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Gene Delivery of Albumin Binding Peptide-Interferon-gamma Fusion Protein with Improved Pharmacokinetic Properties and Sustained Biological Activity

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Received 7 November 2012; revised 31 January 2013; accepted 12 February 2013

Published online 5 March 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23493

ABSTRACT: We have demonstrated that gene delivery of a fusion protein of mouse interferon (IFN) γ with mouse serum albumin (IFNγ–MSA) was effective in prolonging the circulation half-life of IFNγ in mice. However, the fusion to MSA greatly reduced the biological activity of IFNγ to less than 1%. In this study, we designed IFNγ fusion proteins with a 20 amino-acid long albumin-binding peptide (ABP) to prolong the *in vivo* half-life of IFNγ without reducing its biological activity. IFNγ–ABP and ABP–IFNγ, two fusion proteins with the ABP being fused to the C- or N-terminal of IFNγ, retained 40%–50% biological activities determined using a gamma-activated sequence-dependent luciferase assay. These fusion proteins exhibited the ability to bind to MSA. Gene delivery of IFNγ–ABP or ABP–IFNγ to mice using the hydrodynamic injection method resulted in a sustained concentration of IFNγ in the serum compared with gene delivery of IFNγ. In addition, the growth of mouse colon carcinoma CT-26 cells in the lung was efficiently inhibited by gene delivery of the IFNγ fusion proteins. These results indicate that the fusion of ABP is a useful approach to achieving prolonged retention in the blood circulation through binding to serum albumin and retaining biological activity. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:3110–3118, 2013

Keywords: albumin; albumin-binding peptide; biological activity; clearance; fusion protein; gene delivery; hydrodynamic injection; moment analysis; plasmid DNA; pharmacokinetics

INTRODUCTION

Interferon-gamma (IFNγ) is a pleiotropic cytokine with antiviral, antiproliferative, and immunomodulatory activities.[1,2] Despite extensive studies in the last two decades, the clinical application of IFNγ is limited to the treatment of a small number of diseases, including chronic granulomatous disease, osteopetrosis, and renal cancer, largely because of its short *in vivo* half-life.[2–4] Frequent injections are required to maintain effective concentrations, but the fluctuating blood concentration of IFNγ produced by frequent dosing leads to serious adverse toxic effects, including fever, fatigue, and neurotoxicity.[2,5,6]

Gene delivery of a therapeutic protein with unsatisfactory pharmacokinetic properties, such as a short *in vivo* half-life, is a promising approach to achieving sustained therapeutic effects with minimal toxicity. In previous studies, we showed that plasmid-based gene delivery of IFNγ is effective in inhibiting metastatic tumor growth and atopic dermatitis in mice.[7–10] These studies indicated the therapeutic potential of IFNγ gene transfer in several disease models. However, we also found that high doses of IFNγ-expressing plasmids, especially those expressing IFNγ for a long period, induced toxic effects. We hypothesized that controlling the tissue distribution of IFNγ expressed from plasmid vectors would be a promising method of reducing such toxic effects. On the basis of this hypothesis, we designed fusion proteins of IFNγ with mouse serum albumin (MSA) and constructed plasmids encoding the fusion protein,

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Journal of Pharmaceutical Sciences, Vol. 102, 3110–3118 (2013)

IFNγ–MSA.[11] The fusion to MSA resulted in prolongation of the mean residence time of IFNγ after gene delivery. However, the fusion also greatly reduced the biological activity of IFNγ and the IFNγ–MSA exhibited only about 1/200th of the activity of IFNγ.

Marked reduction in the biological activity produced by the fusion of MSA would be due to steric hindrance of the IFNγ–IFNγ receptor interaction by MSA because MSA has a molecular weight of 67,000.[12] Dennis et al.[13–15] reported that the conjugation of a phage-derived 20 amino-acid long albumin-binding peptide (ABP) gave IgG fragments high affinity for serum albumin, leading to an increased *in vivo* circulation half-life.[13–15] The low-molecular weight of the ABP, as well as the noncovalent binding to serum albumin, could prevent it from reducing the biological activity of IFNγ.

Here, we designed fusion proteins of IFNγ with ABP to increase the half-life of IFNγ without any marked reduction in its biological activity. ABP was fused to either the N- or C-terminal end of IFNγ with a short linker peptide. We constructed plasmid vectors encoding these fusion proteins and examined their biological activity and binding to serum albumin, and their serum levels after gene transfer in mice. Then, the therapeutic effects of gene delivery of IFNγ fusion protein were examined in mice with pulmonary metastases of colon carcinoma cells.

