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ORIGINAL ARTICLE

Regulation of immunological balance by sustained interferon-γ gene transfer for acute phase of atopic dermatitis in mice

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Interferon (IFN)-γ, a potent T helper 1 (Th1) cell cytokine, is suggested to suppress Th2 cell responses. Here, we aimed to investigate whether pCpG-Muγ, a plasmid continuously expressing murine IFN-γ, is an effective treatment of atopic dermatitis, a Th2-dominant skin disease. Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) atopic mice with early dermatitis were transfected with pCpG-Muγ by a hydrodynamic tail vein injection at a dose of 0.05 or 0.2 pmol per mouse. The skin lesions improved only in mice receiving the high dose of pCpG-Muγ. IFN-γ gene transfer resulted in a high mRNA expression of IFN-γ and interleukin (IL)-12 and regulatory T cell (Treg) related cytokines, such as IL-10 and transforming growth factor-β, in the spleen, whereas it reduced the IL-4 mRNA expression, and serum levels of immunoglobulin (Ig) G1 and IgE. In addition, the gene transfer markedly inhibited the epidermal thickening, infiltration of inflammatory cells into the skin, the occurrence of dry skin and pruritus. No exacerbating effect on the Th1-mediated contact dermatitis was observed after IFN-γ gene transfer. Taken together, these results indicate that sustained IFN-γ gene transfer induced polarized Th1 immunity under Th2-dominant conditions in NC/Nga mice, leading to an improvement in the symptoms of acute atopic dermatitis without adverse side effects.

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Keywords: atopic dermatitis; interferon-γ; NC/Nga mice; nonviral vector

INTRODUCTION

Atopic dermatitis is a chronic inflammatory skin disorder accompanied by particular skin lesions. The main characteristics of atopic dermatitis are eczematous skin lesions, epidermal hypertrophy, intense pruritus and infiltration by inflammatory cells. Increased serum immunoglobulin (Ig) E and an excessive production of T helper 2 (Th2) cytokines are also frequently observed in patients with atopic dermatitis. The balance of Th1 and Th2 immune responses, which is maintained by their immunoregulatory cytokines under healthy conditions, is considered to be upset in atopic dermatitis. Generally, atopic dermatitis was classified into two phases, acute and chronic. In the acute phase of atopic dermatitis, patients often have raised Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, which induce the class switching of B cells to IgE secretion, but low levels of IFN-γ or IL-12. In contrast, Th1 cytokines, especially IFN-γ, are dominant in chronic phase of atopic dermatitis.[1–6] Therefore, the administration of interferon (IFN)-γ, a typical Th1 cytokine that shifts the differentiation of naive T cells to the Th1 subtype and suppresses the production of Th2 cytokines, appears to be an attractive option for the treatment of acute phase of atopic dermatitis.[7,8]

It has recently been shown that regulatory T cells (Tregs), a subset of T cells, are involved in the modulation of allergic diseases. For example, Tregs and their related cytokines, IL-10 and transforming growth factor (TGF)-β, have been reported to suppress Th2 responses and IgE production.[9–11] Furthermore, IFN-γ as well as the IL-12-specific receptor subunit β2 has important roles in the production of Tregs.[12,13]

Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) mice is a mouse model of human atopic dermatitis. These mice spontaneously develop atopic dermatitis-like skin lesions around the age of 6–8 weeks when raised under conventional conditions but not in specific pathogen-free conditions. The skin lesions of conventional NC/Nga mice are characterized by erythema, hemorrhage, edema, erosion, scaling and dryness of the skin. Hyperproduction of IgE and Th2-type chemokines have also been reported in these mice. These clinical features are similar to those of human atopic dermatitis.[14–17]

We demonstrated in a previous study[18] that sustained expression of IFN-γ is effective in preventing the onset of symptoms of atopic dermatitis in NC/Nga mice. The severity of the atopic lesions and other notable symptoms, such as scratching, dry skin, epidermal thickening, infiltration of inflammatory cells and the raised expression of Th2 cytokines and IgE, were markedly inhibited in mice receiving pCpG-Muγ, a plasmid expressing murine IFN-γ for a long period of time.[18] To use IFN-γ gene transfer in the treatment of atopic dermatitis patients, it is important to show that such gene transfer is effective not only in preventing the onset of the disease but also in improving the symptoms. IFN-γ gene transfer could counteract the progression of atopic dermatitis through the induction of Treg cells.

In this study, we investigated whether IFN-γ gene transfer was able to effectively treat dermatitis induced in NC/Nga mice. To do this, we evaluated the serum levels of IgE and IgG1, the mRNA expression of Th1 and Treg cytokines, transepidermal water loss (TEWL), the number of scratching episodes, the epidermal

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thickening and the infiltration of inflammatory cells into the skin. In addition, the effect of sustained IFN-γ gene transfer on contact dermatitis was also examined to assess the safety of the gene transfer process.

RESULTS

Time-course and pharmacokinetic parameters of IFN-γ after hydrodynamic injection of pCpG-Muγ

Figure 1 shows the time-course of the concentrations of IFN-γ in the serum after hydrodynamic injection of pCpG-Muγ. The serum levels of IFN-γ were dependent on the plasmid dose, and the higher dose of 0.2 pmol resulted in significantly higher concentrations for the first 4 days compared with the lower dose. The profiles were analyzed to obtain area under the concentration-time curve and mean residence time values. Table 1 summarizes the Cmax, area under the concentration-time curve and mean residence time after IFN-γ gene transfer. The Cmax and area under the concentration-time curve were increased by increasing the plasmid dose, whereas the mean residence time was hardly affected by the dose.

Effects of IFN-γ gene transfer on the skin lesions of NC/Nga mice

NC/Nga mice at the age of 7–8 weeks, which already developed atopic dermatitis-like symptoms, were used to assess the severity of skin lesions. Figure 2 shows the time-course of the clinical skin score of NC/Nga mice after injection of pCpG-Muγ. The score of mice treated with the lower dose pCpG-Muγ increased with time and was not significantly different from that of the untreated mice for the first 2 weeks after gene transfer. These results contradicted those of our previous study, in which the lower dose of pCpG-Muγ (0.05 pmol per mouse) was effective in preventing the onset of atopic dermatitis.[18] In contrast, the clinical skin score of mice receiving 0.2 pmol pCpG-Muγ was significantly lower than those of the other two groups over the 5-week observation period. Based on these findings, the dose of the plasmid was set at 0.2 pmol per mouse in the subsequent experiments to study the therapeutic effects of pCpG-Muγ in NC/Nga mice with dermatitis. To confirm the effects of IFN-γ, a control plasmid pCpG-mcs (Invivogen, San Diego, CA, USA) was administered to NC/Nga mice. However, the clinical skin score was hardly affected by this treatment (data not shown).

Serum concentrations of IgG1 and IgE in NC/Nga mice after IFN-γ gene transfer

Figure 3 shows the time-course of the concentration of IgG1 (Figure 3a) and IgE (Figure 3b) in the serum of NC/Nga mice after injection of pCpG-Muγ. Both the serum levels of IgG1 and IgE of the pCpG-Muγ-treated mice were significantly lower than those of the untreated mice at several time points, suggesting that the production of these Th2-mediated Igs in NC/Nga mice was at least partly inhibited by IFN-γ gene transfer.

mRNA expression of Th1, Th2 and Treg cytokines in the spleen of NC/Nga mice after IFN-γ gene transfer

The high concentrations of IFN-γ in the serum were expected to induce the changes in the immunological status of NC/Nga mice. Therefore, the spleen, a major lymphoid organ, was excised and the mRNA expression of IFN-γ, IL-12, IL-4, IL-10 and TGF-β was measured. Figure 4 shows the x-fold increase in mRNA expression by pCpG-Muγ-treated mice compared with untreated mice. The mRNA expression of IFN-γ and IL-12 in the pCpG-Muγ-treated mice was, respectively, 3- and 1.7-fold higher than that in the untreated mice. In addition, the mRNA expression of IL-10 and TGF-β was also significantly higher in the pCpG-Muγ-treated group. On the other hand, the mRNA expression of IL-4, a Th2 cytokine, was reduced to about half in the pCpG-Muγ-treated mice.

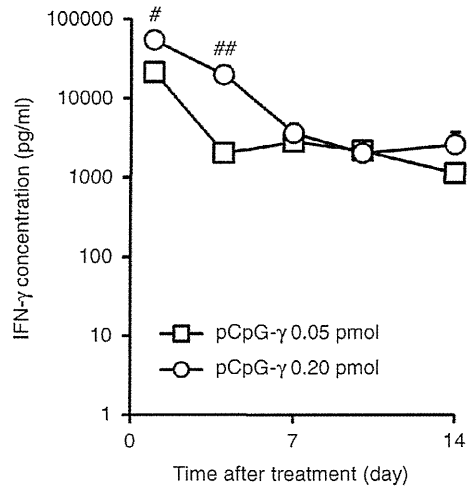

Figure 1. Time-course of the concentration of IFN-γ in serum of NC/Nga mice after hydrodynamic injection of 0.05 or 0.2 pmol pCpG-Muγ. The results are expressed as the mean ± s.e.m. of five mice. $^{\#, \#\#}P<0.05$, 0.01, compared with the lower dose group.

Table 1. Cmax, AUC and MRT of serum IFN-γ after hydrodynamic injection of pCpG-Muγ into mice

Dose (pmol per mouse)	*Cmax (ng ml^{-1})*	*AUC (ng day ml^{-1})*	*MRT (day)*
0.05	21.3 ± 1.5	71.3 ± 7.9	4.67 ± 0.78
0.2	54.0 ± 10[a]	204 ± 35[b]	4.21 ± 0.70

Abbreviations: AUC, area under the concentration-time curve; IFN, interferon; MRT, mean residence time. The Cmax values were obtained at 1 day after hydrodynamic injection of pCpG-Muγ, and are expressed as the mean ± s.e.m. of five mice. The AUC and MRT were calculated by moment analysis, and are expressed as the calculated mean ± s.e.m. of five mice.
[a]$P<0.05$, compared with the lower dose (0.05 pmol per mouse) group.
[b]$P<0.01$, compared with the lower dose (0.05 pmol per mouse) group.

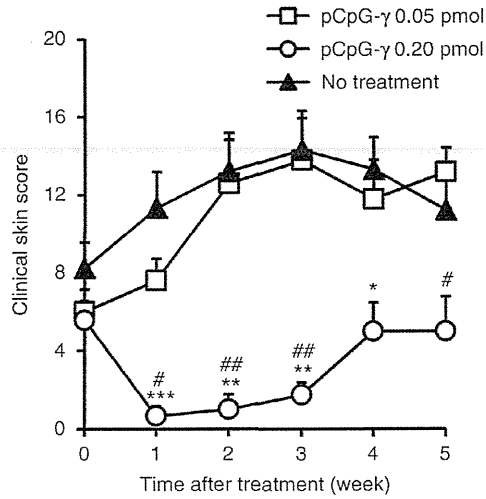

Figure 2. Time-course of the clinical skin score of NC/Nga mice after hydrodynamic injection of pCpG-Muγ. The results are expressed as the mean ± s.e.m. of five (0.05 pmol and 0.2 pmol pCpG-Muγ-treated groups) and nine mice (the untreated group). $^{*, **, ***}P<0.05$, 0.01, 0.001, compared with the untreated group; $^{\#, \#\#}P<0.05$, 0.01, compared with the lower dose group.

