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Binding of Heparin to Human Leukocytes

Sulfated polysaccharides possess a variety of biological functions, the most important compounds with clinical relevance being heparin (1, 2). The non-anticoagulant actions are considered to be in part or completely responsible for the antithrombotic, antiatherosclerotic, and anti-inflammatory potencies (3, 4). The molecular mass of unfractionated heparins (UF) ranges from 3,000 to 30,000 dalton and of low molecular mass (LMM) heparins from 1,200 to 15,000 dalton (5). Heparins exert their anticoagulant actions by enhancing the inactivation of several serine proteases of the coagulation system and by potentiating the activity of antithrombin III (AT III) (6). They also exhibit a variety of AT III independent significant anti-inflammatory and anti-metastatic activities (7, 8). The antithrombotic potency is established in postoperative (9, 10) and general medicine (11, 12) as well as for the treatment of acute thromboembolic diseases (13, 14). However, the AT III independent actions of glycosaminoglycans (GAGs) are less known and currently under investigation.

Heparins can be labeled by radioactivity and fluorescence but this is concerned as a major problem because the biological activity and receptor mediated binding may be modified substantially. Therefore, we have developed a method to label heparins through a tyramine residue, which was linked to the anhydromannosyl group of low molecular mass heparin (LMMH) by endpoint attachment (15). The labeling of tyramine with

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Keywords

Glycosaminoglycans, heparin, low molecular mass heparin-tyramine, leukocytes

Summary

Non-anticoagulant actions of heparins and related sulfated polysaccharides in thrombosis, atherosclerosis and inflammation are currently under investigation. To analyze the involvement of leukocytes in this mechanism, a fluorescein labeled low molecular mass heparin-tyramine has been prepared (LMMH-tyr-Fitc) to investigate the binding of heparin and other sulfated polysaccharides to granulocytes, monocytes, and lymphocytes. The fluorescence intensity on leukocytes was quantified using flow cytometry as detection method. LMMH-tyr-Fitc bound dose-dependently to all three leukocyte populations. Phycoerythrin-labeled CD-antibodies identified the specificity of the binding of LMMH-tyr-Fitc to lymphocytes, monocytes, and granulocytes. Unfractionated heparin and LMM-heparin displaced LMMH-tyr-Fitc dose-dependently from granulocytes, monocytes, and lymphocytes and were more effective compared with dextransulfate. Heparin, LMM-heparin and LMMH-tyr-Fitc bound to leukocytes inhibited factors Xa activity in the S2222 chromogenic substrate assay. The data indicate that negatively charged polysaccharides bind to the surface of granulocytes, monocytes and lymphocytes and that binding is in part depending on the number of negatively charged groups of glycosaminoglycans. After binding to the surface of leukocytes heparin exerts still anticoagulant activity indicating its intact biological function. The binding of heparins to leukocytes may significantly contribute to the antithrombotic and to other biological activities.

Schlüsselwörter

Glycosaminoglycane, Heparin, niedermolekulares Heparin-Tyramin, Leukozyten

Zusammenfassung

Nichtantikoagulatorische Wirkungen von Heparinen und verwandten Verbindungen bei der Thromboseentstehung, Arteriosklerose entwicklung und beim Entzündungsprozeß werden gegenwärtig untersucht. Um die Beteiligung der Leukozyten an diesen Mechanismen zu analysieren, wurde eine Fluoreszein-markierte niedrigmolekulare Heparin-Tyramin-Verbindung hergestellt, um die Heparinbindung an Granulozyten, Monozyten und Lymphozyten zu untersuchen. Die quantitativen Bestimmungen erfolgten mit der Durchflußzytometrie. Das synthetisierte niedermolekulare Heparin-Tyramin-Präparat band sich dosisabhängig an alle drei Leukozytenpopulationen. Unfraktioniertes Heparin und niedermolekulares Heparin konnten diese Verbindung wiederum dosisabhängig von Granulozyten, Monozyten und Lymphozyten verdrängen. Die Bindung negativ geladener Polysaccharide an die Oberfläche von Granulozyten, Monozyten und Lymphozyten hängt von der Zahl negativer Ladungen der Glycosaminoglycane ab. Dieser Prozeß der Heparinbindung an Leukozyten kann bei der antithrombotischen Heparinwirkung und im Rahmen anderer biologischer Heparinaktivitäten von Bedeutung sein.

iodine has been used to develop a sensitive binding assay (16) and to study the renal and liver metabolism in animals (17). Fluorescein-5-isothiocyanate (Fitc) has been specifically tagged to the tyramine group of LMM-heparin without modifying the biological activity (18). Here we report now on the binding of LMMH-tyr-Fitc to human granulocytes, monocytes, and lymphocytes and demonstrate that heparins bound to the surface of leukocytes still exert factor Xa inhibition.

Materials and Methods

Materials

Unfractionated porcine intestinal mucosa heparin was obtained from Braun (Melsungen, Germany). The mean molecular weight was 12,000 dalton, the activity was 160 IU/mg. Low molecular mass heparin (19) Fragmin® was from Kabi Pharmacia (Erlangen, Germany) with a mean molecular weight of 5,600 dalton and 160 anti-factor Xa units and 68 anti-thrombin units/mg. Dextransulfate (molecular weight 500,000 dalton) was from Sigma fine chemicals (St. Louis, USA). Rabbit phycoerythrin conjugated anti-mouse CD3, CD13 and CD14 antibodies were obtained from Becton Dickinson (Heidelberg, Germany).

Preparation of Fitc-conjugated LMM-heparin

Tyramine was specifically bound to LMM-heparin by endpoint attachment and the product was purified by high performance liquid chromatography (5). The specific activities of the product were 108 anti-factor Xa U/mg and 42 antithrombin U/mg. LMMH-tyr was conjugated with fluorescein-5-isothiocyanate (Fitc). The product had a mean molecular weight of 3,600 dalton, and contained 70 anti-factor Xa units/mg and 5 antithrombin units/mg using the first international low molecular weight heparin standard (18) (Fig. 1).

