# RESEARCH ARTICLE

# Prolonged Circulation Half-life of Interferon γ Activity by Gene Delivery of Interferon γ–Serum Albumin Fusion Protein in Mice

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**ABSTRACT:** Gene delivery of mouse interferon (IFN) γ has been shown to inhibit metastatic tumor growth and onset of atopic dermatitis in mouse models. In this study, we tried to increase the circulation half-life of IFNγ after its gene delivery by designing a novel fusion protein of IFNγ with mouse serum albumin (MSA). Western blot analysis confirmed that IFNγ–MSA was expressed as a fusion protein, but hardly formed dimer as IFNγ did. The biological activity of IFNγ–MSA, which was examined using a plasmid expressing luciferase under the control of gamma-activated sequence elements, was about 200-fold lower than the activity of IFNγ. Intravenous injection of the proteins into mice confirmed that the circulation half-life of IFNγ was significantly prolonged by the modification. A hydrodynamic injection of a plasmid expressing IFNγ–MSA resulted in a sustained concentration in mouse serum; it resulted in about sixfold greater area under the concentration–time curve and about threefold longer mean residence time of IFNγ activity than those of IFNγ. Gene delivery of IFNγ–MSA inhibited tumor metastasis to a similar level to that of IFNγ despite the reduced activity of IFNγ–MSA. These results indicate that gene delivery of IFNγ–MSA is a promising approach to prolong the circulation half-life of IFNγ activity. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

**Keywords:** albumin; fusion protein; gene delivery; plasmid DNA; hydrodynamic injection; pharmacokinetics; clearance; moment analysis; dimerization

## INTRODUCTION

Interferon (IFN) γ plays a variety of roles in the immune systems. Despite the potent biological activities of IFNγ, its clinical application is limited to treatment of kidney carcinoma and adult T-cell leukemia and to reduction in the frequency and severity level of serious infection associated with chronic granulomatous disease (CGD) in Japan; in the United States, it was approved only for the treatment of CGD and malignant osteopetrosis by FDA.[1] This limited application of IFNγ is mainly because of the short *in vivo* half-life of the protein, which forces patients to receive frequent subcutaneous injections and to suffer from the adverse effects exacerbated by frequent dosing.[2] It was reported that IFNγ disappears from the systemic circulation with a half-life of less than 3 min after intravenous injection into mice.[3] Its half-life in humans is about 4.5 h after intramuscular injection.[4]

Although a number of technologies have been developed to prolong the short *in vivo* half-life of cytokines or other protein drugs, a few systems have been designed or intended to use for IFNγ.[5] The most studied system to extend the half-life of protein drugs is controlled release formulations. Fujioka et al.[6,7] developed mini-pellets that continuously release IFNα for more than 1 week after subcutaneous implantation. Another approach is the conjugation of polyethylene glycol to IFNα or IFNβ. This pegylation protects the IFNs from proteolytic degradation and reduces their renal clearance and antigenecity.[8] In clinical practice, two types of pegylated human IFNα are used as a standard therapy for hepatitis C virus-infected patients in combination with an antiviral drug rivabirin.[9] A drawback of pegylation is the

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possible variations in the chemical structure as well as the biological activities.

Gene delivery, by which a protein of interest is expressed from a vector within the body, has been studied for more than the last two decades as an alternative strategy to improve the pharmacokinetic properties of protein drugs. We have been studying plasmid-based delivery of IFN genes and reported that gene delivery of IFNγ is effective in inhibiting the metastatic growth of tumor cells[10,11] and in preventing atopic dermatitis in NC/Nga mice, a model mice of human atopic dermatitis.[12] In these previous studies, sustained expression of IFNγ was successfully achieved by modifying the properties of the plasmids.

Controlling the structural properties of IFNγ will provide additional possibilities to increase its therapeutic potency of gene delivery of IFNγ through the changes in the biological activity and pharmacokinetic characteristics. Gene delivery of fusion proteins has been examined for some proteins in animal experiments, but little attention has been paid to the pharmacokinetic characteristics of such fusion proteins adequately, even though it is one of the most important parameters determining therapeutic effects. Therefore, in the present study, we tried to prolong the circulation half-life of mouse IFNγ after *in vivo* gene delivery using nonviral plasmid vectors. To achieve this goal, serum albumin was selected as a partner for the protein because it has a long circulation half-life and is little immunogenic.[13] We constructed plasmids encoding a fusion protein of IFNγ and mouse serum albumin (MSA) and examined whether the conjugation increases the retention time of IFNγ activity after *in vivo* gene transfer to mice. Pharmacokinetic parameters, such as the area under the serum concentration–time curve (AUC) and the mean residence time (MRT), were calculated using a moment analysis method and used for quantitative evaluation.

## MATERIALS AND METHODS

### Cell Culture and Mice

An African green monkey kidney fibroblast cell line, COS-7, was obtained from American Type Culture Collection (Manassas, Virginia). A mouse melanoma cell line,

B16-BL6, was obtained from Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). COS-7 and B16-BL6 cells were cultured in Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Japan Bioserum, Hiroshima, Japan) and penicillin/ streptomycin/ L-glutamine (PSG) (Invitrogen, Carlsbad, California). A mouse colon carcinoma cell line, CT-26, was cultured in RPMI1640 supplemented with 10% FBS and PSG. Male 4-week-old Institute of Cancer Research (ICR) mice, approximately 20 g in weight, were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained on a standard food and water diet under conventional housing conditions. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Science, Kyoto University.

### Plasmid DNA

pcDNA3.1 was purchased from Invitrogen . pCMV-Luc, a firefly luciferase-expressing plasmid DNA, was constructed as described previously.[14] phRL-TK, a renilla luciferase-expressing plasmid DNA under the control of herpes simplex virus thymidine kinase (TK) promoter, was purchased from Promega (Madison, Wisconsin). pC3MSAmH, a plasmid DNA encoding mouse prepro-albumin, was kindly donated by Dr. W. P. Sheffield, McMaster University, Canada.[15] IFNγ-expressing plasmid DNA, pCMV-Muγ was constructed by inserting a mouse IFNγ cDNA fragment into the multicloning site of pcDNA3.1. pCMV-Muγ–MSA, a plasmid encoding the IFNγ–MSA fusion protein, was constructed as follows. An MSA cDNA fragment excluding the secretion signal sequence was amplified by polymerase chain reaction (PCR) from pC3MSAmH. The fragment was inserted into the C-terminal end of IFNγ cDNA in the pCMV-Muγ. The stop codon of IFNγ cDNA was deleted and oligonucleotides coding six amino acids (Gly–Gly–Gly–Ser–Gly–Arg) were inserted between IFNγ cDNA and MSA cDNA as a linker. The sequence of the linker was selected according to a previous report.[16,17] To measure the biological activity of IFNγ–MSA, gamma-activated sequence (GAS)-dependent signal transduction was used. Namely, four copies of GAS element (5′-AGTGATTTCTCGGAAAGAGAG-3′) were inserted into multicloning site of pLuc-MCS (Stratagene, La Jolla, California). Each plasmid DNA was amplified in *Escherichia coli* (DH5α; TOYOBO, Osaka, Japan) and purified using the JETSTAR plasmid purification kit (GENOMED, Löhne, Germany).

