JPP Journal of Pharmacy and Pharmacology

Royal Pharmaceutical Society of Great Britain

JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 985–994

Received February 04, 2010
Accepted April 20, 2010
DOI 10.1111/j.2042-7158.2010.01120.x
ISSN 0022-3573

Research Paper

PEGylation of osteoprotegerin/osteoclastogenesis inhibitory factor (OPG/OCIF) results in decreased uptake into rats and human liver

Motoko Saito-Yabe[a], Yuji Kasuya[b], Yasushi Yoshigae[a], Naotoshi Yamamura[a], Yukie Suzuki[b], Nao Fukuda[b], Masashi Honma[b], Kazuki Yano[c], Shin-ichi Mochizuki[d], Fumihiko Okada[c], Akiko Okada[c], Yuki Nagayama[c], Eisuke Tsuda[c], Thomas Fischer[e], Ursula Höpner[e], Silvia Zaja[e], Juergen Mueller[e], Junichi Okada[f], Atsushi Kurihara[a], Toshihiko Ikeda[a] and Osamu Okazaki[a]

[a]Drug Metabolism and Pharmacokinetics Research Laboratories, [b]Formulation Technology Research Laboratories, [c]Biological Research Laboratories III, [d]Clinical Development Department II, Daiichi Sankyo Co., Ltd., Tokyo, Japan, [e]Drug Metabolism Department, Daiichi Sankyo Europe GmbH, Martinsried, Germany and [f]Daiichi Sankyo Pharma Development, Inc., Edison, NJ, USA

Abstract

Objectives Our aim was to investigate the effect of PEGylation on the uptake of osteoprotegerin/osteoclastogenesis inhibitory factor (OPG/OCIF) into rat liver, kidney and spleen, and human liver.

Methods Copolymer of polyethyleneglycol allylmethylether and maleamic acid sodium salt with OCIF (poly(PEG)-OCIF) (0.5 mg/kg) was administered to rats and the concentrations of poly(PEG)-OCIF in the liver, kidney and spleen at 15 min after administration were measured by ELISA. For human liver uptake, the liver perfusion of OCIF and ^{3}H-labelled poly(PEG)-OCIF was conducted using fresh human liver block.

Key findings The tissue uptake of poly(PEG)-OCIF in rats was significantly lower compared with that of OCIF. In fresh human liver perfusion, ^{3}H-poly(PEG)-OCIF was rarely taken up into the liver. On the other hand, more than 50% of the perfused OCIF was taken up.

Conclusions PEGylation of OCIF using poly(PEG) dramatically suppressed the uptake of OCIF into human liver as well as into rat liver and could be a promising approach for improving the pharmacokinetic and pharmacological effects of OCIF in the clinical setting.

Keywords osteoclastogenesis inhibitory factor; osteoprotegerin; PEGylation; pharmacokinetics; tissue distribution

Introduction

Bone is a dynamic tissue that is morphogenized and maintained by continuous formation and resorption. Disorder of bone formation and resorption causes metabolic bone diseases, such as osteopetrosis and osteoporosis. Osteoprotegerin/osteoclastogenesis inhibitory factor (OPG/OCIF) is a member of the TNF receptor superfamily[1–3] that acts as a soluble secreted receptor for the receptor activator of nuclear factor-kappaB ligand (RANKL) and prevents it from activating the receptor activator of nuclear factor-kappaB (RANK) on the osteoclast surface.[3–5] The pharmacological effect of OCIF was confirmed using recombinant OCIF to increase bone density and strength in rodents and primates.[3,6] In these investigations, OCIF was also found to improve the bone turnover state, and hence would have a therapeutic effect on bone disorders such as osteoporosis or rheumatoid arthritis. However, frequent administration would be needed to obtain a sufficient pharmacological effect due to the short terminal half-life ($t_{1/2\beta}$, 20.0 ± 1.2 min) in the serum after administration to rats.[7] It is reported that OCIF has a highly basic heparin-binding domain (HBD) at the C-terminal,[8] which is supposed to interact strongly with heparin sulfate proteoglycan (HSPG)[9] expressed

Correspondence: Ms Motoko Saito-Yabe, Drug Metabolism & Pharmacokinetics Research Laboratories, Daiichi Sankyo Co. Ltd, 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan.
E-mail: saito.motoko.zf@daiichisankyo.co.jp

on the surface of the liver and other organs. Therefore, modification of the C-terminal region, which has no precise effect on controlling the in-vitro biological activity of OCIF, is a key point to control PEGylation. To prolong the $t_{1/2}$ of OCIF, modification of the HBD seems effective as suggested by Liu *et al*.[10] In addition to OCIF, hepatocyte growth factor (HGF) possesses the HBD, which is positively charged. When HGF is premixed with heparin, a negatively charged polymer, and then given intravenously, its plasma clearance is reduced. This is probably because heparin is electrostatically bound to the HBD of HGF and interferes with the binding of HGF to the liver via HSPG on the cell surface.[11,12] According to this idea, we synthesized copolymer of polyethyleneglycol allylmethylether and maleamic acid sodium salt with OCIF (poly(PEG)-OCIF) and confirmed the reduction of the binding affinity of poly(PEG)-OCIF to heparin, as compared with that of OCIF. In this study, we succeeded in improving the retention of the serum concentration and decreasing tissue uptake of OCIF in rats without modifying the peptide sequence by PEGylation of intact OCIF. In addition, we investigated the human liver uptake of poly(PEG)-OCIF using a radiolabelled conjugate and estimated the liver uptake of poly(PEG)-OCIF in the clinical setting.

Materials and Methods

Reagents

OCIF, poly(PEG)-OCIF and OI-19, a mouse monoclonal antibody against OCIF, were produced by Daiichi Sankyo Co. Ltd (Tokyo, Japan). Peroxidase (POD)-labelled mouse monoclonal antibody against OCIF (POD-labelled OI-4) was synthesized at Daiichi Sankyo Co. Ltd. An enzyme-labelled antibody, anti-human IgG conjugate with horseradish peroxidase, was purchased from Amersham Biosciences (Buckinghamshire, UK). PolyPEG500-MAn(6k) (AM-0510K; molecular weight (M.W.), ca. 6000; M.W. of monomethoxypolyethylenglycol, ca. 500), the precursor of poly(PEG), was purchased from NOF Corporation (Tokyo, Japan). The chemical structure of AM-0510K is shown in Figure 1a. ^3H-$NaBH_4$ was purchased from PerkinElmer Life Sciences Inc. (Wellesley, USA). Streptavidin–horseradish peroxidase conjugate module was purchased from Amersham Biosciences.

