# Heparin Binds to Human Monocytes and Modulates Their Procoagulant Activities and Secretory Phenotypes. Effect of Histidine-Rich Glycoprotein

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**The binding of heparin to human monocytes and the monocytoid cell line U937 was characterized. Heparin binding was rapid, specific, saturable, and reversible. There was a single class of heparin binding sites, with an apparent dissociation constant of 0.19 μmol/L and 1.9 × $10^6$ sites per cell. The binding was not dependent on the anticoagulant property of heparin. Analysis of surface-iodinated cell lysates by heparin affinity chromatography revealed a major 120 Kd cell surface heparin-binding protein. Histidine-rich glycoprotein, a potent heparin antagonist found in human plasma and platelets, decreased the affinity of heparin for cell binding. Cell surface bound heparin was functionally active and markedly accelerated the inactivation of thrombin by antithrombin III. Heparin induced the release of two monocyte secretory proteins of 160 and 17 Kd. Our study supports the thesis that heparin and related glycosaminoglycans interact with monocytes and macrophages, as well as endothelial cells and smooth muscle cells, and play an important and complex role in blood vessel wall biology.**



HEPARIN and heparin-like glycosaminoglycans play an important and complex role in blood vessel wall biology. Anticoagulantly active heparan sulfate on the endothelial cell surface is crucial in the maintenance of the nonthrombogenic property of blood vessels.[1,2] Heparin augments the mitogenic potency of endothelial cell growth factor and is chemotactic for endothelial cells.[3-6] In contrast, both anticoagulantly active and inactive heparin inhibit the growth of smooth muscle cells and regulate a variety of smooth muscle cell activities, including cell migration, biosynthetic phenotype, and the composition of the extracellular matrix.[7-14]

In response to vascular injury, monocytes and macrophages interact with the endothelium and play an active role in the pathogenesis of atherosclerosis, inflammation, and thrombosis.[15,16] Heparin stimulates the production of a mononuclear cell factor by monocytes.[17] However, the interaction of heparin with human monocytes and its potential modulation and functional properties are not well defined. In this study, the specific binding of heparin to human monocytes and the monocytoid cell line U937 cells was characterized. The binding was partially inhibited by histidine-rich glycoprotein (HRGP),[1] a potent heparin antagonist found in human plasma and released from activated platelets.[18-20] Monocyte cell surface bound heparin was functionally active and heparin modulated the secretory phenotype of monocytes.

## MATERIALS AND METHODS

Carrier free [$^{125}$I]sodium iodide and $^{35}$S-methionine were purchased from New England Nuclear, Boston. Phenylmethylsulfonyl fluoride (PMSF), benzamidine, soybean trypsin inhibitor (SBTI), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co, St Louis. Paranitrophenyl paraguanidinobenzoate (pNGB) was purchased from ICN Biomedical Inc (Costa Mesa, CA). Phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) and 3-[(3-cholamidopropyl)-dimethyl ammonio]-1-propane sulfonate (CHAPS) were purchased from Calbiochem-Behring Corp, La Jolla, CA.Thrombin substrate S2238 was purchased from Helena Laboratory (Beaumont, TX). Heparin-sepharose CL 6B, CNBr-activated sepharose 4B and a fast-protein liquid chromatography (FPLC) system, including an anion-exchange Mono-Q column, were purchased from Pharmacia, Piscataway, NJ. Microtitration plates and a Titertek multiscan photometer were purchased from Flow Laboratories, Inc, McLean, VA. All reagents were of analytical grade.

*Human monocytes and U937 cells.* The human monocytoid cell line U937 was a kind gift from Dr Alan Krensky, Stanford University Medical School. The cell line was maintained in a humidified 37°C/5% $CO_2$ environment in RPMI (Gibco Lab) supplemented with 10% fetal calf serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. In some studies, PMA (100 nmol/L for 24 hours at 37°C) was used to stimulate differentiation of U937 cells. Human peripheral blood monocytes were isolated from leukocyte-rich fractions by Ficoll-Hypaque and double percoll density gradients according to published methods.[21] The purity of the monocyte preparation was consistently >90% as judged by immunofluorescence using a monocyte specific monoclonal anti-LeuM3 (Becton-Dickinson, Mountain View, CA).[22]

*Glycosaminoglycans.* [N-sulphonate-$^{35}$S]heparin was purchased from Amersham Corp, Arlington Heights, IL. The specific activity was 17.13 mCi/g with a molecular weight (mol wt) of 14,500 ± 200. Sodium heparin (grade I, 162 United States Pharmacopoeia [USP] U/mL), dermatan sulfate, and chondroitin-4-sulfate were purchased from Sigma. Heparin preparations with a range of anticoagulant activities (sodium heparin, 168 USP U/mL; RD heparin, mol wt 5,127, 98 USP U/mL; DH heparin, mol wt 2,750, 7 USP U/mL) were purchased from Hepar Industries, Franklin, OH.

*Purified proteins.* HRGP was isolated from fresh platelet-poor plasma as previously described with modifications.[20,23-27] Briefly, protease inhibitors pNGB 10 μmol/L, benzamidine 1 mmol/L, PMSF 0.4 mmol/L, SBTI 50 mg/L, and PPACK 1 μmol/L were added to the plasma. The plasma, diluted 1:2 with distilled water with the pH adjusted to 6.3, was batch absorbed with CM-cellulose

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52 (60 g/L plasma) at room temperature for 30 minutes. The CM-cellulose was washed extensively with distilled water, then batch eluted with 0.5 mol/L $NH_4HCO_3$. The eluate was dialyzed overnight against 0.02 mol/L phosphate-buffer with 0.4 mol/L NaCl, pH 6.3, then applied to a heparin-sepharose 6B column preequilibrated with the same buffer. After extensive washings, the column was eluted with phosphate buffer containing 1.0 mol/L NaCl. HRGP eluted from the heparin column was ≥92% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and laser densitometry, with mol wt 63,000 unreduced and 74,000 reduced, as previously reported.[24,25] Microsequencing of the purified protein revealed that the N-terminal 15 residues were identical to that published.[28] In some studies, the purified HRGP was further applied to a Mono-Q column on a FPLC system and eluted at 0.25 mol/L NaCl with a linear 0.1 to 1.0 mol/L NaCl gradient. Protein concentration was determined by absorbance using $E^{1\%} = 5.3$ at 280 nm.[29] Antithrombin III (ATIII) was purified from fresh human plasma or CM-cellulose 52 absorbed plasma following published methods.[30,31] The purified ATIII inactivated thrombin by chromogenic assay and formed a thrombin-ATIII complex as demonstrated by SDS-PAGE. Protein concentration was determined by absorbance at 280 nm ($E^{1\%} = 6.5$).[31] Purified human thrombin was a generous gift of Dr John Fenton II, New York State Department of Health, Albany.