### *In Vitro* Transfection

Cells were seeded at $2 \times 10^5$ cells on six-well culture plates and incubated overnight. Transfection of each plasmid DNA was performed using Lipofectamine2000 (Invitrogen) according to a procedure of the manufacturer's instructions. In brief, 1 μg of plasmid DNA was mixed with 3 μL of Lipofectamine2000 in Opti-MEM at a final concentration of 2 μg plasmid DNA (i.e., 0.48 and 0.40 pmol of pCMV-Muγ and pCMV-Muγ–MSA, respectively) per milliliter and the complex obtained was added to cells. At 4 h after the addition, cells were washed with fresh medium and further incubated for a predetermined period.[18] To


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study the expression profile of IFNγ and IFNγ–MSA in COS-7 cells, the culture media of COS-7 cells transfected with pCMV-Muγ or pCMV-Muγ–MSA were harvested and centrifuged at 430 × *g* for 3 min to remove detached cells and cellular debris.

### Western Blotting

The supernatants of COS-7 cells transfected with pCMV-Muγ or pCMV-Muγ–MSA were collected at 48 h after the transfection. The supernatants were untreated or reduced by the addition of dithiothreitol (0.1 M) and heat treatment at 95°C for 4 min to disrupt disulfide bonds and to dissociate homodimers if they exist. The samples were subjected to 12.5% (IFNγ) or 8% (IFNγ–MSA) sodium dodecyl sulfate-polyacrylamide gel electrophoresis in reducing or nonreducing conditions and transferred to a polyvinylidene fluoride (PVDF) transfer membrane electrophoretically. After blocking with 5% skim milk, the membrane was probed with goat anti-mouse IFNγ polyclonal antibody (R&D System, Inc., Minneapolis, Minnesota) overnight at 4°C and then allowed to react with anti-goat IgG antibody conjugated with horseradish peroxidase (Santa Cruz, Inc., Santa Cruz, California) for 1 h at room temperature. The bands were detected by LAS-3000 (Fuji Film, Tokyo, Japan).

### Measurement of Biological Activity of IFNγ and IFNγ–MSA

B16-BL6 cells were cotransfected with pGAS-Luc (1.4 μg/mL) and phRL-TK (0.6 μg/mL). After 4 h transfection, culture medium was replaced with fresh medium containing serial dilutions of conditioned media of COS-7 cells transfected with pCMV-Muγ or pCMV-Muγ–MSA. After 24 h incubation, cells were lysed with a lysis buffer (0.1 M Tris, 0.05% Triton-X-100, 2 mM EDTA, pH 7.8), and the lysates were mixed with reagents of Dual-Luciferase Reporter Assay System (Promega). Then, firefly and renilla luciferase activities were measured in a luminometer (Lumat LB 9507; EG&G Bethhold, Bad Wildbad, Germany), and the ratio of firefly luciferase activity to renilla luciferase activity was calculated. Here, firefly luciferase activity was used as an indicator of IFNγ-driven transcription and renilla luciferase activity was used for normalization of the transfection efficiency and cell number.[18] The ratio was normalized to give *x*-fold values relative to those of group culture without neither IFNγ nor IFNγ–MSA, and half maximum effective concentration (EC50) of IFNγ and IFNγ–MSA was calculated.

### *In Vivo* Gene Transfer

For gene transfer to mouse liver, mice received a hydrodynamic injection via the tail vein of plasmid DNA dissolved in 1.6 mL saline within 5 s.[19] The doses of plasmids were 0.04, 0.2, or 1 pmol/mouse. At predetermined period, blood was collected from the tail vein, and the blood samples were incubated at 4°C for 2 h and centrifuged at 8000 × *g* for 20 min to obtain serum. This method of gene transfer hardly induced the expression of endogenous IFNγ.[10]

### Measurement of Concentration of IFNγ and IFNγ–MSA

The concentration of IFNγ and IFNγ–MSA in the supernatant of COS-7 cells or mouse serum was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Ready-SET-Go! MuIFN-γ ELISA, eBioscience, San Diego, California). The values estimated by ELISA were used for the calculation of remaining activities, even though the reactivity of the antibody to IFNγ–MSA could be affected by the conjugation. This assumption does not affect the profiles of biological activities of IFNγ and IFNγ–MSA after *in vivo* gene transfer.

### Clearance of IFNγ and IFNγ–MSA Proteins After Intravenous Injection into Mice

To obtain IFNγ and IFNγ–MSA proteins, mice were injected with pCMV-Muγ or pCMV-Muγ–MSA as described above at a dose of 20 μg/mouse. At 12 h after injection, blood was collected from the inferior vena cava, and the serum was obtained in the same manner as described above. The serum samples containing IFNγ or IFNγ–MSA were diluted with saline and were injected to different mice into the tail vein at a volume of 100 μL without further purification. Blood was periodically sampled from interior vena cava. The dose of IFNγ or IFNγ–MSA was set at 8.5 μg IFNγ per kilogram, which was estimated by ELISA.

### Experimental Pulmonary Metastasis

CT-26 cells were trypsinized and suspended in Hanks' balanced salt solution (HBSS). Cell suspensions containing $1 \times 10^5$ CT-26 cells in 200 μL of HBSS were injected into the tail vein of syngeneic BALB/c mice to establish experimental pulmonary metastasis. At 3 days after inoculation of tumor cells, each plasmid DNA was injected into the tail vein by the hydrodynamic injection method at a dose of 0.7 pmol/mouse. pCMV-Luc was used as a control vector. At 14 days after inoculation of tumor cells, mice were sacrificed, and the number of metastatic colonies on the lung surface was counted.[11]

### Data Analysis

The AUC and MRT were calculated for each animal by integration to infinite time.[20] Differences were statistically evaluated by Student's *t*-test, and the level of statistical significance was set at a *p* value below 0.05.