MATERIALS AND METHODS

Cell Culture and Animals

An African green monkey kidney fibroblast cell line, COS-7, was obtained from American Type Culture Collection (Manassas, Virginia). A murine melanoma cell line, B16-BL6, was obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). B16-BL6 and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin–streptomycin–L-glutamine at 37°C. CT-26 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin–streptomycin–L-glutamine at 37°C. Four-week-old male ICR mice and BALB/c 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 Institutional Animal Experimentation Committee.

Plasmid DNA

pcDNA3.1 was purchased from Invitrogen (Carlsbad, California). phRL–thymidine kinase (TK), a renilla luciferase-expressing plasmid under the control of herpes simplex virus TK promoter, was purchased from Promega (Madison, Wisconsin). pGAS–Luc, a firefly luciferase-expressing plasmid under the control of gamma-activated sequence (GAS) promoter, has been described previously.[11,16] pCMV–IFNγ, an IFNγ-expressing plasmid under the control of cytomegalovirus (CMV) promoter, has also been described previously.[11] pCMV–IFNγ–ABP and pCMV–ABP–IFNγ, a plasmid encoding IFNγ–ABP (ABP was fused to the C-terminal of IFNγ) or ABP–IFNγ (ABP was fused to the N-terminal), respectively, were constructed by the following method. An IFNγ–ABP or ABP–IFNγ cDNA fragment was amplified by polymerase chain reaction (PCR) from pCMV–IFNγ using a primer with the addition of ABP sequences (GRLMEDICIPRWGCLWEDDF)[14] to the C- or N-terminal of IFNγ. Oligonucleotides encoding a 4 amino-acid (GGGS) linker were inserted between IFNγ cDNA and ABP sequences according to a previous report.[14,17] The fragment was then inserted into the BamHI/XbaI sites of pcDNA3.1. Each plasmid was amplified in *Escherichia coli* (DH5α, TOYOBO, Osaka, Japan) and purified using a JETSTAR plasmid purification kit (GENOMED, Löhne, Germany).

In Vitro Transfection

Cells were seeded on 10 cm dishes at a density of 1×10^6 cells/dish or 12-well culture plates at 1×10^5 cells/well and incubated overnight and the cells were transfected with the indicated plasmid using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. In brief, 1 μg plasmid was mixed with 3 μL Lipofectamine2000 in Opti-MEM (Invitrogen) at a final concentration of 2 μg DNA/mL and the complex obtained was added to cells. The total amount of plasmid DNA used for transfection was 8 μg/dish for 10 cm dishes and 1 μg/well for 12-well culture plates, respectively.

Collection of Conditioned Medium

COS-7 cells seeded on 10-cm dishes were transfected with pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ as described above. Four hour after transfection, cells were washed with PBS and cultured with serum-free Opti-MEM medium for 48 h. Then, the culture medium was collected as conditioned medium.

Western Blotting

The conditioned medium of COS-7 cells transfected with pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ were collected as described above. The samples were reduced by the addition of dithiothreitol (0.1 M) and heat treatment at 95°C for 4 min to disrupt the disulfide bonds and to dissociate any homodimers that might be present. The samples were

then applied onto 12.5% polyacrylamide gel and were electrophoresed in the presence of sodium dodecyl sulfate (SDS) at the voltage of 200 V for 40 min. After SDS-polyacrylamide gel electrophoresis (PAGE), proteins resolved in the gel were electrophoretically transferred to a PVDF membrane using a wet blotting method in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at the voltage of 200 V for 45 min. After blocking with 5% skimmed milk, the membrane was probed with goat antimouse IFNγ polyclonal antibody (R&D System, Inc., minneapolis, minnesota) overnight at 4°C and then allowed to react with antigoat IgG antibody conjugated with horseradish peroxidase (Santa Cruz, Inc., Santa Cruz, California) for 1 h at room temperature. The bands were detected with LAS-3000 (Fuji Film, Tokyo, Japan).

Measurement of the Biological Activity of IFNγ, IFNγ–ABP, and ABP–IFNγ

B16-BL6 cells were cotransfected with pGAS–Luc (1.4 μg/mL) and phRL–TK (0.6 μg/mL). After a 4 h transfection, the culture medium was replaced with fresh serum-free Opti-MEM containing serial dilutions of the conditioned medium of COS-7 cells transfected with pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ. After a 24 h incubation, cells were lysed with a lysis buffer (0.1 M Tris, 0.05% Triton-X-100, 2 mM ethylenediaminetetraacetic acid, pH 7.8) and the lysates were mixed with reagents of the Dual-Luciferase Reporter Assay System (Promega). Then, firefly and renilla luciferase activity was 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 firefly/renilla ratio of the cells treated with indicated concentrations of IFNγ, IFNγ–ABP, or ABP–IFNγ was divided by the firefly/renilla ratio of the cells cultured without IFNγ, IFNγ–ABP, or ABP–IFNγ to give fold increase in GAS-dependent luciferase activity relative to those of the untreated group. Finally, the half maximum effective concentration (EC50) of IFNγ, IFNγ–ABP, and ABP–IFNγ was calculated. In a separate set of experiments, 100 pg/mL of IFNγ, IFNγ–ABP, or ABP–IFNγ was incubated with or without 0.25 mg/mL of MSA (Sigma; St. Louis, MO, USA) in Opti-MEM at 37°C for 1 h, and added to B16-BL6 cells transfected with pGAS–Luc and phRL-TK. Then, the fold increase in GAS-dependent luciferase activity relative to that of the untreated group was calculated as described above.

