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactive fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, glucose, sodium pyruvate, HEPES and G418.

Five-week-old female C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions and all animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

2.2. Plasmid DNA construction

The pTrc99A expression vector containing the genomic mouse clone Hsp70.1 cDNA was kindly supplied by Dr. Paul Slusarewicz (Mojave Therapeutics, Inc. USA). Murine cDNA of Hsp70.1 was amplified by PCR and inserted into pcDNA3.1 vector using pGEM®-T vector (Promega, USA), subcloning vector system.

An MHC class I epitope peptide of OVA ($OVA_{257-264}$: SIINFEKL-K^b) was selected as a model antigen, and oligonucleotides corresponding to the amino-acid sequence SIINFEKLTEWTS were purchased from Hokkaido System Science (Hokkaido, Japan), and inserted into the C-terminal of Hsp70. TEWTS sequence was added to the epitope because it has been reported to facilitate the liberation of SIINFEKL in proteasomes [31]. The oligonucleotide coding the 25 histidines (polyhistidine) was also synthesized and incorporated into the N-terminal of Hsp70. The original Hsp70 is expressed as a cytosolic protein in mammalian cells. In order to render Hsp70 to be expressed as a secretory protein following transfection, an endoplasmic reticulum (ER) signal sequence amplified from pCMV/mic/ER Shooter vector (Invitrogen, USA) was inserted into the upstream region of each fusion gene. The sequences of these recombinant DNAs were confirmed by Kyoto Science Corporation Technical Service (Kyoto, Japan). The schematic structures of the constructs are shown in Fig. 1A.

2.3. Expression of the fusion proteins in COS-7 cells

Plasmid DNA/LipofectAMINE™ 2000 complexes (DNA-Lipoplex) were prepared in polypropylene centrifuged tubes according to the protocol provided by the manufacturer (Molecular Probes, Invitrogen). COS-7 cells were seeded on 6-well culture plates and incubated

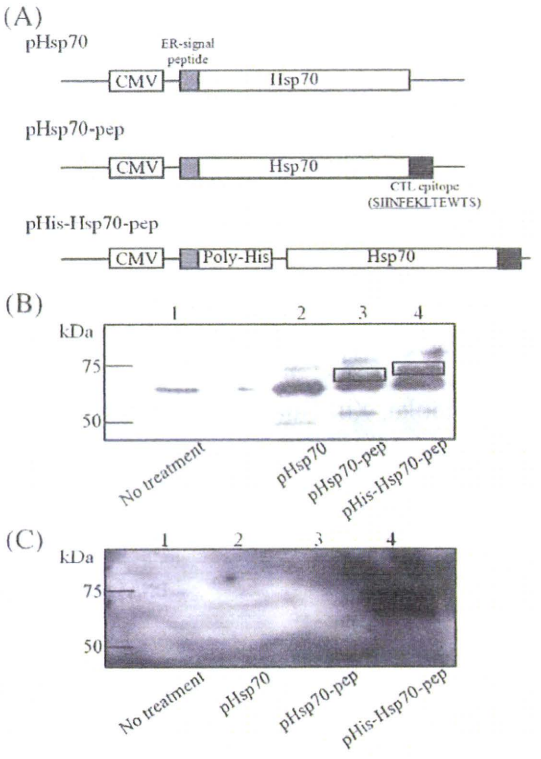

Fig. 1. (A) Schematic representation of Hsp70-CTL epitope fusion constructs. A mini-gene encoding a CTL epitope ($OVA_{257-264}$) was incorporated into the C-terminus of the mouse Hsp70.1 gene; polyhistidine (His_{25}) was further fused to the N-terminus of the Hsp70 gene. These fusion genes were inserted into the pcDNA3.1(+) vector. (B) and (C) Western blot analysis of COS-7 lysates hybridized with monoclonal anti-Hsp70 antibody (B) and anti-histidine antibody (C). The band surrounded by the square indicates fusion protein encoded by pHsp70-pep or pHis-Hsp70-pep, respectively. Lane 1, No treatment; Lane 2, pHsp70; Lane 3, pHsp70-pep; Lane 4, pHis-Hsp70-pep.