## RESULTS

### Expression of IFNγ–MSA in Cultured Cells

Figure 1a shows the Western blot analysis of IFNγ and IFNγ–MSA, both of which were expressed in COS-7 cells. The culture medium of cells transfected with pCMV-IFNγ showed two bands of 15–20 kDa polypeptides under reducing conditions with heat denaturation (lane1), which correspond to a monomeric IFNγ with different degrees of N-glycosylation.[21] The bands of high-molecular weight products (>35 kDa) were considered to be of aggregated products generated in the process of heat treatment. Under nonreducing conditions without heat denaturation, the samples from the pCMV-Muγ-transfected cells had bands of 30–40 kDa (lane2), indicating that IFNγ is in the dimeric form under such conditions. Samples from the pCMV-Muγ–MSA-transfected cells had bands of about 80–100 kDa under both reducing and nonreducing conditions (lanes 3 and 4). The size corresponded to the summation of the size of IFNγ (15–18 kDa)[21] and MSA (approximately 70 kDa),[15] indicating that the fusion protein IFNγ–MSA is expressed from the plasmid. However, few bands with the size of the dimers (170–180 kDa) were detected even in the samples under nonheated and nonreducing conditions, suggesting that the dimerization of the IFNγ–MSA is almost completely blocked.

Figure 1b shows the time course of IFNγ and IFNγ–MSA concentration in culture media of COS-7 cells transfected with pCMV-Muγ or pCMV-Muγ–MSA, respectively. The level of IFNγ–MSA was lower than that of IFNγ for the first 2 days, then increased to a similar level to that of IFNγ in the steady state. There are no significant differences in the stability of IFNγ and IFNγ–MSA proteins in the culture medium (data not shown). These results suggest that the fusion of MSA influences the process of the synthesis and/or secretion of the protein.

### Measurement of Biological Activity of IFNγ–MSA

After binding to IFNγ receptors, IFNγ promotes the expression of IFNγ-responsive genes through recruiting transcription factors that bind to the GAS element of target genes.[22] Therefore, the biological activity of IFNγ was measured using a GAS-dependent expression of firefly luciferase in B16-BL6 cells. The amounts of IFNγ and IFNγ–MSA added to cells were determined by ELISA. As shown in Figure 2, the addition of IFNγ–MSA to cells increased the luciferase activity, indicating that the fusion protein retains biological activity of IFNγ. The analysis of the profiles of IFNγ- or IFNγ–MSA-dependent increase in the luciferase activity resulted in the EC50 values of 30 and 5,920 pg/mL (estimated by ELISA) for IFNγ and

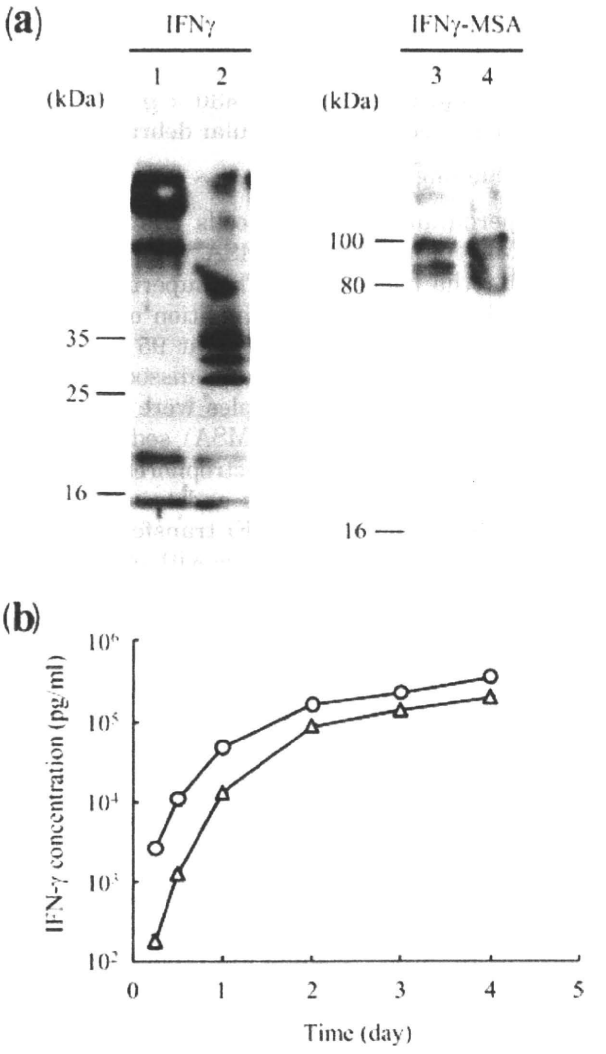


**Figure 1.** Expression of IFNγ and IFNγ–MSA in cultured cells. (a) Western blotting of IFNγ and IFNγ–MSA. Western blotting was performed to confirm the molecular weight of IFNγ (lanes 1 and 2) and IFNγ–MSA (lanes 3 and 4). The culture media of COS-7 cells transfected with pCMV-Muγ amd pCMV-Muγ–MSA were subjected to 12.5% (IFNγ) or 8% (IFNγ–MSA) sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF transfer membrane electrophoretically. IFNγ and IFNγ–MSA were detected with anti-mouse IFNγ polyclonal antibody. Dithiothreitol (0.1 M) was used as a reducing agent to disrupt disulfide bonds. Lanes 1 and 3 were heat denatured under reducing conditions, whereas lanes 2 and 4 were not denatured. (b) Time course of the concentration of IFNγ (open circles) and IFNγ–MSA (open triangles) in the culture medium of COS-7 cells after transfection of pCMV-Muγ and pCMV-Muγ–MSA (2 μg/mL). At the indicated time periods after transfection, the supernatants were collected and the concentration of IFNγ and IFNγ–MSA was measured by ELISA using anti-mouse IFNγ antibody. The results are expressed as the mean ± SE of three independent determinations.


