Low-Molecular-Weight Heparin (LMWH)–Loaded Large Porous PEG-PLGA Particles for the Treatment of Asthma

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Abstract

Background: Heparin-like compounds interrupt leukocyte adhesion and migration, and prevent release of chemical mediators during the process of inflammation. However, little is known whether the anti-inflammatory property of smaller heparin fragments, low-molecular-weight heparin (LMWH), plays any role in the process of airway inflammation. In this study, we sought to evaluate the efficacy of LMWH-loaded large porous polyethylene glycol–poly(D,L-lactide-co-glycolide) (PEG-PLGA) particulate formulations in alleviating the cellular and biochemical changes associated with asthma.

Methods: To study the pharmacological efficacy of LMWH for the treatment of asthma, we have used a previously optimized polymeric formulation of LMWH. The anti-asthmatic efficacy of the optimized formulation was studied in an ovalbumin-sensitized rat model of asthma. The influence of the formulation on asthmatic lungs was assessed by measuring the total protein content and number of inflammatory cells in the bronchoalveolar lavage fluid (BALF). Lungs were also examined for morphological and structural changes that may occur in asthmatic lungs.

Results: Compared with healthy animals, asthmatic animals showed a seven- and threefold increase in the protein content and number of inflammatory cells in BALF, respectively. However, intratracheal LMWH particles reduced the protein content by 2.5-fold and the number of inflammatory cells by 1.8-fold—comparable to those of sham animals. Similarly, LMWH particles reduced the lactate dehydrogenase levels by 2.8- and threefold in BALF and plasma, respectively. The airway wall thickness also decreased from $47.37 \pm 6.02 \mu\text{m}$ to $21.35 \pm 3.60 \mu\text{m}$ upon treatment with PEG-PLGA particles of LMWH. Goblet cell hyperplasia was also reduced in asthmatic rats treated with LMWH particles.

Conclusion: PLGA particles of LMWH were efficacious in improving cellular and histological changes associated with asthma, and thus this polymeric formulation has the potential for further development into a clinically viable anti-asthma therapy.

Key words: PEG-PLGA particles, asthma, low-molecular-weight heparin, anti-inflammatory

Introduction

HEPARIN AND RELATED COMPOUNDS that belong to the glycosaminoglycan family are well characterized and widely used as anticoagulants. Structurally, these compounds are highly sulfated polysaccharides with alternating units of uronic acid and amino sugars linked by glycosidic bonds.⁽¹⁾ A large body of literature suggests that, in addition to anticoagulant properties, heparins including low-molecular-weight heparins (LMWH) possess anti-inflammatory property that

stems from heparins' structural similarities to heparan sulfate, an endogenous macromolecule that participates in inflammatory processes.^(2,3) Further, the efficacy of heparin-like molecules in the treatment of bronchial inflammatory disorders, particularly asthma,^(4,5) has recently been discussed. Importantly, the anti-inflammatory activities of heparins are independent of their anticoagulant property,⁽⁶⁾ but depend on their molecular weight^(7–10) and extent of N-sulfation.⁽¹¹⁾

The anti-asthmatic efficacy of various molecular weight heparins has been evaluated in both preclinical^(7,10–12) and

clinical setups.^(13,14) Heparin-like compounds appeared to reduce allergen and mast cell mediator-induced airway hyper-responsiveness. Although several mechanisms for the anti-inflammatory property of heparins have been reported and are summarized in Figure 1, none of the mechanistic pathways are fully understood. Heparan sulfate is overexpressed by the endothelial cells at inflammation sites and facilitates extravasation of circulating leukocytes toward the inflamed tissue.⁽¹⁵⁾ Because of its structural similarity to heparan sulfate, exogenous heparin interrupts recruitment of inflammatory cells and prevents subsequent inflammatory response. Heparins inhibit activation of neighboring cells by binding with released chemokines and thus prevent propagation of inflammatory responses.⁽³⁾ Heparins also down-regulate expression of adhesion molecules that slow down migration of eosinophils to the site of inflammation.⁽⁶⁾ Further, by preventing release of intracellular calcium, heparins prevent mast cell degranulation and release of histamine-like inflammatory mediators.⁽¹⁶⁾ Data concerning the roles of heparin in the prevention of the inflammatory process prompted many investigators to use these glycosaminoglycans in the treatment of various inflammatory conditions.

To increase the anti-inflammatory effect, unfractionated heparins were separated into ultra-low-molecular-weight segments or chemically modified. Such fractionation and chemical modifications often resulted in reduction of the anti-coagulant properties of heparins.^(7,11,17) We and others have developed drug delivery systems for inhalational delivery of heparin.^(18,19) The goal of the formulation-based approaches was to produce a continuous release of the drug in the lung and reduce bleeding complications associated with systemic exposure. For example, heparin encapsulated in chitosan nanoparticles^(20,21) and inhalable poly(D,L-lactide-co-glycolide) (PLGA) microparticles⁽¹²⁾ is shown to be effica-

cious in the treatment of asthma and other inflammatory conditions. However, these studies evaluated only the influence of unfractionated heparin on the release of mediators from mast cells^(20,21) or airway hyper-reactivity.⁽¹²⁾ There are no systematic studies concerning the use of biocompatible and biodegradable particles as carriers for inhalational delivery of LMWH, smaller fragments of unfractionated heparin, for the treatment of asthma.

