ORIGINAL ARTICLE

The Effect of Heparin on Antigen-Induced Mucus Hypersecretion in the Nasal Epithelium of Sensitized Rats

Takao Ogawa[1], Shino Shimizu[1] and Takeshi Shimizu[1]

ABSTRACT

Background: Heparin is a potential anti-inflammatory drug for allergic airway inflammation. To elucidate the effects of heparin on allergic inflammation, we examined the *in vivo* effects of heparin on antigen-induced mucus hypersecretion and infiltration of eosinophils and neutrophils in the nasal epithelium of sensitized rats.
Methods: We induced hypertrophic and metaplastic changes of goblet cells in the nasal epithelium of ovalbumin (OVA)-sensitized rats by intranasal challenge with OVA. The effects of intranasal instillation with low molecular weight heparin (LMWH; 1-1000 IU/0.1 ml) on mucus production and eosinophil/neutrophil infiltration were examined.
Results: Intranasal instillation with low-dose LMWH (1-10 IU/0.1 ml) at 30 minutes before OVA instillation stimulated OVA-induced mucus production in the nasal epithelium of sensitized rats, whereas treatment with 100 IU/0.1 ml LMWH showed no effect. Intranasal instillation with high-dose LMWH (1000 IU/0.1 ml) significantly inhibited OVA-induced mucus production. Intranasal instillation with LMWH (1-1000 IU/0.1 ml) dose-dependently inhibited eosinophil and neutrophil infiltration into the rat nasal mucosa.
Conclusions: These results indicate that heparin inhibits mucus hypersecretion and infiltration of eosinophils and neutrophils in allergic inflammation, though the inhibitory effect against mucus production is obtained in high-dose heparin. Intranasal instillation with high-dose heparin may provide a new therapeutic strategy for the treatment of nasal allergic inflammation.

INTRODUCTION

Hypersecretion of mucus is an important characteristic of allergic inflammation such as allergic rhinitis and bronchial asthma. Ovalbumin (OVA)-sensitized animals have been used to study the pathophysiologic changes in allergic inflammation in airways, including plasma extravasation, eosinophil infiltration, and mucus hypersecretion.[1] A variety of allergic mediators and inflammatory cells are capable of stimulating mucus hypersecretion. We previously reported that thrombin, an enzyme formed during activation of the coagulation system, plays an important role in airway inflammation by stimulating mucus hypersecretion and production of cytokines and growth factors from epithelial cells.[2] We also demonstrated the presence of thrombin in nasal secretion from patients with allergic rhinitis, and activation of coagulation system is implicated in the pathogenesis of airway allergic inflammation.[3]

Heparin has been used as one of the most important anticoagulant drugs in clinical practice, and it also possesses the anti-inflammatory activities by binding nonspecifically to many proteins involved in the inflammatory process, including cytokines, growth factors, adhesion molecules and tissue-destructive enzymes.[4,5] In the previous study, we revealed that heparin inhibited TNF-α-induced production of mucin and IL-8 in cultured human airway epithelial cells, and that intranasal instillation with hepa-

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Day 1 2 3 4 5 6 7 8 9 10

Intraperitoneal injection of OVA (200 μg) and A1(OH)₃ (5 mg) in a saline solution (1 ml)
(sensitization)

Day 13 14 15 16 17 18 19 20 21 22 23 24

Intranasal instillation of OVA (10 mg) in saline solution (0.1 ml)   sacrificed
(challenge)