*Heparin binding to human monocytes and U937 cells.* Monocytes and U937 cells ($7 \times 10^6$ cells/mL) were incubated in 20 mmol/L phosphate buffered saline, pH 7.4, containing 2% bovine serum albumin (PBS/BSA) for one hour at 4°C. $^{35}$S-heparin, in the presence or absence of 50-fold excess of unlabeled heparin, was added to cell aliquots and incubated for one hour at 4°C. The final volume of the reaction mixture was 300 μL with a cell concentration of $3.6 \times 10^6$ cells/mL. At the end of incubation, cell-bound heparin was separated from unbound heparin by centrifuging the cell suspensions through silicone oil (6.7 parts Dow Corning oil 550, 3.3 parts Dow Corning oil 556, S.G. 1.040) at 14,000 g for ten minutes in a Beckman microfuge. The cell pellets were solubilized with 0.2 N NaOH, added to Biofluor, and cell-bound radioactivity determined in a scintillation counter. Each experiment was repeated at least three times with each data point performed in duplicate. $^{35}$S-heparin binding to U937 cells in the presence of HRGP and to washed human RBCs were performed in an identical manner.

*Internalization of bound heparin by U937 cells.* Aliquots of U937 cells were incubated separately in PBS/BSA buffer for one hour at 4°C and 37°C. Binding of $^{35}$S-heparin (final concentration, 5 μmol/L) was carried out at the two temperatures for one to three hours. At the end of incubation, cell suspensions were washed with cold PBS/BSA and treated with trypsin (0.25%) for 30 minutes at 4°C. Cell-bound heparin, in the presence or absence of trypsin treatment, was determined. Trypsin treatment at this concentration did not cause any cell death as determined by trypan blue exclusion. Alternatively, instead of surface trypsinization, internalized heparin was determined by using 50-fold excess of unlabeled heparin to displace surface-bound $^{35}$S-heparin.

*Fractionation of $^{35}$S-heparin by ATIII affinity chromatography.* Nine milligrams of purified ATIII was coupled to 2.3 mL of CNBr-activated sepharose 4B. Aliquots of $^{35}$S-heparin were applied to the ATIII-sepharose column in PBS and eluted with 20 mmol/L phosphate buffer, pH 7.4, containing 2 mol/L NaCl.[32,33] The column flow-through fractions (low-affinity heparin) and the eluted fractions (high-affinity heparin) were separately pooled, dialyzed, and concentrated.

*Heparin affinity chromatography of surface iodinated U937 cell lysates.* U937 cells were surface iodinated using the immobilized lactoperoxidase bead method (BioRad, Richmond, CA). The labeled cells were washed with phosphate buffer three times and solubilized by 15 mmol/L CHAPS with protease inhibitors at 4°C for one hour. After centrifugation to remove any insoluble materials, aliquots of the cell lysates were applied to a heparin-sepharose 6B column and, after extensive washings, eluted with 1 mol/L NaCl. The eluates were analyzed by SDS-PAGE and autoradiography.

*Inactivation of thrombin by heparin-pretreated U937 cells.* U937 cells and control RBC were first incubated with RPMI/BSA at 4°C for one hour, then incubated with or without heparin (final concentration, 0.24 μmol/L) at 4°C for one hour. Cells were washed once with PBS/BSA and fixed with glutaraldehyde (final concentration, 0.1%) for 20 minutes at room temperature. After fixation, the cells were washed with PBS three times. Using heparin-sepharose beads, it was shown that glutaraldehyde fixation at this concentration did not affect the heparin enhancement effect on thrombin inactivation by ATIII. The fixed U937 cells and RBC, with or without heparin preincubation, were added in various concentrations to thrombin (final concentration, 10 U/mL) and ATIII (final concentration, 10 μg/mL) in 40 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 8.3.[34] After 30 seconds of incubation, thrombin substrate S2238 (final concentration, 0.5 mmol/L) was added. After another 30 seconds of incubation, acetic acid (final concentration, 15.6%) was added to stop the reaction. Acetic acid did not cause any RBC lysis and did not interfere with the absorbance measurement. The reaction mixtures (total volume 200 μL) were centrifuged and the absorbance of the supernates read at 405 nm. Each experiment was repeated three times with each data point done in duplicate.

*Heparin-HRGP interaction.* HRGP (final concentration, 10 μg/mL) was coated on microtitration wells as previously described.[20] Unbound HRGP was removed by washing with Tris/Tween buffer (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, 0.05% Tween 20, pH 7.4) three times. $^{35}$S-heparin in Tris/Tween buffer containing 2 mmol/L $CaCl_2$ or 2 mmol/L EDTA, in the presence or absence of 50-fold excess of unlabeled heparin, was added and incubated for one hour at 37°C. After extensive washings with Tris/Tween buffer, the HRGP-bound heparin was solubilized with 0.2 N NaOH, added to Biofluor, and radioactivity determined. Alternatively, heparin-sepharose beads were incubated with Tris-saline/2% BSA, followed by $^{125}$I-HRGP in the same buffer containing 2 mmol/L $CaCl_2$ or 2 mmol/L EDTA, for one hour at room temperature. The samples were placed into modified 1-mL Eppendorf pipette tips fitted with filter plugs (U-11 plug; Porex Technologies, Fairburn, GA). Unbound proteins were separated by centrifugation for one minute at 2,500 rpm in a table-top centrifuge. Heparin beads retained on the filters were washed three times with Tris/Tween buffer and the amount of $^{125}$I-HRGP bound counted.