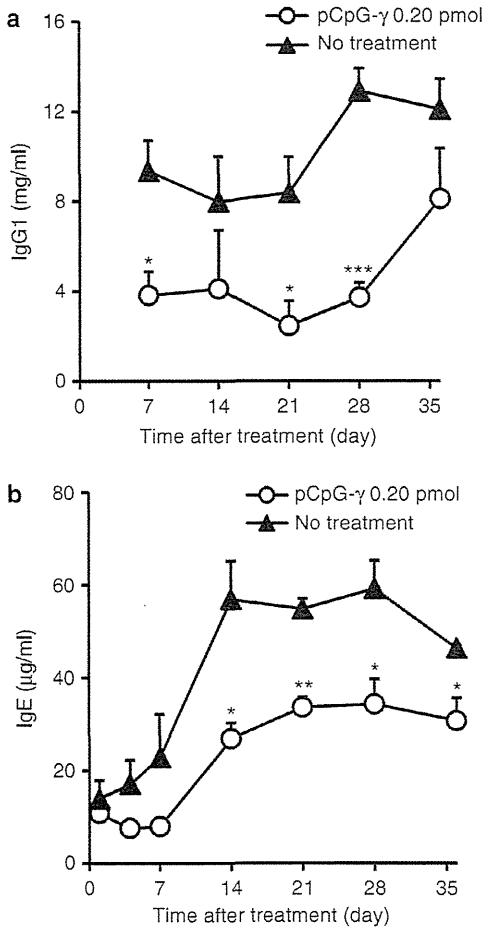

Figure 3. Time-course of the concentration of IgG1 (**a**) and IgE (**b**) in serum of NC/Nga mice after hydrodynamic injection of 0.05 or 0.2 pmol pCpG-Muγ. The results are expressed as the mean ± s.e.m. of four mice. *, **, ***$P<0.05$, 0.01, 0.001, compared with the untreated group.

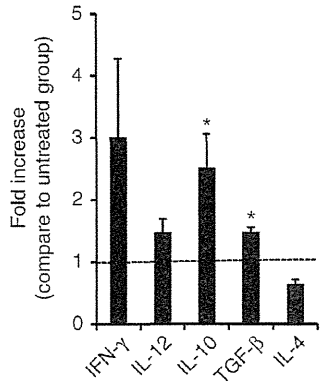

Figure 4. mRNA expression of cytokines in spleen cells of NC/Nga mice. Spleens of untreated mice and pCpG-Muγ-treated mice were excised at day 36 after treatment. mRNA expression was normalized with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are expressed as the mean ± s.e.m. of three (pCpG-Muγ-treated group) or four mice (the untreated group). *$P<0.05$ compared with the untreated group.

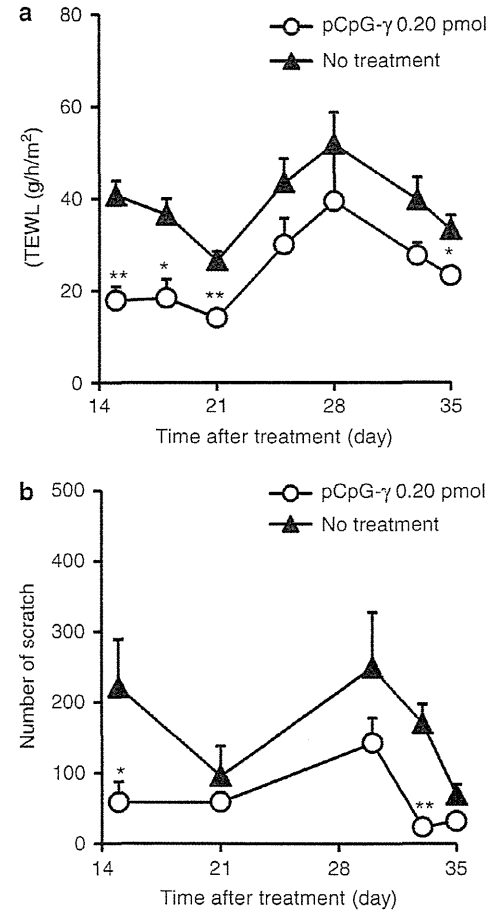

Figure 5. Time-course of the TEWL of the dorsal skin (**a**) and number of scratching episodes (**b**) of NC/Nga mice untreated or after hydrodynamic injection of 0.2 pmol pCpG-Muγ. The results are expressed as the mean ± s.e.m. of four mice. *, **$P<0.05$, 0.01, compared with the untreated group.

TEWL and scratching behavior of NC/Nga mice after IFN-γ gene transfer

To evaluate the severity of skin dryness and itchiness, the TEWL[19] and the number of scratching episodes were measured. Figure 5a shows the time-course of the TEWL of NC/Nga mice from day 15 to day 35 after injection of pCpG-Muγ. The TEWL value of the pCpG-Muγ-treated mice was significantly lower than that of the untreated mice on days 15, 18, 21 and 35 after gene transfer. Figure 5b shows the number of scratching episodes during a 30-min period of observation. The number of scratching episodes exhibited by the mice receiving pCpG-Muγ was fewer than that of the untreated mice at all-time points examined and the difference was significant on days 15 and 33 after injection of pCpG-Muγ. These results indicate that IFN-γ gene transfer relieves the pruritus, a common symptom of atopic dermatitis that may lead to destruction of the skin barrier and is related to the increased TEWL of NC/Nga mice.[20,21]

Histopathological examination of skin sections of NC/Nga mice

Figure 6 shows the hematoxylin and eosin sections (Figures 6a and b) and toluidine blue-stained sections (Figures 6c and d) of the dorsal skin of NC/Nga mice at 36 days after treatment. The sections of the untreated mice showed obvious thickening of the epidermis (Figures 6a and c). However, these characteristic

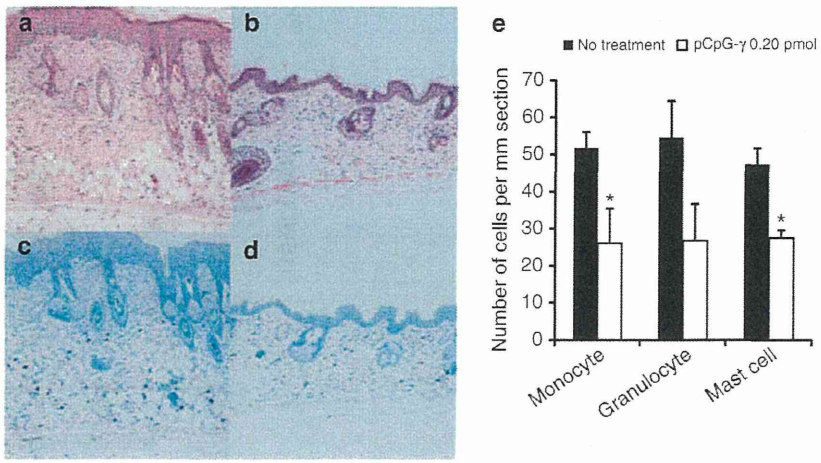

Figure 6. Hematoxylin and eosin (H&E) (**a**, **b**) and toluidine blue (**c**, **d**) sections of the dorsal skin of NC/Nga mice untreated (**a**, **c**) or pCpG-Muγ-treated (**b**, **d**). Numbers of inflammatory cells in skin sections (**e**). The numbers of cells were expressed as the mean ± s.e.m. of three (pCpG-Muγ-treated group) or four mice (the untreated group). $*P<0.05$ compared with the untreated group.

features of skin inflammation were hardly seen in the sections from the pCpG-Muγ-treated mice (Figures 6b and d). Furthermore, the numbers of monocytes, granulocytes and mast cells in the skin sections were counted (Figure 6e). The numbers of these cells infiltrating into the skin of the pCpG-Muγ-treated animals were about a half those in the untreated mice, and there were significant differences between the groups with regard to the number of monocytes and mast cells.

Effect of IFN-γ gene transfer on body temperature and liver injury

The body temperature of the mice was measured to monitor the side effects of the high-dose pCpG-Muγ. No significant difference was observed in body temperature between the treated and untreated groups (data not shown). In addition, there were no significant differences in the serum aspartate aminotransferase and alanine aminotransferase levels between the pCpG-Muγ-treated and the untreated groups (data not shown).

Effect of IFN-γ gene transfer on contact dermatitis

There are some concerns that IFN-γ gene transfer might increase the risk of Th1-associated diseases. To clarify this issue, the ear swelling response in the oxazolone (OX)-induced contact dermatitis model was used to evaluate the contact hypersensitivity response in mice. As shown in Figure 7, OX treatment induced ear swelling but this response was not exacerbated by IFN-γ gene transfer.

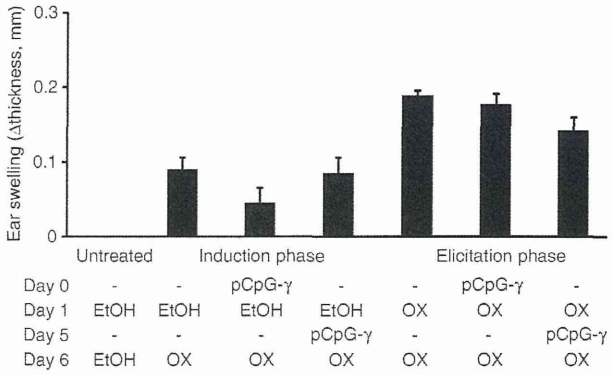

Figure 7. Ear swelling response in OX-treated mice. pCpG-Muγ was injected on day 0 or day 5. Mice were sensitized by OX or ethanol on the shaved abdomen on day 1 and on the right ear on day 6. Ear thickness was measured 24 h after the last OX application. Data are expressed as the mean ± s.e.m. of four mice.

DISCUSSION

NC/Nga mice spontaneously develop atopic dermatitis-like skin lesions around the age of 6–8 weeks when raised under conventional condition. After that, the severity of the skin lesions and the parameters reflecting the severity, such as serum IgE level, increases with age. A previous study reported that the serum IgE levels of conventional NC/Nga mice increased markedly from 6 weeks of age to around 10 weeks and reached plateau at 17 weeks.[16] Thus, the mice with atopic dermatitis at the age of 7–8 weeks, which were used in this study, represent a suitable model to evaluate the therapeutic effect of IFN-γ in the acute phase of atopic dermatitis. In this study, we demonstrated that pCpG-Muγ is effective not only in preventing the onset of this condition but also in alleviating the symptoms of the disease, although a higher dose of pCpG-Muγ was required to treat the symptoms of NC/Nga mice with dermatitis. The mRNA expression of IFN-γ and IL-12, Th1 cytokines, in the spleen of mice receiving pCpG-Muγ was increased and accompanied with a reduced expression of IL-4 mRNA in the spleen and reduced levels of serum IgG1 and IgE (Figure 3). IFN-γ acts in conjunction with IL-12 by mediating a positive feedback loop to drive the Th1 response and inhibits IL-4 production by Th2 cells, resulting in the suppression of IgE and IgG1 secretion by B cells.[22–24] The mRNA expression of IL-10 and TGF- β, two representative Treg cytokines, in the spleen of mice receiving pCpG-Muγ were also increased. It has been reported that Tregs inhibit Th2 cell function in allergic diseases by releasing IL-10 and TGF-β.[11,25] The development of atopic dermatitis-like skin lesions in NC/Nga mice was suppressed by IL-10 expressing plasmid DNA.[26] It is possible that the increased expression of IFN-γ and IL-12 might induce Treg differentiation[12,13] and partially contributed to these immunological changes by suppressing Th2 cell response. Taking all these findings into consideration, it is likely that IFN-γ gene transfer stimulates IL-12 production and these two Th1 cytokines act synergistically to polarize the T-cell response toward Th1 subset and inhibit IgG1 and IgE production by suppressing the Th2 pathway. Further investigation is needed

to prove whether the induction of Treg cytokines is related to these changes.

