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Research Paper

Lactoferrin Conjugated with 40-kDa Branched Poly(ethylene Glycol) Has an Improved Circulating Half-Life

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Purpose. We developed a lactoferrin conjugate by modifying bovine lactoferrin (bLF) with a 40-kDa branched poly(ethylene glycol) (PEG) molecule (designated 40 k-PEG-bLf), and we evaluated its *in vitro* activities and pharmacokinetic properties.
Materials and Methods. We prepared 40k-PEG-bLf by amino conjugation with *N*-hydroxysuccinimide-activated PEG. This conjugate was purified by cation exchange chromatography and its *in vitro* biological activities, such as iron binding, anti-inflammatory effects, and resistance to proteolytic enzymes were investigated. *In vivo* pharmacokinetics analyses, were also performed to examine the rate of clearance from the plasma in rats.
Results. The 40k-PEG-bLf conjugate was fully active in iron binding and exhibited 97.1±5.5% (mean ± S.E., $n=6$) of the original anti-inflammatory activity. The *in vitro* peptic susceptibility of 40 k-PEG-bLf revealed that the proteolytic half-life increased at least 6-fold that of unmodified LF. This PEGylated conjugate demonstrated a plasma half-life that was 8.7-fold longer than that of the unmodified bLF in rats.
Conclusions. The 40k-PEG-bLf exhibited improved *in vitro* bioactivity and stability and enhanced pharmacokinetic properties as compared to those of the unmodified bLF and the 20 k-PEG-bLf conjugate, which was recently developed by PEGylation of bLF with a 20-kDa branched PEG [Nojima Y. *et al.* Bioconjugate Chem. 19:2253–2259 (2008)].

KEY WORDS: bioactivity; branched PEG; lactoferrin; pharmacokinetics; stability.

INTRODUCTION

Lactoferrin (LF) is an 80-kDa iron-binding glycoprotein of the transferrin family, and it is found in various biological fluids (1). LF possesses various biological functions such as immunomodulatory (2), anti-inflammatory (3,4), antiviral (5), antimicrobial (6), and analgesic (7,8) effects and improvement of lipid metabolism (9). This multitude of biological activities suggests that LF is a potential drug candidate. In fact, Agennix Inc. (USA) has reported that orally administered Talactoferrin®, an immunomodulatory recombinant human LF preparation, was effective in the treatment of patients with diabetic neuropathic foot ulcers (10) and in non-small-cell lung cancer (NSCLC) therapy in combination with cisplatin treatment in the phase I/II clinical trial (11). Talactoferrin® has also been used in a recent clinical trial for renal cell carcinoma (12). In addition, Dogru, M. *et al.* have reported that oral administration of bovine LF (bLF) appeared to be an efficient treatment modality in improving tear stability and ocular surface epithelium in dry eye patients with Sjögren's syndrome (13).

PEGylation is defined as the covalent conjugation of poly(ethylene glycol) (PEG) to the protein of interest. This is one of the most promising pharmaceutical technologies used to generate several protein drugs with markedly intensified therapeutic properties as compared to their unmodified species (14). PEGylation imparts diminished immunogenicity and improved pharmacokinetic behavior (e.g., proteolytic resistance due to steric hindrance, inhibition of renal clearance due to the increased molecular mass after PEGylation), culminating in better pharmacological effects.

Recently, we produced 20 k-PEG-bLf by linking bLF to a branched 20-kDa PEG molecule. This conjugate exhibited improved pharmacokinetic properties (a prolonged serum half-life and an increased area under the curve by approximately 5.4-fold and 9.2-fold, respectively compared to those

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ABBREVIATIONS: AUC, area under the curve; bLF, bovine lactoferrin; CBB, Coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; LF, lactoferrin; NHS, *N*-hydroxysuccinimide; PEG, poly(ethylene glycol); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 20k-PEG-bLf, 20-kDa PEG-conjugated lactoferrin; 40k-PEG-bLf, 40-kDa PEG-conjugated lactoferrin.

of unmodified bLF) and enhanced uptake of PEGylated bLF from the rat intestine by approximately 10-fold compared to unmodified bLF, resulting in an increased retention time in the body (15).

The size of PEG is one of the most important considerations in PEGylation (16). In general, PEGylation with larger PEG molecules results in conjugate proteins with improved pharmacokinetic properties, but the resultant protein may exhibit reduced bioactivity. This is largely because larger PEG molecules may cover a greater surface area of protein.

Recently, it has been reported that introducing 40-kDa PEG into the interferon α proteins provided the enhanced *in vivo* efficacy (16–19). Thus, these four reports on successful PEGylated interferon α using 40-kDa PEG encouraged us to focus on the PEGylation of bLF with 40-kDa PEG to achieve greater efficacy than that of 20k-PEG-bLf. In this study, a 2-branched PEG molecule with a molecular mass of 40 kDa was conjugated with bLF (the resultant conjugate was designated 40k-PEG-bLf) to enhance the pharmacokinetic properties and minimize the loss of biological activity. Then, we assessed the *in vitro* bioactivity and pharmacokinetic properties of 40k-PEG-bLf and compared them to those of the unmodified bLF and the recently developed conjugate 20k-PEG-bLf (15).