*Metabolic labeling of monocytes.* Isolated human peripheral blood monocytes were placed in tissue culture wells at 1 to $2 \times 10^6$ cells/mL. After two hours of incubation, the nonadherent cells were removed and the adherent cells cultured in heparin for various periods of time. For the last two hours of heparin incubation, the culture media was replaced by methionine-free RPMI with $^{35}$S-methionine (40 μCi/mL) and 0.4% FCS. At the end of incubation, the culture media were removed and precipitated with 10% trichloroacetic acid. The precipitates were dissolved in sample buffer and analyzed by SDS-PAGE and autoradiography.

## RESULTS

*Heparin binding to U937 cells and human monocytes.* $^{35}$S-heparin bound to U937 cells in a concentration-dependent and saturable manner. Nonspecific binding, as determined by binding in the excess of unlabeled heparin, was <10% (Fig 1). Similar specific binding of heparin to isolated human peripheral blood monocytes was observed

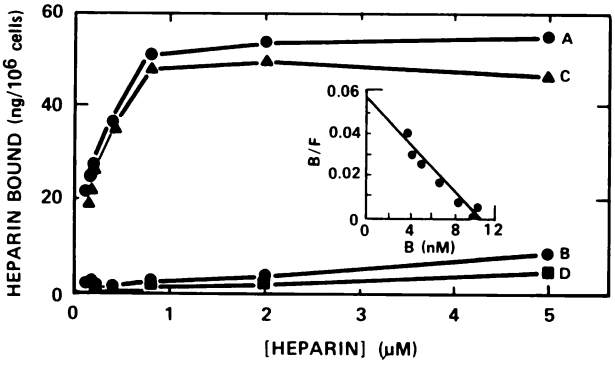


Fig 1. Heparin binding to U937 cells. U937 cells and washed RBC were incubated in PBS/BSA for one hour at 4°C. $^{35}$S-heparin was added, in the presence and absence of 50-fold excess unlabeled heparin, and incubated for one hour at 4°C. The cell suspensions were then spun through silicone oil, the cell pellets solubilized, and the amount of cell-bound radioactivity determined. Each point is the mean of duplicate determinations. A, $^{35}$S-heparin binding to U937 in the absence of unlabeled heparin (total binding); B, $^{35}$S-heparin binding to U937 in the presence of 50-fold excess of unlabeled heparin (nonspecific binding); C, specific binding of heparin to U937 cells (total minus nonspecific binding); D, $^{35}$S-heparin binding to washed RBC in the absence of excess unlabeled heparin. (Insert) Scatchard plot analysis of heparin binding to U937 cells. The specific heparin binding to U937 cells was analyzed. The line represents linear regression by least squares analysis of data points, $Y = -0.00543X + 0.057$, $r = -0.976$.

(data not shown). Heparin binding to control human RBCs was minimal, demonstrating the relative specificity of binding for U937 cells and human monocytes. In these studies, the concentration of RBCs was fivefold greater than that of U937 cells in order to provide an equivalent cell surface area. The binding of heparin to U937 cells was not inhibited by 2 mmol/L EDTA, indicating that it was not divalent cation dependent. The binding reached a steady state at 30 minutes at 4°C and was reversible, with >90% of the surface bound

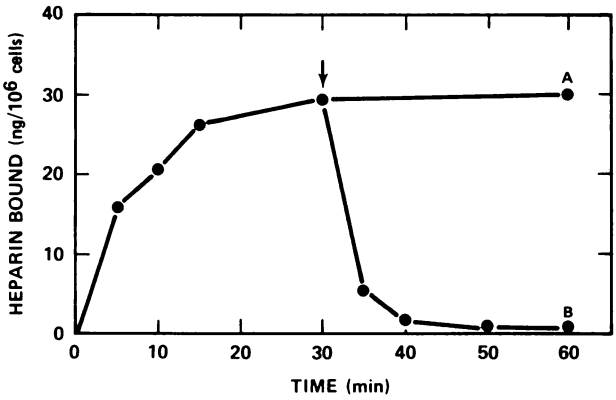


Fig 2. Time course and reversibility of heparin binding to U937 cells. U937 cells were incubated in PBS/BSA for one hour at 4°C. $^{35}$S-heparin (final concentration, 0.26 μmol/L) was added. Duplicate aliquots of the cell suspensions were removed at various time points and spun through silicone oil to determine cell-bound radioactivity (A). After 30 minutes incubation with $^{35}$S-heparin, 100-fold excess unlabeled heparin was added and the cell-bound radioactivity at different time intervals determined (B).

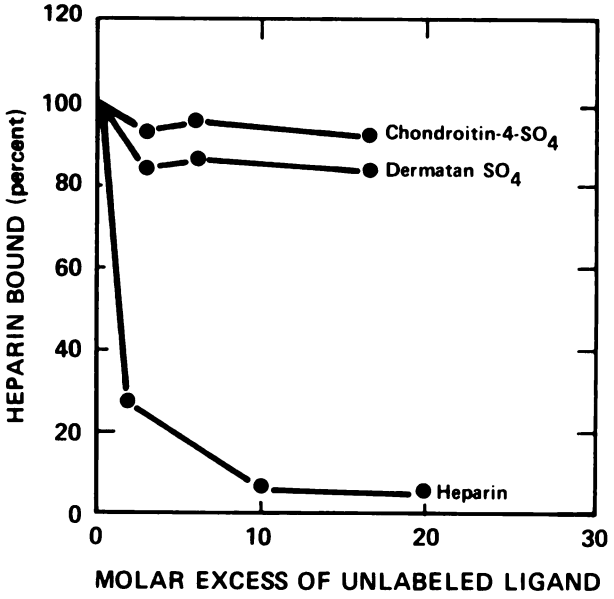


Fig 3. Specificity of heparin binding to U937 cells. U937 cells were incubated with PBS/BSA for one hour at 4°C. $^{35}$S-heparin (final concentration, 0.5 μmol/L) was added in the presence of various molar excess of unlabeled heparin, dermatan sulfate, chondroitin-4-sulfate for 30 minutes at 4°C. Cell-bound radioactivity was then determined. $^{35}$S-heparin bound in the absence of unlabeled glycosaminoglycans was considered 100% binding.

labeled heparin displaced by excess unlabeled heparin (Fig 2). Scatchard plot analysis of the specific heparin binding revealed a single class of heparin binding sites, with an apparent dissociation constant (kd) of 0.19 μmol/L, and $1.92 \times 10^6$ binding sites per cell (Fig 1, insert).

