Heparin Inhibits Ligand Binding to the Leukocyte Integrin Mac-1 (CD11b/CD18)

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Background—The clinical benefits of heparin reach beyond its anticoagulative properties. Recently, it has been described that leukocytes adhere on immobilized heparin mediated by the integrin Mac-1 (CD11b/CD18, αMβ2, or CR3). Because inhibition of this versatile adhesion molecule could explain various aspects of the beneficial clinical effects of heparin, we evaluated whether soluble heparin modulates Mac-1 function in vitro and in vivo.

Methods and Results—Binding of unfractionated heparin to Mac-1 on PMA-stimulated monocytes and granulocytes was directly demonstrated in flow cytometry, whereas no binding of heparin was detected on unstimulated leukocytes. Unfractionated heparin inhibited binding of the soluble ligands fibrinogen, factor X, and iC3b to Mac-1. Adhesion of the monocytic cell line THP-1 and of peripheral monocytes and granulocytes to immobilized ICAM-1 was impaired by unfractionated heparin, to the same extent as with inhibition of Mac-1 by monoclonal antibodies such as c7E3. Low-molecular-weight heparin also inhibits binding of fibrinogen to Mac-1. Additionally, flow cytometry of whole blood preparations of patients treated with unfractionated heparin revealed an inhibitory effect of heparin on the binding of fibrinogen to Mac-1 that correlates (n = 48, r = 0.63, P < 0.001) to the extent of prolongation of the activated partial thromboplastin time.

Conclusions—We describe a pharmacologically relevant property of heparin that may contribute to its benefits in clinical use. The binding of heparin to Mac-1 and the resulting inhibition in binding of Mac-1 ligands may directly modulate coagulation, inflammation, and cell proliferation. (Circulation. 1999;100:1533-1539.)

Key Words: heparin ■ cell adhesion molecules ■ leukocytes ■ fibrinogen

Unfractionated heparin, a heterogeneous mixture of unbranched, acidic glycosamine-glycans rich in N- and O-sulfate groups, acts as an anticoagulant mainly by binding to the circulating protease inhibitor, antithrombin III. The heparin/antithrombin III complex is able to inhibit thrombin, factor Xa, and factor IXa.1 Low-molecular-weight heparins mainly inhibit factor Xa.2 In many clinical settings, heparin has been proven to be of major benefit as an antithrombotic agent.1 In several therapeutic applications (eg, in coronary angioplasty), properties of heparin distinct from its anticoagulative effect may provide further clinical benefit. Inhibition of leukocyte function has been proposed as an additional effect of heparin.1,3

Recently, it was reported that immobilized heparin can mediate cell adhesion via interaction with the leukocyte integrin Mac-1 (CD11b/CD18, αMβ2).4,5 The integrin Mac-1 is expressed predominantly in leukocytes of the myeloid and monocytic lineage and in natural killer lymphocytes.6 Mac-1 is one of the most versatile adhesion molecules with ligands of very different biological functions.6,7 Its interaction with the transmembrane adhesion molecule ICAM-1 mediates cell adhesion on endothelial cells.6,7 Binding of the zymogen factor X to Mac-1 results in the acceleration of its conversion to activated factor Xa and thus constitutes an alternative pathway for the initiation of the coagulation serine protease cascade.8-11 The binding of fibrinogen to Mac-1 can also participate in the coagulation cascade but at the same time mediates cell aggregation and cell adhesion either on immobilized fibrinogen or as a crossbridge between Mac-1 and ICAM-1.12-14 Furthermore, Mac-1 is identical to the complement receptor type 3, which on phagocytes is responsible for the recognition of iC3b-opsonized bacteria and yeast, and thus the initiation of phagocytosis, degranulation, and respiratory bursts.6,7,15,16 The versatile function of Mac-1 is further demonstrated by the recent finding that oligodeoxynucleotides are bound and internalized by this integrin.17 Because both heparin and Mac-1 have a wide variety of binding partners and because immobilized heparin has been described to interact with Mac-1,4,5 we proposed that soluble heparin may bind to Mac-1 and may thus block binding of
other Mac-1 ligands. Moreover, we evaluated whether heparin, in doses commonly used for anticoagulation, inhibits ligand binding to Mac-1 in patients.

Methods

Patients

Blood was collected from 7 healthy volunteers and from 48 patients with coronary artery disease who received intravenous heparin. Informed written consent was obtained from every individual for drawing of 10 mL venous blood. Neither healthy volunteers nor patients had a history of cancer or signs of inflammatory disease.

