Prevention effect of orally active heparin derivative on deep vein thrombosis

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Summary

The use of heparin as the most potent anticoagulant for the prevention of deep vein thrombosis and pulmonary embolism is nevertheless limited, because it is available to patients only by parenteral administration. Toward overcoming this limitation in the use of heparin, we have previously developed an orally active heparin-deoxyclyclic acid conjugate (LMWH-DOCA) in 10% DMSO formulation. The present study evaluates the anti-thrombogenic effect of this orally active LMWH-DOCA using a venous thrombosis animal model with Sprague-Dawley rats. When 5 mg/kg of LMWH-DOCA was orally administered in rats, the maximum anti-FXa activity in plasma was 0.35 ± 0.02, and anti-FXa activity in plasma was maintained above 0.1 IU/ml [the minimum effective anti-FXa activity for the prevention of deep venous thrombosis (DVT) and pulmonary embolism (PE)] for five hours. LMWH-DOCA (5 mg/kg, 430 IU/kg) that was orally administered reduced the thrombus formation by 56.3 ± 19.8%; on the other hand, subcutaneously administered enoxaparin (100 IU/kg) reduced the thrombus formation by 36.4 ± 14.5%. Also, LMWH-DOCA was effectively neutralized by protamine that was used as an antidote. Therefore, orally active LMWH-DOCA could be proposed as a new drug that is effective for the long-term prevention of DVT and PE.

Keywords

Low-molecular-weight heparin, deoxycholic acid, chemical conjugate, oral delivery, deep vein thrombosis

Introduction

Heparin is one of the most potent anticoagulants and is widely used for the treatment and prevention of deep vein thrombosis (DVT) and pulmonary embolism (PE) (1–2). The main disadvantage of heparin treatment, however, is that it is available to patients only by parenteral administration (3–4). On discharge from the hospital, patients are usually switched from heparin to warfarin which can be administered orally. Warfarin, however, has a slow onset and is subjected to a low therapeutic efficacy as well as a high possibility of drug-to-drug interactions. For these reasons there is a great need for oral heparin (5–7). The clinical importance of orally active heparin is that it can be used for a long term treatment for the prevention of several kinds of thrombotic events.

To overcome the poor oral bioavailability of heparin, several research groups have attempted various methods of oral heparin delivery, such as liposomes, oil-water emulsions, complexes of heparin with hydrophobic organic bases, enteric coating, and aerosol formulations (8–11). There also have been attempts to evaluate the enhancing effects of EDTA, acidic buffer, sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC) and sodium N-[10-(2-hydroxybenzoyl) amino] decanoate (SNAD) or sulfated surfactants on heparin absorption in the gastrointestinal (GI) tract (12, 13).

In our previous studies, we synthesized a chemical conjugate (LMWH-DOCA) of low-molecular-weight heparin (LMWH) and deoxyclyclic acid (DOCA), a kind of bile acids (Fig. 1), and it was successfully absorbed in rats when orally administered (7.8% bioavailability in rats) (14–16). These results offered two possibilities for the mechanism of enhanced absorption of LMWH in the intestine: one was due to the added hydrophobic property owing to the conjugated DOCA, and the other was the interaction between the conjugated DOCA and intestinal membrane. However, the orally administered LMWH-DOCA formed self-assembled nanoparticles due to the hydrophobic aggragation of the conjugated DOCA molecules in the aqueous environment of the intestine. Therefore, to maximize the effect of
the conjugated of DOCA in LMWH-DOCA on enhancing the absorption of LMWH-DOCA in the intestine, we proposed an aqueous oral formulation. Among various solubilizers, the dimethyl sulfoxide (DMSO)/water mixture system in particular, which has been used as a dispersing and solubilizing agent, was developed in view of its interesting physicochemical properties such as hydrophobic hydration of water molecules around DMSO methyl groups and hydrophobic association between DMSO molecules. For the purpose of LMHW-DOCA’s solubilization, the dimethyl sulfoxide (DMSO)/water mixture aqueous system, with LMWH-DOCA completely dissolved in 10% DMSO solution, was used, thereby increasing its oral bioavailability up to 17.6% in mice and 16.1% in monkeys (17, 18).

