were then incubated with the complex for 4 h. Then, the cells were washed with phosphate-buffered saline (PBS) and further incubated with the culture medium for specified time periods.

Luciferase assay

B16-BL6 cells were lysed using the cell lysis buffer of an assay kit (Piccagene, Toyo Ink, Tokyo, Japan). Samples were then mixed with the kit luciferase assay buffer, and the chemiluminescence produced was measured in a luminometer (Lumat LB9507; EG and G Berthold, Bad Wildbad, Germany).

mRNA quantification

Total RNA was extracted from cell samples using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan) in accordance with the manufacturer's instructions. To eliminate DNA contamination, the total RNA was treated with DNase I (Takara Bio) before reverse transcription. Reverse transcription was performed using a SuperScript II (Invitrogen) and oligo(dT) primer in accordance with the manufacturer's instructions. For quantitative mRNA expression analysis, real-time polymerase chain reaction was carried out using total cDNA in a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The sequences of the primers used for amplification were: GAPDH forward, 5′-CTGCCAAGTATGATGACATCAAGAA-3′; reverse, 5′-ACCAGGAAATGAGCTTGACA-3′; IFNAR1 forward, 5′-CCCAGAGTTCACCCTCAAGA-3′; reverse, 5′-GTGGGAAGCACACATGACAC-3′; IFNAR2 forward, 5′-TGATGACCCCGCAATAAAAT-3′; reverse, 5′-ATCGATGGCTTCTGAAGGTG-3′; IFNGR forward, 5′-GGGTTCCTGGACTGATTCCT-3′; reverse, 5′-GAACTCTTTTGACCGTGCAT-3′. Amplified products were detected on-line via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit; Roche Diagnostics). The cycling conditions were: 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 15 s Gene-specific fluorescence was measured at 72 °C. The mRNA expression of target genes was normalized by using the mRNA level of GAPDH.

Flouresence activated cell sorting analysis

Two days after siRNA transfection, adherent cells were detached using 2 mM ethylenediaminetetraacetic acid in PBS solution and the cells were then fixed with 4% paraformaldehyde in PBS. After blocking with 10% FBS in PBS, the cells were incubated with one of the following antibodies: anti-IFNAR1 (dilution 1 : 1000 dilution; mouse Anti-Mouse IFNAR1; Leinco Technologies Inc, St Louis, MO, USA), anti-IFNAR2 (dilution 1 : 1000 dilution; rat anti-Mouse IFNAR2; R&D Systems, Minneapolis, MN, USA) and phycoerythrin (PE)-labeled anti-IFNGR1 IFN-γRα (dilution 1 : 10; PE-labeled Anti-IFN-γRα; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). After washing, Alexa Fluor 488 goat anti-mouse secondary antibody (dilution 1 : 600; Invitrogen) or Alexa Fluor 488 goat anti-rat secondary antibody (dilution 1 : 600; Invitrogen) was added to the cells incubated with Mouse Anti-Mouse IFNAR1 or Rat Anti-Mouse IFNAR2, respectively. Samples were analyzed on a flow cytometer (FACSCan; BD, Franklin Lakes, NJ, USA). The Alexa Fluor 488 signal (FL1 channel) and PE signal (FL2 channel) were detected on a logarithmic scale. Forward (FSC) signals were detected in a linear mode. The FL1 and FL2 photomultiplier voltages were set using unstained isotype samples. The threshold using FSC was set to exclude debris without excluding any populations of interest. The flow cytometric data were analyzed with WINMDI 2.8 software® (http://facs.scripps.edu/software.html).

Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were collected at indicated times after transfection. The IFNγ concentration in the supernatants was determined by ELISA using a commercial kit (Ready-SET-Go! Mouse IFNγ ELISA; eBioscience, San Diego, CA, USA). The IFN-β concentration in the supernatant was determined by ELISA as described previously [18].

Measurement of IFN-β concentration in mouse serum

Five-week-old male BALB/c mice (approximately 20 g in body weight), purchased from Japan SLC, Inc. (Shizuoka, Japan), were used for all experiments. The protocols for the animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University. Injections of plasmid DNA and siRNA were carried out using the hydrodynamic injection method [23]. Mice received an injection of siRNA targeting IFNAR1 or GFP at a dose of 30 μg. Three days later, mice received another injection of pGZB-β (3 μg) together with siRNA (10 μg). The protocol of siRNA injections was determined based on the results of preliminary experiments. At indicated periods after injection of pGZB-β, blood was collected from the tail vein. The blood samples were incubated at 4 °C for 2 h to allow clotting and then centrifuged at 8000 *g* to obtain serum. The concentration of IFN-β in the serum was analyzed by ELISA as described above.

Statistical analysis

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

Results

Effect of IFNs on transgene expression of luciferase in B16-BL6 cells

To evaluate the suppressive effect of IFNs on transgene expression, B16-BL6 cells were transfected with pCMV-Luc, which encodes luciferase as a model transgene under the control of CMV promoter, and the luciferase activity was measured after a 24-h incubation in the presence or absence of different concentrations of IFN-β or IFN-γ (Figure 1a). Addition of 0.1 IU IFN-β/ml scarcely affected the activity, although increasing concentrations of IFN-β significantly reduced the activity in a concentration-dependent manner. At a concentration of 100 IU/ml, IFN-β reduced the luciferase activity to approximately 30%. A ten-fold higher IFN-β concentration (1000 IU/ml) induced no further reduction in the activity. Addition of IFN-γ resulted in a similar, but slightly lower, reduction in the luciferase activity. On the basis of these results, the concentration of 100 IU/ml was selected for further experiments. Figure 1b shows the luciferase activity in cells cotransfected with pcDNA, pCMV-β or pCMV-γ (1 μg/ml) and pCMV-Luc (1 μg/ml). Again, the luciferase activity from pCMV-Luc was significantly lower when cells were cotransfected with pCMV-β or pCMV-γ.

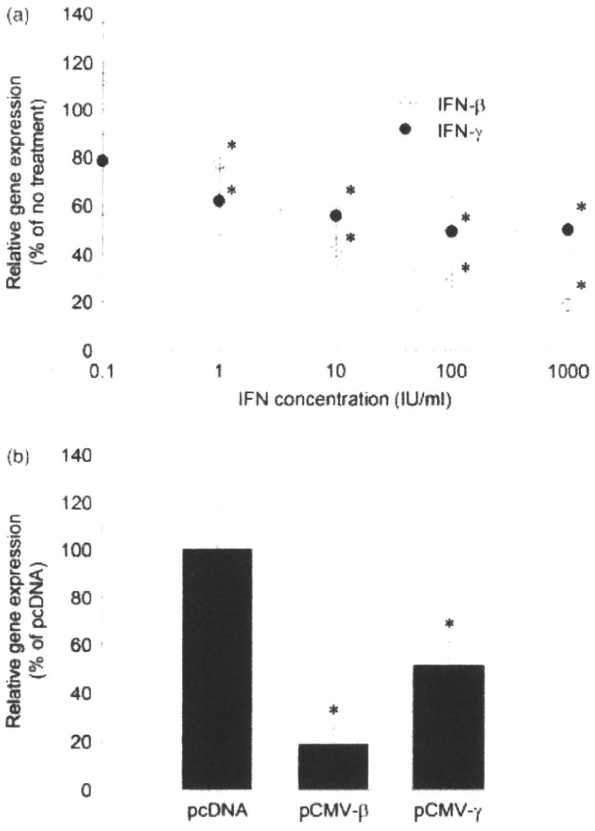

Figure 1. Effect of IFNs on luciferase activity in B16-BL6 cells after transfection of luciferase-expressing plasmid DNA. (a) B16-BL6 transfected cells were incubated with the indicated concentrations of IFN-β and -γ for 24 h, and the luciferase activity was measured. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ compared to no treatment group. (b) B16-BL6 cells were co-transfected with pcDNA, pCMV-β or -γ. Luciferase activity was measured 2 days after the co-transfection. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ (Student's *t*-test) compared to pcDNA group

Knockdown of IFNR in B16-BL6 cells by transfection of siRNA

siRNAs targeting three different mRNA sites were tested for IFNAR1, IFNAR2 and IFNGR1 (Figure 2a). Transfection of siGFP slightly changed the mRNA expression level of IFNAR1, IFNAR2 and IFNGR to approximately 110%, 95% and 120% of the untransfected group, respectively. Transfection of siRNA to cells reduced the mRNA level of the corresponding receptors to 60–20% of the siGFP-treated group. Based on these results, the most effective siRNA for each target was selected, and was used in the subsequent experiments. The selected siRNAs were named siIFNAR1, siIFNAR2 and siIFNGR1, respectively.

