Epithelial Heparin Delivery via Microspheres Mitigates Experimental Colitis in Mice

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ABSTRACT

Low-molecular-weight heparins (LMWH) have been shown to be efficient in the treatment of inflammatory bowel disease (IBD). Parenteral heparin therapy, however, may cause hemorrhagic adverse effects. To reduce this risk, epithelial LMWH delivery in combination with a system ensuring selective drug release to the inflamed tissue was tested here. Enoxaparin loaded microspheres (MS) were administered orally to male BALB mice suffering from a pre-existing experimental colitis, whereas control groups received subcutaneous or rectal LMWH solution. Colon weight/length index and alkaline phosphatase and myeloperoxidase activities were assessed to determine the inflammation. Tissue penetration experiments elucidated the processes involved in the proposed new therapeutic approach. Oral LMWH-MS proved to be equally efficient in mitigating experimental colitis as rectally administered LMWH solution when quantified by myeloperoxidase activity (MS, 10.2 ± 1.5 U/mg tissue; rectal, 9.2 ± 1.6 U/mg) and to be superior to subcutaneous LMWH (s.c., 21.6 ± 5.6 U/mg; untreated colitis control, 30.0 ± 3.8 U/mg). Pharmacokinetic studies found a notably low systemic availability of oral LMWH delivered from MS (<5%) indicating a low potential for adverse effects. The tissue permeability was selectively enhanced in the inflamed regions where a 9-fold higher LMWH penetration was found compared with healthy tissue. Epithelial LMWH delivery has been found a promising anti-inflammatory therapeutic approach. The use of LMWH-MS in this context offers a promising tool for IBD therapy by enhancing specifically drug availability at inflamed tissue sites while reducing the risk for systemic adverse effects to a negligibly low level.

The general principle of pharmacological treatment for inflammatory bowel disease (IBD) is to induce remission of outbreaks and to prevent outbreaks during remission. With this goal in mind, a wide range of anti-inflammatory pharmaceutical products have been commercialized mainly for oral administration with modified delivery profiles in the gastrointestinal tract (Lamprecht et al., 2002; Podolsky, 2002; Hanauer and Present, 2003). In many cases, pharmacotherapy for IBD consists of life-long administration of one or more of aforementioned drugs. Therefore, quality and severity of adverse effects of these therapeutic regimens is an essential issue to address. Consequently, innovative drug delivery strategies have been designed for more selective delivery of drug to sites of inflamed tissue while reducing the risk for systemic adverse effects (Lamprecht et al., 2002).

State-of-the-art drug release strategies such as enzymatically degradable carriers rely on enzymatic activity of colonic bacteria similar to the mechanism of prodrugs. Some drug delivery systems perform time-dependent drug release. Others, among them most of currently commercialized systems, are based on the change of luminal pH during gastrointestinal passage (Lamprecht et al., 2002). Because these systems are known to exhibit a lack of specificity in terms of drug release, newer strategies were developed to increase selectivity of drug deposition toward inflamed colon tissue. Microcarriers were proposed to minimize drug loss related to accelerated carrier elimination by diarrhea associated with IBD (Nakase et al., 2000; Lamprecht et al., 2005b). Systems that are even smaller, e.g., liposomes and nanoparticles, have shown significant improvements (Lamprecht et al., 2001, 2005a; Kesisoglou et al., 2005). The limiting factor in most of aforementioned strategies is premature loss of encapsulated drug during passage in upper parts of the intestine. This compromises the advantage of smaller carrier systems based...
on their ability to mitigate the effect of diarrhea on drug loss (Hardy et al., 1988; Watts et al., 1992). Because this partial drug loss is an unavoidable phenomenon related to the principal physicochemical properties of such microsystems, a closer look at the distinct properties of the drug also seems warranted to find ways to limit undesired early drug absorption and subsequent systemic drug availability. This was partially achieved by use of several budesonide formulations reducing systemic availability by mucosal metabolism of the drug (Klotz and Schwab, 2005). However, the number of such drugs is limited.

