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Long-acting interferon-α 2a modified with a trimer-structured polyethylene glycol: Preparation, in vitro bioactivity, in vivo stability and pharmacokinetics

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Abstract

The proper selection of size and shape for polyethylene glycol (PEG) is one of the most important points in PEGylation technology. Therefore, PEGs of various sizes and shapes have been widely developed to endow specific properties. In this study, a unique, trimer-structured, 43 kDa PEG was conjugated to interferon-α 2a (IFN) by forming an amide bond to improve the pharmacokinetic properties and minimize the loss of IFN bioactivity. Mono-PEGylated IFN (PEG_3–IFN) prepared by utilizing this unique PEG was purified and characterized by cation-exchange chromatography and MALDI-TOF mass spectrometry. The in vitro bioactivity, in vivo stability, and pharmacokinetics of PEG_3–IFN were examined and compared to those of native IFN. PEG_3–IFN exhibited comparable in vitro bioactivities to native IFN and an excellent stability of the conjugation linkage in rat serum and various organs following subcutaneous injection. Furthermore, it showed slow absorption and markedly reduced clearance in rats, thereby increasing the biological half-life by about 40-fold compared to that of native IFN. This is the first report on the application of unique, trimer-structured PEG to bioactive proteins. The results suggest that unique, trimer-structured 43 kDa PEG can provide some advantages to improve the pharmacokinetic properties and to maintain the bioactivity of therapeutic proteins in clinical use.

Keywords: PEG; PEGylation; Interferon; Bioactivity; Stability; Pharmacokinetics

1. Introduction

Interferon is a class of cytokines which displays a wide range of antiviral, antitumoral, and immunomodulatory activities (Pestka, 1983; Wang et al., 2000), and which has been successfully used as a therapeutic tool for a variety of diseases such as skin malignancies, hematologic proliferative diseases, viral infections, and multiple sclerosis. Among the several interferon species, interferon-α possesses the therapeutic efficacy for hepatitis B and C, as well as the above therapeutic indications (Piper et al., 2001).

PEGylation, the covalent attachment of polyethylene glycol (PEG), is one of the most useful pharmaceutical technologies ever developed and has generated a variety of protein drugs with markedly intensified therapeutic properties compared to their unmodified species (Katre, 1993; Bailon et al., 2001). The modification of proteins with PEG increases their molecular size, shields the metabolic sites, and masks the immunogenic sites, thus increases plasma half-life and in vivo stability, and diminishes immunogenicity. Due to these advantages, PEGylated therapeutics can enhance therapeutic efficacy and reduce undesirable effects (Parkinson et al., 2003; Edwards et al., 2003; Moreadith and Collen, 2003; Youn et al., 2005; Lee et al., 2005).

PEGylated interferon-α formulations have already been approved for hepatitis C and were marketed in 2000–2002. Due to the dramatic increase of plasma half-lives achieved by PEGylation, PEG-Intron® (linear PEG_{12K}–Interferon-α 2b) and PEGASYS™ (branched PEG_{40K}–Interferon-α 2a) have allowed a decrease in the weekly dosing frequency from 3 to

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1 and thereby definitely contributed to increased patient compliance and quality of life. They have rapidly made inroads into the markets of their unmodified species such as Intron A and Roferon-A (Youngster et al., 2002; Matthews and McCoy, 2004).

One of the most important points in PEGylation is associated with the proper selection of size and shape of PEG. Advances in PEG synthesis technology have led to a substantial increase of the available PEG size up to 40 kDa. The large-sized PEGs have endowed some therapeutic protein drugs with long-acting capacity. The increasing size of attached PEG, however, may result in a substantial failure of therapeutic application by severe loss of bioactivity. Therefore, the successful development of a PEGylated therapeutic protein requires an optimized balance between enhanced pharmacokinetics and reduced bioactivity by the judicious selection of PEG size (Youngster et al., 2002).

On the other hand, PEG shape also plays a crucial role in bioactivity and pharmacokinetic behavior. Caliceti et al. reported that linear PEG was distributed with a larger distribution volume, whereas branched PEG was distributed with a smaller distribution volume (Caliceti et al., 1999). The report by Yoshioka et al. indicated the influence of attached PEG shape on the in vitro/vivo antitumor activity of tumor necrosis factor-α mutant (Yoshioka et al., 2004). These reports showed the advantages of branched PEG in the therapeutic use of PEGylated proteins by minimizing the loss of in vitro bioactivity and maximizing the blood residence time.