Scratching destroys skin barriers and worsens skin lesions,[20,21] which is closely linked to an increased TEWL. Histological examination of the skin sections clearly showed that IFN-γ gene transfer markedly suppressed skin inflammation and the infiltration of monocytes, granulocytes and mast cells. Mast cells can be sensitized by IgE and regulate the secretion of cytokines, such as IL-4, which subsequently mediates the recruitment of leukocytes,[27] so that the reduced infiltration of these inflammatory cells into the skin could be due to the reduced production of IgE and IL-4. This suppression will prevent mice from scratching, which will accelerate skin repair and reduce TEWL. The number of scratching episodes of mice receiving pCpG-Muγ was lower than that of the untreated mice at all-time points, although it was fluctuated in the both groups. This could be explained by the difference in the time for measurement in each day,[28] which was not be controlled in this study.

The use of IFN-γ as a therapeutic agent would be a double-edged sword. The need for a high dose of 0.2 pmol per mouse to treat, not to prevent, atopic dermatitis symptoms increases the chance of adverse reactions. The most common adverse events of IFN-γ therapy, which have been evidenced in several clinical trials, are 'flu-like' symptoms, including fever, headache, fatigue and myalgia. Diarrhea, erythema at the injection site and the elevation of liver transaminase levels have also been reported in patients receiving the therapy. These common adverse events are generally transient and well tolerated.[29–34] In this study, we measured the body temperature and the liver transaminase levels in serum of mice for monitoring the signs of adverse events and demonstrated that IFN-γ gene transfer is safe for use in mice as long as the dose of the plasmid is 0.2 pmol per mouse or lower. However, the clinical skin score seems to return to baseline level at 4 weeks after treatment with this dose of plasmid (Figure 2). This result may be explained by the reduction of IFN-γ levels in the serum of mice with time, thus increasing the dose of pCpG-Muγ within the safety range could be an option to extend the duration of therapeutic effects. Recently, we have established a plasmid DNA expressing IFNγ in a constant manner with no initial high concentration of IFN-γ that could cause the unwanted effects.[35] This novel IFN-γ expressing plasmid would also be useful to enhance the therapeutic effect of IFN-γ. Another interesting point is that IFN-γ has been reported to be dominant in the skin of patients with chronic atopic dermatitis.[5] Further investigation is required to prove whether IFN-γ gene transfer is suitable for the treatment of chronic phase of atopic dermatitis.

In conclusion, we have demonstrated that sustained transgene expression of IFN-γ is effective in treating the atopic dermatitis in NC/Nga mice without any apparent adverse effects. These results raise a possibility that IFN-γ gene transfer can be a therapeutic option for patients with acute-phase atopic dermatitis.

MATERIALS AND METHODS

Animals

In all, 7- to 8-week-old male NC/Nga mice and 7-week-old female C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were raised and maintained on a standard food and water diet under conventional housing conditions. The NC/Nga group spontaneously developing dermatitis was used as a model of atopic dermatitis. The protocol for the animal experiments was approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

In vivo gene transfer of IFN-γ

The plasmid pCpG-Muγ constructed previously[36] was used for the treatment. The naked plasmid pCpG-Muγ was dissolved in normal saline solution and injected into the tail vein of the mice by the hydrodynamic injection method[37,38] at a dose of 0.05 or 0.2 pmol per mouse on day 0.

Measurement of the concentrations of IFN-γ, IgG1 and IgE

Blood samples were obtained from the tail vein at indicated times after gene transfer, kept at 4 °C for 2 h to allow clotting, and then centrifuged to obtain serum. The concentration of IFN-γ, IgG1 and IgE in the serum was measured using ELISA kits (Ready-SET-Go! Mouse IFN-γ ELISA, eBioscience, San Diego, CA, USA; Mouse IgG1 ELISA Quantitation Set, Bethyl Laboratories, Inc., Montgomery, TX, USA; and Mouse IgE ELISA Set, BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Calculation of the pharmacokinetics parameters of IFN-γ gene transfer

The area under the concentration-time curve and the mean residence time of IFN-γ after gene transfer were calculated from the concentrations of IFN-γ in the serum of NC/Nga mice using moment analysis.[39]

Real-time PCR analysis of cytokine expression

RNA was extracted from approximately 100 mg of spleen samples using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). A mixture of recombinant DNase I-RNase-free (TAKARA, Shiga, Japan) and RNase OUT recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA) was used for DNase treatment. Reverse transcription was performed using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). For a quantitative analysis of mRNA expression, reverse transcribed samples were amplified by PCR using the primers listed below, and a LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) in a LightCycler instrument (Roche Diagnostics GmbH) according to the manufacturer's protocols. The sequences of primers used were as follows (forward and reverse, respectively): IFN-γ (5′-ATGAACGCTACACA CTGCATC-3′ and 5′-CCATCCTTTTGCCAGTTCCTC-3′), IL-12 (5′-CATCGATGAG CTGATGCAGT-3′ and 5′-CAGATAGCCCATCACCCTGT-3′), IL-4 (5′-ACAGGAG AAGGGACGCCAT-3′ and 5′-GAAGCCCTACAGACGAGCTCA-3′), IL-10 (5′-CCA AGCCTTATCGGAAATGA-3′ and 5′-TTTTCACAGGGGAGAAATCG-3′), TGF-β (5′-TTGCTTCAGCTCCACAGAGA-3′ and 5′-TGGTTGTAGAGGGCAAGGAC-3′) and glyceraldehyde 3-phosphate dehydrogenase (5′-CTGCCAAGTATGATG ACATCAAGAA-3′ and 5′-ACCAGGAAATGAGCTTGACA-3′). Individual PCR products were analyzed by melting curve analysis and the length of the products was determined by agarose gel electrophoresis. The mRNA expression of each gene was normalized using the mRNA level of glyceraldehyde 3-phosphate dehydrogenase.

Scoring skin lesions

Skin lesions were scored at indicated times after gene transfer according to the criteria previously reported.[18] In brief, the scoring was based on (i) the eczema severity, (ii) erosion/excoriation, (iii) scale formation, (iv) erythema/hemorrhage, (v) inflammation of the face and (iv) inflammation of the ear. The total clinical skin severity score was defined as the sum of the each of the six signs (none = 0; mild = 1; moderate = 2; and severe = 3).

Measurement of TEWL

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

Observation of scratching behavior

The scratching behavior was recorded on video for 30 min on days 15 and 21. 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, were counted as one bout of scratching. On days 30, 33 and 35 after gene transfer, the scratching behavior was monitored using a SCLABA Real (Noveltec Inc., Kobe, Japan), an automated system to analyze the scratching behavior of small animals. Each mouse was put into an acrylic cage, and its behavior was recorded for 30 min. The number of scratching episodes was automatically quantified.[18]

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 obtained using a microtome and stained with hematoxylin and eosin for histological evaluation and quantification of the numbers of monocytes and granulocytes or with toluidine blue for the detection of mast cells. The number of monocytes, granulocytes and mast cells were manually counted under a microscope, and expressed as the number per unit length of the skin section.

Monitoring of adverse effects of IFN-γ gene transfer

The body temperature of the mice was monitored using a digital rectal thermometer (Physitemp Instruments Inc., Clifton, NJ, USA). To assess liver damage, the serum alanine aminotransferase and aspartate aminotransferase levels of the mice were assayed using commercial test reagents (Transaminase CII-Test Wako, Wako Pure Chemical Industries, Osaka, Japan).

OX-induced contact hypersensitivity

The plasmid pCpG-Muγ was injected into the tail vain of C57BL/6 mice (female, 7-week-old) by the hydrodynamic injection method at a dose of 0.2 pmol per mouse on day 0 or day 5. Mice were sensitized by the application of 100 μl 2% OX in ethanol (elicitation phase group) or ethanol alone (induction phase-group) to the shaved abdominal skin on day 1, followed by an application of 10 μl 1% OX to the right ear on day 6. Ethanol was applied to the left ear. For the untreated group, ethanol was applied instead of OX to the abdomen and right ear on either day 1 or 6. At 24 h after the second application, the thickness of both ears was measured using a digital thickness gauge (Quick Mini, Mitutoyo Co., Kawasaki, Japan). Ear swelling was calculated from the difference in ear thickness between the hapten- and vehicle-treated ears.

Statistical analysis

Statistical significance was evaluated by analysis of variance with a *post-hoc* Tukey–Kramer test for multiple comparisons (clinical skin scores) and Student's *t*-test for comparisons between two given groups. The level of statistical significance was set at $P<0.05$.

ABBREVIATIONS

IFN-γ, interferon γ; Ig, immunoglobulin; IL, interleukin; Th, T helper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Constant and steady transgene expression of interferon-γ by optimization of plasmid construct for safe and effective interferon-γ gene therapy

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Abstract

Background Hydrodynamic injection of pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ, a plasmid DNA (pDNA) expressing murine interferon (IFN)-γ with a murine cytomegalovirus (mCMV) enhancer and a human elongation factor (EF)-1 promoter, has been proven effective for the treatment of cancer and atopic dermatitis in mice. However, the initial peak of IFN-γ soon after injection was quite high compared to the steady level for subsequent periods, which could cause unwanted adverse effects. Therefore, in the present study, aiming to optimize the efficacy/side-effect ratio of IFN-γ gene transfer, we have developed plasmid vectors encoding murine IFN-γ under the control of different combinations of promoter and enhancer sequences.

Methods The promoter and enhancer sequence of pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ, a prototype plasmid expressing human IFN-γ, was replaced or deleted to obtain various pDNAs. To assess the transgene expression profile, each pDNA was delivered to mice by hydrodynamic injection and the serum IFN-γ concentration was measured periodically. On the basis of the results obtained, murine IFN-γ expressing pDNAs were constructed and the body weight change was monitored as an indicator of adverse effects.

Results The prototype pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ showed a high but declining concentration of IFN-γ. Those containing hROSA26 promoter expressed the transgene in a more constant manner with no initial high concentrations and scarcely reduced the body weight.

Conclusions These results indicate that hROSA26 promoter, irrespective of the presence and type of enhancers, is suitable for achieving constant and steady level of transgene expression and effective in avoiding the body weight loss caused by high concentrations of IFN-γ.

Keywords expression profile; hydrodynamic injection; interferon-γ; plasmid DNA; proinflammatory cytokine; promoter

Introduction

Interferon (IFN)-γ is a representative immunomodulatory cytokine that shifts the balance of type I helper T cells (Th1) and Th2 cells of immune responses towards Th1 [1]. In addition, IFN-γ possesses antiviral and antitumor activity. Therefore, IFN-γ has been used to treat several forms of cancer, such as renal cancer and adult T-cell leukemia, and is expected to be used for the treatment of viral infections [2–5] and immune disorders [6–9]. Unfortunately, its short

in vivo half-life of a few hours after either intravenous or intramuscular administration means that frequent dosing is required for therapeutic efficacy [10].