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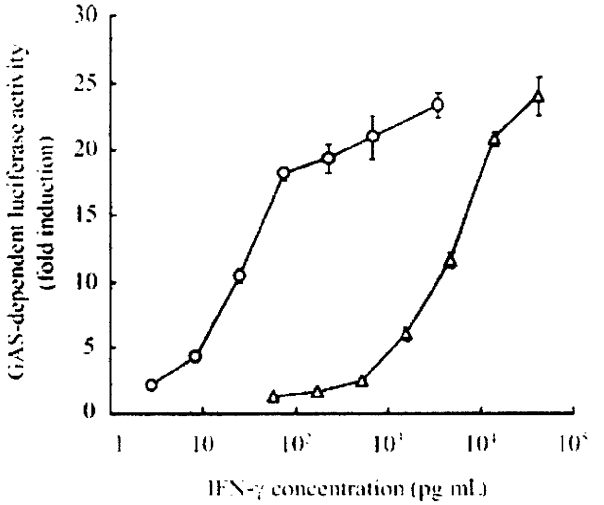


**Figure 2.** Biological activity of IFNγ and IFNγ–MSA. B16-BL6 cells transfected with pGAS-Luc and phRL-TK were incubated with serial dilutions of IFNγ (open circles) or IFNγ–MSA (open triangles) for a further 24 h. pGAS-Luc, plasmid DNA expressing firefly luciferase, was used to assess the degree of activation of GAS signaling pathway. phRL-TK, plasmid DNA expressing renilla luciferase, was used for normalizing the transfection efficiency and cell numbers. The ratio was normalized to give *x*-fold values relative to those of untreated group and half maximum effective concentration (EC50) of IFNγ and IFNγ–MSA was calculated. The results were expressed as the mean ± SE of four independent determinations.

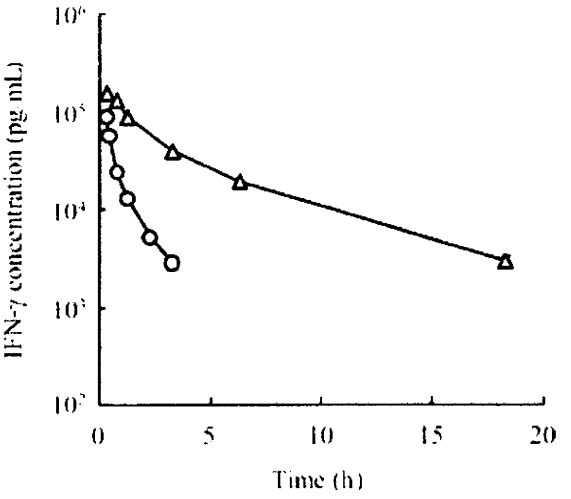


**Figure 3.** Clearance of IFNγ and IFNγ–MSA proteins from the serum after intravenous injection into mice. The concentrations of IFNγ (open circles) and IFNγ–MSA (open triangles) were measured by ELISA using anti-mouse IFNγ antibody and expressed as a percent of dose. The results were expressed as the mean ± SE of four mice.

IFNγ–MSA, respectively, indicating that IFNγ–MSA has approximately 200-fold lower potency than unmodified IFNγ. The activity of IFNγ expressed in COS-7 cells was almost comparable to that of a commercially available recombinant IFNγ (data not shown).

### Blood Clearance of IFNγ and IFNγ–MSA Proteins After Intravenous Injection

To confirm whether the increase in the molecular weight gives any changes in the blood clearance of IFNγ, IFNγ and IFNγ–MSA proteins expressed in and collected from different mice were injected into the tail vein of mice. Figure 3 shows the time course of the concentrations of IFNγ and IFNγ–MSA in the serum after injection of IFNγ and IFNγ–MSA at a dose of 8.5 μg IFNγ/kg bodyweight (estimated by ELISA). The disappearance of IFNγ–MSA from the circulation was much slower than that of IFNγ. The profiles were evaluated by moment analysis to obtain AUC and MRT (Table 1). The AUC of IFNγ–MSA (508 ng·h/mL) was significantly greater than that of IFNγ (51.7 ng·h/mL). The MRT values of IFNγ and IFNγ–MSA were calculated to be 0.937 h and 4.04 h, indicating that albumin fusion resulted in about four-fold increase in the MRT.

**Table 1.** AUC and MRT of Serum IFNγ and IFNγ–MSA after Intravenous Injection into Mice

| Protein | IFNγ | IFNγ–MSA |
|---|---|---|
| AUC (ng·h/mL) | 51.7 ± 2.2 | 508 ± 5* |
| MRT (h) | 0.937 ± 0.020 | 4.04 ± 0.08* |

*Statistically significant ($p < 0.05$) compared with IFNγ.
The AUC and MRT were calculated by integration to infinite time, and the mean ± SE values are expressed.
AUC, concentration–time curve; MRT, mean residence time.

### Time Course of Serum Concentration of IFNγ and IFNγ–MSA After *in Vivo* Gene Transfer

Figure 4 shows the time course of the concentrations of IFNγ and IFNγ–MSA in the serum after hydrodynamic injection of pCMV-Muγ and pCMV-Muγ–MSA at a dose of 0.04, 0.2 or 1 pmol/mouse. In mice receiving pCMV-Muγ, the serum concentration of IFNγ decreased below the detection limit (15 pg/mL) by 3 to 4 days after gene transfer regardless of the dose of the plasmid DNA. In contrast, serum concentration of IFNγ–MSA was observed for at least 14 days after gene transfer. There were little differences in the $C_{max}$ (peak plasma concentration) between the proteins, but the $T_{max}$ (time of maximum serum concentration) values were different; the $T_{max}$ after the injection of pCMV-Muγ–MSA was 24 h, whereas that of pCMV-Muγ was 6 or 12 h. Again, the profiles were subjected to moment analysis. Table 2 summarizes the AUC and MRT of IFNγ and IFNγ–MSA after hydrodynamic injection of pCMV-Muγ and pCMV-Muγ–MSA at varying doses. The AUC values of IFNγ and IFNγ–MSA increased with increasing dose. At any dose, a hydrodynamic injection of pCMV-Muγ–MSA produced