Synthesis of poly(PEG) and poly(PEG)-OCIF

For the synthesis of poly(PEG), AM-0510K was dissolved in 28% of aqueous ammonia and stirred at 25°C for 5 h. After the reaction, 1 N NaOH was added and N_2 bubbling was performed for 2 h to remove excess ammonia. The chemical structure of poly(PEG) and the synthetic scheme of poly(PEG) are shown in Figure 1b and 1d, respectively. For the synthesis of poly(PEG)-OCIF, a portion of poly(PEG) was added to the OCIF solution at a molar ratio of 1.5 (modifier/OCIF). The pH of the mixing solution was adjusted to 4.5 using 1 N HCl and it was then incubated at 37°C for 96 h. To remove a majority of free PEG, PBS (10 mM phosphate buffer, 150 mM NaCl, pH7.4) was added and the mixture was concentrated by ultrafiltration (Centriprep YM-50, Millipore, Billerica, USA). The supposed synthetic scheme of poly(PEG)-OCIF is shown in Figure 1e. The protein concentrations of poly(PEG)-OCIF and OCIF were determined by the Lowry method.[13]

Physicochemical characterization of poly(PEG)-OCIF conjugates

The apparent M.W. of poly(PEG)-OCIF was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reduced conditions. Samples were diluted with sample buffer (4 ×; Invitrogen Life Technology, Carlsbad, USA). Tris-acetate gel (3–8%; Invitrogen Life Technology) was used along with Tris-acetate SDS running buffer (20 ×; Invitrogen Life Technology). The gels were run at 150 V for 60 min. The gels were then fixed and stained with Coomassie Blue (R-250).

In-vitro biological activity of OCIF and poly(PEG)-OCIF

The in-vitro biological activity of OCIF and poly(PEG)-OCIF was determined by mouse osteoclast formation assay using a co-cultured system of ST2 stromal cells and mouse spleen cells, and the tartaric acid-resistant acid phosphatase (TRAP) activity was measured.[14] It was determined by the parallel line method (3×3) using the values of absorbance and concentration of test compounds, and the relative inhibitory activity of poly(PEG)-OCIF to that of OCIF was calculated.

Pharmacokinetics of OCIF and poly(PEG)-OCIF after intravenous administration to rats

Each dosing solution was intravenously administered to female Sprague–Dawley rats obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) at a dose of 0.3 mg/kg (2 ml/kg; $n = 3$–4). After dosing, blood samples were collected from the jugular vein under diethyl ether anaesthesia at 5, 15, 30 min, 1, 2, 4, 8, 24, 48 and 72 h post-dose and prepared to obtain serum samples. The pharmacokinetic parameters of OCIF and poly(PEG)-OCIF were calculated by the noncompartmental approach using WinNonlin Professional Edition (version 3.1; Pharsight Co., Mountain View, USA).

Tissue uptake of OCIF and poly(PEG)-OCIF in rats

Each dosing solution was intravenously administered to female Sprague–Dawley rats ($n = 3$) at a dose of 0.5 mg/kg (2 ml/kg). After dosing, blood samples were collected from the jugular vein under diethyl ether anaesthesia at 2, 5, 10 and 15 min post-dose. After the last blood sampling at 15 min, blood was taken from the aorta and the liver, kidney and spleen were excised from the rat carcass. The excised tissues were weighed in their wet condition. A two-fold volume of PBS containing protease inhibitor (1 mM phenylmethanesulfonylfluoride, 1 μg/ml leupeptin and 1 μg/ml pepstatin) was added to the tissue samples and homogenized. The tissue samples were adequately diluted with dilution buffer (0.2 M Tris-HCl, containing 40% Block Ace, 10 μg/ml mouse IgG and 0.1% Tween 20, pH7.4). The uptake clearance (CL_{uptake}) of each tissue was calculated according to the following equation:

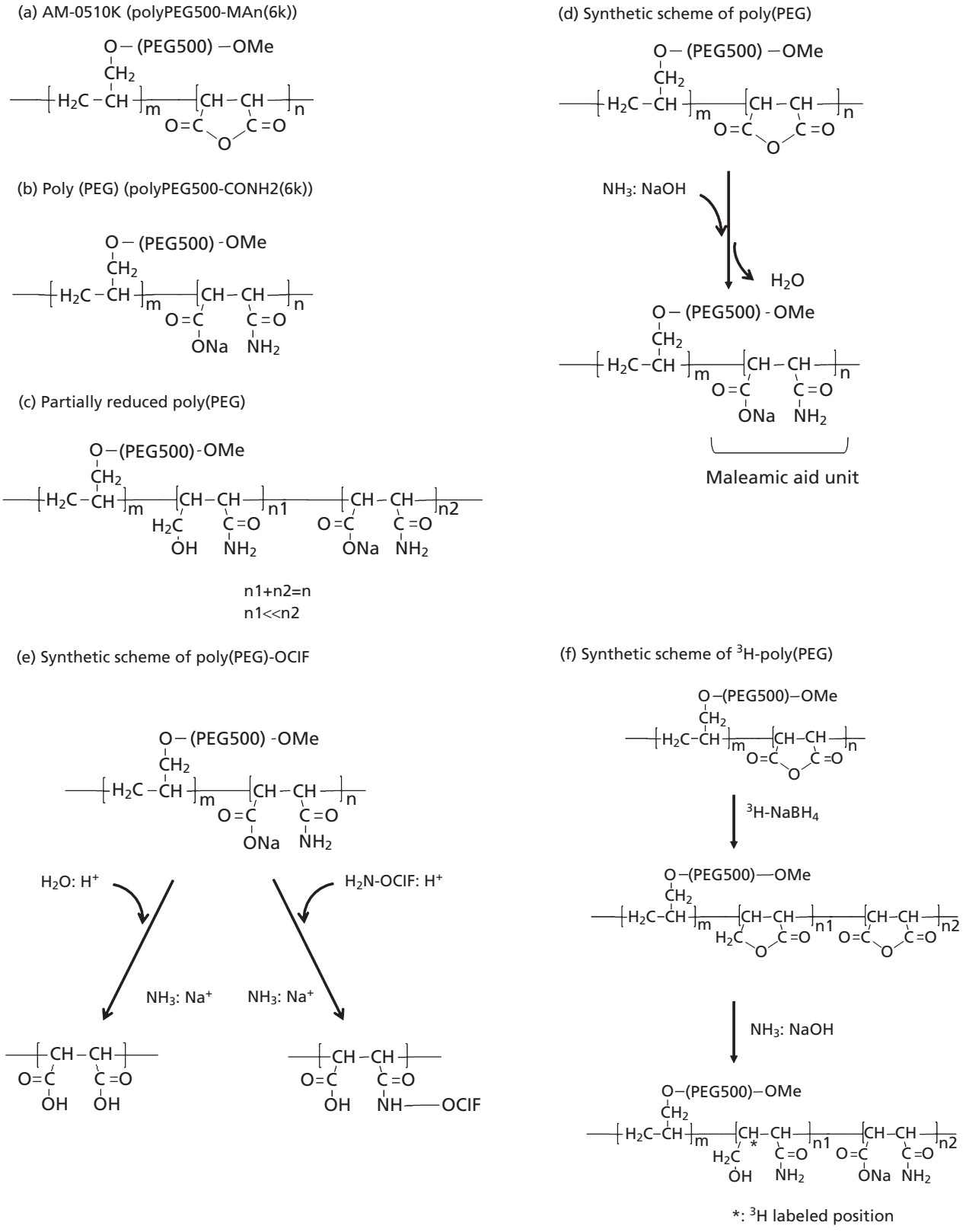

Figure 1 Structures of AM-0510K (a), poly(PEG) (b), partially reduced poly(PEG) (c) and reaction scheme of the synthesis of poly(PEG) (d), poly(PEG)-OCIF (e) and ^{3}H-poly(PEG) (f).

$$CL_{uptake} = \text{OCIF or poly(PEG)-OCIF concn in each tissue} \times \text{tissue weight} / AUC_{0\text{-}15\,min} / \text{body weight (ml/h/kg)} \quad (1)$$

where $AUC_{0–15\,min}$ is the area under the serum concentration–time curve up to 15 min and is calculated using WinNonlin Professional Edition (version 3.1).

Determination of OCIF and poly(PEG)-OCIF

The concentrations of OCIF and poly(PEG)-OCIF in serum and tissue samples were determined by an ELISA method. Each well of a 96-well plate (C96 Maxisorp; Nalge Nunc International, Rochester, USA) was coated with 100 μl of OI-19 solution that had been diluted to 10 μg/ml with 0.1 M sodium hydrogencarbonate buffer (pH 9.6). After overnight storage at 4°C, blocking was conducted with blocking buffer (purified water containing 50% Block Ace, 300 μl/well; Dainippon Pharma Co. Ltd., Suita, Japan) for 2 h at room temperature. After washing the wells with 300 μl of PBS containing 0.1% Tween 20 (washing buffer), 100 μl of serum samples or tissue samples were added to the wells and the plate was incubated at room temperature for 2 h. After washing, 100 μl of POD-labelled OI-4 was added to the wells. Then the plate was incubated at room temperature for 2 h. After washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) soluble reagent (ScyTek Laboratories, West Logan, USA) was added, and the plate was incubated at room temperature for 10–15 min. Finally, 100 μl of TMB stop buffer (ScyTek Laboratories) was added to each well and absorbance on the plate was read at 450 nm.

Synthesis of ^{3}H-poly(PEG) and ^{3}H-poly(PEG)-OCIF

Radio-labelled poly(PEG)-OCIF, which is a conjugate of OCIF and a partially reduced form of poly(PEG) labelled with ^{3}H at the reduction position, and the synthetic scheme of ^{3}H-poly(PEG) are shown in Figure 1c and 1f, respectively. The reduction of maleic anhydride units was carried out according to the method reported by Barton *et al*.[15] AM-0510K was dissolved in anhydrotetrahydrofuran and ^{3}H-$NaBH_4$ was added on ice. The reaction mixture was stirred at room temperature for 24 h. After stirring, 1,4-dioxane containing 0.5 M NH_3 was added to the solution and stirred at room temperature for another 24 h. The procedure described above was conducted in N_2 gas atmosphere. After the production of precipitation by adding diethylether, 5 N NaOH and PBS (100 mM phosphate buffer and 150 mM NaCl, pH7.4) were added to the solution, mixed and left to rest. The solution was separated into two phases and the aqueous layer was collected and concentrated by ultracentrifugation (Centriprep YM-3; Millipore). This concentrated ^{3}H-poly(PEG) was purified by GPC using HiLoad 16/60 Superdex200 (Amersham Biosciences). The fraction containing ^{3}H-poly(PEG) was concentrated by the same procedure.

For the synthesis of ^{3}H-poly(PEG)-OCIF, a portion of ^{3}H-poly(PEG) obtained above was added to the OCIF solution in the molar ratio of 1.7:1 (^{3}H-poly(PEG)/OCIF) and the pH was adjusted to 4.7 using 1 N HCl. The mixture was incubated at 37°C for 96 h. After incubation, 5 N NaOH was added to adjust the pH to 7.4 and then concentrated by ultrafiltration (Centriprep YM-50; Millipore). This concentrated ^{3}H-poly(PEG)-OCIF was purified by GPC using HiLoad 16/60 Superdex200 with HPLC (LC-10A; Shimadzu, Kyoto, Japan) at a wavelength of 280 nm. The fractions containing ^{3}H-poly(PEG)-OCIF were collected and concentrated by ultrafiltration (Centriprep YM-50; Millipore).

Human liver perfusion study

A stock solution of OCIF and ^{3}H-poly(PEG)-OCIF was diluted with Krebs–Henseleit buffer (KHB) containing 50 mM glucose and 4% bovine serum albumin to prepare a test solution containing 200 μg/ml of both OCIF and ^{3}H-poly(PEG)-OCIF. The liver block was weighed in wet condition before the experiment. The human liver block was perfused with KHB via the visible vessels for 10 min at a flow rate of 10 ml/min, followed by perfusion with the test solution for several minutes and was then washed again with KHB for another 10 min at the same flow rate. The outflow from the liver block was collected in 9–11 fractions of 1 ml. A four-fold amount of KHB was added to the liver section, which was then homogenized. The outflow and the homogenate samples were subjected to radioactivity measurement using a liquid scintillation counter (LSC, Tricarb 2900 TR; Packard Instrument Company, Wellesley, USA). In addition to that, the total concentrations of OCIF and ^{3}H-poly(PEG)-OCIF in the outflow and the homogenate samples were determined by the ELISA described above. The amount of OCIF in the samples was determined by the difference in the values calculated from ELISA and LSC because in our ELISA system both OCIF and poly(PEG)-OCIF were detected simultaneously. The human liver tissue was obtained from Hepacult GmbH (Regensburg, Germany) and experimental procedures for human tissue preparation ($n = 3$) were performed according to the guidelines of the charitable state-controlled foundation HTCR, with the informed patients' consent.[16]

Binding of OCIF and poly(PEG)-OCIF to a heparin column

The affinity of OCIF and poly(PEG)-OCIF to heparin was investigated using a heparin column (HiTrap Heparin HP 5 × 1 ml; Amersham Pharmacia Biotech). The column was connected to a 5-ml syringe and washed with 5 ml of PBS via a syringe. After that, 1 ml of the OCIF or poly(PEG)-OCIF solutions adjusted to a concentration of 100 μg/ml, which was diluted with PBS containing 0.1% of Tween20, was then applied and eluted with 5 ml of PBS containing 0.3 M NaCl, followed by 5 ml of PBS containing 2 M NaCl. Eleven eluted fractions of 1 ml each were collected just after applying the samples. The concentration of OCIF or poly(PEG)-OCIF in each fraction was measured by the ELISA as described in the section Determination of OCIF and poly(PEG)-OCIF.