Gene delivery of IFN-γ is a promising approach to extending the time above the threshold concentration following a single administration. In our previous studies, we demonstrated that plasmid DNA with a reduced number of CpG dinucleotides produced a sustained transgene expression compared to conventional counterparts with many CpG motifs when administered to mice by hydrodynamic injection [11–13]. For example, mouse IFN-γ-expressing plasmid pCpG-muIFNγ, which contains human elongation factor (hEF)-1 promoter and murine cytomegalovirus (mCMV) enhancer sequences, produced high levels of IFN-γ in the serum for over 80 days. In addition, there have been several reports in which the number of unmethylated CpG sequences was found to correlate with the rate of transgene silencing under specific conditions. For example, CpG-dependent transgene silencing in the lung was mainly caused by the innate immune response triggered by the recognition of CpG motifs by Toll-like receptor 9-positive cells during cationic liposome-mediated gene transfer [14]. In addition, the level of transgene expression was decreased to 1:100 or less and approximately 70% of CpG motifs in the promoter and enhancer were methylated at 7 days after intramuscular injection of adenovirus vectors [15]. Furthermore, one of our previous studies showed that hydrodynamic gene transfer of naked plasmid DNA elicited less inflammatory cytokine than intravenous injection of the pDNA lipoplex [11]. These observations indicate that the methylation of CpG dinucleotides in plasmid DNA could induce transgene silencing after hydrodynamic injection.

The profile of serum IFN-γ was sustained after hydrodynamic injection of pCpG-muIFNγ, although the initial peak levels were approximately ten- to 100-fold higher than the trough levels [11,12]. Because the efficacy and side-effects of IFN-γ depend on its concentrations, the high concentrations during the initial phase would cause unwanted side-effects [16] when the dose is adjusted based on the steady-state level of IFN-γ. Indeed, we noted that body weight gain was delayed in mice receiving high doses of pCpG-muIFN-γ by hydrodynamic injection (M. Ando, Y. Takahashi, M. Nishikawa, Y. Takakura, unpublished observations). These findings suggest that maintaining a constant concentration of IFN-γ without initial surge after *in vivo* gene transfer is effective in increasing the efficacy/side-effect ratio. Based on these considerations, we attempted to develop plasmid vectors expressing IFN-γ in a constant manner. To evaluate the profile of transgene expression from plasmids, murine IFN-γ does not appear to be suitable because its biological activities could affect the several aspects of transgene expression from plasmids. Therefore, human IFN-γ, which is highly species specific and exhibits no biological activity in mice, was used to compare the properties of plasmid constructs. Because the promoter and enhancer of plasmids are known to greatly affect the profile of transgene expression from plasmid, new plasmids containing a variety of combinations of promoter/enhancer sequences were designed and their expression profiles were evaluated by measuring the serum concentration of human IFN-γ after hydrodynamic injection into mice. Based on a detailed comparison using human IFN-γ-expressing plasmids, the relationship between the transgene expression profile and the biological activity of murine IFN-γ was evaluated by monitoring the body weight change of mice after gene transfer using murine IFN-γ-expressing plasmids.

Materials and methods

Construction of IFN-γ expressing plasmid vectors

In our previous studies, we demonstrated sustained expression of murine IFN-γ from pCpG-muIFN-γ, so that a plasmid containing the same promoter/enhancer sequences and encoding human interferon-γ (huIFN-γ) cDNA, pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ, was constructed as a prototype vector by inserting the *Bgl*II/*Nhe*I huIFN-γ cDNA fragment amplified by polymerase chain reaction (PCR) into the *Bgl*II/*Nhe*I site of the pCpG-mcs vector (InvivoGen, San Diego, CA, USA). pSV40$_{enh}$-hEF-1$_{prom}$-huIFN-γ was constructed by inserting the *Sda*I/*Spe*I SV40 enhancer fragment amplified by PCR from pGL3-control vector (Promega, Madison, WI, USA) into the *Sda*I/*Spe*I site of prototype vector. Prototype vector was digested by *Sda*I/*Spe*I to separate mCMV enhancer from the other component. Next, the product that contains hEF-1$_{prom}$ and huIFN-γ cDNA was treated with T4 polymerase to obtain blunt ends. Then, the product was self-ligated to obtain pΔenh-hEF-1$_{prom}$-huIFN-γ. pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ was digested by *Hind*III and the product was treated with T4 polymerase, blunting the ends to create an *Eco*RV recognition site. Then, the product was digested by *Spe*I to obtain the fragment with the *Spe*I/*Eco*RV site. The fragment from prototype vector that contains cDNA of huIFN-γ and mCMV$_{enh}$ without promoter was ligated with the *Spe*I/*Eco*RV SV40 promoter fragment amplified by PCR from pGL3-control vector to obtain pmCMV$_{enh}$-SV40$_{prom}$-huIFN-γ. pSV40$_{enh}$-SV40$_{prom}$-huIFN-γ was constructed by inserting the *Sda*I/*Spe*I SV40 enhancer fragment amplified by PCR from pGL3-control vector into the *Sda*I/*Spe*I site of pmCMV$_{enh}$-SV40$_{prom}$-huIFN-γ. pmCMV$_{enh}$-SV40$_{prom}$-huIFN-γ was digested by *Sda*I/*Spe*I and the product containing the part of huIFN-γ cDNA was treated with T4 polymerase blunting the ends. Then, the product was self-ligated to obtain pΔenh-SV40$_{prom}$-huIFN-γ. pΔCpG CMV$_{enh/prom}$-huIFNγ was constructed by inserting the *Sda*I/*Hind*III CpG-free CMV enhancer/promoter fragment amplified by PCR from pGZB vector (provided by Dr N. Yew, Genzyme Corporation, Framingham, MA, USA) into the *Sda*I/*Hind*III site of pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ. pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ was digested by *Sda*I and the product that

contains the part of huIFN-γ cDNA was treated with T4 polymerase blunting the ends to the *Nru*I recognition site. Then, the product was digested by *Hind*III to obtain the fragment with the *Nru*I/*Hind*III site. The fragment from pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ with the *Nru*I/*Hind*III site was ligated with the *Nru*I/*Hind*III human CMV enhancer/promoter from pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to obtain phCMV$_{enh/prom}$-huIFN-γ. pΔenh-hROSA$_{prom}$-huIFN-γ was constructed by substituting the *Sda*I/*Hind*III enhancer and promoter sequences of pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ with 2.7 kb *Sda*I/*Hind*III hROSA26 promoter fragment, which was composed of core promoter in the first half of this sequence and a 1200-bp engineered intron in the latter half, from pBROAD2-mcs (InvivoGen). pSV40$_{enh}$-hROSA$_{prom}$-huIFN-γ and pmCMV$_{enh}$-hROSA$_{prom}$-huIFN-γ were constructed by inserting the *Sda*I SV40 enhancer fragment amplified by PCR from pGL3-control vector and the *Sda*I mCMV enhancer fragment amplified by PCR from pCpG-mcs into the *Sda*I site of pΔenh-hROSA$_{prom}$-huIFN-γ, respectively. pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ, which was previously constructed and named pCpG-Muγ [11], was also used as a prototype vector expressing murine IFN-γ (muIFN-γ). Also, pΔenh-hROSA$_{prom}$-muIFN-γ was constructed by substituting the *Sda*I/*Hind*III enhancer and promoter sequences of pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ with a 2.7-kb *Sda*I/*Hind*III hROSA26 promoter fragment. The plasmids used in the present study are summarized in Table 1.

Animals

Four-week-old male ICR mice (approximately 20 g in body weight) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained on a standard food and water diet under temperature- and light-controlled conditions. All protocols for the animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

In vivo gene transfer to mice

Gene transfer to mouse liver was performed by the hydrodynamic injection method [17]. In brief, mice received a rapid injection of naked plasmid DNA dissolved in 1.6 ml of saline solution into the tail vein. In most experiments, the dose of plasmid DNA was set to 0.05 pmol/mouse, which corresponds to 0.18 μg/mouse for plasmids containing the hROSA26 promoter and to 0.12 μg/mouse for other plasmids. Higher doses of 100 and 50 μg/mouse were used for pΔenh-hEF-1$_{prom}$-huIFN-γ and pΔenh-SV40$_{prom}$-huIFN-γ, respectively, because of undetectable expression at the low dose. The dose for muIFN-γ-expressing plasmids were set to 4.5 and 1.2 μg/mouse, which corresponds to 1.3 and 0.5 pmol/mouse for pΔenh-hROSA$_{prom}$-muIFN-γ and pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ, respectively, to obtain a target steady concentration (10^4 pg/ml) of muIFN-γ.

Table 1. Properties of IFN-γ expressing plasmid DNA

	Plasmid DNA	Size (kbp)	Enhancer		Promoter	
			Type	Size (bp)	Type	Size (bp)
huIFN-γ expressing-pDNA	phCMV$_{enh/prom}$-huIFN-γ	3.6	hCMV (viral)		hCMV (viral)	688
	pΔCpG CMV$_{enh/prom}$-huIFN-γ	3.5	CMVsyn (viral)		CMVsyn (viral)	560
	pΔenh SV40$_{prom}$-huIFN-γ	3.1			SV40 (viral)	203
	pSV40$_{enh}$-SV40$_{prom}$-huIFN-γ	3.4	SV40 (viral)	237	SV40 (viral)	203
	pmCMV$_{enh}$-SV40$_{prom}$-huIFN-γ	3.6	mCMV (viral)	423	SV40 (viral)	203
	pΔenh-hEF-1$_{prom}$-huIFN-γ	3.2			hEF-1 (mammalian)	220
	pSV40$_{enh}$-hEF-1$_{prom}$-huIFN-γ	3.4	SV40 (viral)	237	hEF-1 (mammalian)	220
	pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ	3.6	mCMV (viral)	423	hEF-1 (mammalian)	220
	pΔenh-hROSA$_{prom}$-huIFN-γ	5.8			hROSA26 (mammalian)	2740
	pSV40$_{enh}$-hROSA$_{prom}$-huIFN-γ	6.0	SV40 (viral)	237	hROSA26 (mammalian)	2740
	pmCMV$_{enh}$-hROSA$_{prom}$-huIFN-γ	6.2	mCMV (viral)	423	hROSA26 (mammalian)	2740
muIFN-γ expressing-pDNA	pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ	3.5	mCMV (viral)	423	hEF-1 (mammalian)	220
	pΔenh-hROSA$_{prom}$-muIFN-γ	5.7			hROSA26 (mammalian)	2740

Measurement of serum concentrations of IFN-γ

The serum of mice was collected at the indicated time points after gene transfer. Human and murine IFN-γ concentrations were determined using a human IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (Ready-SET-GO! Human IFN-γ ELISA; eBioscience, San Diego, CA, USA) and a murine IFN-γ ELISA kit (Ready-SET-GO! Murine IFN-γ ELISA; eBioscience), respectively.

Statistical analysis

Differences were statistically evaluated by Student's *t*-test and $p < 0.05$ was considered statistically significant. Statistical significance was estimated only when the number of mice per group was three or more. To confirm the reproducibility of the results, we performed three separate sets of experiments and representative results were shown.

