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RESEARCH PAPER

Positive Correlation Between the Generation of Reactive Oxygen Species and Activation/Reactivation of Transgene Expression After Hydrodynamic Injections into Mice

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ABSTRACT

Purpose Hydrodynamic injection has been shown to reactivate silenced transgene expression in mouse liver. In this study, the roles of inflammatory cytokines and reactive oxygen species (ROS) in the reactivation were examined.

Methods Production of inflammatory cytokines and ROS by hydrodynamic injection of saline was examined in mice that had received a hydrodynamic injection of a plasmid expressing Gaussia luciferase. The level of reporter gene expression was used as an indicator of the reactivation. The involvement of cytokines and ROS was examined by depleting Kupffer cells or by pre-administration of antioxidants, respectively.

Results A hydrodynamic injection of saline induced a significant production of interleukin (IL)-6. Depleting Kupffer cells using clodronate liposomes markedly reduced the IL-6 production but had no significant effect on the transgene expression. On the other hand, an injection of catalase or N-acetylcysteine significantly inhibited the hydrodynamic injection-induced reactivation of silenced transgene expression. The silenced expression was also reactivated by carbon tetrachloride, an inducer of oxidative stress in the liver, in a dose-dependent manner, and this reactivation was significantly inhibited by catalase.

Conclusions These findings show a positive correlation between the generation of ROS and the reactivation of silenced transgene expression after hydrodynamic injections.

KEY WORDS hydrodynamic injection · inflammation · liver · plasmid DNA · reactive oxygen species

INTRODUCTION

Hydrodynamic gene delivery, which involves a rapid, large-volume injection of naked plasmid DNA, produces an extremely high level of transgene expression in mouse liver (1,2). Transgene expression in the liver obtained by hydrodynamic injection is several orders of magnitude greater than that obtained by other nonviral methods, including electroporation-mediated gene delivery, direct injection of naked plasmid DNA into the liver and intraportal injection of plasmid DNA/cationic liposome complex (lipoplex) (3). Because such high levels of transgene expression are difficult to explain simply by the difference in the amount of plasmids delivered to the liver (4), some biological changes induced by the injection are thought to play an important role in the high level of transgene expression obtained by hydrodynamic gene delivery.

Recent findings showing that silenced transgene expression in mouse liver is reactivated by rapid, large-volume injection of an isotonic solution containing no plasmid DNA (5,6) have shed light on the mechanism of the hydrodynamic injection. In our previous study, we found that hydrodynamic injection of isotonic solution containing no plasmid DNA activates the transcription factor activator protein (AP)-1 and nuclear factor (NF)-κB in the liver. Plasmids with no binding sequences for these transcription

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factors failed to be reactivated by hydrodynamic injections, strongly suggesting that the activation of these transcription factors is a key process in the reactivation of silenced transgene expression. These findings also suggest an important issue, namely, that the activation of transcription factors in the liver by hydrodynamic injection makes a major contribution to the extremely high transgene expression produced by injection, although the detailed mechanism of how the hydrodynamic injection activates transcription factors is still not clear. The elucidation of the mechanism underlying the reactivation produced by hydrodynamic injection will provide useful information for developing more efficient gene delivery methods.

Hydrodynamic injection induces a variety of changes in mouse liver. Suda *et al.* reported that the size of mouse liver increases soon after hydrodynamic injection up to about 240% compared with the original size (7). They also reported that the blood pressure in both the inferior vena cava and the portal vein of the mice that received a hydrodynamic injection was three-fold higher than that of the control group. These changes in the physiological state would cause tissue damage, leading to a cascade of inflammatory responses. In addition, the oxygen supply to the liver would be reduced because saline or other isotonic solution, which is much less oxygenated than blood, remains in the liver for some time. This could lead to transient hepatic ischemia, which then results in hypoxia. Hypoxia increases the generation of reactive oxygen species (ROS) at mitochondrial complex III (8,9).

These pieces of information suggest that reactivation of silenced transgene expression is mediated by the increased levels of proinflammatory cytokines and/or ROS, because both of these factors are known activators of AP-1 and NF-κB (10,11). Therefore, in the present study, we investigated the roles of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and ROS in the reactivation of silenced transgene expression produced by a hydrodynamic injection of saline.

MATERIALS AND METHODS

Chemicals

Dichloromethylenediphosphonic acid disodium salt (clodronate), lipopolysaccharide (LPS) and bovine liver catalase (40,000–60,000 units/mg protein) were purchased from Sigma Chemical (St Louis, MO, USA). N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade and used without further purification.

Plasmid DNA

pCMV-gLuc encoding Gaussia luciferase under CMV promoter was constructed by subcloning the EcoRV/XbaI Gaussia luciferase cDNA fragment from pGLuc-Basic vector (New England Biolad, Madison, WI, USA) into the multi-cloning site of pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). pCMV-Luc encoding firefly luciferase under the control was obtained as described previously (6). pTA-Luc, pAP-1-Luc and pNF-κB-Luc, plasmid DNA encoding firefly luciferase under the control of TATA-box only, TATA-box and AP-1 binding sites or TATA-box and NF-κB binding sites, respectively were purchased from Clontech (Mountain View, CA). Plasmid DNA was amplified in the *E. coli* strain DH5α, isolated, and purified using JETSTAR 2.0 Plasmid GIGA Plasmid Purification Kits (GENOMED GmbH, Löhne, Germany).

Animals

Male ICR (4-week-old, about 20 g) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were maintained under conventional housing conditions. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

Hydrodynamic Injection

Saline solution with a volume of 8% body weight containing no or only 10 μg naked pCMV-gLuc was injected into the tail vein of mice over 5 s. To reactivate silenced transgene expression of pCMV-gLuc, mice that had received the hydrodynamic injection of pCMV-gLuc were injected with saline in a manner similar to the hydrodynamic injection.

Preparation of Clodronate Liposomes

Phosphatidylcholine (63.5 μmol) and cholesterol (10.3 μmol) were dissolved in chloroform, and a thin lipid film was formed by low-vacuum rotary evaporation. This film was dispersed in 5 ml of phosphate-buffered saline (PBS) in which clodronate was dissolved at a concentration of 0.7 M. The suspension was maintained at room temperature for 2 h followed by ultrasonication under nitrogen gas for 3 min. After incubation for another 2 h at room temperature, the suspension was centrifuged at 22,000 × g

for 1 h at 10°C to remove free clodronate, and then washed four times using centrifugation at 22,000 × g for 25 min at 10°C. The liposomes were then resuspended in PBS and stored at 4°C until use.

Transient Depletion of Kupffer Cells

To deplete Kupffer cells, 100 μl of clodronate liposomes were injected into the peritoneal cavity of mice 48 h prior to the hydrodynamic injection of saline (12). In control mice, PBS was injected into the peritoneal cavity. The existence of Kupffer cells in the liver was evaluated by immunofluorescent staining of liver sections with F4/80, a macrophage-specific antigen. In brief, frozen liver sections (8-μm thick) were obtained by a cryostat (Jung CM 3000, Leica Microsystems AG, Wetzlar, Germany), stained with FITC-conjugated rat anti-mouse F4/80 antibody (1:50 dilution; AbD Serotec, Oxford, UK) and observed using a fluorescence microscope (Biozero BZ-8000, KEYENCE).