In this study, using a venous thrombosis animal model with Sprague-Dawley rats, we evaluated the prevention effect of orally active LMWH-DOCA on thrombosis.

Materials and methods

Materials

Nadroparin (Fraxiparin®), one kind of LMWH, whose average molecular weight is about 4,500 Dalton, was obtained from GlaxoSmithKline Korea (Seoul, Korea), and another LMWH, enoxaparin (Lovenox®), was purchased from Sanofi-Aventis Co. (Bridgewater, NJ, USA). Deoxycholic acid (DOCA), dicyclohexylcarbodiimide (DCC), hydroxysuccinimide (HOSu), 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC), ethylene diamine and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylformamide (DMF) was obtained from Merck (Darmstadt, Germany). Coatest Factor Xa assay kits were purchased from Chromogenix (Milano, Italy). Sprague-Dawley rats (SD male rat, 250–280 g), obtained from the animal care facility at the Korean Animal Center (Seoul, Korea), were kept under a 12:12-hour light-dark cycle and were used after fasting them for 12 hours.

Synthesis of LMWH-DOCA

The chemical conjugate of LMWH (nadroparin) and DOCA was synthesized by conjugating the carboxylic group of DOCA with the carboxylic group of LMWH as described in the previous study (17). Briefly, DOCA (196 mg) was mixed with DCC (165 mg) and HOSu (92 mg) in 15 ml of DMF. The feed mole ratio of DOCA, DCC and HOSu was 1:1.6:1.6. The concentrations of DCC and HOSu were slightly higher than that of DOCA in order to activate DOCA completely. The mixture reacted for 5 h at room temperature in vacuum, and the precipitated dicyclohexylurea was filtered. The unreacted DCC was precipitated by adding 1 ml of distilled water drop-by-drop and filtered, and 15 ml of distilled water was poured into the filtrated solution. The remaining HOSu was dissolved in water, and the activated DOCA was precipitated and filtered. The activated DOCA was mixed with ethylenediamine in DMF and reacted for 5 h at room temperature, thereby forming deoxycholyethylethylamine. The feed mole ratio of activated DOCA to ethylenediamine was 3:1. LMWH (100 mg) was dissolved in 2 ml of formamide and EDAC solution (11.5 mg) was added with LMWH to activate carboxylic acid of LMWH. Deoxycholyethylamine was coupled with activated carboxylic acid of LMWH. The reaction mixture was incubated at 25°C for 12 h. The feed mole ratios of deoxychoelyethylamine to LMWH were 3:1. The produced LMWH-DOCA was purified by washing with acetone for three times, thereby removing completely any ionically bound amineoethyl DOCA. After lyophilizing, LMWH-DOCA conjugate was obtained as white powder.

Absorption of LMWH-DOCA after its oral administration

SD rats were fasted for 12 h before the oral administration of LMWH-DOCA. All animal experiments were carried out in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals. Rats were anesthetized with light diethyl ether and orally administered LMWH-DOCA in DMSO formulation through an oral gavage that was carefully passed down the esophagus into the stomach. The doses were 5 and 10 mg/kg, and the dose volume of the administered LMWH-DOCA solution was 0.4 ml. Blood (450 µl) was collected from a capillary in the retroorbital plexus and directly mixed with 50 µl of sodium citrate (3.8% solution); the sample was then immediately centrifuged at 2,500 x g at 4°C for 15 min. The anti-FXa activity that was induced by LMWH-DOCA in plasma was measured using anti-FXa chromogenic assay.

Venous thrombosis model

The animal model for DVT was prepared as described in the literature (19–22). Briefly, LMWH (enoxaparin) of 100 IU/kg was administered by subcutaneous injection, whereas LMWH-DOCA of 5 and 10 mg/kg in DMSO formulation was orally administered to SD rats. After administration of enoxaparin or LMWH-DOCA via subcutaneous or oral route, animals were anesthetized with ketamine (45 mg/kg) and xylazine (5 mg/kg) by means of intramuscular injection. After rats were anesthetized, both sides of the vena cava of rats were exposed and separated from the surrounding tissue. Each end (2 cm) of the vena cava was loosely tied, and the branched blood vessels were completely tied with 2–0 silk thread. At 60 min after enoxaparin or LMWH-DOCA administration, 1 ml/kg human pooled plasma warmed to 37°C was injected through the tail vein. Fifteen seconds later, the
vena cava was ligated with 2–0 silk thread *in situ* to produce stasis. At 120 min after finishing the surgical operation, the veins were segregated and opened in a Petri dish filled with 3.8% sodium citrate. Thrombus formation was evaluated by measuring the wet weight of the thrombus.

