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Middle Molecular Weight Heparinyl Amino Acid Derivatives (MHADs) Function as Indirect Radical Scavengers *in Vitro*

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Abstract

We conducted the novel synthesis of middle molecular weight heparinyl amino acid derivatives (MHADs) to reduce the adverse effect of heparin (HE) based on its anticoagulant activity. Subsequently, we investigated the radical scavenging effects of 12 kinds of MHAD on cultured human umbilical vein endothelial cells (HUV-ECs) damaged by oxygen free radicals using xanthine and xanthine oxidase *in vitro*. As a result, middle molecular weight heparinyl phenylalanine, middle molecular weight heparinyl leucine, and middle molecular weight heparinyl tyrosine showed significant protective effects on HUV-ECs. In conclusion, these three HE derivatives might be candidates for therapeutic agents to treat diseases attributed to peroxidation.

Keywords

MHAD, Heparin, HUV-EC, Oxygen Free Radical

1. Introduction

Recently, many reports have been published stating that free radicals are associated with diverse diseases, such as cancer (angiogenesis), nephropathy, and myocardial injury, etc. [1]-[5]. So, it is likely that radical scavengers may become useful agents to treat or prevent the above diseases. Heparin (HE), a glycosaminoglycan, is known to exhibit many pharmacological effects. To date, many researchers have already reported its anticoagulant activity, release of lipoprotein lipase, inhibition of complement activation, and anti-inflammatory effects [6]-[9]. In addition, some investigators have shown that HE has an ability to increase the plasma extracellular superoxide

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dismutase (EC-SOD) activity through the release of EC-SOD from such as endothelial cells [10]-[13]. On the other hand, HE had some serious adverse effects causing a bleeding tendency based on its anticoagulant activity.

In this study, we synthesized middle molecular weight heparinyl amino acid derivatives (MHADs) from conventional HE to reduce this adverse effect. Some MHADs successfully reduced the anticoagulant effect evaluated by the activated partial thromboplastin time (APTT) *in vitro* [14], with beneficial effects. Furthermore, we examined the radical scavenging effect of MHADs on cultured human umbilical vein endothelial cells (HUV-ECs) damaged by oxygen free radicals, and three MHADs showed a significant radical scavenging action with decreased bleeding tendency.

2. Materials and Methods

2.1. Materials

Middle molecular weight heparin (MHE), middle molecular weight heparinyl alanine (MHA), middle molecular weight heparinyl aspartic acid (MHD), middle molecular weight heparinyl phenylalanine (MHF), middle molecular weight heparinyl glycine (MHG), middle molecular weight heparinyl histidine (MHH), middle molecular weight heparinyl leucine (MHL), middle molecular weight heparinyl methionine (MHM), middle molecular weight heparinyl proline (MHP), middle molecular weight heparinyl arginine (MHR), middle molecular weight heparinyl serine (MHS), and middle molecular weight heparinyl tyrosine (MHY) were synthesized from heparin (HE)(Scientific Protein Laboratories, Waunakee, WI, USA) at the Research and Development Center of Fuso Pharmaceutical Industries LTD (Osaka, Japan). Amino acids were purchased from Wako (Osaka, Japan).

2.2. Synthesis of MHE and MHADs

Dissolve heparin in water, add a cation exchange resin, and filter after adjustment to pH 3.30 - 3.40. Add hydrogen peroxide solution to the filtrate, pressurize, heat, and depolymerize. After heating, add sodium hydrate aqueous solution to the reaction solution, repeat ultrafiltration, and perform molecular weight fractioning. The molecular weight fraction is recrystallized with ethanol to obtain MHE (mean molecular weight: 8500 - 9500). Dissolve MHE in water (the pH adjusted to 4.75), and add amino acid ester hydrochloride (molar ratio for MHE, 1:100). Furthermore, after gradually adding a aqueous solution of the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (molar ratio for MHE, 1:50), and reacting with stirring for about 4 hours, add water and hexadecyltrimethylammonium bromide aqueous solution until the sedimentation of ammonium salt ceases. After separation of the sediment, add ethanol solution of sodium iodide to the sediment. Filter after stirring, and recrystallize the sediment with ethanol to obtain middle molecular weight heparinyl amino acid ester. Add a sodium hydroxide solution to the middle molecular weight heparinyl amino acid ester, and stir under a nitrogen gas stream (0˚C - 5˚C). Filter the reaction solution after adjustment to pH 5 with acetic acid, and add ethanol to obtain MHADs.