Besides its anticoagulant property, heparin was recently found to possess anti-inflammatory properties and to be effective in the treatment of ulcerative colitis after subcutaneous administration (Törkvist et al., 1999; Dotan et al., 2001). Although mechanisms of action are not completely clear, its clinical application in IBD seems to be interesting. In contrast, long-term administration of heparin increases the risk for hemorrhagic events (Papa et al., 2000). This issue has prevented heparin from becoming a standard therapeutic adjunct in the pharmacotherapy of IBD. We were surprised to find that the therapeutic efficiency of heparins delivered locally to areas of inflammation for epithelial uptake is completely unknown. Thus, a local and defined epithelial delivery of heparin seems to be of high interest to reduce systemic drug availability and hence to lower the risk for adverse effects. This is especially of interest since heparins show a minimal tendency to cross the intact intestinal mucosa, which would further reduce undesired side effects.

In this study, the therapeutic efficiency of low-molecular-weight heparin (LMWH) in IBD treatment delivered by epithelial route was analyzed. Therefore, LMWH-loaded pH-sensitive microcarriers were developed to ensure a selective delivery of the drug toward areas of inflammation in the colon. The mitigating potential of epithelial LMWH in IBD was evaluated in two different colitis models in mice. This study focused specifically on the comparative analysis of the new microsphere (MS) carrier with control groups receiving LMWH as a solution either by rectal or subcutaneous route.

Materials and Methods

Materials

Eudragit P-4135F was a kind gift from Röhm Pharma Polymers (Tokyo, Japan) (for details, see Lehmann and Höss, 2001). For LMWH, enoxaparin sodium (Lovenox 10,000 IU anti-Xa/1 ml) was purchased from sanofi-aventis (Paris, France). Polyvinyl alcohol (PVA), sorbitan monostearate (Span 60), 2,4,6-trinitrobenzene sulfonic acid (TNBS), and oxazolone (OXA) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

Methods

Microparticle Preparation and Characterization. MS were prepared by a water-in-oil-in-water emulsion technique. In brief, 200 mg of Eudragit P-4135F was dissolved in 3 ml of dichloromethane containing 28 mg of sorbitan monostearate. Subsequently, 300 μl of aqueous LMWH (3000 IU) was emulsified in the polymer solution by ultrasonication for 15 s. This primary water-in-oil emulsion was then poured into 75 ml of aqueous 0.5% PVA solution to form a water-in-oil-in-water emulsion. This emulsion was stirred for 1 h with a three-blade propeller at 500 rpm at room temperature until the organic solvent of the internal phase was entirely removed, inducing polymer precipitation and creating solid MS with encapsulated LMWH. After the formulation, MS were filtrated (HA filters; porosity, 0.45 μm; Millipore Corporation, Billerica, MA), washed extensively with deionized water, and dried at room temperature.

MS were analyzed for their size distribution by laser light diffraction (Mastersizer; Malvern Instruments, Malvern, Worcestershire, UK). For scanning electron microscopy, the particles were fixed on a three-blade propeller at 500 rpm at room temperature until...
Cellulose were administered orally. Oral administration was performed by gavage, whereas rectal administration consisted of 4-cm intrarectal catheterization delivering LMWH directly to the site of inflammation. The control groups received saline only (colitis control) or blank MS. The mice were treated once daily for six consecutive days. The animals were sacrificed 24 h after the last drug/particle administration, and their colons were resected.

**Pathophysiological Parameters.** Colitis activity was quantified with a clinical score assessing weight loss, stool consistency, and rectal bleeding as described previously (Hartmann et al., 2000). Resected colon tissue samples were opened longitudinally and rinsed with iced phosphate buffer to remove luminal content. Then, tissue wet weight and colon length were determined and expressed as colon weight/length quotient. Histological assessment was carried out by light microscopy of colon tissue samples. The degree of inflammation was graded using the criteria described previously (Lamprecht et al., 2005b). The score represented the sum of eight individual variables graded 0 to 3 depending upon the severity of the changes (0, no change; 1, mild; 2, moderate; and 3, severe). The variables evaluated were erosion, ulceration, necrosis, hemorrhage, edema, and inflammatory cell infiltration.