Preparation of Cationic Liposomes and Plasmid DNA/Cationic Liposome Complex (Lipoplex)

Cationic liposomes consisting of DOTMA and cholesterol were prepared as previously reported (13). In brief, the lipids mixed in chloroform at a molar ratio of 1:1 were dried as a thin film in a round-bottomed flask using a rotary evaporator, and then hydrated in 5% w/v dextrose by gentle vortexing. After hydration, the dispersions were sonicated for 2.5 min and passed through a Minisart® 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako kit (Wako Pure Chemical Industries). Cationic liposomes and pcDNA3.1 were mixed in 5% dextrose at a charge ratio of +2.24, and the mixture was left for at least 30 min at 37°C to form a lipoplex.

Injection of LPS or Lipoplex into Mice

Five days after hydrodynamic injection of pCMV-gLuc, mice received an injection of LPS or lipoplex. LPS was injected into the peritoneal cavity of mice at the indicated doses. In separate mice, lipoplex was intravenously injected at the indicated doses.

Injection of Catalase or N-Acetylcysteine

Saline (8% of body weight) containing 100–1,000 U bovine liver catalase was hydrodynamically injected into mice that had received pCMV-gLuc transfer 5 days before the catalase administration. Catalase heat-inactivated at 60°C for 30 min was used as a control. In separate mice, N-acetylcysteine (NAC) was injected into the peritoneal cavity of mice that had received pCMV-gLuc transfer 5 days before the NAC administration. Thirty minutes after NAC administration, mice received a hydrodynamic injection of saline.

Carbon Tetrachloride-Induced Reactivation of Transgene Expression

Carbon tetrachloride (CCl_4) dissolved in olive oil was injected into the peritoneal cavity of mice at a dose of 1–3 ml CCl_4/10 ml olive oil/kg body weight. In another group of mice, catalase was injected at a dose of 1,500 U/mouse into the tail vein immediately after administration of 2 ml CCl_4/kg.

Luciferase Assay for Gaussia Luciferase

At indicated time points, blood was collected from the tail vein of mice. The blood samples were incubated at 4°C for 2 h to allow clotting and then centrifuged at 8,000 × g for 20 min to obtain serum. Then, 10 μl of serum was mixed with the sea pansy luciferase assay buffer (PiccageneDual, Toyo Ink, Tokyo, Japan), and the chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany).

Measurement of Serum Concentrations of Inflammatory Cytokines

The concentrations of IL-6 and TNF-α in serum were measured using enzyme-linked immunosorbent assay (ELISA) kits (BD OptEIA Mouse IL-6 ELISA set, BD Bioscience, San Jose, CA, USA; TNF-α Mouse ELISA Kit, R&D systems, Minneapolis, MN, USA).

Evaluation of Transcription Factor-Specific Gene Expression

To examine whether AP-1 and NFκB are activated in the liver, mice received a hydrodynamic injection of 10 μg of pTA-Luc, pAP1-Luc, pNF-κB-Luc or pCMV-Luc. Five days after gene transfer, mice received another hydrodynamic injection of saline or an intraperitoneal injection of CCl_4. At 6 h after the treatment, mice underwent euthanasia, and the liver was excised and homogenized in 5 ml/g lysis buffer (0.1 M Tris, 0.05% Triton-X-100, 2 mM EDTA, pH 7.8). The homogenate was centrifuged at 13,000 g for 10 min at 4°C. Then, the supernatant was mixed with the luciferase assay buffer, and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507).

Detection of Hypoxic Conditions Using a Fluorescent Probe

We used a fluorescent probe QCy5, which emits fluorescence under hypoxic conditions (14). Mice were anesthetized with pentobarbital in phosphate-buffered saline and received an intravenous injection of 10 nmol QCy5 dissolved in 100 μl saline. Soon after the injection of QCy5, a midline abdominal incision was made to expose the liver. Then, mice were left untreated or received a hydrodynamic injection of saline or a hepatic ischemia by clamping the portal vein. Fluorescent images at excitation of 630 nm and detection at 655 nm were repeatedly obtained by using the NightOwl LB 981 Molecular Light Imager (EG&G Berthold) at the indicated time points after the treatment.

Statistical Analysis

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Fisher's PLSD test for multiple comparisons and by Student's *t*-test for two groups. A *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Production of Proinflammatory Cytokines After Hydrodynamic Injection

Fig. 1a shows the concentrations of IL-6 and TNF-α in mouse serum after hydrodynamic injection of saline into mice. A hydrodynamic injection caused a transient increase in serum IL-6 1–2 h after injection (closed symbol) with a peak level of 1,500 pg/ml. In addition, a large volume injection of saline over 30 s or more (a slow injection) had no significant effects on the serum concentration of IL-6 (28.4 pg/ml at 1 h after injection). Then, the level quickly returned to normal while the injection produced hardly any increase in the serum TNF-α level (open symbol).

Effect of IL-6 on the Level of Transgene Expression

IL-6 is mainly produced by Kupffer cells and involved in liver injury and regeneration (15). To examine whether IL-6 plays a key role in the increase in transgene expression by hydrodynamic injection, mice received a hydrodynamic injection of 10 μg pCMV-gLuc. Three days after hydrodynamic injection of pCMV-gLuc, mice received injections of clodronate liposomes into the peritoneal cavity to deplete macrophages including Kupffer cells, and a hydrodynamic injection of saline was performed on day 5. F4/80 staining of liver sections clearly demonstrated that the number of F4/80-positive cells in the liver, most of which are Kupffer cells, was markedly reduced by the injection of clodronate liposomes (Fig. 1b–c). Moreover, mice injected with clodronate liposomes showed decreased IL-6 production after the second hydrodynamic injection (Fig. 1d), indicating that a large fraction of cells producing IL-6 was depleted by the treatment.

Then, the ratio of the expression levels (x-fold increase) before and 6 h after hydrodynamic injection of saline was calculated and used to examine the effect of the clodronate liposomes on the reactivation of silenced transgene expression. The luciferase activity in serum was increased about 20-fold by hydrodynamic injection of saline in the PBS-treated mice (Fig. 1e). A similar ratio was obtained in the clodronate liposome-treated mice, indicating that IL-6 production has no significant effect on the increase in transgene expression.

Effects of LPS and Lipoplex on the Level of Transgene Expression

To examine the effect of inflammatory cytokines, including IL-6, on the level of transgene expression, LPS or lipoplex, both of which are known to induce such cytokines, was administered to mice that had received a hydrodynamic injection of pCMV-gLuc 5 days before. Administration of LPS or pcDNA3.1 lipoplex increased the serum concentration of IL-6 to peak levels of 12,000 and 15,000 pg/ml, respectively, in dose-dependent manner (Fig. 2a). Irrespective of the type of inducer, the luciferase activity in the serum was hardly affected by the treatment (Fig. 2b–c). Taken together, these results indicate that IL-6 produced by hydrodynamic injections contributes very little to the increase in transgene expression.