To determine the specificity of binding for heparin, binding of $^{35}$S-heparin was performed in the presence of excess unlabeled heparin, dermatan sulfate, and chondroitin-4-sulfate (Fig 3). Unlabeled heparin competitively inhibited the binding of $^{35}$S-heparin, with 50% inhibition occurring at a molar ratio of unlabeled:labeled heparin of 1.5, indicating that the binding characteristics of $^{35}$S-heparin was identical to that of unlabeled heparin. In contrast, there was no significant inhibition of heparin binding by the other glycosaminoglycans, even at 16-fold molar excess.

Table 1. Internalization of Surface-Bound Heparin by U937 Cells

| Incubation | Trypsin | Heparin Bound (ng/10⁶ cells) 4°C | 37°C |
|---|---|---|---|
| 1 h | – | 56.4 ± 2.8 | 45.2 ± 3.3 |
| | + | 5.1 ± 0.3* | 8.5 ± 0.6* |
| 3 h | – | 51.9 ± 3.1 | 39.4 ± 2.7 |
| | + | 5.8 ± 0.4† | 11.0 ± 1.4† |

U937 cells were incubated in PBS/BSA buffer for one hour at 4°C and 37°C. Binding of $^{35}$S-heparin (final concentration 5 μmol/L) was carried out at the two temperatures for one hour and three hours. At the end of incubation, cell suspensions were washed with cold PBS/BSA and trypsinized (0.25%) at 4°C for 30 minutes. Cell-bound heparin was then determined. Data presented were the mean ± SD (n = 6).

*$P \leq .01$.

†$P \leq .01$.

*Internalization of bound heparin by U937 cells.* To determine if the bound heparin was internalized by U937 cells, binding of $^{35}$S-heparin was carried out at 4°C and 37°C. Cells were then trypsinized and the residual amount of cell-bound heparin determined (Table 1). At 4°C, ~10% of the cell-bound heparin was not removed by surface trypsinization, consistent with the ~10% nonspecific binding as determined by excess unlabeled heparin, indicating that there was minimal cellular internalization of heparin at 4°C. When binding was performed at 37°C, ~18% and 28% of the respective total cell-bound heparin at one hour and three hours of incubation was not removed by trypsin, demonstrating internalization of heparin by U937 cells at 37°C. Alternatively, when excess unlabeled heparin was added to displace $^{35}$S-heparin from the cell surface, a similar increase in the amount of nondisplaceable cell-bound heparin was observed at 37°C compared with 4°C (data not shown).

*Binding of heparin fractions to U937 cells.* To further characterize the heparin binding to U937 cells, $^{35}$S-heparin was separated into fractions with high or low affinity for ATIII by ATIII affinity chromatography. While the binding of the high-affinity heparin fraction was equivalent to the unfractionated heparin, there was also significant binding of the low-affinity heparin fraction to U937 cells, indicating that the binding was not dependent on the anticoagulant property of heparin (Table 2). Binding of the unfractionated $^{35}$S-heparin was also performed in the presence of 20-fold excess of unlabeled heparin preparations with various mol wts and anticoagulant activities. A low mol wt heparin (RD heparin, mol wt 5,127, 98 USP U/mL) was as effective as regular heparin in inhibiting $^{35}$S-heparin binding (94% inhibition). A heparin fragment with minimal anticoagulant activity (DH heparin, mol wt 2,750, 7 USP U/mL) also caused significant inhibition (73% inhibition), consistent with the observation that heparin binding was not dependent on its anticoagulant property.

*Characterization of heparin-binding proteins on U937 cell surface.* Detergent-solubilized cell surface iodinated U937 cell lysates were applied to a heparin-sepharose affinity column and analysis of the eluate revealed a major cell surface heparin-binding protein with mol wt 120,000 (Fig 4). When the cell lysates were applied in the presence of excess fluid phase heparin, the 120 Kd protein was not detected. Additional control using an albumin-sepharose column was also negative, demonstrating the specificity of this heparin-binding protein. The identity of the 120 Kd cell surface protein was not determined.

*Modulation of heparin binding by HRGP.* HRGP is a

**Table 2. Binding of Heparin Fractions to U937 Cells**

| | Heparin Bound (ng/10$^6$ cells) |
|---|---|
| Unfractionated heparin | 42.7 |
| High affinity heparin | 40.5 |
| Low affinity heparin | 26.8 |

Labeled heparin was separated into high and low affinity fractions by ATIII affinity chromatography. Heparin binding (final concentration, 0.4 μmol/L) was performed as described in Fig 1. Data presented are the mean of two different experiments.

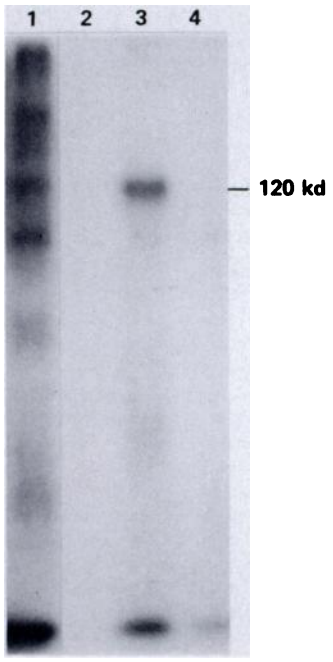


**Fig 4. Heparin-binding proteins on U937 cells. Detergent lysates of surface-iodinated U937 cells were applied, in the presence or absence of excess heparin, to a heparin-sepharose column and, after extensive washings, eluted with 1 mol/L NaCl. The eluates were analyzed by SDS-PAGE and autoradiography. Lane 1, surface-iodinated U937 cell lysates; lane 2, heparin column eluate with lysates applied in the presence of excess heparin; lane 3, heparin column eluate with lysates applied in the absence of fluid phase heparin; lane 4, eluate from control albumin column.**