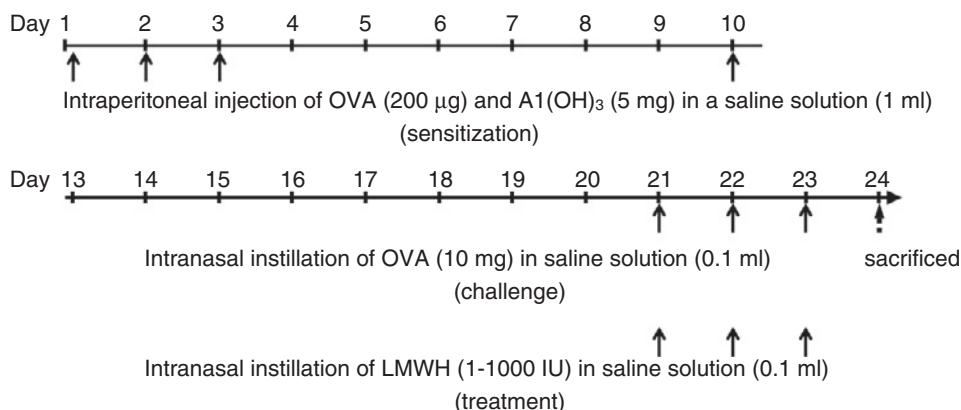Intranasal instillation of LMWH (1-1000 IU) in saline solution (0.1 ml)
(treatment)

Fig. 1 Time line for intraperitoneal sensitization and intranasal challenge with OVA, and intranasal instillation with LMWH at 30 min before OVA challenge.

rin significantly inhibited lipopolysaccharides (LPS)-induced mucus hypersecretion and neutrophil infiltration in rat nasal epithelium.[6] Recently, potential anti-inflammatory effects of heparin in allergic inflammation have been examined by animal studies and several clinical trials on patients with allergic rhinitis and bronchial asthma.[7-11] However, the mechanism responsible for the anti-inflammatory effects of heparin is not well understood, and the effect of heparin on allergic inflammation is still unclear.

In the present study, to elucidate the effects of heparin on mucus hypersecretion in allergic airway inflammation, we induced hypertrophic and mataplastic changes of goblet cells in the nasal epithelium of OVA-sensitized rats by intranasal challenge with OVA. The *in vivo* effects of intranasal instillation with heparin on mucus hypersecretion and on eosinophil and neutrophil infiltration were then examined.

METHODS

SENSITIZATION AND INTRANASAL CHALLENGE WITH OVA
The experimental protocol was approved by the Committee for Animal Care and Ethics of Shiga University of Medical Science and it was carried out following the guidelines of the National Institute of Health. Sensitization and challenge of rats were performed as previously described.[1] Specific pathogen-free male Fisher-344 rats (6 rats in each group) 6 weeks of age were used in this study. Rats were sensitized with an intraperitoneal injection of 1 ml of saline solution containing 200 μg of OVA (grade V; Sigma-Aldrich Corp., St Louis, Missouri, USA) and 5 mg of aluminum hydroxide [Al(OH)₃] on days 1, 2, 3, and 10. On day 21, rats were anesthetized with ether, and 0.1 ml of saline solution containing 10 mg of OVA was instilled into both airways of the nasal cavity once a day for 3 days. Sham-challenged rats received 0.1 ml saline solution in the same manner (Fig. 1). LMWH (enoxaparin;

Clexane, Sanofi-Aventis Pharmaceutical Co., Paris, France); 1-1000 IU in 0.1 ml saline, were intranasally instilled at 30 minutes before the intranasal challenge with OVA for 3 consecutive days.

TISSUE PREPARATION
The rats were painlessly killed with an intraperitoneal overdose of sodium pentobarbital at 24 hours after the last intranasal challenge with OVA. The head of each rat was removed and fixed in 10% neutral buffered formalin for 3 days, then decalcified in 5% trichloroacetic acid for 10 days. The nasal cavity was transversely sectioned at the level of incisive papilla of the hard palate.[12] The tissue block was embedded in paraffin.

MORPHOMETRY
Tissue sections 4 μm thick were stained with Alcian blue (pH 2.6), periodic acid-Schiff (AB-PAS) and hematoxylin. The percent area of AB-PAS stained mucosubstance in the epithelial surface was determined by an image analyzer in nasal epithelium over 2 mm (1 mm each side of the nasal septum) of the basal lamina at the center of the septal cartilage (magnification ×400).[12] The infiltrating eosinophils and neutrophils in nasal mucosa were examined with Hematoxylin-eosin staining. The numbers of eosinophils and neutrophils in the nasal septal mucosa were counted over both sides of the septal cartilage by use of an oil immersion objective lens (magnification ×1,000). All measurements were done by one blinded observer.

