



Heparin Modulates Migration of Human Peripheral Blood Mononuclear Cells and Neutrophils

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Abstract. Evidence has now accumulated that heparin can significantly affect immune response including allergic inflammation. Cell motility is supposed to be very crucial in this process. Thus the aim of our study was to investigate whether heparin is a chemoattractant for some inflammatory cells and is also capable of influencing chemotaxis induced by typical chemoattractants. Peripheral blood mononuclear cells (PBMCs) and neutrophils from 10 healthy subjects were obtained by gradient centrifugation. Chemotaxis assays towards either heparin (molecular weight 16 kDa) or low molecular weight heparin – fraxiparine (molecular weight 5 kDa) were performed in Boyden chambers. We found that both heparin molecules are chemoattractants for both PBMCs and neutrophils in the wide concentration range (0.1–2000 µg/ml). However, maximal chemotaxis was observed at concentrations 50–100 µg/ml (fraxiparine) and 1–50 µg/ml (heparin). We also found that fraxiparine was able to significantly increase chemokinesis and decrease chemotaxis in the gradient of both fMLP and IL-8. These results indicate that heparin is a potent regulator of cell migration.

Key words: peripheral blood mononuclear cells; neutrophils; migration; heparin influence.

Introduction

Heparin, which is a glycosoaminoglycane was first isolated in 1916 from dog livers and thus acquired its name. It has non-homogeneous, polymeric structure containing 3 monosaccharide subunits (α -D-glycosoamine, α -L-iduronic acid, β -D-glucuronic acid), whose amino- and hydroxyl- groups are sulfated. Thus, the polyanionic molecule structure of heparin which contains a certain number of anionic groups enables an easy binding of various organic and non-organic cationic molecules, including amines, aminoacids, peptides, proteins and chemokines. Heparin binds to proteins using ionic linkage. Thus, thanks to its unique structure heparin acts as an universal ionic exchanger¹⁴. Heparin is synthesized and stored mainly in mast cells and to a lesser extent in basophils²³. Heparin is stored

in cell granules in the form of proteoglycan i. e. high molecular weight protein-polysaccharide complexes. Human mast cells contain 2.4–7.8 µg heparin, mol. wt. 20 kDa⁴. Heparin and other granule derived mediators are released due to mast cell degranulation. Heparin has been successfully applied in anticoagulant therapy for more than 50 years. However, in the 70's it has also been demonstrated to possess anti-inflammatory, anti-allergic as well as anti-metastatic properties^{6, 8, 15}. In the recent years, research focused mainly on immunosuppressive and anti-inflammatory effect of heparin^{9-12, 17}. Since the cell migration plays a key role in initiation and progression of inflammatory responses, the aim of our study was to investigate the effect of heparin on motility of important inflammatory cells i. e. peripheral blood mononuclear cells (PBMC) and neutrophils. We investigated the effect of heparin on both random mo-

tility (chemokinesis) and directed movement (chemotaxis) as well as the influence of heparin on typical chemoattractant i. e. fMLP, IL-8 induced chemotaxis.

Materials and Methods

Subjects. Ten normal subjects were healthy adult volunteers with age range 21–44 years, median age 33.1.

Reagents. Gradisol G (Polfa, Poland), Heparinum (16 kDa, Polfa, Poland), low molecular weight heparin – Fraxiparine (5 kDa Sanofi, Winthrop), fMLP(N-formyl methionyl-leucyl-phenyl-alanine methyleste) (Sigma, USA), IL-8 human recombinant (Promega, USA). Medium was composed of Hanks' balanced salt solution (HBSS) containing human albumin (0.4%, ZLB, Switzerland) and Gentamycin (40 µg/ml, Polfa).

Separation of cells. Neutrophils and PBMCs were isolated by the density gradient centrifugation according to ZEMAN et al.²⁷ using Gradisol G. The residual erythrocytes were eliminated by hypotonic lysis. The viability of cells, determined by trypan blue exclusion, was consistently >97%.