For the determination of alkaline phosphatase (AP) activity, tissue samples were weighed and homogenized in at 4°C saline solution. After centrifugation, the homogenates were assayed for protein content. AP activity was determined spectrophotometrically using disodium p-nitrophenyl phosphate as substrate (Bessey et al., 1946). One unit of AP activity was defined as that degrading 1 μmol/min of substrate at 25°C. The results are expressed as AP units per milligram of protein.

The measurement of myeloperoxidase activity (MPO) was performed to quantify the severity of the colitis. It is a reliable index of severity of inflammation caused by infiltration of activated neutrophils into inflamed tissue. Enzymatic activity was analyzed according to a standard method (Krawisz et al., 1984).

**Tissue Penetration.** Control tissue samples were taken from the healthy group. Inflamed or noninflamed tissue samples were taken from the colitis group where noninflamed tissue was resected from areas without macroscopic damage with a distance of approximately 3 cm from sites of major inflammation. The resected mouse tissue samples were washed with ice-cold phosphate buffer, pH 7.4, and full-thickness specimens were mounted in modified Ussing chamber.

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of LMWH-loaded MS after solvent evaporation</th>
<th>MS Batches</th>
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<tbody>
<tr>
<td></td>
<td>Bioavailability (600 IU/kg)</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>126.5 ± 6.6</td>
</tr>
<tr>
<td>Process yield (%)</td>
<td>94.0 ± 2.1</td>
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<tr>
<td>Encapsulation rate (%)</td>
<td>75.8 ± 3.3</td>
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**Fig. 1.** Scanning electron microscopic image of pH-sensitive LMWH-loaded microspheres.

**Fig. 2.** Cumulative LMWH release versus time of LMWH-trapping microspheres in phosphate buffer systems of pH 1.2, 6.8, and 7.4 replaced after 2 h, respectively (n = 3). Data are shown as mean ± S.D.

**Fig. 3.** Clinical activity score in TNBS (A) or OXA (B) model during the whole experimental period after either oral or rectal drug administration (●, colitis control; △, FK506 solution oral; ○, FK506 NP oral; ■, FK506 solution rectal; ●, FK506 NP rectal; NP controls being similar to the colitis control as well as error bars are not shown for clarity; n = 6). * P < 0.05 compared with colitis control rats given saline.
bers. Both chambers were filled with Dulbecco’s modified Eagle’s medium and kept under carbogen bubbles at 37°C by water jackets. Tissues were preincubated for 15 min before the samples were added into the apical compartment, and samples were incubated for 30 min (at final carboxylfluorescein and LMWH-fluoresceinamine concentrations of 1 μM). The LMWH-labeling protocol was adapted to a method described previously (Lamprecht et al., 2006).

**Statistical Analysis.** The data are expressed as means ± S.D. For the analysis of statistical significance, analysis of variance on ranks was applied followed by Dunn’s test for all pairwise comparison. In all cases, \( P < 0.05 \) was considered to be significant.

**Results**

**In Vitro Characteristics of Microspheres.** LMWH-MS were spherical with a particle diameter below 150 μm, and they had a relatively rough surface (Fig. 1). The polymer matrix trapped an internal aqueous phase containing the LMWH with the surrounding polymeric matrix material, resulting in a sponge-like structure. Further MS characteristics such as particle size, LMWH encapsulation efficiency, and drug load are shown in Table 1. In general, in vitro drug release occurred with strong dependence on the pH of the respective buffer system, in which the MS were suspended (Fig. 2). LMWH was retained efficiently inside MS when tested at pH 1.2 and 6.8 where at least 80% of the initial drug load was still present inside the MS after 4 h of incubation. On the contrary, a comparatively fast release was observed at pH 7.4, which delivered nearly 100% of the incorporated drug within 30 min.

