

Effects of Inhaled Low Molecular Weight Heparin on Airway Allergic Inflammation in Aerosol-Ovalbumin-Sensitized Guinea Pigs

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ABSTRACT—Low molecular weight heparin (LMWH) possesses multiple nonanticoagulant properties. In the present study, we observed its anti-airway allergic inflammatory effects by bronchoalveolar lavage in guinea pigs. Guinea pigs were sensitized by repeatedly inhaling aerosolized ovalbumin. LMWH (400 u/l, 800 u/l), dexamethasone (1.2 mg/l) or vehicle (normal saline) was inhaled for 7 days. Then the animals were sacrificed under anesthesia and then lavaged with ice-cold Hank's buffer immediately; bronchoalveolar lavage fluid (BALF) was prepared 24 h after the animals were challenged by antigen exposure. The effects of LMWH on total cell counts, absolute eosinophil counts and cell catalogues in BALF were studied; effects on the activity of eosinophil peroxidase (EPO) and the contents of histamine and eosinophil cationic protein (ECP) in BALF supernatant were detected. Our results showed that compared with the vehicle group, LMWH at 400 u/l and 800 u/l could significantly reduce total cell counts, absolute eosinophil counts and percentage of eosinophils in BALF ($P<0.05$ and $P<0.01$, respectively); LMWH at 800 u/l markedly inhibited the activity of EPO in BALF supernatant ($P<0.05$); LMWH at 400 u/l and 800 u/l remarkably reduced the content of histamine in BALF supernatant ($P<0.05$ and $P<0.01$, respectively), LMWH at 800 u/l decreased the content of ECP ($P<0.05$) significantly. It suggested that LMWH exerted anti-airway allergic inflammatory action by inhibiting infiltration of inflammatory cells and reducing release of inflammatory mediators, as well as antagonizing their activities, and that LMWH could be developed as a potential anti-bronchial asthmatic drug.

Keywords: Low molecular weight heparin (LMWH), Airway allergic inflammation, Bronchoalveolar lavage fluid, Eosinophil, Bronchial asthma

Bronchial asthma, one of the airway allergic inflammatory diseases, is a serious threat human health. Airway allergic inflammation induces mucosal congestion and edema, effusion, formation of intraluminal mucus plugs and degeneration, necrosis, cast-off of airway epithelial cells, and ultimately, causes airway ventilation disorder and airway hyperresponsiveness (AHR). Bronchoalveolar lavage has been generally used in the clinic to detect secretion in asthma patients' airways and to assess seriousness of this disease, by which doctors could evaluate the efficiency of medications and select a proper one to apply to different patients individually.

Heparins, traditionally used as anticoagulants, also possess multiple nonanticoagulant activities such as modify-

ing protein kinases (1, 2), modulating cell proliferation (3, 4), immuno-regulatory activity (5), anti-HIV activity (6) and antiinflammatory activity (7). Recently, it has been shown that heparins could alleviate the acute bronchoconstriction response of allergic asthma by prolonging latent periods of asthmatic response, dilating the airway in guinea pigs (8, 9), which suggested that heparins possessed anti-asthmatic activity. Low molecular weight heparin (LMWH), a newly developed biochemical drug, is low molecular weight components or fragments derived from heparins. LMWH was proved to have airway dilating activity in animals and to be more potent than heparin (10). However, there lacks further investigations on the anti-bronchial asthmatic effects of LMWH. The present study was designed to investigate anti-airway allergic inflammatory effects of LMWH by broncho-alveolar lavage in aerosol-ovalbumin-sensitized guinea pigs so that we could potentially develop a new therapeutic application of LMWH for bronchial asthma.

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MATERIALS AND METHODS

LMWH, mean MW 5,500, was provided to us by the Department of Biochemical Pharmaceutics, College of Pharmacy, Shandong Medical University. Guinea pigs were provided by the Experiment Animal Center of Shandong Medical University. The present study was performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and the Recommendations from the Declaration of Helsinki.

Preparation of animal model and drug treatment (11)

Guinea pigs (150–200 g) were sensitized by inhaling aerosolized-ovalbumin (OA) using an 402 ultrasonic nebulizer (Shanghai, China) once on day 1 and once on day 8. Then the animals were divided into 4 groups and administered LMWH 400 u/l, 800 u/l, dexamethasone (Dex) 1.2 mg/l or vehicle (normal saline, N.S.), respectively, by inhalation, once a day from days 8–14. Immediately after the last administration, diphenhydramine at 10 mg/kg was administered i.p. Thirty minutes later, the animals were placed in a chamber and challenged by aerosolized OA exposure until the appearance of obvious asthmatic seizure. Eight hours later, the animals were administered the agents by inhalation once again.