Measurement of the Binding Affinity of IFNγ, IFNγ–ABP, and ABP–IFNγ for MSA

Mouse serum albumin was immobilized onto 96-well plates at a concentration of 2 μg/mL overnight at 4°C. Wells were added with serially diluted supernatants of COS-7 cells transfected with pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ. After incubation for 2 h at room temperature, each well was washed with phosphate buffered saline-0.05% Tween 20 and proteins bound to MSA were detected by enzyme-linked immunosorbent assay (ELISA) using antimouse IFNγ antibody (eBioscoence, San Diego, California).[13,14]

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.[19,20] The dose of plasmids was set at 0.2 pmol/mouse based on preliminary experiments. At predetermined periods, blood was collected from the tail vein and the blood samples were kept at 4°C for 2 h and centrifuged at 8000*g* for 20 min to obtain serum.

Measurement of mRNA Expression of IFNγ, IFNγ–ABP, and ABP–IFNγ in the Liver

Six hours after gene transfer, mice were sacrificed and total RNA was extracted from approximately 50 mg liver samples using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). After removal of contaminated DNA by DNase I (Takara Bio, Shiga, Japan), reverse transcription was performed using a ReverTra Ace qPCR RT kit (TOYOBO), followed by RNaseH treatment (Ribonuclease H; Takara Bio). For a quantitative analysis of mRNA expression, a real-time PCR was carried out with total cDNA using KAPA SYBR FAST ABI Prism 2× qPCR Master Mix (KAPA BIOSYSTEMS, Boston, Massachusetts). The oligonucleotide primers used for amplification were: *Ifnγ*-sense: 5′-CGGCACAGTCATTGAAAGCCTA-3′, *Ifnγ*-antisense: 5′-GTTGCTGATGGCCTGATTGTC-3′, and *β-actin*-sense: 5′-CATCCGTAAAGACCTCTATGC-3′, *β-actin*-antisense: 5′-ATGGAGCCACCGATCCACA-3. Amplified products were detected via intercalation of the fluorescent dye using a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, California). The mRNA expression of IFNγ was normalized using the mRNA level of β-actin.

Measurement of the Concentrations of IFNγ, IFNγ–ABP, and ABP–IFNγ

The concentrations of IFNγ, IFNγ–ABP, and ABP–IFNγ in the supernatant of COS-7 cells or mouse serum were determined by ELISA using a

commercial kit (Ready-SET-Go! MuIFN-γ ELISA; eBioscience).

Clearance of IFNγ, IFNγ–ABP, and ABP–IFNγ after Intravenous Injection into Mice

To obtain IFNγ, IFNγ–ABP, and ABP–IFNγ proteins, mice received a hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ as described above at a dose of 5 pmol/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, IFNγ–ABP, or ABP–IFNγ were injected into different mice via the tail vein and blood was periodically sampled. The dose of each protein was set at 8.5 μg IFNγ/kg, and this was quantified by ELISA.[11]

Experimental Metastatic Pulmonary Tumor Model

CT-26 cells were trypsinized and suspended in Hanks' balanced salt solution (HBSS). Cell suspensions containing 1×10^5 CT-26 cells in 200 μL HBSS were injected into the tail vein of syngeneic BALB/c mice to establish an experimental metastatic pulmonary tumor model. Then, 3 days after inoculation of the CT-26 cells, each plasmid was injected into the tail vein by the hydrodynamic injection method at a dose of 0.7 pmol/mouse. At 14 days after inoculation, mice were sacrificed and the lungs were collected. The lungs were immersed in 100% methanol to make metastatic colonies more visible. Then, the number of metastatic colonies on the lung surface was counted by the naked eye.[11,21]

Pharmacokinetic Analysis

The peak plasma concentrations (C_{max}) and the time of maximum serum concentration (t_{max}) were obtained from actual data recorded after injection or gene transfer. The area under the serum concentration–time curve (AUC) and mean retention time (MRT) were calculated using a moment analysis method. These parameters were calculated for each animal by integration to infinite time.[22]

Data Analysis

Differences were statistically evaluated by Student's *t*-test, and the level of statistical significance was set at $p < 0.05$.