**Therapeutic Efficiency.** For purposes of testing the therapeutic concept in a pre-existing experimental colitis in rats, LMWH formulations were administered subcutaneously as comparative standard, orally as the new MS formulation, or rectally as solution to deliver a maximum concentration of LMWH to the site of inflammation. LMWH-MS were administrated in two different concentrations (600 and 2000 IU/kg) to detect potential dose-dependent effects.

In the TNBS model, only LMWH-MS lowered clinical activity after a lag time of 24 to 48 h, and it maintained this effect during the whole treatment period, whereas the two solution-receiving groups exhibited a continuous high level (Fig. 3A). Control experiments with blank MS or oral LMWH solution were not significantly different from untreated colitis control (data not shown). The difference between LMWH-MS (600 IU/kg) and colitis became significant on day 5, whereas for the other treated groups, statistically significant differences were not observed. Principally, in line with observations from clinical activity, LMWH-treated groups showed decreased values in the colon weight/length ratio in comparison with the untreated colitis control group (Fig. 4A). Although rectal LMWH administration showed a higher mitigating effect than in clinical activity scores, only levels after the LMWH-MS treatment were found to be significantly lower than the colitis control. Histological sections of the colon demonstrated significant influences by the various treatments (Fig. 5). With LMWH-MS treatment, mucosal and submucosal tissue was found partially intact, whereas in colitis control a complete disintegration of the mucosa occurred. In addition, swelling of the submucosa was reduced in LMWH-treated groups, although not reaching the level of healthy control. Histological damage score again revealed higher treatment efficiency for rectal LMWH and LMWH-MS compared with subcutaneous

![Fig. 4.](https://jpet.aspetjournals.org/)

Colon weight/length ratio (A and B) and histological damage score (C and D) on day 8 in TNBS or OXA colitis model after administration of LMWH and LMWH-MS, respectively. Data are shown as mean ± S.D for \( n = 6 \) animals. * \( P < 0.05 \) compared with colitis control rats given saline.
LMWH, where results were not statistically different from untreated colitis controls (Fig. 4C). Aside from that, MPO activity in samples from inflamed colonic tissue demonstrated similar therapeutic effects for LMWH-MS and rectal LMWH-treated groups, but a significantly lower efficiency of subcutaneous LMWH solution (Fig. 6A). The tissue concentrations of alkaline phosphatase were in line with MPO activity exhibiting significant mitigating effects for oral and rectal LMWH formulations (Fig. 7A). TNF-α values were determined inside the tissue samples of both colitis models; however, changes were not statistically significant (data not shown).

Slightly different tendencies for clinical activity score were found in the OXA model (Fig. 3B). Here, rectal administration of LMWH provided the strongest reduction of clinical activity. It must be noted that differences between all non-parenteral groups were not statistically significant. Generally, the administration of LMWH was noted to be more efficient for the OXA model compared with the TNBS model with marginally better results for LMWH-MS. Although the observed effect after oral and rectal administration of LMWH was similar to the TNBS model, a faster response to treatment was found in the OXA model (different from untreated control, rectal: day 4, LMWH-MS 2000 IU/kg: day 5; \( P < 0.05 \)). Colon weight/length ratios as well as histological damage scores were decreased in all LMWH groups. However, differences were only significant for LMWH-MS as well as for histological damage score after rectal LMWH administration (Fig. 4, B and D). Oppositely, after rectal or subcutaneous administration of LMWH, significant differences were observed in terms of MPO activity (Fig. 6B). Similarly to observations made regarding clinical activity, rectal administration of LMWH solution mitigated colitis to a greater extent, reaching levels of LMWH-MS at values near complete remission. Again, consistent with results of MPO activity, alkaline phosphatase activity was reduced for all LMWH treatments (Fig. 7B).

Systemic LMWH availability after administration of MS formulations was compared with that of subcutaneously administered LMWH solution, which was regarded as 100% value of bioavailability. Relative bioavailability of LMWH-MS at 600 IU/kg was overall less than 3% in healthy animals and less than 5% in animals suffering from TNBS colitis (Table 2). Oral delivery of LMWH solutions led to nondetectable drug absorption with consequent lack of bioavailability (data not shown).