**Neutralization of LMWH and LMWH-DOCA using protamine**

The ability of protamine to neutralize LMWH or LMWH-DOCA was assessed *in vitro*. For this study, 200 µl of human pooled plasma was mixed at the ratio of 4:1 with 100 µg/ml of either LMWH (enoxaparin, nadroparin) or LMWH-DOCA. For the control LMWH, both enoxaparin and nadroparin were used since enoxaparin is the first LMWH used in the clinical therapy and nadroparin was the mother compound of LMWH-DOCA. For each sample, different ratios of protamine to anti-FXa units of heparin, namely, 0, 0.25, 0.5, 1, 1.5, 2, and 3, were prepared. After incubation for 20 min, the anti-FXa activities of the samples were measured using anti-Factor Xa chromogenic assay (Chromogenix kit, Chromogenic® Heparin, Milano, Italy). In this kit, s-2222 (Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA·HCl) was used as the substrate.

**Statistical analysis**

The cumulative data from animal experiments were expressed as mean ± SD, and a paired t-test was used for comparison between groups. A value of *P* < 0.05 was considered as statistically significant.

**Results**

**Characterization of LMWH-DOCA**

As described in our previous study (15), the conjugation of LMWH and DOCA was confirmed by amide bonds formed by coupling carboxylic groups of iduronic acid and glucuronic acid in heparin and the amine group of deoxylchorylamine using FT-IR, 1H-NMR and 13C-NMR. The peaks at 1,720 and 1,585 cm⁻¹ in the Fourier Transform infrared spectrum indicated the presence of amide bonds in LMWH-DOCA. In the 1H-NMR and the 13C-NMR spectrums, the amide peak also occurred at 7.58 and 178 ppm, respectively. The conjugation ratio of DOCA to LMWH (Nadroparin) was 2.4:1, and the anticoagulant activity of LMWH and LMWH-DOCA measured by anti-FXa chromogenic assay was 97 and 86 IU/mg, respectively. The ratio of anti-Xa activity and anti-IIa activity (Xa:IIa ratio) of LMWH and LMWH-DOCA was 3.6:1 and 2.6:1, respectively. LMWH-DOCA formed self-assembled nanoparticles in water since the conjugated hydrophobic DOCA molecules were gathered inside against the hydrophilic aqueous environment; however, LMWH-DOCA was completely dissolved in 10% DMSO solution as confirmed by dynamic light scattering (DSL, Spectra Physics Laser model 127–35, Mountain View, CA, USA) operating at 633 nm.

**Absorption of orally administered LMWH-DOCA in rats**

When 5 mg/kg of LMWH-DOCA in 10% DMSO formulation was orally administered in rats, the maximum anti-FXa activity in plasma was 0.35 ± 0.02, and anti-FXa activities in plasma was maintained above 0.1 IU/ml (the minimum effective anti-FXa activity for the prevention of DVT and PE) for 5 h (Fig. 2). After the oral administration of 10 and 20 mg/kg of LMWH-DOCA, the maximum anti-FXa activity in plasma was 0.49 ± 0.04 and 0.65 ± 0.06 IU/ml, respectively, and this maximum point was observed at 0.5 ~ 1 h. On the other hand, the maximum anti-FXa activity of orally administered LMWH was only about 0.1 IU/ml even when 100 mg/kg of LMWH was orally administered to rats.