Preparation of bronchoalveolar lavage fluid (BALF) (11, 12)

The animals were sacrificed by bleeding from the common carotid artery under anesthesia 24 h after being challenged and were immediately lavaged with ice-cold Hank's buffer, 5 ml each time, for 6 times. The BALF of the first 2 times was mixed together for cell counting and that of the other 4 times was mixed together to detect the contents of histamine and eosinophil cationic protein (ECP) and activity of eosinophil peroxidase (EPO) after centrifugation (4°C, 480 × g for 10 min).

Effect of LMWH on total cell counts, absolute eosinophil (Eos) counts and cell catalogues of BALF (11)

The total cell numbers were counted directly on a blood cell counting plate under a one CH-2 microscope (Olympus Optical Co., Ltd, Tokyo); BALF (0.1 ml) was diluted and stained with diluted Eosin solution (0.9 ml), and then the Eos numbers were counted; an aliquot of the BALF was dropped on a glass slide (two slides per sample) dried at 37°C and then stained with Wright's solution. Then the numbers of Eos, alveolar macrophages (AM), lymphocytes (Lym) and neutrophils (Neu) were counted; the total number of cells counted in each sample was no less than 200.

Effect of LMWH on the activity of EPO in BALF supernatant (13, 14)

Preparation of substrate solution: Substrate solution was prepared according to the method described by Strath et al. (14). Substrate solution contained the following components at the final concentrations indicated in parentheses: *O*-phenylenediamine (OPD, 0.1 mM), Triton X-100 (1 ml/l), H₂O₂ (1 mM) and Tris (0.05 M); the pH was adjusted to 8.0 with 1 M HCl. The substrate solution was kept in the dark at –20°C.

Measurement of EPO activity: BALF (150 µl) and substrate solution (300 µl) were mixed together and incubated in a water bath at 37°C. Thirty minutes later, the reaction was terminated by adding 4 M H₂SO₄ (200 µl), and then the absorbance was measured at the wavelength of 492 nm by U2000 UV spectrophotometer (Hitachi Co., Ltd., Tokyo).

Effect of LMWH on the content of histamine in BALF supernatant (15)

Sample processing: A 0.35-ml aliquot of 1.529 M trichloroacetic acid was gently added to 1.65 ml BALF. Then the mixture was centrifuged at 800 × g for 10 min. The supernatant was removed from the precipitate and kept in new tubes.

Fluorometry and standard curve of histamine: According to the improved method described by Xiang and Chen (15), fluorescence was detected by a 850-Spectrofluorometer (Hitachi Co., Ltd.); a standard curve was made according to the following regression equation, by which fluorescence intensity could be transformed into the histamine content (µg/ml):

$$Y = 0.3430 + X \cdot 5.6422 \times 10^{-3} \quad r = 0.999$$

Y = Fluorescence intensity

X = Concentration of histamine (µg/ml)

Effect of LMWH on the content of ECP in BALF supernatant

The concentration of ECP in BALF supernatant was detected by a CAP Allergen Detecting System (Pharmacia Co., Ltd., Uppsala, Sweden) using immunofluorometry.

Data analysis and statistics

Results were expressed as the mean ± S.E.M. Statistical significance was assessed by the software SAS (Statistical Analysis System, version 6.04), using Dunnett's method. P values less than 0.05 were considered to be statistically significant.

RESULTS

Effect of LMWH on total cell counts, absolute Eos counts and cell catalogues of BALF

As showed in Table 1, we observed that LMWH at 400 u/l and 800 u/l significantly reduced total cell counts and absolute Eos counts compared with those of the vehicle group ($P<0.05$ and $P<0.01$, respectively). The percentages of AM, Eos, Lym and Neu were calculated. LMWH at 400 u/l and 800 u/l significantly reduced the percentage of Eos, major effector cells in allergic airway inflammation, and it significantly increased the percentage of AM, phagocytes in the lung tissue ($P<0.01$ and $P<0.05$, respectively). LMWH had no remarkable effects on the percentages of Lym and Neu. There was no significant difference between the LMWH (800 u/l)- and Dex (1.2 mg/l)-treated groups (Fig. 1).