In this study, we conjugated interferon-α 2a (IFN) to a unique, trimer-structured PEG (PEG_3) of 43 kDa molecular mass, which is comprised of three PEG chains for improving the pharmacokinetic properties and minimizing the loss of bioactivity. In vitro bioactivity, in vivo stability, and pharmacokinetics of mono-PEGylated IFN (PEG_3–IFN) prepared with this PEG_3 were assessed and compared to those of native IFN.

2. Materials and methods

2.1. Materials

Interferon-α 2a was obtained from the Research Laboratories of Dong-A Pharm. Co., Ltd. (Yongin, Korea), and monomethoxy trimer PEG with *N*-hydroxy succinimidyl group ($mPEG_2CONHPEG$-NHS, 43 kDa) from NOF corporation (Tokyo, Japan). Purified IFN gave a single peak (>99%) on reversed-phase HPLC and SDS-PAGE analysis. One milligram of IFN was equivalent to 2×10^8 IU (data not shown).

2.2. Preparation and purification of mono-PEGylated IFN

PEGylation was carried out by mixing of IFN (final concentration of 1 mg/mL) and $mPEG_2CONHPEG$-NHS (43 kDa) reagent at 1:3 molar ratio in 50 mM sodium borate buffer (pH 8.0). After 2 h at 4 °C, the reaction was stopped by adjusting the pH of the mixture to 4.8 with phosphoric acid. The reaction mixture was subjected to high performance size-exclusion chromatography (HPSEC) on a Shodex KW803 column (Showa Denko K.K., Tokyo, Japan) eluted with 50 mM phosphate buffer with 0.5 M sodium chloride (pH 6.0) at a flow rate of 0.8 mL/min, and the PEGylation degree was monitored at the UV wavelength of 220 nm to optimize the condition for the mono-PEGylated IFN. For isolation of the mono-PEGylated IFN species, the reaction mixture was diluted 10-fold with deionized water and applied into a column (4 cm × 15 cm) packed with SP-Sepharose resin (Amersham Biosciences, Sweden), which had previously been equilibrated with 40 mM sodium phosphate (pH 4.8) at a flow rate of 10 mL/min. The column was washed with the equilibration buffer to remove excess PEG reagent. The desired mono-PEGylated IFN was then separated from PEGylated IFN oligomers by a salt gradient from 0 to 0.5 M sodium chloride in the same buffer, collected, and concentrated to approximately 1 mg/mL for further experiments.

2.3. Amino acid composition of PEG_3–IFN

The amino acid composition of PEG_3–IFN and IFN was determined by the Pico-Tag method. With the total number of IFN amino acids assumed to be 165, the number of each amino acid per mole was determined by calculating the ratio of each amino acid in the mixture.

2.4. Circular dichroism spectrometry

Circular dichroism (CD) analysis was used to examine the secondary or tertiary structural conformation of PEG_3–IFN. The concentration was adjusted to 0.5 mg/mL. The spectrum was analyzed and compared to native IFN using a Jasco J600 CD spectropolarimeter (Jasco, Tokyo, Japan).

2.5. MALDI-TOF mass spectrometry

MALDI-TOF MS analyses were performed using a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, Cambridge, MA) with a slight modification of a method described elsewhere (Na et al., 2004). Briefly, samples were prepared by mixing 5 μL of an aliquot with 30 μL of the matrix solution, which was a saturated solution of sinapic acid in 50% of water/acetonitrile with 0.1% trifluoroacetic acid. One microliter of the sample mixture was spotted into a well of the sample plate and dried by vacuum evaporation prior to mass spectrometry. Data for 2 ns pulses of the 337 nm nitrogen lasers were averaged for each spectrum in a linear mode, and positive ion TOF detection was performed by using an accelerating voltage of 25 kV. A mixture of cytochrome C and bovine serum albumin was adapted for the external calibration.