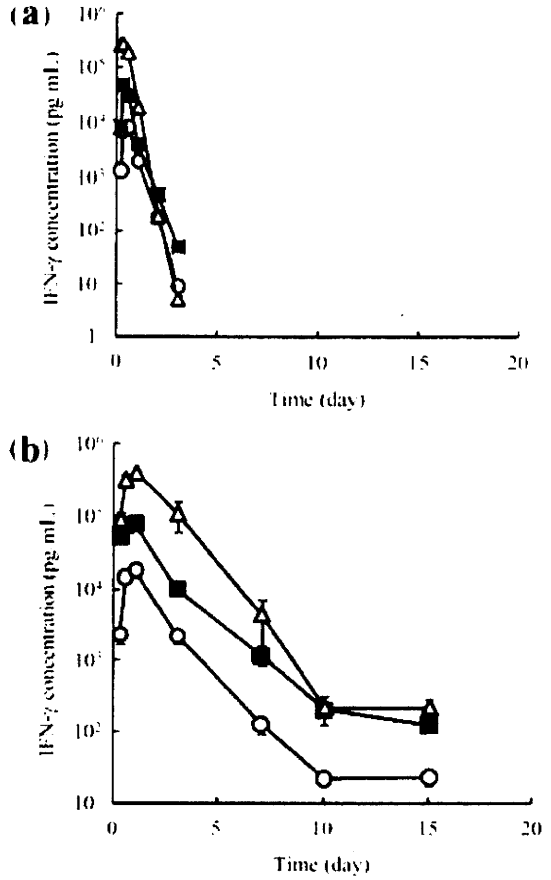


**Figure 4.** Time course of the concentration of IFNγ and IFNγ–MSA in the serum after hydrodynamic injection of pCMV-Muγ or pCMV-Muγ–MSA. Each plasmid DNA was dosed at 0.04 pmol (open ciecles), 0.2 pmol (solid squares) and 1.0 pmol (open triangles), respectively. The concentrations of (a) IFNγ and (b) IFNγ–MSA were measured by ELISA using anti-mouse IFNγ antibody. The results were expressed as the mean ± SE of three mice.

about sixfold greater AUC than that of pCMV-Muγ. The MRT of IFNγ–MSA was significantly longer than that of IFNγ.

### Effects of IFNγ and IFNγ–MSA Gene Transfer on Pulmonary Metastasis of Tumor Cells

Figure 5 shows the number of metastatic colonies on the lung surface measured at 14 days after inoculation of CT-26 cells into the tail vein of mice. A hydrodynamic injection of pCMV-Muγ or pCMV-Muγ–MSA significantly reduced the number of colonies to 70 and 65% of that in the no treatment group, respectively. No significant reduction was observed in the mice injected with pCMV-Luc, a control plasmid, indicating that the reduction is due to the activity of IFNγ or IFNγ–MSA.

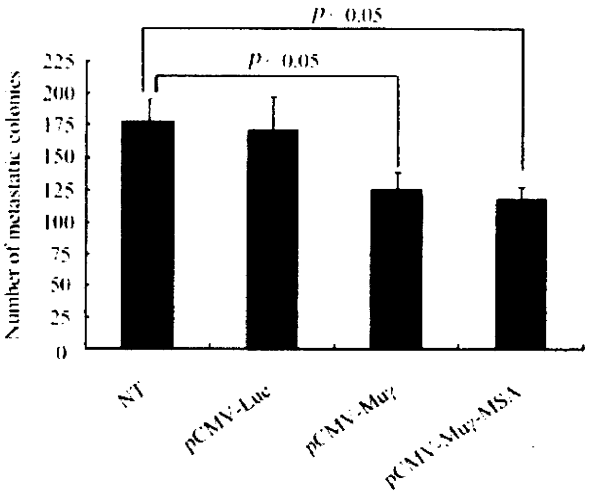


**Figure 5.** Effects of IFNγ and IFNγ–MSA gene transfer on pulmonary metastasis of CT-26 tumor cells in mice. Pulmonary metastasis was induced by inoculation of $1 \times 10^5$ CT-26 cells into a tail vein (day 0). On day 3, pCMV-Muγ, pCMV-Muγ–MSA or pCMV-Luc was injected into mice by the hydrodynamic injection at a dose of 0.7 pmol/mouse. At 14 days after tumor inoculation, mice were sacrificed and the number of metastatic colonies on the lung surface was counted. The results are expressed as the mean ± SE of five mice.

## DISCCUSION

Advances in biotechnology have markedly increased the number of biological pharmaceutics in the market. IFNγ is one of such pharmaceuticals that is now used for CGD and malignant osteopetrosis.[1] Type I IFNs, such as IFNα and IFNβ, have a variety of products used in the clinical practice,[5] but few attempts such as chemical conjugation have been made to reduce the clearance of IFNγ from the body. This may be because of the fact that, unlike the type I IFNs, IFNγ is a homodimeric protein.[23,24] In the case of human IFNα, pegylated derivatives[9] or a fusion protein with serum albumin[25] has been developed as products with a long circulation half-life. However, these modifications were known to be a trade-off between the increased circulation and the biological activity. Thus, these two factors should be simultaneously studied to conclude whether any modification is effective in increasing the therapeutic potency of biological pharmaceuticals, including IFNγ.

A major route of elimination of IFNγ is glomerular filtration.[26,27] Because this process is size-dependent and the molecular weight of IFNγ (about 30–36 kDa in dimeric form) is smaller than the threshold of the filtration, increasing the molecular size of IFNγ can be a rational approach to increasing its therapeutic potency. There are at least two approaches to achieve this. One ?is chemical conjugation, in which a variety of natural or synthetic polymers are covalently bound

**Table 2.** Pharmacokinetic Parameters of Serum IFNγ and IFNγ–MSA after Hydrodynamic Injection of pCMV-Muγ and pCMV-Muγ–MSA into Mice

| | pCMV-Muγ | pCMV-Muγ–MSA | | | | |
|---|---|---|---|---|---|---|
| | 0.04 (pmol) | 0.2 (pmol) | 1.0 (pmol) | 0.04 (pmol) | 0.2 (pmol) | 1.0 (pmol) |
| $C_{max}$ (ng/mL) | 7.63 ± 0.08 | 46.6 ± 3.2 | 269 ± 11 | 18.9 ± 0.9* | 80.9 ± 4.2 | 361 ± 23 |
| $T_{max}$ (day) | 0.5 | 0.25 | 0.25 | 1 | 1 | 1 |
| AUC (ng·day/mL) | 5.82 ± 0.08 | 24.3 ± 1.3 | 168 ± 6 | 37.2 ± 2.2* | 180 ± 3* | 1,010 ± 120* |
| MRT (day) | 0.606 ± 0.006 | 0.492 ± 0.006 | 0.393 ± 0.001 | 1.43 ± 0.03* | 1.45 ± 0.03* | 1.64 ± 0.11* |

*Statistically significant ($p < 0.05$) compared with pCMV-Muγ.