Ussing chamber studies allowed insight into drug penetration for the different tissue samples and subsequent changes depending on the disease state. In this context, carboxyfluorescein was selected as a model compound for low-molecular-weight drugs. LMWH was fluorescently labeled before all

![Fig. 5. Examples for histological colon sections of healthy (A), untreated TNBS colitis (B), and LMWH-treated tissue (C, LMWH s.c.; D, LMWH rectal; E, LMWH-MS 600 IU/kg; and F, LMWH-MS 2000 IU/kg) in mice after the treatment period (magnification, 30x).](image)

![Fig. 6. MPO activity on day 8 in TNBS (A) or OXA (B) colitis model after administration of LMWH and LMWH-MP, respectively. Data are shown as mean ± S.D for \( n = 6 \) animals. *, \( P < 0.05 \) compared with colitis control rats given saline; **, \( P < 0.05 \) compared with rats given LMWH solution subcutaneously.](image)
experiments. Tissue penetration of carboxyfluorescein in colitis tissue was significantly greater than for healthy controls (Fig. 8). LMWH tissue penetration was less than that of carboxyfluorescein. Similarly to observations made for carboxyfluorescein, drug penetration into inflamed tissue was significantly greater compared with tissue from healthy control animals. It was also slightly increased in noninflamed tissue surrounding inflamed regions. In comparison of healthy versus colitis tissue in terms of penetration, differences were immensely increased for LMWH (a 9-fold increase). This finding underlines the notion of LMWH tendency for selective penetration into inflamed tissue.

**Discussion**

LMWH was demonstrated to be a potent approach in the treatment of IBD in animal studies as well as in clinical trials (Törkvist et al., 1999; Dotan et al., 2001). However, long-term routine administration of LMWH increases the risk for hemorrhagic events (Papa et al., 2000), requiring a modification of this early approach to treatment. A selective and local delivery of LMWH could reduce systemic availability of the drug, potentially lowering the risk for adverse effects. Thus, the oral administration pathway might be suggested, however, demanding a significant technological progress in drug formulation science to avoid LMWH loss during its passage through the upper intestinal tract. Currently, standard drug delivery systems release anti-inflammatory drug nonspecifically to the colonic epithelium, regardless from healthy or inflamed state. Therefore, with regard to drug delivery, the optimized strategy would be to combine a higher degree of specificity of drug tissue penetration with an increased selectivity of drug release toward inflamed tissue. In this context, heparin and its derivates are especially interesting considering their minimal tendency to cross the intact intestinal barrier related to their macromolecular structure. This in turn leads to a very low oral bioavailability in healthy subjects (Hoffart et al., 2006; Lamprecht et al., 2006).

The study determining therapeutic efficiency of LMWH was performed in the TNBS and OXA colitis models in mice to analyze therapeutic efficiency on models resembling Crohn’s disease (TNBS) or ulcerative colitis (OXA) in humans, because therapeutic efficiency can vary significantly in both diseases. Despite the fact that the relationship of the TNBS model to human disease is imperfect (Neurath et al., 1995; Fiocchi, 1998), it displays several Crohn’s disease-resembling features. Most notably, comparable is full-thickness transmural mononuclear inflammation driven by T-helper 1-stimulated secretion of IL-2, IL-4, and TNF-α. OXA colitis is a mucosal model of colitis as an IL-4-driven T-helper 2 inflammation with histological similarities to ulcerative colitis (Boirivant et al., 1998). It remains relatively superficial at the microscopic level, affecting mainly the lamina propria of gastrointestinal lumen tissue.

The results of the two models exhibit comparable tenden-
cies with regard to therapeutic effect; however, some considerable exceptions were noted. One essential difference seems to be efficiency of subcutaneous LMWH. A significant therapeutic effect was noted for the OXA colitis model, but it was found to be distinctly less efficient in the TNBS model. The reasons for this observation are not clear, and explanations may range from less mucosal and submucosal swelling with OXA (and subsequently less LMWH penetration hindrance) to other disease-specific mechanisms. Colon weight/length index, MPO, and AP activity results underlined the improved therapeutic efficiency found with LMWH-MS where colitis activity was reduced compared with values from subcutaneous LMWH solution. Rectal administration of LMWH solution reflects local deposition of the drug, avoiding early loss or degradation of the drug during its transport along the intestinal tract. It therefore represents a kind of “best effect value” for epithelial LMWH. Thus, it is a very promising finding that LMWH-MS attained an equivalent level in mitigating efficiency.