Results

Time-courses of the serum huIFN-γ concentration after injection of huIFN-γ-expressing plasmids

To evaluate the gene expression profile without being affected by the biological activities of IFN-γ, plasmids expressing huIFN-γ, which has no biological activity in mice, were used. To evaluate the effect of the type of enhancers on transgene expression, we selected the two most frequently used enhancers: CMV enhancer and SV40 enhancer. Figure 1A shows the time-course of the serum huIFN-γ after hydrodynamic injection of huIFN-γ-expressing plasmids containing one of the CMV enhancers at the dose of 0.05 pmol of pDNA/mouse. An injection of pΔCpG $CMV_{enh/prom}$-huIFN-γ or ph$CMV_{enh/prom}$-huIFN-γ resulted in very high but transient serum levels of huIFN-γ. Similar high huIFN-γ concentrations were observed after injection of pmCMV_{enh}-hEF-1_{prom}-huIFN-γ (the prototype vector) or pmCMV_{enh}-$SV40_{prom}$-huIFN-γ, although the levels remained higher levels at least for the experimental period of 14 days. On the other hand, more constant huIFN-γ levels were obtained after injection of pmCMV_{enh}-h$ROSA_{prom}$-huIFN-γ compared with the other plasmids containing a CMV enhancer.

Next, the expression profiles after injection of plasmids containing SV40 enhancer at the dose of 0.05 pmol of pDNA/mouse were compared (Figure 1B). High and transient serum huIFN-γ levels were observed after injection of p$SV40_{enh}$-hEF-1_{prom}-huIFN-γ or p$SV40_{enh}$-$SV40_{prom}$-huIFN-γ. A steady huIFN-γ concentration was obtained by gene transfer using p$SV40_{enh}$-h$ROSA_{prom}$-huIFN-γ.

Finally, the transgene expression profiles of plasmids with no enhancer were examined. Because no detectable expression was observed after injection of pΔenh-hEF-1_{prom}-huIFN-γ or pΔenh-$SV40_{prom}$-huIFN-γ at the fixed dose of 0.05 pmol (0.12 μg)/mouse used in the above experiments, the dose was increased to 100 and 50 μg for pΔenh-hEF-1_{prom}-huIFN-γ and pΔenh-$SV40_{prom}$-huIFN-γ, respectively. Figure 1C shows the serum huIFN-γ concentration after intravenous injection of enhancer-depleted plasmids. The huIFN-γ concentrations after gene transfer using pΔenh-hEF-1_{prom}-huIFN-γ or pΔenh-$SV40_{prom}$-huIFN-γ exhibited a low peak after 6 h and 1 day, respectively, and then declined quickly. However, the huIFN-γ concentrations in the mice receiving an injection of 0.05 pmol (0.18 μg) pΔenh-h$ROSA_{prom}$-huIFN-γ remained very constant.

Quantitative evaluation of the efficiency and constancy of transgene expression

To compare the efficiency and constancy of transgene expression from each plasmid, the peak/dose and minimum/maximum ratios were calculated as indicators of these parameters, respectively, based on the profiles of the huIFN-γ concentrations (Figure 1D). Plasmids containing the hROSA26 promoter had high minimum/maximum ratios and low peak/dose ratios, irrespective of the type and presence of enhancer. On the other hand, for plasmids containing a CMV enhancer/promoter, the peak/dose ratio was very high but its trough level was approximately 10000-fold less than its peak level. Among the plasmids used, pmCMV_{enh}-hEF-1_{prom}-huIFN-γ and pmCMV_{enh}-$SV40_{prom}$-huIFN-γ showed relatively high and stable (the minimum/maximum was approximately 0.1) transgene expression.

Time-course of serum muIFN-γ concentrations and body weight after injection of muIFN-γ-expressing plasmids

To experimentally confirm the importance of the profile of transgene expression, mice were injected with muIFN-γ-expressing plasmids and the serum muIFN-γ levels and the body weight of mice were determined. Figure 2A shows the time-course of the serum muIFN-γ levels of mice receiving an intravenous injection of each plasmid at various doses. Similar results to those with huIFN-γ-expressing plasmids were obtained. For example, pmCMV_{enh}-hEF-1_{prom}-muIFN-γ produced in high but transient muIFN-γ concentrations, whereas pΔenh-h$ROSA_{prom}$-muIFN-γ produced a constant concentration with a low peak level. To obtain similar steady serum levels of muIFN-γ, these plasmids were injected at different doses and the serum concentrations of muIFN-γ were measured periodically. A target concentration of 10^4 pg/ml was selected based on the published results [11], and the doses were determined to 1.2 μg/mouse (0.5 pmol/mouse) for pmCMV_{enh}-hEF-1_{prom}-muIFN-γ and 4.5 μg/mouse (1.3 pmol/mouse) for

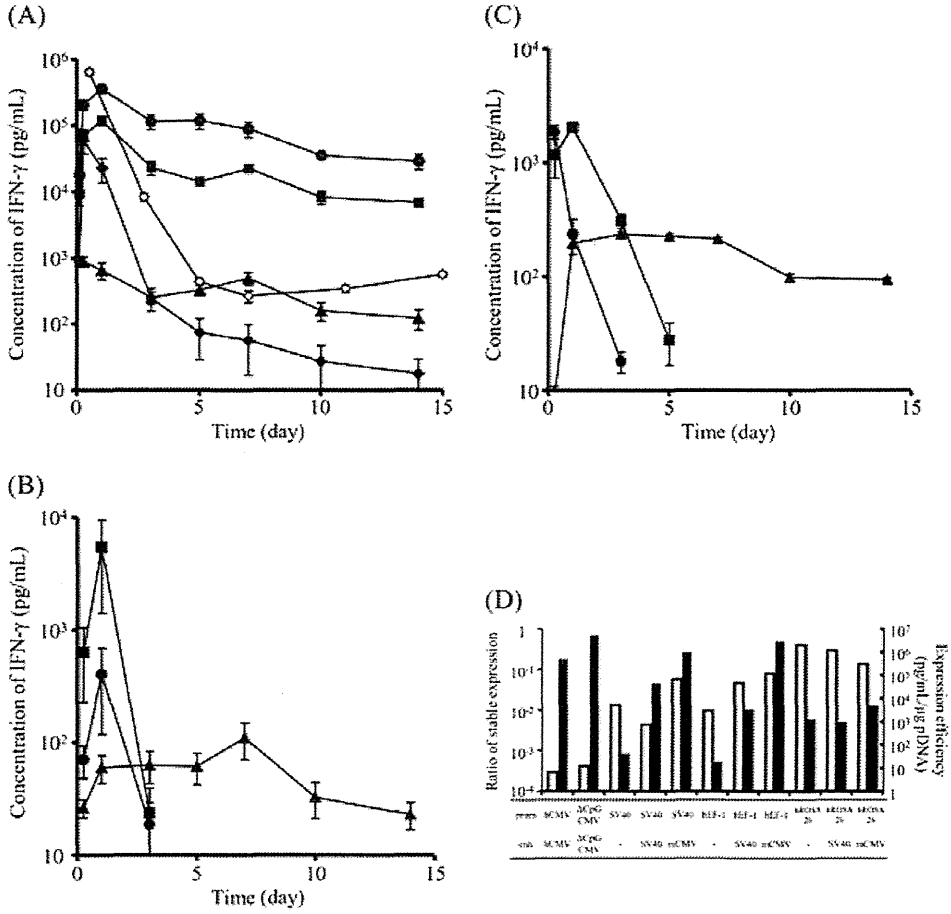

Figure 1. Effect of the promoter and enhancer in pDNAs on the transgene expression profile from the pDNA administered by the hydrodynamics-based procedure. (A) Time-courses of the human IFN-γ concentration in mouse serum after injection of huIFN-γ-expressing pDNA containing CMV enhancer at a dose of 0.05 pmol/mouse; • $pmCMV_{enh}$-$hEF\text{-}1_{prom}$-huIFN-γ (0.12 μg pDNA/mouse); ■ $pmCMV_{enh}$-$SV40_{prom}$-huIFN-γ (0.12 μg pDNA/mouse); ▲ $pmCMV_{enh}$-$hROSA_{prom}$-huIFN-γ (0.18 μg pDNA/mouse); ◊ pΔCpG $CMV_{enh/prom}$-huIFN-γ (0.12 μg pDNA/mouse); ♦ $phCMV_{enh/prom}$-huIFN-γ (0.12 μg pDNA/mouse). Results are expressed as the mean ± SEM ($n = 4$). (B) Time-courses of the human IFN-γ concentration in mouse serum after injection of huIFN-γ-expressing pDNA containing SV40 enhancer at a dose of 0.05 pmol/mouse; •, $pSV40_{enh}$-$hEF\text{-}1_{prom}$-huIFN-γ (0.12 μg pDNA/mouse); ■, $pSV40_{enh}$-$SV40_{prom}$-huIFN-γ (0.12 μg pDNA/mouse); ▲, $pSV40_{enh}$-$hROSA_{prom}$-huIFN-γ (0.18 μg pDNA/mouse). Results are expressed as the mean ± SEM ($n = 4$). (C) Time-courses of the human IFN-γ concentration in mouse serum after injection of huIFN-γ-expressing pDNA without enhancer; •, pΔenh-$hEF\text{-}1_{prom}$-huIFN-γ [100 μg (41.7 pmol) pDNA/mouse]; ■, pΔenh-$SV40_{prom}$-huIFN-γ [50 μg (20.8 pmol) pDNA/mouse]; ▲, pΔenh-$hROSA_{prom}$-huIFN-γ [0.18 μg (0.05 pmol) pDNA/mouse]. Results are expressed as the mean ± SEM ($n \geq 3$). (D) The analysis of gene expression profiles. Stable expression was calculated by dividing the minimum concentration by the maximum concentration (open column). The expression efficiency was calculated by dividing the maximum concentration by the dose of pDNA (solid column).

pΔenh-$hROSA_{prom}$-muIFN-γ. As shown in Figure 2A, the serum concentrations of muIFN-γ from 3–14 days after gene transfer were approximately 10^4 pg/ml as designed for the both groups, and were not significantly different from each other. Figure 2B shows the body weight change in mice under these experimental conditions. We selected $pmCMV_{enh}$-$hEF\text{-}1_{prom}$-huIFN-γ as a control pDNA because huIFN-γ exhibits no biological activity in mice. The body weight of $pmCMV_{enh}$-$hEF\text{-}1_{prom}$-huIFN-γ-injected mice was comparable to that of the untreated mice (data not shown). The body weight of mice receiving 0.12 μg (0.05 pmol) $pmCMV_{enh}$-$hEF\text{-}1_{prom}$-muIFN-γ or 0.18 μg (0.05 pmol) pΔenh-$hROSA_{prom}$-muIFN-γ was comparable to that of mice receiving an injection of 13.5 μg (5.6 pmol) the huIFN-γ-expressing control plasmid. However, mice receiving pΔenh-$hROSA_{prom}$-muIFN-γ at a dose of 4.5 μg (1.3 pmol)/mouse gained more body weight than those receiving 1.2 μg (0.5 pmol) $pmCMV_{enh}$-$hEF\text{-}1_{prom}$-muIFN-γ. Moreover, except for the 1.2 μg $pmCMV_{enh}$-$hEF\text{-}1_{prom}$-muIFN-γ-injected group, no mice died during the experimental period. Therefore, extraordinary high initial concentrations of muIFN-γ could be the reason for the death.