RESULTS

Expression of IFNγ–ABP and ABP–IFNγ in Cultured Cells

Figure 1a shows the Western blot analysis of the expression of IFNγ, IFNγ–ABP, and ABP–IFNγ in

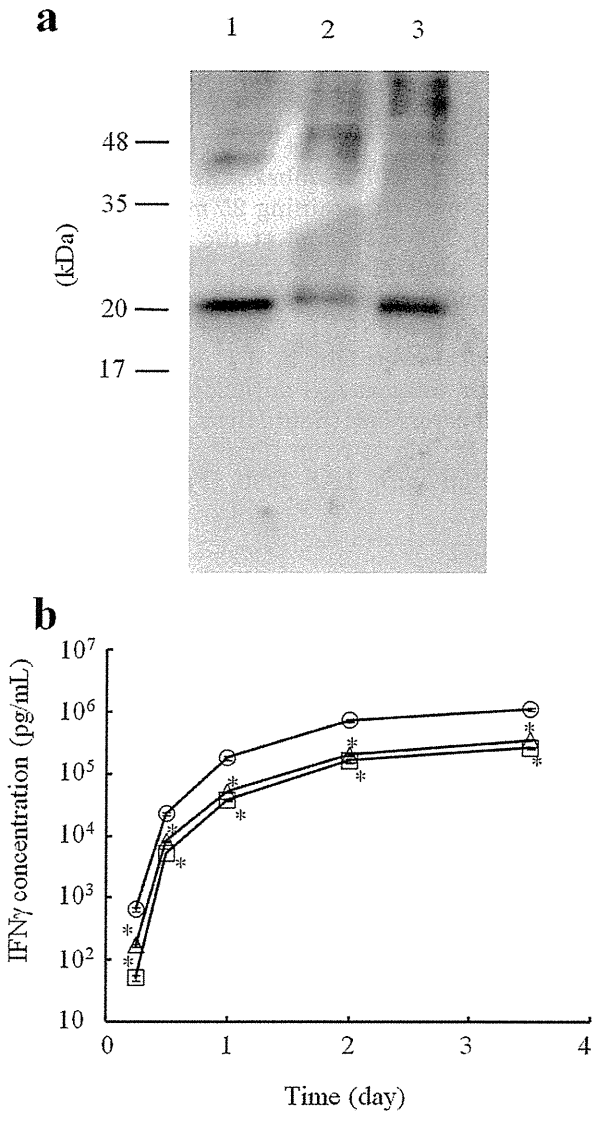

Figure 1. Expression of IFNγ–ABP and ABP–IFNγ in COS-7 cells. (a) Western blot analysis of IFNγ, IFNγ–ABP, and ABP–IFNγ. Western blotting was performed to confirm the molecular weight of IFNγ (lane 1), ABP–IFNγ (lane 2), and IFNγ–ABP (lane 3). Culture media of COS-7 cells transfected with pCMV–IFNγ, pCMV–ABP–IFNγ, and pCMV–IFNγ–ABP were subjected to 12.5% SDS-PAGE under reducing conditions. IFNγ, ABP–IFNγ, and IFNγ–ABP were detected with antimouse IFNγ polyclonal antibody. (b) Time course of the concentration of IFNγ (circles), IFNγ–ABP (squares), and ABP–IFNγ (triangles) in the culture medium of COS-7 cells after transfection of pCMV–IFNγ, pCMV–IFNγ–ABP, and pCMV–ABP–IFNγ (2 μg/mL). At the indicated time periods after transfection, the supernatants were collected and the concentration of IFNγ, ABP–IFNγ, or IFNγ–ABP was measured by ELISA using antimouse IFNγ antibody. The results are expressed as the mean ± standard error of the mean (SEM) of three independent determinations. $*p < 0.05$ compared with pCMV–IFNγ group.

COS-7 cells. Under reducing conditions, a band around 20 kDa was detected in the culture media of cells transfected with pCMV–IFNγ (lane 1), which is in good agreement with the size of monomeric IFNγ.[23] The supernatant of cells transfected with pCMV–IFNγ–ABP or pCMV–ABP–IFNγ also showed a band of slightly higher molecular mass of IFNγ (lanes 2 and 3, respectively), suggesting that the fusion proteins, IFNγ–ABP and ABP–IFNγ, were expressed from the plasmid vectors.

Figure 1b shows the time course of IFNγ, IFNγ–ABP, and ABP–IFNγ concentrations in culture media of COS-7 cells after transfection with pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ, respectively. The concentrations of IFNγ–ABP and ABP–IFNγ were significantly lower than that of IFNγ, although the shapes of the time courses were similar among all the groups.