**Inhibition of venous thrombosis by orally administered LMWH-DOCA**

The doses of 5 and 10 mg/kg of LMWH-DOCA was chosen for the evaluation of the prevention effect of LMWH-DOCA on DVT, since anti-FXa activity in plasma was maintained above the minimum effective anti-FXa activity for the prevention of DVT for 5 h, as shown in Figure 2. When the phosphate buffer only was treated as a control, thrombi were formed weighing as much as 27.7 ± 2.9 mg. A subcutaneous administration of enoxaparin (100 IU/kg) reduced thrombus formation by 36.4 ± 14.5%. The dose of enoxaparin recommended for the prevention of venous thromboembolism is in the range of 50–100 IU/kg, and the highest dose possible in this range was used as the control. On the other hand, 5 mg/kg (430 IU/kg) and 10 mg/kg (860 IU/kg) of LMWH-DOCA that was orally administered reduced thrombus formation by 56.3 ± 19.8 and 69.4 ± 6.9 %, respectively (Fig. 3).

**Neutralization of LMWH-DOCA by protamine**

Among the control LMWH, enoxaparin and nadroparin, nadroparin had better binding affinity to protamine than enoxaparin (Fig. 4), since nadroparin (100 IU) was completely neutralized by protamine when it was mixed with 1.5 mg of protamine. On the other hand, 100 IU of enoxaparin lost about 50% of its bioac-
tivity when it was mixed with 2.5 mg of protamine. The neutralization efficacy of LMWH-DOCA by protamine was greater than that of enoxaparin but lower than that of nadroparin. Additionally, the bioactivity of LMWH-DOCA was effectively decreased up to 80% with the increase of protamine.

Discussion

The development of orally active heparin has been in high demand for the prevention of DVT and PE, for which heparin should be administered over several months. We developed a new orally active heparin derivative, LMWH-DOCA, in a 10% DMSO formulation. In this formulation, LMWH-DOCA was completely solubilized and highly absorbed in rats after its oral administration. A dose of 5 mg/kg LMWH-DOCA was enough to maintain the anti-FXa activity in the plasma above the minimum effective level (0.1 IU/ml) for DVT in rats. In our previous study, we confirmed that LMWH could not be absorbed in the GI tract even though it was orally administered in DMSO solution (17).

Among the various kinds of low-molecular-weight heparin, enoxaparin is widely used for the prevention of DVT; thus, we evaluated the anti-thrombogenic effect of LMWH-DOCA by comparing it with enoxaparin (21, 22). The oral administration of 5 mg/kg (430 IU/kg) of LMWH-DOCA reduced thrombus formation more significantly than the subcutaneous administration of 100 IU/kg of enoxaparin in a rat DVT model. Therefore, 5 mg/kg of LMWH-DOCA in DMSO formulation would be enough to control thrombosis and seems superior to enoxaparin of the recommended dose. LMWH-DOCA showed above 25% activity in preventing DVT compared to enoxaparin in the same anticoagulant unit. Based on this result, it is expected that a 200 mg dose for adults might be applicable for the clinical trials, and LMWH-DOCA could be applied to the long-term treatment for the prevention of DVT and PE.

Positively charged protamine has been used as an antidote for heparin to block the bleeding risk resulting from heparins. The ability to use an antidote for heparin is one of the great merits for heparin, since other anticoagulant drugs cannot be used. The inhibition profile of heparin activity differed slightly according to the kind of heparin, although the bioactivity of all heparins was decreased by adding protamine. In particular, nadroparin, which is LMWH used for the synthesis of LMWH-DOCA, was more completely neutralized by protamine than enoxaparin, and LMWH-DOCA was also effectively neutralized by protamine. Heparin is neutralized by protamine by the binding of sulfate groups of heparin with arginine of protamine. On the other hand, only two to three carboxylic groups of heparin were used for the conjugation with DOCA, and no sulfate groups of heparin were used for the conjugation. Therefore, protamine can be effectively used as an antidote for LMWH-DOCA.

In conclusion, 5 mg/kg of the oral anticoagulant drug, our newly developed LMWH-DOCA conjugate, can prevent DVT more effectively than 100 IU/kg of enoxaparin when administered subcutaneously. Also, LMWH-DOCA is a drug that can be safely used in clinical applications since it can be rapidly neutralized by a protamine. Therefore, orally active LMWH-DOCA could be proposed as an effective drug to use in the long-term prevention of DVT and PE.

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References