Blood sampling

Venous blood was obtained from healthy volunteers, who did not take any medication for the last 10 days. All volunteers had given conformed consent prior to blood sampling. Blood was obtained puncturing an antecubital vein by a 18-gauge butterfly without tourniquet to minimize platelet activation during blood collection. After the first 2 ml of blood were discarded 5 ml was collected in plastic tubes obtaining EDTA for anticoagulation.

Preparation of samples for flow cytometry

One-hundred µl anticoagulated whole blood were incubated with 10 µl LMMH-tyr-Fitc at concentrations ranging from 0.001 to 10 µg/ml for 15 min at dark and at 4° C. Then erythrocytes were lysed by adding 2 ml lysis reagent (Becton Dickinson, Heidelberg, Germany) for 10 min at room temperature and dark. Samples were centrifuged at 500 g for 5 min. The pellet was washed twice with 2 ml 0.05 m tris-NaCl buffer pH 7.4.

Flow cytometry analysis

The fluorescence on the leukocytes was analyzed by FAC-Scan cytometer (Becton Dickinson) equipped with a 15 mW air-cooled 488 nm argon laser. Forward and side scatter as well as green (Fitc) and red (phycoerythrin) signals were acquired by logarithmic amplification with a 585 ± 21 nm filter for collection of FITC and PE signals. Acquisition processing of data from 10,000 cells was carried out with the Consort 30 software (Becton Dickinson) on a Hewlett Packard 300 computer. Based on light scattering properties, each cell was represented by a point in the rectangular coordinate system and according to the forward and side scatter the cell populations were identified as lymphocytes, monocytes and granulocytes. A discrimination frame was placed around the 3 cell clusters. The percentage of positive cells and the mean fluorescence intensity was calculated as median, mean and standard deviation. Each experiment was carried out as duplicate and was repeated three times at different days. Mean values and standard deviations were calculated from the data given in tables and figures. Blood samples were passed through the laser beam through a 70 µm nozzle at a flow rate of 10,000 blood cells per sec. Light scatter and fluorescence data were obtained with gain settings in a logarithmic mode.

Heparin binding to leukocytes

Dilutions of the unfractionated heparin, LMM-heparin, and dextransulfate were used from to 0.01 µg to 0.1 mg/ml. Samples were mixed with 1 µg LMMH-tyr-Fitc/ml and incubated with leukocytes from 1 ml EDTA-anticoagulated blood at dark for 30 min. Fixation of cells was performed in 5% paraform aldehyde as described (20). All experiments were carried out 3-fold and in duplicates.

Identification of LMMH-tyr-Fitc on leukocytes

Granulocytes, monocytes, and lymphocytes were identified by their forward and side scatter characteristics in the flow cytometry analysis. The area of the three cell populations were gated and the Fitc fluorescence was quantified by the software program. Mean values and standard deviations of the relative fluorescence intensities (RFI) of each gated area were calculated separately.

The binding of LMMH-tyr-Fitc on granulocytes, monocytes, and lymphocytes was identified using CD3, CD13 and CD14 antibodies labelled with phycoerythrin (PE). The results were plotted using contour plot diagrams.

Chromogenic S2222 anti-factor Xa assay

The anti-factor Xa activity was determined using 0.36 mmol/l chromogenic substrate S2222 and 0.7 nKat units/ml purified bovine factor Xa (both reagents from Kabi Pharmacia, Erlangen, Germany) (21).

Results

Structure and NMR-analysis of LMMH-tyr-Fitc

The chemical structure of LMMH-tyr-Fitc is depicted in Figure 1. Tyramine is bound by end point attachment to the anhydromannose group by an imide binding in position C8. Fitc is linked to the secondary amino group of C8 of tyramine forming a thiourea group as shown by NMR spectroscopy (Figure 2). The yield of the substitution with Fitc was 80% of LMMH-tyr. The anticoagulant activities are given in Table 1.

Addition of increasing amounts of LMMH-tyr-Fitc to granulocytes, monocytes and lymphocytes

The results of the forward and sideward scatter of the flow cytometry analysis of human granulocytes, monocytes and lymphocytes from EDTA anticoagulated blood is given in Figure 3 (upper panel left). In the upper panel (right), the number of cells per channel of fluorescence intensity is plotted against the relative fluorescence intensity of the lymphocyte population. In the lower panel histograms of monocytes (left) and granulocytes (right) are depicted as number of cells per channel and the relative fluorescence intensity of the cell population. The data in Figure 3 are