Measurement of the Biological Activity of IFNγ–ABP and ABP–IFNγ

The biological activity of IFNγ–ABP and ABP–IFNγ was measured by GAS-dependent luciferase assay (Fig. 2a).[11,24,25] Addition of IFNγ to cells transfected with pGAS–Luc increased the luciferase activity of the cells in a concentration-dependent manner, confirming the validity of the method. IFNγ–ABP or ABP–IFNγ also dose-dependently increased the luciferase activity. The calculated EC50 values of IFNγ–ABP and ABP–IFNγ were 93 and 110 pg/mL, respectively, which indicates that IFNγ–ABP and ABP–IFNγ possess 46% and 40% of the activity of IFNγ (43 pg/mL). Thus, high biological activity remained after the fusion of ABP to IFNγ irrespective of the fusion sites. There was no significant difference in activity between IFNγ–ABP and ABP–IFNγ.

To investigate whether the biological activity of IFNγ, IFNγ–ABP, and ABP–IFNγ is affected by MSA, the GAS-dependent assay was carried out using IFNγ, IFNγ–ABP, or ABP–IFNγ incubated with or without 0.25 mg/mL of MSA (Fig. 2b). Presence of MSA hardly affected the activity of IFNγ, but it significantly reduced that of IFNγ–ABP and ABP–IFNγ.

Binding Affinity of IFNγ–ABP and ABP–IFNγ to Albumin

Figure 3 shows the amount of IFNγ, IFNγ–ABP, and ABP–IFNγ bound to MSA immobilized onto culture plates, determined using ELISA with anti-mouse IFNγ antibody. Both IFNγ–ABP and ABP–IFNγ bound to MSA in a dose-dependent manner. On the contrary, IFNγ hardly bound to MSA. The amount of ABP–IFNγ bound to MSA was significantly higher than that of IFNγ–ABP compared at the same concentrations, indicating the high affinity of the former. The fusion proteins showed much less binding to human serum albumin or bovine IgG (data not shown).

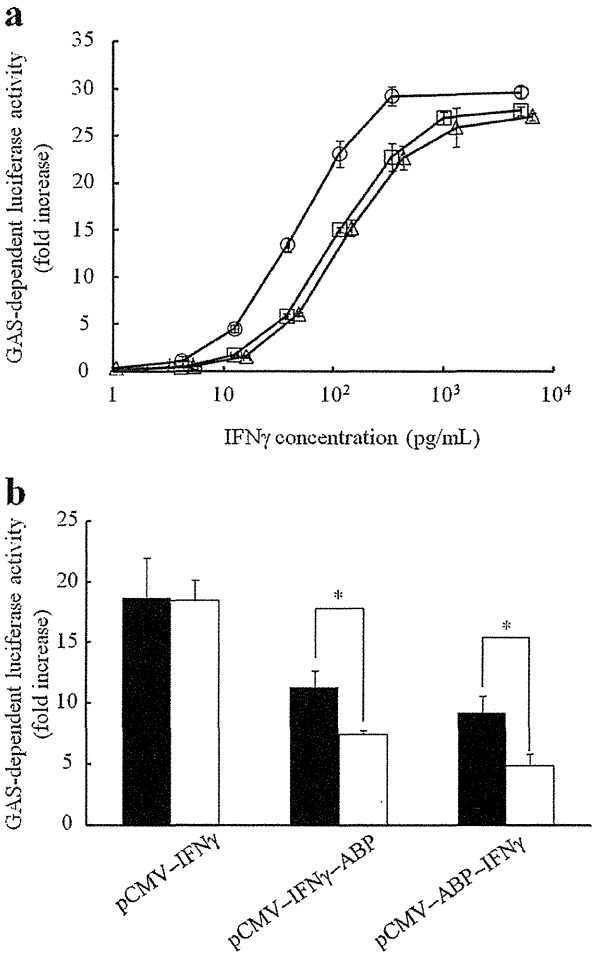

Figure 2. (a) Biological activity of IFNγ–ABP and ABP–IFNγ. B16–BL6 cells transfected with pGAS–Luc and phRL–TK were incubated with serial dilutions of IFNγ (circles), IFNγ–ABP (squares), or ABP–IFNγ (triangles) for a further 24 h. pGAS–Luc, plasmid DNA expressing firefly luciferase, was used to assess the degree of activation of the GAS signaling pathway. phRL–TK, plasmid DNA expressing renilla luciferase was used for normalization of the transfection efficiency and cell numbers. The ratio was normalized to give *x*-fold values relative to those of the untreated group and the half-maximum effective concentration (EC50) of IFNγ, IFNγ–ABP, and ABP–IFNγ was calculated. The results are expressed as the mean ± SEM of four independent determinations. (b) Biological activity of IFNγ, IFNγ–ABP, and ABP–IFNγ with or without MSA. IFNγ, IFNγ–ABP, and ABP–IFNγ (100 pg/mL) incubated with (open column) or without 0.25 mg/mL MSA (closed column) was added to B16–BL6 cells transfected with pGAS–Luc and phRL–TK. The results are expressed as the mean ± SEM of four independent determinations. $*p < 0.05$.