Figures 2b to 2d shows the histograms of the flow cytometry data of B16-BL6 cells after treatment with each siRNA. At 2 days after transfection, IFNAR1 (b), IFNAR2 (c) and IFNGR1 (d) proteins on the cell surface were stained with fluorescent labeled antibodies. The level of IFNAR1 expression on the surface of B16-BL6 cells transfected with siIFNAR1 was lower than that of those transfected with siGFP. Similarly, lower IFNGR1 expression was observed in siIFNGR1-transfected cells. The expression of IFNAR2 was also reduced in the siIFNAR2-transfected cells, although the degree of reduction was only modest compared to the other cases.

Effect of IFNR knockdown on IFN-mediated suppression of transgene expression in B16-BL6 cells

Cells transfected with siRNA (1 μg/ml) and pCMV-Luc (1 μg/ml) were incubated with 100 IU/ml IFN-β or IFN-γ, 24 h after transfection. Figure 3a shows the luciferase activity in cells after a 24-h incubation with IFN-β or IFN-γ. Cells transfected with siIFNAR1 showed a significantly higher luciferase activity than those transfected with siGFP when cells were mixed with IFN-β. Similarly, the siIFNGR1-transfected cells showed a high luciferase activity in the presence of IFN-γ compared to the other groups. However, siIFNAR2-transfected cells showed similar levels of luciferase activity to those of siGFP-transfected cells.

Then, the experiments were repeated by replacing IFN proteins with IFN-expressing plasmids (Figure 3b). pcDNA, pCMV-β or pCMV-γ (0.66 μg/ml) was cotransfected to the cells with pCMV-Luc (0.66 μg/ml) and one

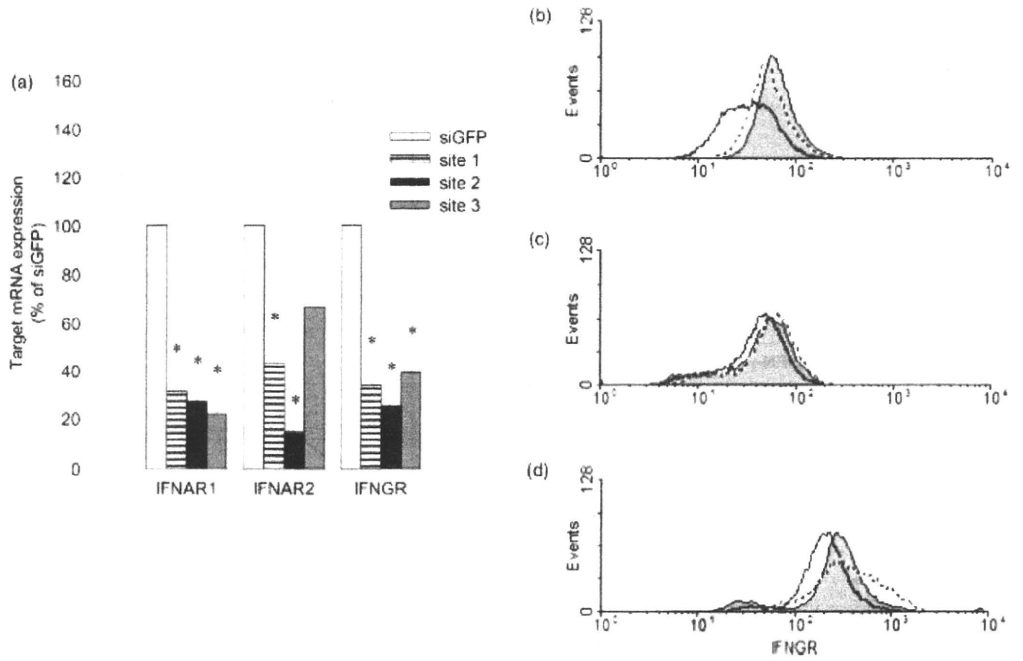

Figure 2. Expression of IFN receptors in B16-BL6 cells after transfection of siRNA. (a) The amount of IFNR mRNA expression was determined 1 day after transfection of siRNA. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ compared to the siGFP group. (b–d) Two days after siRNA transfection, the IFNR on the surface of B16-BL6 cells were fluorescently stained with antibodies specific for IFNAR1 (b), IFNAR2 (c) or IFNGR (d). The fluorescent intensity of stained cells was analyzed by flow cytometry. Dashed lines, Untransfected cells; Solid lines with shade, siGFP-transfeceted cells; solid lines, siIFNR-transfected cells

of siRNA (0.66 μg/ml). Cells cotransfected with pCMV-β and siIFNAR1 showed a significantly higher level of luciferase activity than cells transfected with pCMV-β and the siGFP-transfected group. In addition, a significant increase was observed in the luciferase activity when cells were cotransfected with pCMV-γ and siIFNGR1.

Effect of IFNR knockdown on IFN transgene expression

Cells were cotransfected with siRNA and pCMV-β or pCMV-γ, and the transgene expression of IFNs from plasmid vectors was examined by measuring their concentration in culture medium. The concentration of IFN-β and IFN-γ in the culture media of untransfected B16 cells was under the detection limit (<78.125 pg/ml for IFN-β and <15.625 pg/ml for IFN-γ). Figure 4a shows the concentration of IFN-β in culture media of cells after transfection of pCMV-β. The concentration of IFN-β in the culture media of the cells transfected with pcDNA or pCMV-γ was below the detection limit. The concentration of IFN-β reached a peak 1 day after transfection, and declined thereafter. The concentration slowly declined in the siIFNAR1-transfected cells compared to that in the siGFP-transfected cells. Transfection of siIFNAR2 or siIFNGR1 had no significant effects on the profile of IFN-β. Figure 4b shows the concentration of IFN-γ after transfection of pCMV-γ. The concentration of IFN-γ in the culture media of the cells transfected with pcDNA or pCMV-β was below the detection limit. The concentration of IFN-γ reached a maximum 2 days after transfection. Transfection of siIFNAR1 or siIFNAR2 had little effect on the concentration of IFN-γ, although that of siIFNGR1 markedly increased and sustained the concentration of IFN-γ.

Finally, mice received hydrodynamic injections of siRNA and pGZB-β to investigate whether silencing IFNR expression in transgene-expressing cells is effective in extending the time-period of IFN gene expression in mice. In mice administered with plasmid DNA not expressing IFN-β, serum IFN-β concentration was below the detection limit. Figure 4c shows the serum concentration of IFN-β after hydrodynamic injection of pGZB-β with siGFP or siIFNAR1. No significant differences were observed in the serum concentration of IFN-β for the first 3 days after injection. However, the serum IFN-β concentration in the siIFNAR1-injected mice was significantly higher than that in the siGFP-injected mice 7–14 days after injection. We did not observe any obvious toxicity in siIFNAR1-administered group compared to the siGFP-administered group.

