in vivo *30*: 433-438 (2016)

Inhibitory Effects of Medium Molecular Weight Heparinyl Amino Acid Derivatives on Ischemic Paw Edema in Mice

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Abstract. *We investigated the radical-scavenging effects of heparin (HE), medium molecular weight heparinyl phenylalanine (MHF), and medium molecular weight heparinyl leucine (MHL) using ischemic paw edema in mice. We also examined the activated partial thromboplastin time (APTT) of mice that were administered these compounds as an index of their side-effects. HE had a preventative effect and significant reduced ischemic paw edema. However, its effect was not dose-dependent and the dose–response curve was bell-shaped. The effective dose of HE also exhibited a prolonged APTT. Pretreatment using MHF and MHL were effective against ischemic paw edema without a prolonged APTT. Remarkably, the action of MHF was not only preventively, but also therapeutically active. These results suggest that MHF and MHL are superior to HE as safe radical scavengers in vivo.*

Heparin (HE), a polysaccharide, is one of the most useful anti-coagulants. However, common side-effects of HE are easy bleeding and bruising. Oyanagui and Sato reported that heparin is a potent extracellular superoxide dismutase-releasing agent and it suppresses ischemic paw edema in mice (1). It is, therefore, reasonable to develop HE derivatives that function as radical scavengers without side-effects, such as bleeding or bruising.

In a previous study, we conducted novel synthesis of medium molecular weight heparinyl amino acid derivatives (MHADs) to reduce the adverse effects of anticoagulant activity of HE. Among 12 kinds of MHADs, medium molecular weight heparinyl phenylalanine (MHF), medium molecular weight heparinyl leucine (MHL) and medium molecular weight heparinyl tyrosine (MHY) had significant radical-scavenging action with lesser side-effects than HE *in vitro* (2).

In this study, we examined the effects of MHF and MHL on ischemic paw edema in mice *in vivo*.

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Key Words: Medium molecular weight heparinyl amino acid derivatives, MHADs, ischemic paw edema model, free radical.

Materials and Methods

Animals. Specific pathogen-free male ICR mice (4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan), and used for the experiment after a one-week acclimation. The mice were maintained at 23±2˚C (room temperature) and 50±5% relative humidity under an artificial 12-h light-dark cycle (7:00 on-19:00 off). Food and water were given ad libitum during the experimental period. All procedures followed the office regulations for the Care and Use of laboratory animals approved by the animal experimentation committee of Fuso Pharmaceutical Industries Ltd (approval number: PDS9606).

Materials. MHF and MHL (each mean molecular weight: 8,500-10,000) were synthesized at our Research and Development Center (2), and bulk HE was purchased from Scientific Protein Laboratories (Waunakee, WI, USA).

Measurement of ischemic paw edema. The ischemic paw edema mouse model was prepared after modification of the method by Oyanagui and Sato (1).

The paw thickness of mice was measured with a caliper before rubber ring application as a preliminary value. HE (0.625-40 mg/kg), MHF or MHL (1.25-40 mg/kg) was then injected into the tail vein of mice immediately before ischemic treatment. The right hind leg (ankle) was bound 10 or 13 times with a rubber band (five mice per treatment group). The rubber band was removed after 20 min and the paw thickness was measured with a caliper at 10, 40 and 70 min after recirculation.

Measurement of plasma activated partial thromboplastin time (APTT). HE (0.625, 1.25 and 2.5 mg/10 ml/kg), MHF or MHL (5.0, 10.0 and 20.0 mg/10 ml/kg) were injected into the tail vein of mice, blood was collected from the abdominal aorta at 30 or 90 min after HE, MHF or MHL administration, and anticoagulated with sodium citrate. The plasma was obtained after centrifugation for 10 min at

Table I. *Effect of heparin (HE) on ischemic paw swelling edema in mice.*

Compound	Dose (mg/kg)	Paw thickness (mm)			
		Pre	30 Min	60 Min	90 Min
PBS		2.10±0.04	2.42±0.08	3.21±0.10	3.20±0.06
HE	0.625	2.05±0.10	2.30±0.22	3.04±0.08	2.94±0.17
	1.25	2.17±0.10	2.39±0.14	3.07±0.21	3.04±0.13
	2.5	2.05±0.03	2.11±0.06	2.55±0.11	2.60±0.11*
	5.0	2.06±0.06	2.09±0.11	2.50±0.12*	2.54±0.10**
	10.0	2.22±0.03	2.18±0.14	2.38±0.15*	2.68±0.19
	20.0	2.06±0.04	2.41±0.13	3.07±0.10	3.12±0.14
	40.0	1.98±0.08	2.28±0.16	2.88±0.21	3.20±0.08

PBS: Phosphate-buffered saline. Each value represents the mean±S.E. of 5 animals. Significantly different at *$p<0.05$, **$p<0.01$ *vs.* PBS.