Antibodies, Reagents, and Cells

The mouse anti-heparin monoclonal antibody (mAb) clone H1–18 was purchased from Loxo (Heidelberg, Germany), the fluorescent isothiocyanate (FITC)-labeled polyclonal chicken anti-fibrinogen Ab from Biopool (Umeå, Sweden). Factor X (Sigma, Deisenhofen, Germany) was biotinylated with BAC-SulfoNHS (Sigma) at pH 7.2, resulting in a biotin/protein ratio of 1.6. The anti-CD11b mAbs clone 44 was obtained from Pharmingen (San Diego, Calif) and the human/mouse chimeric antibody fragment c7E3 (ReoPro) from Lilly (Bad Homburg, Germany). The mAbs anti-CD66b (clone 80H3) and anti-CD14 (clone RMO52) were obtained from Coulter-Immunotech (Hamburg). The anti-human iC3b mAb was obtained from Quidel (San Diego, Calif). Unfractionated heparin was purchased from B. Braun (Melsungen, Germany), low-molecular-weight heparin (Fragmin) from Pharmacia (Freiburg, Germany). To prepare zymosan-activated serum (ZAS) samples were resuspended in 50 mmol/L sodium acetate buffer, pH 5. After 1 hour incubation at 37°C, the reaction was terminated by adding 100 μL acid phosphatase solution (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). Monocytes and granulocytes were identified by the forward/side scatter.

Flow Cytometry of Monocytes and Granulocytes in Whole Blood Preparation

Blood was collected by venipuncture with a 21-gauge butterfly needle anticoagulated with citric acid and directly incubated with the detecting mAb or the appropriate ligand. Stimulation of cells was performed by addition of PMA (Sigma) to a final concentration of 100 nmol/L or by zymosan-activated serum containing C5a at a final concentration of 4%. After addition of 4 mL of FACSCytox solution (Becton Dickinson, Mountain View, Calif), centrifugation was performed with 1000 rpm (Sorvall J2-HS, 12 000 rpm, 20 minutes). The supernatant was removed and the adhering monocytes were incubated in the above medium for 3 hours. Floating cells (lymphocytes, erythrocytes) were then removed and the adhering monocytes were incubated in the above medium for 15 minutes at room temperature. Cells were washed twice in modified Tyrode's buffer and resuspended in 500 μL of 1x Cell-Fix (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). Monocytes and granulocytes were identified by forward/side scatter.

Binding of endogenous fibrinogen was determined as described previously.14 Briefly, 10 μL of a FITC-labeled polyclonal chicken anti-fibrinogen Ab was added to 60 μL whole blood preparation and incubated for 30 minutes at room temperature. Factor X binding was evaluated by addition of 10 μL biotinylated factor X to 60 μL whole blood preparation and incubation for 30 minutes at room temperature. After additional centrifugation (1000 rpm, Sorvall 6000) and resuspension in 50 μL modified Tyrode's buffer (150 mmol/L NaCl, 2.5 mmol/L KCl, 2 mmol/L MgCl2, 12 mmol/L NaHCO3, 2 mmol/L CaCl2, 1 mg/mL BSA, 1 mg/mL dextrose; pH 7.4) samples were incubated with 2 μL R-phycocerythrin-conjugated streptavidin (Dianova, Hamburg, Germany) for 20 minutes at room temperature. Heparin binding was measured by the addition of 10 μL anti-heparin Ab to 60 μL whole blood and incubated for 20 minutes at room temperature. After centrifugation and resuspension in 50 μL modified Tyrode's buffer, 2 μL of a dichlorotriaziryl fluorescein (DTAF)-labeled polyclonal goat anti-mouse Ab was added and incubated for 20 minutes at room temperature. For titration of heparin, varying concentrations of heparin were added to whole blood and incubated for 20 minutes at room temperature before the addition of the anti-heparin Ab. Binding of iC3b to Mac-1 was determined by incubation of 50 μL whole blood with 1 μL of the iC3b solution, which was prepared as described above, for 20 minutes at room temperature. After addition of anti-iC3b mAb (10 μg/mL) and an additional incubation for 20 minutes at room temperature, cells were centrifuged, resuspended in 50 μL modified Tyrode's buffer, and incubated with 2 μL DTAF-labeled polyclonal goat anti-mouse Ab for 20 minutes at room temperature. In all experiments, FACs analysis was performed after the addition of FACs-Lysing solution, centrifugation, and resuspension in CellFix as described above.