2.6. Antiviral activity assay for cytopathic effect (CPE)

The in vitro antiviral activity of PEG_3–IFN was determined by cytopathic effect assay (CPE assay) using MDBK cells challenged with vesicular stomatitis virus (VSV), as described by Rubinstein et al. (1981) with some modification. Two-fold dilutions of samples (0.1 mL/well) and MDBK cells (6×10^4 cells/well in 0.1 mL) were added in 96-well microplates. After 2 h, 50 μL of the diluted VSV solution was added to each well. After additional incubation for 20 h, the

supernatants were decanted and the cells were stained with 2% neutral red solution (Sigma, St. Louis, MO). The optical density was read at 540 nm using a microwell plate reader (Tecan, Austria). The potency of PEG_3–IFN was determined by comparing the dose of PEG_3–IFN with that of the IFN standard, which affords 50% protection to infected cells.

2.7. Antiproliferative activity assay

Daudi cells (ATCC CCL 213) were grown in RPMI 1640 medium supplemented with 10% FBS. Cells were collected at a density of 10^6 cells/mL and 100 μL of cell culture was added to each well of 96-well microplates. PEG_3–IFN or interferon was diluted in the medium with varying concentrations and added to the wells in a volume of 100 μL. After 5-day incubation, cell proliferation was quantitated by MTS assay kit (Promega, Madison, WI). The 50% effective concentration (EC_{50}), the concentration required to saturate 50% of the maximal response, was calculated by nonlinear regression method (Mosamann, 1983; Osborn et al., 2002).

2.8. Lymphokine-activated killer (LAK) cytolytic activity assay

Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood by density gradient on Ficoll-Paque (Amersham Biosciences, Sweden). PBMCs were incubated in IMDM medium with 4 U/mL of human interleukin-2 (Sigma, St. Louis, MO) alone or in combination with PEG_3–IFN or interferon, for 3 days to induce effector LAK cells. The LAK-sensitive, Daudi cell line was used as the target cell. Effector cells and target cells were combined for 6 h at an E:T ratio of 50:1. LAK cytolytic activities were examined in a CytoTox 96 non-radioactive cytolytic assay kit (Promega, Madison, WI). The cytotoxicity was calculated by comparing the lactate dehydrogenase (LDH) measurements of drug-treated target cells with those of target cells in maximum LDH release.

2.9. Radioiodination of PEG_3–IFN

Radioiodinated PEG_3–IFN was prepared using the IODO-GEN method described elsewhere (Shin et al., 2005) with a slight modification. Briefly, IODO-GEN (1 mg) (Pierce, Rockford, IL, USA) was dissolved in 1 mL of methylene chloride, and 100 μL of the prepared solution was dispensed to a fresh tube and thoroughly flushed off under a stream of nitrogen gas at room temperature. To this tube were added 0.2 mL of PEG_3–IFN (1 mg/mL) and 5 mCi of $Na^{125}I$ (Perkin-Elmer, Boston, MA, USA) diluted with 10 mM phosphate buffer saline (PBS, pH 7.4). After reaction for 90 min at room temperature, the supernatant was loaded onto a Sephadex G-25 desalting column (Pharmacia LKB, Uppsala, Sweden) eluted with 10 mM PBS (pH 7.4) at a flow rate of 0.4 mL/min. The fractions corresponding to the ^{125}I-PEG_3–IFN were collected and concentrated using Centricon-10 concentrators (Amicon, Beverly, MA, USA), and stored at 4 °C until needed. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The radioactivity of these fractions was measured by gamma counting (Cobra™ Series Auto-Gamma Counting System, Packard Instruments Co., Groningen, The Netherlands). The specific radioactivity of ^{125}I-PEG_3–IFN was approximately 1.1 μCi/μg.

2.10. Stability of conjugation linkage between PEG_3 and IFN

The stability of conjugation linkage between PEG_3 and IFN in rat serum and various tissue organs was evaluated. ^{125}I-labeled PEG_3–IFN (approximately 450 μCi/0.5 mL) was administered to Sprague–Dawley rats (7–8 weeks old, 250–270 g) by a single subcutaneous injection. At 40 h after the injection of ^{125}I-PEG_3–IFN, the rats were sacrificed. Serum and each tissue homogenate of muscle (around the injection site), liver, and kidney were centrifuged at 10,000 rpm for 10 min. After centrifugation, each supernatant was collected for analysis. The supernatant was mixed with SDS sample buffer and applied to Tris–glycine gels of 16.5% polyacrylamide. After electrophoresis at 250 V for 40 min, the gel was fixed by gel dryer and the dried gel was exposed in an image plate (Fuji, Japan) for 48 h. The radioactivities of the bands on the image plate were measured by BAS (Bio-imaging Analyzer System, Fuji, Japan).