Table 1. Effect of LMWH on total cell counts and absolute Eos counts of BALF

Group	Inhalation concentrations	Total cell counts ($10^9/l$)	Absolute Eos counts ($10^8/l$)
N.S.	—	2.9 ± 0.4	2.2 ± 0.3
LMWH	400 u/l	$2.4 \pm 0.6^*$	$1.8 \pm 0.2^*$
	800 u/l	$1.6 \pm 0.3^{**}$	$1.4 \pm 0.7^{**}$
Dex	1.2 mg/l	$1.6 \pm 0.6^{**}$	$1.4 \pm 0.6^{**}$

Data are expressed as the mean $\pm$ S.E.M, $n=7$. LMWH groups and Dex group compared with the vehicle group using Dunnett's method by SAS 6.04: $^*P<0.05$, $^{**}P<0.01$.

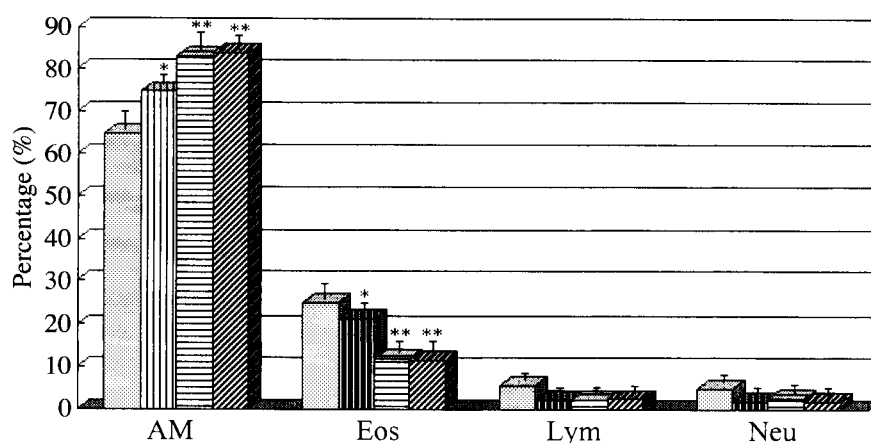


Fig. 1. Effect of LMWH on the percentages of AM, Eos, Lym and Neu in BALF. The cell numbers were counted under a microscope after staining with Wright's solution. The percentages were obtained by dividing the number of each type of cell by the number of the respective type of cell in the vehicle-treated group. $\square$ Vehicle (N.S.) group; |||| LMWH, 400 u/l group; ||||| LMWH, 800 u/l group; ||||| Dex, 1.2 mg/l group. Data represent the mean $\pm$ S.E.M of 7 animals. LMWH groups and Dex group compared with the vehicle group using Dunnett's method by SAS 6.04: $^*P<0.05$, $^{**}P<0.01$.

Effect of LMWH on the activity of EPO in BALF supernatant

As shown in Fig. 2, compared with the vehicle group, LMWH at 800 u/l significantly lowered the absorbance at the wavelength of 492 nm ($P<0.05$), which means that it inhibited the activity of EPO in BALF supernatant significantly. There was no significant difference between the LMWH (800 u/l)- and Dex (1.2 mg/l)-treated groups.

Effects of LMWH on the content of histamine and ECP in BALF supernatant

As indicated by the data of Table 2, compared with the vehicle group, LMWH at 400 u/l and 800 u/l significantly reduced the fluorescence intensity and concentration

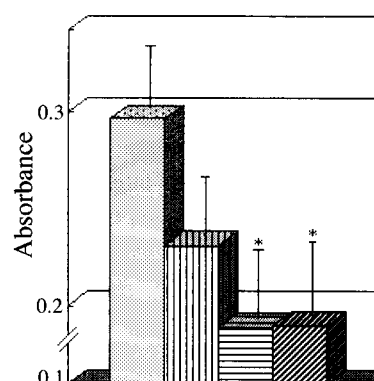


Fig. 2. Effect of LMWH on the activity of EPO in BALF supernatant. $\square$ Vehicle (N.S.) group; |||| LMWH, 400 u/l group; ||||| LMWH, 800 u/l group; ||||| Dex, 1.2 mg/l group. Data represent the mean $\pm$ S.E.M of 7 animals. LMWH groups and Dex group compared with the vehicle group using Dunnett's method by SAS 6.04: $^*P<0.05$.

Table 2. Effects of LMWH on the content of histamine and ECP in BALF supernatant

Group	Inhalation concentration	Content of histamine		Content of ECP
		Fluorescence intensity	Concentration ($\mu\text{g/ml}$)	Fluorescence intensity
N.S.	—	1.540 ± 0.064	0.398 ± 0.020	33.0 ± 1.9
LMWH	400 u/l	$1.450 \pm 0.053^*$	$0.376 \pm 0.022^*$	$28.8 \pm 2.9^*$
	800 u/l	$1.420 \pm 0.059^{**}$	$0.361 \pm 0.022^{**}$	$28.2 \pm 4.1^*$
Dex	1.2 mg/l	$1.406 \pm 0.051^{**}$	$0.354 \pm 0.17^{**}$	—

Data are expressed as the mean $\pm$ S.E.M, $n=7$. LMWH groups and Dex group compared with the vehicle group using Dunnett's method by SAS 6.04: * $P<0.05$, ** $P<0.01$.