with each plasmid DNA/cationic liposome (LipofectAMINE™ 2000, Invitrogen) complexes prepared in Opti-MEM (Invitrogen) for 4 h in 5% CO_2 at 37 °C. Then, the medium was replaced with culture medium. Cells were collected in PBS (300 μl/well) at 24 h after transfection, and lysed by sonication. Then, sonicates were loaded on 8% SDS-PAGE gel for separation. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon PTM, Millipore) and blocking was carried out overnight (o/n) with 9% skimmed milk, 0.1% Tween-20 in PBS buffer. Then, the membrane was incubated with monoclonal mouse anti-Hsp70 antibody (Santa Cruz Biotechnology, Inc., USA) or anti-histidine antibody followed by anti-mouse IgG horseradish peroxidase-conjugated antibody. The immunoblots were developed with an enhanced chemiluminescence reagent kit and exposed to a film (both from Amersham Biosciences, USA).

2.4. Antigen presentation assay

The efficacy of MHC class I presentation activity of the plasmid DNA was assessed by an *in vitro* antigen presentation assay using DC2.4 cells and T hybridoma cells that specifically recognize SIINFEKL-K^b and release interleukin-2 (IL-2). This simple method has been widely used for the evaluation of OVA vaccination systems [32]. Plasmid DNA/LipofectAMINE™ 2000 complexes prepared in Opti-MEM were added to DC2.4 cells (1×10^5/well) which were cultured on 96-well plates overnight. After coincubation for 2 h, medium was replaced with culture medium. Then, 6 h later, CD8OVA1.3 T hybridoma cells (1×10^5/well) were added and coincubated with DC2.4 cells in 5% CO_2 at 37 °C and, 20 h later, the cell culture supernatants were collected and freeze-thawed. The response of CD8OVA1.3 T cells was determined by measuring IL-2 levels in the

supernatants with an enzyme-linked immunosorbent assay (ELISA; OptiEIATM Set Mouse IL-2 and TMB Substrate Reagent Set, BD Biosciences, San Diego, CA).

2.5. CTL assay

C57BL/6 mice were immunized three times at weekly intervals intradermally (i.d.) in the dorsal skin with a total of 50 μl plasmid DNA (2 mg/ml) in normal saline. Electroporation was applied to the injection site (1000 V/cm, 5 ms, 4 Hz, 12 pluses) 30 s after injection. Seven days after the last immunization, splenocytes were isolated from the immunized mice followed by restimulation *in vitro* for 5 days with mitomycin C-treated EG7. Target cells (EG7 or EL4; EL4 was used as a target control) were labeled with ^{51}Cr by incubating with $Na_2^{51}CrO_4$ in culture medium for 45 min at 37 °C. After washing, 2×10^4 of the ^{51}Cr-labeled target cells and serially diluted splenocytes were coincubated in 200 μl culture medium for 4 h at 37 °C. Spontaneous release of ^{51}Cr without effector cells and maximal release in the presence of 1% TritonX-100 were also evaluated. Cells were centrifuged (420 *g*) for 5 min, and 100 μl of each supernatant was collected for radioactivity measurements. The cytosolic activity of CTLs was calculated as [33]:

$$\%\,\text{killing} = (\text{observed release} - \text{spontaneous release}) \div (\text{maximal release} - \text{spontaneous release}) \times 100.$$

2.6. Tumor challenge experiments

C57BL/6 mice were intradermally immunized three times at weekly intervals in the back with each plasmid DNA, followed by electroporation. Eight days after the last immunization, 1×10^6 cells per mouse of EG7 were administered intradermally into the back as a challenge. The mice were monitored for tumor growth every three or four days. The tumor size was measured using the longest and the shortest diameters. The tumor volume was calculated from the equation: $(\text{longest}\times\text{shortest})^{3/2}\times\pi/6$. The survival time of the tumor-challenged mice was also recorded.

3. Results

3.1. Characterization of constructed plasmid DNA

Three types of Hsp70 recombinant plasmid DNA: pHsp70, pHsp70-pep and pHis-Hsp70-pep were constructed. The ER signal sequence, which directs proteins synthesized in the cytosol to the endoplasmic reticulum, inserted at the N-terminal of each fusion gene made them become secreted molecules. Each plasmid DNA was transfected into COS-7 cells to characterize the fusion protein. Western blot analyses of COS-7 cell lysates with the anti-Hsp70 antibody (Fig. 1B) and anti-histidine antibody (Fig. 1C) confirmed each plasmid DNA expressed the designed fusion protein. As shown in figure 1B, the band of approximately 70 kDa (predicted molecular weight: 70 kDa) in lane 1 represented endogenous Hsp70 in cells. The band of 70 kDa in lane 2 represented the mixture of endogenous Hsp70 and Hsp70 translated from pHsp70. The bands in lane 3 and 4 just above 70 kDa represented the fusion proteins of Hsp70-peptide and polyhistidine-Hsp70-peptide, respectively, translated from corresponding plasmid DNAs in COS-7 cells.