STATISTICS
All data are expressed as mean ± SD. The difference between variables was analyzed by the Mann-Whitney U test. Probability (*p*) values of less than 0.05 were considered statistically significant.
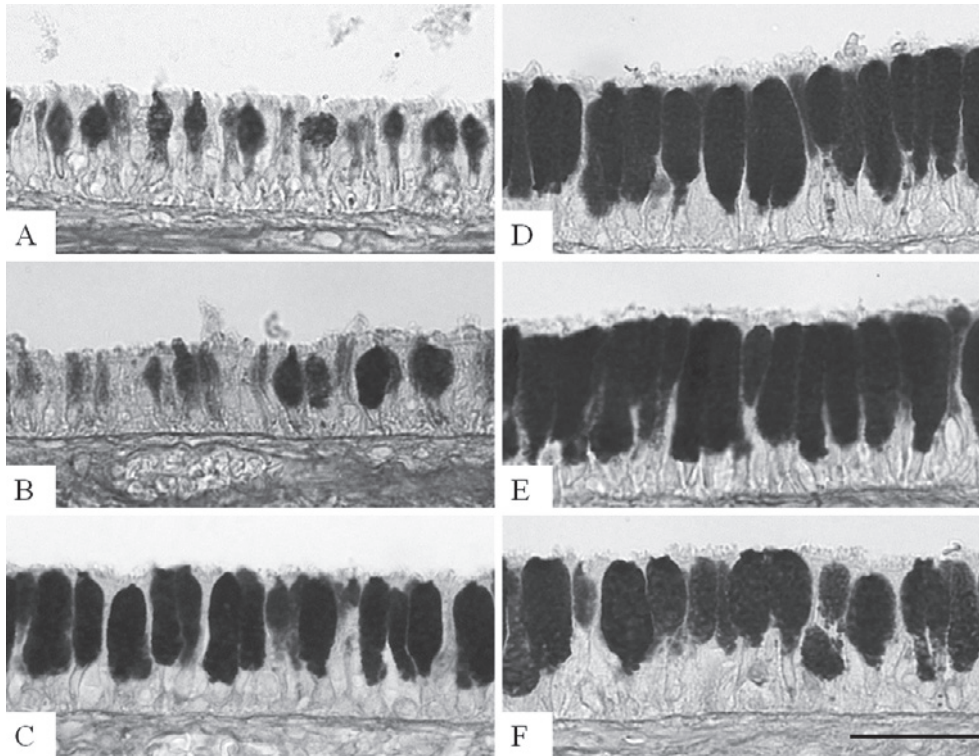
Fig. 2 Effects of LMWH on goblet cell metaplasia and mucus production in the nasal epithelium of sensitized rats. (**A**) Saline-treated saline-challenged control. (**B**) LMWH (100 IU/0.1 ml)-treated saline-challenged rats. (**C**) Saline-treated OVA-challenged rats. (**D**) LMWH (1 IU/0.1 ml)-treated OVA-challenged rats. (**E**) LMWH (10 IU/0.1 ml)-treated OVA-challenged rats. (**F**) LMWH (100 IU/0.1 ml)-treated OVA-challenged rats. Intranasal instillation with OVA induced goblet cell metaplasia and mucus production. Intranasal instillation with LMWH (1-10 IU/0.1 ml) stimulated these changes, whereas LMWH (100 IU/0.1ml) showed no effects. Scale bars represent 30 μm.