Discussion

Cytokine-mediated suppression of transgene expression is a problem frequently associated with gene therapy because cytokines can be induced by gene vectors, such as viral vectors and liposome/DNA complexes. Qin

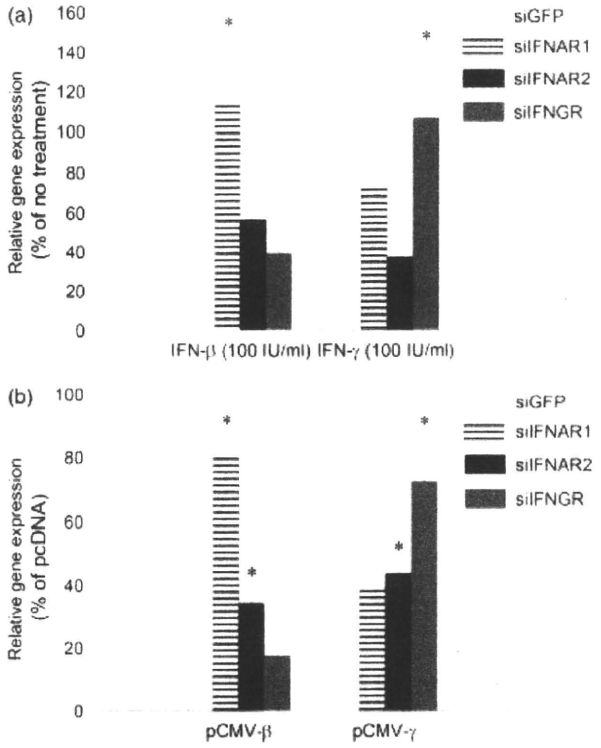

Figure 3. Effect of IFN on luciferase activity in B16-BL6 cells after transfection of luciferase-expressing plasmid DNA and siRNA targeting IFNR. (a) One day after cotransfection of pCMV-Luc (1 μg/ml) with 1 μg/ml of siGFP, siIFNAR1, siIFNAR2 or siIFNGR, transfected cells were treated with or without 100 IU/ml of IFN-β or -γ. Luciferase activity was measured 2 days after transfection and luciferase activities of IFN-treated cells relative to that of cells incubated without IFNs were calculated for each siRNA-transfected group. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ compared to the siGFP group. (b) pcDNA, pCMV-β or pCMV-γ (0.66 μg/ml) were cotransfected to B16-BL6 cells with pCMV-Luc (0.66 μg/ml) and one of siRNA (0.66 μg/ml). Luciferase activity was measured 2 days after transfection and luciferase activities of pCMV-β- or pCMV-γ-transfected cells relative to that of cells transfected with pcDNA were calculated for each siRNA-transfected group. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ compared to the siGFP group

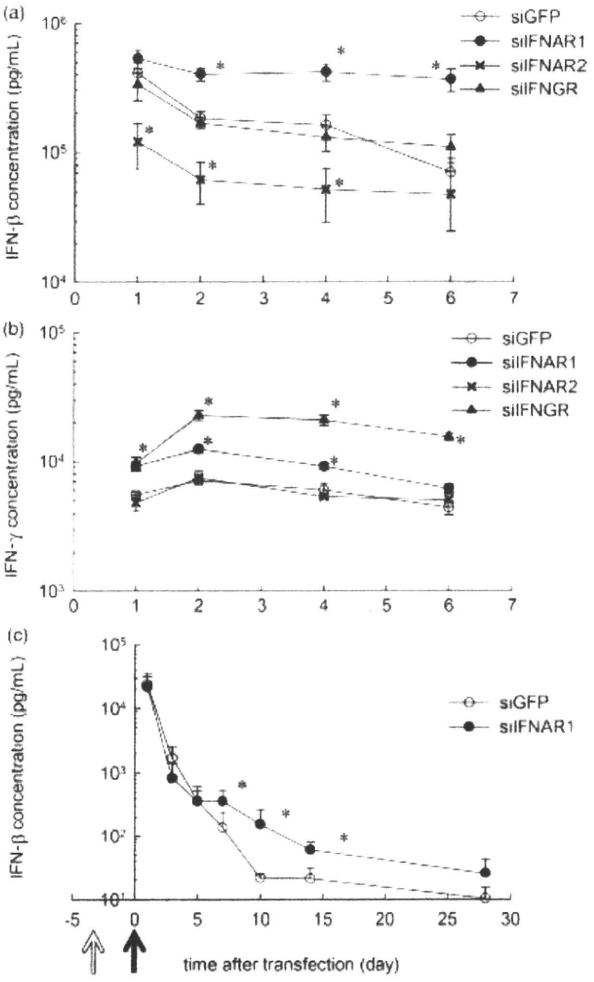

Figure 4. Effect of co-transfection of IFN-expressing plasmid DNA and siIFNR on transgene expression of IFN. B16-BL6 cells were transfected with siRNA (1 μg/ml) and (a) pCMV-β (1 μg/ml) or (b) pCMV-γ (1 μg/ml). Culture supernatant was collected 1, 2, 4 and 6 days after transfection. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ compared to the siGFP group. (c) Mice were hydrodynamically injected with 30 μg of siGFP or siIFNAR1 at the time point indicated by white arrow. Three days later, the mice received another hydrodynamic injection of pGZB-β (3 μg) with siGFP or siIFNAR1 (10 μg) at the time point indicated by black arrow. Serum was collected at the indicated times after the administration of pGZB-β. Results are presented as the mean ± SD of four independent mice. *$p < 0.05$ compared to the siGFP group

et al. [17] reported that IFN-γ suppressed the transgene expression from CMV promoter-driven adenoviral vectors and that anti-IFN-γ monoclonal antibody was effective in prolonging *in vivo* transgene expression from adenoviral vectors. In addition, IFN-α/β has also been proven to suppress *in vitro* and *in vivo* transgene expression from liposome/DNA complexes [18]. Although the exact mechanism by which IFN suppresses transgene expression has not been fully clarified yet, previous studies suggest some possible explanations. Qin *et al.* [17] showed that IFN-γ inhibits transgene expression at the mRNA level and has little effect on the amount of vector DNA, total protein synthesis and viability of the transfected cells. In addition, it has been reported that CMV promoter-driven transgene expression was reduced by IFN-α as a result of the reduced stability of the mRNA transcript [24]. Because cytokines expressed by vectors would also inhibit transgene expression in an autocrine or paracrine manner, such forms of inhibition represent a fundamental problem for cytokine-based gene therapy.

In the present study, we found that transgene expression of firefly luciferase in cells was suppressed when the cells were exposed to IFNs that were added to or expressed by the cells (Figures 1a and 1b). In the experiments investigating the suppressive effect of IFNs on luciferase expression, we did not observe obvious reduction in cell number in the IFN-treated group when

cell number was estimated by protein quantification of cell lysate and MTT assay (data not shown). In our previous study, IFNs exerted cytotoxic and antiproliferative effects on B16-BL6 cells [25]. However, in that previous study, cell number was counted after more than 4 days of incubation with IFNs. Because cells were incubated with IFNs for less than 2 days for luciferase experiment in the present study, reduction in luciferase activity after the incubation with IFNs was mainly a result of the suppressive effects on transgene expression, and not the reduced cell number. Because almost all types of cells, including B16-BL6 cells, express IFNRs, this suppression could be a result of the interaction between IFNs and their receptors. This hypothesis was supported by the experimental results showing that IFN-dependent suppression of luciferase activity almost completely disappeared when the IFNR expression was silenced by the corresponding siRNA (Figure 3). Therefore, these results are the first experimental evidence to show that the interaction of IFN and its corresponding receptor suppresses transgene expression in receptor-positive cells.

In all experiments performed in the present study, we did not observe any significant changes in the level of transgene expression in siIFNAR2-transfected cells in the presence of IFN-β. The degree of reduction in IFNAR2 protein expression after siIFNAR2 transfection was much lower than that in IFNAR1 and IFNGR protein expression after corresponding siRNA transfection, although we found a marked reduction in IFNAR2 mRNA expression after siIFNAR2 transfection. Therefore, the low inhibition of IFNAR2 protein expression by siIFNAR2 could be one reason why siIFNAR2 was not effective. In addition, as Nguyen *et al.* [26] have reported that STAT binding to IFNAR2 was not required for IFN signaling, these results suggest that IFNAR2 does not play an important role in transducing IFN signaling.