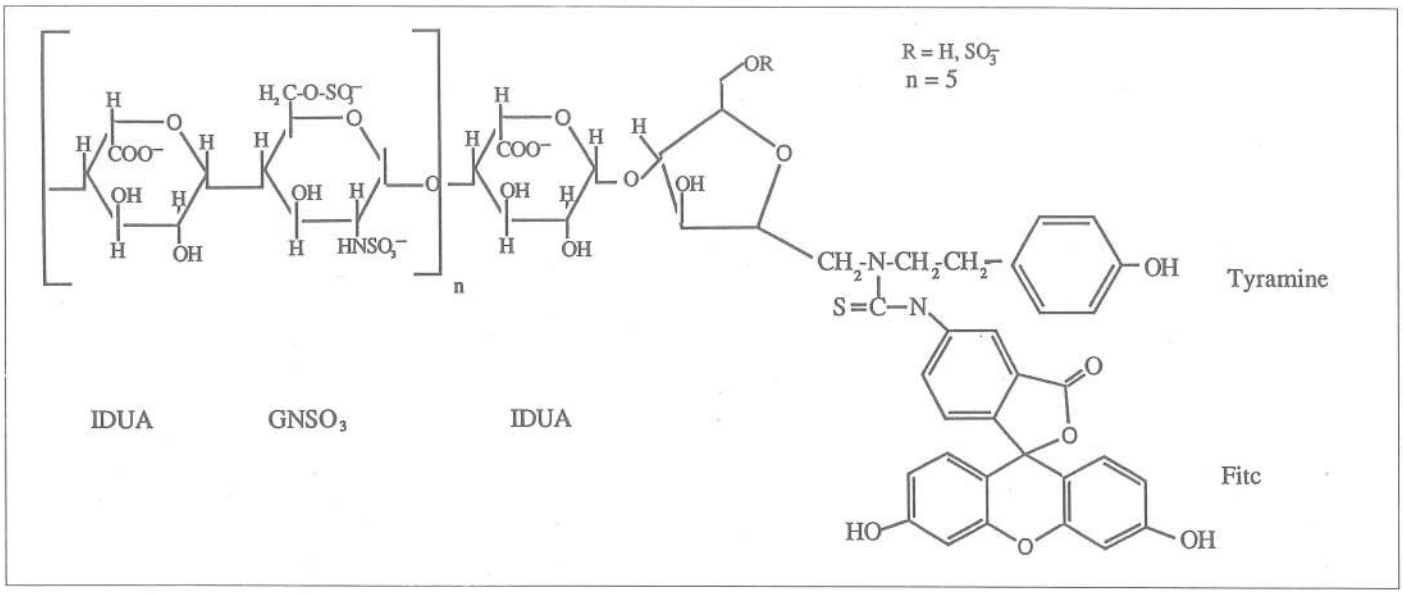

Fig. 1 Chemical formula of fluorescent labeled low molecular mass heparin-tyramine.

Table 1 Anti-factor Xa (aXa) activity and antithrombin (aIIa) activity of the original low molecular mass heparin, LMMH-tyr and LMMH-tyr-Fitc

Compound	aXa U/mg	aIIa U/mg
LMMH	110	44
LMMH-tyr	108	42
LMMH-tyr-Fitc	70	5

from control experiments without addition of LMMH-tyr-Fitc.

Figure 4 shows the results of the addition of 0.1 µg LMMH-tyr-Fitc to 1 ml of EDTA anticoagulated blood. One ml blood contained 6×10^6 leukocytes, which were composed of 65% granulocytes, 28% lymphocytes and 7% monocytes. Compared to the results of Figure 3, the relative fluorescence intensity (RFI) was increased only on granulocytes.

Figure 5 shows the results of addition of 1 µg LMMH-tyr-Fitc/ml EDTA anticoagulated blood. The forward and sideward scatter of the cells remained unaltered after addition of heparin. The RFI increased on lymphocytes and monocytes to a small extent, as compared with the results of Figure 3. The relative fluorescence intensity on granulocytes was considerable enhanced after addition of this amount of LMMH-tyr-Fitc.

Figure 6 shows the results after addition of 10 µg LMMH-tyr-Fitc/ml EDTA anticoagulated blood. No alterations of the forward and sideward scatter of granulocytes, monocytes or lymphocytes were detected again. The RFI of all three cell populations increased and was highest on granulocytes.

Identification of LMMH-tyr-Fitc on leukocytes using CD3, CD13, and CD14 PE-labeled antibodies

To identify the binding of LMMH-tyr-Fitc more specifically on granulocytes, monocytes and lymphocytes, PE-labeled CD3 antibodies were used to identify lymphocytes, CD13 PE-labeled antibody to identify granulocytes and CD14 PE-labeled antibodies to identify monocytes. Antibodies were incubated in separate experiments together with 10 µg LMMH-tyr-Fitc.

Figure 7 shows the results of the binding of CD3 antibody and LMMH-tyr-Fitc on lymphocytes. The data are given as contour plots. In the upper panel controls are given (left) and the results of incubation with CD3 antibody alone (right). In the lower panel the results of the RFI of 10 µg LMMH-tyr-Fitc (left) and of incubation with CD3 antibody together with 10 µg LMMH-tyr-Fitc are shown (right).

Figure 8 shows the respective results using CD14 antibodies to identify monocytes. In the upper panel the control without fluorescent compounds (left) and the addition of CD14 antibody alone (right) are given. In the lower panel RFI of 10 µg LMMH-tyr-Fitc are shown (left) as well as the data obtained after simultaneous incubation of PE-labeled CD14 antibody together with 10 µg LMMH-tyr-Fitc (right).

Figure 9 shows the results of the identification of LMMH-tyr-Fitc on granulocytes using PE-labeled CD13 antibody. In the upper panel the control without fluorescent compounds (left) and the data obtained with incubation of CD13 PE-labeled antibody alone are given (right). In the lower panel the results of the RFI of 10 µg LMMH-tyr-Fitc on granulocytes is shown (left) as well as the data obtained by incubation of PE-labeled CD13 antibody together with 10 µg LMMH-tyr-Fitc (right).

Competition of LMMH-tyr-Fitc with heparin

The competition of LMMH-tyr-Fitc with unfractionated heparin on

Table 2 Inhibition of factor Xa after incubation with 10 µg unfractionated heparin or 10 µg low molecular mass heparin

Compound	U/ml added	U/ml found
UF-heparin	16	0.34
LMM-heparin	16	0.32
LMMH-tyr-Fitc	7	0.13

granulocytes, monocytes, and lymphocytes was analyzed by the incubation of different amounts of LMMH-tyr-Fitc with different amounts of unfractionated unlabeled heparin. As an example the results of the incubation of 1 µg LMMH-tyr-Fitc with 7 µg unlabeled unfractionated heparin are given (Fig. 10). In the upper panel the forward and the sideward scatter of the leukocytes is given (left) together with the relative fluorescence intensity on lymphocytes (right). In the lower panel the results of the relative fluorescent intensity is given as histogram for monocytes (left) and granulocytes (right). A comparison with the results of Figure 3 shows a lower relative fluorescent intensity of all three cell populations. The data are most evident on granulocytes due to the highest RFI.