Effect of Antioxidants on the Increase in Transgene Expression

To examine the role of ROS in the reactivation, catalase, an enzyme-degrading hydrogen peroxide, was injected into mice together with saline by the hydrodynamic injection method 5 days after hydrodynamic administration of pCMV-gLuc. Fig. 3a shows the luciferase activity before the treatment and 6 h after hydrodynamic injection of saline containing catalase or inactivated catalase. About a 17-fold increase in the activity was observed in mice receiving a hydrodynamic injection of saline containing inactivated catalase. The x-fold increase was low when catalase-containing saline was injected; the injection-induced increase in luciferase activity in serum was partially

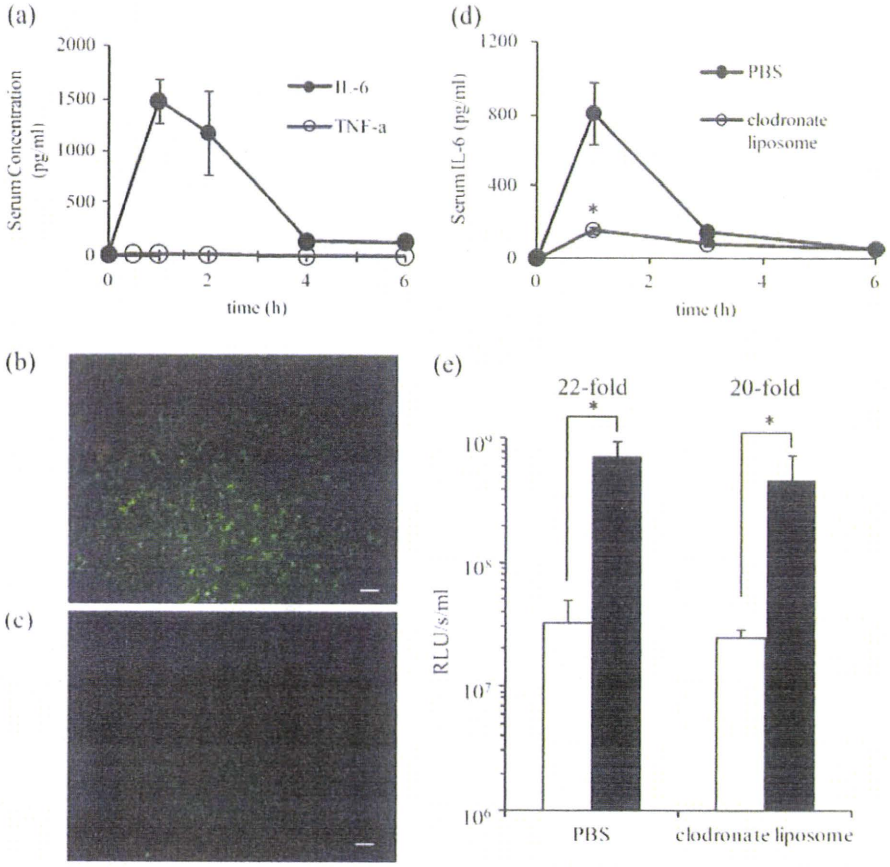

Fig. 1 Inflammatory response following hydrodynamic injection and effect of Kupffer cell depletion on hydrodynamic injection-induced responses. **a** Time-courses of the concentrations of IL-6 and TNF-α in mouse serum after a rapid, large-volume injection of saline. Blood samples were sequentially obtained from the tail vein, and the concentrations of IL-6 (*closed symbols*) and TNF-α (*open symbols*) were measured. Results are expressed as mean ± SEM ($n \geq 4$). **b** and **c** Immunostaining of Kupffer cells in the liver after the administration of clodronate liposome. Liver sections were prepared 2 days after intraperitoneal administration of PBS (**b**) or clodronate liposomes (**c**) and stained with FITC-labeled F4/80 specific antibody. Scale bars = 100 μm. **d** and **e** Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. On day 3, they were intraperitoneally injected with PBS or clodronate liposomes, and on day 5, they received a hydrodynamic injection of saline. **d** Time-course of the concentration of IL-6 in mouse serum after the hydrodynamic injection of saline on day 5. *Closed symbols*: PBS group, open symbols: clodronate liposome group. Results are expressed as mean ± SEM ($n = 4$). $^*P < 0.05$ compared with the PBS-injected group. **e** Gaussia luciferase activity in the serum was measured just before (*open column*) and 6 h after the hydrodynamic injection of saline (*closed column*), and the x-fold increase was calculated by dividing the value 6 h after injection by that before injection. Results are expressed as mean + SEM ($n = 4$).

inhibited by injection of saline containing a high dose of catalase at 1,000 units/mouse (33 μg protein/mouse). Similar results were obtained when NAC was used instead of catalase (Fig. 3b). These results suggest that ROS plays a key role in the increase in transgene expression by hydrodynamic injection.

Increase in Transgene Expression by CCl_4 and its Suppression by Catalase

CCl_4 is metabolized to the trichloromethyl radical (CCl_3) in the liver and induces oxidative stress (16). To examine the effects of oxidative stress on the transgene expression, the change in the level of transgene expression after CCl_4 injection was first examined. Mice that had received a hydrodynamic injection of 10 μg pCMV-gLuc received an intraperitoneal injection of 1–2 ml/kg CCl_4 on day 5. CCl_4 increased the level of luciferase activity in the serum in a dose-dependent manner (Fig. 4a). However, this CCl_4-induced increase in activity was significantly suppressed by an intravenous injection of catalase at the dose of 1,500 units/mouse (50 μg protein/mouse) (Fig. 4b), indicating that ROS, especially hydrogen peroxide, induced by CCl_4 reactivates silenced transgene expression. To determine the time course of the increase in transgene expression after hydrodynamic injection of saline or CCl_4 administration, the luciferase activity in the serum was determined at the indicated time points (Fig. 4c). As some mice did not survive