In the present investigation, we have evaluated the anti-asthmatic efficacy of inhalable large porous polyethylene glycol (PEG)-PLGA particles of LMWH in a rat model of asthma. Particle characterization and optimization have been reported in a previous publication.⁽¹⁸⁾ We and others have shown that large porous particles with relatively low density exhibit favorable aerodynamic behavior and improved deposition efficiency in the lungs. Further, sustained release of LMWH from the particles provides greater concentrations of drug in the lungs and thus an increase in the duration of activity. Thus, we hypothesize that the inhalable large porous PEG-PLGA particles of LMWH produce sustained anti-inflammatory effects in asthmatic animals. We have designed this study to investigate the relative effectiveness of plain LMWH and LMWH-loaded PEG-PLGA particles in reducing inflammation in an ovalbumin-induced rat asthma model.

Materials and Methods

Materials

PEG-PLGA diblock (PLGA, 50:50; PEG, 13–15%) copolymer was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). LMWH with an average molecular weight of 4,493 Da and anti-factor Xa activity of 61 U/mg was purchased from Celsus Laboratories (Cincinnati, OH).

FIG. 1. The role of endogenous heparan sulfate in leukocyte adhesion followed by extravasation during inflammatory response to allergen exposure in asthma and the possible mechanisms of anti-inflammatory activities of heparin and related compounds (redrawn from Li *et al.*⁽⁴²⁾).

Male Sprague-Dawley[®] rats (250–350 g) were supplied by Charles River Laboratories (Wilmington, MA). Lactate dehydrogenase (LDH) and protein assay kits were obtained from Pointe Scientific, Inc. (Canton, MI) and Bio-Rad (Hercules, CA), respectively. Hema-3 manual staining kit was from Fisher Scientific (Pittsburgh, PA). Hematoxylin and eosin (H&E) staining kit, periodic acid–Schiff staining kit, and other analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation and optimization of LMWH-loaded microparticles

LMWH-loaded PEG-PLGA large porous particles were prepared using the “water-in-oil-in-water” ($W_1/O/W_2$) double emulsion–solvent evaporation method as reported earlier.⁽¹⁸⁾ In this study, we have used an optimized formulation prepared with PEG-PLGA diblock copolymers and 8% sodium chloride in the external aqueous phase. In the published study, we evaluated the respirability of this particular formulation in detail.

Development of asthma models and treatment with various formulations

For studying the efficacy of the PLGA particles of LMWH, we have developed an ovalbumin-sensitized/challenged model of asthma using ~300-g male Sprague–Dawley rats (Charles River Laboratories, Charlotte, NC). In brief, the rats were divided into three groups containing four to six rats in each group. The animals were sensitized by intraperitoneal injections of 100 μ g of ovalbumin dispersed in 5 mg of aluminum hydroxide, on days 0, 7, and 14.^(22,23) Twenty-four hours after the last ovalbumin-alum injection, animals were anesthetized with isoflurane; three groups of animals received three treatments—saline, plain LMWH, and porous particles of LMWH—once a day for 3 days (on days 15, 16, and 17) at a dose of 50 IU/kg LMWH or particles containing an equivalent amount of LMWH. Each day, in order to expose animals to allergen, 1 hr after dosing with LMWH or particles, animals were challenged with 100 μ L (1 mg/mL) of intratracheal ovalbumin solution. The formulations and ovalbumin were administered using a microsyringe for rats (PennCentury[®], Philadelphia, PA) as reported previously.⁽¹⁸⁾ A fourth group of animals, used as the negative control, received an intraperitoneal injection of phosphate-buffered saline (PBS), but received no treatment. On day 17, animals were anesthetized for collection of blood samples, and on day 18, animals were sacrificed for collection of bronchoalveolar lavage fluid (BALF) and lung tissue as described below. Study design for the development of the allergen

(ovalbumin)–induced rat asthma model, treatments, and sample collection schedule is summarized in Figure 2. All animal studies were performed in compliance with the NIH Guideline for the Care and Use of Laboratory Animals under an approved protocol (AM-02004).

Collection of blood samples, BALF, and lung tissue from animals

For collection of blood, animals were anesthetized on day 17 with an intramuscular injection of a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg). Upon anesthesia, blood samples were collected from the tail vein into citrated tubes just before the last ovalbumin challenge and at 4, 12, and 24 hr after the challenge. The plasma was then separated by centrifuging blood samples at 9,000 g for 5 min and stored at -20°C until further analysis. On day 18, 24 hr after the last ovalbumin challenge, animals were anesthetized with a cocktail of ketamine and xylazine and sacrificed for collection of lungs and BALF.