MATERIALS AND METHODS

Materials

Bovine lactoferrin (bLF, 95% purity, a molecular weight of approximately 80 kDa) was acquired from MG Nutritionals (Melbourne, Australia). An *N*-hydroxysuccinimide derivative of 2-branched PEG with a molecular mass of 40 kDa (PEG-NHS, SUNBRIGHT GL2-400GS2, 90% purity, an average molecular weight of 42,999) was purchased from NOF Corporation (Tokyo, Japan). MacroCap SP was obtained from GE Healthcare (Uppsala, Sweden). A Pellicon XL 50 ultrafiltration device was supplied by Millipore (Billerica, MA, USA). Titrisol, concentrated iodine solution (0.05 M), was purchased from Merck KGaA (Darmstadt, Germany). Recombinant human IFN-γ (>97% purity, a molecular weight of approximately 17 kDa) was purchased from R&D systems (Minneapolis, MN, USA). Calcitriol (a molecular weight of 416.64) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (LPS, >99% purity) from *Escherichia coli* O127:B8 was purchased from Sigma (St. Louis, MO, USA). Cell counting kit-8 was purchased from Dojindo laboratories (Kumamoto, Japan). A human IL-6 ELISA kit was purchased from Kamakura Techno-Science (Kanagawa, Japan).

Experimental Animals

Male Wistar rats (Institute for Animal Reproduction, Ibaraki, Japan) weighing 250–280 g were used in this study. The animals were maintained at a controlled temperature of 22±2°C with a 12 h-light/12 h-dark cycle (light cycle: 7:00–19:00), and given standard chow (CE-2, Nihon Clea, Tokyo, Japan). All the procedures were approved by the Animal Research Committee of the Tottori University.

Preparation and Purification of 40k-PEG-bLf

The PEGylated reaction mixture consisted of bLF and 40-kDa branched PEG-NHS reagent at a 1:1.3 molar ratio in phosphate-buffered saline (PBS) at a pH of 7.4. The final protein concentration was 26.5 mg/mL. The reaction was carried out for 24 h at 25°C. The reaction mixture was applied to a column packed with MacroCap SP resin, previously equilibrated with 10 mM sodium phosphate, pH 7.6. The column was washed with 5 column volumes of equilibration buffer to remove excess PEG reagent and by-products. Then, 40 k-PEG-bLf and unmodified bLF were eluted with 300 mM sodium chloride and 1 M sodium chloride, respectively, at a flow rate of 10 mL/min. The 40 k-PEG-bLf eluates were desalted and concentrated with the Pellicon XL 50 ultrafiltration device. The protein concentration was determined by the Bradford protein assay procedure with BSA as a standard.

SDS-PAGE Analysis

The reaction mixture and the purified 40k-PEG-bLf compound were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) under non-reducing conditions, followed by staining with Coomassie brilliant blue (CBB). The PEG-bLf and excess PEG reagents were specifically stained with Titrisol iodine solution according to the procedure of Kurfürst with some modifications (20). The SDS-PAGE gel was then rinsed with distilled water and placed in 5% barium chloride solution for 10 min, following which it was washed with distilled water and transferred to 0.1 N Titrisol iodine reagent for 15 min. Excess Titrisol iodine was washed off with distilled water, and the PEG-conjugated proteins and excess PEG reagent were detected as brown bands.

Circular Dichroism Spectrometry

Circular dichroism (CD) analysis was used to examine the secondary structural conformation of 40 k-PEG-bLf. The concentration was adjusted to 0.02 mg/ml. Circular dichroic measurements were taken on a JASCO J-720 spectropolarimeter (JASCO, Tokyo, Japan) using a 1 mm circular quartz cell at room temperature. The spectra were taken from 195 to 250 nm with 0.1 nm step resolution, speed 20 nm/min, response 1 s, and bandwidth 1.0 nm.

MALDI-TOF Mass Spectrometry

All mass spectra were acquired with an UltraflexII MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with smartbeam-1 laser and operated in the positive linear mode. Samples of 1 mg/mL were prepared by mixing of the matrix solution, which was a saturated solution of sinapic acid in 33% acetonitrile with 0.07% trifluoroacetic acid. One microliter of the sample mixture was spotted into a well of the sample plate and dried by air prior to mass spectrometry.

N-terminal Amino Acid Sequence

The 40k-PEG-bLf (600 μg/mL) compound was digested using 60 ng/mL pepsin for 1 h at 37°C. The digested proteins

were subjected to SDS-PAGE and then electroblotted to a poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Protein bands were visualized with CBB and excised from the membrane. The N-terminal amino acid sequence was determined by automated Edman degradation on an Applied Biosystems sequencer (model 470A).