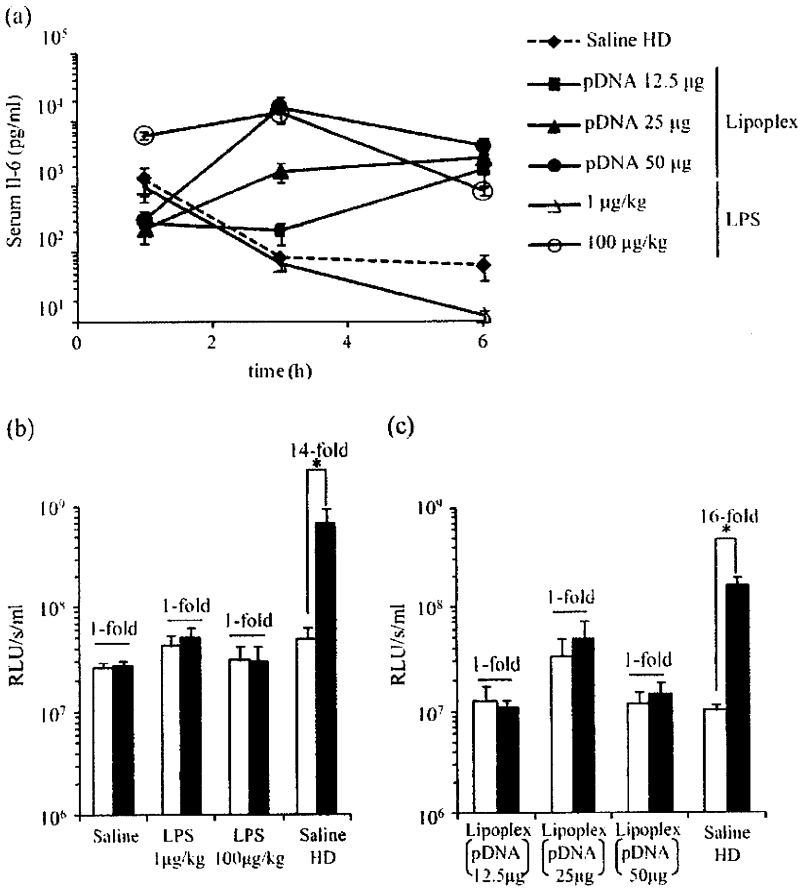

Fig. 2 Effects of LPS and lipoplex on reactivation of silenced transgene expression. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. Five days after the administration of plasmid DNA, mice received a rapid injection of a large volume of saline (saline HD), intraperitoneal injection of indicated doses of LPS or intravenous injection of lipoplex at a dose of 12.5–50 μg as the pcDNA3.1 amount.
a Time-courses of the concentrations of IL-6 and TNF-α in mouse serum after saline HD, LPS administration or lipoplex injection. Results are expressed as mean ± SEM ($n = 4$). **b** and **c** Gaussia luciferase activity in the serum was measured just before (*open column*) and 6 h after saline HD, LPS administration or lipoplex injection (*closed column*). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean + SEM ($n \geq 3$). *$P < 0.05$.

the CCl_4 treatment, the results of survived mice were shown. The luciferase activity had a peak value at 6 h to 1 day after the treatment and decreased thereafter. The profiles were almost identical between the two treatment groups.

To evaluate whether ROS production in the liver activates transcription factors, including NF-κB and AP-1, plasmid DNA with binding sites for these transcription factors was injected. In addition, pCMV-Luc and pTA-Luc were used as a positive and negative control, respectively. A hydrodynamic injection of saline or intraperitoneal injection of CCl_4 increased the luciferase activity in mice that were treated with pCMV-Luc, pAP-1-Luc or pNF-κB-Luc, suggesting that the transcriptional activity of AP-1 and NF-κB is increased by the treatment. On the other hand, neither treatment increased the luciferase activity in mice treated with pTA-Luc, a plasmid DNA lacking the binding sites for transcription factors.

Detection of Hypoxia in Mouse Liver

To investigate whether hydrodynamic injection induces hypoxia in mouse liver, a fluorescent probe QCy5, which emits fluorescence only under hypoxic conditions, was intravenously injected to mice. Only a weak fluorescence, which might be derived from food, was observed in the gastrointestinal tract of untreated mice (Fig. 5b). A time-dependent increase in fluorescent intensity was observed in the liver of mice with the portal vein being clamped. Moreover, hydrodynamic injection also increased the fluorescence intensity of the liver, suggesting that the liver is under hypoxic conditions.

DISCUSSION

Hydrodynamic injections produce a variety of changes in the body, especially within the liver, which include, but are not limited to, a transient increase in the size of the liver, increased blood pressure across the liver, reorganization of the cytoskeleton (unpublished observation), and the activation of transcription factors, such as AP-1 and NF-κB (6,7). In addition to these events, our present study showed that the levels of IL-6 and ROS are increased by hydrodynamic injection.

Partial hepatectomy leads to regeneration of the organ to its original size within a week or so (17). Liver

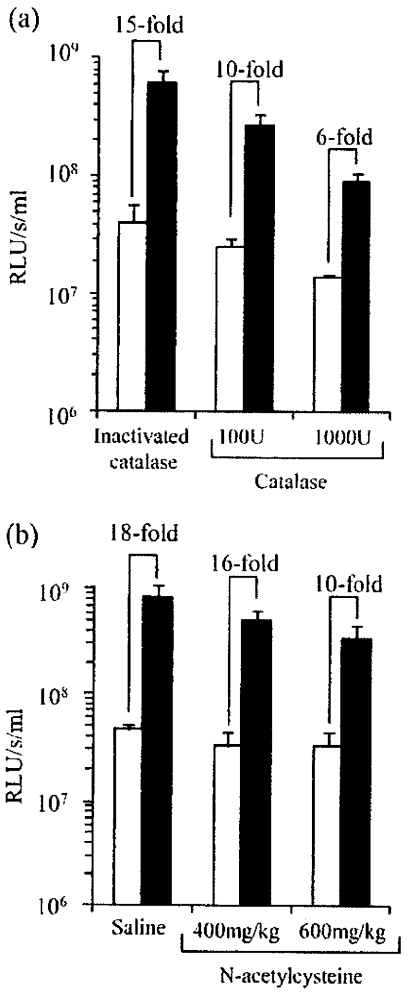

Fig. 3 Effect of antioxidant agents on reactivation of silenced transgene expression. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. **a** On day 5, they received a hydrodynamic injection of saline containing 100 or 1,000 U/shot bovine catalase or its inactivated derivative (inactivated catalase). Gaussia luciferase activity in the serum was measured just before (*open column*) and 6 h after the hydrodynamic injection of saline (*closed column*). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean + SEM ($n=3$). The shown data is representative result from the separate experiments that are performed twice. **b** NAC (400 or 600 mg/kg) was intraperitoneally injected into mice 30 min before a hydrodynamic injection of saline 5 days after pCMV-gLuc transfer. Gaussia luciferase activity in the serum was measured just before (*open column*) and 6 h after the hydrodynamic injection of saline (*closed column*). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean + SEM ($n \geq 4$). The shown data is representative result from the separate experiments that are performed twice.