RESULTS

Intranasal instillation with OVA (10 mg/0.1 ml) for 3 consecutive days significantly induced hypertrophic and metaplastic changes of goblet cells in the nasal septal epithelium of OVA-sensitized rats. Only a few goblet cells were observed in control groups; saline-instilled, or LMWH (100 IU/0.1 ml)-instilled rats. The effects of intranasal instillation with LMWH (1-100 IU/0.1 ml) at 30 minutes before OVA instillation on OVA-induced mucus production are shown in Figure 2. Intranasal instillation with low-dose LMWH (1-10 IU/0.1 ml) stimulated OVA-induced hyperplastic changes of goblet cells. Quantitative changes in the percent area of mucosubstance in this epithelium are described in Figure 3. Low-dose LMWH (1-10 IU/0.1 ml) significantly stimulated OVA-induced mucus production, whereas treatment with LMWH (100 IU/0.1 ml) showed no effect. Intranasal instillation with OVA also induced eosinophil and neutrophil infiltration in the nasal septal mucosa of sensitized rats at 24 hours after the last OVA challenge. Intranasal instillation with LMWH (1-100 IU/0.1 ml) dose-dependently in-
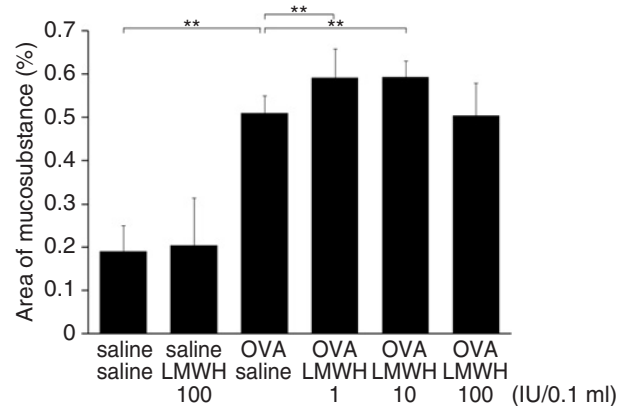

Fig. 3 Effects of LMWH on antigen-induced mucus production in the nasal epithelium of sensitized rats ($n = 6$). Intranasal instillation with OVA significantly induced mucus production. Intranasal instillation with low-dose LMWH (1-10 IU/0.1 ml) stimulated antigen-induced mucus production whereas LMWH (100 IU/0.1 ml) showed no effects. $**P < .01$.

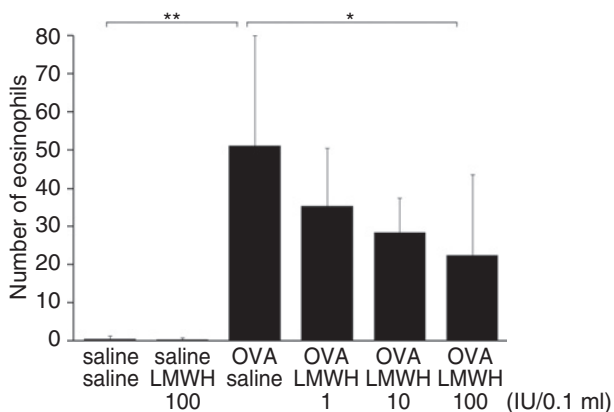**Fig. 4** Effects of LMWH (1-100 IU/0.1 ml) on antigen-induced eosinophil infiltration in the nasal mucosa of sensitized rats (*n* = 6). Intranasal instillation with LMWH (1-100 IU/0.1 ml) dose-dependently inhibited antigen-induced eosinophil infiltration. *P < .05, **P < .01.