Cell migration tests. Cell motility tests were performed by modified BOYDEN⁵ method in 48 well chemotaxis chambers (Neuro-Probe Inc., USA) with nitrocellulose filters (Sartorius, Göttingen, Germany), 5 µm and 8 µm pore size for neutrophils and PBMCs, respectively. The lower chambers were filled with either medium alone or with reagents to be tested. The upper chambers contained 50 µl cell suspensions (neutrophils or PBMC) in medium (0.5×10^6 cells). The chambers were incubated (60 min, 37°C, 5% CO₂). After the incubation the filters were fixed and stained with hematoxylin after Ehrlich. The cell migration was determined using leading front assay based on the measurements of the distance (µm) between upper surface of the filter and the deepest cell layer inside the filter. All cellular tests were performed in duplicates. Individual presented values are means of 10 measurements performed. Checkerboard assays were used to distinguish between chemotaxis (directed migration) and chemokinesis (random migration). For these assays various concentrations of agent were placed in upper wells, lower wells or both upper and lower wells of chemotactic chamber to determine whether migration of cells was greater with positive (chemotaxis) or negative (chemokinesis) gradient of agents.

Statistical methods. We applied Student's *t*-test when analysing the results. The value $p < 0.05$ was considered statistically significant.

Results

Figure 1 shows chemotaxis of PBMCs in various concentration gradients of both low-molecular-weight heparin – fraxiparine, and standard heparin in comparison to positive control (chemotaxis in fMLP gradient). Cell motility was significantly increased in the wide concentration range of both heparin types. However, maximal chemotaxis was observed at concentration 1 µg/ml of standard heparin and higher concentration (50 µg/ml) of fraxiparine. The cell motility at these concentrations was even higher than in the fMLP gradient.

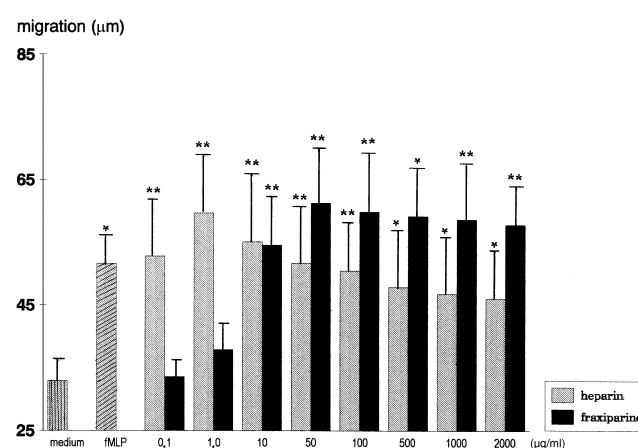


Fig. 1. The effect of standard heparin and low-molecular-weight (fraxiparine) on PBMC motility. Tests were performed in Boyden chambers. Upper chambers contained 0.5×10^6 cells. The lower chambers were filled with either medium (control) or fMLP, positive control or various concentrations of heparin. The cell motility was determined by measurements of the migration distance (µm) between upper surface of the filter and the deepest cell layer inside the filter. Presented values are means of 10 measurements. Asterisks show statistical significance: * $p < 0.05$, ** $p < 0.001$

The migration of neutrophils was also significantly increased at the wide range of concentrations of both heparin types (Fig. 2). However, maximal chemotaxis was observed at the concentration 50 and 100 µg/ml for standard and fraxiparine, respectively. Interestingly, neutrophil motility was higher at these concentrations than in the gradient of fMLP, which is a classical chemoattractant for neutrophils.

The analysis of the cell movement type by the checkerboard assay showed significant dominance of chemotaxis over chemokinesis in the models tested.