Figure 11 shows the displacement of 1 µg LMMH-tyr-Fitc by 7 µg low molecular mass heparin (Fragmin). The forward and sideward scatter of the leukocyte population remained unchanged compared with Figure 3 (upper panel left). The results of the RFI on lymphocytes (upper panel right), monocytes (lower panel left), granulocytes (right) are depicted. The decrease of RFI is similar to the data obtained with unfractionated heparin.

The displacement of 1 µg LMMH-tyr-Fitc by 70 µg dextransulfate is given in Figure 12. The results show only a minimal shift of the RFI values on all three cell populations to the left indicating a very low binding of dextransulfate. It has to be stressed that dextransulfate has been used in a 10-fold higher concentration in this experiment compared with unfractionated heparin or low molecular mass heparin.

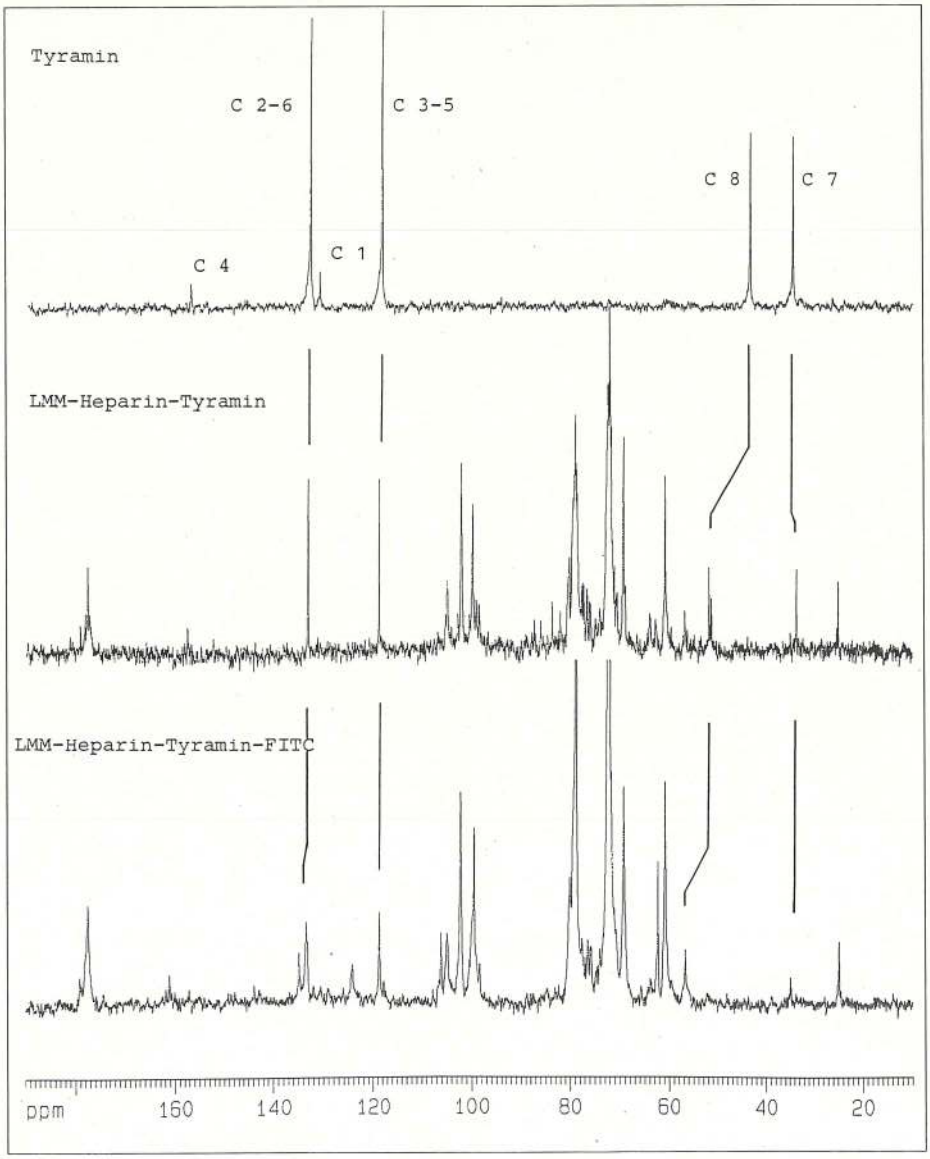

Fig. 2 13C-NMR spectra of tyramine, LMM heparin-tyramine and LMM-heparin tyramine fluorescein-5-isothiocyanate (from reference no. 18, with permission).

Inactivation of factor Xa by heparin treated leukocytes

EDTA-anticoagulated blood was incubated with or without heparin, LMM-heparin or LMMH-tyr-Fitc (final concentration 10 µg/ml) at 4° C for 15 min. Cells were washed and erythrocytes were lysed using lysis reagent (Becton Dickinson). Serial dilutions of leukocytes were added to factor Xa and antithrombin III, as described. Pre-treatment with heparin, LMM-heparin or LMMH-tyr-Fitc inhibited factor Xa activity indicating that cell bound heparin was anticoagulantly functional. The results are given in Table 2.