BALF was collected according to our previously reported procedure.^(18,24) In brief, a tracheotomy was performed and a small syringe containing 5 mL of cold PBS was instilled into the lungs. After 30 sec, the fluid was withdrawn and the procedure was repeated twice. Collected BALF was placed on the ice followed by centrifugation at 500 g for 10 min to separate suspended cells and supernatant. Supernatant was stored at -20°C for subsequent analysis.

Quantitation of protein content, LDH activity, and inflammatory cells

The protein content of BALF supernatant was determined using a commercially available Bio-Rad DC protein assay kit according to a protocol supplied by the manufacturer. In brief, an aliquot of BALF (5 μ L) was added into the 96-well plate containing assay mixture (25 μ L of alkaline copper tartrate solution + 200 μ L of dilute Folin reagent). The reaction mixture was stirred and then incubated in the dark for 15 min, and absorbance was measured at 570 nm using a Synergy MX Microplate Reader (Biotech, Winnoski, VT). Bovine serum albumin was used as a standard for calibration.

LDH levels in plasma (collected over 24 hr after the last ovalbumin challenge on day 17) and BALF supernatant (obtained on day 18) were quantified using an LDH kit from Pointe Scientific. In brief, 50 μ L of plasma or 100 μ L of BALF sample was added into 1 mL of reaction mixture containing lactate and nicotinamide adenine dinucleotide. LDH activity was measured as the change in absorbance per minute at 340 nm.

To count inflammatory cells, BALF was centrifuged and the cell pellet thus obtained was suspended in 5 mL of PBS.

FIG. 2. Study design for the development of the ovalbumin (OVA)-induced asthma model using Sprague-Dawley rats. Animals were sensitized with OVA-alum injections on days 0, 7, and 14 followed by challenges with allergen on days 15, 16, and 17. The treatments were administered once a day 1 hr before the OVA challenges, and blood, BALF, and lung tissue samples were collected as shown in this figure.

An aliquot of cell suspension (100 μ L) was mixed with an equal volume of 0.4% Trypan blue solution. The number of cells was counted using a hemocytometer, and differential leukocyte counting was performed by staining slides containing smeared cells with a Hema-3 manual staining kit (Fisher Scientific). In brief, dried slides were stained by dipping in three different solutions sequentially each for six times (1 sec each) starting with a fixative solution, followed by xanthene solution, and finally in thiazine solution. Slides were washed thoroughly with distilled water and air-dried before viewing under a microscope.

Histological examination of lungs

For measurement of wall thickness and evaluation of goblet cell hyperplasia, the trachea and lungs were surgically exposed by tracheotomy and thoracotomy, respectively. A thin plastic tube, connected to a reservoir containing 10% formalin in PBS placed at 20-cm height, was attached to the trachea, and the lungs were inflated by filling with the liquid for 20 min. Lungs were then detached from the chest cavity and placed in 10% formalin for a day and later in 30% sucrose solution for 2 days. Left lungs were then embedded in TissueTek[®] (Sakura Finetek, Torrance, CA) and stored at -80°C overnight. Lung sections of 25- μ m thickness were prepared after lung blocks were sliced using a Cryostat (Leica Microsystems Inc., Buffalo Grove, IL), and slides were stained using appropriate reagents as discussed below.

The lung histology and the airway wall thickness were assessed after lung sections were stained with the H&E staining method. The wall thickness was measured using ImageJ software (National Institutes of Health, Bethesda, MD). For each section, the thickness of eight to 10 airways was measured and reported as means $\pm$ SD. Means were compared using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. A *p* value of ≤ 0.05 was considered statistically significant.

The extent of goblet cell hyperplasia in rat lungs was evaluated by staining lung sections with periodic acid-Schiff's reagent as recommended by the vendor (Sigma-Aldrich). In brief, the lung sections were first rehydrated followed by incubation with periodic acid solution for 5 min. Then, after washing with deionized water, sections were incubated for 15 min with Schiff's reagent. Following another round of thorough washing and counterstaining with hematoxylin, lung sections were covered with glass coverslips using a mounting medium. Sections were viewed and images were generated using a bright-field microscope (1X-81, Olympus).

Statistical analysis

All data have been presented as means $\pm$ SD. Means were compared using one-way ANOVA followed by Tukey's *post-hoc* test using GraphPad Prism 5 (version 5.01, GraphPad Software, Inc.). A *p* value of ≤ 0.05 was considered statistically significant.