Iron-binding Assay

Native bLF and 40k-PEG-bLf were used to prepare apolactoferrin and iron-rebinding LF according to the method of Shimazaki *et al.* (21). Apolactoferrin was prepared in the presence of citrate. The reconstituted form of LF containing ferric ions was prepared in the presence of bicarbonate. Briefly, native bLF or 40k-PEG-bLf (3 mg/mL) were dialyzed against 0.1 M citric acid solution (pH 2.1) for 24 h at 10°C to remove ferric ions. They were then dialyzed against distilled water for 24 h, followed by dialysis against 50 mM phosphate buffer (pH 6.6) containing 50 mM NaCl to prepare apolactoferrin. Apolactoferrin was dialyzed against 50 mM phosphate buffer (pH 7.5) containing 0.001% (w/v) ferric ammonium citrate and 50 mM bicarbonate for 24 h at 10°C to prepare iron-rebinding LF. It was then dialyzed against distilled water for 24 h, followed by dialysis against 50 mM phosphate buffer (pH6.6) containing 50 mM NaCl for 16 h to remove unbound ferric ions. The iron concentration of each form of LF was determined using the method of Kirkpatrick *et al.* (22) by using the Fe-C test kit (Wako Pure Chemicals, Osaka, Japan).

In-vitro Anti-inflammatory Effects

A cell stimulation assay was carried out using the methods of Mattsby-Balzer *et al.* (3) and Håversen L *et al.* (4) with minor modifications. The human monocyte leukemia cell line THP-1 (JCRB0112) was obtained from the Health Science Research Resource Bank (Osaka, Japan) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM Glutamax (Invitrogen, Carlsbad, CA, USA) in a CO_2 incubator at 37°C. Cells from the logarithmic growth phase were treated with 20 ng/mL IFN-γ and 40 ng/mL calcitriol for 48 h to increase their sensitivity to LPS. Following incubation, the cells were collected and suspended in cell assay medium (RPMI-1640 containing 10% FBS and 2 mM Glutamax) at a concentration of 1×10^6 cells/mL. The cells (400 μL) were then added to a 24-well plate and stimulated with 100 ng/mL LPS, 100 ng/mL LPS plus 100 μg/mL test sample, or 100 μg/mL test sample at 37°C for 24 h in 5% CO_2. After incubation, the culture supernatants were collected and the IL-6 concentration was measured by enzyme-linked immunosorbent assay (ELISA).

Cytotoxicity of 40k-PEG-bLf

THP-1 cell suspension (100 μL of 1×10^6 cells/mL) was dispensed in each well of a 96-well plate, and the cytotoxicity assay was performed in triplicate. Unmodified bLF or 40k-PEG-bLf (10 μg) was added to each well and incubated at 37°C for 24 h under an atmosphere of 5% CO_2. Then, 10 μL of cell counting kit-8 solution was added to each well, and the plate was incubated at 37°C for 1 h in 5% CO_2. Cell viability was determined by measuring the absorbance at 450 nm (reference absorbance at 655 nm) by using a microplate reader.

Proteolytic Digestion

Unmodified bLF or 40k-PEG-bLf was dissolved in 10 mM HCl at a concentration of 0.5 mg/mL and adjusted to a pH of 3.2. Pepsin was added to the protein solution at a final concentration of 20 ng/mL, and the reaction mixture was incubated at 37°C. The reaction mixture was collected at 0, 5, 10, 15, 20, 40, 60, and 100 min and terminated by the addition of SDS-PAGE loading buffer. The samples were heated at 95°C for 5 min and analyzed by SDS-PAGE. The gel was stained with CBB, and the intensity of staining in each band was analyzed using Image J software.

Pharmacokinetic Study

Pharmacokinetic study was conducted according to the methods of Takeuchi *et al.* (23) with some modifications. Eight-week-old Wistar rats ($n=5$–7) were anesthetized with urethane (4 g/kg, s.c.) and sodium pentobarbital (0.1 mL, s.c.), and the external jugular vein was cannulated for blood collection. Unmodified bLF (1 mg/kg or 10 mg/kg) or 40 k-PEG-bLf (1 mg/kg) was injected into the femoral vein. Blood was then collected through the cannula at 0, 1, 5, 10, 15, 30, 60, 120, 180, and 240 min after sample administration. The bLF concentration in the blood was determined quantitatively by polyclonal antibody-based ELISA. The lower limit of quantitation for this ELISA is 2 ng/ml (23). Pharmacokinetic parameters of the test proteins were determined from plasma concentrations using the GraphPad Prism 4 software (San Diego, CA, USA).

Statistical Analysis

The Student's unpaired *t* test was used to evaluate the area under the curve (AUC) in the pharmacokinetic study. *P* values <0.05 were considered significantly different.

RESULTS

Synthesis and Purification of the 40-kDa-PEG-bLF Conjugate

PEGylated bLF was generated by the reaction between *N*-hydroxysuccinimide (NHS) ester derivatives of a 40-kDa 2-branched PEG molecule (Fig. 1) and the free amino groups (ε- and α- amino groups) of bLF. Fig. 2 shows the SDS-PAGE profile of unmodified bLF, the reaction mixture, and the purified PEGylated bLF (40k-PEG-bLf) detected by CBB (Fig. 2 A) and barium iodine, which are stains specific for PEG (Fig. 2 B). The reaction mixture contained unmodified bLF, PEGylated bLF, and an excess of the PEG-NHS reagent. Staining with CBB and barium iodine revealed that 40k-PEG-bLf was successfully purified by ion-exchange chromatography of the PEGylation mixture. The mono-PEGylated bLF was detected as a single band in the gel.