Discussion

The present data show that heparins bind significantly to human granulocytes, monocytes, and lymphocytes and that bound heparins retain in part the anticoagulant activity. This indicates the significance of heparin binding to leukocytes for many biological effects in thrombosis, atherosclerosis, and inflammation. The non-anticoagulant activities of heparins were recently re-emphasized (22) based on a report on the reduction of exercise induced asthma by inhaled heparin (23). The mechanisms of action of this effect is unknown so

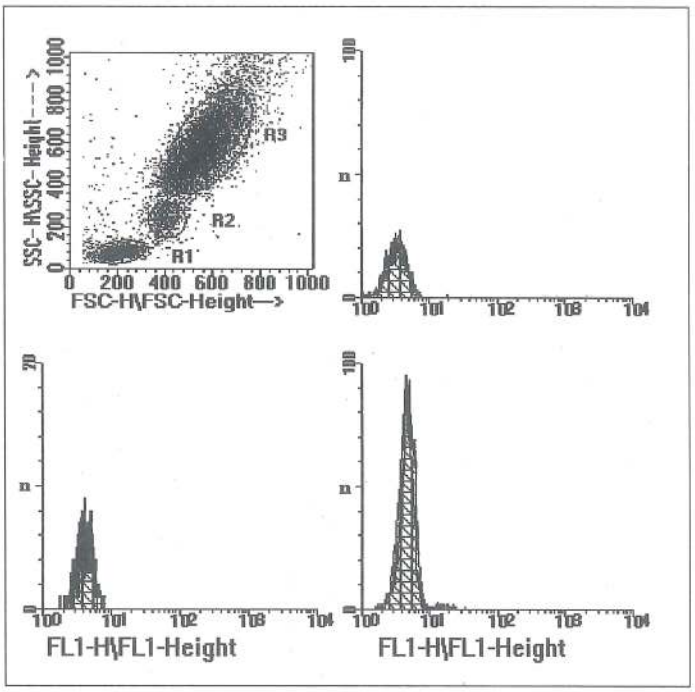

Fig. 3 Histograms of lymphocytes, monocytes, and granulocytes after incubation with saline. Upper panel: forward scatter and sideward scatter (left), number of cells vs. the relative fluorescence intensity (RFI) of lymphocytes (right); lower panel: monocytes (left) and granulocytes (right).

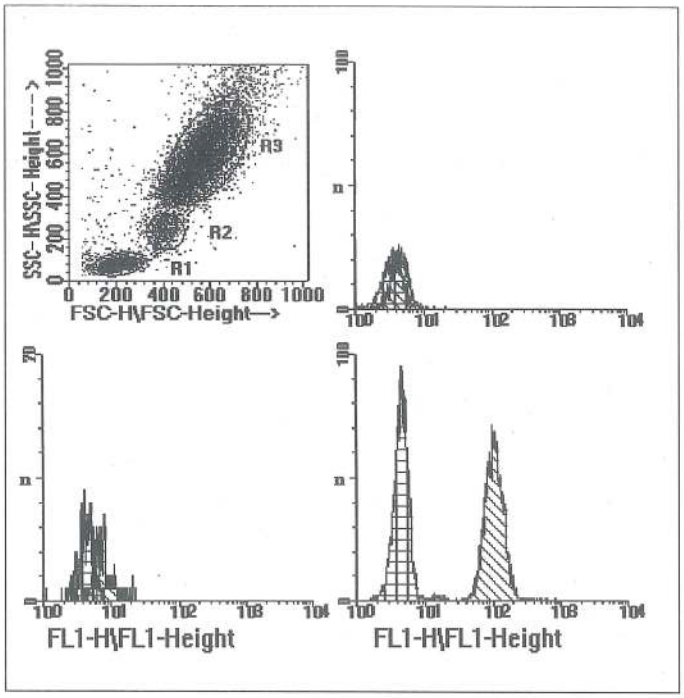

Fig. 5 Histograms of leukocytes incubated with 1 µg LMMH-tyr-Fitc/ml. Upper panel: forward scatter and sideward scatter (left), number of cells vs. the RFI of lymphocytes (right); lower panel: monocytes (left) and granulocytes (right). Square area: saline, diagonal area: 1 µg LMMH-tyr-Fitc.

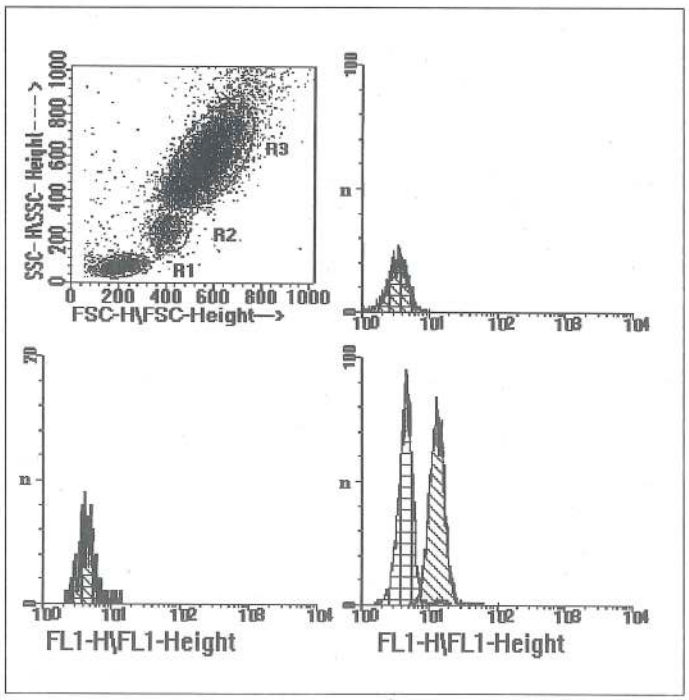

Fig. 4 Histograms of leukocytes incubated with 0,1 µg LMMH-tyr-Fitc/ml. Upper panel: forward scatter and sideward scatter (left), number of cells vs. the RFI of lymphocytes (right); lower panel: monocytes (left) and granulocytes (right). Square area: saline, diagonal area: 0,1 µg LMMH-tyr-Fitc.