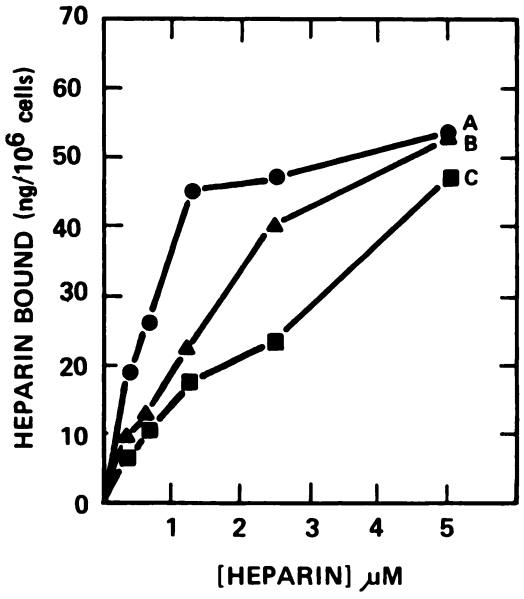


**Fig 5. Effect of HRGP on heparin binding to U937 cells. The binding of $^{35}$S-heparin to U937 cells in the presence of different concentrations of HRGP was performed. A, no HRGP; B, 50 μg/mL HRGP; C, 100 μg/mL HRGP.**

potent heparin-binding protein found in plasma and is released from activated platelets.[18-20] Whether HRGP would modulate heparin binding to U937 cells was examined. HRGP caused a concentration-dependent shift of the heparin binding isotherm to the right, requiring a fourfold increase in heparin concentration to achieve 50% binding in the presence of 100 μg/mL HRGP. The data suggest that HRGP significantly decreased the affinity of heparin for U937 cells (Fig 5). The inhibitory effect was not due to contamination of the HRGP samples by heparin during the preparative procedure since HRGP that had been further purified by anion-exchange (Mono-Q) chromatography had a similar effect on heparin cell binding. While the heparin interaction with HRGP was dependent on divalent cation and inhibited by EDTA (Fig 6A) as previously reported,[19] EDTA did not abolish the HRGP inhibitory effect on heparin binding to U937 cells (Fig 6B).

*Heparin binding to PMA-stimulated U937 cells and modulation by HRGP.* Heparin bound to PMA-stimulated U937 cells with similar characteristics as nonstimulated cells, with a slight decrease in the affinity of heparin binding (50% binding occurring at 0.9 μmol/L instead of 0.4 μmol/L), and a 20% decrease in total heparin cell binding. HRGP also partially inhibited heparin binding to PMA-stimulated cells (data not shown).

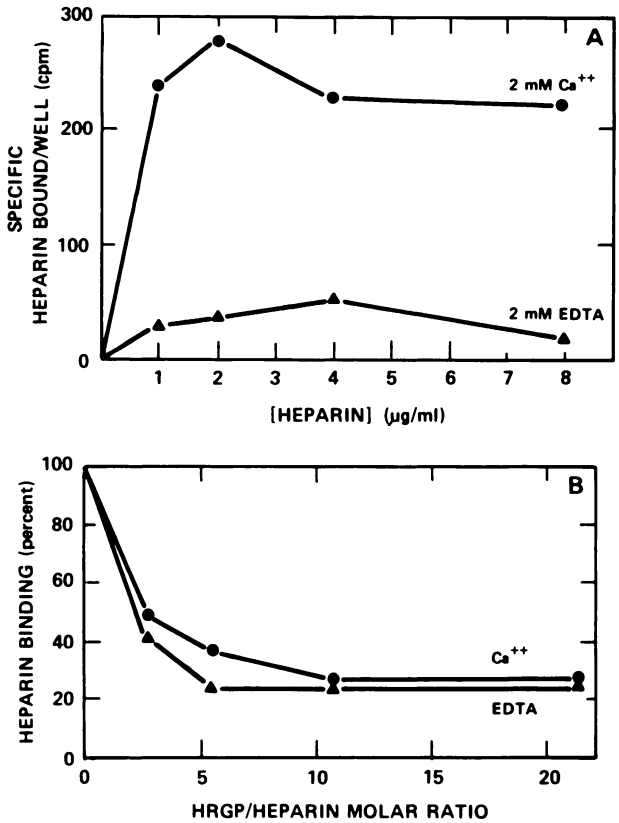


**Fig 6. (A) Effect of EDTA on HRGP-heparin interaction. Purified HRGP (final concentration, 10 μg/mL) was coated on microtitration wells. $^{35}$S-heparin, in the presence or absence of 50-fold excess unlabeled heparin, was added in Tris/Tween buffer containing 2 mmol/L $CaCl_2$ or 2 mmol/L EDTA. After one hour incubation at 37°C, the wells were extensively washed with Tris/Tween buffer and the contents solubilized and radioactivity determined. Specific heparin binding to the HRGP-coated wells was plotted in this figure. (B) Effect of EDTA on HRGP modulation of heparin binding to U937 cells. $^{35}$S-heparin binding (final concentration, 0.25 μmol/L) to U937 cells was carried out in the presence of 2 mmol/L $CaCl_2$ or 2 mmol/L EDTA as described in Fig 1.**

**Table 3. Inactivation of Thrombin by Heparin-Pretreated U937 Cells**

| Reaction Mixture | A450 |
|---|---|
| Thrombin | 0.494 |
| Thrombin + ATIII | 0.344 |
| Thrombin + ATIII + U937 | 0.368 |
| Thrombin + ATIII + Heparin/U937 | 0.085 |
| Thrombin + ATIII + Heparin/RBC | 0.390 |

U937 cells and control RBC were incubated with RPMI/BSA at 4°C for one hour. Cell aliquots were then incubated with heparin (0.24 μmol/L) at 4°C for one hour. Cells with or without heparin preincubation were fixed with glutaraldehyde (0.1%) for 20 minutes at room temperature and washed three times with PBS. Fixed U937 cells and control RBC ($1.2 \times 10^7$/mL) were added to thrombin (10 U/mL), and ATIII (10 μg/mL) and after 30 seconds of incubation, thrombin substrate S2238 was added. After another 30 seconds of incubation, acetic acid was added to stop the reaction and absorbance (A405) of the assay mixture read in a photometer. All reagents were given in final concentrations.