The $C_{max}$ and $T_{max}$ values were obtained from actual data recorded after hydrodynamic injection of pCMV-Muγ and pCMV-Muγ–MSA at a dose of 0.04, 0.2, 1.0 pmol/mouse, respectively. The AUC and MRT were calculated by integration to infinite time, and the mean ± SE values are expressed.

AUC, concentration–time curve; MRT, mean residence time.

to specific amino groups of proteins. The other is to design fusion proteins, in which peptides or proteins are genetically fused to the N- or C-terminus of proteins. The latter is better as far as the homogeneity of protein derivatives is concerned. In the present study, we selected MSA as a modifier of mouse IFNγ because of the following reasons. First, serum albumin is the most abundant serum protein in mice, humans, and other animals. Second, its structural, biochemical, and physiological properties have been extensively studied.[13] Third, it is hardly filtered through the glomerulus and has a long circulation half-life. Last but not least, it has been used to obtain fusion proteins, including IFNα and insulin,[28] with no significant adverse reactions. Then, the site for conjugation, the N- or C-terminal of IFNγ, could be another parameter that affects the outcomes. We selected the C-terminal for the site according to the preliminary results obtained using MSA fusion protein conjugated to the N- or C-terminus of IFNγ (unpublished results). The reduced biological activity by fusion of MSA to IFNγ may indicate that the large size of MSA interferes with the dimerization and/or the interaction of IFNγ to its receptors. Thus, smaller proteins could be better modifiers than MSA if other properties, including but not limited to the biological, pharmacokinetic and immunological ones, are appropriate.

Western blot analysis clearly revealed that the IFNγ–MSA fusion protein does not form homodimers. Dimerization of IFNγ occurs through noncovalent interactions with two monomers being in an antiparallel orientation.[23,24] A possible explanation for the loss of dimerization is that the fusion of MSA increases the steric hindrance for dimerization. In the present study, the biological activity of IFNγ and IFNγ–MSA was measured using an in-house GAS-dependent luciferase assay. Previous studies revealed that the activity of albuferon (recombinant human IFNα-2b-human serum albumin fusion protein) is approximately 1/10 delete of that of IFNα-2b[28] because the steric hindrance induced by fusion to albumin disrupts the interaction with its receptor. The experimental result from GAS reporter assay showed that the IFNγ–MSA has about 1/200 activity of IFNγ. The difficulty in the dimerization of IFNγ–MSA would at least partly explain the low biological activity of the fusion protein because it has been suggested that monovalent IFNγ is much less active than divalent counterpart.[29,30]

An intravenous injection of IFNγ or IFNγ–MSA clearly showed that the fusion of MSA is effective in increasing the circulation time of IFNγ, which resulted in about fourfold increase in the half-life of the elimination phase and the MRT. Because of the low dose, the tissue distribution of these proteins was difficult to be measured in the present study, so the difference in the tissue distribution needs further investigations. In accordance with the altered distribution properties of IFNγ–MSA, its serum concentration after hydrodynamic injection of pCMV-Muγ–MSA was more prolonged than that after injection of pCMV-Muγ. These results clearly show that the gene delivery of IFNγ–MSA is an effective approach to improving the pharmacokinetic properties of IFNγ.

In a previous study, we reported that IFNγ gene transfer is effective in inhibiting the pulmonary metastasis of mouse colon carcinoma CT-26 cells.[11] Similar results were obtained in the present study when the pCMV-Muγ was injected to a mouse model of pulmonary metastasis of CT-26 cells (Fig. 5). Although the activity of IFNγ–MSA was about 1/200 of that of IFNγ, gene delivery of IFNγ–MSA resulted in the inhibition of the metastasis to a level similar to that obtained with that of IFNγ. These results strongly suggest that prolonged circulation half-life of IFNγ is quite effective in increasing its therapeutic potency.

In conclusion, these results indicate that gene delivery of IFNγ genetically fused with albumin could modulate the disposition of IFNγ, which was effective in inhibiting tumor metastasis despite extensive reduction of its biological activity. To further increase the potency of IFN-based therapy, the reduction of biological activity of modified IFNγ needs to be minimized.

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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 (1), 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

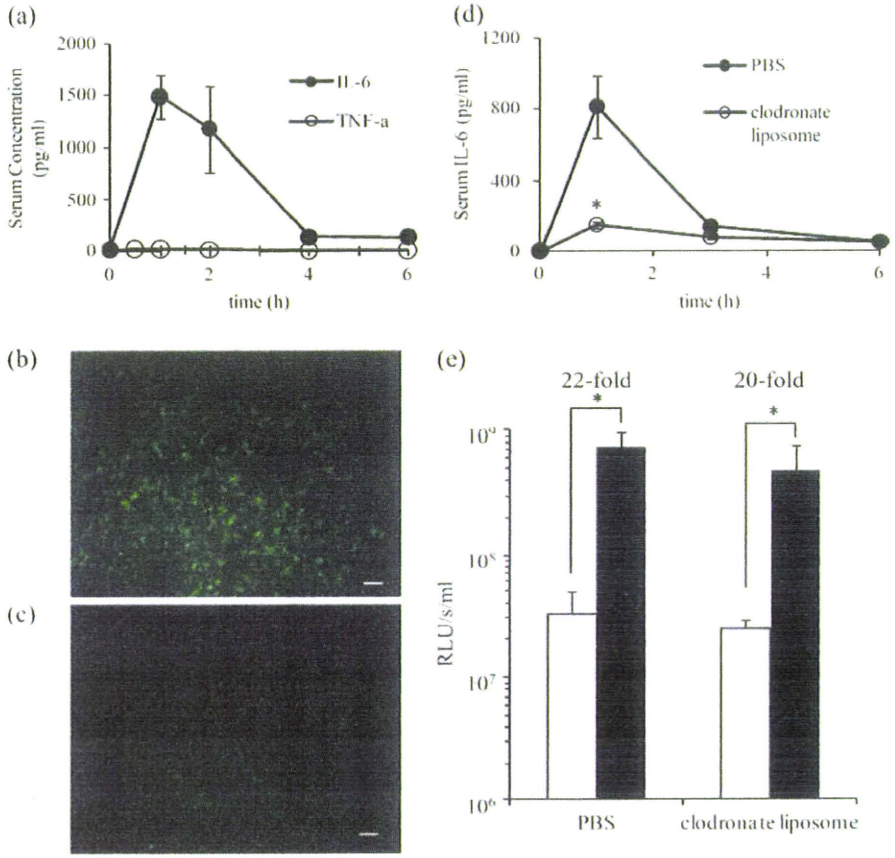


**Fig. 1** Inflammatory response following hydrodynamic injection and effect of Kupffer cell depletion on hydrodynamic injection-induced responses. **a** Time-courses of the concentrations of IL-6 and TNF-α in mouse serum after a rapid, large-volume injection of saline. Blood samples were sequentially 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