Table II. *Effect of medium molecular weight heparinyl phenylalanine (MHF), and medium molecular weight heparinyl leucine (MHL) on ischemic paw edema in mice.*

Compound	Dose (mg/kg)	Paw thickness (mm)			
		Pre	30 Min	60 Min	90 Min
PBS		2.11±0.02	2.61±0.11	3.05±0.09	3.07±0.15
MHF	1.25	2.07±0.02	2.43±0.11	2.75±0.10	2.71±0.08
	2.5	2.11±0.05	2.08±0.04**	2.45±0.05**	2.43±0.07**
	5	2.08±0.07	2.08±0.05*	2.21±0.08**	2.22±0.09**
	10	2.03±0.08	2.07±0.07*	2.28±0.09**	2.19±0.09**
	20	2.17±0.05	2.39±0.13	2.70±0.06	2.67±0.05
	40	2.07±0.03	2.42±0.07	2.90±0.12	2.74±0.11
MHL	1.25	2.07±0.02	2.35±0.10	2.68±0.15	2.70±0.09
	2.5	2.17±0.04	2.14±0.04*	2.43±0.07**	2.59±0.15
	5	2.05±0.08	2.12±0.09	2.29±0.09**	2.23±0.13**
	10	2.05±0.02	2.15±0.08	2.29±0.07**	2.26±0.06**
	20	2.15±0.07	2.32±0.04	2.69±0.08	2.79±0.09
	40	2.08±0.02	2.43±0.10	2.75±0.08	2.68±0.11

PBS: Phosphate-buffered saline. Each value represents the mean±S.E. of 8 animals. Significantly different at *$p<0.05$, **$p<0.01$ *vs.* PBS.

1,000 × *g* at room temperature. APTT was measured using a Wako APTT test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and an Amelung coagulometer KC10A blood coagulation measuring device (Trinity Biotech Plc., Bray, Ireland).

Investigation of a suitable administration time of HE, MHF or MHL. HE, MHF or MHL (10 mg/10 ml/kg) was injected into the tail vein of mice at 5, 10 and 15 min before and immediately after induction of ischemia, and the size of the edema was measured at 0, 30, 60 and 90 min after ischemia induction.

Statistical analysis. Data are represented as the mean±standard error (S.E.) and statistical significance was evaluated by ANOVA followed by Tukey's test in the case of equal variance. We used the Kruskal–Wallis method followed by Tukey's test in cases of unequal variance. When the number of samples among the groups was different, we used the Spjotvoll and Stoline test (corrected Tukey's test). The differences were assessed at a significance level of 0.05.

Results

Time dependency of ischemic paw edema in mice. We measured the paw thickness at 0, 30, 60 and 90 min after ischemia was induced by a rubber band. Simultaneously, we compared the effects between binding 10 or 13 times on paw swelling. The paw thickness increased in a time-dependent manner until 60 min, and almost reached a plateau at 90 min. Since there was no difference in the paw swelling between binding 10 and binding 13 times, we utilized 10 times as the amount of binding for the subsequent experiments (Figure 1).

Effects of different doses of HE, MHF and MHL on ischemic paw edema. Intravenously administered HE, MHF, and MHL reduced the paw thickness significantly (Tables I and II). The effects of various doses of HE, MHF or MHL on paw