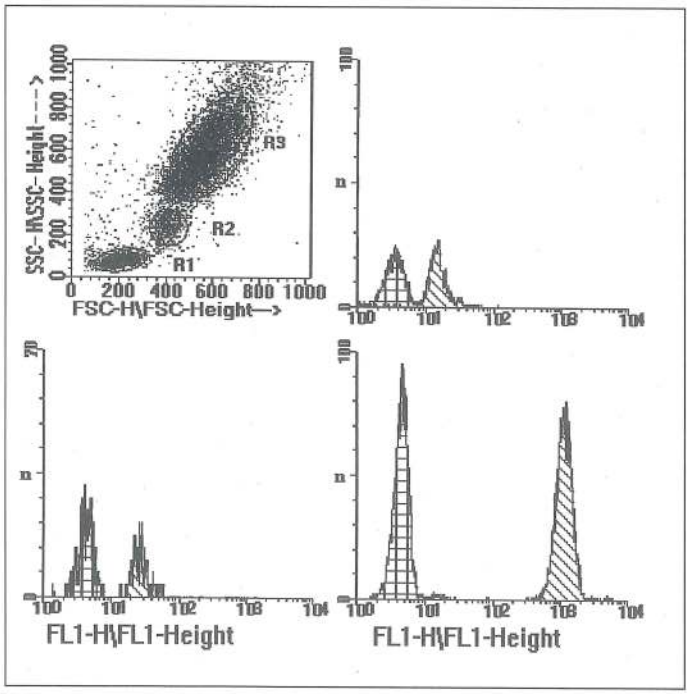

Fig. 6 Histograms of leukocytes incubated with 10 µg LMMH-tyr-Fitc/ml. Upper panel: forward scatter and sideward scatter (left), number of cells vs. the RFI of lymphocytes (right); lower panel: monocytes (left) and granulocytes (right). Square area: saline, diagonal area: 10 µg LMMH-tyr-Fitc.

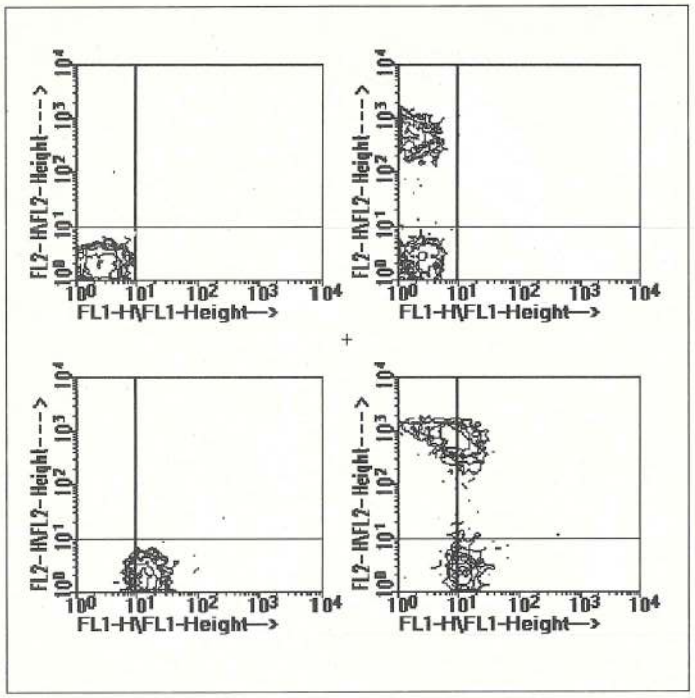

Fig. 7 Contour plot analysis of lymphocytes before and after incubation with PE-labeled CD3 antibody with or without 10 µg LMMH-tyr-Fitc. Upper panel: control without fluorescent labels (left) and with PE-label (right); lower panel: Fitc fluorescence of 10 µg LMMH-tyr-Fitc without (left) and with PE-label (right).

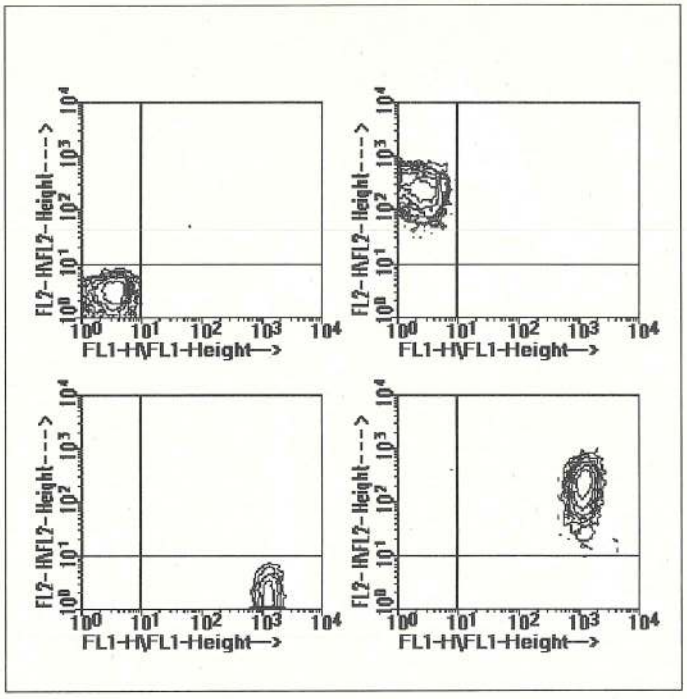

Fig. 9 Contour plot analysis of granulocytes before and after incubation with PE-labeled CD13 antibody with or without 10 µg LMMH-tyr-Fitc. Upper panel: control without fluorescent labels (left) and with PE-label (right); lower panel: Fitc fluorescence of 10 µg LMMH-tyr-Fitc without (left) and with PE-label (right).