Results and Discussion

In an earlier study, we prepared and optimized an inhalable particulate formulation of LMWH, using a 3×3 full factorial design.⁽¹⁸⁾ In brief, nine particulate formulations were prepared by varying two independent variables at

three different levels: (1) polymer type (PLGA, PEG-PLGA, and PLGA-PEG-PLGA), and (2) sodium chloride concentration in external aqueous phase (0%, 5%, and 8% w/v). Detailed physicochemical characterizations, *in vitro* drug release, *in vivo* absorption, uptake by alveolar macrophages, *in vivo* particle deposition, and retention and safety of particulate formulation were studied.⁽¹⁸⁾ The physicochemical characteristics and *in vitro* properties of the optimized formulation are summarized in Table 1. Scanning electron microscopic images (Fig. 3A) show that PEG-PLGA particles have large pores with numerous indentations. LMWH release from particles showed a biphasic profile with $>49\%$ drug release over 2 days. Further, PEG-PLGA particles exhibited reduced uptake by alveolar macrophages due to their large size and presence of PEG chains.^(25,26) The biological half-life of LMWH encapsulated in PEG-PLGA particles was extended by ~ 4.5 -fold following pulmonary administration compared with plain drug given via the same route. Overall, our previous study provided the first proof of concept regarding potential applications of PEG-PLGA block copolymer as a superior carrier system for pulmonary delivery of highly water-soluble drugs such as LMWH. As a continuation of previous studies, we have evaluated the therapeutic efficacy by measuring the anti-inflammatory activity of the optimized LMWH-loaded PEG-PLGA particles in an ovalbumin-induced rat asthma model.

Influence of the formulations on inflammatory markers

The inflammatory process in asthma propagates via various pro-inflammatory cells and an array of chemical mediators. The latter activate many cell types and produce a strong inflammatory response against allergens. Further, inflammation leads to cell death, rupture, and subsequent release of cytoplasmic content. As chemical mediators and intracellular components are chiefly proteins, we measured the amount of total protein in the BALF as a general indicator of inflammatory markers. Compared with the sham group, the total protein content in the BALF of asthmatic animals was increased by sevenfold, suggesting a vigorous inflammatory response to ovalbumin challenge (Fig. 3B). Pretreatment with plain LMWH produced a modest reduction in

TABLE 1. SUMMARY OF THE PHYSICOCHEMICAL AND *IN VITRO* CHARACTERISTICS OF LMWH-LOADED LARGE POROUS PEG-PLGA MICROPARTICLES

Parameters	LMWH-loaded PEG-PLGA particles
Physical characteristics	
Volume-based mean diameter	$9.443 \pm 0.157 \mu\text{m}$
Tapped density	$0.1674 \pm 0.005 \text{ g/mL}$
MMAD	$2.675 \pm 0.4 \mu\text{m}$
GSD	2.52
FPF	$65.05 \pm 3.9\%$
Zeta potential	$-7.546 \pm 0.217 \text{ mV}$
Drug entrapment efficiency	$64.51 \pm 1.78\%$
Cumulative drug release	$49.2 \pm 3.0\%$ over 48 hr

MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation; FPF, fine particle fraction.

These data have been excerpted from one of our previous publications.⁽¹⁸⁾

FIG. 3. (A) Scanning electron micrograph of large porous LMWH-loaded PEG-PLGA particles, and (B) protein content in the BALF collected from the rat lungs 24 hr after the last ovalbumin challenge. Data represent means $\pm$ SD ($n=3-4$). $**p < 0.01$.

protein levels in the BALF of asthmatic animals. However, PEG-PLGA particles loaded with LMWH caused ~ 2.5 -fold reduction in total protein content. These data are consistent with the assumption that LMWH-loaded particles produce anti-inflammatory effects and prevent release of injury markers in the lung.

LDH enzyme is constitutively present in the cytoplasm of all living cells and is specifically released in the extracellular environment following cell injury.⁽²⁷⁾ To assess the extent of lung tissue damage and inflammatory response, we have measured LDH levels in the plasma and BALF collected from sham and asthmatic rats. Systemic levels of LDH were measured over the period of 24 hr after the last ovalbumin challenge (Fig. 4A). Sham animals did not show significant increase in the LDH levels over 24 hr. However, LDH levels in asthmatic animals went up as high as 297% at 4 hr, indicating an early-phase inflammatory response. LDH levels in LMWH-treated asthmatic animals were relatively low (192%) compared with those in untreated animals. Interestingly, LDH levels in LMWH-loaded PEG-PLGA particles-treated animals were comparable to those of normal animals. Similar results were also observed with BALF analyses (Fig. 4B): LDH activities were significantly elevated in asthmatic animals (110.14 ± 1.79 U/L) compared with those of normal animals (19.83 ± 11.34 U/L). But treatment with plain LMWH reduced the LDH levels to 79.76 ± 8.96 U/L, and that was

further reduced to 34.18 ± 1.79 U/L in PEG-PLGA particles-treated animals. Reduced levels of LDH after PEG-PLGA particles treatment suggest protective effects of the formulation against cell injury. Due to rapid systemic absorption and relatively short half-life, plain LMWH was unable to completely inhibit rising LDH levels after allergen challenge. But PEG-PLGA particles provided prolonged protection against inflammation by releasing LMWH in a continuous fashion. In fact, heparins have been shown to reduce leukocyte adhesion and recruitment of pro-inflammatory cells in response to applied inflammatory stimuli⁽²⁸⁾ and protect tissue against injuries due to reactive oxygen species generation.⁽²⁹⁾