*Inactivation of thrombin by heparin-pretreated U937 cells.* To determine the functional role of cell-bound heparin, the effect of heparin-pretreated U937 cells on thrombin inactivation by ATIII was investigated (Table 3). U937 cells not pretreated with heparin had no effect on ATIII inactivation of thrombin. However, pretreatment with heparin markedly accelerated the inactivation of thrombin by ATIII, indicating that the cell-bound heparin was anticoagulantly functional. Using labeled heparin, it was demonstrated that the enhancement effect was not due to the release of cell-bound heparin into the fluid phase. Control studies with human RBC pretreated with heparin did not show any enhancement effect.

The enhancement of thrombin inactivation by heparin pretreated U937 cells correlated positively with the cell concentration, with 50% enhancement occurring at $4.5 \times 10^6$ cells/mL (Fig 7B). An equivalent degree of thrombin inactivation was obtained by 60 ng/mL of heparin in the fluid phase (Fig 7A). Using labeled heparin, the amount of heparin fixed on the U937 cells was determined to be 15 ng/$10^6$ cells or 67.5 ng/mL. The data indicated that the anticoagulant potency of cell-bound heparin was nearly equivalent to that of fluid phase heparin.

*Effect of heparin on monocyte secretory proteins.* When metabolically labeled human monocytes were incubated with heparin, an increase of two secretory proteins, mol wt 160,000 and 17,000, in the culture media was observed (Fig 8). The inductive effect of heparin was dose dependent and occurred quite rapidly (after two hours) with maximal induction observed after 12 to 14 hours. A low mol wt heparin with minimal anticoagulant activity (DH heparin, 7 USP U/mL) was also effective in the induction of these two secretory proteins, whereas dermatan sulfate was much less effective. Of note, the heparin inductive effect was not

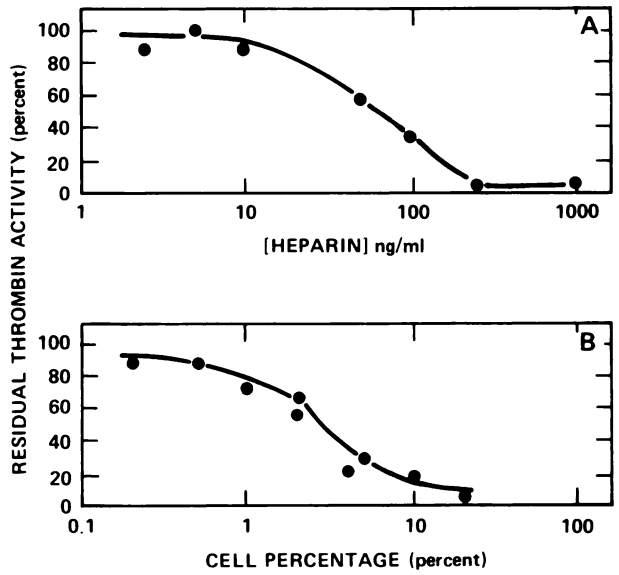


**Fig 7. (A) Inactivation of thrombin by heparin. Increasing concentrations of heparin were added to thrombin (10 U/mL) and ATIII (10 μg/mL) and residual thrombin activities determined as described in Table 2. (B) Inactivation of thrombin by heparin-pretreated U937 cells. Heparin-pretreated U937 cells at 1.7 × $10^8$ cells/mL was added at various dilutions to the thrombin and ATIII mixture and residual thrombin activities determined.**

observed in U937 cells. The identity of these monocyte secretory proteins remained to be determined.

## DISCUSSION

In this study, binding of heparin to human monocytes and the monocytoid cell line U937 was characterized. The binding was specific, time- and concentration-dependent, saturable, and reversible, suggesting a ligand-receptor type interaction on the cell surface (Figs 1 through 3). Since heparin is highly negatively charged, a strong ionic interaction between heparin and cell surface proteins with basic characteristics is anticipated, raising the possibility that heparin may bind to multiple cell surface proteins. However, Scatchard plot analysis of the binding data revealed a single class of heparin binding sites (Fig 1), and heparin affinity chromatography of the surface labeled cell lysates revealed a major 120 Kd heparin-binding protein (Fig 4). These findings demonstrate that heparin only bound to a limited number of specific cell surface proteins. Whether the 120 Kd heparin-binding protein represents a heparin receptor on the monocyte cell surface remains to be determined.

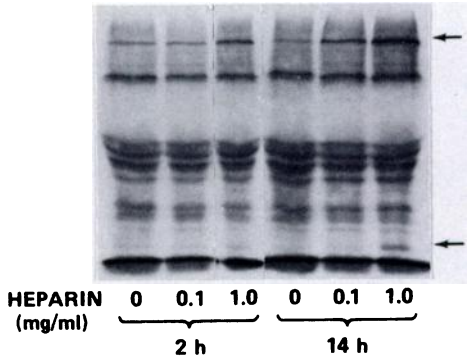


**Fig 8. Induction of monocyte secretory proteins by heparin. Adherent human peripheral blood monocytes were cultured in heparin for different incubation times. For the last two hours of incubation, the cells were metabolically labeled with $^{35}$S-methionine. At the end of incubation, the culture media were removed and precipitated with 10% trichloroacetic acid. The precipitates were dissolved in sample buffer and analyzed by SDS-PAGE and autoradiography. Equal amounts of radioactive counts were applied in each lane.**

The number of heparin-binding sites on U937 cells (1.9 × $10^6$ binding sites per cell) is in the same range as that reported for human endothelial cells (1 × $10^6$ sites per cell) and rat liver cells (1 × $10^7$ sites per cell),[35,36] and higher than that found on smooth muscle cells (1 × $10^5$ sites per cell).[37] Heparin binding to mouse peritoneal macrophages has also been described but the affinity of heparin binding and the number of binding sites were not reported.[38] More recently, heparin binding to human platelets is observed.[39,40] In view of the present findings, it will be of interest to characterize the cell-surface heparin-binding proteins from these various cell types and determine if they are structurally and functionally related.