Discussion

The hydrodynamic injection of naked plasmid DNA produces an extremely high level of transgene expression in mouse liver [17]. This is partly the result of an efficient delivery of plasmid directly to the cytoplasmic

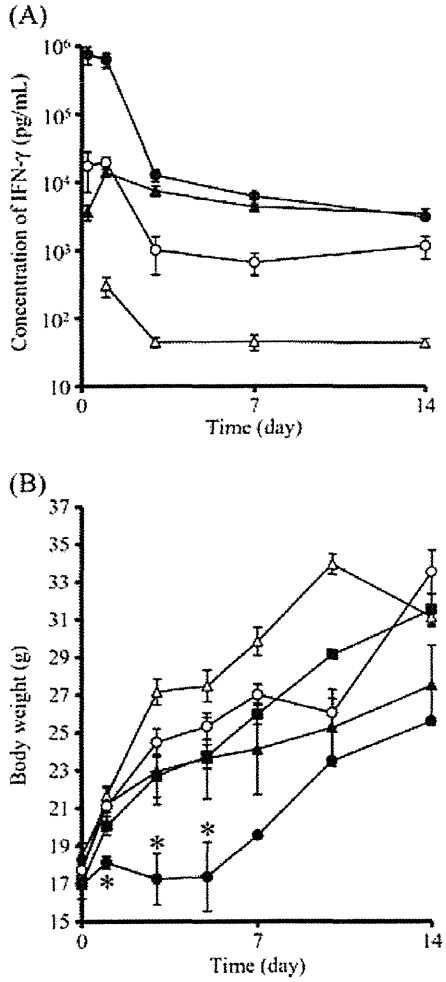

Figure 2. Time-courses of gene expression and body weight of mice given murine IFN-γ expressing pDNA. (A) Time-courses of the concentrations of murine IFN-γ in mouse serum after injection of muIFN-γ-expressing pDNA. Mice received hydrodynamic administration of: •, pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ [1.2 μg (0.5 pmol) pDNA/mouse]; ○, pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ [0.12 μg (0.05 pmol) pDNA/mouse]; ▲, pΔenh-hROSA$_{prom}$-muIFN-γ [4.5 μg (1.3 pmol) pDNA/mouse]; or Δ, pΔenh-hROSA$_{prom}$-muIFN-γ [0.18 μg (0.05 pmol) pDNA/mouse]. Results are expressed as the mean SEM (n = 4 or survived mice). (B) Time-course of the body weight of mice after hydrodynamic injection of pDNAs. The body weight of mice that had received hydrodynamic administration of: •, pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ [1.2 μg (0.5 pmol) pDNA/mouse]; ○, pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ [0.12 μg (0.05 pmol) pDNA/mouse]; ▲, pΔenh-hROSA$_{prom}$-muIFN-γ [4.5 μg (1.3 pmol) pDNA/mouse]; Δ, pΔenh-hROSA$_{prom}$-muIFN-γ [0.18 μg (0.05 pmol) pDNA/mouse]; or ■, pmCMV$_{enh}$-hEF-1$_{prom}$-huIFN-γ [13.5 μg (5.6 pmol) pDNA/mouse] was monitored until 14 days after the hydrodynamic injection. Results are expressed as the mean ± SEM (n = 4 or surviving mice).

compartment of liver cells [17–20]. In addition to this delivery issue, we demonstrated that the hydrodynamic injection transiently activates the transcription factors, including activator protein (AP)-1 and nuclear factor (NF)-κB, in mouse liver [21]. Because most plasmids used in preclinical and clinical studies contain the binding sites for these transcription factors, the injection-induced activation should play an important role in the very high level of transgene expression by hydrodynamic injection of plasmid DNA. One of our important findings was that the activated factors returned to normal levels by 3 days after hydrodynamic injection [21]. This transient activation would affect the profile of transgene expression from plasmids containing the binding sites for such transcription factors. Therefore, any gene transfer method that activates these transcription factors might result in high initial levels of transgene expression.

The CMV enhancer contains the binding sites for AP-1 and NF-κB and the SV40 enhancer region contains the binding sites for AP-1. Therefore, it is reasonable that plasmids containing CMV or SV40 enhancer exhibit a transient profile of transgene expression (Figures 1A and 1B). Plasmids containing CMV enhancer produced more sustained expression than those without enhancer or containing SV40 enhancer when they have an SV40 or EF-1 promoter (Figures 1A and 1B). Although the details need further investigation, these results provide experimental evidence indicating that the total activity of the promoter/enhancer is not a simple sum of the activities of each component.

The hROSA26 promoter contains many binding sites for SP1, a basic transcription factor. In addition, it does not contain a TATA box [22] and the frequency of putative AP-1 and NF-κB-binding sites in hROSA26 promoter is the lowest of the three promoters used in the present study. The transgene expression from plasmids containing hROSA26 promoter was constant without a high initial peak (Figure 1C). These results suggest that the activity of hROSA26 promoter is hardly influenced by AP-1 and NF-κB activated by hydrodynamic injection. In addition, we also found that CMV or SV40 enhancer inserted into plasmids containing hROSA26 promoter hardly affected the profile of transgene expression from these plasmids (Figures 1A and 1B). The size of hROSA26 promoter is approximately ten-fold higher than that of hEF-1 promoter or SV40 promoter. This suggests that the large size of the hROSA26 promoter over-rules the functions of the enhancers. However, this hypothesis is not applicable to a variety of promoters because the expression from plasmids containing the large 1.5-kb PDGF-β promoter was increased by inserting a CMV enhancer [23]. Thus, factors other than the large size, such as the lack of a TATA box and the abundance of SP-1 binding sites, could be important for the constant transgene expression from plasmids containing a hROSA26 promoter.

Several studies have demonstrated that the elimination of CpG sequences from plasmid DNA effectively achieves sustained transgene expression [11,13]. In the present study, the effects of CpG sequences in promoters were also evident when the expression profile was compared between phCMV$_{enh/prom}$-huIFN-γ and pΔCpG CMV$_{enh/prom}$-huIFN-γ. The EF-1 promoter, which contains no CpG sequences, also produced sustained transgene expression (Figure 1A). Although the hROSA26 promoter contains 428 CpG sequences, the transgene expression driven by the hROSA26 promoter was constant and durable (Figure 1D). These results suggest that the number of CpG sequences is not a major factor that determines the kinetics of transgene expression from plasmid. This interpretation could be supported by recent

experimental evidence. Chen *et al.* [24] showed that transgene silencing in liver is independent of CpG methylation of plasmid DNA backbone and Schüttrumpf *et al.* [25] showed that the promoter region in naked plasmid DNA hardly methylated after hydrodynamic gene transfer in mice. Furthermore, we also found that the cDNA and promoter region of plasmid DNA are hardly methylated at least 1 month (L. Zang, M. Nishikawa, M. Ando, Y. Takahashi, Y. Takakura, unpublished observations). Based on these observations and the results of the present study, we conclude that the number of CpG sequences is not a major factor that determines the duration of transgene expression at least when naked plasmid DNA is delivered by the hydrodynamic injection method.

Our previous study of the treatment of atopic dermatitis using IFN-γ gene transfer has indicated that a sustained level of IFN-γ is important for the treatment of this disease [12]. Because the biological activity of IFN-γ is dependent on its concentration, we adjusted the steady-state level of muIFN-γ after gene transfer to compare the adverse effects on body weight. Under these conditions, the peak levels of muIFN-γ exhibited about a 55-fold difference between mice receiving 4.5 μg of pΔenh-hROSA$_{prom}$-muIFN-γ and those receiving 1.2 μg of pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ (Figure 2A), which is in good agreement with the experimental results obtained using huIFN-γ-expressing pDNAs. Retarded weight gain and death in mice receiving pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ is likely a result of the high level of IFN-γ for the first 2–3 days after gene transfer (Figure 2B). This speculation was in agreement with the results that almost no adverse effects was observed in mice that received 0.12 μg of pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ, which showed low levels of muIFN-γ after gene transfer. Therefore, adverse effects of pmCMV$_{enh}$-hEF-1$_{prom}$-muIFN-γ could be avoided by reducing the dose, although this will require frequent dosing. On the other hand, pΔenh-hROSA$_{prom}$-muIFN-γ is more useful for obtaining therapeutic levels of muIFN-γ for a longer period of time without high initial concentrations. For example, we found that intramuscular injection of hROSA promoter-containing pDNA resulted in transgene expression for more than 1 month, and the transgene expression after hydrodynamic injection of the hROSA26 promoter-containing pDNA was constant and durable for more than half a year (Y. Takahashi, Y. Matsui, M. Ando, M. Nishikawa, Y. Takakura, unpublished data). Thus, the steady expression from pΔenh-hROSA$_{prom}$-muIFN-γ will be ideal for diseases that require continuous treatment, such as atopic dermatitis and hepatitis C viral infection. We have found in a previous study that 1000 pg/ml or higher concentrations of IFN-γ was effective in preventing the development of atopic dermatitis or to in suppressing tumor growth in mice [11,12]. In addition, Frese *et al.* [3] reported that 1–10 IU/ml (approximately 200–2000 pg/ml) IFN-γ prevented HCV accumulation in subgenomic HCV replicons. These findings strongly support the hypothesis that IFN-γ concentrations obtained by hROSA promoter-driven pDNA are sufficiently high enough to prevent viral infection, suppress tumor growth and treat chronic diseases.

In conclusion, we have shown that the profile of transgene expression is a function of the type of enhancer and promoter, and the use of hROSA26 promoter is effective in obtaining constant transgene expression with no initial surge. In addition, we have demonstrated that a constant level of IFN-γ is effective for reducing the incidence of toxic side-effects caused by high concentrations of IFN-γ. These findings will help to achieve safe and effective IFN-γ-based gene therapy.

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Saturation of Transgene Protein Synthesis From mRNA in Cells Producing a Large Number of Transgene mRNA

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ABSTRACT: Experimental results have suggested that transgene expression can be saturated when large amounts of plasmid vectors are delivered into cells. To investigate this saturation kinetic behavior, cells were transfected with monitoring and competing plasmids using cationic liposomes. Even although an identical amount of a monitoring plasmid expressing firefly luciferase (FL) was used for transfection, transgene expression from the plasmid was greatly affected by the level of transgene expression from competing plasmids expressing renilla luciferase (RL). Similar results were obtained by exchanging the monitoring and competing plasmids. The competing plasmid-dependent reduction in transgene expression from the monitoring plasmid was also observed in mouse liver after hydrodynamic injection of plasmids. On the other hand, the mRNA and protein expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous gene, in the liver hardly changed even when transgene expression process is saturated. The expression of FL from a monitoring plasmid was significantly restored by siRNA-mediated degradation of RL mRNA that was expressed from a competing plasmid. These results suggest that the efficiency of protein synthesis from plasmid vectors is reduced when a large amount of mRNA is transcribed with no significant changes in endogenous gene expression.