Pharmacological study of OCIF and poly(PEG)-OCIF in ovariectomized rats

The animal experiments were conducted in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals. For the pharmacological study of OCIF, female Sprague–Dawley rats were obtained from Charles River Laboratories Japan, Inc., and bilateral ovariectomy or a sham

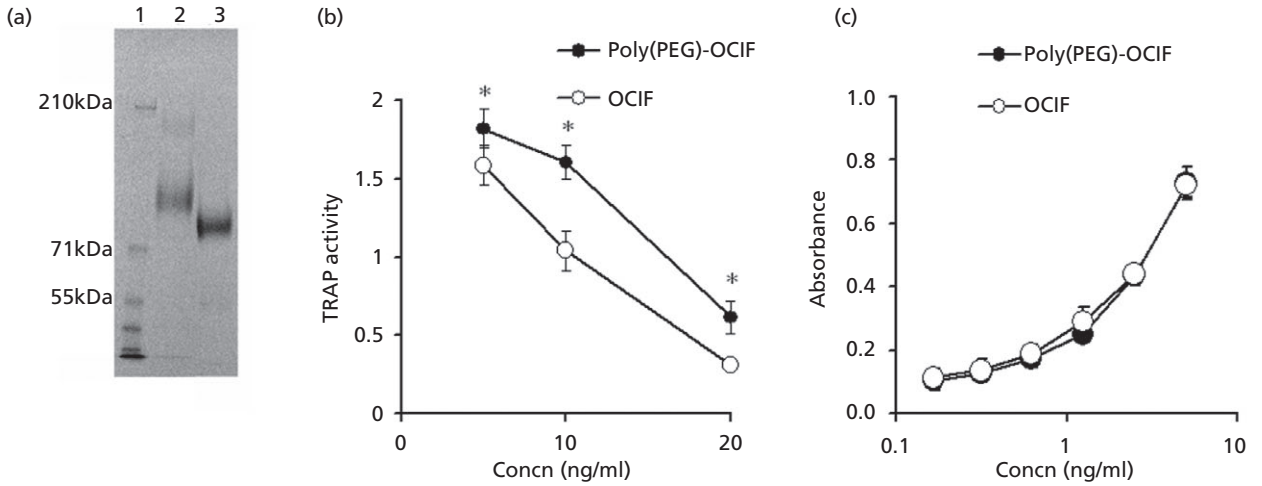

Figure 2 Physicochemical and pharmacological properties of poly(PEG). (a) SDS-PAGE analysis of poly(PEG)-OCIF is shown (lane 1, molecular weight; lane 2, poly(PEG)-OCIF; lane 3, intact OCIF). (b)TRAP activity and (c) immunoreactivity of OCIF and poly(PEG)-OCIF. Each value represents the mean ± SD of three experiments in (b) and (c). $*P < 0.05$ compared with OCIF (Student's *t*-test).

operation was performed at nine weeks of age. The day after the operation, the rats in sham and ovariectomized (OVX)-vehicle groups were intravenously administered with PBS (10 mM phosphate buffer, 150 mM NaCl containing 0.01% (w/v) polysorbate 80, pH7.4) twice a day for 2 weeks ($n = 8$). The rats in the OVX-OCIF group were intravenously administered with OCIF (0.5 mg/kg) twice a day for two weeks ($n = 12$). For the pharmacological study of poly(PEG)-OCIF, female F344 rats were obtained from Charles River Laboratories Japan, Inc., and bilateral ovariectomy or sham operation was performed at 12 weeks of age. The day after the operation, the rats in sham and OVX-vehicle groups were subcutaneously administered with PBS (10 mM phosphate buffer, 150 mM NaCl, containing 0.01% (w/v) polysorbate 80, pH7.4) once a week for 4 weeks ($n = 8$). The rats in the OVX-poly(PEG)-OCIF group were subcutaneously administered with poly(PEG)-OCIF at a dose of 0.3 mg/kg once a week for 4 weeks ($n = 8$). At the end of the administration period, all rats in both groups were sacrificed under anaesthesia and the left and right femurs were excised. The femurs were subjected to measurement of bone mineral density (BMD) by a dual-energy X-ray absorptiometry using DCS-600EX-IIIR (Aloka Co., Ltd, Tokyo, Japan).

Statistical analysis

For the pharmacokinetic parameters of OCIF and poly(PEG)-OCIF, statistical analysis was performed by Student's *t*-test. For AUC_{inf}, CL_{total} and V_d, Student's *t*-test was applied for log-transformed data. For the uptake clearance, a statistical analysis comparing the CL_{uptake} of OCIF with that of poly(PEG)-OCIF was performed by Student's *t*-test. For CL_{uptake}, Student's *t*-test was applied for log-transformed data. For TRAP activity and immunoreactivity of OCIF and poly(PEG)-OCIF, statistical analysis was performed by Student's *t*-test. Statistical analysis comparing the amount of the perfusate of poly(PEG)-OCIF with that of OCIF and the amount of the liver uptake of poly(PEG)-OCIF with that of OCIF was conducted by paired *t*-test. For the femoral BMD values, statistical analyses comparing sham and OVX group, OVX and OCIF or poly(PEG)-OCIF treated group were performed by Student's *t*-test.

Results

Physicochemical characteristics

SDS-PAGE was performed using a series of proteins with known M.W. for the analysis of poly(PEG)-OCIF and poly(PEG). The apparent M.W. of poly(PEG)-OCIF was about 140 kDa as determined by SDS-PAGE (Figure 2a). The difference of 20 kDa in the M.W. between OCIF (about 120 kDa) and poly(PEG)-OCIF is considered to correspond to the amount of poly(PEG) attached to OCIF. Using the apparent M.W. of poly(PEG) (6 kDa), the average number of poly(PEG) chains attached to OCIF was estimated to be about three. On the other hand, as a result of gel permeation chromatography (GPC) analysis, the apparent M.W. of poly(PEG)-OCIF was 300 kDa, much larger than that determined by SDS-PAGE (data not shown). This discrepancy may have resulted from the extent of the expansion of poly(PEG) in GPC medium (PBS). As a result, the hydrodynamic size of poly(PEG)-OCIF is larger than intact protein with a similar molecular mass, as reported by Dhalluin *et al*.[17]

As shown in Figure 2a, the aggregate was detected near 210 kDa, but the aggregate content was very low (approximately 2%, as determined by GPC). From the circular dichroism of OCIF and poly(PEG)-OCIF, the spectra of both compounds were nearly superimposable and modification with poly(PEG) had no significant effect on the secondary structure of OCIF (data not shown).