Isolation of Monocytes and Granulocytes

Blood was collected by venipuncture with a 21-gauge butterfly needle from healthy volunteers and anticoagulated with citric acid. For monocyte preparation, the buffy coat was prepared by centrifugation at 3000 rpm (Sorvall RT 6000) for 20 minutes and a layer of 20 mL was added on 15 mL Ficoll-Paque (Pharmacia). After centrifugation at 2500 rpm for 20 minutes, cells at the interface were removed and incubated in tissue culture flasks in RPMI 1640, 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco, Eggenstein, Germany) for 3 hours. Floating cells (lymphocytes, erythrocytes) were then removed and the adhering monocytes were incubated in the above medium for one day. For adhesion experiments, cells were taken off with a cell scraper. More than 95% of prepared cells released an anti-CD14 mAb staining in flow cytometry. For isolation of granulocytes, blood cells were fractionated by density gradient centrifugation over isotonic Percoll (Pharmacia) with a specific gravity of 1.076 g/mL (Beckman J2-HS, 12 000 rpm, 20 minutes). The supernatant was removed and the pellet containing both erythrocytes and granulocytes was treated for 10 minutes with ice-cold isotonic NH4Cl solution (0.155 mol/L NH4Cl, 0.01 mol/L KHCO3, 0.1 mol/L EDTA; pH 7.4) to lyse the erythrocytes. Cells were centrifuged (1000 rpm for 10 minutes) and residual erythrocytes were lysed in a second lysis step as described above. The remaining granulocytes were resuspended in modified Tyrode's buffer, washed once in this buffer, and finally adjusted to 3000 cells/μL. More than 96% of prepared cells revealed an anti-CD66b mAb staining in flow cytometry.

Adhesion of THP-1 Cells, Monocytes, and Granulocytes on Immobilized ICAM-1

Recombinant soluble ICAM-1 (R&D Systems, Abingdon, UK) was diluted in PBS (pH 7.4) to 10 μg/mL and 100-μL aliquots incubated in the wells of a 96-well plate (Nunc ImmunoPlate, MaxiSorp) overnight at 4°C. Following one washing with PBS, the wells were blocked with 100-μL aliquots of 10 mg/mL heat-inactivated BSA (fraction V, Calbiochem, La Jolla, Calif) for 1 hour at room temperature. Cells were washed twice in modified Tyrode's buffer and preincubated with heparin, c7E3, anti-CD11b mAb clone 44, or nothing for 20 minutes at room temperature. One hundred thousand cells per well were allowed to adhere for 30 minutes at room temperature. The nonadherent cells were washed off with 2 rounds of pipetting. The residual adherent cells were quantified with colormetric assay; the cell-endogenous acid phosphatase activity was used by adding 100 μL of the following substrate/lysis solution to each well: 1% Triton X-100, 6 mg/mL p-nitrophenylphosphate (Sigma) in 50 mmol/L sodium acetate buffer, pH 5. After 1 hour incubation at 37°C, the reaction was terminated by the addition of 50 μL of 1 mol/L NaOH and read in an ELISA plate reader (Titertek Multiscan Plus) with a 405-nm filter. Adherence was determined as the percentage of cells adhering in relation to the unblocked sample.

Activated Partial Thromboplastin Time (aPTT)

The activated partial thromboplastin time was photometrically determined on Electra 1000 MLA (Baxter Diagnostics Inc) using Dade Actin-PS (Baxter). The reference range of control subjects is 25 to 35 seconds.
**Statistical Analysis**

The comparison of multiple groups was performed by 1-way ANOVA followed by the Bonferroni post hoc test. In order to analyze the association of 2 continuous variables, Spearman’s rank-order correlation, as a nonparametric measure of association, and the corresponding P value were calculated. As a graphic method for smoothing noisy data, a cubic spline that minimizes a linear combination of the sum of squares of the residuals of fit and the integral of the square of the second derivative was used.19

**Results**

Binding of soluble, unfractionated heparin to leukocytes can be detected by an anti-heparin mAb in flow cytometry of whole blood. Monocytes and granulocytes were able to bind heparin only after stimulation with PMA (Figure 1). This binding could be blocked by the Mac-1 blocking chimeric Ab fragment c7E3 (Figure 1). Binding of heparin to monocytes increased with increasing concentrations of heparin and demonstrated saturation in binding between 0.1 and 1 U/mL (Figure 2B).