3.2. MHC class I presentation

In order to assess the efficacy of MHC class I presentation activity of the fusion proteins encoded by constructed plasmid DNAs, we performed an *in vitro* antigen presentation assay using DC2.4 cells and T hybridoma cells against SIINFEKL-K^b based on IL-2 production (Fig. 2). High production of IL-2 by CD8OVA1.3 T hybridoma cells were observed because of the effective presentation of the MHC class I epitope encoded by pHis-Hsp70-pep and pHsp70-pep. Furthermore, pHis-Hsp70-pep was more effective than pHsp70-pep in the production of IL-2, suggesting that the MHC class I presentation activity was increased by fusion of polyhistidine, probably because of facilitated cytosolic delivery of the fusion protein. In our previous antigen presentation assay *in vitro*, similar results were obtained for these fusion proteins [17].

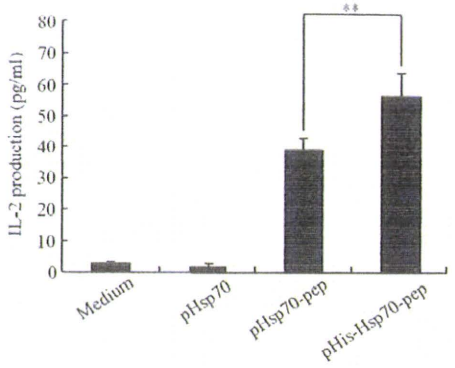

Fig. 2. MHC class I-restricted presentation assay *in vitro*. DC2.4 cells were transfected with each plasmid DNA. At 8 h after transfection, CD8OVA1.3 T cell hybridoma was added to DC2.4 cells. After 24 h, IL-2 production from CD8OVA1.3 cells were measured by ELISA. Results are expressed as mean ± S.D. ($n=3$). Statistically significant differences were assessed using Student's *t*-test against pHsp70-pep (**$p<0.01$).

3.3. Induction of tumor-specific CTLs

To evaluate whether a high level of antigen-specific CTLs was elicited by immunization with pHis-Hsp70-pep, we examined the OVA-specific CTL response using EG7 cells expressing OVA. The splenocytes from the mice immunized with pHis-Hsp70-pep by intradermal injection followed by electroporation showed a higher level of OVA-specific CTL response compared with that from the mice treated with pHsp70-pep (Fig. 3A). No significant CTL activity was seen in the EL4 cells, indicating that the CTL activity was an OVA-specific response (Fig. 3B).

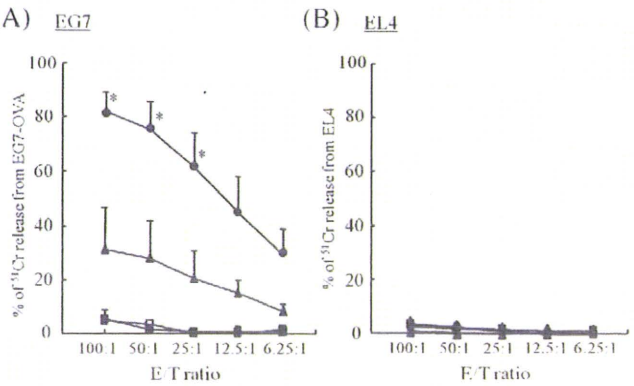

Fig. 3. Generation of OVA-specific CTLs by immunization with pHsp70, pHsp70-pep and pHis-Hsp70-pep. Mice were immunized three times with 100 μg plasmid DNA. 7 days after the last immunization, spleen cells were isolated from the immunized mice followed by restimulation with EG7 cells *in vitro* for 5 days, then standard ^{51}Cr release assay was performed ($n=3$). EG7 (A) and EL4 (B) were used as the target cells. (□) No treatment; (■) pHsp70; (▲) pHsp70-pep; (●) pHis-Hsp70-pep. Results are expressed as mean ± S.D. ($n=4$). Statistically significant differences were assessed using Student's *t*-test against pHsp70-pep (*$p<0.05$).