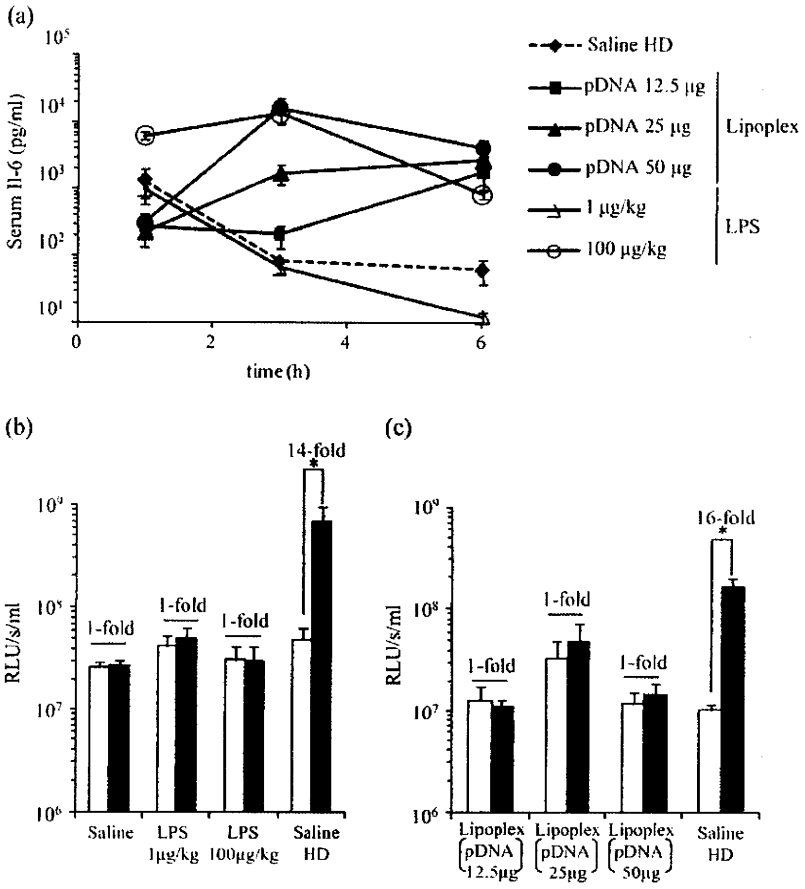


**Fig. 2** Effects of LPS and lipoplex on reactivation of silenced transgene expression. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. Five days after the administration of plasmid DNA, mice received a rapid injection of a large volume of saline (saline HD), intraperitoneal injection of 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

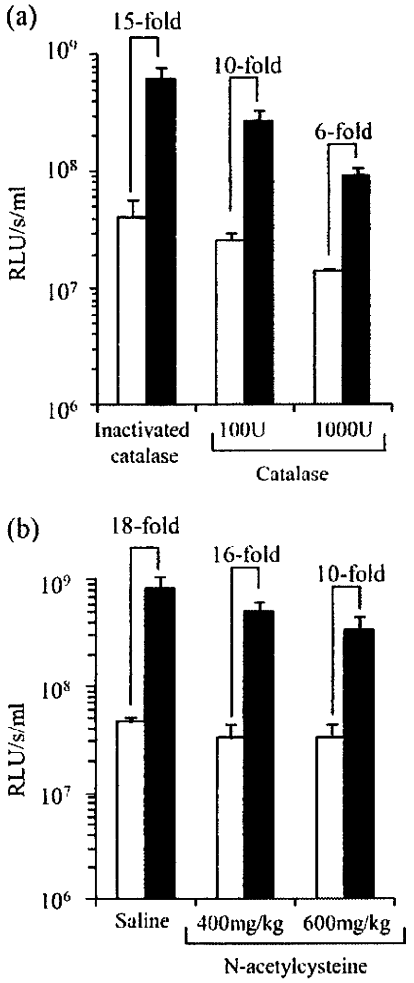


**Fig. 3** Effect of antioxidant agents on reactivation of silenced transgene expression. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. **a** On day 5, they received a hydrodynamic injection of saline containing 100 or 1,000 U/shot bovine catalase or its inactivated derivative (inactivated 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–e). 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

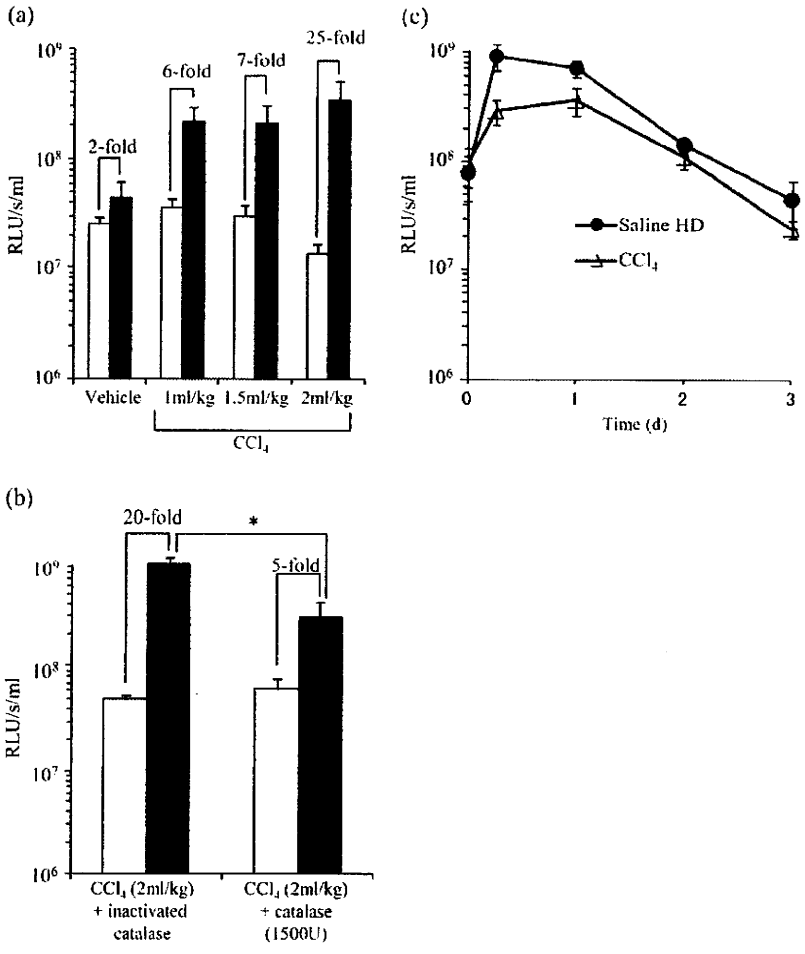


**Fig. 4** Reactivation of transgene expression by $CCl_4$ and its suppression by catalase. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. **a** On day 5, they received an intraperitoneal injection of 1, 1.5 or 2 ml/kg $CCl_4$. Gaussia luciferase activity in the serum was measured just before (*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