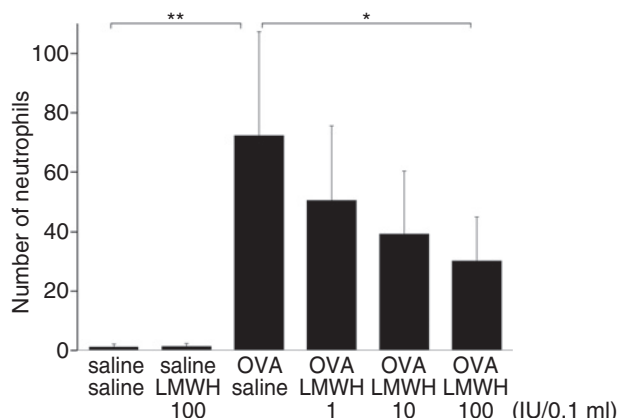**Fig. 5** Effects of LMWH (1-100 IU/0.1 ml) on antigen-induced neutrophil infiltration in the nasal mucosa of sensitized rats (*n* = 6). Intranasal instillation with LMWH dose-dependently inhibited antigen-induced neutrophil infiltration. *P < .05, **P < .01.
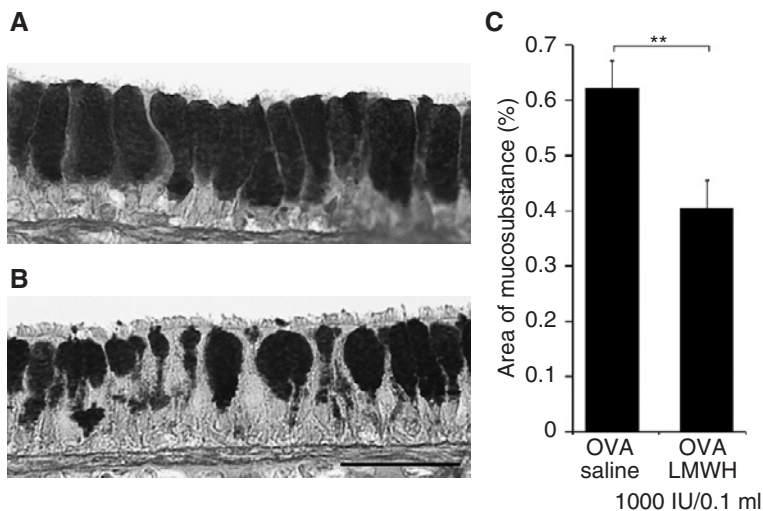

Fig. 6 Effects of high-dose LMWH (1000 IU/0.1 ml) on goblet cell metaplasia and mucus production in the nasal epithelium of sensitized rats. **A**) Saline-treated OVA-challenged rats. **B**) LMWH (1000 IU/0.1 ml)-treated OVA-challenged rats. Intranasal instillation with high-dose LMWH inhibited antigen-induced goblet cell metaplasia and mucus production. Scale bars represent 30 μm. **C**) Effects of high-dose LMWH (1000 IU/0.1 ml) on mucus production in the nasal epithelium of sensitized rats (*n* = 6). Quantitative analysis revealed that intranasal instillation with high-dose LMWH significantly inhibited antigen-induced mucus production. **P < .01.

hibited OVA-induced eosinophil and neutrophil infiltration (Fig. 4, 5).

We next examined the effects of high-dose LMWH (1000 IU/0.1 ml) on OVA-induced mucus production. Intranasal instillation with high-dose LMWH (1000 IU/0.1 ml) significantly inhibited OVA-induced changes of goblet cells (Fig. 6). Quantitative examination

revealed the significant inhibition with high-dose LMWH (1000 IU/0.1 ml) on OVA-induced mucus production. OVA-induced eosinophil and neutrophil infiltration in nasal septal mucosa was also significantly inhibited by intranasal instillation with high-dose LMWH (1000 IU/0.1 ml) (Fig. 7).
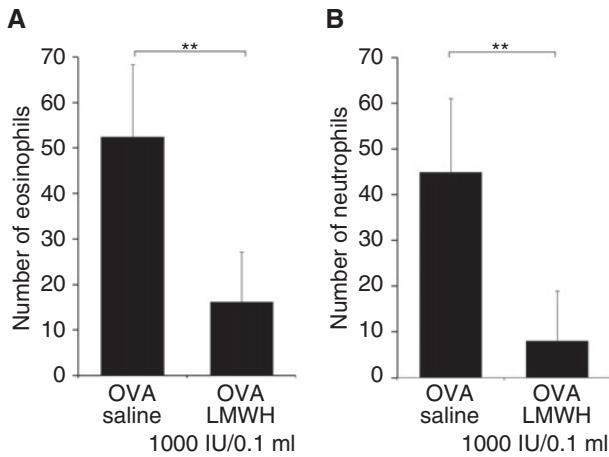
Fig. 7 Effects of high-dose LMWH (1000 IU/0.1 ml) on antigen-induced infiltrations of eosinophils (**A**) and neutrophils (**B**) in the nasal mucosa of sensitized rats ($n = 6$). Intranasal instillation with high-dose LMWH significantly inhibited antigen-induced infiltrations of eosinophils and neutrophils. **$P < .01$.