2.11. Pharmacokinetics

The pharmacokinetics of IFN and PEG_3–IFN were evaluated after a single intravenous or subcutaneous injection in SD rats (7–8 weeks old, 230–250 g). Three rats in each group received 0.5 mL of PEG_3–IFN or native IFN (each 10^7 IU/kg) via each administration route. Blood samples were withdrawn at properly designed time points, and the serum samples were analyzed for interferon activity by CPE assay.

3. Results and discussion

For successful clinical use of various PEGylated proteins, PEG firstly has to be optimized because PEGs with slightly different physicochemical properties can have thoroughly different effects on the pharmaceutical properties of PEG-protein hybrids (Caliceti et al., 1999). This study used a trimer-structured PEG in an effort to improve the pharmacokinetic and biological properties of, and also to characterize, the mono-PEGylated IFN (PEG_3–IFN) prepared by using this unique PEG.

PEGylated IFN was prepared via an amide bond as a result of the reaction between an *N*-hydroxysuccinimide ester derivative of a 43 kDa PEG molecule and the free amino group of IFN. As shown in Fig. 1A, the size-exclusion chromatogram showed that the reaction mixture contained approximately 10% of di-PEG–IFN species, 25% of mono-PEG–IFN species, and 60% of unmodified IFN. Finally, PEG_3–IFN was purified by SP-Sepharose cation-exchange chromatography (Fig. 1B), and the monomer purity of PEG_3–IFN was approximately 97% as determined by HPSEC. Additionally PEG_3–IFN consisted of the four major positional isomers (approximately ∼77%) of mono-PEGylated IFN species at Lys^{31}, Lys^{70}, Lys^{121}, and

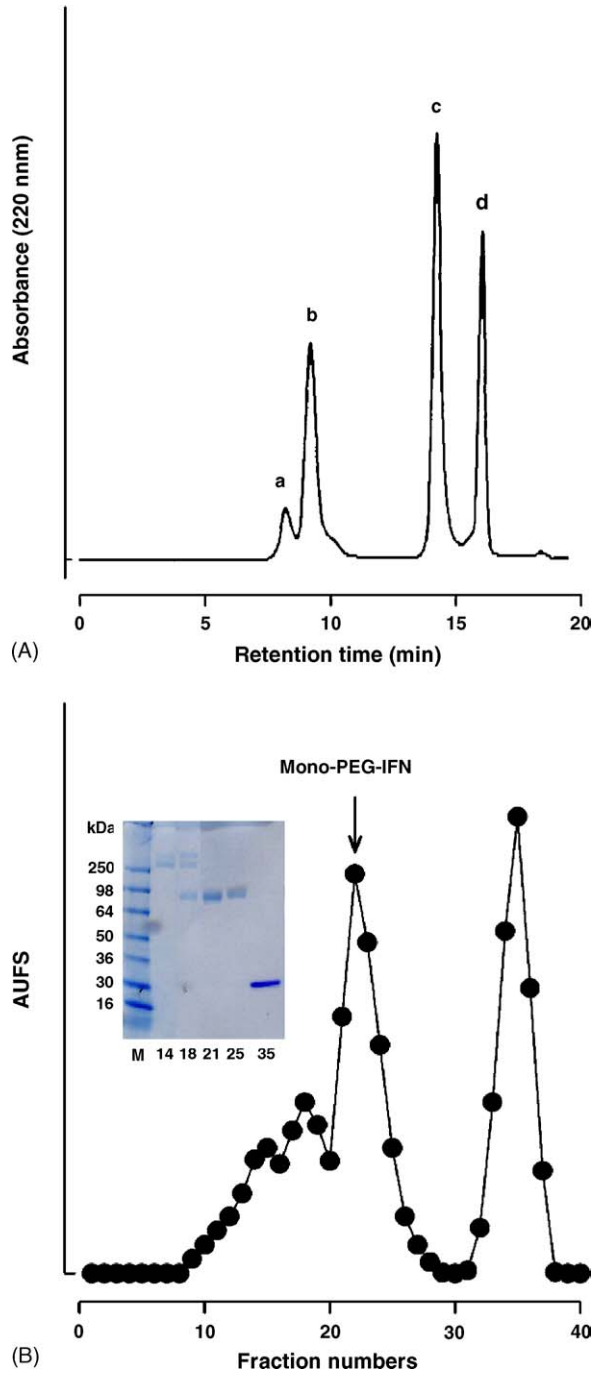

Fig. 1. Size-exclusion chromatogram of PEGylated IFN reaction mixture (A). Peaks: a, di-PEGylated IFN; b, mono-PEGylated IFN; c, unmodified IFN; and d, *N*-hydroxysuccinimide (NHS). Elution profile of PEG–IFN in the ion-exchange chromatography (B). Each number in SDS-PAGE gel corresponds to each fraction.

