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. Low-molecular-weight heparins mainly inhibit factor Xa. In many clinical settings, heparin has been proven to be of major benefit as an antithrombotic agent. 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.

Recently, it was reported that immobilized heparin can mediate cell adhesion via interaction with the leukocyte integrin Mac-1 (CD11b/CD18, αMβ2). The integrin Mac-1 is expressed predominantly in leukocytes of the myeloid and monocytic lineage and in natural killer lymphocytes. Mac-1 is one of the most versatile adhesion molecules with ligands of very different biological functions. Its interaction with the transmembrane adhesion molecule ICAM-1 mediates cell adhesion on endothelial cells. 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. 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. 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. The versatile function of Mac-1 is further demonstrated by the recent finding that oligodeoxynucleotides are bound and internalized by this integrin.

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, 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 Luxo (Heidelberg, Germany), the fluorescein isothiocyanate (FITC)-labeled polyclonal chicken anti-fibrinogen Ab from Biopool (Umeå, Sweden). Factor X (Sigma, Deisenhofen, Germany) was biotinylated with BAC-SulfoNHS (Sigma) at a pH of 7.2, resulting in a biotin/protein ratio of 1.6. The anti-C1d11b mAbs clone 44 were obtained from Pharmingen (San Diego, Calif) and the human/mouse chimeric antibody fragment c7E3 (RedPro from Lilly (Bad Homburg, Germany). The mAbs anti-C66d (clone 80H3) and anti-CDI4 (clone ROM52) were obtained from Coulter-Immunotech (Hamburg). The human anti-C3b 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 containing C5a, human serum (250 mL) was incubated with 2.5-mg zymosan A (Sigma) first at 37°C for 45 minutes and then at 56°C for 30 minutes. Insoluble material was spun down and supernatant was used at a final concentration of 4%. iC3b was prepared by using reagents and protocol of Quidel (San Diego, Calif): (C3 (100 mg/mL) was incubated with factor D (1 mg/mL) and factor B (5 mg/mL) in 2 mmol/L MgCl2 at 37°C for 30 minutes and then incubated for 30 minutes. The monocytic cell line THP-1 that expresses high levels of Mac-1 was obtained from the tumor cell bank of the German Cancer Research Center (DKFZ, Heidelberg).

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. 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 adherent 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 revealed an anti-C1d11b 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-C66d Ab 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 reincubated with heparin, c7E3, anti-C1d11b 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 colorimetric assay; the cell-endogenous acid phosphatase activity was used by adding 100 μL of the following substrate/hypha 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 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 by heparin, monocytes were additionally stimulated with zymosan-activated serum containing C5a.
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 fibrinogen to Mac-1 on stimulated monocytes and granulocytes. This effect is not related to changes in fibrinogen serum levels and is achieved at concentrations of heparin that do not significantly prolong aPTT.
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.\textsuperscript{15,20,21} Based on the ability of a whole set of monoclonal antibodies to inhibit Mac-1-mediated neutrophil adhesion on immobilized heparin, Diamond et al\textsuperscript{5} also suggest the I domain as the binding site for heparin. Therefore, our finding that heparin blocks binding of fibrinogen, iC3b, and factor X 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.\textsuperscript{21} Analyses of factor X suggest several noncontiguous sequences to be involved in ligand binding to Mac-1.\textsuperscript{10} Therefore, our data could be explained by binding of heparin to the I domain which may then cause steric hindrance of the
The ability of c7E3 to inhibit heparin binding site of c7E3 within Mac-1 has also been mapped to the I domain.21,25 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 spatially distinct binding sites within Mac-1. The mAb 7E3, which is as extensively used in patients intravenously treated with unfractionated heparin,22 has been described to bind to Mac-1.23–25 The binding site of c7E3 within Mac-1 has also been mapped to the I domain.21,25 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.8–11 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 (α₃β₃), as well as to the endothelial adhesion molecule ICAM-1.14,26 Furthermore, binding of Mac-1 to immobilized fibrinogen, as it is for example exposed at the injured vessel wall, mediates leukocyte adhesion.14 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.6 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.5

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 IIb/IIIa mediated by fibrinogen.26–28 Angioplasty results in leukocyte activation including an increased Mac-1 expression,29–31 and there is a direct association between late lumen loss and activation status of circulating phagocytes.32 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.3 Furthermore, in ischemia/reperfusion experiments Mac-1 blockade by mAbs reduces myocardial necrosis.33

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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References


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