DISCUSSION

In the present study, intranasal instillation with OVA induced hypertrophic and metaplastic changes of goblet cells and mucosal infiltration of eosinophils and neutrophils in the nasal epithelium of OVA-sensitized rats. Intranasal instillation with high-dose LMWH (1000 IU/0.1 ml) significantly inhibited OVA-induced mucus production, whereas treatment with low-dose LMWH (1-10 IU/0.1 ml) stimulated it. Antigen-induced infiltrations of eosinophils and neutrophils were dose-dependently inhibited by LMWH (1-1000 IU/0.1 ml).

Heparin has been used for a long time in clinical practice as an anticoagulant drug. It has been known that heparin also had anti-inflammatory functions, and animal models have been used to study the regulatory effects of heparin on allergic inflammation.[5] Inhaled heparin reduced antigen-induced airway hyperreactivity in experimental model of asthma in rat[8] and sheep,[7,13,14] and it attenuated the nasal airway pressure and cellular infiltration in nasal lavage fluid in a gunea pig model of nasal allergy.[15] Several clinical studies revealed the anti-inflammatory effects of heparin in patients with bronchial asthma[9,10,16] and allergic rhinitis.[11,17] Inhaled heparin inhibited the bronchoconstrictive response in patients with exercise-induced asthma.[9] Heparin attenuated asthmatic responses in two patients with corticosteroid-resistant asthma.[10] Inhaled intranasal heparin reduced antigen-induced nasal symptoms in patients with allergic rhinitis.[11] However, little is known about the regulatory effects of heparin on antigen-induced mucus production, and this is the first report showing the effect of heparin on goblet cell metaplasia and mucus hypersecretion in nasal epithelium of allergic inflammation.

The mechanism by which high-dose LMWH inhibits antigen-induced mucus production is not well understood. Airway inflammation is associated with increased vascular permeability and leakage of plasma coagulation factors, leading to activation of the coagulation system in the extravascular space.[18] We previously reported that significant concentrations of thrombin and thrombin-antithrombin complex were found in nasal secretion from patients with allergic rhinitis.[3] Thrombin and its agonistic receptor peptide stimulated the mucus secretion from cultured nasal epithelial cells *in vitro*, and intranasal instillation with thrombin induced goblet cell metaplasia and mucus production in rat nasal epithelium *in vivo*.[2] These results indicate that the activation of coagulation system occurs during allergic airway inflammation and that thrombin plays a crucial role in the regulation of mucus hypersecretion. We have reported that the anticoagulant, activated protein C, inhibited thrombin-induced mucus production in rat nasal epithelium.[2] The regulatory effects of heparin on mucus hypersecretion may be partly caused by its anticoagulatory function through the inhibition of thrombin.

Heparin shows anti-inflammatory action by binding to and neutrize a wide variety of inflammatory mediators, such as cytokines, chemokines, complement factors, and extracellular matrix proteins.[4,5] We found that LMWH dose-dependently inhibited antigen-induced infiltrations of eosinophils and neutrophils in rat nasal epithelium. These results are consistent with previous studies showing that heparin inhibited eosinophil infiltration into the lung in animal model of asthma.[8,19] In our previous study, LMWH attenuated LPS-induced neutrophil infiltration in rat nasal epithelium, and it also suppressed TNF-α-induced secretion of IL-8, a neutrophil chemoattractant, from cultured human airway epithelial cells.[6] Leukocyte adhesion and activation play a central role in the inflammatory response, and heparin interferes with the adhesion of leukocyte to the endothelial cells.[20,21] The selectins are responsible for the interaction between leukocyte and endothelium, and it has been reported that heparin inhibits these selectin-mediated cell adhesions.[22,23] Heparin also inhibits the activation of eosinophils and neutrophils by binding the leukocyte adhesion molecule Mac-1.[24]