When pCMV-β or pCMV-γ was cotransfected with siIFNAR1 or siIFNGR1, respectively, the expression of IFN was more sustained in both cases compared to the other siRNA-transfected group, which was in agreement with the results obtained in the experiment using luciferase genes (Figures 4a and 4b). Because cells were exposed to IFNs for time periods that were long enough for IFNs to exert their antiproliferative and cytotoxic effects in this experiment, the greater production in IFNs from the cells that received the transfection of pCMV-β or pCMV-γ with siIFNAR1 or siIFNGR1, respectively, may partly be the result of cells with reduced IFNR expression being less sensitive to the antiproliferative and cytotoxic effects of IFNs. Moreover, it was also shown *in vivo* that silencing IFNAR1 expression is effective in prolonging IFN-β expression (Figure 4c) as a result of administering siIFNAR1 and pGZB-β by the hydrodynamic injection method. These results clearly show that cotransfection of IFN-expressing plasmid DNA and siRNA targeting the receptor is a viable approach to obtaining sustained IFN transgene expression.

Therapeutic effects of IFN gene transfer are obtained through the binding of IFNs to their receptors on the surface of target cells, such as cancer cells, so that the knockdown of IFNR on target cells has little therapeutic benefit. In some cases of *in vivo* gene therapy, healthy cells, including muscle cells [27,28] and hepatocytes [29,30], are used as a factory to produce therapeutic proteins. The knockdown of IFNR would be applicable to such situations to prolong the transgene expression of IFN.

In conclusion, we have demonstrated that silencing IFNR expression in transgene expressing cells can prolong IFN transgene expression by protecting the cells from the autocrine effect of IFNs. The results obtained in the present study provide one strategy for improving the therapeutic effect of IFN-based gene therapy by prolonging IFN gene expression.

Acknowledgments

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

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

Kayoko Hattori,* Makiya Nishikawa,* Kanitta Watcharanurak,* Akihiko Ikoma,† Kenji Kabashima,† Hiroyasu Toyota,* Yuki Takahashi,* Rei Takahashi,‡ Yoshihiko Watanabe,§ and Yoshinobu Takakura*

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

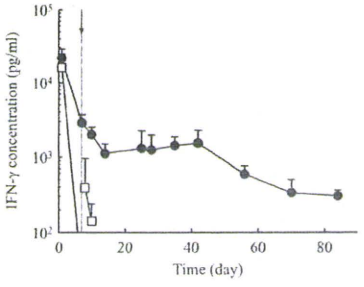

FIGURE 1. Time course of the concentration of IFN-γ in mouse serum after injection of IFN-γ–expressing plasmid DNA. NC/Nga mice were injected i.v. with 0.14 μg pCpG-Muγ (●) or 20 μg pCMV-Muγ (□) by the hydrodynamic injection method. The pCMV-Muγ–injected group received a second injection of 20 μg pCMV-Muγ 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.

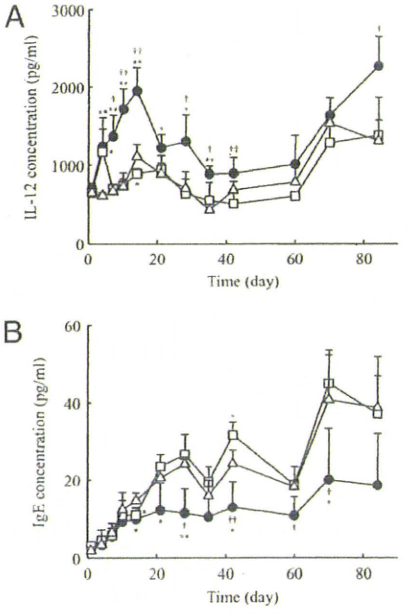

FIGURE 2. Time course of the concentration of IL-12 (*A*) and IgE (*B*) in mouse serum after injection of IFN-γ–expressing plasmid DNA. NC/Nga mice were injected i.v. with 0.14 μg pCpG-Muγ or 20 μg pCMV-Muγ, as described in the legend of Fig. 1. Blood samples from untreated (△), pCpG-Muγ–injected (●), 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.

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).

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

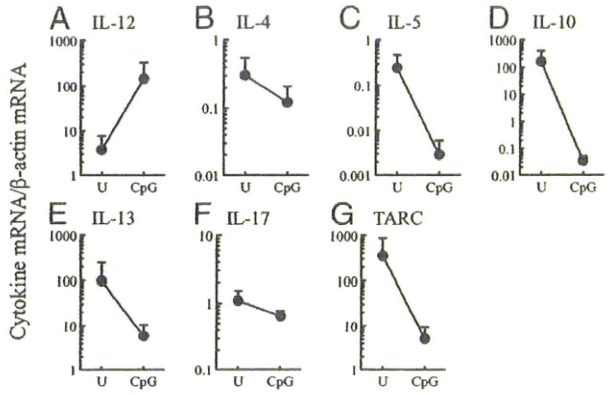

FIGURE 3. mRNA expression of cytokines and TARC in spleen cells. Spleens from NC/Nga mice receiving 0.14 μg pCpG-Muγ (CpG) were collected 14 d after gene transfer, and the mRNA expression of cytokine and chemokine genes was measured by real-time PCR. The mRNA expression of genes was normalized using 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*).

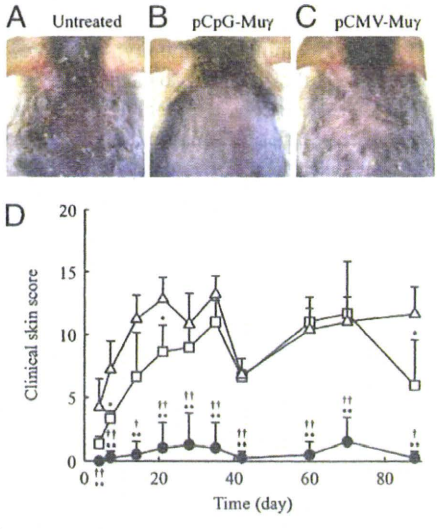

FIGURE 4. Typical images of the back skin of NC/Nga mice (*A–C*) and the time course of the skin clinical score (*D*). Photographs were taken 35 d after the start of treatment. *A*, Untreated mice. *B*, pCpG-μγ–treated mice. *C*, pCMV-μγ–treated mice. *D*, Clinical features of dermatitis were scored 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.

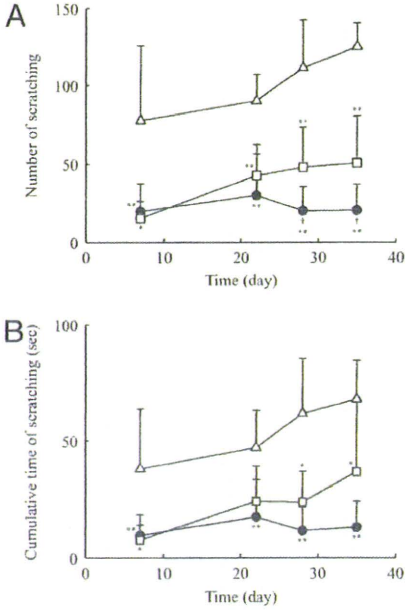

FIGURE 5. Number (*A*) and cumulative time (*B*) of scratching episodes. Scratching behavior of NC/Nga mice treated as described in the legend of Fig. 1 were automatically evaluated using SCLABA-Real. △, untreated mice; ●, pCpG-Muγ–treated mice; □, pCMV-Muγ–treated mice. The results are expressed as 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.