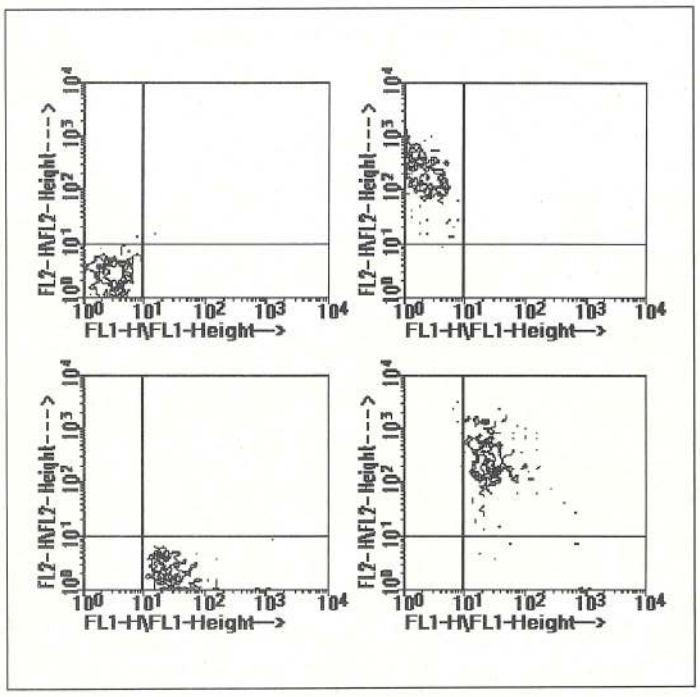

Fig. 8 Contour plot analysis of monocytes before and after incubation with PE-labeled CD14 antibody with or without 10 µg LMMH-tyr-Fitc. Upper panel: control without fluorescent labels (left) and with PE-label (right); lower panel: Fitc fluorescence of 10 µg LMMH-tyr-Fitc without (left) and with PE-label (right).

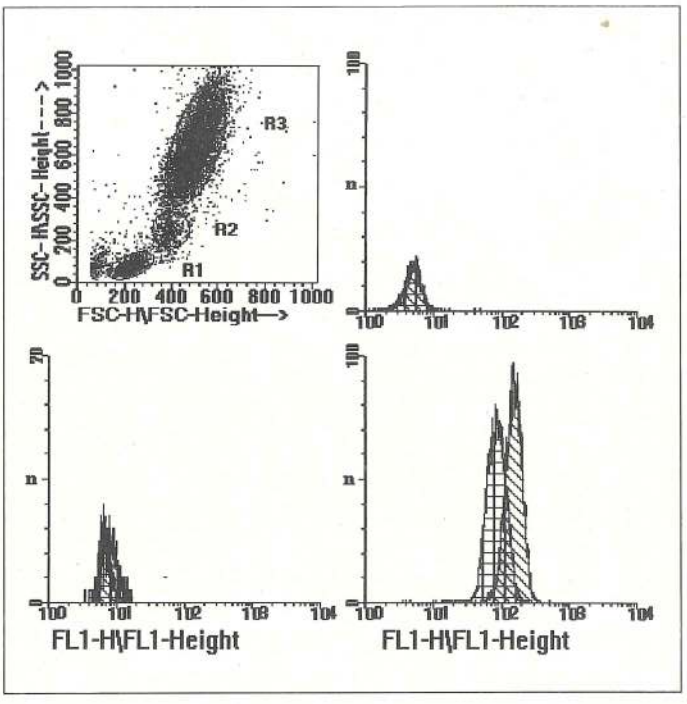

Fig. 10 Decrease of RFI after simultaneous incubation of 7 µg unfractionated heparin together with 1 µg LMMH-tyr-Fitc on lymphocytes, monocytes, and granulocytes. Upper panel: forward and sideward scatter diagram (left), RFI on lymphocytes (right); lower panel: RFI on monocytes (left) and granulocytes (right). Square area: labeled and unlabeled ligand, diagonal area: labeled ligand.

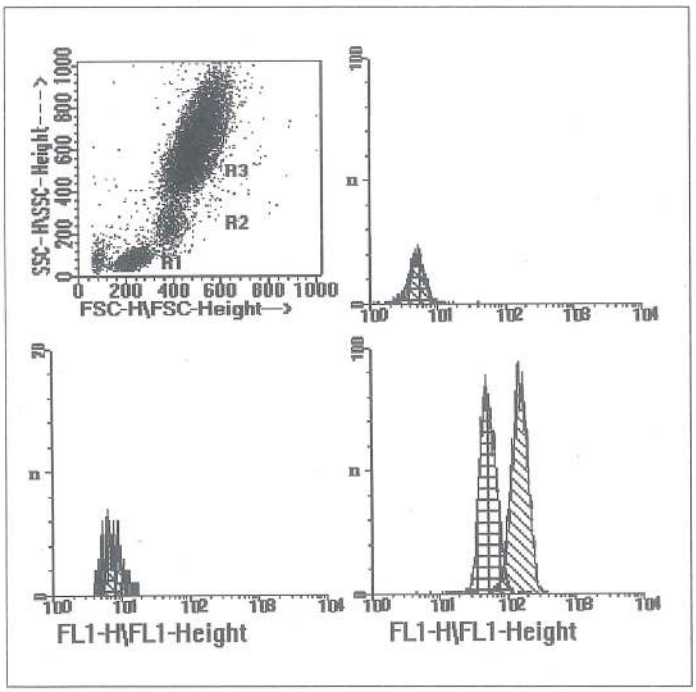

Fig. 11 Decrease of fluorescence after simultaneous incubation of 7 µg low molecular mass heparin together with 1 µg LMMH-tyr-Fitc on lymphocytes, monocytes and granulocytes. Upper panel: forward and sideward scatter diagram (left), RFI on lymphocytes (right); lower panel: RFI on monocytes (left) and granulocytes (right). Square area: labeled and unlabeled ligand, diagonal area: labeled ligand.