Clearance of IFNγ, IFNγ–ABP, and ABP–IFNγ after Intravenous Injection into Mice

To confirm whether the fusion of ABP increases the blood circulation time of IFNγ, IFNγ, IFNγ–ABP, and

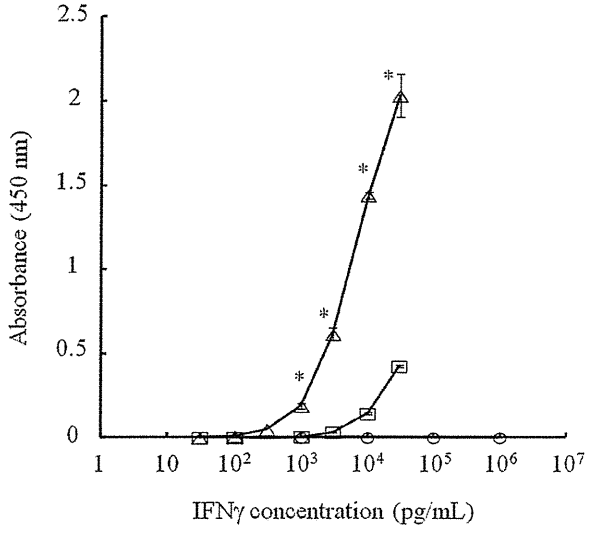

Figure 3. Binding of IFNγ–ABP and ABP–IFNγ to immobilized MSA. MSA was immobilized onto 96-well plates at a concentration of 2 μg/mL overnight. IFNγ (circles), IFNγ–ABP (squares), or ABP–IFNγ (triangles) was serially diluted and 100 μL amounts were added per well. After incubation for 2 h, bound proteins were detected by ELISA using antimouse IFNγ antibody. The results are expressed as the mean ± SEM of three independent determinations. $*p < 0.05$ compared with IFNγ–ABP group at the same concentration.

Table 1. Pharmacokinetic Parameters of IFNγ, IFNγ–ABP, and ABP–IFNγ after Intravenous Injection into Mice

Protein	IFNγ	IFNγ–ABP	ABP–IFNγ
AUC (ng h/mL)	57.6 ± 2.6	270 ± 24*	458 ± 5*
MRT (h)	2.64 ± 0.14	2.64 ± 0.07	4.11 ± 0.20*

The AUC and MRT were calculated by integration to infinite time, and the mean ± SEM values are shown.
*Statistically significant ($p < 0.05$) compared with IFNγ.

ABP–IFNγ were injected into the tail vein of mice. Figure 4 shows the time courses of the concentrations of IFNγ, IFNγ–ABP, and ABP–IFNγ in the serum after intravenous injection into mice at a dose of 8.5 μg IFNγ/kg body weight. The clearance of IFNγ–ABP and ABP–IFNγ was slower than that of IFNγ. The profiles were evaluated by moment analysis to calculate the AUC and MRT (Table 1). The AUC of IFNγ–ABP (270 ng h/mL) and ABP–IFNγ (458 ng h/mL) was significantly greater than that of IFNγ (57.6 ng h/mL). In addition, the MRT of ABP–IFNγ (4.11 h) was significantly longer than that of IFNγ (2.64 h) or IFNγ–ABP (2.69 h).

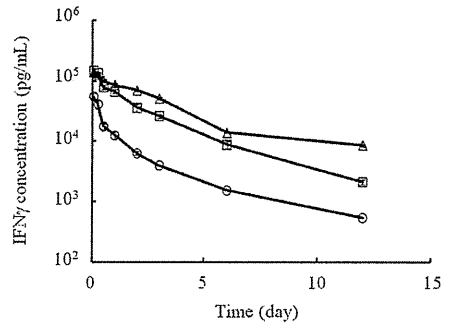

Figure 4. Clearance of IFNγ, IFNγ–ABP, and ABP–IFNγ after intravenous injection into mice. The concentrations of IFNγ (circles), IFNγ–ABP (squares), and ABP–IFNγ (triangles) were measured by ELISA using antimouse IFNγ antibody. The results are expressed as the mean ± SEM of four mice.

mRNA Expression of IFNγ, IFNγ-ABP, and ABP-IFNγ in the Liver and Time Course of the Serum Concentration of IFNγ, IFNγγABP, and ABP-IFN? after In Vivo Gene Transfer