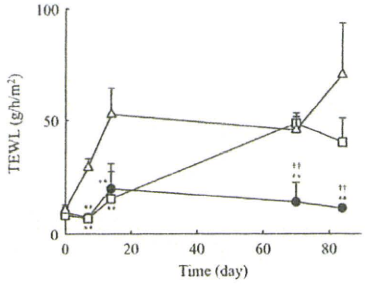

FIGURE 6. Time course of the TEWL of the back skin of mice. TEWL was measured on the back of untreated mice (△), pCpG-Mug–treated mice (●), and pCMV-Mug–treated mice (□). The results are expressed as the mean ± SD of at least three mice. $^{**}p < 0.01$ compared with the untreated group; $^{\dagger\dagger}p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

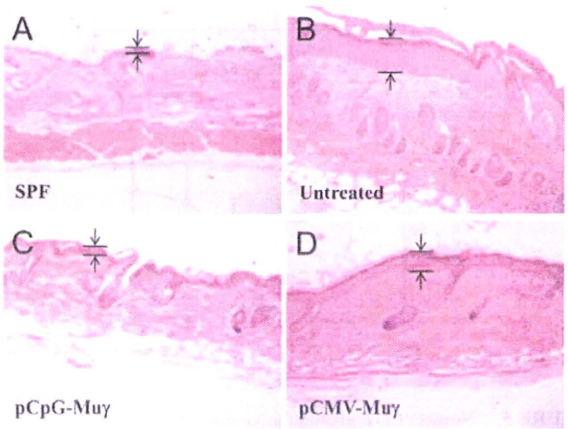

FIGURE 7. H&E sections of the back skin of NC/Nga. Dorsal skin of mice maintained under SPF conditions (*A*), untreated mice (*B*), pCpG-μγ–treated mice (*C*), and pCMV-μγ–treated mice (*D*) were collected at day 14. Skin sections were stained with H&E for histological evaluation and detection of inflammatory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

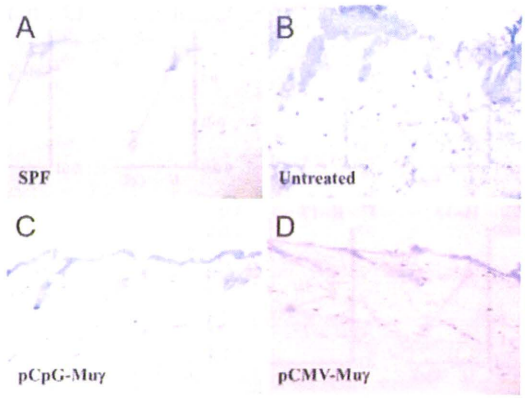

FIGURE 8. Toluidine blue sections of the back skin of NC/Nga mice. Dorsal skin of mice maintained under SPF conditions (*A*), untreated mice (*B*), pCpG-Muγ–treated mice (*C*), and pCMV-Muγ–treated mice (*D*) were collected at day 14. Skin sections were stained with toluidine blue to detect mast cells.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

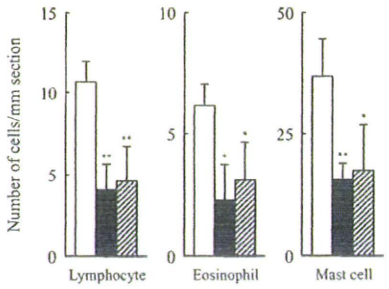

FIGURE 9. Number of lymphocytes, eosinophils, and mast cells in skin sections. Lymphocytes and eosinophils in H&E-stained sections were counted under a microscope. The toluidine blue sections were used for the counting of mast cells. The number of cells was expressed as the mean ± SD of three sections: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

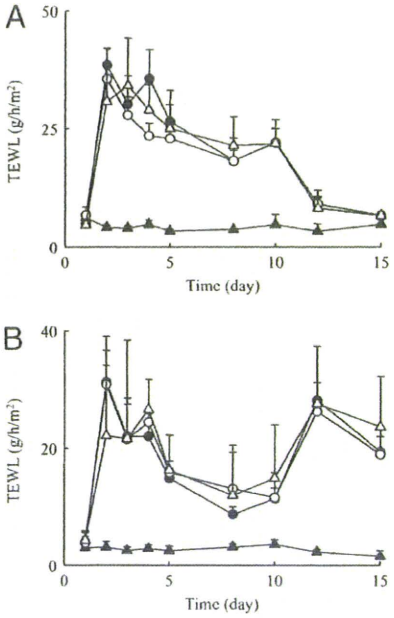

FIGURE 10. Time course of the TEWL of the back skin of mice in a stripped skin and a TNCB-induced dermatitis model. *A*, The back skin of C57BL/6 mice was shaved and tape stripped on days 1 and 8. ▲, untreated mice; △, tape-stripped mice; ○, tape-stripped, saline-treated mice; ●, tape-stripped, pCpG-Muγ–treated 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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RESEARCH ARTICLE

Injection site-dependent induction of immune response by DNA vaccine: comparison of skin and spleen as a target for vaccination

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Abstract

Background The antigen-specific immune response is dependent not only on the properties of the antigens, but also on their encounter with antigen-presenting cells. A previous study showed that the spleen produced a large amount of transgenes after direct tissue injection of plasmid DNA. In addition, the spleen is the largest organ in the lymphatic system and contains a variety of types of immune cells, including lymphocytes, macrophages and dendritic cells. Thus, it can be a promising target for DNA vaccination.

Methods Tissue-dependent properties of transgene expression were examined using a plasmid vector expressing firefly luciferase. Mice received injections of pCMV-Luc into the dorsal skin or spleen followed by electroporation, and the luciferase activity was measured 6 h after injection. Then, plasmids expressing a model antigen ovalbumin (pCMV-OVA) or its typical major histocompatibility complex class I-restricted epitope SIINFEKL (pPep-ER) were injected into C57BL/6 mice twice at an interval of 1 week. Seven days after the second immunization, OVA-specific humoral and cellular immune responses were evaluated.

Results The spleen produced a larger amount of transgenes than the skin after direct tissue injection of plasmid DNA. However, intradermal injection of plasmid DNA resulted in a larger amount of OVA-specific antibodies and a greater cytotoxic T lymphocyte response compared to intrasplenic injection. In addition, intradermal immunization with either pCMV-OVA or pPep-ER generated more protective effects against EG7-OVA tumor challenge.

Conclusions The results obtained in the present study indicate that the spleen is unlikely to be a good target for immunization despite the presence of a large number of lymphocytes and efficient production of transgenes.

Keywords antigen-specific immune response; gene transfer; intradermal injection; intrasplenic injection; plasmid DNA; tumor immunotherapy

Introduction

DNA vaccination is a simple and effective method for inducing protective immune responses against a variety of tumors as well as infectious diseases by the injection of plasmid DNA encoding an antigen of interest. These immune responses include both humoral and cellular immune responses,

each of which can be evaluated through the determination of neutralizing antibodies and antigen-specific cytotoxic T lymphocytes (CTLs), respectively [1]. Because plasmid vectors possess several advantages over recombinant or hybridoma-derived proteins, including their relatively easy and inexpensive preparation, and less toxicity and less immunogenicity compared to viral vectors [2–4], a variety of delivery methods for plasmid DNA have been developed thus far, and some of these are presently undergoing clinical trials [5–8].

The antigen-specific immune response is dependent not only on the physicochemical and immunological properties of antigens, but also on their encounter with antigen-presenting cells (APCs). To increase transgene expression of locally-injected plasmid DNA encoding antigens, electroporation has often been applied to the sites where plasmid DNA is injected. It has been reported that electroporation produces a ten- to 1000-fold increase in the expression of transgene after direct tissue injection of naked plasmid DNA [9,10]. A previous study has shown that the injection of plasmid DNA encoding firefly luciferase (pCMV-Luc) followed by electroporation produced significant amounts of the transgene in a variety of tissues, including the liver, kidney, spleen, skin and muscle [11]. Vaccination with antigens is often performed by intradermal injection because of the easy accessibility of the tissue for administration and the abundant presence of Langerhans cells (LCs), the residual APCs in the epidermis. Upon recognition of antigen, Langerhans cells and dermal dendritic cells migrate to local lymph nodes where they present the antigen to T cells, thus initiating a variety of immunological responses [12].