Influence of the formulations on infiltrating cells

Infiltration of inflammatory cells in response to allergen exposure is one of the classical signs of asthma. Pro-inflammatory mediators released from the activated mast cells set up the stage for inflammatory response by activating and recruiting other cells involved in the process. Quantification of infiltrated cells in alveolar space can give useful information concerning inflammation in the lung as reported by us and others.^(18,30) The number of total infiltrated cells was significantly higher in ovalbumin-challenged animals ($3.43 \pm 0.14 \times 10^6$ cells) compared with normal animals ($1.25 \pm 0.07 \times 10^6$ cells) (Fig. 5A). LMWH-treated animals showed reduced infiltration

FIG. 4. LDH levels in (A) plasma over 24 hr after last ovalbumin challenge, and (B) BALF. Data represent means $\pm$ SD ($n=3$). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

FIG. 5. (A) Total and differential leukocyte cell counts in BALF collected from the rat lungs 24 hr after last ovalbumin challenge. Data represent means $\pm$ SD ($n=3-4$). (B) Histological comparison of lung sections for infiltrated inflammatory cells after Hema-3 staining. A rat lung from each treatment group was fixed without collecting BALF for this experiment. * $p < 0.05$; *** $p < 0.001$.

of inflammatory cells ($2.90 \pm 0.07 \times 10^6$ cells), and that was further decreased in animals treated with LMWH-loaded PEG-PLGA particles ($1.95 \pm 0.11 \times 10^6$ cells). Thus, treatment with plain LMWH and LMWH-loaded PEG-PLGA particles resulted in $>15\%$ and $>43\%$ inhibition in infiltration of inflammatory cells in the lungs compared with untreated animals.

To identify the type of inflammatory cells, Hema-3 stain was used for differential leukocyte counts (Fig. 5). Compared with normal animals, ovalbumin-exposed antigen-sensitized rats exhibited a significant increase in the numbers of various inflammatory cells, particularly macrophages and eosinophils. The increase in the number of eosinophils in asthmatic

animals was ~ 6.5 -fold higher than those observed in sham animals. However, this number was decreased by 1.8-fold in the PEG-PLGA particles-treated rats, suggesting the efficacy of the formulation in reducing the infiltration of common inflammatory cells. These findings were further confirmed by the images of lungs sections stained with Hema-3 stain. A higher level of infiltration of inflammatory cells in the airways of asthmatic animals was observed (Fig. 5B). However, pretreatment with plain LMWH and LMWH-loaded PEG-PLGA particles remarkably reduced the number of infiltrated cells in the airways. As mentioned above, heparin and related compounds exhibit anti-inflammatory effects by inhibiting leukocyte adhesion with the endothelial layer and, thus, interfere in migration of these cells toward the alveolar space. Continuous release of LMWH from the particles has provided sustained concentrations of drug in the lungs and subsequently inhibited or slowed the extravasation of inflammatory cells.

Influence of the formulations on airway wall thickening and goblet cell hyperplasia

The degree of structural changes in the asthmatic lungs, such as airway wall thickening, is associated with the severity of the disease and airway obstruction.⁽³¹⁾ Morphological changes in the airways of asthmatic patients occur due to prolonged inflammation and mucus accumulation. In this experiment, rat lungs collected from different treatment groups were fixed, sectioned, and stained with H&E staining and observed under a microscope (Fig. 6A). Sham animals showed an unaltered airway with fairly smooth epithelial layer. On the other hand, asthmatic airway exhibited significantly altered morphology with lumen narrowing. LMWH-treated lung airways were relatively less remodeled, whereas those of PEG-PLGA particles-treated animals were comparable to those of normal animals. Further, the airway wall thickness was measured using ImageJ software (National Institutes of Health). The airways of normal healthy animals showed a thickness of $18.83 \pm 3.21 \mu\text{m}$, whereas that for asthmatic animals was $47.37 \pm 6.02 \mu\text{m}$ (Fig. 6B). Compared with untreated animals, LMWH and LMWH-loaded PEG-PLGA particles-treated animals showed major reduction in airway wall thicknesses: $32.95 \pm 6.08 \mu\text{m}$ for LMWH and $21.35 \pm 3.60 \mu\text{m}$ in the case of particles containing LMWH. The airway thickness data and images of airways suggest remodeling of airways in ovalbumin-sensitized rats. This may have resulted from inflammation-induced structural alterations and proliferations such as epithelial goblet cell hyperplasia, increased smooth muscle mass, and angiogenesis. However, administration of LMWH reduced the inflammation-induced structural alterations because of its anti-inflammatory and anti-proliferative properties. These observations are consistent with an earlier report that endogenous heparin reduces proliferation of airway smooth muscle cells.⁽³²⁾ Heparin and related compounds have also demonstrated potent anti-angiogenesis effects in preclinical studies.⁽³³⁾