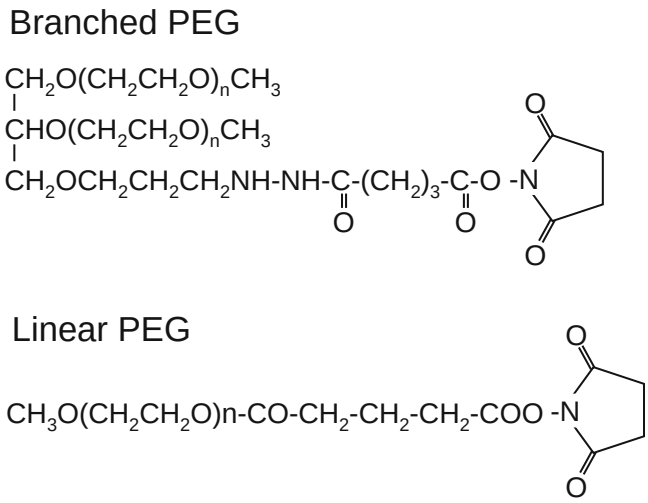

Fig. 1. The structure of branched and linear PEGs. A 2-branched PEG molecule with a molecular mass of 40 kDa was conjugated with bLF in this study.

Characterization of Bioconjugated bLF

The CD spectrum of 40 k-PEG-bLf was very similar to that of unmodified bLF across the near-to-far-UV spectrum, suggesting that PEG attachment to bLF had no effect on the secondary structure of bLF (data not shown). MALDI-TOF-mass spectrometry defined 40k-PEG-bLf as having a major molecular mass of 126,247 Da, which corresponded to the averaged molecular mass of a 40-kDa branched PEG (42,999 Da) plus the molecular mass of bLF determined by MALDI-TOF-mass spectrometry (82,679 Da, data not shown) (Fig. 3).

Iron-binding Assay

LF is composed of 2 globular lobes: the N-lobe and the C-lobe. Each lobe contains an iron-binding site where the ferric ion (Fe^{3+}) is tightly bound in synergistic cooperation with HCO_3^{2-} (24). The iron-scavenging properties of LF are

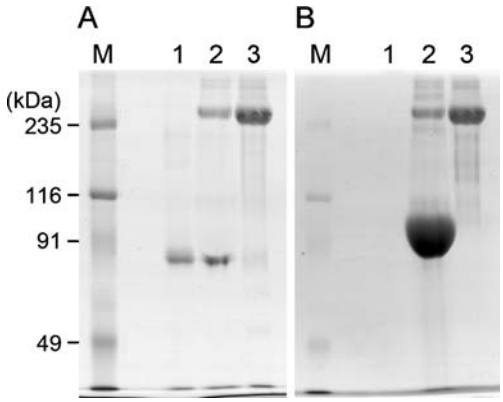

Fig. 2. SDS-PAGE analysis of unmodified bLF and the PEGylated bLF conjugate. (A) Protein staining with Coomassie brilliant blue. Lanes: M, molecular weight marker; 1, unmodified bLF (2 μg); 2, PEGylation reaction mixture; 3, purified 40 k-PEG-bLf (2 μg). (B) PEG-specific staining with barium iodine. Lanes: M and 1–3 are the same as the corresponding lanes in Fig. 2A.

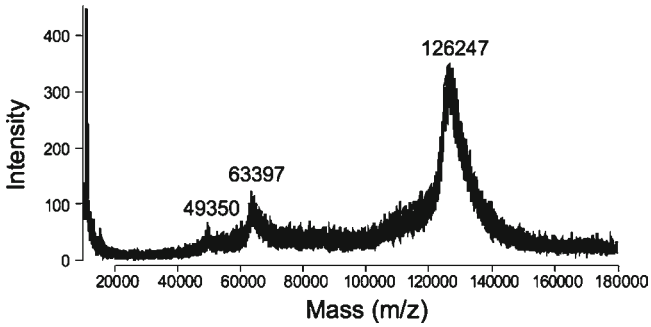

Fig. 3. MALDI-TOF mass spectra of 40 k-PEG-bLf. The molecular mass of 40 k-PEG-bLf was determined to be 126,247 Da.

reportedly involved in anti-oxidative effects because the binding of iron to LF may decrease the rate of conversion of hydrogen peroxide to hydroxyl radicals via the Fenton reaction. We measured the iron content of 40k-PEG-bLf and compared it to that of unmodified bLF. Representative data obtained from 5 independent experiments are shown in Table I. Unmodified bLF and 40k-PEG-bLf bound to iron at concentrations of 833.7 ng and 965.4 ng Fe^{3+} per mg of protein, respectively, revealing the relative binding activity of 40k-PEG-bLf to be 115.8% of unmodified bLF. Thus, 40k-PEG-bLf was fully active in iron binding.