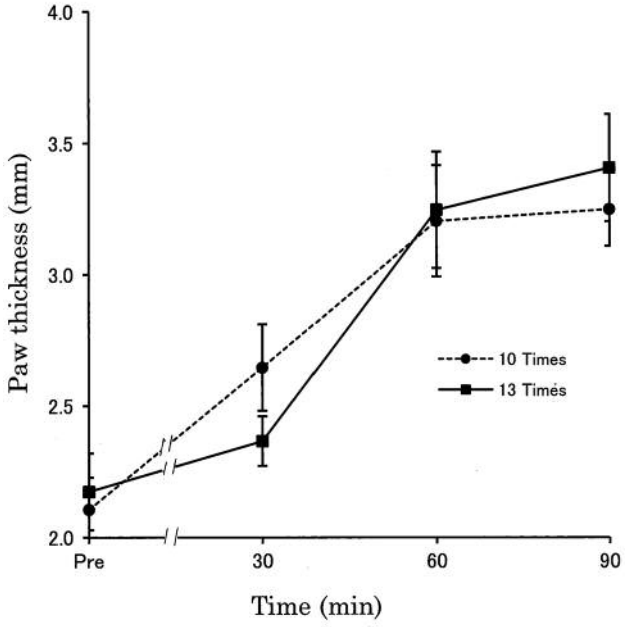

Figure 1. *Time dependency of the ischemic paw swelling edema in the hind leg of mice. when bound 10 or 13 times using an elastic band. Each point represents the mean±S.E. of measurements in five animals.*

thickness were investigated 90 min after ischemic treatment by a rubber ring.

Effects of HE, MHF and MHL on APTT in mice. As an index for side-effects of HE, MHF or MHL, we measured the APTT in mice after HE, MHF or MHL administration. Thirty minutes after HE (2.5 mg/kg), MHF or MHL (20.0 mg/kg) administration, the APTT was prolonged significantly compared to that of the controls; 90 min after MHF (10.0 and 20.0 mg/kg) administration, the APTT was prolonged significantly compared to that of the control (Tables III and IV).

Investigation of a suitable administration time of HE, MHF or MHL. Although HE (10 mg/kg) was injected into the tail vein of mice at 5, 10 and 15 min before and immediately after induction of ischemia, the size of the swelling edema was not inhibited at 0, 30, 60 or 90 min after ischemia (Table V). MHF (10 mg/kg), on the other hand, significantly inhibited paw edema at 30 min after ischemia when administered to mice at 5, 10 or 15 min before and immediately after ischemia. MHF (10 mg/kg) also reduced paw edema significantly at 60 min after ischemia when injected to mice 5 min before ischemia. MHL (10 mg/kg) significantly inhibited the paw edema at 30 min after ischemic treatment when administered to mice 5, 10 or 15 min before ischemia (Table VI).

Table III. *Effects of heparin (HE) on activated partial thromboplastin time (APTT) in mice after induction of ischemia.*

		APTT (s)	
Compound	Dose (mg/kg)	30 Min	90 Min
PBS		23.2±0.7	
HE	0.625	60.4±5.1	23.0±1.9
	1.25	133.8±60.2	23.1±2.0
	2.5	180.0±0.0**	43.9±9.8

PBS: Phosphate-buffered saline. Each value represents the mean±S.E. of 3 or 5 animals. Significantly different at $^{**}p<0.01$ *vs*. PBS.

Table IV. *Effects of medium molecular weight heparinyl phenylalanine (MHF), and medium molecular weight heparinyl leucine (MHL) on activated partial thromboplastin time (APTT) in mice after induction of ischemia.*

		APTT (s)	
Compound	Dose (mg/kg)	30 Min	90 Min
PBS		25.3±0.7	24.7±0.3
MHF	5	32.2±1.6	26.8±0.9
	10	51.7±2.4	33.7±1.2**
	20	125.5±18.8**	39.1±2.1**
MHL	5	27.3±1.0	25.7±1.1
	10	38.7±3.0	25.9±0.8
	20	55.2±2.8*	28.2±1.4

PBS: Phosphate-buffered saline. Each value represents the mean±S.E. of 3 or 5 animals. Significantly different at $^{*}p<0.05$, $^{**}p<0.01$ *vs*. PBS.

Discussion

We utilized the ischemic paw edema mouse model reported by Oyanagui and Sato (1). However, the number of times the rubber band was bound to induce paw edema ranged from 10 to 15 times in other studies (1, 3, 4). Firstly, we compared the degree of paw edema between 10 and 13 bindings; however, the resulting paw thickness did not differ. Accordingly, we defined 10 as the number of rubber band bindings for subsequent experiments.