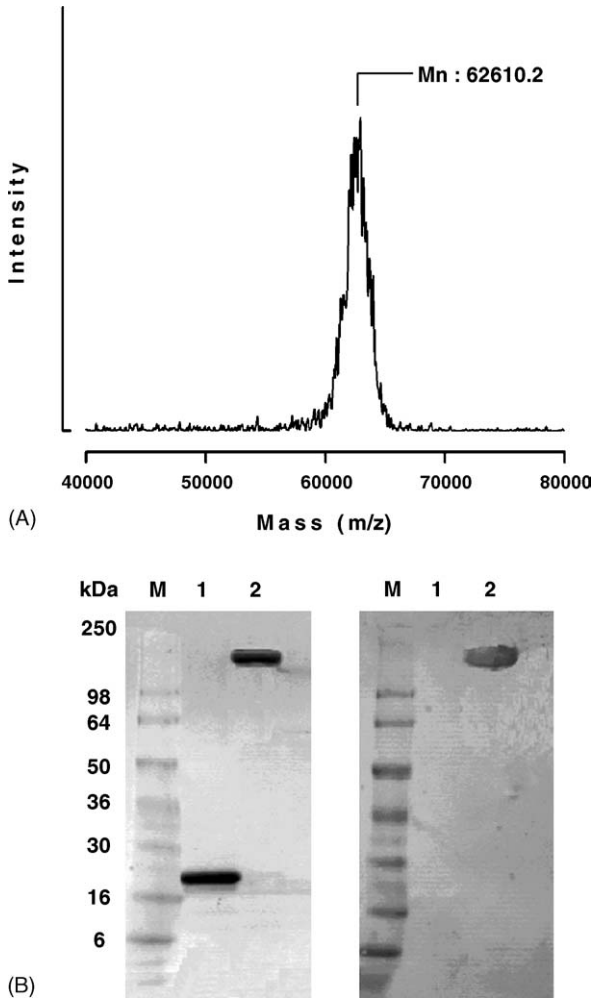

Fig. 2. Characterization of mono-PEGylated interferon-α 2a (PEG_3–IFN). (A) MALDI-TOF MS spectra. (B) SDS-PAGE gel of Coomasie staining (left) and iodine staining (right). Lanes: M, molecular weight marker; 1, IFN; 2, PEG_3–IFN.

Lys^{164}. The minor positional isomers (about ~23%) are the mono-PEGylated IFN species at Lys^{23}, Lys^{83}, and Lys^{112}, and a PEGylated IFN species at the amino terminus was not observed in the reaction mixture (data not shown).

The molecular mass of the purified PEG_3–IFN was determined to be 62610.2 Da by MALDI-TOF mass spectrometry, as shown in Fig. 2A. Considering the IFN mass of about 19.2 kDa, the difference in the mass between IFN and PEG_3–IFN was approximately 43 kDa, which corresponded to the number-averaged molecular mass (M_n) of the trimer-structured PEG. SDS-PAGE analysis with Coomassie staining and iodine staining revealed an intense broad band corresponding to PEG_3–IFN in the gel (Fig. 2B).

From the amino acid composition analysis, PEG_3–IFN appeared to have very similar amino acid composition to native IFN. The CD spectra for IFN and PEG_3–IFN were nearly superimposed across the near-to-far-UV spectrum, suggesting that PEGylation had no significant effect on the secondary or tertiary structure of the IFN protein (data not shown).