Fibrinogen is one of the ligands of Mac-1. Binding of soluble fibrinogen to Mac-1 can be evaluated by a FITC-labeled chicken anti-fibrinogen antibody in flow cytometry. Unstimulated monocytes do not bind fibrinogen (Figure 2A). Stimulation of monocytes by PMA resulted in fibrinogen binding to monocytes that was inhibitable by heparin and c7E3. (Figure 2A). On PMA-stimulated monocytes, fibrinogen binding decreases as heparin binding increases (Figure 2B). Similar data for the blockade of fibrinogen binding to monocytes that was inhibitable by heparin and c7E3. (Figure 2A). On PMA-stimulated monocytes, fibrinogen binding decreases as heparin binding increases (Figure 2B).
With this more physiological method of cell stimulation, similar results for heparin-inhibitable fibrinogen binding could be obtained as with PMA stimulation (Figure 2A).

Factor X was biotinylated and binding to Mac-1 was evaluated by flow cytometry. Mac-1 on PMA-stimulated monocytes and granulocytes bound factor X and this binding could be inhibited by heparin, the anti-CD11b mAb clone 44, and c7E3 (Figure 3). A potential inhibitory effect of heparin on the interaction between Mac-1 and ICAM-1 was studied by adhesion experiments of the monocytic cell line THP-1 and of peripheral monocytes and granulocytes adhering on immobilized ICAM-1. Unfractionated heparin inhibited binding of these cells to ICAM-1, as did anti-CD11b mAb clone 44 and c7E3 (Figure 4). Mac-1 is also described as the complement receptor 3 that binds iC3b. This ligand binding can directly be studied using an anti-iC3b mAb in flow cytometry. iC3b binding to Mac-1 on monocytes and granulocytes could be inhibited by heparin to the same extent as by c7E3 (Figure 5).

To evaluate whether aPTT-directed therapeutic dosing of unfractionated heparin results in Mac-1 blockade in vivo, binding of endogenous fibrinogen to monocytes was determined in patients receiving intravenous heparin. To adjust for individual differences in background binding of the anti-fibrinogen Ab, fluorescence intensity of unstimulated monocytes was subtracted from fluorescence intensity of PMA-stimulated monocytes. A direct reverse correlation \( n = 48, r = 0.63, P < 0.001 \) between aPTT and the binding of endogenous fibrinogen to monocytes could be obtained (Figure 6). Fibrinogen binding to monocytes was not influenced by variations in fibrinogen serum levels of patients \( r = 0.199, P = 0.22 \). Effective inhibition of fibrinogen binding to Mac-1 was already achieved at concentrations of unfractionated heparin that resulted only in a weak prolongation of the aPTT. This finding in patients is consistent with the in vitro data, depicted in Figure 2, demonstrating saturation of heparin binding and inhibition of fibrinogen binding at concentrations between 0.1 and 1 U/mL heparin.

**Discussion**

The present study demonstrates binding of soluble heparin to Mac-1 on stimulated human monocytes and granulocytes, whereas no binding of heparin was detected on unstimulated leukocytes. Thereby, heparin inhibits binding of the soluble

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*Figure 3. Factor X binding to monocytes and granulocytes. Representative flow cytometry histograms of the binding of biotinylated factor X to monocytes and granulocytes with and without PMA stimulation (100 nmol/L) and no addition or the addition of heparin (10 U/mL) or c7E3 (10 μg/mL). Results are representative for experiments with blood drawn from several individuals.*
adhesion on ICAM-1

![Graph showing adhesion of THP-1 and peripheral monocytes and granulocytes on ICAM-1.](image)

**Figure 4.** Adhesion of monocytic cell line THP-1 and of peripheral monocytes and granulocytes on immobilized ICAM-1. Adhesion is depicted as percentage of cells adhering after addition of heparin or mAbs in relation to cells adhering without addition. Mean and SD of 3 determinations are given. *P < 0.01 vs no addition. Representative results of experiments with blood from several individuals are shown for adhesion of monocytes and granulocytes.

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iC3b binding

![Graph showing binding of iC3b to Mac-1 on monocytes and granulocytes.](image)

**Figure 5.** Heparin inhibits binding of iC3b to Mac-1 on monocytes and granulocytes. Flow cytometric determination of iC3b binding to PMA-stimulated monocytes using a mouse anti-iC3b mAb and a DTAF-labeled polyclonal goat anti-mouse Ab. Whole blood was incubated with freshly prepared iC3b (see Methods) alone, in combination with heparin (10 U/mL), in combination with c7E3 (10 μg/mL), or with no iC3b. Monocytes and granulocytes were identified by forward and sideward scatter characteristics. Mean and SD of 3 determinations are given. *P < 0.001 vs all other groups. Representative data of experiments with blood from several individuals are shown.

ligands fibrinogen, factor X, and iC3b to Mac-1 and Mac-1-mediated adhesion of monocytes and granulocytes to immobilized ICAM-1. Furthermore, flow cytometry revealed an inhibitory effect of heparin on fibrinogen binding to Mac-1 that correlates to the prolongation of the aPTT in patients treated with unfractionated heparin.