regeneration is linked with the proliferation of liver cells where the expression of a variety of genes is markedly upregulated. In one of our previous studies, we found that hydrodynamic injection of saline increases the mRNA expression level of c-fos, c-jun and c-myc in the liver, whereas it decreases the level of cyp2e1 (6), which is a similar gene regulation to those observed in the process of liver regeneration. Of the genes upregulated during liver regeneration, IL-6, the expression of which is markedly increased when the liver is damaged (18), plays important roles in the regeneration (15). For example, it was reported that hepatocytes are primed by inflammatory cytokines, such as IL-6, to compete for the replication (19). In the present study, we found that hydrodynamic injections increase the level of serum IL-6 (Fig. 1a), which was comparable with that observed after partial hepatectomy (20). These results imply that hydrodynamic injection induces some liver damages that could trigger responses similar to those occurring during liver regeneration. Therefore, IL-6 induced by hydrodynamic injection may be involved in activating AP-1 and NF-κB, which increases the expression from plasmids. However, the reduced IL-6 level in mice whose Kupffer cells were depleted hardly affected the level of reactivation (Fig. 1b–c). Moreover, increasing the IL-6 production by stimuli other than hydrodynamic injection, such as LPS and lipoplex, was not effective in reactivating the silenced transgene expression (Fig. 2). These results suggest that hydrodynamic injection induces the production of IL-6 from Kupffer cells, but the induced IL-6 has no significant effect on the reactivation of silenced transgene expression by hydrodynamic injection. On the other hand, it is known that LPS or lipoplex treatment activates transcription factors, including NF-κB. These treatments increased the serum concentration of IL-6, indicating that these treatments induced inflammatory responses (Fig. 2a). Considering the fact that hepatocytes are hardly involved in the initiation of inflammatory response, the activation of transcription factors by the treatment of LPS or lipoplex could take place mainly in inflammation-related cells, such as Kupffer cells, but not in hepatocytes.

Oxidative stress is associated with a large number of events in the liver, including hepatitis, viral infection, drug-induced liver injury and ischemia/reperfusion injury (21,22). In the present study, we found that administration of catalase or NAC significantly suppressed the reactivation of transgene expression by hydrodynamic injections (Fig. 3), which strongly suggests that ROS are produced by hydrodynamic injections and that the ROS produced activate the transgene expression. In addition, the experimental results of CCl_4, an inducer of oxidative stress, also supported the hypothesis that ROS production in the liver triggers the reactivation of the transgene expression (Fig. 1). The following is one possible explanation for the mechanism of ROS production by hydrodynamic injections. After hydrodynamic injection, the oxygen supply to the liver would be reduced because isotonic saline solution, which is

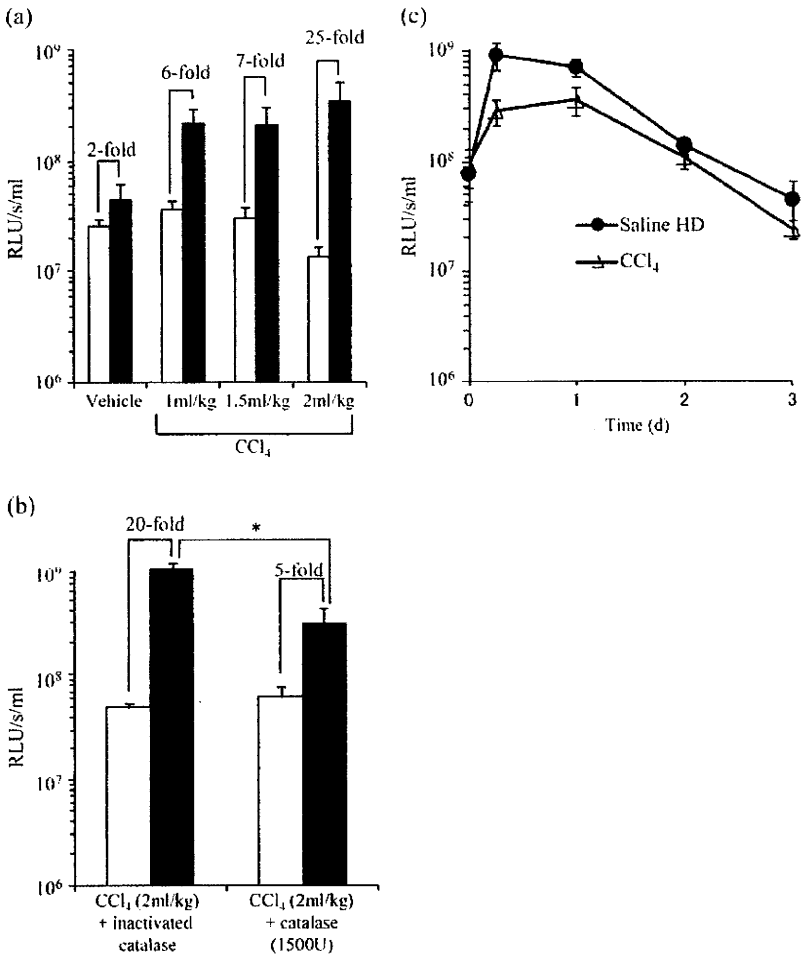

Fig. 4 Reactivation of transgene expression by CCl_4 and its suppression by catalase. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. **a** On day 5, they received an intraperitoneal injection of 1, 1.5 or 2 ml/kg CCl_4. Gaussia luciferase activity in the serum was measured just before (*open column*) and 6 h after the hydrodynamic injection of saline (*closed column*). The x-fold increase was calculated as described in the legend to Fig. 1e. **b** Catalase or inactivated catalase (1,500 U/shot) was intravenously injected into mice soon after an injection of 2 ml/kg CCl_4 into the peritoneal cavity. Gaussia luciferase activity in the serum was measured just before (*open column*) and 6 h after CCl_4 injection (*closed column*). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean + SEM ($n \geq 3$). *$P < 0.05$. **c** On day 5, mice received hydrodynamic injection of saline or intraperitoneal injection of 2 ml/kg CCl_4. At indicated time points after the treatment, Gaussia luciferase activity in the serum was measured. Results are expressed as mean ± SEM ($n = 4$).

much less oxygenated than blood, remains in the liver for some time (7,23). This could lead to transient hepatic ischemia, which then results in hypoxia and increases the generation of ROS at mitochondrial complex III (8,9). In accordance with this hypothesis, we detected that a hydrodynamic injection of saline induced hypoxic conditions in mouse liver (Fig. 5b). As ROS are well-known intracellular second messengers that activate a variety of transcription factors, including AP-1 and NF-κB, it is reasonable that hydrodynamic injection-induced ROS activate these transcription factors, then these factors bind to their sites in the promoter/enhancer regions of plasmids, leading to a high level of transgene expression. In the present study, we also confirmed that CCl_4 administration activated the transcription activity of AP-1 and NF-κB (Fig. 5a).

In our preliminary experiments, we found that hepatic ischemia/reperfusion could also reactivate silenced transgene expression (unpublished observations). In the ischemic liver, xanthine oxidase and hypoxanthine accumulate, and both of them produce a large amount of superoxide anions when the liver is reperfused (24). Therefore, this finding also supports the hypothesis that hepatic ROS production increases the transgene expression in the liver. Therefore, any type of stimuli that produces ROS can be used to increase the transgene expression.