HRGP, a potent heparin antagonist found in human plasma and released from activated platelets,[18-20] decreased the affinity of heparin for U937 cells (Fig 5). Significant modulation of heparin binding was observed with HRGP concentration at 50 to 100 μg/mL, suggesting that plasma HRGP, at 100 to 150 μg/mL,[23,33] would have a similar effect. Recently, HRGP inhibition of the antiproliferative effect of heparin on arterial smooth muscle cells was reported.[41] Taken together, the data suggest that the modulation of heparin binding to cells by HRGP may represent a major function for this protein in vivo.

The mechanism whereby HRGP modulates heparin binding to the monocyte cell surface is not fully defined. The observation that HRGP decreased the affinity of heparin binding suggests that the HRGP-binding site is different from the cell-binding site on the heparin molecule. It should be noted that while EDTA abolished the direct HRGP-heparin interaction as previously reported,[35] it had no effect on the HRGP inhibition of heparin cell binding (Fig 6). The data suggest that, in addition to competition for heparin binding in the fluid phase, HRGP may interact directly with the cell surface and thereby alter its affinity for heparin. Recently, we obtained data demonstrating direct binding of HRGP to U937 cells (K. Saigo, L. Leung, unpublished observation, July 1987).

Cell surface bound heparin was functionally active, and markedly accelerated the inactivation of thrombin by ATIII (Table 3). The anticoagulant potency of the cell-bound heparin was equivalent to that of fluid phase heparin (Fig 7). Human monocytes and macrophages generate potent tissue-factor activity on stimulation and provide an optimal cell surface for the efficient assembly of prothrombinase, the major enzyme complex that catalyzes thrombin generation

in vivo.[42-44] Fibrin deposition is a prominent feature of inflammation and fibrin has been identified on macrophage cell surface.[45-47] The specific binding and localization of functionally active heparin to the same cells may represent a pathway to modulate the procoagulant properties of monocytes and macrophages at sites of inflammation and thrombosis.

The functional consequence of heparin binding to monocytes is not limited to its anticoagulant property. Significant binding of heparin fractions with low affinity for ATIII to U937 cells was demonstrated (Table 2). Heparin induced the release of two monocyte secretory proteins of 160 and 17 Kd (Fig 8). A similar heparin augmentation effect on the secretion of smooth muscle cell proteins has been observed.[12,48] Heparin stimulates macrophages, including the release of a mononuclear cell factor,[17] interferon,[49] and the promotion of lysosomal enzyme secretion.[50] It is of note that native heparin from mast cells also has low anticoagulant activity[51] and that mast cell-macrophage interactions have been implicated in various inflammatory processes in vivo.[17,52-55] Our study supports the thesis that native heparin and heparin-like glycosaminoglycans, derived from cellular matrices or released from mast cells, interact with monocytes and macrophages as well as endothelial cells and smooth muscle cells, and play an important and complex role in blood vessel wall biology.

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## REFERENCES

1. Marcum JA, Reilly CF, Rosenberg RD: The role of specific forms of heparan sulfate in regulating blood vessel wall function, in Coller BS (ed): Progress in Hemostasis and Thrombosis, vol 8. Philadelphia, Grune & Stratton, 1986, p 185

2. Marcum JA, Atha DH, Fritze LMS, Nawroth P, Stern D, Rosenberg RD: Cloned bovine aortic endothelial cells synthesize anticoagulantly active heparan sulfate proteoglycan. J Biol Chem 261:7507, 1986

3. Thorton SC, Mueller SN, Levine EM: Human endothelial cells: Use of heparin in cloning and long-term serial cultivation. Science 222:623, 1983

4. Maciag T, Mehlman T, Friesel R, Schreiber A: Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. Science 225:932, 1984

5. Schreiber AB, Kenney J, Kowalski WJ, Friesel R, Mehlman T, Maciag T: Interaction of endothelial cell growth factor with heparin: Characterization by receptor and antibody recognition. Proc Natl Acad Sci USA 82:6138, 1985

6. Terranova VP, DiFlorio RA, Lyall RM, Hic S, Friesel R, Maciag T: Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. J Cell Biol 101:2330, 1985

7. Clowes AW, Karnovsky MJ: Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature 265:625, 1977

8. Hoover RL, Rosenberg R, Haering W, Karnovsky MJ: Inhibition of rat smooth muscle cell proliferation by heparin. II. In vitro studies. Circ Res 47:578, 1980

9. Castellot JJ, Beeler DL, Rosenberg RD, Karnovsky MJ: Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. J Cell Physiol 120:315, 1984

10. Fritze LMS, Reilly CF, Rosenberg RD: An anti-proliferative heparan sulfate species produced by postconfluent smooth muscle cells. J Cell Biol 100:1041, 1985

11. Majack RA, Clowes AW: Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. J Cell Physiol 118:253, 1984

12. Majack RA, Bornstein P: Heparin and related glycosaminoglycans modulate the secretory phenotype of vascular smooth muscle cells. J Cell Biol 99:1688, 1984

13. Majack RA, Cook SC, Bornstein P: Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix of smooth muscle cells. J Cell Biol 101:1059, 1985

14. Majack RA, Cook SC, Bornstein P: Control of smooth muscle cell growth by components of the extracellular matrix: Autocrine role for thrombospondin. Proc Natl Acad Sci USA 83:9050, 1986

15. Ross R: The pathogenesis of atherosclerosis—an update. N Engl J Med 314:488, 1986

16. Harlan JH: Leukocyte-endothelial interactions. Blood 65:513, 1985

17. Yoffe JR, Taylor DJ, Woolley DE: Mast-cell products and heparin stimulate the production of mononuclear-cell factor by cultured human monocyte/macrophages. Biochem J 230:83, 1985

18. Haupt H, Heimburger N: Humanserumproteine mit hoher affinitat zu carboxymethylcellulose I. Hoppe-Seyler's Z Physiol Chem 353:1125, 1972

19. Lijnen HR, Hoylaerts M, Collen D: Heparin binding properties of human histidine-rich glycoprotein. Mechanism and role in the neutralization of heparin in plasma. J Biol Chem 258:3803, 1983