The spleen is the largest organ in the lymphatic system, and plays an important role in humoral and cellular immune responses. It contains a variety of immune cells, including B and T lymphocytes, macrophages and dendritic cells [13], which provide the organ with a cytokine-rich environment [14,15]. Although the spleen-targeted delivery or expression of antigen appears to be an attractive strategy for increasing the antigen-specific immune response, its applicability as a target site of DNA vaccination has received little attention.

In the present study, we selected the spleen as an injection site of DNA and examined its usefulness as the target for DNA vaccination in comparison with the skin, a very popular target. Two types of plasmid DNA, pCMV-OVA and pPep-ER, which are able to undergo different presentation pathways, were used as DNA vaccines. pCMV-OVA is considered to mainly undergo cross-presentation, because the model antigen, OVA, is secreted from transduced cells and taken up by professional APCs for antigen presentation [16–19]. On the other hand, pPep-ER was developed as a novel vector in our laboratory, by which an antigen peptide is expressed and bound to the major histocompatibility complex (MHC) class I molecule without being secreted from transduced cells [20].

Materials and methods

Animals

Four-week-old female ICR mice and 5-week-old female C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). ICR mice were used for luciferase experiments and C57BL/6 mice were used for DNA vaccination studies. Animals were maintained under conventional housing conditions and received humane care according to the criteria outlined in the US National Institutes of Health 'Guide for the Care and Use of Laboratory Animals'. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

Plasmid DNA

pCMV-Luc, encoding firefly luciferase cDNA under the control of the cytomegalovirus (CMV) promoter, was constructed as described previously [11]. pCMV-OVA was a kind of gift from Dr Shoshana Levy (Department of Medicine/Oncology, Stanford University Medical Centre, Stanford, CA, USA) [21]. The construction of pPep-ER has been reported elsewhere [20]. In brief, oligodeoxynucleotides corresponding to the sequence of SIINFEKL peptide were annealed, then inserted into the *SalI-NotI* restriction site of the multiple cloning site of pCMV/myc/ER Shoot vector (Invitrogen, Carlsbad, CA, USA). All the plasmid DNA samples were amplified in the *Escherichia coli* strain DH5α, then isolated, and purified using a Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide straining and the DNA concentration was measured by ultraviolet absorption at 260 nm. The sequences of the recombinant DNA were confirmed by Kyoto Science Corporation Technical Service (Kyoto, Japan).

Cell culture

EL4 (C57BL/6, H-2b, T lymphoma) and EG7-OVA (EL4 cells transfected with OVA cDNA) [22] were purchased from American Type Culture Collection (Manassas, VA, USA). EL4 was cultured in Dulbecco's modified Eagle's medium (Nissui Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactive fetal bovine serum (FBS). EG7-OVA was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, glucose, sodium pyruvate, HEPES and G418.

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Gene transfer with luciferase-expressing plasmid DNA

Female ICR mice were anesthetized with diethyl ether, then, pCMV-Luc solution in saline (20 μg/20 μl) was injected into the dorsal skin or spleen. For the spleen injection, a 1-cm incision was made on the left flank and the spleen was withdrawn from the peritoneal cavity. Then, pCMV-Luc was injected and the incision was closed with metal clips. Each pCMV-Luc injection was followed by electroporation of the injection site. Electroporation was performed using a pair of 1 cm^2 forceps-type electrodes connected to a rectangular direct current generator (CUY-21; Nepagene Chiba, Japan). Electric pulses were delivered to target tissue using the electrodes approximately 30 s after tissue injection of plasmid DNA. Six hours or 6 days after injection, mice were killed and the skin or spleen receiving an injection of plasmid DNA was excised, homogenized in a lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM ethylenediaminetetraacetic acid, pH 7.8), and subjected to three cycles of freezing in liquid nitrogen (−190 °C) and thawing (37 °C). Then, the homogenates were centrifuged at 10 000 *g* for 10 min at 4 °C and 5 μl of the supernatant was mixed with 50 μl of a luciferase assay buffer (PGL1500; Tokyo Ink, Tokyo, Japan) and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG&G Berthhold, Bad Wildbad, Germany). The luciferase activity was expressed as relative light units (RLU) per tissue (RLU/tissue). In separate mice, imaging of luciferase activity was carried out using a NightOwl LB 981 Molecular Light Imager (Berthold Technologies, Bad Wildbad, Germany) as reported previously [23].

DNA immunization in mice

Female C57BL/6 mice were anesthetized with diethyl ether, and then injected with plasmid DNA into the dorsal skin or spleen twice with an interval of one week. For injection into the skin, plasmid DNA solution in saline (100 μg/50 μl) was injected intradermally. For injection into the spleen, a 1-cm incision was performed on the left flank of the back and the spleen was withdrawn from the peritoneal cavity. Then, pDNA solution in saline (100 μg/20 μl) was injected and the incision was closed with metal clips. Each injection was followed by electroporation. The parameters of the electric pulses were: 2 ms/pulse, 12 pulses, 4 Hz, 1000 V/cm. A group of mice received a single injection of 100 μg OVA protein (Sigma, St Louis, MO, USA) emulsified in complete Freund's adjuvant (CFA) (ICN Biomedicals Inc., Aurora, OH, USA) into the dorsal skin [24].

Anti-OVA antibody production

Five days after the last immunization, serum samples were collected from the tail vein of mice to measure anti-OVA IgG antibodies by an enzyme-linked immunosorbent assay (ELISA). OVA (1 mg/ml) in carbonate/bicarbonate buffer (0.05 M, pH 9.6) was placed in each well of 96-well flat-bottom polystyrene plates (100 μl/well). Following overnight incubation at 4 °C, the wells were blocked with 5% BSA-containing Tween-20 (ICN Biomedicals Inc.) phosphate-buffered saline (T-PBS; 0.5 w/w% Tween-20 in PBS) for 1 h at 37 °C. After washing three times with T-PBS, serially diluted 100-μl serum samples were added to each well. After 2 h of incubation at 37 °C, the wells were washed five times with T-PBS, and 100 μl of anti-IgG-HRP conjugate (Sigma), diluted 2000 : 1 with 5% BSA-containing T-PBS, was added to each well. After 1 h of incubation at 37 °C, each well was washed with T-PBS and then 200 μl of freshly prepared *O*-phenylenediamine dihydrochloride (Wako Pure Chemical Industries Ltd, Osaka, Japan) solution containing 0.08 μl of hydrogen peroxide in phosphate-citrate buffer (0.075 M, pH 5.0) was added to each well. After 10 min of incubation, 50 μl of 10% H_2SO_4 was added and then the absorbance was measured at 490 nm. Serum total IgG titers were estimated by the dilution ratio at which an absorbance value of 0.2 was obtained.

Interferon (IFN)-γ production

Seven days after the last immunization, spleens were isolated from the immunized mice and single cell suspensions were prepared. Cells were placed in 96-well plates and adjusted to a concentration of 1×10^6 cells/well in a RPMI 1640 medium supplemented with 10% FBS, 1% penicillin, streptomycin, and glutamine and 50 μM 2-mercaptoethanol. They were stimulated by addition of 1 mg/ml OVA solution for 4 days in 5% CO_2 at 37 °C. After the cells were centrifuged at 420 *g* for 10 min, the supernatants were harvested and freeze-thawed. Then, IFN-γ levels in the supernatant were measured by ELISA using antibodies for capture and detection in accordance with the manufacturer's instructions (Ready-SET-Go! Mouse IFN-γ ELISA; eBioscience, San Diego, CA, USA).