Reactivation of silenced transgene expression is an interesting event. It suggests that plasmid DNA delivered to the liver remains for a long time in a form that can be activated transcriptionally by some stimuli. A previous study in our laboratory showed that the reactivation of transgene expression occurs even 3 months after hydrodynamic gene delivery (6). Thus, the reactivation of transgene expression could be an effective approach to achieving desirable transgene expression without repeated gene transfer. The requirement of ROS for reactivation may raise some concern about tissue damage, but the damage induced when the reactivation stress is applied should be less than that when plasmid DNA is delivered to cells by

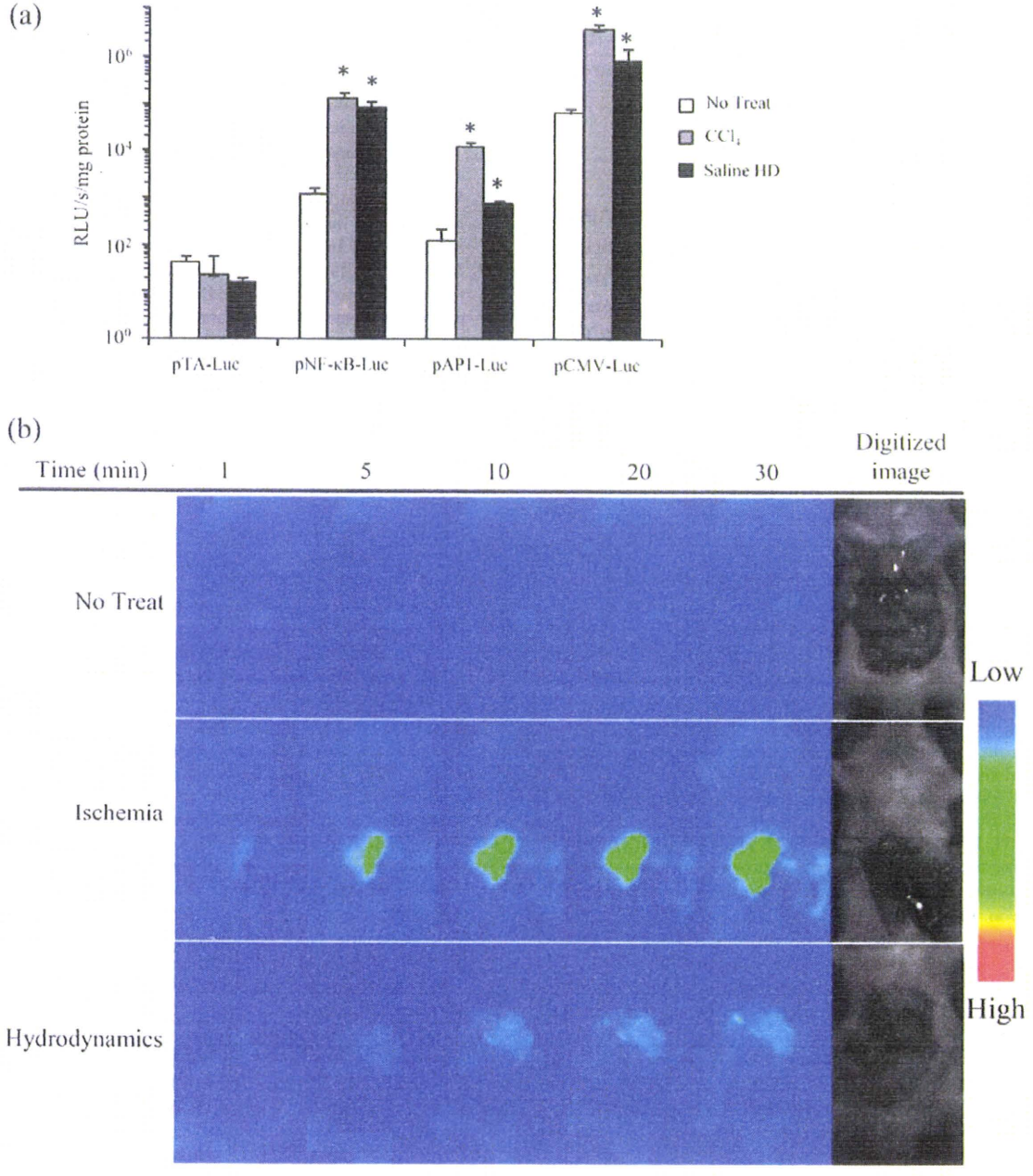

Fig. 5 Effect of hydrodynamic injection of saline on transcription factors and oxygen supply to the liver. **a** Mice received a hydrodynamic injection of 10 μg of pTA-Luc, pAP1-Luc, pNF-κB-Luc or pCMV-Luc. On day 5, they received an intraperitoneal injection of 3 ml/kg CCl_4 or hydrodynamic injection of saline. Six hours after the treatment, luciferase activity in the liver was determined. Results are expressed as mean + SEM ($n \geq 3$). *$P < 0.05$ *vs* No Treat groups. **b** Mice received intravenous injection of 10 nmol QCy5 dissolved in 100 μl saline. Soon after QCy5 administration, mice were untreated or received hydrodynamic injection of saline or hepatic ischemia by clamping the portal vein. At the indicated time periods after the initiation of the treatments, the fluorescent images at excitation of 630 nm and detection at 655 nm were obtained by using the NightOwl LB 981.

other means. Developing methods that increase oxidative stress topically at the site where plasmid DNA is delivered will increase the availability of the reactivation technique for prolonged transgene expression without repeated administration of plasmid DNA.

CONCLUSION

The present study clearly demonstrates that hydrodynamic injection induces the production of both inflammatory cytokines and ROS, but only ROS is associated with

reactivation of silenced transgene expression by hydrodynamic injection, whereas inflammatory cytokines are less likely to be associated with the reactivation.

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RESEARCH ARTICLE

Persistent interferon transgene expression by RNA interference-mediated silencing of interferon receptors

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Abstract

Background The *in vivo* half-life of interferons (IFNs) is very short, and its extension would produce a better therapeutic outcome in IFN-based therapy. Delivery of IFN genes is one solution for providing a sustained supply. IFNs have a variety of functions, including the suppression of transgene expression, through interaction with IFN receptors (IFNRs). This suppression could prevent IFNs from being expressed from vectors delivered. Silencing the expression of IFNAR and IFNGR, the receptors for type I and II IFNs, respectively, in cells expressing IFNs may prolong transgene expression of IFNs.

Methods Mouse melanoma B16-BL6 cells or mouse liver were selected as a site expressing IFNs (not a target for IFN gene therapy) and IFN-expressing plasmid DNA was delivered with or without small interfering RNA (siRNA) targeting IFNRs.

Results Transfection of B16-BL6 cells with siRNA targeting IFNAR1 subunit (IFNAR1) resulted in the reduced expression of IFNAR on the cell surface. This silencing significantly increased the IFN-β production in cells that were transfected with IFN-β-expressing plasmid DNA. Similar results were obtained with the combination of IFN-γ and IFNGR. Co-injection of IFN-β-expressing plasmid DNA with siRNA targeting IFNAR1 into mice resulted in sustained plasma concentration of IFN-β.