Goblet cell hyperplasia and elevated mucus secretion are major markers for bronchial inflammatory diseases such as asthma and cystic fibrosis. Thus, we measured the number of goblet cells in the lungs of various treatment groups and examined the lung samples under a microscope. Sham animals, which were not sensitized with ovalbumin, did not

FIG. 6. (A) Photomicrographs of rat lung sections following H&E staining for airway wall thickening. (B) Airway wall thickness. Data represent means $\pm$ SD ($n=8-10$ airway measurements). ** $p<0.01$; *** $p<0.001$.

show that many stained cells upon treatment with periodic acid-Schiff reagent, indicating that goblet cells and the mucus production mechanism underwent no pathological changes (Fig. 7). However, lung sections from asthmatic animals demonstrated substantial increase in the number of goblet cells and mucus production as depicted by an intense pinkish stain. Intratracheal treatment with plain LMWH showed reduced goblet cell-associated pink stain compared with untreated asthmatic rats, and that was further reduced in the animals treated with LMWH-loaded PEG-PLGA particles. These data agree with the observations that mild to moderate asthma is associated with goblet cell hyperplasia with abnormal mucin gene expression⁽³⁴⁾ and with the report that heparin reduces mucus hypersecretion in airway epithelial cells.⁽³⁵⁾ Although the mechanisms of inhibitory effects of heparin on mucus secretion are unknown, it may stem from reduction of goblet cell counts.

FIG. 7. Photomicrographs of rat lung sections following periodic acid-Schiff staining of goblet cells and mucus.

Based on the efficacy data, one may question that the anti-asthmatic effect of the formulations may be attributed, in part, to the polymer (PEG-PLGA) itself rather than LMWH. But both PLGA and PEG are FDA-approved inert pharmaceutical additives used in commercially available medications, and thus they are unlikely to produce any drug-like effects in asthma.^(36,37) Further, PLGA-based particulate carrier systems have been investigated in ovalbumin-induced animal asthma models with the objective of improving the therapeutic efficacy of plain drug by producing sustained drug levels in the lungs. Consistent with the data presented in this article, budesonide-loaded PLGA particles also showed superior anti-asthmatic effects compared with plain drug in a rat model of asthma.⁽³⁸⁾ Similarly, in a mouse model, unfractionated heparin-loaded PLGA particles showed significantly heightened pharmacodynamic response against airway hyper-reactivity and inflammation compared with plain heparin.⁽¹²⁾

Heparins are reported to significantly reduce bronchial hyper-reactivity in asthmatic patients challenged with external stimuli.^(39,40) Owing to shorter duration and mechanisms involved in anti-inflammatory activities of plain heparin, these effects were largely limited to the early-phase response. In this study, particulate formulation exhibiting prolonged drug release was synthesized and investigated for sustained anti-inflammatory effects in an allergen-induced rat asthma model. Selection of a formulation with 20% burst release for pharmacodynamic studies in the present investigation was based on the assumption that this set of particles will provide an initial bolus dose and subsequently produce continuous release of the drug. In principle, all prolonged-release dosage forms should release a certain amount of drug for immediate effect, and the remaining should be released in a continuous fashion. Further, it has been reported earlier that the minimum anti-factor Xa levels for generation of anticoagulant effects in rats is 0.15 IU/mL.⁽⁴¹⁾ In our previous study, pulmonary administered LMWH-loaded PEG-PLGA particles produced anti-factor Xa activity levels predominantly close to the minimum effective concentration with a

$C_{\max}$ of only 0.19 IU/mL.⁽¹⁸⁾ As anticoagulant therapy-related bleeding complications are observed at relatively higher anti-factor Xa levels, optimized particulate formulation is unlikely to cause major bleeding complications. Although this preliminary study provides encouraging evidence regarding the protective efficacy of LMWH-loaded particles in preventing structural alterations of airways and reducing infiltration of inflammatory cells, further studies are warranted to understand the effects of particle treatment on airway hyper-reactivity.

Conclusions

An LMWH-loaded large porous PEG-PLGA particulate formulation appeared to be more effective than plain drug in producing sustained anti-inflammatory effects following once-a-day intratracheal administration. Histological and *in vivo* studies suggest that particulate formulations could alleviate allergen-induced inflammation in the lungs. Overall, this short-term study establishes large porous PEG-PLGA particles of LMWH as a potential therapeutic alternative for asthma therapy.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