The average, specific antiviral activity of PEG_3–IFN was approximately 10% of that of the native IFN (Fig. 3). This decreased in vitro antiviral activity was challenging rather than discouraging because 40 kDa, branched PEG-modified IFN-α 2a (PEG_{40K}–IFN) has been reported to have an antiviral specific activity of approximately 7% that of native IFN-α 2a (Bailon et al., 2001). Furthermore, the prolonged in vivo half-life of PEGylated IFN-α species has over-compensated for the decreased bioactivity, eventually leading to a markedly enhanced in vivo therapeutic efficacy (Reddy et al., 2002). Therefore, despite

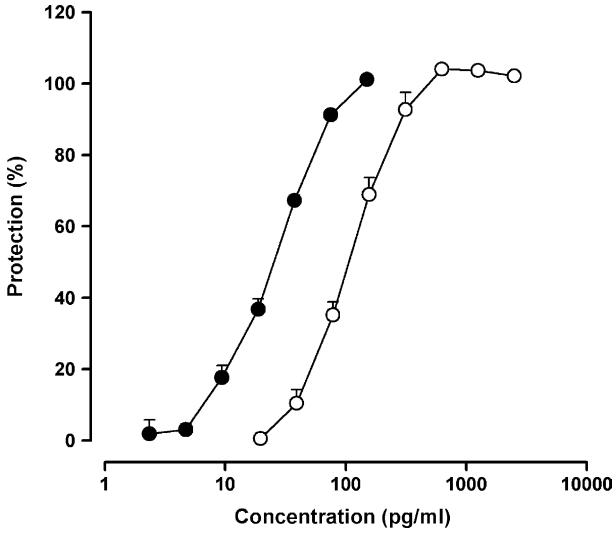

Fig. 3. In vitro antiviral activity of IFN (●) and PEG_3–IFN (○). The error bar represents the standard deviation at each data point.

the 10% residual in vitro antiviral activity of PEG_3–IFN, it would give a promising result in a future clinical study. On the other hand, the in vitro antiproliferative activities of IFN and PEG_3–IFN were described in terms of the concentration where 50% of the saturating maximal response is observed. The EC_{50}s of IFN and PEG_3–IFN were 0.044 and 0.507 ng/mL, respectively (Fig. 4). These results were in contrast to the patent description for PEG_{40K}–IFN; however, Monkarsh et al. (1997) reported that 5 kDa PEG–IFN had an antiproliferative activity that was more reduced than that of unmodified IFN. No prominent difference in LAK cell cytolytic activity was observed between PEG_3–IFN and native IFN, as determined by *t*-test ($p < 0.05$) (Fig. 5). These results of pharmacological tests demonstrated that the biological activity of PEG_3–IFN was maintained in both antiviral and immunotherapeutic activity, despite the conjugation with 43 kDa PEG.

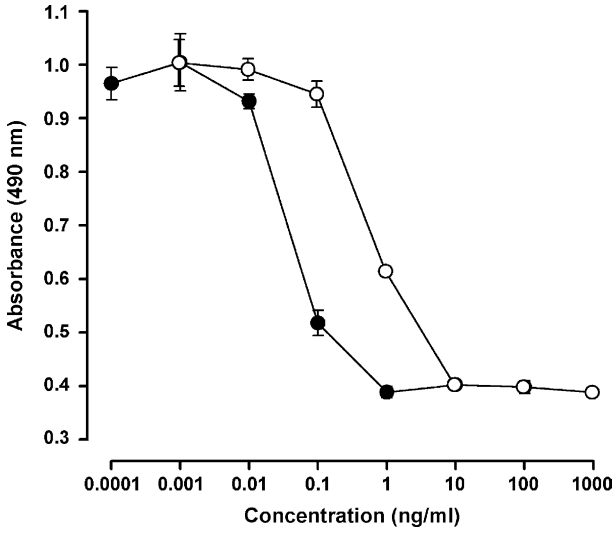

Fig. 4. In vitro antiproliferative activity of IFN (●) and PEG_3–IFN (○). The error bar represents the standard deviation at each data point.