Conclusions These results provide experimental evidence that the RNAi-mediated silencing of IFNRs in cells expressing IFN, such as hepatocytes, is an effective approach for improving transgene expression of IFNs when their therapeutic target comprises cells other than those expressing IFNs. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords interferon; interferon receptor; RNA interference; small interfering RNA

Introduction

Interferons (IFNs) are a class of cytokines with a variety of biological activities, such as antiviral, antitumor and immunomodulatory effects. Therefore, IFNs have been used to treat a variety of diseases, including cancer, viral infections, multiple sclerosis and allergy [1–3]. Because IFN proteins are quickly degraded and eliminated from the blood circulation, it has been claimed that frequent administration is necessary so that they can exert their therapeutic

effects [4]. Therefore, an improvement in the retention time of IFNs would greatly increase their therapeutic efficacy and the quality of life of patients.

Various methods have been developed to increase the residence time of IFNs in the body. Controlled release formulations have been developed by using liposomes, silicon nanospheres and other delivery systems [5]. Another method that has been approved for clinical use is chemical conjugation of IFNs. IFN-α2a and IFN-α2b conjugated with polyethylene glycol is now used as a standard therapeutic agent for the treatment of hepatitis C. Such modification of IFN proteins, however, is a double-edged sword because it is effective in prolonging residence time of IFNs in the systemic circulation [6,7], although, at the same time, it hinders the interaction of IFNs with their receptors [8,9]. Thus, chemical modifications unavoidably reduce the biological activity of IFNs, so that modified IFN-based therapy needs to be optimized by achieving a balance between the modification-dependent enhanced pharmacokinetics and reduced biological activities.

Delivery of IFN genes is an alternative method of prolonging the retention time of IFNs without reducing their biological activities. Studies have reported that gene delivery of IFNs is an effective approach to treating a variety of diseases [10–14]. We have demonstrated that hydrodynamic injections of plasmid DNA encoding IFN-β or IFN-γ inhibited metastatic tumor growth of mouse colon carcinoma cells in mice [11,15,16]. However, recent experimental evidence indicates that IFNs inhibit transgene expression through binding to IFN receptors (IFNRs) [17–20]. Therefore, transgene expression of IFNs may be inhibited when they bind to the receptors on the surface of cells, in which IFNs are expressed from delivered vectors. This might explain why transgene expression of IFNs is very transient in previous studies [11,15].

IFNs are classified into two major subfamilies based on their biological activities, receptors and their producer cells. IFN-α and IFN-β are major type I IFNs, which are secreted from almost all types of cells. IFN-γ is the sole type II IFN and is mainly produced by Th-1 lymphocytes, natural killer cells and professional antigen-presenting cells in response to cytokines, such as interleukin (IL)-12 and IL-18 [21]. IFNAR, a type I IFN receptor (IFNR), consists of two different subunits, IFNAR1 and IFNAR2 [22], and the type II IFNR, IFNGR, also consists of two different subunits, IFNGR1 and IFNGR2 [22]. In the present study, we hypothesized that the knockdown of IFNR on transgene expressing cells would prevent IFN-mediated suppression of the transgene expression, which would be effective in obtaining more sustained transgene expression profile of IFNs. This approach would be valid only when transgene expressing cells are different from target cells for IFN-mediated therapy. To this end, small interfering RNA (siRNA) targeting either IFNAR1, IFNAR2 or IFNGR1 was used to suppress the expression of IFNRs in transgene-expressing cells, not in therapeutic target cells, and their effects on IFN transgene expression were examined in cultured cells and in mice.

Materials and methods

Plasmid DNA

pCMV-Luc, pCMV-β and pCMV-γ encoding firefly luciferase, mouse IFN-β and mouse IFN-γ, respectively, under the control of the cytomegalovirus (CMV) promoter, were constructed as described previously [11] and pGZB-β encoding mouse IFN-β was also constructed as described previously [15]. pcDNA3.1 (pcDNA) was purchased from Invitrogen (Carlsbad, CA, USA) and used as a control plasmid DNA without IFN expression. Each plasmid DNA was amplified in the DH5α strain of *Escherichia coli* and purified using JETSTAR 2.0 Plasmid GIGA Plasmid Purification Kits (Genomed GmbH, Löhne, Germany).

siRNA and IFN

Pre-designed, but not validated, siRNAs targeting murine *IFNAR1*, *IFNAR2* and *IFNGR1* were purchased from Invitrogen. siRNA targeting green fluorescent protein (siGFP) was purchased from Takara Bio (Otsu, Japan). Recombinant mouse IFN-β and IFN-γ were purchased from PBL Biomedical Laboratories (New Brunswick, NJ, USA).

Cell culture

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

Transfection

B16-BL6 cells were placed on culture plates at varying densities in varying types of culture plates depending on the experiments: 2×10^4 cells/well in 24-well-plates (luciferase experiments); 1×10^5 cells/well in 24-well-plates (mRNA measurement experiments); 3×10^5 cells/well in six-well-plates (Facs analysis); and 1×10^5 cells/well in 12-well-plates (measurement of IFN concentration in the medium). After an overnight incubation, cells were transfected with plasmid DNA and siRNA using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. In brief, 1 μg of nucleic acids was mixed with 3 μg of Lipofectamine 2000 at a final concentration of 6 μg of Lipofectamine 2000/ ml OPTI-MEM I (Invitrogen). In the cotransfection experiments, the mixture of siRNAs and pDNAs in OPTI-MEM I was added with Lipofectamine 2000. The resulting complex was added to cells which

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.

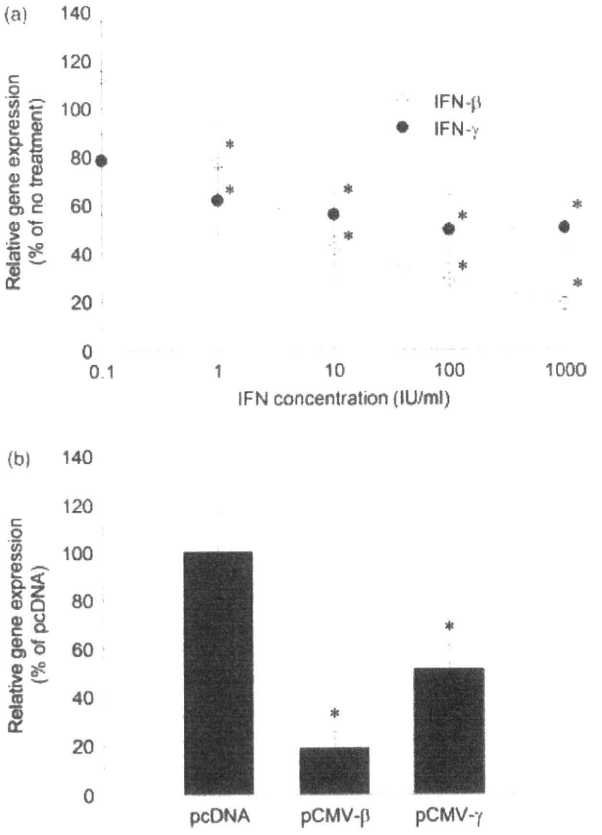

Figure 1. Effect of IFNs on luciferase activity in B16-BL6 cells after transfection of luciferase-expressing plasmid DNA. (a) B16-BL6 transfected cells were incubated with the indicated concentrations of IFN-β and -γ for 24 h, and the luciferase activity was measured. Results are presented as the mean ± 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