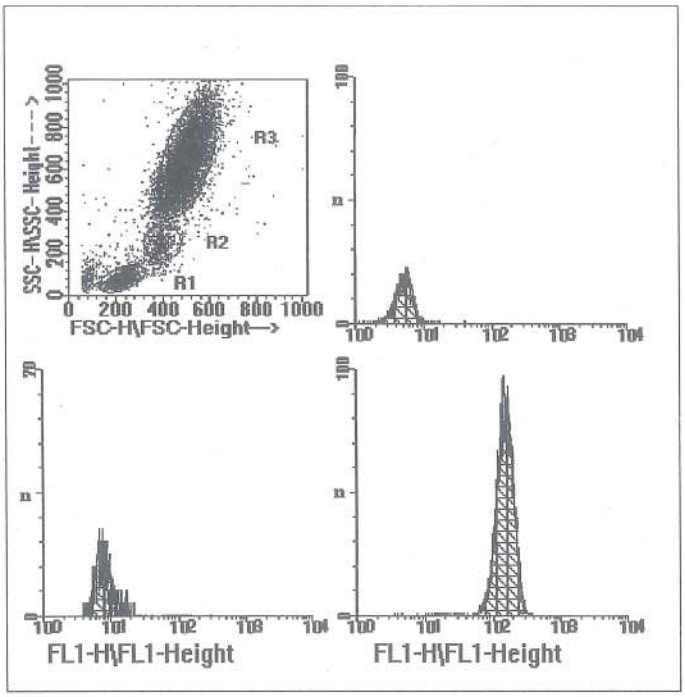

Fig. 12 Fluorescence intensity after simultaneous incubation of 70 µg dextransulfate together with 1 µg LMMH-tyr-Fitc on lymphocytes, monocytes, and granulocytes. Upper panel: forward and sideward scatter diagram (left), RFI on lymphocytes (right); lower panel: RFI on monocytes (left) and granulocytes (right). Square area: labeled and unlabeled ligand, diagonal area: labeled ligand.

far. The role of sulfated polysaccharides in inflammation is thought to be caused by binding to positively charged groups of matrices and peptides, which are released from activated granulocytes.

Heparins can bind to and inactivate cationic proteins such as elastase (24), eosinophilic cationic protein (ECP) and major basic protein (MBP). ECP and MBP are released from granulocytes in asthma and atopic dermatitis (25, 26). The highly basic content of these proteins (pH 9–11) is too toxic to cell surfaces, induce damage and promote diseases. The release of superoxide dismutase from granulocytes is particularly important, because this enzyme effectively detoxifies one of the most aggressive oxygen radicals produced by activated granulocyte superoxide anion (27). As a consequence, the endothelial damage inflicted by the release of these proteins is reduced by heparin and other sulfated polysaccharides (28, 29).

Heparin and sulfated polysaccharides stimulate lymphocyte migration (30) and enhance the proliferating responses of murine T cells in allogeneic mixed leukocyte reaction. Heparin also enhances generation of cytotoxic T lymphocytes against allogeneic cells and histocompatible tumors. The mechanism of the immunoenhancing activity of heparin was reported to be based on a synergistic response of heparin or dextransulfate and interleukin-1 by upregulating IL-1 receptor expression (31). Thus it can be anticipated from our studies that heparin plays an important role in these pathophysiological conditions and that it is transported to the site of action via binding to granulocytes, monocytes as well as to lymphocytes.

The clinical relevance of lymphocytes, monocytes and granulocytes in the pathogenesis of thrombosis, arteriosclerosis and inflammation is well documented. The consecutive healing process leads to the formation of collagen and fibrin fibres and tissue repair. These processes destroy the original cell system and lead to secondary destruction of the organ. Inhibition of these processes may be beneficial. It can be speculated from the present results that heparin may be beneficial in these senses by inhibiting the fibrous repair and by promoting angiogenesis (32).

The experiments of the present study characterize the binding of heparins to granulocytes, monocytes, and lymphocytes. The binding was specific, time- and concentration-dependent, saturable and reversible for all three leukocyte populations. Granulocytes bound heparin at about 50-fold lower concentrations compared with monocytes and lymphocytes. Heparin binding was saturable on all three cell populations suggesting a ligand-receptor type interaction on the cell surface. Heparins are highly sulfated negatively charged polyanions which interact with clusters of basic regions of aminoacids. Hence it

may possibly bind to multiple cell surface proteins if no specific receptor can be identified.

Non-heparin glycosaminoglycans have been shown to be recognized by lectin-like surface receptors on lymphocytes (33, 34, 35) and granulocytes (36). The binding of lymphocytes to endothelial venules has been described to be selectively blocked by heparin (37). This is in agreement with our studies. Accordingly, it can be speculated that heparin and heparin related oligosaccharides in part bind to lectin-like surface receptors of leukocytes.

In summary, the present data demonstrate that heparin and low molecular mass heparin bind specifically to human granulocytes, monocytes and lymphocytes. Sulfated polysaccharides with lower degree of sulfation bind to a lower extent indicating the importance of the sulfation of polysaccharides for binding. It has been demonstrated that heparin bound to the surface of leukocytes is in part still anticoagulantly active. Furthermore, as the non-anticoagulant active heparin was not determined so far in the present study, it can be assumed that clinical significant actions of heparin in thrombosis, arteriosclerosis, and inflammation are mediated by binding to leukocytes. Further studies are currently undertaken to find more precisely these non-anticoagulant actions of heparins bound to leukocytes.

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