Biological activity and immunoreactivity of poly(PEG)-OCIF

Figure 2b shows the in-vitro biological activity determined by TRAP assay. The TRAP activity of poly(PEG)-OCIF was

significantly higher than that of OCIF ($P < 0.05$) and the ratio of the biological activity of poly(PEG)-OCIF to OCIF was calculated to be 67.4%, according to the parallel line method. The ELISA response curves of OCIF and poly(PEG)-OCIF are shown in Figure 2c. The immunoreactivity of poly(PEG)-OCIF was comparable with that of intact OCIF, suggesting that modification by poly(PEG) did not interfere with the binding of OCIF and OI-19 or OI-4 (mouse monoclonal antibody against OCIF for ELISA).

Pharmacokinetics of OCIF and poly(PEG)-OCIF after intravenous administration

The serum concentrations of OCIF and poly(PEG)-OCIF after intravenous administration of each compound are shown in Figure 3. The pharmacokinetic parameters are summarized in Table 1. The AUC_{inf} value of poly(PEG)-OCIF was about 58 times higher than that of OCIF, and the CL_{total} value dramatically decreased due to PEGylation. Poly(PEG)-OCIF disappeared from the serum gradually and bi-exponentially with a $t_{1/2}$ value of 7.59 ± 0.34 h. On the other hand, OCIF was eliminated from serum with a $t_{1/2}$ value of 3.91 ± 0.24 h.

Tissue uptake of OCIF and poly(PEG)-OCIF in rats

Tissue uptake clearance (CL_{uptake}) and % of dose of tissue uptake of OCIF and poly(PEG)-OCIF after intravenous administration of each compound are summarized in Figure 4 and Table 2. About 70% of the administered OCIF was taken up into the liver as compared with only about 6% of administered poly(PEG)-OCIF. OCIF and poly(PEG)-OCIF were scarcely taken up into the kidney and spleen. The CL_{uptake} of poly(PEG)-OCIF in the liver was about 140 times lower than that of OCIF, and a significant difference between CL_{uptake} of OCIF and that of poly (PEG)-OCIF was observed.

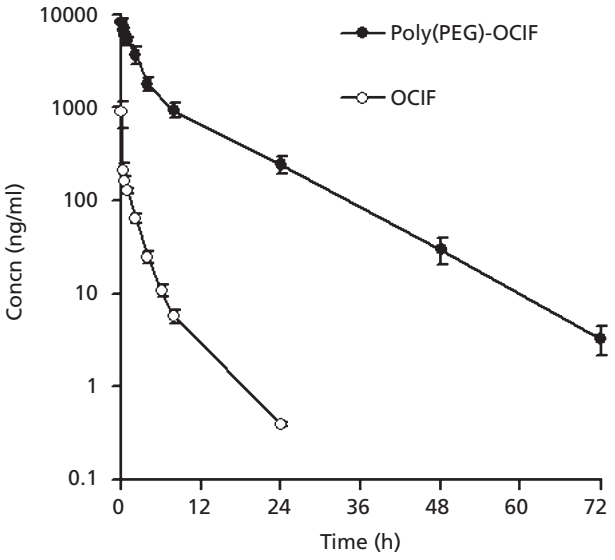

Figure 3 Serum concentrations of OCIF and poly(PEG)-OCIF after intravenous administration to rats at a dose of 0.3 mg/kg of each compound. Each value represents the mean ± SD of three or four rats.

Human liver uptake of OCIF and ^{3}H-poly(PEG)-OCIF

To estimate the liver uptake of poly(PEG)-OCIF in the clinical situation, we investigated the liver uptake of poly(PEG)-OCIF using fresh human liver block. The amount of ^{3}H-poly(PEG)-OCIF and OCIF in liver after perfusion of both compounds is shown in Figure 5. The ratio of liver uptake to perfusate for ^{3}H-poly(PEG)-OCIF was 0.006 and that for OCIF was 0.613, suggesting that the PEGylation of OCIF significantly reduced the uptake into fresh human liver ($P < 0.05$). The recovery of OCIF and ^{3}H-poly(PEG)-OCIF was 90.4 ± 5.6% and 77.5 ± 9.5% of the dose, respectively, and the perfusion was well conducted.

Binding of OCIF and poly(PEG)-OCIF to heparin column

The concentrations of OCIF and poly(PEG)-OCIF in each fraction from the heparin column are shown in Figure 6. Poly(PEG)-OCIF was eluted just after being applied to the column and almost all the poly(PEG)-OCIF was eluted with PBS containing 0.3 M NaCl (fraction 0–5). On the other hand, OCIF was hardly eluted with PBS containing 0.3 M NaCl, but was eluted by PBS containing 2 M NaCl, at a higher salt concentration (fraction 6–10). This result suggests that OCIF easily binds to a heparin column via an HBD in the OCIF module. Poly(PEG)-OCIF, whose HBD is expected to be masked by poly(PEG), is considered to bind to the heparin matrix with much less affinity than OCIF.

Pharmacological study of OCIF and poly(PEG)-OCIF in OVX rats

The effects of poly(PEG)-OCIF and OCIF on the BMD values were examined using OVX rats. The BMD values of femurs after the administration of OCIF or poly(PEG)-OCIF are shown in Figure 7a and 7b, respectively. The BMD values in the OVX group were significantly lower than those in the sham group ($P < 0.01$ vs OVX group) in both studies. Subcutaneously injected OCIF did not ameliorate the BMD of the femurs in the OVX rats even with daily injections for two weeks at a dose of 5 mg/kg (unpublished data). In contrast, intravenously injected OCIF increased the BMD of the femurs

Table 1 Pharmacokinetic parameters of OCIF and poly(PEG)-OCIF after intravenous administration to rats at a dose of 0.3 mg/kg of each compound

Compound	AUC_{inf} (ng h/ml)	$t_{1/2}$ (h)	CL_{total} (ml/h/kg)	V_d (ml/kg)
OCIF	609 ± 99.5	3.91 ± 0.24	502 ± 78.9	1030 ± 277
Poly(PEG)-OCIF	35 100 ± 5300*	7.59 ± 0.34*	8.67 ± 1.21*	66.7 ± 6.9*

*$P < 0.05$, compared with OCIF (Student's t-test). Student's t-test was applied for log-transformed data of AUC_{inf}, CL_{total} and V_d.