In Vitro Anti-inflammation

As an anti-inflammatory agent, bLF downregulates LPS-induced IL-6 secretion in human monocytic cells (3,4). The effect of 40k-PEG-bLf on this response was analyzed in THP-1 cells. Representative data obtained from 6 independent experiments are shown in Fig. 4A. No IL-6 was detected in the culture fluid of cells that were not treated with LPS, whereas IL-6 was detected at low levels in the supernatants of cells treated with 40k-PEG-bLf alone or with unmodified bLF. Both 40k-PEG-bLf and unmodified bLF inhibited the LPS-induced IL-6 secretion, with mean reductions of 134.5 pg/mL and 137.7 pg/mL IL-6, respectively. Thus, the relative inhibitory activity of 40k-PEG-bLf was approximately 97.7% of that of unmodified bLF. Six independent experiments revealed that the residual anti-inflammatory activity of 40k-PEG-bLf was 97.1±5.5% (means ± S.E., $n=6$) of the native bLF. In order to rule out possible cell toxicity of the added samples, which could account for the observed inhibitory effect, we evaluated the effect of 40k-PEG-bLf on THP-1 cell viability. Neither 40k-PEG-bLf nor bLF affected cell viability, suggesting that the added samples themselves did not exert any cytotoxic effect (Fig. 4B).

Resistance to Peptic Degradation

Fig. 5 shows the proteolytic profiles of native bLF and 40k-PEG-bLf in artificial gastric fluid containing pepsin. Native bLF was rapidly degraded with a half-life of approximately 17 min, whereas 40k-PEG-bLf was not at all degraded at 100 min post-digestion (Fig. 5A and 5B), suggesting that 40k-PEG-bLf has extremely high stability in the gastrointestinal tract.

Table I. Iron-binding Activities of 40k-PEG-bLf

Protein	Fe concentration (ng/mg protein)			Relative binding ratio (% of unmodified bLF)
	apo-LF	holo-LF	bound Fe [a]	
Unmodified bLF	79.7	913.4	833.7	100.0
40k-PEG-bLf	128.9	1094.3	965.4	115.8
BSA [b]	67.0	119.6	52.6	6.3

[a] The concentration of bound Fe was calculated by subtracting the concentration of apo-LF from that of the holo-LF.
[b] BSA was used as a negative control.

N-terminal Amino Acid Sequence of Pepsin-digested 40 k-PEG-bLf

The N-terminal amino acid sequences of pepsin-digested fragments from 40k-PEG-bLf were determined to estimate the PEG conjugation sites on it. Digestion of 40k-PEG-bLf with pepsin gave rise to 4 digestive fragments (Fig. 6A, lane 2). Of these, the 3 fragments indicated by arrows specifically stained with iodine (data not shown), suggesting the presence of PEG-attached fragments. The following N-terminal amino acid sequences were obtained for the 3 PEG-attached fragments: subfragment 1 (approximately 171 kDa), APRKN VRWXT (X indicates an unknown residue); subfragment 2 (approximately 131 kDa), APRKNVRWXT; and subfragment 3 (approximately 114 kDa), APRKNVRWXX. These N-terminal amino acid sequences correspond to amino acids 1–10 of mature bLF (length, 689 amino acids) (Fig. 6B), and all these sequences are present in the N-terminal portion of bLF. These results permit us to speculate that the N-terminal portion of bLF is not the main target site for PEG modification.

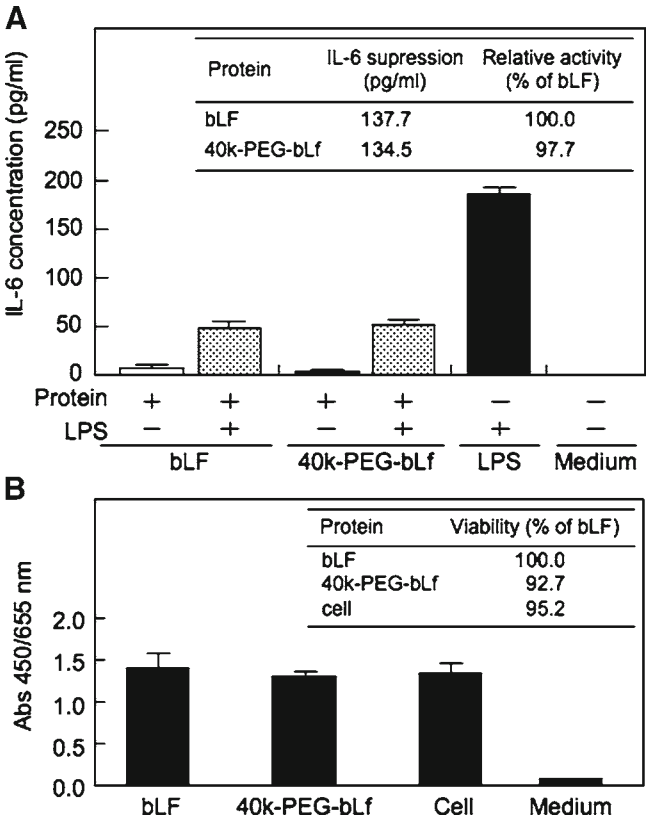

Fig. 4. Inhibition of the LPS-induced IL-6 response in THP-1 cells by 40k-PEG-bLf. (A) ELISA comparison of IL-6 levels in supernatants from LPS-stimulated THP-1 cells. The cells were treated with 100 μg/mL of bovine LF (bLF) or 40k-PEG-bLf after LPS (100 ng/mL) stimulation. All data represent means±S.D. from assays performed in triplicate. The inset shows the mean relative anti-inflammatory activity of 40k-PEG-bLf as compared to that of bLF. The suppressed IL-6 concentrations were calculated by subtracting the values of "bLF+LPS" or "40 k-PEG-bLf+LPS" from "cells treated with LPS alone." (B) Viability of THP-1 cells treated with 40 k-PEG-bLf. bLF or 40 k-PEG-bLf (100 μg/mL) was added to THP-1 cells. All data represent means±S.D. from assays performed in triplicate. The inset shows the mean relative cell viability compared to that of bLF.