CTL assay

Seven days after the last immunization, spleens were isolated from the immunized mice and single cell suspensions were prepared. Spleen cells were restimulated with mitomycin C-treated EG7-OVA in 5% CO_2 at 37 °C for 5 days. Target cells, EL4 and EG7-OVA, were labelled with ^{51}Cr by incubating with $Na_2{}^{51}CrO_4$ (Daiichi Radioisotope Labs, Tokyo, Japan) in culture medium for 45 min at 37 °C. After washing, 2×10^4 of the ^{51}Cr-labelled target cells and serially diluted spleen cells were co-incubated in

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200 µl of culture medium for 4 h at 37 °C. Spontaneous release of ^{51}Cr without effector cells and maximal release in the presence of 1% Triton X-100 were also evaluated. Cells were centrifuged at 420 *g* for 5 min, and 100 µl of each supernatant was collected for radioactivity measurements. The cytolytic activity of CTL was calculated [25] as:

$$\% \text{ of killing} = (\text{observed release-spontaneous release})/(\text{maximal release-spontaneous release}) \times 100$$

Tumor challenge

Eight days after the last immunization, 1×10^6 cells per mouse of EG7-OVA were administered intradermally into the back as a challenge. The mice were monitored for tumor growth every 3–4 days. The longest and shortest diameter of the tumor was measured, and the tumor volume was calculated from the equation: (longest × shortest)$^{3/2} \times \pi/6$. The survival time of the tumor-challenged mice was also recorded.

Results

Optimization of parameters for electroporation

To optimize the parameters for electroporation, pCMV-Luc was injected into the dorsal skin or spleen. According to the results obtained in a previous study [11] and those obtained in preliminary experiments, the following parameters were fixed: number of pulses = 12; frequency of pulses = 4 Hz. The electric field and the length of the pulse remained variable. Electric pulses were applied to the injection site approximately 30 s after injection using forceps-type electrodes. We evaluated the effect of the pulse length (2 and 5 ms), and of the electric field (750 and 1000 V/cm) on the luciferase activity and tissue damage. The luciferase activity in the skin was slightly greater at 1000 V/cm than at 750 V/cm (Figure 1A). The luciferase activity in the skin obtained using pulses with a duration of 2 ms was not significantly different from that using pulses of 5 ms. A similar tendency of luciferase activity was observed for injection into the spleen. According to these results, the short pulse length of 2 ms was selected to avoid possible tissue damage associated with longer electric pulses. The parameters of the electric pulses used in the subsequent experiments were: 2 ms/pulse, 12 pulses, 4 Hz and 1000 V/cm. Using these fixed parameters, a higher transgene expression was obtained after gene transfer to the spleen compared to that after gene transfer to the skin (Figure 1A). The expression in the skin was sustained for a long period of time and the luciferase activity in the skin was approximately 10 000-fold or greater than that in the spleen at 6 days after injection, indicating that the expression in the skin prolongs far longer than that in the spleen. The *in vivo* imaging data in white ICR mice also confirmed the long expression in the skin (Figures 1B and 1D). The expression in the skin was detectable even at 6 days after injection, whereas that in the spleen was not detectable at day 3 or later after injection.

Production of anti-OVA IgG antibody in mouse serum

To examine whether there were any differences in the antigen-specific antibody production between the injection sites, we measured the amount of anti-OVA IgG antibodies in immunized mice. Figure 2 shows the total anti-OVA IgG antibody level after two immunizations. A significant level of anti-OVA antibody was detected in mice immunized with pCMV-OVA irrespective of the site of injection. The level of antibody in mice immunized by the intradermal route was approximately 2.6-fold higher than that in mice immunized by the intrasplenic route. No significant antibody production was observed in mice immunized with pPep-ER, because it does not express any MHC class II epitopes required for antibody production.

Production of IFN-γ from spleen cells of immunized mice

We investigated IFN-γ production in spleen cells of immunized mice in response to the stimulation with OVA. Spleen cells from mice immunized with pCMV-OVA generated a large amount of IFN-γ, regardless of intradermal or intrasplenic immunization (Figure 3). A slightly greater amount of IFN-γ was produced in mice immunized by the intradermal route than by the intrasplenic route, although the difference was not statistically significant. Immunization with pPep-ER resulted in only a minor amount of IFN-γ production because of the absence of MHC class II epitope in the plasmid.

Induction of OVA-specific CTLs

We examined the OVA-specific CTL response using EG7-OVA cells expressing OVA and EL4 cells as target cells, respectively. The spleen cells from the mice immunized with pPep-ER intradermally had the highest level of OVA-specific CTL activity among all groups examined (Figure 4A). The level obtained was even comparable with OVA in CFA, which is highly effective but too severely toxic to use in humans. Little OVA-specific CTL activity was observed in the spleen cells from the mice intrasplenically immunized with pPep-ER. In the case of pCMV-OVA, spleen cells from mice immunized either intradermally or intrasplenically showed high OVA-specific CTL activity. In particular, intradermal immunization induced higher OVA-specific CTL activity than intrasplenic immunization. No significant CTL activities were seen against EL4

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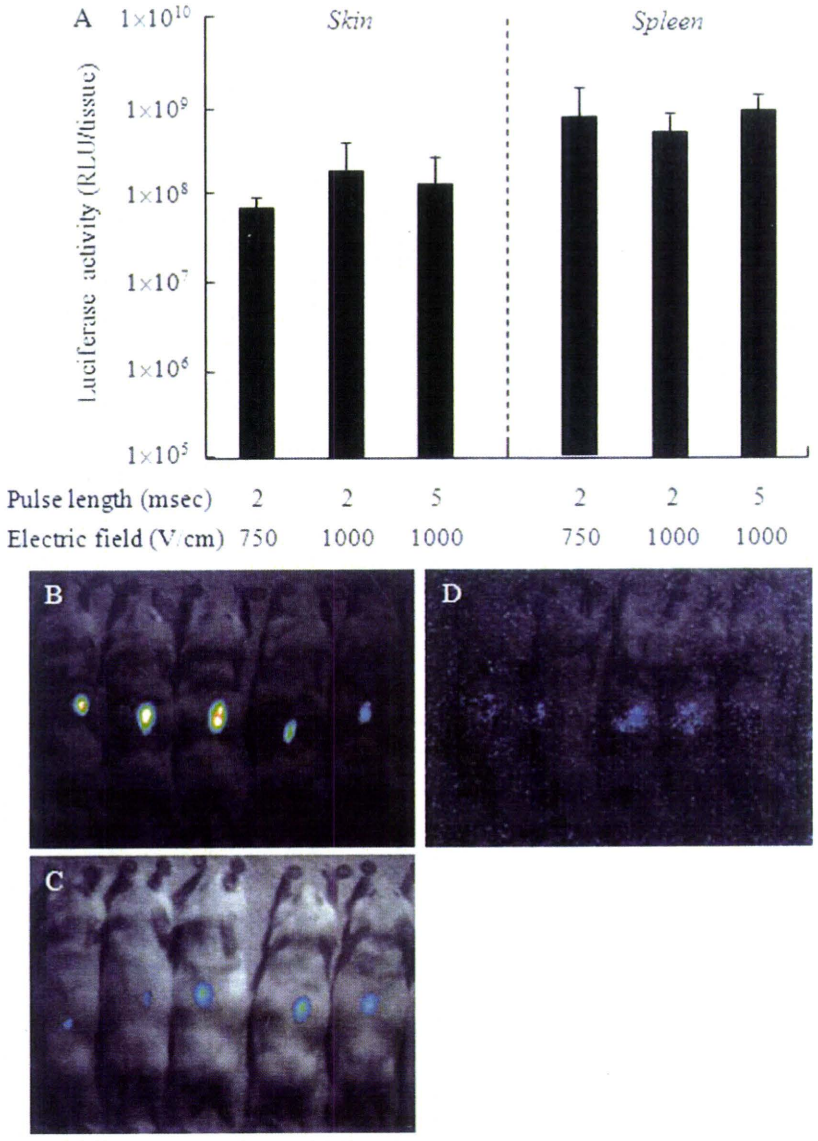

Figure 1. Transgene expression after direct tissue injection of pCMV-Luc into the skin or spleen. (A) Effect of electric pulse parameters on the level of luciferase activity after direct tissue injection of pCMV-Luc into the skin or spleen. Female ICR mice were injected intradermally or intrasplenically with 20 μg pCMV-Luc in 20 μl of saline and received 12 electric pulses (4 Hz). The pulse length and electric field are as shown. Six hours after injection, mice were killed and the luciferase activity of the skin or spleen was measured as relative light units. The results are expressed in RLU/tissue as the mean ± SD of four mice. (B–D) Time course of transgene expression after direct tissue injection of pCMV-Luc into the skin or spleen. Female ICR mice were injected intradermally or intrasplenically with 20 μg pCMV-Luc in 20 μl of saline and received 12 electric pulses (2 ms, 1000 V/cm, 4 Hz). Luminescent images were taken at (B) 1 day, (C) 6 days after intradermal gene transfer, or at (D) 1 day after intrasplenic gene transfer. No detectable luminescence was observed in mice at 3 or 6 days after intrasplenic gene transfer

cells, indicating that the CTL activities were OVA-specific responses (Figure 4B).