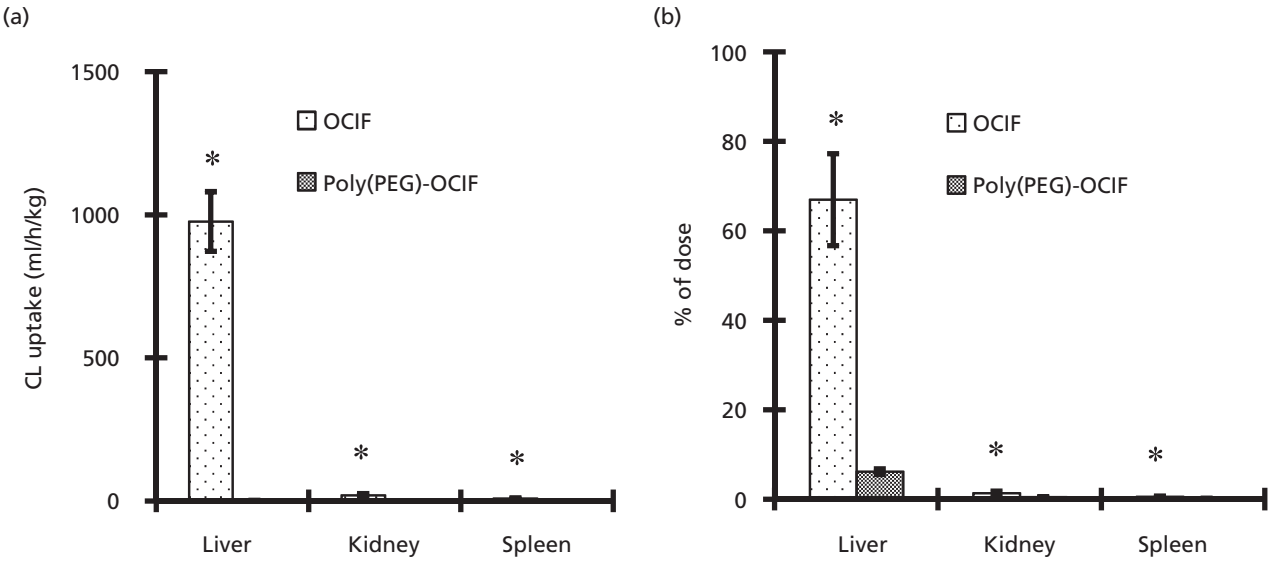

Figure 4 Tissue uptake clearance (a) and % of dose (b) of OCIF and poly(PEG)-OCIF after intravenous administration of each compound to rats at a dose of 0.5 mg/kg. Each value represents the mean ± SD of three rats. *$P < 0.05$ compared with poly(PEG)-OCIF (Student's *t*-test).

Table 2 Tissue uptake clearance (CL_{uptake}) and % of dose of tissue uptake of OCIF and poly(PEG)-OCIF after intravenous administration to rats at a dose of 0.5 mg/kg of each compound

	CL_{uptake} (ml/h/kg)			**Tissue uptake (% of dose)**		
	Liver	Kidney	Spleen	Liver	Kidney	Spleen
OCIF	977 ± 104	20.4 ± 5.5	9.21 ± 2.46	67.0 ± 10.3	1.41 ± 0.47	0.626 ± 0.145
Poly(PEG)-OCIF	6.87 ± 0.49*	0.604 ± 0.066*	0.231 ± 0.079*	6.23 ± 0.61*	0.547 ± 0.074*	0.208 ± 0.064*

*$P < 0.05$, compared with OCIF (Student's *t*-test). Student's *t*-test was applied for log-transformed data of CL_{uptake}.

of the OVX rats ($P < 0.001$ vs OVX group) to almost the same level as the sham group by frequent (twice a day) administration at a dose of 0.5 mg/kg. Poly(PEG)-OCIF significantly increased the BMD of the femurs ($P < 0.001$ vs OVX group) to a level slightly higher than the sham group by intermittent subcutaneous administration, once a week, at a dose of 0.3 mg/kg. Comparing the increase of BMD after subcutaneous administration of OCIF and poly(PEG)-OCIF, it is assumed that the pharmacologically effective dose of OCIF is about 100 times higher than that of poly(PEG)-OCIF.

Discussion

In this study, we have shown that the liver uptake of poly(PEG)-OCIF was significantly reduced and the systemic exposure of poly(PEG)-OCIF was substantially improved in rats. To estimate the liver uptake of poly(PEG)-OCIF in the clinical setting, as well as in preclinical observation, we performed a human liver perfusion study using fresh human liver block. As the liver uptake of OCIF and poly(PEG)-OCIF was not observed in the hepatocyte system, we adopted the liver perfusion system. PEGylation of OCIF significantly reduced the uptake of OCIF into fresh human liver compared with

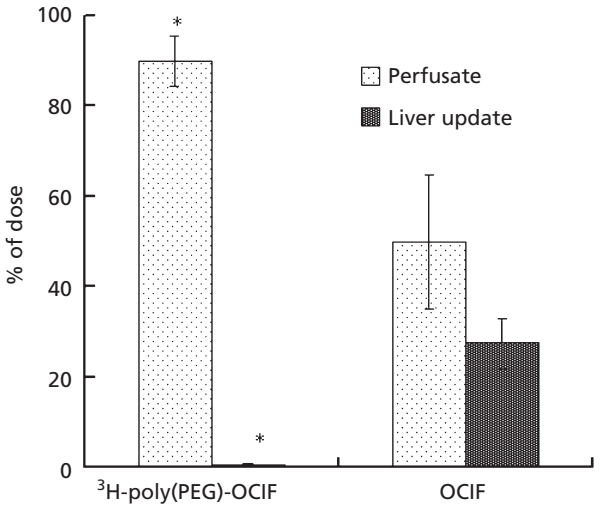

Figure 5 Percent of dose of perfusate and liver uptake of ^{3}H-poly(PEG)-OCIF and OCIF after perfusion of both compounds in human liver. Each value represents the mean ± SD of three experiments. *$P < 0.05$ compared with OCIF (paired *t*-test).

intact OCIF, suggesting that the liver uptake of poly(PEG)-OCIF will be reduced in the clinical situation.