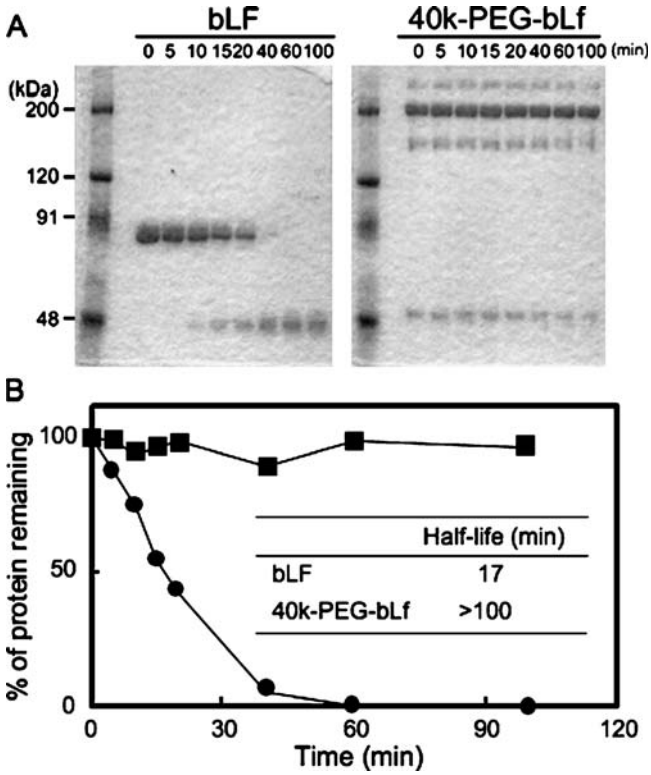

Fig. 5. Degradation profiles of unmodified bLF (●) and 40k-PEG-bLf (■) after pepsin hydrolysis. (A) SDS-PAGE profile of pepsin digested proteins. (B) Degradation rates of pepsin-digested unmodified bLF and 40k-PEG-bLf. Proteins were visualized with Coomassie brilliant blue, and residual protein bands were quantified using Image J software by using reference values of untreated bLF and 40k-PEG-bLf, which were arbitrarily set at 100%. Each data point was derived from 1 experiment and all the time points were from the same incubation. The inset shows the half-life of resistance against pepsin digestion.

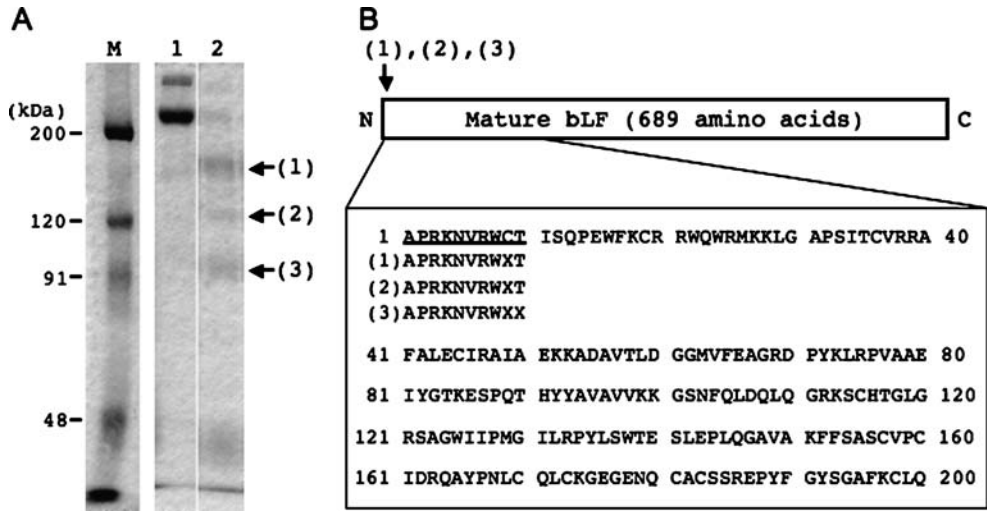

Fig. 6. Deduced N-terminal amino acid sequences of pepsin-digested 40k-PEG-bLf fragments. (A) 40k-PEG-bLf was treated with pepsin at a concentration of 60 ng/mL for 60 min at 37°C. Lanes: M, molecular weight markers; 1, undigested 40k-PEG-bLf; 2, 40k-PEG-bLf digested with pepsin. (B) Schematic diagram showing the location of the N-terminal amino acid sequences from pepsin-digested 40k-PEG-bLf.