The I (also called A) domain of the αM subunit has been demonstrated to be a direct binding site of Mac-1 for the ligands ICAM-1, fibrinogen, and iC3b. Based on the ability of a whole set of monoclonal antibodies to inhibit Mac-1-mediated neutrophil adhesion on immobilized heparin, Diamond et al also suggest the I domain as the binding site for heparin. Therefore, our finding that heparin blocks binding of fibrinogen, ICAM-1, and iC3b to Mac-1 is consistent with the mapping of the ligand binding sites to the I domain of Mac-1. Additionally, we demonstrate that binding of factor X is also inhibited by heparin. Binding experiments with a recombinant form of the I domain did not show a direct single binding site for factor X on this αM fragment. Analyses of factor X suggest several noncontiguous sequences to be involved in ligand binding to Mac-1. Therefore, our data could be explained by binding of heparin to the I domain which may then cause steric hindrance of the
binding of factor X to spatially distinct binding sites within Mac-1. The mAb 7E3, which is as extensively used in patients as human/mouse chimeric mAb fragment, c7E3, or abciximab, has been described to bind to Mac-1. The binding site of c7E3 within Mac-1 has also been mapped to the I domain. The ability of c7E3 to inhibit heparin binding on monocytes and granulocytes further implies the I domain as binding site of heparin within Mac-1.

Binding of factor X to Mac-1 is considered to be an important part of a cell bound alternative pathway of initiation of the coagulation system; it results in acceleration of the conversion to factor Xa and the release of proteases that activate coagulation factors. Besides the anticoagulative effects of heparin mediated by its association with antithrombin III, the described blockade of factor X binding to Mac-1 may participate in the anticoagulative properties of heparin. Also, the inhibition of fibrinogen binding to Mac-1 by heparin may directly influence the coagulation system. Binding of soluble fibrinogen to Mac-1 is part of the cell membrane–bound coagulation system and constitutes a bridging function to platelet integrin GP IIb/IIIa (αIIbβ3), as well as to the endothelial adhesion molecule ICAM-1. Furthermore, binding of Mac-1 to immobilized fibrinogen, as it is for example exposed at the injured vessel wall, mediates leukocyte adhesion. The inhibition of Mac-1-mediated cell adhesion on ICAM-1 by heparin is expected to impair Mac-1-dependent leukocyte adhesion on endothelial cells and thus to attenuate inflammatory and proliferative stimuli associated with the emigration of leukocytes. Furthermore, binding of soluble heparin to Mac-1 in patients treated with heparin may inhibit leukocyte adhesion, which was recently described to be partially mediated by binding of Mac-1 to heparin-like structures bound on endothelial cells.

Inhibition of Mac-1 may be a part of the beneficial effects of heparin in various clinical settings, as suggested by the following findings: unstable angina is associated with activation of circulating monocytes including upregulation of Mac-1 expression and crossbridging to platelet GP Ib/IIa mediated by fibrinogen. Angioplasty results in leukocyte activation including an increased Mac-1 expression, and there is a direct association between late lumen loss and activation status of circulating phagocytes. After stenting in rabbit iliac arteries, the number of infiltrating monocytes correlates to intimal thickening and cell proliferation, and indeed, heparin drastically decreases monocyte adhesion and infiltration. Furthermore, in ischemia/reperfusion experiments Mac-1 blockade by mAbs reduces myocardial necrosis.

In conclusion, we demonstrate binding of soluble heparin to the integrin Mac-1 on stimulated monocytes and granulocytes. Thus, heparin inhibits binding of the ligands fibrinogen, factor X, IC3b, and ICAM-1 to Mac-1. Furthermore, we describe that anticoagulative dosing of unfractionated heparin in patients is sufficient to inhibit ligand binding to Mac-1. Thereby, heparin may alter leukocyte function such as participation in coagulation, inflammation, and initiation of cell proliferation. The described pharmacological property of heparin may contribute to the various clinical benefits of heparin therapy.

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