2.3. Determination of the Molecular Weight of HE, MHE, and MHADs

HE, MHE, and MHADs were dissolved at 5 mg/mL in a sodium sulfate aqueous solution as sample solutions. Polyethylene glycol, (molecular weight; 20,000 and 10,000, Sigma-Aldrich Co., St. Louis, MO, USA), molecular weight; 8500 and average molecular weight; 3000 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), average molecular weight; 1450 (KANTO CHEMICAL CO., INC., Tokyo, Japan), were used as a standard. Determinations of the molecular weight of HE, MHE, and MHADs were performed by HPLC (LC-10A, Shimadzu, Kyoto, Japan) with a refractive index detector (RI-8020, Tosoh, Tokyo, Japan) using the calibration curve method. The conditions were as follows: column, 7.8 × 300 mm (40˚C) (TSK gel G3000PWXL Tosoh, Tokyo, Japan); flow rate, 1.0 mL/min; and room temperature.

2.4. Measurement of the Activated Partial Thromboplastin Time (APTT)

Human normal plasma [Pacific Hemostasis, Coagulation control plasma (normal) Level 1, Thermo Fisher Scientific Inc., Waltham, MA, USA] and HE, MHE, or MHADs (prepared in various concentration) were mixed at a ratio of 9:1 as a test plasma, and phosphate buffered saline (PBS) (-) was prepared like the test plasma instead of HE, MHE, or MHADs as a control plasma. APTTs of HE, MHE, and MHADs were measured using test

or control plasma and APTT-TEST-WAKO (Wako, Osaka, Japan) and an Amelung-coagulometer (Trinity Biotech Plc., Ireland).

2.5. Culture Cells

HUV-ECs (DS Pharma Biomedical Co., LTD. Osaka, Japan) were cultured in 75 cm^2 tissue culture flasks with Medium 199 which contained 20% fetal bovine serum and an EGM-MV set (additional and growth factor set: Sanko Junyaku Co., LTD, Tokyo, Japan) and then they were placed in a CO_2 incubator (95% O_2 and 5% CO_2) at 37˚C. Subconfluent cells were used for all experiments.

2.6. Assay of Oxidative Stress-Induced Cell Death

The damage of HUV-ECs was examined according to the method of Hiebert and Liu [15]. Briefly, HUV-ECs were divided at 1.5×10^5 cells/well each into 6 wells of tissue culture plates with Medium 199 containing 1% fetal bovine serum and then xanthine, xanthine oxidase (Wako, Osaka, Japan), and each compound prepared in PBS(-) were added to each well at concentrations of 0.01 μmol/mL, 0.2 U/mL, and 50 μg/mL, respectively. After 24 hours, cells were harvested by 0.025% trypsin: 0.01% EDTA-2Na = 1:1 solution. Then, an equal volume of 0.2% trypan blue solution was added to the cell suspension. After 2 minutes, cells were differentiated as dead or living, and the number of living cells was counted using a hemocytometer under a light microscope.

2.7. Statistical Analyses

The data are presented as the means ± standard error (S.E.), significance was tested by ANOVA followed by Tukey's test, and the differences were assessed at a 0.05 significance level.

3. Results

3.1. Determination of the Molecular Weights of HE, MHE, and MHADs

The mean molecular weight of MHE was smaller than that of HE, and MHADs were synthesized from MHE (mean molecular weight: 8500 - 9500, and the molecular weight between 5000 and 10,000 constituted above 70%) to reduce the anticoagulant action. The mean molecular weight of MHD was 9000 - 10,000, and those of the other derivatives were 8500 - 9500 (**Table 1**).

3.2. Measurement of the Activated Partial Thromboplastin Time (APTT)

The APTT-prolonging actions of MHE in the human normal plasma were weaker than those of HE. Moreover,

Table 1. The molecular weight of HE, MHE and MHADs.

Compounds	Maximum molecular weight	Minimum molecular weight	Mean molecular weight
HE	18,000	7000	13,000 - 14,000
MHE	12,500	4500	8500 - 9500
MHA	12,500 - 13,500	3500 - 4500	8500 - 9500
MHD	12,500 - 135,00	3500 - 4500	9000 - 10,000
MHF	12,500 - 13,500	3500 - 4500	8500 - 9500
MHG	12,500 - 13,500	3500 - 4500	8500 - 9500
MHH	12,500 - 13,500	3500 - 4500	8500 - 9500
MHL	12,500 - 13,500	3500 - 4500	8500 - 9500
MHM	12,500 - 13,500	3500 - 4500	8500 - 9500
MHP	12,500 - 13,500	3500 - 4500	8500 - 9500
MHR	12,500 - 13,500	3500 - 4500	8500 - 9500
MHS	12,500 - 13,500	3500 - 4500	8500 - 9500
MHY	12,500 - 13,500	3500 - 4500	8500 - 9500

the APTT-prolonging actions of MHADs were weaker than those of MHE and the concentration of MHADs yielding an APTT of 100 seconds was about 6 - 17 times higher than that of HE (**Table 2**).

3.3. Assay of Oxidative Stress-Induced Cell Death

MHD and MHP did not show any effects on the damage of HUV-ECs induced by oxygen free radicals. However, HE or other MHADs showed a tendency to inhibit the decrease in cell viability induced by free radicals. MHF, MHL, and MHY showed significant inhibition of the decrease in cell viability (**Figure 1**).