Growth inhibition of EG7-OVA in immunized mice

Finally, we compared the intradermal and intrasplenic immunization in terms of the protection against EG7-OVA tumor challenge *in vivo*. Similar to the results obtained with respect to CTL activities, stronger inhibitory effects were observed in the intradermally immunized mice compared to those immunized intrasplenically, irrespective of the plasmid used (Figure 5). At day 90 after EG7-OVA cells were transplanted, 50% (three of six) and 33.3% (two of six) of the mice that had been intradermally immunized with pPep-ER and pCMV-OVA, respectively, were still alive. All the mice that received intrasplenic immunization with pPep-ER and pCMV-OVA died at days 49 and 61, respectively (Figure 5B).

Discussion

To evaluate whether the intrasplenic immunization with plasmid DNA vaccines can be an alternative to conventional intradermal immunization, we compared the

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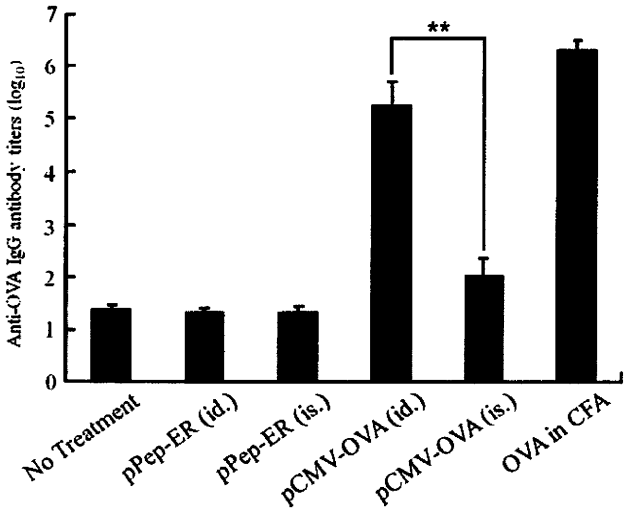

Figure 2. Production of total anti-OVA IgG antibody after immunization with pCMV-OVA or pPep-ER via the intradermal (id.) or intrasplenic (is.) route. Mice were immunized twice with 100 μg of plasmid DNA followed by electroporation (12 pulses, 4 Hz, 2 ms, 1000 V/cm). Five days after the last immunization, the serum samples from immunized mice were collected and the antibody titers were measured by ELISA. The results are expressed as the mean ± SD of four to ten mice. **$p < 0.01$

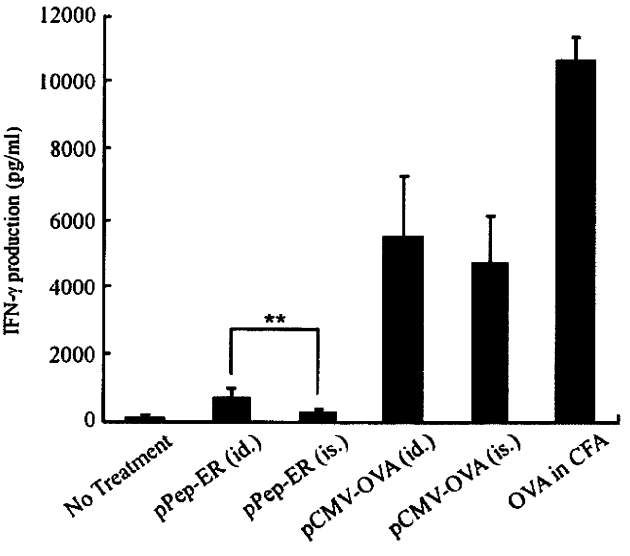

Figure 3. Production of IFN-γ from spleen cells of mice immunized with pCMV-OVA or pPep-ER via the intradermal (id.) or intrasplenic (is.) route. Mice were immunized twice with 100 μg of plasmid DNA followed by electroporation. Seven days after the last immunization, spleen cells isolated from immunized mice were co-cultured with OVA protein at 37 °C for 4 days. The levels of IFN-γ released into the culture medium were measured by ELISA. The results are expressed as the mean ± SD of four mice

antigen-specific immune response induced by intradermal and intrasplenic immunization using plasmid DNA followed by electroporation. In a different set of experiments, we found that two or more immunizations with plasmid DNA result in high humoral and cellular immune responses systemically. On the basis of these observations, we selected two immunizations in the present study, in order to obtain significant immune responses and to avoid possible tissue damages by electroporation, which was especially obvious in the spleen when more harsh conditions of electroporation were used. Another parameter that could be optimized was the injection volume of DNA solution. The injection volume to spleen was limited because of the small size of the organ, so that the volume was fixed to 20 μl. In the case of skin, and to make more solution diffuse into the dermis, the volume was fixed to 50 μl. We found that there were no significant differences in the level of transgene expression in the skin when a same amount of DNA was injected in 20 or 50 μl of solution (data not shown).

Two types of plasmid DNA, pCMV-OVA and pPep-ER, which mainly undergo cross-presentation and direct-presentation pathways, respectively, were used as vaccines. Generally, the generation of antibody is associated with helper T cells [26], which recognize and are activated by MHC class II molecule-antigen complex presented by APCs. Therefore, immunization with pCMV-OVA or OVA in CFA, both of which possess MHC class II epitopes, is able to induce anti-OVA antibody. By contrast, pPep-ER, which harbors only a typical MHC class I epitope of OVA, SIINFEKL, cannot generate antibodies. In the case of IFN-γ production from spleen cells after restimulation of OVA protein *in vitro*, significant production was observed in the mice immunized with pCMV-OVA or OVA in CFA, but not in the mice immunized with pPep-ER. When the site for immunization was compared, more IFN-γ production was observed after intradermal injection compared to intrasplenic injection. Similar trends were observed in the antigen-specific CTL activities and antigen-specific anti-tumor effects; a higher immune response was induced by intradermal immunization than intrasplenic immunization, although the differences depended on the type of vectors used. These results strongly suggest that the peak amount of antigens expressed is not the only parameter determining the generation of the immune response, including humoral and cellular immune responses, because the peak amount of antigens expressed is similar for the intrasplenic injection and intradermal injection of plasmid DNA. The use of adjuvants, such as double-stranded RNA, would increase the potency of DNA vaccination without changing the level of transgene expression, which would appear to be a promising approach, although it is beyond the scope of the present study.

A previous study [11] demonstrated that the transgene expression in the spleen and skin after tissue injection reached a peak at 6 h after injection, although the expression in the spleen quickly decreased with an apparent half-life of approximately 16 h. Seven days after injection, the expression in the skin was approximately 25-fold greater than in the spleen. Similar results were observed in the present study when electroporation was applied to the injection sites (Figure 1). This rapid decrease in the transgene expression in the spleen might be one factor responsible for the poor results obtained with respect to intrasplenic immunization, whereas the

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