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KEYWORDS: saturation; transgene expression; plasmid vector; mRNA expression; RNA interference

Introduction

Development of efficient gene delivery methods is needed not only for gene therapy but also for the functional studies of genes. Of the various vectors and gene delivery/transfer methods developed thus far, rapid injection of naked plasmid DNA in a large volume of isotonic solution, the so-called the hydrodynamic injection method, is one of the most effective and promising gene delivery methods because of its simplicity, reproducibility, and high efficiency (Herweijer and Wolff, 2007; Kobayashi et al., 2005; Lewis and Wolff, 2007; Liu et al., 1999). Therefore, this gene delivery technique has frequently been used as an experimental tool to investigate the gene of interest in mouse liver, the organ that most efficiently expresses the gene products after hydrodynamic delivery of naked plasmid DNA.

In a previous study, we found, using a firefly luciferase (FL)-expressing plasmid DNA, that the level of luciferase activity in mouse liver increased in parallel with the increasing dose of the plasmid up to about 1 μg DNA/mouse (Kobayashi et al., 2004). However, we also noticed that the luciferase activity reached a plateau at doses around 10 μg DNA/mouse, suggesting that the transgene expression from plasmid DNA is saturated at such high doses. These experimental results could be explained by assuming the presence of one or more saturable processes in the transgene expression after hydrodynamic delivery of plasmid DNA to mouse liver. One obvious possibility is that the amount of plasmid DNA delivered to the nucleus of transfected cells is not proportional to the increasing dose of the DNA. However, this can be rejected by the previous finding that 20 μg empty plasmid co-administered had no significant effect on the level of transgene expression from 0.2 μg luciferase-expressing plasmid (Kobayashi et al., 2004). The empty plasmid used contained the same promoter/enhancer and other components as those in the luciferase-expressing plasmid except for the cDNA region. Therefore, these results

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strongly support the hypothesis that the efficiency of the cellular uptake and intracellular trafficking of plasmid DNA, including the nuclear entry, is not a function of the amount or concentration of the DNA, even although several reports have indicated that transcription factors and other proteins were involved in the nuclear entry of plasmid DNA microinjected into the cytosol (Dean, 1997; Dean et al., 1999; Miller and Dean, 2008).

There are several steps in the process of transgene expression, including RNA synthesis (transcription), RNA modification (splicing), export of mRNA from the nucleus into the cytosol, protein synthesis (translation), post-translational modification of protein, and transport/trafficking of protein. Considering the fact that all these steps in the gene expression are capacity-limited, it is reasonable that transgene expression becomes saturated when a large amount of DNA is introduced into cells. In good agreement with this hypothesis, saturation in transgene expression has been discussed in several studies involving transgene expression in cultured cells. Tachibana et al. (2002) investigated the relationship between the dose of plasmid DNA added to cells and the amount of transgene product. They showed that the copy number of plasmid DNA in the nucleus correlated with its dose. Furthermore, they demonstrated that the copy number in the nucleus did not correlate with the amount of transgene product, especially when the copy number was high. A recent study by Cohen et al. (2009) also showed that nuclear uptake of plasmid DNA was linearly related to its dose whereas the level of transgene expression was not. Carpentier et al. (2007) investigated the limiting factors involved in the process of transgene expression. They found that both transcriptional and translational processes were saturated under optimal transfection conditions in which quite a high level of gene expression was obtained. These previous studies strongly suggest that transgene expression can be saturated under certain conditions.

Detailed understanding of this saturable mechanism of transgene expression from plasmid vectors will provide new insights into how gene expression is regulated in cells. The understanding could also lead to the development of methods to achieve high level of transgene expression without saturation. In addition, from the viewpoint of safety, it is important to investigate whether endogenous gene expression is affected when transgene expression process is saturated, because it may cause unexpected side effects, such as cell death. For example, Lin et al. (2007) reported that adenoviral vector-mediated high transgene expression of serum response factor resulted in nonspecific reduction of transcription of endogenous genes and caused cell death. Therefore, in the present study, we examined whether the synthesis of transgene products is actually saturated when a large amount of plasmid DNA is delivered to cells. To this end, cultured cells were transfected with two types of plasmid vectors, each of which encodes a reporter protein, and transgene expression from the vectors was used to investigate whether the expression from one plasmid (monitoring plasmid) is affected by that from the other plasmid (competing plasmid). Then, similar experiments were carried out using mice that received a hydrodynamic injection of the two plasmid vectors. In addition, the mRNA and protein levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mouse liver were evaluated as a model endogenous gene to evaluate the effect of transgene expression on endogenous gene expression. Small interfering RNA (siRNA) was used to reduce the amount of mRNA without altering the amount of plasmids for transfection.

Materials and Methods

Plasmid DNA and siRNA

Salmon testes DNA (stDNA) was purchased from Sigma (St Louis, MO). Plasmid DNA encoding FL under the control of CMV promoter (pFL-CMV) was constructed as described previously (Nomura et al., 1999). The following plasmids were purchased from the sources in brackets: pGL3-control vector (Promega, Madison, WI), pLuc-mcs (Stratagene, La Jolla, CA), pRL-CMV (Promega) encoding renilla luciferase (RL) under the control of CMV promoter, pRL-SV40 (Promega), pRL-TK (Promega), pEGFP-F (BD Biosciences Clontech, Palo Alto, CA) encoding farnesylated enhanced green fluorescent protein (GFP) under the control of CMV promoter. These plasmids were renamed according to the cDNA and promoter of each plasmid as follows: pFL-SV40, pFL-TATA, pRL-CMV, pRL-SV40, pRL-TK, and pGFP-CMV. siRNA targeting RL mRNA (siRL) or GFP mRNA (siGFP) was purchased from Takara Bio (Otsu, Japan). Target sites in the RL and GFP mRNA are as follows: RL, 5′-GUAGCGCGGUGUAUUAUAC-3′; GFP, 5′-GGCUAC-GUCCAGGAGCGCA-3′.

Cell Culture

A murine melanoma cell line, B16-BL6, was obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Poste et al., 1980). B16-BL6 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 μg/mL)/L-glutamine (2 mM) at 37°C and 5% CO_2.

In vitro Transfection

B16-BL6 cells were plated on 24-well culture plates at the density of 2×10^4 cells/well. After an overnight incubation, transfection of plasmid DNA was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, 1 μg of nucleic acids (plasmids with or without siRNA), was mixed with 3 μg Lipofectamine 2000 in Opti-MEM I (Invitrogen) at a

final concentration of 6 μg Lipofectamine 2000 mL^{-1}. The resulting complex was added to cells and the cells were incubated with the complex for 4 h. Then, cells were washed with PBS and further incubated with the culture medium.

Luciferase Assay in B16-BL6 Cells

B16-BL6 cells were lysed using the cell lysis buffer of an assay kit (PiccageneDual, Toyo Ink, Tokyo, Japan). Then, samples were 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). The luciferase activities were converted to the amount of firefly and RL using recombinant proteins as the standard.

Flow Cytometric Analysis of GFP Expression in B16-BL6 Cells

B16-BL6 cells were transfected with pGFP-CMV as described above. One day after transfection, adherent cells were detached by trypsinization and resuspended in PBS. Resuspended cells were analyzed on a flow cytometer (FACSCan, BD, Franklin Lakes, NJ). The threshold on FSC was set to exclude cell debris without excluding any populations of interest.

mRNA Quantification in B16-BL6 Cells

One day after transfection, total RNA was extracted from cells using Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan). Following RNase-free DNase I treatment (Takara Bio), reverse transcription was performed using a SuperScript II (Invitrogen) and 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 Light-Cycler instrument (Roche Diagnostics, Basle, Switzerland) as reported previously (Takahashi et al., 2005). The mRNA expression of target genes was normalized using the mRNA level of GAPDH.

Animal Experiments

Four-week-old male ICR mice (approximately 20 g body weight), purchased from Japan SLC, Inc. (Shizuoka, Japan) were used for all experiments. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University. Mice received an intravenous injection of plasmid DNA by the hydrodynamic injection method as described previously (Liu et al., 1999). At 6 h after gene transfer, the peak time of the expression of firefly and RLs from the plasmid vectors used, the mice were killed by cervical dislocation and the amount of luciferase mRNA and protein in the liver was determined as described below.

Quantification of the Amounts of Luciferase and GAPDH in Mouse Liver

The liver was excised and homogenized in a lysis buffer (0.1 M Tris (pH 7.8), 0.05% Triton X-100, 2 mM EDTA, 0.1% protease inhibitor cocktail (Sigma)), and centrifuged at 13,000*g* for 20 min at 4°C. The amount of luciferase in the supernatant was determined by the method described in the above section. The amount of GAPDH protein in the lysate was measured by ELISA by using a commercial kit (GAPDH Whole-cell Normalization kit, Active Motif, Carlsbad, CA).

mRNA Quantification in Mouse Liver

Total RNA was extracted from approximately 50 mg liver samples using Sepasol RNA I Super. After purification of the extracted RNA sample using an RNeasy mini kit (Qiagen, Hilden, Germany), reverse transcription and real-time PCR analysis were performed as described in the above section.

Statistical Analysis

The normality of the data was evaluated by using Shapiro–Wilk test. Data of normal distribution were analyzed by Student's *t*-test for two independent samples or one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons. Data without normal distribution were analyzed using Mann–Whitney rank sum test for two independent samples or Kruskal–Wallis one-way ANOVA on ranks followed by the Tukey's test for multiple comparisons. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Time-Course and Dose-Dependent Luciferase Expression in B16-BL6 Cells

Figure 1A shows the time-courses of the amounts of firefly and RLs in cells after cotransfection with pFL-CMV and pRL-CMV. The expression of both luciferases was detectable as early as 6 h after transfection, reached maximum values at 24 h and then eventually decreased with time. As the highest luciferase expression was detected at 24 h after transfection under the conditions used, this time point was selected and used in the following experiments to examine transgene expression in B16-BL6 cells.

Figure 1B shows the amount of FL in cells transfected with different amounts of pFL-TATA, pFL-SV40, or pFL-CMV. pFL-CMV showed the highest FL, followed by pFL-SV40, then pFL-TATA, reflecting the strength of these promoters used. When cells were transfected with one of the RL-expressing plasmid DNA, pRL-CMV showed the highest RL activity, followed by pRL-SV40 and pRL-TK (data not shown).