References

- Casu B: Structure of heparin and heparin fragments. *Ann N Y Acad Sci.* 1989;556:1–17.
- Tyrell DJ, Kilfeather S, and Page CP: Therapeutic uses of heparin beyond its traditional role as an anticoagulant. *Trends Pharmacol Sci.* 1995;16:198–204.
- Young E: The anti-inflammatory effects of heparin and related compounds. *Thromb Res.* 2008;122:743–752.
- Diamant Z, and Page CP: Heparin and related molecules as a new treatment for asthma. *Pulm Pharmacol Ther.* 2000; 13:1–4.
- Lever R, and Page C: Glycosaminoglycans, airways inflammation and bronchial hyperresponsiveness. *Pulm Pharmacol Ther.* 2001;14:249–254.
- Seeds EAM, and Page CP: Heparin inhibits allergen-induced eosinophil infiltration into guinea-pig lung via a mechanism unrelated to its anticoagulant activity. *Pulm Pharmacol Ther.* 2001;14:111–119.
- Ahmed T, Smith G, Vlahov I, and Abraham WM: Inhibition of allergic airway responses by heparin derived oligosaccharides: identification of a tetrasaccharide sequence. *Respir Res.* 2012;13:6.
- Ahmed T, Gonzalez BJ, and Danta I: Prevention of exercise-induced bronchoconstriction by inhaled low-molecular-weight heparin. *Am J Respir Crit Care Med.* 1999;160:576–581.
- Molinari JF, Campo C, Shakir S, and Ahmed T: Inhibition of antigen-induced airway hyperresponsiveness by ultralow molecular-weight heparin. *Am J Respir Crit Care Med.* 1998; 157:887–893.
- Campo C, Molinari JF, Ungo J, and Ahmed T: Molecular-weight-dependent effects of nonanticoagulant heparins on allergic airway responses. *J Appl Physiol.* 1999;86:549–557.
- Ahmed T, Smith G, and Abraham WM: Effect of oral and intravenous heparin tetrasaccharide on allergic airway responses: critical role of N-sulfation. *Pulm Pharmacol Ther.* 2013;26:180–188.
- Yildiz A, John E, Ozsoy Y, Araman A, Birchall JC, Broadley KJ, and Gumbleton M: Inhaled extended-release microparticles of heparin elicit improved pulmonary pharmacodynamics against antigen-mediated airway hyper-reactivity and inflammation. *J Control Release.* 2012;162:456–463.
- Stelmach I, Jerzynska J, Stelmach W, Majak P, Brzozowska A, Gorski P, and Kuna P: The effect of inhaled heparin on airway responsiveness to histamine and leukotriene D₄. *Allergy Asthma Proc.* 2003;24:59–65.
- Pavord I, Mudassar T, Bennett J, Wilding P, and Knox A: The effect of inhaled heparin on bronchial reactivity to sodium metabisulphite and methacholine in patients with asthma. *Eur Respir J.* 1996;9:217–219.
- Ekre HP, Naparstek Y, Lider O, Hyden P, Hagermark O, Nilsson T, Vlodavsky I, and Cohen I: Anti-inflammatory effects of heparin and its derivatives: inhibition of complement and of lymphocyte migration. *Adv Exp Med Biol.* 1992;313:329–340.
- Baram D, Rashkovsky M, Hershkoviz R, Drucker I, Reshef T, Ben-Shitrit S, and Mekori YA: Inhibitory effects of low molecular weight heparin on mediator release by mast cells: preferential inhibition of cytokine production and mast cell-dependent cutaneous inflammation. *Clin Exp Immunol.* 1997;110:485–491.
- Duong M, Cockcroft D, Boulet LP, Ahmed T, Iverson H, Atkinson DC, Stahl EG, Watson R, Davis B, Milot J, Gauvreau GM, and O'Byrne PM: The effect of IVX-0142, a heparin-derived hypersulfated disaccharide, on the allergic airway responses in asthma. *Allergy.* 2008;63:1195–1201.
- Patel B, Gupta V, and Ahsan F: PEG-PLGA based large porous particles for pulmonary delivery of a highly soluble drug, low molecular weight heparin. *J Control Release.* 2012;162:310–320.
- Qi Y, Zhao G, Liu D, Shriver Z, Sundaram M, Sengupta S, Venkataraman G, Langer R, and Sasisekharan R: Delivery of therapeutic levels of heparin and low-molecular-weight heparin through a pulmonary route. *Proc Natl Acad Sci USA.* 2004;101:9867–9872.
- Oyarzun-Ampuero FA, Brea J, Loza MI, Alonso MJ, and Torres D: A potential nanomedicine consisting of heparin-loaded polysaccharide nanocarriers for the treatment of asthma. *Macromol Biosci.* 2012;12:176–183.
- Oyarzun-Ampuero FA, Brea J, Loza MI, Torres D, and Alonso MJ: Chitosan-hyaluronic acid nanoparticles loaded with heparin for the treatment of asthma. *Int J Pharm.* 2009; 381:122–129.
- Onoue S, Matsui T, Kuriyama K, Ogawa K, Kojo Y, Mizumoto T, Karaki S, Kuwahara A, and Yamada S: Inhalable sustained-release formulation of long-acting vasoactive intestinal peptide derivative alleviates acute airway inflammation. *Peptides.* 2012;35:182–189.
- Onoue S, Aoki Y, Matsui T, Kojo Y, Misaka S, Mizumoto T, and Yamada S: Formulation design and *in vivo* evaluation of dry powder inhalation system of new vasoactive intestinal peptide derivative ([R^{15, 20, 21}, L¹⁷, A^{24,25}, des-N²⁸]-VIP-GRR) in experimental asthma/COPD model rats. *Int J Pharm.* 2011;410:54–60.
- Gupta V, Rawat A, and Ahsan F: Feasibility study of aerosolized prostaglandin E₁ microspheres as a noninvasive therapy for pulmonary arterial hypertension. *J Pharm Sci.* 2010;99:1774–1789.