Hiebert and Liu reported that pretreatment with HE 24 h beforehand protected cultured arterial endothelial cells from damage by toxic oxygen metabolites (5). Several researchers evaluated the mechanism of HE action, and demonstrated that HE functioned as an indirect radical scavenger by raising the level of extracellular superoxide dismutase (EC-SOD) in the blood (6-9). Moreover, Oyanagui and Sato demonstrated that HE (2000 U/kg, *i.v.*) increased the release of EC-SOD and prevented ischemic paw edema in mice, whereas a high dose of HE (4,000 and 10,000 U/kg, *i.v.*) led to a smaller increase in the release

Table V. *Influence of timing of heparin (HE) administration on inhibition of ischemic paw edema.*

Compound	Timing	Pre	Paw thickness (mm)		
			30 Min	60 Min	90 Min
PBS		2.10±0.04	2.42±0.08	3.21±0.10	3.20±0.06
HE	15 Min before	2.15±0.07	2.44±0.14	3.02±0.10	2.95±0.08
	10 Min before	2.12±0.07	2.27±0.07	3.05±0.13	3.17±0.11
	5 Min before	2.25±0.06	2.55±0.10	3.01±0.08	3.24±0.05
	Immediately after	2.27±0.11	2.68±0.11	3.41±0.26	3.16±0.08

PBS: Phosphate-buffered saline. Each value represents the mean±S.E. of 5 animals.

Table VI. *Influence of timing of medium molecular weight heparinyl phenylalanine (MHF), and medium molecular weight heparinyl leucine (MHL) administration on inhibition of ischemic paw edema.*

Compound	Timing	Pre	Paw thickness (mm)		
			30 Min	60 Min	90 Min
PBS		2.11±0.02	2.61±0.11	3.05±0.09	3.07±0.15
MHF	15 Min before	2.13±0.05	2.33±0.07**	2.61±0.14	2.82±0.15
	10 Min before	2.04±0.04	2.34±0.06**	2.89±0.08	2.96±0.16
	5 Min before	2.08±0.04	2.40±0.06*	2.52±0.12*	2.66±0.10
	Immediately after	2.02±0.05	2.37±0.07*	2.90±0.12	2.84±0.09
MHL	15 Min before	2.05±0.04	2.23±0.07**	2.56±0.16	2.68±0.16
	10 Min before	2.10±0.05	2.24±0.09**	2.57±0.07	2.67±0.12
	5 Min before	2.07±0.04	2.25±0.05**	2.62±0.15	2.62±0.10
	Immediately after	2.05±0.05	2.49±0.08	2.71±0.10	2.94±0.13

PBS: Phosphate-buffered saline. Each value represents the mean±S.E. of 8 animals. Significantly different at *$p<0.05$, **$p<0.01$ *vs.* PBS.

of SOD (1). We also obtained similar results. In our experiments, although HE (2.5, 5.0 and 10.0 mg/kg) had inhibitory action against ischemic paw edema, 2.5 mg/kg of HE also significantly prolonged APTT. Furthermore, HE was only effective on ischemia paw edema when administered to mice before induction of ischemia. In other words, HE acts preventatively against ischemic paw edema, but does not have a therapeutic effect on this pathogenesis.

In our previous study, we synthesized 12 kinds of MHADs to reduce the adverse effects of the anticoagulant activity of HE. In the present study, from among the 12 kinds of MHADs, we investigated the effects of MHF and MHL on ischemic paw edema mice. As a result, MHF (2.5 and 5.0 mg/kg) and MHL (2.5, 5.0 and 10.0 mg/kg) had inhibitory action against ischemic paw edema without significantly prolonging APTT. Moreover, even when MHF was administered to mice after ischemia, it had an inhibitory action.

One of the mechanisms involved in ischemia/reperfusion injury is ischemia/reperfusion-mediated Ca^{2+} overload (5, 10, 11). Boffi *et al.* suggested that the ischemic condition induced changes in membrane permeability and an increase of intracellular Ca^{2+}, both of which led to cell membrane damage (12). Inserte *et al.* reported that calpains, a large family of non-lysosomal neutral cysteine proteases contribute to myocardial ischemia/reperfusion injury, require Ca^{2+} for activity (13). Our previous data also suggested that MHF exhibited an inhibitory effect on rat homologous passive cutaneous anaphylaxis reactions (unpublished data). Systemic anaphylaxis induces activation of mast cells and macrophages (14). Mast cell activation occurs via an increase in the intracellular Ca^{2+} concentration followed by a release of chemical mediators (15). MHF may inhibit the increase of intracellular Ca^{2+} in cells damaged after ischemia and thereby inhibit ischemic paw edema.

In conclusion, MHF and MHL are superior to HE as safe radical scavengers *in vivo*. MHF in particular, showed not only a preventative effect, but also a therapeutic effect on ischemic paw edema.

Conflicts of Interest

The Authors declare no conflict of interest associated with this study.

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Received April 6, 2016
Revised May 11, 2016
Accepted May 12, 2016