3.4. In vivo growth inhibitory effect of EG7 tumor cells

We finally examined the effect of immunization with pHis-Hsp70-pep to protect mice from an EG7 tumor challenge. Tumor growth was significantly inhibited in the pHis-Hsp70-pep-treated group, followed by group treated with the pHsp70-pep (Fig. 4A). The survival of mice challenged intradermally with EG7 tumor cells was also prolonged in the mice immunized with pHis-Hsp70-pep. Untreated mice or those treated with pcDNA3.1 (Mock) or pHsp70 all died by day 57, whereas about 30% and more than 80% of the mice immunized with pHsp70-pep or pHis-Hsp70-pep, respectively, survived the first 100 days (Fig. 4B).

4. Discussion

Based on our previous study on protein vaccine [17], the Hsp70-based DNA vaccine, pHis-Hsp70-pep, was designed as a multifunctional antigen delivery system to induce the antigen-specific immune response in our present study. The multiple functions include: the antigen-Hsp70 complex can be taken up by APCs via Hsp receptors, such as CD91 and LOX-1; Hsp70 can also activate the innate immunity through CD40 and Toll-like receptor-2 (TLR-2) and TLR-4 to induce cytokine secretion [7,8]; polyhistidine can enhance the cytosolic delivery to improve MHC class I antigen presentation [13,17]. Moreover, in addition to the functions described above, which also are possessed by the protein vaccine, the bacterial immunostimulatory unmethylated CpG motifs in the structure of plasmid DNA are potent activators of innate immunity and induce cellular response mediated by TLR-9 [34]. Furthermore, plasmid DNA possesses of relative easy and inexpensive nature for preparation, quality stability and long-time storage period, compared with protein vaccines. Therefore, it would be an attractive alternative for delivering His-Hsp70-pep vaccines.

DNA vaccine has shown promise in eliciting an effective CTL response to antigens since Wolff and colleagues first observed that an intramuscular injection of naked DNA led to the expression of encoded genes in myofiber cells [35]. Direct transfection of DCs with DNA is one way to promote antigen presentation by APCs, but only a few DCs are transfected after DNA injection, because of the relatively low amount of APCs, especially dendritic cells (DCs), present at the sites for administration. Another way is to enhance the cross-presentation, in which APCs exogenously take up antigenic protein which is expressed in DNA-transfected non-APCs. It seems that the latter is more effective from the perspective of gene expression. In the present study, we added the endoplasmic reticulum (ER) signal sequence at the upstream region of each fusion gene inserted into pcDNA3.1 vector, which made Hsp70 a secretory protein, in order to increase the probability of cross-presentation. In fact, the vector is different from the one we used for protein vaccine in our previous study in terms of backbone, promoter and form of the expressing protein [17]; pGEX-6P-2. GST-tag expression plasmid was used to express Hsp70 fusion proteins in *E. coli* DH5α cells as a recombinant protein.

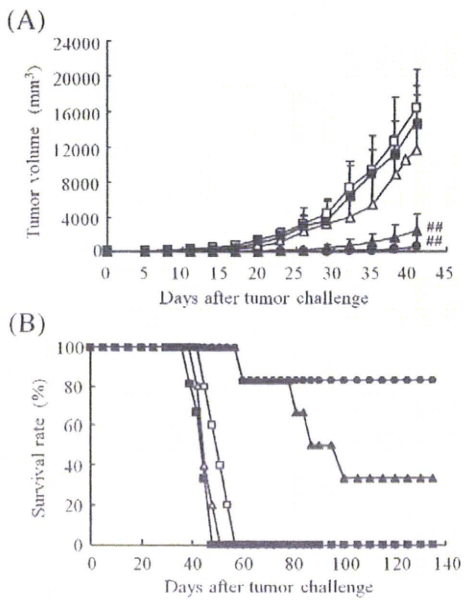

Fig. 4. *In vivo* growth inhibitory effect (A) and prolongation of survival time (B) obtained by immunization with plasmid DNA. Mice were immunized intradermally twice with 100 μg plasmid DNA and 7 days after the last immunization, 1×10^6 cells of EG7 were injected intradermally ($n = 5$ or 6). (□) No treatment; (△) Mock (pcDNA3.1); (■) pHsp70; (▲) pHsp70-pep; (●) pHis-Hsp70-pep. Results are expressed as mean ± S.D. ($n = 5$ or 6). Statistically significant differences were assessed using Student's *t*-test against Mock (## $p < 0.001$).