The short $t_{1/2}$ of OCIF in the circulation is considered to be due to tissue uptake via HSPG. The HBD of OCIF at the C-terminal domain is highly basic and it is supposed to interact strongly with HSPG. From the heparin binding study, we confirmed the reduction of the binding affinity of poly(PEG)-OCIF (Figure 6). By masking the HBD with poly(PEG), it becomes difficult for poly(PEG)-OCIF to interact with HSPG, therefore the tissue uptake of poly(PEG)-OCIF is reduced and the serum concentration of poly(PEG)-OCIF is improved compared with that of OCIF. Poly(PEG) is negatively charged and may bind to the C-terminal region of OCIF electrostatically. After the formation of the electrostatic complex, covalent bonds are formed between OCIF and poly(PEG), as shown in Figure 2a by SDS-PAGE analysis. The estimated mechanism of conjugate formation between poly(PEG) and OCIF is illustrated in Figure 8.

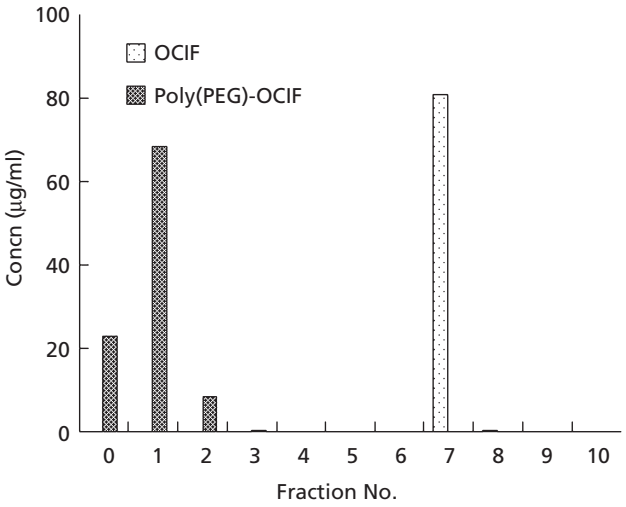

Figure 6 Concentrations of OCIF and poly(PEG)-OCIF in each fraction eluted from a heparin column. Fraction 0 was collected immediately after sample application. Fractions 1–5 were eluted by PBS containing 0.3 M and fractions 6–10 were eluted by PBS containing 2 M NaCl.

There are many reports about PEGylation of proteins using various kinds of PEG.[18–22] Some of them improved the retention of proteins in the circulation system; however, they significantly reduced the pharmacological activity. For example, PEGylated recombinant interferon alpha-2b (PEG Intron) is already on the market for the treatment of hepatitis. PEG Intron is a conjugate of interferon and 12 kDa mono-methoxy polyethylene glycol with a longer in-vivo circulating half-life and a substantial reduction of bioactivity.[23] The difference in the half-life value between that found in our study and that reported in a previous paper[7] was probably due to the content of sialic acid. We have already found that OCIF with higher sialic acid content demonstrates higher $t_{1/2}$ (data not shown). Consistent with this trend, the content of sialic acid in OCIF was 38.6 μg/ml and in the previous paper was 19.9–27 μg/ml.

Modification of OCIF with poly(PEG) increased the serum concentrations of OCIF while maintaining the pharmacological activity of OCIF *in vitro* (Figure 2b). In addition, poly(PEG)-OCIF dramatically increased the BMD of the femurs in OVX rats compared with OCIF. As shown in Figure 7, the pharmacological effectiveness of intact OCIF to ameliorate BMD was solely achieved by frequent intravenous administration, twice a day. It has been shown in our

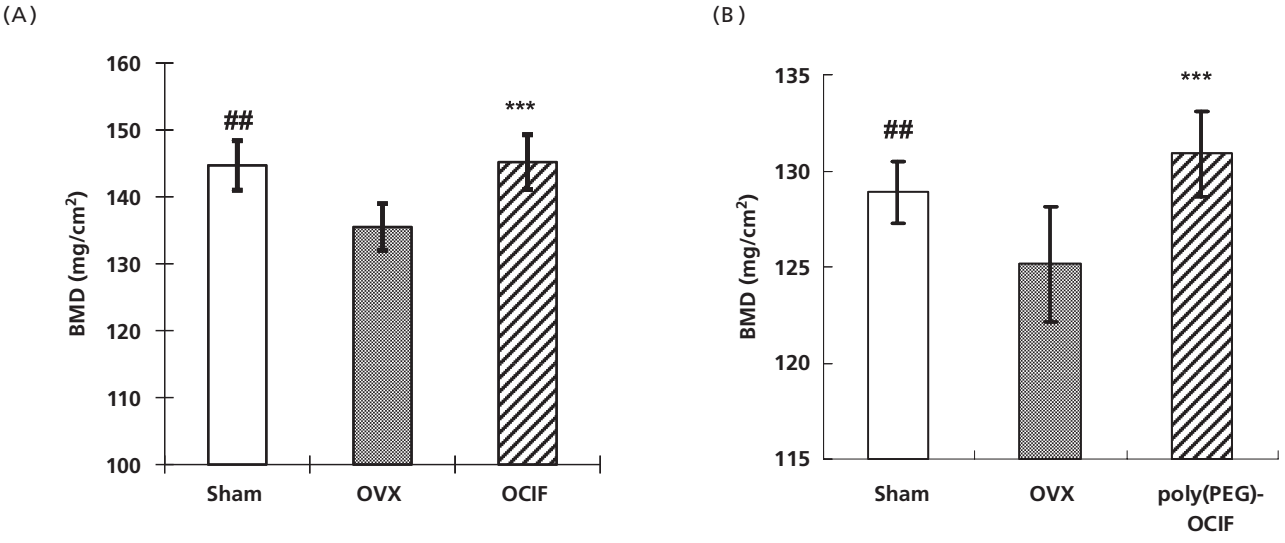

Figure 7 Effect of OCIF (a) and poly(PEG)-OCIF (b) on bone mineral density (BMD) of femurs in ovariectomized (OVX) rats. OCIF: 0.5 mg/kg was administered intravenously twice a day for two weeks. Poly(PEG)-OCIF 0.3 mg/kg was administered subcutaneously once a week for four weeks. Each value represents the mean ± SD of eight rats (except OCIF-treated group: 12 rats). $^{\#\#}P < 0.01$, $^{***}P < 0.001$, compared with OVX group (Student's *t*-test).