The anti-allergic effects of heparin may be related to the inhibition of mast cell functions. Heparin inhibits inositol triphosphate-mediated intracellular calcium release and mast cell degranulation.[14] Histamines and lukotrienes are important mast cell mediators in the initiation and development of antigen-induced airway responses. In our previous study, H1-antagonist inhibited the antigen-induced eosinophil infiltration in nasal mucosa of sensitized rats, and

cysLT1 antagonist attenuated the antigen-induced goblet cell metaplasia and mucus production,[1] suggesting that LMWH suppresses the allergic inflammation partially through the inhibition of mast cell degranulation.

In the present study, intranasal instillation with high-dose LMWH (1000 IU/0.1 ml) significantly inhibited antigen-induced mucus hypersecretion, whereas the treatment with low-dose LMWH (1-10 IU/0.1 ml) stimulated it. We previously reported that LMWH dose-dependently inhibits LPS-induced mucus production in rat nasal epithelium, and that it suppressed TNF-α-induced secretion of anti-MUC5AC reactive mucin and mRNA expression of MUC5AC in cultured human airway epithelial cells.[6] The inhibitory effects of high-dose LMWH on mucus hypersecretion may be caused by the direct inhibitory effects on mucus secretion from the epithelial cells and indirectly through the anti-inflammatory actions against inflammatory cells and mediators. However, the mechanism of the adverse response against low-dose LMWH on antigen-induced mucus production is unclear.

These biphasic responses of LMWH on mucus production may be caused by the complex and heterogeneous actions of heparin. Nitric oxide (NO) is a potent vasodilator, and heparin has been shown to promote NO formation in cultured endothelial cells,[25] and to preserve myocardial contractility after ischemia-reperfusion.[26] Nasal blood flow is regulated by the NO released from parasympathetic nitrergic nerves innervating the nasal mucosal arteries. We previously reported that the NO contributes an increase in nasal mucosal blood flow induced by sensory and parasymthetic nerve stimulation in rat.[27] Recently, NO has been shown to be an important mediator that stimulates nasal blood flow, vascular permeability, inflammatory cell infiltration and mucus production in allergic inflammation.[28,29] There is a possibility that the adverse effect of heparin on mucus production may be related to the heparin-induced increase of NO release after parasymthetic nerve stimulation caused by histamine in allergic inflammation.

In the present study, intranasal instillation with high-dose LMWH (1000 IU/0.1 ml) inhibited mucus hypersecretion in allergic inflammation of rat nasal epithelium, though low-dose LMWH showed opposite effects. Many researchers have been used high-dose LMWH or heparin (more than 10000 IU) for the clinical trials on allergic rhinitis and bronchial asthma.[4,9-11,13,16] Vancheri et al. reported that 15000 IU/4 ml of heparin nebulizer was effective for patients with allergic rhinitis.[11] Bendstrup et al. reported that the highest dose of inhaled heparin to use safely for bronchial asthma was 32000 IU.[30] In these studies, no adverse effect such as bleeding or thrombocytopenia has been reported. Our results suggest that topical application of high-dose LMWH (more than 10000 IU) may be useful for the clinical treatment of allergic inflammation in upper airways.

In conclusion, we have induced hypertrophic and metaplastic changes of goblet cells in nasal epithelium of OVA-sensitized rats by intranasal OVA challenge, and we have demonstrated in this model that antigen-induced mucus production and mucosal infiltrations of eosinophils and neutrophils are significantly inhibited by intranasal instillation with high-dose LMWH. Although antigen-induced infiltrations of eosinophils and neutrophils were dose-dependently inhibited by LMWH, mucus production was stimulated by low-dose LMWH. These results indicate that LMWH has heterogeneous anti-inflammatory effects in allergic inflammation, and that high-dose LMWH may provide a new therapeutic strategy for allergic inflammation. It is hoped that such study will improve the understanding and treatment of mucus hypersecretion in allergic airway inflammation.

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