Pharmacokinetic Profiles of 40k-PEG-bLf in Rats

Pharmacokinetic parameters of unmodified bLF and 40 k-PEG-bLf were determined by the intravascular administration of these proteins in male Wistar rats. The mean plasma concentrations of bLF *versus* time curves of 40k-PEG-bLf (1 mg/kg) and unmodified bLF (1 mg/kg, 10 mg/kg) are shown in Fig. 7. No bLF was detected in the plasma before injection. The maximum plasma levels of 6.9±1.3 μg/mL (means ± S.E., n=7) for unmodified bLF (1 mg/kg), 40.5± 5.4 μg/mL (means ± S.E., n=5) for unmodified bLF (10 mg/kg) and 6.6±0.1 μg/mL (means ± S.E., n=7) for 40 k-PEG-bLf (1 mg/kg), were observed 1 min postinjection. The concentration of bLF rapidly decreased and became undetectable at 60 min and 120 min after administration of 1 mg/kg and 10 mg/kg unmodified bLF, respectively. In contrast, 40 k-PEG-bLf continued to be detected up to 240 min after intravenous administration. The AUC levels were 80.5± 8.3 (μg·min)/mL (means ± S.E., n=7) for unmodified bLF (1 mg/kg), 908.1±40.6 (μg·min)/mL (means ± S.E., n=5) for unmodified bLF (10 mg/kg), and 921.3±33.0 (μg·min)/mL (means ± S.E., n=7) for 40k-PEG-bLf (1 mg/kg). Thus, the AUC of 40k-PEG-bLf was approximately 11.4-fold higher than that of unmodified bLF (1 mg/kg treatment group). The 40k-PEG-bLf conjugate had a half-life of 67.9±0.9 min, whereas unmodified bLF (1 mg/kg) had a half-life of 7.8± 0.2 min. Therefore, PEGylation of bLF led to an 8.7-fold prolongation of the half-life as compared to that of unmodified bLF (1 mg/kg).

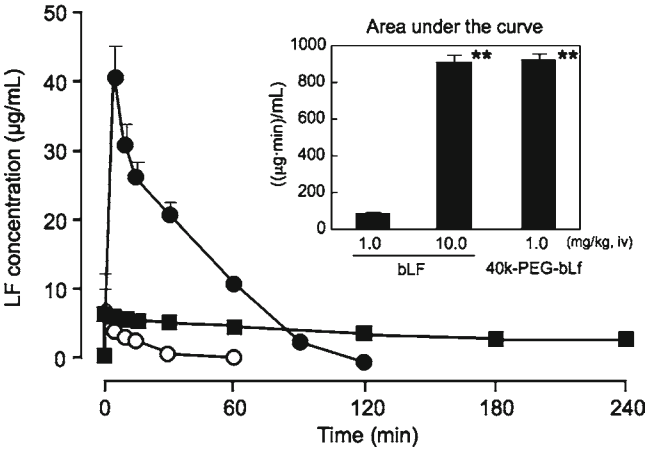

Fig. 7. Pharmacokinetic profiles of unmodified bLF and 40 k-PEG-bLf after intravenous injection. Open circles, closed circles, and closed squares show the data from rats injected at a dosage of 1 mg/kg (unmodified bLF), 10 mg/kg (unmodified bLF) and 1 mg/kg (40 k-PEG-bLf), respectively. The inset presents data of the area under the curve (AUC). Blood was collected at the indicated time points and assayed for bLF plasma levels by ELISA. Data are represented as means±S.E. of 5–7 rats. **Significant difference from unmodified bLF (1 mg/kg) administration (P<0.01).

DISCUSSION

There has been increasing interest in the PEGylation procedure as it can be used to enhance the therapeutic and biotechnological potential of peptides and proteins. Many PEG conjugates have been developed and approved for the treatment of disease (14). Recently, we developed a conjugate by PEGylation of bLF with 20-kDa branched PEG. This conjugate, designated 20k-PEG-bLf, possesses a high level of biological activity and exhibits enhanced pharmacokinetic properties (15). In this study, we selected a branched PEG with a molecular mass of 40 kDa to generate a PEGylated bLF that is more potent than 20k-PEG-bLf. Comparison of the *in vitro* bioactivity, *in vitro* peptic susceptibility, and pharmacokinetic properties between 20k-PEG-bLf and 40k-PEG-bLf is summarized in Table II.

NHS active esters of PEG (PEG-NHS) are frequently used for amino group modification of target proteins. NHS-activated esters produce stable amide linkages between PEG and primary amines such as N-terminal α-amine and lysine ε-amine residues. Since bLF has 54 internal lysine residues that are potential attachment sites, linear PEG-NHS with average molecular weights of 5 kDa and 30 kDa could be used for the

Table II. Comparison of the *In Vitro* Bioactivity, Peptic Susceptibility and Pharmacokinetic Properties Between 20 k-PEG-bLf and 40 k-PEG-bLf

Protein	Iron-binding activity (% of unmodified bLF)	Anti-inflammatory activity (% of unmodified bLF)	Resistance to peptic degration (Half-life, min)[d]	Pharmacokinetic parameters [b,c]	
				Half-life (min)	AUC ((μg·min)/mL)
Unmodified bLF	100	100	17	7.8±0.2	80.5±8.3
20k-PEG-bLF [a]	100	69.6±2.9 [b]	35	42.4±0.1	739.7±72.1
40k-PEG-bLF	100	97.1±5.5 [b]	>100	67.9±0.9	921.3±33.0

[a] Nojima, Y. *et al.* (15).
[b] Data represents mean±S.E. (n=6-7).
[c] Data from rats injected intravenously at a dosage of 1 mg/kg.
[d] Representative data obtained from two independent experiments. AUC, area under the curve.