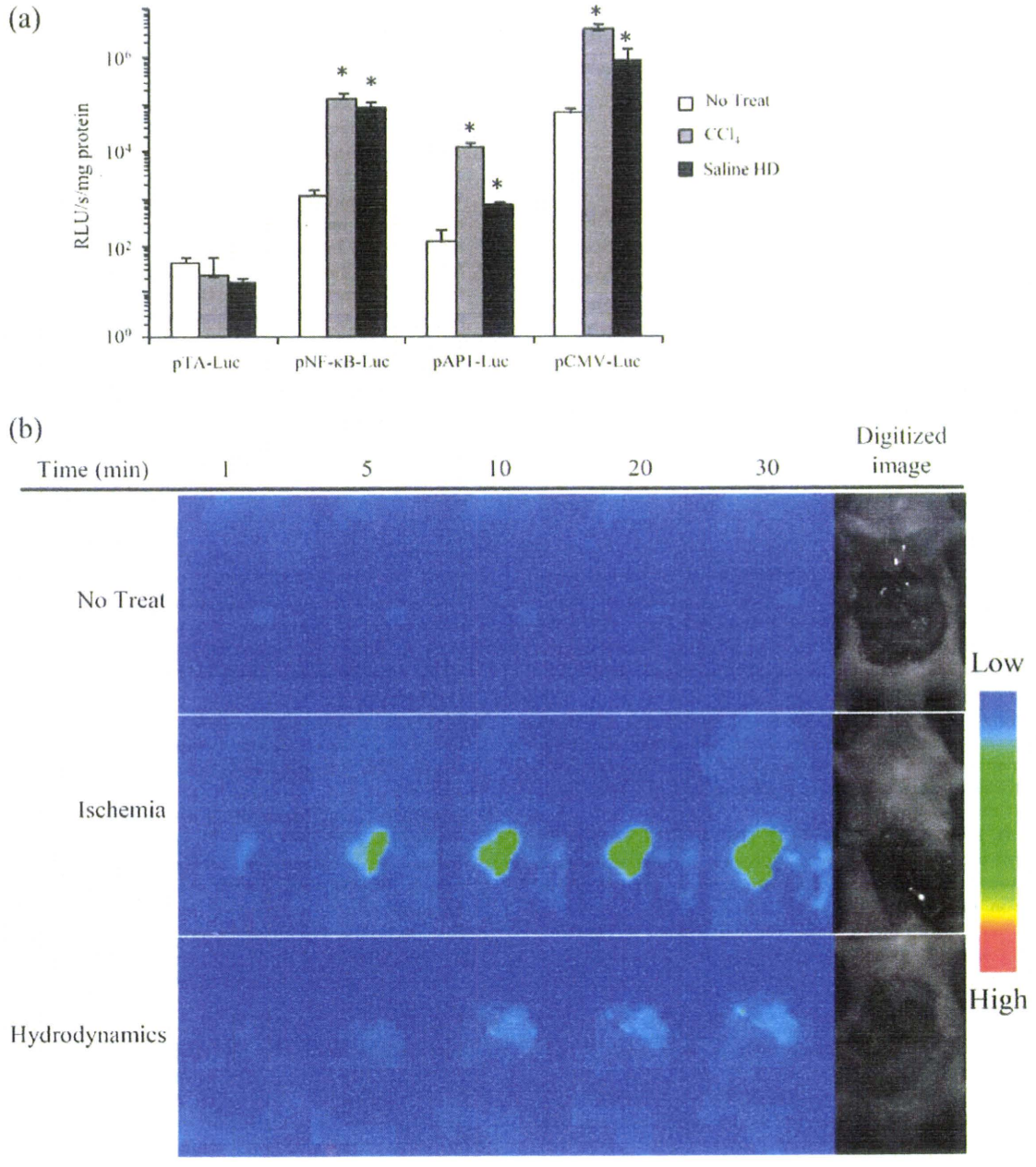


**Fig. 5** Effect of hydrodynamic injection of saline on transcription factors and oxygen supply to the liver. **a** Mice received a hydrodynamic injection of 10 $\mu$g of pTA-Luc, pAP1-Luc, pNF-κB-Luc or pCMV-Luc. On day 5, they received an intraperitoneal injection of 3 ml/kg $CCl_4$ or hydrodynamic 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 $\mu$l 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.

## ACKNOWLEDGMENTS

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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-$\beta$ production in cells that were transfected with IFN-$\beta$-expressing plasmid DNA. Similar results were obtained with the combination of IFN-$\gamma$ and IFNGR. Co-injection of IFN-$\beta$-expressing plasmid DNA with siRNA targeting IFNAR1 into mice resulted in sustained plasma concentration of IFN-$\beta$.

**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-$\alpha$2a and IFN-$\alpha$2b 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-$\beta$ or IFN-$\gamma$ 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-$\alpha$ and IFN-$\beta$ are major type I IFNs, which are secreted from almost all types of cells. IFN-$\gamma$ 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-$\beta$ and pCMV-$\gamma$ encoding firefly luciferase, mouse IFN-$\beta$ and mouse IFN-$\gamma$, respectively, under the control of the cytomegalovirus (CMV) promoter, were constructed as described previously [11] and pGZB-$\beta$ encoding mouse IFN-$\beta$ 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 DH5$\alpha$ 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-$\beta$ and IFN-$\gamma$ 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: $2 \times 10^4$ cells/well in 24-well-plates (luciferase experiments); $1 \times 10^5$ cells/well in 24-well-plates (mRNA measurement experiments); $3 \times 10^5$ cells/well in six-well-plates (Facs analysis); and $1 \times 10^5$ 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