20. Leung LLK, Harpel PC, Nachman RL, Rabellino EM: Histidine-rich glycoprotein is present in human platelets and is released following thrombin stimulation. Blood 62:1016, 1983

21. Burdach S, Shatsky M, Wagenhorst B, Levitt L: The T cell CD2 determinant mediates inhibition of erythropoiesis by the lymphokine cascade. Blood 72:770, 1988

22. Burdach SEG, Levitt LJ: Receptor-specific inhibition of bone marrow erythropoiesis by recombinant DNA-derived interleukin-2. Blood 69:1368, 1987

23. Lijnen HR, Hoylaerts M, Collen D: Isolation and characterization of a human plasma protein with affinity for the lysine binding sites in plasminogen. J Biol Chem 255:10214, 1980

24. Koide T, Odani S, Ono T: Human histidine-rich glycoprotein: Simultaneous purification with antithrombin III and characterization of its gross structure. J Biochem 98:1191, 1985

25. Lijnen HR, Rylatt DB, Collen D: Physicochemical, immunochemical and functional comparison of human histidine-rich glycoprotein and autorosette inhibition factor. Biochim Biophys Acta 742:109, 1983

26. Leung LLK, Nachman RL, Harpel PC: Complex formation of platelet thrombospondin with histidine-rich glycoprotein. J Clin Invest 73:5, 1984

27. Leung LLK: Interaction of histidine-rich glycoprotein with fibrinogen and fibrin. J Clin Invest 77:1305, 1986

28. Koide T, Foster D, Yoshitake S, Davie EW: Amino acid sequence of human histidine-rich glycoprotein derived from the nucleotide sequence of its cDNA. Biochemistry 25:2220, 1986

29. Morgan WT: The histidine-rich glycoprotein of serum has a domain rich in histidine, proline, and glycine that binds heme and metals. Biochemistry 24:1496, 1985

30. Miller-Andersson M, Borg H, Andersson LO: Purification of antithrombin III by affinity chromatography. Thromb Res 5:439, 1974

31. Olson ST: Heparin and ionic strength-dependent conversion of antithrombin III from an inhibitor to a substrate of $\alpha$-thrombin. J Biol Chem 260:10153, 1985

32. Höök M, Bjork I, Hopwood J, Lindahl U: Anticoagulant activity of heparin: Separation of high-activity and low-activity heparin species by affinity chromatography on immobilized antithrombin. FEBS Lett 66:90, 1976

33. Danishefsky I, Pixley R: Preparation of highly stable antithrombin-sepharose and utilization for the fractionation of heparin. Thromb Res 26:129, 1982

34. Abildgaard U, Lie M, Odegard OR: Antithrombin (heparin cofactor) assay with "new" chromogenic substrates (S2238 and Chromozym TH). Thromb Res 11:549, 1977

35. Glimelius B, Busch C, Höök M: Binding of heparin on the surface of cultured human endothelial cells. Thromb Res 12:773, 1978

36. Kjellen L, Oldberg A, Rubin K, Höök M: Binding of heparin and heparan sulfate to rat liver cells. Biochem Biophys Res Comm 74:126, 1977

37. Castellot JJ, Wong K, Herman B, Hoover RI, Albertini DF, Wright TC, Caleb BL, Karnovsky MJ: Binding and internalization of heparin by vascular smooth muscle cells. J Cell Physiol 124:13, 1985

38. Bleiberg I, MacGregor I, Aronson M: Heparin receptors on mouse macrophages. Thromb Res 29:53, 1983

39. Gogstad GO, Solum NO, Krutnes M-B: Heparin-binding platelet proteins demonstrated by crossed affinity immunoelectrophoresis. Br J Hematol 53:563, 1983

40. Horne MK, Hart JS: Heparin binding to platelets. Blood 68:317a, 1986 (abstr) (suppl 1)

41. Hajjar DP, Boyd DB, Harpel PC, Nachman RL: Histidine-rich glycoprotein inhibits the antiproliferative effect of heparin on smooth muscle cells. J Exp Med 165:908, 1987

42. Tracy PB, Rohrbach MS, Mann KG: Functional prothrombinase complex assembly on isolated monocytes and lymphocytes. J Biol Chem 258:7264, 1983

43. Gregory SA, Edgington TA: Tissue factor induction in human monocytes. J Clin Invest 76:2440, 1985

44. Schwartz BS: Antigen-induced monocyte procoagulant activity. J Clin Invest 76:970, 1985

45. Colvin RB, Dvorak HF: Fibrinogen/fibrin on the surface of macrophages: Detection, distribution, binding requirements, and possible role in macrophage adherence phenomena. J Exp Med 142:1377, 1975

46. Sherman LA, Lee J: Specific binding of soluble fibrin to macrophages. J Exp Med 145:76, 1977

47. Dvorak HF: Tumors: Wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 315:1650, 1986

48. Castellot JJ, Cochran DL, Karnovsky MJ: Effect of heparin on vascular smooth muscle cells. I. Cell metabolism. J Cell Physiol 124:21, 1985

49. Schultz RM, Papamatheakis JD, Chirigos MA: Interferon: An inducer of macrophage activation by polyanions. Science 197:674, 1977

50. Schorlemmer HU, Burger R, Hylton W, Allison AC: Induction of lysosomal enzyme release from cultured macrophages by dextran sulfate. Clin Immunol Immunopathol 7:88, 1977

51. Yurt RW, Leid RW, Austen KF, Silbert JE: Native heparin from rat peritoneal mast cells. J Biol Chem 252:518, 1977

52. Roche WR: Mast cells and tumors. The specific enhancement of tumor proliferation in vitro. Am J Pathol 119:57, 1985

53. Azizkhan RG, Azizkhan JC, Zetter BR, Folkman J: Mast cell heparin stimulates migration of capillary endothelial cells in vitro. J Exp Med 152:931, 1980

54. Kokkonen JO, Kovanen PT: Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein. Proc Natl Acad Sci USA 84:2287, 1987

55. Serafin WE, Austen KF: Mediators of immediate hypersensitivity reactions. N Engl J Med 317:30, 1987