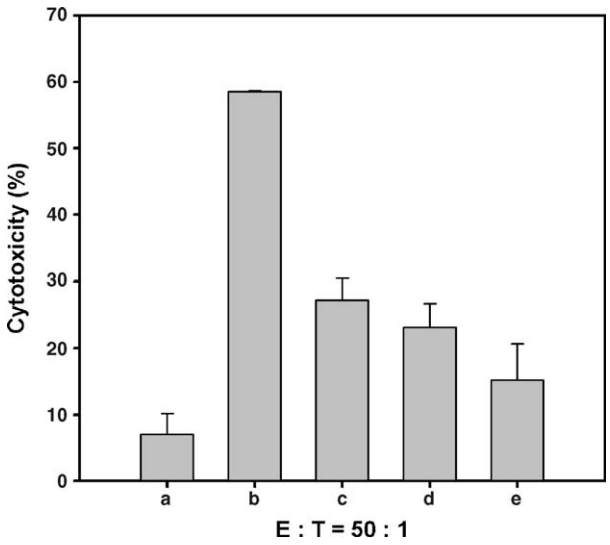

Fig. 5. In vitro LAK cytotoxic activity of IFN and PEG_3–IFN. Data are relative percents of cytolysis to maximum lysis of the target cell and the error bar represents the standard deviation of results in two independent experiments: a, 4 U/mL IL-2; b, 200 U/mL IL-2; c, 4 U/mL IL-2 in combination with 5 ng/mL IFN; d, 4 U/mL IL-2 in combination with 50 ng/mL PEG_3–IFN; e, 4 U/mL IL-2 in combination with 5 ng/mL PEG_3–IFN. All treatments were significantly different from that of 4 U/mL IL-2 (a), except for the treatment of 4 U/mL IL-2 in combination with 5 ng/mL PEG_3–IFN (e). However, no significant difference was observed between IFN treatment (c) and PEG_3–IFN treatment (d), as determined by *t*-test ($p < 0.05$).

It is of great importance to maintain the stability of the conjugation linkage between the drug and PEG because covalently attached PEG-drug conjugate can guarantee the sustained in vivo efficacy due to an increased plasma half-life. Moreover, unstable linkage may sometimes lead to degradation of the PEG-drug conjugates during the manufacturing process or drug injection (Harris, 1985). When ^{125}I-labeled PEG_3–IFN was injected into

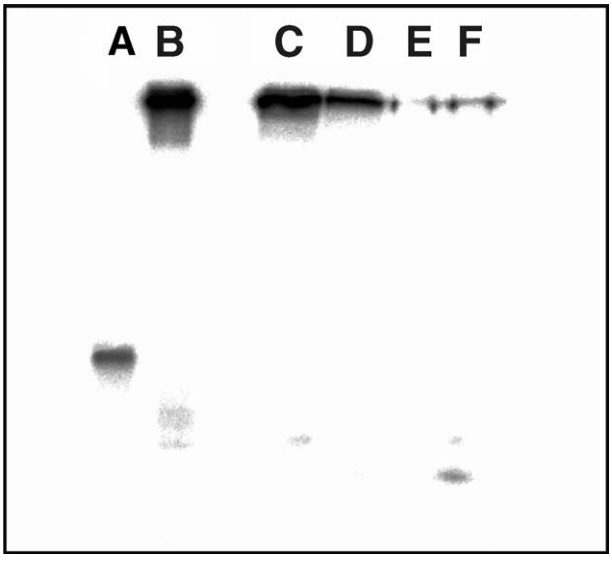

Fig. 6. Monitoring of the stability of the linkage between PEG_3 and IFN in rat serum (C), muscle (injection site) (D), liver homogenate supernatant (E), and kidney (F). (A) and (B) denote ^{125}I-IFN and ^{125}I-PEG_3–IFN standards, respectively.

Table 1
Summary of pharmacokinetic parameters of IFN and PEG_3–IFN ($n = 3$–4)

	PEG_3–IFN		IFN	
	s.c.	i.v.	s.c.	i.v.
MRT (h)	90.5 ± 13.7	59.4 ± 5.1	2.4 ± 0.3	1.1 ± 0.5
CL (mL/h/kg)	6.8 ± 2	2.7 ± 0.4	199 ± 72.2	121 ± 22.4
V_{ss} (mL/kg)	606 ± 120	161 ± 120	468 ± 153	127 ± 75.8
Terminal, $t_{1/2}$ (h)	47.4 ± 6.8	37.3 ± 2.3	1.3 ± 0.1	1.2 ± 0.1
AUC (IU h/mL)	$1.6 \times 10^6 \pm 4.1 \times 10^5$	$3.7 \times 10^6 \pm 6.1 \times 10^5$	$5.7 \times 10^4 \pm 2.6 \times 10^4$	$8.5 \times 10^4 \pm 1.7 \times 10^4$

rats via subcutaneous route, it exhibited the excellent stability of the conjugation linkage, as well as the protein portion itself, both in serum and various tissue organs, as shown in Fig. 6.