Because antigen internalized into endosome is degraded by various protease or likely loaded on MHC class II molecules [36], enhancing release of antigen from endosomes into cytoplasm is required to increase antigen-specific CTLs. In our previous study, we successfully used polyhistidine to enable antigen to escape from endosomes into cytoplasm in order to increase the potency of the vaccine based on Hsp70 [17]. Examination of the intracellular location of fusion protein by confocal microscopy showed that His-Hsp70-pep fusion protein was widely distributed in DC2.4 cells, suggesting that the fusion protein was transferred into the cytoplasm. It has been reported that exogenously-administered PEI/DNA particles undergo normal endocytotic trafficking and complexes are distributed to the cytoplasm and enter nuclei in the form of large, discrete structures and imidazole-containing polymers also exhibit these functions [13,37,38]. Therefore, it is considered that His-Hsp70-pep fusion protein follows the same course and escapes from endosomes by the imidazole groups of histidine.

When DC2.4 cells were transfected with plasmid DNA encoding fusion protein to examine the antigen presentation efficiency, pHis-Hsp70-pep was more effective than pHsp70-pep in the production of IL-2 (Fig. 2), suggesting that the MHC class I presentation activity was increased by polyhistidine fusion. We speculate that the presentation procedure is as follows: the proteins translated from the plasmid DNA are directed into the endoplasmic reticulum by the ER signal peptide at the N-terminal of the plasmid DNA, and are then transported into the extracellular space through the Golgi apparatus. Then, the fusion proteins can be taken up again by the DC2.4 cells even including those secreting the fusion protein, where polyhistidine can play its designed role.

Intradermal and intramuscular injections have been often used for DNA vaccination [39]. In this study, we selected intradermal injection to immunize mice for the presence of abundant APCs, such as Langerhans cells and dendritic cells, in the epidermis [40]. Activated Langerhans cells and dendritic cells can traffic to draining lymph nodes where presentation of the encoded antigens to T cells and B cells occur, thereby initiating a variety of immune responses. We also applied electroporation after intradermal injection to increase the level of transgene expression.

In our previous study, we demonstrated that the protein vaccine of His-Hsp70-pep can generate strong antigen-specific CTL responses and antitumor activity in mice [17]. Here, we demonstrate that the DNA vaccine using pHis-Hsp70-pep can also strongly enhance the antigen-specific CTL responses and antitumor activity in mice even compared with its protein vaccines. These results indicate that the control of intracellular trafficking of Hsp70-antigen using polyhistidine is also a useful strategy to enhance the antigen-specific immune response induced by DNA vaccine. The inhibition effect of tumor growth by DNA vaccine in this study was stronger than that by the protein vaccine, although the survival rate was similar between them

(Figs. 3 and 4), suggesting the advantage of the DNA vaccine over the protein vaccine. In addition to the vector encoding the fusion protein having 25 polyhistidine repeats, we also developed the DNA vector encoding the fusion protein with more polyhistidine repeats (50 repeats). In the case of protein vaccine, the fusion protein with 50 repeats was much less effective probably due to inactivation during protein purification [17]. On the other hand, the DNA vaccine expressing the fusion protein with 50 repeats showed antigen presentation activity and antigen-specific CTL activities comparable with the vector the fusion protein with 25 repeats, pHis-Hsp70-pep (data not shown). These results also suggest DNA vaccine encoding the fusion protein is more advantageous than the same fusion protein vaccine.

In summary, this study demonstrates for the first time that the plasmid DNA vector encoding antigen fused to polyhistidine and Hsp70 is a potent vaccine compared with the vaccine encoding antigen only fused to Hsp70. Thus, the usefulness of the novel vector as a multifunctional antigen delivery system has been shown. An advantage of DNA vaccine with plasmid DNA, through altering the antigen sequence inserted into the multiple clone site of a plasmid DNA, is that the vector constructed can also be used to treat other tumors and infectious diseases.

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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.