Table 2. Effect MHADs on APTT using human plasma.

Compounds	Concentration (μg/mL) of compounds that APTT is 100 seconds	Ratio to the HE
HE	4.79	1.00
MHE	14.79	3.09
MHA	30.90	6.45
MHD	31.56	6.59
MHF	40.27	8.41
MHG	53.70	11.21
MHH	57.54	12.01
MHL	35.96	7.51
MHM	35.48	7.41
MHP	28.18	5.88
MHR	85.11	17.77
MHS	26.86	5.61
MHY	38.90	8.12

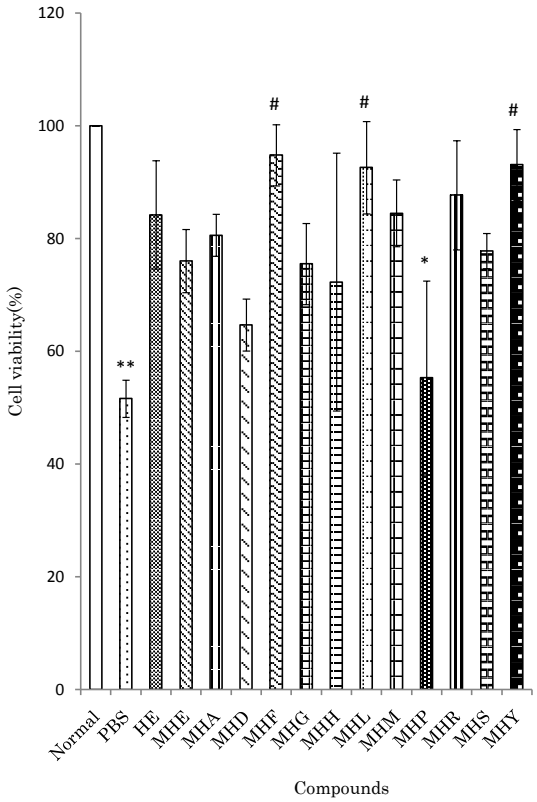

Figure 1. Effect of heparinyl amino acid derivatives on endothelial cells treated with xanthine and xanthine oxidase. All compounds were added to the culture medium at 50 μg/mL. This experiment was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Normal, $^{\#}P < 0.05$ vs PBS (by Tukey's test).

4. Discussion

A number of studies have reported a close relationship between SOD and diverse diseases [16]-[19]. However, few ethical drugs with a radical scavenging function, like "edaravone", have been developed since there are some obstacles to using radical scavengers as medicines. For example, one of the radical scavengers, SOD, synthesized artificially, exhibits some immunogenicity after administration to humans [20] [21]. On the other hand, HE functions as an indirect radical scavenger and induces the release of EC-SOD from endothelial cells *in vivo* [10]-[13]. EC-SOD is one kind of SOD [22] [23], containing Cu^{2+} [24], and we do not have to be concerned about immunogenicity since it is an endogenous enzyme [25]. Accordingly, we have focused on its beneficial effect as a radical scavenger.

MHADs (excluding MHD and MHP) tended to inhibit free radical-induced reduction of cell viability of HUV-EC, and MHF, MHL, and MHY significantly protected HUV-EC more strongly than HE. For HE to induce EC-SOD and remove radicals, it has to first bind to HUV-EC. Originally, the HUV-EC surface is positively charged. HE is a negatively charged proteoglycan and readily binds to HUV-EC [26]. MHF, MHL and MHY were superior to HE in the action on cell viability. These may be strongly negatively charged or likely to bind to HUV-EC compared with HE.

However, the useful dose of HE is limited because of its hemorrhage-promoting activity. It is known that HE is a mixture of variously sized sulfated aminoglycans, and its hemorrhage-promoting activity is correlated with its molecular weight [27]. In other words, the high molecular weight of HE indicates a high hemorrhage-promoting activity. To resolve this, we have synthesized MHADs to reduce the hemorrhage activity of HE with beneficial effects. Recently, low-molecular-weight heparins (LMWHs) are being used as anticoagulants and these are much safer than conventional HE [28]-[30]. In this study, we did not only try to decrease the molecular weight of HE, but also conjugate amino acids to reduce its high hemorrhagic tendency. From the results of APTT, antithrombin activation ability of MHADs is thought to be weaker than HE or MHE due to the lower molecular weight and addition an amino acid.

5. Conclusion

In conclusion, we examined the radical scavenging effect of MHADs, and the protective effect of MHF, MHL, and MHY on HUV-ECs damaged by oxygen free radicals was stronger than HE at the same concentration. Although we do not have enough data on the pharmacological mechanisms of MHADs, a number of MHADs might be promising candidates for clinical use as oxygen radical scavengers with few side effects.

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