Figure 3 shows motility of cells incubated (30 min, 37°C) in various concentrations of fraxiparine (1–2000 µg/ml). After incubation the cells were washed, resuspended in medium, and cell motility tests were per-

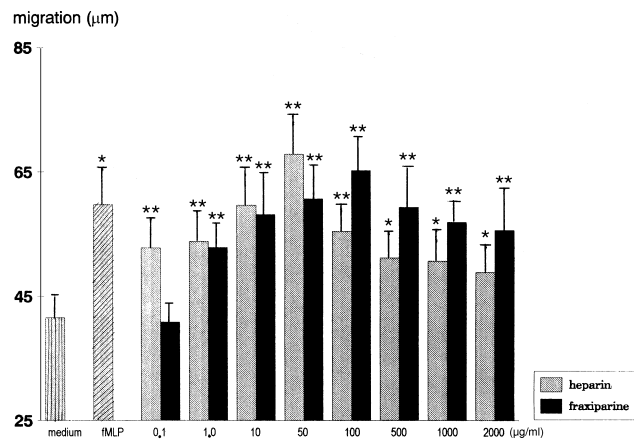


Fig. 2. The effect of standard heparin and low-molecular-weight (fraxiparine) on neutrophil motility. Explanations see under Fig. 1

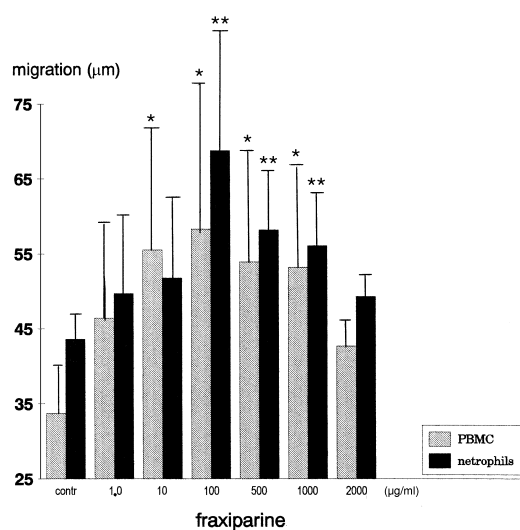


Fig. 3. Spontaneous migration of PBMC and neutrophils after incubation with fraxiparine. PBMC and neutrophils were incubated with various concentration of fraxiparine (30 min, 37°C). Upper chambers contained 0.5×10^6 cells. The lower chambers were filled with medium alone. The cell motility was determined by measurements of the migration distance (μm) between upper surface of the filter and the deepest cell layer inside the filter. Presented values are means of 10 measurements. Asterisks show statistical significance: * $p < 0.02$, ** $p < 0.01$.

formed in medium alone. Both PBMC and neutrophils showed significantly increased chemokinesis.

The same fraxiparine incubated (50 $\mu\text{g/ml}$, 30 min, 37°C) cells when tested in the gradient of chemoattractants (fMLP, IL-8) showed decreased chemotaxis as compared to control cells which were not incubated with fraxiparine (Fig. 4).

Discussion

Our data indicate that both low-molecular weight (fraxiparine) and standard heparin are potent chemoat-

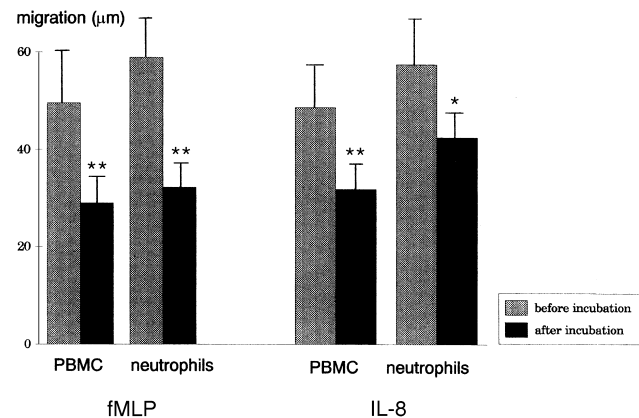


Fig. 4. Chemotaxis of PBMC and neutrophils in the gradient of fMLP or IL-8 before and after incubation with fraxiparine. PBMC and neutrophils were incubated with fraxiparine 50 $\mu\text{g/ml}$, (30 min, 37°C). Control (not incubated) or incubated cells were tested simultaneously in Boyden chambers. Chemotaxis of PBMC and neutrophils is significantly inhibited by fraxiparine incubation. Asterisks show statistical significance: * $p < 0.01$, ** $p < 0.001$