Contents

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

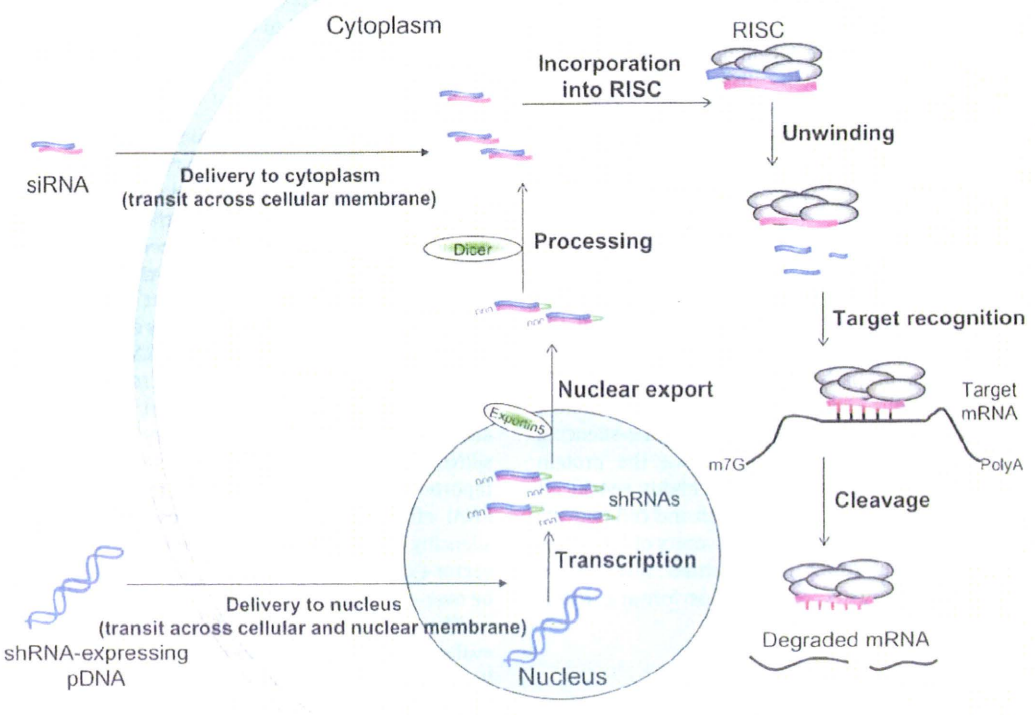

Fig. 1. Schematic image of gene silencing by siRNA and shRNA-expressing pDNA. As the RNAi effect is limited in the cells that have siRNA, both siRNA and shRNA-expressing pDNA should be delivered to the target cells. shRNA-expressing pDNA should be delivered to the nucleus to transcribe shRNA while siRNA 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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RESEARCH ARTICLE

Effect of the content of unmethylated CpG dinucleotides in plasmid DNA on the sustainability of transgene expression

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Abstract

Background Nonviral gene transfer generally suffers from short-term expression of transgenes. We have previously demonstrated that plasmids with reduced CpG content exhibited a more prolonged expression of murine interferon (IFN)-β or IFN-γ, which was effective in inhibiting metastatic tumor growth. A further extension of the duration of transgene expression could be achieved by controlling the number and location of CpG motifs in plasmid DNA.

Methods Luciferase-expressing plasmids with differing CpG content were injected into the tail vein of mice by the hydrodynamic injection method. The effects of CpG content on the duration of transgene expression were examined, focusing on cytosine methylation and pro-inflammatory cytokines. Based on the findings, IFN-γ-expressing plasmids were constructed and their transgene expression and inhibitory effect on pulmonary metastasis were evaluated.

Results Plasmids with a few CpG motifs showed a prolonged luciferase activity in the liver. Methylation of CpG motifs in plasmids reduced the expression and the extent of this reduction was greater for plasmids with a high CpG content. Pro-inflammatory cytokines hardly affected the expression. pCpG-Muγ, the IFN-γ-expressing plasmid, which contains 20 CpG motifs only in the cDNA region, exhibited a sustained IFN-γ concentration at therapeutic levels, and had a great inhibitory effect on the pulmonary metastasis of tumor cells.

Conclusions The duration of transgene expression of IFN-γ was successfully increased by reducing the CpG content of IFN-expressing plasmid vector, which resulted in an increased anticancer activity of IFN gene transfer.

Keywords cancer gene therapy; CpG motif; hydrodynamic injection; interferon-γ; methylation; pro-inflammatory cytokine

Introduction

A number of approaches have been developed to obtain the expression of therapeutic genes after the *in vivo* application of vectors encoding the gene of interest. Both viral and nonviral vectors have been used in clinical trials for human gene therapy. Nonviral approaches using plasmid vectors is considered safer than those using viral vectors [1]. However, very few