Figure 5a shows the mRNA expression of IFNγ, IFNγ–ABP, and ABP–IFNγ in the liver at 6 h after hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ, respectively. The expression of IFNγ, IFNγ–ABP, and ABP–IFNγ was not significantly different from one another. Figure 5b shows the time courses of the serum concentrations of IFNγ, IFNγ–ABP, and ABP–IFNγ after hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ, respectively, at a dose of 0.2 pmol/mouse. The disappearance of IFNγ–ABP and ABP–IFNγ from the circulation was much slower than that of IFNγ. The C_{max} of IFNγ–ABP (516 ng/mL) and ABP–IFNγ (1940 ng/mL) was markedly greater than that of IFNγ (46.6 ng/mL). The time to reach the maximum serum concentration (t_{max}) of the fusion proteins was 0.5 (IFNγ–ABP) and 1 (ABP–IFNγ) day, which was later than that of IFNγ (0.25 day). Table 2 summarizes the AUC and MRT values after hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, and pCMV–ABP–IFNγ. A hydrodynamic injection of pCMV–IFNγ–ABP or pCMV–ABP–IFNγ produced about a 25- or 140-fold greater AUC and a 1.5- or twofold longer MRT than seen with pCMV–IFNγ.

Inhibition of the Growth of Metastatic Pulmonary Tumor by Gene Transfer of IFNγ, IFNγ–ABP, or ABP–IFNγ in Mice

Figure 6 shows the number of metastatic colonies of CT-26 cells on the lung surface at 14 days after inoculation of CT-26 cells into the tail vein of mice. A hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ signifi-

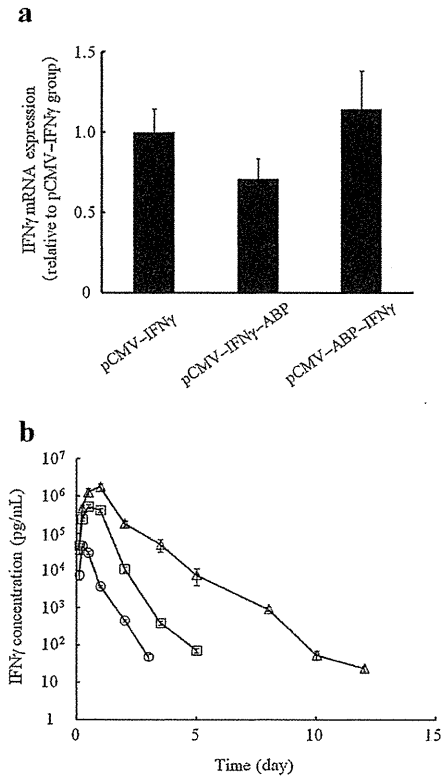

Figure 5. (a) mRNA expression of IFNγ, IFNγ–ABP, and ABP–IFNγ in the liver. mRNA expression of IFNγ, IFNγ–ABP, and ABP–IFNγ in the liver was measured 6 h after hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ into mice. The results are expressed as the mean ± SEM of three mice. (b) Time courses of the concentrations of IFNγ, IFNγ–ABP, and ABP–IFNγ in the serum after hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ into mice. Each plasmid DNA was administered at a dose of 0.2 pmol/mouse. The concentrations of IFNγ (circles), IFNγ–ABP (squares), andABP–IFNγ (triangles) were measured by ELISA using antimouse IFNγ antibody. The results are expressed as the mean ± SEM of three mice.

Table 2. Pharmacokinetic Parameters of IFNγ, IFNγ–ABP, and ABP–IFNγ after Hydrodynamic Injection of pCMV–IFNγ, pCMV–IFNγ–ABP, and pCMV–ABP–IFNγ, Respectively, into Mice

	IFNγ	IFNγ–ABP	ABP–IFNγ
AUC (ng h/mL)	24.3 ± 2.6	569 ± 58*	3430 ± 430*
MRT (h)	0.49 ± 0.01	0.79 ± 0.01*	1.03 ± 0.02*
C_{max} (ng/mL)	46.6 ± 3.2	516 ± 60*	1940 ± 240*
t_{max} (h)	6	12	24

The C_{max} and t_{max} values were obtained from actual data recorded after hydrodynamic injection of pCMV–IFNγ, pCMV–IFNγ–ABP, and pCMV–ABP–IFNγ at a dose of 0.2 pmol/mouse. The AUC and MRT were calculated by integration to infinite time, and the mean ± SEM values are shown.

*Statistically significant ($p < 0.05$) compared with pCMV–INFγ.