25. Edwards DA, Ben-Jebria A, and Langer R: Recent advances in pulmonary drug delivery using large, porous inhaled particles. *J Appl Physiol.* 1998;85:379–385.
26. Fontana G, Licciardi M, Mansueto S, Schillaci D, and Giammona G: Amoxicillin-loaded polyethylcyanoacrylate nanoparticles: influence of PEG coating on the particle size, drug release rate and phagocytic uptake. *Biomaterials.* 2001;22:2857–2865.
27. Glick JH, Jr: Serum lactate dehydrogenase isoenzyme and total lactate dehydrogenase values in health and disease, and clinical evaluation of these tests by means of discriminant analysis. *Am J Clin Pathol.* 1969;52:320–328.
28. Salas A, Sans M, Soriano A, Reverter JC, Anderson DC, Piqué JM, and Panés J: Heparin attenuates TNF- α induced inflammatory response through a CD11b dependent mechanism. *Gut.* 2000;47:88–96.
29. Dandona P, Qutob T, Hamouda W, Bakri F, Aljada A, and Kumbkarni Y: Heparin inhibits reactive oxygen species generation by polymorphonuclear and mononuclear leukocytes. *Thromb Res.* 1999;96:437–443.
30. Misaka S, Aoki Y, Karaki S, Kuwahara A, Mizumoto T, Onoue S, and Yamada S: Inhalable powder formulation of a stabilized vasoactive intestinal peptide (VIP) derivative: anti-inflammatory effect in experimental asthmatic rats. *Peptides.* 2010;31:72–78.
31. Kosciuch J, Krenke R, Gorska K, Zukowska M, Maskey-Warzechowska M, and Chazan R: Relationship between airway wall thickness assessed by high-resolution computed tomography and lung function in patients with asthma and chronic obstructive pulmonary disease. *J Physiol Pharmacol.* 2009;60 Suppl 5:71–76.
32. Panettieri RA, Yadavish PA, Kelly AM, Rubinstein NA, and Kotlikoff MI: Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. *Am J Physiol.* 1990;259:L365–L371.
33. Folkman J, and Shing Y: Control of angiogenesis by heparin and other sulfated polysaccharides. *Adv Exp Med Biol.* 1992;313:355–364.
34. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, and Fahy JV: Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med.* 2001;163:517–523.
35. Ogawa T, Shimizu S, Tojima I, Kouzaki H, and Shimizu T: Heparin inhibits mucus hypersecretion in airway epithelial cells. *Am J Rhinol Allergy.* 2011;25:69–74.
36. Banerjee SS, Aher N, Patil R, and Khandare J: Poly(ethylene glycol)-prodrug conjugates: concept, design, and applications. *J Drug Deliv.* 2012;2012:103973.
37. D'Souza SS, and DeLuca PP: Methods to assess *in vitro* drug release from injectable polymeric particulate systems. *Pharm Res.* 2006;23:460–474.
38. Oh YJ, Lee J, Seo JY, Rhim T, Kim SH, Yoon HJ, and Lee KY: Preparation of budesonide-loaded porous PLGA microparticles and their therapeutic efficacy in a murine asthma model. *J Control Release.* 2011;150:56–62.
39. Diamant Z, Timmers MC, van der Veen H, Page CP, van der Meer FJ, and Sterk PJ: Effect of inhaled heparin on allergen-induced early and late asthmatic responses in patients with atopic asthma. *Am J Respir Crit Care Med.* 1996;153:1790–1795.
40. Stelmach I, Jerzynska J, Bobrowska M, Brzozowska A, Majak P, and Kuna P: [The effect of inhaled heparin on airway responsiveness to metacholine in asthmatic children]. *Pol Arch Med Wewn.* 2001;106:567–572.
41. Bianchini P, Bergonzini GL, Parma B, and Osima B: Relationship between plasma antifactor Xa activity and the antithrombotic activity of heparins of different molecular-mass. *Haemostasis.* 1995;25:288–298.
42. Li P, Sheng J, Liu Y, Li J, Liu J, and Wang F: Heparosan-derived heparan sulfate/heparin-like compounds: one kind of potential therapeutic agents. *Med Res Rev.* 2013;33:665–692.

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