tractants for inflammatory cells (PBMCs, neutrophils). On the other hand, we found that heparin (fraxiparine) was capable of inducing chemokinesis in medium alone and inhibit chemotaxis induced in the gradients of both chemotactic peptide (fMLP) and chemotactic cytokine IL-8.

Previous studies indicate that heparin is capable of inducing both lymphocytosis and to a lesser extent leukocytosis^{13, 22}. It was also demonstrated³ that heparin (10–50 $\mu\text{g/ml}$) induced migration of other cell types including endothelial cells. Heparin is also a strong chemoattractant for human spermatozoons²⁴. Heparin has no chemotactic effect on eosinophils, however it has been shown that chemotaxis induced by factors like zymosan-activated serum, bacterial factor, eosinophil chemotactic factor, RANTES and fMLP was inhibited by heparin^{2, 7}. We also found that heparin is able to inhibit chemotaxis of both PBMCs and neutrophils, which might indicate its anti-inflammatory effect. Recently, immunosuppressive and anti-inflammatory effects of heparin has again attracted significant interest. Numerous studies in both animal and human models indicate that heparin inhibited the immediate reaction to antigens in the human lung and skin⁴. Heparin is capable of preventing bronchospasm¹ as well as inhibiting allergen and exercise induced bronchoconstriction²¹. It also inhibited delayed sensitivity in mouse¹⁰ and suppressed allograft rejection and experimental autoimmune diseases¹⁸. Our *in vitro* data from the current study are conform with *in vivo* study⁶, showing anti-inflammatory action of heparin in rodents, ex-

perimentally provoked with SO₂. Both injected and inhaled heparin prevented bronchial inflammation assessed in biopsies and bronchoalveolar lavage fluid (BALF). Thus, heparin might prevent inflammation by inhibition of cell chemotaxis.

It has been demonstrated that heparin might influence the immune response by binding and neutralization of chemokines. Since all chemokines are basic molecules they can easily bind to polyanionic heparin molecules^{19, 25}. Despite many observations the exact mechanism of action of heparin remains obscure. Biological activities of heparin are exerted through binding of various proteins leading to their activation, deactivation or stabilization. Beside its well known anticoagulant effect, which is very dependent upon the negative charge of the molecule and polymerization of saccharide chains, heparin also binds and stores various positively charged biogenic agents, like histamine, chemokines and lactins^{20, 23, 25}. Under physiological conditions heparin cannot be detected in serum. Heparin released from tissue mast cells might interact in microenvironment with both basic molecules, like chemoattractants, and positively charged cell surface molecules like selectins.

The chemotactic effect of heparin on lymphocytes or neutrophils has not been described. We found that the effect of heparin on cell motility was different in various concentrations. Maximal chemotaxis was observed at low concentrations (1–50 µg/ml). However, at high concentrations (100 µg/ml) maximal chemokinesis was found. Ambiguous effect of heparin dependent upon its concentration was described by YAMASHITA et al.²⁶, who found that heparin at 1 µg/ml induced proliferation of fibroblasts, whereas 100 µg/ml of heparin inhibited proliferation.

Our data indicate that heparin modulates migration of inflammatory cells by inhibition of chemotaxis induced by common chemotactic factors. Interestingly, heparin by itself can increase the motility of lymphocytes and neutrophils. These mechanisms might be responsible for anti-inflammatory and immunosuppressive actions of heparin.

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