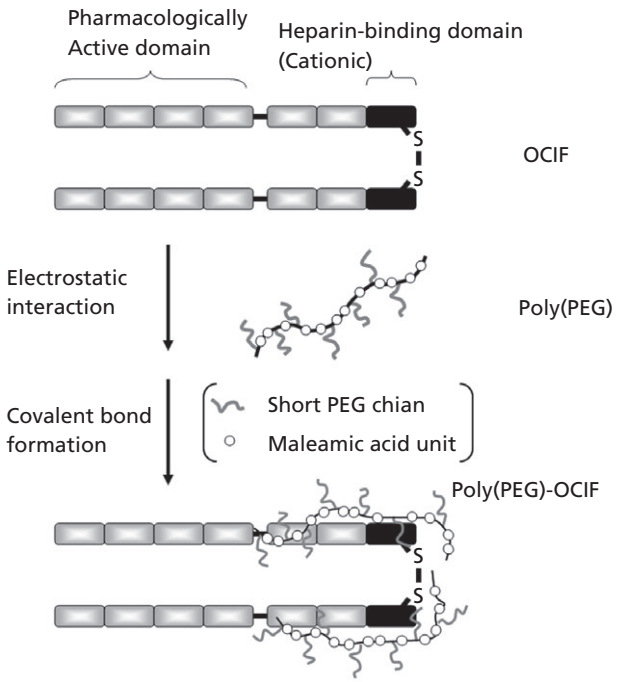

Figure 8 Estimated mechanism of conjugate formation between poly(PEG) and OCIF.

previous study that OCIF increased BMD and bone strength in a rat model of immobilization-induced osteopenia by a daily, or twice a week, intramuscular administration.[24] It was also reported that OCIF improved bone volume and bone strength in a tail-suspended growing rat model by daily intra-muscular administration of OCIF.[25] Thus, frequent administration of OCIF by intravenous or intramuscular injection is vital to achieve in-vivo effectiveness of OCIF in the improvement of bone. In contrast, poly(PEG)-OCIF showed pharmacological activity *in vivo* even using intermittent administration scheduled once a week and subcutaneous injection.

We demonstrated that liver uptake is a major contributor to the rapid clearance of OCIF from the circulation in rats. As shown in Figure 4b, about 70% of OCIF administered was taken up by the liver. To estimate the liver uptake of poly(PEG)-OCIF in the clinical setting, we evaluated the liver uptake of poly(PEG)-OCIF using fresh human liver block. Test solutions of OCIF and ^{3}H-poly(PEG)-OCIF were perfused simultaneously to negate the individual variability of human livers. As the immunoreactivity of OCIF and poly(PEG)-OCIF are similar to each other (Figure 2c), it is possible to determine the amount of OCIF in the samples by subtracting the values calculated by LSC from that by ELISA. The characteristics of human liver uptake of poly(PEG)-OCIF were similar to those of biodistribution in rats and the uptake of poly(PEG)-OCIF in human liver was significantly suppressed (Figure 5). This suggests that poly(PEG)-OCIF will extend the pharmacokinetic profile with a greater half-life in the circulation system in humans and will have a therapeutic effect on bone disorders.

Conclusions

We have evaluated the human liver uptake of poly(PEG)-OCIF using fresh human block and determined that PEGylation of OCIF with poly(PEG) has a large effect on human liver uptake. Thus, this improvement may translate into clinical benefits.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgements

The study was supported by the charitable state controlled foundation HTCR supplying human tissue for research purposes.

References

1. Khosla S. Minireview: the OPG/RANKL/RANK system. *Endocrinology* 2001; 142: 5050–5055.
2. Yasuda H *et al*. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* 1998; 139: 1329–1337.
3. Yasuda H *et al*. A novel molecular mechanism modulating osteoclast differentiation and function. *Bone* 1999; 25: 109–113.
4. Yasuda H *et al*. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci* 1998; 95: 3597–3602.
5. Simonet WS *et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997; 89: 309–319.
6. Smith BB *et al*. A toxicity profile of osteoprotegerin in the cynomolgus monkey. *Int J Toxicol* 2003; 22: 403–412.
7. Tomoyasu A *et al*. Characterization of monomeric and homodimeric forms of osteoclastogenesis inhibitory factor. *Biochem Biophy Res Com* 1998; 245: 382–387.
8. Yamaguchi K *et al*. Characterization of structural domains of human osteoclastogenesis inhibitory factor. *J Biol Chem* 1998; 273: 5117–5123.
9. Standal T *et al*. Osteoprotegerin is bound, internalized, and degraded by multiple myeloma cells. *Blood* 2002; 100: 3002–3006.
10. Liu KX *et al*. Protamine enhances the proliferative activity of hepatocyte growth factor in rats. *Am J Physiol Gastrointest Liver Physiol* 1998; 274: 21–28.
11. Liu KX *et al*. Decrease in the hepatic uptake clearance of hepatocyte growth factor (HGF) in CCl_4-intoxicated rats. *Hepatology* 1993; 17: 651–660.
12. Kato Y. *et al*. Heparin-hepatocyte growth factor complex with low plasma clearance and retained hepatocyte proliferating activity. *Hepatology* 1994; 20: 417–424.
13. Lowry OH *et al*. Protein measurement with the folin phenol reagent. *J. Biol. Chem* 1951; 193: 265–275.

14. Tsuda E *et al*. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophy Res Comm* 1997; 234: 137–142.
15. Barton DHR *et al*. Studies related to the synthesis of maytansinoids. *Tetrahedron Let* 1982; 23: 651–654.
16. Thasler WE *et al*. Charitable state-controlled foundation human tissue and cell research: ethic and legal aspects in the supply of surgically removed human tissue for research in the academic and commercial sector in Germany. *Cell Tissue Banking* 2003; 4: 49–56.
17. Dhalluin C *et al*. Structural and biophysical characterization of the 40 kDa PEG-interferon-alfa2a and its individual positional isomers. *Bioconjugate Chem* 2005; 16: 504–517.
18. Pettit DK *et al*. Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modeling. *J Biol Chem* 1997; 272: 2312–2318.
19. Chapman AP *et al*. Therapeutic antibody fragments with prolonged in vivo half-lives. *Nat Biotechnol* 1999; 17: 780–783.
20. Tsunoda S *et al*. Selective enhancement of thrombopoietic activity of PEGylated interleukin 6 by a simple procedure using a reversible amino-protective reagent. *Br J Haematol* 2001; 112: 181–188.
21. Czupryn MJ *et al*. Structure-function relationships in human interleukin-11. *J Biol Chem* 1995; 270: 978–985.
22. Bell SJ *et al*. Enhanced circulating half-life and antitumor activity of a site-specific pegylated Interferon-α protein therapeutic. *Bioconjugate Chem* 2008; 19: 299–305.
23. Wang YS *et al*. Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. *Adv Drug Deliv Rev* 2002; 54: 547–570.
24. Mochizuki S *et al*. Osteoclastogenesis inhibitory factor/osteoprotegerin ameliorates the decrease in both bone mineral density and bone strength in immobilized rat. *J Bone Miner Metab* 2002; 20: 14–20.
25. Ichinose Y *et al*. Osteoclastogenesis inhibitory factor/osteoprotegerin reduced bone loss induced by mechanical unloading. *Calcif Tissue Int* 2004; 75: 338–343.