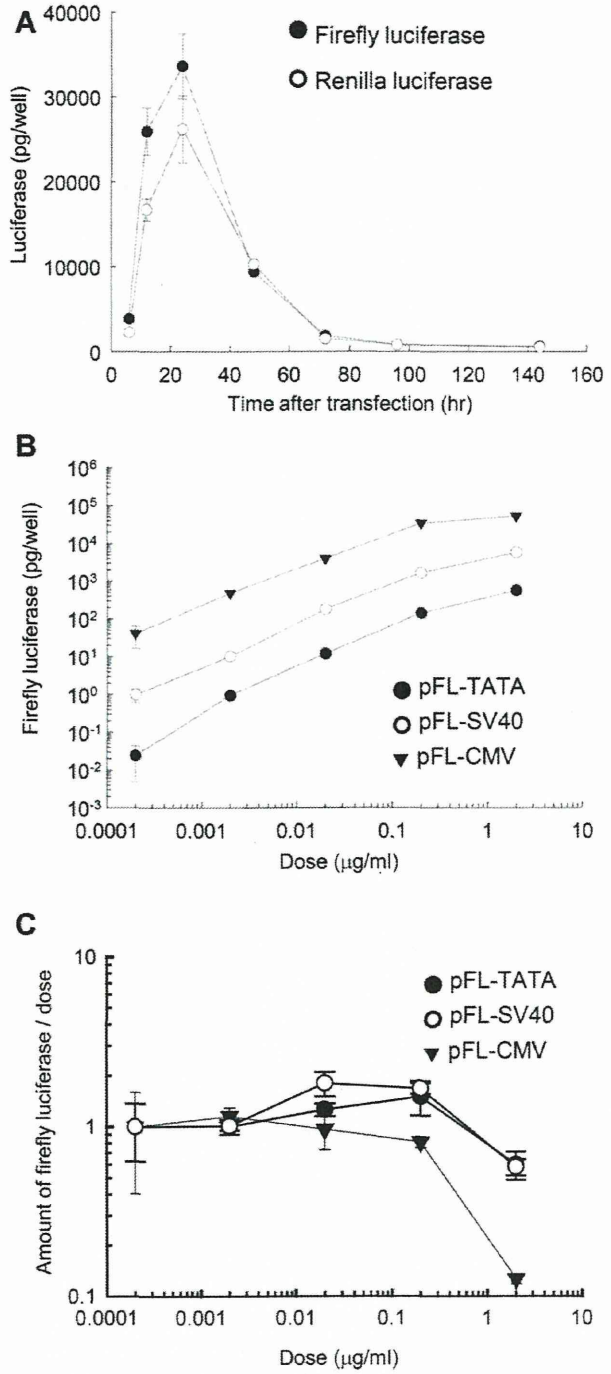

Figure 1. Time- and dose-dependent luciferase expression in B16-BL6 cells after transfection of luciferase-expressing plasmid DNA. **A**: The firefly (closed symbols) and renilla (open symbols) luciferase activities were measured at indicated times after transfection of B16-BL6 cells with 1 μg/mL pFL-CMV and 1 μg/mL pRL-CMV. The results are expressed as the mean ± SD ($n=4$). **B**: The luciferase activity was measured at 1 day after transfection of B16-BL6 cells with the indicated dose of pFL-CMV (open triangle), pFL-SV40 (open circle), or pFL-TATA (closed circle). The results are expressed as the mean ± SD ($n=3$). **C**: The amount of firefly luciferase protein in (B) was divided by the dose of plasmid DNA. The results are expressed as the mean ± SD of the relative value to that of 0.0002 μg/mL (pFL-CMV and pFL-SV40) or to that of 0.002 μg/mL (pFL-TATA) ($n=3$).

To statistically evaluate whether the expression is saturated with an increasing amount of plasmid DNA, the expression efficiency was estimated by dividing the luciferase activity by the dose (Fig. 1C). The value was about 40,000 pg/well/μg plasmid DNA at the lowest dose of 0.0002 μg/mL pFL-CMV. Increasing the dose to 2 μg/mL significantly reduced the value to 5,000 pg/well/μg plasmid DNA.

Luciferase Expression in B16-BL6 Cells Transfected With Two Different Plasmids

Figure 2 shows the amounts of firefly (Fig. 2A) and RL (Fig. 2B) in cells 24 h after cotransfection with 1.8 μg/mL pFL-TATA, pFL-SV40, or pFL-CMV and 0.2 μg/mL pRL-TK, pRL-SV40, or pRL-CMV. The amount of FL was the highest in cells transfected with pFL-CMV, which was about 4- and 20-fold higher than that in cells transfected with pFL-SV40 or pFL-TATA, respectively. In all cases examined, the amount of FL was hardly affected by the cotransfection with renilla-expressing plasmid DNA (Fig. 2A). This was probably because the dose of the RL-expressing plasmid DNA was only a tenth of the total amount of plasmids used for transfection. In marked contrast, the RL activity in B16-BL6 cells was affected not only by the type of RL-expressing plasmid DNA, but also by the type of competing, FL expressing plasmid DNA (Fig. 2B). RL activity in cells transfected with pRL-CMV and pFL-CMV (pRL-CMV/pFL-CMV) or pRL-SV40/pFL-CMV was significantly lower than that in cells transfected with pRL-CMV/pFL-TATA or pRL-SV40/pFL-TATA, respectively. The amount of RL in B16-BL6 cells transfected with pRL-TK was quite low compared with cells transfected with pRL-CMV or pRL-SV40.

Similar experiments were performed using 0.2 μg/mL FL-expressing plasmid DNA and 1.8 μg/mL RL-expressing plasmid DNA (Fig. 2C and D). The amount of FL in cells cotransfected with pFL-CMV/pRL-CMV was significantly lower than that in cells cotransfected with pFL-CMV/pRL-TK (Fig. 2C). Again, the amount of RL was hardly affected by the type of FL expressing plasmid vectors cotransfected (Fig. 2D).

Fluorescent Histogram of GFP in B16-BL6 Cells Transfected With pGFP-CMV and Renilla Luciferase-Expressing Plasmid

Figure 2E shows typical histograms of the fluorescence intensity of B16-BL6 cells transfected with pGFP-CMV and one of the following plasmids: pRL-CMV, pRL-SV40, or pRL-TK. In all cases examined, there was a large variation in the level of fluorescence intensity of B16-Bl6 cells transfected with pGFP-CMV. There were no obvious differences in the histograms of cells transfected with pGFP-CMV/pRL-TK or pGFP-CMV/pRL-SV40. On the other hand, the histogram of cells transfected with pGFP-CMV/pRL-CMV was different from the others; the percentage of cells with a

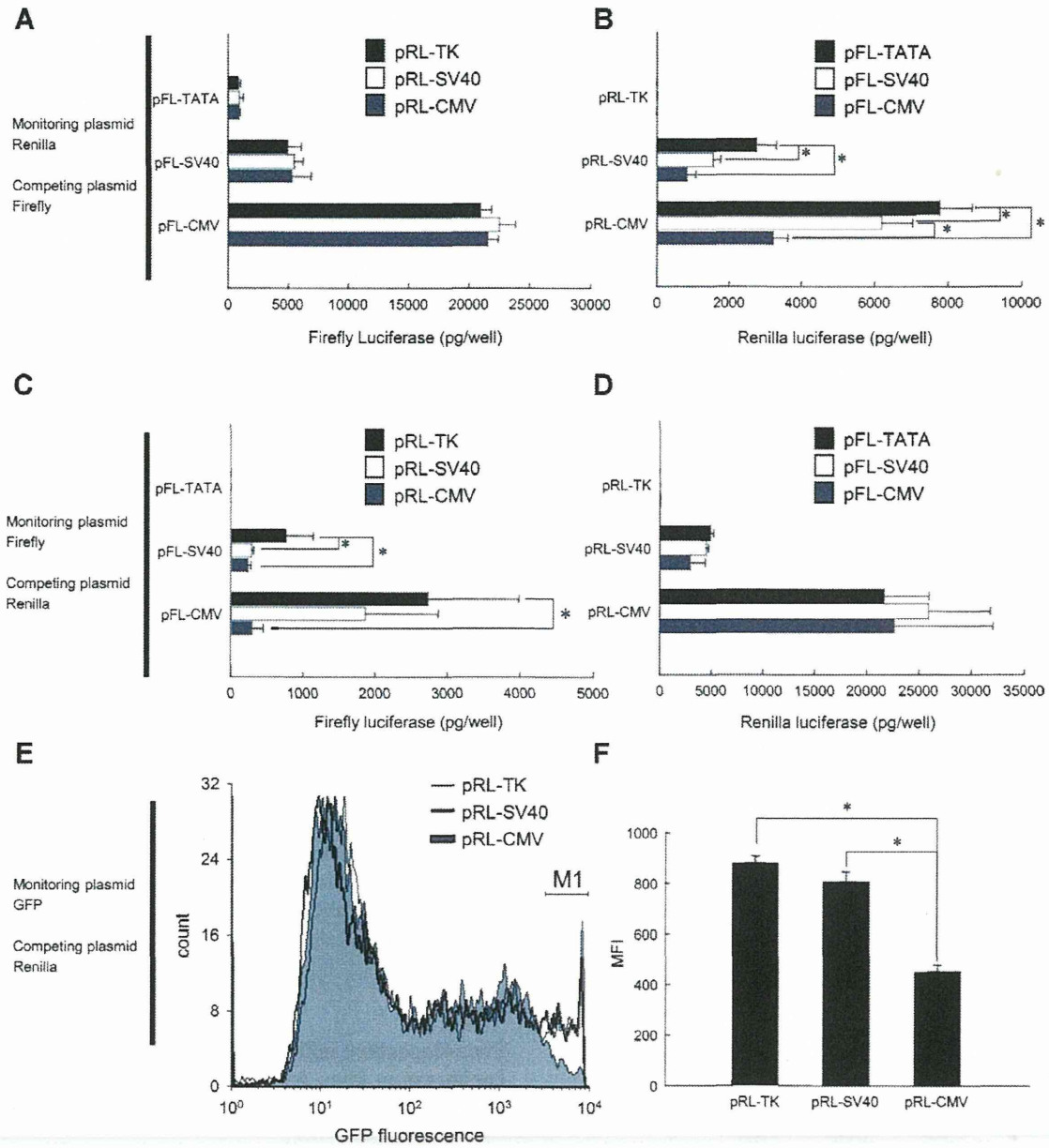

Figure 2. Effect of the level of transgene expression from competing plasmid DNA on transgene expression from monitoring plasmid DNA. **A and B**: B16-BL6 cells were cotransfected with 1.8 μg/mL pFL-TATA (closed bar), pFL-SV40 (open bar), or pFL-CMV (gray bar) and 0.2 μg/mL pRL-TK (closed bar), pRL-SV40 (open bar), or pRL-CMV (gray bar). One day after transfection, firefly (A) and renilla (B) luciferase activities were simultaneously measured. The results are expressed as the mean ± SD ($n = 4$). $^*P < 0.05$, compared with the pFL-TATA-transfected group. **C and D**: B16-BL6 cells were cotransfected with 1.8 μg/mL pRL-TK (closed bar), pRL-SV40 (open bar), or pRL-CMV (gray bar) and 0.2 μg/mL pFL-TATA (closed bar), pFL-SV40 (open bar), or pFL-CMV (gray bar). One day after transfection, firefly (C) and renilla (D) luciferase activities were simultaneously measured. The results are expressed as the mean ± SD ($n = 4$). $^*P < 0.05$ (vs. pRL-TK transfected group). **E and F**: B16-BL6 cells were cotransfected with 1.8 μg/mL pRL-CMV (line with shade), pRL-SV40 (bold line), or pRL-TK (solid line) and 0.2 μg/mL pGFP-CMV. One day after transfection, the fluorescence intensity histogram (E) and mean fluorescence intensity (F) of cells were analyzed by flow cytometry. The results are expressed as the mean ± SD ($n = 4$). $^*P < 0.05$.

high fluorescence intensity (the M1 region in the histogram) was 3%, and this value was smaller than the 8% and 9% for the pGFP-CMV/pRL-SV40- and pGFP/pRL-TK-treated cells, respectively. These findings indicate that cotransfection of cells with pRL-CMV, the plasmid with the strongest promoter among those used, markedly reduced the expression of GFP from pGFP-CMV only in cells that received efficient delivery of these two plasmids. In accordance with these histograms, the mean fluorescence intensity of cells transfected with pGFP-CMV/pRL-CMV