production of hyper-modified PEGylated bLF as judged from smear patterns on an SDS-PAGE gel (data not shown). On the other hand, branched PEG-NHS forms with average molecular weights of 20 kDa (15) and 40 kDa (present study) led to the successful preparation of PEGylated bLF with lower heterogeneity, because they gave discrete bands on an SDS-PAGE gel. The branched shape of PEG may lead to its attachment to fewer binding sites on bLF due to its higher steric hindrance as compared to linear PEG molecules (25). The SDS-PAGE profile and MALDI-TOF mass spectra revealed that the 40k-PEG-bLf conjugate consisted of mono-PEGylated bLF, which was judged by the appearance of a single band in the gel and a molecular mass of its conjugate. On the other hand, 20k-PEG-bLf consisted of a mixture of mono-, di-, tri- and tetra-PEGylated bLF molecules (15). This is probably because not all PEGylation sites in bLF are readily accessible to the 40-kDa branched PEG due to its larger size and branched structure. The 40k-PEG-bLf conjugate with lower heterogeneity would be a more suitable drug candidate as compared to 20k-PEG-bLf, although the former is probably composed of different positional isomers.

One of the most important considerations in PEGylation is selecting the appropriate size of PEG derivatives. The therapeutic utility of PEGylated proteins requires an optimized balance between enhanced pharmacokinetics and reduced bioactivity by the proper selection of PEG size. In general, PEGylation with larger PEG molecules results in conjugate proteins with improved pharmacokinetic properties, but the resultant protein may exhibit reduced bioactivity (16). As expected, 40k-PEG-bLf showed enhanced pharmacokinetic properties as compared to 20k-PEG-bLf. The AUC values increased from 739.7±72.1 (μg·min)/mL for 20k-PEG-bLf (1 mg/kg treatment group) to 921.3±33.0 (μg·min)/mL for 40k-PEG-bLf (1 mg/kg). The plasma half-life increased from 42.4±0.1 min for 20k-PEG-bLf (1 mg/kg) to 67.9±0.9 min for 40 k-PEG-bLf (1 mg/kg). The maximum plasma levels of 9.1±1.0 μg/mL for 20k-PEG-bLf (1 mg/kg) and 6.6±0.1 μg/mL for 40k-PEG-bLf (1 mg/kg) were observed 5 min and 1 min postinjection, respectively. On the other hand, 40k-PEG-bLf showed higher residual bioactivity than 20k-PEG-bLf. The iron-binding activity was equivalent to that observed in the native bLF and 20k-PEG-bLf (100%). The anti-inflammatory activity of 40k-PEG-bLf was comparable to that of native bLF (97.1±5.5%) and was approximately 1.4-fold higher than that of 20k-PEG-bLf. Higher bioactivities of 40k-PEG-bLf may be explained by the distinct PEG attachment site from that of 20k-PEG-bLf. Furthermore, 40k-PEG-bLf showed a significant improvement in resistance to pepsin treatment as compared to native bLF (>5.9-fold) and 20k-PEG-bLf (>2.9-fold). Therefore, as compared to 20k-PEG-bLf, 40k-PEG-bLf is a more potent drug candidate for *in vivo* pharmacological studies.

LF is an orally administered protein. It should be stressed, however, that no information is available on oral bioavailability for bLF except for the studies by Takeuchi, T. *et al.* They have reported that the intraduodenally administred bLF was transported into blood circulation via the thoracic duct lymph fluid in adult rats (23). Following intraduodenal administration of bLF (1 g/kg), the transported bLF was detected in the plasma and reached a peak value (221.6 ng/mL) 2 h postadministration. The relationship between the pharmacologic actions of bLF and its plasma levels were also uncertain, mainly due to the difficulties in quantitating low bLF concentrations in the blood. The pharmacokinetic characteristics of orally administered native and PEGylated bLF remain to be elucidated.

The N-terminal region of LF contains unique clusters of basic amino acid residues that are involved in various biological functions such as antimicrobial and antiviral activities and affinity to heparin and LPS (26–28). The N-terminal amino acid sequences of pepsin-digested fragments from 40k-PEG-bLf indicate that PEGylation did not modify the N-terminal portion of bLF. This also explains the high residual activity of 40k-PEG-bLf.

The 20 k-PEG-bLf conjugate exhibited enhanced absorption from the rat intestine as compared to native bLF (15). Although we did not examine the uptake of 40k-PEG-bLf from the intestine in this study, oral administration of 40k-PEG-bLf exhibited enhanced analgesic effects in mice as compared to oral administration of native bLF performed in a preliminary study (Takeuchi, T. *et al.*, unpublished data). This result indicates that 40k-PEG-bLf can be delivered into the systemic circulation via the enteric route.

CONCLUSION

We have developed PEGylated bLF with 2-branched 40-KDa PEG (40k-PEG-bLf). This conjugate showed higher residual bioactivity (100% and 97.1% of the original iron-binding and anti-inflammatory activities, respectively) and enhanced pharmacokinetic properties as compared to those of the recently developed 20k-PEG-bLf (a 1.6-fold prolongation of the half-life). We believe that 40k-PEG-bLf is a potential drug candidate with high clinical performance.

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