($\mu\text{g/ml}$) of histamine ($P<0.05$ and $P<0.01$, respectively). There were no significant differences between the LMWH (400 u/l)-, LMWH (800 u/l) and Dex (1.2 mg/l)-treated groups. LMWH at 400 u/l and 800 u/l significantly decreased the content of ECP in BALF supernatant ($P<0.05$).

DISCUSSION

Airway allergic inflammation, now regarded as one major pathologic changes of bronchial asthma, is more closely related to airway ventilation disorder and AHR, which are the two major features of bronchial asthma. Several categories of inflammatory cells play important roles in bronchial asthma, such as mast cells and Eos, which can release a variety of inflammatory mediators if they are activated by antigens or non-allergic stimuli. Histamine, released from degranulated mast cells, binding to H_1 -receptors, leads to spasm of tracheal smooth muscle, mucosal edema and vasodilation. ECP and EPO, two major toxic eosinophil-derived cationic proteins, which were proven to be cytotoxic to airway epithelium (16), damage the airway epithelium at different levels, including scaling of cilia and brush cells, inhibiting mucus-clearing function of cilia, resulting in denuded epithelium. Activated Eos also infiltrate through airway tissue and induce a serious allergic response in the airway.

Our results showed that LMWH could lower the content of histamine in BALF, which may be related to its inhibitory effect on the degranulation of mast cells induced by antigen or non-antigen (17, 18). Release of Ca^{2+} from reticulum conducted by inositol-1,4,5-triphosphate (IP_3), which induces a transient increase of intracellular free Ca^{2+} , was necessary for degranulation of mast cells (19). Heparin has been shown to act as a specific blocker of IP_3 and to inhibit IP_3 -mediated Ca^{2+} release (20), and as a consequence, inhibits degranulation of mast cells and release of histamine (17, 21). This observations indicate that the effect of LMWH to lower the content of histamine may possibly be related to its ability to block IP_3 -mediated re-

lease of intracellular Ca^{2+} in mast cells.

Eosinophils are the major effector cells in the late phase asthmatic response and eosinophil infiltration is one of the characteristic features of asthmatic airway and differentiates asthma from other inflammatory conditions of the airway. In the late phase asthmatic response, eosinophils recruit markedly in peripheral blood, airway secretion and BALF, and there was a close relationship between eosinophil counts and seriousness of allergic airway blockage and nonspecific AHR (22). It was found that eosinophils also release a variety of mediators and cytokines conducting the immunologic response in the local tissue of the airway. Heparins have been shown to inhibit the eosinophil infiltration through the airway and to reduce eosinophil counts in BALF in allergen-sensitized guinea pigs (23). Our results showed that LMWH could significantly reduce total cell counts, eosinophil absolute counts and percentage of eosinophils in BALF, suggesting that LMWH could inhibit inflammatory cells infiltrating through lung tissue and reduce inflammatory effusion. On the other hand, LMWH significantly inhibited the activity of EPO and reduced the content of ECP in BALF. It suggested that LMWH also could inhibit release of inflammatory mediators from activated Eos and had an antagonistic effect against inflammatory mediators, especially ECP and EPO, two main toxic cationic proteins; these actions lead to alleviation of airway epithelium damage and enables recovery of the function of the airway epithelium as one barrier between the inside of the body and the outside environment.

In conclusion, the results of the present study suggested that LMWH can exert anti-airway allergic inflammatory activity by inhibiting infiltration of inflammatory cells and reducing the release of inflammatory mediators, and also by antagonizing their activity, and that LMWH can potentially be developed as an anti-bronchial asthmatic drug.