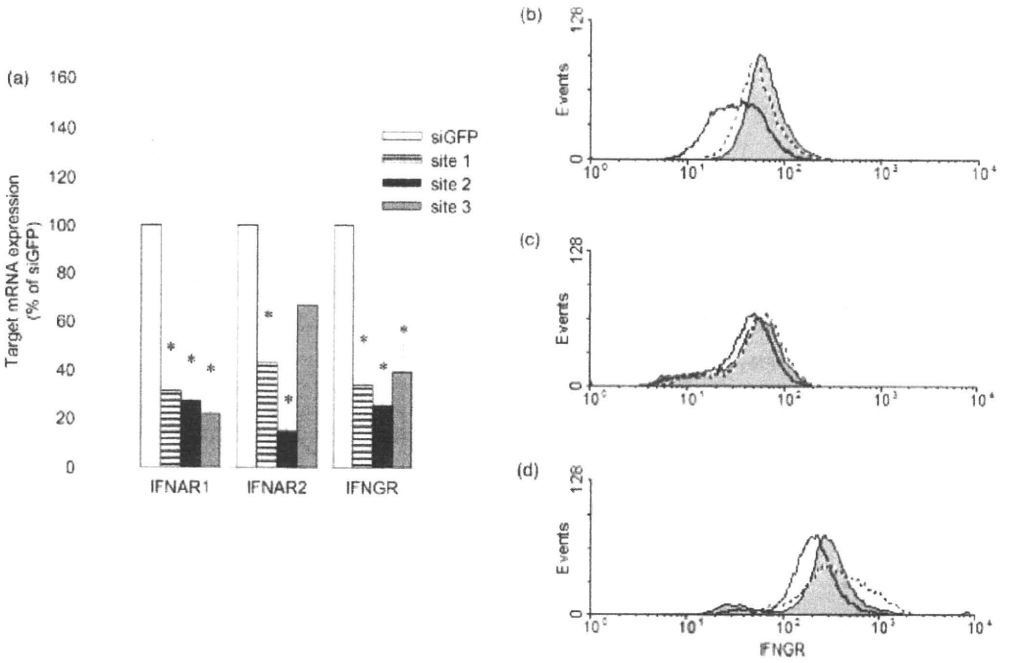

Figure 2. Expression of IFN receptors in B16-BL6 cells after transfection of siRNA. (a) The amount of IFNR mRNA expression was determined 1 day after transfection of siRNA. Results are presented as the mean ± SD of four independent determinations. *$p < 0.05$ compared to the siGFP group. (b–d) Two days 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

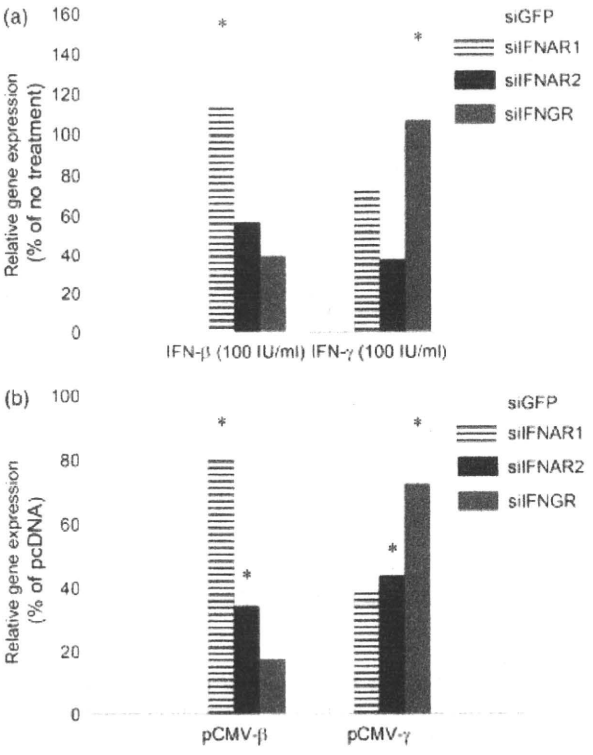

Figure 3. Effect of IFN on luciferase activity in B16-BL6 cells after transfection of luciferase-expressing plasmid DNA and siRNA targeting IFNR. (a) One day after cotransfection of pCMV-Luc (1 μg/ml) with 1 μg/ml of siGFP, siIFNAR1, siIFNAR2 or siIFNGR, transfected cells were treated with or without 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

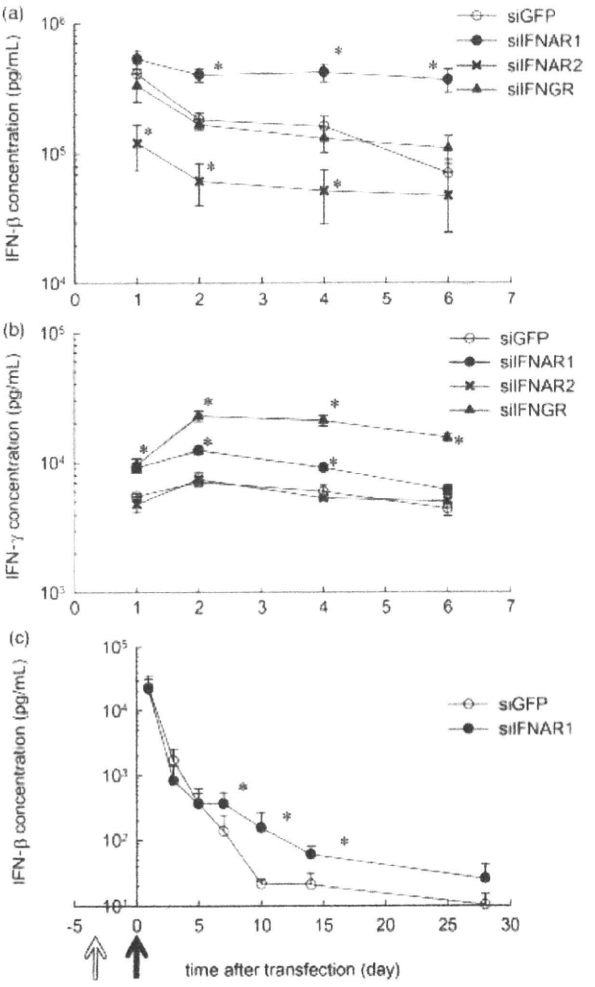

Figure 4. Effect of co-transfection of IFN-expressing plasmid DNA and siIFNR on transgene expression of IFN. B16-BL6 cells were transfected with siRNA (1 μg/ml) and (a) pCMV-β (1 μg/ml) or (b) pCMV-γ (1 μg/ml). Culture supernatant was collected 1, 2, 4 and 6 days after transfection. Results are presented 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.

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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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