An in-depth comparison of the different treatments elucidates the advantages of the developed MS system. After parenteral administration of LMWH, targeted areas of inflamed colonic tissue were not reached by a sufficiently high drug dose. This administration pathway seems particularly inappropriate, because suboptimal drug doses may be used to decrease the risk for adverse effects. When LMWH is administered orally, all drug undergoes intraluminal enzymatic inactivation in the upper parts of the gastrointestinal tract. Rectally administered LMWH, however, showed significant improvements of inflammation in both tested animal models. Likewise, LMWH-MS can protect the drug from early degradation during passage through the upper intestine, and it may allow its intact passage until reaching colonic tissue near sites of inflammation.

In terms of efficiency, this microparticulate system might be compared in the context of other studies applying LMWH. However, relevant information derived from preceding studies is limited, because those studies dealt exclusively with parenteral LMWH administration (Fries et al., 1998; Xia et al., 2004). Improvements of colonic inflammation were observed after a 14-day treatment with dalteparin and suggested time- and dose-dependent effects of LMWH accompanied by severe intestinal bleeding (Xia et al., 2004). This highlights advancements that may be obtained through epithelial LMWH delivery.

The very low oral bioavailability demonstrates that, in terms of adverse effects, epithelial LMWH may allow significant progress compared with existing oral delivery approaches. Although oral bioavailability was mentioned to be altered in active state of IBD (Fries et al., 1999; Schurmann et al., 1999), no significant impact was determined in this study with LMWH-MS. This was determined to be due to efficient retention inside the particle matrix until delivery to the colon.

Apparently, LMWH effect is mainly local, because its systemic concentration is negligible after administration of LMWH-MS and therapeutic effect is limited after subcutaneous administration. Aside from possible effects on microcirculation (Vrij et al., 2001), several other mechanisms of action may explain the therapeutic action of heparins. They were found to interact with a variety of biological proteins such as proinflammatory chemokines, leukocyte proteases, growth factors, and extracellular matrix proteins (Tyrrell et al., 1995). Inhibition of IL-1 production (Jones and Geczy, 2005) may be considered as a specific mechanism as well as the nonspecific plain physicochemical interaction between heparins and a variety of interleukins, namely IL-2, IL-6, IL-10, and IL-12 (Hasan et al., 1999; Salek-Ardakani et al., 2000). In contrast, the inhibition of TNF-α production by macrophages (Cahalon et al., 1997) seems improbable, because TNF-α levels remained unchanged with LMWH treatment, which is a similar finding to results from other groups (Wan et al., 2002). Also an interaction with P- and L-selectin, the adhesion molecules responsible for the leukocyte recruitment, from the endothelial side (Nelson et al., 1983; Koenig et al., 1998) is also not a likely explanation due to the rather long diffusional transport distance for the drug. These aspects may require further in-depth studies to elucidate whether the inhibitory effect of epithelial LMWH is the result of a nonspecific binding of LMWH to one or more cytokines or whether it involves a selective inactivation mechanism.

Selective epithelial LMWH delivery seems to be a promising approach in the therapy of IBD. LMWH-MS allow the desired drug to be released with a high degree of selectivity in areas of inflamed tissue, ensuring a therapeutic concentration of the entrapped drug near the site of action. Furthermore, the entrapped LMWH enhanced the phenomenon of specificity by its very own minimal tendency to cross intact intestinal barrier, resulting in extremely low oral bioavailability and a specific tissue penetration at the inflammation site. This approach proposes the clinical use of LMWH for the oral treatment of IBD with enhanced therapeutic efficiency of LMWH by selective drug delivery combined with negligible systemic adverse effects due to particularly low systemic drug absorption.

References


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