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REFERENCES

- 1 Schwartz LB and Bradford TR: Regulation of tryptase from human lung mast cells by heparin. *J Biol Chem* **261**, 7372–7379 (1986)
- 2 Naclerio RM and Baroody F: Understanding the inflammatory processes in upper allergic airway disease and asthma. *J Allergy Clin Immunol* **101**, s345–s351 (1998)
- 3 Garg HG, Joseph PA, Yoshida K, Thompson BT and Hales CA: Antiproliferative role of 3-*O*-sulfate glucosamine in heparin on cultured pulmonary artery smooth muscle cells. *Biochem Biophys Res Commun* **224**, 468–473 (1996)
- 4 Shao NS, Wang HX, Zhou TC, Xue YN and Liu C: Heparin potentiation of the effect of acidic fibroblast growth factor on asrtocytes and neurons. *Life Sci* **54**, 785–789 (1994)
- 5 Lagodzinski Z, Gorski A and Waski M: Immunosuppressive action of low-dose heparin: Effect on skin allograft survival. *Transplantation* **50**, 714–715 (1990)
- 6 Rusnati M, Coltrini D, Oresta P, Zoppetti G, Albini A, Noonan D, Fagagna F, Giacca M and Presta M: Interaction of HIV Tat protein with heparin: Role of the backbone structure, sulfation and size. *J Biol Chem* **272**, 11313–11320 (1997)
- 7 Jaques LB: Heparins anionaic polyelectrolyte drugs. *Pharmacol Rev* **337**, 717–720 (1979)
- 8 Polosa R, Magri S, Vanchari C, Armato F, Santonacito G, Mistretta A and Crimi N: Time course of changes in adenosine 5'-monophosphate airway responsiveness with inhaled heparin in allergic asthma. *J Allergy Clin Immunol* **99**, 338–344 (1997)
- 9 Ming L, Zhang Y, Li W, Wang Y and Xu SY: Antiasthmatic effect of heparin. *Chin Pharmacol Bul* **13**, 361–364 (1997)
- 10 Ahmed T, Campo C, Abraham MK, Molinari JF, Abraham WM, Askin D, Syryste T, Andersson LO and Svahn CM: Inhibition of antigen-induced acute bronchoconstriction, airway hyperresponsiveness, and mast cell degranulation by a nonanticoagulant heparin comparison with a low molecular weight heparin. *Am J Respir Cirt Care Med* **155**, 1848–1855 (1997)
- 11 Wang CZ, Wang CX, Qian GS and Guo XJ: Modulation of platelet-activating factor antagonist BN 52021 on hypodense eosinophils in airway of allergic guinea pigs. *Chin Pharmacol Bull* **12**, 238–240 (1996)
- 12 Wang CZ, Pu Y, Wu GM, Li SP and Guo XJ: Inhalation of platelet-activating factor induced guinea pigs airway hyperresponsiveness: Role of eosinopils activation. *Chin J Tubcul and Respir Dis* **18**, 155–157 (1995)
- 13 Kroegel C, Yukawa T, Dent G, Venge P, Chung KF and Barnes PJ: Stimulation of degranulation from human eosinophils by platelet-activating factor. *J Immunol* **142**, 3518–3526 (1989)
- 14 Strath M, Warren DJ and Sanderson CJ: Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. *J Immunol Methods* **83**, 209–215 (1985)
- 15 Xiang JJ and Chen HC: Modified method of fluorometric determination of blood histamine. *Acta Acad Med Sin* **3**, 183–186 (1981)
- 16 Cottin V, Deviller P, Tardy F and Cordier JF: Urinary eosinophil-derived neurotoxon/protein X: A simple method for assessing eosinophil dagranulation in vivo. *J Allergy Clin Immunol* **101**, 116–123 (1998)
- 17 Ahmed T, Syryste T, Mendelssohn R, Sorace D, Mansour E, Lansing M, Abraham WM and Robinson MJ: Heparin prevents antigen-induced airway hyperresponsiveness: interference with IP3-mediated mast cell degranulation? *J Appl Physiol* **74**, 893–901 (1993)
- 18 Wang QL, Shang XY and Zhang SL: Anti-allergic effects of low molecular weight heparin. *J Chin Pharm Sci* **8**, 163–166 (1999)
- 19 Somlyo AV, Bond M, Somlyo AP and Scarpa A: Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. *Proc Natl Acad Sci USA* **82**, 5231–5235 (1985)
- 20 Kobayashi S, Somlyo AV and Somlyo AP: Heparin inhibits the inositol 1,4,5-trisphosphate-dependent, but not independent, calcium release induced by guanine nucleotide in vascular smooth muscle. *Biochem Biophys Res Commun* **153**, 625–631 (1988)
- 21 Lucio J, D'brot J, Guo CB, Abraham WM, Lichtenstein LM, Sobotka AK and Ahmed T: Immunologic mast cell-mediated response and histamine release are attenuated by heparin. *J Appl Physiol* **73**, 1093–1101 (1992)
- 22 Burrows B, Hansan FM and Barbee RA: Epidemiologic observations on eosinophilia and its relation to respiratory disorders. *Am Rev Respir Dis* **122**, 709–715 (1980)
- 23 Weller PF: Updates on cells and cytokines: Human eosinophils. *J Allergy Clin Immunol* **100**, 283–287 (1997)