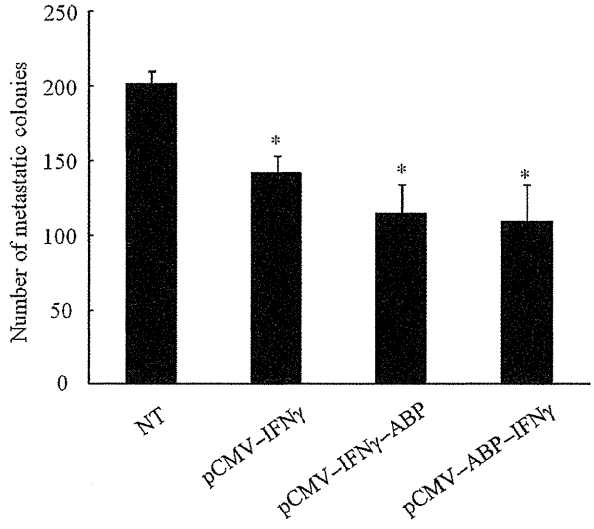

Figure 6. Number of metastatic colonies of CT-26 cells on the lung surface of mice. Pulmonary metastasis was induced by inoculation of 1×10^5 CT-26 cells into a tail vein (day 0). On day 3, pCMV–IFNγ, pCMV–IFNγ–ABP, or pCMV–ABP–IFNγ was injected into mice by hydrodynamic injection at a dose of 0.7 pmol/mouse. A group of control mice was left untreated (NT). 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 ± SEM of five mice. $*p < 0.05$ compared with the NT group.

cantly reduced the number of colonies to 70%, 56%, or 53% that in the no treatment group, respectively. There was no significant difference in the number of metastatic colonies among the groups.

DISCUSSION

Our previous study showed that the fusion of MSA to IFNγ dramatically improved the pharmacokinetic properties of IFNγ. However, the fusion also greatly reduced the biological activity of IFNγ.[11] Such reduction in the activity is similar to that of polyethylene glycol-conjugated human IFNα products used in clinical settings.[26] These results suggest that the use of serum albumin or other macromolecules is not suitable for the control of the pharmacokinetics of IFNγ. The present study clearly demonstrates that the fusion of a much smaller peptide (20 amino acids) than MSA is useful for controlling the tissue distribution of IFNγ without significantly reducing its biological activity.

Both IFNγ–ABP and ABP–IFNγ exhibited about 50% the activity of IFNγ, suggesting that the site of the fusion of ABP is of little importance. In addition, the reduced activity of IFNγ–ABP and ABP–IFNγ

in the presence of MSA (Fig. 2b) strongly suggests that the binding of IFNγ–ABP and ABP–IFNγ with MSA somewhat interferes with the binding of these fusion proteins to IFNγ receptor. It has been reported that both the N- and C-terminals of IFNγ are critical for the receptor interaction.[27,28] Because bioactive IFNγ is an antiparallel dimer,[1,29] fusion at either end would have a similar impact on the physiochemical properties of IFNγ. On the contrary, ABP–IFNγ exhibited higher binding affinity to MSA than IFNγ–ABP (Fig. 3), indicating that the fusion site did affect the interaction with MSA. A crystal structure analysis of human IFNγ revealed that both the N- and C-terminals are located on the surface of the molecule and the N-terminal is more flexible than the C-terminal which has some adjacent side chains.[30,31] Although there is little information on the structural properties of mouse INFγ, the helix of IFNγ is strongly conserved between mice and humans.[30,32] Therefore, the stronger binding activity of ABP–IFNγ to MSA, compared with IFNγ–ABP, is probably due to the greater flexibility of the N-terminal domain compared with the C-terminal.

Reflecting the binding to MSA, the fusion of ABP could prolong the circulation time of IFNγ. Similar results were observed after gene delivery of these derivatives. An injection of pCMV–ABP–IFNγ produced a higher AUC and longer MRT than that of pCMV–IFNγ–ABP (Table 2). These differences would be due to the difference in the binding affinity to MSA between these fusion proteins, as was the case with the protein injection, because the mRNA expression of IFNγ in the liver was comparable among all the groups (Fig. 5a). Taken together, these results clearly show that the gene delivery of IFNγ–ABP or ABP–IFNγ is an effective approach to improving the pharmacokinetic properties of IFNγ.

Effective inhibition of metastatic tumor growth by gene delivery of IFNγ–ABP or ABP–IFNγ indicates that these fusion proteins are pharmacologically active and their gene delivery is a promising therapeutic method for the treatment of a number of diseases. Although the affinity of IFNγ–ABP for MSA was higher than that of ABP–IFNγ, and no significant difference was observed in the number of metastatic colonies. A possible explanation of this is that the high affinity of the fusion protein for MSA disturbs its binding to the IFNγ receptor, as observed in the in vitro experiment (Fig. 2b). The results of the present study indicate that selecting or designing ABP with proper affinity for serum albumin can maximize the therapeutic effects of IFNγ by achieving optimal balance between biological activity and retention in blood circulation.

CONCLUSION

We have demonstrated that the fusion of ABP to IFNγ is a useful approach to achieving prolonged retention in blood circulation through binding to serum albumin and this prolonged retention is effective in inhibiting metastatic tumor growth in mouse lung.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, and by a Grant-in-Aid for Research on Hepatitis from the ministry of Health, Labour, and Welfare of Japan.

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