Considering the 70 kDa cutoff of the kidney, a PEG mass of approximately 40–50 kDa is required to retard the renal filtration of small molecules (Bailon and Berthold, 1998; Harris and Chess, 2003). In practice, it has been reported that the renal clearance rate of PEG_{40K}–IFN was significantly lower and that the maximal serum concentration of PEG_{40K}–IFN was more prolonged than that of PEG_{12K}–IFN (Nieforth et al., 1996). As expected, the trimer-structured, 43 kDa PEG–modified IFN showed a dramatic improvement in various pharmacokinetic parameters. The mean serum antiviral activity–time curves obtained after intravenous or subcutaneous injection of native IFN and PEG_3–IFN to rats are shown in Fig. 7. The individual pharmacokinetic parameters of each species are summarized in Table 1. The serum activity of PEG_3–IFN peaked 1 h after the subcutaneous injection, whereas the maximal activity of PEG_3–IFN was reached at 23–36 h after the injection. The serum PEG_3–IFN concentration remained over 1000 IU/mL for 7 days while the serum IFN concentration was decreased to below 100 IU/mL after 1 day, irrespective of the administration route. Serum PEG_3–IFN concentrations after intravenous administration remained slightly higher than those after subcutaneous administration. After the subcutaneous injection, the half-life of PEG_3–IFN was increased 40-fold compared to that of IFN.

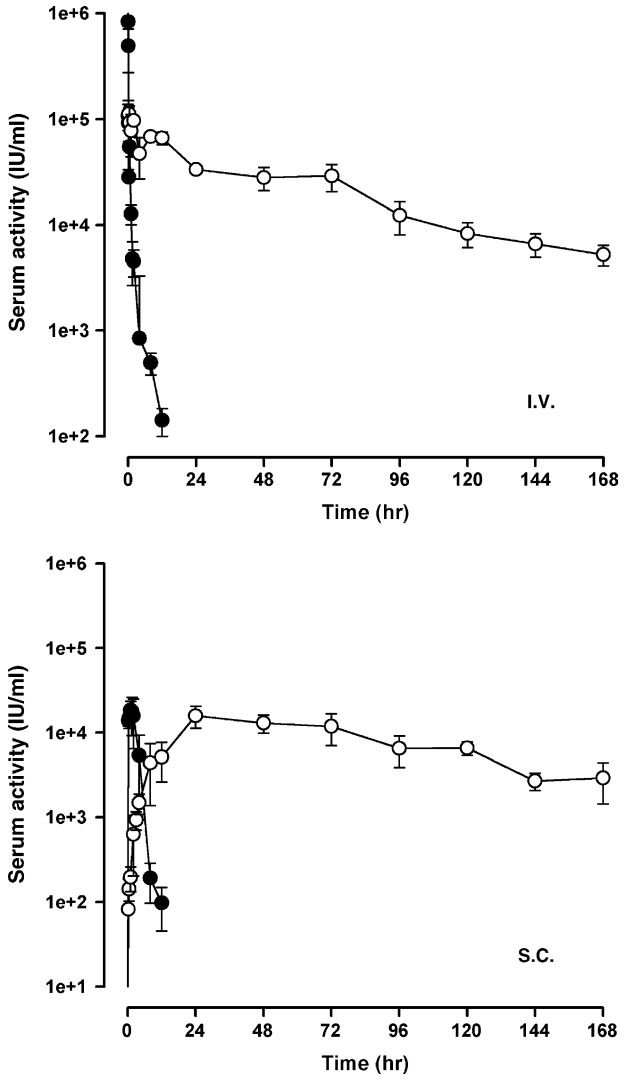

Fig. 7. Mean serum antiviral activity–time curves following intravenous (i.v.) or subcutaneous (s.c.) injection (10^7 IU/kg) of IFN (●) and PEG_3–IFN (○) in rats.

This study was focused on the unique, trimer-structured PEG and its use for bioactive proteins. The long-acting IFN prepared by utilizing this unique PEG showed dramatically enhanced properties compared to those of native IFN. In particular, for antiviral activity, which is one of the most important factors for hepatitis therapy, PEG_3–IFN exhibited superior performance to branched 40 kDa PEG–IFN. These results appeared to be attributable to the differences of the size and shape of PEG_3. Consequently, the trimer-structured PEG has the potential to endow promising therapeutic proteins with clinically useful properties.

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